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Development of a high-yielding bioprocess for 11-α hydroxylation of canrenone under conditions of oxygen-enriched air supply

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ABSTRACT

A high yielding bioprocess for 11-α hydroxylation of canrenone (**1a**) using *Aspergillus ochraceus* ATCC 18500 was developed. The optimization of the biotransformation involved both fermentation (for achieving highly active mycelium of *A. ochraceus*) and biotransformation with the aim to obtain 11-α hydroxylation with high selectivity and yield. A medium based on sucrose as C-source resulted particularly suitable for conversion of canrenone into the corresponding 11-hydroxy derivative, whereas the use of O_2-enriched air and dimethyl sulfoxide (DMSO) as a co-solvent for increasing substrate solubility played a crucial role for obtaining high yields (>95%) of the desired product in high chemical purity starting from 30 mM (10.2 g/L) of substrate. The structure of the hydroxylated product was confirmed by a combination of two-dimensional NMR proton-proton correlation techniques.

1. Introduction

The regio- and stereoselective hydroxylation of steroids by fungal strains is a well-known method for the preparation of corticosteroids and progestogens having an oxygen substituent at C-11 position [1,2]. To overcome synthetic laborious steps, different species of eumycetes (i.e., *Rhizopus* and *Aspergillus*) are employed to perform the 11-hydroxylation of steroids because of the high regio- and stereoselective specificity of their oxygenating enzymes [3]. Although microbial oxidation is a good method for the simplification of the steroid drugs production, large-scale biotransformations are sometimes limited by low yields because of the poor solubility of substrates in the reaction medium and formation of different oxygenated by-products, which sometimes are very-difficult to separate [4,5]. Another problem is that many fungal species are very specific in their nutritional needs, so the use of cultural growing media is drastically limited. Microbial 11-hydroxylation of steroids using *Aspergillus ochraceus* is a well-known process and applicable on different substrates; the stereoselectivity of the hydroxylation mostly depends on the substrate employed. α-Hydroxylation is preferred with progesterone [6–10], 2-oxatestosterone [11], 13β-ethyl-gona-4-en-3,17-dione [12], canrenone [13], estr-4-en-3,17-dione [13], 16α, 17-epoxyprogesterone [14], 5α-androstan-3-one [15], whereas β-hydroxylation was found with cortexolone [16]. Generally, the bioconversion yield and selectivity is affected by different parameters, such as substrate concentration, aeration, and agitation; the chosen strain can be also important for regio- and enantioselectivity [2], as observed for other steroid biotransformation as well [17]. High substrate concentrations are needed to achieve industrially relevant biotransformations, but often dramatically limit conversion and selectivity. In the case of 11α-hydroxylation of canrenone, good yields and selectivity were obtained only when pure substrate concentration was kept below 5–6 g/L (14.7–17.6 mM) [13,18]; higher substrate concentrations not only limited overall conversions, but also yielded undesired by-products due to hydroxylation of different positions [19].

In this study we have investigated an efficient, economical and industrially scalable method for the conversion of canrenone into the corresponding 11α-canrenone using *Aspergillus ochraceus* ATCC 18500 as the biocatalyst. This microorganism is able to grow on

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different inexpensive nutrient media and to be stored, after lyophilisation, without losing its oxygenating skills [6,7]. *Aspergillus ochraceus* ATCC 18500 was employed for the preparation of 11α-hydroxy-canrenone, a key intermediate for the preparation of eplerenone (an important potassium sparing diuretic useful in the heart failure treatment) [20].

2. Materials and methods

2.1. Materials

All the solvents and reagents were purchased from Sigma-Aldrich, whereas canrenone and reference products were kindly furnished by Industriale Chimica (Saronno, Varese, Italy).

2.2. Microorganism and cultivation

Aspergillus ochraceus ATCC 18500 was routinely maintained on M5YE agar (malt broth, yeast extract 0.5%, agar 1.5% pH 5.6).

Growth in shake flasks: Erlenmeyer flasks (1 L) containing 100 mL of liquid medium (malt extract 3%, sucrose 2%, yeast extract 0.3%, pH 5.6) were inoculated from slants prepared with M5YE-agar medium. Each flask was inoculated with one slant, suspending the culture with 5 mL of sterile water. The flasks were incubated on a rotatory shaker at 28 °C, 150 rpm, for 48 h. Culture media were prepared using different combinations of the following products:

C-sources: glucose (1–5%), sucrose (1–5%), lactose (1–5%), glycerol (1–5%), maltose (1–5%), soluble starch (1–5%).

C- and *N*-sources: malt extract (0–1%), yeast extract (0–1%), corn steep liquor (0–1%).

Growth in stirred tank reactor: inoculum was prepared using two 1 L baffled Erlenmeyer flasks containing 100 mL of liquid medium (malt extract 3%, sucrose 2%, yeast extract 0.3%, pH 5.6) pre-inoculated from slants prepared with M5YE-agar medium. Each flask was inoculated with one slant, suspending the culture with 5 mL of sterile water and incubated on a rotatory shaker at 28 °C, 150 rpm, for 48 h. A 20 L stirred-tank bioreactor Applikon Biobench 20 L (Applikon Biotechnology B.V.) equipped with an on-line data acquisition, control system, gas mixer, and pH and O_2 electrodes was employed as stirred tank reactor (STR). The STR was filled with 5 L of liquid medium (malt extract 3%, sucrose 2%, yeast extract 0.3%, pH 5.6). The bioreactor was inoculated with 200 mL of inoculum and incubated at 28 °C for 48 h with air inlet 300 vvm, stirring 300 rpm, pH maintained at 5.6. Dry weight was determined after filtration of the mycelium with a Buchner funnel and dried for 24 h at 110 °C. Results were an average of five replicates.

2.3. Biotransformations

After 48 h of growth, the substrate (neat or solubilized in solvents) was added to the whole culture to start the biotransformation. Pure air and different air/O_2 ratios (5/1, 3/1, and 1/1) were used during the biotransformation. The reaction was followed by HPLC. When the bioconversion was over, mycelium was filtered and washed with dichloromethane; the supernatant was extracted 3 times with dichloromethane and the organic extracts collected, dried over Na_2SO_4 and the solvent was removed; the crude product was purified by flash chromatography (*n*-hexane/EtOAc 1:1).

2.4. Analyticals

Molar conversions were evaluated by HPLC (Purospher Star RP18e 250 * 4.6 mm, 5 μm column (Merck, Darmstadt, Germany), mobile phase: CH_3CN/water 60:40, flow rate 0.5 mL/min, detection at 299 nm for **1a,** and at 240 nm for **2a**. The mobility of substrate and product was: **1a** = 16.3 min, **2a** = 7.9 min.

NMR spectroscopy. ^1H NMR and ^{13}C NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer using the residual signal of the deuterated solvent as internal standard. ^1H and ^{13}C chemical shifts (δ) are expressed in ppm, and coupling constants (J) in hertz (Hz).

Two-dimensional proton-proton NOESY experiments were performed using standard pulse sequences, present in the spectrometer library.

2a ^1H NMR (300 MHz,$CDCl_3$): δ = 1.05 (s, 3H), 1.25 (s, 3H), 1.29–1.59 (m, 4H), 1.62 (s, br, OH), 1.86–2.06 (m, 5H), 2.28–2.46 (m, 4H), 2.5–2.65 (m, 4H), 4.12 (sest, J = 4.8 Hz, 1H), 5.7 (s, 1H), 6.03 (dd, J = 9.7, 1.8 Hz, 1H), 6.03 (dd, J = 9.7, 2.2 Hz, 1H) ppm; ^{13}C NMR (300 MHz, $CDCl_3$): δ = 15.6, 17.2, 22.5, 29.1, 31.1, 34.2, 35.5, 35.5, 35.8, 36.3, 37.7, 43.8, 46.6, 55.7, 67.9, 94.8, 124.7, 128.8, 138.0, 162.9, 176.4, 199.9 ppm.

3. Results

3.1. Optimization of the growth conditions in shake flasks

Firstly, an optimization of the growth conditions of *A. ochraceus* in shake flasks was performed for obtaining high and selective activity towards canrenone **1a.** The substrate (15 mM, 5.1 g/L) was added after 48 h of growth. Yields of 11α-canrenone (**2a**) (expressed as g/L $g_{mycelium}$ of **2a** produced after 24 h) and chemical purity of **2a** produced (evaluated by HPLC) were used as response parameters. It is known that hydroxylation of different position of **1a** may decrease the selectivity of 11-α hydroxylation with *A. ochraceus* [19]. Simultaneous evaluation of different combinations and concentrations of C-sources and *N*-sources (glucose, sucrose, lactose, glycerol, maltose, hydrolysed starch, malt extract, yeast extract, corn steep) were evaluated using the Multisimplex experimental design, already used for optimizing fermentations and biotransformations [21,22]. The best hydroxylation conditions were found when *A. ochraceus* was grown for 48 h at 28 °C, 300 rpm in a medium composed with 2% sucrose, 3% malt extract, 0.3% yeast extract at pH 5.6, giving the highest molar conversion (68–70%, analytical yield) of **1a** into **2a** (chemical purity 99.5%), starting from 15 mM substrate concentration.

3.2. Optimization of 11-α hydroxylation of canrenone in stirred tank (bio)reactor

Once found the best conditions of growth in shake flask, the fermentation and biotransformation were carried out in a conventional stirred tank reactor (STR). *A. ochraceus* was grown in the STR employing the medium optimized in shake flasks (2% sucrose, 3% malt extract, 0.3% yeast extract at pH 5.6 rpm); the first experiment was carried out with an agitation of 400 rpm and aeration of 2.0 vvm. Biotransformation was started by addition of 15 mM of **1a** after 48 h of growth, furnishing a molar conversion of 82% into **2a** (chemical purity 99.5%). The growth and biotransformation in STR allowed for a remarkable increase of the yield of **2a** with respect to what obtained in shake flasks (maximum yield 70% from 15 mM substrate concentration). This first positive result in STR led us to study different conditions of aeration and agitation during the process of growth and biotransformation. O_2 is poorly soluble in water (7.5 mg/L at 1 atm and 30 °C [23]) and, therefore, transfer of O_2 from the gas to the liquid phase is often the limiting step in aerobic bioprocesses, including enzymatic hydroxylations; different conditions of aeration and agitation may have a strong impact on dissolved oxygen. Neat substrate (15 mM) was added after 48 h and pH maintained at 5.6 (Table 1).