Process Biochemistry 48 (2013) 1822–1830

Contents lists available at ScienceDirect

Process Biochemistry

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

Performance improvement of cephalosporin C fermentation by *Acremonium chrysogenum* with DO-Stat based strategy of co-feeding soybean oil and glucose

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ARTICLE INFO

Article history:
Received 25 July 2013
Received in revised form
10 September 2013
Accepted 22 September 2013
Available online 29 September 2013

Keywords:
Cephalosporin C
Co-substrates feeding
DO-Stat
Fermentation

ABSTRACT

Cephalosporin C (CPC) fermentation by *Acremonium chrysogenum* featured with two major problems: (1) high raw materials cost (low CPC yield from soybean oil) and (2) low oxygen transfer rate between gaseous/aqueous phases leading to low CPC productivity and quality instability of CPC fermentation product due to the accumulation of deacetoxycephalosporin C (DAOC). To solve the problems, in this study, we proposed a novel DO-Stat based co-substrates feeding strategy by simultaneously supplementing soybean oil and glucose, and testified the effectiveness of the strategy in a 7 L bioreactor. The CPC fermentation performance were significantly improved when co-feeding soybean oil and glucose at a weight ratio of 1:0.7, as compared with those when feeding pure soybean oil: (1) final CPC concentration and yield reached higher levels of 37 g/L and 23.5%, the increments were 46% and 82%, respectively; (2) oxygen transfer rate was largely improved, oil consumption rate and CPC productivity were enhanced by 31% and 136%, respectively; and (3) DO could be controlled at adequately high levels so that DAOC accumulation could be minimized and the quality of CPC fermentation product be ensured. The proposed strategy showed application potential in improving the economics of industrial CPC productions.

1. Introduction

The filamentous fungus *Acremonium chrysogenum* (*syn. Cephalosporium acremonium*) is the natural strain for β-lactam antibiotic cephalosporin C (CPC) fermentation [1]. CPC is an important intermediate for synthesizing other semi-synthetic cephalosporins, and CPC concentrations in stirred-tank scaled fermentations by *A. chrysogenum* range around 30 g/L with 40 g/L as the maximum [2,3]. The global sales amounts of cephalosporins based products reached 11.9 billion USD in 2009, however, the sales growth stayed at a lower level of 3.4% in the past 5 years because of price rise of raw materials and strict control of antibiotics utilization in feedstuff in many countries [4]. In a most recent global marketing report [5], it was estimated that the sales amounts of CPC based pharmaceutical products will exceed 13–15 billion USD in 2016.

In CPC fermentation industry, starches such as glucose and plants oils like soybean oil are the main carbon sources or raw materials for CPC production. Soybean oil containing fatty acids has been recognized as the most efficient carbon source for CPC synthesis, as it promotes the formation of CPC precursors by fatty acids catabolism, as well as the enlargement of carbon flux in TCA and glyoxylate by-pass [6–8]. On the other hand, the metabolites formed when fermenting starches based carbon sources such as glucose may greatly repress CPC synthesis, particularly when concentrations of those carbon sources are high [9]. As a result, soybean oil is dominantly used for CPC fermentation during the main CPC synthesis phase. Seidel et al. reported that CPC concentration and productivity could be maximized to the levels of 29.77 g/L and 0.191 g/L/h, respectively, when using 150% enriched complex medium containing soybean oil in a fermentor with 30 L working

Abbreviations: Glc, glucose; Gly, glycerol; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; GAP, glyceraldehyde-3-phosphate; G-3-P, 3-phosphoglycerate; Ru-5-P, ribulose-5-phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; Ac-CoA, acetyl-CoA; ICIT, isocitrate; α-KG, α-ketoglutarate; OAA, oxaloacetic acid; α-AAA, α-aminoadipate; ACV, α-aminoadipyl-cysteinyl-valine; PEN, penicillin N; DAOC, deacetoxycephalosporin C; DAC, deacetylcephalosporin C; CPC, cephalosporin C; Metex, extracellular methionine; ATP, adenosine triphosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; REE, ring-expansion enzyme.

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http://dx.doi.org/10.1016/j.procbio.2013.09.021

volume [10]. Soybean oil is expensive, and its price keeps rising. During 2008–2012, soybean oil price at global market averaged at $1270–1376/ton [11,12]. The raw materials costs almost occupy more than 60% of the entire production cost [13]. Therefore, raw materials cost has become one of the biggest issues for further development in antibiotic industries, including CPC [13]. It would be of great commercial interest to produce CPC based antibiotic products by using low-valued raw materials [14]. Recently, utilization of cheap raw materials such as beet molasses, glycerol, etc. for CPC fermentation by *A. chrysogenum* was reported [15,16]. Shin et al. reported that CPC concentration reached a level of 8.45 g/L when using mixed carbon sources of glucose and glycerol in shake-flask cultures [16]. Glucose ($400/ton, industrial class) could also be considered as a cheap and alternative carbon source for CPC production, but it was reported that a high glucose concentration of 6 g/L could markedly inactivate the ring-expansion enzyme leading to the dramatic reduction in CPC production [9].

Another factor which deteriorates the economics of industrial CPC fermentation is the operation cost. CPC fermentation is an extremely high oxygen-consuming process and a large amount of oxygen is required either as an electron acceptor or as substrate for biosynthetic oxygenases [17]. In addition, the high viscosity feature in CPC fermentation also deteriorated oxygen transfer ability [18]. In CPC fermentation, dissolved oxygen concentration (DO) must be controlled over a certain levels, otherwise, deacetoxycephalosporin C (DAOC), the main by-product in CPC fermentation would accumulate. 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 is the only way in controlling the quality of CPC fermentation product [19–21]. A ratio of <0.5% in DAOC/CPC (w/w) is an enforced standard for the qualified CPC fermentation products. In CPC fermentation, soybean oil and the aqueous broth forms a stable emulsion system [13], oil is difficult to permeate into the aqueous bulk leading to a very low oxygen transfer coefficient of K_La. During main CPC synthesis phase, K_La is dominantly controlled by the oil diffusion/permeation rate into the aqueous bulk. As a result, rigorous agitation or extensively high power supply must be applied to maintain a minimum K_La required. The low oxygen transfer coefficient K_La causes not only high operation costs but also automatic control problems. In our previous study, we proposed a DO-Stat based soybean oil feeding strategy in couple with ammonium sulfate addition for efficient CPC fermentation [22]. The proposed control strategy stabilized CPC fermentation performance by suitably controlling mycelium differentiation and DAOC/CPC ratio, but CPC concentration and yield (from soybean oil) finished at lower levels (30.7 g/L and 14.4%) under ordinary air-aeration condition.

In this study, focusing on solving the above-mentioned problems in CPC fermentation, we proposed a novel DO-Stat based co-substrates feeding strategy for CPC production, attempting to produce CPC in efficient, high-quality, economical and automatic ways.

2. Materials and methods

2.1. Microorganism and media

A. chrysogenum HC-3, the previous generation of industrial CPC production strain, provided by CSPC Hebei Zhongrun Pharmaceutical Co., Ltd., was used. The strain was preserved in frozen suspension containing 20% glycerol at −20 °C. The slant agar medium consisted of (in g/L): wort (12°) 200, maltose 40, peptone 10, agar 17, and pH 7.0. Seed medium contained (g/L): sucrose 35, glucose 5, corn steep liquor 31, D,L-methionine 0.5, $CaCO_3$ 5, soybean oil 5, and pH 6.5. Fermentation medium contained: (g/L): cornstarch 35, dextrin 70, corn steep liquor 50, D,L-methionine 6, urea 3, $(NH_4)_2SO_4$ 13, $CaCO_3$ 10, KH_2PO_3 9, soybean oil 50, α-amylase (20,000 U/mL) 0.2, and pH 6.2. Feeding medium: pure soybean oil (purchased at local supermarket), glucose (500 g/L), glycerol (500 g/L), $(NH_4)_2SO_4$ (200 g/L), and ammonia water 25% (w/w).

2.2. Batch 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 240 r/min and 28 °C for 84 h. CPC fermentation was implemented in a 7 L mechanically stirred fermentor (BIOTECH-7BG, Baoxing Bio-Engineering Equipment Co., Ltd., China) equipped with on-line DO/pH measurement probes, with initial medium volume of 3 L. Air aeration rate was maintained at 1.5 vvm throughout the fermentations. The inoculation amount in all fermentations was 15% (v/v). The temperature was controlled at 28 °C in the first 50 h, and then shifted to 25 °C. pH was maintained at a range of 5.60 ± 0.05 by automatically adding either diluted H_2SO_4 solution or ammonia water. The agitation initiated at 300 r/min, and it was manually increased by an increment of 50 r/min when DO dropped down to a low limit (20% saturation).

2.3. Fed-batch fermentation

The proposed combinational ammonium sulfate and DO-Stat based carbon sources feeding strategy in previous study [22] was used for carbon/nitrogen sources supplements during the fed-batch phase. When carbon sources in the initial medium were completely consumed out at about 105 h and DO suddenly rose up to an upper-limit (about 50% saturation), the feeding strategy was initiated for feeding carbon/nitrogen sources via a multi-channels A/D–D/A converter (PCL-812PG, Advantech Co., Ltd., Taiwan), with the aid of the self-developed control programs (Visual Basic, Ver.6.0) embedded in an industrial computer. In this study, three carbon sources feeding strategies were investigated, namely pure soybean oil feeding, co-feeding soybean oil and glycerol, and co-feeding of soybean oil and glucose. Glycerol and glucose were prepared as concentrated solution of 500 g/L (50%, w/w). The carbon sources feeding media were: (1) pure soybean oil (run #A); (2) soybean oil + glycerol (run #B, oil/glycerol weight ratio of 1:0.5); (3) soybean oil + glucose (run #C, oil/glucose weight ratio of 1:0.5); and (4) soybean oil + glucose (run #D, oil/glucose weight ratio of 1:0.7). The soybean oil/glucose and soybean oil/glycerol weight ratios were so selected to satisfy the principle of "co-substrates simultaneous consumption", which attempted to consume out the co-substrates simultaneously within one DO-Stat feeding cycle.

A modified DO-Stat strategy featured with a certain specified delay period (5 min) was adopted for carbon sources feedings: when DO rose up over its upper-limit, the programmable peristaltic pump(s) was switched on and then continued to run during the specified feeding delay period even though DO had dropped down below its upper-limit. The peristaltic pump(s) was switched off when DO declined below this upper-limit and the delay time was run out of as well. In the cases of co-feeding mixed carbon sources, two peristaltic pumps were accurately switched on and off at the same instant. Two electronic balances (JA1102, Haikang Instrument Co., Ltd., China) connected to the industrial computer via the multi-channels A/D–D/A converter were used to on-line monitor carbon sources (soybean oil, glycerol or glucose) addition amounts by measuring the weight losses in their feeding reservoirs. The speed of the peristaltic pump (when it was on) for soybean oil feeding was fixed. The speeds of the pump for glycerol or glucose additions (when it was on) were manually regulated to satisfy the required weight ratios (1:0.5, 1:0.7, etc.) at the beginning moment of carbon sources feeding, and then they were kept at the rates after the targeted weight ratios were reached. The O_2 partial pressure in exhaust gas was on-line measured by a gas analyzer (LKM2000A, Lokas Co., Ltd., Korea). The exhaust gas data and DO/pH signals from the fermentor control cabinet were collected into the computer via RS232. Oxygen uptake rate (OUR) was then calculated on-line by the standard calculation formula.

2.4. Analytical methods

20 mL of fermentation broth was accurately taken at each sampling time. The samples were then centrifuged at 8000 r/min for 15 min. The supernatants were collected and properly stored for the measurements of CPC, DAOC and glucose. 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). CPC and DAOC were measured by an Agilent 1200 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 = 15/85/0.05 (v/v); detection at 254 nm with an UV detector. The standards of CPC and DAOC were supplied by CSPC Co., Ltd. Glucose concentration in supernatant was measured by a SBA-40C glucose analyzer (Shandong Academy of Sciences, China).

3. Results and discussion

3.1. Enhancing soybean oil feeding rate and CPC productivity by co-feeding soybean oil and glucose

The four fermentations using different carbon sources feeding strategies were carried out. Fig. 1 depicted the basic fermentation curves, including the amounts and rates of soybean oil feeding, the