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Production of testosterone from phytosterol using a single-step microbial transformation by a mutant of *Mycobacterium* sp.

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A testosterone (TS)-producing mutant, ST2, was derived from a phytosterol-assimilating and androst-4-ene-3,17-dione (AD)-producing bacterium, *Mycobacterium* sp. B-3805S, using nitrosoguanidine (NTG) mutagenesis. Production of TS from phytosterol using a single-step microbial transformation process by ST2 was investigated in a 5-l surface-aeration microprocessor-controlled fermentor loaded with a synthetic medium supplemented with 0.1% phytosterol, 2% glucose and 1% peptone at 30°C. An increase in dissolved oxygen at the initial stage of fermentation favored the side-chain degradation of phytosterol to AD. Later in the fermentation, a decrease in the dissolved oxygen to zero resulted in a decrease in pH to 6.0 as well as the reduction of AD to TS. Under optimal fermentation conditions, the maximum conversion ratio of phytosterol to TS was 31% after 120 h cultivation. It was concluded that the control of dissolved oxygen in the fermentation culture is the most important parameter for production of TS from phytosterol via AD. TS was isolated from the fermentation culture by addition of Amberlite XAD-7 resin and was further purified by flash chromatography on a silica gel column. After crystallization, TS was obtained as needle crystals with the correct melting point.
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Introduction

Testosterone (TS) is the principal hormone released by the testes, and is produced by the interstitial cells. It acts as a powerful androgen and is responsible for the male sex characteristics. The final step in the biosynthesis of TS is reduction of androst-4-ene-3,17-dione (AD) to TS by 17-ketosteroid reductase (17β-hydroxysteroid: NADP 17-oxidoreductase, EC 1.1.1.64) [12]. The enzymatic reduction of AD to TS by 17β-hydroxysteroid dehydrogenase (17β-hydroxysteroid: NAD 17-oxidoreductase, EC 1.1.1.63) occurs in bacteria [11], yeast [15,18], and fungi [5]. Although the microbial transformation of sterols to AD is now utilized on an industrial scale, commercial production of TS from AD is still carried out using a chemical synthesis [7].

We have previously reported [8] a novel, single-step microbial transformation process for the production of TS from cholesterol by *Mycobacterium* sp. NRRL B-3805 in a synthetic medium supplemented with peptone and glucose. It was found that the reduction of AD, an intermediate metabolite, to TS was catalyzed by an inducible 17β-hydroxysteroid dehydrogenase of *Mycobacterium* sp. NRRL B-3805. As a cofactor, generation of NADH by metabolism of another substrate was necessary for the reduction of AD to TS in the microbial transformation process [7,10]. This single-step microbial transformation process could also be used to transform phytosterol to TS via AD [9]. The purpose of this study was to further elucidate important parameters involved in the transformation of phytosterol to TS during fermentation by *Mycobacterium* sp. NRRL B-3805.

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Materials and methods

Microorganism

A phytosterol-assimilating and AD-producing mutant strain, B-3805S, derived from *Mycobacterium* sp. NRRL B-3805 was used as parental strain.

Materials

Yeast extract, nutrient broth dehydrate, peptone and agar were obtained from Difco (Detroit, MI). AD, glucose and nitrosoguanidine (NTG) were obtained from Sigma (St. Louis, MO). The TS and phytosterol (campesterol:stigmasterol:β-sitosterol=8:1:11, mole ratio) used were products of Tokyo Kasei (Tokyo, Japan). Inorganic salts and other chemicals were all of reagent grade.

Cultivation methods

The stock culture was maintained on an enriched nutrient broth (ENB) agar medium, which contained (per liter): nutrient broth dehydrate 8 g, yeast extract 1 g, glucose 10 g and agar 20 g (pH 6.8). A glucose nutrient broth (GNB) medium consisting of 16 g of nutrient broth dehydrate and 40 g of glucose per liter of distilled water (pH 6.8), and a synthetic medium consisting of 1.5 g of ammonium acetate, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.4 g of K_2HPO_4, 0.8 g of KH_2PO_4, 5 mg of $FeSO_4 \cdot 7H_2O$, 2 mg of $ZnSO_4 \cdot 7H_2O$ and 0.5 mg of $MnCl_2 \cdot 4H_2O$ per liter of distilled water (pH 6.5) were used, respectively, as seed and basal media for the production of TS. Five milliliters of the seed culture was inoculated into 100 ml of a synthetic medium supplemented with 0.1% phytosterol, 2% glucose and 1% peptone in a 500-ml Hinton flask. The culture was incubated at 30°C on a rotary shaker (120 rpm) for 120 h. This was then used as inoculum in the fermentation experiments, which were carried out in a 5-l surface-aerated, microprocessor-controlled fermentor (Hotech, Taiwan) [7,9] loaded with 2.5 l of

Table 1 Production of androstenones from phytosterol by *Mycobacterium* sp. B-3805S and its mutants

Strain	Androstenones (mM)[a]			Total conversion ratio (%)[b]
	AD	TS	Total	
B-3805S	0.71	0.32	1.03	42.7
ST1	0.35	0.67	1.02	42.3
ST2	0.13	1.19	1.32	54.7

[a]Androstenones: AD, androst-4-ene-3,17-dione; TS, testosterone.
[b]A synthetic medium consisting of 1.5 g of ammonium acetate, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.4 g of K_2HPO_4, 0.8 g of KH_2PO_4, 5 mg of $FeSO_4 \cdot 7H_2O$, 2 mg of $ZnSO_4 \cdot 7H_2O$ and 0.5 mg of $MnCl_2 \cdot 4H_2O$ per liter of distilled water (pH 6.5) supplemented with 0.1% phytosterol, 2% glucose and 1% peptone was used for cultivation. The cultures were incubated in a 500-ml Hinton flask at 30°C on a rotary shaker (120 rpm) for 120 h. The working volume was 100 ml. The conversion ratio was calculated on the basis of 0.1% phytosterol (2.21 mM).

synthetic medium supplemented with 1% peptone, and the fermentor was operated at 30°C for 168 h. At the initial stage, 2% glucose and 0.1% phytosterol were added as carbon sources.

Isolation of TS-producing mutants

Mutagenesis was performed by an NTG-induced mutation [16]. Mid-log phase cells of strain B-3805S freshly grown in GNB medium at 30°C were harvested, washed and resuspended in Tris–maleic buffer (0.05 mol/l Tris, pH 6.0, 0.04 mol/l maleic acid) containing 100 μg of NTG/ml. After incubation at 37°C for 30 min, the NTG-treated bacteria were washed, resuspended in GNB medium with 0.1% Tween-80, and appropriate dilutions were plated on ENB agar plates containing 4 g/l of phytosterol. Plates were incubated at 30°C for 120 h. Visible colonies were regarded to become potentially tolerant to phytosterol through a mutation. These mutants were isolated and cultured in shake flasks containing a synthetic medium supplemented with 0.1% phytosterol, 2% glucose and 1% peptone at 30°C for 120 h. The culture filtrates were tested for AD and TS.

Isolation and purification of TS

TS in the fermentation culture was isolated by the addition of Amberlite XAD-7 resin (20 g/l of culture) as an adsorbent. The resin particles were filtered and washed with distilled water, TS was then eluted with acetone (2 ml/g of resin), and the solvent was dried over sodium sulfate and evaporated to dryness. The crude TS was further purified by flash chromatography on a silica gel column (15×2.5 cm) eluted with ethyl acetate/*n*-hexane (50/50, v/v). The fractions containing TS were collected and rechromatographed on a silica gel column using ethyl acetate/chloroform (20/80, v/v) as the developing solvent. The effluent containing TS was evaporated under reduced pressure.

Analytical methods

Steroids: The fermentation culture 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 determined by HPLC using a C_{18} column and a mobile phase composed of acetonitrile and water (50/50, v/v) [6]. Phytosterol was measured according to the Lieberman–Burchard color reaction [19]. The conversion rate of TS was calculated on the basis of phytosterol added to the medium.

Glucose and cell growth: Glucose was determined using the dinitrosalicylic acid method [1]. Cell growth was determined using a turbidimetric method ($OD_{600\ nm}$) as described in a previous paper [6].

Relative dissolved oxygen: Dissolved oxygen was measured using a dissolved oxygen probe (Ingold, Messtechnik, CH-8606, Switzerland). The relative dissolved oxygen was calculated from the initial dissolved oxygen (100%) of the medium before inoculation under the same fermentation conditions [7].

Results

Isolation of TS-producing mutants

One hundred and thirty phytosterol-tolerant mutants were initially isolated and tested for productivity of AD and TS. The productivities of two mutants, ST1 and ST2, as potential TS producers are shown in Table 1. The productivity of TS and the conversion ratio of phytosterol to AD and TS by mutant ST2 was superior to those of mutant ST1 and parental strain B-3805S. Therefore, mutant ST2 was used in the following experiments.

Effect of glucose on the accumulation of androstenones from phytosterol

The effect of glucose on the accumulation of androstenones, AD and TS from phytosterol by mutant ST2 was examined. The rapid consumption of phytosterol and glucose were in parallel with an increase in cell growth (Figure 1). The maximum accumulation of AD occurred at 72 h. From that point, there was a reduction of AD as TS increased. Most TS was oxidized to AD after 144 h cultivation.

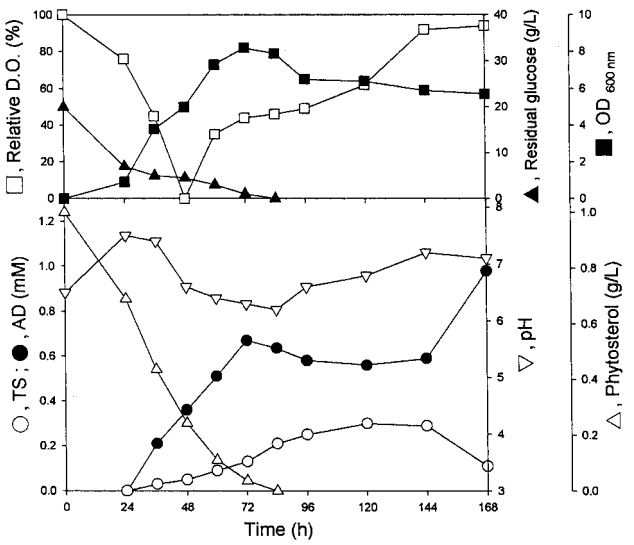

Figure 1 Accumulation of androstenones from phytosterol in a 5-l surface-aeration fermentor. The same synthetic media were used as shown in Table 1. The operating conditions were: working volume, 2.5 l/5-l fermentor; inoculum size, 5%; surface aeration rate, 1 vvm; agitation speed, 300 rpm; temperature, 30°C; 0.1% phytosterol, 2% glucose and 1% peptone were added at the initial stage.

Since the mutant ST2 consumed phytosterol rapidly in the presence of glucose, the fermentation conditions for the production of TS were changed to a synthetic medium supplemented with 0.1% phytosterol and 1% peptone. After incubation at 30°C for 24 h, 2% glucose was added. The rapid consumption of phytosterol and the added glucose were paralleled by an increase in the cell growth over the first 60 h cultivation (Figure 2). At the same time, the pH and the relative dissolved oxygen in the fermentation culture decreased to 6.0 and zero, respectively. The maximum AD accumulation occurred at 72 h cultivation. From that point, there was a reduction of AD as TS increased. By 96 h, there was an increase in dissolved oxygen, pH and AD accumulation in the fermentation culture, which paralleled a decrease in cell growth and TS accumulation. From the above results, it was obvious that the changes in relative dissolved oxygen, pH and cell growth in the fermentation culture were apparently important parameters for production of AD and TS from phytosterol.

Effect of agitation speed on the accumulation of androstenones from phytosterol

A linear-program control of agitation speed was used to control dissolved oxygen in the fermentation culture. The agitation speed fixed at 300 rpm for 24 h, and then linearly increased to 400 rpm between 24 and 72 h. As shown in Figure 3, the rapid consumption of phytosterol and glucose were parallel with increases in cell growth and pH. During this period, the relative dissolved oxygen was always maintained at a high level. When the agitation speed was linearly decreased from 400 to 250 rpm between 72 and 96 h, the dissolved oxygen was gradually reduced to zero. Maximum accumulation of AD appeared at 96 h, and by then, most of the AD was reduced to TS after cell growth decreased, and the pH decreased to 6.0. The maximum accumulation of TS was at 120 h. The conversion ratio of phytosterol to TS was about 31%. After 120 h cultivation, cell lysis occurred and thereafter most of the TS was oxidized to AD.

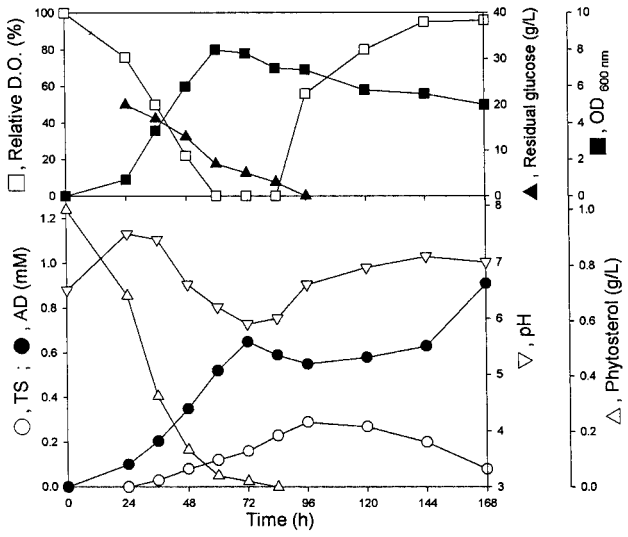

Figure 2 Effect of later addition of glucose on the accumulation of androstenones from phytosterol in a 5-l surface-aeration fermentor. The fermentation conditions are the same as shown in Figure 1, except that 2% glucose was withheld until 24 h after cultivation.

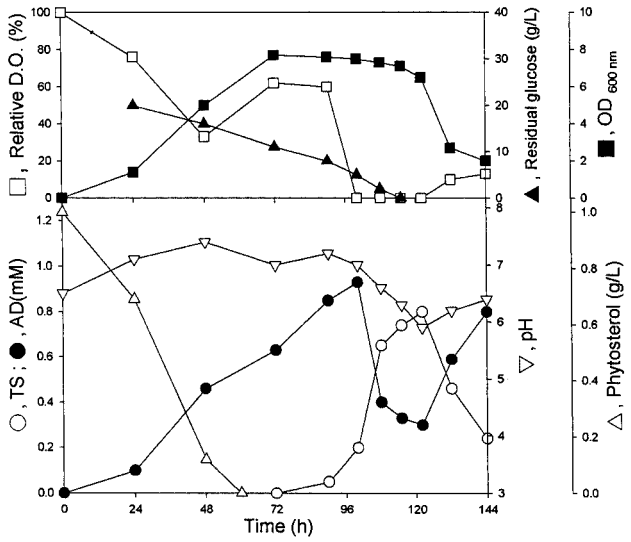

Figure 3 Effect of agitation speed on the accumulation of androstenones from phytosterol in a 5-l surface-aeration fermentor. The fermentation conditions are the same as shown in Figure 2, except that agitation speed was controlled by a linear program as follows: 0–24 h, 300 rpm; 24–72 h, linear speed-up from 300 to 400 rpm; 72–96 h, linear speed-down from 400 to 250 rpm; 96–144 h, 250 rpm.

Purification of TS from the fermentation

A TS powder with a purity over 98% was obtained from the fermentation using the method described earlier. After recrystallization in acetone, needle crystals were obtained. The melting point of these crystals was 153–154°C, which was identical to that of authentic TS.

Discussion

Microbial degradation of the side chain of sterols has become an important transformation process for production of the 17-ketosteroid intermediate, AD [4]. Four inducible groups of catabolic enzymes are involved in the side-chain degradation pathway; the fatty acid β-oxidation system, the ω-oxidase reaction, a methyl-crotonyl-CoA carboxylation system and the propionyl-CoA caboxylase system [17]. Enzymatic reduction of AD to TS has also been found in some microorganisms [2,14,15,18]. However, the available information concerning the production of TS from sterols via a single-step microbial transformation process is still very limited [3,13,17].

We have previously reported [7] that the reduction of AD to TS was catalyzed by an inducible 17β-hydroxysteroid dehydrogenase in *Mycobacterium* sp. NRRL B-3805. The optimal pHs for the reaction between AD and TS in the presence of NADH and NAD were 6.0 and 8.9, respectively. The oxidation of TS to AD was preferred to the reverse reduction reaction. Therefore, the supply of reducing power, NADH, by metabolism of another substrate (e.g., glucose) was necessary for reduction of AD to TS in a fermentation of *Mycobacterium* sp. NRRL B-3805 [10]. As shown in Figure 2, rapid consumption of phytosterol and the added glucose resulted in an increase in cell growth and accumulation of AD. However, reduction of AD to TS was depressed by the increase in relative dissolved oxygen and pH in the fermentation culture. Consequently, most of the TS was oxidized to AD. The results of the

reversible reaction between AD and TS in the fermentation culture agrees with the reversibly catalytic properties of 17β-hydroxysteroid dehydrogenase in *Mycobacterium* cells [7,10].

Since microbial transformation of phytosterol to TS occurred via the side-chain degradation of phytosterol to AD and the further reduction of AD to TS by 17β-hydroxysteroid dehydrogenase, the oxygen supply seemed to be the most important parameter for a multistep biotransformation process. In the present study, a linear-program control of agitation speed was used to control dissolved oxygen in the fermentation culture. As shown in Figure 3, the increase of relative dissolved oxygen by linearly increased agitation speed from 300 to 400 rpm resulted in an increase in cell growth and pH. These conditions were favorable for accumulation of AD by the side-chain degradation of phytosterol.

On the other hand, the decrease of relative dissolved oxygen to zero level by linearly decreased agitation speed from 400 to 250 rpm resulted in a decrease in cell growth and of pH to 6.0. Under these conditions, most of the AD was reduced to TS by a 17β-hydroxysteroid dehydrogenase in the *Mycobacterium* cells. Although the fermentation conditions for the side-chain degradation of phytosterol were different from that of cholesterol, the conditions for the reduction of AD to TS in fermentation culture of phytosterol were similar to cholesterol [7,10].

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