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Effect of two-steps substrate addition on steroids 11β-hydroxylation by *Curvularia lunata* CL-114

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Abstract

In this paper, the effect of substrate 17α-hydroxypregn-4-ene-3,20-dione-21-acetate (RSA, Cortexolone-21-acetate) on the expression of cytochrome P450 and the production of hydrocortisone by *Curvularia lunata* CL-114 was studied. Meanwhile the effect of pH on the production of hydrocortisone was observed. Based on the effect of substrate RSA two-steps addition on cytochrome P450 expression and hydrocortisone production, a novel fermentation process was established as follows: 0.3 g/L RSA was added for the first time after 16 h of inoculation, followed by the second addition of 0.7 g/L RSA after 8 h later, then pH was regulated to constant 6.5 after another 8 h till the end of fermentation. The results showed that the novel process was much better than the original one on improving the induction of cytochrome P450 and production of hydrocortisone, and the hydrocortisone yield had achieved an improvement of 17.6% higher than that of the original process correspondingly.

Keywords: *Curvularia lunata*; Hydrocortisone; Cytochrome P450; Steroid; Induction

1. Introduction

11β,17α,21-Trihydroxypregn-4-en-3,20-dione (hydrocortisone, cortisol or Substance F of Kendal), as an important medical steroid and also a starting substrate for the manufacture of several other potent steroids (such as 1-dehydrocortisol), is biotransformed by fungi from 17α, 21-dihydroxypregn-4-en-3,20-dione (Reichstein's Substance S, RS or cortexolone) or 17α-hydroxypregn-4-en-3,20-dione-21-acetate (RSA, Cortexolone-21-acetate) according to steroids 11β-hydroxylation reaction by *Curvularia lunata* [1–3].

Fungal steroid 11β-hydroxylation was catalyzed by cytochrome P450 monooxygenases (i.e. cytochrome P450) [4,5]. Cytochrome P450 is responsible for the oxygen insertion in the steroid substrate molecule [4,6,7]. It was shown that intact protoplasts of *C. lunata* can be successfully used for determining the cytochrome P450 content in the cells by carbon monoxide difference spectra method as an indicator of the steroid 11β-hydroxylation systems [4]. It was found that substrate cortexolone presence in the culture could induce the expression of cytochrome P450 [4,8,9].

The traditional 11β-hydroxylation process for hydrocortisone production was as follows: pH of culture was adjusted to optimal value at late logarithmic phase (or early stationary phase), and then substrate was added into the culture in a lump.

In this paper, based on the induction of cytochrome P450 by substrate RSA, a two-steps substrate addition mode was investigated. Effect of pH on improving the yield of hydrocortisone and maintaining the cytochrome P450 activity was studied. A comparison of the efficiencies of novel and traditional process was carried out with the findings that this process could give more efficient production of hydrocortisone under the same substrate addition concentration.

2. Materials and methods

2.1. Organism and medium

C. lunata CL-114 was conserved in our lab and maintained on PDA slant agar (glucose 20.0 g, agar 20.0 g, dissolved in 200 mL 20% (w/v) potato decoction, with natural pH value). The fermentation medium for hydrocortisone production was as follows: glucose 20.0 g, peptone 5.0 g, yeast extract 5.0 g,

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soybean powder 10.0 g, dissolved in 200 mL distilled water, with initial pH adjusted to 6.5. The protoplast regeneration medium was as follows (g/L): glucose 10.0, yeast extract 4.0, agar 15.0, 0.6 mol/L KCl, dissolved in 6° Bx malt extract juice.

2.2. Reagents

Steroids: RSA (17α-hydroxypregn-4-en-3,20-dione-21-acetate) was the substrate of steroid transformation; hydrocortisone (11β,17α,21-trihydroxypregn-4-en-3,20-dione) was the standard main product for the 11β-hydroxylation. RSA and hydrocortisone were obtained from Tianjin pharmacy Ltd. (China) and Sigma, respectively. The other chemicals came from Serva or Sigma.

2.3. Protoplast preparation

The protoplasts were prepared according to the methods described by Dlugonaski et al. [10], with later modification by Lu et al. [11].

2.4. Culture conditions

2.4.1. Flask cultivation

One milliliter spores suspension with the concentration of 3×10^6 spores/mL sterile water was inoculated into a 250 mL Erlenmeyer flask containing 30 mL fermentation medium and incubated at 180 r/min and 28 °C. RSA, dissolved in 80% (v/v) ethanol with the ratio of 1:18 (w/v) was added into the 24 h culture broth (adjusting pH to 6.5 before addition) up to the RSA final concentration of 1.0 g/L. Steroid transformation was terminated after a further 48 h cultivation.

2.4.2. 7 L bioreactor cultivation

The fermentor fermentation experiments were carried out in a 7 L jar fermentor (Gaoji Shiye Ltd., Shanghai, China) with a working volume of 4 L. The fermentation medium used was the same as that in flask cultivation. The seed medium contained (g/L distilled water): glucose 22.5, beef extract 5.6, yeast extract 5.3 and KH_2PO_4 3.0, with initial pH adjusted to 6.5. Thirty milliliters of seed medium in 250 mL Erlenmeyer flasks was inoculated with 1 mL spores suspension (3×10^6 spores/mL), incubated at 28 °C and 180 r/min for 22 h, and 4% (v/v) inoculums were inoculated into the fermentor. Fermentations were performed under controlled operational conditions as follows: temperature 30 °C, initial pH 6.5, aeration rate 4 L/min and agitation speed from 160 to 400 r/min. Dissolved oxygen tension (DOT) was measured with a dissolved oxygen probe. The DOT level was maintained within 20–30% at logarithmic phase and 30–40% at stationary phase. RSA addition and pH regulation were the same as that in the flask cultivation.

2.5. Cytochrome P450 determination

Cytochrome P450 content was determined according to reduced carbon monoxide difference spectrum method described by Omura and Sato [12], with later modification by Dlugonaski et al. [13]. Protoplasts suspended in buffer C containing Tris–HCl buffer (pH 7.5) 0.1 mol/L, glucose 0.25 mol/L, KCl 1 mmol/L, glycerol 10% (v/v) and DTT 5 mmol/L were counted and used for the experiments. Antimycine A (4 mg/L) was added to the protoplasts samples and conducted at 0 °C for 5 min. The suspensions were then poured into two vessels averagely. One of the samples was saturated with CO by bubbling the gas for 5 min, and the other without CO saturation was treated as the reference solution. The CO difference spectra were recorded on a Shimadzu UV-3000 spectrophotometer, using the cuvettes with 1 cm light path. Cytochrome P450 content was calculated as follows:

$$\text{P450 (mmol/L)} = \frac{\text{A450 nm} - \text{A490 nm}}{91} \quad (1)$$

where A450 nm and A490 nm were the absorbency at 450 nm and A490 nm, respectively, and 91 $\text{cm}^{-1}\,\text{mM}^{-1}$ was the molar extinction increment between 450 and 490 nm. The cytochrome P450 content in this paper meant the value per 10^8 protoplasts.

2.6. The presence position of cytochrome P450

0.5 g/L RSA was added to the broth after 16 h of inoculation and conducted with a further 8 h cultivation. The broth was centrifuged, and the supernatant was filtrated with G4 filler. 0.5 g/L RSA was added to the two reaction systems, respectively, as follows: one containing 3.0 g collected wet mycelia in 250 mL Erlenmeyer flask with 30 mL 0.2 mol/L NaH_2PO_4–Na_2HPO_4 buffer (pH 6.5), the other containing the same volume of supernatant in 250 mL Erlenmeyer flask. The hydrocortisone yield was determined after a further 10 h cultivation.

2.7. Assays

HPLC analysis of samples was conducted according to the methods described by Pharmacopoeia of PRC [14], with modification by Lu et al. [15]. The broth was centrifuged at 20,000 × *g* for 10 min, and the precipitates mycelia were collected and washed twice with distilled water. Washed mycelia samples were dried at 80 °C till constant weight. pH was measured by PHS-2C acidity meter.

2.8. Statistics

All experiments were repeated three times. The data shown in the corresponding tables and figures of Section 3 were the mean values of the experiments, and it was indicated that the relative standard deviations were all within ±3%.

3. Results and discussion

3.1. Carbon monoxide difference spectra of cytochrome P450 in C. lunata

Carbon monoxide difference spectra of protoplasts are the effective method to determine the cytochrome P450 content in