CHAPTER 2

Improvement of Microbial Production
of Ursodeoxycholic Acid from Lithocholic Acid

Part 1   Conditions for Ursodeoxycholic Acid
            Production by *Fusarium equiseti* M41

Introduction

As described in the previous chapter, the fungus *Fusarium equiseti* M41 was found to be capable of transforming LCA to UDCA, but in a yield of only 35%. However, a preliminary investigation into the effect of oxygen supply on the production suggested an important characteristic of the strain with respect to the induction of the hydroxylation activity. This chapter deals with the effects of medium components and culture conditions such as temperatures, pH as well as dissolved oxygen tension on the production of UDCA. These factors were examined with respect to the induction of hydroxylation activity of the organism and the maximization of expression of the activity, with a view to improvement of UDCA production by *F. equiseti* M41 both in the resting system and in the growing culture. The information obtained was integrated into a fed-batch culture in a 10-ℓ jar fermentor, and a remarkable increase of product accumulation in the culture broth was achieved.

Materials and Methods

Chemicals   Chenodeoxycholic acid and cholic acid were purchased from

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Sigma Chemical Co., St. Louis, Mo. Hexafluoroisopropanol was obtained from E. Merck AG, Darmstadt, West Germany. Trifluoroacetic anhydride was purchased from Gasukuro Kogyo Co. Ltd., Tokyo. Sabouraud dextose broth (0382-15-1) was the product of Difco Laboratories, Detroit, Mich. Yeast extract was obtained from Daigo Nutritive Chemicals, Ltd., Osaka. [Carboxyl-^{14}C] LCA was purchased from Amersham International, Amersham, U.K. (specific radioactivity, 55 mCi/m mol). All other reagents were the highest grade available from Nakarai Chemicals, Ltd., Kyoto, or Wako Pure Chemical Industries, Ltd., Osaka.

Medium   Sabouraud dextrose broth was used to prepare the stock and seed cultures of *F. equiseti* M41 througout this work. The basal supplement contained 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of $CaCl_2 \cdot 2H_2O$, 1 g of yeast extract, and 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 $ZnSO_4 \cdot 7H_2O$ in 1 ℓ of deionized water. Medium A, used for the survey of carbon and nitrogen sources, contained the basal supplement, 10 g of carbon source, 5 g of nitrogen source, 3 g of KCl, 2 g of KH_2PO_4, 3 g of $CaCO_3$ and 1 g of LCA in 1 ℓ of deionized water. Medium B contained the basal supplement, 40 g of carbon source, 12 g of nitrogen source and 1 g of LCA in 1 ℓ of 0.1 M potassium phosphate buffer (pH 7.0). Medium C, used for jar fermentation, was medium B containing L-asparagine and dextrin as carbon and nitrogen sources.

Cultivation   For the inoculum preparation, 100 ml of Sabouraud dextrose broth in a 500-ml Sakaguchi flask was inoculated with 5 ml of the stock culture (stored at -80°C) and incubated at 28°C on a reciprocating shaker (120 rpm) for 72 h. Flask cultivations were performed with 100 ml of medium A or B in 500-ml Sakaguchi flasks, inoculated with 5 ml of 72-h culture, and incubated at 28°C on a reciprocating shaker (120 spm) for 7 or 14 days. The fermentor cultivation was performed by inoculating 100 ml of

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the inoculum into a 2.5-ℓ jar fermentor (type MD-250-3S; L.E. Marubishi,

Tokyo) containing 1.5 ℓ of Medium C. The temperature was maintained at

28°C, the aeration rate was adjusted to 1 vvm, and the pH was controlled

at 7.0 unless otherwise indicated. Dissolved oxygen tension (DOT) was

maintained at the desired value ±5% deviation by automatically changing the

agitation speed. The levels of DOT and pH during cultivation were monitored

with a membrane-type dissolved oxygen electrode (type DX-26; L.E. Marubishi)

and a pH electrode (type D-26; L.E. Marubishi).

Assay   Bile acid content was determined by gas chromatography after

derivatization with hexafluoroisopropanol and trifluoroacetic anhydride

(59) as described previously (54), using cholic acid as an internal standard.

The relative retension times of UDCA, LCA, and by-product with respect to

cholic acid were 0.67, 0.29 and 0.89, respectively.

Cell mass during cultivation was expressed as dry weight (g/ℓ).

Total sugar content was determined as glucose by the phenol-H_2SO_4 method

(60).

The production of UDCA by resting cells   Fungal mycelium was

separated from 40-h culture broth and washed thoroughly with deionized

water in a Büchner funnel. One gram of mycelium was suspended in 20 ml of

0.1 M Tris-hydrochloride (pH 8.0) in a 100-ml Erlenmeyer flask, 20 mg of

LCA was added, and the mixture was incubated at 28°C on a reciprocating

shaker at 180 strokes per min (M-100-N, Taiyo Scientific Industries Co. Ltd.,

Tokyo). Samples of 1 ml were taken at appropriate intervals and assayed

for bile acid.

Uptake of [carboxy-^{14}C] LCA   Mycelia were cultivated in a 2.5-ℓ jar

fermentor containing 1.5 ℓ of medium C under the following conditions: pH

7.0, 28°C, aeration rate of 1 vvm, and 0% DOT. These cultivation conditions,

under which oxygen consumption by the fungus was almost equal to the oxygen supply

from air at an aeration rate of 1 vvm with a varied agitation speed, are represented by the term "DOT 0%". The agitation speed at DOT 0% was the maximum agitation speed at which the indicator of the DOT meter (Type DX-26; L.E. Marubishi) remained at 0%. After 40 h of cultivation, the mycelium was collected by centrifugation (15000 rpm, 10 min, 4°C) and washed three times with ice-cold 0.01 M Tris-hydrochloride (pH 7.0). The mycelium was finally collected by suction filtration on a Büchner funnel. One gram of wet mycelium was suspended in 20 ml of ice-cold 0.1 M Tris-hydrochloride (pH 8.0) with or without 0.5 M KCl. After preincubation for 5 min at 28°C, 20 μl of [^{14}C] LCA dissolved in ethanol was added. At intervals, 1-ml samples were withdrawn, mixed with 50 ml of ice-cold 0.01 M Tris-hydrochloride (pH 7.0), filtered through a 0.45 μm membrane filter (type TM-2; Toyo Kagaku Sangyo Co. Ltd., Osaka), and washed five times with 10-ml portions of ice-cold 0.01 M Tris-hydrochloride (pH 7.0). After drying the membrane filter at 60°C for 20 min, the radioactivity was measured in the presence of 10 ml of toluene scintillant containing 2,5-diphenyloxazole (9 g/ℓ) with a liquid scintillator (Beckman LS 7500; Beckman Instruments, Inc., Irvine, Calif.).

Results

1. Induction of enzyme activity by LCA

To investigate whether LCA 7β-hydroxylation is catalyzed by an inducible enzyme, *F. equiseti* M41 was cultivated in the presence and absence of LCA and measured the appearance of 7β-hydroxylation activity in the presence of 250 μg/ml of cycloheximide, which has little effect on the activity. Mycelium cultivated in the presence of LCA catalyzed 7β-hydroxylation (375 μg/g of wet cells·h), whereas mycelium cultivated without LCA possessed no

activity, despite showing the same growth curve as mycelium grown in the presence of LCA. When cycloheximide was present, the hydroxylation activity was not observed in spite of the presence of LCA. Therefore, it can be concluded that the enzyme(s) was induced by the substrate LCA.

2. Determination of medium composition

In Chapter 1, in which we described *F. equiseti* M41 as a transformer of LCA into UDCA, we used modified Czapek-Dox medium for screening and oatmeal medium for production. However, the oatmeal medium gave a rather low production yield ($\sim$35%), and its high viscosity prevented cultivation conditions such as DOT from being determined satisfactorily. Therefore, we sought a suitable medium composition providing higher UDCA production and lower viscosity. First the effects of carbon and nitrogen sources on UDCA production were investigated (Tables 1 and 2). Of the 18 carbon sources tested, UDCA production from 18.5 mg of UDCA per g of dry cells with dextrin to 2.07 mg per g of dry cells with sodium succinate and no activity with salicin. The eight nitrogen sources produced an even wider range of results, e.g., 27.7, 21.3, and 3.07 mg of UDCA per g of dry cells with NH_4Cl, L-asparagine, and tryptone, respectively.

Since the pH values of media after cultivation were between 8.4 and 9.1, except for lactose (pH 7.8), sodium succinate (pH 9.3), and meat extract (pH 6.8, Table 2), the nature of the carbon or nitrogen source rather than the pH seemed to be the primary reason for the differences in UDCA production.

To determine the best combination of carbon and nitrogen sources for UDCA production, the combined effect of some carbon and nitrogen sources was investigated (Table 3). In this experiment we used 0.1 M potassium phosphate buffer (pH 7.0) instead of 0.3% (w/w) $CaCO_3$ to maintain the pH during cultivation in Sakaguchi flasks. In the presence of a higher con-

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Table 1. Effect of carbon source on formation of UDCA
          by *F. equiseti* M41.*

Carbon source (10 g/ℓ)	UDCA production (mg of UDCA per g of dry cells)
D-Xylose	2.83
L-Arabinose	3.14
D-Glucose	2.60
D-Fructose	2.87
D-Galactose	2.98
D-Mannitol	3.69
Salicin	0
Maltose	4.73
Lactose	7.43
Raffinose	7.05
Sucrose	5.03
Starch (soluble)	7.68
Carboxymethyl cellulose	10.5
Dextrin	18.5
Malt extract	5.88
Sodium acetate	7.60
Glycerol	7.60
Sodium succinate	2.07

*Cultivation was performed with medium A contain-
ing the indicated carbon source in a 500-ml
Sakaguchi flask on a reciprocating shaker (120 rpm)
for 7 days at 28°C. $NaNO_3$ (5 g/ℓ) was used as the
nitrogen source. UDCA production is expressed as
UDCA formed from added LCA (100 mg per flask).
Cell mass ranged from 0.4 to 0.8 of dry cells per
flask.

Table 2. Effect of nitrogen source on formation of UDCA
          by *F. equiseti* M41.*

Nitrogen source (5 g/ℓ)	UDCA production (mg of UDCA per g of dry cells)
NH_4Cl	27.7
$(NH_4)_2SO_4$	20.2
L-Asparagine	21.3
Casamino Acids	4.59
Peptone	4.67
Tryptone	3.07
Yeast extract	6.77
Meat extract	0

*Sodium succinate (10 g/ℓ) was used as the
carbon source; other conditions are as in
the footnote to Table 1.

Table 3. Combined effect of carbon and nitrogen sources
         on formation of UDCA by *F. equiseti* M41 during culti-
         vation with Sakaguchi flasks.*

Carbon source	UDCA production (mg of UDCA per g of dry cells)		
	NH_4Cl	$(NH_4)_2SO_4$	L-Asparagine
Starch (soluble)	0.28 (3.6)	0 (3.2)	34.4 (7.9)
Dextrin	0.27 (3.0)	0 (2.9)	39.3 (7.7)

*Cultivation was performed with 100 ml of medium B containing
the indicated carbon (40 g/ℓ) or nitrogen (12 g/ℓ) source in a
500-ml Sakaguchi flask for 14 days at 28°C. The pH at the end
of cultivation is indicated in parentheses; the initial pH was
adjusted to 7.0. Other experimental conditions are as described
in the footnote to Table 1. Cell mass ranged from 1.5 to 1.7 g
of dry cells per flask.

centration of potassium phosphate, UDCA production became higher and constant.

This stimulating effect of potassium phosphate will be described later.

In the medium composed of dextrin and L-asparagine, a very high conversion

of up to 39.3 mg of UDCA per g of dry cells was observed after 14 days of

cultivation (Table 3), whereas very poor production was obtained with in-

organic ammonium salts as the nitrogen source. The low pH in these cultures

may indicate that a rapid pH decrease was the reason for poor UDCA produc-

tion. However, even under pH control in a jar fermentor (pH was controlled

as described in Fig. 7a), NH_4Cl combined with either dextrin or sodium

succinate was found to be ineffective (< 5% UDCA production after 120 h

compared with 80% conversion with dextrin and L-asparagine). Therefore,

the use of inorganic ammonium salts as medium constituents was excluded.

As the results indicated that dextrin and L-asparagine were good

medium constituents for UDCA production, they were selected for further

studies. Examination of the optimal relative amounts of L-asparagine

and dextrin showed that 12 to 20 g of L-asparagine per liter with 40 g of

dextrin per liter resulted in similar UDCA production, whereas more or less

L-asparagine was less effective.

3. Reaction conditions for LCA hydroxylation

To investigate various reaction conditions for UDCA production, it became necessary to assay the 7β-hydroxylation activity of the mycelium. Because we aimed to improve the UDCA production of living mycelia, and because measurements with cell-free extract revealed the instability of 7β-hydroxylation activity, of which the recovery from cells was less than 1%, we developed an assay method for resting mycelia of *F. equiseti* M41. UDCA formation by resting mycelia proceeded linearly for up to 4 h (Fig. 1). The use of Tris-hydrochloride buffer was essential in this assay because with other buffers the pH during the assay changed rapidly and frequent pH adjustment became necessary. The reaction rate was 375 μg/g of wet cells/h with resting mycelium, which agreed well with that during cultivation, 330 μg/g of wet cell/h. This indicated that the activity measured by this method was a good indication of the UDCA-producing activity of the mycelium.
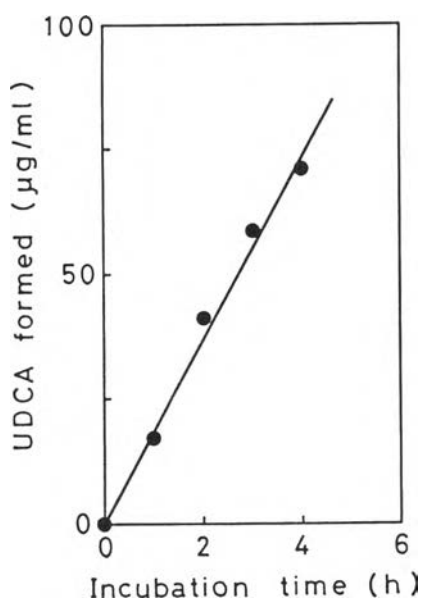

Fig. 1. Time course of UDCA formation by resting cells of *F. equiseti* M41. After 40 h of cultivation with medium C (1.5 ℓ) in a 2.5-ℓ jar fermentor, mycelia were collected and assayed as described in the text. During the cultivation, pH was controlled at 7.0 and DOT at 0% saturation. Other experimental conditions are described in the text.

First the effects of pH and temperature on UDCA production were investigated (Fig. 2). The optimum temperature for UDCA production as well as for growth was found to be 28°C. Higher or lower temperature caused a
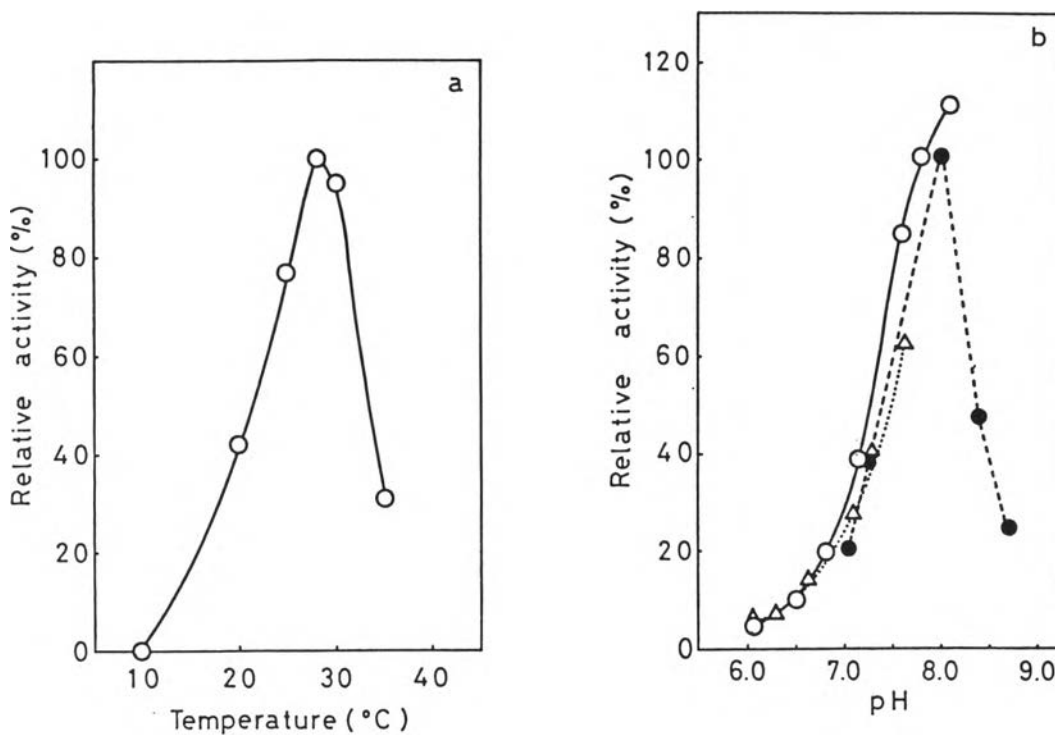
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Fig. 2. Effects of temperature (a) and pH (b) on formation of
UDCA by resting cells of *F. equiseti* M41. Experimental
conditions are identical to those described in the legend
to Fig. 1, except that temperature or pH was varied. In
(b) 0.1 M potassium phosphate buffer (●), 0.1 Tris-
hydrochloride buffer (○), or 0.1 M PIPES [piperazine-
N,*N*'-bis(2-ethanesulfonic acid)]-hydrochloride (△) buffer
was used to obtain the desired pH value.

sharp decline in conversion activity. The optimum pH for UDCA production

was found to be pH 8.0 with a narrow range, i.e., production increased five-

fold from pH 7.0 to 8.0, whereas the fungus grew well between pH 5 and 7.

This suggested that a pH shift to 8.0 (UDCA production phase) from pH 7.0

or lower (growing phase) would enhance UDCA production during the cultivation

in a fermentor.

As with other fungal hydroxylases, it is probable that atmospheric

oxygen is incorporated directly into the substrate, and the oxygen concen-

tration during conversion may affect the reaction rate (57,61). Indeed, as

shown in Fig. 3, the conversion activity increased when DOT was increased,

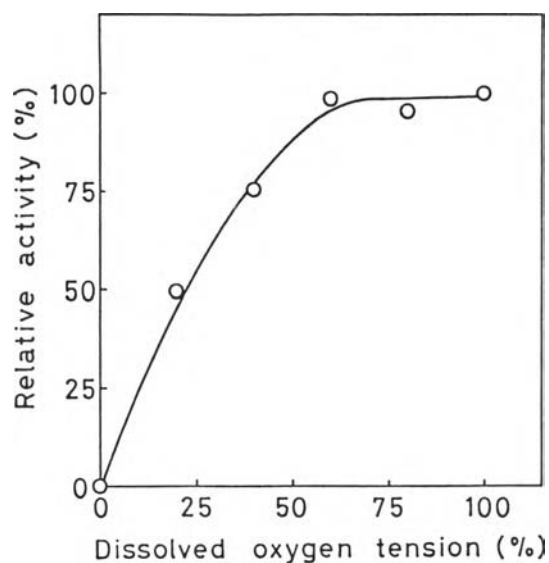and reached maximum at 60% saturation (Fig. 3). The results indicated that

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Fig. 3. Effect of DOT on formation of UDCA by resting cells of
F. equiseti M41. Mycelia cultivated in medium C (6 ℓ) in
a 10-ℓ jar fermentor were harvested at 40 h, washed, and
suspended in 0.1 M Tris-hydrochloride (pH 8.0) (50 g of
wet mycelia per ℓ). The conversion reaction was performed
at 28°C with 1-ℓ portions of the suspension in a 2.5-ℓ
jar fermentor equipped with a membrane-type DOT electrode.
The DOT was controlled at the indicated value by changing
the agitation speed (aeration rate, 1 vvm).

the fungus required a rather high DOT, in the range of 60 to 100% saturation,

for maximal UDCA production.

In preliminary experiments in Sakaguchi flasks, it was observed that

high concentrations of potassium phosphate buffer (0.1 M, pH 7.0)

facilitated higher UDCA production. In the cultures in a fermentor with pH

control, a high concentration of potassium phosphate was also effective

(1.8-fold UDCA in the presence of 0.1 M potassium phosphate, pH 7, compared

with 0.04 M potassium phosphate, pH 7), excluding the possibility that the

buffer effect was the reason for higher production. Therefore, the depend-

ence of UDCA production on KCl or K_2HPO_4 concentration was examined using

resting mycelia (Fig. 4). Both compounds enhanced UDCA production by two-

fold at a K^+ concentration of 0.5 M, suggesting that K^+ rather than HPO_4^{2-}

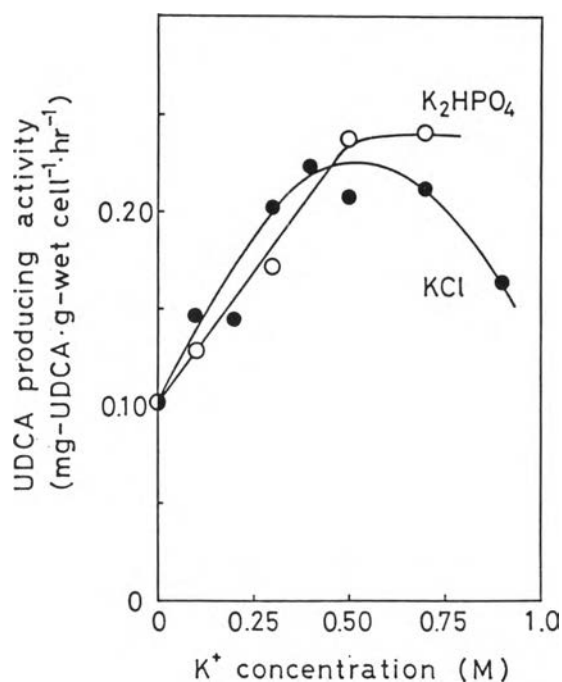was effective in the activation. To investigate this activation further,

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Fig. 4. Effect of potassium ion concentration on formation of UDCA by resting cells of *F. equiseti* M41. Experimental conditions are essentially identical to those described in the legend to Fig. 1, except that the indicated amount of potassium ion was added as either KCl (●) or K_2HPO_4 (○).

the effect of several ions on UDCA production was tested (Table 4). Na^+ and Rb^+ were about as effective as K^+, whereas Cs^+ was effective only at higher concentrations. Li^+ was most effective at lower concentrations but was inhibitory at 0.5 M, probably due to its well-known toxicity (62). NH_4^+ and all of the divalent cations were inhibitory. Anions were tested by use of their sodium salts. In the presence of Na_2SO_4, Na_2HCO_3, or $NaNO_3$, UDCA production was less than that obtained in the presence of NaCl, indicating the inhibitory nature of those anions. In the case of HPO_4^{2-}, the promoting effect seemed to come from coexisting Na^+. Indeed, when $Tris-H_3PO_4$ was used instead of Tris-hydrochloride as the reaction buffer to check the effect of HPO_4^{2-} alone, the phosphate anion was found to be slightly in-hibitory. Thus, it can be concluded that UDCA production was specifically activated by monovalent cations.

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Table 4. Effect of ions on UDCA formation by resting cells of *F. equiseti* M41.*

Addition	Relative activity (%) at concentration of		
	0.1 M	0.25 M	0.5 M
None	100		
Li^+	189		81
Na^+	156		211
K^+	161		211
Rb^+	147		280
Cs^+	104		223
NH_4^+	85		0
Ca^{2+}	0		0
Mg^{2+}	0		0
Mn^{2+}	0		0
Zn^{2+}	0		0
HPO_4^{2-} ($2Na^+$)	162	202	
HPO_4^{2-} (Tris)*	74		70
SO_2^{2-} ($2Na^+$)	124	80	
HCO_3^- (Na^+)	105		44
NO_3^- (Na^+)	120		14

[a]Experimental conditions are essentially identical to those described in the legend to Fig. 1. Cations were added as chlorides at the indicated concentrations. Anions were added as sodium salts, in the case of phosphate ion (indicated by asterisk) the pH of the reaction mixture was adjusted to 8.0 by changing the concentration of Tris. Therefore, at 0.1 and 0.5 M phosphate ion concentrations, the concentrations of Tris buffer were 0.06 and 0.32 M, respectively, instead of 0.1 M as in the other cases.

Several possible mechanisms for the K^+-dependent increase in UDCA production were considered: (i) increased solubility of LCA in the presence of K^+; (ii) increased LCA uptake into the mycelium in the presence of K^+; and (iii) direct activation of LCA 7β-hydroxylation by K^+. The first possibility was discounted because no remarkable difference was found in the solubility of LCA upon addition of K^+, i.e., 13 and 10 mg/ℓ in the absence and presence of 0.5 M KCl. And the third possibility was discounted because 7β-hydroxylation with cell-free extract was not affected by the addition of 0.5 M KCl. Therefore, LCA uptake was investigated in the absence and presence of K^+ (Fig. 5), and the addition of 0.5 M KCl was found clearly to increase
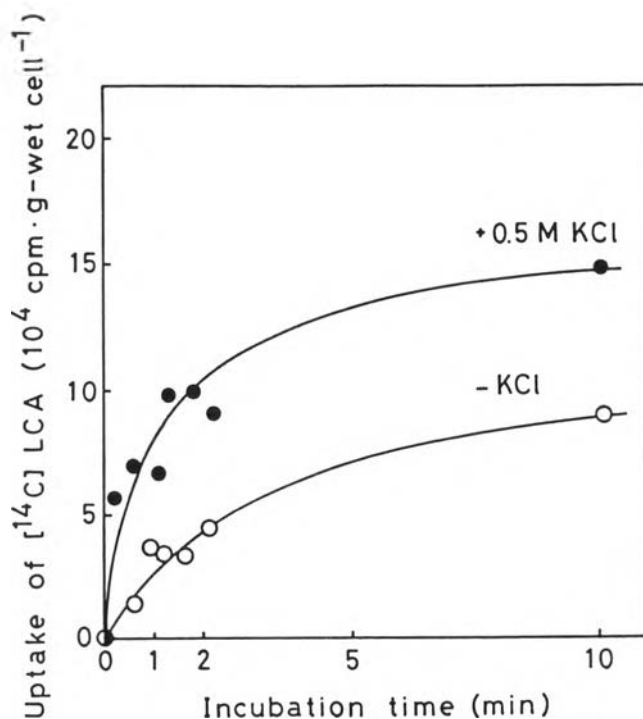
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Fig. 5. Uptake of [^{14}C]LCA by resting cells of *F. equiseti*
M41 in the presence (●) and absence (○) of 0.5 M KCl.
Experimental conditions are described in the text.

LCA uptake. The initial uptake rate and equilibrium concentration of LCA

of the mycelia were enhanced by 5.7 and 1.7-fold, respectively. Thus,

enhanced UDCA production in the presence of K^+ can be attributed to the

increased LCA uptake into the mycelia of *F. equiseti* M41.

4. Effect of DOT on hydroxylating activity during cultivation

As mentioned before, the optimum DOT for conversion of LCA was observed

between 60 and 100% saturation. However, DOT may also affect the synthesis

or degradation of the enzyme. To clarify this point, we investigated the

effect of DOT during cultivation on 7β-hydroxylation activity (Fig. 6).

When the fungus was cultivated at a DOT of 15% or higher, the enzyme activity

reached the maximum after 25 h of cultivation, whereas the peak was delayed

to 34 and 50 h at 5 and 0% DOT, respectively. Cell growth was not the reason

for this phenomenon because both the growth rate and the cell mass at the

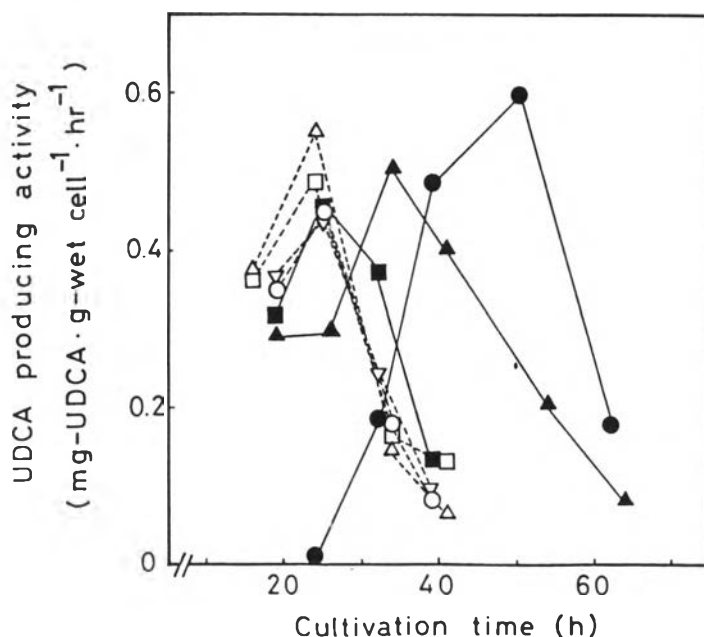stationary phase were identical under all DOT values.

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Fig. 6. Effect of DOT during cultivation on the amount of LCA
7β-hydroxylating activity. Cultivation was performed with
medium C (1.5 ℓ) in a jar fermentor (2.5 ℓ) at a tempera-
ture of 28°C, aeration rate of vvm, and pH of 7.0. DOT
was controlled at the indicated value by changing
agitation speed. At the indicated cultivation time, a
sample was withdrawn, and the 7β-hydroxylating activity
of the mycelia was assayed as described in the text.
DOT during cultivation: ●, 0%; ▲, 5%; ■, 15%; O, 30%;
△, 40%; □, 60%; ▽, 100% saturation.

Having reached its maximum, the activity in the mycelium decreased

rapidly irrespective of the DOT, falling by 70% in 10 h at pH 7.0. However,

this decline in enzyme activity was retarded by increasing the pH to 8.0

(15% reduction in 10 h). Also, the addition of 0.5 M KCl retarded the

decline in activity, although less remarkably than the pH shift.

5. Production of UDCA in a jar fermentor

From the evidence obtained with resting mycelia, the optimum conditions

for UDCA production could be summarized as follows:

i) pH 7 or lower during the growth phase, and pH 8.0 during the

production phase,

ii) addition of 0.5 M K$^+$ during the production phase,

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iii) 60 to 100% DOT during the production phase,

iv) initiation of production phase by changing conditions at 40 to 50 h of growth phase controlled at 0% DOT or at 25 h of growth phase controlled at 15% DOT or higher.
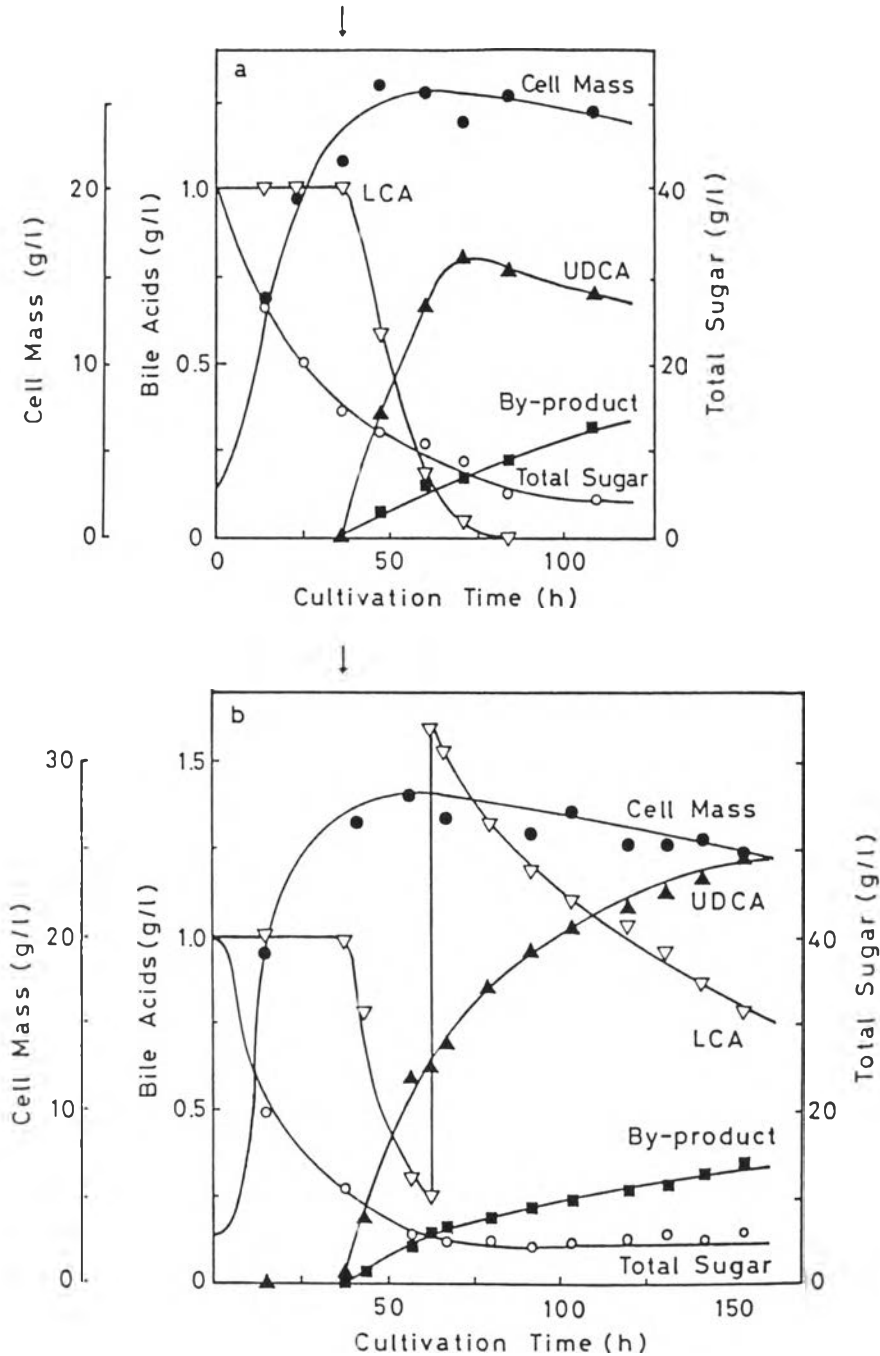

Fig. 7. UDCA production in jar fermentors (a) with pH shift from 7.0 to 8.0 and addition of 0.5 M KCl at 40 h of culti- vation, and (b) with the same condition shift at 40 h and LCA addition at 60 h. Cultivation conditions are described in the text. At the arrow, production was initaited by chaning conditions.
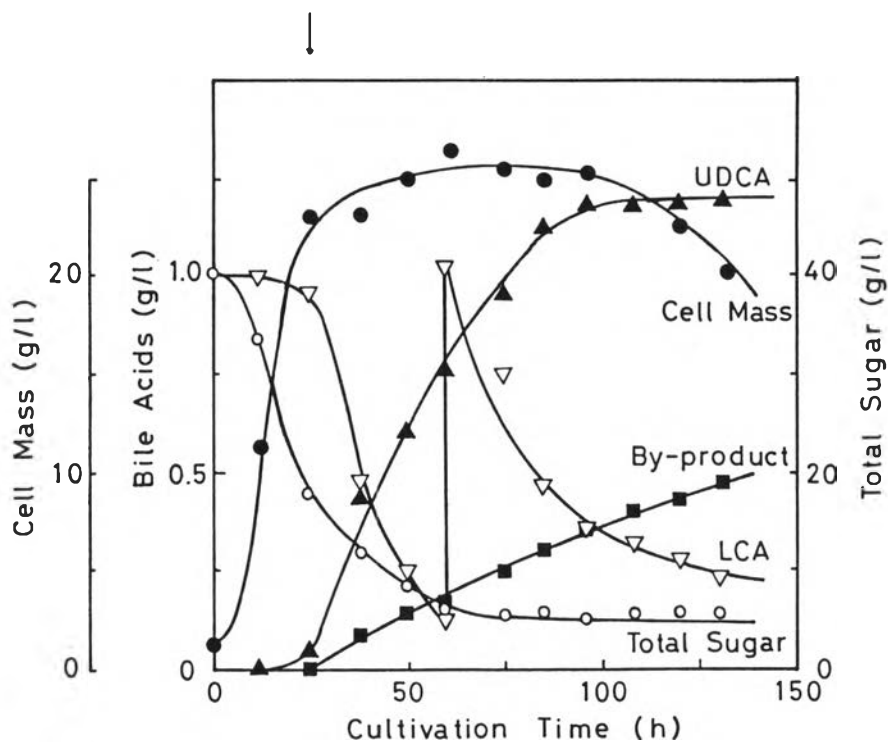
Fig. 8. UDCA production in a jar fermentor with 40% DOT during
growth phase and 100% DOT during production phase.
Experimental conditions are essentially the same as those
described in the legend to Fig. 7, except that DOT was
controlled at 40% saturation until 40 h of cultivation and
another portion of LCA (1 g/ℓ) was added at 60 h. The
production phase was initiated at the arrow.

Figures 7 and 8 show the time courses of cultivation with these shifts

in condition. In the control experiment (not shown), in which the pH was

maintained at 7.0 throughout the cultivation without KCl addition, UDCA

remained at 0.2 g/ℓ even after 150 h of cultivation. In contrast, 0.8 g

(80% yield) of UDCA per liter was produced within 72 h (Fig. 7a) when the

DOT was controlled at 0% saturation during cell growth and conditions

were shifted at 40 h: pH to 8.0, DOT to 100% saturation, and addition of

0.5 M KCl. In this cultivation, the substrare, LCA, was almost completely

consumed at 70 h, and its depletion seemed to be the main reason why UDCA

production ended at 0.8 g/ℓ. Indeed, by providing additional LCA, UDCA pro-

duction reached 1.23 g/ℓ at 146 h of cultivation (Fig. 7b). Next, to

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shorten the cultivation time, we applied a higher DOT (40% saturation) during the growth phase and performed the same condition shifts at 25 h (Fig. 8). As predicted, almost the same amount of UDCA (1.20 g/ℓ) was produced in the shorter cultivation time of 96 h.

Discussion

Almost all steroidal hydroxylases of microbial origin are known to be inducible (63-65), although a few have been reported to be constitutive (46,66). Little, however, is known about the hydroxylation of bile acids, which possesses very similar structures to steroid hormones. In this study we confirmed that the enzyme catalysing LCA 7β-hydroxylation was inducible, which suggests that inducibility of the enzymes involved may be a common feature of bile acid hydroxylation.

A survey of potential medium constituents showed that dextrin and L-asparagine were very effective for UDCA production (Tables 1-3). It was observed that slowly utilizable carbon sources, such as dextrin or soluble starch, tended to facilitate UDCA production. A high concentration of glucose greatly inhibited UDCA production by resting mycelium (88% inhibition in the presence of 20 g of glucose per ℓ), suggesting probably enzyme repression or inhibition by glucose. In contrast, organic ammonium rather than nitrate was a preferred nitrogen source. L-Asparagine is known to be a highly required nutrient for the growth of *Fusarium* species (67), and well-balanced growth in the presence of L-asparagine might be a cause of higher UDCA production. However, the mechanisms by which dextrin and L-asparagine facilitated UDCA production remain unclear, and further investigation is needed.

By using resting mycelium the optimum conversion conditions were determined to be pH 8.0, temperature of 28°C, and DOT of 60 to 100% saturation; and two-fold activation was obtained by the addition of 0.5 M KCl (Fig. 4). The results shown in Fig. 5 suggested that K^+ increased UDCA production by increasing the uptake rate or equilibrium concentration of LCA. When bile acids are absorbed by the liver (68-70) or small intestine (71-73)), transport of bile acids has been reported to be Na^+ dependent. This effect was explained by the cotransport of bile acids with Na^+ uptake catalyzed by Na^+, K^+-ATPase. A similar mechanism may operate in hydroxylation of LCA by *F. equiseti* M41. Another possibility is that monovalent cations disturb the membrane structure, thereby enhancing passive transport. Li^+ was the most effective of the monovalent cations at a lower concentration, whereas Rb^+ or Cs^+ became more effective at a higher concentration (Table 4). Ito *et al.* (74) reported that the entrance of plasmid DNA into *Saccharomyces cerevisiae* was enhanced by treatment with a monovalent cation, especially Li^+ or Cs^+. The optimum concentration of Cs^+ was rather high (1.0 M). Considering that a rather high concentration (0.5 M) was also necessary for the optimum activation of 7β-hydroxylation activity, enhanced passive transport of LCA due to membrane disarrangement seemed more likely, although further investigation is necessary to clarify the mechanism.

F. equiseti M41 converted LCA into UDCA, but UDCA was not the sole product. The ratio of UDCA to a by-product was 4:1 (Figs. 7a and 8). After UDCA production ended due to lack of LCA (Fig. 7), by-product formation continued with a concomitant decrease in UDCA. From this phenomenon and the R_f value of the by-product on gas chromatography and thin-layer chromatography, the by-product seemed to be a 3α,7β,12α-trihydroxy derivative of 5β-cholanic acid, although it was not identified completely. UDCA production by this fungus would be improved further by eliminating or inhibit-

ing the pathway by which the by-product is formed.

As mentioned previously, LCA hydroxylation activity was induced by the
presence of the substrate, LCA. The amount of LCA initially contained in
the media was 1 g per ℓ, but the solubility of LCA, which is highly hydro-
phobic, is only about 15 mg per ℓ of aqueous solution. This raises the ques-
tion of what is the effective concentration of LCA for induction and expres-
sion of hydroxylation activity. As shown in Figs. 7(b) and 8, the second
addition of LCA stimulated the production of UDCA. This together with the
retardation of LCA consumption and UDCA production with the decrease of LCA
concentration after 60 h of cultivation, shown in Fig. 7(a), indicated that
the production rate of UDCA depended on the concentration of LCA. This
phenomenon will be discussed further in the following section, Part 2.

Summary

The present study dealt with the optimum conditions for ursodeoxycholic
acid production by *Fusarium equiseti* M41. Resting mycelia of *F. equiseti*
M41 showed maximum conversion at 28°C, pH 8.0, and dissolved oxygen tension
of higher than 60% saturation. Monovalent cations, such as Na^+, K^+, and
Rb^+, stimulated the conversion rate more than twofold. In the presence of
0.5 M KCl, the initial uptake rate and equilibrium concentration of litho-
cholic acid were enhanced up to 5.7-fold and 1.7-fold, respectively. Our
results confirmed that the enzyme catalyzing 7β-hydroxylation of lithocholic
acid was induced by substrate, lithocholic acid. The activity of the
mycelium was controlled by dissolved oxygen tension during cultivation.
With dissolved oxygen tensions of 15% and over, the activity peak was
observed at 25 h of cultivation, whereas it was delayed to 34 and 50 h with

5 and 0% dissolved oxygen tension, respectively. After reaching the
maximum, the 7β-hydroxylation activity in the mycelium declined rapidly at
pH 7.0, but the decline was retarded by increasing the pH to 8.0. A
combination of several operations, including pH shift (from 7 to 8), addi-
tion of 0.5 M KCl, and dissolved oxygen control, was applied to the produc-
tion of ursodeoxycholic acid in a jar fermentor, and a much larger amount
of ursodeoxycholic acid (1.2 g/ℓ) was produced within 96 h of cultivation.

Part 2   Bio-adsorption of Lithocholic Acid
         in the Production of UDCA by *F. equiseti* M41

Introduction

As described in the previous part of this chapter, the concentration of
LCA should be kept much higher than its solubility in water solution to
secure a high production of UDCA.   In this situation, most of the LCA re-
mains insoluble during the biotransformation.   Although much information
has been accumulated on the biotransformation of steroidal compounds, little
is known about how insoluble substrates interact with the microorganisms
involved.

In the course of the studies on the biotransformation of LCA by *F.*
equiseti M41, it was noticed that the turbidity caused by the insoluble LCA
rapidly disappeared.   This could not be explained by UDCA formation, and it
was found that the insoluble LCA bound to the mycelia of *F. equiseti* M41.

This part describes the relationship between the LCA binding and the
UDCA producing activity, and the characterization of the LCA binding to the
mycelia to clarify the mechanism of UDCA production.

Materials and Methods

Chemicals   Bile acids, hexafluoroisopropanol, trifluoroacetic acid
anhydride, and acetonitril were purchased from commercial sources as men-
tioned in the first part of this chapter.   Lithocholic acid - 3α sulphate
was synthesized from LCA by the method of Palmer and Bolt (75).   9-Anthranyl

methyl esters of lithocholic acid and acetic acid were synthesized and purified by the method of Baker (76) and the purity was checked by high pressure liquid chromatography. Other chemicals were all reagent grade.

The preparation of cells   The cultivation was performed in a 2.5-jar fermentor (Type MD-250-3S; L.E. Marubishi, Tokyo) containing 1.5 ℓ of medium B, described in Part 1. The cultivation medium was inoculated with 100 ml of 72-h culture. The inoculum preparation is described in Part 1 of this Chapter. The temperature was maintained at 28°C, aeration rate at 1 vvm, pH at 7.0, and DOT at 0 - 2% saturation. Agitation rate was varied to adjust DOT. After 40 h of fermentation, pH and DOT were changed to 8.0 and 60 - 100% saturation, respectively, followed by the addition of KCl to a final concentration of 0.5 M. The cultivation was then continued for 10 h, and mycelia were collected by suction filtration and washed thoroughly with deionized water.

Assay   Bile acid was assayed as described in the first part of this chapter. Cell mass was expressed as grams of dry weight per ℓ after drying at 80°C for 24 h.

Determination of bound bile acid   One and a half g of wet cells was suspended in 30 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing the indicated amount of each bile acid in a 100-ml Erlenmeyer flask. The same buffer, 0.1 M Tris-HCl (pH 8), was used for suspending and washing cells unless otherwise mentioned.   The binding reaction was performed at 28°C at a shaking speed of 180 spm for 20 min, then mycelia were filtered through a stainless-steel sieve (mesh 100, pore size 149 μm, Iida Co. Ltd.) and washed twice with 30-ml portions of the buffer. To completely eliminate the un-bound bile acid, the mycelium remaining on the sieve was subjected to five cycles of resuspension in 30 ml of the buffer, filtering and washing. The 6th suspension was divided into 10-ml and 20-ml portions for cell mass and

bile acid analyses, respectively.

    Hydrophobicity determination of bile acid derivative   Hydrophobicity
of each bile acid was estimated from the retention time in reverse-phase
HPLC, using methanol:water as the mobile phase. The ratio of two solvents
was varied from 90/10 to 75/25. HPLC analysis was performed with a Trirotar
II (Japan Spectroscopic Co. Ltd.) equipped with Zorbax-ODS column (Dupont
Instrument), flow rate of 1 ml/min and detection at 210 nm.

    Surface area of the mycelia was determined by measuring the average
length and average diameter of the mycelia on photographs taken at 300-fold
magnification, and assuming that mycelia can be regarded as cylindrical.
For fluorescent microscopic analyses, a fluorescent microscope (type BH-RFL,
Olympus Co.) was used.

    Particle size of LCA in the incubation buffer was determined using
Coulter Counter (type ZBI, Coulter Electronics) with latex particles
(average particle size 4.86 μm) as standard.

Results and Discussion

1. Binding of LCA to the mycelia of *F. equiseti* M41

    When substrate LCA was used at a concentration of 1 g per liter or
higher, more than 60% conversion to UDCA was attained. It was not clear,
however, how or in what state LCA interacted with the mycelia during the
conversion, because LCA was almost insoluble in water (solubility = ca. 15
mg per ℓ) and more than 98% of the LCA would have been undissolved at the
concentration of 1 g/ℓ. To clarify this point, the amount of LCA attached
to mycelia was first determined (Fig. 1). At concentrations at which LCA
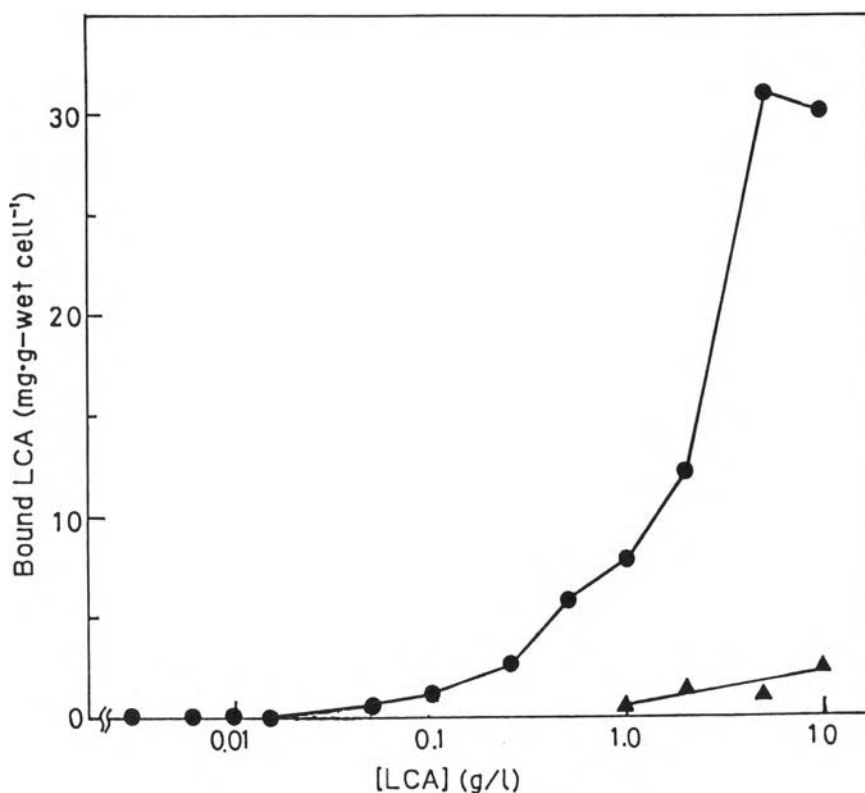dissolves in water, i.e., up to 15 mg per ℓ, no LCA was found bound to the

Fig. 1. Effect of LCA concentration on LCA binding to the
mycelia of *F. equiseti* M41. (●), Mycelia were suspended
in the buffer containing the indicated concentration of
LCA as described in Materials and Methods; (▲) mycelia
(1.0 g-wet cell) were suspended in 20 ml of the buffer
in a dialysis tube and the tube was incubated with 200 ml
of the buffer containing the indicated concentration of
LCA in a 500-ml Erlenmeyer flask. Incubation was per-
formed for 4 h.

mycelia; but as LCA concentration was increased up to 5 g per ℓ, the amount

of bound LCA also increased. At 5 g/ℓ of LCA, 31.8 mg of LCA/g-wet cell

was found attached to the mycelia, which was equivalent to the binding of

3.0 µg of LCA/cm^2 of surface area. From a Lineweaver-Burk type plot, the

apparent saturation constant, $K_{1/2}$ (binding) of 1.9 g/ℓ for LCA was obtained.

At concentrations higher than 1 g/ℓ, almost all LCA should exist as insoluble

particles. This suggests two possible modes of LCA binding: direct binding

of the insoluble LCA, and binding of only soluble LCA, which is continuously

supplied from insoluble LCA.

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To distinguish between these two possible processes, mycelia were put in a dialysis tube to preclude their direct contact with insoluble LCA. As shown in Fig. 1, less than 10% of LCA was found with mycelia at each LCA concentration tested, compared with the amounts observed in the direct contact with insoluble LCA. This indicated that most of the bound LCA resulted from direct binding of insoluble LCA. To confirm this, the fluorescent analog of LCA (9'-anthranylmethyl lithocholate) was synthesized and its mode of binding was observed under a fluorescent microscope (Fig. 2).
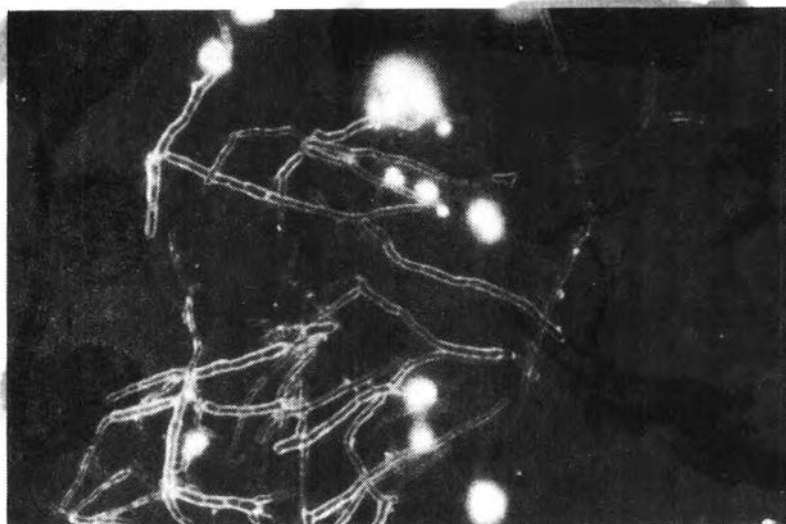

Fig. 2. Fluorescent microscopy of the mycelia incubated with fluorescent analog of LCA. Mycelia (50 mg) were incubated with 9'-anthranylmethyl ester of LCA (1 g/ℓ) in 2 ml of 0.1 M Tris-HCl buffer (pH 8.0) for 1 hr at 28°C, followed by repeated washing as described in Materials and Methods. The sample was observed under a fluorescent microscope (Type BH-RFL, Olympus Co.).

Particles of the fluorescent LCA derivative were found attached to the surface of the mycelia, while the mycelia incubated with control compound (9-anthranylmethyl acetate) did not show any difference from free mycelia. Therefore, it can be concluded that particles of insoluble LCA bind to the

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surface of the mycelia.

2. Characterization of the LCA binding in the mycelia

To learn more about the binding of insoluble LCA to the mycelia, several features were investigated.

Time course study of the LCA binding (Fig. 3) showed that the binding was very rapid, occurring within 20 seconds, and almost constant value was obtained until 60 min. From a practical reason, 20 min incubation was used for further study.
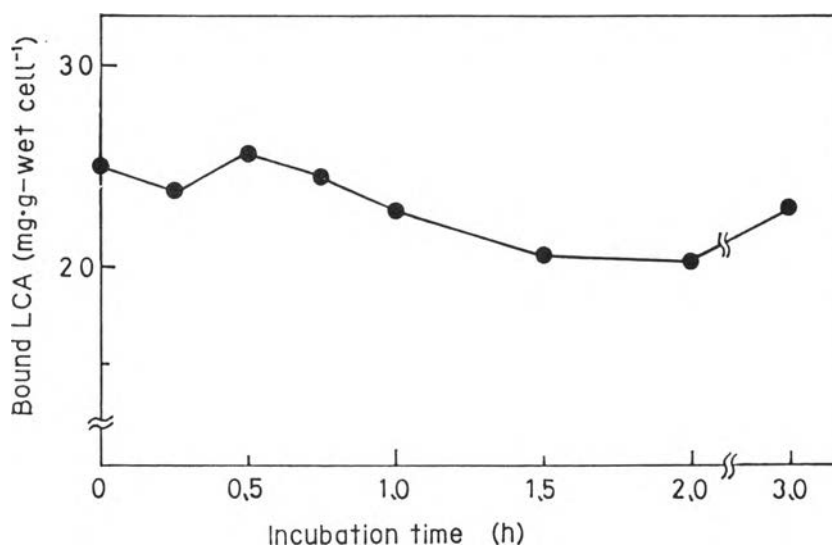

Fig. 3. Time course of LCA binding to the mycelia of *F. equiseti* M41. Experimental conditions are identical to those described in Fig. 1, except that the incubation period was varied.

Two possibel modes of interaction between the mycelia and LCA were next considered: i) ionic interaction with the side-chain COO^- group in LCA molecule, and ii) hydrophobic interaction with the polycyclic 5β-cholane moiety. If the ionic iteraction plays the main role in the binding, the binding should be lowered at the acidic pH where COOH rather than COO^- becomes dominant. But from the pH dependence of the LCA binding (Fig. 4), it is apparent that in the acidic range LCA binding increased rather than
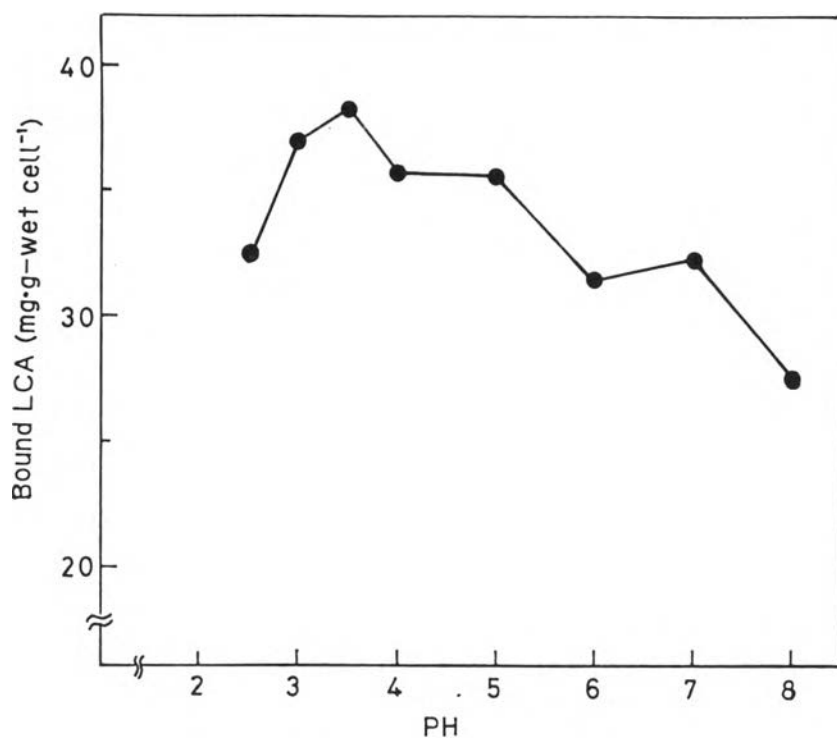
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Fig. 4.   Effect of pH on the binding of LCA to the mycelia of *F. equiseti* M41.   Experimental conditions were essentially the same as those described in Fig. 1, except that pH was varied. For pH 2.5 - 6.0, 0.1 M Na-citrate buffer was used, and for pH 6.0 - 8.0, 0.1 M Tris-HCl buffer.   As washing buffer, 0.1 M Tris·HCl buffer (pH 8.0) was used at each pH.

decreased.   This together with the results that the bound LCA was not washed off with a high concentration of KCl (up to 1 M) and that the presence of monovalent cations during the incubation with LCA did not affect the amount of bound LCA suggests that ionic interaction does not participate in the binding of LCA to *F. equiseti* M41 and that hydrophobic interaction is probable.

The results obtained by using 9'-anthranylmethyl lithocholate which was blocked its carboxyl group as ester group also supports non ionic mechanism.

To clarify this point further, the temperature dependence of the LCA binding was investigated.   As shown in Fig. 5, LCA binding increased
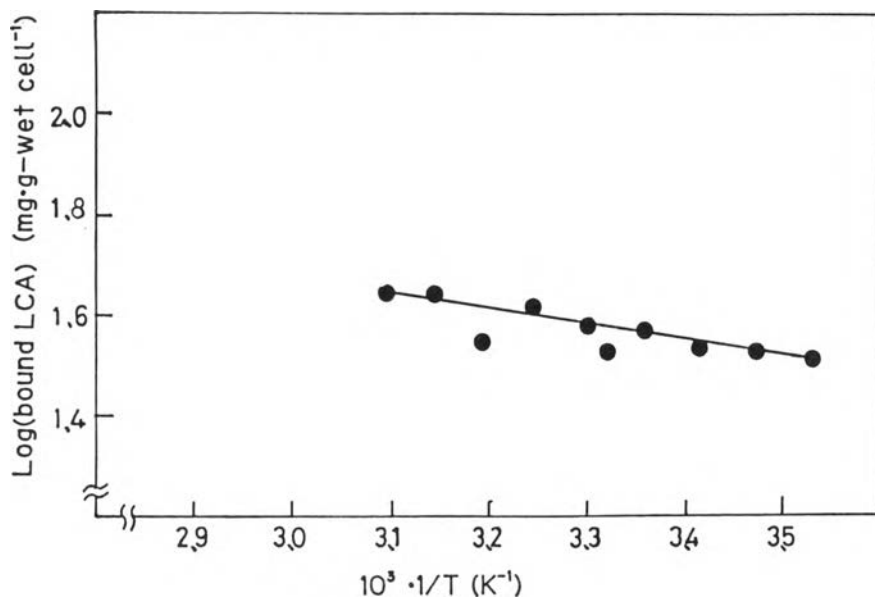
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Fig. 5. Temperature dependence of LCA binding to the mycelia
of *F. equiseti* M41. Experimental conditions were es-
sentially identical to those described in Fig. 4, except
that the incubation temperature was varied in the range
10 - 45°C.

slightly with the increase of temperature with an E_a value of 0.9 kcal/mole,
indicating that hydrophobic interaction played the major role in the LCA
binding to the mycelia. Ea values for hydrophobic interactions are usually
less than 10 kcal/mole, and from 10 to 100 kcal/mole for ionic interactions.

Next, we investigated the binding capacity of several bile acid
derivatives with different hydrophobicities (Table 1). A close relation-
ship was observed between the hydrophobicity of the bile acids and their
binding capacity, i.e., the more hydrophobic a bile acid was, the more of
it bound to the mycelia. However, there were some exceptions, such as 5β-
cholanic acid and 3-keto-lithocholic acid. 5β-Cholanic acid has no OH-
group at the 3α-position, and its hydrophobicity is 3 times that of LCA;
but it showed less binding than LCA. The reverse was the case for 3-keto-
lithocholic acid, in which 3α-OH group is oxidized to a keto group and the
hydrophobicity is decreased. It showed increased binding to the mycelia,

Table 1. Correlation between hydrophobicity and the binding
capacity of bile acid derivatives.

	Hydrophobicity	Amount of bound LCA (mg/g wet cell)
Lithocholic acid (3α-OH)	4.28	22.6
Deoxycholic acid (3α, 12α-OH)	2.11	1.8
Chenodeoxycholic acid (3α, 7α-OH)	1.97	2.3
Ursodeoxycholic acid (3α, 7β-OH)	0.58	0.9
Hyodeoxycholic acid (3α, 6α-OH)	0.99	4.0
Cholic acid (3α, 7α, 12α-OH)	0.86	0.2
Dehydrocholic acid (3, 7, 12-keto)	0	0.05
5β-Cholanic acid	14.9	13.0
3-Keto-lithocholic acid	1.76	54.1
Lithocholic acid 3α-sulfate	0	1.4

Reverse-phase HPLC was performed as described in Materials and
Methods using mobile phases for LCA, deoxycholic acid,
chenodeoxycholic acid, UDCA, cholic acid, and dehydrocholic
acid of methanol:H_2O = 6:4 (pH 3.0 with conc. H_3PO_4); and for
LCA, 3-keto-lithocholic acid, lithocholic acid 3α-sulfate and
hyodeoxycholic acid of methanol:H_2O = 75:25 (pH 5.0 with 5 mM
H_3PO_4). Three μl of each bile acid solution (1 mg/ml) was injected.
Hydrophobicity was determined by the method of Armstrong and
Carey (78). Other experimental conditions are described in
Materials and Methods.

indicating that the functional group at C-3 was important in the binding.
Actually, bulky substituents at C-3 greatly reduced the binding, as in the
case of lithocholic acid 3α-sulfate, although the hydrophobicity of this
compund was also decreased. Thus, the oxygen atom at C-3 seems to play
an important role in the binding, probably through hydrogen bond formation
of bile acids or dipole-dipole interaction.

In the binding of bile acids to the mycelia of *F. equiseti* M41, there
thus appear to be two essential factors affecting the binding; i) the
hydrophobicity of the bile acid molecule, and ii) the presence of an oxygen
atom at C-3.

3. Correlation between the LCA binding and UDCA production (7β-hydroxylating

   activity)

   Because the LCA binding showed saturation kinetics and a specific requirement in the structure of target molecule, the possibility that LCA binding is the active transport coupled with 7β-hydroxylating activity was examined by using several inhibitors of electron transport (Table 2). However, none of the inhibitors significantly affected the amount of bound LCA, while 7β-hydroxylating activity was significantly reduced (KCN or

Table 2. Effects of several inhibitors on LCA binding and UDCA production by the mycelia of *F. equiseti* M41.

	Final concentration	Producing activity	Bound LCA
		(%)	(%)
None		100	100
Antimycin A	3 µg/ml	00.0	88.0
NaN$_3$	5 mM	57.9	87.2
AsO$_4$	10 mM	66.5	105.0
Dicyclohexyl carbodiimide	2 mM	183.0	87.3
2,4-Dinitro-phenol	1 mM	41.8	85.2
KCN	1 mM	29.2	91.0
Ouabain	1 mM	38.0	112.0
Carbonylcyamide m-chlorophenol hydorazone (CCCP)	2 µM	30.1	115.0

Experimental conditions are essentially identical to those described in Fig. 1 and Materials and Methods. Each inhibitor was added at the indicated concentration during the reaction.

CCCP) or was completely abolished (antimycin A). This suggests that the main part of the LCA binding is not mediated by active transport, although the possibility cannot be excluded of carrier-mediated transport. Direct coupling of the LCA binding with 7β-hydroxylating activity is unlikely.

To further investigate the correlation between the LCA binding to the mycelia and the 7β-hydroxylating activity of the fungus, LCA concentration dependence of the 7β-hydroxylating activity was measured using resting cells (Fig. 6). The 7β-hydroxylating activity showed a similar concentration dependence to that of the LCA binding: at lower LCA concentration than the solubility, no activity was observed, but at higher LCA concentration than the solubility, the 7β-hydroxylation increased with the increase of LCA concentration. A Lineweaver-Burk type plot gave the K_m'apparent for LCA of 1.85 g/ℓ, which agreed well with that obtained for LCA binding to the mycelia. Therefore, insoluble LCA attached to the mycelia seems to correlate strongly with the 7β-hydroxylating activity of the mycelia. This together with the data in Table 2 on the relation between 7β-hydroxylating activity and the LCA binding suggests that LCA binding is prerequisite for high 7β-hydroxylating activity.
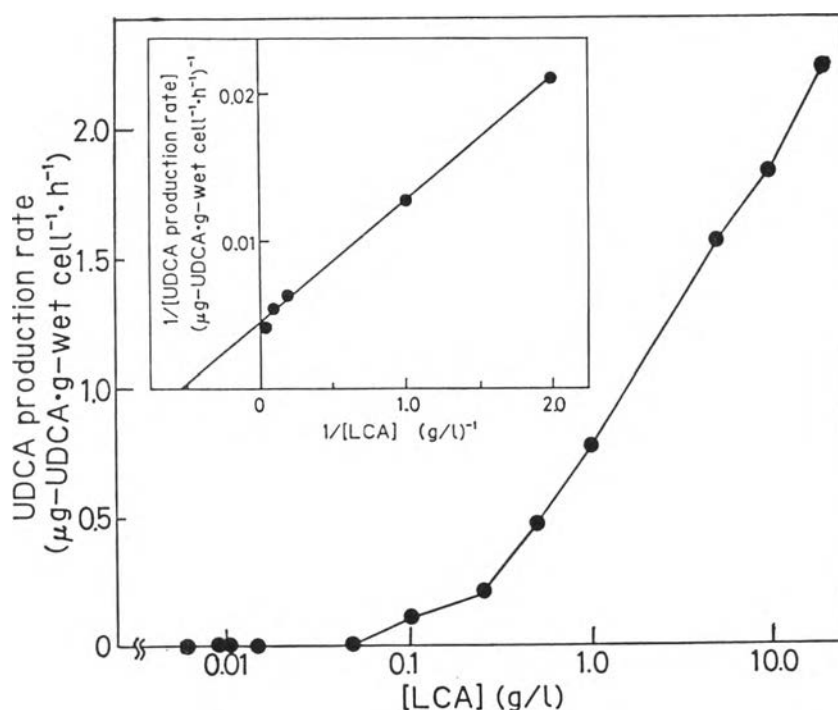

Fig. 6. Effect of LCA concentration on UDCA formation by the resting cells of *F. equiseti* M41. Experimental conditions are similar to those described in Fig. 1. Other experimental conditions are described in the text.

To investigate the mechanism by which insoluble LCA attached to the mycelia stimulated the 7β-hydroxylation, release of bound LCA by repeated washing with 30% dimethyl sulfoxide was examined (Fig. 7). The amount of bound LCA decreased gradually by repeated washing with 30% DMSO, but levelled off at 20 mg LCA bound/g wet-cell. This amount of binding represented 40% of the amount of LCA bound after repeated washing with 0.1 M Tris·HCl buffer. Therefore, of the total amount of bound LCA, 60% seemed to be attached to the surface of the mycelia and to be removable by mild washing,
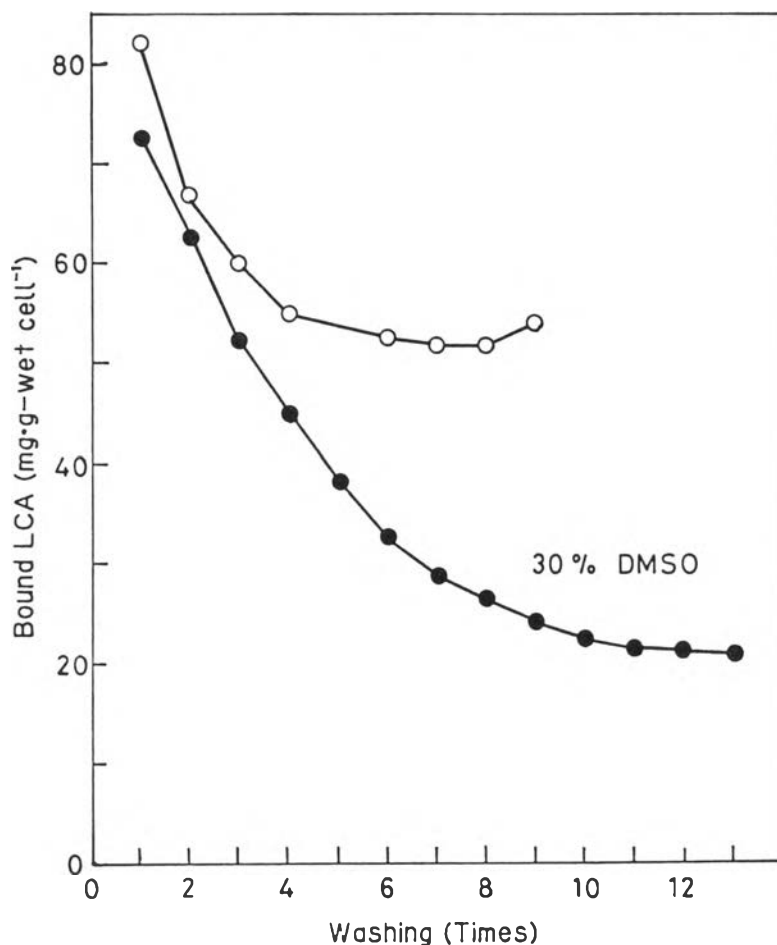

Fig. 7.   Release of bound LCA by repeated washing with 30% dimethyl sulfoxide (●) and the buffer described in Materials and Methods (○). Mycelia were incubated at an LCA concentration of 5 g/ℓ, then repeatedly washed either with the buffer containing 30% dimethylsulfoxide or with just the buffer.

and 40% to be enclosed within the cell. From the average cell volume determination, the intracellular LCA concentration was calculated to be 21.5 g of LCA/ℓ, assuming that the tightly bound LCA is distributed evenly within the cell. This LCA concentration of 21.5 g/ℓ was 1433 times higher than the solubility of LCA in the reaction mixture (0.015 g/ℓ). Thus, it can be concluded that the binding of insoluble LCA to the surface of the mycelia greatly increased the intracellular concentration of LCA, and resulted in enhanced enzyme activity.

Discussion

The rate of UDCA production was found to be dependent on the concentration of substrate, LCA, when the concentration of LCA was in the range of 0.2 - 1.0 g/ℓ, which is much greater than the saturation concentration of LCA of about 15 mg/ℓ. It was also determined that when the concentration of LCA in the fermentation broth was less than 15 mg/ℓ, UDCA was not produced. Maintenance of LCA concentration in the fermentation broth at a certain level therefore appears to be requisite for increasing the concentration of product. In the case of $3\alpha,15\beta$-DHC production, which will be described in Chapter 4, product formation was stimulated by feeding additional LCA into the fermentation broth when it became depleted. Since UDCA production involves the same cultivation medium and the same substrate, LCA, one might expect the similar result in improving the production of UDCA, although the strain was not same. In the last experiment described in Part 1, UDCA production decreased as the concentration of LCA decreased, and finally ceased, presumably when LCA concentration fell below the saturation concentration value. But when 1 g/ℓ of LCA was fed before its concen-

tration had fallen to the saturation concentration, UDCA production continued and reached a higher level.

Summary

Bioadsorption of a hydrophobic precursor, LCA, to the mycelia of *F. equiseti* M41 was studied in relation to the conversion of LCA to UDCA. It was revealed that the binding of LCA to the mycelia involved a hydrophobic interaction, not ionic. The amount of bound LCA increased with increase in LCA concentration, although almost all LCA existed as insoluble particles in the higher concentration region. The amount of LCA bound to the mycelia was negligible when its concentration was lower than its solubility in water (0.015 g/ℓ). The essential nature of the binding of insoluble LCA for UDCA formation was demonstrated by the agreement of the half saturation constant in the binding of LCA with the $K_{m'apparent}$ for the transformation of LCA to UDCA. From the amount of tightly bound LCA which resisted removal by dimethylsulfoxide, the internal LCA concentration was found to be about 1400-fold higher than its saturated concentration in the reaction mixture.

Thus, it can be concluded that *F. equiseti* M41 binds insoluble particle of LCA by hydrophobic interaction. The bound LCA stimulates the UDCA producing activity of *F. equiseti* M41 by raising the internal LCA concentration far higher than its solubility.

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