Kor. J. Appl. Microbiol. Biotech.
Vol. 18, No. 4, 394-400 (1990)

Optimization of Switching Time from Growth to Product Formation for Maximum Productivity of Recombinant *Escherichia coli* Fermentation

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유전자 재조합 대장균 발효의 최대 생산성을 위한 생육에서 제품 생성으로의 전환시기의 최적화

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Maximization of productivity of recombinant cell fermentations requires consideration of the inverse relationship between the host cell growth rate and product formation rate. The problem of maximizing a weighted performance index was solved by using optimal control theory for recombinant *E. coli* fermentation. Concentration of a growth inhibitor was used as a control variable to manipulate the specific growth rate, and consequently the cloned-gene expression rate. Using a simple unstructured model to describe the main characteristics of this system, theoretical analysis showed that the optimal control profile results in an initial high growth rate phase followed by a low growth rate and high product formation rate phase. Numerical calculations were done to determine optimal switching times from the growth to the production stage for two representative cases corresponding to different dependency of the product formation rate on the growth rate. For the case when product formation rate is sensitive to the specific growth rate, the optimized operation yields about 60% increase in the final product concentration compared with a simple batch fermentation.

A rapid emergence of biochemical methodologies in the late 1970s made it possible to construct recombinant organisms with desired properties. Recombinant DNA technology is not only a powerful tool for studying the complex molecular mechanisms of gene expression but also a method for producing proteins of medical and industrial importance. Developmental work has already led to the production of several biologically active human agents including insulin, interferon and growth hormone in significant amounts (1, 2).

Successful transition from gene manipulation in the laboratory to large scale commercial production requires careful investigation of the influence of growth conditions upon gene expression. Several previous studies indicate an inverse relationship between specific growth rate and cloned-gene expression levels (3, 4). This can be explained by considering the competition for common biosynthetic machinery between the plasmid and the host cell DNA. If more biosynthetic potential is channeled into plasmid-directed macromolecular synthesis, less of its is available for cell growth. Overexpression of cloned proteins is often fatal to the host cell (4, 5). For maximizing the productivity of recombinant systems one needs to weight the relative importance of gene expression and growth of the host cell. Thus, it is important to control cloned-gene expression levels through manipulation of growth conditions.

Key words: Recombinant *E. coli*, optimization, fermentation
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Optimization of growth conditions as a function of time in fermentation processes requires the use of the maximum principle, first stated by Pontryagin *et al.* (6) and singular control theory (7). Most efforts on optimization of fermentations have focussed on non-recombinant cell fermentations. Previous work in this area has been reviewed extensively (8, 9). Menawat *et al.* (10) used the technique of augmenting the performance index by addition of a quadratic term in the control vector to solve a singular control ploblem. Modak (11) solved the problem of determining the optimum feed rate profiles in a fed-batch operation for many biochemical systems of interest. Park and Ramirez (12) determined the optimal substrate feed-rate policy for maximization of a secreted protein in yeast.

Seressiotis and Bailey (13) determined optimal operation trajectories for maximum productivity of unstable recombinant organisms in batch and continuous flow reactors. They used a model based on molecular mechanisms to simulate cell growth and cloned product formation. Their results indicate the presence of an optimum copy number or an optimum transcription efficiency of the cloned gene for maximizing reactor productivity.

In this work, the problem of maximizing a weighted performance measure was solved for recombinant *Escherichia coli* strains by using the concentration of a growth inhibitor as a control variable. A simple unstructured mathematical model was used to explain the key features of this system. Using optimal control theory for this model, the maximization problem is reduced to numerical determination of optimal switching time between two control arcs.

Mathematical Model

It is necessary, in general, to take into account the effects of plasmid instability when modeling recombinant cell growth. However, in some recombinant systems plasmids are stably maintained even in non-selective media (3). The growth of stable recombinant cells in the presence of a growth inhibitor can be modeled by a single differential equation,

$$\frac{dX}{dt} = \mu(S, I)X \tag{1}$$

The yield of cell mass $Y_{X/S}$ is assumed to be constant. Hence,

$$\frac{dS}{dt} = -\frac{\mu(S, I)X}{Y_{X/S}} \tag{2}$$

The growth rate is assumed to follow the following functionality.

$$\mu(S, I) = \frac{\mu_{max}S}{K_s + S + I/K_I} \tag{3}$$

where I is the concentration of the inhibitor. Such functional dependence has previously been proposed by Seo and Bailey (3) for recombinant *E. coli* strains containing closely related copy number mutant plasmids. They used α-methylglucoside, a competitive inhibitor of glucose transport, to change the specific growth rate of the strains so that the effect of specific growth rate on plasmid copy number and cloned gene expression could be studied.

For describing the product formation kinetics an empirical form suggested by Leudeking and Piret (15) has been quite popular. In this form, the specific product formation rate is considered as the sum of growth-associated and growth-independent terms. A variation of this has been used here to account for the inverse relationship between specific growth rate and product formation rate.

$$\frac{dP}{dt} = (-\alpha\mu + \beta)X, \tag{4}$$

where α and β are positive constants. The negative coefficient of μ in the above expression assures this dependence. For the recombinant *E. coli* strains described above, Seo and Bailey (3) observed that with an increase in the specific growth rate, (1) the plasmid content decreases almost linearly, and (2) the specific activity of the cloned-gene product (β-lactamase) decreases. The product formation data obtained by them can be fit quite well using this dependence.

At this point, it is important to realize some limitations of this model. First, the product formation rate does not go to zero, when the substrate concentration is zero as one would expect intutively. But, because of unavailability of data at low growth rates it is difficult to guess the functional dependence at low substrate concentrations. The calculations described later were done within the limits imposed by the model. Second, the substrate consumption rate is assumed to be independent of the product formation rate.

Optimization Problem

For optimization purposes, the problem can be

restated in state formulation as

$$\dot{x}=f, \tag{5}$$

where

$$x=\begin{bmatrix} x_1 \\ x_2 \\ x_3 \end{bmatrix} \tag{6}$$

and

$$f=\begin{bmatrix} \mu x_1 \\ -\dfrac{\mu x_1}{Y_{X/S}} \\ (-\alpha\mu+\beta)x_1 \end{bmatrix} \tag{7}$$

The initial conditions for state variables are specified.

$$x(0)=x_0 \tag{8}$$

The aim is to maximize the performance index II defined by

$$\max_{0\le I(t)\le I_{max}} \Pi = x_{3f} - \frac{k_1 x_{1f}}{x_{3f}} \tag{9}$$

The first and the second terms in Eq. (9) signify the value of the product and separation cost, respectively. The separation cost is assumed to be inversely proportional to the intracellular product concentration (x_{3f}/x_{1f}). The concentration of the growth inhibitor is used as the control variable to manipulate specific growth rate. It is also assumed that the inhibitor is not metabolized (14) and that the addition of inhibitor does not change the reactor volume significantly. Assuming the final time t_f to be fixed, the Hamiltonian can be defined as

$$H(x,\lambda,I)=\lambda^T f=(z\mu+\lambda_3\beta)x_1, \tag{10}$$

where λ^T given in Eq. (11)

$$\lambda^T=[\lambda_1,\lambda_2,\lambda_3] \tag{11}$$

represents the adjoint variables, and

$$z=\lambda_1-\frac{\lambda_2}{Y_{X/S}}-\lambda_3\alpha \tag{12}$$

The adjoint differential equations can now be written.

$$\dot{\lambda}=-\frac{\partial H}{\partial x}=\begin{bmatrix} -z\mu-\lambda_3\beta \\ -z\mu_2 x_1 \\ 0 \end{bmatrix} \tag{13}$$

where

$$\mu_2=\frac{\partial\mu}{\partial x_2} \tag{14}$$

The transversality conditions are given by the following set of final conditions of the adjoint variables.

$$\lambda(t_f)=\frac{\partial\Pi}{\partial x(t_f)}=\begin{bmatrix} -\dfrac{k_1}{x_{3f}} \\ 0 \\ 1+\dfrac{k_1 x_{1f}}{x_{3f}^2} \end{bmatrix} \tag{15}$$

To maximize the performance index Pontryagin's maximum principle can now be used. Thus, for given 'x' and 'λ' values at an instant of time, the control 'I' should be picked so as to maximize the Hamiltonian within the constraints imposed on the control variable. The gradient of the Hamiltonian is,

$$\frac{\partial H}{\partial I}=\mu_I z x_1, \tag{16}$$

where

$$\mu_I=\frac{\partial\mu}{\partial I}=-\frac{\mu_{max}SK_I}{(K_sK_I+SK_I+I)^2}<0 \tag{17}$$

Clearly, the gradient is non-zero for non-zero substrate concentrations. Hence, the optimal control profile must lie on the boundary of the allowed region for the control variable, unless 'z' becomes zero for a finite interval of time. The optimal control sequence I* is determined by the sign of the gradient of the Hamiltonian.

$$\text{If } z>0, \text{ then } \frac{\partial H}{\partial I}<0, \text{ and } I^*=0$$

$$\text{If } z<0, \text{ then } \frac{\partial H}{\partial I}>0, \text{ and } I^*=I_{max}$$

To get more information about the control profile, the time derivative z is considered.

$$\dot{z}=\frac{dz}{dt}=(-\mu+\frac{\mu_2 x_1}{Y_{X/S}})z-\lambda_3\beta \tag{18}$$

This is a linear differential equation in z, with final conditions given by,

$$z(t_f)=-\frac{k_1}{x_{3f}}-\alpha-\frac{\alpha k_1 x_{1f}}{x_{3f}^2}<0 \tag{19}$$

To check for the possibility of sign change of 'z', setting 'z' to zero in Eq. (18) we get

$$\dot{z}=-\lambda_3\beta \tag{20}$$

Since 'λ_3' is a positive constant (by Eqs. (13) and (15)), 'z' is negative at the point of switching . It follows

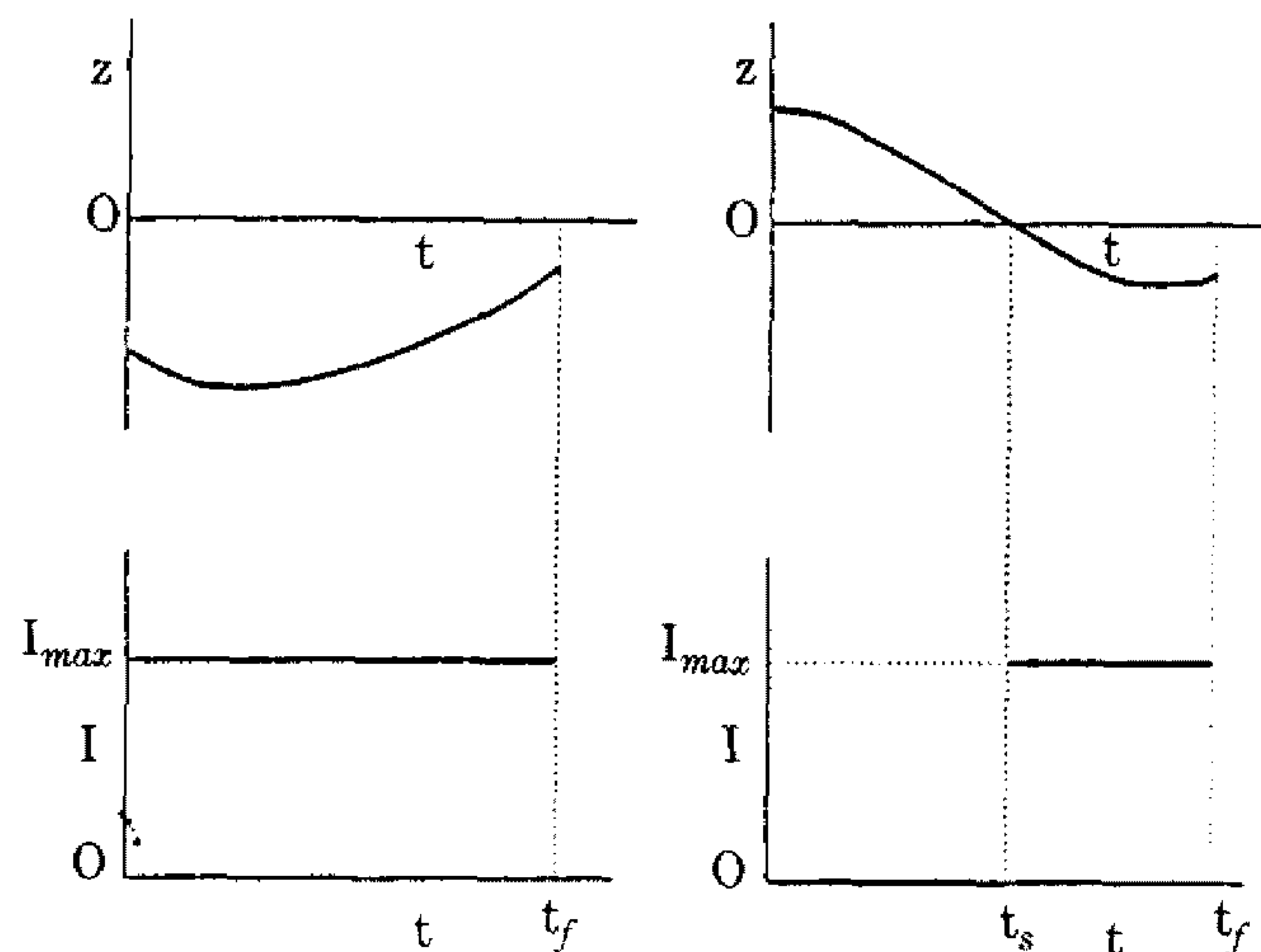

Fig. 1. Two possible optimal inhibitor concentration profiles corresponding to two different 'z' profiles.

that 'z' cannot be zero over a finite interval of time. Also, there can be at the most one switch in the sign of 'z'. Thus there are only two possibilities for the optimal inhibitor concentration profile: (1) a switch from initial zero value to a constant maximum value, and (2) a constant maximum value (Fig. 1). It is difficult to get an explicit analytical expression for the optimum switching time, which needs to be determined numerically.

Numerical Results

It was shown from theoretical arguments that the only control variable profiles that are candidates for being optimum are those which have a single switch from zero to the maximum allowed value. Calculations were performed, using the IMSL integration routine IVPAG, to determine the optimal switching time. Two cases corresponding to different α and β values were considered. The cases thus differ in the sensitively of specific product formation rate to specific growth rate. The parameters used for simulations are summarized in Table 1.

Case (A) $\alpha = 460$, $\beta = 250$

Since the value of β (representing the growth-independent part of specific product formation rate) is smaller than that of α, product formation rate is somewhat sensitive to changes in specific growth rate. Hence, the optimal operating strategy should result in a significant improvement over a simple batch fermentation.

In Fig. 2, the profiles of cell mass and product for

Table 1. Parameter values and constants used for computer simulation

Kinetic parameters*		Simulation parameters	
μ_{max}	0.38	x_0	0.01
K_s	0.0119	s_0	2.0
K_I	0.00911	p_0	0.0
$Y_{X/S}$	0.32	I_{max}	1.0-12.0
		t_f	11.0

*Kinetic parameters listed here are adapted from the data of Seo and Bailey (3).

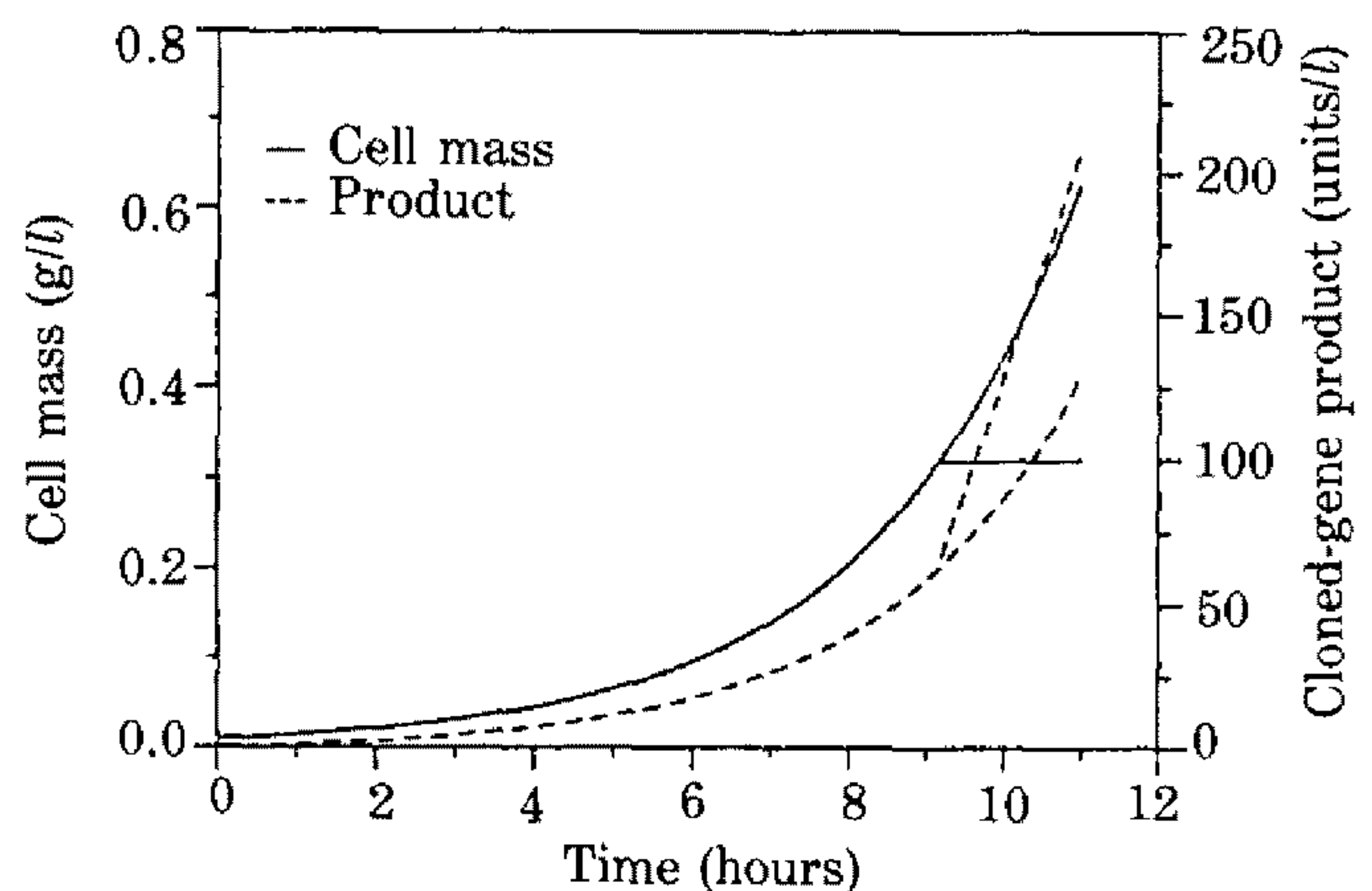

Fig. 2. Comparison of cell mass and product concentration profiles.
Profiles resulting from the use of optimal inhibitor concentration profile for maximum final product ($k_1 = 0$) are compared with those for simple batch fermentation. The profiles are identical till the switching time of 9.16 hours. After that the discontinuous curves corresponding to the optimal case.
$I_{max} = 1$ g/l, $\alpha = 460$, $\beta = 250$.

the operating strategy which gives maximum final product are compared with those for a simple batch fermentation without the addition of the growth inhibitor. The final product obtained using the optimal inhibitor feeding policy is about 60% greater. Moreover, the amount of cell mass at the end of fermentation is 49% less. The effect of changing the maximum inhibitor concentration was also investigated. A change in the maximum inhibitor concentration from 1 g/l to 12 g/l results in less than 1% change in the optimum switching time. The reason for this insensitivity is that even at a concentration of 1 g/l the inhibitor decreases the specific growth rate quite drastically, thus increasing the specific product formation rate. Further increase in the inhibitor concen-

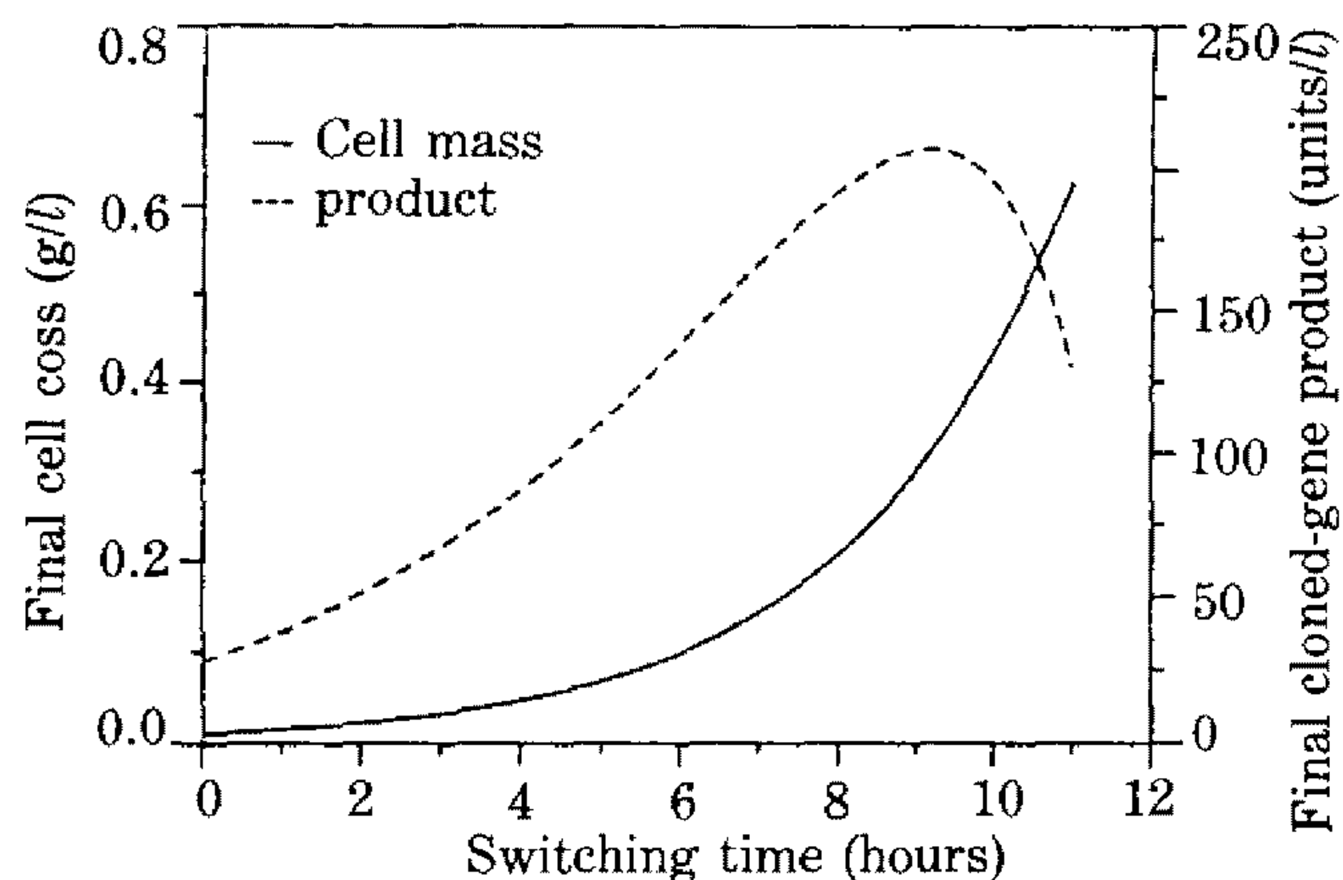

Fig. 3. Final cell mass and product concentration as a function of switching time.
I_{max} = 1 g/l, α = 460, β = 250.

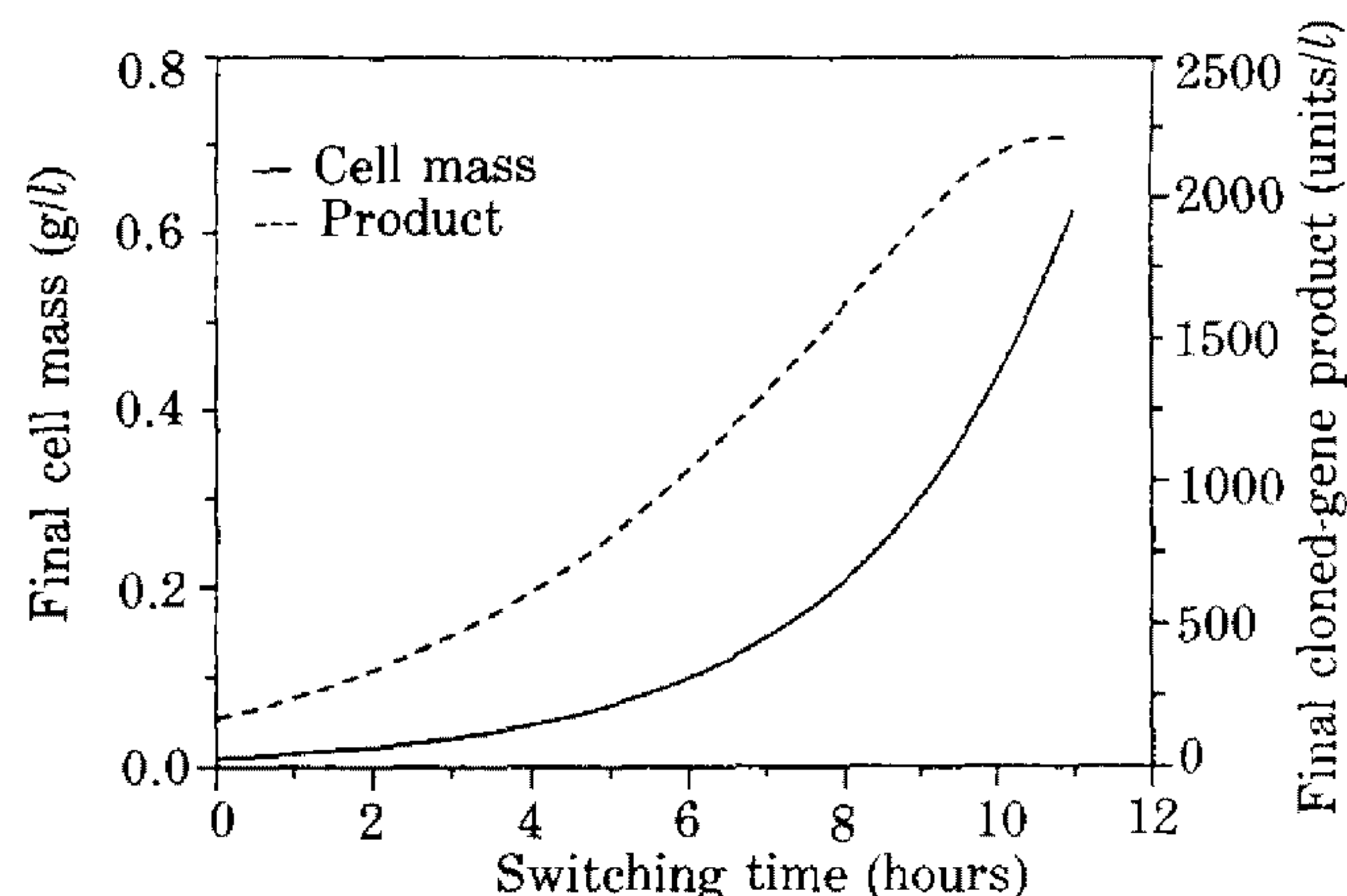

Fig. 4. Final cell mass and product concentration as a function of switching time.
I_{max} = 1 g/l, α = 460, β = 1,500.

Table 2. Effect of operating cost and maximum inhibitor concentration on optimal switching time for growth-sensitive strain.

k_1	Optimal switching time (h) I_{max} = 1 g/l	Optimal switching time (h) I_{max} = 12 g/l
0	9.16	9.16
1000	9.14	9.14
5000	9.07	9.07

tration does not cause a significant increase in the product formation rate.

The concentrations of cell mass and product at the final time as a function of the switching time are shown in Fig. 3. The product concentration profile shows a maximum at a switching time of 9.16 hours. The cell mass concentration, however, incereases monotonically with an increase in switching time. If the aim is to maximize the cell mass, it is intuitively evident, and can be proved theoretically using arguments similar to those described in the prevcious section, that the best performance is achieved when the inhibitor is not added at all. If the separation cost is higher, it is expected that the optimal switching time should be lower, yielding a shorther cell growth phase. Calculations were performed for different k_1 values, which reflect differing importance of separation cost. The optimum switching time does not change significantly for small k_1 values, but starts decreasing as k_1 is increased further (Table 2).

Case (B) $\alpha = 460$, $\beta = 1500$

This corresponds to a relatively low sensitivity of product formation rate to specific growth rate. In fact, this is the case for recombinant *E. coli* strain with the plasmid pDM247. Calculations show that the optimal operating strategy results in very little improvement compared to a simple batch fermentation, as expected. However, the cell concentrations do change significantly (Fig. 4). When separation costs are sufficiently higher compared to the product value, use of the optimal control profile is still necessary.

Discussion

Pontryagin's maximum principle was used to determine optimal profiles of growth conditions for batch cultures of recombinant *E. coli*. Theoretical analysis indicates that the optimal operating strategy consists of an initial high growth rate stage followed by a low growth but high product formation rate stage. Simple numerical calculations were performed to determine the exact optimal switching time and to confirm the theoretical argument. When product formation rate is sensitive to specific growth rate, the optimal operation policy yields more than five-fold increase in the final product concentration compared with a simple batch fermentation. For the case of relatively low sensitivity of product formation rate to specific growth rate, there is little improvement in the process performance.

This work employed a growth inhibitor to separate the cell growth phase from the cloned gene expression phase. Uncoupling of these two phases can also be achieved by using regulated promoters, which allow control of product levels by manipulation of environmental parameters such as medium composition

or temperature. Previous studies (16, 17) indicate that even in these systems an initial growth stage followed by product formation stage should be optimal. The optimization method developed in this work would be applied to recombinant systems containing a cloned regulated promoter if model equations describing cell growth and product formation in terms of a control parameter are available.

For the system described herein, the specific product formation rate is a linear decreasing function of specific growth rate, resulting in a bang-bang type of control. For some recombinant systems, however, the specific product formation rate shows a maximum at a certain specific growth rate (18). For these systems intermediate (off-boundary) control arcs will, in general, be part of the complete optimal control trajectory.

요 약

유전자 조작된 세포 발효공정의 생산수율을 최대화하기 위하여 세포의 성장속도와 제품 생성속도간의 상반관계를 고려하여야 한다. 유전자 조작된 *E. coli* 발효에 있어, 최적화 이론을 적용하여 두 속도의 가중치를 결정함으로써 생산수율의 최대화를 꾀하였다. 성장저해제의 농도는 비성장속도를 조절하고 결국 융합된 유전자의 발현속도를 조절하는 변수로 사용된다. 이런 system의 특성을 위하여 간단한 unstructured model을 사용하였다.

이론적 해석에 의하면, 최적조절곡선은 초기의 높은 세포성장속도에 이어서 낮은 성장속도와 더불어 높은 물질생성속도를 보이는 두 단계로 구성된다. 물질생성속도가 세포성장속도에 대해 서로 다른 의존도를 보이는 대표적인 두 가지 경우에 대해, 수식계산을 통해 세포성장에서 물질생성으로 전환시키는 시기를 결정하였다. 물질생성속도가 세포성장속도에 민감한 경우 최적화 조작을 시행했을 때 단순 회분식 발효에 비해 최종생산물 농도가 약 60% 증가함을 알 수 있었다.

Acknowledgement

This work was supported by the National Science Foundation.

Notation

H : Hamiltonian
I : concentration of the growth inhibitor (g/l)
I_{max} : maximum allowed inhibitor concentration (g/l)
K_s : constant (g/l)
K_I : inhibition constant (g inhibitor/g substrate)
k_1 : a weighing factor for operating cost in the performance index
P : concentration of cloned-gene product (units/l)
S : concentration of substrate (g/l)
t : time (h)
t_f : final time (h)
x : vector of state variables
X : cell mass concentration (g cell dry weight/l)
$\mathbf{x}_0$: vector of initial concentrations for state variables
x_1 : cell mass concentration (g cell dry weight/l)
x_2 : substrate concentration (g/l)
x_3 : cloned-gene product concentration (g/l)
x_{if} : value of state x_i at final time t_f
$Y_{X/S}$: yield of cell mass (g cell dry weight/g substrate)
z : a variable defined to simplify analysis
α : constant (units/g cell dry weight)
β : constant (units (g cell dry weight)$^{-1}$ h^{-1})
λ : vector of adjoint variables
μ : specific growth rate (/h)
μ_{max} : maximum specific growth rate (/h)
Π : performance index

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(Received May 31, 1990)