Biochemical Engineering Journal 61 (2012) 1–10

Contents lists available at SciVerse ScienceDirect

Biochemical Engineering Journal

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

Enhanced cephalosporin C production with a combinational ammonium sulfate and DO-Stat based soybean oil feeding strategy

Shengbing Duan[a], Guoqiang Yuan[b], Yanli Zhao[b], Hongfei Li[b], Weijia Ni[a], Meina Sang[a], Liming Liu[a], Zhongping Shi[a,*]

[a] Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi, Jiangsu 214122, China
[b] CSPC Hebei Zhongrun Pharmaceutical Co. Ltd., Shijiazhuang, Hebei 050041, China

ARTICLE INFO

Article history:
Received 13 July 2011
Received in revised form 8 November 2011
Accepted 26 November 2011
Available online 13 December 2011

Keywords:
Acremonium chrysogenum
Antibiotic
Bioprocess monitoring
Cephalosporin C production
Fermentation
Optimization

ABSTRACT

A novel substrates feeding strategy by coupling ammonium sulfate addition with soybean oil feeding was proposed for efficient cephalosporin C (CPC) production by *Acremonium chrysogenum* and testified in a 7 L fermentor to maintain NH_4^+—N concentration stably for normal mycelium differentiation. On the base of the coupling strategy, fermentation performance during main CPC production phase with different soybean oil feeding methods was compared. The results indicated that the modified DO-Stat soybean oil feeding strategy, namely, the method of adopting DO-Stat for soybean oil feeding and using O_2-enriched air for aeration, could maintain CPC synthesis rate at an appropriately high level and reduce deacetoxycephalosporin (DAOC, the major by-product of CPC fermentation) accumulation simultaneously. The modified DO-Stat soybean oil feeding strategy could raise final CPC concentration up to a high level of 35.77 g/L and lower DAOC concentration down to a level of 0.178 g/L, so that the quality standard of CPC fermentation product could be satisfied by controlling DAOC/CPC ratio below 0.5%. The metabolic analysis revealed that a weakened carbon flux distribution in TCA was beneficial for reducing DOAC overflow.

1. Introduction

Cephalosporin C (CPC) and its semi-synthetic derivatives (7-aminocephalospranic acid, 7-ACA, etc.) are very important and widely used β-lactam antibiotics [1,2].

Cephalosporins are produced by several *Streptomyces* spp. and filamentous fungi. Among them, *Acremonium chrysogenum* is widely used in industrial CPC production for its high CPC yield [3]. CPC production by *A. chrysogenum* is characterized with feature of morphological differentiation [4]. Maximum CPC production rate coincides with differentiation of filamentous hyphae to wide-highly swollen and metabolically active hyphal fragments [5,6]. During CPC fermentation, organic nitrogen sources are used at early stage and then ammonia nitrogen (NH_4^+—N) is consumed at late phase. Ammonium sulfate ($(NH_4)_2SO_4$) is a kind of ammonia nitrogen sources, and generally used in industrial CPC fermentation as it supplies both N source for cells/CPC synthesis and S source for CPC production. However, excessive NH_4^+—N severely inhibits mycelium differentiation and CPC synthesis [7,8]. As a result, it is important to control ammonium sulfate concentration at certain adequate level throughout fermentations. Carbon sources and their feeding control are also important for CPC synthesis. Soybean oil containing fatty acids has been considered as the most efficient carbon source for CPC synthesis, as it promotes the formation of CPC precursors (α-aminoadipic acid, valine and cysteine) by fatty acids degradation, as well as the enlargement of carbon flux in TCA and glyoxylate by-pass [9–11]. The morphological structure models based optimal soybean oil feeding profile was reported [12,13], which suggested that the profile is featured with a continuous increase in feeding rate during early fermentation stage followed by a constant feeding rate in late phase. Dissolved oxygen (DO) control is another important factor for CPC fermentation because of the extremely high oxygen consuming feature of the process. Large amount of oxygen is required either as an electron acceptor or as substrate for biosynthetic oxygenases [14]. In addition, the weakening oxygen supply ability associated with high broth viscosity in CPC fermentation is also very difficult to deal with [15].

In CPC fermentation, the major by-product, deacetoxycephalosporin C (DAOC) could accumulate up to a level of 1–2% DAOC/CPC (w/w) [16]. DAOC has similar molecular weight and structure as those of CPC. Separation of the two substances is very difficult, so that repressing DAOC accumulation during CPC fermentation has become the only way in controlling the quality of CPC fermentation product [2,17,18]. Soybean oil over-feeding might cause DAOC accumulation. DO-Stat and its derived feeding

* Corresponding author at: School of Biotechnology, Jiangnan University, 1800 Lihu Ave., Wuxi 214122, China. Tel.: +86 510 85918292; fax: +86 510 85326276.
E-mail address: zpshi@jiangnan.edu.cn (Z. Shi).

1369-703X/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bej.2011.11.011

methods by limiting substrate consumption rate are widely applied in recombinant substances production to prevent the formation of inhibitory by-products [19,20], and might also be applicable in CPC fermentation to reduce DAOC accumulation.

In this study, a novel feeding strategy by coupling $(NH_4)_2SO_4$ addition with soybean oil feeding for efficient CPC fermentation was proposed and testified in a 7 L fermentor, aiming at simultaneously obtaining higher CPC concentration and lower DAOC/CPC ratio under mild aeration/agitation environments, to supply a prototype for large-scaled CPC production.

2. Materials and methods

2.1. Microorganism

A. chrysogenum HC-3, an industrial CPC producing strain, supplied by Hebei Zhongrun Pharmaceutical Co. Ltd., was used in this work.

2.2. Fermentation medium

Seed medium contained (w/w): sucrose 3.5%, glucose 0.5%, corn steep liquor 3.1%, D,L-methionine 0.05%, $CaCO_3$ 0.5%, soybean oil 0.5%. pH 6.5.

Fermentation medium contained: (w/w): cornstarch 3.5%, dextrin 7.0%, corn steep liquor 5.0%, D,L-methionine 0.6%, urea 0.3%, $(NH_4)_2SO_4$ 1.3%, $CaCO_3$ 1.0%, KH_2PO_3 0.9%, soybean oil 5.0%, α-amylase (20,000 U/mL) 0.02%, and trace element solution. pH 6.2.

Feeding medium (w/w): soybean oil (purchased at local supermarket), $(NH_4)_2SO_4$ 20% (200 g/L), ammonia water 25%.

2.3. Cephalosporin C fermentation

Seed cultures were carried out in 500 mL Erlenmeyer flasks containing 50 mL seed medium. The flasks were placed on a rotary shaking incubator, at 280 rpm and 28 °C for 72 h. The fed-batch CPC fermentation was implemented in a 7 L mechanically stirred fermentor (BIOTECH-7BG, Baoxing Co., China) equipped with on-line DO/pH measurements, with initial medium volume of 5 L and air aeration rate of 2.5 vvm. The temperature was controlled at 28 °C in the first 50 h, and then shifted to 25 °C. pH was maintained in a range of 5.5–5.6 by automatically adding either diluted H_2SO_4 solution or ammonia water. The agitation started at 300 rpm. When DO dropped down to a critically low level (20% saturation), agitation rate was then manually increased at an increment of 50 rpm. If maximum agitation rate (980 rpm) still could not maintain DO above the critical level, then O_2-enriched air (O_2 content about 50%) was aerated into the fermentor. Three electronic balances (JA1102, Haikang Instrument Co., China) connecting with an industrial computer via a multi-channels A/D-D/A converter (PCL-812PG, Advantech Co., Taiwan) was used to on-line measure the weight losses of the feeding reservoirs for ammonium sulfate, ammonia water and soybean oil, their addition amounts and consumption rates were then monitored/calculated at specified instant (measurement interval adjustable, 1–4 h). The CO_2 and O_2 partial pressure in exhaust gas were on-line measured by a gas analyzer (LKM2000A, Lokas Co. Ltd, Korea). The exhaust gas data and DO/pH/agitation rate (AG) signals from the fermentor control cabinet were collected into the industrial computer via RS232. Average agitation rate (AG) during soybean oil feeding period was calculated as:

$$\text{Ave. AG} = \frac{\int_{t0}^{tf} \text{AG}(t)\,dt}{t_f - t_0}$$

where t_f and t_0 represented final fermentation time and the time when soybean oil feeding was initiated, respectively. Oxygen uptake rate (OUR) and CO_2 evolution rate (CER) were calculated on-line by the standard calculation formula. Based on the collected data of DO, ammonia water and soybean oil supplement amounts, two self-designed control programs (Visual Basic, Ver. 6.0) embedded in the computer were then activated (as described in the next section) to feed $(NH_4)_2SO_4$ and soybean oil, by driving two peristaltic pumps (BT00-50M, Langer Co., China) with the aid of the multi-channels A/D-D/A converter.

2.3.1. Ammonium sulfate feeding strategies

Two kinds of ammonium sulfate feeding strategies were considered.

Strategy #1: Ammonium sulfate feeding coupling with ammonia water addition. When pH began to drop down and automatic ammonia water addition for pH adjustment started, this ammonium sulfate feeding strategy was activated. In this case, $(NH_4)_2SO_4$ feeding was empirically set at a constant rate of 0.25 g/(L h).

Strategy #2: Ammonium sulfate feeding coupling with soybean oil addition. When DO rose up suddenly and sharply, which indicated complete exhaustion of carbon sources, various soybean oil feeding strategies were then initiated. Once initiating a soybean oil feeding method, $(NH_4)_2SO_4$ feeding was also activated and its feeding amount in the current control interval (1–4 h) was associated with the measured soybean oil feeding amount during the previous interval, by an empiric coefficient K. In this way, $(NH_4)_2SO_4$ feeding during control interval k were carried out as: $A_{AS}(k) = K \times A_{SO}(k-1)$, where $A_{AS}(k)$ was ammonia sulfate to be fed in the current interval and $A_{SO}(k-1)$ were the measured soybean oil addition amounts during the previous interval, respectively.

2.3.2. Soybean oil feeding strategies

Three kinds of soybean oil feeding strategies, namely intermittent, constant rate and DO-Stat based feeding, were considered.

Strategy #1: Intermittent soybean oil feeding (fermentation run #a). When carbon sources were completely consumed out and DO rose up to an up-limit (80% in this case), 50 g soybean oil was added intermittently using the self-programmed control software. When soybean was used out and DO rose up to the up-limit again, the same measures were repeatedly taken until the end of fermentation.

Strategy #2: Soybean oil feeding at constant rate (run #b). Once carbon sources in fermentation medium were completely consumed out, oil feeding was activated at a constant rate. Feeding rate was set at 2.25 g/(L h) during the first 34 h after initiating feeding, and then shifted to 1.15 g/(L h) until the end of fermentation.

Strategy #3: Soybean oil feeding with DO-Stat based methods (runs #c & #d).

2.4. Analytical methods

20 mL of fermentation broth was accurately taken at each sampling time. The samples were then centrifuged at 8000 rpm for 15 min. The supernatants were collected and properly stored for the measurements of CPC, DAOC, NH_4^+—N and amino acids. The wet cell weight (WCW) was calculated by weighing the solid residual of each sample. The WCW was converted into dry cell weight (DCW) by a pre-calibrated relationship (1 DCW = 0.190 WCW) for the purpose of metabolic analysis. After properly diluting the supernatants, NH_4^+—N concentrations was measured by a spectrophotometer (UV-2100, Unico, China) at 625 nm using indophenol blue reaction [21]. CPC and DAOC were measured by an Agilent 1100 HPLC, under the following conditions: reverse column ODS-C18 4.6 mm × 250 mm; temperature 30 °C; flow rate 0.8 mL/min and mobile phase methanol/distilled water/phosphate = 15/85/0.15 (v/v); detection at 254 nm with an UV detector. The standards of CPC and DAOC were supplied by CSPC Co. Ltd. Amino acids