CHAPTER 1

Microbial Conversion of Lithocholic Acid to Ursodeoxycholic Acid by *Fusarium equiseti* M41

Introduction

Ursodeoxycholic acid (UDCA) was first isolated from bear bile in 1927 by Shoda (48). It has been used as a chemotherapeutic agent and has recently been reported to solubilise cholesterol in gallstones (43). It is prepared at present by a seven-step chemical synthesis (Fig. 1), but the yield have been fairly low (9 to 14%) (46,47).

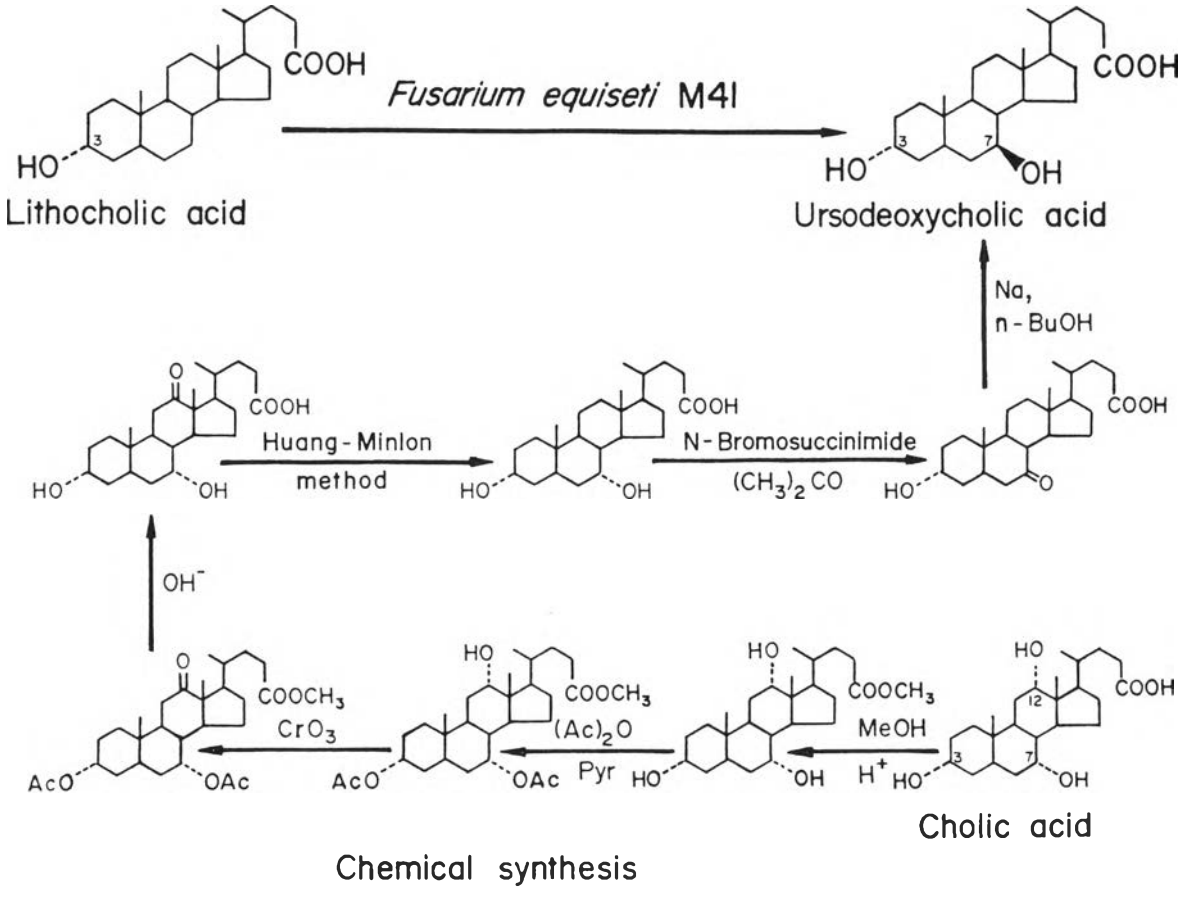

Fig. 1. UDCA production by chemical and microbial processes.

We found a soil fungus, *Fusarium equiseti* M41, which transformed lithocholic acid (LCA) to UDCA in a one-step reaction with 35% yield. This process might have economic appeal since LCA is inexpensive and commercially available.

Until this study, the conversion of LCA to UDCA by fungi has not been reported, although several microorganisms have been reported to hydroxylate steroids at the C-7-position: *Curvularia lunata* converts progesterone to 7α,14α-dihydroxyprogesterone (49), *Curvularia fallax* and *Curvularia pallescens* convert 11-deoxycortisone to 7α,21-dihydroxypregn-4-ene-3, 20-dione (50), *Rhizopus nigricans* transforms 4-methyltestosterone to 4-methyl-7β-hydroxytestosterone (51), and *Rhizopus nigricans* ATCC 62276 hydroxylates digitoxigenin to 7β-hydroxydigitoxigenin (52).

This chapter describes the taxonomic identification of strain M41, the chemical identification of the product, and product preparation in a 10-ℓ fermentor.

Materials and Methods

Chemicals LCA, which was extracted and purified from ox bile, was purchased from Sigma Chemical Co., St. Louis, Mo. Since it contained a small amount of deoxycholic acid, it was recrystallized from ethanol before use. UDCA was purchased from Gasukuro Kogyo Co. Ltd., Tokyo.

Medium The basal medium used for the isolation of microorganisms contained 30 g of glucose, 5 g of yeast extract, 2 g of KH_2PO_4, 3 g of K_2HPO_4, 5 g of $NaNO_3$, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 0.5 g of LCA in 1 ℓ of tap water. The pH of the medium was adjusted to 7.0. The production medium contained 50 g of oatmeal, 5 g of $NaNO_3$, 3 g of K_2HPO_4, 2 g of KH_2PO_4, 0.5 g

of $MgSO_4 \cdot 7H_2O$, 20 mg of $FeSO_4 \cdot 7H_2O$, 20 mg of $MnSO_4 \cdot 6H_2O$, and 1 g of LCA in 1 ℓ of tap water. The pH of the medium was adjusted to 7.0.

Screening of microorganisms Diluted soil suspensions were spread on the basal agar medium in Petri dishes, which were incubated at 27°C for 2 to 6 days. Isolated fungal colonies were transferred to 100-ml conical flasks containing 20 ml of the basal medium and 0.5 g/ℓ LCA. The flasks were incubated at 27°C on a rotary shaker for 5 days. The ethyl acetate extracts of the culture broths were analyzed for UDCA by thin-layer chromatography (TLC).

Cultivation Five liters of culture medium in a 10-ℓ jar fermentor (type MD-500; L. E. Marubishi) was inoculated with 250 ml of a 48-h seed culture. The pH was kept at 7.0, the temperature at 27°C, the aeration rate at 0.5 vvm, and the agitation speed at 300 rpm unless otherwise indicated.

Analysis and assay Bile acids were esterified with hexafluoro-isopropanol and trifluoroacetic acid anhydride and analyzed by gas-liquid chromatography. The operating conditions for gas-liquid chromatography (Hitachi 165) were: column size, 3 mm by 1 m; packing material, uniport HP coated with silicon DC QF-1 (2%); Gasukuro Kogyo, Tokyo; column temperature, 220°C; injection temperature, 270°C; nitrogen gas flow rate, 40 ml/min; detection, flame ionization detector; sample size, 10 μl. The retention times were 6 min for LCA and 16 min for UDCA. Cell mass in the basal medium was expressed as dry weight, grams per liter. In the oatmeal-containing production medium, the cell growth was monitored as nucleic acid complex content determined by the method of Schneider (53). Glucose was determined by the Somogyi-Nelson method. The total sugar content was determined as glucose by the same method after acid hydrolysis.

Gas chromatography was performed by use of Hitachi 165. Mass,

infrared, and nuclear magnetic resonance spectra were obtained with Hitachi RMU-6E, Hitachi 215, and JEOL JUM-TS-100 spectrometers, respectively.

Thin layer chromatography Twenty-ml portions of culture broth were acidified to pH 3.0 and extracted two times with 50-ml portions of ethyl acetate. The extracts were concentrated to dryness in a rotary evaporator and the residue was dissolved in 5 ml of ethanol. Aliquots of 10 µl were spotted on thin-layer plates (Kiesel gel 60 F_{254}; E. Merck AG), which were developed with chloroform-acetone-acetic acid (100:100:1, by vol.). After drying, the plates were sprayed with concentrated sulfuric acid and heated at 160°C for 5 min. The R_f values of LCA (yellow) and UDCA (green) were 0.62 and 0.49, respectively.

Results

1. Screening of microorganisms

None of the steroid-hydroxylating fungi listed in the American Type Culture Collection Catalogue of Strains is registered as a transformer of LCA to UDCA. Eight strains, namely, *C. lunata* ATCC 12017 and 13432, *C. pallescens* 12018, *C. fallax* ATCC 38579, *R. nigricans* ATCC 34121, *Rhizopus stolonifer* ATCC 6227 b, *Thamnicium elegans* ATCC 18191, and *Verticillium theobromate* ATCC 12474, were examined for the ability to produce UDCA from LCA. The last two strains were registered as hydroxylated-steroid producers, but no more details are given in the American Type Culture Collection Catalogue. Of the 8 strains, only *C. lunata* ATCC 13432 produced UDCA from LCA, but in a yield of less than 5% (54).

To obtain UDCA producing fungi, we screened 609 fungal strains isolated from soil. Only one strain, M41, showed the ability to convert LCA to

UDCA efficiently. Strain M41 gave a higher yield than *C. lunata* ATCC 13432, so it was selected for further studies.

2. Identification of strain M41

When strain M41 was cultivated on potato-dextrose agar at 30°C, the mycelium was white initially, pinkish after 3 days, and finally dark brown. In the early stage of cultivation, macroconidia were formed from a single phialide (2.5 to 3 by 10 to 12.5 μm), which was formed in addition to the conidiophores. These conidiophores formed no sporodochia, and obclavate phialides (3 to 4 by 12 to 17 μm) were formed on top of the conidiophores after 2 weeks (Fig. 2). Macroconidia were crescent shaped and variable in size. They usually had five septa (3.8 to 4.5 by 50 to 63 μm), but sometimes three (3.8 to 4.8 by 30 to 38 μm). When microconidia were formed, they were oval to cylindrical (3.0 to 3.7 by 3.7 to 5.0 μm). Intercalary chlamydospores in the mycelium were formed singly or in chains and were globose (7- to 9-μm diameter) or oval (5 to 8.7 by 11 to 12.5 μm). Asci

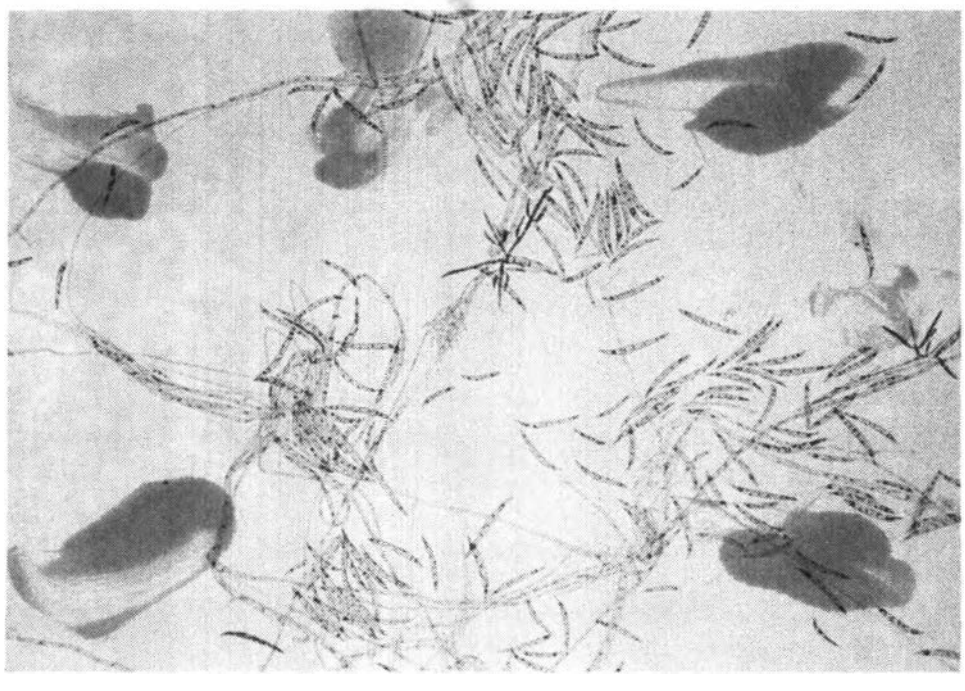

Fig. 2. Photomicrograph of *Fusarium equiseti* M41 grown on oat-meat agar slant for 2 weeks (×200).

and ascopsores were not formed, even after 2 months of cultivation on potato-dextrose and oatmeal agars.

From these morphological characteristics, strain M41 was identified as *Fusarium equiseti* (Corda) Sacc. Sylloge Fung (55).

3. Identification of the product

Strain M41 was cultivated in Czapek Dox broth (Difco) with 2.5 g of LCA (0.5 g/ℓ) in a jar fermentor as described in Materials and Methods. After 60 h of cultivation, UDCA was detected by TLC, and the transformation was terminated after 90 h (Fig. 3). After 90 h of cultivation, the culture broth was acidified with 5 N hydrochloric acid to pH 2.0 and extracted three times with 15 liters of ethyl acetate. The extracts were pooled, and the solvent was evaporated at 50°C, yielding 1,917 mg of an oily substance.

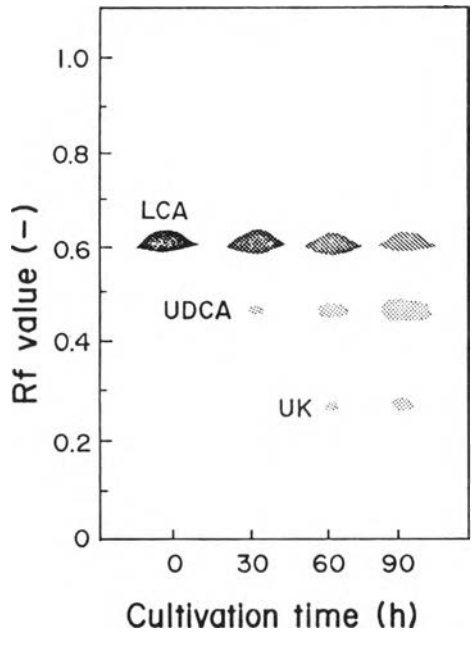

Fig. 3. Thin-layer chromatogram of bile acids in *F. equiseti* M41 culture broth. LCA, Raw material, UDCA, spot of a product with the R_f value of UDCA, UK, unknown product.

This was further purified by silica gel column chromatography using 2,2,4-trimethyl pentane-ethyl acetate-acetic acid (5:5:1, by vol.) as a solvent system and 258 mg of product was recovered. This was further purified by preparative silica gel TLC (2-mm thickness) using chloroform-acetone-acetic acid as a solvent system, as described in Materials and Methods. The band

located at the R_f 0.49 was collected and eluted with acetone, yielding 31.4 mg of white crystals, which were recrystallized from ethanol-water. The crystals showed the same R_f value as UDCA in 17 TLC solvent systems (56) and had the expected retention time of 16 min by gas-liquid chromatography. The melting point of the purified product was 198 to 199.5°C which was equal to that of authentic UDCA and the mixture of authentic UDCA and the product. Elemental analysis gave: C, 73.42%; H, 10.22%; and the elemental composition calculated for $C_{24}H_{40}O_4$ (UDCA) was C, 73.43%; H, 10.27%. The parent peak in the mass spectrum was at 392 m/e (Fig. 4), which corresponds to 392.56, the theoretical molecular weight of $C_{24}H_{40}O_4$.

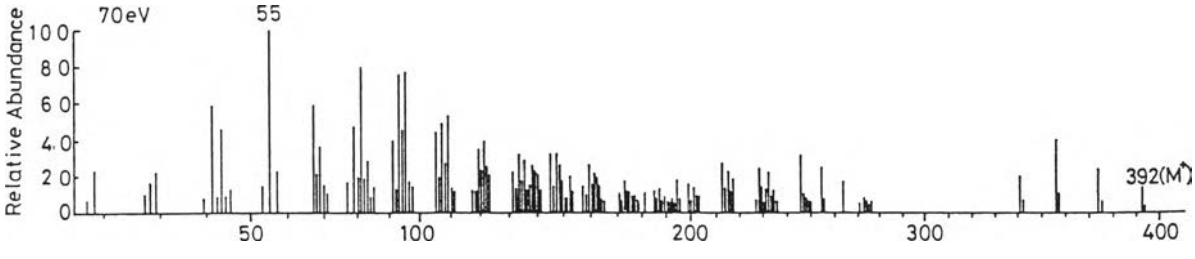

Fig. 4. Mass spectrum of the product converted from LCA by *F. equiseti* M41.

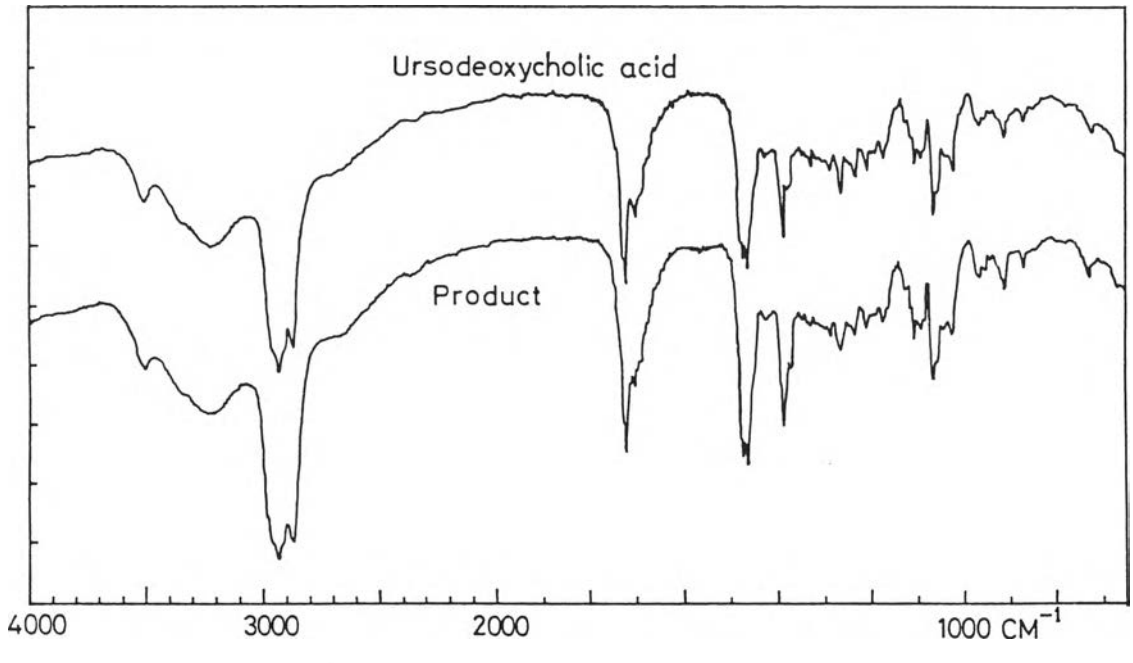

Fig. 5. Infrared spectra (in nujol) of ursodeoxycholic acid and the product transformed from LCA by *F. equiseti* M41.

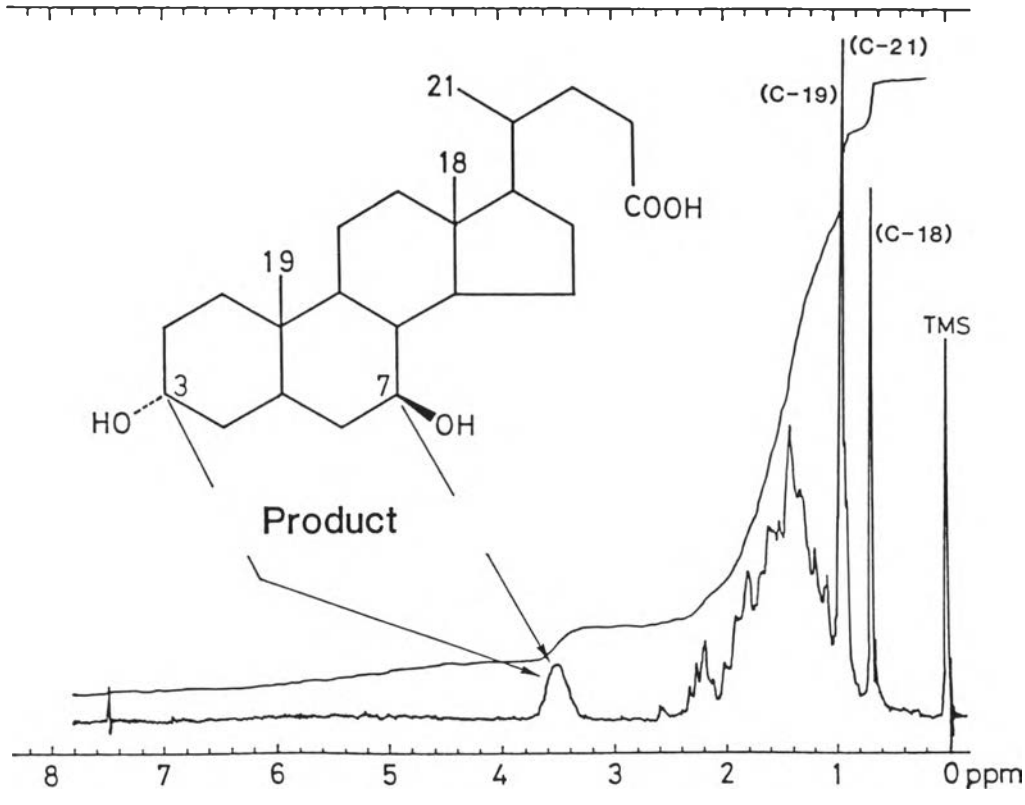

Fig. 6. NMR spectrum of the product obtained from LCA by *F. equiseti* M41.

The infrared spectrum (in nujol) showed absorbances at 1,720 cm^{-1} (C = O) and around 3,200 cm^{-1} (C-3-OH) (Fig. 5). The nuclear magnetic resonance spectrum had signals at 0.92 ppm (C-19, C-21-CH_3), 0.66 ppm (C-18-CH_3), and 3.5 ppm (C-3, C-7-H) (Fig. 6). These spectra were completely identical to those of standard UDCA. From these results, the product was identified as UDCA.

4. Production of UDCA The organism grew well between 15 and 30°C, with optimum growth at 27°C. An acidic medium (pH 4 to 7) allowed good growth, and the highest production of UDCA was observed at pH 6 to 7. UDCA production was not detected under fully aerobic conditions in the medium containing oatmeal, and reduction of oxygen supply during growth was necessary for induction of the conversion activity (see Fig. 7). UDCA production started with an increase in dissolved oxygen tension after 64 h of oxygen limited

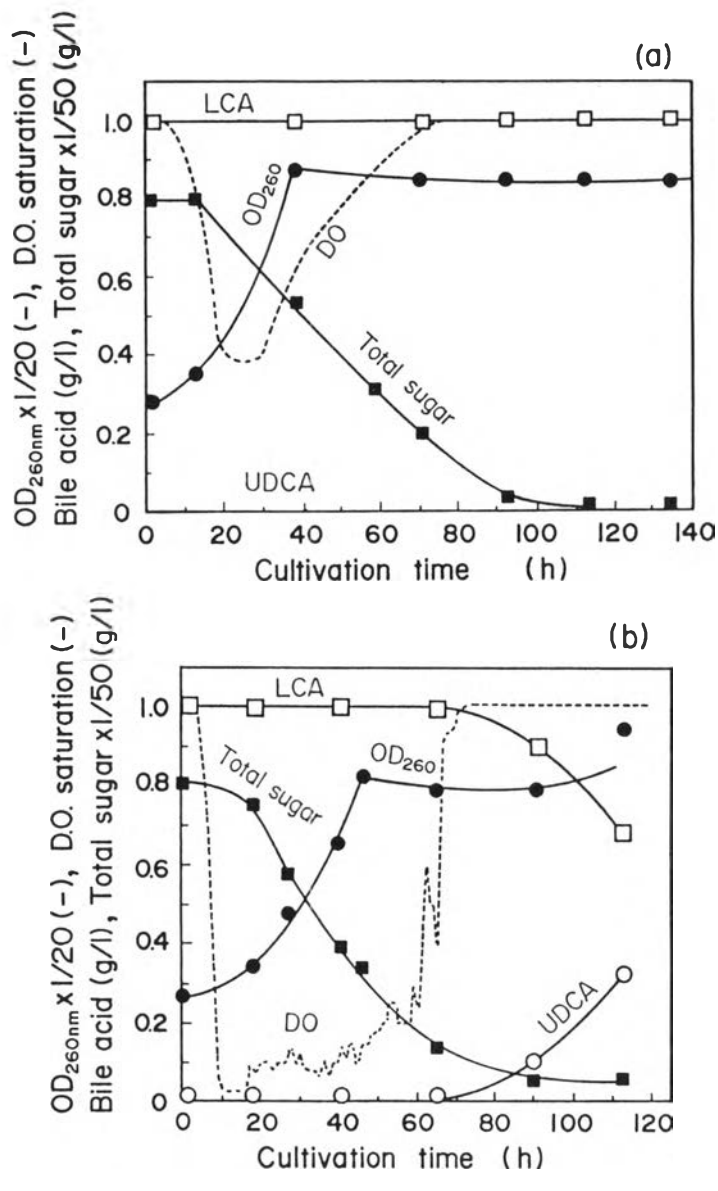

Fig. 7. UDCA production under aerobic conditions (a) and dissolved oxygen control (b) by *F. equiseti* M41 in oatmeal medium. Culture conditions are described in the text. Symbols: □, LCA; ■, total sugar; ● optical density at 260 nm; ○, UDCA; ----, saturation degree of dissolved oxygen.

cultivation. The amount of product accumulated reached 350 mg/ℓ after 112 h of cultivation.

Discussion

The strain M41 produced UDCA in a medium containing LCA. The thin-layer chromatogram in Fig. 3 shows that the spot with the same R_f value as UDCA grew concomitantly with the fading of the LCA spot. UDCA was not, however, the sole product of LCA hydroxylation; other LCA derivatives were also detected. Gas-liquid chromatography, a more sensitive method for detection showed several products. Although these were not identified completely, their R_f values on thin-layer chromatograms and retention times on gas-liquid chromatograms suggested that they might be derivatives of LCA with α- or β-oriented hydroxyl group(s) at the C-7 or C-12 position (or both positions).

UDCA has been produced from cholic acid by chemical synthesis involving seven reaction steps as mentioned before (47). The efficiency of selective acetylation of hydroxyl groups at the C-3 and C-7 positions is not high; therefore, the total yield is rather low. For this reason, a microbial one-step method for the production of UDCA from LCA is promising.

It was found that production of UDCA by *F. equiseti* M41 was greatly affected by the oxygen supply. We speculate that the oxygen atom of the hydroxyl group introduced at the C-7 position is incorporated from molecular oxygen (57).

Hanisch *et al.* (58) found that oxygen has a major effect on the amount of progesterone 11α-hydroxylase synthesized by *R. nigricans* ATCC 62276 in a medium containing progesterone, an inducer of the enzyme. They also reported that the hydroxylase was optimally induced at a dissolved oxygen tension of 10% air saturation, which was much lower than the optimal value for the maximum hydroxylation rate. Our results (Fig. 7) also suggested that oxygen plays an important role in the induction of LCA 7β-hydroxylase

in oatmeal medium with LCA.

Summary

Strain M41, fungus identified as *Fusarium equiseti*, was isolated from soil and found to convert lithocholic acid to ursodeoxycholic acid by 7β-hydroxylation. The optimum pH for production of ursodeoxycholic acid was between 6 and 7. The production of ursodeoxycholic acid was markedly affected by the oxygen supply in a medium containing oatmeal: the organism produced ursodeoxycholic acid in a batch culture in which oxygen supply was limited during growth, but not in a fully aerobic culture. The amount of product accumulated reached 350 mg/ℓ after 112 h of cultivation.