Production of 3-Ketosteroid-delta-1-Dehydrogenase by a Two-stage Continuous Culture

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INTRODUCTION

Batch processes are widely used for production of industrial enzymes. Production of constitutive enzymes usually involves primarily the cell growth. On the other hand, optimizing the productivity of inducible enzymes requires a good understanding of regulatory mechanisms involved in the biosynthesis of the enzymes. In some cases, the productivity of inducible enzymes can be increased by a factor as much as one-thousandfold by means of controlling the regulatory mechanisms of enzyme.[1,2]

The continuous process may well be employed for production of enzymes if the optimal process conditions are known.[3,4,5] For certain inducible enzymes, however, the optimal conditions for cell growth do not often coincide with those for induction and production of enzymes.[1,2,6,7] When the optimal set of conditions for cell growth and that for biosynthesis of enzymes are different, the two-stage continuous culture system can be used very efficiently for production of the enzyme.[8~11] For this reason, we have studied the applicability of the principles and inherent advantages of two-stage continuous culture system for the purpose of optimizing the productivity of 3-ketosteroid-delta-1-dehydrogenase. The first stage is used as a growth stage where cells are grown at the optimal growth rate which corresponds to the maximal cell productivity and the second stage is used as the enzyme production stage where the optimal conditions for induction and production of the enzyme are used.

In this paper, some salient design features and optimal conditions of the two-stage continuous process for production of the enzyme are discussed in terms of such parameters as specific growth rate, dilution rate, mode of induction. space time of fermentors, enzyme productivity, specific activity of the enzyme and other environmental conditions.

EXPERIMENTAL METHODS

The organism used is *Arthrobacter simplex* (ATCC 6946). The medium consists of Sheffield-NZ-Amine-A (enzyme-hydrolyzed casein, produced by Sheffield, Kraft Co.) 2%, glucose 0.5%, KH_2PO_4 0.1% and Antifoam-Ucon LB625 (Dow Chemical, Mich.) 0.01%.

The inoculum development procedure is the same as that used by Ryu, et al.[12] Progesterone is used as an inducer. The progesterone dissolved in dimethylformamide is added to the second stage continuously or intermittently to maintain its concentration in the 2roth at a level of 200mg per liter.

The first stage is inoculated with 5% inoculum and is allowed to proceed as a batch culture for about 4 doubling times for initial cell growth. After this initial growth phase phase, the valxe on the transfer line connecting the two stages is opened, the feed of the fresh medium into the first stage is started, and the valve on the over-

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flow line from the second stage is opened. Approximately 8 additional generation times is allowed to establish steady state condition of the two-stage continuous culture system.

Batch culture and single-stage continuous culture systems are also used to study the effect of certain parameters on the enzyme productivity.

The typical operating conditions for the two-stage continuous culture system are:

a. First stage(5-1 New Brunswick Scientific jar fermentor).

Culture Volume	2.0~3.0 liters
Temperature	25±1°O
pH	7±0.1
Agitation	400~600 RPM
Aeration	0.5 VVM
Dilution Rate	0.1~0.18hr^{-1}

b. Second stage(14-1 New Brunswick Scientific jar fermentor).

Culture Volume	8.5~13.5 liters
Temperature	25±1°C
pH	7.4±0.2
Agitation	250~450 RPM
Aeration	0.5 VVM
Dilution Rate	0.02~0.06

The arrangement of the two-stage continuous enzyme process is schematically shown in Figure 1.

ENZYME ASSAY

Enzyme activity was determined spectrophotomentrically by measuring the rate of dehydrogenation of steroid substrate(9α-fluoro-11β, 16α, 17α, 21-tetrahydroxy-4-pregnene-3, 20-dione) in a 50ml reaction mixture. Cells were first centrifuged at 5°C, washed with 1% Tris buffer, washed with acetone and dried under vacuum. The enzyme prepared in the form of whole cell will be referred to as the whole-cell enzyme in this paper. The reaction mixture contained 12.5mg dry cell per ml, 4mg steroid substrate per ml, and 0.67μg menadione per ml. The reaction is carried out on a rotary shaker at 35°C, pH 7.0 to 7.5. The specific activity of the enzyme in the form of whole cell is defined as units per gram of dry cell weight, where one unit of enzyme activity is defined as the amount of enzyme necessary to produce one μ mol of dehydrogenated product, triamcinolone, per minute. The enzyme activity can be calculated from the first order reaction rate constant by following the dehydrogenation spectrophotometrically. The procedures of enzym

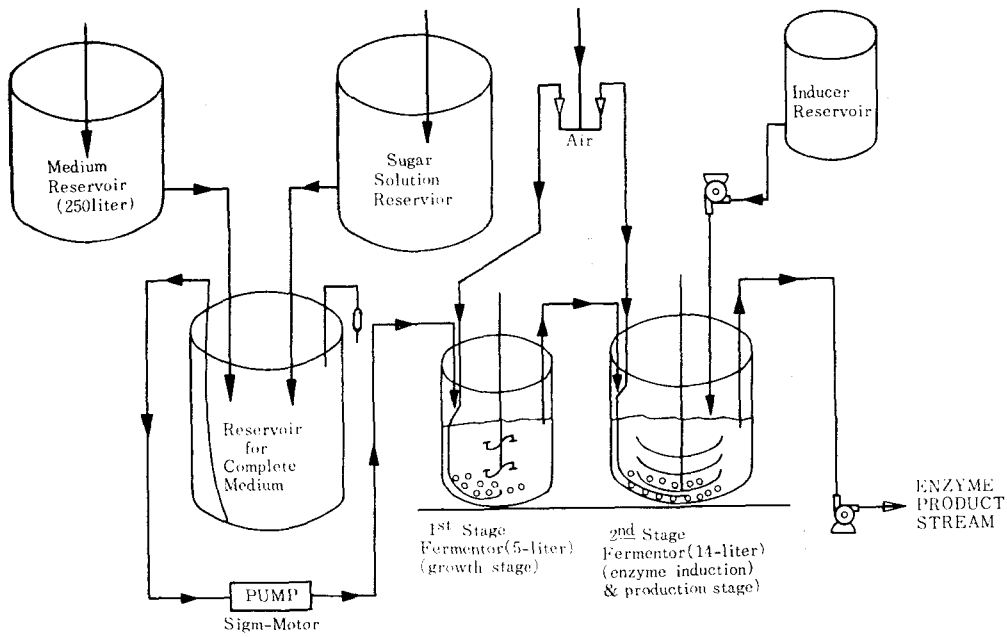

Fig. 1. Schematic Flow Sheet of Two-Stage Continuous Dehydrogenase Process

assay and the steroid assay used in this experiment are the same as those used by Ryu, et al.,[12] Lee, et al.,[13] and Ivashkiv.[14,15]

The method described by Erickson, et al.[16] was used to prepare acetone dried dehydrogenase in the form of wholecell enzyme.

RESULTS AND DISCUSSION

Batcch Culture System

The effect of the solvent, dimethylformamide, used to dissolve the inducer on cell concentration was studied. The solvent without the inducer is added to the flask culture and the growth in terms of cell concentration is compared with that of control flask which contained on solvent. A similar result was found from continuous culture experiment when an excessive amount(greated than 0.05% by volume) of solvent is accumulated in the vessel. The result indicated that the solvent lowers the cell concentration by as much as 1/2. This inducer solvent is found to be incompatible with a good growth of cells.

Also studied was the effect of the mode of addition of inducer on the enzyme activity. was examined in a batch cultre without the inducer solvent. The results are shown in Figure 2. The relative enzyme activity is the highest when the inducer is added at about five generation times after the inoculation, and when no longer than egiht generation times is allowed for the inducer to be in contact with the cells. The relative enzyme activity is defined as the ratio of enzyme activity to the maximum enzyme activity. Practcally no activity is found when inducer is added at the time of inoculation. Very low enzyme activity is found if longer than eight generation times is allowed for cells to remain in the fermentation broth prior to separation of cells.

The effect of medium concenration was examined also. In a batch culture, the high concentration of medium 5 times as much as the control experiment resulted in little or on enzyme activity as shown in Figure 3 despite the high cell concentration. This result indicated a possibility of repression effect of medium on the enzyme biosynthesis.

Based on these results obtained from the batch

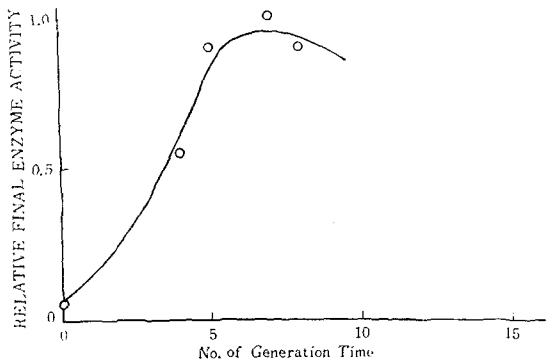

Fig. 2. Mode of Induction:
Relative enzyme activity and the time of inducer addition

culture experiments, we were able to conclude that:

1. Too high a concentration of medium causes little or no production of enzyme. This appears to be due to the catabolite repression.

2. The time of induction, the inducer solvent and the time repuired for the enzyme to be induced fully are all important to the productivity of enzyme.

These findings have led us to consider the two-stage continuous culture system as a potential production tool for the dehydrogenase. When the growth stage and the enzyme production stage are separated, we can readily provide a different set of optimal operating conditions for each.

The attractive features of two-stage continuous culture system considered were:

1. We can operate the first-stage continuous culture system at the optimal dilution rate that corresponds to the maximum cell productivity without the adverse effect of inducer solvents on the enzyme productivity.

2. We can adjust and control the medium conceutration of the feed as well as the feed rate in such a way that the repression effect of the medium can be eliminated or reduced in the second stage.

3. We can readily control and adjust the mode of enzyme induction in the second stage. The space time of the second stage should correspond

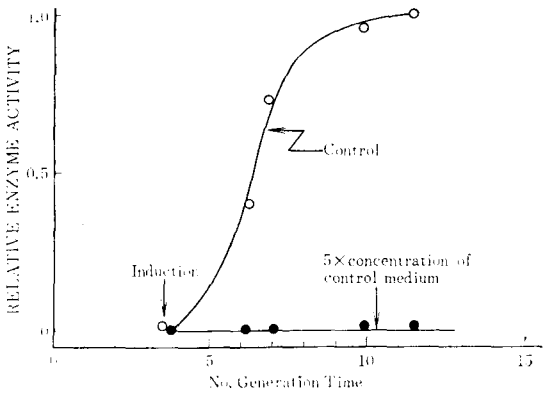

Fig. 3. Effect on Medium Concentration on Enzyme Activity

to the time required for the enzymes to be fully induced.

Single-Stage Continuous Culture System

Single-stage continuous culture system was used to determine the optimal dilution rate that corresponds to the maximal cell productivity. This experiment was carried out without the inducer or the solvent for theinducer. Using the single-stage continuous culture system, the cell at varying dilution rates were measured and the cell productivity, defined here as $[(X_1)(D_1)]$, was calculated and plotted in Figure 4. The optimal range of dilution rate for the first stage was found to be $0.16 \sim 0.18\ hr^{-1}$.

These results suggest that for the cell growth stage(first stage) the value of dilution rate should be about $0.17 hr^{-1}$, if cell productivity is to be maintained at its maximum level.

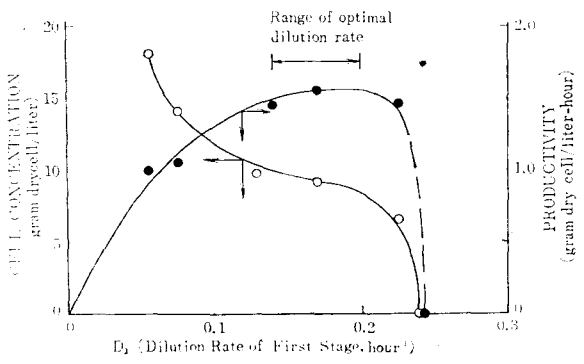

Fig. 4. First-Stage Cell Productivity

Two-Stage Continuous Culture System

The mathematical expression for a two-stage continuous culture system can be given as Equations (1) and (2).

$$Fx_0+\mu x_1 V_1-Fx_1=V\cdot\frac{dx_1}{dt} \tag{1}$$

$$Fx_1+\mu_2 x_2 V_2-Fx_2=V_2\frac{dx_1}{dt} \tag{2}$$

Where F=flow rate(liter/hr)

x_0=cell concentration in feed stream(g/liter).

x_1&x_2=cell concentration in the second stage fermentor, respectively(g/liter).

μ_1&μ_2=specific growth rate in the first and second stage fermentor, repectively (g/liter).

V_1&V_2=the culture volume in the first and second stage fermentor, respectively (liter).

t=time(hr)

Once the system attains a steady state.

$$\left.\begin{aligned}\mu_1&=D_1\\ \theta_1&=\frac{1}{D_1}\end{aligned}\right\}\ \text{for the first stage}\quad (3),\ (4)$$

and,

$$\left.\begin{aligned}\mu_2&=D_2\left(1-\frac{x_1}{x_2}\right)\\ \theta_2&=\frac{1}{D_2}\end{aligned}\right\}\ \text{for the second stage}\quad (5),\ (6)$$

Where:

D_1 and D_2=dilution rate for the first and second stage, respectively(hr^{-1}).

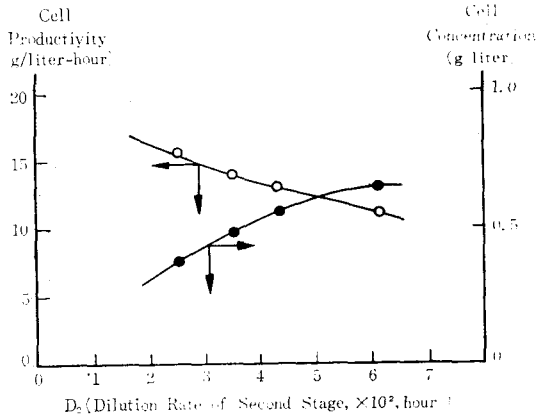

Fig. 5. Cell Concentration and Productivity in the Second Stage.

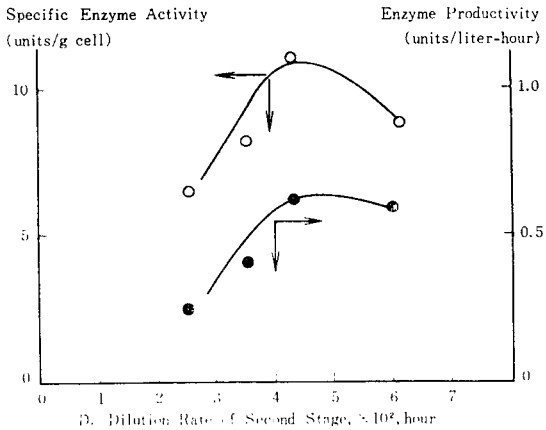

Fig. 6. Enzyme Productivity

θ_1 and θ_2=the space time of the first and second stage, respectively(hr).

Since the range of the optimal dilution rate of the first stage(D_1) was found to be about 0.17 hr^{-1}, this value of D_1 was maintained constant while the enzyme productivities at varying dilution rates of the second stage(D_2) were experimentally measured.

Since $D_2=F/V_2$, the value of D_2 may be varied by changing either the flow rate, F, or the culture volume V_2.

For the low dilution rater of the second stage ($D_2<0.03$) 2-liter culture volume in the first stage was used, and 3-literuculture volume in the first stage was used for the higher dilution rates($0.03<D_2<0.06$). From this experiment, the cell concentration and the cell productivity of the second-stage were measured and the results plotted in Figure 5. In the same experiment, the specific enzyme activity (measured as units perg of dry cell) was determined, and the effect of D_2(dilution rate in the second-stage) on the enzyme productivity is calculated and plotted in Figure 6. This result suggests that the range of D_2 0.04 and 0.05 hr^{-1} gives the maximum enzyme productivity.

The specific growth rate in the second stage, μ_2, that corresponds to the optimal enzyme productivity (i.e. at $D_2=0.045$ hr^{-1}), was found to be about 0.14hr^{-1} according to Equation (5). At this growth rate the time required for cells to double its concentration is estimated to be 49 hours according to Equation (6). The dilution rate in the second stage, 0.045hr^{-1}, represents space time of about 22 hours which in turn corresponds to the mean residence time. Thus the space time allowed in the second stage is approximately 1/2 of the doubling time under the given operating conditions. This represents, as a bulk average, only 31% increase in cell concentration during the period of 22 hours in the second stage. This result is in good agreement with Penasse.[17] They reported that the thim required to bring about the maximum beneficial eflect of inducer is about 20 hours.

The optimal ratio of first and second stage fermentor sizes can be estimated. Since the optimal values of D_1 and D_2 are found to be 0.17 and 0.045hr^{-1} respectively, for a desired throughput (i.e. given flow rate, F), the volumetric ratio should be,

$$V_2/V_1=D_1/D_2=3.8 \quad (7)$$

Thus, the fermentor size of the second stage should be 3.8 times greater than the first stage fermentor if the optimal set of conditions are to be satisfied.

The observation that the enzyme productivity goes through a maximum in the continuous culture system is highly likely due to the balance between the opposing effects of induction and repression as was suggested by Clarke, et al.[18] for the case of amidase system. They observed a similar phenomenon and attributed this to the induction effect at lower side of dilution rate and to the catabolite repression effect at the higher side of dilution rate. There is a possibility that the nutrient may begin to accumulate in the second stage as dilution rate increases even in this two-stage continuous culture system, although it is by far lower than that likely to be found in a single-stage continuou culture system.

Under this optimal set of operating conditions, the two-stage continuous culture process was tested for a prolonged period of(10 days) operation, and the enzyme productivity was maintained near the maximal level for the period tested. The summary of this experimental run is shown in Table 1.

TABLE 1 RESULTS OF TWO-STAGE CONTINUOUS CULTRE EXPERIMENT

Sample Number	X_2 Cell Conc. (g/l)	D_2 Dil. Rate h(r^{-1})	Cell Productivity (g/l-hr)	Spec. Enz. Activity (u/g-cell)	Enz. Productivity (u/l-hr)	Relative Enz. Product*
1—26—70 (0 day)	Experiment	Commenced				
1—28—70 (2 days)	13.1	0.043	0.56	11.0	6.15	1.0
2—3—70 (8 days)	14.0	0.05	0.7	6.4	4.5	0.73
2—6—70 (11 days)	12.4	0.046	0.57	8.8	5.0	0.81

* Relative enzyme productivity=(enzyme productivity/maximum enzyme productivity)

This result indicates that the two-stage continuous culture system can be employed satisfactorily for production of the dehydrogenase for a priode of about 85 generation times. Although the enzyme productivity tends to fall somewhat after about 6 days, a reasonably high enzyme productivity was mainatined for the period of 10 days. This result obtained from two-stage continuous culture system indicates that a significant advantage in terms of process time required can be found if compared with a batch process. As an indicator of comparison between the batch and continuous system, the ratio of production rate of continuous to that of a batch process (G) was calculated by using the formula of the type given in Aiba et al. [9]

$$G=\ln\frac{x_m}{x_i}+t_L\mu_m \qquad (7)$$

where: t_L=the batch cycle time minus the period of logarithmic growth (48 hours).

μ_m=maximum specific growth rate (0.23 hr^{-1}).

x_m/x_i=the ratio of maximum to initial cell concentration (20)

This value of the ratio of production rate is estimated to be about 14 in favor of the continuous process.

SUMMARY

We have studied the applicability of the principles and inherent advantages of the two-stage dontinuous uclture technique to an enzyme process for the purpose of improving and optimizing the productivity of 3-ketosteroid-delta-1-dehydrogenase.

By using a two-stage continuous culture system, the growth st ageand enzyme produdtion stage are separated. In each stage an optimal set of toperaing conditions was determined, and this was tested for feasibility for the period of 10 days. During this period, at least 70% of the maximum enzyme productivity could be maintained. The important design parameters studied are: (1) optimal specific growth rate in the first stage which corresponds to the maximal cell productivity, (2) the optimal dilution rate in the second stage which in turn determines the size of second stage fermentor and the mean residence time of cells in the second stage, (3) cell concentration in both stages, and (4) the specific enzyme productivity and enzyme productivity of the second stage. In addition, by using two-stage continuous culture system we have been able to reduce or eliminate the effect of catabolite repression due to high medium concentration and the adverse effect of the solvent used to dissolve the inducer. We have found the balance between the opposing effects of induction and repression in the second stage judging from the observation that the enzyme productivity goes through a maximum.

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