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Enhancement of microbial hydroxylation of 13-ethyl-gon-4-ene-3,17-dione by *Metarhizium anisopliae* using nano-liposome technique

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Abstract The introduction of 11α-hydroxy to 13-ethyl-gon-4-ene-3,17-dione (GD) by microbial transformation is a key step in the synthesis of oral contraceptive desogestrel, while low substrate solubility and uptake into cells are tough problems influencing biotransformation efficiency greatly. Nano-liposome technique was used in the hydroxylation of GD by *Metarhizium anisopliae*. The substrate GD was processed to be GD-loaded nano-liposomes (GNLs) with high stability and encapsulation efficiency, and then applied in microbial hydroxylation by *M. anisopliae*. The results proved that the yield of the main product 11α-hydroxy-13-ethyl-gon-4-ene-3,17-dione (HGD) tripled compared to regular solvent dimethylformamide dispersion method at 2 g/l of substrate feeding concentration, and the HGD conversion rate showed no obvious reduction when the substrate feeding concentration increased from 2 to 6 g/l, which indicated the improvement of GNL addition method on biotransformation. Furthermore, the main byproduct changed from 6β-hydroxy derivative of GD (with similar polarity to HGD) to 6β,11α-dihydroxy derivative, which benefits the following purification of HGD from fermentation broth. These advantages suggest a great potential for the application of nano-liposome technique in microbial steroid transformation.

Keywords Hydroxylation · Nano-liposome · *Metarhizium anisopliae* · 13-Ethyl-gon-4-ene-3, 17-dione · Biotransformation

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Introduction

Microbial steroid transformation is a powerful tool for the generation of novel steroidal drugs, as well as for efficient production of steroid-active pharmaceutical ingredients and key intermediates [5]. Several microbial transformations of steroids and sterols have been reported, mostly on steroid hydroxylation, Δ1-dehydrogenation and sterol side chain cleavage [4, 6]. Microbial hydroxylation is possibly the most widespread type of steroid biotransformation, which can be used to build intermediates for further chemical synthesis, by offering access to otherwise inaccessible sites of the steroid molecule [13]. Compared to chemical synthesis, microbial transformations are generally carried out under mild conditions, following the principles of green chemistry, and have the advantage of high regio- and stereoselectivity [27].

The introduction of 11α-hydroxy to 13-ethyl-gon-4-ene-3,17-dione (GD, CAS 21800-83-9) is a key step for the preparation of desogestrel (17α-ethynyl-13β-ethyl-11-methylene-4-gonen-17-ol) [14], a synthetic progestogen used in therapy as contraceptives, either alone or associated with estrogens, which can minimize the unwanted side effects mainly represented by an increased risk of cardiovascular diseases and by some hepatic disorders [1, 7]. The microbial 11α-hydroxylation of 13-ethyl-gon-4-ene-3,17-dione (GD) had been reported to be conducted by *Aspergillus ochraceous* [7].

Microbial transformations of hydrophobic steroid compounds are often hindered by two obstacles: limited substrate accessibility to microorganisms because of the low aqueous solubility of steroids, and inhibition or toxicity of both substrate and product exerted upon the microorganisms [11, 23]. Many biotransformation systems, such as aqueous organic two-phase systems [3], aqueous two-phase polymer systems [19], cloud point systems [21, 22,

24], and ionic liquid aqueous systems [16, 26], have been reported to alleviate or overcome these problems. Although these approaches provide advantages over aqueous media, the high toxicity or the low tolerance of cells to organic solvents and other additives is still a limiting step in the process. It is imperative to develop more biocompatible and effective substrate supply approaches for widespread application of microbial steroid transformation.

Nanotechnology focuses on the design, synthesis, characterization, and application of materials and devices at the nanoscale, and it has been widely applied in medicine, ranging from in vivo imaging and diagnosis to therapeutics such as drug delivery and gene therapy. Because of their small size, nanoparticles (NPs) can readily interact with biomolecules on the cell surface or inside cells. An NP-mediated drug delivery system allows the continuous and controlled release of therapeutic drugs to maintain drug levels within a desired level rather than a burst of drugs so that a high therapeutic efficiency can be achieved without side effects [10]. Liposomes are spherical lipid particles in an aqueous compartment formed by an enclosing lipid bilayer [17]. The fatty layer of the liposome is expected to protect and confine the enclosed drug until the liposome binds to the outer membrane of target cells. The hydrophilic core and hydrophobic phospholipids bilayer coat of liposomes improve the solubility of many amphiphilic drugs. Further, because of their structure, liposomes can entrap hydrophilic pharmaceutical agents in their internal aqueous compartment and lipophilic drugs within the lipid membrane [8, 9]. NPs made with liposomes are the simplest form of NPs and have the advantage of excellent biocompatibility, reduced systemic toxicity, increased uptake into cells, and steady release of their payload. They have been widely used in clinics for decades [15]. It was also reported that the liposomal medium allows performing steroid conversions at high substrate concentrations with non-toxic for microorganisms [12]. All of these promising advantages prompted us to investigate the efficacy of nano-liposomes in microbial steroid transformation.

We have screened a *Metarhizium anisopliae*, which has the ability to introduce 11α-hydroxy to 13-ethyl-gon-4-ene-3,17-dione (GD). In this work, the nano-liposome technique was used in the biotransformation of 13-ethyl-gon-4-ene-3,17-dione (GD) to 11α-hydroxy-13-ethyl-gon-4-ene-3,17-dione (HGD) by *M. anisopliae*. The substrate GD was firstly processed to be GD-loaded nano-liposomes (GNLs) with lecithin by thin-film method connected with a high-pressure homogenizer, and then applied in microbial hydroxylation by *M. anisopliae*. The effects of this GNL addition method on HGD yield, biotransformation process, product compositions, and substrate concentration were investigated to evaluate its improvement on steroid biotransformation. To our knowledge, this is the first report on the application of nano-liposome in microbial steroid transformation.

Materials and methods

Microorganism

M. anisopliae 11490 used in this work was isolated from the silkworm by our research group and now preserved in the China Center for Type Culture Collection (Wuhan, China) with CCTCC No. M 2011240. The strain was stored on agar slant at 4 °C.

Chemicals

13-ethyl-gon-4-ene-3,17-dione (GD) of 99.3 % purity was kindly provided by Beijing Zizhu Pharmaceutical Co., Ltd. Egg yolk lecithin and soybean lecithin were purchased from Toshisun International Pty Ltd. (Ludwigshafen, Germany). HPLC-grade methanol and acetonitrile were obtained from Dikma. All other reagents were of chromatographic pure or analytical grade and available commercially.

Culture media

Microorganisms were stored at 4 °C on potato dextrose agar (PDA) added with 0.25 % (w/v) silkworm chrysalis meal. PDA and spore suspensions were prepared as previously described [28]. The fermentation medium (pH 6.5) was composed of 10 g of soymeal, 10 g of glucose, and 5 g of silkworm chrysalis meal per liter broth.

Cultivation and biotransformation

Flasks (250 ml) containing 40 ml of sterilized (20 min, at 121 °C) medium were inoculated with 3 % (v/v) of fungal spore suspensions from *M. anisopliae* 11490 and incubated at 28 °C on a rotary shaker at 220 rpm. After 24 h, 10 ml of the cultured mycelia was transferred to 50-ml shake flasks for biotransformation, and then the prepared GD-loaded nano-liposomes (GNLs, lyophilized and stored at −20 °C) were added into the matured mycelia broth to initiate biotransformation under the same conditions. The GD concentration (gram weight of GD per liter of the matured mycelia broth) used was in the range of 2–7 g/l. In a control experiment, solid substrate was dissolved in a suitable solvent prior to addition into the reaction medium (solvent dispersion). Particularly, equivalent amounts of GD dissolved in 200 μl of dimethylformamide (DMF) were added into 10 ml of matured mycelia broth in 50-ml shake flasks and the biotransformation conditions were the same as those described above. After biotransformation, 2 ml of ethyl acetate was added into 1 ml of broth to extract the substrate and product and the organic layer was applied directly to thin-layer chromatography (TLC). For high-performance liquid chromatography (HPLC) analysis, 200 μl

of supernatant was dried and dissolved in 1 ml methanol of chromatographic grade and analyzed. In the determination of the conversion curve, ten flasks were used simultaneously, i.e., parallels, where three samples at random were analyzed every 6 h and the average was adopted.

Preparation and characterization of GD-loaded nano-liposomes (GNLs)

Liposome preparation

Egg yolk lecithin or soybean lecithin (1 g) and GD (0.2 g) were dissolved in 60 ml of ethanol. The mixture was evaporated in a rotary evaporator under reduced pressure at 30 °C and a thin film was formed. The resulting dried lipidic film was then dispersed in 200 ml of PBS (pH 7.4). After being sonicated for 10 min, the liposomal suspension was treated by a high-pressure homogenizer (ATS Engineering Inc.) at 600 bar for five times to reduce the liposome size. The prepared GNL suspension added with 5 % (w/v) of sucrose as protective agent was freeze-dried with a lyophilizer and stored at −20 °C.

Morphological investigation

GNL morphology was investigated using a transmission electron microscope (TEM) (JEM-1230, JEOJ, Tokyo, Japan). A drop of the liposome suspension was placed onto a carbon-coated copper grid, forming a thin liquid film. The films were negatively stained with a 2 % phosphotungstic acid solution (w/w; pH 7.1) for 1 min. The excess of phosphotungstic solution was removed with a filter paper and stained samples were characterized by TEM.

Size and zeta-potential analysis

Mean diameters and polydispersity index (PI) of GNLs was determined by photon correlation spectroscopy (PCS) using Malvern Zetasizer Nanoseries (3000HS; Malvern Instruments, UK), after sample dilution in water. Zeta potential was measured on the principle of electrophoretic mobility of charged particles in an electric field [20] and was performed on the same instrument. All measurements were performed in triplicate at 25 °C.

Encapsulation efficiency

The liposome encapsulation efficiency was determined from the amount of entrapped substrates using the dialysis method. Briefly, 10 ml of the GNL sample (V) was placed in a dialysis bag. In order to separate the unloaded substrate, the dialysis bag was put into 100 ml of PBS (pH 7.4) and the dialysis was processed for 24 h with 40 rpm magnetic stirring. The dialysate in the bag was then dissolved in equal volume of ethyl acetate in order to release the encapsulated amount of substrate ($E_{\text{substrate}}$). An equal volume of the liposome suspension (V) was used in order to assess the total amount of the substrate ($T_{\text{substrate}}$) present in the suspension. $T_{\text{substrate}}$ was measured after having dissolved and disrupted the liposomes in ethyl acetate using an ultrasound bath for 5–10 min. The substrate-encapsulation efficiency (EE%) was calculated as the percentage of the encapsulated amount ($E_{\text{substrate}}$) to the total amount ($T_{\text{substrate}}$), as follows:

$$\text{EE}\,\% = \frac{E_{\text{substrate}}}{T_{\text{substrate}}} \times 100$$

Total and encapsulated substrate-amount determination was carried out by using HPLC.

Analytic methods

TLC was performed using a Silica Gel GF254 plate (Huiyou Silica Gel Development Co. Ltd., Yantai, China) and a solvent system of dichloromethane:methanol (20:1, v/v) as the lower phase. After TLC, the samples were detected by CAMAG TLC Scanner.

HPLC analysis was performed on an Agilent 1100 series HPLC system (Germany). A Plastisil C18 analytical column (4.6 × 250 mm, 5 μm, Dikma Technologies) was used at a column temperature of 25 °C. The flow rate was set at 1.2 ml/min, and the sample injection volume was 10 μl. The mobile phase consisted of water (A) and acetonitrile (B) using the following gradient program: 0–5 min, 70:30 (v/v) A/B; 5–15 min, liner decrease to 40:60; and 15–25 min, 40:60. The system was retuned to initial conditions after 6 min of re-equilibration time. The detection wavelength was 215/254 nm. HGD was eluted at 12.8 min and GD was eluted at 22.6 min.

Each sample was analyzed in triplicate. Bioconversion rate of substrate GD was calculated as follows:

$$\text{Bioconversion rate}\,\% = \frac{\text{Weight of HGD}/\text{MW of HGD}}{\text{Weight of GD}/\text{MW of GD}} \times 100$$

where MW is the molecular weight (i.e., the MW of GD is 286 and 302 for HGD). All of the experiments were conducted in triplicate and the results are expressed as mean values ± SD.

Results

Preparation and characterization of GNLs

Two kinds of phospholipid, egg yolk lecithin and soybean lecithin, were selected to prepare GNLs by thin-film

method connected with a high-pressure homogenizer. The properties such as zeta potential, mean diameters, polydispersity indexes, and its encapsulation efficiency of resulted GNLs made of these two kinds of phospholipid are listed in Table 1. It is obvious that there were no clear differences in these properties between these two kinds of phospholipids made GNLs. Moreover, the encapsulation efficiencies of both kinds of GNLs in liposomes were all above 90 %.

Negative-stain TEM images (Fig. 1) revealed that GNLs obtained using both kinds of phospholipid were elliptically shaped. According to TEM images, liposomes ranged in size from 200 to 300 nm. Indeed, microscopic size results correlated well with the size values obtained by PCS (Table 1). No notable differences were found in morphology between egg yolk lecithin- and soybean lecithin-made liposomes, and no substrate crystals were visible in TEM images regardless of the phospholipids used. These results indicated that both kinds of phospholipid successfully achieved stable, nanosized liposomes with suitable encapsulation efficiencies.

Effect of GNL addition method on biotransformation

To investigate the improvement of GNL addition method on biotransformation, the GD hydroxylation conducted by *M. anisopliae* was examined with the two kinds of phospholipids made GNLs and the comparison with solvent (DMF) dispersion method (control) was also made. The results are shown in Fig. 2.

Both egg yolk lecithin-made GNL and soybean lecithin-made GNL displayed very encouraging results in biotransformation of GD, where the HGD yield reached 14.44 and 14.08 mg (2 g/l of substrate feeding concentration in 10 ml of culture medium), respectively, at 66 h transformation time, while its value was only 5.21 mg in the control. To elucidate the effect of phospholipids on biotransformation, the GD hydroxylation was also conducted in the medium added with equivalent phospholipids (egg yolk lecithin or soybean lecithin). The result indicated that the HGD yield in phospholipids-added medium was almost the same as that in the control at 66-h transformation time. However, when the transformation time extended to 116 h, the HGD yield increased to 8.44 mg. Although phospholipids contained in medium showed somewhat improvement on steroid biotransformation in our study, the efficiency was lower than that of the GNL addition method, whether in transformation time or in HGD yield.

Furthermore, GNLs exhibited high biocompatibility to *M. anisopliae*. The mycelium dry weight was 80 mg/10 ml in the GNL addition method, while the value was only 15 mg/10 ml in the control. All of these results indicated that nano-liposomes may be an excellent substrate addition method in steroid biotransformation. Because of the similar characterizations and effects of two kinds of phospholipids made GNLs on biotransformation, soybean lecithin was selected to prepare GNLs in the following experiments, which is much cheaper than egg yolk lecithin.

Effect of substrate supply method on product composition

Our preliminary research showed that there were two byproducts (M and D) except for the main product HGD (Rf 0.41 in TLC) in the hydroxylation of GD by *M.*

Table 1 Mean size, polydispersity index (PI), zeta potential, and encapsulation efficiency (EE %) of prepared GNLs

Phospholipids	Mean diameters (nm)	PI	Zeta potential (mV)	EE (%)
Egg yolk lecithin	244.3	0.410	−31.1	93.6 ± 1.68
Soybean lecithin	266.4	0.372	−32.6	90.7 ± 1.04

Encapsulation efficiencies are expressed as mean values ± standard deviations ($n = 3$)

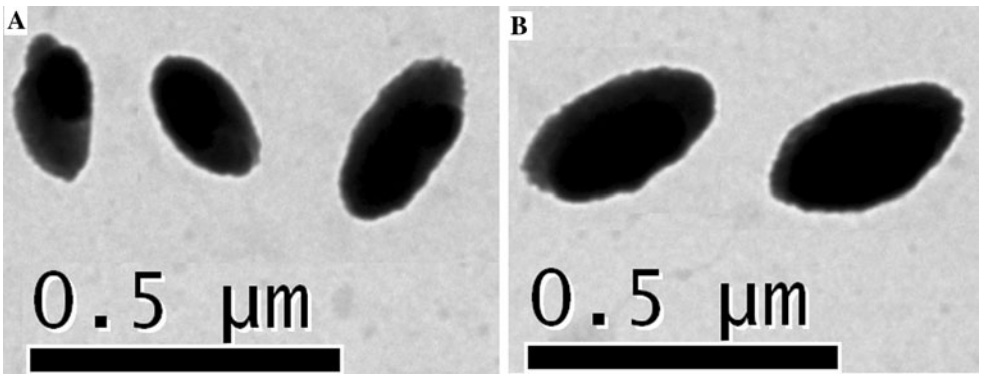

Fig. 1 TEM micrographs of GD-loaded nano-liposomes (GNLs) prepared with egg yolk lecithin (**a**) and soybean lecithin (**b**)

anisopliae (Fig. 3). Spectroscopic analysis proved that M (Rf 0.56 in TLC) and D (Rf 0.22 in TLC) were 6β-hydroxy derivative and 6β,11α-dihydroxy derivative of GD, respectively (data not shown). In the course of the purification of product HGD from fermentation broth by column chromatography on silica gel, we found that it was very difficult to isolate byproduct M from HGD because of their similar polarity.

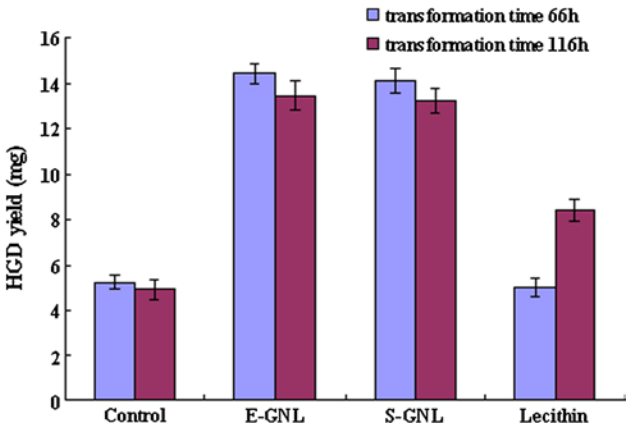

Fig. 2 Effect of GNL addition method on hydroxylation of 13-ethyl-gon-4-ene-3,17-dione (GD) by *Metarhizium anisopliae*. GD concentration was 2 g/l in 10 ml of culture medium. E-GNL, GNLs made of egg yolk lecithin; S-GNL, GNLs made of soybean lecithin; Lecithin, transformation in medium added with equivalent egg yolk lecithin. In the control, GD was dispersed in medium using DMF. Data were expressed as mean ± SD from three independent experiments

A very exciting result came up with the comparison of HPLC profiles of the transformation products under two kinds of substrate addition method, nano-liposomes and DMF dispersion (Fig. 4). Under the nano-liposome addition method, substrate was almost completely transformed to HGD and a small amount of byproduct D, and scarcely any M was found, which would benefit the HGD purification from fermentation broth. In the control, there were still many residual substrates, and the amount of byproduct M was three times that of byproduct D, which would cause many difficulties in the following purification.

Biotransformation curve of GD

The kinetic of biotransformation of GD under the GNL addition method was compared with that under control in Fig. 5. After approximately 12 h of hydroxylase induction, about half of GD was transformed rapidly to HGD and small amounts of byproducts M and D within the following 12 h transformation time (from 12 to 24 h) under

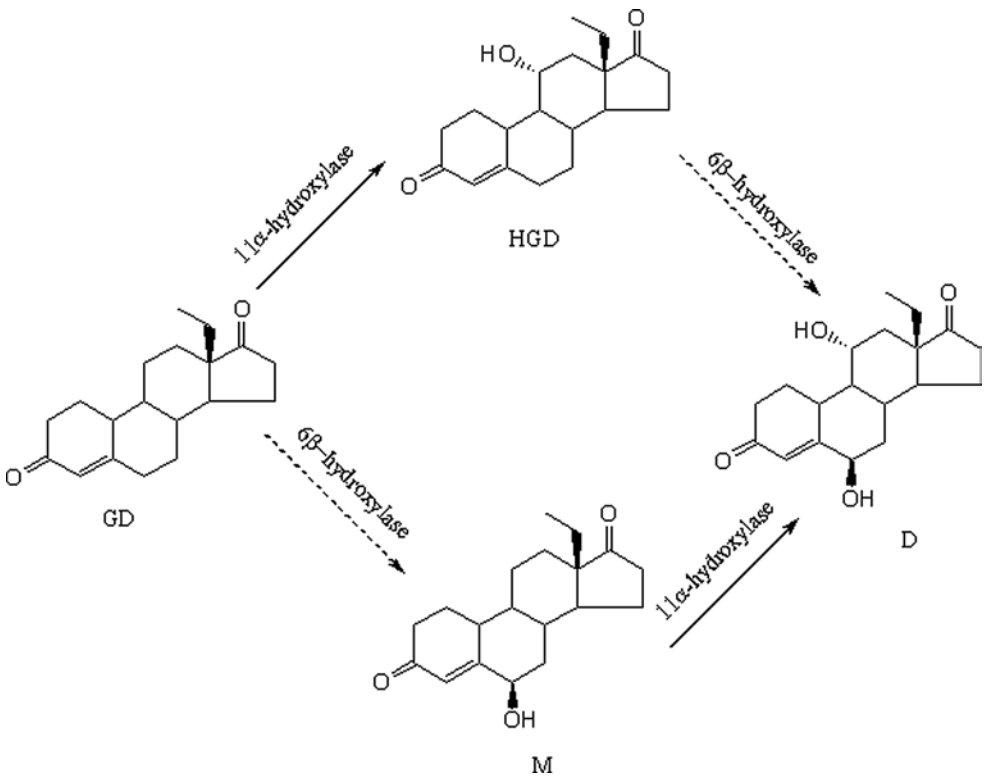

Fig. 3 Biotransformation pathway of 13-ethyl-gon-4-ene-3,17-dione (GD) by *Metarhizium anisopliae*. *Full lines* indicate main reactions whereas *dashed lines* indicate side reactions

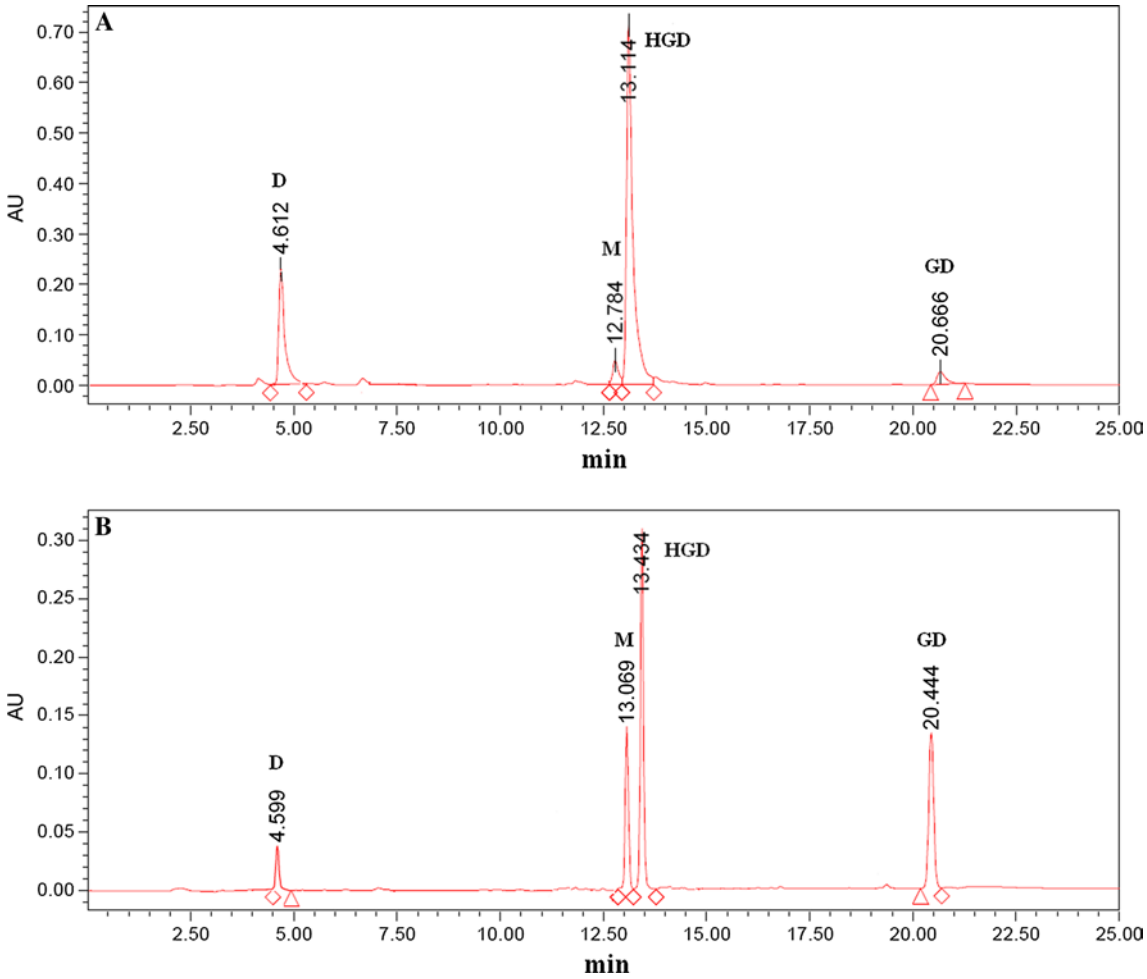

Fig. 4 HPLC profiles of the transformation products under two kinds of substrate addition method. **a** GNL addition method and **b** DMF dispersion method. *GD* 13-ethyl-gon-4-ene-3,17-dione, *HGD* 11α-hydroxy-13-ethyl-gon-4-ene-3,17-dione, *M* 6β-hydroxy-13-ethyl-gon-4-ene-3,17-dione, *D* 6β,11α-dihydroxy-13-ethyl-gon-4-ene-3,17-dione

GNL addition method. At this time, the main byproduct was M (1.50 mg) rather than D (0.68 mg). From 24 to 60 h, the remaining GD was continuously transformed and HGD yield increased steadily. At 60 h, substrate GD was almost transformed completely. However, the amount of M reached the highest level at 48 h and then began to be transformed to D by higher 11α-hydroxylase activity, which led to the rapid increase of the amount of D. Especially in the last 6 h transformation time (from 60 to 66 h), most of M was transformed to D, while the yield of main product HGD hardly increased. In control, substrate GD was transformed slowly and the amount of M was higher than that of D throughout the whole biotransformation process. At the end of transformation, the HGD yield was only one-third of that under the GNL addition method and the main byproduct was M. Furthermore, still about 30 % of GD was not transformed. These results provide further confirmation that nano-liposome can improve the solubility of steroid and accelerate its transport into cells, which facilitates the biotransformation process.

Effect of substrate concentration

In order to improve the biotransformation efficiency, the substrate feeding concentration should be adequately high. The effect of different substrate feeding concentrations on biotransformation was investigated in GD hydroxylation. Figure 6 shows that the increase of substrate in fermentation broth from 2 to 6 g/l had little adverse effect on hydroxylation of GD under the GNL addition method. Although the substrate feeding concentration was increased three times from 2 to 6 g/l, the conversion rate of 11α-hydroxylation did not decrease significantly (from 71.4 to 61.2 %, respectively). However, when the substrate concentration reached 7 g/l, the conversion rate decreased rapidly to 34.3 %. In the control, the substrate concentration had a great negative

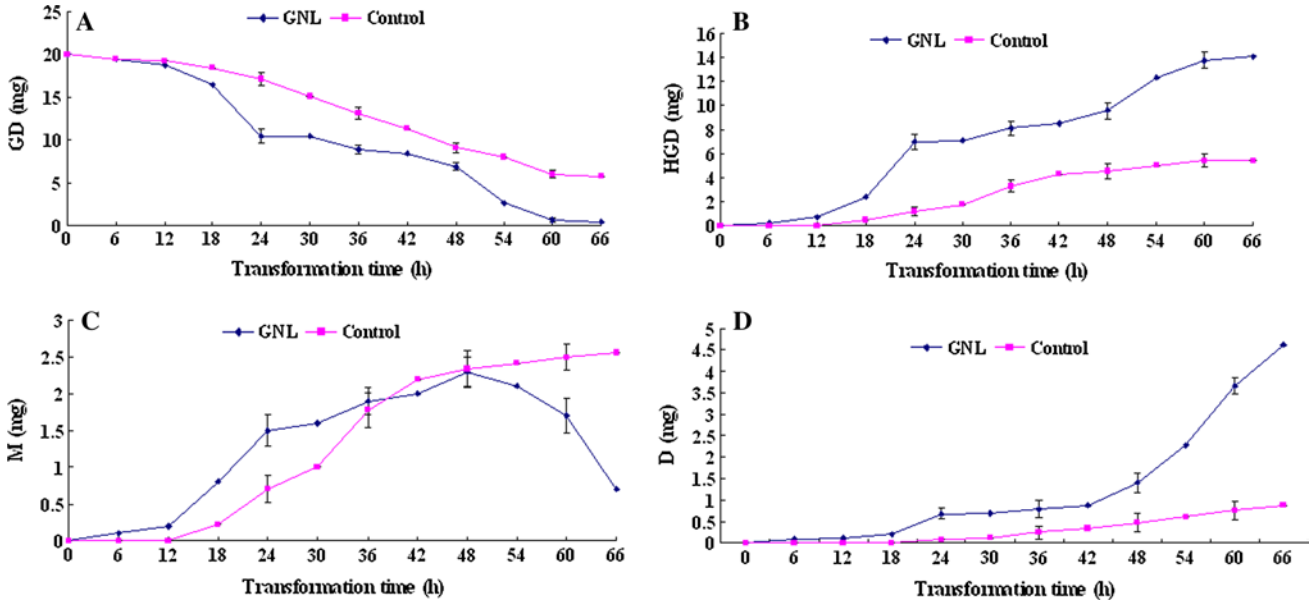

Fig. 5 Kinetics of 13-ethyl-gon-4-ene-3,17-dione (GD) hydroxylation by *Metarhizium anisopliae* under two kinds of substrate addition method. GNL, nano-liposome addition method; Control, DMF dispersion method. **a** Biotransformation curve of GD (13-ethyl-gon-4-ene-3,17-dione); **b** biotransformation curve of HGD (11α-hydroxy-13-ethyl-gon-4-ene-3,17-dione); **c** biotransformation curve of M (6β-hydroxy-13-ethyl-gon-4-ene-3,17-dione); **d** biotransformation curve of D (6β,11α-dihydroxy-13-ethyl-gon-4-ene-3,17-dione). Substrate feeding concentration was 2 g/l in 10 ml culture medium. Data were expressed as mean ± SD from three independent experiments

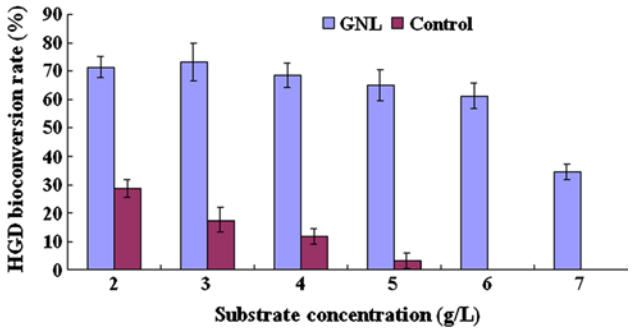

Fig. 6 Effect of substrate feeding concentration on 11α-hydroxylation of 13-ethyl-gon-4-ene-3,17-dione (GD) by *Metarhizium anisopliae*. GNL, nano-liposome addition method; Control, DMF dispersion method. Data are expressed as mean ± SD from three independent experiments

impact on the conversion rate. In 2 g/l of substrate concentration, the HGD conversion rate was 28.6 %. The substrate was hardly transformed when its concentration was above 5 g/l, indicating toxicity and inhibition of excess substrate on fungus and enzyme.

Discussion

Using biotransformation for some chemical synthesis steps has unique advantages, such as achieving higher product selectivity, the use of milder reaction conditions, as well as the potential for a reduction in the total number of synthetic steps. However, a common limitation with such systems is the inhibition or toxicity posed by the starting substrate as well as limited aqueous solubility in many cases. The supply and method of delivery of a given substrate are key determinants in the effectiveness of a given biotransformation [11]. In this article, the nano-liposome technique was adopted to supply substrate for GD hydroxylation by *M. anisopliae*.

As the basis of the following experiments, the substrate GD was successfully processed to be stable GNLs with about 250 nm particle size and suitable encapsulation efficiencies. Factors affecting encapsulation efficiency within liposomes are varied and mainly rely on liposome composition and encapsulated substrate properties. Concerning the encapsulated substrates, the encapsulation efficiency is commonly affected by the hydrophilic/lipophilic substrate character [2]. The water-insoluble substrate encapsulation efficiencies are usually high, which may indicate a high association of the substrate with phospholipid bilayers of liposomes. However, water-soluble substrate encapsulation efficiencies are limited by liposome aqueous core volume, since they are mostly entrapped in the aqueous cavities. The high GD encapsulation efficiencies (>90 %) were believed to be due to the high lipophilicity of GD.

Zeta potential is a key factor to evaluate the stability of colloidal dispersion. In general, particles could be dispersed

stably when the absolute value of zeta potential was above 30 mV due to the electric repulsion between particles [29]. As shown in Table 1, both of the absolute values of zeta potential for egg yolk lecithin-made GNL and soybean lecithin-made GNL were higher than 30 mV, and no obvious difference was found between these two kinds of liposome. This demonstrated that the GNLs obtained by the present method were physically stable.

Being a lipophilic substrate, GD can be embedded within the liposome bilayers in GNLs, which will improve its solubility in fermentation medium and decrease the inhibition or toxicity of free substrate on microorganism cells and enzymes [8, 9]. Lecithin was also biocompatible and showed no adverse effect on cell growth. Furthermore, because of their small size, GNLs can readily interact with cells and then facilitate GD transport into the cells [10]. However, in the solvent (DMF) dispersion method, the substrate in fermentation broth existed in solid form, which generated a heterogeneous system and exerted inhibition on microorganisms. Moreover, organic solvents have been shown to affect microorganism growth and catalytic activity [11]. Because of these advantages of GNLs, our experiments demonstrated the improvement of GNLs on microbial hydroxylation by *M. anisopliae*. The yield of the main product HGD tripled with a substrate feeding concentration of 2 g/l compared with the regular solvent (DMF) dispersion method. In addition, the substrate feeding concentration increased to 6 g/l with a satisfied HGD conversion rate, which greatly improved the biotransformation efficiency.

Our experiments also revealed that lecithin showed somewhat improvement on GD microbial hydroxylation by *M. anisopliae*. It has been reported that lecithin contained in a medium could increase the penetration of the sterol through the cell envelope barrier and then improve the biotransformation of β-sitosterol to androstenedione by *Mycobacterium vaccae* by changing the lipid composition of the cell envelope [18]. Used as a solubilizing agent, lecithin was much more efficient in dispersing cholesterol in water and then improved the biotransformation of cholesterol to androstenedione by *Mycobacterium* sp. when compared with Tween 80 [25]. Our results are in accord with these previously published data. Because of the similarity in structure and composition, the fusion between the liposomal membrane and the cell one is likely the other probable mechanism to explain the improvement of the nano-liposome addition method on biotransformation.

Recently, ionic liquids (ILs), as green solvents, present the potentials for improving the biotransformation process [16]. It was reported that in 11α-hydroxylation of 16α,17-epoxyprogesterone by *Rhizopus nigricans* in a biphasic IL aqueous system, the highest substrate concentration in IL phase was 18 g/l and then the feeding concentration in whole fermentation broth was 3 g/l (phase ratio, Aq/IL, 5:1) [26]. As far as substrate feeding concentration is concerned, the nano-liposome addition method has a great advantage because of the high biocompatibility and small particle size. However, we also found that when the substrate concentration reached 7 g/l, the conversion rate decreased rapidly. Steroid hydroxylases belong to cytochrome P450 monooxygenases (CYP, P450), which are heme-containing enzymes and oxygen dependent [30]. At 7 g/l of substrate concentration, the fermentation broth became thick and sticky because of the abundant addition of lecithin, which affected the oxygen transfer in fermentation system and then depressed the hydroxylase activity.

The product HPLC profiles and biotransformation curve suggested that the hydroxylases in *M. anisopliae* had two kinds of activity, 11α-hydroxylation activity and 6β-hydroxylation activity, and the 11α-hydroxylation activity was higher then 6β-hydroxylation activity. Substrate entrapped in GNLs had few negative impacts on enzyme activity and cell growth. GD was then transformed mainly to HGD and the small amount of M was transformed to D quickly by high 11α-hydroxylation enzyme activity, which would benefit the HGD purification from fermentation broth. On the contrary, in the control, GD could not be transformed completely and the 6β-hydroxy derivative M could not be transformed to 6β,11α-dihydroxy derivative D effectively because of the inhibition of both substrate and DMF on enzyme activity and cell growth.

In summary, the nano-liposome technique has been successfully applied in GD hydroxylation by *M. anisopliae*. The yield of the main product HGD and substrate feeding concentration were greatly increased because of the high biocompatibility and substrate solubility compared with the regular solvent (DMF) dispersion method. Furthermore, the change in byproduct composition benefits the following purification. These desirable properties indicate the potential of the nano-liposome technique in microbial steroid transformation.

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