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Controlled Fed-Batch Cultivation of *Escherichia coli* Mutant for L-Tryptophan Production

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대장균 변이주의 조절식 유가배양법에 의한 L-트립토판 생산

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For optimal production of L-tryptophan using a regulatory mutant of *Escherichia coli* the relationship between product formation and acid production was investigated. Experimental results showed that the production level of L-tryptophan was lowered as the specific acid production rate increased. In order to reduce the amount of acid produced during the fermentation, a controlled fed-batch fermentation was employed. In this fed-batch process, the feed rate of the nutrient feed medium was controlled in relation to the oxygen level in the culture and thus the growth of the cells was regulated in such a way that the oxygen demand of the culture could not exceed the oxygen supply. When *E. coli* cells were cultivated in a controlled fed-batch mode of fermentor operation, the specific acid production rate was significantly reduced and L-tryptophan production was increased as much as five times that obtained in a conventional fed-batch fermentation.

The first step in developing a fermentation process to produce a desired metabolite in high yields might be to define conditions that would provide efficient cell growth and product formation. In increasing the cell mass or product concentration, however, there are several constraints such as limitations in dissolved oxygen and by-product formation. To overcome such problems various techniques have been developed by many investigators, including use of oxygen-enriched air (1,2), semi-batch culture with cell separator (3), dialysis culture (4), and optimal feeding of nutrients in fed-batch culture system (5,6).

The principal rationale employed in growing cells to high cell concentrations is to force the cells to utilize the nutrients as efficiently as possible. However, cells grown near the maximum specific growth rate or under dissolved-oxygen limitation frequently produce partially oxidized and usually toxic metabolites (7). Particularly in the case of *Escherichia coli* acetic acid is frequently accumulated in the culture broth to an inhibitory level for the growth (4,8). In order to reduce the accumulation of organic acids it is therefore desirable to restrict the growth of the cells so that the oxygen demand of the culture does not exceed the oxygen supply in the fermentation system.

Most works reported until now, however, have focussed on the effects of acid production on cell growth. As a consequence, we have investigated the

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relationship between the production of organic acids and metabolic product formation, in our case L-tryptophan production, using a regulatory mutant of *Escherichia coli*. In order to circumvent the production of acids a controlled fed-batch mode of fermentor operation has been employed for the cultivation of *E. coli* and the results were compared with those obtained in a conventional fed-batch fermentation.

Materials and Methods

Bacterial strains and media

E. coli strain TA-40-10 was used throughout the work. Details on the characteristics of *E. coli* TA-40-10 have been described previously (9). The compositions of fermentation media used in this study are summarized in Table 1.

Analytical methods

Glucose and tryptophan concentration were determined by DNS method and DMAB method respectively, as reported previously (10). Optical density (OD) of the culture broth was determined at 540 nm with a spectrophotometer (Baush & Lomb Spectronic 20) and dry cell weight (DCW) was determined as previously described (10).

Culture method

Fed-batch fermentation was carried out in a 5-liter jar fermentor. A loopful of cells grown on agar slant was transferred into 100 m*l* of Luria broth and precultured overnight at 31 °C in an incubator shaker. Seed culture broth was then aseptically transferred into a fermentor. The pH of the culture was controlled automatically at 7.0 with 4N NaOH and the temperature was maintained at 30 °C. Air flow rate and agitation speed were changed depending on the experiments. The dissolved oxygen (DO) concentration and the pH were monitored continuously. In case that the fermentation was carried out in a conventional fed-batch mode of operation, glucose (30g) and phosphate (1.5g) were supplemented to the culture broth when the glucose concentration in the medium was reduced to about 10g/*l*. In a controlled fed-batch mode of operation the feed medium was supplied with a pump which was connected to the DO control system. When the oxygen level in the culture broth rose, due to slowing down of growth and deminished demand for oxygen, the feeding pump was actuated. As soon as the oxygen level dropped below a predetermined value, resulting from the demand generated by accelerated growth, the feed rate of the medium was decreased. Thus, the concentration of glucose was kept at a level that restricted growth and thereby reducing the oxygen demand and the required oxygen supply. The regulated growth phase was preceded by an

Table 1. Medium composition for fed-batch and controlled fed-batch fermentation

Component	Fed-batch fermentation[a]		Controlled fed-batch fermentation	
	Base medium (g/*l*)	Feed medium (g)	Base medium (g/*l*)	Feed medium (g/*l*)
Glucose	50.0	30.0	5.0	400.0
NH_4Cl	8.0		3.0	50.0
$MgCl_2 \cdot 6H_2O$	1.0		1.0	8.0
K_2SO_4	0.4		1.0	
$FeCl_3 \cdot 6H_2O$	0.015		0.05	
K_2HPO_4	3.0	1.5	7.0	
KH_2PO_4			8.0	
Sodium citrate	1.2		1.0	
Yeast extract	1.0		5.0	
Trace element[b]	A		B	

(a) Base medium of fed-batch fermentation is identical to F medium used in previous experiments (9).

(b) A: 1 m*l* of stock solution composed of $(NH_4)_6Mo_7O_{24}$: 30 μM, H_3BO_3; 4 mM, $CuSO_4$; 0.1 mM, $MnCl_2$; 0.8 mM, $ZnSO_4$; 0.1 mM

B: $CaCl_2 \cdot 2H_2O$; 50 mg/*l*, $ZnCl_2 \cdot 4H_2O$; 10 mg/*l*, $CoCl_2 \cdot 6H_2O$; 10 mg/*l*, $Na_2MoO_5 \cdot 2H_2O$; 10 mg/*l*, $CuSO_4 \cdot 5H_2O$; 10 mg/*l*, H_3BO_3; 10 mg/*l*.

initial uncontrolled growth in the basal medium in which the concentration of glucose was lower than that used in a conventional fed-batch culture (see Table 1 for composition of the medium). During the regulated growth phase the feed medium was added to the culture to an extent that would not cause the culture to enter into a state of oxygen deficiency by regulating the feed rate depending on the oxygen level.

Results and Discussion

Acid production during the fermentation

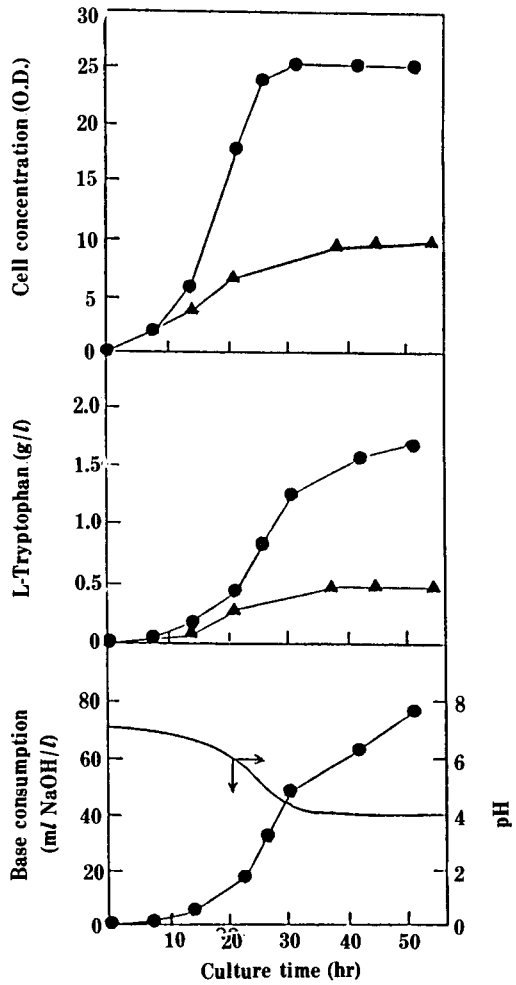

Fig. 1. Profiles of cell growth, tryptophan formation and acid production.

(●) with pH control at 7.0, (■) without pH control (pH change is shown in a solid line)

Fed-batch fermentations were carried out at 30 °C, 400 rpm, and 0.3 vvm.

The results of fed-batch fermentation with and without pH control are presented in Fig. 1. When the fermentation was carried out without pH control, the pH of the culture broth dropped gradually until the cells stopped the growth and then remained relatively constant at pH4.0. This suggests that a large amount of acids was produced during the fermentation and that the amount of acid production was highly dependent on the cell growth rate.

More direct evidences for acid production during the fermentation were provided by the pH-controlled fermentation experiment. In this experiment the cells were cultivated in the fermentor equipped with a pH controller, and the amount of base (4N NaOH) added to the fermentor was continuously monitored so as to measure the amount of acid produced by the microorganism. Since the pH of the culture broth was tightly regulated at a constant value (7.00 ± 0.05), the amount of base consumed to control pH could be set equal to the amount of acid produced during the fermentation. As shown in Fig. 1, the rate of acid production was increased as the cell concentration increased and then became constant after the cell growth reached its maximum. Since fermentation characteristics of microorganisms strongly depend upon environmental conditions the effects of environmental variables on acid production were further examined.

Effect of aeration rate on acid production and product formation

The production of acid by *E. coli* cells might be related to oxygen level in the fermentor. To examine this possibility fed-batch experiments were carried out at different aeration rates, keeping the other fermentation variables constant. The results are shown in Fig. 2. Under anaerobic condition both cell growth and tryptophan production were very low as expected. But under aerobic conditions the accumulation of L-tryptophan was higher at the aeration rate of 0.3 vvm than at 0.6 vvm while the growth patterns of the cells were almost identical in both cases. It was noticed, however, that the amount of acid produced during the fermentation was different depending on the aeration rates. As shown in Fig. 2, more acids were produced when the cells were cultivated at the aeration rate of 0.6 vvm.

The experimental results in Fig. 2 were reanalyz-

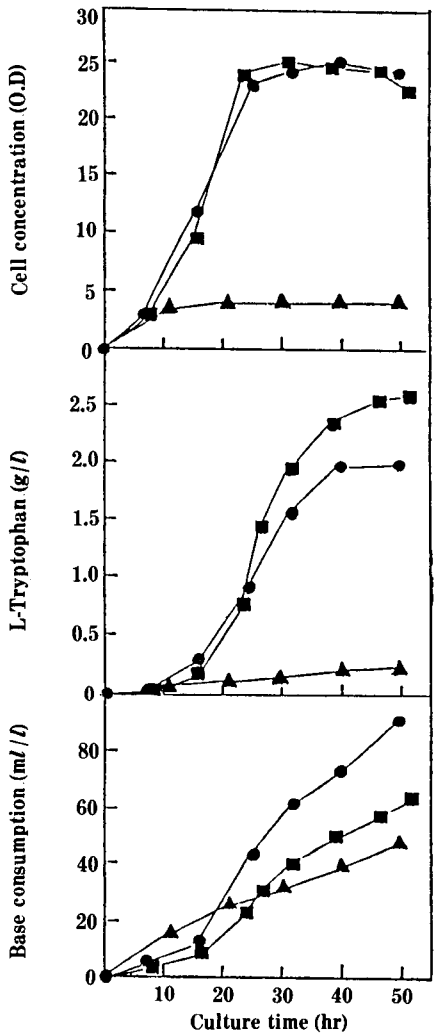

Fig. 2. Effect of aeration on cell growth, tryptophan production and base consumption.
(●) 0.6 vvm, (■) 0.3 vvm, (▲) anaerobic culture
Fed-batch fermentations were carried out at 30°C, 600 rpm and pH 7.0.

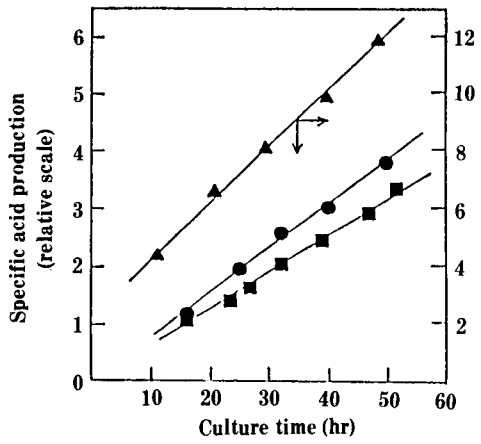

Fig. 3. Specific acid production at different aeration rates.
Symbols are the same as in Fig. 2.

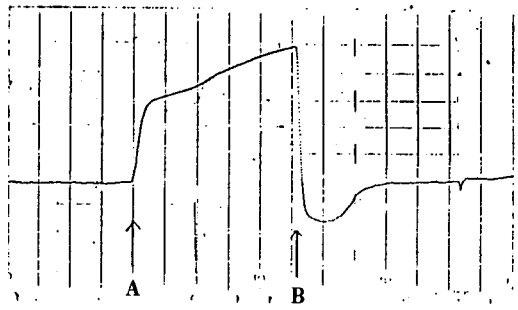

Fig. 4. A typical response of the dissolved oxygen concentration to the variations in glucose concentration.
The arrows A and B indicate the time when glucose is depleted in the medium (A) and when additional glucose is fed into a fermentor (B) respectively.

ed by introducing the concept of "specific" acid production. As mentioned earlier, acid production was closely associated with the growth of the cells and therfore the use of specific acid production would be desirable to eliminate the effect of cell mass on the acid production level. In Fig. 3 profiles of specific acid production during the fermentation are presented to illustrate the differences in the rate of acid production at different aeration rates. Comparision of specific acid production rate with tryptophan production levels reveals that a decrease in specific acid production rate causes an increase in tryptophan production. In view of this analysis, reduction of acid production might be a key factor in increasing the production level of L-tryptophan. As a consequence, we decided to employ a controlled fed-batch mode of fermentor operation to prevent the oxygen limitation and thereby reducing the amount of acid produced during the fermentation.

Controlled fed-batch fermentation

In this work, oxygen-level-dependent control technique was used for regulating the nutrient feed rate in the controlled fed-batch fermentation. This method was originally proposed by Gleiser and Bauer (11), but modified in this experiment. The feed rate of the nutrient feed medium (see Table 1 for medium composition) was adjusted in relation

to the oxygen level in the culture and the culture was sparged with air instead of using oxygen-enriched air. Also the concentration of carbon source in the culture broth was maintained in the range of 0.5-1.5 g/*l* during the regulated growth phase, which was an order of magnitude lower than that employed by Gleiser and Bauer.

The basic principle of the oxygen-level linked nutrient feeding system is as follows. When glucose is depleted in the medium the dissolved oxygen concentration in the culture rises due to diminished oxygen demand. If additional glucose is introduced in the culture then the level of dissolved oxygen gradually decreases due to further growth of the cells. Fig. 4 illustrates a typical example. The regulation of cell growth could be therefore achieved by adjusting the nutrient feed rate in such a way that the feed rate was increased when the oxygen level rose above the predetermined value and decreased when the oxygen concentration in the culture fell below it.

In Fig. 5 the profiles of dissolved oxygen concentration, cell density, tryptophan concentration, acid production, glucose consumption and product yield are shown for a culture of *E. coli* strain TA-40-10 grown in a controlled fed-batch mode of fermentor operation. For comparision, the results obtained in a conventional fed-batch mode of ope-

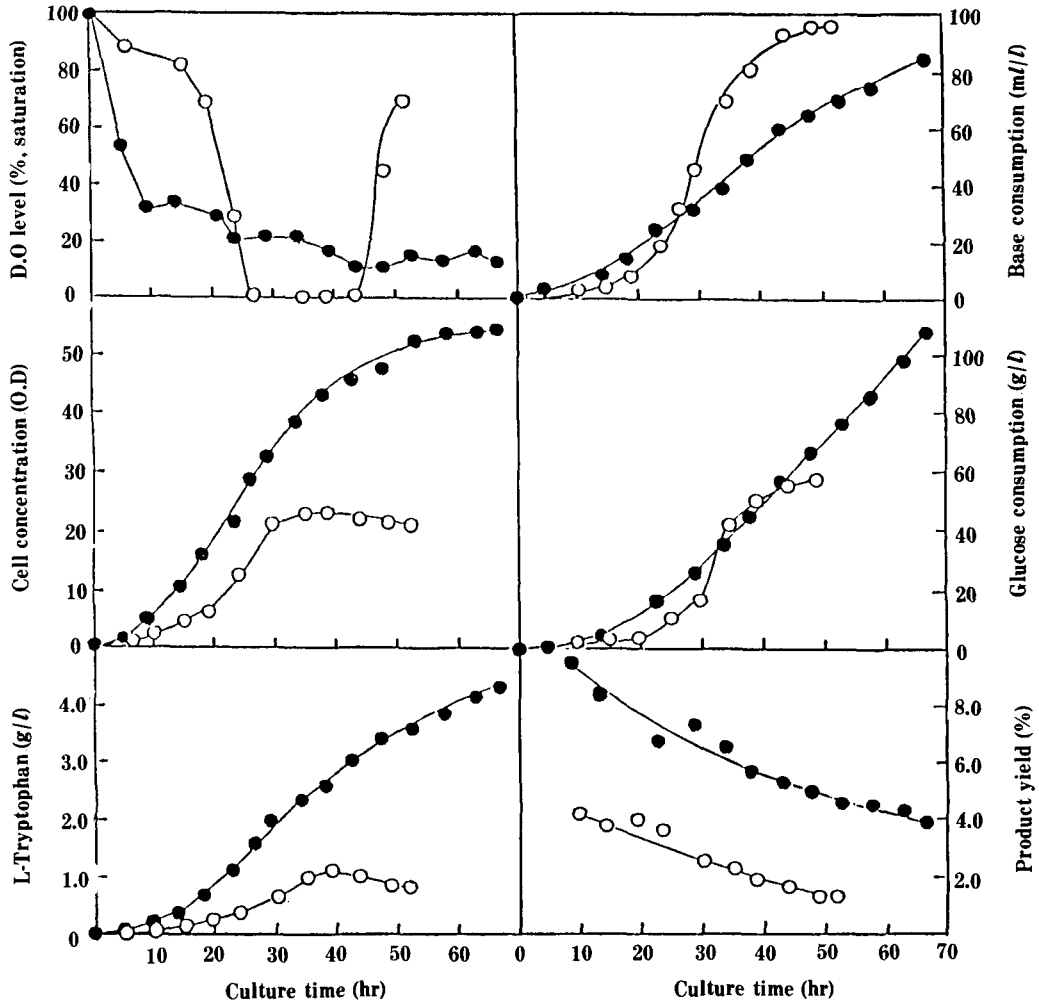

Fig. 5. Comparison of the changes in fermentation parameters related to tryptophan production under different mode of fermentor operation.

(○); conventional fed-batch operation, (●); controlled fed-batch operation.

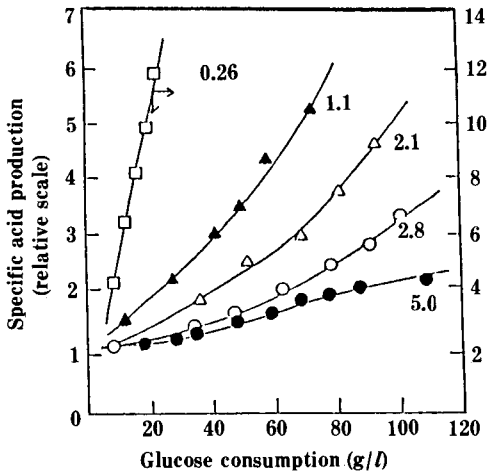

Fig. 6. Plots of specific acid production versus glucose consumption for fed-batch fermentations of *E. coli* under different culture conditions.

(□) anaerobic culture (conventional fed-batch)
(▲) 1.15 vvm (〃), (△) 0.6 vvm (〃), (○) 0.3 vvm (〃), (●) 1.15 vvm (controlled fed-batch)
Numbers inside the figure represent the maximum tryptophan production level at specified culture conditions. (unit; g/*l*).

ration are also presented. The environmental conditions (30 °C, 600 rpm, 1.15vvm, pH 7.0) were identical in both cases while the nutrient feeding strategy and medium composition were different as described before.

In the conventional fed-batch fermentation, limitations in dissolved oxygen were observed after 25 hr cultivation and at this time the amount of base added to control pH was increased rapidly. This indicates that acid production is elevated under oxygen deficiency conditions. On the other hand, in the controlled fed-batch fermentation the dissolved oxygen concentration was maintained at 20 ± 10% of air saturation and both cell growth and tryptophan production were enhanced significantly as compared to those obtained in the conventional fed-batch culture. The maximum cell density and tryptophan concentration in the controlled fed-batch fermentation were about 2.5- and 4.5-fold higher than those in the traditional fed-batch fermentation respectively. After 85-hr cultivation of cells in a controlled fed-batch mode of operation as much as 5.0g/*l* of L-tryptophan was accumulated in the culture broth while in the conventional fed-batch system the maximum tryptophan concentration was only 1.1g/*l*. Also the product yield on glucose was found to be enhanced more than twice with a controlled glucose-feeding system.

At the initial stage of fermentation the amount of base added to the culture was higher in the controlled fed-batch fermentation than in the conventional fed-batch fermentation. The amount of acid produced per cell mass i.e. specific acid production is, however, much lower in the controlled fed-batch culture system, as illustrated in Fig. 6 (closed symbols). In Fig. 6, where the data shown in Fig. 3 (open symbols) are also provided for comparision, the variations of specific acid production are plotted against the amount of glucose consumed instead of culture time (cf. Fig. 3) because glucose consumption pattern of the controlled fed-batch culture system is significantly different from that of the conventional fed-batch fermentation (see Fig. 5). From Fig. 6 it can be found that the production level of L-tryptophan is lowered as the ratio of specific acid production to total glucose consumption is enhanced irrespective of culture conditions such as aeration rates and the modes of fermentor operation. One simple interpretation of this result might be that the conversion of glucose to organic acids resulted in a corresponding reduction of L-tryptophan production. However, it is also possible that the accumulation of acid during the fermentation inhibits the formation of L-tryptophan. For discriminating these possibilities further works are needed to be done.

요 약

대장균 변이주를 이용한 L-트립토판의 최적생산을 위해 목적산물의 생산과 유기산의 생성과의 상호관계를 조사한 결과 비 산생성속도(specific acid production rate)가 증가할수록 L-트립토판의 생산이 감소하는 것으로 나타났다. 따라서 L-트립토판 발효시 산의 생성량을 줄이기 위해 조절식 유가배양법을 도입하였는데 이 배양공정에서는 배양액내의 용존산소농도에 따라 영양배지의 유가속도를 조절하는 방식을 사용하여 세포증식속도를 제한, 세포의 산소요구량이 공급량을 초과하지 않도록 하였다. 이러한 조절식 유가배양법으로 대장균 세포를 배양한 결과 기존의 유가배양식에 비해 비 산생성속도가 현저하게 감소하였으며 L-트립토판의 생산은 5배 정도나 증가되었다.

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