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Comparative production of a monoclonal antibody specific for enrofloxacin in a stirred-tank bioreactor

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ABSTRACT

A hybridoma producing monoclonal antibody (MAb) specific for enrofloxacin was cultivated in a 2-L stirred-tank bioreactor in various modes and the performances of each mode were compared. In batch mode, a maximum viable cell and MAb concentration of 9.21×10^5 cells mL^{-1} and 67.3 mg L^{-1}, respectively, were obtained. When the hybridoma was cultivated in a fed-batch culture with the addition of specific nutrients, no improvement in either the viable cell number or MAb concentration was observed. On the other hand, an increase in the production of toxic metabolites, i.e. ammonia and lactate, was observed with growth inhibition of the hybridoma cells occurring at ammonia and lactate concentrations of 2.0 mM and 2.0 g L^{-1}, respectively. However, the best performance of hybridoma cultivation was achieved in a perfusion culture mode using a spin filter, which was installed in the stirred-tank reactor as a cell retention device with a perfusion rate of 0.80 vvd. Under these conditions a steady viable cell concentration of 1.57×10^6 cells mL^{-1} was obtained within five days with an overall productivity and yield of 73.7 mg L^{-1} d^{-1} and 61.4 mg d^{-1}, respectively, which was a significant increase over that attained with the batch process.

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1. Introduction

Monoclonal antibodies (MAbs) produced by hybridoma cell lines are currently used in many applications, an increasing number of which now require high yields, such as the diagnosis and treatment of certain diseases, and the purification and detection of substances. The growing demand for MAbs in large quantities means it is necessary to optimize antibody production processes and to maximize mammalian cell bioreactor productivity.

For greater cost effectiveness, large-scale MAb production is often performed in suspension culture, with the bioreactor operated in either a batch, fed-batch or perfusion mode. Although all of these modes are widely used in practice, each one has its own disadvantages due to different working principles [1]. The fed-batch method involves the addition of nutrient supplements into the bioreactor to replenish depleted nutrients so as to enable the production of MAbs for a prolonged period of time, but is subject to the subsequent inhibition of hybridoma growth and metabolism by the accumulating toxic waste metabolites which are not depleted. The fed-batch operation, in general, produces antibodies at a higher final concentration due to the accumulation of product [2]. However, the volume throughput per run is restricted intrinsically by the size of a bioreactor vessel and is rate-limited by its prolonged operation time. To compensate for the low-volume throughput, high yields often require a much larger volume bioreactor vessel, with the resulting set up costs and aseptic operating demands. Improvements of the economics of mammalian cell production systems are, therefore, likely to be achieved by better medium design and process control. In the perfusion mode, medium is perfused through the reactor at a high rate whilst cells are retained or recycled back into the reactor by some type of retention devices using sedimentation, centrifugation or filtration principles [2–4], and the cell-free permeate containing the MAb product is removed continuously from the bioreactor. In this perfusion mode, the medium is maintained at a constant volume. As a result, nutrients are constantly replenished whilst toxic metabolites are removed, but MAb product is diluted making for a less efficient and more demanding purification due to the larger volume to process and the lower product (MAb) to impurity (e.g. sera and cellular proteins) ratio. Perfusion cultures have also

Abbreviations: vvd, volume of fresh media/working volume of reactor/day.

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been used in attempts to achieve high cell concentrations in a bioreactor and have attained significantly higher productivity than that in conventional batch cultures. Generally, the highest product output can be achieved in continuous stirred tank bioreactors performed in a perfusion mode [5]. In this cultivation mode, the volumetric productivity can be almost two orders of magnitude higher than in batch mode, and in some cases product concentrations are increased by up to five-fold [6–9]. Since the pioneering work of Himmelfarb et al. [10], spin filter systems have become the preferred method for retaining cells since they are both simple and convenient for the operation of continuous perfusion cultivations in stirred bioreactors [11–13].

In this article, we cultivated a hybridoma cell line, producing IgG_1 against the antibiotic enrofloxacin, in various modes to investigate the optimal conditions for MAb production. Batch, fed-batch and perfusion modes were performed in a stirred-tank bioreactor using a spin filter as a retention device to evaluate the optimal process for hybridoma cell culture.

2. Experimental

2.1. Cell line and culture medium

A hybridoma, generated by cell–cell fusion, which produces a monoclonal antibody in the subclass of IgG_1 against enrofloxacin [14], a synthetic antibiotic belonging to the fluoroquinolone group, was used in this study. RPMI-1640 (Invitromex) supplemented with glucose (2.0 g L^{-1}), L-glutamine (0.1 g L^{-1}), sodium pyruvate (0.11 g L^{-1}) and sodium penicillin G-streptomycin sulfate (1000 U mL^{-1} and 1 g L^{-1}, respectively), was used in all the experiments as basal media for cell cultivation and was additionally supplemented with 10% (v/v) fetal bovine serum (Invitromex) unless stated otherwise. Cells were grown and maintained in T-flasks (Nunc) at 37 °C, pH 7.2 and 5% (v/v) CO_2 and precultures to seed the stirred-tank bioreactor were grown in 200-mL spinner flasks in a CO_2 incubator to mid to late log phase, harvested by centrifugation and resuspended in fresh media such that the initial cell density in the bioreactor was about 1.00×10^5 cells mL^{-1} (1.2 L total final volume) after inoculation.

2.2. Bioreactor systems

All of the cultures were performed in 2-L BIOSTAT B-plus bioreactor system (B. Braun Biotech, Germany) with a working volume of 1.2 L. Aeration was performed through a membrane tube basket at 0.03–0.05 vvm for bubble free aeration. The dissolved oxygen concentration (DO) was controlled at 50% (v/v) of air saturation by a Gas Mix Unit, which can adjust the proportions of air, O_2, CO_2 and N_2 automatically. The cultivation temperature was maintained at 37 °C by use of a water circulating system. Media pH was kept constant at 7.20 through the controlled addition of either CO_2 (g) or 8% (w/v) sodium bicarbonate solution as appropriate. Agitation was provided by a 3-blade segment impeller rotated at 100 rpm. The 20-μm spin filter was attached to the stirrer shaft. Therefore, stirrer speed and spin filter rotation velocity were coupled. In fed-batch mode, the hybridoma culture was fed with glucose (1.2 g L^{-1}) or glutamine (0.2 g L^{-1}) or serum (100 mL) after two days of cultivation. Perfusion was accomplished by using the spin filter and performed with two peristaltic pumps installed between the fresh media tank and the reactor, and between the harvest tank and the reactor, respectively. Cells were retained by the spin filter whilst the cell-free permeate containing the product was continuously withdrawn from the bioreactor by one peristaltic pump and transferred to a sterile product tank. The volume of withdrawn permeate was equally replenished with the addition of fresh medium at the same rate by the other peristaltic pump into the outer space of the spin filter. The feed rate of fresh media (perfusion rate, D) was set at one of 0.1, 0.2, 0.4 and 0.8 d^{-1} (vvd) constantly throughout the operation. The rotation speeds of both feed and harvest pumps were adjusted with respect to the assigned perfusion rate.

2.3. Analytical methods

Samples (5 mL) were taken aseptically once daily and centrifuged at 5000 rpm for 5 min. The supernatants were filtered through 0.22 μm pore membranes, divided into 1 ml aliquots and stored at 4 °C for later analysis. Simultaneously, cell concentration and viability were determined using a hemacytometer and the trypan blue dye exclusion method. The concentrations of glucose and lactate in the culture supernatants were determined using high-performance liquid chromatography (HPLC) with a C-18 column (BIO-RAD) [15]. The mobile phase was 2 mM H_2SO_4 with a flow rate of 6 mL min^{-1}. The concentration of ammonia was quantified using the method previously reported by Fawcett and Scott [16]. The antibody concentration was determined by an indirect enzyme-linked immunosorbent assay (ELISA).

3. Results and discussion

The culture was performed at a 2-L scale in a batch, fed-batch, semi-continuous perfusion and continuous perfusion mode, respectively.

3.1. Batch experiment

A batch operation was conducted to investigate the cell growth and MAb production in the 2-L stirred-tank bioreactor. As shown in Fig. 1A, cells grew out of the lag phase during the first day with an increase in the viable cell density from 1.30 to 1.79×10^5 cells mL^{-1}. During the logarithmic growth phase, the viable cell density increased to the maximum value of 9.21×10^5 cells mL^{-1} at the third day of cultivation, with a total cell viability of over 90% during the first two days, and then decreasing at the late logarithmic phase to only about 80% (the time when the highest viable cell density was obtained). Although the total cell density slightly increased to 12×10^5 cells mL^{-1} at day four, the viable cell density decreased dramatically during the last two days of cultivation. The specific viable cell growth rate during the first 48 h was evaluated as 0.0317 h^{-1}. The MAb concentration profile (Fig. 1B) matched the total cell density profile well. The MAb concentration in the culture increased slowly during the lag phase and rapidly in the log phase to the highest concentration of 67 mg L^{-1} at day four of cultivation. The productivity and yield of monoclonal antibody were 11.2 mg L^{-1} d^{-1} and 13.5 mg d^{-1}, respectively. The average specific MAb production rate was 1.12 ng $cell^{-1}$ h^{-1}.

3.2. Fed-batch experiment

In fed-batch mode, the hybridoma culture was fed in separate runs with glucose (1.2 g L^{-1}) or glutamine (0.2 g L^{-1}) or serum (100 mL) to replenish the key nutrient. The time of feeding, which was derived from the batch mode growth profile, was at day two of culture. The results showed that the maximum viable cell and final MAb concentrations of all fed-batch studies were slightly less than that of the batch mode (Table 1). This could be due to the fact that there were some unidentified inhibitory factors that restrict the increase of viable cells and MAb concentration. In general, hybridoma cell cultures are well known for being susceptible to ammonia as well as lactate. Previous reports have suggested that the inhibitory level for ammonia and lactate ranged from 3 mM to

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