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Production of testosterone from cholesterol using a single-step microbial transformation of *Mycobacterium* sp

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A novel single-step microbial transformation process for the production of testosterone (TS) from cholesterol by *Mycobacterium* sp was investigated. It was found that the supply of reducing power, NADH, from the metabolism of glucose was necessary for the reduction of androst-4-en-3,17-dione (AD) to TS. The cultivation time for the maximum accumulation of TS and the residual glucose increased in parallel with the amount of glucose supplemented in fermentation cultures. After the glucose in the fermentation culture was completely consumed, most of the TS was oxidized to AD. Adding a larger amount of glucose could prevent oxidation of TS to AD. Under optimal fermentation conditions, the maximum molar conversion rate of TS from cholesterol was 51% in a 5-L surface-aerated fermentor after 120 h cultivation.

Keywords: testosterone; androst-4-en-3,17-dione; cholesterol; *Mycobacterium*

Introduction

Microbial degradation of the side chain of sterols, such as cholesterol and sitosterol, has become an important transformation process for the production of 17-ketosteroid intermediates, such as androsta-1,4-diene-3, 17-dione (ADD) and androst-4-en-3,17-dione (AD) [5]. The reduction of AD to testosterone (TS) is catalyzed by the microsomal enzyme 17-ketosteroid reductase (17β-hydroxysteroid : NADP 17-oxidoreductase, EC 1.1.1.64) in the testes [1]. Enzymatic reduction of AD to TS by 17β-hydroxysteroid dehydrogenase (17β-hydroxysteroid : NAD 17-oxidoreductase, EC 1.1.1.63) has also been found in *Pseudomonas* [13], *Saccharomyces* [14,17], *Marchantia* [3] and *Mycobacterium* [7]. Although the microbial transformation process has been used on an industrial scale for the production of AD from sterols, commercial production of TS from AD is carried out via a four-step chemical synthesis [2].

We have previously reported [7,10,11] a novel single-step microbial transformation process for the production of TS from sterols in a synthetic medium supplemented with peptone and glucose. The purpose of this study was to further elucidate the important parameters involved in the transformation of cholesterol to TS in fermentation cultures of *Mycobacterium* sp.

Materials and methods

Microorganism

A cholesterol-assimilating and AD-producing mutant of *Mycobacterium* sp NRRL B-3805 was used throughout this study.

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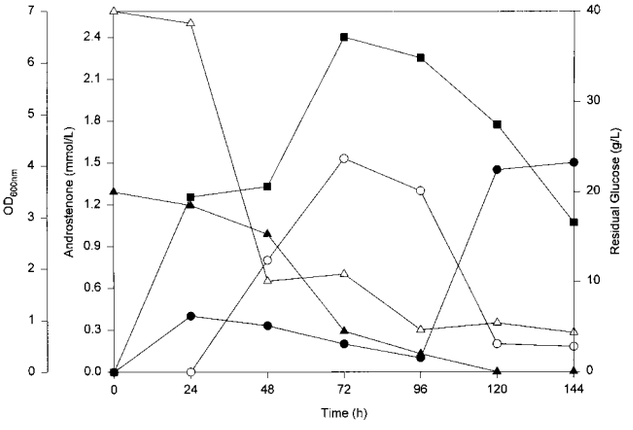

Figure 1 Effect of 2% glucose on the accumulation of androstenones from cholesterol by *Mycobacterium* sp in a 500-ml shaking culture. The fermentation conditions were the same as those shown in Table 1. At the initial stage, 2% glucose and 0.1% cholesterol were added. ○—○, TS; ●—●, AD; △—△, cholesterol; ▲—▲, glucose; ■—■, cell growth ($OD_{600\ nm}$).

Materials

Yeast extract, nutrient broth dehydrate, peptone and agar were obtained from Difco (Detroit, MI, USA). Testosterone (T1500) and androst-4-ene-3,17-dione (A9630) were obtained from Sigma (St Louis, MO, USA). The cholesterol used was a product of Tokyo Kasei Inc (Tokyo, Japan). Inorganic salts and other chemicals were all of reagent grade.

Cultivation methods

The stock culture was maintained on an enriched nutrient agar medium which contained (per liter): nutrient broth dehydrate 8 g, yeast extract 10 g, glucose 10 g, and agar 20 g (pH 6.8). A glucose nutrient broth medium consisting of 16 g of nutrient broth dehydrate and 40 g of glucose per liter of distilled water (pH 6.8), and an AD-producing synthetic medium consisting of 1.5 g of ammonium acetate,

Table 1 Effect of glucose on the production of androstenones from cholesterol

Glucose[a] (%)	Maximum accumulation of TS (h)	Residual glucose (g L^{-1})	Androstenones (mmol L^{-1})[b]		Total molar conversion rate (%)[c]
			TS	AD	
0	–	0	0	0.64	25
1	48	0	0.80	0.48	50
2	72	0	1.47	0.18	64
3	96	0.9	1.51	0.16	65
4	120	2.5	1.34	0.06	54
5	144	3.4	1.07	0.15	47

[a]A synthetic medium consisting of 1.5 g ammonium acetate, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.4 g K_2HPO_4, 0.8 g KH_2PO_4, 5 mg $FeSO_4 \cdot 7H_2O$, 2 mg $ZnSO_4 \cdot 7H_2O$ and 0.5 mg $MnSO_4 \cdot 4H_2O$ per liter of distilled water (pH 6.8) supplemented with 1% peptone was used as the basal medium. The cultures were incubated in a 500-ml Hinton flask at 30°C on a rotary shaker (125 rpm) for 144 h. Various concentrations of glucose and 0.1% cholesterol were added at the initial stage. The working volume was 100 ml.
[b]Androstenones: TS, testosterone; AD, androst-4-ene-3,17-dione.
[c]The molar conversion rate was calculated on the basis of 0.1% cholesterol (2.586 mmol L^{-1}).

0.2 g of $MgSO_4 \cdot 7H_2O$, 0.4 g K_2HPO_4, 0.8 g KH_2PO_4, 5 mg $FeSO_4 \cdot 7H_2O$, 2 mg $ZnSO_4 \cdot 7H_2O$ and 0.5 mg of $MnSO_4 \cdot 4H_2O$ per liter of distilled water (pH 6.8) [9] were used as seed and basal media for the production of TS, respectively. Five milliliters of the seed culture were inoculated into 100 ml of a medium supplemented with 0.1% cholesterol and 1% peptone in a 500-ml Hinton flask. The culture was incubated at 30°C on a rotary shaker (120 rpm) for 5 days. Fermentation experiments were carried out in a 5-L surface-aerated fermentor (Hotech, Taiwan) [8].

Analytical methods

Steroids: The culture filtrate was extracted with ethyl acetate. The organic phase was evaporated, and the residue was dissolved in methanol. AD and TS in the methanol solution were then determined by HPLC using a C_{18} column and a mobile phase composed of acetonitrile and water (50/50, v/v) [8]. Cholesterol was measured according to the Lieberman–Burchard color reaction [18]. The molar conversion rate of TS was calculated on the basis of cholesterol added to the medium.

Glucose and cell growth: Glucose was determined using the dinitrosalicylic acid method [6]. Cell growth was determined using a turbidimetric method (O.D. 600) as described in a previous paper.

Relative dissolved oxygen: Dissolved oxygen was measured using a dissolved oxygen probe (Ingold, Messtechnik AG, Switzerland). The relative dissolved oxygen was calculated on the basis of the initial dissolved oxygen of the medium before inoculation under the same fermentation conditions.

Results

Effect of glucose on the accumulation of androstenones from cholesterol

The effect of glucose on the accumulation of androstenones, AD and TS from cholesterol in a synthetic medium supplemented with 1% peptone was examined in shaking

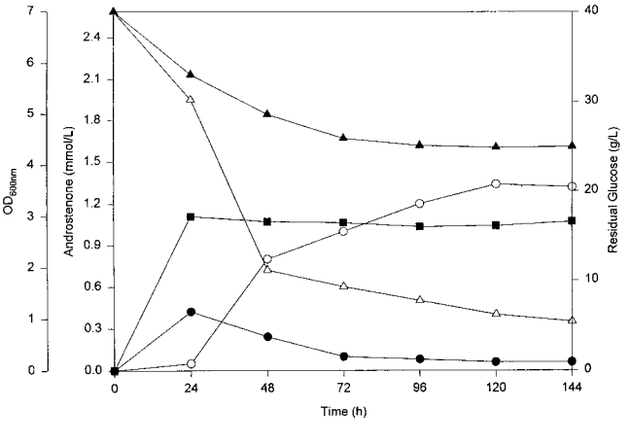

Figure 2 Effect of 4% glucose on the accumulation of androstenones from cholesterol by *Mycobacterium* sp in a 500-ml shaking culture. The fermentation conditions and the symbols are the same as those shown in Figure 1 except that 4% glucose was added at the initial stage.

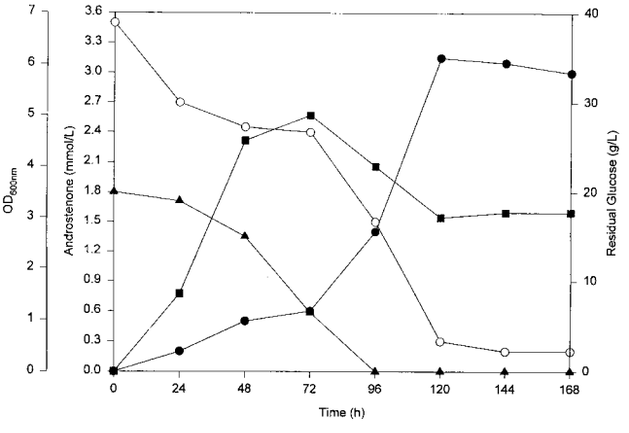

Figure 3 Effect of 2% glucose on the oxidation of TS to AD in the fermentation culture of *Mycobacterium* sp. The fermentation conditions and the symbols are the same as those shown in Figure 1 except that 0.1% TS and 2% glucose were added at the initial stage.

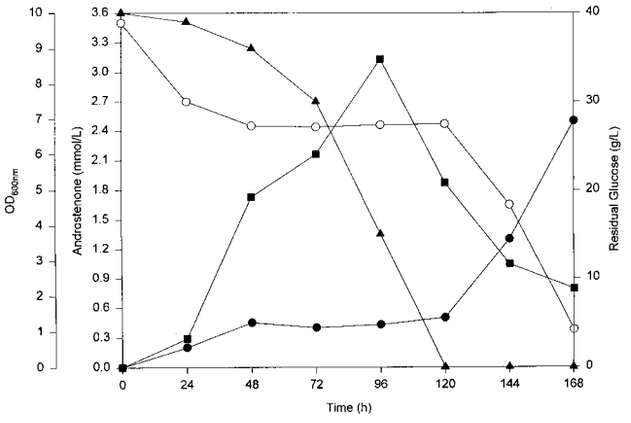

Figure 4 Effect of 4% glucose on the oxidation of TS to AD in the fermentation culture of *Mycobacterium* sp. The fermentation conditions and the symbols are the same as those shown in Figure 3 except that 4% glucose was added at the initial stage.

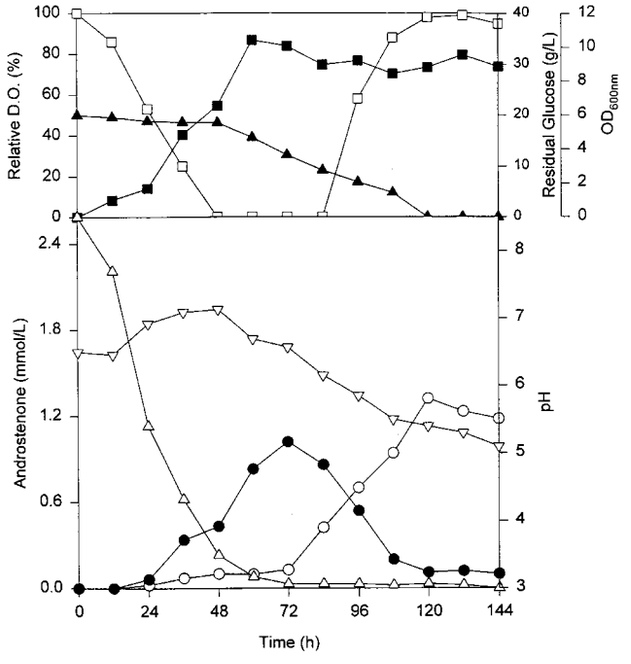

Figure 5 Effect of 2% glucose on the accumulation of androstenones from cholesterol in a 5-L surface-aerated fermentor. The basal medium was the same as that shown in Figure 1. The operation conditions were as follows: working volume, 2.5 L per 5-L fermentor; inoculum size, 5%, surface aeration rate, 1 vvm; agitation speed, 300 rpm; temperature, 30°C; 0.1% cholesterol and 2% glucose were added at the initial stage. ○—○, TS; ●—●, AD; △—△, cholesterol; ▲—▲, glucose; ■—■, cell growth ($OD_{600\ nm}$); □—□, relative DO; ▽—▽, pH.

cultures incubated at 30°C for 144 h. Various concentrations of glucose and 0.1% cholesterol were added at the initial stage as carbon sources. As shown in Table 1, it was found that accumulation of TS occurred only in the glucose-supplemented cultures. The increase of the glucose concentration was in parallel with the decrease of AD accumulation. When the concentration of glucose was higher than 3%, the residual glucose in the fermentation cultures gradually increased. The maximum accumulation of TS in the 2% glucose-supplemented medium occurred at 72 h-cultivation. However, most of the TS was oxidized to AD after the glucose was completely consumed during further cultivation (Figure 1). On the other hand, it was found that the main product of androstenone that accumulated in the 4% glucose-supplemented medium was TS. The maximum accumulation of TS occurred at 120 h of cultivation, and the residual glucose was about 2.5% (Figure 2). From the above results, it was apparent that the cultivation time for the maximum accumulation of TS and the residual glucose increased in parallel with the amount of glucose supplemented in the fermentation cultures (Table 1).

Effect of glucose on the oxidation of TS to AD

The effect of glucose on the oxidation of TS to AD in a synthetic medium supplemented with 1% peptone was examined in shaking cultures incubated at 30°C for 168 h. Various concentrations of glucose and 0.1% TS were added at the initial stage as carbon sources. Almost no cell growth could be detected in the glucose-free medium. This indicated that TS could not be used as a carbon source for cell growth. Within 72 h of cultivation, most of the TS in the fermentation cultures was oxidized to AD (data not shown). As shown in Figure 3, the rapid consumption of glucose in the 2% glucose-supplemented medium was in parallel with the increase of cell growth. Within 72 h of cultivation, most of the TS was still retained in the fermentation culture. However, after glucose in the fermentation culture was completely consumed, most of the TS was oxidized to AD. The pattern of the oxidation of TS to AD in the 4% glucose-supplemented medium was similar to that in the 2% glucose-supplemented medium except that TS was retained in the fermentation culture within 120 h of cultivation (Figure 4). From the above results, it was apparent that adding a larger amount of glucose could prevent the oxidation of TS to AD in the fermentation culture.

Production of TS in fermentor

The fermentation conditions for the production of TS were investigated in a 5-L surface-aerated fermentor loaded with 2 L of synthetic medium supplemented with 1% peptone. At the initial stage, 0.1% cholesterol and 2% glucose were added as carbon sources. As shown in Figure 5, cell growth and the pH of the culture increased in parallel with the rapid consumption of cholesterol. After most of the cholesterol was consumed, rapid consumption of glucose as well as a decrease of the pH in the fermentation culture occurred. The accumulation of AD first appeared at 24 h, and then most of the AD was reduced to TS after 72 h of cultivation. It was apparent that the consumption of glucose and the decrease of pH to below 7.0 were favorable for the accumulation of TS in the fermentation culture. The maximum molar conversion rate of TS from cholesterol was about 51% after 120 h of cultivation.

Discussion

The microbial transformation of cholesterol to 17-ketosteroid intermediate, AD, is now utilized on an industrial scale [5]. AD is a very useful starting compound for the synthesis of a pharmacologically active steroid, TS. However, the amount of available information concerning the

production of TS from sterols via a single-step microbial transformation process is still very limited [4,12,15,16].

As shown in Table 1, the accumulation of TS appeared only in the glucose-supplemented cultures. After the glucose in the fermentation cultures was completely consumed, most of the TS was oxidized to AD during further cultivation (Figure 1). Adding a larger amount of glucose prevented the oxidation of TS to AD in the fermentation cultures. However, the cultivation time for the maximum accumulation of TS and the residual glucose increased in parallel with the amount of glucose supplemented in the fermentation cultures (Figure 2 and Table 1). We have previously reported [7] that *Mycobacterium* sp used in the present study could consume cholesterol in preference to glucose in the presence of these two substrates. As shown in Figure 5, cell growth increased in parallel with the rapid consumption of cholesterol. The accumulation of AD first appeared at 24 h, and then most of the AD was reduced to TS after rapid consumption of glucose as well as a decrease of the pH in the fermentation culture occurred. At the same time, the relative amount of dissolved oxygen in the fermentation culture rapidly decreased to zero level. Since the reduction of AD to TS is catalyzed by 17β-hydroxysteroid dehydrogenase coupled with a reduced nicotinamide cofactor, NADH, at pH 6.0 in microorganisms, it is suggested that the supply of reducing power, NADH, from the metabolism of glucose is necessary for the reduction of AD to TS in fermentation cultures of *Mycobacterium* sp [3,7,13,14,17].

Acknowledgements

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References

1 Bogovich K and AH Payne. 1980. Purification of rat testicular microsomal 17-ketosteroid reductase. J Biol Chem 255: 5552–5559.
2 Ercoli A and PD Ruggierii. 1953. An improved method of preparing testosterone, dihydrotestosterone and some of their esters. J Am Chem Soc 75: 650–653.
3 Hamada H and S Kawabe. 1991. Biotransformation of 4-androstene-3,17-dione by green cell suspension of *Marchantia polymorpha*: stereoselective reduction at carbon 17. Life Sci 48: 613–615.
4 Hung B, A Falero, N Llanes, C Perez and MA Ramirez. 1994. Testosterone as biotransformation product in steroid conversion by *Mycobacterium* sp. Biotechnol Lett 16: 497–500.
5 Imada Y, H Ishikawa and D Nishikawa. 1981. The new development of steroid fermentation. Nippon Nogeikagaku Kaishi 55: 713–721.
6 Lee CY and WH Liu. 1992. Production of androsta-1,4-diene-3,17-dione from cholesterol using immobilized growing cells of *Mycobacterium* sp NRRL B-3683 adsorbed on solid carriers. Appl Microbiol Biotechnol 36: 598–603.
7 Liu WH, CW Kuo, KL Wu, CY Lee and WY Hsu. 1994. Transformation of cholesterol to testosterone by *Mycobacterium* sp. J Ind Microbiol 13: 167–171.
8 Liu WH and CY Lee. 1990. Microbial transformation of cholesterol to androsta-1,4-diene-3,17-dione by *Mycobacterium* sp NRRL B-3683 in a cholesterol-synthetic medium. J Chin Agric Chem Soc 28: 166–174.
9 Liu WH and CY Lee. 1992. Production of androst-4-ene-3,17-dione from cholesterol by *Mycobacterium* sp in a synthetic medium. J Chin Agric Chem Soc 30: 52–58.
10 Liu WH, TF Wu and CK Lo. 1997. Transformation of sitosterol to testosterone by *Mycobacterium* sp. J Chin Agric Chem Soc 35: 124–131.
11 Liu WH, KL Wu and CK Lo. 1997. Microbial transformation of cholesterol to testosterone. J Chin Agric Chem Soc 35: 236–242.
12 Sarmah U, MK Roy and HD Singh. 1989. Steroid transformation by a strain of *Arthrobacter oxydans* incapable of steroid ring degradation. J Basic Microbiol 29: 85–92.
13 Schultz RM, EV Groman and LL Engel. 1977. 3(17) β-Hydroxysteroid dehydrogenase of *Pseudomonas testosteroni*. J Biol Chem 252: 3775–3783.
14 Singer Y, H Shity and R Bar. 1991. Microbial transformations in a cyclodextrin medium. Part 2. Reduction of androstenedione to testosterone by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 35: 731–737.
15 Szentirmal A. 1990. Microbial physiology of sidechain degradation of steroids. J Ind Microbiol 6: 101–116.
16 Wang KC, CS Gau and RL Chen. 1982. Microbial oxidation of sterol. I. Conversion of cholesterol and sitosterol to 17-hydroxy steroids. J Taiwan Pharm Assoc 34: 129–137.
17 Ward OP and CS Young. 1990. Reductive biotransformations of organic compounds by cells or enzymes of yeast. Enzyme Microb Technol 12: 482–493.
18 Zurkowski P. 1964. A rapid method for cholesterol determination with a simple reagent. Clin Chem 10: 451–453.