CHAPTER 4

Conditions for the Production of 3α,15β-Dihydroxy 5β-Cholanic Acid by *Cunninghamella blakesleeana* ST22

Introduction

As described in the previous chapter, 3α,15β-DHC, the product of the hydroxylation of LCA by *Cunninghamella blakesleeana* ST22, was found as a new bile acid which possesses cholesterol-solubilizing properties *in vitro* similar to those of UDCA. There is a strong possibility that this compound might be usable therapeutically for cholesterol-gallstone disease. Its toxicity in mice was higher than that of UDCA, but not seriously so. Therefore, it has a high potential for future exploitation as a cholesterol-gallstone solubilizer.

As reported in Chapter 2, the amount of UDCA produced by *Fusarium equiseti* was increased from 0.35 g/ℓ to 1.2 g/ℓ by improving cultivation conditions. For these reasons, further study was undertaken to improve 3α, 15β-DHC production by *Cunninghamella blakesleeana* ST22.

Materials and Methods

Chemicals LCA, UDCA, CDCA, DCA, CA were purchased from Sigma Chemicals Co., St. Louis, Mo. Hexafluoro-isopropanol was obtained from E. Merck, Damstadt. Trifluoroacetic anhydride was the product of Wako Pure Chemical Ind. Ltd., Osaka. The other reagents were all reagent grade and

were used without further purification.

Medium The medium used for investigation on the effect of carbon and nitrogen sources on the production of 3α,15β-DHC contained 1 g of LCA, 2 g of KH_2PO_4, basal supplement (0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of $CaCl_2 \cdot 2H_2O$, 1 g of yeast extract, 0.01 g each of $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $CuSO_4 \cdot 5H_2O$, $Na_2MoO_4 \cdot 2H_2O$ and $ZnSO_4 \cdot 7H_2O$) and test carbon and nitrogen sources. The medium used for the production in a 10-ℓ jar fermentor has the same composition except that 40 g of dextrin and 12 g of L-asparagine were used as carbon and nitrogen sources, respectively.

Cultivation Seed culture was prepared by inoculating spore suspension of *C. blakesleeana* ST22 into 100 ml of Sabouraud dextrose broth in a 500-ml Sakaguchi flask and incubating for 72 h at 30°C on a reciprocating shaker (120 strokes/min). For the cultivation in a 10-ℓ jar fermentor (type MD-500, L.E. Marubishi), 6 ℓ of the medium was inoculated with 300 ml of the seed culture. The aeration rate was maintained at 1 vvm, agitation speed at 330 rpm, and temperature at 33°C. Unless stated otherwise, pH was controlled at 8.0 throughout the fermentation.

Assay Cell mass was expressed as gram of dry weight per ℓ after drying at 90°C for 12 h. The total sugar content was determined as glucose by the phenol-H_2SO_4 method as described previously. Bile acid content was measured by gas chromatography, after derivatization with hexafluoroisopropanol and trifluoroacetic anhydride as described in Chapter 2, using cholic acid as internal standard.

The 15β-hydroxylating activity of the resting cells was assayed as follows: cells were harvested by suction filtration in a Büchner funnel and washed thoroughly with deionized water. One gram of wet cells was suspended in 20 ml of 0.1 M Tris-HCl (pH 8.4) in a 100-ml Erlenmeyer flask. The conversion reaction, initiated by adding 20 mg of LCA, was performed at

36°C with shaking at a speed of 140 strokes/min. At appropriate intervals, 1-ml aliquots of reaction mixture were withdrawn and subjected to bile acid analysis.

Gas chromatography for assay of bile acids was performed as described in Chapter 3 Part 3.

Results and Discussion

1. Medium composition

As reported in Chapter 3, the production of 3α,15β-DHC in a 10-ℓ jar fermentor yielded only 0.17 g/ℓ in 55 h, which is quite low. Therefore, first, the effect of carbon and nitrogen sources on the product formation was investigated (Table 1). Of 11 carbon sources tested, dextrin was found to be the best, while L-asparagine and $NaNO_3$ seemed to be good nitrogen sources. However, in the medium containing $NaNO_3$, rapid degradation of 3α, 15β-DHC was observed at the late phase of the growth, although the maximum conversion was rather high (∿50%). In contrast, in the medium containing dextrin and L-asparagine, the degradation was retarted and high conversion (40 ∿ 50%) was maintained after 7 days of cultivation. Therefore, the combination of dextrin as carbon source and L-asparagine as nitrogen source was selected as the main medium composition for further study.

2. The effect of temperature on cell growth and biotransformation

As shown in Fig. 1-A, optimum temperature for cell growth of *C. blakesleeana* ST22 was found to be 33°C, while optimum temperature for 15β-hydroxylation ranged from 33 - 40°C (Fig. 1-B). Therefore, the temperature selected for growth and hydroxylation in a jar fermentor was 33°C.

As for pH, 15β-hydroxylating activity showed the maximum at pH 8.4

Table 1. Effect of carbon and nitrogen sources on the formation of 3α,15β-DHC by *C. blakesleeana* ST22.

Exp. I

Carbon source (40 g/ℓ)	3α,15β-DHC production (%)	Carbon source (40 g/ℓ)	3α,15β-DHC production (%)
Glucose	30.0	Glycerol	10.0
Galactose	30.3	Starch (soluble)	30.2
Lactose	23.0	Dextrin	54.6[a]
Raffinose	16.7	Carboxymethyl cellulose	2.5
Sucrose	16.7		
Sodium acetate	0	Malt extract	27.0

Exp. II

Nitrogen source (15 g/ℓ)	3α,15β-DHC production (%)	Nitrogen source (15 g/ℓ)	3α,15β-DHC production (%)
$NaNO_3$	11.3[a]	Yeast extract	2.2
NH_4Cl	1.8	Glycine	27.7
$(NH_4)_2SO_4$	0.9	L-Glutamic acid	0.9
L-Asparagine	38.2	Casamino acid	3.5
L-Aspartic acid	5.6	Peptone	6.2

[a] The large difference between the yields was due to the rapid degradation of 3α,15β-DHC.
Culitvation was performed in a 500-ml Sakaguchi flask. In Exp. I, $NaNO_3$ (15 g/ℓ) was used as nitrogen source, and in Exp. II dextrin (40 g/ℓ) was used as carbon source. 3α,15β-DHC production was expressed as a percentage of the added LCA (100 mg/flask).

(Fig. 2-B). Since alkaline pH is generally unfavorable for the growth of fungi, the effects of pH on both cell growth and the hydroxylation were further investigated using a jar fermentor (Fig. 2-A).

As expected, *C. blakesleeana* ST22 grew well at pH 5 to pH 7, but at alkaline pH the growth was reduced, by 20% at pH 8.0 and 70% at 8.5. In contrast, 3α,15β-DHC production was maximal, 0.49 g/ℓ at 96 h, at pH 8.0. Compromizing this discrepancy, pH 8 was chosen for constant pH cultivation in a jar fermentor.

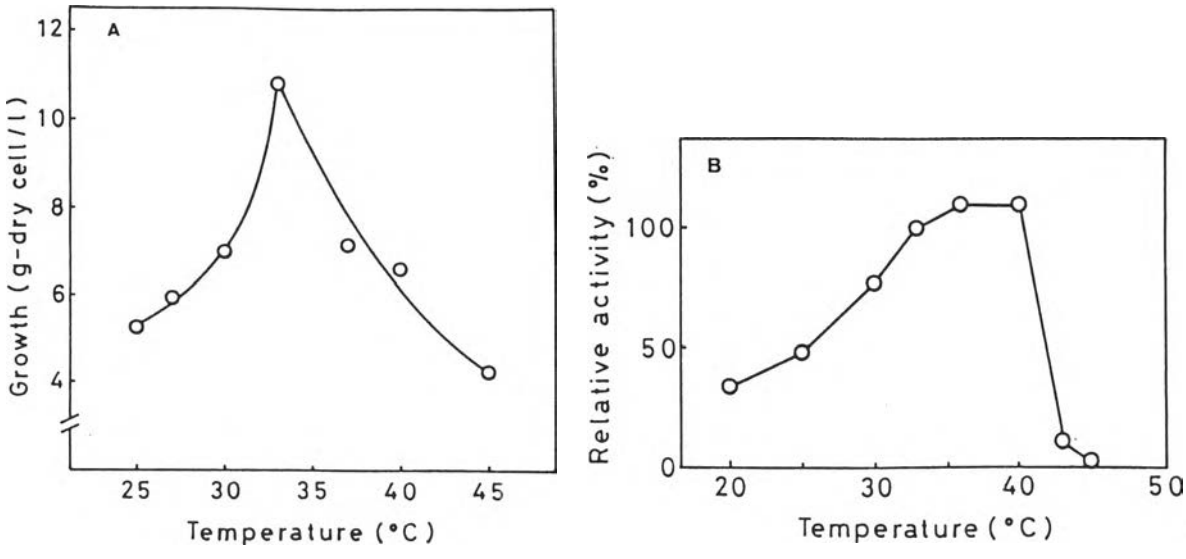

Fig. 1. Effect of temperature on (A) cell growth and (B) 15β-hydroxylation. In (A), *C. blakesleeana* ST22 was cultivated with 50 ml of medium in a 250-ml conical flask for 3 days. The medium was composed of dextrin (40 g/ℓ) and L-asparagine (12 g/ℓ) as carbon and nitrogen sources, respectively. In (B), the activity at 33°C was taken as 100% (0.44 mg 3α,15β-DHC·h^{-1}·g-wet $cell^{-1}$).

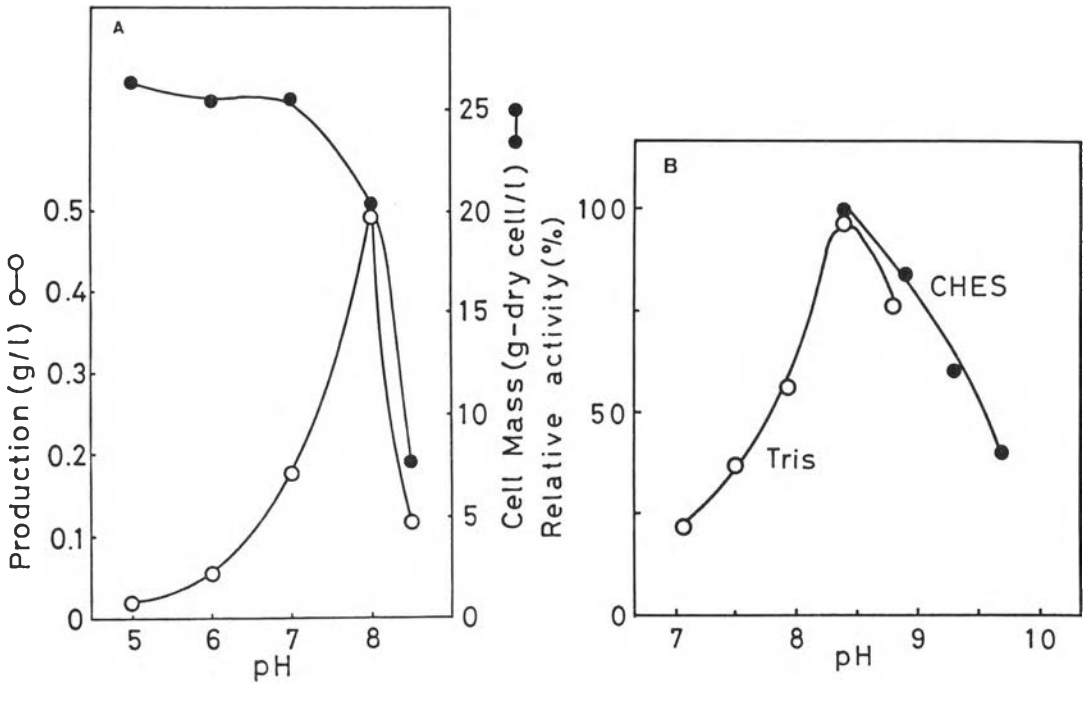

Fig. 2. Effect of pH on (A) cell growth and 3α,15β-DHC production during cultivation in a fermentor and (B) 15β-hydroxylating activity of resting cells. In (B), 0.1 M Tris·HCl (O) or 0.1 M CHES.HCl (●) was used to obtain the desired pH value. The reaction was performed at 36°C. The activity at pH 8.4 was taken as 100% (0.25 mg 3α,15β-DHC·h^{-1}·g-wet $cell^{-1}$).

3. Effect of organic solvents on 15β-hydroxylation

The stimulatory effect of organic solvents on steroid transformation has been reported previously by several investigators (93,94). In the 15β-hydroxylation by *C. blakesleeana* ST22 some organic solvents were also found to enhance 15β-hydroxylation by resting cells: in the presence of 1% (v/v) of DMSO, ethanol, dimethyl formamide or dioxane, 15β-hydroxylation activity increased to 200%, 172%, 168% and 114%, respectively. Fukui and Tanaka (95) reported the use of organic solvents to improve aqueous solubility of hydrophobic substrates. In the case of 15β-hydroxylation by *C. blakesleeana* ST22, DMSO may work similarly by increasing the solubility of LCA. In the presence of 2% DMSO, the solubility of LCA increased from 13 mg/ℓ to 18 mg/ℓ. The optimum concentration of DMSO for 15β-hydroxylation was in the range of 2 to 4% (Fig. 3). The product formation decreased when the concentration of DMSO was lower than 2% or higher than 4%.

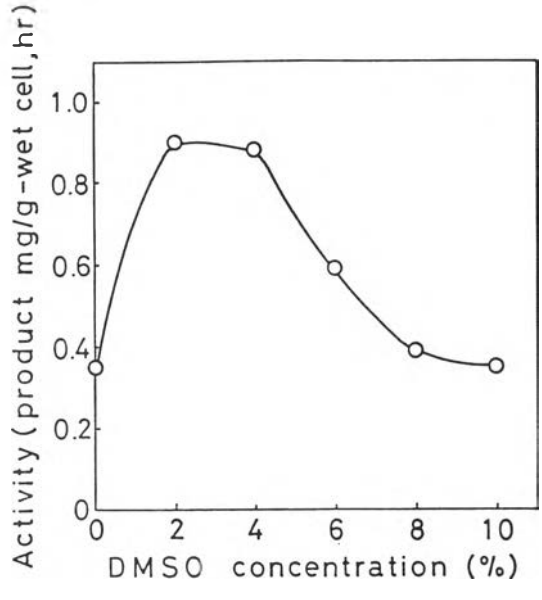

Fig. 3. Effect of DMSO concentration on 15β-hydroxylating activity by resting cells. Experimental conditions are similar to those described in Fig. 2-B except that the reaction was performed at 36°C and pH 8.4 with the indicated amount of DMSO.

4. Production of 3α,15β-DHC in a jar fermentor

The production of 3α,15β-DHC in a jar fermentor was investigated under the optimum conditions obtained from the previous experiments (Fig. 4-A). During the cultivation, the concentration of LCA gradually decreased from 1 g/ℓ to 0.3 g/ℓ at 72 h, and 0.36 g/ℓ of 3α,15β-DHC was detected at this stage. By feeding additional LCA (1 g/ℓ) at 72 hr of cultivation, the amount of 3α,15β-DHC was increased to 0.8 g/ℓ at 12 h after LCA supply, suggesting that LCA concentration higher than the saturated concentration of soluble LCA is important for the production. Next, LCA (1 g/ℓ) was added on three occasions to maintain the substrate concentration (Fig. 4-B). 3α,15β-DHC production continued until 108 h of cultivation, reaching 1.2 g/ℓ. Finally the effect of DMSO (final 2%) was tested in jar fermentation (Fig. 4-C). Additional LCA (final 1 g/ℓ) and DMSO (final 2%) were added at 72 h, as a result of which the amount of 3α,15β-DHC increased from 0.49 g/ℓ to 0.98 g/ℓ in 6 h after the DMSO addition. A high specific hydroxylation rate, 4.2 mg of 3α,15β-DHC h^{-1} g-dry $cell^{-1}$, was obtained in the presence of DMSO, in comparison with 2.2 mg of 3α,15β-DHC h^{-1}·g-dry $cell^{-1}$ in the absence of DMSO, indicating that DMSO was highly effective. On the other hand, poor production (70% reduction) and poor cell growth (59% reduction) were observed when DMSO was present from the beginning of cultivation. This result suggests that DMSO may have a toxic effect on *C. blakesleeana* ST22 in the early phase of growth but less or even none at the stationary phase.

As described in Chapter 2, Part 2, LCA concentration in the fermentation broth determined the amount of LCA bound to mycelia of strain M41 and, in turn, the extent of product formation. The binding of LCA to the mycelia of strain ST22 may play a similar role. Although strains M41 and ST22 produce different kinds of product, they both hydroxylate the same substrate,

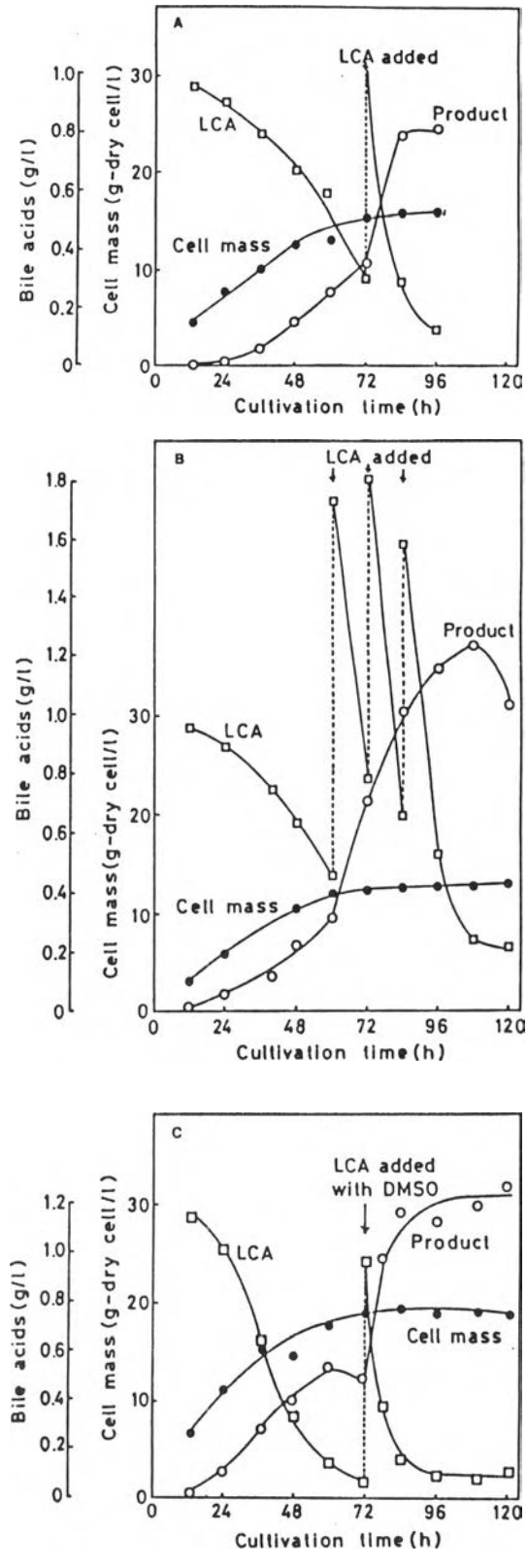

Fig. 4. 3α,15β-DHC production in a jar fermentor with additional LCA supply at 72 h (A), with additional LCA supply at 60, 72 and 84 h (B), and with additional LCA supply and DMSO addition (2%) at 72 h (C).

LCA. Also, both strains are mycelial molds whose cell wall component are similar to each other.

The remarkable enhancing effect of DMSO on hydroxylation might be the result of DMSO facilitating incorperation of insoluble LCA into the cells by promoting the interaction of LCA with the cell membrane.

Summary

The combination of dextrin and L-asparagine was found to be best in the cultivation medium for the conversion of lithocholic acid into 3α,15β-dihydroxy 5β-cholanic acid by *C. blakesleeana* ST22. The optimum temperature for cell growth was 33°C, while optimum temperature for 15β-hydroxylation ranged from 33 - 40°C. The maximum 15β-hydroxylating activity by resting cells was found at pH 8.4. *C. blakesleeana* ST22 grew well at pH 7 or lower, but poorly at alkaline pH. In contrast, the maximum production of 3α,15β-DHC, 0.49 g/ℓ at 95 h, was obtained at pH 8.0. Dimethyl sulfoxide was found to be the most effective solvent, and the optimum concentration of DMSO for the enhancement of 15β-hydroxylation was in the range of 2 - 4%. By applying the optimum medium composition, temperature, pH and addition of LCA together with DMSO, production was increased to 0.98 g/ℓ at 6 h after adding LCA, and reached 1.2 g/ℓ at 84 h of cultivation.

CONCLUSION

A remarkable rise in the incidence of gallstone disease has highlighted the need to find an efficient process for the production of a cholesterol-gallstone solubilizer. At present, effective cholesterol-gallstone solubilizers, ursodeoxycholic acid and chenodeoxycholic acid, are produced by chemical processes which involve high production costs. This problem might be overcome by developing a production process employing microbial conversion. This thesis describes the microbial production of ursodeoxycholic acid (UDCA) and a new bile acid, 3α,15β-dihydroxy-5β-cholanic acid (3α,15β-DHC) from lithocholic acid (LCA).

The first chapter deals with the production of UDCA from LCA by a fungus strain M41. This strain was selected from 609 strains isolated from soil and was identified as *Fusarium equiseti*. The product of *F. equiseti* M41 was extracted with ethyl acetate at pH 2 and purified by silica gel column chromatography with trimethylpentane-ethyl acetate-acetic acid, and preparative silica gel thin-layer chromatography (TLC) with chloroform-acetone-acetic acid. Then it was crystallized from acetone and recrystallized from ethanol-water. The product was identified as UDCA from the results of TLC, gas-liquid chromatography, melting point, elemental analysis, and mass, infrared (IR), and ^{1}H-nuclear magnetic resonance (NMR) spectra. The production of ursodeoxycholic acid by *F. equiseti* M41 in a jar fermentor using oatmeal medium was investigated. The product accumulated to a level of 350 mg/ℓ at 112 h of cultivation.

Part 1 of Chapter 2 looks at the improvement of microbial production of UDCA, the effect of some factors on hydroxylation, and the production of UDCA in a jar fermentor. Dextrin and L-asparagine were found to be the best carbon and nitrogen sources for UDCA production. In a resting-cell

system of *F. equiseti* M41, the maximum conversion of LCA to UDCA was found at 28°C, pH 8.0, and dissolved oxygen tension (DOT) of 60 to 100% saturation, and a two-fold activation was observed on addition of 0.5 M KCl. This increase in UDCA production by K^+ was suggested to result from an increase in the uptake rate or equilibrium concentration of LCA. Monovalent cations such as Na^+, K^+, Li^+, Cs^+ and Rb^+ stimulated the conversion rate more than twofold. The activity of the mycelium was affected by DOT during cultivation in a fermentor. With 15% DOT or over, the activity peak was observed at 25 h of cultivation, whereas the peak was delayed to 34 to 50 h when DOT was reduced to 5% and 0%, respectively. After reaching the maximum, 7β-hydroxylation activity in the mycelium declined rapidly at pH 7.0, but the rate of decline was reduced by raising the pH to 8.0. On applying the optimum conditions, i.e., pH shift (from 7.0 to 8.0), addition of 0.5 M KCl, the DOT control, 1.2 g/ℓ of UDCA was produced within 96 h of cultivation.

Part 2 deals with the binding characteristics of LCA to the mycelia of *F. equiseti* M41. It was found that concentration of LCA in the cultivation medium affected the formation of the product: the production rate of UDCA was proportional to the concentration of LCA in the range of 0.2 to 10.0 g/ℓ. Most of the LCA bound to the mycelia of *F. equiseti* M41 was in an insoluble form. The binding of LCA to the mycelia was shown to be a hydrophobic interaction, for the following reasons: i) the binding of LCA to the mycelia was not affected by the ionic strength of the medium, ii) the activation energy of LCA binding was low (0.9 kcal/mole), iii) more bound LCA was in a hydrophobic form than an ionic form, and iv) the amount of bile acid bound depended on the hydrophobicity of the acid.

Chapter 3 deals with the transformation of LCA to a new bile acid, 3α, 15β-DHC, by *Cunninghamella blakesleeana* ST22. Part 1 describes the isola-

tion and identification of strain ST22. Strain ST22, which was selected from 110 fungal isolates, converted LCA to the dihydroxy bile acid derivative efficiently. From its morphological and cultural characteristics, it was identified as *Cunninghamella blakesleeana*.

Part 2 describes a structural analysis of the product. From the results of m.p., $[\alpha]_D^{25}$, elemental analysis, and mass, IR, and ^{13}C-NMR spectra, the product was identified as dihydroxy derivative of 5β-cholanic acid. By comparing the m.p., $[\alpha]_D^{25}$, retention times on GC, and HPLC R_f value of the product with those of known dihydroxy derivatives, the product was found to be a new compound. From the result of ^{1}H-NMR it was shown that the second hydroxyl group, other than that at the 3α position, was located at either the 15β or 16β position. The results of the two-dimensional ^{1}H-NMR narrowed down its position to 15β.

Part 3 of this chapter describes the biological activity of the product, 3α,15β-DHC. In its hydrophilicity and the *in vitro* cholesterol solubilizing activity test, 3α,15β-DHC showed similar properties to UDCA. The toxicity test in mice indicated that the toxicity by intraperitoneal injection of 3α,15β-DHC was slightly higher than that of UDCA.

Chapter 4 examines the optimum conditions for 3α,15β-DHC production. The combination of dextrin and L-asparagine was found to provide the best basis for the medium. The most suitable temperature and pH were 33°C and 8.0. Some organic solvents, of which 2 - 4% DMSO was the most effective, enhanced the product formation. Product formation was also remarkably increased by feeding LCA to maintain its level at 1 g/ℓ, and if LCA was added together with 2% DMSO, only one addition was sufficient to give a high concentration of product. By applying these optimum conditions, 1.2 g/ℓ of product was obtained at 84 h of cultivation.

The result of the studies in this thesis provide a basis for develop-

ment of microbial production processes of cholesterol-gallstone solubilizers, which might replace the conventional chemical conversion processes now employed to supply these therapeutic drugs. The finding of a novel bile acid, 3α,15β-dihydroxy-5β-cholanic acid, which possesses the ability to solubilize cholesterol-gallstones, opens up the prospect of an alternative process to ursodeoxycholic acid production. Analysis of the interaction between microbial cells and LCA in the conversion of LCA to ursodeoxycholic acid opened the studies on the kinetics of bioadsorption of a lipophilic substance and its microbial conversion.