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Conversion of Plant Sterols to Androsta-4-ene-3,17-dione by a mutant of *Mycobacterium sp.* NRRL B-3805

Lee, Kang-up
Technology Research Institute, Sun-Hill Glucose Co. Inchon, Korea

*Mycobacterium*종 (NRRL B-3805)의 변이종에 의한 식물스테롤의 androsta-4-ene-3,17-dione(AD)으로의 전환

이 강 업
선일포도당 기술연구소

ABSTRACT: A mutant was selected by NTG treatment of *Mycobacterium* sp. NRRL B-3805, which was capable of degrading plant sterol to androsta-4-ene-3, 17-dione and yields was higher than NRRL B-3805. Also this mutant produced androst-4-ene-3, 17-dione faster than NRRL B-3805.

It described the mode of sitosteroidal degradation, and the interrelation between cell membrane and its attachment to substrate during the sterol degradation process by this mutant and it was compared with *Mycobacterium* sp. NRRL B-3805.

KEY WORDS□ Mycobacterium sp. Sitosterol Mutants.

Use of microorganisms to convert readily available sterols, such as cholesterol, β-sitosterol, and compesterol, into clinically important steroids had its beginnings in the work of Turfitt (Turfitt, 1944, 1946, 1948). He found that incubation of the actionmycete *Nocardia erythropolis* with cholestenone yielded androst-4-ene-17β-carboxylic acid. The significance of this and its possible economic implications did not begin to be seen until the structure and clincal uses of the naturally occuring animal corticosteroids and sex hormones had been elucidated.

Much efforts have been put into devising methods by which sterols may be microbially degraded only to the steroid nucleus and in yields high enough to qualify as industrial processes (Charnev *et al.*, 1967; Iizuka *et al.*, 1967).

One approach has been either to screen many different species of fungi, yeast, and bacteria or isolate an unknown culture which is capable of using the sterol as a sole carbon source, hoping to chance upon one which naturally metabolizes only the side chain. Another approach has been the modification of the starting sterols. Sih *et al.* (1965) noted that estrone was resistant to microbial attachment, and reasoned that, if a substrate was arranged so that A-ring aromatization could occur without the retro-aldol reaction which would lead to a 9, 10-seco steroid, estrone should be produced in good yield. Third approach was also made to improve the yields of steroidal products from natural sterols by inclusions in the incubation medium of chemical agents, such as chelation agents, metals, and redox dyes, which apparently inhibit essental steps in nuclear degradation. In this way, yields of up to 57% of androsta-1, 4-diene-3, 17-dione from sterols have been obtained. The final approach was the generation and isolation of mutant, and this method is a well established process in microbial genetics.

An increasing amounts of the pharmaceutically active steroid compounds annually produced in industry was derived from readily available sterol sources such as plant sterol or cholesterol (Kieslish, 1980). It has been the history of the fermentation industry that the major increases in product yields hae resulted

from modification of the producing organisms by mutation of physical or chemical means.

Marsheck *et al.* (1972) reported a process, in which naturally isolated soil bacteria degraded the cholesterol and plant sterols to produce androsta-1, 4-diene-3, 17-dione and androst-4-ene-3, 17-dione. These isolates were described as *Mycobacterium* sp. NRRL-B-3683 and mycobacterium sp. NRRL-B-3805. These mycobacteria produce substantial amounts of 17-Ketonic compounds without appreciable degradation of the steroid nucleus. No ring degradation inhibitory agent was used for this process. The mutagen of the choice for this study was NTG, a very efficient mutagenic agent. Nitrosoguanidine has widely been used as a potent mutagen in the field of microbial genetics, since mandell *et al.* (1960) discovered its mutagenicity. Cerda-olmedo *et al.* (1967) described the effect of nitrosoguanidine on macromolecules and their synthesis, in connection with the lethal and mutagenic action. They found that repair replication of DNA took place after the treatment with nitrosoguanidine.

In prokaryotes the mutagen NTG was thought to induce mutation selectively at the DNA replication fork and NTG mutagenesis at different stage of the cell cycle has provided information on the order and timing of individual gene replication. (Adelberg *et al.*, 1965) This report describes a process for the mutating a mycobacterium sp. NRRL-B-3805, improving AD production as the results of the mutation. And also it describes the mode and parameters of sitosterol degradation by a mutant strain.

METHODS & MATERIALS

Bacterial strain

Mycobacterium sp. NRRL-B-3805, was used in the current studies. This strain lacked the ability to 1-dehydrogenate steroids and was found to produce androst-4-ene-3, 17, dione from sterols. Approximately 1000 isolates which were picked at random from the survivors of a single NTG treatment has been screened, in the initial 3 runs for the first step, for the production of degradation of plant sterol. The best 10 AD producers from each of these 3 runs also have been screened in the second step. The 5 AD over-producers were selected from first 3 run of the second step screen. (with some of the first 10 AD producers 20 α-hydroxy methyl-pregna-1, 4, dien, 3-one (SC30571) was also produced). First NTG treatment of the bacterium produced an isolate (B-1) with increased ability to produce AD as a major product and 20 α-hydroxy methyl-pregna-1, 4, dine, 3-one as a minor product (Fig. 1). The second step treatment of NTG produced an isolate with AD over production ability and it was designated *Mycobacterium* sp. B-2.

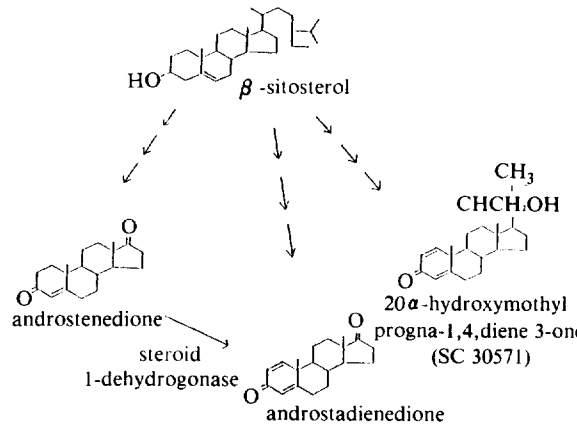

Fig. 1. *Biodegradation of β -sitosterol to several steroidal intermediates by mycobacterium sp.*

Mutagenesis.

Methods of mutagenesis was modified from the paper reported by Radochova *et al* (9) and Konicek *et al* (4). Medium A consisting of NH_4NO_3, 1 g; K_2HPO_4, 0.25 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $FeSO_4 \cdot 7H_2O$, 0.05 g; glycerol, 20 g; Tween 80, 0.1 g; and 1 liter of tap water was adjusted to pH 7.7 with potassium hydroxide. The resultant medium was added as 50 m*l* portion to shake flasks (250 m*l*) and sterilized by heating 20 minutes at 121 °C. This medium was inoculated with 2.5 m*l* of a culture of *Mycobacterium* sp. NRRL-B-3805 grown in the same medium. The culture was incubated at 30 ± 1 °C on a rotary shaker with a 2 inch circular orbit at 200 RPM for 2 days. The culture was pelleted by centrifugation and cells are suspended in an equal volume of 0.05M Tris-maleic buffer (pH 8.0) containing 0.01% tween 80, 15 m*l* of Tris-maleic buffer containing 1 mg/m*l* of nitrosoguanidine were added to 15 m*l* of the cell suspension to give a final NTG concentration of 500 ug/m*l*. The cell suspension was incubated in a water bath shaker for 15 minutes at 30°C, after which the suspension was centrifuged once more. The cells were washed three times with a solution of 0.85% NaCl and 0.01% tween 80, and then resuspended in medium A.

Isolation of mutant mycobacterium sp B-2

Following incubation on the shaker for one day at 30 ± 1 °C to allow for segregation of putative mutant from cell clumps, the culture was diluted serially in-tris maleic buffer with tween 80. Aliquots from ap-

propriate dilution are spread on the surface of previously prepared plates containing 20 to 26 m/ of medium B which has the following formula: peptone, 5 g; beef extract, 3 g; yeast extract, 1 g; glycerol, 20 g; agar, 15 g; and 1 liter of tap water, and which was sterilized for 20 min. at 121 °C. The plates were then incubated at 30 ± 1 °C for 7 days.

Shake tube evaluation.

Resultant colonies which appear on the agar plates are picked at random and transferred to test tubes containing 10 m/ of medium B. The cultures were incubated statically for 7 days at 30 ± 1 °C. One-half m/ portions of the inoculum cultures were then transferred to test tubes (25 × 125 mm) containing 10 m/ of A medium which contained 100 mg of plant sterols. The substrate was added before sterilizing. After 7 days incubation on the shaker at 30 ± 1 °C, the shake tube cultures were extracted with 10 m/ of methylene chloride. The methylene chloride extracts were analysed by thin layer chromatography using silica gel and a solvent system consisting of 90:10 (by volume) toluene-ethanol. The evidence of the presence of significant amounts of AD confirm the selective degradation of plant sterols by the novel mutant produced from the parent *Mycobacterium* sp B-1.

Shake culture growth and fermentation condition.

500 m/ erlenmyer flasks containing 100 m/ of media A were inoculated with 3 m/ of a cell suspension from a 7-day old agar slant culture of *Mycobacterium* sp. The seed culture flask was incubated in a controlled environment on a rotary shaker (New Bruinswick Scientific Co) at 200 rec/min. After 72 hours at 31°C 10 m/ of the inoculum was taken from the seed culture flask and transferred to 100 m/ of medium B in a 500 m/ erlenmyer flask. For the preparation of sterol substrate in media B, 1000 mg of powdered sitosterols, N.F. (The up John Co; Contanining 55% β-sitostero and 45% campesterol) prepared pulverizing with a mortar and pestal, was added to each 500 m/ erlenmyer flask containing 100 m/ of media B in a 500 m/ erlenmyer flask containing 100 m/ of media. The inoculated medium B was incubated in a controlled environment on a rotary shaker at 200 rec/min. for 168 hours at 31 °C.

Extraction and analysis of products.

The bioconversion beers were extracted twice with one-half volume of methylene chloride, and the extracts were combined. Preliminary analysis of the fermentation was performed by thin-layer chromato graphy on silica gel G-plates. The plates were developed using toluene-ethanol (85:15, v/v) as the developing solvents. The spots were visualized by spraying the plates with 50% sulphuric acid and heating at 60 °C.

Table 1. *The Production of AD and SC30571 by Three-different Mutants of NRRL B-3805, B-2, and B-1.*

Organisms	Conc. 1 g/l	AD[a] g/l	SC 30571[a] g/l
NRRL B-3805	1 g/l	0.37	trace[b]
NRRL B-2	1 g/l	0.65	trace
NRRL B-1	1 g/l	0.5	0.3g

[a] Measured by GLC, [b] Less than 0.01g

Isolation and identification of the products.

After removal of the methylene chloride under reduced pressure, the residue from a 5 g fermentation broth was subjected to column chromatography on 500 g of silica gel CC-7 (J.T. Baker). Elution with benzene containing successively increasing proportion of ethyl-acetate afforded the following products: Androst-4-ene-3, 17-dione (15 to 20%ethyl acetate); androsta-1, 4-diene-3, 17-dione (20 to 25% ethyl acetate); and 20 α-hydroxymethyl pregna-10,4-dine-3-one (30 to 50% ethyl acetate) (Fig. 1.)

The fraction containing each of the aforementioned products were combined and recrystalized: Androst-4-ene-3, 17-dione from ethyl acetate-hexane, melting point (mp) 170 to 172 °C; Androsta-1, 4, diene-3, 17-dione from ether, mp 139 to 141 °C; and 20 α-hydroxymethyl pregna-1, 4-diene-3-one from aceton-ether, mp 179 to 182 °C. Androst-4-ene-3, 17-dione, and androsta-1, 4-diene-3, 17-dione were identified by comparison of their physical properties, including mp, mixed mp, optical rotation, TLC, and GLC with those of authentic samples.

Synthesis and structural proof of 20 $^{\alpha}$-hydroxymethylpregna-1, 4-dien-3-one: A sample of 20 α-hydroxymethylpregna-1, 4-dien-3-one obtained by column chromatography and recrystallization was further recrystallized twice from acetone-ethyl acetate, giving pure material: mp 181 to 183.5 °C; [α] D + 28.17 (C 1, chloroform); ultraviolet maxima (methanol), 245 nm infrared maxima (KBr), 3438 (OH), 1669, 1621, 1601, and 887 cm^{1} (Δ^{14}-3-ketone); NMR (Table 8); molecular weight, 328 (M.S.). Analysis: $C_{22}H_{32}O_2$ (328.48); calculated: C, 80.44: H, 9.83; found: C, 80.09; H, 9.9.

Identification of 20 α-hydroxymethy-preg-4-en-3-one: It was identified by comparison of their physical properties, including mp, mixed mp, optical rotation, thin-layer chromatography, and GLC.

Determination of fatty acid composition

Cultures grown in various types of media were first acidified with HCl to prevent enzymatic degradation of celular lipids and then saponified with methanolic KOH at room temperature. The methyl esters of the free fatty acid in an ether extract of the saponified cells were formed by reaction with 14% boron trifluoride methanol reagent. Suosequently, the methyl esters were analysed by a GC procedure (Fig. 6&7).

The various fatty acid methyl ester peaks were tentatively identified by matching retention times with those in a calibrated standard containing 23 bacterial fatty acid methyl esters (Supelco).

Microscopy procedures

Phase-contrast microscopy. Wet mounts were prepared from conversion samples after first allowing the larger sitosterol particles to settle to the bottom of the container. Th mounts were examined using a Leitz Dialux microscope fittd with 12.5X widefield oculars and a 100X phaco 3 phase-contrast objective.

Conversion samples were centrifuged and the pellets in Karnovsky's fixative (4% paraformaldehyde, 5% glutaraldehyde in 0.2 M phosphate buffer, pH 7.2). The fixed preparation were freeze-dried and further processed by routine S.E.M. procedures before being examined on a scanning electron microscope.

RESULTS & DISCUSSION

Time course studies

The time course of AD production by Mycobacterium B-2 and B-3805 in shake flasks containing various amounts of sitosterol was presented in Table 2 and Fig. 2. Total AD yiels by both strains were directly proportional to sitosterol concentration with very limited yields obtained with concentration from 0.5 to 2 g/L. Both strains produced significant AD yields only at plant sterols concentration of 5 g/L and above. The time-course and total amounts of AD produced by a mutant and B-3805 was similar over the range from 0.5 to 5.0 g/L of sitosterol. However, commensurate with the greater growth and sterol degradation achieved by a mutant in the presence of higher levels of sitosterol, a mutant produced AD faster and to a greater extent than B-3805 with sitosterol at 10 and 15 g/L.

The effect of citric acid on AD production

AD yields by a mutant increased proportionally with increasing citric acid concentration over the range from 0.25 to 1 g/L (Table 3 and Fig. 3). Increasing the concentration from 1 g to 2 g/L resulted in a further slight increase over most of this range. Very low AD yields and pH were obtained in media containing on or 0.25 g/L of citric acid. Growth was also greatly decreased in such media as indicated by micro and macroscopic observation. The apparent

Table 2. *Effect of Plant Sterol Concentration on the Time-course of AD Production by B-2 and B-3805 in Shake Flask*[a]

Strain	Sitosterol Conc. (g/l)	AD yields (g/l) Day 1	2	3	4	5	6	7	Micoscopic Observat Free Cells	Sitosterol Colonization[d]
B-2	0.5	0.31	0.25	0.25	0.25	0.25	0.25	0.21[a]	heavy	ess. none
	1.0	0.37	0.28	0.33	0.43	0.43	0.43	0.54[a]	moderate	ess. none
	2.0	0.42[a]	0.53[a]	0.64[a]	0.68[a]	0.62	0.62	0.62[a]	moderate	overgrown
	5.0	0.40[a]	0.88[a]	1.29[a]	0.93	0.24	1.02	1.74[b]	moderate	Sl Overgrown
	10.0	0.42	1.24	1.83	2.34	2.85	2.14	3.04[a]	very few	Complete
	15.0	0.31	0.84	1.74	2.42	3.91	3.44	4.04[c]	very few	Complete
B-3805	0.5	0.12	0.25	0.32	0.25	0.25	0.25[a]		heavy	ess. none
	1.0	0.16	0.28	0.34	0.31	0.53	0.53	0.53[a]	moderate	ess. none
	2.0	0.31[a]	0.53[a]	0.48[a]	0.62	0.74	0.84	0.8[a]	moderate	overgrown
	5.0	0.43	0.59[a]	0.82[a]	1.15[a]	1.43	1.24	1.45[b]	moderate	Sl. Overgrown
	10.0	0.39	0.74	1.43	2.02	2.29	2.33	2.79[a]	few	Complete
	15.0	0.31	0.93	1.24	1.83	2.73	2.54	2.65[c]	very few	Complete

[a]Average of two sets of data

[b]Average of three sets of data

[c]Average of seven sets of data

[c]As determined after 7 days of incubation

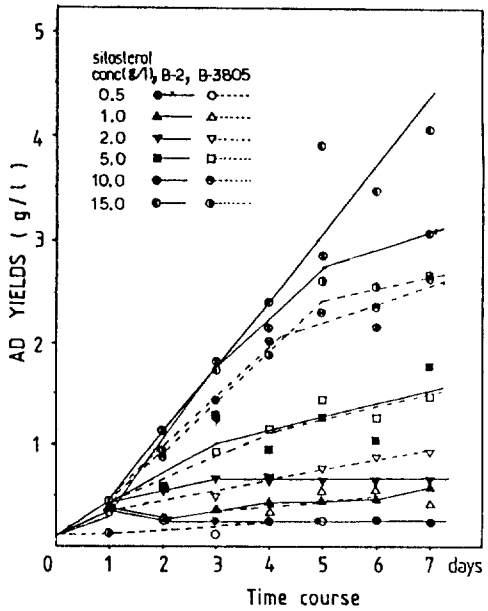

Fig. 2. *Time course of AD production by B-2 and B-3805 in shake flasks.*

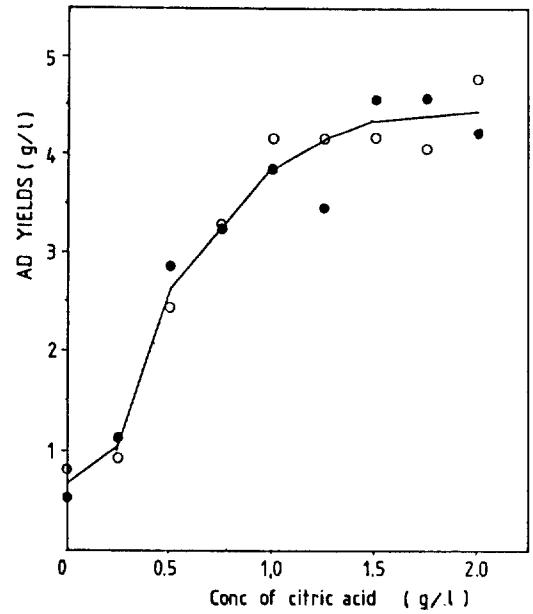

Fig. 3. *The effect of citric acid concentration on AD yields by B-2.*

Table 3. *The Effect of Citric Acid Concentration on AD Yields by B-2 in Minimal Media*[a]

Citric acid conc. (g/l)	1 st Run Final pH	1 st Run AD yield (g/l)[b]	2nd Run Finsl pH	2nd Run AD yield (g/l)[b]
0.00	5.2	0.53	5.0	0.81
0.25	5.3	1.24	5.4	0.93
0.50	7.0	2.85	7.0	2.42
0.75	7.2	3.22	7.4	3.32
1.00	7.4	3.84	7.1	4.15
1.25	7.3	3.44	7.1	4.15
1.50	7.3	4.56	7.1	4.15
1.75	7.4	4.56	7.2	4.03
2.00	7.4	4.20	7.3	4.73

[a]AD yields were determined by the routine shake flask procedure except that the citric acid concentration of the medium was varied as idicated.
[b]The concentration of Sitosterol in minimal media was 15 g/l.

Table 4. *The Effect of Varying Concentrations of Calcium Chloride on AD Production by B-2*[a]

$CaCl_2$ conc. (mM)	1 st Run Final pH	1 st Run AD yield (g/l)[b]	2nd Run Finsel pH	2nd Run AD yield (g/l)[b]
0	7.3	4.74	7.2	4.77
25	6.9	1.64	7.2	1.44
50	6.9	1.74	7.0	1.44
75	7.1	1.43	6.7	1.06
100	7.1	2.64	6.7	0.86

[a]AD yields were determined by the routine shake flask assay procedure except that the $CaCl_2$ was added as indicated.
[b]The concentration of Sitosterol in minimal media was 15 g/l.

citric acid requirement of a mutant presumably reflects an absolute requirement for iron where the amounts of iron available to the organism depends on the amounts of citric acid present.

The effect of $CaCl_2$ on the AD production

As shown on table 4, adding various concentration of $CaCl_2$ over the range from 25 to 100 mM decreased AD production by a mutant. Final pH value of the $CaCl_2$ containing flasks were also decreased slightly. Microscopic observation indicated that sitosterol crystals were well colonized in the presence or absence of $CaCl_2$. Very few free cells were observed in the absence of $CaCl_2$. However, all four levels of $CaCl_2$ tested appeared to stimulate cell growth resulting in the presence of many highly-aggregated cells free in the medium. These observations suggest

Table 5. *The Effect of Tween 80 and Yeast Extract on Plant Sterol Transformation by Mycobacterium B-2 and NRRL B-3805*[a]

Strain	Tween 80 Conc. (%)	Yeast extract Conc. (%)	1st Run AD yield[b]	2nd Run AD yield[b]
B-2	0.1	0.0	4.43	4.30
	0.5	0.0	3.63	3.06
	0.0	0.1	4.65	4.16
	0.0	1.0	4.65	4.16
	0.0	1.0	3.04	4.04
	0.5	0.1	5.05	4.24
	0.0	0.0	5.44	4.85
B-3805	0.1	0.0	3.53	3.32
	0.5	0.0	2.95	2.74
	0.0	0.1	4.34	3.45
	0.0	1.0	3.44	3.52
	0.0	1.0	3.44	3.52
	0.5	0.1	3.63	3.12
	0.0	0.0	3.39	3.21

[a]AD yiels were determined by the routine shake flask assay procedure with Tween 80 and yeast extract added as indicated.

[b]The concentration of Sitosterol in minimal media was 15 g/l.

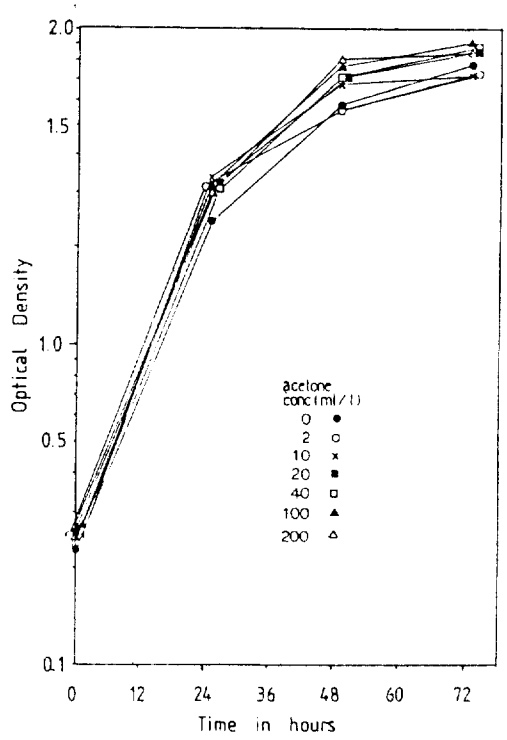

Fig. 4. *The effect of acetone concentration on growth of B-2.*

that even though $CaCl_2$ was capable of stimulating cell growth it did so in a way which was not fully compatible with sitosterol conversion.

The effect of tween 80 and yeast extract on AD production

As shown in table 5 the lower concentration of tween 80 (0.1%) or yeast extract (0.1%) and the combination of tween 80 (0.5%) and yeast extract (0.1%) has primarily stimulatory effects on product formation by a mutant (B-2) and B-3805. The higher tween 80 (0.5%) and yeast extract (1.0%) concentrations inhibited AD production by mycobacterial strains.

The effect of acetone and ethanol-solubilized sitosterols on AD production

AD production by a mutant was substantially reduced when sitosterol was dissolved in acetone and added to the production medium at 2 g/L before autoclaving. Essentially no AD was produced when the sitosterol was dissolved similarly in ethanol. Although the solvent concentration in both cases was high (20%), microscopic observation on the production flasks and the growth experiments discussed below indicated that the acetone at this concentration has a slightly stimulatory effect on growth. Ethanol at the same concentration somewhat inhibited the growth according to optical density data (Fig. 4 and

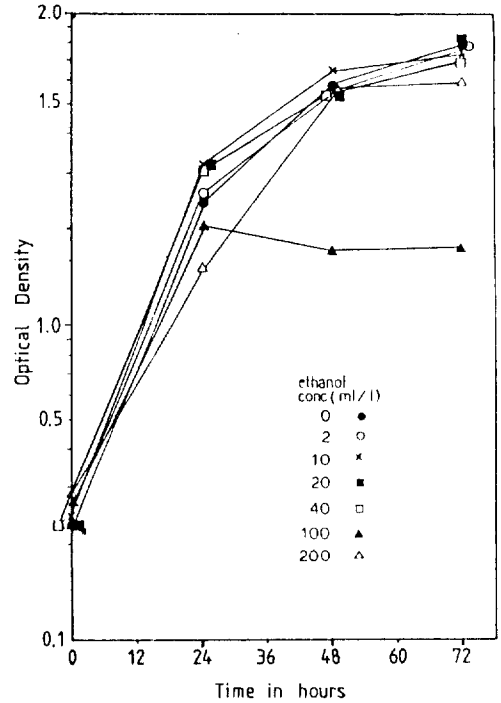

Fig. 5. *The effect of ethanol concentration on growth of B-2.*

Table 6. *The Effect of Phosphate Concentratin on AD Production by Mycobacterium B-2 and NRRL B-3805*[a]

Phos. conc. (g/l)	First Run B-3805 AD yield[b] (g/l)	First Run B-2	Second Run B-3805 AD yield[b] (g/l)	Second Run B-2
0.10	—	3.55	3.78	4.17
0.25	3.60	—	—	—
0.50	3.33	5.08	4.07	5.08
1.00	3.53	5.08	2.74	4.62
2.50	3.23	4.58	2.84	4.36
5.00	3.03	4.14	2.96	3.71
7.00	—	4.06	2.51	4.30
10.00	2.05	—	—	—
20.00	2.10	—	—	—

[a]AD yields were determined by the routine shake flask assay procedure in minimal Medium with phosphate varied as indicated.

[b]The concentration of Sitosterol in minimal medium was 15 g/l.

5). The effects of several concentrations of acetone or ethanol on the growth of B-2, in production medium was shown in Fig. 4 and 5, respectively. Acetone generally stimulated growth over the range tested (2 to 200 ml/L) with the higher concentrations supporting the most growth. Results with ethanol were more variable with maximum growth supported by lower concentrations (2 to 20 ml/L) and growth leveled off after 24 hours with 100 ml/L of ethanol (This phenomena probably due to extensive clumping), but remained relatively high in the presence of 200 ml/L of ethanol through the incubation period. Final growth levels obtained in ethanol-containing media were only slightly lower than those obtained in acetone containing or slovent free media. These results suggest that factors other than solvent inhibition growth probably are responsible for the poor AD yields obtained in solvent-containing production medium as discovered above.

The effect of phosphate concentration on AD production by a mutant (B-2) and NRRL B-3805.

As shown in Table 6, the phosphate concentation in the range from 0.1 g to about 1.0 g/L resulted in essentially comparable AD yields which higher concentration especially in the 10 to 20 g/L range were inhibitory.

The studies show that the phosphate concentration over the range of 0.1 to 1.0 g/L supported the maximum AD yields.

Table 7. *The Effect of Plant Sterol Concentration on AD Yields and Conversion Efficiencies by NRRL B-3805 and Mycobacterium B-2*[a]

Sitosterol Conc. (g/l)	B-3805 AD Yield (g/l)	B-3805 Conversion Efficiency (% of Theoretical Yield)	B-2 AD Yield (g/l)	B-2 Conversion Effeciency (% of Theoretical Yield)
1.0	0.57	82.6	0.54	78.3
2.0	0.80	60.9	0.62	47.0
5.0	1.94	54.4	1.74	48.9
10.0	2.68	38.9	3.04	43.5
15.0	2.97	28.8	4.04	39.2

[a]The routine shake flask procedure was used except that the amount of Sitosterol was varied as indicated.

Table 8. *Comparisons of Yields Supprted by Medium B and Minimal Medium*[a]

Medium	Sitosterol Conc. (g/l)	AD Produced by B-1 in 7 days (g/l)	(% Conv.)[b]	SC 30571 procuced by B-1 in 7days (g/l)	(% Conv.)[c]
Medium B	1	0.5	73.8	0.44	55.0
	15	4.39	42.6	3.60	29.9
Minimal	1	0.39	56.4	0.51	58.8
Medium	15	3.47	33.5	3.78	31.5

[a]AD and SC-30571 yields were determined by the routine shake flask assay procedure.

[b]The % conversion was calculated using a factor of 0.69 to account for the relative formula weight of AD and sitosterol.

[c]The % conversion was calculated using a factor of 0.80 to account for the relative formula weight of SC 30571 and sitosterol.

The effect of plant sterols in medium A on AD production by a mutant (B-2) and NRRL B-3805.

As shown in table 2, at least 5 g/L of sitosterol were required to support significant AD production and provide sufficient surface area for efficient colonization by both the mutant (B-2) strain and NRRL B-3805 in the routine shake flask system. The difference between B-2 strain and NRRL B-3805 shows up in higher and more rapid AD production from the larger amounts of plant sterols tested which reflects better growth and greater degradation of sitosterol crystals.

The effect of sitosterol concentratin on AD yields and conversion efficiency by a mutant (B-2) and NRRL B-3805.

As shown in table 7, AD yields produced by NRRL B-3805 and the mutant (B-2) in shake flask increased with increasing sitosterol conc. over the range from 1.0 to 10.0 g/L. No or very little further increase of AD yields was obtained with NRRL B-3805 when 15 g/L of sitosterol was substrated, however the mutant (B-2) strain produced fairly larger amounts of AD with same conc. of sitosterol used.

As reported by Jiu, J and M.J. Kvetkas (1980) the SC 30571 producing mutant was identified as being of interest. Mycobacteriem B-1 identical to the mutant B-2 was isolated at the first step of the first set isolates. The mutant mycobacterim B-1 was tested during the first mutagenesis/screening cycle. According to G.C.analysis this mutant produced approximately 30% of the theoretical yields of SC 30571 and 8% of 20 α-hydroxymethyl-pregna-4-ene-3-one (SC 25873) (Fig. 1. and Table 1) based on the amounts of sitosterol recovered.

Considerably higher SC 30571 yields (50%) had been obtained with the mutant B-1 in shake flask assays. This suggest that B-1 mutant which reverted to AD production might also produce AD at almost same high rate (Table 8). The attempts to isolate such revertants following the treatment with NTG was quite successful. As mentioned above the mutant (B-2) was AD over producer according to the data shown previously.

Microscopic Description of the Sterol Conversion process.

Sitosterol conversions are initiated when one or more mycobacterial cells from the inoculum culture attach to the surface of a sitosterol particle. Following attachment these cells multiply and form microcolonies on the surface of the particle. Subsequent cell growth and colonization is accompanied by a gradual dissolution and etching of the sterol particle during which small pieces break off from the outer edges. This process culminates in the essentially complete disintegration of the entire sterol particle when-

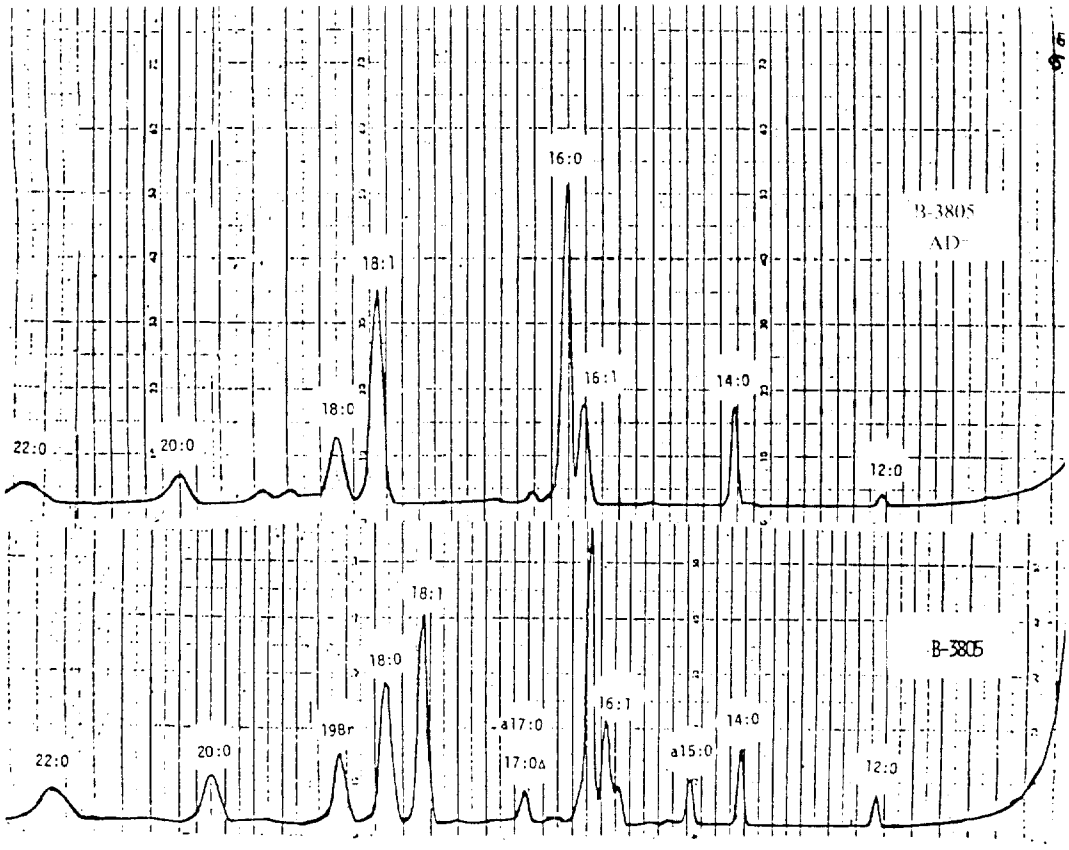

Fig. 6. *Comparison of the Fatty Acid composition of M. sp. B-3805 AD⁻ and B-3805.*

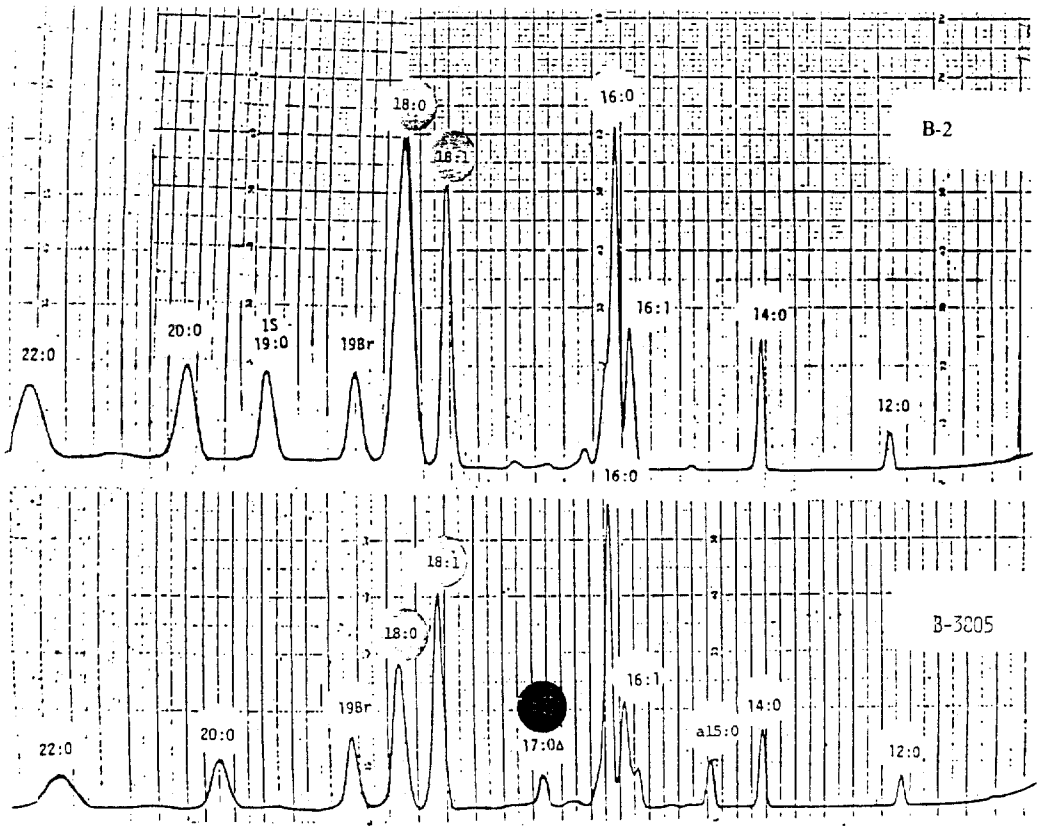

Fig. 7. *Comparison of the Fatty Acid Composition and M. sp. B-2 and B-3805.*

ever the process continues long enough. The process is quite rapid whenever the initial size of the particle is comparatively small relative to the size of the mycobacterial cell.

AD crystals appear to be formed in two ways during the conversion process. Much of this AD, especially in poorer converisons, remains associated with the cell/sitosterol complex and may gradually cover the exterior of these complexes as the crystals continue to grow. In the better conversion, free AD crystals also appear when the sitosterol concentration in the medium exceeds its solubility. Some of the free crystals grow to be quite large. Phase contrast and scanning electron photomicrographs which illustrate this process are presented in Fig. 8 to 15. The scanning electron micrograph were particularly helpful in that they provided a dramatically different insight into the intimate association of AD production with cell/sitosterol complexes.

Based on microscopic observations, the amount of AD produced in any given sitosterol conversion appears to reflect the extent of cell growth which occurred during the conversion process. How much growth actually does occur depends, in turn, on two additional factors (i.e., the genetic nature of the strain and the composition of the conversion medium). For example, both the original AD production strain, B-3805, and the improved strain, B-2, grow better when stimulants such as yeast extract, surfactants, and lipids are added to the conversion medium. However B-2 consistently grows to a greater extent and produces more AD than B-3805 when tested under the same conditions.

Microscopic observation made during the course of these studies established that at least 5 g/L of sitosterol were required to provide sufficient surface area for efficient colonization by both B-2 mutant and B-3805 cells in the growing population. A substantial proportion of free cells were released into the medium by the second day for B-2 and by the third day for B-3805 cultures. Increasing the amounts of sitosterol to higher levels decreased the proportion of free cells. Use of 2 g/L of sitosterol still allowed the efficient colonization early in the incubation period

Fig. 8. *Initial stage of attachment of a cell on the surface of the sitosterol particle.*

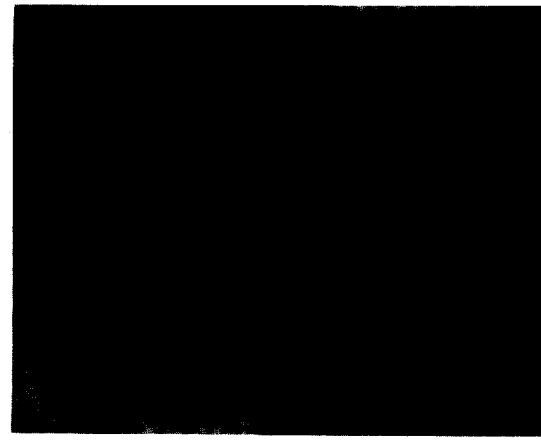

Fig. 11. *Later stage of colonization showing extensive sitosterol degradation and etching.*

Fig. 9. *Early stage of microcolony formation on the surface of the sitosterol particle.*

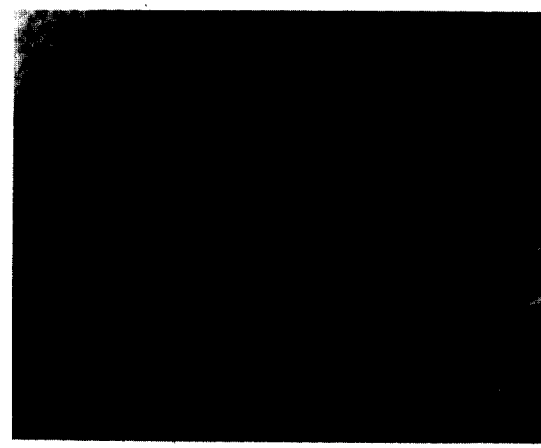

Fig. 12. *Still later stage of colonization showing chunks which had broken off larger particles.*

Fig. 10. *Early stage of colonization over the entire sitosterol particle surface.*

Fig. 13. *Still later state of colonization showing AD crystals covering an entire cell/sitosterl complex.*

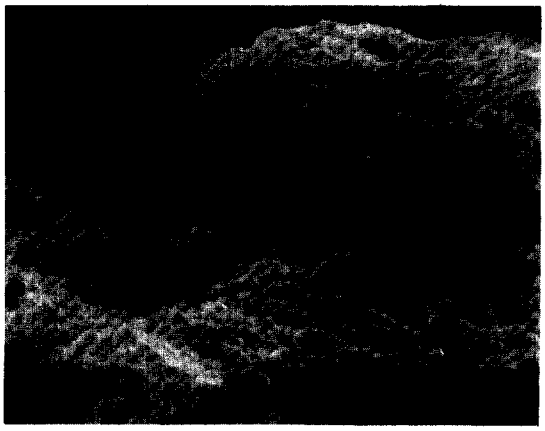

Fig. 14. *Scanning electron micrograph (S.E.M.) showing an intermediate stage of colonization.*

Fig. 15. *S.E.M. showing a complex of cells which have overgrown a small sitosterol particle.*

although both B-2 and B-3805 cells overgrew the sitosterol surface later on. When only 0.5 to 1 g/L of sitosterol was present, colonization by both strains was markedly less efficient with most of cell multiplication occuring free in the medium.

Role of Lipids in the Cell Envelope.

Many of the organisms active in sterol conversions (e. g., Arthrobacter, Corynebacterium, Mycobacterium, and Norcardia) have similar cell walls which typically contain very high levels of lipid compared to other microorganisms (Table 9). These lipids presumably play an important role in both attachment to and solubilization of the sterol which makes it available to the sterol-converting enzyme. Many of these sterol-converting enzyme are lipoproteins which are located in the cell membrane.

Table 9. *Lipid Contents of Several Bacteria*

Organism	Gram Stain	% Cell Dry Wt.	% Cell Wall Dry Wt.
Escherichia coli	Negative	9.6	22
Serratia marcescens	Negative	7.2	—
Bacillus megaterium	Positive	4.0	—
Staphylococcus aureus	Positive	2.5	4
Mycobacterium BCG	Positive	36.0	52
M. Phlei[a]	Positive	25.0	60

[a]Arthrobacter, Corynebacterium and Nocardia are very closely related taxonomically of rapidly-growing Mycobacterium species such as M. phlei and presumably contain similar amounts of lipid in their cell walls.

Table 10. Minimal Mediu For Plant Sterol Conversion To AD

Ingredient	g/l
K_2HPO_4	0.5
$MgSO_47H_2O$	0.5
Citric Acid	2.0
Ferric Ammonium Citrate	0.05
NH_4NO_3	1.0
Glycerol	20.0
Tap water	Q.S. to 1,000 ml

It has been known for some time that amounts of lipids in mycobacterial cell walls can be varied by change in the environmental conditions (Gusev *et al.*, 1980). For example, change in the medium constituents (i.e., carbon source, carbon/nitrogen ratio; addition of fats, oils or surfactants, etc), in the incubation temperature and in the age of the culture will all affect both the total amounts and fatty acid composition of cell envelope lipids.

The presence of large amounts of lipids in the walls of B-3805 and B-2 cells has serveral practical consequences which must be dealt with in working with cultures of these organisms (Ratledge, 1976). First, these cells typically grow in clumps which often become so large at early stages in the growth cycle that they are visible macroscopically, especially in unsupplemented minimal medium (Table 10). While older cultures appear to be considerably less granular by eye, microscopic examination reveals that cell clumps of various sizes are retained throughout the entire growth cycle.

The tendency of B-3805 and B-2 cells to form clumps can be minimized by growing them in rich media

or in minimal media supplemented with lipids. For example, adding Mazu 60 p, a lipoidal type organic antiform, to B-2 seed flasks results in more extensive and dispersed growth of this organism (Marsheck *et al.*, 1975). Similarly, exponential phase culture of B-3805 or B-2 grown in the presence of 1% Tween 80 can be dispersed by sonication to give the single cell distribution which are needed for mutagenesis procedures. As mentioned above, lipids in mycobacterial cell walls also appear to be responsible for the attachment of these cells to sitosterol particles. This interpretation is supported by the observation that cells of a non-AD producing mutant of B-3805 (Kvetkas and Jiu, 1977) grow somewhat more dispersely than B-3805 cells and also unable to attach to sitosterol particles. Differences in the fatty acid composition of these two strains and of B-2 grown in the same medium are shown Fig. 6 and Fig. 7.

In addition, changes in the fatty acid compostion and increases in total cell lipids reported to occur in mycobacterial cells with increasing culture age presumably are responsible for changes in the ability of B-3805 cells to attach to and convert sitosterol to AD (Table 2). These data illustrate the necessity of having sitosterol present at the begining of the fermentation.

As shown in Table 2, cells which had multiplied freely in the medium for much more than 16 hours in the absence of sitosterol would not be able to attach to additionol sitosterol which was added after that time. Conversely, adding sitosterol at later times to fermentation in which sufficiently large amounts of sitosterol were present initially would also appear to be useless. Under these conditions, essentially all of the cells from the original inoculum culture would already be attached to sitosterol particles.

Sterol transformation by several strains of NRRL B-3805.

TLC assay of mycobacterium B-1951-99 and one of its derivative (Lee and Jiu, 1990) indicated that these two strains produced approximately equal amounts of AD and 9 α OHAD when tested in small scale shake flask system with 10 g/L of sitosterol. The derived strain produced only trace amounts of two (9 α-OH SC30571 and SC25813), presumably hydroxylated intermediates with SC30571 as substrate and essentially on products with AD as substrate, however B-1951-99 produced good amounts of 9 α OHAD and 9α-OH SC30571 from AD and SC30571 respectively. B-1951-99 strain attached efficiently to sitosterol, but the derivative strain did not attach to SC30571 and AD according to microscopic observations made after 7 days of incubation.

It is still not clear whether attachment is necessary for substrate utilization by mycobacteria in steroid as well as in sterol transformation. While attachment appears to correlate with acceptance of sitosterol as substrate (non-AD producing mutants do not attach to sitosterol).

As reported by Kvetkas. M.T. and J. Jiu (1977) one of mutant (B-HB-33) generated from NRRL B-3805 produced testosterone efficiently from AD even though the cells did not attach to this substrate.

However, P.C. Goswami (1983) reported that growth and substrate transfer took place primarily by attachment of cell to substrate in the case of β-sitosterol.

In conclusion, all of the results presently available have been in conformity with the hypothesis that mycobacteria derived from or similar to NRRL B-3805 utilize as substrate only those compounds (primarily sterols) which have a 3-hydroxy, Δ5,6 configuration. Most of these strains, with the exception of HB-33, and B1951-99 appear to utilize steroids or steroidal intermediates with 3-Keto, Δ 4,5 configuration poorly if at all. With mutant HB-33 and B-1951-99 as the examples, it is likely that other strains able to use steroidal substrates could be produced by mutation. This consideration suggerted that mycobacterium B-1951-99 would be a good candidate organism for strain improvement efforts for the production of steroidal derivatives. Secondly, as shown on Table 2, and 7, B-2 was used to convert 10 g/L of sitosterol under routine shake flask condition, there were considerable number of free cells present and relatively rapid and complete sitosterol colonization and degradation. When the sitosterol concentration was increased to 15 g/L, there were essentially no free cells and increasingly poor colonization and degradation of sitosterol particles. The same pattern held true with NRRL B-3805 but with sitosterol concentration shifted down by about 5 g/L. Thirdly the B-2 and B-3805 cells may attach to macroscopic substrate particle very well because of its higher lipid content with cell envelope, and substrate transfer to cells may place presumably through absorption-adsorption of substrate molecules on the cell envelope when substrated by a 3-hydroxy, Δ configuration sterol.

적 요

*Mycobacterium*속 균주의 하나인 NRRL B-3805를 NTG로 처리하여 AD 생산성이 증가된 새로운 돌연변이주인 B-2 균을 선별하였다. B-2 균주의 AD 생산성은 모균인 NRRL B-3805보다 약 50%가 향상되었으며 배양시간도 단축되었다. 또한 이 연구에서는 세포벽에 함유되어 있는 lipid의 양에 있어서 B-2 균이 다른 *Mycobacterium*속의 균주나 모균인 B-3805보다 많이 가지고 있었으며 따라서 세포벽에 존재하는 lipid의 증가에 따라 sitosterol의 매체에 의한 용해성이 향상되어 sterol converting enzyme에 의한 이용을 쉽게 한다는 점과 substrate가 균주의 세포벽에 부착하는데 도움을 준다는 점 때문에 AD의 생산성이 향상된 것으로 생각되었다. 이러한 점에서 substrate가 B-2 균주의 세포벽에 부착하는 mechanism을 설명하고 NRRL B-3805 균주와 비교하였다.

REFERENCE

1. **Carda-olmedo, E., P.C. Hanawalt:** *Biochim. Biophys. Acta.*, **142:** 450 (1967).
2. **Charney, W., H.L. Herzog:** P. 11.*Academic press Inc.* New York (1967).
3. **Iizuka, H., A. Naito:** Microbial transformation of steroids and alkaloids. University park press, state college, pa (1967).
4. **Konicek, J., G.Y. Daraselia and Konic Kova-Radochova:** *Fol. Microbiol.*, **26:** 62 (1981).
5. **Kieslich, K:** *J. Basic Microbiol.*, **25:** 461 (1985).
6. **Lee, K.U., and J. Jiu:** *Kor. J. Appl. Microbiol. Biotech.* **18:** 12-17 (1990).
7. **Mandell, J.D., and J. Greenberg:** *Biochem. Biophys. Res. Commun.* **3:** 575 (1960).
8. **Marsheck, W.J., S. Kraychy, and R.D. Muir:** *Microbiol.* **23:** 72 (1972).
9. **Radochova, M., J. Konicek, and I. Malek:** *Fol. Microbiol.* **11:** 163 (1966)
10. **Sih, C.J., Y.Y. Tson, S.S. Lee, K.C. Wang, and F.N. Chang:** *J. Amer. Chem. Soc.* **87:** 2765 (1965).
11. **Sih, C.J., and K.C. Wang:** *J. Amer. Clhem. Soc.* **87:** 1387 (1965).
12. **Turfitt, G.E.:** *J. Bacteriol.* **47:** 487 (1944), *Biochem. J.* **40:** 79 (1946), *Biochme. J.* **42:** 376 (1948).
13. **K. Kieslish:** *Steroid Conversion. Economic Microbiology*, **5** (p 369-413). Academic press (1980).
14. **KvetKas, M.J., and J. Jiu:** *Chemical Development Report of Searle Lab.* (unpublished paper) (1977).
15. **J. Jiu and M.J. KvetKas:** *Microbiology Reportof Searle Lab.* (unpublished paper) 1980.
16. **Ratledge, C.:** The physiology of Mycobacteria: *Adv. Microbial physiology*, **13** (115-244). Academic press (1976).
17. **Marsheck, W.J., and P. Wang:** *Microbiology Development Report of Searle Lab.* (unpublished paper) (1975).
18. **Gusev, M.V., T.V. Koronelli, Y.N. Korolev, and T. I. Komarova:** *Microbiologiya*, **49**, 619 (1980).
19. **Schomer, U., and F. Wagner:** *European J. of Appl. Microbiol. Biotechnol.*, **10** (1980)
20. **Goswami, P.C., H.D. Singh, S.D. Bhagt, and J.N. Baruah:** *Biotechnol. Bioeng.*, **25.** 2929 (1983).
21. **Adelberg, E.A., M. Mandel, and G.C.C. Chan:** *Biochem. Biophys. Res Commun.* **18:** 788-795 (1965).

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