Performance of Recombinant Fermentation and Evaluation of Gene Expression Efficiency for Gene Product in Two-Stage Continuous Culture System

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In order to develop a general methodology for evaluation of the gene expression efficiency for gene product, theoretical and experimental studies were undertaken using a recombinant *Escherichia coli* K12ΔH1Δ*trp*/pPLc23*trp*A1 as a "gene–host cell" model system in a two-stage continuous-culture system. For this, a genetically structured kinetic model proposed earlier for biosynthesis of gene product in batch cultivation was extended to the two-stage continuous-culture system. A partial list of key parameters of the model includes the rate of plasmid segregation, specific growth rate of recombinant cell, plasmid content, rates of transcription and translation, and other parameters related to product biosynthesis. The dynamics of heterogeneous cell population containing plasmid-harboring and plasmid-free cells were also studied. Theoretical analysis of cell population dynamics shows that the recombinant cells could be maintained stably for a prolonged time in a two-stage continuous-culture system. Fermentation performance of the recombinant *E. Coli* cells in a two-stage continuous bioreactor system was examined experimentally, and the gene expression efficiency of a cloned gene product was determined based on the genetically structured kinetic model proposed. Based on our experimental results, the gene expression efficiency of the model gene–host cell system was found to be about twofold more efficient (i.e., 41.8 mg TrpA protein/mg plasmid DNA) as compared to the average rate of protein biosynthesis by *E. coli* cells. The performance of two-stage recombinant fermentation was also simulated using a mathematical model developed. General trends obtained from the model simulation agree reasonably well with the currently available experimental data, although further refinements need to be made. The methodology illustrated in this article could be used for evaluation of the gene expression efficiency of other genetically engineered recombinants once such recombinants with certain gene–host cell systems are constructed.

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INTRODUCTION

The stability of certain recombinant microorganisms is one of the most important problems in scaling-up recombinant fermentation processes.[1–3] It has been reported that the presence of certain plasmids has a deleterious effect on host cell growth, especially in the case of high-expression plasmids.[4–6] Growth of recombinant cells in selective medium may allow the stable maintenance of the plasmid-carrying cell population by inhibiting the growth of plasmid-free cells, but very often the use of direct selection pressure is impractical in an industrial scale production.

Several modes of bioreactor operation have been proposed to maximize the concentration of cloned gene product and/or the productivity of a bioreactor system for the unstable recombinant cell cultures.[7–10] One strategy to deal with the recombinant cells that are unstable due to an increased productivity of cloned gene is to separate the growth stage from the production stage by controlling the levels of cloned gene expression using a genetic switch.[10] A two-stage fermentation system in combination with a temperature-sensitive gene-switching system offers the possibility of minimizing the instability problem of high-expression recombinants in continuous production of cloned gene product. The instability problem associated with the high expression of the cloned gene has been addressed in an earlier report.[10]

The use of a thermoinducible promoter operator enables us to separate the growth and production stages by simply adjusting the culture temperature. We can then grow the cells under the repressed state with very little or no expression of the cloned gene product in the first growth stage and thereby minimize the expression-related instability problem. When expression is desired, it can easily be turned on by a temperature shift in the second production stage. Thus, a two-stage system makes it very convenient

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to study the fermentation variables under both the repressed and derepressed conditions by operating the first stage at a low temperature and the second stage at a slightly higher temperature. Since the recombinant cells can be maintained stably even under high-expression conditions, as will be discussed in this article, the two-stage continuous bioreactor system also permits the study of the effects of fermentation parameters on gene expression of recombinant organisms under better controlled conditions than is possible in a single-stage continuous or batch culture systems.

Recently, models for product fermentation in recombinant systems have been formulated based on the gene regulation involved at the molecular level. In this work, the previously developed genetically structured model for product synthesis by the recombinant cells in a batch culture system was extended to describe the kinetics of cell growth, substrate consumption, and product formation in a two-stage continuous-culture system. Based on a proposed kinetic model, several important kinetic parameters related to gene expression and productivity of cloned gene product were determined from experimental data. In this work, our interest was focused on (1) development of a genetically structured mathematical model for product formation in a two-stage continuous bioreactor, (2) application of such a predictive kinetic model to the evaluation of performance of recombinant fermentation, (3) examination of the dynamics of heterogeneous cell populations in a two-stage continuous culture system, and (4) the development of a general methodology for evaluation of the gene expression efficiency for gene product of new recombinants constructed having certain gene–host cell systems.

THEORETICAL ANALYSIS

Kinetic Model

A kinetic model for product formation in unstable recombinant cells in batch and single-stage continuous cultures was discussed elsewhere.[11] In this work, the model was extended to describe the kinetics of cell growth including the population dynamics of heterogeneous cells with and without plasmids and product formation in a two-stage continuous bioreactor system.

Batch Culture

A brief summary of the model equations describing cell growth, substrate concentration, and product formation in a batch culture system is given in what follows.

Cell Growth. If θ denotes the relative segregation rate, namely, the ratio of the specific rate of plasmid-free cell generation by segregation to the specific growth rate of plasmid-harboring cells, the following equation can be derived from the growth of plasmid-harboring cells (X^+) and plasmid-free cells (X^-):

$$\frac{dX^+}{dt} = \mu^+X^+(1 - \theta) = \overline{\mu}^+X^+ \tag{1}$$

$$\frac{dX^-}{dt} = \mu^+X^+\theta + \mu^-X^- \tag{2}$$

where

$$\mu^+ = \frac{\mu_m^+ S}{K_s^+ + S} \quad \text{and} \quad \mu^- = \frac{\mu_m^- S}{K_s^- + S} \tag{3}$$

In equation (1), $\overline{\mu}^+$ represents the overall specific growth rate of the plasmid-harboring cell populations, defined as

$$\overline{\mu}^+ = \frac{1}{X^+}\frac{dX^+}{dt} = \mu^+(1 - \theta) \tag{4}$$

Substrate Consumption. The rate of substrate consumption can be described as

$$\frac{dS}{dt} = -\left(\frac{1}{Y_x^+}\frac{dX^+}{dt} + \frac{1}{Y_x^-}\frac{dX^-}{dt}\right) - \frac{1}{Y_p}\frac{dp}{dt} - (m^+X^+ + m^-X^-) \tag{5}$$

where Y_x, Y_p, and m denote the cell yield coefficient, product yield coefficient, and maintenance coefficient, respectively. If the yield coefficient and maintenance coefficient are not significantly different for each cell type, i.e., $Y_x^+ \approx Y_x^- = Y_x$ and $m^+ \approx m^- = m$, equation (5) becomes

$$\frac{dS}{dt} = -\frac{1}{Y_x}(\mu^+X^+ + \mu^-X^-) - \frac{1}{Y_p}\frac{dp}{dt} - m(X^+ + X^-) \tag{6}$$

Product Formation. The rate of product formation is described based on molecular mechanisms involved. The *intracellular* concentration of the cloned gene product, $\hat{p}$, is given by

$$\frac{d\hat{p}}{dt} = \varepsilon f(\mu)\hat{G}_p - k_{-p}\hat{P} - \mu\hat{p} \tag{7}$$

where ε, $\hat{G}_p$, and k_{-p} denote the gene expression efficiency, intracellular concentration of plasmid DNA, and decay constant of protein, respectively. The function $f(\mu)$ is a nonlinear function of specific growth rate and can be approximated by the linear function

$$f(\mu) \cong k_0(\mu + b) \tag{8}$$

where k_0 and b are constants. [A more complete form of $f(\mu)$ for batch culture is given in Ref. 11.]

In order to assess the bioreactor performance, it is important to convert the intracellular product concentration to product concentration in the culture volume. Since the product formation occurs only in plasmid-harboring cell populations, the required relationship is

$$p = \hat{p}X^+/\rho_b \tag{9}$$

where ρ_b is the density of the recombinant cell. If ρ_b is assumed to be constant, differentiating equation (8) and substituting equations (1), (6), and (7) gives

$$\frac{dp}{dt} = k_0\varepsilon G_p(\overline{\mu}^+ + b)X^+ - k_{-p}P \tag{10}$$

where G_p is equivalent to $\hat{G}_p/\rho_b$.

Two-Stage Continuous-Culture System

The batch culture kinetic equations were extended to describe the cell concentration, substrate concentration, and product formation in a two-stage continuous-culture system. The two-stage continuous cultivation system is schematically shown in Figure 1, and the mass balances for each stage can be written as follows: For the first-stage (growth stage),

$$\frac{dX_1^+}{dt} = -D_1X_1^+ + \mu_1^+X_1^+(1 - \theta_1) \tag{11}$$

$$\frac{dX_1^-}{dt} = -D_1X_1^- + \mu_1^+X_1^+\theta_1 + \mu_1^-X_1^- \tag{12}$$

$$\frac{dS_1}{dt} = D_1(S_0 - S_1) - \frac{1}{Y_{x1}}(\mu_1^+X_1^+ + \mu_1^-X_1^-) - m_1(X_1^+ + X_1^-) \tag{13}$$

and for the second stage (production stage),

$$\frac{dX_2^+}{dt} = -D_2X_2^+ + D_{12}X_1^+ + \mu_2^+X_2^+(1 - \theta_2) \tag{14}$$

$$\frac{dX_2^-}{dt} = -D_2X_2^- + D_{12}X_1^- + \mu_2^+X_2^+\theta_2 + \mu_2^-X_2^- \tag{15}$$

$$\frac{dS_2}{dt} = -D_2S_2 + D_{12}S_1 + D_{02}S_0 - \frac{1}{Y_{X2}}(\mu_2^+X_2^+ + \mu_2^-X_2^-) - m_2(X_2^+ + X_2^-) - \frac{1}{Y_p}\frac{dp}{dt} \tag{16}$$

$$\frac{dp}{dt} = -D_2P + k_0\varepsilon G_p(\overline{\mu}_2^+ + b)X_2^+ - k_{-p}p \tag{17}$$

where D and S_0 denote the dilution rate and inlet substrate concentration, respectively, and $\overline{\mu}_2^+ = \mu_2^+(1 - \theta_2)$. As in the case of a batch culture system, the specific growth rate at each stage, μ_j, may be written in the form of equation (3).

For the sake of simplicity, here the rate of product formation in the growth stage was assumed to be negligible. Experimental data show that the maximum specific TrpA1 enzyme activity at 40°C (production stage) was about 100 units/mg cell while that at 38°C (growth stage) was lower than 2 units/mg cell. In the experimental system used, the host cell *E. coli* K12ΔH1Δ*trp* contains the temperature-sensitive *cI* 857 repressor gene in the chromosome. This mutant repressor blocks transcription of the cloned gene from the P_L promoter carried on the plasmid pPLc23*trp*A1 at lower temperature (see Fig. 2 for the structure of the recombinant plasmid used in our experiments). Increasing the culture temperature above 38°C denatures the *cI* repressor and causes expression from the P_L promoter.[10]

Dynamics of Heterogeneous Cell Population

To determine the feasibility of using a two-stage continuous-culture system to minimize the destabilizing effects of high-expression recombinants, a kinetic model was further analyzed to examine the changes in plasmid-harboring cell populations in a two-stage bioreactor system. Although a number of previous investigators have analyzed models describing genetic instability,[1-3] the dynamic behavior of recombinant cell populations in a two-stage culture system has not yet been studied in detail.

Let X_T and μ^{app} denote the total cell concentration and the apparent specific growth rate, respectively. Then equations (18) and (19) can be derived from equations (11) and

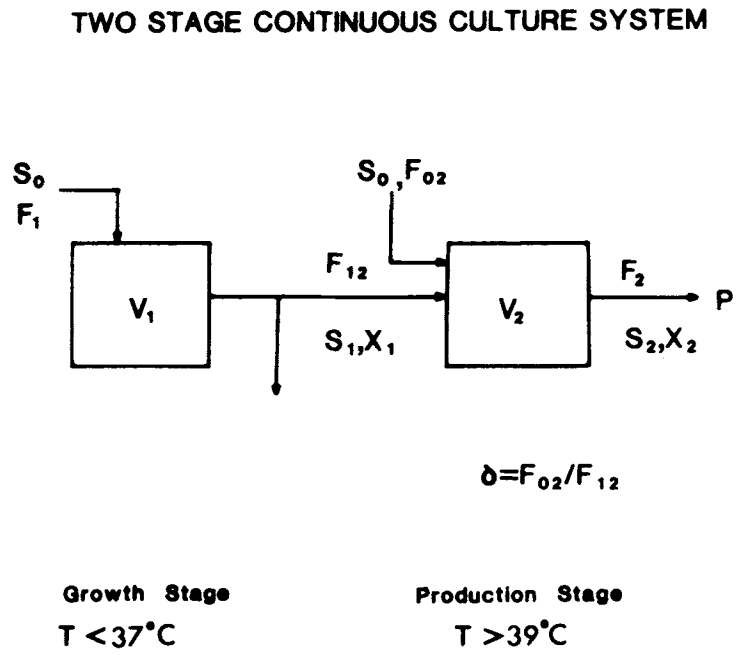


Figure 1. Schematic diagram of two-stage continuous-culture system employed in this study.

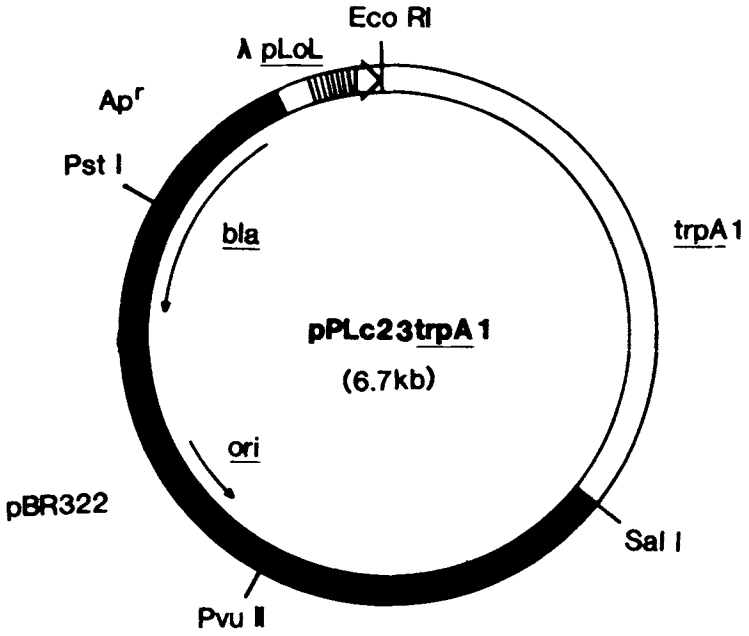


Figure 2. "Plasmid-host cell" model system used in this study.

(12) for the first stage and also from (14) and (15) for the second stage, respectively:

$$\frac{dX_{1T}}{dt} = (\mu_1^{app} - D_1)X_{1T} \tag{18}$$

$$\frac{dX_{2T}}{dt} = D_{12}X_{1T} + (\mu_2^{app} - D_2)X_{2T} \tag{19}$$

where

$$X_{1T} = X_1^+ + X_1^- \tag{20.1}$$

$$X_{2T} = X_2^+ + X_2^- \tag{20.2}$$

$$\mu_1^{app} = (\mu_1^+ X_1^+ + \mu_1^- X_1^-)/X_{1T} \tag{21.1}$$

$$\mu_2^{app} = (\mu_2^+ X_2^+ + \mu_2^- X_2^-)/X_{2T} \tag{21.2}$$

At the apparent steady-state, which is defined as

$$\frac{dX_{1T}}{dt} = \frac{dx_{2T}}{dt} = 0 \tag{22}$$

equations (18) and (19) become

$$\mu_1^{app} = D_1 \tag{23}$$

$$\mu_2^{app} = D_2 - D_{12}\frac{X_{1T}}{X_{2T}} = D_2\left(1 - \frac{1}{1+\delta}\frac{X_{1T}}{X_{2T}}\right) \tag{24}$$

In equation (24), δ denotes the ratio of the flow rate of fresh medium into the second stage to that of the fermentor broth coming from the first stage (see Fig. 1). From the material balance one can find that

$$D_{12} = D_2/(1+\delta) \quad \text{and} \quad D_{02} = D_2\delta/(1+\delta) \tag{25}$$

when the reactor volume of the first stage is identical to that of the second stage ($V_1 = V_2$), as is the case for our experimental system. It is of interest to find that equations (23) and (24) are identical to the steady-state solutions for the cultivation of *homogeneous* cell populations in a two-stage chemostat. Although the cell populations of each species (i.e., X^+ or X^-) can change as a function of cultivation time, the *total* cell concentration and the *apparent* specific growth rate can be kept constant if the system reaches apparent steady state.

Next, the changes in cell population of each species in a two-stage continuous-culture system will be examined. Defining the fraction of plasmid-harboring cells at each stage as

$$\Phi_j = \frac{X_j^+}{X_{jT}} \tag{26}$$

the changes in the fraction of the productive plasmid-harboring cells can be written as

$$\frac{d\Phi_j}{dt} = \frac{d}{dt}\frac{X_j^+}{X_{jT}} = \frac{1}{X_{jT}}\left(\frac{dX_j^+}{dt} - \frac{X_j^+}{X_{jT}}\frac{dX_{jT}}{dt}\right) \tag{27}$$

Substituting equations (11), (14), (18), and (19) in equation (27) gives the following differential equations: For the first stage,

$$\frac{d\Phi_1}{dt} = \Delta_1\Phi_1^2 - (\Delta_1 + \Theta_1)\Phi_1 \tag{28}$$

and for the second stage,

$$\frac{d\Phi_2}{dt} = \Delta_2\Phi_2^2 - (\Delta_2 + \Theta_2 + \Gamma)\Phi_2 + \Gamma\Phi_1 \tag{29}$$

where

$$\Delta_1 = (\mu_1^- - \mu_1^+) \qquad \Delta_2 = (\mu_2^- - \mu_2^+) \qquad \Theta_1 = \mu_1^+\theta_1$$
$$\Theta_2 = \mu_2^+\theta_2 \qquad \Gamma = D_{12}X_{1T}/X_{2T} \tag{30}$$

It may be assumed that Δ_j, Θ_j, and Γ are constants after the system reaches apparent steady state, as mentioned earlier. In equation (30), Δ_j and Θ_j represent the difference in specific growth rates between plasmid-harboring cells and plasmid-free cells and the rates of segregational plasmid loss, respectively. From equation (24), the expression for Γ in equation (30) can be rewritten as

$$\Gamma = D_{12}X_{1T}/X_{2T} = D_2 - \mu_2^{app} \tag{31}$$

and, hence, Γ represents the difference between dilution rate and apparent specific growth rate in the second stage.

The changes in the fraction of productive cells during cultivation can be predicted by solving equations (28) and (29) with appropriate initial conditions.

Assuming the initial condition in the first stage is

$$\Phi_1(0) = 1 \tag{32}$$

analytical solution of equation (28) becomes

$$\Phi_1(t) = \frac{\Delta_1 + \Theta_1}{\Delta_1 + \Theta_1 e^{(\Delta_1+\Theta_1)t}} \tag{33}$$

At infinite culture time the fraction of plasmid-harboring cells in the first stage, $\Phi_1(\infty)$, becomes either zero or a finite value depending upon the sign of the exponent in equation (33):

$$\text{Case I:} \quad \Delta_1 + \Theta_1 > 0, \qquad \Phi_1(\infty) = \lim_{t\to\infty} \Phi_1(t) = 0 \tag{34}$$

$$\text{Case II:} \quad \Delta_1 + \Theta_1 < 0, \qquad \Phi_1(\infty) = 1 + \frac{\Phi_1}{\Delta_1} = 1 - \theta_1 \cong 1.0 \tag{35}$$

Case I corresponds to the situation where there is no selective pressure. If the growth of plasmid-free cells in the presence of selective pressure is negligible (i.e., $\mu_1^- \approx 0$), then $\Delta_1 + \Theta_1 = -\mu_1^+(1 - \theta_1) < 0$, and consequently, this case corresponds to case II. Hence, the recombinant cells could be maintained stably in the first stage, at least in principle.

Assuming that Φ_1 is kept at a constant value of ϕ_1 and that the initial condition in the second stage is

$$\Phi_2(0) = \phi_1 \tag{36}$$

equation (29) becomes the so-called Riccatti equation. The solution of equation (29) can be obtained in the form

$$\Phi_2(t) = -\frac{1}{\Delta_2}\frac{\lambda_1 + c\lambda_2 e^{(\lambda_2-\lambda_1)t}}{1 + ce^{(\lambda_2-\lambda_1)t}} \tag{37}$$

where

$$c = -\frac{\lambda_1 + \Delta_2\phi_1}{\lambda_2 + \Delta_2\phi_1} \tag{38}$$

and λ_1 and λ_2 are the roots of the equation ($\lambda_2 < \lambda_1$)

$$\lambda^2 + (\Delta_2 + \Theta_2 + \Gamma)\lambda + \Delta_2\Gamma\phi_1 = 0 \tag{39}$$

Since $\lambda_2 - \lambda_1 < 0$, Θ_2 at infinite time becomes

$$\Phi_2(\infty) = \lim_{t\to\infty} \Phi_2(t) = -\frac{\lambda_1}{\Delta_2} \tag{40}$$

or

$$\Phi_2(\infty) = \frac{2\Gamma\phi_1}{(\Delta_2 + \Theta_2 + \Gamma) + \sqrt{(\Delta_2 + \Theta_2 + \Gamma)^2 - 4\Delta_2\Gamma\phi_1}} \tag{41}$$

From this equation it can be seen that the fraction of plasmid-harboring cells in the second stage (Φ_2) will not be zero *even without selection pressure* as long as the first stage continuously supplies the plasmid-harboring cells to the second stage (i.e., $\Gamma\phi_1 \neq 0$).

Introducing the dimensionless variables

$$\alpha_2 = \frac{\mu_2^+}{\mu_2^-} \qquad \theta_2 = \frac{\Theta_2}{\mu_2^+} \qquad \gamma = \frac{\Gamma}{\mu_2^-} \tag{42}$$

equation (41) can be rewritten as

$$\Phi_2(\infty) = \frac{2\gamma\phi_1}{(1 - \alpha_2 + \alpha_2\theta_2 + \gamma) + \sqrt{(1 - \alpha_2 + \alpha_2\theta_2 + \gamma)^2 - 4(1 - \alpha_2)\gamma\phi_1}} \tag{43}$$

When $\phi_1 = 1.0$, equation (43) can be simplified for the following special cases:

Case I, $\alpha_2 = 0$:

$$\Phi_2(\infty) = \begin{cases} \gamma & \text{when } \gamma < 1 \\ 1.0 & \text{when } \gamma \geq 1 \end{cases} \tag{44}$$

Case II, $\alpha_2 = 1$:

$$\Phi_2(\infty) = \frac{\gamma}{\theta_2 + \gamma} \cong 1.0 \tag{45}$$

Case III, $\gamma = 1 - \theta_2$:

$$\Phi_2(\infty) = \gamma = 1 - \theta_2 \cong 1.0 \tag{46}$$

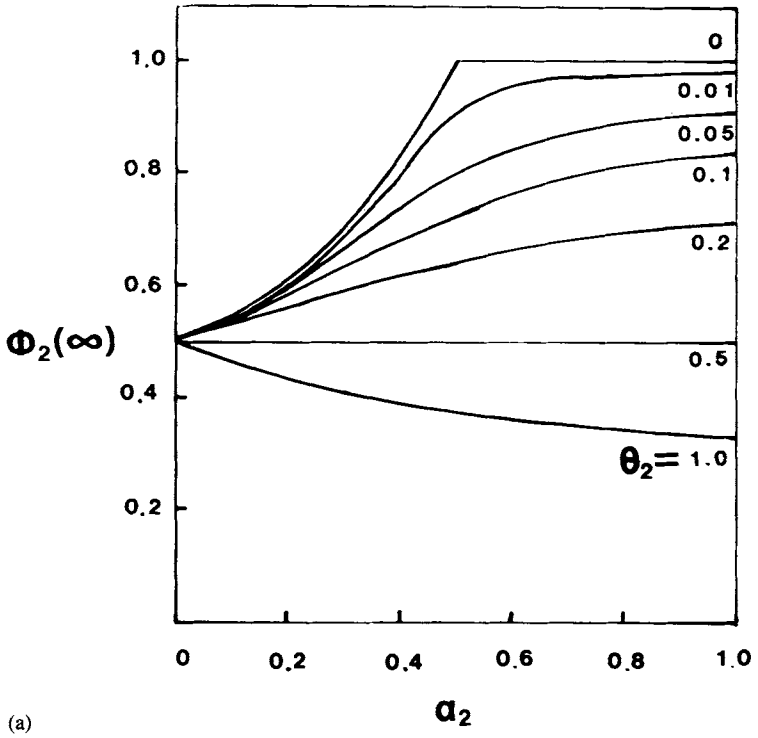
Case IV, $\theta_2 \ll (1 - \alpha_2 + \gamma)/\alpha_2$:

$$\Phi_2(\infty) \cong \begin{cases} \dfrac{\gamma}{1 - \alpha_2} & \text{when } \gamma < 1 - \alpha_2 \\ 1.0 & \text{when } \gamma \geq 1 - \alpha_2 \end{cases} \tag{47}$$

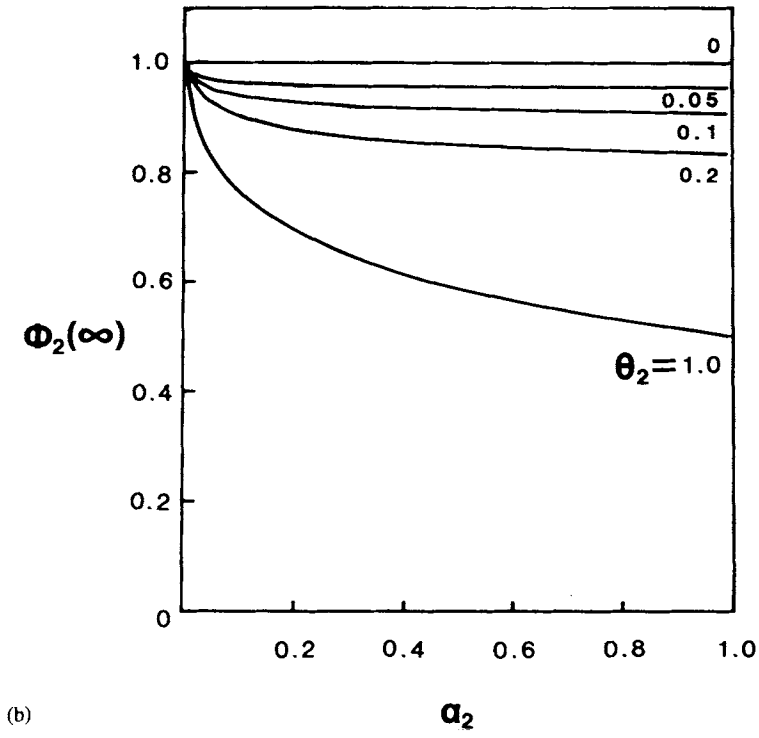
In Figure 3, the calculated values of $\Phi_2(\infty)$ at various α_2 and θ_2 are shown. The influence of γ on $\Phi_2(\infty)$ at various values of α_2 are also shown in Figure 4. From these analyses it can be found that the fraction of plasmid-harboring cell populations can be maintained at nearly 1 in a two-stage continuous-culture system by adjusting or controlling γ appropriately depending upon recombinant instability parameters such as α_2 and θ_2.

Determination of Gene Expression Efficiency

As shown in equation (17), the rate of product formation in the second stage is given by



(a)



(b)

Figure 3. Graphical solution of dynamics of heterogeneous population consisting of plasmid-harboring and plasmid-free cells at varying conditions of growth ratio (α_2) and segregation coefficient (θ_2): (a) $\gamma = 0.5$, $\phi_1 = 1.0$; (b) $\gamma = 1.0$, $\phi_1 = 1.0$.

$$\frac{dp}{dt} = -D_2 P + k_0 \varepsilon G_p (\bar{\mu}_2^+ + b) X_2^+ - k_{-p} P$$

Assuming $D_2 \gg k_{-p}$ (in most cases the rate constant of protein decay is smaller than 0.01 h^{-1}, and the lowest dilution rate of the second stage in our experiments was 0.40 h^{-1}), equation (17) can be rewritten as

$$\frac{dp}{dt} = -D_2 P + q_p X_2^+ \tag{48}$$

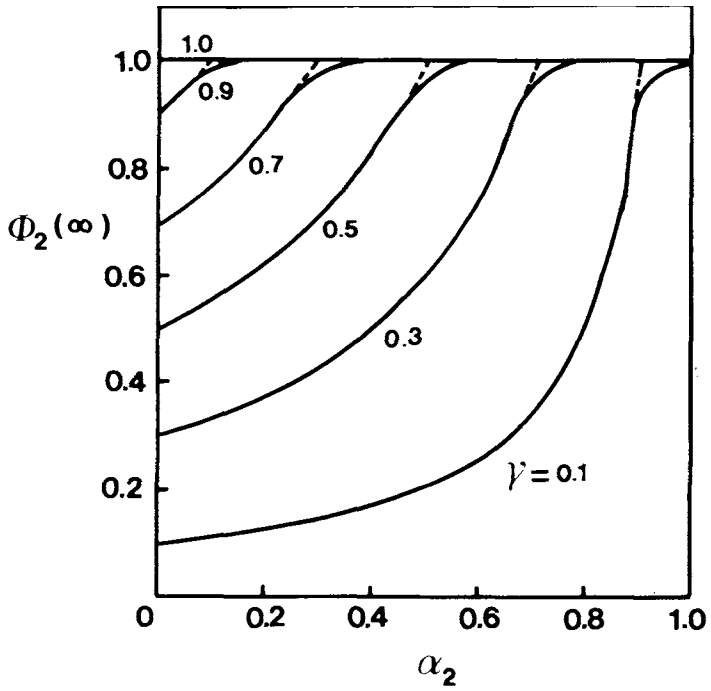


Figure 4. Effect of γ on $\Phi_2(\infty)$ at various conditions of α_2. Solid line, $\theta_2 = 1 \times 10^{-3}$; dotted line, $\theta_2 = 0$.

where

$$q_p = k_0 \varepsilon G_p (\bar{\mu}_2^+ + b) \tag{49}$$

At apparent steady state, the changes in the rates of product concentration and the total cell concentration in the second stage become zero. From equation (48) the specific production rate q_p after the system reaches an apparent steady state can now be expressed as

$$q_p = \frac{D_2 P}{X_2^+} = \frac{D_2 P}{\Phi_2 X_{2T}} \tag{50}$$

On the other hand, $\bar{\mu}_2^+$ in equation (49) is given by

$$\bar{\mu}_2^+ = \frac{1}{\Phi_2}[\mu_2^{app} - \bar{\mu_2}(1 - \Phi_2)](1 - \theta_2) \tag{51}$$

Since the fraction of plasmid-harboring cells can be maintained at a finite value near 1 ($\Phi_2 \approx 1.0$) in a two-stage continuous-culture system as discussed in the previous section, equations (50) and (51) are now simplified in the form

$$q_p \approx \frac{D_2 P}{X_{2T}} \tag{52}$$

$$\bar{\mu}_2^+ \approx \mu_2^{app} \tag{53}$$

In equation (53) we assumed that $(1 - \theta_2) \approx 1$, based on the fact that θ_2 values are usually much lower than 10^{-2}.[12,13] Equation (49) becomes

$$\frac{q_p}{G_p} = k_0 \varepsilon (\mu_2^{app} + b) \tag{54}$$

Accordingly, experimental data were analyzed using equations (54), (52), and (24). The results indicate that the plot of q_p/G_p vs. μ_2^{app} yields the straight-line relationship, and the slope and intercept correspond to $k_0\varepsilon$ and $k_0\varepsilon b$, respectively.

MATERIALS AND METHODS

The bacterial strain *E scherichia coli* M72 (Sm^R, *lac*Zam, Δ*bio-uvrB*, Δ*trp*EA2[λ*N*am7, *N*am53*cI*857, ΔH1]) was used as the host cell and a source of the temperature-sensitive *cI* 857 repressor. This strain, designated as K12ΔH1Δ*trp*, was used as a host cell for the recombinant plasmid pPLc23-*trp*A1 (see Fig. 2). Both host and plasmid were kindly shared by Remaut.[7]

Recombinant cells were grown on M-56 minimal medium supplemented with 10 μg/L biotin and 20 mg/L tryptophan (designated as M56) or an M56 enriched with 1g/L yeast extract (M56YE).

Continuous fermentation systems have been described previously, as were analytical procedures for determination of cell concentration, tryptophan synthetase activity (TrpA), plasmid loss indicated by ampicillin sensitivity of replicated colonies, specific growth rates, and other experimental protocols.[10]

RESULTS

Stability of Recombinant Cells in Two-Stage Continuous-Culture System

The experimental results obtained from both the single-stage and two-stage fermentation system are compared in Figure 5. For the single-stage continuous-culture system, the plasmid-harboring cell fraction decreases rapidly and

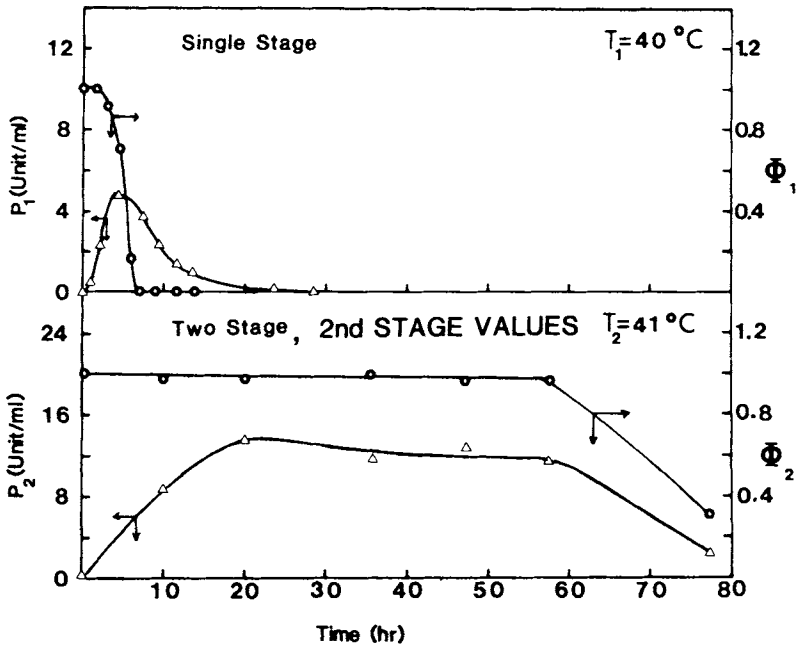


Figure 5. Profiles of product concentrations and plasmid-harboring cell fractions for single-stage continuous-culture system (upper) and second stage in two-stage continuous-culture system (lower).

disappeared completely within about 7 h cultivation time, and the product concentration goes through a maximum and decreases to practically zero within about 22 h (Fig. 5). For the two-stage continuous-culture system with the gene-switching system design, the fraction of productive cells (plasmid-harboring cells) (Φ_2) and the product concentration in the second stage were maintained at steady and high levels for a period of approximately 60 h or longer in several runs (Fig. 5). Due to stable maintenance of recombinant cells, the product concentration in the two-stage fermentation system was about 2.5 times higher than the maximum product level in a single-stage continuous-culture system. These results indicate that the unstable plasmid-harboring recombinants due to high expression of a cloned gene product could be stabilized, and hence, productivity could be improved considerably by the use of a two-stage continuous-culture system.

In a later culture period, however, decrease in the fraction of plasmid-harboring cells was observed, which is caused by the appearance of plasmid-free cells in the first stage. For a recombinant cell used in this study the plasmid-free cells appeared after 50–70 h cultivation time depending upon culture conditions such as dilution rate and medium composition.[10] To improve the reactor performance using a two-stage fermentation system, further efforts should be made for maintaining the recombinant cells stably in the growth stage. As one of such efforts, recently, a low-segregant strain was isolated and found to be more stable than the parent strain.[10]

Evaluation of Gene Expression Efficiency for Gene Product

The two-stage continuous-culture system makes it possible to study the effects of fermentation parameters on gene expression kinetics of recombinant microorganisms under well-controlled conditions, since the recombinant cells can be maintained stably for a prolonged period even under high-expression conditions. For determination of the kinetic parameters related to the product formation under the fully derepressed (or expressed) condition, the second-stage temperature was maintained at 41°C while the first stage was operated at 37°C.

Data from 23 runs of the two-stage continuous culture at different dilution rates of the first stage and of the second stage, D_1 and D_2 and two different media, M56YE and M56, are summarized in Table I. Total cell concentrations in the first and second stage, X_{1T} and X_{2T}, product concentrations in terms of TrpA activity, P, and the fraction of plasmid-harboring cells, Φ_2, are also listed in Table I.

Using equations (24) and (52), the apparent specific growth rate in the second stage (μ_2^{app}) and the specific product formation rate (q_p) can be calculated from the data given in Table I. In order to calculate q_p/G_p, information on the relationship between specific growth rate and plasmid content is required, as shown in equation (54). In Table II, the effect of specific growth rate on plasmid content (G_p) and the number of plasmid molecules per bacterial cell (N_p) that are obtained from the experiments are

Table I. Summary of experimental data.

D_1 (h^{-1})	D_2 (h^{-1})	δ	X_{1T} (g/L)	X_{2T} (g/L)	P (μ/mL)	Φ_2
0.62	0.42	3.77	0.368	0.240	19.1	1.00
(M56YE)	0.61	3.78	0.363	0.235	15.0	1.00
	0.79	4.81	0.348	0.213	8.39	0.99
	1.02	3.52	0.368	0.193	5.64	0.96
	1.20	3.89	0.363	0.185	5.09	1.00
	1.41	3.92	0.368	0.147	2.84	1.00
	1.60	3.83	0.362	0.168	3.74	0.98
	1.77	3.88	0.343	0.145	2.65	0.98
	2.00	3.85	0.367	0.143	2.47	1.00
0.12	0.40	14.0	0.510	0.662	29.8	1.00
(M56YE)	0.59	10.9	0.480	0.333	16.3	0.99
	0.81	9.22	0.453	0.330	13.6	0.97
	1.01	9.75	0.441	0.162	4.95	1.00
	1.41	10.2	0.466	0.098	2.55	0.98
	1.62	10.6	0.490	0.103	1.90	1.00
0.12	0.42	7.85	0.539	0.119	5.69	1.00
(M56)	0.60	5.81	0.490	0.123	5.06	1.00
	0.80	9.88	0.495	0.069	2.08	0.99
	1.03	6.60	0.613	0.115	2.94	1.00
	1.20	10.6	0.502	0.048	0.92	0.98
	1.40	10.6	0.490	0.048	1.08	1.00
	1.61	10.6	0.502	0.053	1.12	1.00
	1.76	10.6	0.510	0.046	1.31	1.00

Table II. Effect of specific growth rate on plasmid content.

μ (h^{-1})	G_p (mg plasmid DNA/ g bacteria)	N_p (molecules/ bacterium)
0.13	1.345	67
0.25	1.010	61
0.495	0.532	44
0.72	0.358	40
0.79	0.309	38
0.97	0.194	31
1.08	0.180	32

summarized. From the experimental data shown in Table II, the following empirical relationship between the specific growth rate and plasmid content was obtained:

$$G_p = 0.175/\mu + 0.103 \quad \text{(correlation coefficient 0.0968)} \tag{55}$$

Equation (55) was then used to calculate q_p/G_p at various specific growth rates in the second stage.

The calculated values of μ_2^{app}, q_p, and q_p/G_p for 23 experimental runs are summarized in Table III, and the calculated q_p/G_p values are plotted against μ_2^{app} in Figure 6. The experimental data agreed well with the theoretical model equation [eq. (54)], which was derived from the modeling analysis as an approximation. The plot of q_p/G_p vs. μ_2^{app} gave a straight-line relationship (correlation coefficient 0.973), showing the independence of these parameters to the first-stage dilution rate and medium composition. From Figure 6, two important parameters related to product formation kinetics, $k_0\varepsilon$ and b, were found, and they were 41.8 mg protein/mg DNA and 0.036 h^{-1}, respectively. Consequently, the specific rate of product formation, q_p, can be expressed by

$$q_p = 41.8G_p(\mu_2^{app} + 0.036) \tag{56}$$

The relatively smaller value of the constant b indicates that the product formation by recombinant pPLc23*trp*A1 DNA is highly dependent on cell growth, and the relative effect of b on q_p is only about 0.036 h^{-1} for a given value of μ_2^{app}.

Since we know the size of the TrpA protein (268 amino acids) and of pPLc23*trp*A1 (6.7 kb), we can calculate the molecular weights of the protein (30,000 daltons) and the plasmid DNA (4.1×10^6 daltons). The parameter $k_0\varepsilon$ for the recombinant cell can be expressed in terms of the number of protein molecules produced per plasmid molecule:

$$k_0\varepsilon = \frac{41.8 \text{ mg protein}/3.0 \times 10^4}{\text{mg plasmid DNA}/4.1 \times 10^6} = 5.7 \times 10^3 \text{ molecules TrpA protein/plasmid DNA}$$

Performance of Recombinant Fermentation in Two-Stage Continuous Culture

Using the kinetic model developed in this article and the kinetic parameters listed in Table IV, the changes in the fraction of plasmid-harboring cell (Φ), product concentration (P), and total cell concentration (x_T) with culture time were obtained from the numerical solution of equations (11)–(17). The model simulation results indicate that the fraction of productive cells in the second stage (Φ_2) is maintained at approximately 1 for a long period of operation and that the changes in X_{2T} and μ_2^{app} with respect to D_2 are basically identical to the case of classical two-stage fer-

Table III. Calculated values of μ_2^{app}, q_p, and q_p/G_p.

D_1 (h^{-1})	D_2 (h^{-1})	μ_2^{app} (h^{-1})	q_p mg protein/g cell h	q_p/G_p mg protein/mg DNA h
0.62	0.42	0.284	11.0	15.3
(M56YE)	0.61	0.411	13.2	25.0
	0.79	0.568	10.8	26.4
	1.02	0.591	10.1	25.4
	1.20	0.722	11.4	33.0
	1.41	0.695	9.3	26.3
	1.60	0.880	12.3	40.7
	1.77	0.909	11.4	38.5
	2.00	0.940	11.9	41.2
0.12	0.49	0.380	6.5	11.6
(M56YE)	0.59	0.518	10.1	23.0
	0.81	0.699	11.4	32.2
	1.01	0.750	10.6	31.4
	1.41	0.804	12.5	39.0
	1.62	0.854	10.2	33.1
0.12	0.42	0.202	6.9	7.1
(M56)	0.60	0.247	8.5	10.5
	0.80	0.238	8.4	10.0
	1.03	0.305	9.1	13.5
	1.20	0.105	8.0	4.5
	1.40	0.158	10.9	9.0
	1.61	0.295	11.7	16.8
	1.76	0.066	17.4	6.3

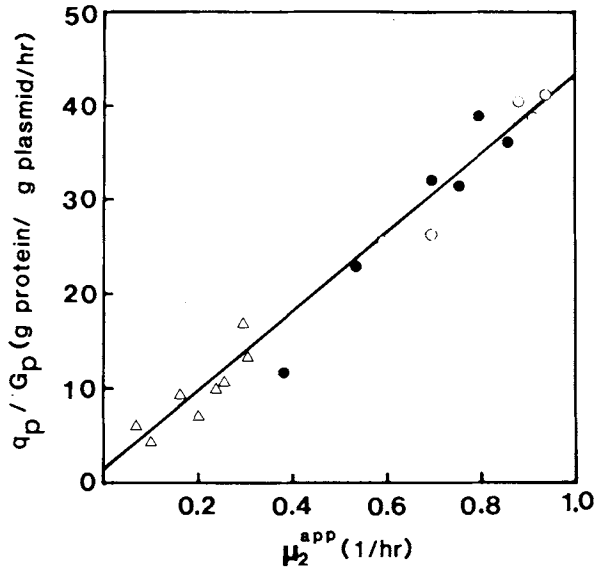


Figure 6. Empirical correlation between specific production rate per plasmid content (q_p/G_p) and apparent specific growth rate in second stage (μ_2^{app}) in two-stage continuous-culture system: (●) D_1 = 0.12 h^{-1} (M56YE); (○) D_1 = 0.62 h^{-1} (M56YE); (△) D_1 = 0.12 h^{-1} (M56).

Table IV. Model parameters used for simulation study.[a]

Parameters	Values	Unit
μ_{m1}^{-}	0.50	h^{-1}
μ_{m1}^{+}	0.45	h^{-1}
μ_{m2}^{-}	0.70	h^{-1}
μ_{m2}^{+}	0.63	h^{-1}
K_{s1}, K_{s2}	0.10	g/L
Y_{X1}, Y_{X2}	0.50	Dimensionless
θ_1	1×10^{-5}	Dimensionless
θ_2	1×10^{-2}	Dimensionless
$K_0\varepsilon$	40	g protein/mg plasmid
G_p	$0.20/\mu_2^{app} + 0.10$	mg plasmid/g cell
D_1	0.20	h^{-1}
S_0	10.0	g/L

[a] Other parameter values assumed to be negligible.

mentation (Fig. 7). Such model simulation results are qualitatively consistent with the experimental observations shown in Figure 8.

The results of model simulation studies also suggest that the productivity profile of a cloned gene product as a function of dilution rates shows a maximum, suggesting that there exists an optimal second-stage dilution rate that maximizes the productivity (D_2P) in the two-stage continuous-culture system (Fig. 7). Experimental data shown in Figure 8 supports this prediction.

Previously, the influence of growth rate on plasmid content and expression of a cloned gene product has been analyzed in detail using a mathematical model based upon the molecular mechanism of plasmid replication and known relationships between growth rate and gene expression activities.[15] The existence of an optimum growth rate that corresponds to the maximum productivity in the recombinant fermentation system is confirmed experimentally (Figs. 8 and 9).

In order to assess the relationship between the specific growth rate and the product protein content, the specific activity of enzyme protein TrpA1 (P/X_{2T}) was calculated from the experimental data listed in Table I and was plotted against the apparent specific growth rate in the second (production) stage, μ_2^{app}. The data shown in Figure 9 indicate that the calculated protein content of the recombinant cell exhibits a maximum when it is plotted against the growth rate, and this result supports the theoretical prediction. The simulation of the kinetic model agrees reasonably well with the experimental results. However, further studies need to be pursued since some experimental data are scattered, although a clear trend was observed.

DISCUSSION

In this article, the gene expression efficiency for a cloned gene product in a recombinant cell has been evaluated using a proposed kinetic model. The model used here for estimation of gene expression efficiency has been derived from a genetically structured model that provides a relationship between product formation kinetics and fundamental biological processes such as plasmid replication, transcription of a cloned gene, and synthesis of protein via translation process.[11,15] The experimental data from 23 runs agreed well with theoretically predicted relationships, as illustrated in Figure 6. The linear relationship between q_p/G_p and μ_2^{app} shown in Figure 6 is basically originated from the linear approximation of the function $f(\mu)$, which is a nonlinear function of specific growth rate.[11] Such a linear approximation leads to the final product formation expression, which is identical in form to the empirical correlation of the type given by the Leudeking–Piret equation, but the plasmid content or gene concentration is included in our model equation, and the coefficients shown in the Leudeking–Piret equation now have biological and physical meaning.

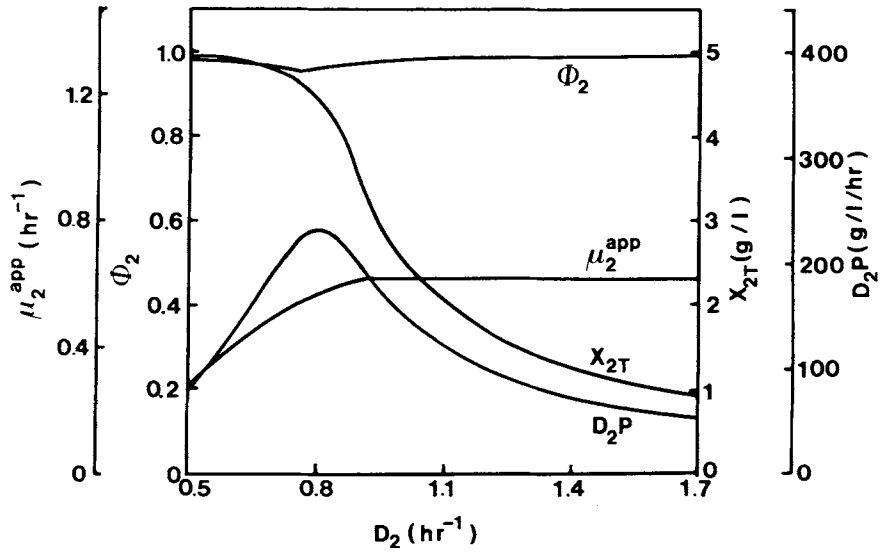


Figure 7. Model simulation results for performance of recombinant fermentation in two-stage continuous-culture system. Culture time 100 h.

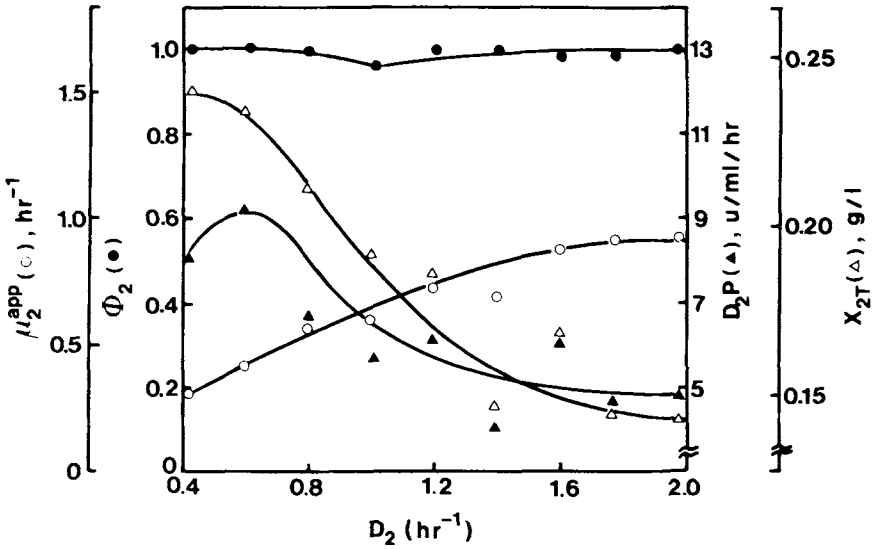


Figure 8. Experimental data for performance of recombinant fermentation in two-stage continuous-culture system (D_1 = 0.62 h^{-1}). (Results should be compared with computer simulation profiles shown in Fig. 7).

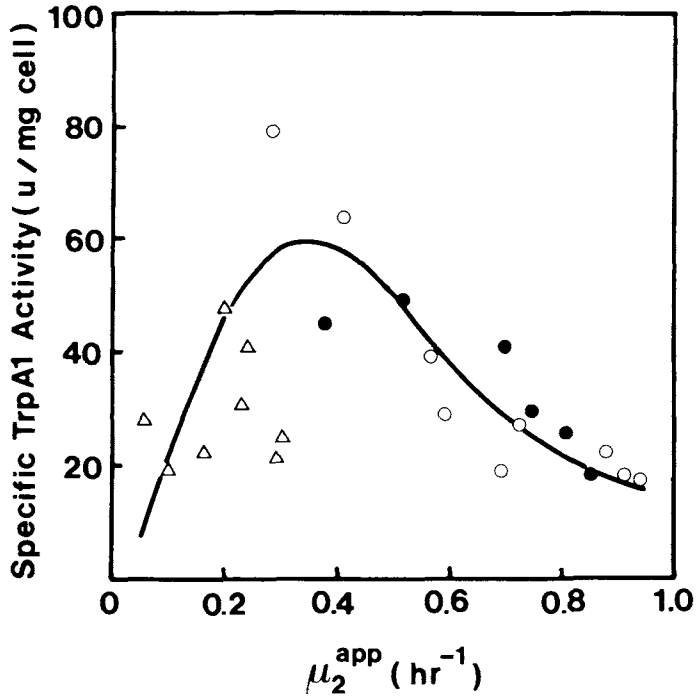


Figure 9. Effect of apparent specific growth rate in second stage (μ_2^{app}) on specific activity of TrpA1 protein product: (●) D_1 = 0.12 h^{-1} (M56YE); (○) D_1 = 0.62 h^{-1} (M56YE); (△) D_1 = 0.12 h^{-1} (M56).

However, several important points should be noted. Due to the differences in the growth rate effects on plasmid DNA and the chromosome, a simple Leudeking–Piret equation could not be applied to the recombinant cell system where the product is synthesized from the cloned gene in the plasmid. Most product formation kinetics studied so far are confined to the cases where the product is formed from the chromosomal gene expression. In such cases, the empirical Leudeking–Piret equation, in which the gene dosage concept is not incorporated, may be applied successfully because the chromosomal DNA content varies very little with the specific growth rate (see Ref. 14). On the other hand, for the recombinant cell system the gene dosage effect should be considered for analyzing the relationship between the growth rate and product formation since plasmids have quite different replication mechanism from chromo-

somal DNA. In the case of pPLc23*trp*A1, e.g., the plasmid content decreases significantly as the growth rate increases: When the growth rate is 1.08 h^{-1}, the plasmid content (mg plasmid DNA/g bacteria) is only about 13% of that observed at the specific growth rate of 0.13 h^{-1} (see Table II).

Previously, the effect of growth rate on plasmid content has been analyzed theoretically using a mathematical model based upon the molecular mechanism of plasmid replication,[15] and the model simulation seems to agree reasonably well with our experimental observation that the plasmid content decreases with increasing growth rate.[10] From the model simulation of the expression of a cloned gene product in combination with plasmid replication, it has also been proposed that we might be able to find an optimal growth rate that corresponds to the maximum intracellular product accumulation.[15] Experimental data shown in Figure 9 seem to agree with the theoretical prediction, although further experiments need to be pursued.

The use of a two-stage continuous-culture system with the gene-switching system was proven to be very useful in that it offers the possibility of minimizing the plasmid instability problem of high-expression recombinants. Since the recombinant cells can be maintained stably under the high-expression condition, the two-stage continuous bioreactor system of the type studied and evaluated in this work also enables us to study the product formation kinetics under well-controlled conditions without being impeded by the problems associated with the plasmid instability.

Based on the proposed kinetic model, the method for evaluation of the gene expression efficiency of recombinant cells using a two-stage continuous-culture system has been illustrated, and the cloned gene expression efficiency of the model recombinant cell *E. coli* K12ΔH1Δ*trp*-(pPLc23*trp*A1) was found to be 41.8 mg TrpA protein/mg plasmid DNA, which corresponds to about 5700 molecules TrpA protein/plasmid molecule under the derepressed condition.

It is usually found that for nonrecombinant cells the total protein content per genome is approximately constant and independent of growth rate. The literature data, which shows the ratio of total protein to chromosomal DNA on weight basis (P/D) as measured values under the condition of balanced growth at different growth rates in batch cultures of *E. coli* and *Saccharomyces typhimurium,* are summarized in Table V. Although the values of P/D are slightly different between strains and species, most of the data show a relatively constant P/D over a wide range of specific growth rate. Assuming that the value of P/D for the recombinant cell used in our experiments is equal to that for the *E. coli* K12 strain without the plasmid, it can be found that the gene efficiency of TrpA protein encoded in recombinant DNA is about 2.4-fold higher than the average gene expression efficiency of total protein encoded in chromosomal DNA.

When the gene expression efficiency of a cloned-gene product is compared based on P/D, however, the value could vary depending upon the size of the protein product and the recombinant DNA because P/D is determined on a weight basis. To avoid this problem, it is desirable to compare the relative gene expression efficiency in terms of the number of protein molecules per DNA molecule. In Table VI, the gene expression efficiency of the λP_L expression system of our model recombinant cell was compared with two very common gene expression systems, the *lac* and *trp* systems, on the basis of molar ratio. Table VI shows that the efficiency of the λP_L expression system is comparable to the *lac* or *trp* expression system, indicating that our estimation of gene expression efficiency is quite reasonable. We must point out that the overall gene expression efficiency is related to both transcription and translation efficiency.

Table V. Ratio of total cytoplasmic protein to chromosomal DNA for *E. coli* and *S. typhimurium*.

Microorganism	μ[a] (h^{-1})	Protein/DNA[b] (mg/mg)	Comments	Reference
E. coli K12	0.17–1.10	17.5 (1.4)	calculated from data points; data for other *E. coli* strains (B, ML30, ML308, and C600) also available	16
E. coli 15T⁻	0.30–1.87	21.6 (3.1)	calculated from data points	17
E. coli B/r	0.83–1.66	19.2	calculated from reported value, 4.1×10^8 amino acids/genome; $(4.1 \times 10^8) \times (110)/[(3.8 \times 10^6) \times (617)] =$ 19.2 mg protein/mg DNA; when $\mu < 0.83$ h^{-1}, protein–DNA ratio increases with μ.	18
S. typhimurium	0.14–1.66	21.3 (0.7)	calculated from DNA and protein contents measured at four different growth rates	19

[a] Experimental ranges where ratios of protein to DNA are approximately constant.
[b] Average value of experimental data, with standard deviation shown in parentheses.

Table VI. Comparison of gene expression efficiency for three different promoter systems under induced or derepressed condition.

Promoter	Gene product	Number of molecules per DNA molecule	Features related to gene expression	Reference
lac	β-galactosidase	~1500	wild-type *lac* operon; induced with IPTG	20
	λcro	~4000	promoter mutant (*lac P* UV5); cloned in pBR322 derivative	21
trp	anthranilate synthetase	4100	constitutive mutant (*trp R*$^-$); chromosomal *trp* operon	22
	tryptophan synthetase β	7700	constitutive mutant (*trp R*$^-$); chromosomal *trp* operon	23
λP_L	tryptophan synthetase α	5700	λ repressor mutant (*cI* 857); induced at 42°C; cloned in pBR322 derivative	this study

Thus, the efficiency of cloned gene expression can be dependent upon the structure of the ribosome binding site (RBS), distance between RBS and initiation codon, codon usage, strength of promoter, and others.[24] This implies that the gene expression efficiency can be altered even when the gene expression is controlled by the same promoter–operator system and that the gene expression efficiency shown in Table VI may not be directly proportional to the promoter strength.

Next, we will further discuss the reasons why the recombinant cells can be maintained stably even under the high-expression condition in a two-stage continuous-culture system.

Theoretical analyses presented in this article indicate that when the plasmid segregation parameter θ_2 is very small, the fraction of plasmid-harboring cell populations in the second stage can be maintained at or near 1 [$\Phi_2(\infty) = 1.0$]:

$$\gamma \geq 1 - \alpha_2 \tag{57}$$

[see eq. (47)]. Since the value of θ_2 is usually smaller than 10^{-2},[12,13] equation (57) may be applied to most recombinant cell systems. With the aid of equation (42), equation (57) can be rewritten as

$$D_2 - \mu_2^{app} \geq \mu_2^- - \mu_2^+ \tag{58}$$

or simply [see eqs. (30) and (31)] as

$$\Gamma \geq \Delta_2 \tag{59}$$

When $\Phi_2 \cong 1.0$, the apparent specific growth rate is equal to μ_2^+, i.e.,

$$\mu_2^{app} = \mu_2^-(1 - \Phi_2) + \Phi_2\mu_2^+ \cong \mu_2^+ \tag{60}$$

As a consequence, equation (58) may be further simplified:

$$D_2 \geq \mu_2^- \tag{61}$$

and combining equation (60) with equation (61) yields the following useful criteria for stabilizing the recombinant cells in the second stage:

$$\frac{D_2}{\mu_2^{app}} \geq \frac{1}{\alpha_2} \tag{62}$$

In equation (62), the parameters D_2, μ_2^{app}, and α_2 can be estimated from the experiments. The condition shown by equation (61) is a necessary condition for stable maintenance of recombinant cells in the second stage. Since there is no wash-out dilution rate for the second stage in a two-stage continuous culture, it is possible to adjust and control the second-stage dilution rate, which satisfies the condition given by equation (61).

The importance and implication of equation (61) are as follows. If the mean residence time (the reciprocal of the dilution rate) (see Ref. 25) of the recombinant cells in the fermentor is, on the average, less than the mean culture generation time (the reciprocal of the specific growth rate) of the plasmid-free cell populations, the plasmid-free cells may leave the fermentor before the plasmid-free cells divide and propagate. It is very important to distinguish between the culture generation time and the cell generation time (see Ref. 26). This indicates that the two-stage continuous-culture system gives another type of selective pressure against the growth and generation of plasmid-free cells if the condition given in equation (61) is satisfied. The specific growth rate of plasmid-free cells is usually higher than that of plasmid-harboring cells ($\mu^- \geq \mu^+$) in the presence of a cloned gene[12,13] and even in the absence of a cloned gene.[1-3] Consequently, plasmid-harboring cell populations may leave the fermentor even earlier than plasmid-free cells and thus plasmid-harboring cells cannot divide and generate the plasmid-free cells due to irregular segregation at cell division within the residence time at which the fermentor is operated.

As mentioned earlier, however, the stable maintenance of recombinant cells in the first stage (i.e., under the repressed condition) is a prerequisite for maintaining the stability in the production stage (i.e., under the expression condition). For stabilizing the recombinant cells, several methods such as insertion of the genes involved in the partitioning of plasmids (*par*, *cer*, etc.), introduction of internal and/or external selection pressure, and genetic modification of the host–cell genotype have been suggested (see Ref. 26 for a review). One of the easiest ways among several possible methods may be the selection of a

low-segregant strain. Previously, we reported that one strain designated as pPLc23*trp*A1 (1.s.) can be maintained stably for a much longer time period than the parent strain.[13]

Once the recombinant cells are maintained stably in the first stage, the cells can be maintained stably under the high-expression condition in the production stage by adjusting the second-stage dilution rate appropriately depending upon the parameter values μ_2^{app} and α_2 in such a way that equation (62) is satisfied. Thus, if the stable condition in a two-stage continuous-culture system is to be maintained, careful estimation of parameters such as α_2 and θ_2 is required.

Finally, the performance of recombinant fermentation in a two-stage continuous-culture system has been examined from model simulation of the proposed kinetic model. Although we did not try to fit the calculated values to experimental data, the qualitative trends obtained from model simulation agree reasonably well with the experiments (Figs. 7 and 8). Further refinement of the model simulation and comparative experimental study should be carried out after the model parameters are carefully determined from the separate experiments.

In summary, the advantage of employing the two-stage continuous-culture system with the gene-switching system design is that it allows a high expression of the cloned gene for a prolonged period of culture time despite the instability caused by the high-expression gene system, and this technique as a strategy for recombinant fermentation process analysis and development was demonstrated at least in principle in this study. Further studies may need to be pursued for the practical application of the concepts and principles illustrated in the future development of recombinant fermentation technology.

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NOMENCLATURE

b	parameter defined in eq. (8)
c	constant defined in eq. (38)
D	dilution rate
F	feed rate
$\hat{G}_p$	intracellular plasmid concentrations (mg plasmid/mL cell volume)
G_p	plasmid content (mg plasmid/g cell)
k_0	constant defined in eq. (8)
k_{-p}	decay constant of protein product (tryptophan synthetase)
K_s	Monod constant
m	maintenance coefficient
N_p	number of plasmid molecules per cell
P	concentration of protein product
$\hat{p}$	intracellular protein concentration
q_p	specific product formation rate
s	concentration of limiting nutrient
T	culture temperature
t	culture time
V	fermentor volume
X	concentration of cell
x_T	total cell concentration ($= x^+ + x^-$)
Y_x	cell yield coefficient
Y_p	product yield coefficient

Greek symbols

α	ratio of specific growth rate of plasmid-harboring cells to that of plasmid-free cells ($= \mu^+/\mu^-$), or growth ratio
γ	dimensionless parameter defined as γ/μ_2^-
Γ	difference between dilution rate and apparent specific growth rate in second stage ($= D_2 - \mu_2^{app}$)
δ	ratio of flow rate of fresh medium into second stage to that of fermentor broth coming from first stage
Δ	difference between specific growth rate of plasmid-free cells and that of plasmid-harboring cells ($= \mu^- - \mu^+$)
ε	gene expression efficiency
θ	ratio of specific rate of generation of plasmid-free cell to specific growth rate of plasmid-harboring cell ($= \Theta/\mu^+$), or relative segregation rate
Θ	specific rate of generation of plasmid-free cells ($= \mu^+\theta$), or plasmid loss rate
μ	specific growth rate
μ^{app}	apparent specific growth rate
$\bar{\mu}$	overall specific growth rate, $=\mu(1 - \theta)$
μ_m	maximum specific growth rate
ρ_b	cell density
Φ	fraction of plasmid-harboring cells ($= x^+/x_T$)
ϕ	particular value of Φ

Subscripts

j	culture stage (1, first stage, or 2, second stage)
0	inlet feed concentration
T	total value of cell population

Superscripts

+	plasmid-harboring cells
−	plasmid-free cells
app	apparent value

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