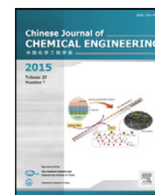
Chinese Journal of Chemical Engineering 23 (2015) 1178–1182

Contents lists available at ScienceDirect

Chinese Journal of Chemical Engineering

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



Biotechnology and Bioengineering

Reduction of foaming and enhancement of ascomycin production in rational *Streptomyces hygroscopicus* fermentation☆

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A R T I C L E I N F O

Article history:
Received 16 December 2013
Received in revised form 26 March 2014
Accepted 26 April 2014
Available online 2 April 2015

Keywords:
Acetate accumulation
Fermentation optimization
Ascomycin
Foaming
Oxygen deficiency

A B S T R A C T

Foaming reduces the working volume and limits the biosynthesis of macrolide immunosuppressant ascomycin (FK520) in the batch fermentation process of *Streptomyces hygroscopicus* FS-35 in a 7.5 L bioreactor. To find the relation between FK520 production and foaming, effects of 10 fermentation parameters including organic acids and membrane permeability were investigated. The results suggest that acetate accumulation caused by short period oxygen deficiency and fast consumption of glucose is the reason for increased foaming and declined FK520 production. Therefore, a fed-batch fermentation strategy was developed to reduce the accumulation of acetate. After optimization, the maximum acetate concentration dropped from 320 $mg\cdot L^{-1}$ to 157 $mg\cdot L^{-1}$, decreased by 50.8%, and the maximum foam height reduced from 5.32 cm to 3.74 cm, decreased by 29.7%, while the maximum FK520 production increased from 375 $mg\cdot L^{-1}$ to 421 $mg\cdot L^{-1}$, improved by 12%.
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1. Introduction

Foaming is harmful to fermentation processes [1,2]: lowering the working volume of bioreactor and leading to overflow, contamination of fermentation broth, and even strain mutations. Traditional consideration for foaming control is to improve fermentation equipment or add chemical antifoams, so various new antifoams and bioreactors have been developed [1,3–6]. These methods suppress foaming effectively but bring some negative effects on fermentation process, such as increasing fermentation costs, suppressing cell growth [1,7], and making the downstream separation of targeted compounds more difficult [2,8]. To understand the foaming mechanism in fermentation process, more researchers focus on the effects of cell metabolism and phenotypes on foaming. For example, Patnaik *et al.* [9] found that the efflux of L-tyr facilitated foam formation, Brey *et al.* [10] explored the effect of proteinase A on foaming, and Shimoi *et al.* [11] knocked out the awa1 gene of sake yeast, then efficiently controlled the foaming during fermentation. According to these investigations, the metabolic activity of cells is important for foaming behavior, so it is necessary to analyze the components in the fermentation broth to reveal the metabolic changes of strains with foaming behavior. It has been proved [12] that acetate produced in cell metabolism could affect the membrane permeability apparently, causing a leakage of intracellular metabolites, which may facilitate foaming [7].

In the fermentation study from shake flask to a 7.5 L bioreactor, ascomycin (FK520) production by *Streptomyces hygroscopicus* FS-35 in the bioreactor was much lower. As the FK520 production rate reduced in the middle stationary phase (96–132 h), the foaming increased. Two traditional approaches were used: installing an antifoaming paddle and adding 4 different antifoams in the fermentation broth separately. Although foaming was effectively controlled, the production of FK520 was not improved. The addition of chemical antifoams even suppressed FK520 production further. Therefore, foaming may reduce the working volume of bioreactor but may not be the direct reason for declined FK520 production in this study. If we know the underlying causes for increasing foaming, it is possible to understand the reasons for declined FK520 production and make corresponding improvement for FK520 biosynthesis.

In present study, dynamic changes of 10 fermentation parameters, including organic acids and membrane permeability, are determined. The effect of each parameter on foaming is assessed. The results suggest that acetate accumulation may be the reason for increased foaming and

☆ Supported by the National Basic Research Program of China (2013CB733600), the Key Program of National Natural Science Foundation of China (21236005), the Natural Science Foundation of Tianjin (12JCZDJC21900) and the Program of Introducing Talents of Discipline to Universities (B06006).
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http://dx.doi.org/10.1016/j.cjche.2014.04.006
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declined FK520 production. Thus a preliminary fed-batch fermentation mode is developed, reducing acetate accumulation and foam height, while improving FK520 production.

2. Materials and Methods

2.1. Strain, mediums, and shake flask culture conditions

FK520 producing strain used in this study was *S. hygroscopicus* FS-35, a mutant strain of *S. hygroscopicus* ATCC 14891 [13]. The slant and seed medium were prepared as reported [13]. The fermentation medium contains ($g\cdot L^{-1}$) starch 20, dextrin 40, yeast powder 7, peptone 5, corn steep liquor 2, K_2HPO_4 0.5, $MnSO_4$ 0.5, $(NH_4)_2SO_4$ 1.5, $CaCO_3$ 1, and $MgSO_4$ 1, as well as Tween 80 0.07% (by volume) and soybean oil 11 $ml\cdot L^{-1}$. The original pH values of fermentation media were maintained at 6.7 by the addition of 5 $mol\cdot L^{-1}$ NaOH. Spore solution (1×10^7 spores$\cdot ml^{-1}$) was added to 250 ml shake flask containing 40 ml seed medium with a 5% (by volume) inoculum, shaking (200 $r\cdot min^{-1}$) at 28 °C for 48 h to obtain the seed.

2.2. Bioreactor culture conditions

The working volume of 7.5 L bioreactor (NBS BioFlo 110, USA) is 4.4 L with a 10% inoculum. The batch fermentation was lasted for 168 h at 28 °C. The aeration rate and stirring speed were obtained from previous optimization experiments. The aeration rate was maintained at 1.5 vvm. Dissolved oxygen (DO) was maintained at 50% in lag phase (0–24 h) and 30% in early log phase (24–48 h), then maintained at 10% by setting the maximum stirring speed at 500 $r\cdot min^{-1}$ during middle and late log phases (48–72 h) to get moderate oxygen supply and avoid cell injury by vigorous stirring [2,6]; in stationary phase (72–168 h), the stirring speed was 400 $r\cdot min^{-1}$ to meet the basic demand of oxygen. No antifoams, acid or base was added in the batch fermentation process.

2.3. Dynamic monitoring of fermentation parameters

Cell density, FK520 concentration, and residual sugar were measured as reported [13]. The average production rate of FK520 in 6 h is calculated by

$$R_{FK520} = (C_2 – C_1)/6 \quad (1)$$

where C_1 and C_2 are the concentration at the beginning and the end of one period, respectively.

DO and pH values were reflected by the detector instantly. Protein concentration in fermentation broth was quantified through Bradford method. Phosphorus was determined according to Fiske and Subbarow [14]. Foaming was measured as follows: 20 ml fresh fermentation broth was added to a 100 ml tube, then aerated at the rate of 50 $ml\cdot min^{-1}$ and stirred with a glass rod for 1 min. The height of foam was measured to evaluate the foaming ability of the fermentation broth.

2.4. HPLC analysis of organic acids and permeability of cell membrane

HPLC method was used to find the changes of organic acids in the fermentation broth. The organic acids were extracted as follows: 3 ml fermentation broth was mixed with 3 ml 6 $mol\cdot L^{-1}$ HCl, reacted in 80 °C water for 20 min, then cooled to room temperature; the supernatant was filtrated through 0.22 μm membrane, follows by a centrifugation at 10000 $\times g$ for 10 min. The organic acids were determined as reported [15].

To determine whether acetate accumulation directly relates to membrane permeability changes during *S. hygroscopicus* fermentation, the extracellular and total FK520 were detected separately. The membrane permeability was measured from 24 h to 168 h according to [13].

2.5. Strategies to improve bioreactor culture conditions

A preliminary fed-batch fermentation mode was established according to the analysis of fermentation parameters in fermentation broth for *S. hygroscopicus* FS-35 batch fermentation in the bioreactor. The carbon source in fermentation medium was changed to 20 $g\cdot L^{-1}$ dextrin. 100 $g\cdot L^{-1}$ glycerol was fed at the rate of 0.1 $ml\cdot min^{-1}$ from 48 h to 144 h. A mixture of 30% oxygen and 70% air was used to supply oxygen from 48 h to 96 h. The stirring and aeration were maintained constant.

In this study, DO and pH values were instant values automatically detected and recorded. Each of the rest experiments was repeated three times. The data shown in the result are their mean value and error bars reflect the standard deviation.

3. Results and Discussion

To describe the process more accurately, the fermentation process was divided into four phases (lag phase, 0–24 h; log phase, 24–72 h; stationary phase, 72–166 h; and decline phase, 168 h later) in this study.

3.1. Relations between fermentation parameters and foaming

To investigate the influences of fermentation parameters on foaming, dynamic changes of cell density, FK520, foam height, pH value, DO value, extracellular protein concentration, residual sugar, and phosphorous of this fermentation process are shown in Fig. 1. In 0–84 h, foam height [Fig. 1(a)] decreased continuously and reached the lowest point at 84 h, so the increased foaming in stationary phase was not caused by components in initial medium; in 84–132 h, foam height increased significantly from 0.62 cm to 5.07 cm; in 132–168 h, it was basically unchanged. As foaming increased, cell density and phosphorus were stable while extracellular protein concentration and residual sugar decreased dramatically, suggesting that cell lysis and phosphorus are not the main reason for foaming, and proteins and carbohydrates are not foaming agents here.

In present study, to control oxygen supply efficiently, the stirring speed was first connected to DO value from 0 h to 72 h, with the lowest and highest stirring speeds at 200 and 500 $r\cdot min^{-1}$, respectively; then the stirring speed was set to 400 $r\cdot min^{-1}$ from 72 h to 168 h to meet the oxygen demand and avoid cell injury. The actual DO value was close to the setting value from 0 h to 48 h, so oxygen supply was sufficient. However, the DO value dropped quickly and less than 10% (the setting value) for more than 12 h (60–72 h), during this period the pH value was 5.08 (60 h), 4.81 (66 h), 4.72 (72 h) and 4.77 (78 h) [Fig. 1(c)]. From the changes of DO and pH, it was noted that the deficiency of oxygen occurred first, then the pH value decreased continuously and maintained at low level for more than 18 h. In this situation, *S. hygroscopicus* FS-35 cells got insufficient oxygen and consumed glucose to produce organic acids such as lactate and acetate [16], which may cause pH dropping in the fermentation broth. Interestingly, foaming occurred and increased [Fig. 1(a)] in early stationary phase (72–96 h) while oxygen was insufficient and the pH value was low. Therefore, the deficiency of oxygen and lower pH value may increase foaming.

3.2. Relations between acetate accumulation, membrane permeability and foaming

pH value is basically determined by medium components and their changes in the fermentation broth. Among them, organic

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