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MATHEMATICAL MODELING AND OPTIMIZATION OF PLASMID-HARBORING AND CHROMOSOME-INTEGRATED RECOMBINANT YEAST CULTURE PROCESSES

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Abstract—For the enhanced secretion of foreign proteins, the mathematical modeling for overall recombinant yeast culture considering protein secretion dynamics is important because we can understand and predict the behavior of biosynthesis and secretion of foreign protein and can develop control strategies for feeding with these model equations. In this research, the mathematical modeling and simulation considering protein secretion dynamics for recombinant yeast cultures that contain multicopy plasmids or chromosomally integrated genes were performed for optimization of foreign glucoamylase production. The optimal feeding policy for maximizing glucoamylase production was suggested in fed-batch culture. By introducing this optimal feeding policy, final glucoamylase activity and productivity of fed-batch culture were significantly increased compared with those of batch culture in both recombinant yeasts.

Key words: Plasmid-Harboring Recombinant Yeast, Chromosome-Integrated Recombinant Yeast, Glucoamylase, Mathematical Modeling, Optimization

INTRODUCTION

Recombinant DNA technology enables us to identify, clone, and transform the desired genes and to have the host cell express the genes very efficiently. On the other hands, bioprocess technology, if and when properly developed and optimized, enables us to produce gene products economically by making good use of recombinant fermentation, purification, and bioseparation technologies. Although many had underestimated the importance of bioprocess technology, during the early period of biotechnological development, it is now widely recognized and many workers are now focusing on these problems, namely, scale-up problems related to recombinant fermentation and bioseparation of gene products.

Yeast *Saccharomyces cerevisiae* is often used as a host for the expression of foreign genes and secretion of proteins because of a number of advantages [Kingsman et al., 1987]. Glucoamylase (EC 3.2.1.3) which is used to saccharify starchy feed stocks in commercial processes for glucose and ethanol productions, is not produced by *S. cerevisiae*. Having this in mind, the *STA* gene (glucoamylase gene of *Saccharomyces diastaticus*) was chosen as a glucoamylase gene source for this research [Yamashita et al., 1985]. The *STA* gene that was used in this work was cloned from a wild type *S. diastaticus* DS101. Since the restriction maps of three *STA* genes were known to be identical, it could not be determined whether the *STA* gene in this study was *STA1* or *STA2* or *STA3*. In this research, the *SUC2* promoter that is regulated by glucose in the culture broth was used as a promoter [Chu and Maley, 1980; Carlson and Botstein, 1982]. The *STA* signal sequence is a sequence of yeast *S. diastaticus* glucoamylase that is secreted into the culture broth and was used in this work for glucoamylase secretion.

Mathematical models can represent the behavior of a process on a simplified level and adequately for a given purpose. The principal aims of mathematical models are as follows. First, models are built to allow prediction of the conversion or production of any system. Second, models are built to examine the nature and behavior of a plant's operation under a variety of operation conditions and to examine the regions where the model is valid, including how far from this region extrapolation is permitted. Third, models allow generalization to other situations within the boundaries of their validities. Fourth, models are mathematical formulas that can be manipulated to optimize processes. Fifth, computer simulations are types of mathematical models. Sixth, models are used to identify unknown or previously disregarded process variables and parameters that may be significant variables. Seventh, models serve as controls in examining whether an effective separation of biological and physical phenomena has been achieved. Eighth, as an indirect results, models may also help in clarifying reaction mechanisms. Modeling of recombinant systems has received considerable attention in the past few years. These studies have addressed both stoichiometric and kinetic issues. Segregated, unstructured models have dealt with plasmid content distributions in recombinant *Saccharomyces cerevisiae* [Hjortso and Bailey, 1984; Schwartz et al., 1987] and *Escherichia coli* [Seo and Bailey, 1985; Bentley and Kompala, 1987].

In recent years, many researches have been directed toward investigation of optimal control problems involving fed-batch fermentations for enhancement of desired product production [Modak et al., 1986; Park and Ramirez, 1988; Park et al., 1989; Patkar et al., 1993]. Medium feed rate is often used as a control variable in these studies. Computation of the optimal feed rate requires a mathematical model able to describe the kinetics of cell growth and product formation.

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In the case of secreted foreign protein, the mathematical modeling for overall recombinant yeast culture considering protein secretion dynamics was very important because we can understand and predict the behavior of biosynthesis and secretion of foreign protein and enhance the secretion of protein by feeding control using these model equations. In this research, formulation of mathematical models for overall two type recombinant yeasts (plasmid-harboring recombinant yeast and chromosome-integrated recombinant yeast) fermentations considering secretion dynamics was performed. To get maximum foreign glucoamylase production, optimal feeding policy was suggested for maximizing glucoamylase production.

EXPERIMENT

1. Strains and Culture Medium

MMY2 (a, *ura3-52, sta*^{0}, *sta10*) strain was used as a host. Recombinant plasmids YEpSUCSTA and YIpSUCSTA that contained the glucoamylase coding *STA* gene fused with the *SUC2* promoter and original *STA* signal sequence were transformed into host. The transformants were named MMY2SUCSTA (plasmid-harboring recombinant yeast) [Cha et al., 1992] and MMY2SUCSTA-I (chromosome-integrated recombinant yeast) [Cha and Yoo, 1994]. Yeast was grown at 30℃ in semi-synthetic minimal medium containing 0.2% $MgSO_4 \cdot 7H_2O$, 0.2% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.025% $CaCl_2 \cdot 2H_2O$, 0.2% yeast extract, 0.3% bacto-peptone, 1% glucose, 1% starch, and 50 mM succinate. Starch was used as a carbon source for glucoamylase biosynthesis [Cha et al., 1992].

2. Measurements of Cell Mass and Glucoamylase Activity

The measurements of cell mass were carried out with spectrophotometer (Kontron; UVICON930) at 600 nm wavelength. To measure glucoamylase activity, 0.7 ml culture supernatant was incubated in 0.1 ml 1 M sodium acetate buffer (pH 5.0) and 0.2 ml 8% soluble starch (Junsei) at 50℃ for 30 min and boiled at 100℃ for 5 min for inactivating glucoamylase. Glucose produced by the action of glucoamylase on soluble starch was assayed by using a glucose-diagnostic kit (Sigma, No. 510). Glucoamylase activity was expressed as unit and 1 unit is 1 μmol of glucose released per 1 min at above conditions.

3. Cell Fractionation

Cells were harvested and washed with 10 mM sodium azide. Then, cells were suspended in lysis buffer [0.1 M sodium acetate (pH 5.0), 10 mM sodium azide, 1 mM EDTA and 0.1%(v/v) Triton X-100] and mechanically lysed by vortexing with glass bead (Sigma, 425-600 microns). After intermittent vortexing and cooling on ice, the suspension was harvested by centrifugation with 12,000 rpm at 4℃ for 15 min, and the supernatant was used for intracellular glucoamylase assay.

4. Simulation of Foreign Glucoamylase Production in Recombinant Yeast Culture

The parameters of model equations were estimated from experimental data of batch culture using least-square regression method. Determination of optimal glucose concentration and simulation of recombinant yeast culture were performed using software MATLAB (version 4.0, The MathWorks, Inc.). Ordinary differential equations of model equations were solved using 2nd and 3rd order Runge-Kutta method.

MODELING OF PROTEIN SECRETION DYNAMICS FROM RECOMBINANT YEAST

Preceding the design of a process and the control scheme to maximize the production of desired protein, it is important to investigate the secretion dynamics. An appropriate mathematical modeling of foreign protein secretion dynamics is needed but has been studied scarcely until now [Park and Ramirez, 1988]. The secretion events are a series of complicated and compartmentalized enzymatic reactions and intercompartmental transport. The proteins produced in the ribosome are transported in lumen of the endoplasmic reticulum (ER). Then the core-glycosylation and folding of proteins occur in the ER and the proteins are transported in the Golgi body. The outer chain glycosylation of secreted proteins occurs in the Golgi body. After the secreted proteins are transferred to secretory vesicles, they are secreted into the culture broth or periplasmic space by plasma membrane fusion. This secretion pathway can be represented schematically as shown below.

$$ER(P_E) \xrightarrow{k_e} Golgi\ (P_G) \xrightarrow{k_g} Periplasm\ (P_P) \xrightarrow{k_p} Culture\ broth\ (P_M)$$

Here, P_E, P_G, P_P, and P_M are amount of foreign protein in the ER, Golgi body, periplasm, and culture broth [units/mg-cell], respectively and k_e, k_g and k_p are rate constants [hr^{-1}] of translocation from one secretory apparatus to next one. The postulations for modeling of heterologous protein secretion are indicated down.

1. Postulation

(1) The translocation of protein to the next secretory apparatus is irreversible first order reaction. Protein that is made in cytoplasm is modified and processed in secretion pathway. Since the reactant is only protein itself, assumption for first order reaction of translocation is valid to simplify description of translocation reaction. Also, secretion is ATP consumption reaction and carrier-involved transport. Thus, protein is always transferred to the front secretory apparatus. So, assumption of irreversible reaction of translocation is valid. (2) The instability (or degradation) of proteins does not exist during secretion.

The protein balance in the ER secretory apparatus can be expressed as following first-order system.

$$\text{ER: } \frac{d(P_E X)}{dt} = F_P X - k_e P_E X \tag{1}$$

Here, P_E is expressed as units/mg-cell and $P_E X$ is expressed as units/mL. F_P is specific expression rate. Similarly, dynamics of the Golgi compartment can be expressed as

$$\text{Golgi: } \frac{d(P_G X)}{dt} = k_e P_E X - k_g P_G X \tag{2}$$

Also, the protein balance in the periplasm compartment can be expressed as

$$\text{Periplasm: } \frac{d(P_P X)}{dt} = k_g P_G X - k_p P_P X \tag{3}$$

Then, accumulation of the foreign secretory protein in the culture broth compartment can be expressed as

$$\text{Culture broth: } \frac{d(P_M X)}{dt} = k_p P_P X \tag{4}$$

In this system, the translocation of glucoamylase from the ER to the Golgi apparatus was rate-limiting step [Cha, 1995]. Therefore, the translocations in the other secretory apparatus are assumed as steady state. So,

$$\frac{d(P_G X)}{dt} = \frac{d(P_P X)}{dt} = 0 \quad (5)$$

From Eqs. (2), (3), and (5), the relationship between proteins in different secretory apparatus becomes

$$k_e P_E X = k_g P_G X = k_p P_P X \quad (6)$$

There is the overall conservation relationship among the pools of the concerned protein as

$$\begin{aligned} P_T &= P_M + P_I \\ &= P_M + P_E + P_G + P_P \end{aligned} \quad (7)$$

Inclusion of P_E, P_G, and P_P in the secretion model will cause practical problems since they are unmeasurable and inestimable. On the contrary, P_M and P_T are measurable quantities. Therefore, the above model equations are modified as the model that includes measurable P_M and P_T. By Eq. (6), Eq. (7) becomes

$$\begin{aligned} P_T &= P_M + P_E + \frac{k_e}{k_g} P_E + \frac{k_e}{k_p} P_E \\ &= P_M + \left(1 + \frac{k_e}{k_g} + \frac{k_e}{k_p}\right) P_E \end{aligned} \quad (8)$$

Rearranging Eq. (8) gives

$$P_E = \frac{P_T - P_M}{1 + k_e/k_g + k_e/k_p} \quad (9)$$

By Eqs. (6) and (9), Eq. (4) becomes

$$\begin{aligned} \frac{d(P_M X)}{dt} &= k_e P_E X \\ &= \frac{k_e}{1 + k_e/k_g + k_e/k_p} (P_T X - P_M X) \\ \frac{d(P_M X)}{dt} &= K(P_T X - P_M X) \end{aligned} \quad (10)$$

, where

$$K = \frac{1}{1/k_e + 1/k_g + 1/k_p} \quad (11)$$

Here, K is specific secretion rate. Also, there is conservation relationship among the pools of the intracellular protein as

$$P_I = P_E + P_G + P_P \quad (12)$$

Differentiating Eq. (12) with respect to t and Eq. (5) leads to Eq. (13)

$$\begin{aligned} \frac{d(P_I X)}{dt} &= \frac{d(P_E X)}{dt} + \frac{d(P_G X)}{dt} + \frac{d(P_P X)}{dt} \\ &= \frac{d(P_E X)}{dt} \end{aligned} \quad (13)$$

By differentiation and Eq. (13), Eq. (7) becomes

$$\begin{aligned} \frac{d(P_T X)}{dt} &= \frac{d(P_M X)}{dt} + \frac{d(P_I X)}{dt} \\ &= \frac{d(P_M X)}{dt} + \frac{d(P_E X)}{dt} \end{aligned} \quad (14)$$

By Eqs. (1)-(4) and Eq. (6), Eq. (14) becomes

$$\frac{d(P_T X)}{dt} = F_P X \quad (15)$$

MODELING FOR BATCH CULTURE OF TWO TYPE RECOMBINANT YEASTS

Plasmid instability has been considered for modeling of plasmid-harboring recombinant microorganism culture [Ollis and Chan, 1982; Seo and Bailey, 1984; Lee et al., 1985; Wittrup and Bailey, 1987; Bentley and Kompala, 1987; Schwartz et al., 1987; Hong, 1988]. In this work, segregation coefficient θ was introduced for the plasmid instability. Only one limiting substrate is assumed to be used in recombinant yeast culture. Plasmid-harboring cells (X^+) and plasmid-free cells (X^-) are competitive in utilizing the single substrate. Since plasmid-harboring cells are subject to severe segregational loss of plasmids, they revert to plasmid-free cells. This can be written as belows.

$$aG + X^+ \rightarrow (2-\theta)X^+ + \theta X^- \quad (16)$$

$$bG + X^- \rightarrow 2X^- \quad (17)$$

Here, a and b are coefficients of substrate that is utilized by one plasmid-harboring cell and one plasmid-free cell, respectively. The mass balance for cell growth of plasmid-harboring recombinant yeast is described as below by segregation coefficient θ.

$$\frac{dX^+}{dt} = \mu^+ X^+ (1-\theta) \quad (18)$$

, where

$$\mu^+ = \mu_m^+ \left(\frac{G}{k_G^+ + G}\right) \quad (19)$$

The model equation for cell growth of plasmid-free yeast can be written in Eq. (20).

$$\frac{dX^-}{dt} = \mu^+ X^+ \theta + \mu^- X^- \quad (20)$$

, where

$$\mu^- = \mu_m^- \left(\frac{G}{k_G^- + G}\right) \quad (21)$$

Specific growth rate (μ) is described as simple Monod equation that is a function of glucose concentration. Glucose inhibition on the cell growth is observed when medium is highly concentrated with glucose. High glucose as a substrate is not recommendable for protein production because higher glucose concentration than a certain point does not contribute significantly to the cell growth rate. Also, ethanol that is produced in high glucose medium inhibits protein expression as well as cell growth. Thus, the effect of high glucose on the cell growth rate was disregarded. The kinetics of ethanol inhibition on yeast growth have been investigated by many workers [Aiba et al., 1968; Novak et al., 1981; Luong, 1985]. The effect of accumulated ethanol on the cell growth was also disregarded to avoid additional complexity since this does not change the overall pattern. The mass balance for substrate (glucose) consumption is described using cell growth and cell maintenance terms.

$$\frac{dG}{dt} = \frac{-1}{Y_{XG}} (\mu^+ X^+ + \mu^- X^-) - m(X^+ + X^-) \quad (22)$$

The product glucoamylase can be regarded as total (intracellular and secreted) glucoamylase and secreted glucoamylase that is se-

creted into the culture broth. The mass balance for total glucoamylase protein can be written down.

$$\frac{d(P_T X^+)}{dt} = F_P X^+ \quad (23)$$

The *SUC2* promoter that was used in this work, is regulated by glucose concentration in the culture broth. When glucose concentration is high, the expression of *SUC2* promoter is repressed. However, when glucose concentration is decreased to a certain point, the expression is derepressed. Thus, to consider glucose repression on the expression of *SUC2* promoter, glucose repression term (G^2/k_{PI}) is introduced into specific expression rate F_P as below.

$$F_P = \frac{F_{Pm} G}{(k_P + G + G^2/k_{PI})} \quad (24)$$

Also, the mass balance for secreted glucoamylase can be written down.

$$\frac{d(P_M X^+)}{dt} = K(P_T X^- - P_M X^+) \quad (25)$$

Specific secretion rate K is related with cell growth condition. So, it was assumed that specific secretion rate is function of specific growth rate μ^+ of plasmid-harboring recombinant cells.

In the case of chromosome-integrated recombinant yeast, it is not necessary to consider plasmid instability since the genetic stability of chromosome-integrated yeast is almost perfect. Thus, the mass balance for cell growth of chromosome-integrated yeast is described as below.

$$\frac{dX}{dt} = \mu X \quad (26)$$

, where

$$\mu = \mu_m \left(\frac{G}{k_G + G} \right) \quad (27)$$

The mass balance for substrate (glucose) consumption is described as below.

$$\frac{dG}{dt} = -\frac{\mu X}{Y_{XG}} - mX \quad (28)$$

The mass balance for total glucoamylase protein can be written down.

$$\frac{d(P_T X)}{dt} = F_P X \quad (29)$$

, where

$$F_P = \frac{F_{Pm} G}{(k_P + G + G^2/k_{PI})} \quad (30)$$

The mass balance for total glucoamylase protein can be written down.

$$\frac{d(P_M X)}{dt} = K(P_T X - P_M X) \quad (31)$$

PARAMETER ESTIMATION IN MODEL EQUATIONS

The parameters that were required for simulation of recombinant yeast culture model were estimated from experimental results of batch fermentations by using least squares method. Recombinant yeasts utilized yeast extract and bacto-peptone as energy source for cell growth and glucoamylase biosynthesis after depletion of glucose. Therefore, glucoamylase was continuously produced for a long time although glucose in the culture broth was exhausted. However, since glucose concentration was considered only for glucoamylase biosynthesis and cell growth models, parameters of model equations were estimated from the data that were obtained till glucose in the culture broth was depleted.

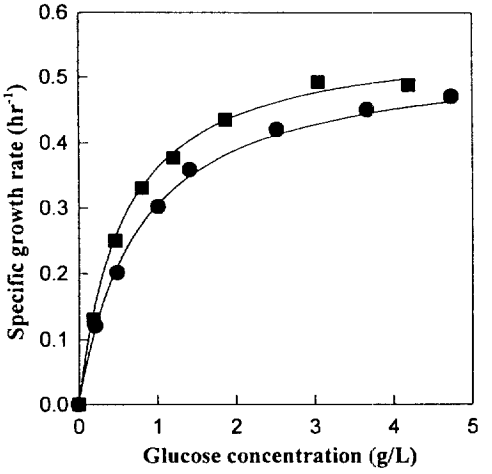

Fig. 1. Dependence of specific growth rate on glucose concentration in plasmid-harboring recombinant yeast MMY2SUCSTA (●) including plasmid-free cells and chromosome-integrated recombinant yeast MMY2SUCSTA-I (■).
Symbols; ●, ■ : experimental data and — : simulation of model

The specific growth rate μ of total plasmid-harboring recombinant yeast including plasmid-free cells was indicated in Fig. 1 and its maximum value was 0.536 hr^{-1}. To obtain the specific growth rate μ^+ of only plasmid-harboring cells, it was calculated using the fraction of plasmid-harboring cells F and the specific growth rate μ^- of host MMY2 as shown in Eq. (33).

$$\bar{\mu} = \mu^+ \left(\frac{X^-}{X} \right) + \mu^- \left(1 - \frac{X^+}{X} \right)$$
$$= \mu^+ F + \mu^- (1 - F) \quad (32)$$

$$\mu^- = \frac{\bar{\mu} - \mu^+ (1 - F)}{F} \quad (33)$$

The maximum specific growth rates of plasmid-harboring cells and plasmid-free cells were 0.531 hr^{-1} and 0.566 hr^{-1}, respectively. Also, the maximum specific growth rate of chromosome-integrated cells was 0.564 hr^{-1} as indicated in Fig. 1.

The segregation coefficient θ of plasmid-harboring yeast was determined by numerical calculation of Eq. (36) and its mean value was 0.102.

$$X^- = X_0^+ e^{\mu^+ (1-\theta) t} \quad (34)$$

$$X^- = \frac{\mu^+ \theta X_0^+}{\mu^+ (1-\theta) - \mu^-} \left(e^{\mu^+ (1-\theta) t} - e^{\mu^- t} + X_0^- \right) \quad (35)$$

$$F = \frac{X^-}{X^+ + X^-}$$
$$= \frac{\{\mu^+ (1-\theta) - \mu^-\} e^{\mu^+ (1-\theta) t}}{(\mu^+ - \mu^-) e^{\mu^+ (1-\theta) t} - \mu^- \theta (e^{\mu^- t} - X_0^-)} \quad (36)$$

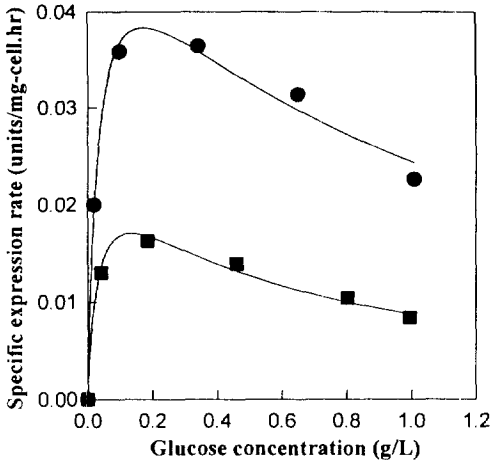

Fig. 2. Dependence of specific glucoamylase expression rate on glucose concentration in plasmid-harboring recombinant yeast MMY2SUCSTA (●) and chromosome-integrated recombinant yeast MMY2SUCSTA-I (■).
Symbols; ●, ■ : experimental data and — : simulation of model

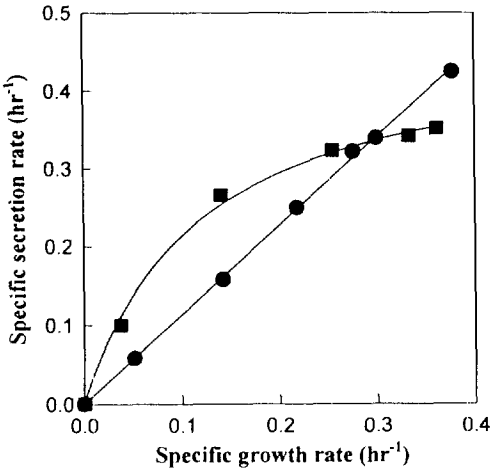

Fig. 3. Dependence of specific glucoamylase secretion rate on specific growth rate in plasmid-harboring recombinant yeast MMY2SUCSTA (●) and chromosome-integrated recombinant yeast MMY2SUCSTA-I (■). Specific growth rate of MMY2SUCSTA is that of plasmid-harboring cells.
Symbols; ●, ■ : experimental data and — : simulation of model

The specific glucoamylase expression rate F_P of plasmid-harboring recombinant yeast MMY2SUCSTA was indicated in Fig. 2 and its maximum value was 0.054 units/mg-cell·hr. The maximal expression of plasmid-harboring yeast occurs at glucose concentration of 0.17 g/L in the culture broth. Also, the maximum specific glucoamylase expression rate of chromosome-integrated recombinant yeast MMY2SUCSTA-I was 0.026 units/mg-cell·hr. In this case, the maximal expression occurs at glucose concentration of 0.13 g/L.

As shown in Fig. 3, the specific secretion rate K of plasmid-harboring recombinant yeast MMY2SUCSTA and the specific growth rate μ^+ as cell growth condition had proportional relationship (linear coefficient r was 0.9993) and its proportional coefficient was 1.137. Since specific growth rate was a function of glucose concentration, the specific secretion rate could be described as a function of glucose concentration as Eq. (37).

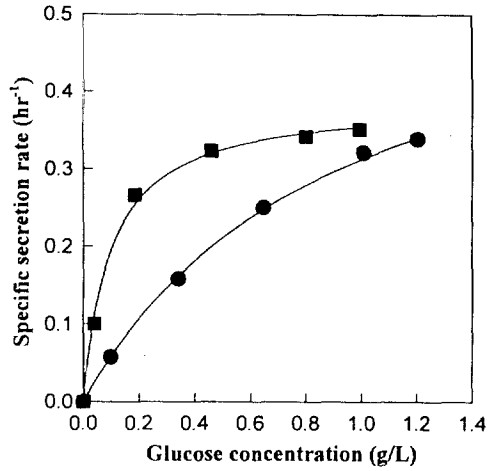

Fig. 4. Dependence of specific glucoamylase secretion rate on glucose concentration in plasmid-harboring recombinant yeast MMY2SUCSTA (●) and chromosome-integrated recombinant yeast MMY2SUCSTA-I (■).
Symbols; ●, ■ : experimental data and — : simulation of model

$$K = a\mu^+ = \frac{KmG}{\alpha + \beta G} \tag{37}$$

, where

$$Km = a\mu_m^+$$
$$\alpha = k_G^+$$
$$\beta = 1$$

Therefore, the specific secretion rate depending on glucose concentration was indicated in Fig. 4. On the other hands, the specific secretion rate of chromosome-integrated recombinant yeast MMY2SUCSTA-I depending on the specific growth rate was indicated in Fig. 3. In this case, its relationship was not linear. Its pattern was similar to Monod equation type. Thus, the specific secretion rate K of chromosome-integrated yeast depending on the specific growth rate could be described as below equation.

$$K = \frac{a\mu}{b + \mu} = \frac{KmG}{\alpha + \beta G} \tag{38}$$

, where

$$Km = a\mu_m$$
$$\alpha = bk_G$$
$$\beta = b + \mu_m$$

So, it could be assumed that linear relationship of the specific secretion rate [Eq. (37)] was resulted from Eq. (38) since b was much larger than μ in the case of plasmid-harboring recombinant yeast MMY2SUCSTA. The parameters of the model equations for batch culture of plasmid-harboring recombinant yeast MMY2SUCSTA and chromosome-integrated recombinant yeast MMY2SUCSTA-I were summarized in Table 1.

Table 1. Parameters for simulation of plasmid-harboring recombinant yeast MMY2SUCSTA culture model (A) and chromosome-integrated recombinant yeast MMY2SUCSTA-I culture model (B)

(A)

Parameter	Value	Remarks
μ_m^+	0.531	Experimental
μ_m^-	0.566	Experimental
k_G^+	0.929	Experimental
k_G^-	0.558	Experimental
θ	0.102	Experimental
Y_{XG}	0.158	Experimental
F_{Pm}	0.054	Experimental
k_P	0.035	Experimental
k_{Pi}	0.864	Experimental
K_m	0.603	Experimental
α	0.929	Experimental
β	1.000	Experimental
m	0.01	Literature

(B)

Parameter	Value	Remarks
μ_m	0.564	Experimental
k_G	0.557	Experimental
Y_{XG}	0.157	Experimental
F_{Pm}	0.026	Experimental
k_P	0.034	Experimental
k_{Pi}	0.521	Experimental
K_m	0.267	Experimental
α	0.068	Experimental
β	0.685	Experimental
m	0.01	Literature

The model simulation results of plasmid-harboring recombinant yeast and chromosome-integrated recombinant yeast agreed very well with the experimental results as shown in Fig. 5. Because this mathematical model was not a structured model, it was unable to predict the lag period before starting to grow on glucose. So, the simulation was performed from 3 hour as starting culture time.

OPTIMIZATION OF GLUCOAMYLASE PRODUCTION

To maximize the extracellular glucoamylase productivity in fed-batch culture, the optimal feeding policy was suggested as shown in Fig. 6. Since the specific growth rate is monotonic, cells are grown optimally in high glucose medium. From Fig. 1, the optimal glucose concentration for cell growth was set at 5 g/L. Cell growth may be inhibited at above this concentration due to produced ethanol and glucose effect. So, glucose feed rate is set for maintaining of 5 g/L optimal glucose concentration at start (t_0). When cell mass reaches appropriate concentration that is optimal for maximum glucoamylase production, feeding is shut off (t_1) until the glucose concentration reaches at optimal glucose concentration (t_2) for maximum extracellular glucoamylase productivity. After then, optimal feeding for glucoamylase production continues until fermentor vessel is full (t_3). This optimal feeding profile was named feeding policy II. This optimal feeding policy contains three unknown switching times (t_1, t_2, and t_3). Because of final vessel volume constraint, final switching occurs when vessel is full. Thus, t_3 is readily determined. If final time (t_f) is smaller than t_3, fed-batch operation is shut off at t_f. Also, second switching occurs when glucose concentration in the culture broth reaches at the appropriate optimal glucose concentration for glucoamylase production [Cha, 1995] (0.16 g/L for plasmid-harboring yeast MMY2SUCSTA and 0.09 g/L for chromosome-integrated yeast

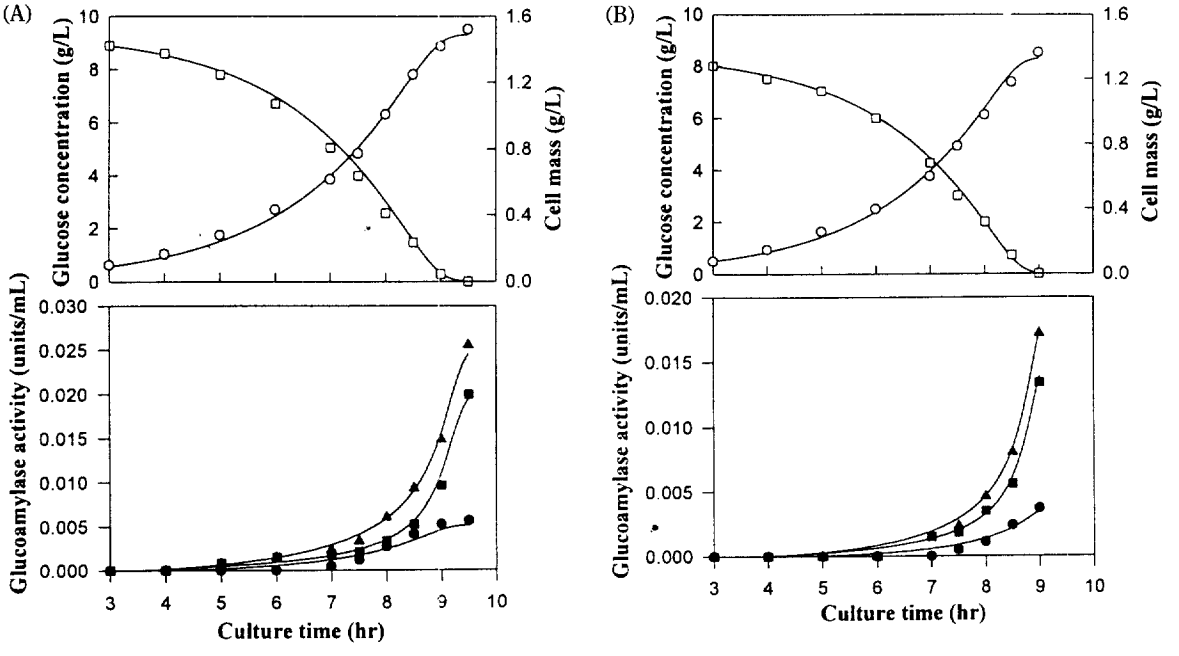

Fig. 5. Experimental and model simulation results for batch fermentation of plasmid-harboring recombinant yeast MMY2SUCSTA (A) and chromosome-integrated recombinant yeast MMY2SUCSTA-I (B) in 10 g/L initial glucose medium.
Symbols; ○ : cell mass, □ : glucose concentration, ● : extracellular glucoamylase activity, ■ : intracellular glucoamylase activity, ▲ : total glucoamylase activity, and — : simulation results.

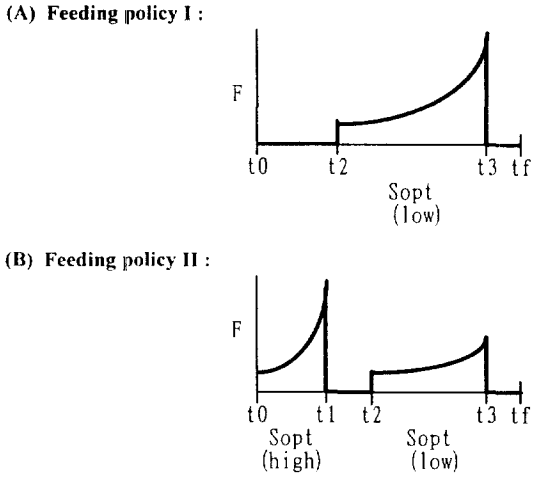

Fig. 6. Optimal feeding policy II for fed-batch culture of recombinant yeast.

MMY2SUCSTA-I). Thus, t_2 can be also readily determined if glucose concentration in the broth can be determined as real-time measuring. On the basis of this assumption, the sole unknown time is t_1. Thus, optimal switching time t_1 for maximum final extracellular glucoamylase productivity was determined by using numerical methods. On the other hands, the feeding policy I is the mode without initial feeding for maximizing cell mass. Thus, switching time t_1 is unnecessary. In this research, the feed flow rate was calculated by the following well-known equation in a singular region.

$$f=\frac{\left(\frac{\mu}{Y_{XG}}+m\right)XV}{(G_f-G_{OPT})} \tag{39}$$

This equation includes state variables (cell mass and vessel volume). So, after measuring the state variables, feed rate was calculated using these state variables.

In the case of plasmid-harboring recombinant yeast MMY2SUCSTA fed-batch culture using the feeding policy II and final operation time of 30 hour, the optimal switching time t_1 was determined as 5 hour and the switching time t_2 was 7 hour as shown in Fig. 7(A). The glucoamylase productivity was 14.84 units/hr. On the contrary, when feeding policy I was used, the switching time t_2 was 5 hour and the glucoamylase productivity was 12.99 units/hr as shown in Fig. 7(B). Thus, the glucoamylase productivity was 14.2% increased by introducing the optimal feeding policy II. Thus, the usage of optimal feeding policy that was suggested in this work, was effective in enhancing glucoamylase productivity from fed-batch culture using recombinant yeast. In the case of chromosome-integrated recombinant yeast MMY2SUCSTA-I fed-batch culture using the optimal feeding policy II, the optimal switching time t_1 was 5.5 hour, the switching time t_2 was 7 hour, and the glucoamylase productivity was 20.97 units/hr as shown in Fig. 8(A). On the contrary, when feeding policy I was used, the switching time t_2 was 5 hour and the glucoamylase productivity was 11.88 units/hr as shown in Fig. 8(B). In this recombinant yeast culture, the glucoamylase productivity was increased (76.5%) by introducing the optimal feeding policy.

Overall comparison of simulation results for glucoamylase production from plasmid-harboring and chromosome-integrated recombinant yeast cultures was summarized in Table 2. By intro-

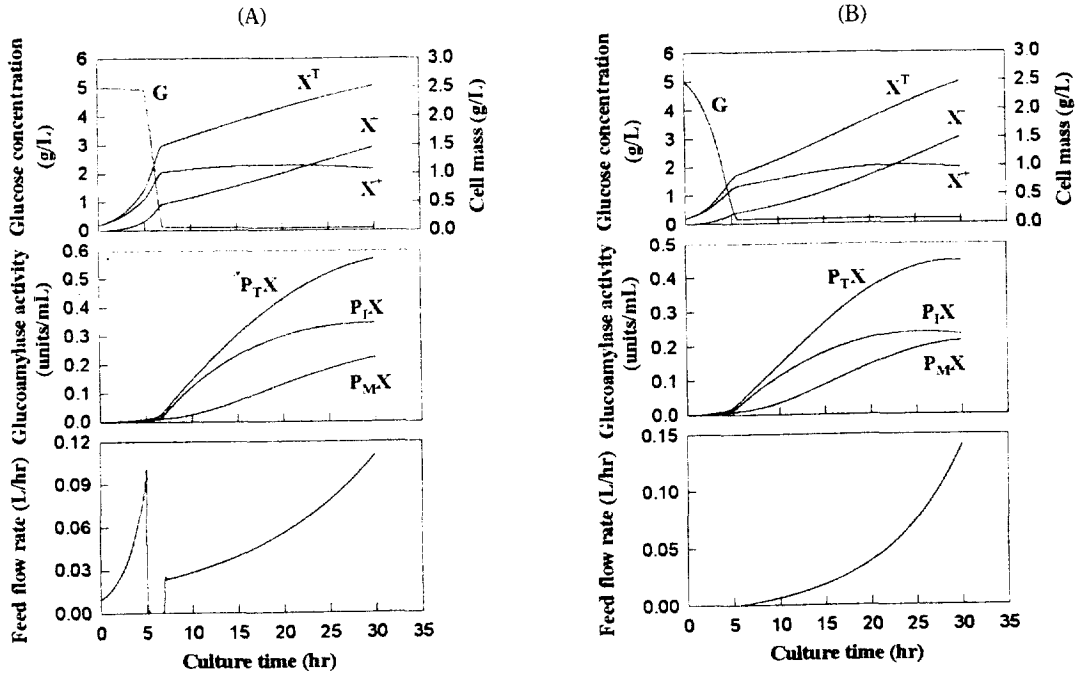

Fig. 7. Simulation results for fed-batch culture of plasmid-harboring recombinant yeast MMY2SUCSTA using feeding policy II (A) and feeding policy I (B).

Initial and final conditions; $X^+=0.1$ g/L, $X^-=0.0$ g/L, $G=5$ g/L, $G_f=20$ g/L, $V_0=0.5$ L, and $t_f=30$ hours. Abbreviation; X^+ : plasmid-harboring cell, X^- : plasmid-free cell, X^T : total cell, G : glucose concentration, V : vessel volume, P_IX : intracellular glucoamylase activity, P_MX : extracellular glucoamylase activity, and P_TX : total glucoamylase activity

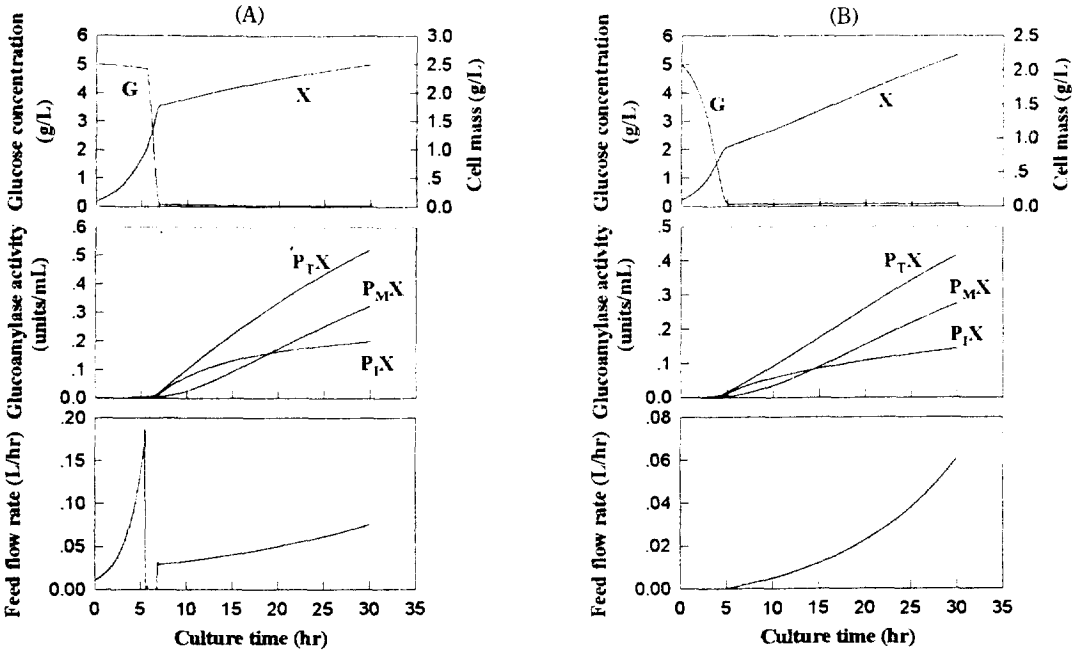

Fig. 8. Simulation results for fed-batch culture of chromosome-integrated recombinant yeast MMY2SUCSTA-I using feeding policy II (A) and feeding policy I (B).

Initial conditions; X=0.1 g/L, G=5 g/L, G_f=20 g/L, V_0=0.5 L, and t_f=30 hours. Abbreviation; G : glucose concentration, V : vessel volume, P_IX : intracellular glucoamylase activity, P_MX : extracellular glucoamylase activity, and P_TX : total glucoamylase activity

Table 2. Summary of simulation results for glucoamylase production from plasmid-harboring and chromosome-integrated recombinant yeasts

Culture type	Plasmid-harboring recombinant yeast MMY2SUCSTA				Chromosome-integrated recombinant yeast MMY2SUCSTA-I				Remarks
	Volumetric activity (units/mL)	Relative increase (folds)	Productivity (units/hr)	Relative increase (folds)	Volumetric activity (units/mL)	Relative increase (folds)	Productivity (units/hr)	Relative increase (folds)	
Batch	0.0056	1.0	1.17	1.0	0.0037	1.0	0.82	1.0	10 g/L initial glucose
Fed-batch	0.2146	38.3	12.99	11.1	0.2742	74.1	11.88	14.5	30 hr culture time
Fed-batch with optimal feeding policy	0.2254	40.3	14.84	12.7	0.3225	87.2	20.97	25.6	30 hr culture time

ducing the optimal feeding policy, final glucoamylase activity and productivity of fed-batch culture were largely increased (40.3 and 12.7 folds in plasmid-harboring recombinant yeast MMY2SUCSTA and 87.2 and 25.6 folds in chromosome-integrated recombinant yeast MMY2SUCSTA-I, respectively) than those of batch culture in both recombinant yeasts. Therefore, this optimal feeding policy can be applied successfully to the recombinant protein production system using *SUC2* promoter.

NOMENCLATURE

f : feed flow rate [L/hr]
F : fraction of plasmid-harboring cells [-]
F_P : specific expression rate [units/mg-cell·hr]
F_{Pm} maximum specific expression rate [units/mg-cell·hr]
G : glucose concentration in the culture broth [g/L]
G_f : glucose concentration in the feed [g/L]
G_{OPT} : optimal glucose concentration for maximizing productivity [g/L]
K : specific secretion rate [hr $^{-1}$]
Km : maximum specific secretion rate [hr $^{-1}$]
k_G : saturation constant [g/L]
k_G^- : saturation constant of plasmid-harboring cell [g/L]
k_G^+ : saturation constant of plasmid-free cell [g/L]
k_P : constant of specific expression rate [g/L]
k_{Pi} : repression constant of specific expression rate [g/L]
m : maintenance coefficient [-]
P_E : specific endoplasmic reticulum enzyme concentration [units /mg-cell]
P_G : specific Golgi body enzyme concentration [units/mg-cell]
P_I : specific intracellular enzyme concentration [units/mg-cell]
P_M : specific extracellular enzyme concentration [units/mg-cell]
P_P : specific periplasmic enzyme concentration [units/mg-cell]
P_T : specific total enzyme concentration [units/mg-cell]
P_EX : endoplasmic reticulum enzyme concentration [units/mL]
P_GX : Golgi body enzyme concentration [units/mL]

P_IX : intracellular enzyme concentration [units/mL]
P_MX : extracellular enzyme concentration [units/mL]
P_PX : periplasmic enzyme concentration [units/mL]
P_TX : total enzyme concentration [units/mL]
t : operation time [hr]
t_0 : initial operation time [hr]
t_1 : first switching time [hr]
t_2 : second switching time [hr]
t_3 : third switching time [hr]
t_f : final operation time [hr]
V : vessel volume [L]
X : cell mass [g/L]
X^+ : plasmid-harboring cell mass [g/L]
X^- : plasmid-free cell mass [g/L]
X^T : total cell mass [g/L]
Y_{XG} : yield coefficient [-]

Greek Letters

α : constant of specific secretion rate [g/L]
β : constant of specific secretion rate [-]
μ : specific growth rate [hr^{-1}]
μ^+ : specific growth rate of plasmid-harboring cell [hr^{-1}]
μ^- : specific growth rate of plasmid-free cell [hr^{-1}]
$\bar{\mu}$: average specific growth rate [hr^{-1}]
θ : segregation coefficient [-]

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