Production of Prednisolone by a Pseudo-Crystallo-Fermentation Technique: Effect of Fermentation Parameters

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Pseudo-Crystallo-Fermentation 기법에 의한 prednisolone의 생산
— 발효변수들의 영향 —

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Effect of various fermentation parameters was investigated on the production of prednisolone by microbial Δ'-dehydrogenation of hydrocortisone. The microbial conversion process was conducted by using pseudo-crystallo-fermentation techniques. The optimum temperature for the bioconversion process was found to be 35°C. It was noted that the production rate of prednisolone was little affected within the initial pH range of 6.5-7.8, and also by the use of surfactant, Tween 80. Production rate of prednisolone was significantly reduced by the use of the antifoam agent, neolin. In a fermentor operation, however, large amount of antifoam agent should be used to remove foams generated by the high aeration rate, which resulted in a lower production rate of prednisolone than that from the shake flask experiment.

Most of steroids have poor solubilities in aqueous solution, resulting in low effective concentration of the substrate. In some cases, this could cause serious diffusional limition in the reaction mixture.

Several attempts have been made to compensate for this problem. Addition of organic solvents, miscible or immiscible with water, enhances the solubility of substrates and products which are hardly soluble in aqueous systems. It generally has a good influence on the productivity of the reactions catalyzed by microbial cells and enzymes. However, the fact that many organic solvents are toxic to microorganisms or enzymes has limited the number of solvents and their amount which can be used (1-8).

In order to obviate the use of organic solvents, pseudo-crystallo-fermentation processes have been suggested (9-11). These were run at considerably higher substrate levels to achieve acceptable economics. They are reported to employed in many industrial processes for steroid bioconversions (12, 13).

This paper reports the effect of environmental conditions such as pH and temperature in the process of Δ'-dehydrogenation of hydrocortisone to prednisolone by using a pseudo-crystallo-fermentation technique. In addition, the effect of antifoam agent on this process was investigated in further detail.

Materials and Methods

Microorganism

Arthrobacter simplex (ATCC 6946) was used throughout this study.

Chemicals

Hydrocortisone and prednisolone were obtained from Sigma Chemical Co. (St. Louis, U.S.A.) and Prodotti Gianni (Milano, Italy). Chloroform, methanol, glacial acetic acid and ethyl acetate of HPLC grade were purchased from E. Merk (Darmstadt, F.R.G.). The other chemicals used were of analytical grade.

Culture conditions

A. simplex was cultivated in a medium containing glucose (2%), yeast extract (1%), KH_2PO_4 (0.1%), K_2HPO_4 (0.1%). The medium was adjusted to pH 7.3 with 2N NaOH. The seed culture was grown on a shaking incubator (Korea Scientific Instrument Co., Seoul, Korea) for 20 hrs at 30 °C (160-180 rpm).

Bioconversion

Bioconversion was carried out in a 500 ml Erlenmyer flask containing 100 ml liquid medium at 160-180 rpm and 30 °C unless otherwise specified. During the jar fermentor operation (working volume, 1.2 liter), the agitation speed and the air flow rate were maintained at 300 rpm and 1 vvm, respectively. The medium used in the bioconversion consisted of casamino acid 0.2%, KH_2PO_4 0.05%, K_2HPO_4 0.05%, $MgCl_2$ 0.001%. The pH was adjusted to 7.3 with 2N NaOH before autoclave. Hydrocortisone at a concentration of 10 or 50 g/l was used as the substrate in a crystalline state. It was added to the fermentation broth containing growing cells, and then converted to prednisolone.

Analytical methods

The samples from the bioconversion experiment were immediately treated with ethyl acetate to extract steroids. The ethyl acetate phase was then subjected to HPLC (Hitachi Model 655A-12, Tokyo, Japan) analyses for the detection of hydrocortisone and prednisolone.

The separation of steroids was achieved on the Lichrosorb SI100 column (E. Merck, Darmstadt, F.R.G.) having 10 μm particle size by eluting with a mobile phase of 97.3% chloroform, 2.5% methanol and 0.2% glacial acetic acid at a flow rate of 2 ml/min. The compounds were detected by absorbance at 254 nm on a UV detector. Under these conditions, the retention times of hydrocortisone and prednisolone were found to be 6.1 and 7.7 min, respectively.

The particle size of hydrocortisone obtained from Sigma Chem. Co. was analyzed by using the particle size analyzer (Elzone 180 Systems, Particle Data Inc., U.S.A.). The arithmetic and geometric mean diameters determined were 20.17 μm and 17.60 μm, respectively. Standard deviation of the data was 11.29%.

Results and Discussion

Effect of initial pH on the bioconversion

The effect of initial pH of the culture broth on the bioconversion of hydrocortisone to prednisolone is shown in Table 1, where prednisolone content is defined as

Prednisolone content(%) =

$$\frac{prednisolone(g/l)}{hydrocortisone(g/l) + prednisolone(g/l)} \times 100$$

Hydrocortisone and prednisolone hardly dissolved in the fermentation broth due to their low solubilities. Since they were suspended in crystalline states, sampling with a pipette was not easy to make from the flask and the jar fermenter. Therefore, total steroid concenterations determined by sampling often demonstrated quite large fluctuations. Prednisolone content defined as above was thus adopted as an important parameter to determine the bioconversion efficiency.

No significant effect of initial pH of the culture broth was observed in the range of 6.5-7.8. The

Table 1. Effect of initial pH on Δ^1-dehydrogenation of hydrocortisone

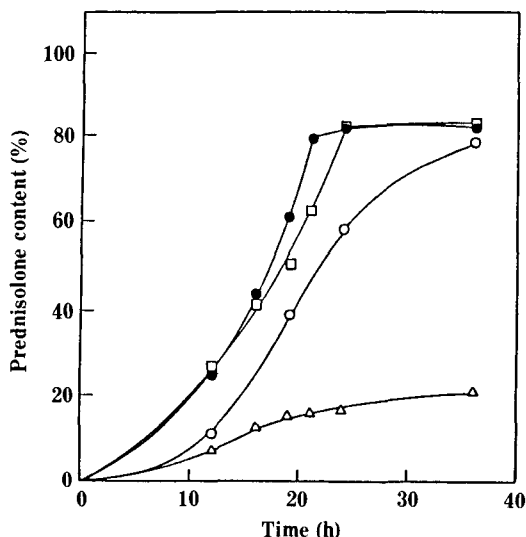| Initial pH	prednisolone content(%)		
	after 12h	after 24h	after 32h
6.5	14.6	66.5	79.1
7.0	15.9	70.3	79.5
7.3	15.7	65.9	79.6
7.8	15.3	68.8	78.4

Fig. 1. Time courses of prednisolone production by Δ'-dehydrogenation of hydrocortisone at various temperatures.
30 °C(○); 35 °C(●); 37 °C(□); 40 °C(△)
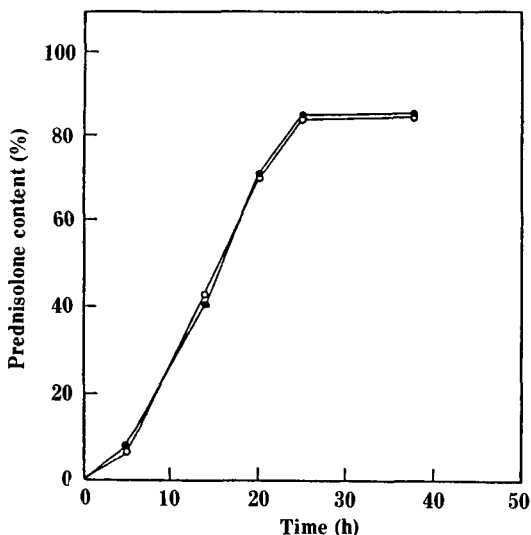Initial substrate concentration = 50 g/l

Fig. 2. Time courses of prednisolone production by Δ'-dehydrogenation of hydrocortisone with (○) or without (●) a surfactant (Tween 80).
Initial substrate concentration = 50 g/l

control of pH during the fermentor operation was considered unnecessary since the pH of the culture broth did not overrun this range.

Effect of temperature
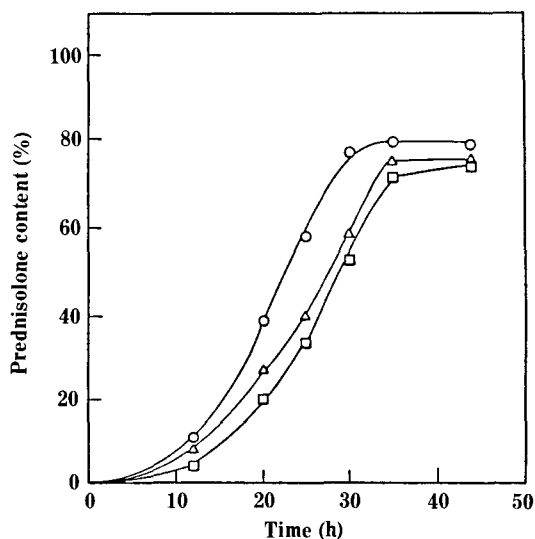
Fig. 1 shows the effect of temperature on the

Fig. 3. Effect of the antifoam agent(neolin) on the bioconversion.
Blank (○); 200 μl/100 ml (△); 400 μl/100 ml (□)
Initial substrate concentration = 50 g/l

bioconversion. Most of the studies on Δ'-dehydrogenation of hydrocortisone by *A. simplex* ATCC 6946 have been carried out at 30 °C. In this study, the bioconversion rate at 35 and 37 °C was higher than that at 30 °C which was known as an usual practice in the earlier publications on the similar bioconversion processes. However, the bioconversion rate was very low at 40 °C. After 20h bioconversion at 35 °C, the prednisolone content reached at ca. 80% and no more prednisolone was produced. This phenomenon has been observed in many studies on the steroid bioconversion. It is believed possible that the reaction can not proceed to 100% because of the mixed crystal formation(12).

Therefore, the further studies should focus on developing the processes which are capable of increasing the yield of final prednisolone.

Effect of surfactant(Tween 80)

In the steroid bioconversion by pseudo-crystallo-fermentation, the surfactants such as Tween 80 are added to the fermentation broth in order to achieve high degree of dispersion of the solid particles, which, in general, has yielded some increase in the bioconversion rate. However, use of the surfactants often results in some operational problems such as foaming with high aeration.
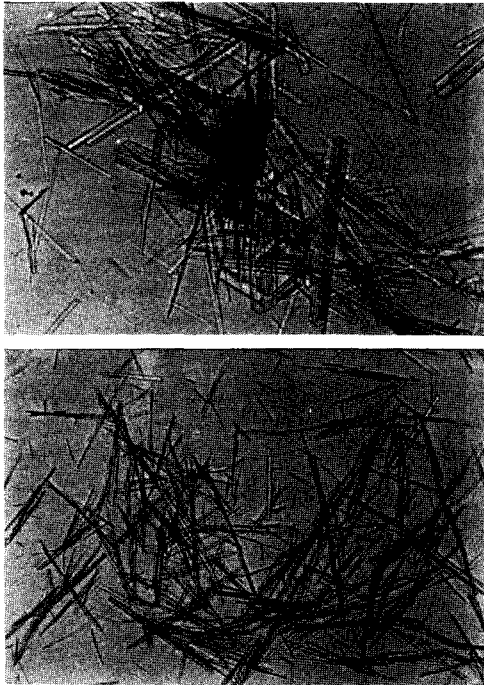
Fig. 2 shows the effect of the surfactant, Tween

Fig. 4.Microphotographs of prednisolone particles produced in the medium with (upper) or without (lower) the antifoam agent, neolin (Magnification; ×400).
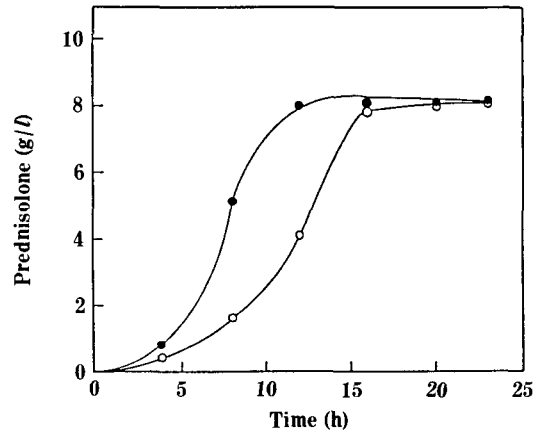

Fig. 5. Time courses of prednisolone formation during Δ'-dehydrogenation of hydrocortisone in the shake flask (●) and jar fermenter (○).
Initial substrate concentration = 10 g/l

80, on the bioconversion. No significant improvement in the bioconversion rate was observed with Tween 80 (0.05% v/v) when compared to that without the surfactant. Therefore, it was considered possible to operate the fermentor without any surfactants.

Effect of antifoam agent(neolin)

It was inevitable to use the antifoam agent in the fermentor operation because of severe foaming. As shown in Fig. 3, use of the antifoam agent resulted in a significant decrease in the bioconversion rate. The bioconversion rate with neolin at a concentration of 400 μl/100 ml was only 0.7 times to that without the antifoam agent.

The crystals of prednisolone observed through microscope with and without the antifoam agent were shown in Fig. 4. It is noted that the size of prednisolone crystals in the presence of neolin was bigger than that without the antifoam agent.

Weaver *et al.*(14,15) reported in their studies on 11 α-hydroxylation of progesterone that a higher degree of interfacial contact between the substrate

particles and the microorganisms, and consequently higher conversion yield, could be achieved from the substrate particles in a ground form. It was therefore most probable that the antifoam agent affected the bioconversion rate in such a way that it changed the substrate particle size.

Biocnversion in a jar fermentor

Fig. 5 shows the time courses of prednisolone formation in the shake flask and the jar fermentor. Production rate of prednisolone in the shake flask was higher than that in the jar fermentor. It should be noted that quite large amount of antifoam agent (neolin) had to be used during the jar fermentor operation because of heavy foaming. As mentioned above, addition of the antifoam agent was considered as the main reason for the lower conversion rate in the jar fermentor; however, at the present point it is not clear yet whether the lower conversion is due to the poor contact between the microorganisms and the substrate particles. Further detailed investigation is currently underway since it may be a serious problem in a large scale process for the same bioconversion.

요　약

미생물에 의해 hydrocortisone을 Δ'-dehydrogenation하여 prednisolone 생산을 위한 공정에 있어 여러 발효변수들의 영향이 조사되었다. 미생물 전환

공정은 pseudo-crystallo-fermentation 방법을 이용하였으며, 생물 전환을 위한 최적 온도는 35℃였다. 발효배지의 초기 pH 6.5-7.8 범위에서는 prednisolone 생산에 pH의 영향이 거의 없었으며, 또한 계면활성제 Tween 80의 영향도 거의 없는 것으로 나타났다. Prednisolone의 생산속도는 소포제 neolin 을 첨가함으로써 현저하게 감소하였다. 발효조 조업 시에는 높은 공기주입 속도로 인해 발생되는 기포를 제거하기 위해 많은 양의 소포제가 사용되었으며, 따라서 shake flask 조업시보다 prednisolone 생산 속도가 낮은 것으로 나타났다.

References

1. Buckland, B.C., P. Dunnill and M.D. Lilly: *Biotechnol. Bioeng.* **17**, 815 (1975).

2. Larsson, P.O., S. Ohlson and K. Mosbach: *Nature* **263**, 796 (1976).

3. Sonomoto, K., A. Tanaka, T. Omata, T. Yamane and S. Fukui: European *J. Appl. Microbiol. Biotechnol.* **6**, 325 (1979).

4. Sonomoto, K., I.N. Jin, A. Tanaka and S. Fukui: *Agric. Biol. Chem.* **44**, 1119 (1980).

5. Kaul, R. and B. Mattiasson: *Appl. Microbiol. Biotechnol.* **24**, 259 (1986).

6. Carrea, G., F. Colombi, G. Mazzola and P. Cremonsi: *Biotechnol. Bioeng.* **21**, 39 (1979).

7. Cremonsi, P., G. Carrea, L. Ferrara and E. Antonini: *Biotechnol. Bioeng.* **17**, 1101 (1975).

8. Omata, T., A. Tanaka and S. Fukui: *J. Ferment. Technol.* **58**, 339 (1980).

9. Kondo, E. and E. Masuo: *J. Gen. Appl. Microbiol.* **7**, 113 (1961).

10. Kondo, E.: *Agr. Biol. Chem.* **27**, 69 (1962).

11. Constantinides, A.: *Biotechnol. Bioeng.* **7**, 815 (1980).

12. Miller, T.L.: Comprehensive Biotechnology (M. Moo-Young, ed.), Pergamon Press, Oxford, Vol. **3**, 297 (1984).

13. Smith, L.L.: Biotechnology-Biotransformations (K. Kieslich, ed.), Verlag Chemie, Weinheim, Vol. 6a, 31 (1984).

14. Weaver, E.A.: U.S. Patent 3,019,335 (1962).

15. Weaver, E.A., H.E. Kenney and M.E. Wall: *Appl. Microbiol.* **8**, 345 (1962).