ELSEVIER

Biochemical Engineering Journal 40 (2008) 460–464

Biochemical Engineering Journal

www.elsevier.com/locate/bej

Production of hyaluronic acid by repeated batch fermentation

Wei-Chih Huang [a], Shu-Jen Chen [b], Teh-Liang Chen [a,*]

[a] *Department of Chemical Engineering, National Cheng Kung University, Tainan 701, Taiwan*
[b] *Department of Chemical and Material Engineering, National Kaohsiung University of Applied Sciences, Kaohsiung 807, Taiwan*

Received 26 September 2007; received in revised form 9 December 2007; accepted 11 January 2008

Abstract

The production of hyaluronic acid (HA) by *Streptococcus zooepidemicus* with repeated batch fermentation has been investigated. It was found that, with conventional operation, both maximal specific growth rate (μ_m) and specific HA productivity ($Y_{P/X}$) decreased with increasing seed volume, suggesting that there exist some inhibitors in the broth. The removal of liquid in the seed was first attempted by installing nonwoven fabrics (NWF) in the fermentor to retain some of the cells when draining the broth. However, this resulted in a loss of HA productivity, which in turn was attributed to the growth of a sticky, non-HA-producing mutant on the NWF. Using an external cartridge filter to partially retain the cells, followed by back-washing the filter with fresh medium for seeding, μ_m and $Y_{P/X}$ could be maintained successfully at their batch levels during the repeated cycles. In an operation that seeded 31% cell, the volumetric production rate of the repeated batch culture (0.59 g HA L^{-1} h^{-1}) was found to be 2.5-fold of the batch culture (0.24 g HA L^{-1} h^{-1}).

Keywords: Fermentation; Batch processing; Submerged culture; Biosynthesis; Hyaluronic acid; *Streptococcus zooepidemicus*

1. Introduction

Hyaluronic acid (HA) is a mucopolysaccharide consisting of alternating *N*-acetyl-D-glucosamine and D-glucuronic acid, which has received great interest in the medical and cosmetic markets [1–3]. In recent years, HA from microbial fermentation, rather than extraction from animal sources, is receiving increased attention for avoidance of cross-species viral infection.

HA fermentations have been mostly by *Streptococci* spp., where HA is a capsular biopolymer shedding to the medium [4]. To aid the competence of the fermentation process, the development of an economical process for mass production is necessary. In the literature, most reports on HA fermentation have been based on batch culture [5–9]. A major drawback of batch culture is a long turnaround time, which greatly decreases the volumetric production rate, and this in turn results in a high fixed cost per unit product.

There are two approaches that could be employed to increase the volumetric production rate. One is to increase HA concentration in the fermentor, which can be accomplished either through high-cell-density fermentation or through a high-yield strain. However, we found that, as the HA concentration higher than 4 g/L, the broth became too viscous to achieve efficient agitation and aeration; as a result, the benefit of high HA concentration is worn down by the defect of low efficiency of HA synthesis. The other approach is to skip the turnaround phase by using continuous culture. Continuous culture could also offer two benefits. First, cell growth can be maintained at the exponential phase, so that the excretion of cell wall proteins at the stationary phase [4] can be avoided. Second, the cells can be controlled to grow at a lower specific growth rate, which might result in HA of higher molecular weight [5]. However, continuous culture has its inherent defect—low efficiency of substrate utilization; and this is the primary reason why continuous culture is seldom used in a commercial process. Another adverse factor concerned is that the efficiency of HA production would decrease during prolonged operation [10,11].

Accordingly, repeated batch culture seems a promising mode for HA fermentation, because it skips the turnaround time and the lag phase. Unfortunately, no report on such subject has been published to date. The aim of this work was to explore the problems encountered during HA production by repeated batch culture, to rationalize the problems, and to propose a feasible operation strategy.

* Corresponding author. Tel.: +886 6 2757575x62660; fax: +886 6 2344496.
E-mail address: t62660@mail.ncku.edu.tw (T.-L. Chen).

1369-703X/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bej.2008.01.021

2. Materials and methods

S. zooepidemicus ATCC 39920 was used as the HA producer. The cells were maintained at −30 °C in 50% (v/v) glycerol. The isolation of a pure culture was achieved by streaking onto TSB agar plates, which contained (per liter) 17 g of tryptone, 3 g of soytone, 2.5 g of glucose, 2.5 g of K_2HPO_4, 5 g of NaCl, and 15 g of agar. Precultures were prepared in a 500-mL shaking flask, with 100 mL of the TSB medium, at 37 °C for 12 h.

HA production was performed in a 3 L fermentor (MDL-300, B. E. Marubishi, Japan), with a working volume of 2 L. The medium comprised of (per liter) 20 g of glucose, 10 g of yeast extract, 1.7 g of tryptone, 0.3 g of soytone, 2 g of NaCl, 2.5 g of K_2HPO_4 and 1.5 g of $MgSO_4{\cdot}7H_2O$. The fermentor was inoculated with 5% (v/v) preculture, and operated at 37 °C and pH 7.0 (with 5N NaOH). The aeration rate was 1 vvm, and the concentration of dissolved oxygen (DO) was controlled above 10% air saturation by mixing the inlet air with oxygen when necessary. Agitation was achieved with a gate impeller similar to that of Hiruta et al. [12], and an agitator speed of 400 rpm was used.

In repeated batch culture, the end of each cycle was determined when there was a rise in DO level; it is a sign that the cells cease to grow. The medium concentration was adjusted according to the amount of cell in the seed, such that the repeated cycles were supposedly to achieve the same cell concentration as the batch level. The nonwoven fabric (NWF) used to retain the cells consists of a polypropylene core and a polyethylene surface. The fabric has a unit weight of 60 g/m^2 and a specific gravity of 0.90. To equip the NWF in the fermentor, three pieces of the NWF (4 cm × 17 cm) were tied around the three baffles, unless otherwise noted. The cartridge filter used to retain the cells outside the fermentor had a diameter of 3 cm and a length of 30 cm, where the NWF was used as the filter medium. After draining the broth, the retained cells were released by back-washing with fresh medium to seed the fermentor. The back-wash procedure was ended with air-purge to avoid microbial growth in the cartridge. The amount of cells in the seed was adjusted by the amount of the NWF inside the filter as well as the number of the cartridges.

Cell concentration was measured from optical density (OD) of the broth at 660 nm using a spectrophotometer. Owing to a change in cell morphology after entry into the stationary phase [13], the correlations of OD with dry cell weight (DCW) were DCW (g/L) = 0.399 × OD − 0.003 for the exponential growth phase, and DCW (g/L) = 0.456 × OD − 0.012 for the stationary phase. HA concentration was determined by the carbazole method [14], in which the optical density was measured at 525 nm and D-glucuronic acid was used as the standard.

3. Results and discussion

The preliminary results of batch culture showed that the cells entered the stationary phase at 9 h, with a cell concentration of 3.2 g/L and a HA concentration of 2.3 g/L; in addition, the formation of HA was a growth-associated matter (data can also be seen in Fig. 1). The maximal specific growth rate (μ_m) was

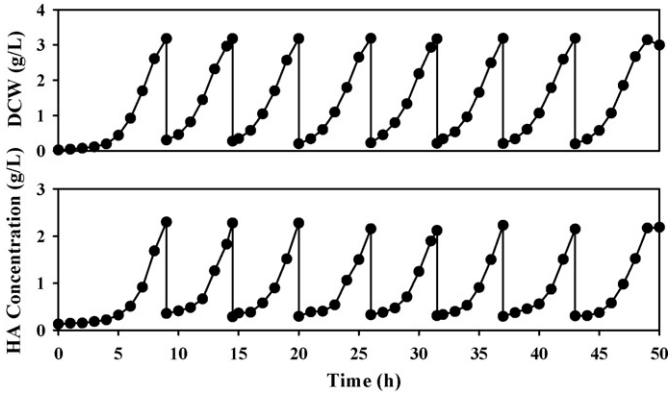

Fig. 1. HA production by repeated batch culture, using 5% broth as the seed. Symbols: (●) cell density and (◯) HA concentration.

estimated to be 0.59 h^{-1}; and the specific productivity ($Y_{P/X}$), defined as the ratio of total increment of HA to total increment of cell mass, was 0.72 g HA/g cell. μ_m together with $Y_{P/X}$ are good indicators for assessing the efficiency of the repeated batch culture.

The repeated batch culture was first performed using a seed of 5% broth. As can be seen in Fig. 1, both cell and HA concentrations could be maintained as in the batch culture for at least seven repeated cycles. However, μ_m and $Y_{P/X}$ are reduced to 0.51 h^{-1} and 0.62 g HA/g cell, respectively. To increase the volumetric production rate, volumes of the seed were further increased to 10, 20, 30 and 40% of the broth, respectively. Fig. 2 depicts the profiles of using 40% broth as the seed. Unfortunately, although cell concentration could be maintained, HA production was reduced gradually to 1.3 g/L. The influence of seed volume in the repeated batch culture is summarized in Fig. 3, which shows both μ_m and $Y_{P/X}$ decreased with increasing seed volume. It thus suggests that in the broth there exist some inhibitors that hinder cell growth and HA synthesis.

It might also be possible that the inhibitors hinder cell growth, and a smaller specific growth rate results in a smaller specific HA productivity; in other words, the inhibitors might not function directly on HA synthesis. We therefore performed a fed-batch culture that was operated at a specific growth rate of 0.30 h^{-1},

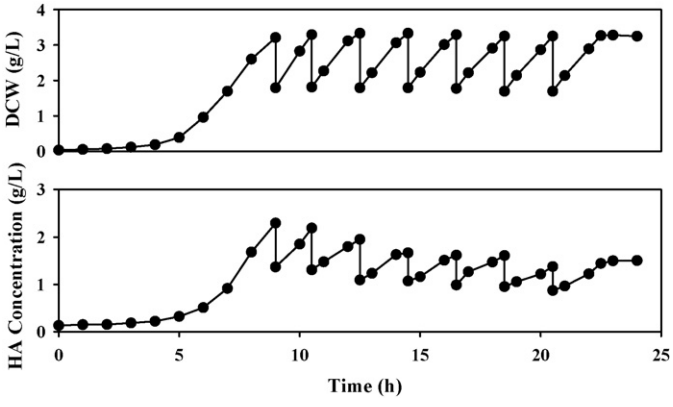

Fig. 2. HA production by repeated batch culture, using 40% broth as the seed. Symbols: (●) cell density and (◯) HA concentration.