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Modeling and Optimization of Steroid Transformation in a Mixed Culture

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Summary. Maximization of microbial two-step conversion of 9α-fluorohydrocortisone to Δ^1-dehydro-16α-hydroxy-9α-fluorohydrocortisone (triamcinolone) in a mixed culture of two microorganisms, *Arthrobacter simplex* and *Streptomyces roseochromogenus*, was attempted by a digital simulation to optimize an operational parameter, pH, during batch cultivation.

A mathematical model of the steroid transformation in the mixed culture was constructed with nine differential equations. The kinetic parameters other than the Michaelis-Menten constants in the mathematical model varied with pH of the culture medium. The mathematical model facilitated the simulation of the effect of pH on steroid conversion in the mixed culture. A modified Simplex method of direct search was applied to optimize the switching time and the combination of pH values in the batch culture with several step-changes of pH. Varied-pH processes showed much higher conversion yields than the constant pH process. A process of 3-phase pH change is expected to be advantageous in practical operation because of its lower susceptibility to pH perturbation, though the yield is almost the same as that of the 2-phase process.

Introduction

Many microorganisms have been isolated as effective transformers of steroids (Iizuka and Naito 1967). Several papers have described the application of entrapped cells (Mosbach and Larsson 1970; Larsson et al. 1976; Ohlson et al. 1978; Yamane et al. 1979; Ohmata et al. 1979; Sonomoto et al. 1979) and mixed culture (Lee et al. 1969; Ryu et al. 1969; Lee et al. 1970) to steroid transformation. The mixed-culture method affords a reduction in the number of processes and the process cost.

Lee et al. (1969) reported a double transformation of 9α-fluorohydrocortisone to Δ^1-dehydro-16α-hydroxy-9α-fluorohydrocortisone in a single-stage mixed culture of *Arthrobacter simplex* and *Streptomyces roseochromogenus*. They claimed that the 20-ketoreductase responsible for production of undesired by-products was repressed in the mixed culture.

In this research, we further investigated the kinetics of cell growth, enzyme production and enzyme reaction to obtain a mathematical model and optimize the mixed-culture process. In the mixed-culture system investigated, we found that pH has a remarkable effect on growth rate, synthesis rate of enzymes and the rate of enzyme reaction. An optimal profile of pH change in batch cultivation was investigated through simulation studies. Optimization studies of operational conditions for several pure culture systems have been reported. The temperature profile in penicillin fermentation was optimized by using the Pontryagin principle (Constantinides et al. 1970). For a very complex system like a mixed culture, which includes many interactions between organisms and other variables, the Pontryagin principle appeared to be inadequate. This paper shows a way of handling an optimization problem for a complicated fermentation process by using a simple procedure of direct search, a modified Simplex method (Himmelblau 1968).

Materials and Methods

Organisms

The organisms used were *Arthrobacter simplex* IFO 12069 (ATCC 6946) for 3-ketosteroid-Δ^1-dehydrogenase and *Streptomyces roseochromogenus* (ATCC 13400), which was supplied by Dr. Lee, for 3-ketosteroid-16α-hydroxylase. *A. simplex* was maintained on yeast beef agar slopes and *S. roseochromogenus* on glucose yeast extract agar slants.

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Media

The germination medium E_{33} of the Lee et al. (1969) was used for precultures, and the fermentation medium used was E_{34} of Lee et al. (1969) modified by omitting $CaCO_3$ to facilitate determination of microbial cell concentration.

Cultivation

Cultures for flask experiments were grown in 100-ml portions of medium in 500-ml Erlenmeyer flasks at 28 °C on a rotary shaking machine operating at 150 rpm. The inoculum was grown in two 48-h stages. The inoculum levels of *A. simplex* and *S. roseochromogenus* were 3 and 2% by volume, respectively. Fermentations were carried out in 1.6 l of culture medium in a 2-l jar fermentor (Model M-100, Tokyo Rikakikai Co.) with air flow of 1 vvm and agitation of 500 rpm.

Measurement of Cell Concentration

1. Pure Culture. Cell concentration in the pure culture was determined as dry cell weight.

2. Mixed Culture. The amount of *A. simplex* cells in a mixed culture was determined by density gradient centrifugation. The centrifuged (10 min at 6,000 G) and washed cells from 20 ml of culture broth were resuspended in 5 ml of distilled water. *A. simplex* cells were separated from *S. roseochromogenus* by density gradient centrifugation (10 min at 36,500 G) with Percol solution (Pharmacia Fine Chemicals, Sweden). Five ml of Percol was used for each 2.7 ml of suspension of the washed cells, which had the same cell contents as 4 ml of the original culture broth. *A. simplex* cells were found in the bottom fraction. The cell concentration of *A. simplex* was determined from the optical density reading at 610 mn. The complete seperation of *A. simplex* cells from *S. roseochromogenus* cells was certified by microscopical observation. Furthermore, it was confirmed that the cell mass of each organism was quantitatively recovered from several preparations of mixed cells of two organisms at known ratios. The amount of *S. roseochromogenus* cells was estimated from the difference between the total cell weight and the *A. simplex* cell fraction.

Steroid Assay

The steroid contained in a 5-ml sample was extracted with the same volume of dimethylformamide (DMF) and assayed by high pressure liquid chromatography (HPLC with constant flow pump, Tri-Rotor, Nihon Bunko Co.). The column material used was Nucleosil $7C_{18}$ (Machery-Nagel Co., Gemany). The steroid was eluted with 50% methanol.

Enzyme Assay

The cells obtained from a 30-ml broth sample were washed and resuspended in 29 ml of fresh medium. To this medium, in a 100-ml Erlenmeyer flask, was added 1 ml of steroid substrate solution. The rate of steroid transformation was determined by following the change in substrate concentration during incubation of the reaction mixture at 28 °C. The substrate and pH for each reaction were as follows: Δ^1-dehydrogenation, 9α-fluorohydrocortisone at pH 8; 20-ketoreduction, Δ^1-dehydro-9α-fluorohydrocortisone or Δ^1-dehydro-16α-hydroxy-9α-fluorohydrocortisone at pH 7; and 16α-hydroxylation, Δ^1-dehydro-9α-fluorohydrocortisone at pH 7.

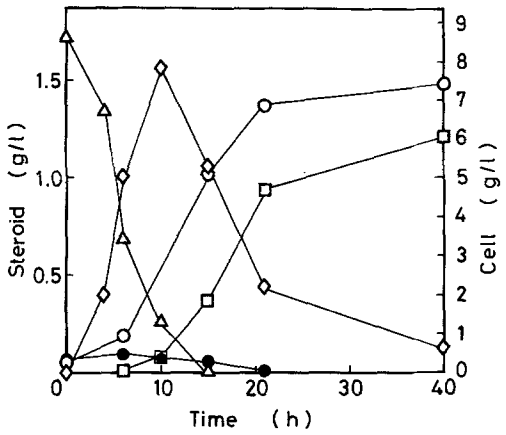

Fig. 1. Time course of the mixed culture of *Arthrobacter simplex* and *Streptomyces roseochromogenus* for the steroid transformation of 9α-fluorohydrocortisone to Δ^1-dehydro-16α-hydroxy-9α-fluorohydrocortisone. •, *A. simplex;* ○, *S. roseochromogenus;* △, 9α-fluorohydrocortisone (F2); ◇, Δ^1-dehydro-9α-fluorohydrocortisone (Δ^1F2), □, Δ^1-dehydro-16α-hydroxy-9α-fluorohydrocortisone (F4)

Results and Discussion

Characteristics of the Mixed Culture

Figure 1 shows a typical result of a batch mixed culture. The growth of *A. simplex* was remarkably suppressed by a product of the competitor, *S. roseochromogenus*. The first steroid substrate, 9α-fluorohydrocortisone (F2), was converted to Δ^1-dehydro-9α-fluorohydrocortisone (Δ^1F2) by the Δ^1-dehydrogenase produced by *A. simplex*. After a 10-h lag, *S. roseochromogenus* produced 16α-hydroxylase, at which time almost all F2 had been converted to Δ^1F2 by the enzyme of *A. simplex*. The 16α-hydroxylase acted on Δ^1F2 to produce the final product, Δ^1-dehydro-16α-hydroxy-9α-fluorohydrocortisone (F4). *A. simplex* also produced 20-ketoreductase, which reduced the carbonyl radical at the 20-position of the steroid F2, Δ^1F2 and F4, resulting in the loss of these steroids as by-products. Thus, suppression of synthesis and inhibition of 20-ketoreductase is necessary for high yield of the product.

Kinetics of Steroid Transformation

All the steroid transformations followed Michaelis and Menten kinetics, as shown in Figs. 2–4. The Michaelis-Menten constant for each reaction remained constant in the pH range from 6 to 8. The substrate dependency of 20-ketoreductase varied with the substrate, as shown in Fig. 3; it catalyzed the reduction of Δ^1F2 faster than that of F4, and the Michaelis-Menten constant for Δ^1F2 was larger than that for F4. Figure 4 shows an important

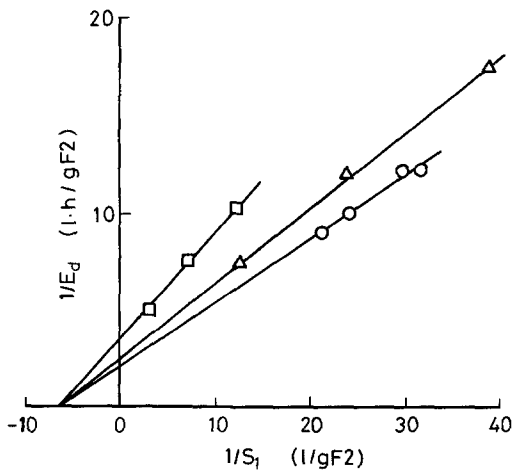

Fig. 2. Double reciprocal plot of substrate dependency of Δ^1-dehydrogenation on F2. □, pH 6; ○, pH 7, △, pH 8

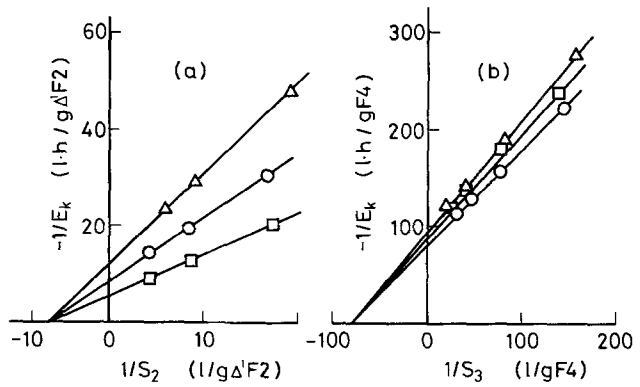

Fig. 3a and b. Double reciprocal plot of substrate dependency of 20-ketoreduction on Δ^1F2 and F4. (**a**) Δ^1F2, (**b**) F4. □, pH 6; ○, pH 7; △, pH 8

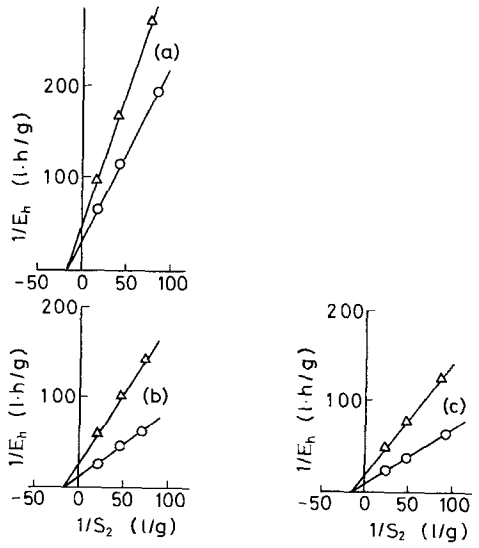

Fig. 4a–c. Difference between the comsumption rate of Δ^1F2 and the production rate of F4 in 16α-hydroxylation. (**a**) pH 6, (**b**) pH 7, (c) pH 8. *Circles* represent consumption and *triangles*, production

feature of 16α-hydroxylation. While the yield was constant, 0.6, irrespective of pH, formation rate of F4 by *S. roseochromogenus* was obviously lower than the consumption rate of Δ^1F2. The reason for this phenomenon is unknown, though it can be speculated that the product F4 was partially converted to another compound, or that Δ^1F2 was converted by another enzyme with the same Michaelis-Menten constant as 16α-hydroxylase. Whatever the case, the consumption rate of Δ^1F2 and the formation rate of F4 clearly had the same K_m values, and this fact led us to use rate equations of the same form for consumption of Δ^1F2 and production of F4.

The action of 20-ketoreductase was inhibited by the product of *S. roseochromogenus*, and the extent of inhibition was remarkably affected by pH; the supernatant of 24-h culture broth showed 69 percent inhibition at pH 6, though only 3.1% at pH 8.

The following equations were derived for the changes in concentration of steroids F2, Δ^1F2 and F4, or S_1, S_2, and S_3, respectively.

$$\frac{dS_1}{dt} = -\frac{f_d E_d S_1}{K_{md} + S_1} - \frac{f_{k1}(1 - \epsilon X_r) E_k S_1}{K_{mk1} + S_1} \tag{1}$$

$$\frac{dS_2}{dt} = \frac{f_d E_d S_1}{K_{md} + S_1} - \frac{f_{k2}(1 - \epsilon X_r) E_k S_2}{K_{mk2} + S_2} - \frac{f_h E_h S_2}{K_{mh} + S_2} \tag{2}$$

$$\frac{dS_3}{dt} = \frac{p f_h E_h S_2}{K_{mh} + S_2} - \frac{f_{k3}(1 - \epsilon X_r) E_k S_3}{K_{mk3} + S_3} \tag{3}$$

where E is volumetric enzyme activity which is equivalent to enzyme concentration, K_m is the Michaelis-Menten constant, f is an activity coefficient, ϵ is a coefficient of inhibition of 20-ketoreductase, which is related to the concentration of *S. roseochromogenus* X_r, and p is the yield factor of 16α-hydroxylation. Both f and ϵ were affected by pH, but p was not.

Kinetics of Growth, Enzyme Synthesis, and Inhibitor Production

Several batch runs of pure cultures of *A. simplex* and *S. roseochromogenus* were carried out at constant pH to investigate the effect of pH on (1) the growth of these organisms, (2) the syntheses of Δ^1-dehydrogenase and 20-ketoreductase by *A. simplex* and 16α-hydroxylase by *S. roseochromogenus*, and (3) the production of the inhibitor by *S. roseochromogenus*.

A quotient of inhibition, γ, was determined as follows. It was assumed that the growth rate of *A. simplex* was suppressed by an inhibitor produced by the competitor and it could be expressed as:

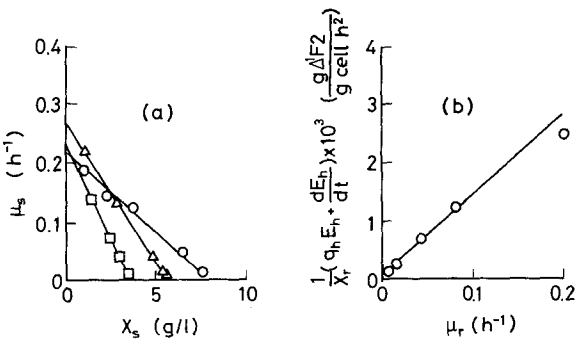

Fig. 5a and b. Examination of the model equations of the growth of *A. simplex* and the production of the enzyme, 16α-hydroxylase. (a) the growth of *A. simplex;* $dX_s/dt = a_s X_s\ (1 - X_s/b_s)$. □, pH 6; ○, pH 7; △, pH 8. (b) the production of 16α-hydroxylase; $dE_h/dt = (\alpha_h \mu_r + \beta_h)X_r - q_h E_h$

$$\frac{dX_s}{dt} = \left(\frac{dX_s}{dt}\right)_c (1 - \gamma) \tag{4}$$

where $(dX_s/dt)_c$ is the growth rate of pure *A. simplex* culture, and dX_s/dt, the growth rate in presence of the inhibitor produced by *S. roseochromogenus.* The concentration of the inhibitor (I) in an arbitrary unit was estimated from the following correlation.

$$\gamma = f_i I \tag{5}$$

where f_i is a relative factor for inhibitory activity as a function of pH. The value of f_i was taken as unity at pH 7.

Many kinetic models for cell growth and product synthesis have been proposed. Kono (1968) and Kono and Asai (1969) developed a simple expression for growth and production in batch cultivation by introducing a time-variable coefficient of growth activity and saturation concentrations of cells and products. Their model held good for batch cultivation in many fermentation processes, but a simpler expression is desired for the system discussed here since it is far more complex than are conventional fermentation systems. For this reason, the logistic model was chosen for the cell growth, and a Luedeking-type expression for the production of the enzymes and the inhibitor. First-order kinetics of enzyme decay was also assumed.

Figure 5 shows the examination of the kinetic models to judge their fitness. For the growth kinetics, the specific growth rate, μ, was plotted against the cell concentration of *A. simplex*, X_s. The linear correlation indicates the good prediction of growth by the logistic model. The values of parameters a_s and b_s were determined from the points of intersection with the ordinate and the abscissa, respectively. The growth pattern of *S. roseochromogenus* could similarly be predicted by the logistic model, although the plots are not shown.

The model equation for the enzyme synthesis was examined, and the result of 16α-hydroxylase synthesis by *S. roseochromogenus* is shown as an example also in Fig. 5. The value of the first-order decay constant, q_h, was estimated by trial and error. A linear correlation between $(1/X_r)(q_h E_h + dE_3/dt)$ and the specific growth rate, μ_r, was obtained from the data of the enzyme activity, E_h, and the cell concentration, X_r, in a pure culture of *S. roseochromogenus.* The values of parameters, α_h and β_h, were determined from the slope of the straight line and the point of intersection with the ordinate. The changes in the activities of the other enzymes, Δ^1-dehydrogenase and 20-ketoreductase of *A. simplex*, and the inhibitor produced by *S. roseochromogenus* could also be predicted from the Luedeking model.

The repression of 20-ketoreductase synthesis by a product of *S. roseochromogenus* was observed, and therefore a repression term was introduced into the model equation of the enzyme synthesis. The repression term was linearly correlated to the cell concentration of *S. roseochromogenus*. The whole mathematical model was developed according to the above discussion.

For the cell concentrations of *A. simplex*, X_s, and *S. roseochromogenus*, X_r, Eqs. (6) and (7) were derived,

$$\frac{dX_s}{dt} = a_s X_s \left(1 - \frac{X_s}{b_s}\right)(1 - \gamma) \tag{6}$$

$$\frac{dX_r}{dt} = a_r X_r \left(1 - \frac{X_r}{b_r}\right) \tag{7}$$

where a and b are the parameters which are affected by pH.

Equations for the volumetric activities equivalent to the concentration of Δ^1-dehydrogenase, 20-ketoreductase and 16α-hydroxylase, E_d, E_k, and E_h, respectively, and the inhibitor, I, are as follows:

$$\frac{dE_d}{dt} = (\alpha_d \mu_s + \beta_d)X_s - q_d E_d \tag{8}$$

$$\frac{dE_k}{dt} = (\alpha_k \mu_s + \beta_k)(1 - \delta X_r)X_s - q_k E_k \tag{9}$$

$$\frac{dE_h}{dt} = (\alpha_h \mu_r + \beta_h)X_r - q_h E_h \tag{10}$$

$$\frac{dI}{dt} = (\alpha_i \mu_r + \beta_i)X_r - q_i I \tag{11}$$

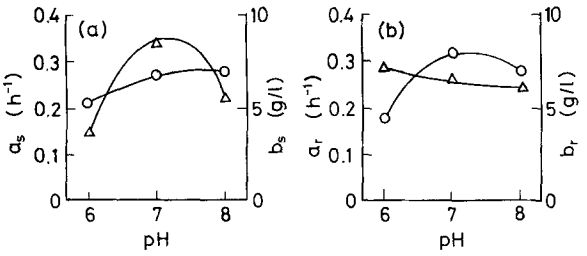

Fig. 6a and b. Effect of pH on the parameters of the rate equation of cell growth. **(a)** *A. simplex*, **(b)** *S. roseochromogenus*. ○, a; △, b; $dX/dt = aX(1 - X/b)$

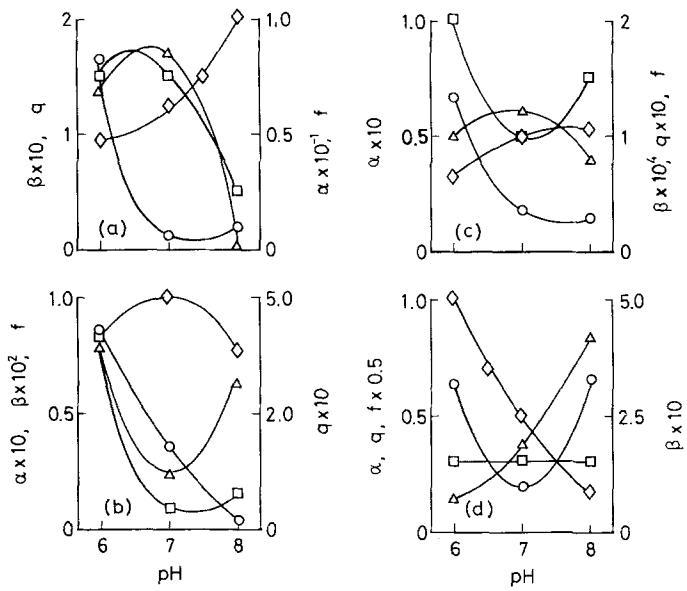

Fig. 7a–d. Effect of pH on the parameters of the activities of the enzymes and inhibitor, and the rate equations of enzyme production and inhibitor formation. **(a)** Δ^1-dehydrogenase, **(b)** 20-ketoreductase, (c) 16α-hydroxylase, (d) inhibitor. ○, α; △, β; □, q; ◇, f

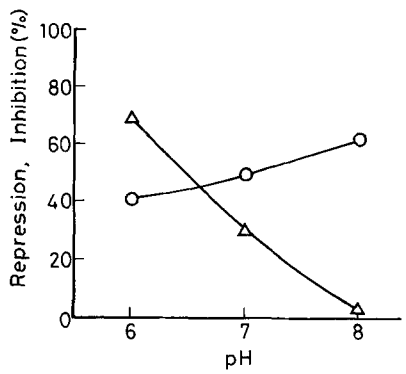

Fig. 8. Effect of pH on the factors of repression of synthesis and inhibition of 20-ketoreductase. ○, repression factor, δ; △, inhibition factor, e

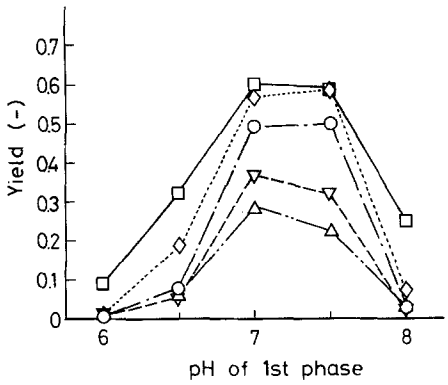

Fig. 9. The maximum yield of F4 during batch culture at various combinations of two pH (simulated results). The pH shift was made when 80% of the substrate steroid had been converted. □, pH 6; ◇, pH 6.5; ○, pH 7; △. pH 7.5; △, pH 8 at the second phase

where μ is the specific growth rate, and α, β, δ and q are the parameters which are affected by pH.

Effect of pH on Kinetic Parameters

Figures 2–4 show that the maximum reaction rate of each enzyme varied with pH in the range from 6 to 8, and that pH did not affect the Michaelis-Menten constant. Also, pH affected the growth rates of the two organisms (see, for example, Fig. 5), and the synthesis rates of the enzymes and the inhibitor.

Selection of an appropriate operational variable is one of the most important steps in optimization of a process. As mentioned above, pH affected all the synthesis rates and enzymatic reaction rates, resulting in big differences in process performance as evaluated by the process time or the final yield of the product steroid. Furthermore, pH is a practically adoptable operational variable, being readily controllable through the widespread installation of pH-meters on fermentors. Thus, pH was selected as the operational variable for the optimization of this steroid conversion process.

Figures 6 and 7 illustrate respectively the pH dependency of the parameters in the rate equations of cell growth, and of those in the synthesis of Δ^1-hydroxylase, 20-ketoreductase, 16α-hydroxylase and growth inhibitor. The pH dependency of the factors of repression of synthesis and inhibition of 20-ketoreductase are shown in Fig. 8. Each parameter must depend on pH for a particular reason, though in some cases the reason is unknown. For our present purpose of optimization, however, it is enough to formulate the pH dependencies of the parameters in a simple mathematical expression without examining the underlying reasons in detail. All the correlations were expressed by second-order or first-order algebraic equations.

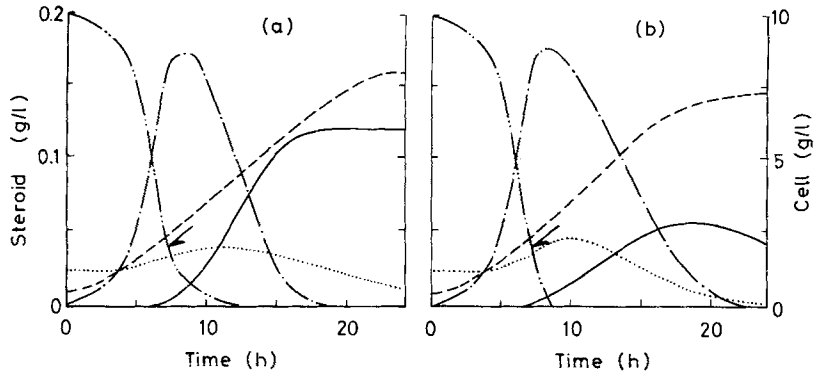

Fig. 10
Simulated results for mixed culture with shift of pH from 7 to 6 (**a**) or 8 (**b**). pH was shifted at the point indicated by the *arrow*, when 80% of F2 had been converted.
..., *A. simplex*; ---, *S. roseochromogenus*; -·-·-, F2; -·- Δ^1F2; ——, F4

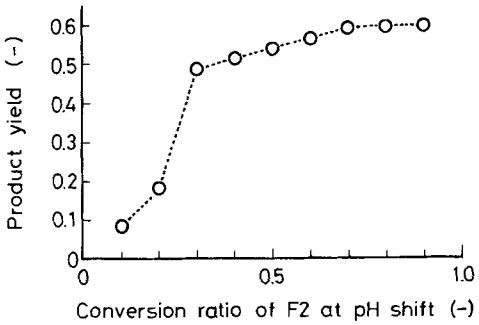

Fig. 11. Effect of the time of pH shift on the yield of the product steroid. pH was shifted from 7 to 6

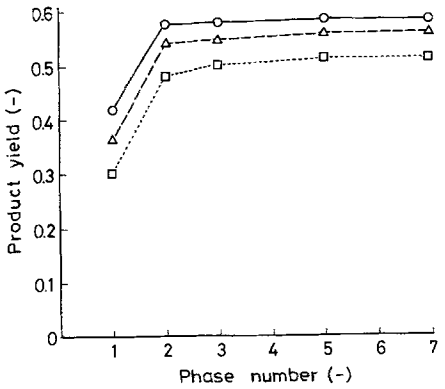

Fig. 12. Effect of the phase number on the final product yield at various cultivation times. ○, 22 h culture; △, 21 h culture; □, 20 h culture

Simulation of Two-pH-Phase Process

The steroid transformation in the batch culture was simulated by use of the mathematical model developed. The time lags of cell growth and the enzyme syntheses were taken into account in calculating the changes in the variables during the cultivation.

A process with two pH-phases was simultaed by dividing the cultivation time into two phases, in each of which pH was kept constant. Figure 9 shows the maximum yield from batch cultures with different combinations of pH. The maximum yield was obtained from the combination of pH 7 and 6 in the first and second phases, respectively. Figure 10 shows the time courses of two simulated batch cultures with first-phase pH of 7 and second-phase pH of 6 or 8. The pH shift was made when 80% of the substrate steroid, F2, had been converted. When pH was shifted to 6, the higher synthesis rate of 16α-hydroxylase (Fig. 7c) caused faster decrease of the intermediate, Δ^1F2, and faster production of F4. Furthermore, the production of 20-ketoreductase was suppressed. In contrast, the shift to pH 8 retarded the conversion of Δ^1F2. The higher pH in the second phase allowed additional growth of *A. simplex* because of lower effect of the inhibitor (Fig. 7d), and the production of 20-ketoreductase by *A. simplex* reduced the yield of F4.

The product yield was also affected by the timing of the pH shift, as shown in Fig. 11. When pH was shifted before 30% of F2 had been converted, the final product yield was remarkably reduced, because of the appearance of 20-ketoreductase and the slow production of 16α-hydroxylase.

Optimization of Multiphase-pH Batch Culture

As mentioned above, batch culture with two pH-phases improved the process performance, especially the product yield, which was much higher than that in batch culture at constant pH. The optimal combination of pH and the optimal switching time of a multiphase-pH batch culture were sought by means of a modified Simplex method (Himmelblau 1968).

Figure 12 shows the product yields of constant-pH and multiphase-pH batch cultures. Batch culture with two pH-phases under the optimal conditions gave much higher product yield than the batch culture at constant pH, but further increase in the number of pH-phases afforded only small increases in the product yield. That is, the yield from a batch culture with two pH-phases is

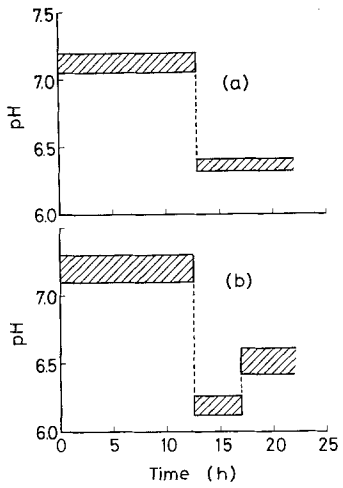

Fig. 13a and b. Tolerable breadth of pH fluctuation for a product yield of 58% in processes with two or three pH-phases. (**a**) 2 pH-phases, (**b**) 3 pH-phases

very close to the marginal value of the yield of 0.6. Consequently, division of the period of batch culture into two phases was enough to improve the product yield.

This process of steroid transformation is, however, highly sensitive to pH, and the value of pH in an actual plant usually fluctuates to some extent. Therefore, for practical application, it is important to examine how the product yield is affected by pH fluctuation. Figure 13 shows the tolerable breadth of pH fluctuation in batch cultures with two and three pH-phases that would guarantee a certain product yield, in this case 58%. This result indicates that a three-phase process is superior to a two-phase process in terms of stability of production, even though only a small increase in product yield could be expected.

In conclusion, from kinetic investigation and simulation of a process of two-step steroid transformation in a mixed culture of two organisms, *A. simplex* and *S. roseochromogenus*, we found: (1) pH of broth showed a remarkable effect on the rate processes: cell growth, enzyme production and enzyme reaction, (2) the process could be simulated by a mathematical model comprising a combination of a simple models: the logistic model, the Luedeking model and the Michaelis-Menten model, (3) batch culture with pH shift can guarantee the maximum yield of the product steroid up to the marginal value by suppressing by-product formation, and (4) multistep pH change in a batch culture allows looser pH control without decreasing the yield.

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Nomenclature

a: rate constant in the logistic model (1/h)
b: saturation constant in the logistic model (g/l)
E: volumetric enzyme activity (unit/l)
f: activity coefficient of an enzyme or an inhibitor (−)
I: volumetric inhibitor activity (unit/l)
k_m: Michaelis-Menten constant (g/l)
p: yield factor of 16-hydroxylation by *S. roseochromogenus* (−)
q: first-order decay constant of an enzyme or an inhibitor (1/h)
s: steroid concentration (g/l)
X: cell concentration (g/l)

Greek Letters

α: constant in the growth-associated term of the Luedeking model (unit/g)
β: constant in the non-growth-associated term of the Luedeking model (unit/g · h)
γ: inhibition coefficient for the growth of *A. simplex* (l/unit)
δ: repression coefficient for 20-ketoreductase synthesis (l/g)
ϵ: inhibition coefficient for 20-ketoreductase activity (l/g)
μ: specific growth rate (1/h)

Subscripts

1: 9α-fluorohydrocortisone (F2)
2: Δ^1-dehydro-9α-fluorohydrocortisone (Δ^1F2)
3: Δ^1-dehydro-16α-hydroxy-9α-fluorohydrocortisone (F4)
d: Δ^1-dehydrogenase
h: 16α-hydroxylase
i: inhibitor
k: 20-ketoreductase
r: *Streptomyces roseochromogenus*
s: *Arthrobacter simplex*

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