Bioresource Technology 134 (2013) 173–179

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

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

Significant decrease of broth viscosity and glucose consumption in erythromycin fermentation by dynamic regulation of ammonium sulfate and phosphate

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HIGHLIGHTS

- The effects of different nitrogen sources on broth viscosity were evaluated.
- High ammonium sulfate decreased broth viscosity and glucose consumption.
- Partial substitution of soybean meal with inorganic nitrogen and phosphate improved erythromycin A production.

ARTICLE INFO

Article history:
Received 1 January 2013
Received in revised form 8 February 2013
Accepted 9 February 2013
Available online 16 February 2013

Keywords:
Erythromycin
Broth viscosity
Glucose consumption
Ammonium sulfate
Medium modification

ABSTRACT

In this study, the effects of nitrogen sources on broth viscosity and glucose consumption in erythromycin fermentation were investigated. By controlling ammonium sulfate concentration, broth viscosity and glucose consumption were decreased by 18.2% and 61.6%, respectively, whereas erythromycin biosynthesis was little affected. Furthermore, erythromycin A production was increased by 8.7% still with characteristics of low broth viscosity and glucose consumption through the rational regulations of phosphate salt, soybean meal and ammonium sulfate. It was found that ammonium sulfate could effectively control proteinase activity, which was correlated with the utilization of soybean meal as well as cell growth. The pellets formation contributed much to the decrease of broth viscosity. The accumulation of extracellular propionate and succinate under the new regulation strategy indicated that higher propanol consumption might increase the concentration of methylmalonyl-CoA and propionyl-CoA and thus could increase the flux leading to erythromycin A.

1. Introduction

Erythromycins, a group of important broad-spectrum antibiotics, are produced by *Saccharopolyspora erythraea* through industrial fermentation (Mironov et al., 2004). Since erythromycin A was isolated in 1952, erythromycin A production has ascended for 10–13 folds through stain improvement and cultivation optimization (Minas, 2005). Recently, the wide use of erythromycin derivatives (e.g., azithromycin, clarithromycin, and telithromycin) in clinic promoted the efforts to improve erythromycin A production (Chen et al., 2008), and billions of annual sales of erythromycin and its derivatives highlighted the significance of erythromycin industrial fermentation (Chng et al., 2008). In the past decades, high viscosity has remained to be a key challenge in large-scale erythromycin fermentation. High broth viscosity resulted in the problems of high power consumption, oxygen supply limitation and low mixing efficiency, which affected cell metabolism and production (Lara et al., 2006). Furthermore, high broth viscosity led to a decrease of membrane fluxes during separation process (Davies et al., 2000). In addition, the main impurity component erythromycin B was accumulated especially under the condition of oxygen deficiency, which was difficult to be removed from erythromycin product due to its similar structure with erythromycin A (Chen et al., 2009). Generally, dissolved oxygen concentration (DO) was controlled at not less than 40% of air saturation during erythromycin production phase in order to avoid the accumulation of erythromycin B. However, there was still not any effective way to reduce broth viscosity in erythromycin fermentation. Recently, a new approach to reduce broth viscosity was reported by Bhargava et al. (2003a–c) who found that pulsed feeding of the limiting substrate could effectively decrease the broth viscosity with *Aspergilllus*

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http://dx.doi.org/10.1016/j.biortech.2013.02.023

oryzae (Bhargava et al., 2003a,b,c, 2005). So far, the most common approaches to reduce broth viscosity were to add water and/or increase agitation. These methods often lead to either the lower production or the heavier power consumption.

Modification of medium was reported to be an effective way to improve the broth properties. Kanda et al. (2010) reported that the broth viscosity was decreased by 50% with inorganic nitrogen source rather than the conventional organic nitrogen source in industrial-scale production of FR901379 (Kanda et al., 2010). Davies et al. (2000) found that the residual soybean meal in erythromycin fermentation broth had negative effects on the microfiltration characteristics, suggesting that lowering soybean meal concentration was beneficial to the microfiltration process (Davies et al., 2000). Our preliminary experiments showed that the reduction of soybean meal effectively decreased broth viscosity, whereas erythromycin A production was also significantly decreased. Soybean meal and glucose were successfully replaced with biological nitrogen source and molasses (cheaper substrates) to reduce product cost and increase erythromycin A (EI-Enshasy et al., 2008; Zou et al., 2010). However, partial substitution of soybean meal with inorganic nitrogen and inorganic phosphate was not reported yet. Many reports focused on the improvement of erythromycin production by adding (or feeding) easily-metabolized nitrogen sources and modifying the genes involved in erythromycin biosynthesis (Chen et al., 2008; Reeves et al., 2004, 2006, 2007; Wang et al., 2007), but little attention was paid to the relation between broth viscosity and nitrogen source utilization. Hence, with the aim to decrease broth viscosity and glucose consumption, the effects of organic nitrogen source and inorganic nitrogen source on broth viscosity were investigated. Furthermore, the integrated regulations of phosphate source, soybean meal and ammonium sulfate were carried out to increase erythromycin A production still with characteristics of low broth viscosity and glucose consumption.

2. Methods

2.1. Microorganism

The strain used in this study was *S. erythraea* E4 which was kindly provided by Yidu HEC Pharmaceutical Co., Ltd., (Hubei province, China). Spore stocks were stored in 20% (v/v) glycerol solution at −70 °C.

2.2. Experimental design

Primary seed culture was transferred into 15 L fermenter containing 8 L of the secondary seed medium and cultivated for 41 h at 34 °C. Then the secondary seed culture was inoculated into 50 L fermenter containing 30 L of fermentation medium and cultivated at 34 °C for 190 h. The agitated fermenter was equipped with three turbine impellers and devices to monitor and control more than 14 on-line measurable parameters (FUS-50, Shanghai Guoqiang Bioengineering Equipment Co., Ltd. China). DO (dissolved oxygen) was monitored with polarographic DO electrode (Mettler, Toledo) and controlled in 40–60% of air saturation by adjusting agitation and aeration during fermentation. On-line pH was measured with pH electrode (Mettler, Toledo). Glucose was continuously fed and the feed rate was regulated automatically once per hour according to the pH tendency. Propanol was fed once per hour manually and the feed rate was set at a constant value (0.2 g/(L h)).

2.2.1. Experiment-1

The fed-batch fermentations with three different media were conducted simultaneously in parallel with the same seed culture in a 15 L seed fermenter. The original medium (g/L) consisted of starch 35, dextrin 5, soybean meal 30, yeast powder 10, ammonium sulfate 3.0, NaCl 2.0, $CaCO_3$ 7.0, antifoam agent 56 mL. The high yeast powder medium contained 18 g/L yeast powder and the high ammonium sulfate medium contained 8 g/L ammonium sulfate. The other components of the two media were the same to the original medium.

2.2.2. Experiment-2

The fed-batch fermentations under three strategies were carried out. In strategy 1, potassium dihydrogen phosphate was added to high ammonium sulfate medium to 0.2 g/L. In strategy 2, the concentration of soybean meal was decreased from 30 to 20 g/L based on strategy 1. Furthermore, the concentration of ammonium sulfate was decreased from 8 to 6 g/L in strategy 3.

2.3. Determination of process parameters

Fermentation broth was centrifuged at 4000*g* for 10 min and PMV (packed mycelium volume) was the percentage of the precipitation (v/v). The supernatant was collected for analysis. The concentration of amino nitrogen was detected by formol titration method (Zou et al., 2009b). Glucose was detected with glucose-oxidase kit (Shanghai Kexin biotechnology Co., Ltd.). Soluble phosphate was detected by Molybdenum blue method (Yuan et al., 2004). Ammonium ion was detected by Berthelot color reaction method (Ma et al., 2006).

The concentration of erythromycin was measured by the modified colorimetric method (Zou et al., 2009a). The components of erythromycin was analyzed by HPLC (JASCO PU2080, Japan) with acetonitrile and 0.025 mol/L potassium dihydrogen phosphate (60:40, v/v) as the mobile phase at 0.9 mL/min (column temperature 30 °C). The concentration of amino acids and organic acids in the broth were measured with HPLC (Zou et al., 2009b).

Proteinase activity was measured as described by Zou et al. (2011) and broth viscosity was measured using Brookfield DV-II + - PRO rheometer with LV3 rotor at 34 °C. The contents of oxygen and carbon dioxide in the exhaust gas were determined by a process mass spectrometer (MAX300-LG, Extrel, USA). Oxygen uptake rate (OUR) was calculated on-line (Liang et al., 2011).

3. Results and discussion

3.1. Effects of nitrogen sources on erythromycin fermentation

Fig. 1a showed that PMV under high yeast powder medium was comparable to that under the original medium. However, PMV under high ammonium sulfate medium was only 62.7% of that under the original medium. Fig. 1b indicated that high ammonium sulfate (8 g/L) led to 18.2% decrease of broth viscosity compared with that under the original medium. The images of mycelia revealed that some pellets were formed after 23 h under high ammonium sulfate medium. But no pellets were found under high yeast powder medium and the original medium. The similar mycelial pellets were also observed in erythromycin seed cultivation (Zou et al., 2011) and in erythromycin fermentation with chemical medium (Potvin and Peringer, 1994). Mycelial pellets formation often occurred in the transition from trophophase to idiophase in antibiotic fermentation and was dependent on the process conditions. Reimann et al. (2011) demonstrated that cell aggregation during continuous culture with immobilized cell was induced by feeding medium with $CaCl_2$, high sugar concentration and steep pH gradients. In our experiments, the possible explaination of pellets formation was that high ammonium sulfate decreased proteinase acitvity and so less soybean meal was degraded and utilized (Fig. 1d), which decreased the PMV and OUR (Fig. 1a and c). Zou et al. (2011) also