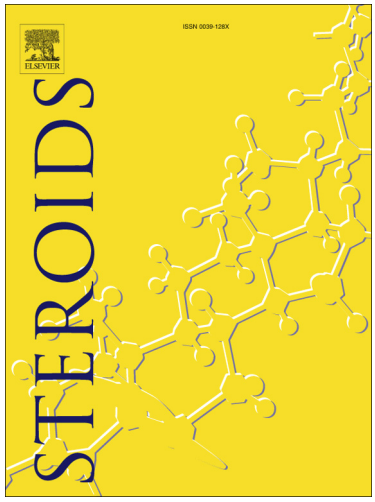

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Regio- and stereoselective reduction of 17-oxosteroids to 17β-hydroxysteroids by a yeast strain *Zygowilliopsis* sp. WY7905

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Abstract

The reduction of 17-oxosteroids to 17β-hydroxysteroids is one of the important transformations for the preparation of many steroidal drugs and intermediates. The strain *Zygowilliopsis* sp. WY7905 was found to catalyze the reduction of C-17 carbonyl group of androst-4-ene-3,17-dione (AD) to give testosterone (TS) as the sole product by the constitutive 17β-hydroxysteroid dehydrogenase (17β-HSD). The optimal conditions for the reduction were pH 8.0 and 30°C with supplementing 10 g/l glucose and 1% Tween 80 (w/v). Under the optimized transformation conditions, 0.75 g/l AD was reduced to a single product TS with > 90% yield and > 99% diastereomeric excess (de) within 24 h. This strain also reduced other 17-oxosteroids such as estrone, 3β-hydroxyandrost-5-en-17-one and norandrostenedione, to give the corresponding 17β-hydroxysteroids, while the C-3 and C-20 carbonyl groups were intact. The absence of by-products in this microbial 17β-reduction would facilitate the product purification. As such, the strain might serve as a useful biocatalyst for this important transformation.

Keywords: *Zygowilliopsis* sp; 17β-Reduction; Steroid biotransformation; Androst-4-ene-3,17-dione; Testosterone

1. Introduction

17β-Hydroxysteroids such as testosterone (TS), estradiol, 1,2-dehydrotestosterone are of pharmaceutical importance and one of the straightforward methods for their synthesis is the stereoselective reduction of 17-oxosteroids [1-3]. The ability of microorganisms to reduce 17-oxo- to 17β-hydroxysteroids was firstly reported nearly a century ago and the 17β-reduction reaction was widely observed and played important roles in microorganisms [3]. A variety of 17-oxosteroids were reported to be reduced to 17β-hydroxysteroids by microorganisms of different taxonomy: bacteria, fungi, yeasts, algae or protozoa [3-6]. As such, microbial 17β-reduction of 17-oxosteroids was envisaged as a promisingly convenient and effective method for the production of 17β-hydroxysteroids [3].

However, microbial 17β-reduction of 17-oxosteroids were usually companied with other reactions such as hydroxylation, reduction of double bond and dehydrogenation [3]. For example, 17-carbonyl reduction and hydroxylation at C-6β, C-14α, C-15α positions occurred in the biotransformation of androst-1,4-dien-3,17-dione (ADD) by filamentous fungus *Mucor racemosus*, resulting in two monohydroxylated and three dihydroxylated products as well as 17β-hydroxyandrost-1,4-dien-3-one [7]. A double reduction of both 17-oxo group and 1,2-C=C bond of ADD by *Mycobacterium* sp. NRRL B-3683 was observed [8]. Therefore, we initiated an effort to search for new microbial strains for effectively converting 17-oxosteroids to 17β-hydroxysteroids without concurrence of other reactions. In this study, a new *Zygowilliopsis* yeast in our laboratory was identified and found to effectively transform androst-4-ene-3,17-dione (AD) to TS. In addition, the microbial reduction

conditions were optimized and the ability of this strain to reduce other 17- oxosteroids was described.

2. Experimental

2.1. Materials and analytical methods

The 17-oxosteroids (≥97% of purity), standard samples of 17β-hydroxysteroids (≥98% of purity) and other chemical reagents and solvents were purchased from chemical companies. 17α-Hydroxy-androst-4-en-3-one was prepared by the reduction of AD with a carbonyl reductase available in our laboratory according to the precedure described in the supplementary materials (section 3). Silica gel (Qingdao Haiyang ChemicalCo., 200-300 mesh) was used for the column chromatography. Thin layer chromatography (TLC) was developed with ethyl acetate and petroleum ether (6:4, v/v) and visualized by UV light (254 nm) and then by phosphomolybdic acid colored. High performance liquid chromatography (HPLC) was performed with the mixture of water and methanol (35:65, v/v) as eluent at a flow rate of 0.5 ml/min and detection wavelength of 254 nm, 210 nm, 230 nm. The retention times for AD and TS were 12.3 and 14.4 min, respectively. HPLC analysis was performed with the column CHIRALPAK OD-H [Daicel Chemical Industries Ltd, 4.6 mm × 250 mm, *n*-hexane/isopropanol (7:3, v/v), flow rate: 0.5 ml/min, UV254 nm]. The retention times were 6.4 and 7.2 min for 17β-hydroxy-androst-4-en-3-one (TS) and 17α-hydroxy-androst-4-en-3-one.

2.2. Microorganisms, conditions of cultivation and preparation of resting cells

Strains were isolated from soil samples in our previous studies [9,10] and stored in our laboratory at -80°C with 20 % glycerol (v/v). The strains (5%, v/v) were inoculated into 20 ml of liquid growth culture medium (15 g/l glucose, 5 g/l yeast extract, 5 g/l peptone, 1 g/l K_2HPO_4, 1 g/l KH_2PO_4, 1 g/l NaCl, 0.5 g/l $MgSO_4 \cdot 7H_2O$, pH 7.0) and grown at 30°C on a rotatory shaker at 200 rpm. After 12 h growth, 1 ml of pre-culture liquid was transferred into 50 ml of fresh liquid growth medium with same composition as described above and continued to grow at 30°C on a rotatory shaker at 200 rpm for 24 h. The cells were harvested by centrifugation at 7875 $\times g$, for 10 min (4°C) and washed twice with phosphate buffer (100 mM, pH 7.0). The resulting resting cells were used for the steroid transformation.

2.3. Screening for TS-producing strains

The resting cells (100 g/l, 0.2 g wet weight) obtained as described above were resuspended in phosphate buffer (100 mM, pH 7.0) and added into 10 mL erlenmeyer flask, containing glucose (10 g/l, 30 mg) and AD (1.5 g/l, 3 mg) dissolved in N,N-dimethyl-formamide (DMF) (1%, v/v of DMF/buffer). The final reaction volume was 2 ml and the reactions were carried out at 30 °C, 200 rpm for 24 h under aerobic conditions in the shaker. The whole reaction mixtures were extracted with an equal volume of ethyl acetate, centrifuged, and the 1ml of supernatant was dried and subjected to HPLC analysis. The yield (%) of product, the conversion (%) of substrate and the de value were determined and calculated based on the areas of related peaks. For the yield (%) of product, the conversion (%) of substrate, the amounts of product and substrate in the reaction mixture were calibrated by the standard curve obtained with known concentration of standard samples of product and substrate.

2.4. Phenotypic, biochemical characterization and 26S rDNA sequence analysis

Morphocytology of strain WY7905 was observed by light microscope (Leica DM5000B, Germany). Morphological, biochemical, and physiological tests of strain WY7905 were carried out as described in the literature [11]. Assimilation and fermentation tests were conducted as described in the reference [12]. Total genomic DNA was extracted using TIANamp Yeast DNA kit (Tiangen Biotech, Beijing, China). The 26S rDNA was amplified by PCR (95°C for 3 min, one cycle; 95°C for 30 s/65°C for 30 s/72°C for 1 min 25 cycles; 72°C for 10 min ,one cycle) using dNTPs mix (0.2 $mmol \cdot l^{-1}$), universal primers NL1: 5'-GCATATCAATAAGCGGAGGAAAAG-3', NL4: 5'-GGTCCGTGTTTCAAGACGG-3'; 0.5 $\mu mol \cdot l^{-1}$ each), genomic DNA template (100 ng) and polymerase (2.5 U) in a final volume of 50 µl. PCR products were purified by using Cycle-pure kits (Tiangen Biotech, Beijing, China) and sequenced. Highly homologous strains were obtained by comparing this sequence with the GenBank database using nucleotide BLAST. A phylogentic tree was constructed by using ClustalX version 2.0, MEGA version 4.0 based on the homologous 26S rDNA sequences.

2.5. The inductive effect of steroidal compounds on the 17β-HSD activity

Strain WY7905 was inoculated in 20 ml of culture medium at 30°C on a rotatory shaker at 200 rpm. After growing for 12 h, 1 ml of the culture was transferred into 50 ml of growth medium containing 100 µg/ml of different steroidal compounds (estrone, hydrocortisone, cortodoxone, 3β-hydroxyandrost-5-en-17-one (DHEA), AD, TS, progesterone) and grown at 30°C for 24 h. The cells were harvested as described in section 2.2. The resting cells (0.2 g, wet weight) were resuspended in phosphate buffer (100 mM, pH 7.0) and added into 10 mL erlenmeyer flask, containing glucose

(15 g/l, 30 mg) and AD (1.5 g/l, 3 mg) dissolved in DMF (1%, v/v of DMF/buffer). The final reaction volume was 2 ml and the reactions were carried out at 30°C, 200 rpm for 24 h under aerobic conditions in the shaker. The whole reaction mixtures were extracted with an equal volume of ethyl acetate. Then the extract was dried and analyzed by HPLC to determine the conversion and yield.

2.6. Time course of 17β-HSD production of Zygowilliopsis sp. *WY7905*

Zygowilliopsis sp.WY7905 was cultivated as described in section 2.2. The OD_{600} of the cell culture was measured by ultraviolet spectrophotometer at various time intervals. The cells were collected at these time intervals as described in section 2.2 and used to determine the 17β-HSD activity by measuring the yield of TS as described in section 2.5.

2.7. Optimization of reaction conditions for the 17β-reduction of AD by the resting cells

The resting cells (0.2 g, wet weight) resuspended in different pH buffers (100 mM, sodium acetate buffer for pH 4.0-6.0, phosphate buffer for pH 6.0-8.0, Tris-HCl buffer for pH 8.0-9.0, Na_2CO_3/$NaHCO_3$ buffer for pH 9.0-10.0) were added into 10 ml erlenmeyer flask containing glucose (15 g/l, 30 mg) and AD (1.5 g/l, 3 mg) dissolved in DMF (1 %, v/v of DMF/buffer). The final reaction volume was 2 ml and the reactions were carried out at 30°C, 200 rpm for 24 h under aerobic conditions. The reaction samples were worked-up and analyzed to evaluate the pH effect on the transformation as described in section 2.5. To optimize the reaction temperature, the reaction was carried out in phosphate buffer (100 mM, pH 8.0) at 20, 25, 30, 35, 37 or 40°C for 24 h, respectively. The reaction mixture was worked-up and analyzed as described in section 2.5.

Similarly, the reaction was performed with different final concentrations of glucose (0, 5, 10, 15, 20 or 25 g/l) in phosphate buffer (100 mM, pH 8.0). The reaction mixture was shaken at 30°C for 24 h, worked-up and analyzed as described in section 2.5.

AD (1.5 g/l, 3 mg) dissolved in different kinds of co-solvents (1%, v/v) or Tween 80 (0.5%, w/v) was added into 10 ml erlenmeyer flask containing glucose (10 g/l, 20 mg) and resting cells (0.2 g, wet weight) resuspended in phosphate buffer (100 mM, pH 8.0). The reaction mixture (2 ml) was carried out at 30°C, 200 rpm for 24 h. Similarly, AD (1.5 g/l, 3 mg) dissolved in different concentrations of Tween 80 was added to the reaction mixture, and the reaction was carried out at 30°C for 24 h. The reaction mixture was worked-up and analyzed as described in section 2.5.

AD (0.25-1.5 g/l) dissolved in Tween 80 (1%, w/v) were added into 10 ml erlenmeyer flask containing glucose (10 g/l, 20 mg) and resting cells (0.2g, wet weight) resuspended in phosphate buffer (100 mM, pH 8.0), and the resulting mixtures (2 ml) were shaken at 30°C and 200 rpm for 24 h. Then the reaction mixtures were worked-up and analyzed as described in section 2.5. Under the optimized substrate concentration, the time course of bioreduction of AD by *Zygowilliopsis* sp. WY7905 was investigated. Samples were drawn at the different intervals, worked-up and analyzed as described in section 2.5.

2.8. Biotransformation of AD by growing cells of Zygowilliopsis sp. *WY7905*

Zygowilliopsis sp. WY7905 was inoculated into 20 ml growth medium and incubated at 30°C for 12 h. Then 1 ml of pre-culture liquid was added into 20 ml fermentation medium (pH 7.0) consisted of 30 g/l of glucose, 5 g/l of yeast extract, 5 g/l of peptone, 10.23 g/l of K_2HPO_4, 9.05 g/l of KH_2PO_4, 1 g/l of NaCl and 0.5 g/l of

$MgSO_4.7H_2O$. The resulting medium was cultivated at 30°C for 12 h. AD (0.5-2 g/l) dissolved in the Tween 80 (1%, w/v) was added into the culture. 5 g/l of glucose was added into the culture at intervals of 12 h within 72 h and the transformation was implemented at 30°C under aerobic conditions. Samples were drawn at the time intervals of 12 h, worked-up and analyzed as described in section 2.5.

2.9. Transformation of C-18, C-19 and C-21 steroids by the resting cells

C-18, C-19 and C-21 steroids (0.75 g/l, 1.5 mg), such as estrone (**3**), 9-dehydroandrostenedione (**7**), norandrostenedione (**9**), estra-4,9-diene-3,17-dione (**11**), 19-hydroxyandrost-4-ene-3,17-dione (**13**), etc separately dissolved in Tween 80 (1% w/v) were added into 2 ml of reaction mixtures containing glucose (10 g/l, 10 mg) and resting cells (0.2 g wet weight) resuspended in phosphate buffer (100 mM, pH 8.0).The mixture was shaken at 30°C for 24 h, then worked-up and analyzed as described in section 2.5.

For the preparative scale reactions, different substrates (0.75 g/l, 150 mg) in Tween 80 (1% w/v) were added into the reaction mixtures (200 ml) containing glucose (10 g/l, 2 g) and resting cells (20 g wet weight) resuspended in phosphate buffer (100 mM, pH 8.0). The reactions were carried out at 30°C for 24 h in shaker. The whole reaction mixtures were extracted three times with ethyl acetate (200 ml), and the organic extracts were dried over anhydrous sodium sulfate. The solvents were evaporated under vacuum to give crude products, which were purified by silica gel chromatography with ethyl acetate / petroleum ether as eluent to give pure products. In order to determine the configuration of 17-hydroxyl, NMR analyses of the products were performed (see below and supplementary materials section 4). ^{1}H NMR data of isolated products testosterone (**2**) (137.2 mg, 93% yield), 17β-estradiol (**4**) (15.2 mg,

11% yield) and 19-nortestosterone (**10**) (75.6mg, 53% yield) were compared with those of authentic standard samples, ^{1}H NMR data of androst-5-ene-3β,17β-diol (**6**) (64.2 mg, 47% yield) were compared to the literature data [13]. ^{1}H, ^{13}C and 2D NMR (^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY, ^{1}H-^{13}C HMBC, ^{1}H-^{13}C HMQC) of products Δ-9(11)-testosterone (**8**) (76.5 mg, 52% yield), 17β-hydroxy-estra-4,9-diene-3-one (**12**) (90.45 mg, 61% yield) and 19-hydroxytestosterone (**14**) (123.7 mg, 84% yield) were conducted, and the results were summarized in Table 1, which showed that 17β-hydroxyl products were obtained in all the cases.

*2.9.1 Testosterone (**2**)*

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.72 (s, 1H), 3.65 (t, 1H *J* = 4.0 Hz), 2.25-2.46 (m, 4H), 1.99-2.13 (m, 2H), 1.82-1.89 (m, 2H), 1.66-1.73 (m, 1H), 1.54-1.66 (m, 3H), 1.39-1.52 (m, 2H), 1.27-1.34 (m, 1H), 1.19 (s, 3H), 1.05-1.13 (m, 1H), 0.89-1.05 (m, 3H), 0.79 (s, 3H). The ^{1}H NMR spectrum was indentical to the standard sample.

*2.9.2 17β-Estradiol (**4**)*

^{1}H NMR (400 MHz, CD_3OD) δ 7.00 (d, *J* = 8.0 Hz, 1H), 6.46 (dd, *J* = 5.6, 8.0 Hz, 1H), 6.41 (d, *J* = 2.4 Hz, 1H), 3.58 (t, *J* = 8.8 Hz, 1H), 2.61-2.79 (m, 2H), 2.14-2.28 (m, 1H), 1.74-2.20 (m, 4H), 1.55-1.68 (m, 1H), 1.02-1.51 (m, 7H), 0.69 (s, 3H). The ^{1}H NMR spectrum was indentical to the standard sample.

*2.9.3 Androst-5-ene-3β,17β-diol (**6**)*

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.25 (d, *J* = 5.2 Hz, 1H), 3.42 (m, 1H), 3.24 (m, 1H), 2.01-2.20 (m, 2H), 1.62-1.97 (m, 5H), 1.28-1.58 (m, 7H), 1.01-1.06 (m, 1H), 0.95-1.05 (m, 2H), 0.94 (s, 3H), 0.80-0.90 (m, 2H), 0.64 (s, 3H). The ^{1}H NMR spectrum was indentical to that in the literature [13].

*2.9.4 19-Nortestosterone (**10**)*

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.82 (s, 1H), 3.66 (t, *J* = 8.0 Hz, 1H), 2.35-2.53 (m, 2H), 2.18-2.37 (m, 3H), 2.01-2.51 (m, 2H), 1.76-1.92 (m, 3H), 1.20-1.69 (m, 6H), 0.95-1.18 (m, 3H), 0.82-0.80 (m, 1H), 0.80 (s, 3H). The ^{1}H NMR spectrum was indentical to the standard sample.

3. **Results and discussion**

3.1 Identification of TS-producing strains

A total of eighty strains from our laboratory were tested for their ability to produce TS from AD. As shown in Table 2, eight strains were able to reduce AD to TS with > 99% de and one strain (WY97-3) reduced AD less stereoselectively. The strain WY7905 showed higher conversion for 17-ketone reduction. On the basis of these results, the strain WY7905 was selected for further studies. Colonies of the strain WY7905 on nutrient agar were milky, wet and smooth. Cells of the strain were oval, non-motile, non-spore. The physiological characteristic of WY7905 was investigated (supplementary materials Table S1 and Fig. S1). Comparative database analysis of 26S rDNA sequences suggested that strain WY7905 was very similar with strain in the yeast of *Zygowilliopsis* sp. The sequence similarity between WY7905 and some *Zygowilliopsis* strains was ≥98%, revealing that strain WY7905 belongs to the yeast *Zygowilliopsis*. Until now there was been no report about 17β-reduction of 17-oxosteroids by *Zygowilliopsis* sp. The strain WY7905 was deposited at CGMCC under accession number 2.5599.

3.2 The induction effect of steroidal compounds on the 17β-HSD activity

The inducible character of 17β-HSD from *Comamonas testosteroni* [14], *Streptomyces hydrogenans* [15] and other bacteria had been reported, and only small amounts of constitutive enzymes (1-5%) were found in cells grown in the absence of steroidal compounds [3]. The possible inducibility of 17β-HSD from *Zygowilliopsis* sp. WY7905 was examined by adding different steroidal compounds into the culture medium. The strain did not grow on androstenedione or testosterone as a sole source of carbon. As shown in Fig. 1, addition of steroidal compounds into the growth medium did not enhance the 17β-HSD activity, indicating the 17β-HSD of WY7905 might be constitutive enzyme. For the fungal, *Cochliobolus lunatus* [16], besides having inducible 11β-HSD and 20β-HSD activities, this strain was also found to have a constitutive 17β-HSD activity by the reduction reaction with intact cells. A 17β-HSD from *Schizosaccharomyces pombe* was also reported as a constitutive enzyme [17].

3.3 Time course of 17β-HSD production of Zygowilliopsis sp.*WY7905*

The cell growth and 17β-HSD production of *Zygowilliopsis sp*. WY7905 were monitored by measuring the OD_{600} and yield of TS at different cultivation times. As shown in Fig. 2, the 17β-HSD activity increased in parallel with cell growth during the period of 24 h. The 17β-HSD activity of *Zygowilliopsis* sp. WY7905 reached the highest at 24 h, and then decreased slightly. This is different from *Williopsis saturnus var. mrakii* AJ-5620 [18], in which reductase activity decreased obviously after about 20 h. At all stages of WY7905 cell growth, the stereoselectivity of product was unchanged (de >99%).

3.4 Reaction condition optimization of the 17β-reduction of AD by resting cells

The reaction conditions were optimized by using resting cells of *Zygowilliopsis* sp. WY7905 grown until optimal culture stage. The effects of buffer pH and temperature on the reduction of AD were studied, and the results are presented in Figs 3 and 4. The yield was < 10% when pH was below 5.0. The conversion of AD to TS improved with the increase of buffer pH from 5.0 to 8.0. The strain showed high 17β-reduction activity at a broad pH range with the optimal pH being 8.0. The 17β-reduction of AD by *Zygowilliopsis* sp. WY7905 reached highest conversion when the biotransformation was carried out at 30°C.

Cofactor recycling is one of the most crucial issues encountered in bioreductions and the cofactor regeneration with glucose as a co-substrate is preferred because of its availability and low cost [19]. In the reduction reaction of AD by *Zygowilliopsis* sp. WY7905, as shown in Fig. 5, the addition of glucose was able to accelerate the 17β-reduction of AD and the substrate conversion reached maximum with 10 g/l glucose in the reaction system. Higher glucose concentration had no effect on reduction, suggesting that 10 g/l glucose was enough for cofactor regeneration.

Steroids have very low aqueous solubility which is in the range of 1–100 μM [20]. The low steroid aqueous solubility results in poor availability of the substrate to whole-cell biocatalysts. In order to improve the solubility of steroids and further increase the biocatalytic efficiency, the effects of various co-solvents besides Tween 80 on the 17β-reduction of AD were evaluated. As shown in Fig. 6, compared to the organic co-solvents, higher yield of TS was obtained when Tween 80 was used to improve the availability of the substrate. The effect of different concentrations of Tween 80 on the bioconversion of AD by *Zygowilliopsis* sp. WY7905 was studied

(Fig. 7). The yield of TS reached the highest when substrate was dissolved in 1% (w/v) Tween 80.

The 17β-reduction of AD by *Zygowilliopsis* sp. WY7905 at different substrate concentration was studied, and the results are presented in Fig. 8. When the concentration of AD was 0.5 g/l and 0.75 g/l, the yields of TS achieved 96% and 93% within 24 h, respectively. As the substrate concentration was increased up to 1.0 g/l, the yield of TS decreased to below 80% at 24 h. The time course of 17β-reduction of AD (0.75 g/l) catalyzed by *Zygowilliopsis* sp. WY7905 under the optimized reaction conditions is presented in Fig. 9. Within 24 h, the bioreduction of AD was almost completed and only TS was detected. With longer reaction time, the yield and configuration of the product were unchanged (de >99%) .

3.5. 17β-reduction of AD by growing cells of Zygowilliopsis sp.*WY7905*

17β-reduction of AD with growing cells of *Zygowilliopsis* sp. WY7905 was also investigated. As shown in Fig. 10, at 0.5 g/l, 1 g/l and 2 g/l substrate concentration, the yield of TS achieved above 90% within 120 h and TS was the only product. At the substrate concentration of 2.5 g/l, the yield of TS was above 80% within 120 h.

Currently, some other microbial strains have been reported to reduce 17-oxosteroids to 17β-hydroxysteroids by growing cells. For example, *Aspergillus brasiliensis* converted ADD to 17β-hydroxyandrost-1,4-dien-3-one at the final substrate concentration of 1 g/l in 5 days. During this conversion, two hydroxylated metabolites were detected [21]. *Mucor piriformis* could transform DHEA to androst-5-ene-3β,17β-diol with other by-products [22]. Similarly, *Mucor racemocus* transformed AD to TS and produced other metabolite including C-6β, C-14α, C-15α hydroxylated products [7]. *Nostoc ellipsosporum* and *Nostoc muscorum* transformed

AD and ADD to the corresponding 17-hydroxysteroids at substrate concentration of 0.5 g/l in 7 days [23, 24]. Some yeasts could also produce TS from AD. For instance, *Saccharomyces cerevisiae* could reduce AD to TS in the absence and presence of cyclodextrin at substrate concentration of 2 g/l in 5 days with 27% and 90% yield of product [25]. For *Zygowilliopsis* sp. WY7905, the growing cells of strain could transform AD to TS in the absence and presence of Tween 80 at subtrate concentration of 2 g/l in 5 days with 35% and 90% yield of product. *Candida albicans* and *Schizosaccharomyces pombe* could reduce 17-ketosteroids to 17β-hydroxysteroids, but the conversion efficiency was at low level [26, 27]. The generation of by-products commonly observed in the microbial reduction of 17-oxosteroids to 17β-hydroxysteroids resulted in the difficulty in product purification, thus limiting their synthetic applications. In this study, no by-product was detected in the 17β-reduction of AD by the resting cells and growing cells of *Zygowilliopsis* sp. WY7905, resulting in TS as the sole product in greater than 90% yields at the substrate concentration of 0.75 g/l and 2 g/l within 24 h and 120 h, respectively.

3.6. Transformation of other oxo-steroids by the resting cells

Biotransformation of other C18-, C19- and C21-steroids were also studied under the optimized reaction conditions by using the resting cells of *Zygowilliopsis* sp. WY7905. The results are summarized in Table 3. It can be seen that steroids containing C-17 carbonyl groups were reduced to the corresponding 17β-hydroxysteroids. 17β-reduction reactions of some steroids by microorganism were reported for the first time, such as 9-dehydroandrostenedione, 19-hydroxytestosterone, estra-4,9-diene-3,17-dione, the products of which are significant drug intermediates.

As shown in Table 3, TS and ethisterone, only containing C-3 carbonyl group, were not reduced by *Zygowilliopsis* sp. WY7905, indicating that the strain did not exhibit 3α/3β-HSD activity. In addition, the TS was not oxidized to AD in the presence of glucose, indicating that the oxidative activity was much weaker than reductive activity. C-20 carbonyl groups of C21-steroids were also barely reduced by this strain. These results indicated that the strain WY7905 had highly selective 17β-HSD activity under the conditions studied.

4. Conclusion

In this study, a 17β-HSD producing strain was identified as *Zygowilliopsis* sp. WY7905, based on physiological biochemical characteristics, and 26S rDNA gene sequence. *Zygowilliopsis* sp. WY7905 reduced AD to TS without the formation of by-products. The reaction conditions (pH, temperature, amount of glucose, co-solvent) were optimized for the bio-reduction with the resting cells of *Zygowilliopsis* sp. WY7905. Under the optimized conditions, 0.75 g/l AD was transformed to TS with 93% yield within 24 h. The strain also reduced other 17-oxosteroids giving the corresponding 17β-hydroxysteroids with no by-product being detected, but showed highest activity toward AD. Almost no reduction activity was observed for steroids with only C-3 or C-20 carbonyl group. The 17β-HSDs in *Zygowilliopsis* sp. WY7905 existed as constitutive enzymes. Cloning of the new 17β-HSD genes from the strain, and characterization and synthetic applications of the recombinant enzymes are in progress in our laboratory. These results will be reported in due course.

Abbreviations

TS Testosterone

AD	4-Androstene-3,17-dione
HSD	Hydroxysteroid dehydrogenase
ADD	Androst-1,4-dien-3,17-dione
DHEA	3β-Hydroxyandrost-5-en-17-one
DMF	N,N-Dimethylformamide
DMSO	Dimethyl sulfoxide
EA	Ethyl acetate
MTBE	Methyl tertiary butyl ether

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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/…

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Table 1. ^{1}H-NMR and ^{13}C-NMR data of compounds (δ in ppm).

Carbon	8		12		14	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	31.7	2.01, 1.06	37.2	2.49	34.6	2.41, 1.77
2	32.8	2.55, 2.34	25.9	2.90, 2.50	36.0	2.77, 2.29
3	199.4		199.9		203.4	
4	123.9	5.74	122.2	5.69	126.8	5.87
5	169.9		157.4		172.0	
6	34.2	2.47, 2.48	30.9	2.42, 2.35	34.7	2.52, 2.38
7	33.8	2.10,	27.2	1.90, 1.27	33.3	1.93, 1.05
8	37.5	2.21	39.5	2.25	37.6	1.73
9	144.7		146.5		52.3	0.95
10	41.0		125.5		45.5	
11	118.6	5.53	25.7	2.80, 2.14	24.3	1.63, 1.32
12	38.4	2.12, 1.94	36.6	1.95, 1.25	38.2	1.86, 1.03
13	41.3		43.0		44.1	
14	47.6	1.25	51.2	1.15	55.8	1.06
15	24.1	1.78, 1.38	23.5	1.68, 1.39	22.3	1.72, 1.54
16	30.6	2.11, 1.53	30.7	2.12, 1.52	30.7	1.98, 1.48
17	81.6	3.75	81.4	3.70	82.3	3.58
18	10.5	0.76	10.6	0.94	11.8	0.83
19	26.1	1.35			66.1	4.04, 3.83

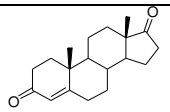
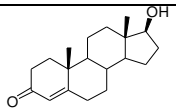
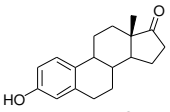
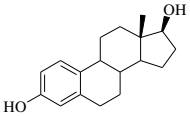
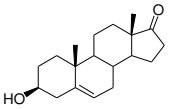
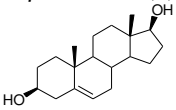
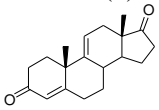
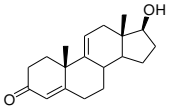
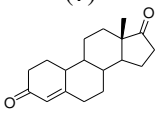
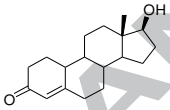
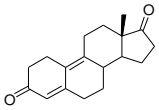
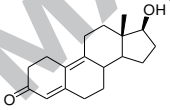
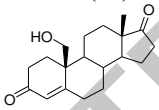
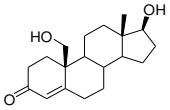
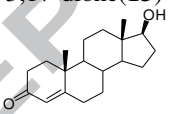
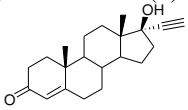
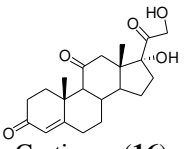
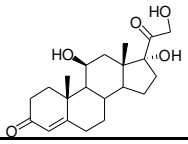
Table 2. Transformation of AD to TS by resting cells of different strains.

Strain number	8101	97-3	8002	0902	0802	0801	2401	7905	93-3
Yield (%)	16.68	52.2	17.77	18.01	11.82	18.23	17.04	30.59	10.21
de (%)[a]	99	97	99	99	99	99	99	99	99

[a] The diastereomeric excess values were measured by HPLC analysis.

Table 3. Transformation of oxo-steroids by the resting cells of *Zygowilliopsis* sp. WY7905.

Entry	**Substrate**	**Product**	**Conversion (%)[a]**	**De (%)[b]**

1	 Androst-4-ene -3,17-dione (**1**)	 Testosterone (**2**)	96	99
2	 Estrone (**3**)	 17β-estradiol (**4**)	11.8	99
3	 3β-hydroxyandrost-5-en-17-one (**5**)	 androst-5-ene-3β,17β-diol (**6**)	48	99
4	 9-Dehydroandrostenedione (**7**)	 Δ-9(11)-testosterone(**8**)	52.5	99
5	 Norandrostenedione(**9**)	 19-nortestosterone (**10**)	53	99
6	 Estra-4,9-diene-3,17-dione(**11**)	 17β-hydroxy-estra-4,9-diene-3 one(**12**)	61.5	99
7	 19-hydroxyandrost-4-ene-3,17-dione(**13**)	 19-hydroxytestosterone (**14**)	85.3	99
8	 Testosterone (**2**)	-	-[c]	
9	 Ethisterone (**15**)	-	-[c]	
10	 Cortisone (**16**)	-	Trace	
11		-	Trace	

Hydrocortisone (**17**)	

[a] The conversions were determined by HPLC analysis.

[b] The diastereomeric excess values were measured by NMR.

[c] No conversion was observed.

Fig. 1. The effect of different steroids on the 17β-reduction activity of *Zygowilliopsis* sp. WY7905 (□) Conversion of AD; (■) yield of TS.

Fig. 2. Time-course of cell growth and enzyme production by *Zygowilliopsis* sp. WY7905(●) cell growth;(□) yield of TS.

Fig. 3. Effect of pH on the 17β-reduction of AD by *Zygowilliopsis* sp. WY7905 (□) Conversion of AD; (●) yield of TS.

Fig. 4. Effect of reaction temperature on the 17β-reduction of AD by *Zygowivlliopsis* sp. WY7905 (□) Conversion of AD; (●) yield of TS.

Fig. 5. Effect of glucose addition on the 17β-reduction of AD by *Zygowivlliopsis* sp. WY7905 (□) Conversion of AD; (■) yield of TS.

Fig. 6. Effect of different co-solvents and on Tween 80 the 17β-reduction of AD by *Zygowivlliopsis* sp. WY7905 (□) Conversion of AD; (■) yield of TS.

Fig. 7. Effect of various Tween 80 concentrations on the 17β-reduction of AD by *Zygowivlliopsis* sp. WY7905 (□) Conversion of AD; (■) yield of TS.

Fig. 8. Effect of substrate concentration on the 17β-reduction of AD by *Zygowivlliopsis* sp. WY7905 (□) Conversion of AD; (●) yield of TS.

Fig. 9. Time course of the 17β-reduction of AD by *Zygowivlliopsis* sp. WY7905 (□) Conversion of AD; (●) yield of TS.

Fig. 10. Biotransformation of AD by the growth cells of *Zygowivlliopsis* sp. WY7905

Fig. 1

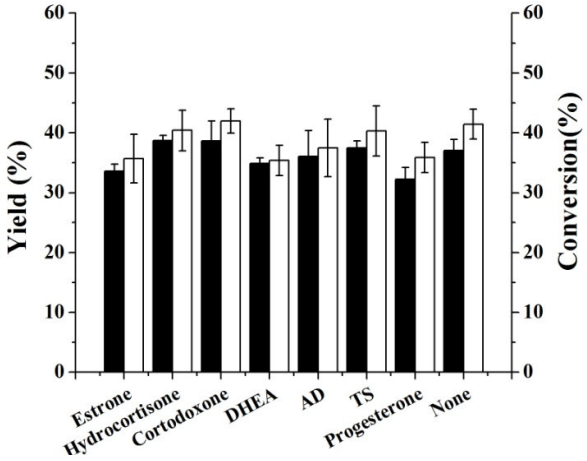


Fig. 2

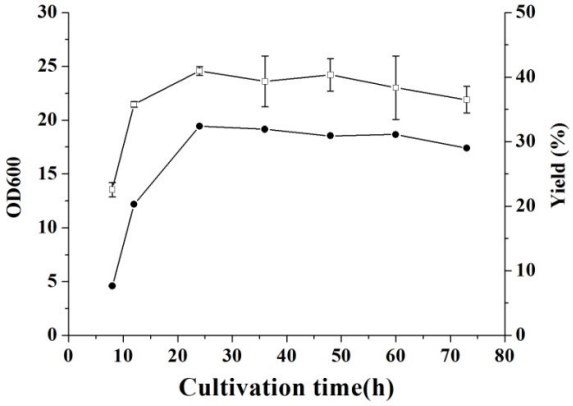


Fig. 3

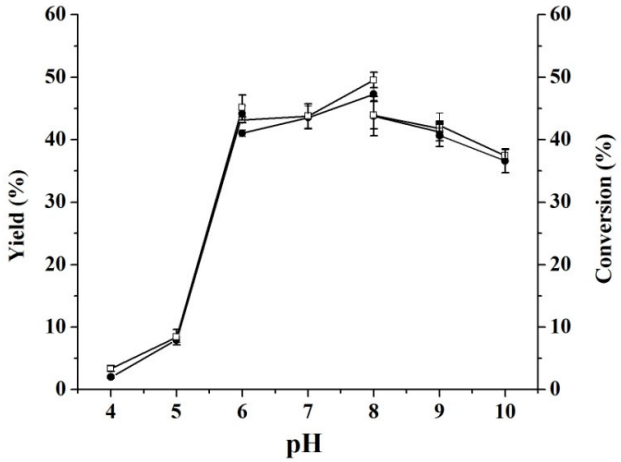


Fig. 4.

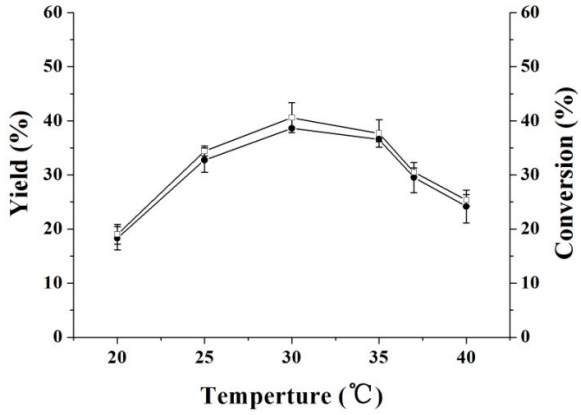


Fig. 5.

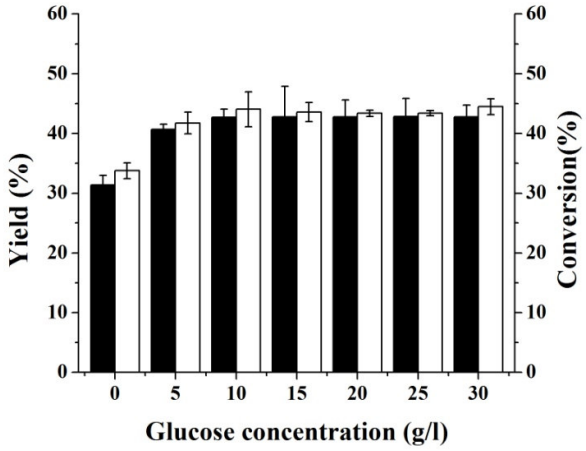


Fig. 6

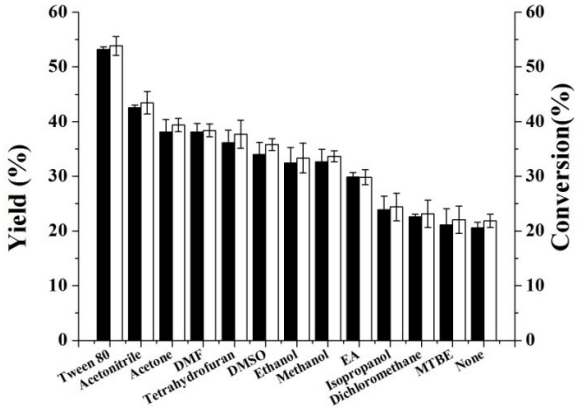


Fig. 7.

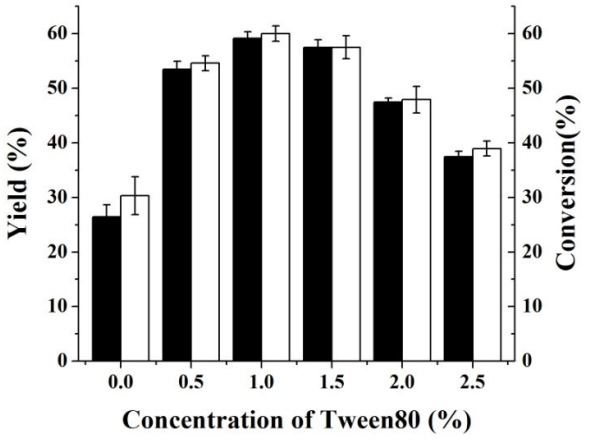


Fig. 8.

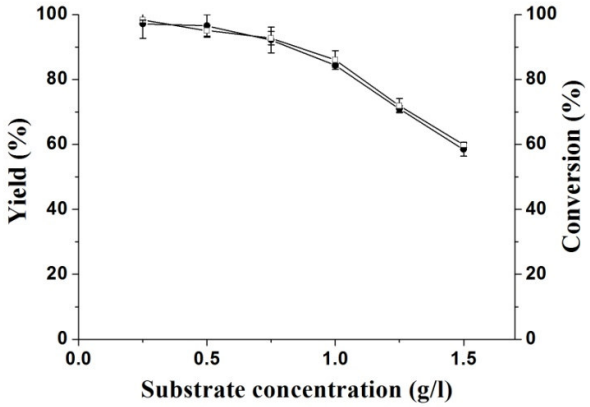


Fig. 9

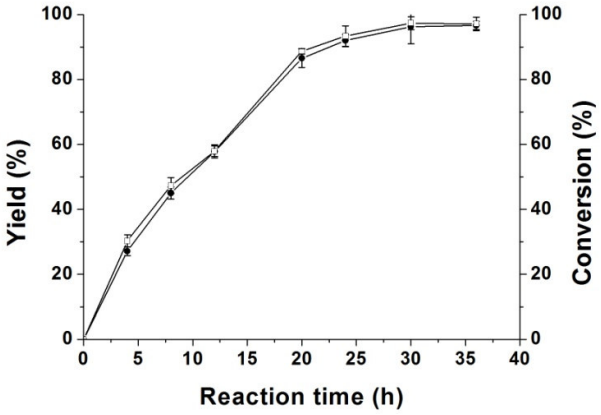
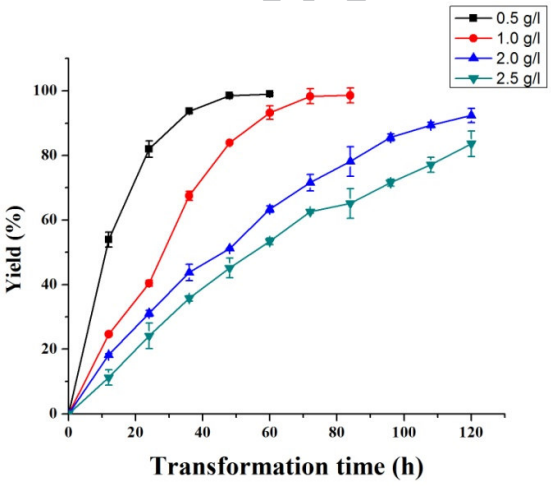
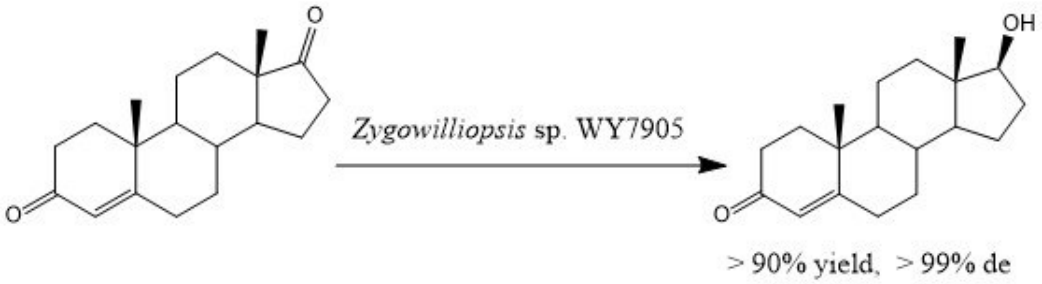


Fig. 10





> 90% yield, > 99% de

Highlights

- *Zygowilliopsis* sp. WY7905 contained constitutive 17β-hydroxysteroid dehydrogenases.
- It effectively catalyzed the reduction of androst-4-ene-3,17-dione to testosterone.
-
- It also reduced other 17-oxosteroids to the corresponding 17β-hydroxysteroids.
- It was not active toward steroids with only C-3 or C-20 carbonyl groups.