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A novel control scheme for inducing angiostatin-human IgG fusion protein production using recombinant CHO cells in a oscillating bioreactor

Ing-Kae Wang^{a,**}, Sing-Ying Hsieh^a, King-Ming Chang^a, Yu-Chi Wang^a,
Andy Chu^a, Shyh-Yu Shaw^b, Jung-Jung Ou^b, Lewis Ho^{c,*}

^a *Cesco Bioengineering, Co., Ltd., Rm. 537, Bldg. 53, #195, Sec. 4, Chung-Hsing Rd., Hsin-Chu, Taiwan*

^b *Department of Chemistry, College of Science, National Cheng Kung University, No. 1, Ta-Hsueh Road, 701, Taiwan*

^c *IDL Bioservices, LLC., 46 Twin Lakes Dr., Waterford, CT 06385, USA*

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Abstract

In this study, a novel control scheme for inducing protein production using a recombinant CHO cell line in a BelloCell® bioreactor was developed. This control scheme was applied in a simple regular semi-batch process. Production of angiostatin-human IgG fusion protein in a suspension recombinant CHO cell culture and a protein-free medium was used for this study.

The bottom holding time (BH) was the sole operating variable to control the exposure time of the cells immobilized on the carriers to the air and allow the nutrient remained on the liquid film of the carriers to be consumed to a threshold level so that the cells can be arrested and promoted for protein production. In the cell cultures with various BH (1.5–90 min), final cell densities of $1.6\text{--}4.0 \times 10^9$ have been obtained in 20 days while total angiostatin-human IgG production of 228–388 mg have been harvested. In general, low BH will minimize the nutrient limitation and favor the cell growth, while high BH will restrict the nutrient and promote the production in this type of non-growth associated production systems. It was found that specific production rate was generally inversely proportional to the specific growth rate. In this case of study, BH of 30 and 60 min were found to be about 72% better than BH of 1.5 min and 35% better than BH of 9 and 90 min in term of the total angiostatin-human IgG production. In comparison to a conventional spinner flask study, a 3.8-fold increase of the total angiostatin-human IgG production was realized in a 35-day culture. This study illustrated that a simple method of using BH in a semi-batch process can effectively control the apparent nutrient concentration to the cells, and thus regulate the cell growth and protein production in a novel oscillating bioreactor.

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* Corresponding author. Fax: +1 775 766 1971.

** Corresponding author.

E-mail address: lewisho@gmail.com (L. Ho).

1. Introduction

Cell culture technologies play essential roles in the production of recombinant proteins. Mammalian cell cultures are used extensively to produce proteins for therapeutic use because of their ability to perform post-translational modifications, including glycosylation (Gawlitsek et al., 1995; Goochee et al., 1991). The Chinese hamster ovary (CHO) cell line is one of the most widely used mammalian production cell lines. Several cultivation processes have been commonly developed for mass production using CHO cells in bioreactor including batch, fed-batch, and perfusion processes (Ho et al., 2004b).

The biosynthesis of secondary metabolite production in the microbial culture is often occurred by the cells entering the production phase at a special growth rate rather than association with the maximal growth rate (Bu'Lock, 1965, 1979). Similarly, many protein productions in cell cultures occur in the production phase where the cells are arresting or slower specific growth rate instead of the maximal specific growth phase (Mazur et al., 1999). Batch process is one of the most widely used methods for large-scale protein production, because it is relatively simple, reproducible and easier to validate. However, it is difficult to control the nutrient concentration and retain a desirable growth rate as well as a long production phase in a batch process. Therefore, fed-batch process is the most commonly adopted in industry because it is able to control the nutrient concentration and growth rate by slowly feeding the nutrient during the culture (Bibila and Robinson, 1995; Ogata et al., 1994; Zhou et al., 1997). More sophisticatedly Fussenegger et al. (1997) and Mazur et al. (1999) applied a controlled proliferation technology by implementing a multicistronic expression genes to control the non-productive growth phase and proliferation inhibited production phase to improve the production in a batch process.

However, both batch (Hayter et al., 1991) and fed-batch (Duval et al., 1992; Xie and Wang, 1994) processes have a common problem of waste accumulation during the culture, which would be toxic and result in the death of cells. Because of this problem the perfusion process (Buntemeyer et al., 1992) was developed. It can not only control the nutrient concentration and growth rate as a fed-batch process, but also continuously remove the wastes during the culture. Thus, the

life of culture can be substantially prolonged. However, perfusion process requires special bioreactors which are capable of retaining the cells while constantly withdrawing the spent broth. BelloCell[®] bioreactor (Cesco Bioengineering Co.) is one of the simplest devices for this application. The cells are immobilized on the matrix inside of bioreactor, and thus conveniently allow it to run as a perfusion mode or a semi-batch mode with repeated medium exchanges. However, in order to achieve a desirable nutrient concentration for regulation of growth and production, it requires careful in-process monitoring and control. In this study, we have developed a simple method of running a semi-batch process with repeated medium exchanges without requirement of stringent control. Selection of BH is the only key operating variable for this method. In this study the cultivation of a recombinant CHO cells for production of angiostatin-human IgG (angiostatin-hIgG) fusion protein was used to illustrate the effectiveness of this method.

2. Materials and methods

2.1. Cell culture

Angiostatin-human IgG fusion protein gene was cloned and expressed in CHO K1-4 (Du, 2001) which was grown in HyQ PF-CHO MPS protein-free medium (Hyclone) containing 1% penicillin/streptomycin/amphotericin (GIBCO) and 200 nM methotrexate (MTX) (Sigma). Cells were incubated at 37 °C with 5% CO₂ and 70% humidity.

2.2. BelloCell[®]-500 and BelloStage[®]-3000

BelloCell[®]-500 bioreactor, a disposable bioreactor produced by Cesco Bioengineering Co., Ltd., has been described and used previously by Hu et al. (2003), Ho et al. (2004b) and Chen et al. (2005) for cultivation of HEK293, BHK and insect cells. The bioreactor bottle contains two chambers and holds 500 ml of medium. The upper chamber contains 6.5 g of BioNOC[®] II matrix (with surface area of about 2000 cm² g⁻¹) and the lower one is a compressible bellow. The bottle is compressed and expanded by BelloStage[®] 3000, a control console as described previously, to control the up/down speed of compression/expansion on the bel-

low of bioreactor bottle. It can also control the time span at the bottom position (full expansion), which is referred to as bottom holding time (BH). The full expansion position would allow the matrix to expose fully to the air.

2.3. BelloCell[®]-500 operation

As described previously by Hu et al. (2003) and Ho et al. (2004b) for the operation of BelloCell[®]-500, the bottle was filled with 500 ml HyQ PF-CHO MPS culture medium containing 200 nM MTX, 5 g l⁻¹ glucose and 4 mM glutamine and inoculated with 2.0×10^5 sus-

pension cells ml. After the cells were >95% attached to the matrix in 3–5 h, the BelloStage[®] was set at up/down speed of 1.5 mm s⁻¹ and various BH of 1.5, 9, 30, 60 and 90 min. During the culture, pH, residual glucose and glutamine concentration and other metabolites were monitored each day. When glucose concentration below 2.0 g l⁻¹ or glutamine concentration below 0.50 mM, the culture was replenished by a fresh medium. pH was controlled by % CO₂ of the incubator and/or increasing NaHCO₃ concentration in the fresh medium. It can also be controlled by replenishing the medium as it dropped before 6.9. For convenience of operation in this study, the cultures were replenished

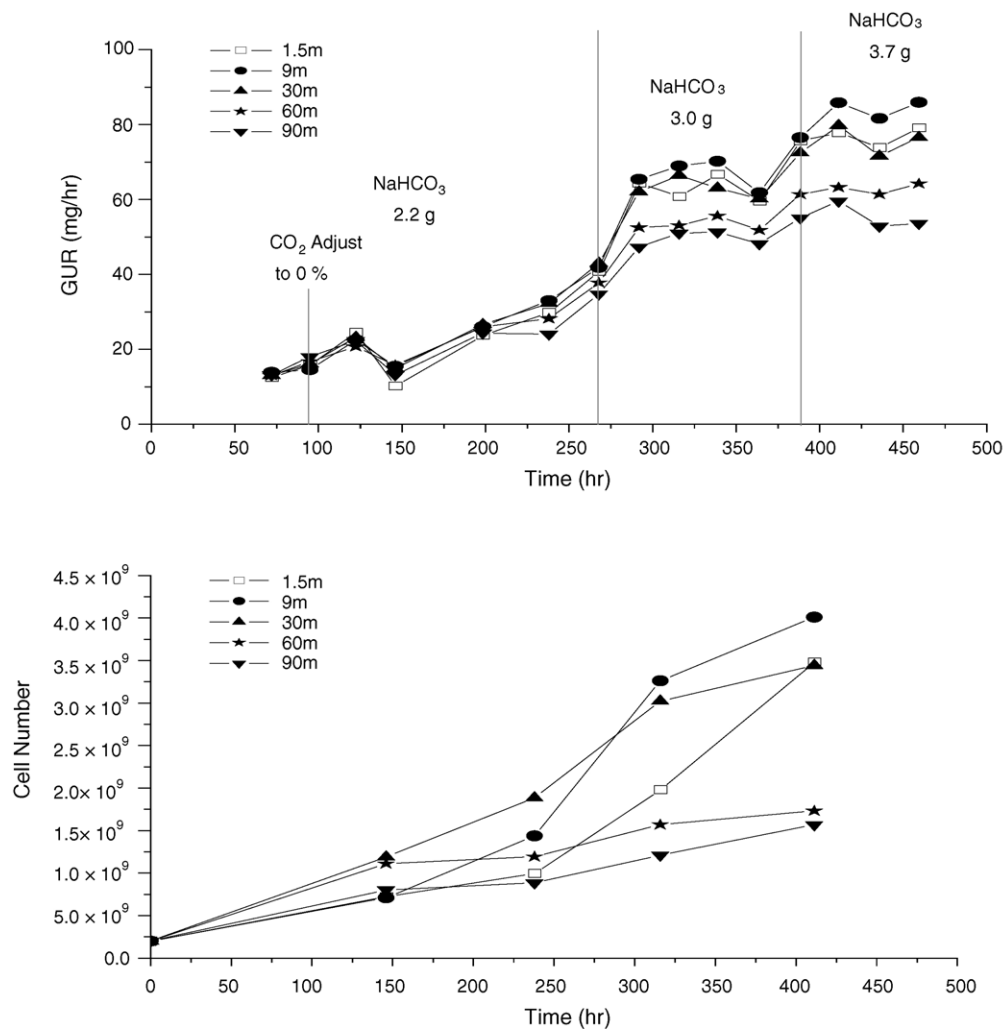


Fig. 1. GUR and cell number profiles for various BH runs.

with fresh medium once a day from the 3rd day until the end of run. NaHCO_3 concentration had been increased from 2.2 up to 3.7 g l^{-1} during the run.

2.4. Spinner flask operation

Prepare one sterilized 1000-ml spinner flask and inoculate with 4.0×10^5 suspend cells ml^{-1} in 500 ml HyQ PF-CHO MPS culture medium containing 200 nM MTX. Culture at 60 rpm, 37°C for 3 days. Then replaced one-fourth medium (125 ml) of the flask every 24 h until the end of run.

2.5. Analytical methods

pH, glucose, lactate, glutamine, glutamate and ammonia concentrations in the culture supernatant were measured offline using a NOVA bioprofile 100 biochemical analyzer (Nova Biomedical Corporation, Waltham, MA).

Angiostatin-hIgG protein of CHO secretion was detected with Sandwich ELISA Kit (Bethyl laboratories Inc., Montgomery, TX) in the supernatant. Briefly, ELISA plates were coated with anti-human IgG pAb ($100 \mu\text{l}$ of $10 \mu\text{g/ml}$) for 1 h at room temperature and

blocked with 50 mM Tris buffer saline/1% BSA (blocking buffer) for 30 min at room temperature. Culture supernatants ($100 \mu\text{l}$) were added and incubated at room temperature for 1 h. Wash each well with washing solution (50 mM Tris buffer saline pH 8.0/0.05% Tween 20) five times, add $100 \mu\text{l}$ of HRP conjugated of anti-human IgG pAb and incubate for 1 h at room temperature. After washed with washing solution for five times, $100 \mu\text{l}$ of TMB solution with 0.1% H_2O_2 were applied for 10 min at room temperature. The reaction was stopped with $100 \mu\text{l}$ of 2 M H_2SO_4 and the absorbance was measured at 450 nm. The concentration of angiostatin-hIgG protein was calibrated by a standard angiostatin-hIgG protein, which was prepared by purification using an affinity column.

For cell number, a crystal violet dye (CVD) nucleus staining method was used. Six carrier chips were sampled from different locations of the bottle and placed in three 1.5 ml Eppendorf vial with two carrier chips in each vial. 1.0 ml CVD reagent was added. Then vortex and incubate at 37°C for 20 min. Repeat three times to ensure that the cell membrane ruptured and the nucleus released from the disks. Then used the hemocytometer to do the nucleus count, from which the cell number was attained.

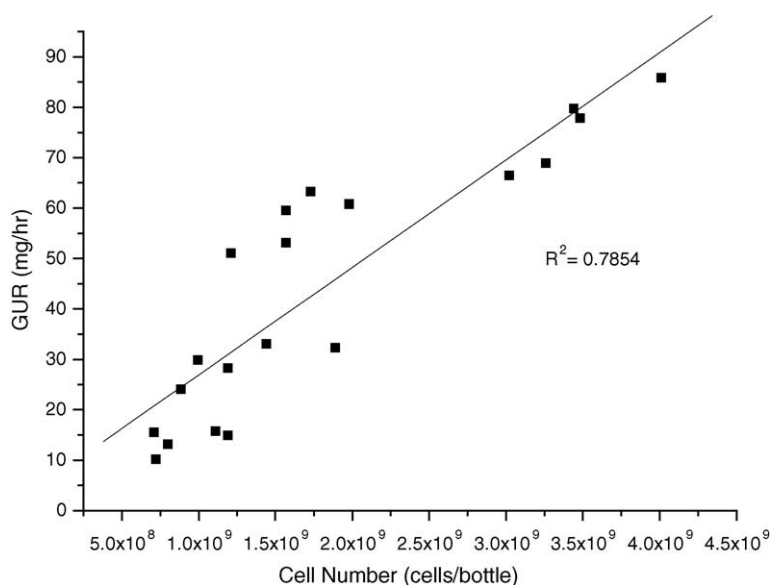


Fig. 2. Correlation between GUR and cell number by CVD method.

3. Results and discussion

In Fig. 1 is shown the glucose uptake rate (GUR) and cell density profiles for the 20-day cultures using various BH. It is known (Miller et al., 1988; Portner et al., 1994) that GUR correlates well with cell density in the cell culture and often is used to estimate the cell density. Since CVD nucleus staining method for cell number may be more subject to manual errors, the data obtained may not be as accurate as that GUR data by glucose assay. However, they are generally in agreement as shown in Fig. 2 with R -value of 0.785.

Fig. 3 shows the pH, glutamine and lactate concentration profiles of these cultures. It shows that all those basic metabolite concentrations in the BelloCell[®] have been reasonably controlled within generally desirable ranges for all running conditions as suggested in the literature (Ho et al., 2004a). Glucose concentration of

the bulk medium was controlled between 1 and 5 g l⁻¹s throughout the run, which was not limiting to the cells. However, the actual glucose concentration available to the cells in the surrounding thin liquid film generated by various BH control was significantly lower and different from that in the bulk medium.

Figs. 4 and 5 show the profiles of specific growth and production rates for various BH. The specific rates were simply calculated as the rate of cell density and product concentration changes per an average cell density between two data points. The general problem associated with numerical differentiation and compounding effect of assay error would result in scattered data as expected. However, the profiles of specific rates shown have provided clear indication and relationship as expected in this study. In Figs. 1 and 4 significant differences of growth and specific growth rates were noticed after the cell density reached 1–1.5 × 10⁹ for various

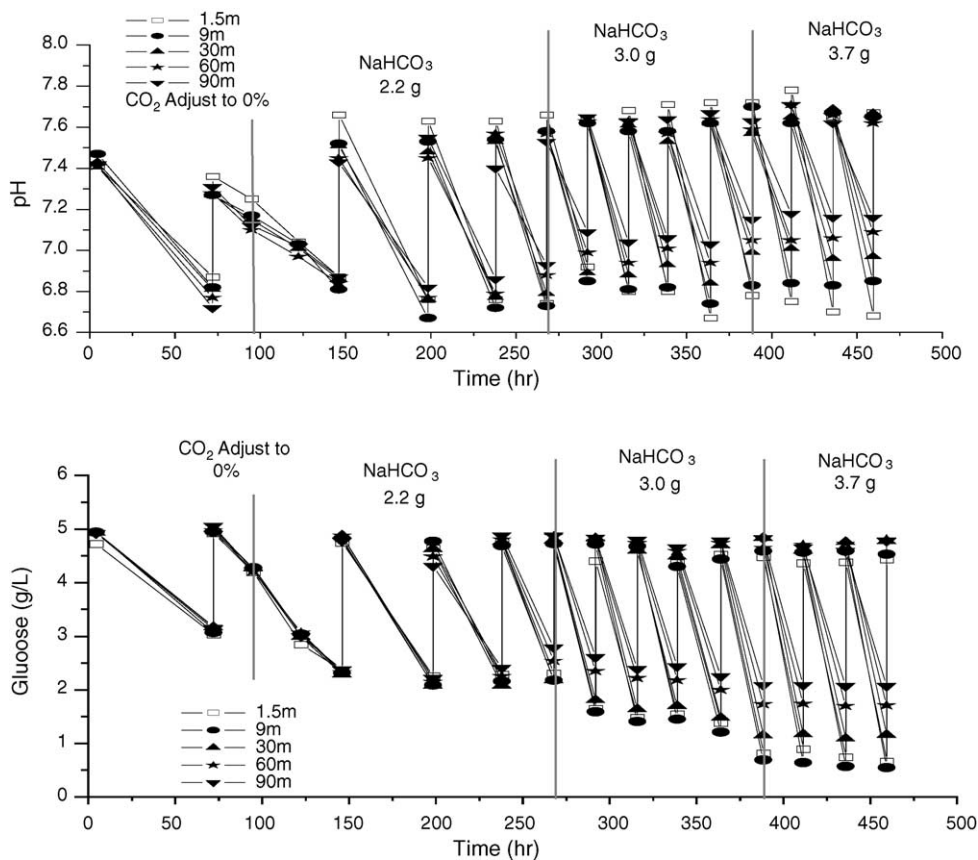


Fig. 3. pH, glucose, glutamine, ammonium and lactate profiles for various BH runs.

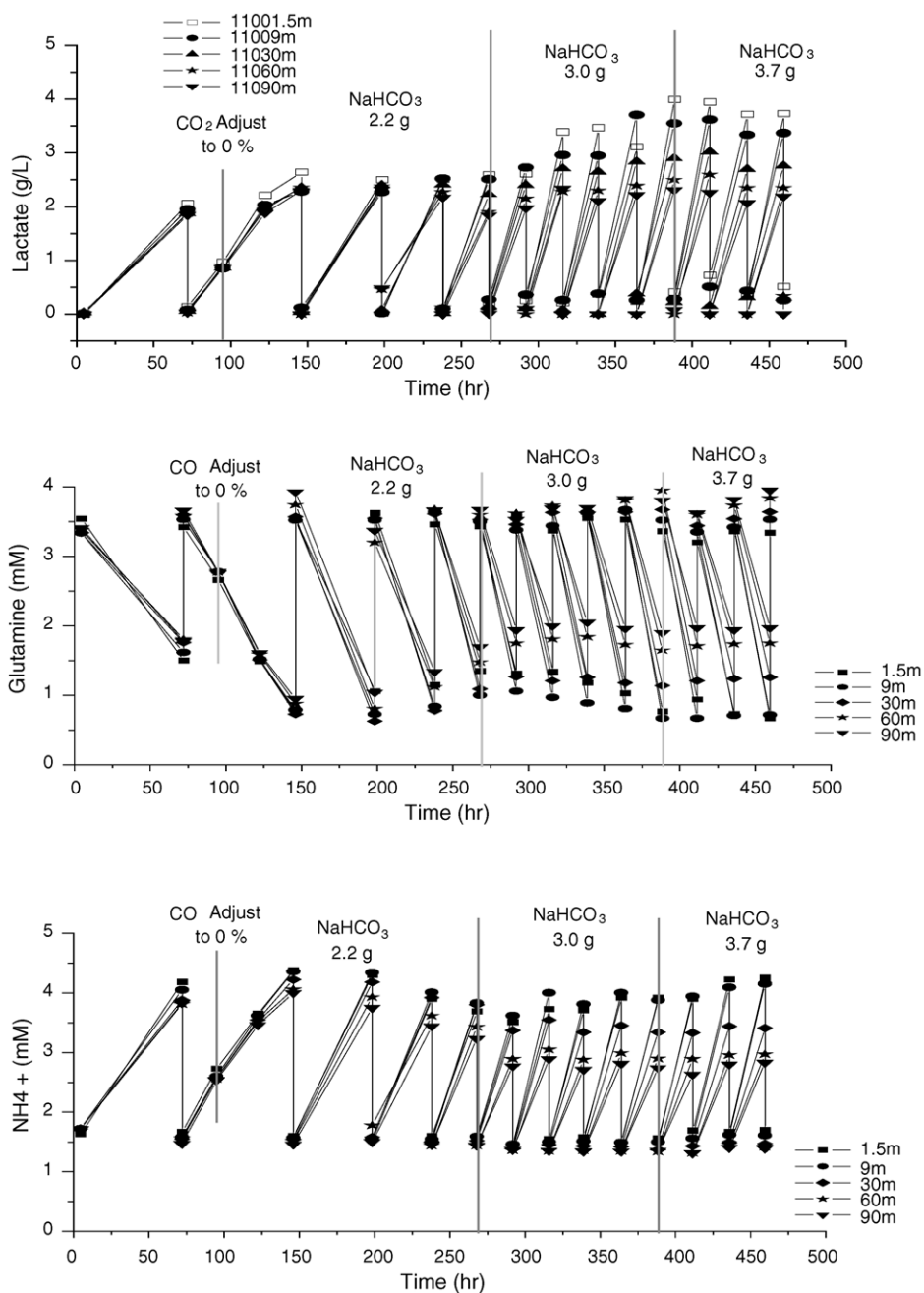


Fig. 3. (Continued).

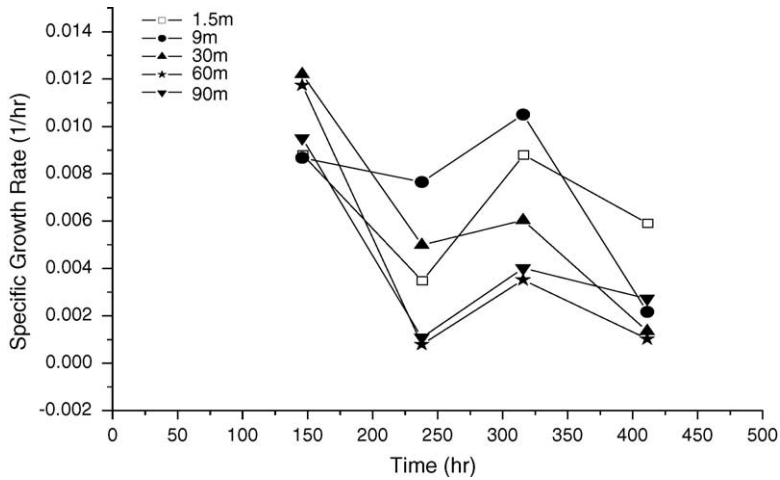


Fig. 4. Specific growth rate profiles of various BH runs.

BH. It indicates that the nutrient concentration of the liquid film on the carriers available to the cells more frequently became limiting as the BH increased and the growth was thus restricted. The specific production rate was plotted against specific growth rate as shown in Fig. 6. The pattern reveals that specific production rate was inversely proportional to specific growth rate for this specific system. Therefore, there exists an optimal specific growth rate associated with a specific production rate for a maximum total production.

Fig. 7 shows the angiostatin-human IgG concentration profiles during the course of the culture. Since the same amount of medium (500 ml) was exchanged at each sampling point for all BH studies, the product collected was proportional to the concentration. Fig. 8 shows the accumulated product profiles for various BH runs. In term of the total angiostatin-human IgG production, BH of 30 and 60 min were shown to be about 72% better than BH of 1.5 min and 35% better than BH of 9 and 90 min. Apparently there exists an optimal

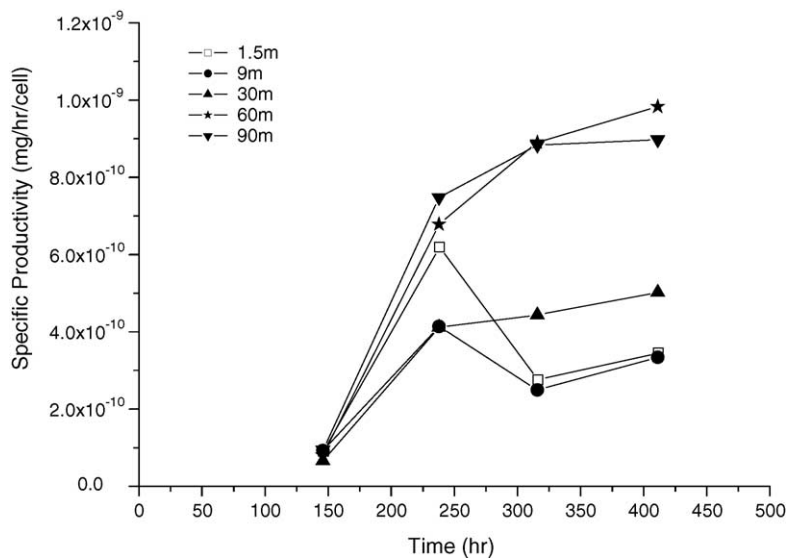


Fig. 5. Specific production rate profiles of various BH runs.

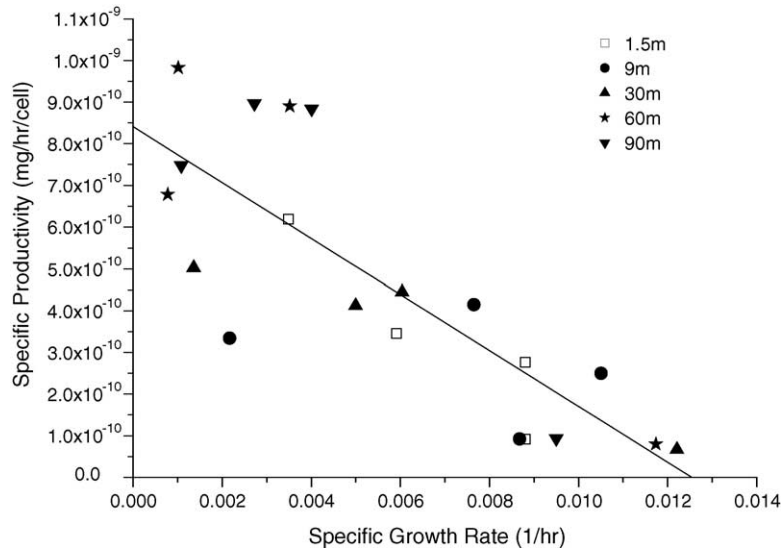


Fig. 6. Relationship between specific growth and production rates for various BH runs.

BH between 30 and 60 min, or theoretically an optimal time-varied BH profile along the course of culture. No effort was made in this study to seek for the optimum condition for this specific culture system. The primary objective of this study was to demonstrate the effect of BH on the specific growth and production rates. The similar effect can be achieved by a perfusion process to control the nutrient concentration at a desirable lim-

iting level. However, it is much more complicated and required significantly more effort than that required by this simple control scheme presented in this study.

In Fig. 9 is shown the cell density and product concentration profiles of a 35-day culture in a BelloCell[®] bioreactor and a spinner flask bioreactor under similar operating conditions. Both bioreactors had the same volume capacity (500 ml) and used the same amount

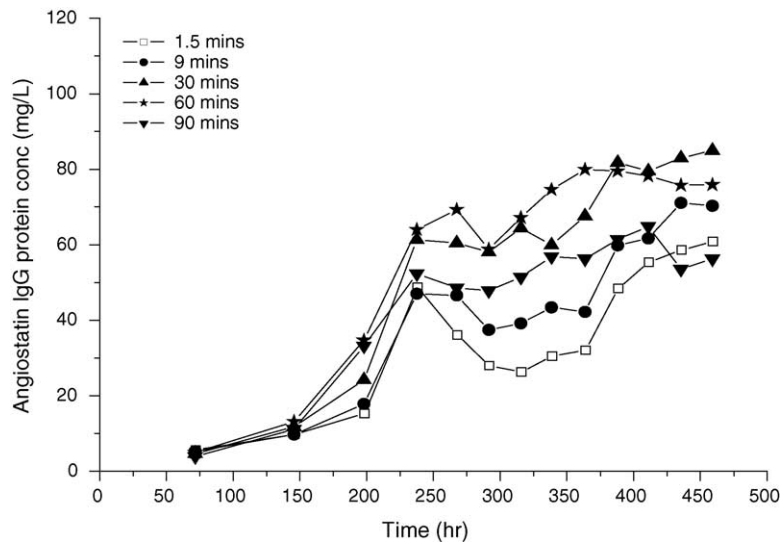


Fig. 7. Angiotstatin-human IgG protein concentration profiles for various BH runs.

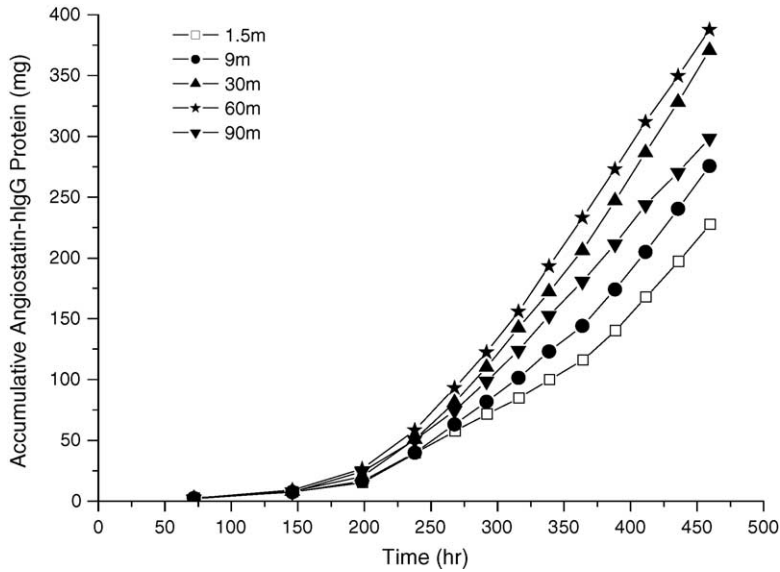


Fig. 8. Accumulative product profiles for various BH runs.

of inoculum (2.0×10^8 cells). Fig. 10 compares the profiles of accumulated product produced by BH of 30 min in a BelloCell[®] versus that in a spinner flask in a 35-day culture along with the accumulated volume of medium harvested for the product in both cases. It reveals that BelloCell[®]-500 could achieve more than 3.8-fold increase of angiostatin-hIgG production than a spinner flask. It was noticed that the cell density pro-

file of the spinner flask remained relatively constant throughout most of the run because the process was run under a semi-continuous mode where cells were not immobilized and removed partially from the flask as it was replaced daily by fresh medium. Wu (2003) used the identical cell line, medium and procedure as our study in a spinner flask except retaining the cells by settlement before partial medium was replaced each

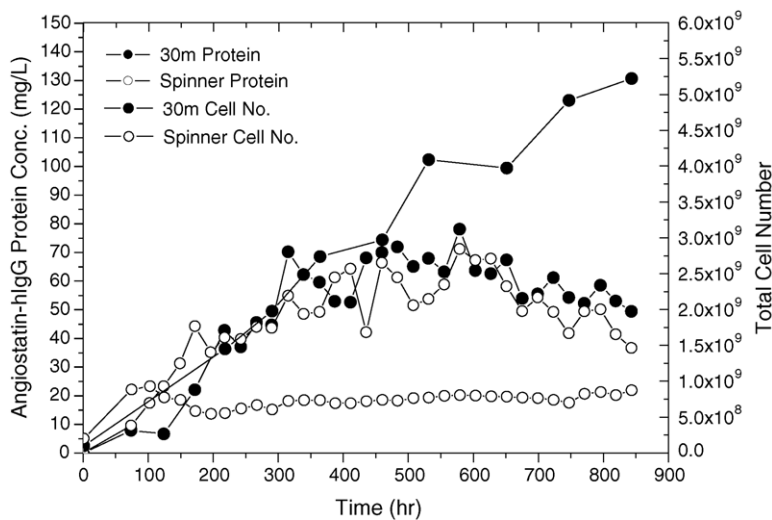


Fig. 9. Cell number and product concentration profiles of the 35-day cultures of a BelloCell[®]-500 (BH of 30) and a spinner flask runs.

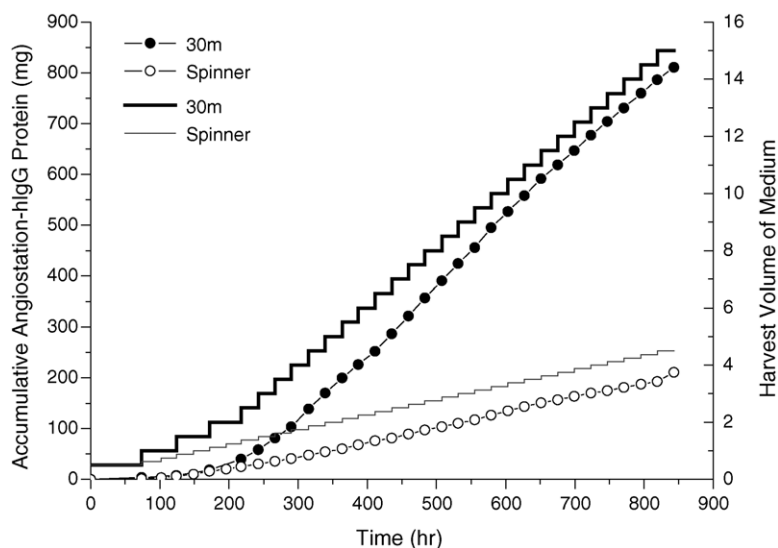


Fig. 10. Profiles of accumulated product and harvested volume of the 35-day cultures of a BelloCell[®]-500 (BH of 30) and a spinner flask runs.

time. As a result the cell density was higher. However, the total protein production was about the same as our study. It indicates that specific production rate and/or the viability of cells in the spinner utilizing the settlement can be significantly inferior to that in a BelloCell[®] with BH of 30 min.

4. Conclusion

The recombinant CHO cell cultures for production of angiostatin-human IgG using a semi-batch process with repeated medium exchanges in BelloCell[®] bioreactor, various bottom holding time have shown significant effect on the total yield of product. BH of 30 and 60 min were found to be about 72% better than BH of 1.5 min and 35% better than BH of 9 and 90 min in term of the total angiostatin-human IgG production. In comparison to conventional spinner flask study, a 3.8-fold increase of the total angiostatin-human IgG production was noticed in a 35-day culture.

The specific production rate was shown to be related to BH and inversely proportional to the specific growth rate for this culture system. Higher BH is favorable to specific production rate but less favorable to specific growth rate.

This study illustrated that a simple method of using BH in a semi-batch process can effectively control the

apparent nutrient concentration available to cells as that achieved by a complicated perfusion process in regulation of cell growth and protein production.

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