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# Hydroxylation of lithocholic acid by selected actinobacteria and filamentous fungi

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ABSTRACT

Selected actinobacteria and filamentous fungi of different taxonomy were screened for the ability to carry out regio- and stereospecific hydroxylation of lithocholic acid (LCA) at position 7β. The production of ursodeoxycholic acid (UDCA) was for the first time shown for the fungal strains of *Bipolaris*, *Gibberella*, *Cunninghamella* and *Curvularia*, as well as for isolated actinobacterial strains of *Pseudonocardia*, *Saccharothrix, Amycolatopsis*, *Lentzea*, *Saccharopolyspora* and *Nocardia* genera. Along with UDCA, chenodeoxycholic (CDCA), deoxycholic (DCA), cholic (CA), 7-ketodeoxycholic and 3-ketodeoxycholic acids were detected amongst the metabolites by some strains.

A strain of *Gibberella zeae* VKM F-2600 expressed high level of 7β-hydroxylating activity towards LCA. Under optimized conditions, the yield of UDCA reached 90% at 1 g/L of LCA and up to 60% at a 8-fold increased substrate loading. The accumulation of the major by-product, 3-keto UDCA, was limited by using selected biotransformation media.



## 1. Introduction

Bile acids (BAs) are 5β(H)-steroid compounds showing a *cis*-A/B ring juncture of the cyclopentanoperhydrophenanthrene nucleus, angular methyl group at C19, $C_5$-acyl side chain attached to C17, and functional groups which are mostly limited to hydroxyl groups in the nucleus and a terminal carboxylic acid group of the isopentanoic side chain (Fig. 1) [1].

In humans, BAs are produced from cholesterol *via* the introduction of a hydroxyl group at C7 catalyzed by cholesterol 7α-hydroxylase. During enterohepatic cycling, BAs undergo 7-dehydroxylation by intestinal bacteria resulting in the formation of the corresponding 7-deoxy derivatives. The third hydroxyl group in bovids and primates is usually present at C12.

The structural features of BAs determine their amphipathic properties and make them powerful biological detergents. In fact, they play an essential role in digestion by emulsifying and solubilizing fats, in cholesterol balancing, apoptosis, colonic salvage, etc. The dysfunctions in their metabolism may result in various pathologies, such as cholelithiasis, hepatitis, liver cirrhosis, cholesterol level misbalances, digestive system disorders, and carcinogenesis [2,3].

Microbial transformation of BAs was mostly studied for the exclusively high role of intestinal microflora in their metabolism [4,5]. The reactions include hydrolysis of conjugated BAs, 7α-dehydroxylation, dehydrogenation of the α-OH groups at C-7, C-3 and C-12 and/or their epimerization to the corresponding β-OH derivatives.

On the other hand, BAs are attractive raw materials for the pharmaceutical industry. It has been estimated that for each million cattle slaughtered, more than 25 tons of BA conjugates become available as by-products [6]. Some of these compounds, e.g., lithocholic acid (LCA, **I**, Fig. 1) and deoxycholic acid (DCA, **II**), do not find any real application and are destroyed after their separation from valuable BAs such as ursodeoxycholic acid (UDCA, **III**) and chenodeoxycholic acid (CDCA, **IV**) which are known for their high therapeutic action against many human diseases [7–11].

Therefore, effective transformation of BAs into valuable steroid derivatives, either key steroid intermediates like 3-oxo-steroids of androstane moiety or pharmaceutically active BAs such as UDCA, is a challenge of the modern biotechnology.

Soil actinobacteria were reported to carry out degradation of the isopentanoic side chain, oxidation of the 3α-hydroxyl function as well as introduction of C–C-double bonds [11]. However, in the current literature no data on hydroxylation of BAs by actinobacteria have been reported so far.

At variance, filamentous fungi are known to carry out hydroxylation of steroids at different positions of the steroid core [12], but hydroxylase activity towards lithocholic acid (LCA) has been reported for a restricted number of fungal strains. The fungus *Rhizoctonia solani* hydroxylated LCA at positions C-7, C-12 and C-15 [13], whereas *Helicostylum piriforme* ATCC-8992 formed

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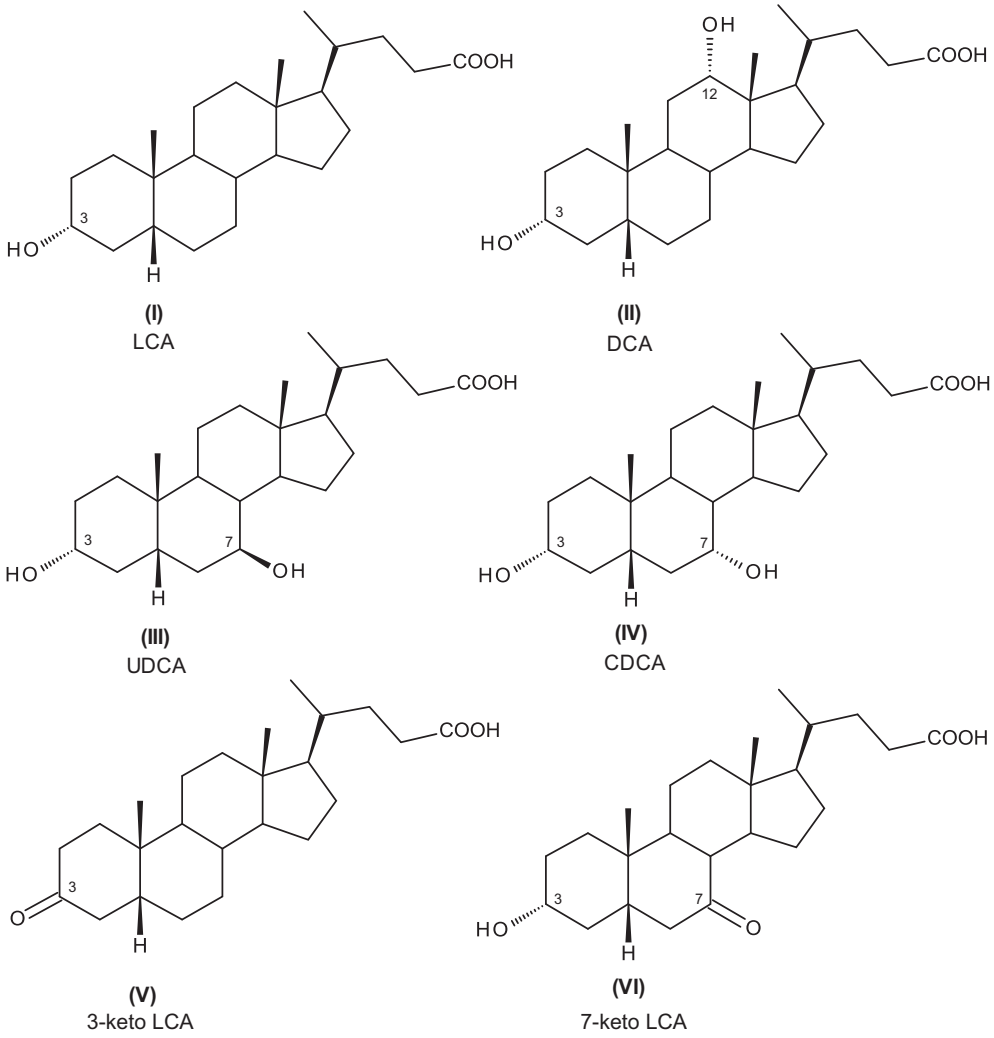


**Fig. 1.** Structure of representative bile acids: I – lithocholic acid (3α-hydroxy-5β-cholanic acid, LCA); II – deoxycholic acid (3α,12α-dihydroxy-5β-cholanic acid, DCA); III – ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanic acid, UDCA); IV – chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanic acid, CDCA); V – 3-keto-5β-cholanic acid (3-keto-LCA); VI – 7-keto-lithocholic acid (3α-hydroxy-7-keto-5β-cholanic acid, 7-keto-LCA).

12β-hydroxy and 12α,15α- as well as 12β,15β-dihydroxy derivatives [14]. A strain of *Cunninghamella blakesleeana* carried out 15β-hydroxylation of LCA [15]. Hydroxylation at C-15 and C-1, both in β configuration, of LCA was reported for *Curvularia lunata* NRRL-2380 [16].

Interestingly, a strain of *Fusarium equiseti* M-41 was reported to transform LCA to UDCA [17]. Strains belonging to this taxon were confirmed to be the most effective UDCA producers as revealed by extensive screening of about 200 fungal strains [18]. However, the bioconversion efficiency was not high even at optimized conditions [19,20], thus evidencing the necessity to find out novel strains capable of effective UDCA production.

During our previous work, the capability of more than 450 fungal strains to carry out regio- and stereospecific hydroxylation of dehydroepiandrosterone (DHEA, 3β-hydroxy-androst-5-en-17-one) has been studied and the strains with maximal 7α- and 7β-hydroxylating activity were selected [21]. The search has been recently extended to actinobacteria and strains capable of 7α- and 7β-hydroxylation of DHEA were firstly revealed among the isolated representatives of *Pseudonocardia*, *Amycolatopsis*, *Saccharopolyspora*, *Saccharothrix*, *Streptomyces*, *Nocardiopsis* and *Nonomuraea* [22].

In the present work we investigated the ability of selected bacterial and fungal strains showing 7-hydroxylating activity towards DHEA to produce UDCA from LCA and studied the distinctive features of LCA hydroxylation by the most active selected strain.

## 2. Experimental

### 2.1. Materials

Lithocholic acid (3α-hydroxy-5β-cholanic acid (LCA), ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanic acid, UDCA), and deoxycholic acid (3α,12α-dihydroxy-5β-cholanic acid, DCA) were obtained from ACROS Organics (USA). Other bile acids and their derivatives were a kind gift from Prodotti Chimici e Alimentari S.p.A. (Basaluzzo, Italy). Dehydroepiandrosterone (DHEA, 3β-hydroxy-androst-5-en-17-one) and cholesterol were from Sigma–Aldrich (USA). Yeast extract was purchased from Difco (USA), corn steep solids from Sigma–Aldrich (USA), and soy flour (Cultimed) from Panreac (Spain). All other reagents were of the best purity grade from commercial suppliers.

### 2.2. Microorganisms

The strains were obtained from All-Russian Collection of Microorganisms (VKM IBPM RAS) and working collection of Laboratory MTOC (IBPM RAS).

### 2.3. Screening of actinobacteria strains

The strains were grown on nutrient medium (50 mL) containing (g/L): glucose – 7, yeast extract – 4.5, malt extract – 5, $CaCO_3$ – 0.05, dissolved in deionized water (pH 7.0) in 750 mL shaker flasks. Seed cultures were prepared in two stages for 24 and 48 h, respectively, by strain cultivation on a rotary shaker (220 rpm) at 29 °C. For LCA bioconversion, 5% (v/v) of the second stage seed culture was inoculated in the transformation medium (medium P or R). Medium P composition (g/L): glycerol – 5; $KH_2PO_4$ – 1, $K_2HPO_4$ – 4, $(NH_4)_2SO_4$ – 3, carbamide – 0.25, $MgSO_4$ – 0.2, $FeSO_4$ – 0.01, $ZnSO_4$ – 0.002; deionized water (pH 7.0). Medium R composition (g/L): soy flour – 20, malt extract – 10, yeast extract – 5, $KH_2PO_4$ – 15.6, $K_2HPO_4$ – 3, $MgSO_4$ – 0.2, $FeSO_4$ – 0.01, $ZnSO_4$ – 0.002, deionized water (pH 7.0). LCA (1–2 g/L) was added as methanol solution. Final concentration of the solvent did not exceed 4% (v/v). Biotransformation samples (1 mL) were taken every 24 h. Steroids were extracted with EtOAc (1 × 5 mL) after 120–144 h of bioconversion and analyzed by TLC as described below.

### 2.4. Screening of fungal strains

If not otherwise mentioned, fungal strains were grown on the nutrient medium F (50 mL) containing (g/L): glucose – 30, yeast extract – 5, $K_2HPO_4$ – 2, $KH_2PO_4$ – 3, $NaNO_3$ – 5, $MgSO_4$ – 0.5, $FeSO_4$ – 0.02, $MnSO_4$ – 0.02, pH 7.0. Cultures were carried out aerobically on a rotary shaker (220 rpm) at 29 °C for 48 h in Erlenmeyer flasks (750 mL).

For LCA bioconversion, medium 1, containing (g/L): yeast extract – 5, corn steep – 10, $K_2HPO_4$ – 3, $KH_2PO_4$ – 2, $NaNO_3$ – 5, $MgSO_4$ – 0.5, $FeSO_4$ – 0.02 (pH 7.0) was inoculated with a second stage mycelium (10% (v/v)). LCA was added as a fine powder to a final concentration of 1 g/L. Bioconversion was carried out on a rotary shaker (220 rpm) at 29 °C for a maximum of 144 h and monitored daily by TLC as described below. Conversions were estimated by GC as described below.

### 2.5. LCA bioconversion by Gibberella zeae VKM F-2600

#### 2.5.1. Bioconversion by growing mycelium

Two kinds of growth medium (designated as A and B) were used for cultivation of the strain. Medium A composition (g/L): starch – 45, corn extract – 10, dried yeast – 30, $CaCO_3$ – 3 (pH 6.5); medium B composition (g/L): glucose – 7, starch – 10, dried yeast – 10, yeast extract – 4.5, $CaCO_3$ – 0.05 (pH 6.5).

For LCA bioconversion, mycelium grown for 48 h (5% (v/v)) was inoculated in the transformation media 1–6, pH 7.0 (Table 1).

LCA was added as methanol solution (2% (v/v)), or as 0.1 M potassium phosphate stock solution (15 g/L, pH 10.5) to a final concentration of 1–8 g/L.

Strain cultivation and LCA bioconversion was carried out aerobically on a rotary shaker at 220 rpm and 29 °C using rotary flasks (750 mL) containing 50–100 mL of the medium.

#### 2.5.2. Bioconversion by washed mycelium

The second stage mycelium was separated by filtration and washed with 0.1 M potassium phosphate buffer, and the wet mycelium (4 g) was incubated in 50 mL of the same buffer at pH 5.0–8.0. LCA was added as 2% (w/v) methanol solution to a final concentration of 1 g/L.

### 2.6. Isolation of metabolites

Metabolites were isolated from the culture medium by EtOAc extraction. After evaporation of the solvent under vacuum, crude residues were fractionated by silica gel column chromatography [21]. The individual compounds were analyzed by MS and NMR techniques.

**Table 1**
Medium compositions used for LCA bioconversion by *G. zeae*.

| Component | Medium No. / concentration of component, g/l | | | | | |
|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** |
| Yeast extract | 5 | | | 3 | 4.5 | 5 |
| Corn steep | 10 | 10 | | 5 | | 10 |
| Soya flour | | | | 17 | | 20 |
| Dextrin | | | 40 | | | |
| Starch | | | | | 30 | |
| L-Asparagine | | | 12 | | | |
| Glucose | | 30 | | | 7 | |
| $NaNO_3$ | 5 | 5 | | | | |
| $KH_2PO_4$ | 3 | 3 | 3 | 3 | 3 | 3 |
| $K_2HPO_4$ | 2 | | 2 | | | |
| $MgSO_4$ x $7H_2O$ | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| $FeSO_4$ | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |

### 2.7. Analyses

#### 2.7.1. Thin layer chromatography (TLC)

Samples of cultivation broth (1 mL) were taken every 24 h and extracted with 5 mL of EtOAc. The extracts were applied to Sorbfil UV 254 (Russia) TLC plates and developed in a mixture of $CHCl_3$-acetone-$CH_3COOH$ 50:50:0.5 (System A), or $CHCl_3$-MeOH-$CH_3$-COOH 70:30:0.5 (System B). Staining of TLC plates was carried out by using $MnCl_2$ [23] or the Komarowski reagent [24] and heating to 105 °C.

#### 2.7.2. Gas chromatography (GC)

Samples (20 mL) of the cultivation broths were extracted with EtOAc (3 × 40 mL), the solvent was evaporated under vacuum and the residue was redissolved in EtOH (5 mL). BAs were analyzed by the following method with some modifications [14]. Trimethylsilyl derivatives of methyl esters of BA were obtained as follows: 700 μL of 2 N solution of hydrogen chloride in methanol and 70 μL of 2,2-dimethoxypropane were added to the dry residue which was obtained after the evaporation of ethanol solution, the mixture was heated for 1 h at 85 °C and evaporated to dryness under vacuum. Then, 100 μL of dehydrated methanol was added to the dried residue and again evaporated to dryness. Silanization was carried out by treatment with 600 μL of pyridine and bis (*N,O*-trimethylsilyl) trifluoracetamide (BSTFA) (2:1, v/v) for 40 min at 80 °C.

Analyses were performed on HP 5890 chromatograph (USA), quartz column (15 m × 0.25 mm) with SPB-1, carrier gas (helium) flow – 1.4 mL/min, column temperature – 150–290 °C, and plasmic ionization detector with signal registration on HP 3396A integrator.

#### 2.7.3. Mass-spectrometry (MS) and ($^1$H) NMR

MS spectra were recorded on a Bruker Esquire 3000 Plus spectrometer.

$^1$H-NMR spectra were recorded on UNITY + 400 «Varian» (USA) with working frequency 300 MHz for $^1$H NMR nucleus in DMSO-$d_6$ and $CDCl_3$. Tetramethylsilane was used as an internal standard.

Compound **III** (ursodeoxycholic acid); mp 199 °C [lit. [25] mp 196 °C]; $^1$H-NMR (solvent DMSO-$d_6$), selected signals, δ: 3.494 (2H, m, H-3β and H-7α; 0.980 (3H, s, $CH_3$-19); 0.977 (3H, d; $J$ = 6.2 Hz, $CH_3$-21); 0.734 (3H, s, $CH_3$-18) [25]. $^{13}$C-NMR (solvent DMSO-$d_6$) δ: 176,882; 70.901; 70.721; 56.269; 55.335; 43.554; 43.292; 42.817; 40.340; 39.494; 37.379; 36.792; 35.432; 34.871; 33.947; 31.138; 30.798; 29.810; 28.358; 26.691; 22.690; 21.160;

17.687; 11.406. ESI-MS: 391.20 (calculated for $[C_{24}H_{40}O_4 + H]^+$: 391.56); $[\alpha]_D$ = +58 [lit. [25] $[\alpha]_D$ = +59].

Compound **VII** (3-keto ursodeoxycholic acid); mp 74 °C [lit. [26] mp 73 °C]; $^1$H-NMR (solvent $CDCl_3$), selected signals, δ: 3.624 (1H, m, H-7α; 1.064 (3H, s, $CH_3$-19); 0.963 (3H, d; *J* = 8.0 Hz, $CH_3$-21); 0.730 (3H, s, $CH_3$-18) [26]. $^{13}$C-NMR (solvent DMSO-$d_6$) δ: 211.357; 178,349; 70.407; 55.223; 54.455; 43.873; 43.325; 42.955; 42.632; 39.541; 39.010; 36.513; 35.889; 35.734; 34.695; 33.937; 30.352; 30.321; 28.031; 26.322; 22.179; 21.183; 17.883 11.672.. ESI-MS: 413.30 (calculated for $[C_{24}H_{39}O_4 + Na]^+$: 413.54); $[\alpha]_D$ = +66 [lit. [27] $[\alpha]_D$ = +67].

## 3. Results and discussion

### 3.1. LCA conversion by actinobacteria

Actinobacteria of different taxa (totally 32 strains) were selected based on their metabolic capacity to carry out the 7-hydroxylation of DHEA [22] and tested for the activity towards LCA. The capability of transforming LCA was shown for strains belonging to the *Agromyces*, *Amycolatopsis*, *Catellatospora*, *Lentzea*, *Nocardia*, *Nocardiopsis*, *Nonomuraea*, *Pseudonocardia*, *Saccharopolyspora*, *Saccharothrix*, and *Streptomyces* genera (Table 2).

Preliminary TLC analyses of bioconversion broths in comparison with authentic BAs standards showed that UDCA (**III**, Fig. 1), CDCA (**IV**), DCA (**II**), ursocholic acid (3α,7β,12α-trihydroxy-5β-cholanic acid, UCA), cholic acid (3α,7α,12α-trihydroxy-5β-cholanic acid, CA), as well as their oxidized derivatives, were produced from LCA by several strains, thus indicating the presence of both hydroxylation and oxidation activities.

At a deeper analysis, it is noteworthy that some strains showed only 7β-hydroxylating activity towards LCA, thus forming UDCA, e.g., *Nocardia nova* VKM Ac-1971, while others, such as *Saccharothrix longispora* VKM Ac-907 and *Catellatospora* sp. VKM Ac-122, hydroxylated LCA mostly at the 7α-position to form CDCA.

In addition to 7-hydroxylation, a significant 12α-hydroxylating activity was shown with different actinobacteria strains, leading to the formation of DCA as the main product, as in the case of *Amycolatopsis coloradensis* VKM Ac-1732, or to the trihydroxylated derivatives UCA and CA obtained in the bioconversions performed with *Streptomyces hydroscopicus* VKM Ac-831.

Moreover, the activity towards LCA seemed to be strain specific as different strains of the same taxa showed different activities. For example, unlike the Ac-907 strain, *Saccharothrix longispora* Ac-1265 inserted hydroxyl functions both in positions 7β and 12α in addition to 7α, thus forming a mixture of UDCA, CDCA, UCA, and other derivatives. On the contrary, other strains of the same genus showed no activity at all towards LCA.

It should be noted that the literature data on BAs transformation by actinobacteria are scarce and concern restricted species mostly belonging to *Mycobacterium*, *Rhodococcus*, or *Arthrobacter*. The metabolic pathway of BA by these organisms mainly included oxidation of 3α-hydroxyl group, desaturation of A-ring to form 3-keto-1,4-diene structures and cleavage of the isopentanoic side chain at C17 [11].

To our knowledge, no LCA hydroxylation was so far reported for actinobacteria of *Amycolatopsis*, *Lentzea*, *Nocardia*, *Nocardiopsis*, *Nonomuraea*, *Pseudonocardia* and *Saccharothrix* genera. Instead, the results obtained evidence a broad metabolic capacity of actinobacteria which express hydroxylating activity towards not only

**Table 2**
LCA bioconversions by selected actinobacteria.

| Strain | | LCA conversion | Products | | |
|---|---|---|---|---|---|
| | | | UDCA | CDCA | Others |
| *Agromyces* | *fucosus* ssp. *fucosus* VKM Ac-1346 | – | – | – | – |
| | *fucosus* ssp. *serinus* VKM Ac-1341 | + | + | – | Traces |
| *Amycolatopsis* | *coloradensis* VKM Ac-1732 | ++ | – | – | DCA, UCA |
| | *lurida* VKM Ac-1242 | ++ | + | – | UCA, CA, 12-keto CA |
| *Catellatospora* | sp. VKM Ac-122 | + | – | + | Traces |
| *Clavibacter* | *insidiosus* ssp. *insidiosus* VKM Ac-1402 | – | – | – | – |
| *Glycomyces* | *rutgersensis* VKM Ac-1248 | – | – | – | – |
| *Dactylosporangium* | *matrsurakiense* VKM Ac-1321 | – | – | – | – |
| *Lentzea* | *waywayandensis* VKM Ac-1970 | ++ | + | + | 7-Keto CA |
| *Microbacterium* | *lacticum* VKM Ac-1145 | – | – | – | – |
| *Nocardia* | sp. VKM Ac-1059 | – | – | – | – |
| | *nova* VKM Ac-1971 | + | + | – | Traces |
| *Nocardiopsis* | *albirubida* VKM Ac-1962 | – | – | – | – |
| | *albirubida* VKM Ac-1882 | – | – | – | – |
| | "*aha*" VKM Ac-1757 | – | – | – | – |
| | sp. TB-1353[a] | ++ | + | + | Traces |
| | *dassonvillei* VKM Ac-836 | ++ | – | | Non identified |
| *Nonomuraea* | *roseoviolacea* ssp. *carminata* VKM Ac-1780 | ++ | – | + | UCA |
| *Pseudonocardia* | *alni* VKM Ac-901 | + | + | + | UCA, 12-keto UCA |
| | *alni* VKM Ac-914 | – | – | – | – |
| | *autotrophica* VKM Ac-1067 | + | + | + | Traces |
| | *petroleophila* VKM Ac-799 | – | – | – | – |
| | *petroleophila* VKM Ac-865 | + | – | – | CA, UCA |
| *Saccharopolyspora* | *erythraea* VKM Ac-1189 | – | – | – | – |
| | *hirsuta* VKM Ac-666 | ++ | – | – | Non identified |
| | *rectivirgula* VKM Ac-810 | ++ | + | + | 12-keto LCA |
| *Saccharothrix* | *coeruleolusca* VKM Ac-855 | – | – | – | – |
| | *espanaensis* VKM Ac-1969 | – | – | – | – |
| | *longispora* VKM Ac-907 | + | – | + | Traces |
| | *longispora* VKM Ac-1265 | ++ | + | + | UCA, DCA, 7-keto LCA |
| *Streptomyces* | *endus* VKM Ac-129 | + | – | – | UCA, DCA |
| | *hydroscopicus* VKM Ac-831 | ++ | – | + | UCA, CA, 12-keto UCA |

⟨⟨–⟩⟩ – No activity; ⟨⟨+⟩⟩ – Less than 15%; ⟨⟨++⟩⟩ – More than 20%.
[a] Strain from working collection of Laboratory MTOC (IBPM RAS).

5-en-3β-ol or 4-en-3-one steroids [22], but also towards 3α-hydroxy-5β(H)-cholanoic acids. Therefore, even if conversions with these strains did not exceed 10–15%, these results could be applied for the generation of novel producers by strain improvement. Moreover, whereas it has been generally assumed that bacteria usually utilize BAs as a mere carbon and energy source, actinobacteria showed a detoxification activity similar to that observed with fungi. In fact, it is known that LCA can exert toxic effects on living systems. Our results show that actinobacteria can also detoxify LCA by insertion of one or more hydroxyl, or keto-functions thus making it more available for the further degradation.

### 3.2. LCA conversion by fungal strains

Screening of LCA bioconversions in the presence of filamentous fungi was carried out with 31 different strains belonging to 29 species of 12 genera (Table 3). About half of them, including selected strains of *Aspergillus*, *Acremonium*, *Gongronella*, *Nigrospora*, *Trichothecium* and *Phoma* which were reported earlier as capable of hydroxylating DHEA [21,28], showed no appreciable activity towards LCA.

As shown in Table 3, the capability to convert LCA to different BAs derivatives was observed for 16 strains of the genera *Bipolaris*, *Cunninghamella*, *Curvularia*, *Fusarium*, and *Gibberella*.

UDCA (**III**) was detected among the products by 9 of the 16 active strains. LCA to UDCA conversion rate was estimated by GC analyses of the culture broths as 3–15% for *Bipolaris australiensis* VKM F-3040, *Cunninghamella* sp. MTOC 6, *Cunninghamella japonica* VKM F-1205, *Curvularia protuberata* VKM F-3708, and *Fusarium merismoides* VKM F-2310. Maximal level of UDCA production (27–30% conversion under non-optimized conditions) was observed with the strains belonging to the *Gibberella* genus, in particular with the three *G. zeae* strains tested.

Along with UDCA, DCA was detected among the metabolites by *Bipolaris australiensis* VKM F-3040, *Curvularia protuberata* VKM F-3708 and *G. zeae* VKM F-2600 thus evidencing the presence of 12α-hydroxylating activity towards LCA. The conversion of LCA to DCA was low and varied in a range 1.5–4%.

The strains of *G. zeae* and all the tested strains of the *Cunninghamella* genus were found to produce 3-keto UDCA (**VII**, Fig. 2) from LCA whose structure was confirmed by MS and $^1$H-NMR analyses (see Materials and methods section for details) and by comparison with an authentic sample.

Based on metabolites analysis, the pathway by *G. zeae* was proposed to include LCA 7β-hydroxylation followed by oxidation of the 3α-hydroxy function since no accumulation of the intermediate 3-keto LCA was observed (Fig. 2, pathway 1). Unlike *G. zeae*, the strains of *Cunninghamella* did not accumulate UDCA from LCA (except for *C. japonica* F-1205). The formation of 3-keto-UDCA was proposed to proceed *via* oxidation of the 3α-hydroxyl group of LCA followed by 7β-hydroxylation of 3-keto-LCA (Fig. 2, pathway 2).

Interestingly, oxidation of 3α-hydroxyl function was not so far reported for mycelial fungi, while the presence of 3α-hydroxysteroid dehydrogenase activity was previously described for various anaerobic [29] and aerobic bacteria [11].

Finally, a different pathway was shown with the strain *Cunninghamella echinulata* VKM F-470, which accumulated 7-keto-LCA (**VI**, Fig. 2, pathway 3) along with 3-keto-UDCA (**VII**). The formation of this compound could be the result of 7-dehydrogenation of 7-hydroxyl function. Most likely, UDCA could be formed by this strain as a short-lived intermediate. Again, the presence of 7-hydroxysteroid dehydrogenases was reported earlier for several bacterial strains of human intestinal flora (*Eubacterium lentum*, *Bacteroides uniformis*, *Bacteroides fragilis* and others) [8], but it has been only predicted by sequence analyses in filamentous fungi. Other products were observed in minor amounts and not identified at this stage.

Based on the comparative analysis of the strain activity towards LCA, the strain of *G. zeae* VKM F-2600 was selected for further investigation.

### 3.3. LCA bioconversion by Gibberella zeae VKM F-2600

#### 3.3.1. Inoculum dosage and medium composition

Inoculum dosage was varied from 0.04–0.18 g of dry mycelium per lL of the nutrient medium. Mycelium of 48-h age grown on the medium F was used in the experiment; LCA bioconversion was carried out in the medium 1 (see the Materials and methods section for details about the media).

**Table 3**
Conversion of LCA by selected fungal strains.[a]

| Strain | | LCA conversion[b] | Products[b] | | |
|---|---|---|---|---|---|
| | | | UDCA | 3-Keto-UDCA | Others |
| *Bipolaris* | *australiensis* VKM F-3040 | + | + | – | + |
| *Cunninghamella* | *echinulata* VKM F-470 | ++ | – | ++ | + |
| | *japonica* VKM F-662 | ++ | – | ++ | – |
| | *japonica* VKM F-1205 | ++ | + | + | – |
| | *blakesleeana* VKM F-981 | + | – | ++ | – |
| | sp. MTOC 6[c] | ++ | + | ++ | – |
| *Curvularia* | *protuberata* VKM F-3708 | + | + | – | + |
| | *lunata* VKM F-644 | ++ | – | – | + |
| *Fusarium* | *culmorum* VKM F-2303 | + | – | – | – |
| | *merismoides* VKM F-2310 | ++ | + | – | ++ |
| | *moniliforme* VKM F-670 | + | – | – | + |
| | *proliferatum* VKM F-136 | + | – | – | + |
| *Gibberella* | *zeae* VKM F-2600 | ++ | ++ | ++ | + |
| | *zeae* VKM F-2599 | + | ++ | + | + |
| | *zeae* VKM F-2598 | + | ++ | + | + |
| | *fujikuroi* VKM F-1014 | ++ | + | – | ++ |

[a] No appreciable conversion of LCA was observed with the following strains: *Acremonium egypticum* VKM F-199; *A. felinum* VKM F-1300; *Aspergillus* sp. MTOC 159*; *A. niger* VKM F-212; *A. flavus* VKM F-1024; *A. sydowi* VKM F-441; *Fusarium sporotrichiella* VKM F-1600; *F. graminearum* VKM F-2306; *F. equiseti* VKM F-848; *F. oxysporum* VKM F-931; *Gongronella butleri* VKM F-1033; *Nigrospora oryzae* VKM F-1939; *Trichothecium roseum* VKM F-843; *Phoma glomerata* VKM F-1890.

[b] ⟨⟨–⟩⟩ – No activity; ⟨⟨+⟩⟩ – Less than 15%; ⟨⟨++⟩⟩ – More than 20%.

[c] Strains from working laboratory collection of Laboratory MTOC IBPM RAS.

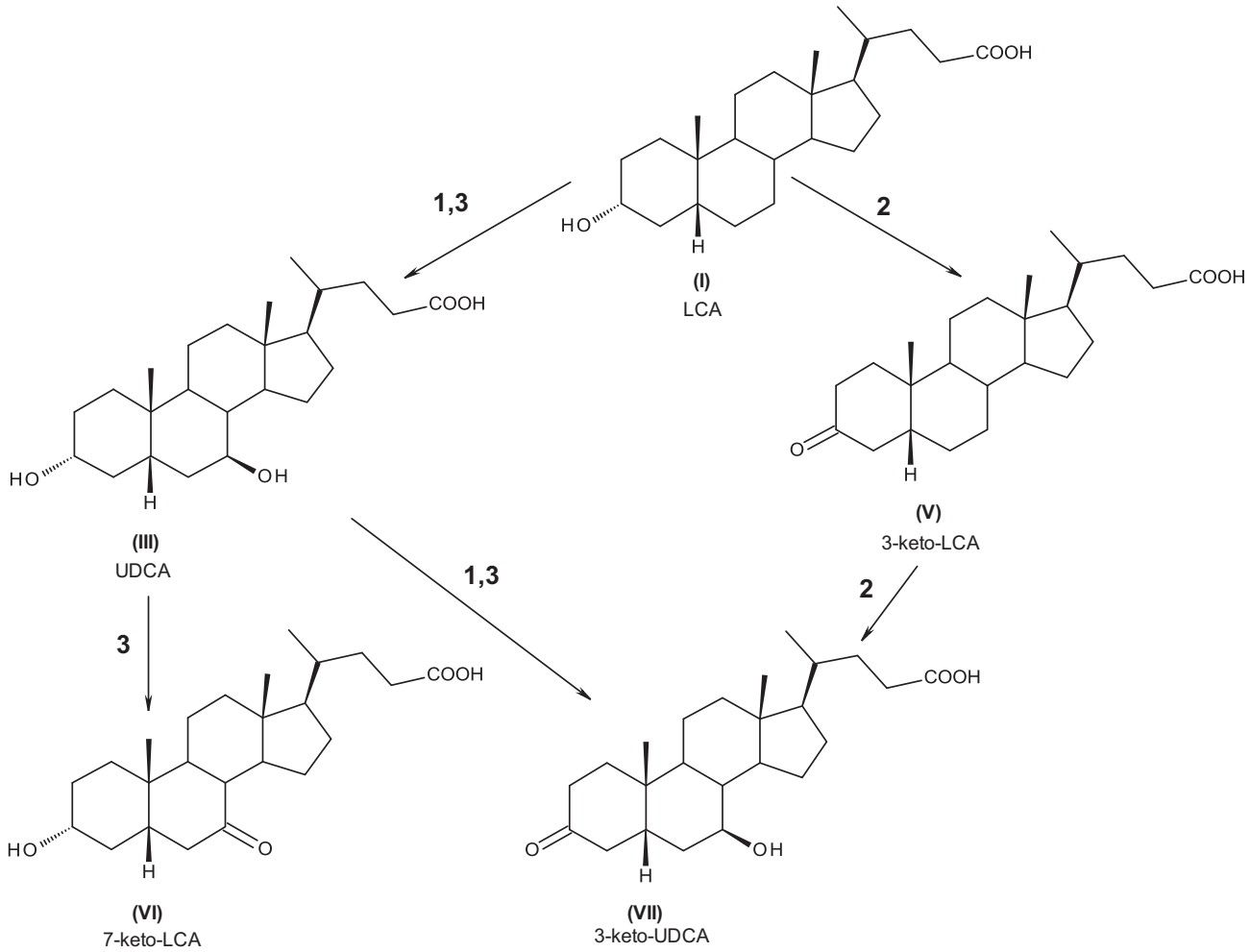


**Fig. 2.** Proposed pathways of LCA bioconversion by: I – *Gibberella zeae* strains; II – *Cunninghamella* strains; III – *C. echinulata* VKM F-470. The scheme is based on the time-courses of metabolites at the conversion of LCA (1 g/L) by growing mycelia. Medium F and medium 1 were used for seed culture preparation and LCA conversion, respectively.

Preliminary bioconversion experiments were carried out with medium F. The optimal dosage was determined as 0.06 g/L (generally corresponding to 5% (v/v) of inoculum), giving up to 65% conversions in about 90 h (Table 4, entry 1) while a 3-fold increase or a decrease to 0.04 g/L resulted in at least 10% lower UDCA conversion from LCA. Supplement of glucose (up to 30 g/L) and removal of yeast extract resulted in 2.5-fold decrease of UDCA accumulation (entry 2).

Further investigations of medium composition effects on UDCA conversion were carried out on the basis of our preliminary studies and literature data obtained for the relative strains [15]. Experiments were performed using mycelium grown on medium A or B because these media were found to provide the formation of small filamentous pellets (entries 3–5). Mycelium grown on medium A generally provided higher LCA to UDCA conversion as compared with those grown on medium B.

Finally, maximal LCA to UDCA conversion (83% in 120 h) was observed by inoculating a mycelium grown on medium A into the bioconversion medium 6 (entry 6).

*3.3.2. Influence of pH, inducers and detergents on bioconversion yields*

During LCA transformation by *G. zeae*, a pH increase from 7 to ~9 was observed. To clear the influence of pH on UDCA accumulation by the strain, the experiments were carried out using washed mycelium in a buffered medium.

Maximal levels of UDCA (about 40% conversion after 48 h) were observed at the use of an initial pH value of 8 followed by its decrease to ~7.4 during mycelium incubation. Bioconversion performance at pH 5–7 resulted in lower UDCA accumulation (< 5% conversion after 96 h, see Supplementary Materials for details). As BAs solubility reaches maximal level at the alkaline range, the increase of UDCA formation at pH >7 could be explained by the higher solubility of the substrate LCA in these conditions [30]. The results generally correspond to the data reported for LCA to UDCA conversion by *F. equiseti* [14]. *C. blakesleeana* ST-22 performed the 15β-hydroxylation of LCA at pH 8.0 [31]. The same substrate was converted to the 12β-hydroxy derivative by *Helicostylum piriforme* at pH 7.0 [14].

As previously shown, *G. zeae* is capable of effectively 7α-hydroxylating DHEA and the activity can be induced by DHEA as well as by other steroid substrates [28]. It is of interest that not only 3β-hydroxy-5-ene-steroids, but also LCA and DCA induced 7α,15α-dihydroxylation of DHEA by *G. zeae* (data not shown).

In this study, LCA, DCA, DHEA and cholesterol were tested as possible inducers of the 7β-hydroxylating activity of *G. zeae* towards LCA. Maximal inducing effect was observed for LCA and DCA, but the latter showed longer biotransformation times to achieve comparable UDCA yields (Fig. 3). On the contrary, the tested 5-en-3β-ol steroids negatively affected LCA to UDCA conversion.

To improve the solubility of hydrophobic substrates like steroids, detergents are often used in preparative scale bioconversions [32]. The accelerating effect of Tween 80 was reported for the 9α-hydroxylation of androstenedione (AD) by *Rhodococcus* sp. whole

**Table 4**
LCA to UDCA conversion on different media.

| Medium No. | Major components | Medium for seed culture growth | Maximal LCA to UDCA conversion (%) | Duration of reaching maximal UDCA level (h) |
|---|---|---|---|---|
| 1 | Yeast extract, corn steep, $NaNO_3$ | F | 65 ± 3 | 88–90 |
| 2 | Corn steep, glucose, $NaNO_3$ | F | 25 ± 2 | 96–98 |
| 3 | Dextrin, L-asparagine | A | 45 ± 3 | 96–98 |
| | | B | 20 ± 3 | 120–122 |
| 4 | Soya flour, corn steep, yeast extract | A | 75–80 | 120–122 |
| | | B | 55 ± 3 | 120–122 |
| 5 | Glucose, starch, yeast extract | A | 55 ± 3 | 120–122 |
| | | B | 9 ± 1 | 144–148 |
| 6 | Yeast extract, soya flour, corn steep | A | 83 ± 5 | 120–122 |

The data given are the average of at least three experiments.

cells [33]. Enhancement of β-sitosterol bioconversion to AD and androsta-1,4-diene-3,17-dione (ADD) by *Fusarium solani* was obtained by Tween 80 addition to an aqueous-organic solvent system [34].

Therefore, the effect of Tween 80 on UDCA formation from LCA was tested at Tween 80 concentration of 0.02–0.6% (v/v). Best conversions were observed at the presence of 0.1–0.2% (v/v) Tween 80 (about 10% improvement with respect to the control, see Supplementary Materials for details).

### 3.3.3. LCA bioconversion under optimized conditions

Based on the results obtained, the following conditions were determined to be favorable for effective LCA to UDCA conversion by *G. zeae*: growth of the mycelium on medium A for 48 h, induction for 3–6 h by 0.2 g/L LCA, bioconversion with medium 6 supplemented with 0.1–0.2% (v/v) Tween 80, initial pH value – 8.0.

The dynamics of LCA (1 g/L) conversion under these optimized conditions is presented in Fig. 4, whereas Fig. 5b shows the conversion to UDCA reached after 60 h (about 90%). The amount of non converted LCA was estimated to be less than 5%.

Increase of substrate loading from 1 to 8 g/L resulted in the decrease of LCA to UDCA conversion from 90 to 20%. This problem was overcome by substrate addition as an alkaline (pH 10–11) water solution (15 g/L) instead of methanol solution, which provided an increase of UDCA yield to 60% at high (8 g/L) LCA concentration. Therefore, approximately 4.8 g UDCA were recovered from 8 g of LCA under optimized conditions after 67 h of biotransformation (Fig. 6). Further strain incubation (over 72 h) resulted in the decrease of UDCA level.

In comparison, 1.2 g/L of UDCA were reported to be obtained from 2 g/L LCA using *Fusarium equiseti* M-41 [14]. UDCA yield of 48–50% was reported from 0.2–0.6 g/L LCA for the same strain [18]. The conversion of LCA to UDCA by *Penicillium* sp. TTUR 422 (FERM BP-5410) did not exceed 5.1% at the loading of 5 g/L LCA [19].

### 3.3.4. By-products accumulation during UDCA synthesis by Gibberella zeae VKM F-2600

During LCA bioconversion by *G. zeae* at the optimized conditions, 3-keto UDCA (**VII**, Fig. 2) was detected among the metabolites in small amounts. Increase of corn extract content up to 10 g/L in the nutrition medium shifted the ratio of the products formed towards preferable accumulation of 3-keto UDCA.

It should be noted that no 3-keto LCA was observed during LCA conversion by *G. zeae* thus indicating the capability of the strain to oxidize the 3α-hydroxyl function only in the presence of additional hydroxyl group in ring B and confirming the pathway of 3-keto

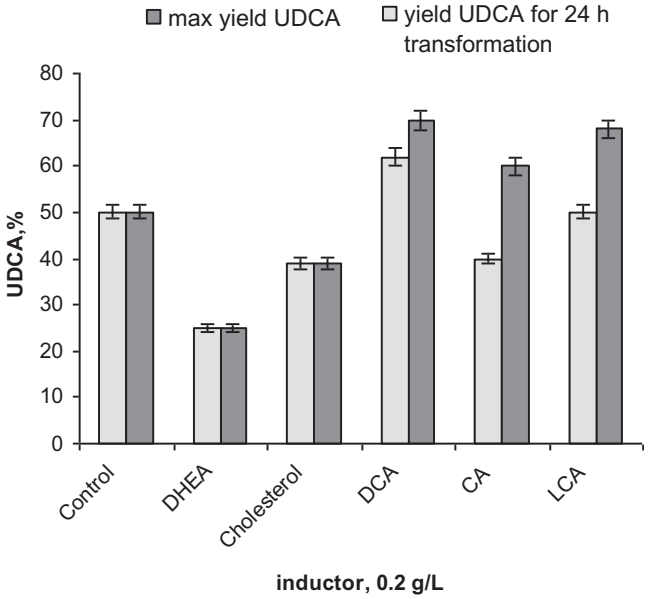


**Fig. 3.** Influence of DHEA, cholesterol, DCA, CA and LCA as possible 7β-hydroxylase inducers on UDCA yield from LCA by *G. zeae* VKM F-2600. Possible inducers were added at the concentration of 0.2 g/L; no inducer was added in control. The results are representative from several independent experiments.

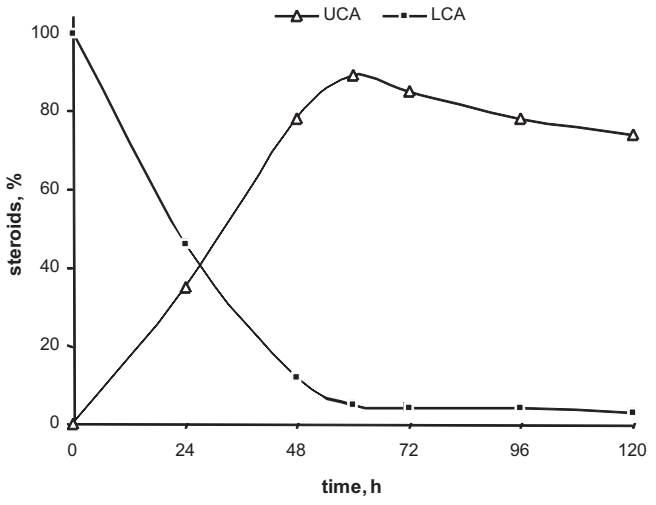


**Fig. 4.** Time course of LCA to UDCA conversion by *G. zeae* VKM F-2600 under the optimized conditions. Mycelium was grown on medium A for 48 h; 0.2 g/L of LCA was added for induction of 7β-hydroxylase activity for 3–6 h; the inoculate obtained (10%, v/v) was incubated with LCA (1 g/L) aerobically in medium 6 supplemented with 0.1–0.2% (v/v) Tween 80; initial pH was adjusted to 8.0. The data are the average from three independent experiments.

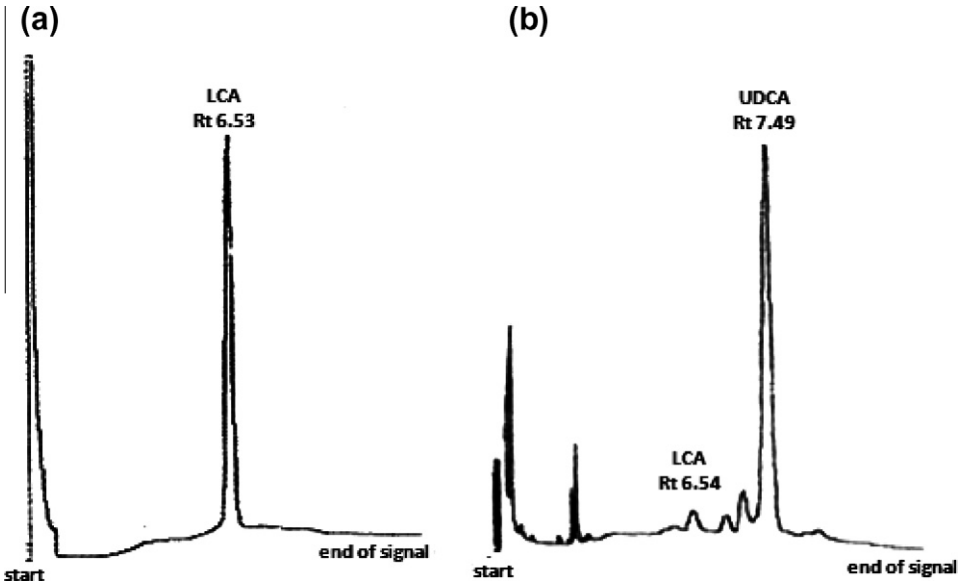


**Fig. 5.** GC chromatograms of the extracts obtained at LCA (1 g/L) transformation by *G. zeae* VKM F-2600 under the optimized conditions. The data are presented for the samples taken at: (a) 0 h; (b) 60 h of conversion.

UDCA formation via UDCA (Fig. 2). Probably the presence of the 7β-hydroxyl group in the UDCA molecule is necessary for expression of the 3α-hydroxysteroid dehydrogenase activity in this *G. zeae* strain.

The data are in correlation with those reported for bacterial strains. As shown, 3α-hydroxysteroid dehydrogenase activity expressed only towards bile acids with two or three hydroxyl groups. For instance, *Clostridium perfringens* formed 7α,12α-dihydroxy-3-oxo-cholanoic acid as a major product from CA [35]. The oxidation at C-3 was found during fermentation of DCA with *Actinobacter simplex* whereas the same bacterial strain showed no activity towards LCA [36]. Broad substrate specificity of 3α-hydroxysteroid dehydrogenase from *Pseudomonas* sp. B-0831 allowed its adaptation to various substrates including BAs [37].

Activation of the 3α-hydroxysteroid dehydrogenase enzyme(s) in *G. zeae* at high corn extract concentration is not fully clear. Probably, some components of this nutrient could induce this activity. In any case, the results confirmed the importance of medium composition for controlling process selectivity in fungal biotransformations.

Furthermore, the monitoring of metabolite composition by TLC showed the appearance of some more polar by-products in minor amounts. Three of these compounds were isolated by preparative TLC and flash chromatography and analyzed by mass analyses. ESI-MS data suggest the presence on the steroidal nucleus of one keto and two hydroxyl groups in two of them (MW = 407.56) and of three hydroxyl groups in the third one (MW = 408.57) (see Supplementary Materials for details). These data are consistent with preliminary NMR investigations and further studies are currently in progress to define the exact position of the functional groups.

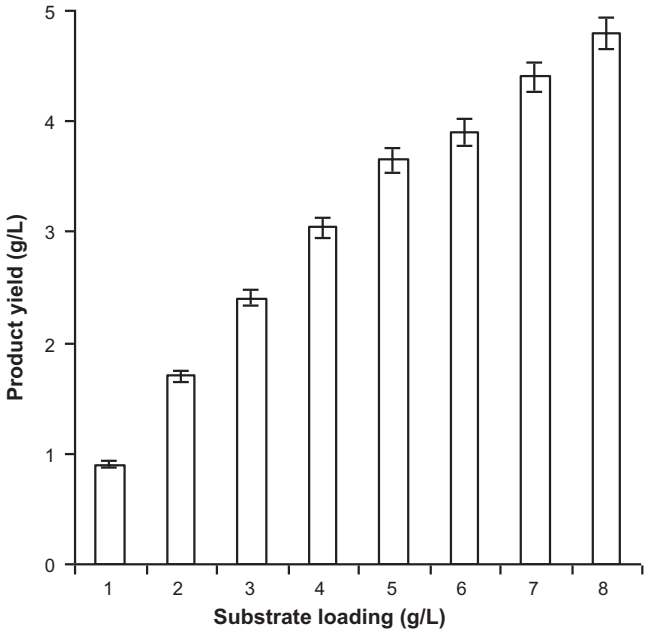


**Fig. 6.** Dependence of UDCA yield by *G. zeae* VKM F-2600 on LCA concentration. Bioconversion was carried out under the optimized conditions; LCA was added as a stock alkaline solution. Data are given for 67 h transformation and representative from several independent experiments.

## 4. Conclusions

In this work, novel organisms of actinobacteria and filamentous fungi have been revealed capable of converting LCA to high-value UDCA. In spite of the fact that the activity and process selectivity of actinobacterial strains towards LCA was found to be not sufficient for direct exploitation, the discovery of hydroxylating activities in actinobacterial strains is of importance as it could be applied to the generation of novel and more effective strains. Moreover, the data confirm the broad metabolic capacity of actinobacteria which were found to carry out not only oxidative degradation of BAs like rhodococci and mycobacteria, but also hydroxylation at different positions of the steroid core.

Novel data were obtained in this study concerning the ability of filamentous fungi to transform LCA to UDCA with maximum activity for *G. zeae* VKM F-2600. Along with UDCA, 3-keto UDCA can be formed as a product from LCA. The pathway was shown to include the oxidation of the 3α-hydroxy group of UDCA, and can be regulated by fine-tuning of the biotransformation conditions.

Under the optimized conditions, the yield of UDCA reached ~90% at a LCA loading of 1 g/L, while an 8-fold increase of substrate concentration resulted in 4.8 g/L UDCA at one batch. The results are considerably more positive than those previously published and they might be suitable for preparative-scale exploitation of the selected fungal strains for UDCA production.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2012.12.010.

## References

[1] Alvarez M, Jover A, Carrazana J, Meijide F, Soto VH. Vazquez Tato J. Crystal structure of chenodeoxycholic acid, ursodeoxycholic acid and their two 3β,7α- and 3β,7β-dihydroxy epimers. Steroids 2007;72:535–44.
[2] Lazaridis KN, Gores GJ, Lindor KD. Ursodeoxycholic acid «mechanisms of action and clinical use in hepatobiliary disorders. World J Hepatol 2001;35:134–46.
[3] Hofmann AF, Hagey LR. Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics cell. Mol Life Sci 2008;65:2461–83.
[4] Prabha V, Ohri M. Bacterial transformation of bile acids. World J Microbiol Biotechnol 2006;22:191–6.
[5] Begley M, Gahan CG, Hill C. The interaction between bacteria and bile. FEMS Microbiol Rev 2005;29:625–51.
[6] Mahato S, Mukherjee E, Banerjee S. Advances in microbial biotechnology of bile acids. Biotech Adv 1994;12:357–61.
[7] Sharma R, Prichard D, Majer F, Byrne AM, Kelleher D, Long A, et al. Ursodeoxycholic acid amides as novel glucocorticoid receptor modulators. J Med Chem 2011;54:122–30.
[8] Bortolini O, Medici A, Poli S. Biotransformations on steroid nucleus of bile acids. Steroids 1997;62:564–77.
[9] Ikegami CI, Matsusaki Y. Ursodeoxycholic acid. Mechanism of action and novel clinical applications. Hepatol Res 2008;38:123–31.
[10] Williams CI, Shaffer EA. Gallstone disease: current therapeutic practice. Curr Treat Options Gastroenterol 2008;48:692–4.
[11] Philipp B. Bacterial degradation of bile salts. Appl Microbiol Biotechnol 2011;89:903–15.
[12] Donova M, Egorova O. Microbial steroid transformations: current state and prospects. Appl Microbiol Biotechnol 2012;94:1423–47.
[13] Carlstroem K, Kirk DN, Sjoevall J. Microbial synthesis of 1β- and 15β-hydroxylated bile acids. J Lipid Res 1981;22:1225–34.
[14] Hayakawa S, Yao K, Lijima M, Sasaki K. Hydroxylation of litchoholic acid using the fungus *Helicostylum piriforme*. J Chem Soc Chem Commun 1980;3:84–5.
[15] Kulprecha S, Ueda T, Nichira T, Yochida T, Tagushi H. Optimum conditions for ursodeoxycholic acid production from lithocholic acid by *Fusarium equiseti* M-41. Appl Environ Microbiol 1985;49:338–44.
[16] Hayakawa S. Microbial transformation of bile acids. A unified scheme for bile acid degradation, and hydroxylation of bile acids. Z Allg Mikrobiol 1982;22:309–26.
[17] Sawada H, Kulprecha S, Nilubol N, Yoshida T, Kinoshita S, Taguchi H. Microbial production of ursodeoxycholic acid from lithocholic acid by *Fusarium equiseti* M41. Appl Environ Microbiol 1982;44:1249–52.
[18] Li S, Fa Y. Screening of strains producing ursodeoxycholic acid from lithocholic acid and identification of product. Acta Microbiol Sinica 1995;35:197–203.
[19] Sawada H, Taguchi H. Method for production of ursodeoxycholic acid by means of microbial transformation. US Patent 4,579,819; 1986.
[20] Okamura A, Matsui H. Bile acid converting microorganism and process for preparing bile acid. US Patent 5,989,855; 1999.
[21] Lobastova TG, Gulevskaya SA, Sukhodolskaya GV, Turchin KV, Donova MV. Screening of mycelial fungi for 7α- and 7β-hydroxylase activity towards dehydroepiandrosterone. Biocatal Biotransform 2007;25:434–42.
[22] Donova MV. Steroid bioconversion by actinobacteria. Pushchino: ONTI PSC RAS; 2009.
[23] Jork H, Funk W, Fischer W, Wimmer H. Thin-layer chromatography. Reagents and detection methods. Weinheim: FRG; 1990.
[24] Macdonald IA. Detection of bile acids with Komarowskýreagent and group specific dehydrogenases. J Chromatogr 1977;136:348–52.
[25] Dangate PS, Salunke CL, Akamanchi KG. Regioselective oxidation of cholic acid and its 7β epimer by using *o*-iodoxybenzoic acid. Steroids 2011;76:1397–9.
[26] Bortolini O, Fantin G, Fogagnolo M, Forlani R, Maietti S, Pedrini P. Improved enantioselectivity in the epoxidation of cinnamic acid derivatives with dioxiranes from keto bile acids. J Org Chem 2002;67:5802–6.
[27] Riva S, Bovara R, Zetta L, Pasta P, Ottolina G, Carrea G. Enzymatic α/β inversion of C-3 hydroxyl of bile acids and study of the effects of organic solvents on reaction rates. J Org Chem 1988;53:88–92.
[28] Lobastova TG, Gulevskaya SA, Sukhodolskaya GV, Donova MV. Dihydroxylation of dehydroepiandrosterone in positions 7α and 15α by mycelial fungi. Appl Biochem Microbiol 2009;45:617–22.
[29] Edenharder R, Schneider J. 12β-Dehydrogenation of bile acids by *Clostridium paraputrificum*, *C. tertium*, and *C. difficile* and epimerization at carbon-12 of deoxycholic acid by cocultivation with 12α-dehydrogenating *Eubacterium lentum*. Appl Environ Microbiol 1985;49:964–8.
[30] Alan F. Hofmann l, Karol J. Bile acid solubility and precipitation *in vitro* and *in vivo*: the role of conjugation, pH, and Ca ions. J Lipid Res 1992;33:617–26.
[31] Kulprecha S, Nihira T, Yamada K, Yoshida T, Nilubol N, Taguchi H. Transformation of lithocholic acid to a new bile acid, 3α,15β-dihydroxy-5β-cholanic acid by *Cunnighamella blakesleeana* ST-22. Appl Microbiol Biotechnol 1985;22:211–6.
[32] Wang Z, Zhao F, Hao X, Chen D, Li D. Microbial transformation of hydrophobic compound in cloud point system. J Mol Catal B: Enzym 2004;27:147–53.
[33] Avramova T, Spassova D, Mutafov S, Momchilova S, Boyadjieva L, Damyanova B, Angelova B. Effect of Tween 80 on 9α-steroid hydroxylating activity and ultrastructural characteristics of *Rhodococcus* sp. cells. World J Microbiol Biotechnol 2010;26:1009–14.
[34] El Refai HA, Abd-elslam IS. Enhancement of β-sitosterol bioconversion by *Fusarium solani* using aqueous-organic solvent system. Aust J Basic Appl Sci 2010;4:4107–12.
[35] Macdonald IA, Forrest TP, Costain GA, Rao BG. Identification of 7α,12α-dihydroxy-3-oxocholanoic acid as the major degradation product from cholic by *C. perfringens*. J Steroid Biochem 1978;9:353–8.
[36] Kimura H, Okamura A. Kawaide H, Yamaura T. Oxidation of bile acids with *Bacillus*. European Patent 518,661; 1992.
[37] Shigeru U, Masayuki O, Shigeyuki I, Masatake O. Steady-state kinetic properties of 3α-hydroxysteroid dehydrogenase from *Pseudomonas* sp. B-0831: steroid substrate specificity and nucleotide cofactor dependency. J Biol Macromol 2004;4:23–8.