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BIOCATALYSIS

Enhanced biotransformation of dehydroepiandrosterone to 3β,7α,15α-trihydroxy-5-androsten-17-one with *Gibberella intermedia* CA3-1 by natural oils addition

Heng Li · Zhenzhen Fu · Hui Li · Xiaomei Zhang · Jinsong Shi · Zhenghong Xu

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Abstract Dihydroxylation of dehydroepiandrosterone (DHEA) is an essential step in the synthesis of many important pharmaceutical intermediates. However, the solution to the problem of low biohydroxylation conversion in the biotransformation of DHEA has yet to be found. The effects of natural oils on the course of dihydroxylation of DHEA to 3β,7α,15α-trihydroxy-5-androsten-17-one (7α,15α-diOH-DHEA) were studied. With rapeseed oil (2 %, v/v) addition, the bioconversion efficiency was improved, and the 7α,15α-diOH-DHEA yield was increased by 40.8 % compared with that of the control at DHEA concentration of 8.0 g/L. Meantime, the ratio of 7α,15α-diOH-DHEA to 7α-OH-DHEA was also increased by 4.5 times in the rapeseed oil-containing system. To explain the mechanism underlying the increase of 7α,15α-diOH-DHEA yield, the effects of rapeseed oil on the pH of the bioconversion system, the cell growth and integrity of *Gibberella intermedia* CA3-1, as well as the membrane composition were systematically studied. The addition of rapeseed oil enhanced the substrate dispersion and maintained the pH of the system during bioconversion. Cells grew better with favorable integrity. The fatty acid profile of *G. intermedia* cells revealed that rapeseed oil changed the cell membrane composition and improved cell membrane permeability for lipophilic substrates.

Heng Li · Hui Li · X. Zhang · J. Shi · Z. Xu (✉)
School of Pharmaceutical Science, Jiangnan University,
1800 Lihu Avenue, 214122 Wuxi, People's Republic of China
e-mail: zhenghxu@jiangnan.edu.cn

Hui Li
e-mail: lihui@jiangnan.edu.cn

Z. Fu
School of Biotechnology, Jiangnan University, Wuxi 214122,
People's Republic of China

Keywords Biotransformation · *Gibberella intermedia* CA3-1 · Hydroxylation · Dehydroepiandrosterone · Natural oils

Introduction

Steroid pharmaceuticals are among the most marketed medical products available today and represent the second large category of pharmaceuticals next to antibiotics [30]. For producing novel steroidal drugs and generating active pharmaceutical ingredients, microbial transformation is employed as a novel, efficient and economical tool. The technique is particularly useful because it presents higher chemo-, regio- and stereo-selectivity than the chemical synthesis route [31]. Hydroxylation is among the most important reactions of steroid functionalization, while hydroxylated steroids usually exhibit higher biological activities than their less polar non-hydroxylated analogs [5, 14].

Dehydroepiandrosterone (DHEA) could be transformed to diverse steroids, of which 3β,7α,15α-trihydroxy-5-androsten-17-one (7α,15α-diOH-DHEA) is a key intermediate in the synthesis of pharmacologically significant steroids, such as antagonists of aldosterone and drospirenone [13]. This process could be achieved by several microorganism species, including *Colletotrichum lini* [26], *Gibberella zeae* [18] and *Fusarium oxysporum* [23]. However, 3β,7α-Trihydroxy-5-androsten-17-one (7α-OH-DHEA) is also produced as a byproduct accompanied with the manufacture of 7α,15α-diOH-DHEA, which leads to the reduction of product yield and the increase of separation cost. As DHEA is remarkably hydrophobic, solubilization of DHEA is considered as the rate-limiting step of steroid biotransformation [15].

For aqueous bioconversion systems involving hydrophobic compounds, organic solvents are usually used as the substrates and products pools for improving the low volumetric productivity [11]. However, the use of organic compounds in the industry is now facing restrictions because of environmental constraints and adoption of green chemistry guidelines [32]. Green solvents, including ionic liquids [4], liquid polymers [3] and natural oils [9], can be used as alternatives to organic solvents for their high substrate solubility and great biocompatibility with biocatalysts. However, high costs of most of the green solvents limit their applications in bioconversion. Natural oils are more economical than the above green solvents. Phase and Patil [24] reported an increase in the bioconversion efficiency of soybean sterols to 17-ketosteroids in a two-phase oil-buffer system. Gulla et al. [9] found that soybean oil could promote the conversion of soy sterols to androstenedione via a subspecies of *Mycobacterium fortuitum*. Nevertheless, studies on the effects of natural oils on steroid bioconversion remain scarce.

In this work, the dihydroxylation of DHEA to 7α,15α-diOH-DHEA by *Gibberella intermedia* CA3-1 [17] was selected as a model to evaluate the effects of natural oils on steroid bioconversion. The effects of natural oils on substrate solubility, systematical pH, cell growth, cell features and hydroxylation activity were systematically investigated. These results might provide some important information for studies on steroid biotransformation.

Materials and methods

Chemicals

Dehydroepiandrosterone and 3β,7α,15α-trihydroxy-5-androsten-17-one (7α,15α-diOH-DHEA) were obtained from Xianju Pharmaceutical Company Ltd. (Taizhou, Zhejiang, China) with purity of 98 %. Natural oils were purchased from Yihai Kerry Investment Company Ltd. (Shanghai, China). Ethyl acetate was analytical grade, while acetonitrile was high-performance liquid chromatography grade, and they were both purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Microorganism and culture media

Gibberella intermedia CA3-1 (China General Microbiological Culture Collection Center, CGMCC 4903) was cultured at 30 °C on agar medium consisting of the following composition (g/L): glucose 10.0, yeast extract 7.5, NaCl 2.72, KH_2PO_4 1.73 and agar 20.0 at natural pH. The seed medium formula was the same without agar with original pH 6.5. The transformation medium contained (g/L): glucose 15.0, yeast extract 15.0, corn steep liquor 2.0, NaCl 2.0 and KH_2PO_4 1.5 with original pH 6.5.

Culture condition and biotransformation procedure

Mycelium was transferred aseptically into 500 mL Erlenmeyer flasks each containing 50 mL seed medium. After being incubated on a rotary shaker (200 rpm) at 30 °C for 24 h, the culture was inoculated into 30 mL transformation medium in 250 mL flasks with the inoculums amount of 5 %. After being cultivated for a further 24 h, the fungus went into the growth stationary phase, and 8.0 g/L DHEA dispersed in proper amount of natural oils was added into the cell culture broth. The process without natural oils served as the control. Biotransformation runs were performed for 70 h on a rotary shaker with 220 rpm at 30 °C.

Biomass and product analysis

Biomass was determined as described by Lu et al. [20]. Cells were harvested and washed with deionized water. The washed cells were dried at 80 °C to a constant weight. Biomass was expressed as grams of dry weight per liter of culture medium.

The transformation products were analyzed by HPLC. Samples were taken at regular time intervals. 1 mL sample of the fermentation broth was withdrawn and then extracted with 1 mL ethyl acetate for 4 times. The extract (the ethyl acetate layer) was evaporated under vacuum at 80 °C for 5 h. Subsequently, the residue was redissolved in acetonitrile and analyzed by HPLC (Agilent 1200 series, Agilent Technologies, USA) with an Agilent TC-C18 column (4.6 × 250 mm, 5 μm) at 206 nm. The acetonitrile and distilled water at ratio of 70:30 were used as the mobile phase, with a flow rate of 0.5 mL/min at 30 °C. The peak area correction factor was 1.0.

Conversion of DHEA was expressed with 7α,15α-diOH-DHEA yield calculated according to the following formula:

$$7\alpha, 15\alpha - \text{diOH - DHEA yield}\,(\%) = \frac{\text{moles of } 7\alpha, 15\alpha \text{ - diOH - DHEA yield}}{\text{moles of DHEA transformed}} \times 100\,\%$$

Determination of solubility

To assess the solubility of DHEA in the five natural oils, given amounts of DHEA were added into 10 mL of natural oil each until saturation, which could be checked visually by formation of insoluble phase. The process was performed in magnetically stirred vessels under controlled temperatures of 90–100 °C, and then cooled to 30 °C. The supernatant was diluted in acetonitrile and assayed for steroid concentrations by HPLC.

Table 1 Parameters evaluated for the screening of natural oils

Solvents	DHEA solubility (g/L, 30 °C)	Biomass (g/L)	7α,15α-diOH-DHEA yield (%)	Conversion (%)
Control	0.01	28.53 ± 0.45	45.34 ± 1.31	67.20 ± 1.53
Turpentine	28.02	32.70 ± 0.87	30.23 ± 0.90	46.21 ± 1.56
Olive oil	115.7	33.12 ± 1.02	61.78 ± 1.89	83.31 ± 2.35
Rapeseed oil	110.1	32.34 ± 0.67	62.98 ± 2.11	82.45 ± 3.12
Soybean oil	90.7	34.23 ± 1.23	55.77 ± 1.52	78.43 ± 2.12
Sunflower oil	15.98	30.45 ± 0.67	18.09 ± 0.51	50.09 ± 0.98

The additional amount of natural oils was 4 % (v/v). The concentration of DHEA was 8 g/L

Fatty acid extraction and analysis

Total lipids were extracted according to the method of Shen et al. [28] after residual natural oil removal with hexane washing. The extracted fatty acids were analyzed as methyl esters by gas chromatograph (GC-2010, Shimadzu, Japan). The analytical parameters were as follows: CP-WAX capillary column (30 m long, 0.32 mm i.d., Varian-Chrompack); the injector and detector temperatures were both 250 °C; the carrier gas was nitrogen with a flow rate of 3 mL/min; and the column temperature was maintained initially at 100 °C for 3 min, followed by a 10 °C/min ramp to 180 °C and a secondary ramp of 3 °C/min to 240 °C. For quantification, standard C_{14}–C_{20} fatty acids were used.

Scanning electron microscopy preparation (SEM)

Cells were fixed in 3 % glutaraldehyde at 4 °C for 2 h. The fixed cells were washed with distilled water and dehydrated in water–ethanol ascending series from 30 to 100 % (v/v), followed by air-drying for 2–3 h. The samples were coated with a thin layer of gold. Observations were obtained using a Hitachi S-4800 scanning electron microscope equipped with a camera for digital micrographs operated at the accelerating voltage of 1.0 kV.

Results

Selection of natural oils for the bioconversion of DHEA

The solubility of DHEA in the five selected natural oils was measured. All of the assayed natural oils showed high substrate solubility and satisfactory biocompatibility. The effects of natural oils on the growth of *G. intermedia* CA3-1 and dihydroxylation of DHEA at 7α and 15α were investigated (Table 1). Application of rapeseed oil and olive oil resulted in the 7α,15α-diOH-DHEA yields of 62.98 and 61.78 %, respectively. Correspondingly, DHEA conversion reached 82.45 and 83.31 %, respectively. The results were obviously higher than that obtained in the control. Other oils, such as turpentine, sunflower oil and soybean oil, showed high substrate solubility but lower conversion rates. Because rapeseed oil has the advantage of inexpensiveness

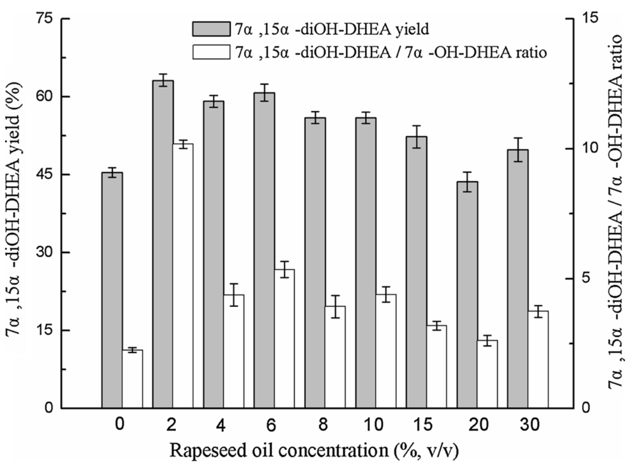


Fig. 1 Effects of concentrations of rapeseed oil on DHEA dihydroxylation efficiency. The initial substrate concentration was 8 g/L

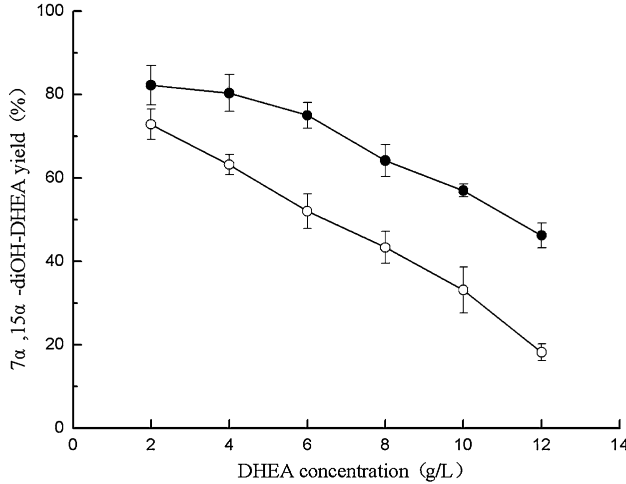


Fig. 2 Variations in dihydroxylation efficiency under different substrate concentrations in systems with (*filled circles*) and without (*open circles*) rapeseed oil

over olive oil, rapeseed oil was selected as the suitable solvent for the bioconversion of DHEA.

For further improving 7α,15α-diOH-DHEA yield, the effects of rapeseed oil concentration (0–30 %, v/v) were investigated. As shown in Fig. 1, the amount of rapeseed oil affected the 7α,15α-diOH-DHEA yield and the ratio of 7α,15α-diOH-DHEA to 7α-OH-DHEA significantly. Compared with the original conversion process without oil addition, the yield of 7α,15α-diOH-DHEA increased by 22.3–33.3 % in the presence of less than 10 % rapeseed oil. A volumetric concentration of 2 % rapeseed oil produced the highest 7α,15α-diOH-DHEA yield of 63.34 %. Besides, the 7α,15α-diOH-DHEA/7α-OH-DHEA ratio was enhanced dramatically and reached 10.2:1, which was 4.5 times higher than that in the control.

Effect of initial substrate concentration

The effect of substrate concentration on the dihydroxylation of DHEA was studied (Fig. 2). The product yield was higher than 80 % in the system containing 2 % rapeseed oil when the substrate concentration was less than 4 g/L. The yield of objective product gradually declined with the increase of substrate concentration. The similar trend was also observed in the original process. However, the rate of decline was more rapid than the system with rapeseed oil. The results indicated that a large amount of substrate led to incomplete dissolution and inhibition of intracellular enzymatic activity. Rapeseed oil showed potential use in the biotransformation of DHEA with high concentration, and higher product yields were obtained in the system containing 2 % rapeseed oil at the same substrate concentration.

DHEA bioconversion process and growth of *G. intermedia* CA3-1 in the system containing rapeseed oil

The time course of dihydroxylation of DHEA in the system containing rapeseed oil is shown in Fig. 3. Biomass was promoted and increased in the presence of rapeseed oil. Cell growth continued for 80 h in the presence of rapeseed oil, whereas biomass production began to decrease after 48 h in the control. The initial bioconversion rate and productivity in the system containing rapeseed oil also increased. The maximum 7α,15α-diOH-DHEA yield was 63.12 %, which was 40.3 % higher than the yield obtained in the original process.

Variation in pH in the system containing rapeseed oil

Figure 4 shows pH changes in the original and rapeseed oil-containing systems during the course of DHEA transformed. After the addition of DHEA, a steady increase in pH was observed in both processes. In the control

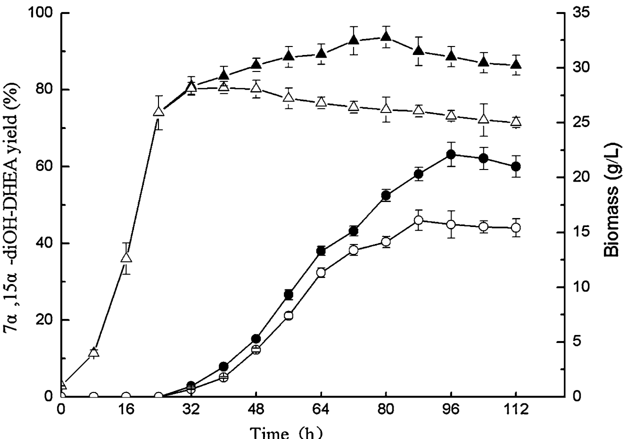


Fig. 3 Time course of DHEA 7α and 15α dihydroxylation by *G. intermedia* CA3-1 in the system containing rapeseed oil. *Filled circles* 7α,15α-DiOH-DHEA yield in the system containing rapeseed oil; *open circles* 7α,15α-DiOH-DHEA yield in the control system. *Filled triangles* Biomass in the system containing rapeseed oil; *opened triangles* biomass in the control system. The initial substrate concentration was 8 g/L

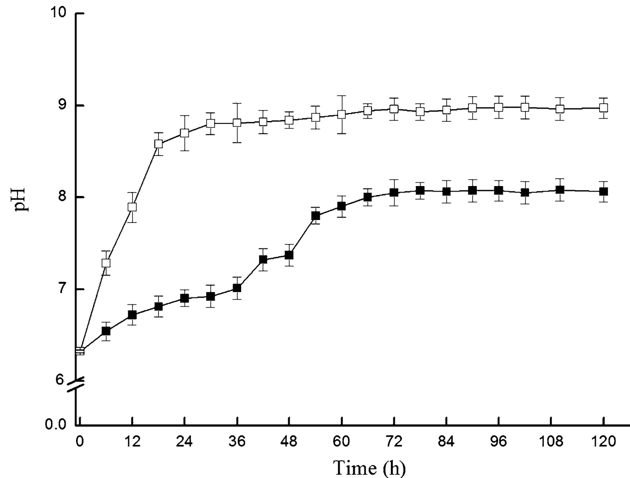


Fig. 4 Variation of pH in systems with (*filled squares*) and without (*open squares*) rapeseed oil

Table 2 Effect of constant pH on hydroxylation of DHEA in the original process

pH	7α,15α-diOH-DHEA yield(%)
3.5	25.78 ± 0.90
4.5	32.95 ± 0.86
5.5	40.64 ± 0.99
6.5	47.70 ± 1.35
7.5	52.57 ± 1.42
8.5	44.02 ± 1.11
9.5	20.38 ± 1.86

The substrate concentration was 8 g/L

process, the pH value showed a sharp increase initially until a stable pH of 9.0 was achieved. By contrast, the pH increased slowly and remained between 7.0 and 8.0 in the system containing rapeseed oil. After 72 h, pH values in systems with and without rapeseed oil were both not increasing anymore as the cell growth and transformation both stopped. Rapeseed oil could obviously stabilize the pH around 8.0, which might affect the bioconversion of DHEA. To verify this conjecture, the effect of constant pH ranging from 3.5 to 9.5 on DHEA bioconversion in the original process was investigated. As shown in Table 2, pH was indeed correlated with the hydroxylation efficiency. The pH value of 7.5 appeared to be optimal as high product yield of 52.3 % was obtained. This pH was close to the range of pH variation in the system containing rapeseed oil.

Changes in fatty acid profiles of the cells

The fatty acids of cells grown with and without rapeseed oil were maintained after lyophilization (Fig. 5). Analysis of the fatty acid compositions in the control and rapeseed oil-treated systems displayed several interesting differences. For the controlled cells, 31.83 % of the fatty acids were saturated, 62.45 % unsaturated, and lower than 5 % unidentified. Comparatively, cells grown in the presence of rapeseed oil showed marked changes in composition. The amount of unsaturated fatty acids in cells incubated with rapeseed oil reached 79.33 %, which was nearly 17 % higher than that in the control, while the amount of saturated fatty acids decreased to 16.38 %. Specifically, the percentage of C18:1 and C18:3 fatty acids were both improved noticeably.

Effect of natural oil on cell integrity

SEM images revealed considerable changes in the cell surface structures of microorganisms grown in rapeseed oil. In the control system, cells appeared rough and showed numerous concavities, resulting in an uneven cell outline. By contrast, cells grown in rapeseed oil had a dense surface with distinct and even outlines (Fig. 6).

Discussion

Natural oils have a wide variety of applications in the food, cosmetics, and lubrication industries [4, 10, 24]. Although natural oil has been employed as an alternative green solvent, specific information about the influences of natural oils on steroid biotransformation are still limited.

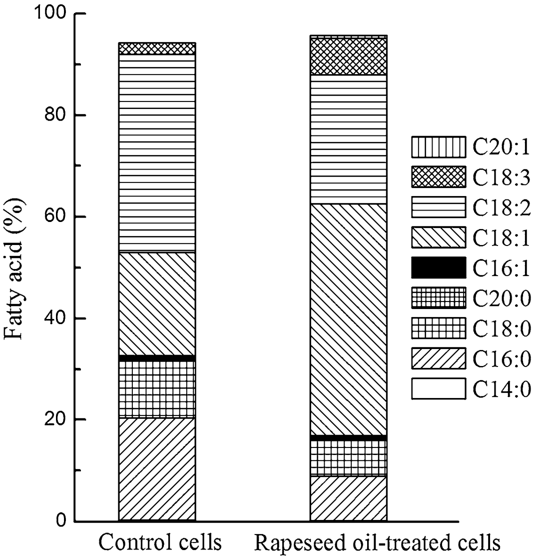


Fig. 5 Compositions of fatty acids extracted from the membranes of control and rapeseed oil-treated cells of *G. intermedia* CA3-1

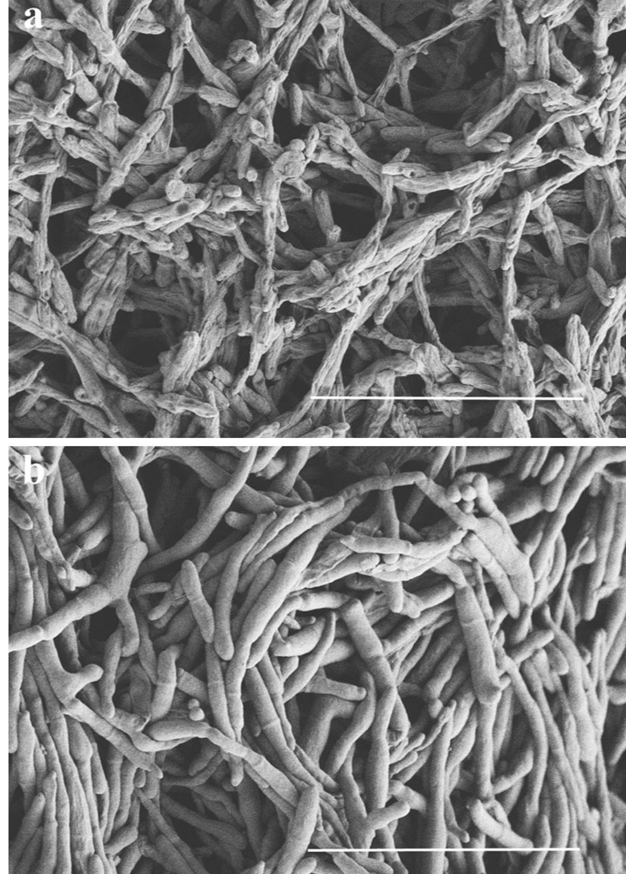
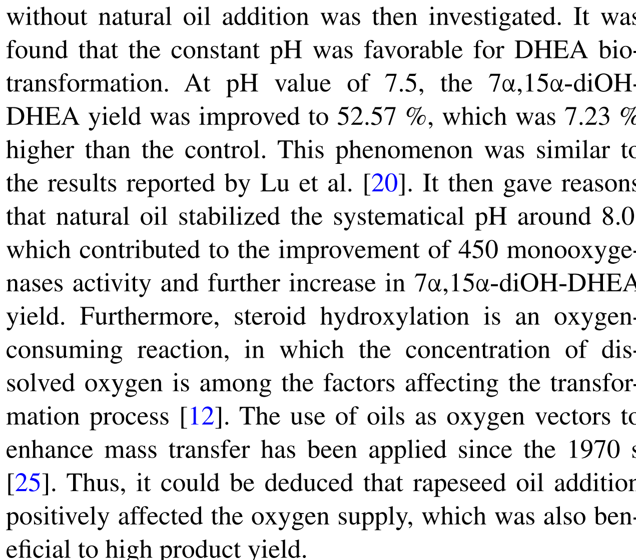


Fig. 6 SEM images of *G. intermedia* CA3-1 cells after 64 h of bioconversion **a** without (control) and **b** with rapeseed oil. *Bar* 50 μm

In this study, natural oils were used to improve the 7α- and 15α-dihydroxylation of DHEA by *G. intermedia* CA3-1. As substrate solubility is one of the key factors affecting steroid bioconversion, natural oils were added during DHEA biotransformation and showed satisfactory results (Table 1). Both olive oil and rapeseed oil performed high DHEA solubility up to 110 g/L. During the transformation process, DHEA feeding with addition of natural oils showed favorable dispersion, which was beneficial to enhancing the contact of substrate and cells. Compared with the control, the yield of 7α,15α-diOH-DHEA was increased by 40.8 % and the ratio of 7α,15α-diOH-DHEA/7α-OH-DHEA was increased by 4.5 times in the system containing 2 % rapeseed oil. The parameter of 7α,15α-diOH-DHEA/7α-OH-DHEA ratio is an indicator for biotransformation activity of the cells. A high ratio indicates the high accumulation of 7α,15α-diOH-DHEA, which might be favorable for product separation during downstream processing. 7α,15α-diOH-DHEA yield of approximately 63 % was obtained after incubation for 72 h with the initial substrate concentration of 8 g/L. The substrate concentration was higher than that reported in the literatures. Lobastova et al. [18] reported that, with the initial DHEA concentration of 2.0 g/L, the molar output of 7α,15α-diOH-DHEA transformed by *F. oxysporum* VKM F-1600 and *Gibberella zeae* BKM F-2600 reached 58.5 and 68 %, respectively. In a biotransformation process catalyzed by *C. lini*, the 7α,15α-diOH-DHEA yield reached 69.8 % from 2.5 g/L DHEA [26]. It could be seen that with the increase of substrate concentration, the gap of the 7α,15α-diOH-DHEA yield between the system with and without oil addition was greater and the superiority of oil addition thus became obvious.

Fungal steroid hydroxylation at positions of 7α and 15α is catalyzed by cytochrome 450 monooxygenases, which promote oxygen insertion in the steroidal substrate molecules [16]. Therefore, application of biotransformation conditions appropriate for 450 monooxygenases would benefit hydroxylation efficiency. The pH values have been found exerting great influences on the production of hydrocortisone [20] and 1,4-androstadiene-3,17-dione [29]. In the present studies, pH variation indeed impacted DHEA dihydroxylation. As shown in Fig. 4, the pH value changed in a wide range during the course of DHEA transformation in the control, especially during the initial 16 h, whereas the trend in the oil-added system varied relatively gently. After transformation for 72 h, both the pH values of systems with and without oil addition showed little changes. The yield of 7α,15α-diOH-DHEA was not increased anymore mainly because the strain had stopped growing and transforming. Therefore, the addition of rapeseed oil into the fermentation broth could stabilize the system pH at suitable levels. The influence of constant pH on DHEA biotransformation



without natural oil addition was then investigated. It was found that the constant pH was favorable for DHEA biotransformation. At pH value of 7.5, the 7α,15α-diOH-DHEA yield was improved to 52.57 %, which was 7.23 % higher than the control. This phenomenon was similar to the results reported by Lu et al. [20]. It then gave reasons that natural oil stabilized the systematical pH around 8.0, which contributed to the improvement of 450 monooxygenases activity and further increase in 7α,15α-diOH-DHEA yield. Furthermore, steroid hydroxylation is an oxygen-consuming reaction, in which the concentration of dissolved oxygen is among the factors affecting the transformation process [12]. The use of oils as oxygen vectors to enhance mass transfer has been applied since the 1970 s [25]. Thus, it could be deduced that rapeseed oil addition positively affected the oxygen supply, which was also beneficial to high product yield.

Lipids and oils are essential components for many fermentation media because they could supply supplemental nutrients for strain growth and cell activity maintenance [6]. As shown in Fig. 3, rapeseed oil performed significant effects on the production of 7α,15α-diOH-DHEA and cell growth. After cultivation in the system containing 2 % rapeseed oil for 24 h, glucose was nearly completely consumed when the substrate was fed into the broth. Biomass was continuously accumulated and the maximum was achieved until 80 h, which was nearly 1.6-fold of that in the control. Comparatively, in the original system, biomass remained constant between 32 and 48 h and then decreased. SEM (Fig. 6, b) analysis showed that the mycelium cells in the system containing 2 % rapeseed oil presented better characteristics, such as smooth surface, plump appearance, and excellent shape. These results revealed that rapeseed oil could be utilized and promote the active growth of cells.

For enhancing the biotransformation of DHEA, direct contact between cell and substrate was required. Therefore, improving the permeability of the cell wall or cell membrane could promote steroid uptake and further enhance the bioconversion efficiency. Various agents, such as D,L-norleucine [27], glycine [8], polycations [21], antibiotics [22], Tween-80 [2] and cyclodextrin [7] have been applied in cell wall/membrane permeability enhancing. Rapeseed oil was found to have the similar effect. Cellular fatty acids' composition, especially the unsaturated fatty acids' content, has been widely recognized as a factor influencing membrane fluidity and permeability significantly, and thus endowing cells several special properties [1, 19] You et al. [33] demonstrated that ethanol tolerance in yeast resulted from incorporation of oleic acid into lipid membranes, effecting a compensatory decrease in membrane fluidity that counteracts the fluidizing effects of ethanol. Rumijowska-Galewicz et al. [27] found that D,L-norleucine and m-fluorophenylalanine inhibited the biosynthesis of the

complex lipids in the *Mycobacterium vaccae* cell wall outermost layer. The rate of transformation of β-sitosterol to 4-androsten-3,17-dione (AD) increased considerably due to the cell wall permeability to β-sitosterol alteration. Analysis of the fatty acid components of the cells in the control and rapeseed oil-containing systems revealed significant changes of the relative proportions of saturated to unsaturated fatty acids (Fig. 5). Increase of the unsaturated fatty acids improved the hydrophobicity and permeability of cell membrane. Therefore, the cell-substrate contact and DHEA transformation activity were promoted.

In conclusion, the yield of 7α,15α-diOH-DHEA and DHEA conversion were both enhanced through addition of rapeseed oil. It exerted multiple effects on the DHEA hydroxylation process through substrate solubility and dispersion increase, pH stabilization, cell growth promotion and cell membrane permeability strengthened. The results contribute to the understanding of the effects of natural oil on steroid biotransformation, and provide a convenient and powerful method for enhancing the efficiency of steroid biotransformation.

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