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Production of androsta-1,4-diene-3,17-dione from cholesterol using two-step microbial transformation

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Abstract. A novel two-step transformation process for the production of androsta-1,4-diene-3,17-dione (ADD) from a high concentration of cholesterol by microorganisms is proposed. Cholesterol (20 g/l) was initially converted to cholest-4-en-3-one (cholestenone) by an inducible cholesterol oxidase-producing bacterium, *Arthrobacter simplex* U-S-A-18. The maximum productivity of cholestenone was 8 g/l per day and the molar conversion rate was 80%. Subsequently, a fine suspension of cholestenone (50 g/l), which was prepared directly from the fermentation broth of *A. simplex*, was converted to ADD by *Mycobacterium* sp. NRRL B-3683 in the presence of an androstenone adsorbent, Amberlite XAD-7. The maximum productivity of ADD was 0.91 g/l per day and the molar conversion rate was 35%.

Introduction

Microbial transformation of cholesterol and other sterols into 17-ketosteroids has received much attention, since it allows the use of inexpensive sterols as raw material for the production of steroid drugs (Imada et al. 1981; Lenz 1983). The major step in this bioconversion is the degradation of the C-17 side chain of sterols without concomitant degradation of the steroid nucleus. This process has been shown to be composed of multistep enzymatic reactions (Szentirmai 1990).

Sterol solubility in aqueous solution is very low (Haberland and Reynolds 1973). Several methods of enhancing its solubility have been proposed. The use of water-immiscible organic solvents may be unfavorable, as the side-chain degradative activity is easily inactivated by organic solvents (Buckland et al. 1975). The use of cyclodextrins (Hesselink et al. 1989) or aqueous two-phase systems (Flygare and Larsson 1989) seems to be more attractive in respect of the stability of the transformation activity, but the concentration of substrate in the reaction system is still limited. Therefore the use of insoluble substrate is still imperative and finer particles of sterols must be prepared in order to provide a larger surface area for the reaction.

Various methods of preparing sterol suspensions for bioconversion were used in the past, such as mechanical pulverization (Marsheck et al. 1972), sonication of sterol powder (Nagasawa et al. 1969), chemical pulverization using water-miscible organic solvent (Conner et al. 1976), homogenization (Srivastava et al. 1985), sonification of lyophilized powder prepared from an organic solvent of sterol and detergent (Martin and Wagner 1976), and adding the solution of sterol in an organic solvent to an aqueous system and removing the organic solvent by heating (Beaton 1978). A systematic study of the effect of the preparation method on production, however, has not been reported.

When they studied the mode of uptake of sterols, Goswami et al. (1983) found pseudosolubilization of sterols during the growth of cells and proposed that the production of sterol bioemulsifier/biosolubilizer by the cells was implicated. Similarly, Liu et al. (1983) also observed the same phenomenon in the study of the production of cholesterol oxidase by *Arthrobacter simplex* U-S-A-18.

The purpose of this study was to investigate the effect of the substrate-preparation method on the production of androsta-1,4-diene-3,17-dione (ADD) from a high concentration of substrate by microorganisms. A two-step process comprising the transformation of cholesterol to cholest-4-en-3-one (cholestenone) and followed by the transformation of cholestenone to ADD was proposed as a novel method for the bioconversion of steroid using a high concentration of substrate.

Materials and methods

Microorganisms. Mycobacterium sp. NRRL B-3683 was used as an ADD-producing strain (Marsheck et al. 1972). *A. simplex* U-

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S-A-18, an inducible cholesterol oxidase-producing strain (Liu et al. 1983), was used as a cholestenone-producing strain in this study.

Materials. Yeast extract, nutrient broth dehydrate, peptone and agar were purchased from Difco (Detroit, Mich., USA). Cholesterol and soy-bean lecithin were obtained from Tokyo Kasei Industries (Tokyo, Japan). Cholest-4-en-3-one was obtained from E. Merck (Darmstadt, FRG). Amberlite XAD-7, ADD, androst-4-ene-3,17-dione (AD) and Tween 80 were obtained from Sigma (St. Louis, Mo., USA). Inorganic salts and other chemicals were all of reagent grade.

Medium. Stock cultures were maintained on glucose- and yeast-extract-supplemented nutrient agar slants and the supplemented nutrient broth was used as the seed culture medium (Lee and Liu 1992). The basal medium for the production of cholestenone was composed of 20 g cholesterol, 2 g ammonium acetate, 5 g yeast extract, 1 g K_2HPO_4, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$ and 1 l distilled water (pH 7.2) (Liu et al. 1983). Cholesterol powder was used directly without any pretreatment. The basal medium for the production of ADD consisted of 1 g cholesterol, 1.5 g ammonium acetate, 0.8 g KH_2PO_4, 0.4 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 5 mg $FeSO_4 \cdot 7H_2O$, 2 mg $ZnSO_4 \cdot 7H_2O$, 0.5 mg $MnCl_2 \cdot 4H_2O$, 0.1 g Tween 80 and 1 l distilled water (pH 7.5) (Liu and Lee 1990). Standard cholesterol suspension was prepared by adding a solution consisting of 10 g cholesterol and 200 ml acetone dropwise into 200 ml of an aqueous Tween 80 solution (0.5%, w/v) under constant stirring. Distilled water was then added to give a final cholesterol concentration of 10 g/l.

Cultivation. Unless stated otherwise, a cotton-plugged 500-ml Hinton flask containing 100 ml medium was used as the reaction vessel. It was inoculated with a 2-day-old seed culture (10 ml for *Arthrobacter* sp. and 5 ml for *Mycobacterium* sp.) and incubated at 30° C with reciprocal shaking. Transformation of cholesterol to cholestenone was also carried out in a 5-l fermentor (Model FS-605, New Brunswick Scientific Co., N.J., USA). The operating conditions were as follows: working volume, 2 l; agitation speed, 500 rpm; surface aeration rate, 1.0 vvm; and inoculum size, 10% (Liu and Lee 1990).

Analytical methods. Steroids in fermentation broth and those adsorbed on Amberlite XAD-7 were extracted with ethyl acetate and acetone, respectively, for analysis. Cholestenone was determined by HPLC using a C_{18} column (Chemosorb I-7 C_{18}, 4.6 mm × 150 mm) and methanol as the mobile phase (flow rate, 3 ml/min). Androstenones (ADD and AD) were also determined by HPLC using the same column and a mobile phase composed of acetonitrile and water (50/50, v/v) (flow rate, 2 ml/min). Acetone in the reaction medium was determined by HPLC under the same conditions as that for androstenones. Cholesterol was determined by the method of Zurkowski (1964). Cell mass was determined by a turbidimetric method and expressed as total optical density at 600 nm (Lee and Liu 1992).

Results

Transformation of cholesterol to cholestenone

Under the optimum conditions for production of cholesterol oxidase (Liu et al. 1983), 1.4 g/l (3.6 mmol/l) of cholestenone was obtained from 20 g/l of cholesterol after incubating *A. simplex* U-S-A-18 in shaking flasks for 2 days. The production of cholestenone increased by a factor of four to six when 0.1 g/l of surfactant was added to the medium (Table 1). Although a low concentration of Triton X-100 showed the best stimulating effect on production, an strongly adverse effect was observed when its concentration increased above 0.1 g/l. On the other hand, increasing the concentration of soybean lecithin could enhance the production of cholestenone, although most of the product adhered to the wall of the flasks (Table 2).

Table 1. Effect of various surfactants on the production of cholestenone by *Arthrobacter simplex* U-S-A-18

Surfactant[a]	Cholestenone (mmol/l)[b]
Control	3.60
Lecithin (soy bean)	17.41
Triton X-100	20.62
Tween 80	15.89
Silicon	15.28
Span 80	14.58

[a] The concentration of surfactant was 0.1 g/l
[b] The initial concentration of cholesterol was 20 g/l and the cultivation time was 5 days

Table 2. Effect of the concentration of Triton X-100 and lecithin on the production of cholestenone by *A. simplex* U-S-A-18

Surfactant	Concentration (g/l)	Cholestenone (mmol/l)[a]	
		I[b]	II[c]
Triton X-100	0.05	20.56	—[d]
Triton X-100	0.10	21.61	—
Triton X-100	0.25	14.87	—
Triton X-100	0.50	1.69	—
Lecithin	0.10	17.40	—
Lecithin	0.50	18.10	37.78
Lecithin	1.00	19.31	32.18
Lecithin	5.00	9.16	36.13
Lecithin	10.00	3.52	31.12

[a] Production concentration after 5 days cultivation
[b] Samples were taken directly from the broth
[c] Samples were taken after the flask-adhering material was scraped and mixed with the broth
[d] No flask-adhering material was found

When the bioconversion was carried out in a fermentor using surface aeration, the amount of adherent product could be diminished and a much higher concentration (5 g/l) of lecithin was necessary for a higher and reproducible production of cholestenone (Fig. 1). Although the optimal pH of cholesterol oxidase was 7.5 (Liu et al. 1988), controlling the medium pH at 7.5 during the bioconversion had an adverse effect on the production of cholestenone (data not shown). After 48-h cultivation under optimal conditions, 16 g/l (42 mmol/l) of cholestenone was produced from 20 g/l of cholesterol (Fig. 2). The maximum productivity of cholestenone was 8 g/l per day and the molar conversion rate was 80% based on the substrate added.

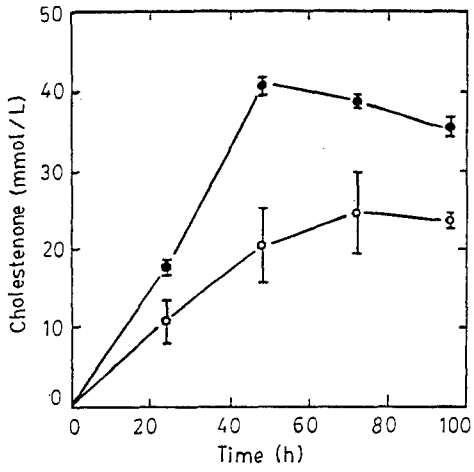

Fig. 1. Effect of the concentration of lecithin on the production of cholestenone by *Arthrobacter simplex* U-S-A-18 in a 5-l fermentor. The fermentation conditions were as follows: agitation speed, 500 rpm; surface aeration rate, 1.0 vvm; inoculum size, 10%. The lecithin concentrations were 0.5 g/l (○) and 5 g/l (●). Each test was carried out three times

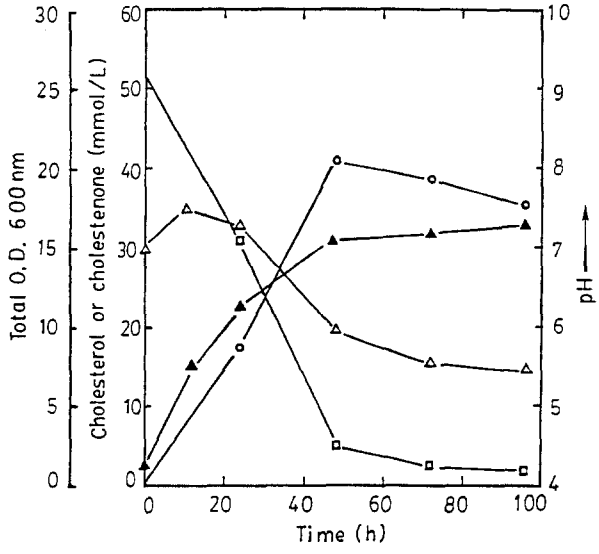

Fig. 2. Time course of the production of cholestenone from cholesterol by *A. simplex* U-S-A-18 in a 5-l fermentor under optimal conditions (the same as in Fig. 1) using 5 g/l of lecithin in the medium: □, cholesterol; ○, cholestenone; △, pH; ▲, total optical density at 600 nm (OD_{600})

Production of ADD with a high concentration of cholesterol

Standard cholesterol suspension proved to be a good preparation for the production of ADD when the substrate concentration was 1 g/l (Liu and Lee 1990). The production of ADD, however, decreased when the initial concentration of cholesterol was higher than 2.5 g/l (Fig. 3). Acetone in the standard cholesterol suspension could be removed by means of heating and stirring. When the heat-treated cholesterol suspension was used as substrate, the consumption of cholesterol increased in accordance with the increase in substrate loading, but not the production of ADD (Fig. 4).

Production of ADD from cholestenone

When the cholestenone suspension obtained by the transformation of cholesterol by *A. simplex* U-S-A-18 was used as the substrate, the production of ADD increased significantly (Table 3). It was also found that the consumption of cholestenone and the accumulation of ADD stopped after 2 days of incubation (Fig. 5). Because ADD had inhibitory effects on both the growth of

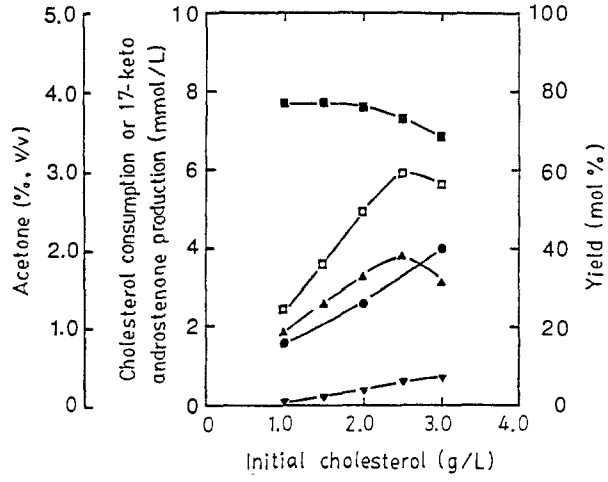

Fig. 3. Effect of the concentration of cholesterol and acetone on the production of androsta-1,4-diene-3,17-dione (ADD) by *Mycobacterium* sp. NRRL B-3683 in a synthetic medium with standard cholesterol suspension. The inoculum size was 1% and the cultivation time was 5 days: □, cholesterol consumption; ▲, ADD; ▼, androst-4-ene-3,17-dione (AD); ■, yield of total 17-keto androstenones (ADD and AD); ●, acetone

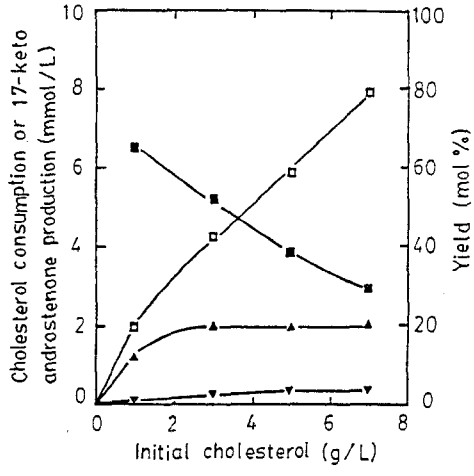

Fig. 4. Effect of the concentration of heat-treated cholesterol suspension on the production of ADD by *Mycobacterium* sp. NRRL B-3683 in a synthetic medium. Standard cholesterol suspension (10 g/l) was heated at 90° C for 5 h under stirring to remove acetone before preparing the medium: □, cholesterol consumption; ▲, ADD; ▼, AD; ■, yield of total 17-keto androstenones (ADD and AD)

Table 3. Effect of various substrates and the addition of Amberlite XAD-7 on the production of androsta-1,4-diene-3,17-dione (ADD) by *Mycobacterium* sp. NRRL B-3683

Substrate	Amberlite[a] XAD-7	Residual substrate (mmol/l)	Androstenones (mmol/l)[b]	
			ADD	AD
Cholestenone[c]	–	13.24	6.53	2.21
Cholestenone	+	4.44	13.65	1.26
Precipitated cholestenone[d]	+	5.64	10.84	1.26
Cholesterol suspension[e]	–	ND	2.44	0.56
Cholesterol powder[f]	–	ND	1.83	0.11

AD, androst-4-ene-3,17-dione; ND, not determined
[a] Amberlite XAD-7 loading was 1.5 g per 50 ml medium
[b] Product concentration after 6 days cultivation
[c] Diluted fermentation broth of *A. simplex* U-S-A-18 containing 10.4 g/l of cholestenone and 1.0 g/l of cholesterol
[d] Solid materials in fermentation broth of *A. simplex* U-S-A-18 were recovered by centrifugation and resuspended to the original volume
[e] Heated standard cholesterol suspension containing 10 g/l of cholesterol
[f] 10 g/l of cholesterol powder

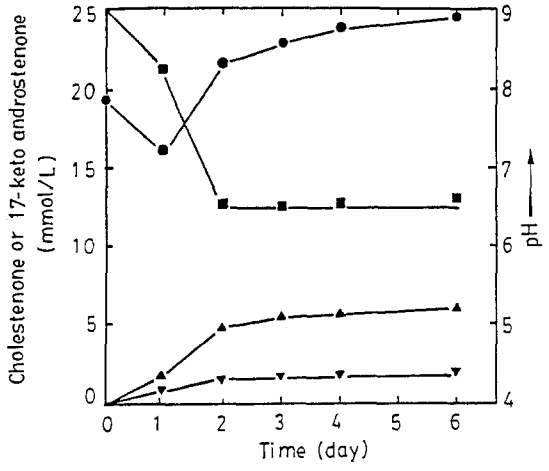

Fig. 5. Time course of the production of ADD from cholestenone by *Mycobacterium* sp. NRRL B-3683: ■, cholestenone; ▲, ADD; ▼, AD; ●, pH

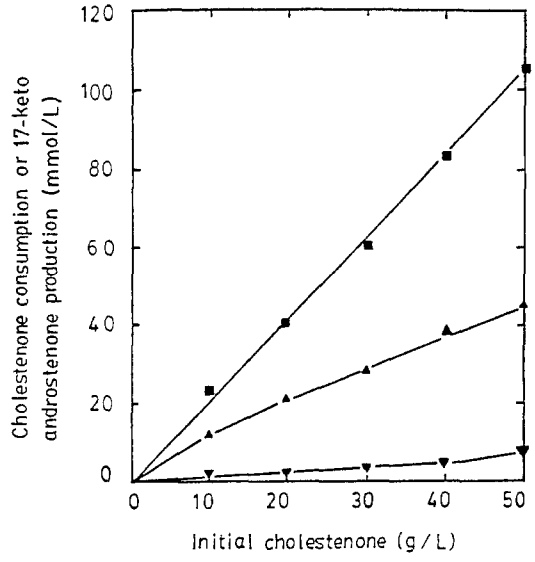

Fig. 6. Effect of the concentration of cholestenone on the production of ADD by *Mycobacterium* sp. NRRL B-3683 in the presence of Amberlite XAD-7. The cultivation time was 14 days. The amount of Amberlite XAD-7 was 5 g per 50 ml medium: ■, cholestenone consumption; ▲, ADD; ▼, AD

cells and the degradation of cholesterol (Lee and Liu 1992), polymeric adsorbent Amberlite XAD-7 was added to trap ADD (Martin and Wagner 1976). As a result, in the presence of Amberlite XAD-7 the production of ADD was promoted twofold (Table 3) and the concentration of substrate could be elevated to a level as high as 50 g/l without any adverse effect (Fig. 6). After 14 days cultivation, 12.8 g/l of ADD was obtained from 50 g/l of cholestenone. The maximum productivity of ADD was 0.91 g/l per day and the molar conversion rate was 35%.

Discussion

In order to increase the product concentration of microbial side-chain cleavage of sterols, the substrate concentration should be increased. Owing to the limitation of methods in enhancing solubility, the use of large amounts of finer particles of sterols, which provides an increased substrate surface area, is necessary. Sterols tend to be waxy and do not undergo milling very well, hence it is difficult to obtain finely ground powders. Sterols also tend to clump and do not disperse well in the medium. Ultrasonic breaking of cholesterol particles into finer ones during bioconversion could enhance the conversion rate (Bar 1988), but ultrasound had an adverse effect on the microbial slurry when the cholesterol loading was higher than 5.0 g/l. Beaton (1978) described a process for preparing high concentration sterols for bioconversion, which comprised dissolving the sterols in an organic diluent forming a solution, adding the solution to an aqueous system and removing the organic diluent. Although slurry with a sterol concentration more than 100 g/l could be obtained, the bioconversion was

performed only with diluted substrate suspension with a concentration no more than 5 g/l.

In our study it was found that 20 g/l of cholesterol could be converted efficiently to cholestenone by an inducible cholesterol-oxidase-producing bacterium, *A. simplex* U-S-A-18 (Fig. 2). Cholesterol powder was added to the medium containing lecithin without any pretreatment. Although the addition of small amounts of Triton X-100 (0.05–0.1 g/l) was of some advantage to the production of cholesterol oxidase (Liu et al. 1983), the addition of surfactants to the medium, however, had a highly stimulating effect on the production of cholestenone (Table 1). Therefore, the disintegration of cholesterol aggregates in aqueous medium by surfactant is crucial for success when a potent cholesterol-oxidase-producing strain was used in the transformation. On the other hand, the use of a harmless surfactant to the cell is also very important. After incubation for 2 days with incorporation of lecithin into the medium, a fine particle suspension of cholestenone at a high concentration could be obtained.

In a previous study we developed a synthetic medium for the production of ADD (Liu and Lee 1990). The substrate used was the standard cholesterol suspension, which was obtained by adding an acetone solution of cholesterol to an aqueous solution of Tween 80 under stirring. This preparation was good for production when 1 g/l of cholesterol was used, whereas an inhibitory effect emerged when a higher concentration of cholesterol was used. The inhibitory effect might be due to an increase in the concentration of acetone, which remained in the cholesterol suspension and was incorporated into the medium while preparing it. When acetone was removed by heating, the inhibitory effect could be eliminated. At least, the consumption of cholesterol was not limited. The heated cholesterol suspension, however, did not prove to be a good substrate preparation because the production of ADD did not increase in accordance with the increase in substrate loading and both the consumption of cholesterol and the accumulation of ADD in the system using heated substrate were less effective than those using the standard cholesterol suspension (Fig. 4).

When the fine cholestenone suspension, which was directly prepared from the fermentation broth of *A. simplex* U-S-A-18, was used as the substrate, a marvellously good result could be obtained in the production of ADD by *Mycobacterium* sp. NRRL B-3683. A substrate loading as high as 50 g/l could be used and the production of ADD was proportional to the increase in substrate loading as long as Amberlite XAD-7 was presented in the medium (Fig. 6). On the other hand, the amount of ADD produced from our bio-produced cholestenone suspension was greater than that produced from chemically pulverized cholesterol powder when a high concentration of substrate was used (Table 3). This result implied that the determinant of the preference for substrate was the degree of fineness rather than the structural difference between cholesterol and cholestenone. This is in contrast to the statement of Marsheck et al. (1972), who said that *Mycobacterium* sp. NRRL B-3683 displayed a definite preference for substrate possessing the 3β-hydroxy-Δ^5 system.

In a previous study we demonstrated that the productivity of ADD could be enhanced threefold, from 0.1 g/l per day to 0.3 g/l per day, when a surface-aerated fermentor was used in place of shaking flask as the reaction vessel (Liu and Lee 1990). In the present study the maximum productivity of ADD in the process using 50 g/l of cholestenone as substrate was 0.91 g/l per day. This figure was obtained by using a shaking flask as the reactor and there should be still a lot of space for increase if the reaction is carried out in fermentor. On the other hand, although the productivity obtained by using 50 g/l of cholestenone as substrate was nine times that obtained by using 1 g/l of cholesterol as substrate, this achievement, however, is not satisfied considering the huge increase of substrate loading. Because the yield of ADD based on substrate consumption decreased when the substrate concentration increased (Fig. 3), improving the yield becomes an important target for the enhancement of productivity of ADD when a high substrate concentration is used.

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