Process Biochemistry 46 (2011) 1663–1669

Contents lists available at ScienceDirect

Process Biochemistry

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

High efficient production of recombinant human consensus interferon mutant in high cell density culture of *Pichia pastoris* using two phases methanol control

Dan Wu, Ju Chu*, Yu-You Hao, Yong-Hong Wang, Ying-Ping Zhuang, Si-Liang Zhang*

State Key Laboratory of Bioreactor Engineering, National Engineering Center of Biotechnology, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, People's Republic of China

ARTICLE INFO

Article history:
Received 13 January 2011
Received in revised form 4 May 2011
Accepted 25 May 2011

Keywords:
Recombinant human consensus interferon mutant
Pichia pastoris
Specific growth rate
Disulfide bond
Cell physiology

ABSTRACT

A recombinant human consensus interferon mutant (cIFN) producing Mut$^+$ *Pichia pastoris* strain was used to study the influence of the methanol concentration on the cIFN production level and quality. The data showed that a high methanol concentration at initial induction phase was important for the expression level, while the specific growth was critical for the cIFN product quality at following induction phase. A high cell density fed-batch culture was performed by two phases methanol control, which composed of 5 g L^{-1} methanol at initial 6 h induction (the first induction phase) and maintaining of 0.02 h^{-1} specific growth rate (the second induction phase) by controlling methanol concentration at 0.25%, yielding high cIFN product level with the high quality. Our results indicated that it preferred to produce cIFN doublets containing incomplete disulfide bond and cIFN aggregates when higher methanol concentration was maintained at second induction phase. Chemostat cultures indicated that the formation of disulfide bonds in cIFN was strongly growth-dependent, and cIFN aggregates were easily induced at high specific growth rate. These results provide insights into how cell growth rate could affect the recombinant protein quality, indicating the importance of application of a physiologically suitable strategy.

1. Introduction

The *Pichia pastoris* expression system has been used successfully for the production of more than 500 proteins [1]. For recombinant protein expression in *P. pastoris*, methanol concentration is the most important factor to maximize protein production because methanol concentration directly affects cellular growth and protein yield, and determines growth and uptake rates [2,3]. Maintaining a constant methanol concentration is a simple, easy to scale-up, and cost-effective for industrial fermentations [4]. Tight control of methanol inducer concentration leads to an increase in recombinant protein productivity by avoiding the buildup of methanol to cytotoxic levels and depletion of methanol to non-inducing levels [5], and fermentation process that uses methanol feeding to control the concentration can show much higher productivity, especially at low dilution rates, for the industrial scale production of recombinant proteins by *P. pastoris* [6].

Recombinant human consensus interferon-α (IFN-Con1) is a wholly synthetic type I interferon (IFN) with two intramolecular disulfide linkages that have been determined to reside between Cys^1–Cys^{98} and Cys^{29}–Cys^{138} [7]. Studies *in vitro* show that IFN-Con1 has the merit of multi-interferon-α [8] and a 5–20 times higher anti-viral efficacy than IFN-α [9] which makes it be a more stronger interferon with more broader antivirus spectrum. Many publications have reported high-level expression of IFN by *P. pastoris*. To the best of our knowledge, no study has reported the optimization of methanol concentration to control induction and expression of cIFN.

During induction the influence of cell physiology on quality of recombinant protein can be easily overlooked. For example, recombinant proteins of scFv [10,11], HBsAg [12,13] and IFN [14,15] were unstable when expressed in *P. pastoris*. Many aggregates were formed in supernatant despite high level protein expression, and no more detailed studies were carried out to explain why the stability of these proteins were low and investigate the interaction of cell physiology (e.g. cell growth) on the protein expression. To date, little work has been done to reveal potential operational strategies for alleviating the effects of cellular stress responses on the production of foreign proteins in the presence of constant methanol concentrations, this makes it extremely difficult to draw comparisons between productions of particular proteins using *P. pastoris* expression systems, resulting in the difficulty in the establishment of an appropriate control strategy on high efficient expression of recombinant proteins. In this work, we investigated the influence of methanol concentration on the expression of cIFN by a Mut$^+$

* Corresponding authors. Tel.: +86 2164253021; fax: +86 2164253702.
E-mail addresses: juchu@ecust.edu.cn (J. Chu), siliangzhang@yahoo.cn (S.-L. Zhang).

1359-5113/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2011.05.015

transformant of *P. pastoris*. We examined cell growth kinetics to see if it would improve product formation and quality, and we established a simple and physiologically suitable control strategy. In addition, our research investigated how the methanol concentration could influence cIFN monomer concentration in supernatant and cIFN disulfide bond formation. We found that optimizing the steady state methanol concentration could control the specific growth rate and protein expression to form cIFN.

2. Material and methods

2.1. Strains

The Mut$^+$ recombinant strain *P. pastoris* GS115/pPIC9k-cIFN used in this study was constructed by Liu et al. [16]. The cIFN gene was integrated into the genome of the yeast and under control of AOX1 gene promoter with the *Saccharomyces cerevisiae* alpha factor leader for its extracellular production.

2.2. Fermentation

For the cultures before fermentation, the preparation was performed according to Wu et al. [17], except that PMSF 0.0174% (w/v) and EDTA 0.05% (w/v) were added in initial BMY fermentation medium. A 5 L bioreactor (National Engineering Center of Biotechnology, Shanghai, China) containing 1.8 L of BMY with a methanol sensor and controller (FC-2002, East China University of Science and Technology, China) was used. Three fed-batch cultivations were conducted with control of residual methanol concentration at levels ranging from 0.25 to 1% (w/v). The fermentation was divided into three phases, e.g., the glycerol batch phase, glycerol fed-batch phase, and the methanol fed-batch induction phase [3]. The glycerol batch phase was completed within 20 h of inoculation, and complete consumption of glycerol in the culture was detected by monitoring the dissolved oxygen (DO) spike. Then there was a glycerol-fed batch phase, in which 50% (v/v) glycerol was fed for about 4 h until an OD_{600} at 50 was reached. After that the induction was performed with a continuous feed containing 100% methanol. The feeding rate for methanol was automatically controlled by the methanol sensor and controller so that it was maintained at 0.25%, 0.5%, 0.75% and 1% methanol according to the experiment requirement. The DO level in the bioreactor was maintained at 20–40% for the entire fermentation by adding O_2 as needed. The pH in bioreactor was kept at 5.4 during the glycerol growth phase and at 5.0 during the methanol induction phase by adding 28% NH_4OH. The temperature was set at 30 °C for growth in glycerol and 25 °C for methanol induction.

For the fed-batch fermentation with constant methanol concentration, the specific growth rates were controlled with methanol control by the following equation:

$$\mu = \frac{d(XV)}{(XV)dt} \tag{1}$$

where X, V, and t are cell density (g DCW L^{-1}), culture volume (L), and time (h), respectively, and μ (h^{-1}) is determined from the slope of ln(XV) versus t. At each sampling point t, V is determined from the sum of the initial broth volume, the volume of ammonium hydroxide, glycerol, and methanol added up to that time, minus the volume sampled.

In case of the methanol concentration and pH are constant from t_0 to t, the specific methanol consumption rate (q_s, g g^{-1} h^{-1}), and the specific cIFN production rate (q_p, mg g^{-1} h^{-1}) are expressed as [18–20]

$$Q_{MeOH} = q_s \int_{t_0}^{t} (XV)dt \tag{2}$$

$$J = q_p \int_{t_0}^{t} (XV)dt \tag{3}$$

where Q_{MeOH} is the total amount of methanol consumed, and J is the total protein produced from t_0 to t. q_s is determined by measuring the amount of methanol added to the system.

For chemostat cultures, after the initial batch growth phase, the supply of the feed-medium was started at a dilution rates (D) of 0.02 and 0.025 h^{-1}, respectively. At each dilution rate, at least two culture volumes were allowed to pass prior to beginning steady-state measurements, as signalled by a constant cell density, DO and cIFN concentrations at the output. The methanol concentration at different specific growth rate was also found to be near to zero too. The medium for continuous cultures in 0.1 M phosphate buffer adjusted to pH 6.0 contained, in g L^{-1}: methanol (SCR, China), 150; yeast extract (OXOID, England), 10; peptone (OXOID, England), 20; YNB (Difco, USA), 13.4.

3. Analytical assay

3.1. SDS-PAGE

The protein was fractionated by SDS-PAGE system as described by Blackshear [21] and SDS-PAGE was performed as described by Wu et al. [22].

3.2. Western blotting

Western blotting was performed according to Wu et al. [22]. A rat anti-human IFN-α monoclonal antibody (Abcam, UK) was used as the primary antibody.

3.3. Alcohol oxidase activity

The alcohol oxidase activity was determined according to Suye et al. [23].

3.4. Quantity of cIFN

Quantity of cIFN was determined by scanning the area of each band on reduced SDS-PAGE gels, and then calculating with Image-Master TotalLab software (Amersham Biosciences) using Infergen (Amgen, USA) as a reference.

4. Results

4.1. Effect of the methanol concentration on the specific rates

To find the most appropriate methanol concentration, three experiments were performed with methanol setpoint concentration of 0.25, 0.5, 0.75, or 1.0%, respectively. Because maximum cIFN concentrations were always attained by about 60 h of total induction time, the comparison of cIFN productivity and quality was made within 66-h after induction. The specific cell growth rate, specific methanol consumption rate and specific cIFN production rate were calculated based on Eqs. (1)–(3), respectively. These parameters are displayed in Fig. 1 and summarized in Table 1. Cell grew fastest at 0.75% methanol, followed by 0.5, 0.25 and 1.0% (Fig. 1A), and a typical growth inhibition profile was observed at methanol concentration of 1.0%. Methanol consumption rate was the highest at 1% methanol during initial 6 h induction (Fig. 1B), reached 0.12 g g^{-1} h^{-1}. After that the methanol consumption rate decreased gradually and was close to those induced at other methanol concentrations. Since methanol acts both as carbon source and as inducer for the AOX promoter, increasing methanol consumption rate should help cell growth and cIFN accumulation. However, our results showed that the specific growth rate at 1% methanol was lower than that of at 0.75% methanol before 6 h induction (Fig. 1A), while specific cIFN production rate was the highest at 1% methanol before 6 h induction (Fig. 1C), indicating that the energy for protein production may compete with that needed for cell growth at initial induction. After the first 6 h induction period, the specific cIFN production rate at 1% methanol decreased quickly and reached the very low value at about 15 h induction (Fig. 1C). This may be attributed to that the methanol concentration is high enough to harm *P. pastoris*, resulting in the decrease of cINF production. Then the specific cIFN production rate (Fig. 1C) showed the similar profile with the specific growth rate (Fig. 1A) during 20–66 h induction. Therefore, it is important to maintain the methanol concentration at a value that is suitable to maintain the activity of cells and control the specific growth rate to optimize product formation at following phase. We have demonstrated that higher induction temperature is harmful to cell viability [17], in this study, we found that the cell