Bioresource Technology 100 (2009) 2271–2277

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Novel biotransformation process of podophyllotoxin to produce podophyllic acid and picropodophyllotoxin by *Pseudomonas aeruginosa* CCTCC AB93066, Part II: Process optimization

Yan Li [a], Yuan-Yuan Li [a], Zhi-Yuan Mi [a], Dong-Sheng Li [a], Ya-Jie Tang [a,b,c,*]

[a] Hubei Provincial Key Laboratory of Industrial Microbiology, College of Bioengineering, Hubei University of Technology, Room 310, Wuhan 430068, China
[b] National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100080, China
[c] State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China

ARTICLE INFO

Article history:
Received 18 July 2008
Received in revised form 22 October 2008
Accepted 27 October 2008
Available online 12 December 2008

Keywords:
Biotransformation process optimization
Podophyllotoxin
Podophyllic acid
Fed-batch biotransformation
Pseudomonas aeruginosa

ABSTRACT

This work optimized the novel biotransformation process of podophyllotoxin to produce podophyllic acid by *Pseudomonas aeruginosa* CCTCC AB93066. Firstly, the biotransformation process was significantly affected by medium composition. 5 g/l of yeast extract and 5 g/l of peptone were favorable for podophyllic acid production (i.e. 25.3 ± 3.7 mg/l), while not beneficial for the cell growth of *P. aeruginosa*. This indicated that the accumulation of podophyllic acid was not corresponded well to the cell growth of *P. aeruginosa*. 0 g/l of sucrose was beneficial for podophyllic acid production (i.e. 34.3 ± 3.9 mg/l), which led to high podophyllotoxin conversion (i.e. 98.2 ± 0.1%). 1 g/l of NaCl was the best for podophyllic acid production (i.e. 47.6 ± 4.0 mg/l). Secondly, the production of podophyllic acid was significantly enhanced by fed-batch biotransformation. When each 100 mg/l of podophyllotoxin was added to the biotransformation system after 4, 10 and 25 h of culture, respectively, podophyllic acid concentration reached 99.9 ± 12.3 mg/l, enhanced by 284% comparing to one-time addition (i.e. 26.0 ± 2.1 mg/l). The fundamental information obtained in this study provides a simple and efficient way to produce podophyllic acid.

1. Introduction

Podophyllotoxin, the main cyclolignan component of *Podophyllum* sp. resin, possesses good anti-tumor activity but failed in the clinical utility for its unacceptable gastrointestinal toxic side-effects (Imbert, 1998). In spite of the severe toxicity, podophyllotoxin became a leading compound for the design of new drugs with improved pharmacological profile (Castro et al., 2003). Biotransformation is a good choice for the structure modification of podophyllotoxin with the advantages of strict stereo- and region-selectivity, mild reaction condition and simple operation procedure (Riva, 2001). A novel biotransformation process of podophyllotoxin to produce picropodophyllotoxin and podophyllic acid has been developed in our laboratory (Li et al., 2008), which was the first report for the production of podophyllic acid by podophyllotoxin biotransformation. Podophyllic acid was reported to show impressive in vitro activity against ovarian and melanoma cell lines (Subrahmanyam et al., 1998), and furthermore podophyllic acid could be used as a leading compound to synthesize podophyllic acid [4-(2,2,6,6-tetramethyl-piperidinooxyl)] hydrazone (GP-1) and so on, which possess good anti-tumor activity (Tian et al., 2002; Li et al., 2002). While, the anti-tumor activity of picropodophyllotoxin decreased because its *cis*-lactone was not favor to its inhibition on tubulin polymerization (Bohlin and Rosen, 1996).

In order to enhance podophyllic acid production, this work focused on the optimization of the above-mentioned novel biotransformation. For the biotransformation process of podophyllotoxin, the key factors may be the cell growth of *Pseudomonas aeruginosa* and substrate addition mode. For the cell growth, the significances of culture medium have been stated in other biotransformation processes, and it was concluded that there was a correlation between the cell growth and biotransformation product yield (Zhou et al., 2008; Chattopadhyay et al., 2002). For substrate supply (Kim et al., 2007), a common limitation is the inhibition or/and toxicity of substrate as well as the limited aqueous solubility (Carragher et al., 2001; Held et al., 1999; Wick et al., 2002). General speaking, substrate concentration need to be controlled at a low level, and the slow addition of the substrate into the biotransformation system is a common solution, which named after fed-batch biotransformation (Hack et al., 2000; Çelik et al., 2004; ďAnjou and Daugulis, 2001). Fed-batch biotransformation has been successfully applied to other biotransformation processes to obtain a better yield (Amanullah et al., 2002; Doig et al., 2002).

* Corresponding author. Address: Hubei Provincial Key Laboratory of Industrial Microbiology, College of Bioengineering, Hubei University of Technology, Room 310, Wuhan 430068, China. Tel./fax: +86 27 88015108.
E-mail address: yajietang@hotmail.com (Y.-J. Tang).

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doi:10.1016/j.biortech.2008.10.044

In this work, the novel biotransformation process of podophyllotoxin to produce podophyllic acid was optimized. The influences of culture medium on podophyllic acid production were investigated and the optimal medium compositions were identified. Effects of initial substrate concentration on the biotransformation process were also studied. On the basis of the results, a fed-batch process was proposed, and the approach was found to be very useful for the efficient enhancement of podophyllic acid accumulation.

2. Methods

2.1. Maintenance and preculture of P. aeruginosa

The strain of *P. aeruginosa* CCTCC AB93066 was purchased from China Center for Type Culture Collection (Wuhan, China). It was maintained on agar slants, stored at 4 °C and subcultured periodically. Peptone was kindly provided by Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Beef extract was purchased from Beijing Shuangxuan Microbe Culture Medium Products Factory (Beijing, China). Liquid medium consisted of the following components (g/l): sucrose, 10; peptone, 5; beef extract, 5; NaCl, 5, and pH was adjusted to 7.0 with 1.0 M NaOH. For the liquid seed culture, 50 mL medium was prepared in a 250 ml shake flask, and then two loops of fresh spores were inoculated, and followed by 7 h incubation at 37 °C on a rotary shaker (200 rpm).

Cell density was measured as the unit of optical density (UOD = OD × dilution rate). The culture broth was diluted with deionized water after the OD of the diluted sample reached approximately 0.5 at 600 nm.

2.2. Effect of nitrogen source

In order to enhance podophyllic acid production, a 3^2 full factorial design (FFD) experiment was conducted around the optimum vicinity to locate the optimum concentrations of yeast extract and peptone. The biotransformation medium (at an initial pH of 7.0) was composed of 10 g/l of sucrose, 5 g/l of NaCl, and a certain combination of yeast extract and peptone as investigated. The FFD experimental results were fitted with a second-order polynomial equation of Eq. (1) by a multiple regression technique.

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i x_i + \sum_{i=1}^{2} \beta_{ii} x_i^2 + \sum \sum_{\substack{i=1 \\ i<j}}^{2} \beta_{ij} x_i x_j + \varepsilon \quad (i \neq j;\ \ i = 1,2;\ \ j = 1,2) \tag{1}$$

Where Y is the predicted response, β_0 is the offset term, β_i is the linear terms, β_{ii} is the quadratic terms, β_{ij} is the interaction terms, ε is an experimental error. And x_i and x_j represent the independent variables in the form of coded values as following equation:

$$x_i = \frac{X_i - X}{\Delta X_i} \quad i = 1,\ \ 2,\ \ 3 \tag{2}$$

Where x_i and X_i are the dimensionless and actual values of independent variable i, X is the actual value of the independent variable i at the central point, and ΔX_i is the step change of X_i corresponding to a unit variation of the dimensionless value. The fitness of the second-order model was expressed by the determination coefficient R^2 and its statistical significance was tested by an F-test. The regression significance was tested by a t-test. The computer software used was "Statistica" (trial version 6.0, StatSoft, Tulsa, OK).

A 5 ml aliquot of liquid seed culture was used to inoculate the biotransformation in a 250 ml shake flask containing 44 ml medium, and followed by 4 h incubation at 37 °C on a rotary shaker (200 rpm). Podophyllotoxin was dissolved in alcohol and diluted to 5.0 mg/ml before used as stock solution. After 4 h of culture, 1 ml of the prepared substrate solution was added to each shake flask, then the initial concentration of podophyllotoxin in the culture broth was diluted to 100 mg/l. Incubation was then allowed to proceed for 0–48 h. The dynamic profiles of the cell growth, podophyllotoxin biotransformation, podophyllic acid and picropodophyllotoxin production were monitored during the biotransformation process. Multiple flasks were run at the same time, and three flasks were taken at each sampling point. Each data point was expressed by an average with an error bar (i.e. standard deviation from three independent samples).

2.3. Effect of initial sucrose concentration

For the investigation on the initial sucrose concentration, its levels of 0, 2.5, 5.0 and 10.0 g/l were tested to obtain a maximal podophyllic acid production. The biotransformation medium (at an initial pH of 7.0) was composed of 5 g/l of yeast extract, 5 g/l of peptone, 5 g/l of NaCl, and a certain initial concentration of sucrose as investigated. The other culture conditions were the same as above.

2.4. Effect of NaCl concentration

For the investigation on the NaCl concentration, its levels of 0, 1.0, 2.5 and 5.0 g/l were tested to obtain an optimal podophyllic acid production. The biotransformation medium (at an initial pH of 7.0) was composed of 5 g/l of yeast extract, 5 g/l of peptone, and a certain concentration of NaCl as investigated. The other culture conditions were the same as above.

2.5. Effect of podophyllotoxin addition mode on the biotransformation process

Effect of initial podophyllotoxin concentration on the biotransformation process was studied with its levels of 120, 200, 300 and 400 mg/l. Based on the above initial podophyllotoxin concentration experiment result, podophyllotoxin adding modes was selected as follows. For the first podophyllotoxin addition mode, each 100 mg/l of podophyllotoxin was added to the biotransformation process after 4 and 10 h of culture, respectively. For the second podophyllotoxin addition mode, each 100 mg/l of podophyllotoxin was added to the biotransformation process after 4, 10 and 25 h of culture, respectively. For the third podophyllotoxin addition mode, 100, 50 and 50 mg/l of podophyllotoxin was added to the biotransformation process after 4, 10 and 25 h of culture, respectively. The biotransformation medium (at an initial pH of 7.0) was composed of 5 g/l of yeast extract, 5 g/l of peptone, and 1 g/l NaCl. The other culture conditions were the same as above.

2.6. High performance liquid chromatography (HPLC) analysis

At the end of the biotransformation process, the total broth taken from the shake flask was centrifuged at 10,000 rpm for 30 min, after which 2 ml of this supernatant was added acetonitrile to a constant volume of 5 ml. Then the diluted solution was centrifuged at 13,000 rpm for 30 min to remove protein and so on. The clear supernatant was collected and then filtered with 0.45 μm micropore filter for HPLC analysis.

HPLC analysis was carried out on a Waters 600 Series HPLC system, equipped with 2487 ultraviolet detector. An Akasil C18 column (5 μm, 4.6 mm × 250 mm) was used. Mobile phase was 0.05 M KH_2PO_4–methanol–acetonitrile (52:32:16) and pH was adjusted to 3.00 with phosphoric acid. The HPLC oven temperature was maintained at 40 °C, and the detection wavelength was 230 nm. The flow rate was 0.8 ml/min.