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SHORT COMMUNICATION

OPTIMIZATION OF TWO-STAGE CONTINUOUS CULTURE SYSTEM FOR PRODUCTION OF POLY-β-HYDROXYBUTYRATE

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Abstract—The optimization of poly-β-hydroxybutyrate (PHB) fermentation process is essential to obtain a high PHB productivity. An optimization of two-stage continuous culture system by complex method for PHB production is presented. PHB production rate of the system obtained in the present study was chosen as the objective function. The PHB production rate of the system was higher than that of the phasewise optimization method which maximizes the growth rate of residual biomass in the first stage and the PHB production rate in the second stage. When the inlet glucose concentration in the first stage increased, PHB content and yield also increased. When the fermentor volume ratio (V_2/V_1) was 0.5, maximum PHB productivity in the second stage was 2.86 g/L·h, which is highest compared with the reported value on PHB.

Key words: *PHB, Two-stage Continuous Culture, Optimization, Productivity*

INTRODUCTION

Poly-β-hydroxybutyrate (PHB) is a biodegradable thermoplastic which can be used as an alternative of synthetic plastics for the conservation of environment. The polymer is a carbon and energy storage material accumulated inside a wide range of microorganisms under the limitation of nutrients such as nitrogen, oxygen, or trace elements. The use of a two-stage continuous culture system which separates the growth and PHB production stages and its optimization is desirable to obtain a high PHB productivity.

Lee et al. [1] showed that the recombinant cells could be maintained stably for a prolonged time in a two-stage continuous culture system by theoretical analysis of cell population dynamics. Park et al. [1989] determined an optimal operating condition for recombinant *Escherichia coli* in a two-stage continuous system. They reported that the productivity is more sensitive to the combination of the dilution rates than to the volume ratio of two reactors. Ramsay et al. [1990], also, applied a two-stage continuous culture system to P(3HB-co-3HV) copolymer production.

Even though multi-stage continuous systems have not been widely used in commercial bioprocesses, potential advantages on theoretical back grounds are considerable. Luyben and Tramper [1982] derived an analytical formula for the optimal design of CSTR's in series, where Michaelis-Menten kinetics were applied. Under the assumption that the activity of biocatalyst in a reactor is constant, They optimized the total reactor size to perform a specified conversion. Ong [1986] optimized a series of CSTR's using dynamic programming for the maximization of profit or the minimization of investment cost. Malcata [1989] reported that only a few reactors in series should be considered if total investment cost was the objective function. Preoptimization of the reactors by minimizing the total reactor space time gave faster convergence in achieving minimum investment cost.

Optimizations of CSTR's in series mainly have been directed to minimize the total reactor volume with the desired conversion to find the number of reactors required for the maximization of profit or to minimize the investment cost for simple Michaelis-Menten or Monod type kinetics.

In this study, an optimization strategy of two-stage continuous culture system for PHB production is proposed. The operation variables, such as dilution rates and inlet nutrients concentrations were optimized to maximize the PHB production rate.

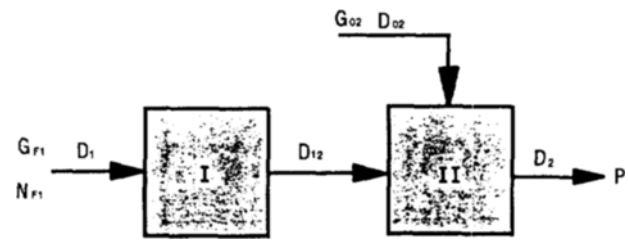

Fig. 1. Schematic diagram of the two-stage continuous culture system for PHB production.

MODELING AND OPTIMIZATION

A schematic diagram of two-stage continuous culture system is shown in Fig.1. Both carbon and nitrogen sources were fed as a mixture at the first stage. Only the carbon source was, however, fed at the second stage for the accumulation of PHB. Reactor volume ratio of the first and second stage (V_2/V_1) was 1 in the model equations, otherwise mentioned.

The kinetic model equations reported in the previous paper [Lee and Yoo, 1991] was employed as simulation equations. Eqs. (1)-(8) represent the unsteady state mass balances of residual biomass, PHB, and carbon and nitrogen sources. Eqs. (1)-(4) represent the mass balances of the first stage, and Eqs. (5)-(8), those of the second stage, where residual biomass concentration was calculated as the difference between cell mass concentration and

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PHB concentration.

$$\frac{dR_1}{dt} = \mu_1 R_1 - D_1 R_1 \quad (1)$$

$$\frac{dP_1}{dt} = \nu_1 R_1 - D_1 P_1 \quad (2)$$

$$\frac{dG_1}{dt} = D_1(G_{F1} - G_1) - \left(\frac{\mu_1}{Y_{R/G}} + \frac{\nu_1}{Y_{P/G}} + m\right)R_1 \quad (3)$$

$$\frac{dN_1}{dt} = D_1(N_{F1} - N_1) - \frac{\mu_1}{Y_{R/N}} R_1 \quad (4)$$

$$\frac{dR_2}{dt} = \mu_2 R_2 + D_{12} R_1 - D_2 R_2 \quad (5)$$

$$\frac{dP_2}{dt} = \nu_2 R_2 + D_{12} P_1 - D_2 P_2 \quad (6)$$

$$\frac{dG_2}{dt} = D_{12} G_1 + D_{02} G_{02} - D_2 G_2 - \left(\frac{\mu_2}{Y_{R/G}} + \frac{\nu_2}{Y_{P/G}} + m\right)R_2 \quad (7)$$

$$\frac{dN_2}{dt} = D_{12} N_1 - D_2 N_2 - \frac{\mu_2}{Y_{R/N}} R_2 \quad (8)$$

,where $D_1 = D_{12}$ and $D_2 = D_{12} + D_{02}$.

At steady state, the specific growth rate of the residual biomass in the first stage is equal to the dilution rate as shown in Eq. (1). One can also deduce from Eq. (2) that the specific PHB production rate is equal to the product of dilution rate, and the ratio of PHB concentration and residual biomass concentration. The specific growth rate of residual biomass and the specific PHB production rate reported in the previous paper [Lee and Yoo, 1991] are as follows;

$$\mu = \hat{\mu}\left(\frac{G}{K_G + G + G_2/K_{GI}}\right)\left(\frac{N}{K_N + N + N^2/K_{NI}}\right) \quad (9)$$

$$\nu = \hat{\nu}\left(1 - \frac{P/X}{(P/X)_m}\right)^{\alpha}\left(\frac{G}{K_{GP} + G}\right)\left(\frac{K_I}{K_I + N}\right) \quad (10)$$

At normal stable steady state, the dilution rate of the first stage is less than the maximum value of μ that can be calculated by Eq. (11), which is a necessary condition, and is less than the dilution rate at washout, which is a sufficient and necessary condition. At washout, substrate concentrations in the reactor approach the inlet substrate concentrations.

$$D_1 \le \mu_{max} = \frac{\hat{\mu}}{[1 + 2\sqrt{K_G/K_{GI}}]\,[1 + 2\sqrt{K_N/K_{NI}}]} \quad (11)$$

Inlet concentrations of carbon and nitrogen sources have constraints as shown in Eqs. (12) and (13), for they have to be less than their solubilities.

$$0 \le G_F \le \rho_G \quad (12)$$

$$0 \le N_F \le \rho_N \quad (13)$$

, where ρ_G and ρ_N denote solubilities of carbon and nitrogen sources, respectively. The necessary and sufficient condition for stability suggested by Paviou [1987] must be satisfied as in Eq. (14).

$$Q = \sum_{i=1}^{n} \frac{1}{Y_i} \frac{\partial \mu}{\partial S_i} > 0, \text{ for every } S_i \ge 0 \quad i = 1, \cdots, n \quad (14)$$

Eq. (9) is substituted to Eq. (14).

$$Q = \frac{\mu^2}{\hat{\mu} Y_G Y_N G^2 N^2}\,[Y_G G(K_N - N^2/K_{NI})(K_G + G + G^2/K_{GI}) + Y_N N(K_G - G^2/K_{GI})(K_N + N + N^2/K_{NI})] > 0 \quad (15)$$

In order to satisfy $Q > 0$, the value within the parenthesis must be positive as shown in Eqs. (16) and (17).

$$0 \le G \le \sqrt{K_G K_{GI}} \quad (16)$$

$$0 \le N \le \sqrt{K_N K_{NI}} \quad (17)$$

These inequalities represent that G and N are less than the substrate concentrations at which μ have a maximum value. G_1 and N_1 must be in the range of $0 \le G_1 \le 57.6$ and $0 \le N_1 \le 0.91$ in order for the process to be stable in the first stage. The parameter values obtained from the continuous cultures were used for the optimization [Lee and Yoo, 1991].

In order to optimize the two-stage continuous culture system for PHB production, four operation variables which were inlet carbon and nitrogen source concentrations and dilution rate at the first stage and dilution rate at the second stage-were selected as the manipulated variables. The inlet carbon source concentration to the second stage was assumed constant (800 g/l) which was lower than glucose solubility. Nitrogen source was not fed to the second stage in order to accumulate PHB in the cell by lowering nitrogen source level. The fermentor volumes of first and second stage were assumed to be equal. The system has a nonlinearity, multivariables, and also constraints as shown in Eqs. (11), (12), (13), (16) and (17). We used the complex method which is known to be adequate for the optimization of nonlinear-multivariable processes which are subject to nonlinear inequality constraints [1973]. The objective function is to maximize PHB production rate as shown in Eq. (18);

$$J = \max\ [D_2 P_2] \quad (18)$$

In order to use complex method, an original complex of $A \ge B + 1$ points consisting of a feasible starting point must be generated, where A is the number of points in the complex, and B is the number of explicit independent variables. In this case, $A = 6$ and $B = 4$. The objective function initially was evaluated at each point. The point having the lowest function value was replaced by a point which was located at a distance 1.3 times from the centroid of the remaining points as the distance of the rejected point on the line joining the rejected point and the centroid. Convergence was assumed when the maximum difference of the objective function values at each point was within 1×10^{-4} for 5 consecutive iterations. More detailed procedure of the complex method is described in the reference. The initial complex points were regenerated to confirm the optimization results.

RESULTS AND DISCUSSION

1. Optimization of the Two-stage Continuous Culture System

The values of four manipulative variables, [D_1, G_{F1}, N_{F1}, and D_{02}] were optimized by complex method to maximize PHB production rate of the system (D_2P_2). Optimal conditions of D_1, G_{F1}, N_{F1}, and D_{02} were 0.026 h^{-1}, 343 g/L, 29.3 g/L, and 0.0058 h^{-1}, respectively as shown in Fig. 2. The growth of residual biomass and PHB production were fully acquired in the first stage, the increase of cell concentration in the second stage was due to PHB production. The glucose concentration in the second stage was three times higher than that in the first stage. The concentration of nitrogen source was low in the first stage and completely depleted in the second stage. The presence of optimal D_{02} coincided

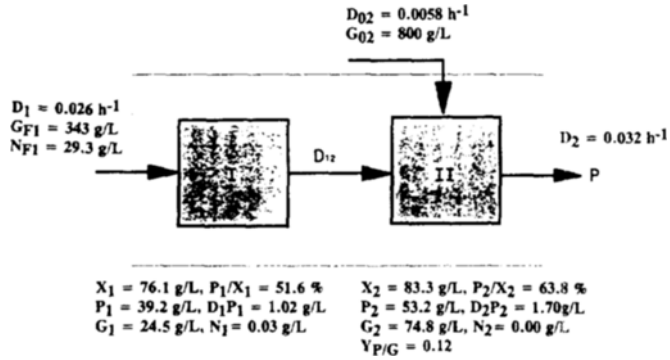

Fig. 2. Optimization results from the two-stage continuous culture system for PHB production by complex method.

Table 1. Comparison of the general and phasewise optimization results in two-stage continuous culture of *Alcaligenes eutrophus* by complex method

Method	P_2/X_2 (%)	D_2R_2 (g/L/h)	D_2P_2 (g/L/h)	$Y_{P/G}$
General	43.80	0.96	1.70	0.12
Phasewise	43.94	1.58	1.24	0.07

with the report by Park et al. [1990]. They observed optimal cell growth rate in the second stage giving the maximum value in overall productivity. The PHB production rate (DP) and PHB content (P/X) in the first stage were 1.02 g/L/h, and 51.6% of dry cell weight, and in the second stage, 1.70 g/L/h and 63.8% of dry cell weight, respectively. By fed-batch culture of *Protomonas extorquens*, PHB productivity of 1.12 g/L/hr was reported [Suzuki et al., 1986]. The productivity obtainable from the two-stage continuous culture was 70% higher than that obtained from the fed-batch culture.

2. Comparison with the Phasewise Continuous Culture

The phasewise continuous culture process which devide cell growth phase and production phase of secondary metabotites has been applied to many fermentation processes compared to non-phasewise process which do not devide cell growth and production phase in order to increase secondary metabolite production [Godin and Engasser, 1988]. This concept was tested and compared with the optimization results.

D_1, G_{F1}, and N_{F1} which maximize the growth rate of residual biomass (D_1R_1) in the first stage, and D_{02} which maximize the PHB production rate (D_2P_2) in the second stage were searched by complex method. The optimal values of D_1, G_{F1}, and N_{F1} for the phasewise continuous culture were determined to be 0.083 h^{-1}, 117.8 g/L and 15.10 g/L, respectively. The maximum PHB production rate was 1.24 g/L/h.

The optimization results in Fig. 2 were compared with that of the phasewise continuous culture which cellular productivity should be maximized at this stage and PHB production rate in the second stage. As shown in Table 1, the optimization results of phasewise culture gave lower values than that shown in Fig. 2. The reason for the difference was as follows; In the condition that the maximum specific growth rate of residual biomass is higher than maximum specific PHB production rate, the dilution rate of the second stage cannot be lowered than that of the first due to $D_2 = D_1 + D_{02}$ when each fermenter volume of the first and second stages is the same. Therefore, the calculation results from the phasewise continuous culture were not optimal and thus lower than the non-phasewise optimization results. When the volume of second stage is larger than that of the first stage in order to lower the dilution rate of the second stage than that of the first stage, PHB content and yield were increased, while PHB production rate per unit volume was decreased as shown in Table 3.

Table 2. Optimization results for various inlet glucose concentrations at the first stage by complex method

G_{F1} (g/L)	N_{F1} (g/L)	D_1 (h^{-1})	D_{02} (h^{-1})	P_1/X_1 (wt %)	D_1P_1 (g/L/h)	P_2/X_2 (wt %)	D_2P_2 (g/L/h)	$Y_{P/G}$* (g/g)
200	21	0.049	0.008	39.5	0.86	54.3	1.57	0.10
500	36	0.016	0.005	59.3	1.06	69.4	1.65	0.14
700	42	0.011	0.004	65.2	1.05	73.6	1.55	0.15
800	45	0.009	0.003	67.3	1.03	75.0	1.49	0.16

*Based on total amounts of inlet glucose

Table 3. Effects of fermentor volume ratio (V_2/V_1) in the two-stage continuous culture of *Alcaligenes eutrophus* NCIMB 11599

V_2/V_1	X2 (g/L)	P_2 (g/L)	G_2 (g/L)	P_2/X_2 (%)	D_2P_2 (g/L/h)	$Y_{P/G}$
0.5	75.0	44.8	118.8	59.8	2.86	0.105
1.0	83.3	53.2	74.9	63.8	1.70	0.125
2.0	92.9	62.7	28.6	67.5	1.00	0.147
3.0	95.0	64.9	11.0	68.3	0.69	0.152
5.0	92.0	61.9	3.4	67.2	0.40	0.145
10.0	80.5	50.3	0.7	62.5	0.16	0.118

By these optimization results, we found that phasewise continuous culture method was not always optimal for the production of secondary metabolites. This concept can be applied effectively to the production of secondary metabolites by microorganisms where specific cell growth rate is greater than specific production rate of secondary metabolites.

3. Effects of Operation Variables

Effects of the inlet glucose concentrations of the first stage on the PHB production rate were investigated. For various inlet glucose concentrations of the first stage, the manipulated variables, D_1, N_{F1}, and D_{02} were optimized as shown in Table 2. The PHB production rate became lower from the inlet glucose concentrations of the first stage became higher or lower than the optimal value (343 g/L), but PHB content and PHB yield increased as the inlet glucose concentration of the first stage increased. This is due to the fact that optimal dilution rate of the first stage became lowered, in other words, the retention time of the cell increased as the inlet glucose concentrations of the first stage increased. High PHB content and yield could be achieved by increasing the inlet glucose concentrations of the first stage, although PHB production rate were not maximized.

The fermentor volume ratio (V_2/V_1) was varied from 0.5 to 10 as shown in Table 3. As the volume ratio increased, the glucose concentration and the PHB production rate in the second stage decreased rapidly. PHB concentration, PHB content, and PHB yield from glucose had their maximum value at the fermentor volume ratio of 3. In the fermentor volume ratio of 3, the PHB content, PHB yield, and PHB production rate were 68.26% of dry cell weight, 0.15 (g/g), and 0.69 g/L/h, respectively. The reason that there is an optimal fermentor volume ratio to maximize PHB concentration, PHB content, and PHB yield in the second

Table 4. Comparison of the optimization results for the single-stage and the two-stage continuous culture systems of *Alcaligenes eutrophus* NCIMB 11599

System	D_1 (h^{-1})	G_{F1} (g/L)	N_{F1} (g/L)	D_{02} (h^{-1})	P/X (%)	DP (g/L)
Single-stage	0.016	521	35	-	60.4	1.07
Two-stage	0.026	343	29	0.006	63.8	1.70

stage is as follows; As the fermentor volume ratio of the second stage increased, PHB content increased due to the increase of the cell residence time. However, PHB accumulation rate became less than PHB degradation rate due to the decrease of carbon source concentration in the second stage by increasing of fermentor volume ratio and then PHB content and PHB yield decreased. At the volume ratio of 0.5, the maximum PHB productivity was 2.86 g/L/hr, which is the highest among the reported values on PHB [Suzuki et al., 1986; Lee and Chang, 1993].

4. Comparison of the Performances of the Single-stage and the Two-stage Continuous Culture Systems

In order to compare the efficiency of the two-stage continuous culture with that of the single stage continuous culture, the optimum dilution rate, inlet glucose concentration, and inlet nitrogen source concentration of the single stage continuous culture which maximize PHB production rate were searched. The simulation results of two systems were compared in Table 4. The optimal values of dilution rate, inlet glucose concentration, and inlet nitrogen source concentration of the single-stage continuous culture were 0.016 h^{-1}, 521 g/L, and 35 g/L, respectively. In this condition, PHB production rate, PHB content, and PHB yield were 1.07 g/L/h, 60.4% of dry cell weight, and 0.13 (g/g), respectively. Comparing the optimization results of the two-stage continuous culture with that of the single-stage continuous culture, the PHB production rate and PHB content were higher in the two-stage continuous culture than in the single-stage continuous culture.

NOMENCLATURE

D : dilution rate [h^{-1}]
d : group of manipulative variable [-]
F : volumetric feed rate [L/h]
G : carbon source concentration [g/L]
G_F : inlet carbon source concentration [g/L]
K_G : saturation constant of carbon source for the growth rate of residual biomass [g/L]
K_{GI} : inhibition constant of carbon source for the growth rate of residual biomass [g/L]
K_{GP} : saturation constant of carbon source for the PHB production rate [g/L]
K_I : inhibition constant of nitrogen source for the PHB production rate [g/L]
K_N : saturation constant of nitrogen source for the growth rate of residual biomass [g/L]
K_{NI} : inhibition constant of nitrogen source for the growth rate of residual biomass [g/L]
m : maintenance energy coefficient [hr^{-1}]
N : nitrogen source concentration [g/L]
N_F : inlet nitrogen source concentration [g/L]
P : PHB concentration [g/L]
$(P/X)_m$: maximum PHB content [g-PHB/g-dry cell]
R : residual biomass concentration [g/L]
S : substrate concentration [g/L]
s : group of state variables [-]
t : culture time [h]
V : fermentor volume [L]
X : total cell mass concentration [g/L]
Y : yield coefficient [g/g]

Greek Letters

α : exponent in specific PHB production rate
μ : specific growth rate of residual biomass [h^{-1}]
$\hat{\mu}$: proportional coefficient of specific growth rate of residual biomass [h^{-1}]
ν : specific PHB production rate [h^{-1}]
$\hat{\nu}$: proportional coefficient of specific PHB production rate [h^{-1}]
ρ_G : solubility of carbon source [g/L]
ρ_N : solubility of nitrogen source [g/L]

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