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Microbial Conversion of Cholesterol to 4-Androstene-3,17-dione by Intermittent Addition of Substrate

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간헐적으로 첨가된 Cholesterol로 부터 미생물전환에 의한 4-Androstene-3, 17-dione의 생산

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Production of 4-androstene-3,17-dione(AD) from cholesterol by microbial conversion was investigated. To facilitate the solubilization of cholesterol in the fermentation broth, ethanol was used as an organic solvent. Inhibition on cell growth by ethanol was observed to be negligible upto 2% (V/V) concentration. Microbial conversion was successfully carried out with high yield when the cholesterol was added at early logarithmic growth phase with pH control at 7.0. In order to improve the process productivity, bioconversion was conducted at various mode of cholesterol addition; 0.1% (V/W) of cholesterol was found to be most appropriate for solubilization in ethanol and was added intermittently. When added three time(total 3 g/*l*), overall bioconversion yield reached upto 65% while single addition of same amount of cholesterol (3 g/*l*) yielded about 40% conversion.

As the natural source of steroids such as diosgenin became more scare and expensive, selective degradation of sterols has become more attractive for preparation of various steroid compounds. (1-3) Much research efforts have been focused on developing microbial strains which are able to selectively degrade side chains of sterol without breaking steroid ring nucleus. (4-7) In these cases, most fermentation products of sterols are mainly androstane compounds, which are starting materials for the synthesis of testosterone, estrone and 9 -halogen-substituted corticoids (8).

Low solubility of sterols in water was considered as one of the major drawbacks in the microbial conversion of cholesterol. (9) Fermentation at high concentration of sterol is, therefore, considered essential for economy of the bioconversion process. Various organic solvents and surfactants have been used to increase the substrate concentration in the fermentation broth. However, it has been known that the use of detergents caused serious problems in separation processes and organic solvents even has toxic effects on cell growth. Choice of suitable organic solvent or surfactant and their concentrations are thus important factors for successful operation of the bioconversion process.

In our laboratory, we have been investigating the microbial conversion of cholesterol to 4-androstene-3,17-dione(AD).

It was found that intermittent addition of the cholesterol considerably increased the product yield of AD in the process. The results are reported in this communication.

Key words: Bioconversion, cholesterol, 4-androstene-3,16-done
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Materials and Methods

Materials

Cholesterol, 4-androstene-3,17-dione and Tween 80 were purchased from Sigma (St. Louis, Mo., U.S.A.); n-hexane, ethylacetate, iso-propanol and ethanol of HPLC grade were obtained from Merck(Darmstadt, F.R.G.). All other chemicals used were of analytical grade.

Cultivation of microorganisms

Mycobacterium sp. NRRL B3805, which has low activity for degradation of sterol nucleus, (4) was used to transform cholesterol to AD.

Medium composition for cultivation of the microorganism was; NH_4NO_3 0.3%, K_2HPO_4 0.0025%, $MgSO_4 \cdot 7H_2O$ 0.0025%, $FeSO_4 \cdot 7H_2O$ 0.0001%, yeast extract 0.5% and glycerol 1.0%. The pH was adjusted to 7.0.

Bioconversion in shake flask

100 m*l* of culture medium in a 500 m*l* Erlenmyer flask was inoculated with a 36 hr culture of the microorganism, and cultivated on a reciprocal shaker (200 strokes per min., Kukje Sci. Co., Seoul, Korea) at 30°C. After 24 hr of cultivation, 100 mg of cholesterol dissolved in 2 m*l* ethanol was added to the broth and incubated for 72 hr.

Bioconversion in a jar fermenter

2.6 liter fermenter(Marubishi Co., Tokyo, Japan) was used with a working volume of 1.2 liter. After sterilization, 60 m*l* of preculture of the microorganism was inoculated, and cholesterol dissolved in ethanol was added at desired time during cultivation. The operating conditions were; temperature, 30°C, agitation speed, 250 rpm, aeration rate, 1 vvm and pH, 7.0.

Analysis of steroid compounds

Fermentation broth was extracted with two volumes of methylene chloride, and organic phase was filtered through a membrane filter(pore size 0.45 um, Millipore, MA., U.S.A.) before injection into HPLC.

The HPLC used was equipped with a programmable, variable-wavelength(Hitachi Model 655A-12, Tokyo, Japan). The column(30 cm × 4.6 mm I.D.) was packed with nominal 10 um silica gel (u-porasil, Waters Assoc., Milford, MA., U.S.A.). The mobile phase was a mixture of n-hexane and isopropanol(85:15(V/V)) and the flow rate was 1 m*l* per min..

The column eluate was monitered at 208 nm for cholesterol, and at 250 nm for 4-androstene-3.17-dione. This was done by using the time programming function for the detection wavelength of the model. Peak areas were calculated with an integrator(Model D-2000 Hitachi Co., Tokyo, Japan).

Ten microliter of the sample solution was injected by microsyringe (Hamilton, Reno, NV, U.S.A.). All analysis were conducted at room temperature. Bioconversion yield of AD from cholesterol was calculated by the following equation;

$$\text{Conversion yield (\%)} = \frac{\text{Wt. of product formed}}{\text{Wt. of substrate added}} \times \frac{\text{M.W. of substrate}}{\text{M.W. of product}} \times 100$$

Results and Discussion

Determination of cholesterol and AD by HPLC

A typical elution pattern of the fermentation broth after extraction with methylene chloride was shown in Fig. 1. Extraction of culture broth with other solvents such as ethyl acetate, chloroform and ether was also tested, but they were found to be less efficient than methylene chloride (10).

Calculated peak area and concentrations of steriod compounds were shown to be linearly correlated in the range of 1 μg to 10 μg. The lowest limit for the analysis of cholesterol and AD were 20 ng and 2 ng, respectively. This analytical method could practically be applied to determine cholesterol and AD when methylene chloride was used as an extaction solvent.

Efect of ethanol on cell growth

One of the most serious problem in sterol bioconversion was known to be the low solubility of cholesterol in water. Various organic solvents and detergents have been used to increase the sterol concentration in the broth. However, it is also well

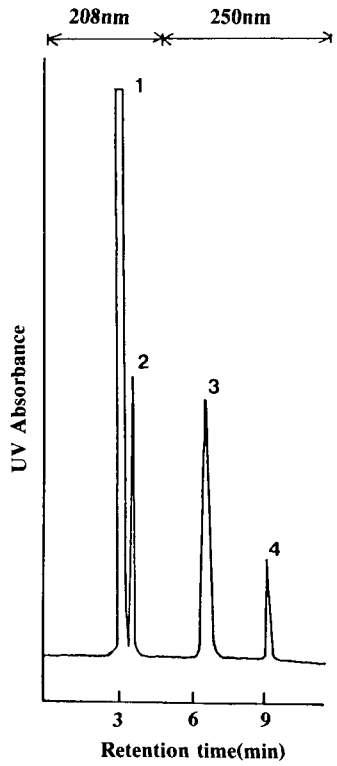

Fig. 1. Quantitative analysis of cholesterol and AD by HPLC.
1. ethylacetate
2. cholesterol
3. 4-androstene-3,17-dione(AD)
4. 1,4-androstadiene-3,17-dione(ADD)

known that organic solvents have the toxic effect on cell growth (9). In our study, N,N-dimethylformamide (DMF), acetone, ethylene glycol, ethyl acetate and dimethyl sulfoxide (DMSO) were tested, and ethanol was found to be least inhibitory on cell growth. As shown in Fig. 2, cell growth was seriously inhibited at ethanol concentrations higher than 4% (V/V). The optimal concentration of ethanol in broth was determined to be 2% (V/V) at which the microorganism was most tolerable.

Effect of addition time of cholesterol in AD production

The enzymes involved in side chain degradation of cholesterol are believed to be inducible by the presence of cholesterol (11), and the efficiency of bioconversion is generally proportional to the amount of cell mass. In view of this point, mode of cholesterol addition could be a useful means to increase bioconversion yield. As shown in Fig. 3, addition of cholesterol at exponential growth phase resulted in the highest AD production. This probably indicates that both cholesterol uptake and induction of the necessary enzymes system were most efficient at exponential growth phase. It is also observed that cholesterol was not efficiently utilized during the early growth phase, which was considered due to the presence of other more easily utilizable carbon source, glycerol. Low AD production was observed when cholesterol was added at the early beginning stationary phase.

Time course of cholesterol bioconversion

AD production in jar fermenter was carried out with 0.1% (W/V) cholesterol. As shown in Fig. 4, AD was mostly produced at late exponential phase. From the results, it seems most rational to say that cholesterol is transported into the cell during the exponential growth phase, and AD released to the broth after the side chain is degraded. The conversion yield based on the initial input of cholesterol was about 70% when pH was controlled at 7.0. Dissolved oxygen was observed to drop to zero level during the exponential growth phase. Effects of dissolved oxygen on cell growth and AD production should be investigated in further detail.

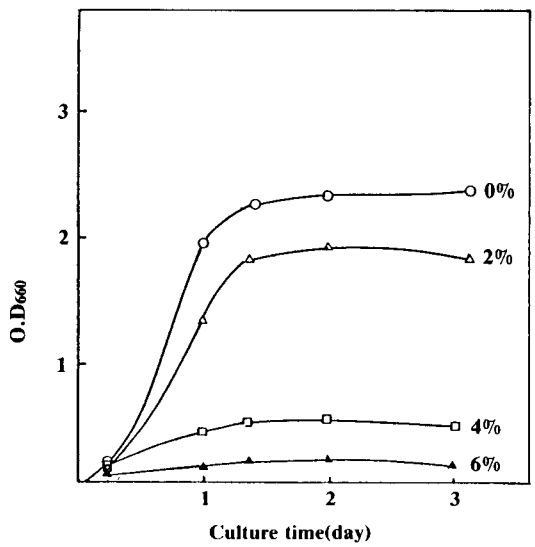

Fig. 2. Effect of ethanol concentration on cell growth.

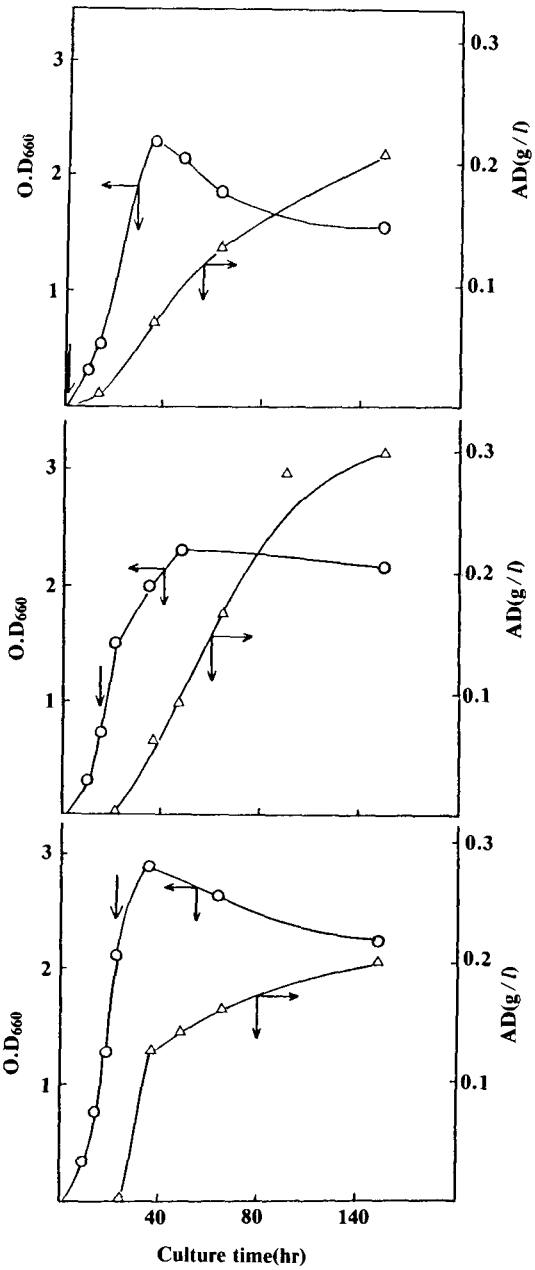

Fig. 3. Effect of cholesterol addition time on AD production.

Cholesterol was added at arrow indicated.

AD production by intermittent addition of cholesterol

Despite the low solubility of cholesterol, bioconversion at high concentration of cholesterol is considered to be most important for the economy of

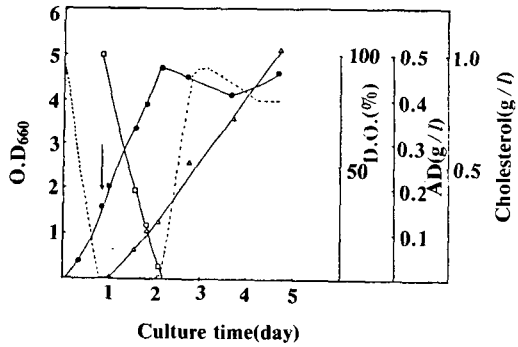

Fig. 4. Time course of cholesterol bioconversion in jar fermentor.

Cholesterol was added at arrow indicated. Optical density (●); Dissolved oxygen (---); Cholesterol (□); AD (△)

AD production. However, when cholesterol concentration was increased to 0.2%, conversion yield was only about 40.5% (Fig. 5). This could be explained by following observation. The microorganisms aggregated on a cholesterol particles dispersed in the culture broth during bioconversion (Fig. 6a). As cholesterol was used up, the cell-cholesterol aggregates disappeared (Fig. 6b). Compared with 0.1% of cholesterol, cholesterol particles were not finely dispersed in the case of 0.2% of cholesterol, and uptake of cholesterol by cells was not efficiently achieved. In order to improve the conversion yield at increased concentrations of cholesterol, it was added intermittently during the transformation process. As shown in Fig. 7, total conversion yield

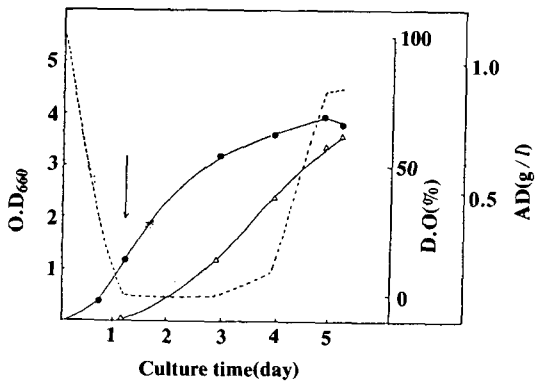

Fig. 5. AD production by addition of 0.2% concentration of cholesterol.

Cholesterol was added at arrow indicated. Optical density (●); Dissolved oxygen (---); AD (△)

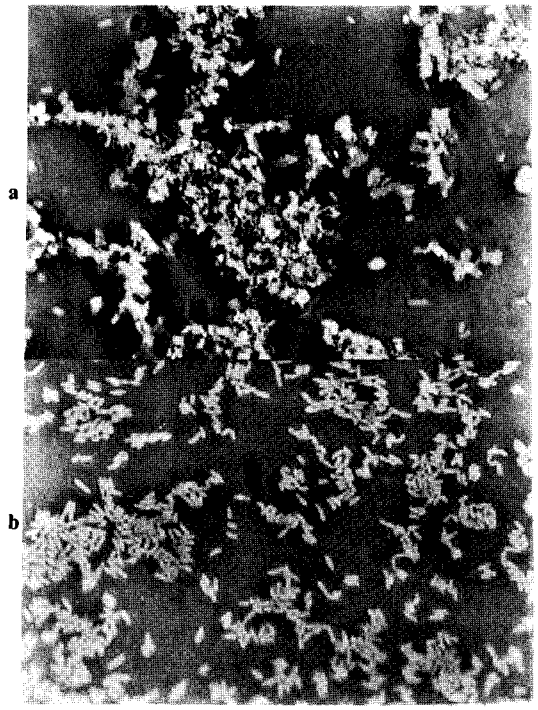

Fig. 6. Aggregation of microorganisms on a cholesterol particles during bioconversion
(a) in the presence of cholesterol particles
(b) after depletion of cholesterol

was about 67% when 0.1% cholesterol was added twice. This conversion yield was as high as the single addition of 0.1% cholesterol. Similar conversion yield was obtained by further addition of cholesterol; when 0.1% cholesterol was added three times, the conversion yield was about 65% and final concentration of AD in the culture broth was determined to be 1.5 g/*l* (Fig. 8). This process could be well applied to other bioconversion system of water insoluble substrate. Recently, it was reported the continuous feeding of hydrocortisone suspension into the fermenter to produce prednisolone by using immobilized *Arthrobacter simplex* greatly improved the conversion yield in pilot-plant scale(12).

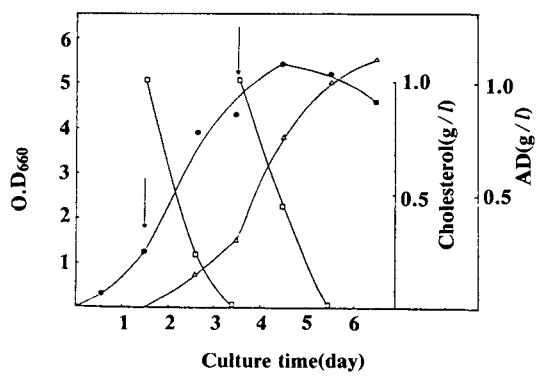

Fig. 7. AD production by adding 0.1% cholesterol two times.
Cholesterol was added at arrow indicated. AD (△); Optical density (●); Cholesterol (□)

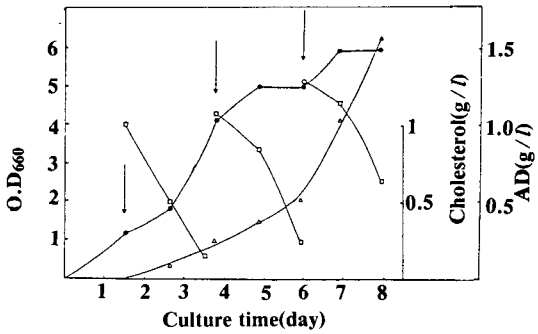

Fig. 8. AD production by adding 0.1% cholesterol three times.
Cholesterol was added at arrow indicated. AD (△); Optical density (●); Cholesterol (□)

It is believed that such an innovative effort on process engineering can greatly improve the overall productivity in the cholesterol bioconversion process.

요 약

Cholesterol로 부터 미생물전환 방법에 의한 4-androstene-3, 17-dione(AD)의 생성에 대해 연구를 수행했다. 발효액중에 cholesterol의 용해도를 증가시키기 위해 ethanol을 용매로 사용하였는데, ethanol 농도가 2%(v/v) 까지는 세균성장이 크게 저해되지 않았다.

미생물전환은 pH를 7.0으로 조절하고, 초기대수증식기에 cholesterol을 첨가했을 때 효율적으로 AD가 생성되었다. AD 생성을 높이기 위해 cholesterol 첨가방법을 여러가지로 변환시켰다. 즉, 최종 cholesterol 농도를 0.1% 하여 ethanol에 녹여 간헐적으로 첨가했을 때 가장 높은 수율을 얻었다. Cholesterol을 세번(전체 3g/*l*) 첨가했을 때 최종 전환수율이 65%에 도달한 반면, 같은 양의 cholesterol(3g/*l*)을 한번에 넣었을 때 40%의 생성수율을 얻었다.

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