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Increased CHO cell fed-batch monoclonal antibody production using the autophagy inhibitor 3-MA or gradually increasing osmolality

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ABSTRACT

Modulating autophagy provides a new method to increase CHO cell protein production. A fed-batch protocol using the autophagy inhibitor 3-methyl adenine (3-MA), developed for a tissue-plasminogen activator (t-PA) expressing DHFR based CHO cell line, was successfully adapted to a monoclonal antibody (MAb) expressing CHOK1-SV based CHO cell line. By optimizing the timing and dose of 3-MA treatment, the cell-specific productivity was increased 4-fold, resulting in 2-fold increased total MAb production. The positive effect of the 3-MA treatment appeared to be reduced when the amino acid feed concentration was increased 5-fold. Further investigation revealed that by slowly increasing osmolality up to ~450 mOsm/kg, both the cell-specific productivity and the total MAb almost doubled. This effect was replicated with a DUXB-based CHO cell line expressing a human–llama chimeric antibody. The positive effect of gradually increasing osmolality was then combined with the positive effects of the 3-MA treatment, however their combined effect were not additive. Thus, either increased osmolality or 3-MA treatment were equally effective in increasing MAb-CHO cell fed-batch production on the cell lines tested. Analysis of protein glycosylation showed that both of these fed-batch modifications did not substantially influence the overall glycan profiles of the MAb product.

1. Introduction

The performance and yield of mammalian fed-batch cell cultures used to produce therapeutic recombinant proteins depend on cellular responses to the changing culture environment. Apoptosis and autophagy are two major responses that can influence process performance, limiting in particular the maximum viable cell concentrations. Inhibition of apoptosis has been widely explored as a means to increase cell culture productivity [1,2]. Autophagy (derived from Greek words for 'self-eating') is another stress response where cellular components are degraded for reuse and cell survival, and may also lead to cell death [3]. Modulating autophagy has more recently been explored as an additional means to increase cell culture productivity [4–7], but whether induction or inhibition should be used to improve process performance remains an open question. Induction of autophagy by rapamycin was shown to increase monoclonal antibody (MAb) production in Chinese hamster ovary (CHO) cells by up to 21% and cell-specific productivity by up to 6% [6]. Conversely, inhibition of autophagy with 3-methyl adenine (3-MA) resulted in a 2.8-fold increase in t-PA production in fed-batch cultures of CHO cells [5]. 3-MA is an inhibitor of mammalian Vps34, a class III phosphatidylinositol 3-kinase (PI3K), a protein that is required to initiate the formation of autophagic vesicles [2,8–11].

Another strategy widely reported to increase mammalian cell productivity is based on culturing cells under hyperosmotic conditions [12–17], for example by increasing the osmolality with the addition of inorganic or organic osmolytes (such as NaCl or sorbitol). However, this increase in cell-specific productivity combined with a reduction in cell growth often results in no overall benefit in terms of product yield [15,18,19]. Biphasic batch culture strategies or gradual increases of osmolality [17,20,21], osmoprotective components [22] and consequent sub-culturing [23] have been used with some success to overcome the problem

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of cell growth suppression. However, most of these studies were performed under batch conditions and the effect of high osmolality on fed-batch culture needs further study.

In this work, we report exploring and combining the positive effects of 3-MA treatment and of using a gradual increase in culture osmolality to increase MAb productivity in a CHO cell fed-batch culture. We first adapted a CHO cell fed-batch protocol using the autophagy inhibitor 3-MA developed for t-PA production [5], to the production of a MAb expressed by a different CHO cell line. Then we modified the CHO cell fed-batch protocol to explore the effects of a gradual increase in osmolality as well as its combination with 3-MA treatment on overall MAb production. Finally, since autophagy inhibition, hyperosmolality and other fed-batch conditions may influence product glycosylation [24,25], glycan profiles of the fed-batch MAb products were analyzed to assess effects of such treatments on protein product quality.

2. Materials and methods

2.1. Cell line and maintenance culture

This study used two Chinese hamster ovary (CHO) clonal cell lines (based on CHOK1SV) expressing a human IgG1 anti-interleukin 1β monoclonal antibody (referred to as C44 and C56, originally named ChK2 437.90.44 and ChK2 437.89.56, respectively) [26] and a CHO cell line based on CHO-DUKXB (CHO-EG2) expressing a human–llama chimeric antibody for epidermal growth factor receptor (EG2-hFc) [27].

Frozen cells in 1 mL vials were thawed rapidly at 37 °C and placed in a 125 mL shake flask containing 20 mL of maintenance medium, CD CHO (Invitrogen, Burlington, ON) supplemented with 6 mM glutamine (Invitrogen), at 140 rpm. In the case of the MAb expressing CHOK1SV cell lines, selection pressure was maintained by addition of 4.5 μg/mL bleocin (Calbiochem, La Jolla, CA) and 100 μg/mL hygromycin B (Invitrogen). For the CHO-EG2 cell line, the CD CHO maintenance medium was supplemented with 4 mM glutamine, 25 μg/L IGF-1 and 4X anti-clumping agent (Invitrogen). Newly thawed cells were passaged to 3.0×10^5 cells/mL at least three times before use in experiments.

2.2. Batch and fed-batch culture

All cultures were performed in 125 and 500 mL shake flasks using the basal medium of CD CHO plus 4 mM glutamine. The initial fed-batch protocol for CHO-MAb was based on the protocol established for CHO-tPA [5], inoculated at 1 or 2×10^6 cells/mL in basal medium. These cultures were then fed daily with CHO CD Efficient Feed™ A (Feed A, Invitrogen) supplemented with two amino acid mixtures of EN and CYD at either 1× or 5× concentration. The EN and CYD solutions were prepared separately at 5× and diluted in cell culture grade water to 1×. EN (1×) consisted of 10 mM L-glutamic acid and 75 mM L-asparagine (Sigma, St. Louis, MO) dissolved in 0.1 M NaOH. CYD (1×) consisted of 10 mM L-cystine disodium salt hydrate (MP Biomedicals, Solon, OH), 15 mM L-tyrosine disodium salt (Sigma) and 10 mM aspartic acid (Invitrogen) dissolved in 0.1 M HCl. The feed volumes were a percentage of the initial culture volume (25 mL). The fed-batch protocol started with the daily addition of 4% Feed A plus 2% EN 1× and 2% CYD 1×. In the case of the high osmolality experiments, EN and CYD were added undiluted at 5× and thus their volumes reduced to 0.4% EN 5× and 0.4% CYD 5×. When the cell concentration reached greater than 5×10^6 cells/mL, the daily feed volume was increased by 3-fold. Sample volumes of 6% of the initial culture volume were removed daily. Since there were considerable volume changes, total MAb production (mg) and cell-specific productivity, q_p (pg/cell/day) were reported. When needed, osmolality was increased using addition of NaCl to the cell culture medium.

2.3. 3-MA treatment

3-methyl adenine (3-MA) (Sigma) was used to chemically inhibit autophagy. A 500 mM stock was dissolved in 0.5 M HCl. The culture pH was adjusted to 7.0 after 3-MA addition with a solution of 7.5% w/v $NaHCO_3$ (Invitrogen). The 3-MA was tested from 0 to 15 mM and usually added one day after the peak cell concentration.

2.4. Analytical methods

2.4.1. Cell concentration and viability

The viable cell concentration was determined by mixing equal volumes of sample and 0.25% trypsin-EDTA solution (Invitrogen, Burlington, ON) at 37 °C for 10 to 15 min to disperse cellular aggregates, then analyzed using a Cedex automated cell counter (Innovatis AG, Bielefeld, Germany). This device uses an automated trypan blue dye exclusion method and digital imaging to count the cells.

2.4.2. MAb concentration and cell productivity

The MAb concentration was determined using a non-specific IgG ELISA assay [26]. To determine the product titer of the CHOK1SV cell line, an IgG FCγ antibody fragment (Jackson Immuno Research, West Grove, PA) that binds IgG heavy chains was used as the capture antibody, and goat anti-human IgG (H + L)-alkaline phosphatase conjugated antibody (Jackson Immuno Research) as the detection antibody. In the case of EG2-hFc, a goat anti-human IgG (Fc specific) antibody (Sigma) was used as the capture antibody, and a goat anti-human IgG (Fc specific)-peroxidase (Sigma) as the detection antibody. Standard ELISA protocols were followed in 96-well plates and using a microplate reader (V_{max} Kinetic, Molecular Devices, Sunnyvale, CA or Tecan M200, Männedorf, Switzerland). The IgG content of samples was calculated from a standard curve at: 3.9–1000 mg/L of purified human IgG (Sigma) in the case IgG1 MAb or 3.2–100 μg/L of purified EG2-hFc. Cell-specific productivity (q_p) was determined from the total MAb accumulated divided by the integral of viable cells (IVC). IVC was calculated using the following equation in which X is the viable cell concentration (VCC), V is the volume and t is the time: $\mathrm{IVC} = \int_0^t XV\,dt$.

2.4.3. Osmolality

Osmolality was measured according to the manufacturer's instructions using a freezing point depression osmometer (Advanced Devices, Norwood, MA).

2.4.4. Glycan analysis

Glycan analysis was performed using an in-gel method [28]. By running the IgG1 on a reducing gel the glycan structures of the heavy and light chain were analyzed separately. PNGase F was used to cleave the glycans from the protein before fluorescent labeling with 2-aminobenzamide (Sigma). The glycan samples were then purified using a GlycoClean S cartridge (Prozyme, Hayward, CA) and analyzed by Normal Phase-HPLC (Waters, Milford, MA) [29]. Glycan peaks were quantified using Empower Pro software. Using a dextran ladder standard the glucose units (GU) values were determined for all profile peaks making up more than 2% of the total integration area. The peaks were then assigned to structures based on their GU value and information from an exoglycosidase digest using Glycobase (Dublin-Oxford Glycobiology, NIBRT). To compare the identified glycan characteristics in the different samples the antennarity, sialylation, galactosylation and fucosylation indices