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Biphasic addition strategy of hypoxanthine and thymidine for improving monoclonal antibody production

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In our previous study, we demonstrated that combinatorial addition of hypoxanthine (10 mg/L) and thymidine (2 mg/L) was able to stimulate initial cell growth and elevate volumetric concentration of antibody by 22% (Chen et al., Appl. Microbiol. Biotechnol., 93, 169–178, 2012). In this study, a systematic study was carried out to investigate the effects of hypoxanthine and thymidine (H&T) on cell growth and antibody production in a much wider range of concentration. In addition, we pursued to establish a highly productive fed-batch culture via rationally designing H&T addition regime. It was found that both cell growth and antibody production in batch cultures were H&T concentration-dependent. Specifically, a low concentration stimulated cell growth while exerting no influence on specific productivity (q_{mAb}), and a high concentration inhibited cell growth, however, significantly enhancing q_{mAb}. Subsequent experiments with fed-batch shaking flasks demonstrated the feasibility of improving antibody production using a biphasic addition strategy for H&T: supplementing a low concentration of H&T during initial cell growth phase and a high concentration of H&T at the production phase. By applying the optimized feeding regime, a maximum viable cell density (VCD) of 6.45×10^6 cells/mL and volumetric antibody production of 632 mg/L were achieved in a 2 L-B.Braun bioreactor. Taken together, in this study, a biphasic H&T addition strategy for cell culture was developed, which hold great promise to improve antibody production.

[**Key words:** Chinese hamster ovary cells; Hypoxanthine; Thymidine; Monoclonal antibody production; Fed-batch culture]

The ever-increased market demands for therapeutic monoclonal antibodies (mAb) necessitate optimized processes to improve manufacture efficiency of mAb. Development of advanced culture medium as one of the most efficient strategies has met with great success. Additives such as amino acids, vitamins (1), hormones (2), and metal ions (3,4) have all been identified as important components for improved mAb production. However, few reports have documented the application of exogenous nucleotides (nucleosides or bases) for process optimization.

Several studies have investigated the influence of nucleotides (nucleosides or bases) on cell growth, while giving conflicting results. Carvalhal et al. (5) reported that 1 mM adenosine (or guanosine) significantly led to growth arrest of Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells. However, Wong et al. (6) observed no significant effects on CHO cell growth with 5 mM uridine (or 10 mM cytidine). Recently, our study (7) revealed that a combination of 10 mg/L hypoxanthine and 2 mg/L thymidine stimulated growth of CHO cells. The varied growth responses of cells to nucleotide addition may be attributed to the differences in cell lines and cell culture process. In addition, attention was paid to the effect of nucleotide (nucleoside or base) on product quality (especially, glycosylation). Gramer et al. (8) demonstrated that the addition of uridine in culture medium improved the galactosylation of antibody product. Wong et al. (6) also found that combinatorial feeding of nucleosides (uridine and cytidine) with sugar precursors was able to enhance intracellular nucleotide sugar levels. But, till now, the utilization of nucleotide (nucleoside or base) addition as an effective tool to elevate antibody production has rarely been explored.

In our previous study, a 22% enhancement of antibody volumetric production was achieved through the combinatorial addition of 10 mg/L hypoxanthine and 2 mg/L thymidine in batch cultures (7). In the current study, we aimed to perform a more comprehensive study concerning the effects of hypoxanthine and thymidine (H&T) on cell growth and antibody production in a wider concentration range. Furthermore, as a continuing endeavor, we developed a highly productive fed-batch culture using a rational feeding strategy of H&T. These extensive works would provide a new insight perspective into understanding the roles of H&T supplementation in dihydrofolate reductase (DHFR)-CHO cell cultures, which might be very informative to develop an optimized mAb production process in more rational design.

MATERIALS AND METHODS

Cell line and maintenance of cell culture A CHO cell line expressing a chimeric anti-human CD20 monoclonal antibody (anti-CD20 mAb) under a DHFR expression system was used in this study (7). This cell line had previously been successfully adapted to suspension culture and cultivated in in-house developed animal component-free medium (named as CHO232 medium, Chinese patent

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TABLE 1. Culture media represented for batch and fed-batch cultures.

Abbreviation	Culture mode	Medium specification
H0T0	Batch	CHO232 medium with 0 mg/L hypoxanthine and 0 mg/L thymidine
H10T2	Batch	CHO232 medium with 10 mg/L hypoxanthine and 2 mg/L thymidine
H50T10	Batch	CHO232 medium with 50 mg/L hypoxanthine and 10 mg/L thymidine
H100T20	Batch	CHO232 medium with 100 mg/L hypoxanthine and 20 mg/L thymidine
F1	Fed-batch	Cells cultivated in H0T0 growth medium, and maintained in the medium without H&T addition when shifted to production phase.
F2	Fed-batch	Cells cultivated in H0T0 growth medium, and then the medium was fed with H&T with final concentration of 100 mg/L hypoxanthine and 20 mg/L thymidine when shifted to production phase
F3	Fed-batch	Cells cultivated in H10T2 growth medium, and maintained in the medium without H&T addition when shifted to production phase
F4	Fed-batch	Cells cultivated in H10T2 growth medium, and then the medium was fed with H&T with final concentration of 100 mg/L hypoxanthine and 20 mg/L thymidine when shifted to production phase

published: CN101603026). Cell culture was routinely maintained on a rotary shaker at 50 rpm in a humidified 37°C incubator supplied with 5% CO_2.

Batch and fed-batch shaking flask cultures Batch and fed-batch cultures were both carried out using shaking flasks (Corning, NY, USA) on a rotary shaker at 110 rpm, with an initial working volume of 30 mL. For batch cultures, exponentially growing CHO cells were collected by centrifugation (1200 rpm, 5 min) and inoculated at 3×10^5 cells/mL in fresh CHO232 medium supplemented with 4 different concentrations of H&T (H0T0, H10T2, H50T10 and H100T20, as defined in Table 1). For fed-batch cultures, cells were inoculated at 5×10^5 cells/mL and subjected to 4 different H&T addition strategies (F1, F2, F3 and F4, also as defined in Table 1). Feeding was operated using a concentrated mixed solution of glucose, amino acid and vitamin between day 2 and day 12 on a daily base to maintain the glucose concentration within the range of 10–20 mM. The culture temperature was shifted from 37°C to 30°C on day 6 to initiate the production phase in all fed-batch cultures. For F3 and F4 fed-batch cultures, additional feedings of H&T (hypoxanthine and thymidine at 100 mg/L and 20 mg/L final concentrations, respectively) on day 6 were performed. Cell suspension samples were taken every day to determine cell density and mAb concentration.

Fed-batch culture in bioreactor The fed-batch process in a bioreactor (2 L-B.Braun bioreactor with 1 L working volume) was similar as that for the above defined F4 fed-batch culture in shaking flask. Cells were seeded at 2.5×10^5 cells/mL, and samples were taken every 12 h for off-line analysis of viable cell density (VCD) and antibody concentration. Nutrient feeding started from 55.5 h and was replenished every 12 h. The process parameters for bioreactor control were set as following: pH at 7.0, dissolved oxygen (DO) at 50%, and an agitation speed at 80 rpm.

Routine analytical methods VCD and cell viability were determined by counting cells with a hemocytometer using the trypan blue dye exclusion method. The anti-CD20 mAb was purified by Protein A affinity chromatography (HiTrap Protein A HP Columns, GE) and subsequently, the purified mAb concentration was quantified by reversed-phase HPLC as previously described by Chen et al. (7). Lactate dehydrogenase (LDH) activity in the culture supernatant was detected by a commercial enzyme test kit (Nanjing JianCheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. Glucose and glutamine concentrations were measured on NOVA 400 BioProfile automated analyzer (Nova Biomedical, Waltham, MA). Osmolality was determined on an auto-freezing-pointing osmometer (FM-8P, Shanghai Medical University Instrument Factory, China). The specific mAb production rate (q_{mAb}) was calculated by plotting the mAb concentration against the integral of viable cell concentration (IVCC) (9).

RESULTS

Shaking flask batch cultures with different H&T concentrations Batch cultures in shaking flasks at different concentrations of H&T were carried out and results were summarized in Fig. 1. Supplementation with H&T at concentrations of 10 mg/L and 2 mg/L (defined as H10T2) improved initial cell growth, though the maximum VCD is comparable to the culture without H&T supplementation (H0T0) ($4.16 \pm 0.19 \times 10^6$ cells/mL versus $3.80 \pm 0.39 \times 10^6$ cells/mL). However, it was found that the supplementation of H&T at concentrations of 50 mg/L and 10 mg/L

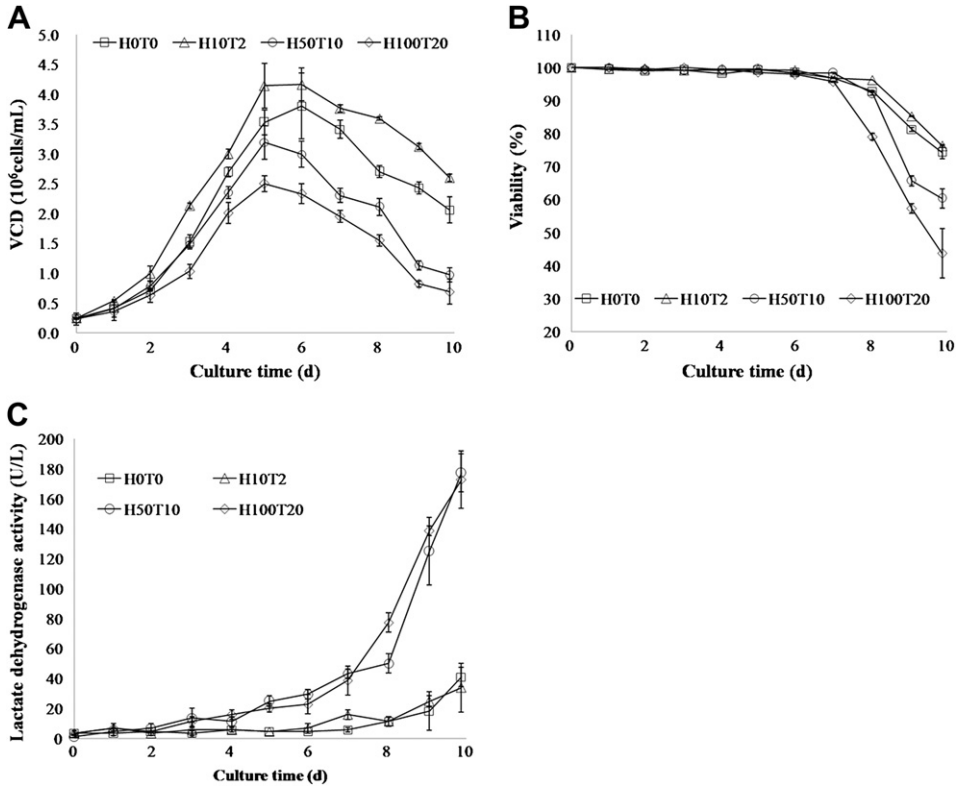

FIG. 1. Growth (A), viability (B) and LDH activity (C) of CHO cells in batch cultures at different concentrations of H&T. The error bars represented standard deviation of two independent experiments.