DEVELOPMENT OF A FEEDING STRATEGY FOR A FED-BATCH CULTURE OF A MONOCLONAL ANTIBODY-PRODUCING AGARABI CHO CELL LINE

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DEVELOPMENT OF A FEEDING STRATEGY FOR A FED-BATCH CULTURE OF A MONOCLONAL ANTIBODY-PRODUCING AGARABI CHO CELL LINE

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Abstract: Animal cell culture is the most extensively used platform for producing novel, recombinant DNA-based therapeutics as these cells have the required machinery to make human-like post-translational modifications. In 2017, therapeutic antibodies became the main product produced by the biopharmaceutical industry, with antibodies making up approximately 20% of the entire global pharmaceutical market. Mammalian cells, such as baby hamster kidney cells, human embryonic kidney cells, and mouse myeloma cells, are common choices for hosts to produce complex recombinant therapeutic proteins. Chinese Hamster Ovary (CHO) cells, however, are considered the gold standard when it comes to large-scale manufacturing processes. Currently, fed-batch culture is the preferred operation mode to produce monoclonal antibodies (mAbs) in mammalian cells. An ideal feeding regimen prevents the depletion of critical medium components and maximizes final product concentration, which depends on the viable cell concentration and the specific production rate of that cell line. The main objective of this work was to develop a feeding strategy for a fed-batch culture with SFM4CHO basal medium growing chimeric IgG1-producing cells, Agarabi CHO. One of our goals of the strategy was to maintain the glucose and glutamine levels within a specified range while adding Cell Boost 7a/7b at fixed times with different volumetric ratios to the cell culture basal medium. Operating as fed-batch and adding Cell Boost 7a containing glucose to the SFM4CHO basal medium led to an increase in the accumulation of glucose and by-products, especially in the early stage of the culture. The replacement of the feed supplement with Cell Boost 7a lacking glucose and substituting the L-glutamine with dipeptide glutamine led to reduce accumulation of glucose and by-products, especially ammonia and lactate. Our feeding strategy developed for a fed-batch culture led to a 4-fold increase in the maximum cell density and increased the longevity of the culture by more than 2-fold compared to batch culture conditions.

TABLE OF CONTENTS

Chapter Page

Chapter Page

Chapter Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Figure Page

CHAPTER I

LITERATURE REVIEW

1.1 Introduction

The manufacturing of biopharmaceutical products is one of the most sophisticated and elegant achievements of modern science. These biopharmaceutical products function extraordinarily well, offering high efficacy and few side effects. The ability of biopharmaceutical products to address previously untreated conditions, combined with their efficacy and safety, have led to the major pharmaceutical companies to shifting their focus from small molecule to large molecules products. The world market for biopharmaceuticals was over $186 billion in 2017 and is projected to reach $526 billion by 2025 [1]. For major biopharmaceutical companies, the biggest challenge has been producing enough product for clinical trials and establishing a large-scale manufacturing process to meet the market demand. The advancement in single-use technology has helped to shorten the time from research and development (R&D) to production as well as the time from lab-scale to a large-scale production. Single-use bioreactors also decrease the time for process development and manufacturing of the biopharmaceutical products by eliminating the required time for cleaning and sterilization between batches.

The living machines that produce recombinant therapeutics are genetically modified mammalian cells that must be frozen for storage, thawed while maintaining cell viability, and made to thrive in the stressful environment of a bioreactor vessel. The desired biomolecules produced by the cells must then be separated from the cells and the medium in which they were produced, all without destroying their complex, fragile structures. Currently, Chinese Hamster Ovary (CHO) cells are the gold standard for the manufacturing of recombinant proteins. Most manufacturers of single-use bioreactors, therefore, use a process based on CHO cells to validate their product and to prove that their system will maximize productivity of the cells without affecting the quality of the biopharmaceutical product. Although advancement of upstream process development has led to a decrease in the time from lab to market, the process optimization and scaling up of the process are still cell line dependent and also differ from one product to another. Each new biopharmaceutical product candidate, therefore, needs a dedicated team of scientists for the optimization and up-scaling process [2].

The focus of his study was to establish an optimized medium and feeding strategy for a fed-batch culture of CHO cells producing a model chimeric IgG 1 antibody. The fed-batch culture used the SFM4CHO as a basal medium for the culture while Cell Boost 7a/7b was added as feed media. The process development was based on finding the optimum concentration of feed media with the ideal feeding schedule. The optimization process of the feeding regimen was depended on finding the nutritional requirement for the CHO cells by performing a metabolite analysis of the nutrients and by-products. In the near future, the optimized fed-batch culture process will be used as a part of the validation process for an innovative multi-chamber single-use bioreactor with 3 L minimum working volume and 200 L maximum capacity. The 200 L multi-chamber single-use bioreactor is based on a prototype by Momen et al. with a 50 L maximum working volume [3]. This chapter provides a comprehensive literature review including monoclonal antibody production using CHO systems, cell culture media advancements, cell culture process

development, cell culture operation modes, metabolites analysis, the relationship between the nutrients in media and the process by-products, and common methods for the optimization of fed-batch culture.

1.2 Chinese Hamster Ovary Cells

Mammalian cells have the required intracellular machinery to perform post-translational modification to produce recombinant proteins similar to the native proteins found in the human body. These modifications and proper protein folding are essential to retaining the biological activity of complex proteins such as monoclonal antibodies (mAbs) [4]. The most common cell lines used as a platform for biopharmaceutical manufacturing are murine myeloma lymphoblastoid-type cells such as NS0 cells, Chinese Hamster Ovary (CHO) cells, Baby Hamster Kidney (BHK) cells, and Human Embryonic Kidney epithelial cells (HEK-293) [5]. In the early ages of recombinant protein manufacturing, cell lines such as NS0 were initially used. Despite successful manufacturing using NS0, ~70% of the recombinant biological have been produced with CHO systems. The ability of CHO cells to make human-like post-translational modifications and a long history of regulatory approval make it more popular in the biopharmaceutical industry. Further, CHO cell lines are non-permissive to most human viruses and easily adapted to grow in suspension conditions using serum-free and chemically defined media required for the large scales needed to meet market demands [6, 7]. In 1956, Theodore Puck recovered an immortalized population of fibroblast cells from cultured ovarian cells of the Chinese hamster. Then, Puck generated CHO-K1 from the original cell line in 1957 [8]. CHO cells, however, were not used for large-scale protein production until the 1980s, when the cells were modified to include a selection capability for cells containing recombinant DNA plasmids. In 1980, Urlaub and Chasin used ethyl methanesulfonate with the CHO-K1 cell line to produce CHO-DXB11, a cell line with a deletion of one of the dihydrofolate reductase (DHFR) allele and an inactivating mutation in the second allele [9]. Cells lacking a functional copy of the DHFR gene cannot survive in a

thymidine-free medium. Using these DHFR deficient cells and thymidine-free medium, one can select for cells that were co-transfected with a linearized plasmid containing both the DHFR gene and a gene-of-interest (GOI). Further, varying the concentration of methotrexate (MTX) in the medium can be used to identify cells with high expression levels of the GOI. In 1983, Urlaub and his lab members used gamma radiation on a different cell population to produce a second cell line, CHO-DG44, in which the DHFR gene was deleted in both alleles [10]. In 1986, using CHO cells, human tissue plasminogen activator (t-PA) marketed as Activase® becomes the first therapeutic protein from recombinant mammalian cells to obtain approval from the Federal Drug Administration (FDA). Nowadays, the most widely used CHO systems for large-scale production of therapeutic protein are derived using the DHFR/ MTX-based selection because it results in high levels of gene amplification and expression [11-13].

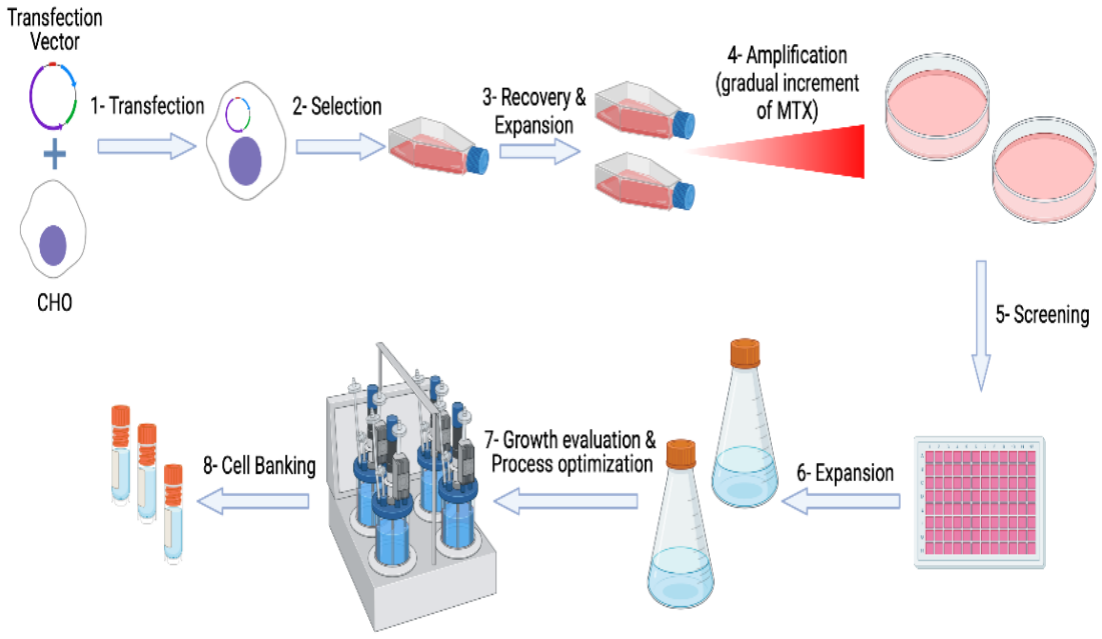

Figure 1. A Schematic of steps of CHO cell line development for recombinant protein production.

The generation of recombinant cell lines, as presented in Fig.1, begins with DHFR-knockout CHO cells that lack the essential metabolic enzyme, DHFR [10]. DHFR (-) cells have a particular nutritional need for growth, which is the basis for isolating and selecting stable clones.

The GOI may be cloned in the same expression vector with the DHFR gene, as in Fig. 2, followed by subsequent growth in a medium lacking glycine, hypoxanthine, and thymidine (Step1 and 2) [7, 11, 12, 14, 15]. Through these steps, only clones with active expression vectors will survive. Using cationic polymers such as polyethyleneimine with mild-hypothermia (27 – 34 °C) or deacetylase inhibitors has made the transfection process more efficient and cost-effective [16, 17]. The surviving cells that successfully integrate the DNA vector are eventually recovered and expanded (Step 3). To enhance the cell line productivity, selected clones are exposed to increasing concentrations of MTX (typically ramped over 0.5 – 1 µm). MTX inhibits DHFR enzyme activity resulting in a significant increase in the number of copies of both the DHFR gene and the GOI (Step 4) [18].

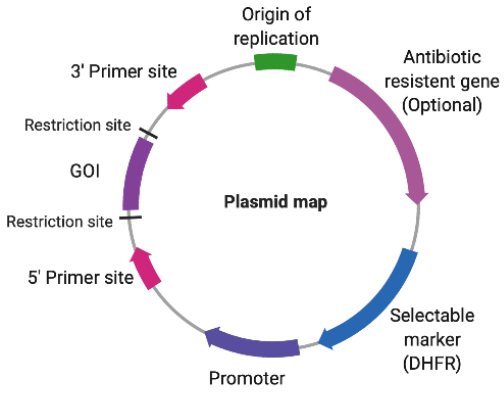

Figure 2. DHFR based expression vector.

Usually, integration of a DNA vector occurs randomly, so the resulting pool of cells is heterogeneous, with different integration sites, with varying cell numbers and cell productivities. Therefore, a series of limiting dilutions in multi-well plates are used to isolate the single colonies with the highest productivity, growth rates, and uniform cell populations (Step 5). The clones that are picked are further passaged (Step 6), and each colony is assessed in lab-scale bioreactors under conditions mimicking large-scale production facilities (Step 7). After evaluation, a single

cell line is chosen and banked as frozen vials (Step 8) for future use [2, 19-22]. The most critical selection criteria are growth rate, cell productivity, glycosylation profiles, development of charge variants, and clone stability [7, 23, 24].

Large-scale manufacturing of therapeutic proteins using the chosen clone requires more upstream process (USP) optimization after the cell line development and engineering. Media and feed optimization, metabolic characteristics, bioprocess development, and scale-up are essential parts of each biopharmaceutical company's large-scale manufacturing process development after choosing the optimal single cell line [25]. Although the transfection process to produce a stable recombinant cell line takes several months, optimization of a new process in the shortest time possible presents a significant challenge especially during the scale-up because there are no reliable methods for predicting the growth profile of the cell line in the large-scale bioreactor [2, 25, 26]. Although further research is needed to fully understand the genomic and proteomic information of CHO cells, it is viewed as the mammalian cell equivalent of the bacterial workhorse, *E. coli*, in biotechnology research.

1.2.1. Agarabi CHO (ATCC® CRL-3440™)

Here, we utilized the Agarabi CHO cell line (referred to as CHO-3440 for the rest of the work) bought from American Type Culture Collection (ATCC). CHO-3440 derived from a genetically modified CHO-DG44 cell line using gamma rays to cause deletion and inversion in DHFR genes [27]. The cell line was stably transfected with a vector system expressing a model mouse-human chimeric IgG1 antibody. Like mentioned in the previous section, single cell-derived clones were isolated by limiting dilution and expanding. CHO-3440 was subjected to further amplification by treatment with 2 µM methotrexate for up to 42 days and subsequently frozen. Then, in the absence of methotrexate, CHO-3440 was serially subcultured on a 3-4 day regime for 2 – 3 weeks and cryopreserved [28].

1.3 Monoclonal antibodies production

Immunoglobulins (antibodies) are multifunctional, large multimeric (typical molecular weight, approximately 150 kilodaltons [150 kD]) components of the immune system. Antibodies trigger many cellular and humoral reactions to multiple foreign substances (antigens). Most antibodies are produced by several distinct B-lymphocytes (polyclonal), and as a result, they have a slightly different specificity for the target antigen. Also, production of large quantities of monoclonal antibodies (from a single B-cell clone) is possible, making them monospecific, homogenous, and effective tools for developing bio-therapeutics diagnostics [29].

Since 1975 when Köhler and Milstein introduced the hybridoma technique to produce mAbs [30], the potential clinical use of mAbs was obvious. Over the last couple of decades, many new antibodies have been produced without animals as intermediates to induce antigen-specific B-cells. In 1986, the FDA approved the first therapeutic monoclonal antibody (Orthoclone OKT3) to prevent kidney transplant rejection. The sales growth and approval of additional mAbs were slow until the first chimeric mAbs were approved in the late 1990s. Since then, the rate of approval of mAbs has increased dramatically, leading to 106 therapeutic mAbs approved by the FDA by 2019. In 2013, the global sales revenue for all mAbs was approximately half of the total sales of all biopharmaceutical products. The use of mAbs has become the predominant treatment for various diseases such as cancer, cardiovascular, autoimmune, and metabolic disorders [31].

In the past, monoclonal antibodies were murine (Fig. 3a) in origin and produced by immunizing the mice and taking the naturally produced immune cells from the spleen of the mice, then fusing them with cancerous cells such as myeloma to obtain hybridoma cells with the ability to secrete the mAbs. The possibility of adverse immunological reactions in the recipient limited the murine mAb therapy. Recombinant engineering techniques permit customization of mAb binding sites, size, and structures so that the mAbs are more similar to human antibodies without

loss of binding properties. Engineered mAbs occur in two distinct forms, chimeric and humanized, with particular advantages in terms of low immunogenicity, effective functionality, and improved specificity [29, 30, 32].

In 1994, the FDA approved the first chimeric antibody (Abciximab) for the inhibition of platelet aggregation in cardiovascular disease. Chimeric antibodies (Fig. 3b) are usually produced by combining the human constant region domain sequence with the murine variable domain to decrease the immunogenic effects without losing binding properties [33, 34]. The second breakthrough in therapeutic mAbs production was using the complementary-determining region (CDR) grafting technique to produce humanized mAbs. CDR grafting was used to transplant non-human antibody CDR sequence into a human framework sequence (Fig. 3c) to maintain target specificity. The first humanized mAb approved by the FDA was Daclizumab, an anti-IL-2 receptor, in 1997 [35, 36]. In 2018, George P. Smith and Sir Gregory P. Winter were awarded the Noble Prize in Chemistry for their work on developing phage-displayed peptides which can be used to evolve new proteins. In 1990, the first fully human mAb (fig. 3d) was generated using the phage display technique [37-39].

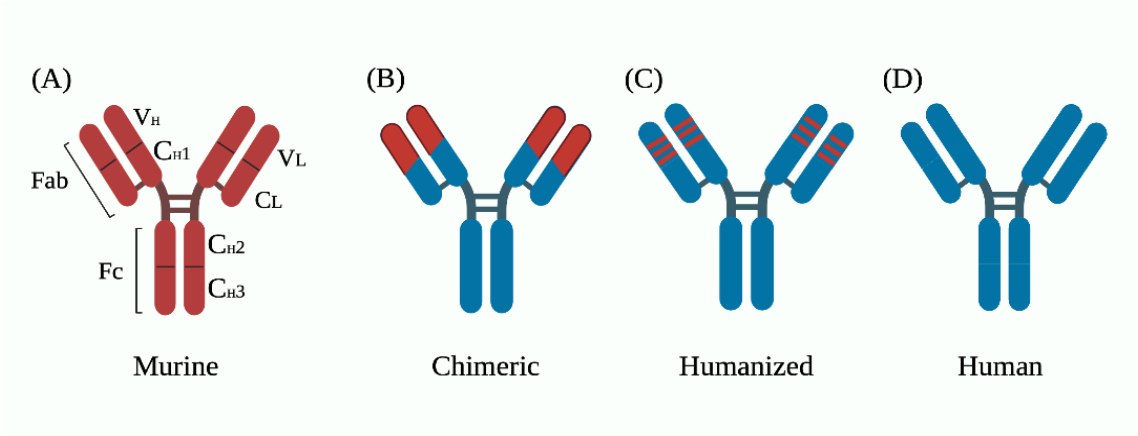

Figure 3. A Schematic overview for engineered antibodies from murine antibodies (red domains) to fully human antibodies (blue domains). (A) murine monoclonal antibody. (B) chimeric monoclonal antibody. (C) humanized monoclonal antibody. (D) human monoclonal antibody. C_H: domain of the constant region of the heavy chain; V_H: Variable domain of the heavy chain; C_L: Constant domain of the light chain; V_L: The Variable domain of the light chain. Fab and Fc fragments are resulting from proteolysis.

Nowadays, recombinant expression technology in mammalian cell culture is the primary method for the commercial production of therapeutic mAbs. *In vitro* production of mAbs in mammalian cells, such as CHO, provides a high degree of control, safety, consistency, and production of large amounts of mAbs without usage of animals [40]. Biopharmaceutical companies work to minimize the time required for manufacturing process development through two approaches. The first approach is to rapidly produce a sufficient amount of the mAbs for the clinical studies. The second approach is to develop a large-scale process that can deliver adequate amounts of the mAbs to meet market demand. The approved process should be scalable, robust, and meet quality standards [41].

Advancements in producing serum-free, chemically defined, and animal-derived component-free media have finally provided platform technologies to use standard media, feeds, and growth conditions[42, 43]. Therefore, the productivity of mammalian cell processes has improved dramatically in recent years and can achieve high antibody concentrations[44]. The media and feed optimization, however, differ from one cell line to another, and there is no predictive model for the growth profile and cell productivity. Consequently, each new therapeutic monoclonal antibody candidate needs a dedicated team of scientists for the development and optimization of the new process, especially during scale-up [2].

1.4 Cell culture process development

Manufacturability and scalability of therapeutic proteins using mammalian cells have been considered difficult due to low yield, serum requirement, and shear sensitivity. After more than two decades of research on using mammalian cell systems, cell-specific productivity over 20 pg/cell/day is routinely achieved with high titers up to 10 g/L and cell densities of over 20 million cells/mL. The improvement of product titers using fed-batch processes has been achieved not only by selecting highly productive clones but also by optimizing media, feed, and bioreactor

operation conditions. Also, process optimization must take into account possible product changes, such as altered glycosylation or degradation from the implemented modifications to the process[45, 46]. For example, Ivarsson et al. [47] showed the effect of pH on IgG1 monoclonal antibody N-glycosylation, and Bechmann et al. [48] demonstrated the impact of the concentration of manganese in cell culture media on the fucosylation pattern.

While the media and feed optimization process are considered cell line-dependent and based on the metabolism and nutrient consumption of specific cell lines, the process development using standard media and feeds could be used with minimal modifications for different cell lines [49, 50]. Culture media are designed using different systemic approaches, such as single-component titration, spent medium analysis, and medium blending [51]. This rational approach to designing the medium can be used to meet the aggressive requirements of process development in the shortest amount of time. In general, the optimization process is labor-intensive and time-consuming and the most common approach to shorten development time is to use a combination of high-throughput cell culture systems with statistical design of experiment (DOE) [52, 53].

1.4.1. Mode of operation

While bioreactors available nowadays can operate in different modes of culture, the following modes are the most commonly mentioned in the literature: batch, fed-batch, and continuous culture [54]. The choice of the best mode of operation depends on the cell culture application or the intended production scale.

1.4.1.1. Batch culture

In batch culture operation mode, the bioreactor is inoculated with cells and all required nutrients at the beginning of the cultivation, without adding any nutrients during the subsequent bioprocess, with the exception of control elements such as gases, acids, and bases [55]. After the

cells reach a certain density and product concentration, the cell supernatant is collected, and the recovery process for the product of interest begins. Due to batch culture simplicity, it is the most common operation mode in the biotech industry [56].

As the batch culture is a closed system, the concentration of nutrients will gradually decrease, whereas the waste product levels increase, leading to a deteriorating cell culture environment. The maximum cell density possible is dictated by limitation in the nutrients (*e.g.*, glucose, glutamine) and inhibitory effects of the waste products (*e.g.*, ammonia, lactate, and CO_2). Consequently, product concentration is usually low. This limitation contributes to the significant disadvantages of batch culture mode, which can only be overcome through addition of nutrients and/or removal of waste products [54, 55, 57]. The end of the bioprocess run, the harvest time, is based on product formation kinetics, which is associated with growth or nongrowth. At the harvesting step, the biomass or medium is processed to obtain the desired product. From the prospective of the bioreactor, biomass is produced in stages that are interrupted by cleaning and sterilization steps [58, 59].

The primary advantages of this mode are simplicity, reduced chances of contamination resulting from addition of nutrients, and the short duration of the bioprocess. Batch culture is suitable, therefore, for rapid experiments such as strain characterization or the optimization of medium composition. In spite of the simplicity of batch processes, they are less common in large-scale manufacturing process due to waste accumulation and nutrient limitations, which can be addressed using other more efficient modes such as fed-batch [60].

1.4.1.2. Fed-batch culture

The fed-batch operation mode is considered a partly open system with a basal medium to support initial growth and a feed to prevent nutrient depletion and sustain the production phase. The addition of a feed is done to prevent some nutrients from becoming limiting factors, thereby

increasing the longevity of the culture, cell yield, and overall productivity. The limiting nutrients should be identified through the exponential phase of the culture, so that the optimal concentration of required nutrients is added at particular intervals. Also, the fed-batch mode offers a wide range of strategies for nutrients addition; the user can either manually set the feed addition at any time (linear, exponential, or pulse-wise) or add nutrients when a specific biomass concentration is reached or when a certain nutrient is depleted [55, 61, 62]. While a stoichiometric fed-batch strategy has been employed effectively to optimize monoclonal antibody production using CHO cells by adding feeds manually every 12-24 h, this approach may subject the cells to nutrient concentrations much higher than required. Hence, the high rates of glycolysis and glutaminolysis result in the accumulation of waste metabolites such as ammonia and lactate. A dynamic feeding approach can, therefore, further control the accumulation of metabolites and result in a more efficient metabolism [63-65].

Currently, fed-batch processes are used in all areas of biotechnology and specifically in large-scale production of mAbs. Fed-batch process are popular because of their scalability, ease of operation, and high volumetric productivity. Also, the fed-batch mode can switch genes on or off by changing a particular substrate in the culture media [66]. One of the major drawbacks of the fed-batch process is that the accumulation of waste products to toxic levels is not controlled. This drawback has been overcome with continuous culture, which can operate for periods much longer than are typical with batch or fed-batch culture [67].

1.4.1.3. Continuous culture

The principles of continuous culture first appeared near the middle of the 20th century [68]. Continuous culture is an open system with the constant addition of a fresh media feed at a steady flow rate and the removing of used medium at the same rate. The continuous mode can be divided into two main types: chemostat culture and perfusion culture [55].

Chemostat culture is a simple way to have a continuous process where fresh medium is added at a constant flow rate, usually referred to as dilution flow rate, and the bioreactor content is removed at the same flow rate without cell retention. Cell growth can be controlled by the flow rate of the fresh media with a typical flow rate of 0.2 – 1.0 vol/day for mammalian cells. Steady-state equilibrium is reached when the cell growth rate equals the dilution rate, so that the concentration of cells, nutrients, and products in the bioreactor remain constant. Although used for mammalian cell culture, chemostat operation is most common for microbial fermentation [55, 69, 70].

In perfusion culture, there is a constant flow of medium both in and out of the bioreactor, just as with the chemostat culture. The major difference between the two culture techniques, however, is that even though the spent medium is continuously replaced with a fresh supply of medium the cells are retained within the bioreactor. Some cell retention devices, such as gravity settlers and centrifuges, operate based on cell density. Other cell retention devices, such as hollow fibers or spin filters, function based on cell size. Today, the most widely used retention devices for large-scale perfusion systems are based on tangential flow filtration (TFF) or hollow fiber systems [67, 71-73].

The continuous supply of fresh nutrients and removal of waste products results in increased process longevity, improved cell density, and higher volume productivity. Also, the residence time of the product is reduced, which improves product quality. Despite the advancements of the perfusion mode, the following issues may complicate its implementation: high cost and time needed for process development, the scalability of the cell retention instruments, and mixing difficulties due to high cell densities. In addition, process development over the last decade focused mostly on fed-batch processes, which are currently available in most companies [69, 73-75].

1.4.2. Cell culture media

Early cell culture media like Ham's F10 and Dulbecco's Modified Eagle Medium (DMEM) mainly consisted of carbohydrates, inorganic salts, amino acids, vitamins, and co-factors. These types of culture media were supplemented with animal serum, mostly fetal bovine serum (FBS), at a 1 – 20% concentration to support and promote cell growth. Serum includes a complex mixture of hormones, growth factors, proliferation factors (such as insulin), and trace elements. While serum is essential for propagating the mammalian cells, there are undesirable effects of the serum, including lot-to-lot inconsistency due to undefined components, risk of contamination, and high cost [51, 76, 77]. In addition to these technical issues of using serum in the cell culture media, there are safety concerns related to transmissible spongiform encephalopathy (TSE) and other contaminants that lead to regulatory problems for manufacturing biological therapeutics [78].

The early trials to grow cells in serum-free media go back to the 1980s. The motive of these studies was to understand the role of serum in cell culture and replace serum with selected hormones that would lead to serum-free, chemically defined media (see table 1). Not only would removing serum address the drawbacks raised above, but eliminating proteins from the media would facilitates the downstream processing of the recombinant proteins [79, 80]. After two decades of media development, driven principally by concerns about using animal-derived serum, fully chemically defined, protein-free, serum-free media consisting of amino acids, vitamins, trace elements, lipids, and growth factors, have been developed for large-scale mAbs production. Currently, over 100 different serum-free media formulations are available in the market and can be readily used.

Table 1. Culture media types.

Serum-free media	Media do not require serum supplementation but may contain proteins
Protein-free media	Serum-free media with no high molecular weight proteins but may contain peptide fractions (*e.g.*, protein hydrolysates)
Animal-derived component-free media	Contain no components of animal or human origin but may have bacterial or yeast hydrolysates
Chemically defined media	Media do not contain proteins, hydrolysates, or any other components of unknown composition

1.4.2.1. Source of energy

For CHO cells, glucose and glutamine are two significant sources of energy in culture medium. Half of the energy comes from glucose, while the other half comes from L-glutamine. Glucose is catabolized to produce adenosine tri phosphate (ATP) through the tricarboxylic acid (TCA) cycle under aerobic conditions; due to the limitation of oxygen absorption, glucose is converted to lactate via anaerobic glycolysis. The generation of lactate as a by-product can alter pH and subsequently increase osmolality due to the addition of base (*e.g.*, sodium hydroxide and sodium bicarbonate) to control the pH [81, 82]. Due to the metabolic shift that usually happens from lactate production to net consumption with CHO cells, the accumulation of lactate decreases in the late stages of CHO cultures. Hence, the monitoring of glucose and lactate concentrations has become a routine practice in biopharmaceutical manufacturing. Monitoring the level of glucose provides an assessment of energy consumption, and measuring an increase in the concentration of lactate indicates the accumulation of metabolic by-products and is a sign of deteriorating culture conditions [83, 84].

Glutamine is a primary source of energy, nitrogen, and carbon for CHO cells. Also, glutamine provides precursors for different biosynthetic processes and stimulates the utilization of glucose through glycolysis. Cells oxidize glutamine to carbon dioxide either entirely or partially *via* glutaminolysis. [85, 86]. A high consumption rate of glutamine is typically observed for CHO cells, and glutamine decomposes with the culture age and temperature to ammonia and pyrrolidone carboxylic acid. The decomposition rate of glutamine is accelerated at room temperature (15 – 25 °C) and higher temperatures. Although the storage of glutamine solution at refrigerated temperature (2 – 8 °C) slows down the decomposition rate, about 30% of the glutamine solution will be gone by 6 months, and at 37 °C glutamine is depleted in 2 – 3 days. In a cell culture with 6 mM L-glutamine, CHO cells will produce as much as 6 mM ammonia in just 3 days. Levels as low as 2 mM of ammonia can be toxic to some mammalian cells, while most of cells are affected by levels greater than 10 mM. Besides the toxic effect on the cells, high levels of ammonia affect protein glycosylation and protein activity [87-89]. Due to the stability issues of L-glutamine in cell culture media, the best practice is to prepare media without L-glutamine and to add it at the time of use. The media with freshly added L-glutamine should be good for up to a month if stored refrigerated [81].

While the cell culture media provides high levels of glucose and glutamine to achieve a high growth profile, these two feeds also result in high lactate and ammonia levels. The accumulation of lactate and ammonia inhibits the growth and productivity of cells. The optimization of fed-batch culture focuses on minimizing the generation of these by-products by limiting the addition of glucose and glutamine. Substitution of glucose with other sugars, such as fructose or galactose, leads to decreasing accumulation of lactate, but these sugars are less efficient. While maintaining the concentration of glucose at low levels, approximately 4 g/L, provides the cells with the optimum sugar, it limits the accumulation of lactate by-products [90,

91]. A concentrated glucose solution is added to the culture media when the level of glucose reaches the critical concentration.

Using stabilized glutamine-containing dipeptides, such as alanyl-L-glutamine, have reduced ammonia by 60 – 90%. Alanyl-L-glutamine has the advantage of good stability even at 37 °C. The decomposition of glutamine dipeptide does not begin until the cells break the dipeptide bond using a cytosolic peptidase, and the cells will not break all of them at one time. In the studies investigating glutamine replacement, two strategies are used; the first strategy is a complete replacement, where the glutamine supplement is replaced in both the basal medium and feed. The second approach is replacing the glutamine in the feed media while retaining the L-glutamine in the basal medium. The second approach helps to shorten the lag phase and allow the cells to reach high densities before adapting them to the glutamine substitute [92-94].

1.4.3. Repressing metabolites

In order to maximize cell productivity, several key process parameters, including viable cell density, the duration of culture, and specific production rate are the focus for process development [95]. As approximately 50% of proteins produced from eukaryotic cells are glycosylated, the control of consistency in protein post-translational modification plays a critical role in product quality and stability [96]. The elevation of lactate, ammonia, osmolality, and carbon dioxide concentration to inhibitory levels is well known to affect cell growth, protein production, and post-translational modification in mammalian cell culture [97-99]. Under ideal culture conditions, all four repressing metabolites are, therefore, maintained below the inhibitory levels.

1.4.3.1. Ammonia

Ammonia is one of the potential growth inhibitors that has been reported to accumulate in cell cultures. Cellular metabolism of glutamine (glutaminolysis) is the primary source of ammonia accumulation. A portion of the accumulated ammonia is a result of the non-enzymatic chemical degradation of glutamine in culture medium [97]. Typically, ammonia accumulates to concentrations of 2 – 5 mM during batch cultures, and when ammonia concentration reaches levels >5 mM, it decreases the cell density by 10% [100]. When ammonia reaches a higher concentration, >10 mM, the glycosylation pattern is altered [95].

Ammonia affects the glycosylation process mainly by decreasing terminal sialylation, a phenomenon observed in the production of various recombinant proteins [100]. The first possible cause of the ammonia effect is increasing the incorporation of ammonia into glucosamine, a precursor for uridine diphosphate N-acetylglucosamine (UDP-GlcNAc). This nucleotide-sugar, UDP-GlcNAc, competes with the transport of cytidine-5'-monophosphate-N-acetylneuraminic acid (CMP-NANA) into the Golgi and decreases the available substrate concentration for sialylation. The second possible mechanism is that ammonia raises the pH of the Golgi, thereby shifting the optimum pH of the sialyltransferase enzymes [101].

Initially, at low cell concentration, most of glutamine content in the medium undergoes chemical decomposition. Since the decomposition of the glutamine cannot be avoided, it is recommended to begin the cell culture with the lowest concentration of glutamine that is accebtable to the cells, usually 4 – 6 mM. As the decomposition of glutamine proceeds even at 4 °C and accelerated by the temperature increase, it is always recommended to use freshly prepared media for kinetic analysis [102]. Commercial products of stabilized glutamine-containing dipeptides have the advantages of high stability even at 37 °C, and using them can decrease the accumulation of ammonia by 60 – 90% [92].

1.4.3.2. Lactate

Glucose metabolism is the primary source of lactate in the cell culture; however, lactate can also be produced in small amounts from glutamine. The accumulation of lactate depends on various factors, including glucose concentration, cellular activity, and culture conditions in the bioreactor. Typically, the concentration of lactate can be as high as 35 mM in the batch mode. With pH control systems, lactate has a dual effect, direct inhibition due to the media acidification and increasing the level of osmolality due to the addition of bases for controlling the pH. Generally, the accumulation of lactate has a lesser inhibitory effect in CHO cell culture because of the metabolic shift from lactate production to net consumption.

Xing et al. [95] evaluated the effect of lactate and other repressing metabolites in 45 cell culture runs in 50-L bioreactors, and lactate above 58 mM was found to inhibit cell growth by 25% and affect cell viability. Wilkens et al. showed that the rate of lactate generation is not significantly affected by glucose concentration in the early stages of cell culture; however, high glucose concentration in the later stages can increase lactate accumulation in the culture media [103]. In the exponential phase, CHO cells produce lactate regardless of oxygen concentration *via* aerobic glycolysis, while cells in the stationary phase mostly consume lactate and perform oxidative phosphorylation [104]. These findings have led to feeding strategies that use a basal medium with high glucose concentration to support the cell culture in the early stages while using feeds without glucose and adding the glucose separately to provide a limited glucose concentration in the culture [63, 105].

1.4.3.3. Osmolality

Although fed-batch culture using CHO cells and commercial feed leads to an increase in maximum cell density and mAb titer, this system leads to the accumulation of toxic metabolites. These metabolites, especially lactate, lead to an increase in extracellular osmolality, a measure of

the osmotic pressure of cell culture, in the late stage of the culture. High osmolality levels can affect specific mAb productivity and the glycosylation pattern [106, 107]. The four major cations in cell culture media are sodium (Na^+), potassium (K^+), calcium (Ca^{++}), and magnesium (Mg^{++}). The balanced salt solution that contains these cations is important to maintain membrane potential and nutrient transport. The amounts of Na^+ and K^+ have a key role in determining the initial osmolality and maintaining osmotic balance. The major anions are phosphate ($PO4^-$), chloride (Cl^-), and bicarbonate (HCO_3^-). The most important role for these anions is to form a buffer system to maintain an optimum pH range of 7.2 – 7.4 for mammalian cells [81].

The accumulation of lactate alters the culture pH and subsequently increases the amount of base required to maintain pH. The addition of base leads to an increase in the culture osmolality levels. When the osmolality is increased from 316 to 445 mOsm/kg, the specific cell growth rate is reduced by 60%. Also, osmolality levels >400 mOsm/kg and accumulation of toxic by-products, such as ammonia and free radicals, accelerate reaching the stationary and death phases of the cell culture. Besides the addition of base, the osmolality of culture media can rise due to the medium evaporation, so that the incubators must be humidified to prevent evaporation from culture flasks. The best practice to maintain optimum osmolality levels through mammalian cell culture is to start the culture at a lower osmolality, such as 265 - 320 mOsm/kg, so that the addition of feeds and base through the exponential phase would not raise the osmolality to toxic levels [81, 108, 109].

1.4.3.4. Carbon dioxide

In animal cell culture, carbon dioxide (CO_2) is the end product of the oxidation of pyruvate and fatty acids to produce acetyl CoA [110]. In small-scale studies and under normal conditions in an incubator, CO_2 diffuses from the cell into the medium where it decreases the pH. In a large-scale bioreactor and under conditions where the pH is controlled using CO_2 and

carbonate base (see Equation 1), the amount of CO_2 produced by the cells may exceed the capacity of the bioreactor to remove CO_2 from the medium. This accumulation of CO_2 in the medium increases the partial pressure of CO_2 (pCO_2) and results in a decrease of the pH. To control the pH, carbonate based is added, which inturn, leads to an increases in the osmolality of the culture medium [107].

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{-2} \qquad \text{(Equation 1)}$$

Elevated pCO_2 have been shown to affect the growth and productivity of CHO cells. For example, Gray et al. demonstrated that pCO_2 within the range of 105 to 150 mmHg inhibited cell growth, cell viability, and decreased the production of a viral antigen in CHO-DG44 cells [111]. Similarly, Kimura and Miller found that pCO_2 in the range of 140 to 250 mmHg inhibited CHO cell growth and production of a recombinant protein, (t-PA) [112]. Generally, when pCO_2 increases from 50 to 150 mmHg, the cell growth rate has been shown to decrease by 9%. In addition, pCO_2 levels at or above 111 mmHg reduce the quality of recombinant proteins [95].

A wide deadband around the pH setpoint allows the culture pH to drift without the addition of base or CO_2, which leads to less dissolved CO_2 and a lower osmolality. Using a wide dead band, however, is not preferred as a slight deviations of 0.1 units from the optimal pH value can significantly impact the cell growth rate of some cell lines [25]. Balancing the effect of pH, osmolality, and dissolved CO_2 on cell growth, therefore, must be a crucial step during process optimization.

1.4.4. Non-nutritional components

During CHO cell culture process development, some non-nutritional components need to be included and adjusted in the cell culture media to provide more favorable conditions. These components include surfactants, antifoam, and anti-clumping agents.

1.4.4.1. Surfactant Pluronic F-68

The size and lack of a rigid cell wall for mammalian cells increase their sensitivity to shear forces, which impact their survival in the bioreactors. Also, CHO cells in most bioreactor systems are exposed to fluid-mechanical shear stress through agitation and aeration. CHO cells have a relatively large cell size, and consequently, sparged gases can damage cells by subjecting them to high shear forces at the air/liquid interface [113-115]. Shear stress effects have been found vary depending on the cell type and the duration of exposure. For example, CHO cells are more susceptible to shear stress during cell-cycle stages S_1 and G_2 than during the G_1 phase, especially when subjected to intense hydrodynamic forces found with turbulent flow, constant agitation, and aeration [116].

Pluronic F68 (P-F68) is a non-ionic surfactant commonly used in suspension cell culture media to protect mammalian cells from the shear stress in the bioreactor or shake flasks. P-F68 belongs to a familt of amphiphilic polymers that have both hydrophilic and hydrophobic polymer strands, and is made from polyethylene and polypropylene [117]. P-F68 can protect the cell membrane through several mechanisms, including forming a protective layer on the cell membrane. This layer that formed on the surface of the cell reduces the hydrophobicity of the cell membrane surface, stabilizes the foam layer at the top of the cell culture, and/or strengthens the cell membrane [118, 119]. P-F68 can significantly enhance cell growth, cell viability, productivity, and protein quality by working as a shear stress protector. CHO cells can take up P-F68 and degrade it in lysosomes, so that in addition to including the P-F68 in the basal medium, the continuous addition of this surfactant as a part of the feed may be necessary to maintain the optimum concentration in the cell culture [120].

Generally, the effect of P-F68 is concentration-dependent, and the optimum concentration is 0.3 – 3 g/L [121, 122]. Also, there is a direct relationship between the volumetric convective

mass transfer coefficient, K_La, and PF-68 at the concentration range of 1 – 3 g/L. Increasing the concentration of P-F68 will decrease the bubble size, which results in a high surface area for mass transfer [117]. The concentration 1 g/L of the P-F68 is most common in industrial cell culture processes; however, this concentration can vary depending on the cell line and the cell culture process [123].

1.4.4.2. Antifoaming agents

While agitation and sparging are necessary to supply enough oxygen to the cells, the formation of bubbles and foam in large-scale bioreactors can damage the sensitive mammalian cells [124]. The results of many investigations in the literature show that cell damage in sparged bioreactors occurs mainly in the region of bubble disengagement [125, 126]. Furthermore, excessive foaming can damage the proteins from bursting bubbles, block the exhaust filter, stop the whole culture system, and lead to loss of system sterility from escaping foam [127, 128]. From these considerations, the control of foam formation is a vital part of mammalian cell culture process development, especially in stirred and sparged bioreactors.

Antifoaming agents are strong hydrophobic surfactants composed of solid particles, an oil, or a mixture of these components. The solid particles can disrupt foam as the hydrophobicity of the particles causes perforations on the surface of foam bubbles. Oil-based antifoams can also form bridges in the foam lamella that leads to foam rupture [129, 130]. While using antifoaming agents has a positive effect on the cell culture conditions, high concentrations of antifoaming agents affect the growth and productivity of the cells. Antifoams can alter cell permeability and results in cell content leakage [131]. The addition of antifoams should be carefully controlled so as to use the minimal effective amount. Silicon-based antifoams such as Antifoam C, SE-15, and Y-30 are widely used in CHO cell culture due to their high efficiency and low toxicity [132].

1.4.4.3. Anti-clumping agents

While CHO cell lines have been adapted to grow in free suspension culture, some CHO cells may attach to one another and form clumps for various reasons. The presence of free DNA and cell debris in the medium are the most common causes for the aggregation of cells. The sticky nature of DNA causes cells and other debris to aggregate into large clumps. Environmental stress, such as mechanical forces and repeated freeze/thaw cycles, and cell overgrowth are the most common causes of cell lysis and release of DNA. additionally, certain suspension cell types aggregate when they are not under optimal conditions [133, 134].

The aggregation of cells hinders accurate cell counting, culture monitoring, controlling of the cellular environment, and impairs the transport of nutrients into the cells. The aggregation of cells has been shown to reduce growth by up to 50% and increase the rate of cell death [133, 135]. The addition of an anti-clumping reagent to the basal medium is the most common approach to prevent the aggregation of cells. DNase I can be used to decrease the effect of free DNA on the formation of cell aggregates. Some chelators, such as citrate and EDTA, can remove bivalent cations that accelerate the formation of cells clumps. Currently, there are some commercially available anti-clumping agents that are animal-origin-free and chemically defined. GIBCO® Anti-Clumping Agent and LONZA® Anti-Clumping Agent A and B are concentrated solutions that can be added to the culture medium at a specific concentration to prevent the formation of cells clumps.

1.4.5. Feeding strategy development

In general, the addition of feed media begins when an essential nutrient(s) is exhausted. The most common approach is to concentrate the basal medium and use this concentrated solution as a feed medium. The feed composition and feeding strategy are often considered cell line-dependent as the consumption of nutrient(s) and the accumulation of by-product(s) differ

from one process to another [136]. As discussed, the accumulation of by-products such as lactate and ammonia can be minimized by maintaining low concentrations of glucose and glutamine through the fed-batch culture. Typically, most of the salts and glutamine that are found in the basal medium are not included in the feed solution.

Stepwise addition of the concentrated feed solution at a stoichiometric ratio to the total culture volume is most often used due to the simplicity and scalability of this approach. Meanwhile, when glutamine and/or glucose reach a particular concentration, a precise amount of stock solution of these nutrients is added to reach glutamine and/or glucose setpoints. Usually, the setpoint is the amount of nutrients that should provide enough nutrients to support cell growth while avoiding the accumulation of by-products to toxic levels [63, 137, 138]. While feeding strategy development is cell line dependent, labor-intensive, and time-consuming, a combination of high throughput cell culture systems with statistical design of experiment (DOE) approaches can be applied to shorten the timeline [52, 53]. Monitoring the cell culture by measuring the relevant parameter is crucial for reaching the optimal feeding strategy. While some parameters should be monitored online, other parameters can be measured using an offline method.

1.4.5.1. Online monitoring

Dissolved oxygen (DO), pH, temperature, agitation, and the flow rates of various gases are the most common parameters that should be monitored using online methods. As even a slight deviation of the setpoints of these parameters can significantly impact the growth and metabolism of cells. Currently, most bioreactors include non-invasive optical sensors to measure pH and DO coupled with an automated control system to adjust these parameters continuously [139]. Besides the advantage of monitoring the cell culture continuously, the non-invasive sensor decrease the risk of contamination due to decreasing human interaction with the culture vessels.

1.4.5.2. Offline monitoring

Cell culture metabolites, such as glucose, lactate, glutamine, glutamate, and ammonia, are commonly measured offline using enzymatic biosensors. The measurement of these metabolites is essential for maintaining the levels of substrates above their critical setpoints and monitoring the accumulation of toxic by-products. Among the available instruments, Nova analyzers, such as the Nova Bioprofile 400 analyzer, can measure multiple metabolites with one sample in addition to measuring pH, oxygen and carbon dioxide partial pressure [140]. The Trypan Blue exclusion method using a microscope is the most common method for measuring the cell concentration and viability. This method depends on the ability of the live cells (*i.e.*, cells with intact membranes) to exclude Trypan Blue stain. There are automated analyzers that use this method and are coupled to image analysis. Among the commercially available analyzers, Vi-Cell and Cedex are the most widely used. While there are currently online optical cell density probes in the market to monitor cell cultures, these methods are generally linear with cell concentration only at high viabilities and deviate from linearity in cultures with low viability [141].

1.5 Summary

While 70% of the recombinant protein production uses the CHO expression system, cell culture process development is still cell line dependent and differs from one process to another. The recent development of the cell culture media coupled with concepts of rebalancing basal and feed media compositions helps to optimize nutrient delivery while controlling the accumulation of by-products. This advancement in cell culture technology has increased the expression of recombinant proteins from milligrams per liter to several grams per liter. The commercially available serum-free media and feeds can be used to find the optimum feeding strategy for CHO cells as a first step of the process development, and this can help decrease the timeline for the process development.

CHAPTER II

EFFECTS OF USING FEED MEDIA CONTAINING GLUCOSE ON THE PERFORMANCE OF CHO-3440 CELLS IN FED-BATCH CULTURE WITH HYCLONE™ SFM4CHO™ MEDIUM AND HIGH GLUTAMINE CONCENTRATIONS

2.1 Introduction

CHO cells are the most used system for large-scale manufacturing of recombinant proteins [6, 7]. The advancement in cell culture media and fed-batch operation mode have led to increases in cell productivity, reaching product amounts of several grams/liter; however, the optimization of a new process is still cell line dependent and takes several months [2]. The fed-batch optimization depends on adding nutrients that have been depleted, such as glucose and glutamine, while avoiding overfeeding to control the formation and accumulation of by-product [137, 138].

Glucose is a significant energy source for mammalian cells, as half of the required energy comes from glucose through the TCA cycle. Most basal media, therefore, contain large amounts of glucose to support a high growth rate. Despite the importance of glucose in the basal medium, high levels of unused glucose increase the accumulation of toxic by-products, especially lactate

[81, 82]. A common approach to overcome the accumulation of these by-products is to limit the availability of glucose in the late stages of the culture [138].

Glutamine is another significant energy source for CHO cells; however, its instability at high temperatures leads to decomposition and the accumulation of ammonia to toxic levels. The basal medium, therefore, is provided in powder form and prepared without glutamine. The required amount of glutamine is added at the time of use. Additionally, since glutamine undergoes chemical decomposition at low initial cell concentration, the cell culture is with the lowest possible concentration of glutamine [102]. Currently, most feed media are provided without glutamine so that the concentration of glutamine can be controlled separately. Similar to how glucose is managed, the concentration of glutamine is reduced in the late stages of cell culture to minimize the accumulation of ammonia.

A common approach to developing a new bioreactor process as quickly as possible is to find the optimum combination of commercially available basal medium and feed media. This approach can be achieved through trying different types and concentrations of feed media while monitoring cell density, viability, and the concentration of essential nutrients and by-products in the culture medium. The purpose of the work that will be presented here was to optimize the feed strategy of a CHO-3440 cell line with a SFM4CHO basal medium that has a relatively high concentration of glutamine. Firstly, the CHO-3440 cell line was adapted to grow in the serum-free basal medium, SFM4CHO, with 8 mM L-glutamine. Secondly, a study was performed to understand the effects of two different types and concentrations of feed media, Cell Boost 7a/7b and Cell Boost 5 (CB5), on the maximum cell density, accumulation of by-products, and longevity of the culture. Lastly, using the optimized feed media, the starting concentration of SFM4CHO basal medium was varied in an attempt to reduce the amount of essential nutrients remaining at the end of the cell culture.

2.2 Materials and methods

2.2.1. Cell line

The CHO-3440 cell line was purchased from ATCC (Manassas, VA). CHO-3440 cells were prepared from a CHO-DG44 cell line that was genetically modified by deletion of the DHFR gene [10] and was stably transfected with a vector system expressing a model mouse-human chimeric IgG1 antibody [28]. The base medium for CHO-3440 cells is CD FortiCHO medium that was purchased from Thermo Fisher Scientific (Waltham, MA). CD FortiCHO medium was supplemented with 8 mM L-glutamine. The L-glutamine was purchased from Sigma Aldrich (St. Louis, MO), and a 200 mM stock solution was prepared and stored at -20 °C to decrease the decomposition rate of L-glutamine. Once the vial of CHO-3440 cells was received, it was thawed rapidly, and the cells were recovered in a 125 mL vented flat-bottom shaker flask with a seeding density of 0.5×10^6 viable cells/mL at a rocking speed of 130 rotation per minute (rpm) in an 8% CO_2 humidified incubator at 37 °C. Every 2 – 3 days, when the cells reach ~ 2 $\times 10^6$ viable cells/mL, the cells were passaged to $0.45 – 0.55 \times 10^6$ viable cells/mL. A working cell bank was prepared in cryopreservation medium, complete culture medium + 5% dimethylsulfoxide (DMSO), with $1 – 2 \times 10^7$ viable cells/mL and stored in the vapor phase of liquid nitrogen.

2.2.2. SFM4CHO medium adaptation

According to ATCC, the base medium for CHO-3440 cell line is CD FortiCHO medium, however this medium is commercially available from Thermo Fisher Scientific in a 1 L volume only. The main objective of this work is to develop a feeding strategy for a fed-batch culture that can be used in large-scale culture applications; therefore, the SFM4CHO medium is a suitable choice as it is commercially available with different volumes and up to 100 L. CHO-3440 cells growing in CD FortiCHO medium were adapted to grow in SFM4CHO medium (Cytiva,

Marlborough, MA) through a process were the ratio of SFM4CHO medium to the CD FortiCHO medium was sequentially increased. The complete SFM4CHO medium was supplemented with 8 mM L-glutamine, 1 g/L P-F68, and 2.2 g/L sodium bicarbonate. Frozen cells in 1 mL vials were thawed rapidly into 15 mL of CD FortiCHO supplemented with 8 mM L-glutamine in a 75 cm^2 T-flask. The cells were maintained at 37 °C in an 8% CO_2 humidified incubator. After 2 days, the cells were transferred to a 125 mL shake flask containing 30 mL of CD FortiCHO medium supplemented with 8 mM L-glutamine and maintained at 37 °C in an 8% CO_2 humidified incubator at a rocking speed of 130 rpm. Cells were cultured in the following ratios of SFM4CHO medium to CD FortiCHO medium: 25:75, 50:50, 75:25, 87.5:12.5, and finally 100:0. The whole process is a series of individual adaptations to different media mixtures, so the cells were passaged at least 3 times in a particular mixture of media to make sure that cells were fully adapted to that mixture before increasing the percentage of SFM4CHO medium. The successfulness of adaptation was determined by comparing the growth curve of the adaptation culture with the reference growth curve for cells growing in the CD FortiCHO medium. Once the growth kinetics of adaptation culture match the reference culture in terms of length of lag phase, the growth rate in the exponential growth phase, and maximum cell density, cells were considered adapted and shifted to the following mixture with a higher percentage of SFM4CHO medium.

Once cells were growing in 100% SFM4CHO medium, the CO_2 percentage of the atmosphere inside the incubator was reduced to 5% due to the difference in sodium bicarbonate concentration within the two media. When the growth rate and maximum cell density in 100% SFM4CHO medium met or exceeded expected values from the reference growth curve, aliquots of the culture with $1 – 2 \times 10^7$ viable cells/ mL were cryopreserved in SFM4CHO medium + 5% DMSO in the vapor phase of liquid nitrogen.

2.2.3. Batch and fed-batch culture media

The SFM4CHO medium was used for batch and fed-batch culture studies. To complete the SFM4CHO medium, it was supplemented with 8 mM L-glutamine, 1 g/L P-F68, and 2.2 g/L sodium bicarbonate. The feed media for fed-batch culture were Cell Boost 5 and 7a/7b (Cytiva, Marlborough, MA). Each feed medium was used with different concentrations and different addition schedules. The batch and fed-batch studies were performed in 125 mL shaker flasks with initial working volumes of 30 mL/flask. The cells that were used in either batch or fed-batch culture studies were from the cell bank that was completely adapted to SFM4CHO medium. A 1 mL vial of cells was thawed rapidly in 15 mL complete SFM4CHO medium in a 75 cm^2 T-Flask. The cells were maintained at 37 °C in a 5% CO_2 humidified incubator. After 2 days, the cells were transferred to a 125 mL shake flask containing 30 mL complete SFM4CHO medium with a seeding density of 0.45 – 0.55 $\times 10^6$ viable cells/mL at 37 °C in a 5% CO_2 humidified incubator at a rocking speed of 130 rpm. Every 2 – 3 days, when the cells reach ~2 $\times 10^6$ viable cells/mL, the cells were subcultured to 0.45 – 0.50 $\times 10^6$ viable cells/mL. Before using cells in batch and fed-batch culture studies, cells were passaged at least two times to ensure the growth rate and viability of the cells.

D-glucose stock solution with a concentration of 450 g/L was used in the fed-batch culture studies to maintain the concentration of glucose at or above 4 g/L. For the preparation of glucose stock solution, 450 g/L D-glucose was dissolved in ultrapure water under low heating conditions; the solution was then sterilized using a 0.22 µm filter and stored at room temperature.

L-glutamine stock solution with a concentration of 200 mM was used in the fed-batch culture studies to maintain the concentration of L-glutamine within the range of 2 – 4 mM after the addition of feed media. For the preparation of L-glutamine stock solution, 2.92 g/L L-

glutamine was dissolved in 0.85% saline; the solution was then sterilized using a 0.22 µm filter and stored in the freezer (-20 °C).

2.2.3.1. Batch culture

For batch culture studies, cells were transferred to a 125 mL shaker with a seeding density of 0.45 – 0.55 $\times 10^6$ viable cells/mL with at least 99% viability. Every day, the culture was monitored by taking a 1 mL sample to measure the cell density, viability, and concentration of metabolites. The batch culture was maintained without any feed addition until the cell viability reach <70%. The maximum cell density, cell viability, and metabolites profile through the cell culture period were recorded. All experiments were performed in duplicate.

2.2.3.2. Cell Boost 5 feed media

Cell Boost 5 (CB5) supplement was provided as a free sample from Cytiva (Marlborough, MA). The feed media CB 5 is chemically defined, animal-derived component-free, protein-free, and does not contain L-glutamine or P-F68. The use of CB 5 as a feed media provides nutrients such as lipids, amino acids, vitamins, and growth factors as a part of the fed-batch cell culture. Cytiva tested CB 5 with different cell lines, including CHO. CB 5 was prepared according to the provided preparation protocol from Cytiva with 39 g/L in deionized water and a pH of 7 – 7.4.

Fed-batch culture studies using CB 5 were performed in 125 mL shake flasks with starting volumes of 30 mL. Cells were inoculated at 0.45 – 0.55 $\times 10^6$ viable cells/mL, and CB 5 was added on day 3 and every other day until day 9 of the culture at volumes listed in Table 2. Cells were maintained at 37 °C in a 5% CO_2 humidified incubator at a rocking speed of 140 rpm. The concentrations of glucose and L-glutamine were monitored and supplemented separately as needed to maintain their concentrations at 4 g/L and 2 – 4 mM, respectively. Samples were taken

daily and cell density, viability, and metabolite content were measured. The fed-batch culture was maintained until the cell viability reach <70%. All experiments were performed in duplicate.

Table 2. Setup of shake flask experiment using Cell Boost 5.

Shake flask volume (mL)	SFM4CHO starting working volume (mL)	Cell Boost 5 (% of working volume)	Cell Boost 5 (mL)
125 mL	30 mL	5.0	1.50
125 mL	30 mL	2.5	0.75

2.2.3.3. Cell Boost 7a and 7b

Cell Boost 7a has a pH close to neutral and contains a mixture of amino acids, vitamins, glucose, and trace elements, while Cell Boost 7b has an alkaline pH and is a concentrated solution of amino acids. The two supplements, therefore, complement each other and are used in combination with the basal medium, SFM4CHO. Cytiva (Marlborough, MA) provided these supplements as a free sample for our studies. Cell Boost 7a and 7b are chemically defined, animal-derived component-free, and do not contain any growth factors, peptides, hydrolysates, phenol red, or 2-mercaptoethanol, ensuring batch-to-batch consistency. The recommended ratio of cell boost 7a to 7b is 10 to 1 (v/v) and should be added to the cultivation vessel as individual solutions and should not be mixed in advance as mixing will cause precipitation. The two feeds were provided as a dry concentrated powder to be hydrated before use according to the recommended protocol.

Fed-batch culturing in shake flasks were conducted to obtain an optimal feeding strategy. Studies were performed in 125 mL shake flasks with a starting volume of 30 mL SFM4CHO medium. Cells were inoculated at 0.45 – 0.55 viable cells/mL and maintained at 37 °C in a 5% CO_2 humidified incubator at a rocking speed of 140 rpm. Two different approaches were used to find the optimum feeding strategy. The first approach was to add Cell Boost 7a and 7b on day 3 once daily until day 9 at volumes listed in Table 3.

Table 3. Setup of shake flask experiment using Cell Boost 7a/7b every day.

Shake flask volume (mL)	SFM4CHO starting working volume (mL)	Cell Boost 7a/7b (% of working volume)	Cell Boost 7a (µL)	Cell Boost 7b (µL)
125 mL	30 mL	2.50/0.25	750	75
125 mL	30 mL	2.0/0.2	600	60

The second approach required the addition of Cell Boost 7a and 7b at day 3 and once every other day until day 9 at the volumes listed in Table 4. In both approaches, glucose and L-glutamine were monitored and supplemented separately as needed to maintain their concentration at 4 g/L and 2 – 4 mM, respectively. Samples were taken daily and cell density, viability, and metabolite content were measured. The fed-batch culture was maintained until the cell viability reach <70%. All experiments were performed in duplicate.

Table 4. Setup of shake flask experiment using Cell Boost 7a/ 7b every other day.

Shake flask volume (mL)	SFM4CHO starting working volume (mL)	Cell Boost 7a/7b (% of working volume)	Cell Boost 7a (µL)	Cell Boost 7b (µL)
125 mL	30 mL	2.50/0.25	750	75
125 mL	30 mL	1.5/0.1	450	30

2.2.3.4. SFM4CHO (80%, 15.9 g/L) with Cell Boost 7a/7b

To study the effect of the initial glucose concentration in the basal medium. The SFM4CHO medium was prepared at a concentration of 15.9 g/L instead of 19.83 g/L, representing a concentration that was 80% of the recommended concentration. The decrease in medium concentration led to a decrease in the initial glucose concentration. Cells were inoculated at 0.45 – 0.55 $\times 10^6$ viable cells/mL in a shaker flask with starting volume of 30 mL of 80% SFMFCHO medium. The cells were maintained at 37 °C in a 5% CO_2 humidified incubator at a rocking speed of 140 rpm. Starting on day 3, 1.5%:0.1% Cell boost 7a/7b were added once every

other day until day 9. Like the other fed-batch culture experiments, glucose and L-glutamine were monitored and supplemented separately as needed to maintain their concentration at 4 g/L and 2 – 4 mM, respectively. Samples were taken daily and cell density, viability, and metabolite content were measured. The fed-batch culture was maintained until the cell viability reach <70%. All experiments were performed in duplicate.

2.2.3.5. Cell clumping

GIBCO® Anti-clumping agent was added to the initial basal medium to decrease the cell aggregation. The use of anti-clumping agent is recommended at a dilution ranging from 1:250 to 1:1000 in culture media. To find the optimum concentration of the anti-clumping agent, the fed-batch culture was started with the lowest concentration of the anti-clumping agent, 1:1000 in culture media, and the concentration of anti-clumping agent was increased to find the lowest concentration that would prevent aggregation of the cells. The anti-clumping agent study used the same protocol of fed-batch culture using 80% SFM4CHO with the addition of 1.5%:0.1% Cell boost 7b/7b on day 3 and every other day until day 9.

2.2.4. Analytical methods

2.2.4.1. Cell density and viability

A hemocytometer-based trypan blue dye exclusion assay was used to count the number of viable and non-viable cells. After mixing the culture to ensure the uniformity of the sample, 200 µL of culture was aseptically transferred to a 1.5 mL clear Eppendorf tube as a representative sample. The sample (50 µL) was mixed with an equal volume of 0.4% trypan blue in isotonic salt solution. Viable (seen as bright cells) and non-viable (stained blue) cells were counted using a dual-chamber hemocytometer and a light microscope. Ideally, 100 – 200 cells should be counted in order to increase the accuracy of the measurement. If the number of cells was above 200, the

sample was diluted with medium before adding the trypan blue. The concentration of viable and non-viable cells was calculated using Equation 2, where viability is the percentage of viable cells relative to the total cell count (viable and non-viable). Each sample was repeated three times and the results were used to calculate the average and standard deviation (SD). The error bars on the cell growth profile graphs show ± 1 SD of the average.

$$Cell\ Count\ (Cells/mL) = \frac{Number\ of\ cells\ counted}{4} \times Dilution\ factor \times 10^4 \qquad \text{(Equation 2)}$$

2.2.4.2. Metabolite analysis

A Bioprofile 400 automated chemistry analyzer was used to measure the concentrations of glucose, lactate, glutamine, glutamate, and ammonia, as well as the concentration of potassium and sodium cations in cell culture supernatant. This multi-channel analyzer also offers the option to measure pH, PO_2, and PCO_2, plus calculating the osmolality. Some of the cell culture (1 mL) was aseptically transferred to a 1.5 mL Eppendorf tube; then, the cell suspension was centrifuged for 4 minutes at 10,000 rpm at room temperature. The supernatant was used for the Bioprofile 400 analyzer test.

2.3 Results and discussion

2.3.1. Batch culture using CD FortiCHO medium

Growing the CHO-3440 cell line using a CD FortiCHO medium under batch culture conditions was used to establish a reference growth curve for the cell line (Figure 4). This reference growth curve was used to gauge the progress of the adaptation process to the new serum-free medium, SFM4CHO. The curve shows the four phases of growth in cell culture: lag phase, exponential phase, stationary phase, and decline phase. The maximum cell density was $2.97 \pm 0.23 \times 10^6$ cells/mL, and the longevity of the culture was 6 days before the cells reached viability < 70%.

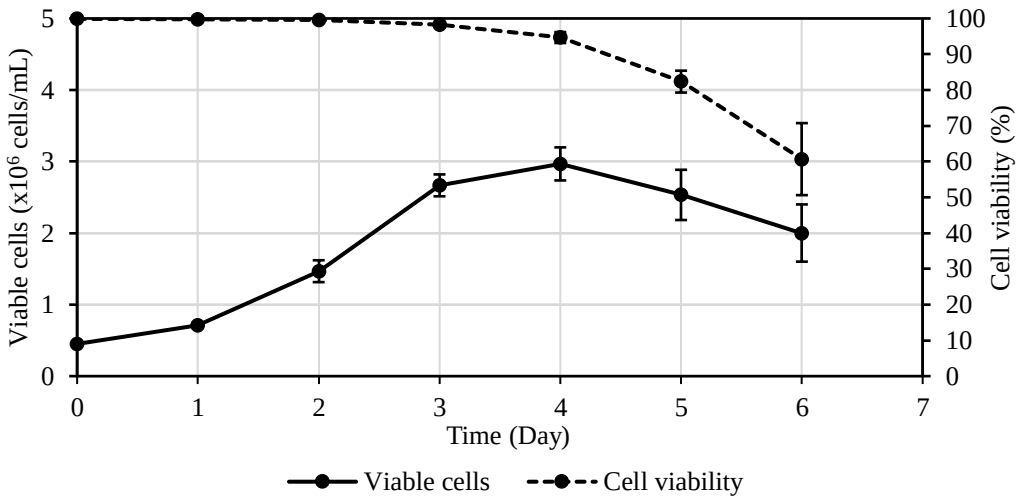

Figure 4. Growth profile for batch shake flask culture with CD FortiCHO medium.

2.3.2. SFM4CHO medium adaptation

The protocol for the adaptation process was based on increasing the ratio of SFM4CHO medium to CD FortiCHO medium from one step to another. Through each step of the sequential adaptation process, the goal was to achieve a growth rate similar to the reference profile. When the ratio of SFM4CHO medium to CD FortiCHO medium increased to 87.5:12.5, however, the cell size and cell aggregation increased (Figure 5). The rocking speed, therefore, was increased from 130 rpm to 140 rpm to decrease the tendency of the cells to aggregate through the adaptation process.

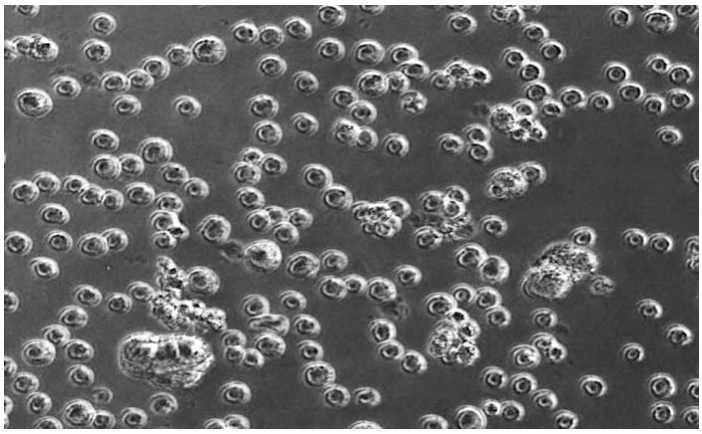

Figure 5. CHO-3440 cells aggregation in 87.5% SFM4CHO + 12.5% CD FortiCHO.

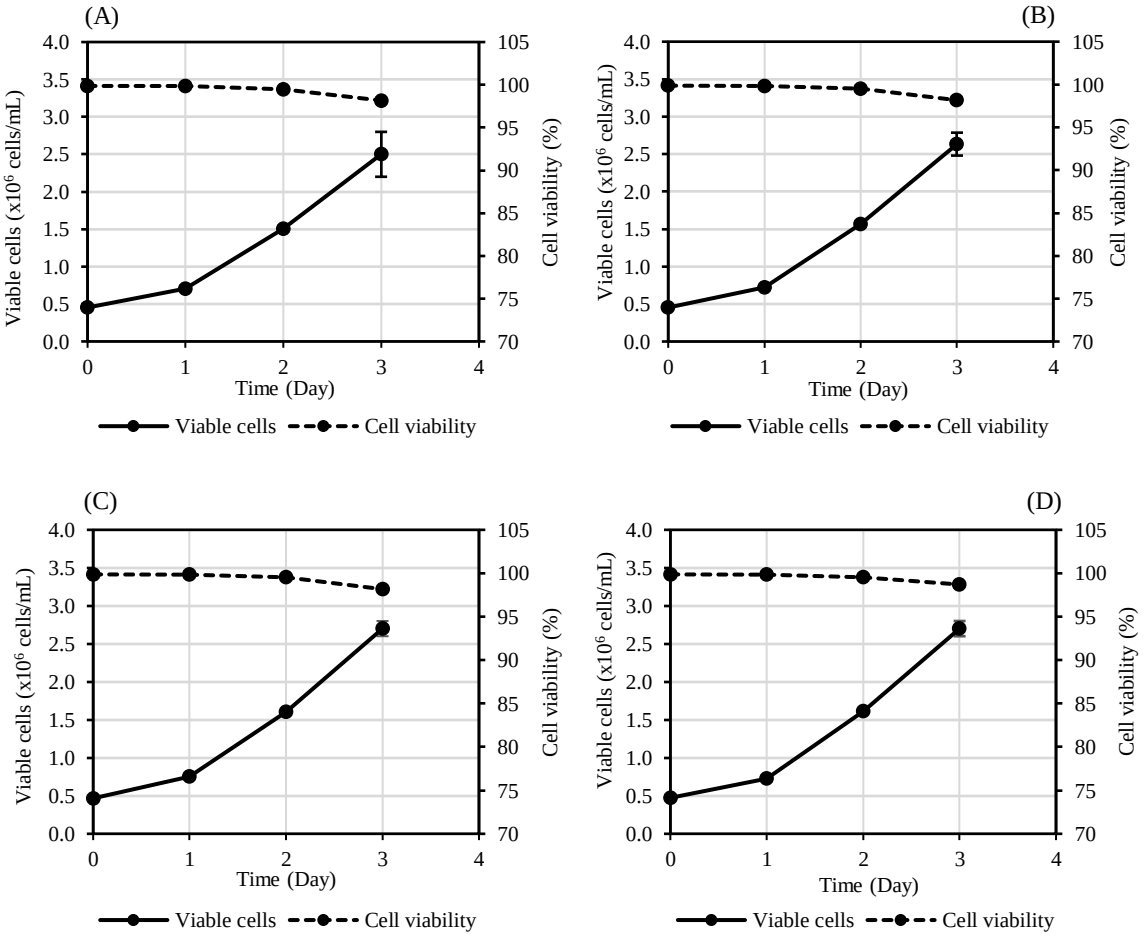

Figure 6. CHO-3440 cell line sequential adaptation for SFM4CHO medium in shake flask culture. (A) Growth profile in 25% SFM4CHO+75% CD FortiCHO. (B) Growth profile in 50% SFM4CHO+50% CD FortiCHO. (C) Growth profile in 75%SFM4CHO+25% CD FortiCHO. (D) Growth profile in 87.5% SFM4CHO+12.5%CD FortiCHO.

Figure 6 represents the growth profile of the first four steps of SFM4CHO medium sequential adaptation process. In each step, from A to D, the cells had the same cell density as the reference growth profile at day 3, indicating good adaptation of the cells in the new media mixtures. Gowth curves for the batch cultures with the CD FortiCHO and SFM4CHO basal medium (Figure 7) were compared for the assessment of adaptation process from CD FortiCHO medium to SFM4CHO medium. Although the two cultures have the same growth phases and longevity, the maximum cell density was significantly increased ($3.53 \pm 0.25 \times 10^6$ vs. $2.97 \pm 0.23 \times 10^6$ viable cells/mL) for the SFM4CHO culture. The similarity between the two cultures and the

higher maximum cell density in the SFM4CHO medium shows a successful adaptation of the cells to the new medium.

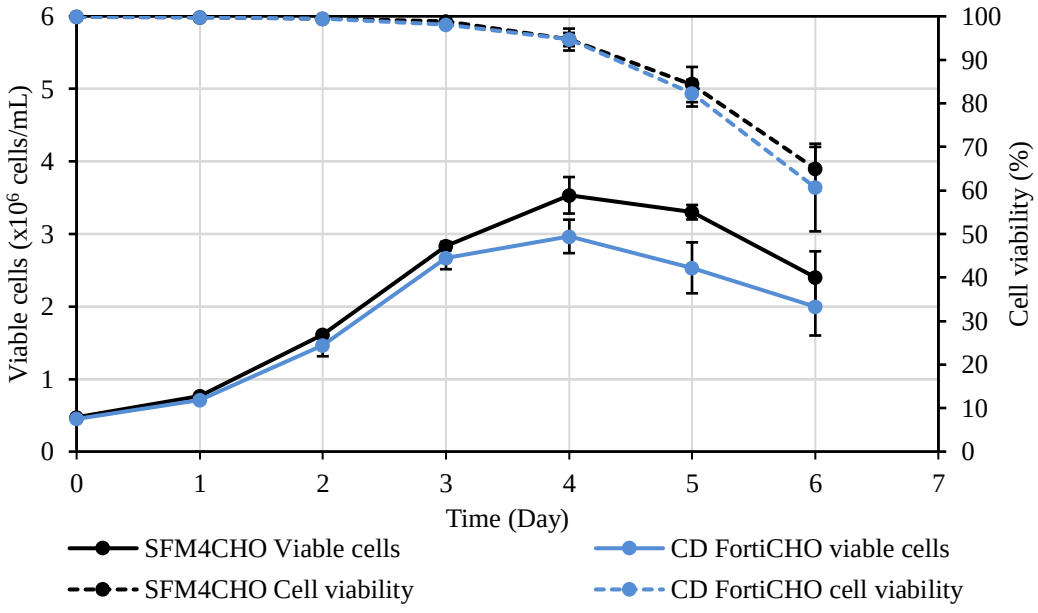

Figure 7. Growth profile for CHO-3440 batch culture using SFM4CHO and CD FortiCHO.

2.3.3. Every day feed regimen

The first trial for the optimization of feed media was adding feeds once daily beginning at day 3. To determine the effect of the every day feed regimen on culture metabolites, shake flask cultures were supplemented with Cell boost 7a/7b in ratios of either 2.5%:0.25% or 2%:0.2% of total culture volume. The growth and viability of cells for the tested feed volumes are shown in Figure 8. The results show that using a fed-batch culture instead of a batch culture increased the maximum cell density from $3.53 \pm 0.25 \times 10^6$ to $4.47 \pm 0.15 \times 10^6$ and $4.6 \pm 0.20 \times 10^6$ viable cells/mL with 2.50%:0.25% and 2.0%:0.2% Cell boost 7a/7b, respectively. Additionally, the every day feed regimen increased the longevity of the culture to 9 days instead of 6 days observed with batch culture.

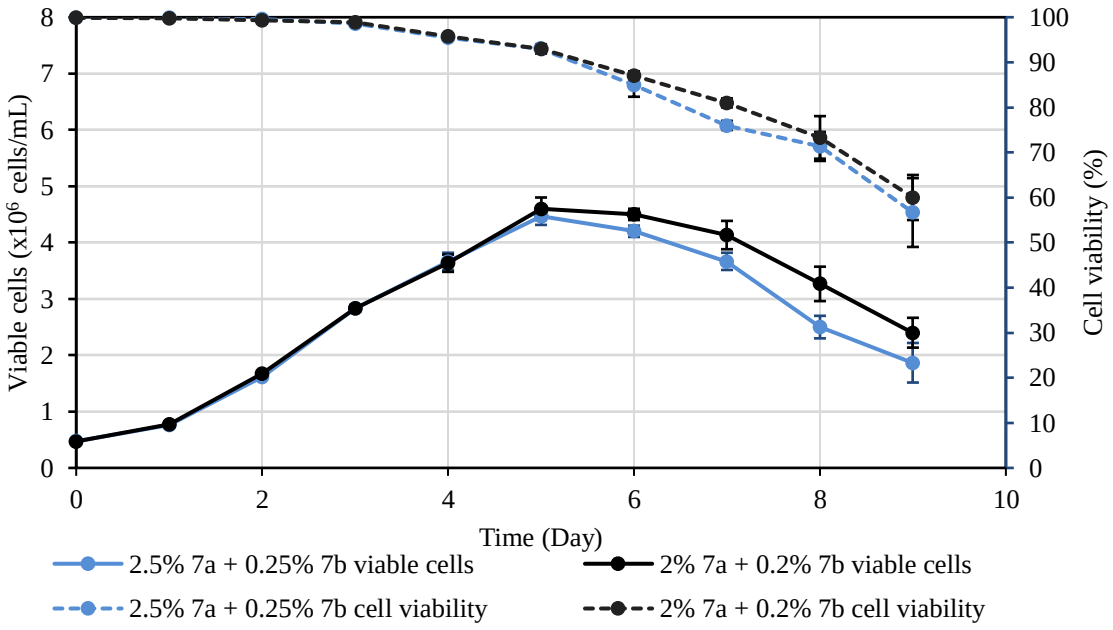

Figure 8. Growth profile for fed-batch culture using Cell boost 7a and 7b starting on day 3 every day.

Although using Cell boost 7a/7b feeds led to an increase in the maximum cell density and the longevity of the culture, the level of ammonia increased rapidly two days after the addition of feed. Figure 9 shows that ammonia reached inhibitory concentrations of 11.5 and 10.7 mM in 2.5%:0.25 and 2%:0.2% Cell boost 7a/7b fed-batch culture, respectively, on day 5, and by day 9 ammonia had reached 15.2 and 14 mM in 2.5%:0.25% and 2%:0.2 Cell boost 7a/7b culture, respectively.

The profile of glucose and lactate for the tested feed concentrations are shown in Figure 10. The results show a high level of glucose through all stages of the culture, with the glucose concentration being highest at the end of the culture period. As the glucose did not reach the threshold level of 4 g/L, this indicates that the addition of feeds every day has increased the concentration of glucose above the consumption rate of cells. The accumulation of glucose and the inhibitory concentration of ammonia on day 5 poses an inhibitory effect on cell growth [95, 142]. As the CHO cells consume lactate in extended fed-batch culture, lactate accumulation reached a peak of 2.8 g/L between days 7 and 8 and then began to decrease.

Table 5 shows an elevation of the osmolality above the inhibitory level of 400 mOsm/Kg in both fed-batch cultures. The high level of osmolality is an indication of the accumulation of metabolites, salts, and amino acids above the consumption rate of cells. The rise of osmolality levels above 400 mOsm/Kg shortened the culture period as it drove the cells to the stationary phase by day 5.

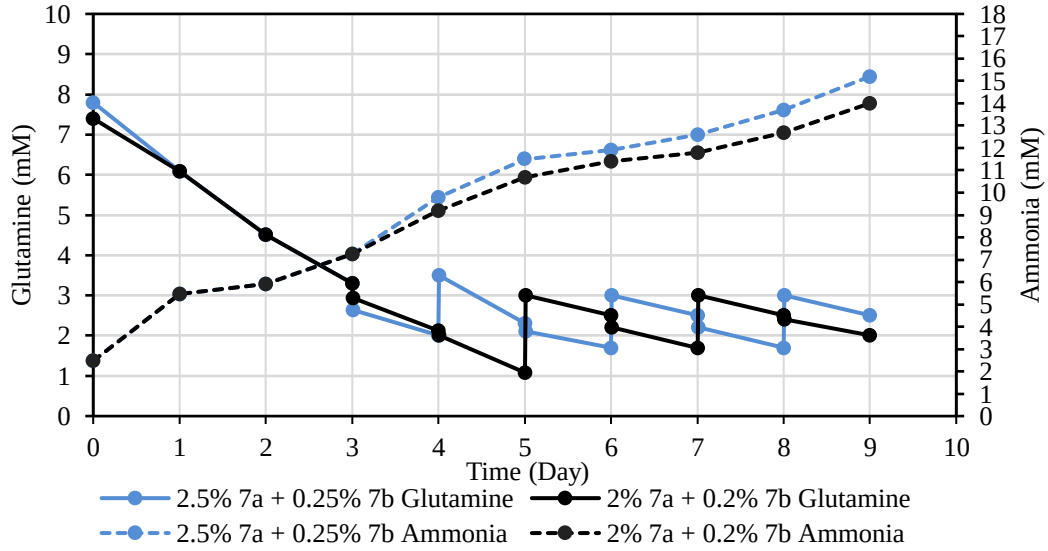

Figure 9. Glutamine and ammonia concentration profile for fed-batch culture using Cell boost 7a and 7b on day 3 every day.

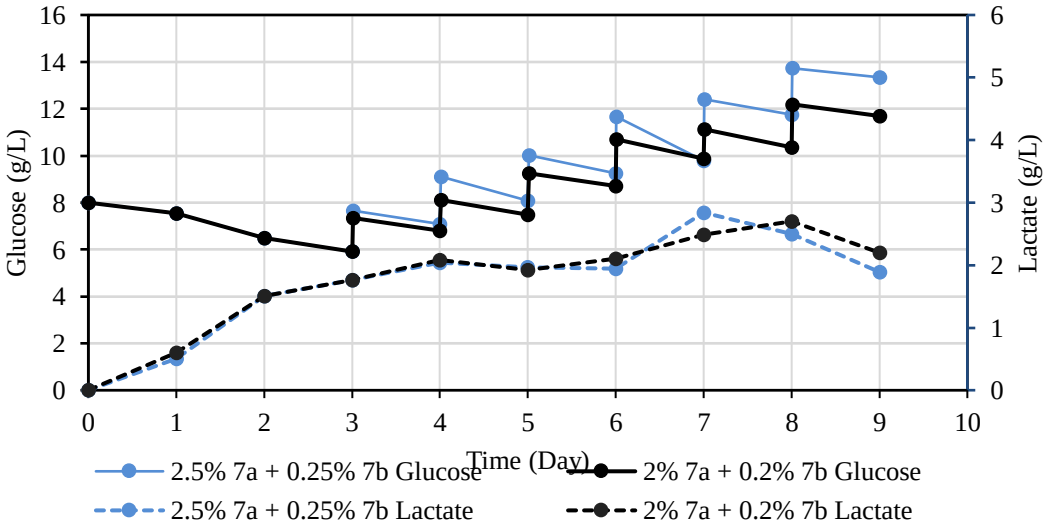

Figure 10. Glucose and lactate concentration profile for fed-batch culture using Cell boost 7a and 7b on day 3 every day.

Table 5. Osmolality for fed-batch culture using Cell boost 7a/7b every day.

Day	2.50/0.25 % Cell boost 7a/7b fed-batch culture osmolality (mOsm/Kg)	2.0/0.2 % Cell boost 7a/7b fed-batch culture osmolality (mOsm/Kg)
1	395	396
2	388	400
3	399	395
4	424	414
5	440	415
6	430	432
7	441	440
8	448	444
9	440	448

The results of using the every day feeding regimen strategy with Cell boost 7a/7b and SFM4CHO medium as a basal medium reflect the importance of determining an appropriate feeding regimen based on the nutritional requirements of a specific cell line so as to avoid overfeeding the culture. SFM4CHO medium is serum-free medium with a high concentration of glucose, and using a feed containing glucose, such as Cell boost 7a, once daily provides the culture with amounts of glucose above the nutritional requirements. Overfeeding the culture with glucose poses a direct inhibitory effect on cell growth by increasing the accumulation of lactate, pyruvate kinase and lactate dehydrogenase [142]. Overfeeding the culture, therefore, shortened the exponential growth phase and increased the chemical decomposition of unused glutamine in culture that led to the accumulation of ammonia above inhibitory levels [95].

2.3.4. Every other day feed regimen

In order to overcome the overfeeding of SFM4CHO culture, the feeding regimen was changed to every other day instead of adding the feeds daily. Cell boost 7a/7b in ratios of either 2.5%:0.25 or 1.5%:0.1% of total culture volume were added at day 3 and every other day till day 9. In addition to testing Cell boost 7a/7b supplements, the feed medium CB5 was tested as an alternative. CB5 was at ratios of either 5% or 2.5% of the total culture volume and was added on

day 3 and every other day until day 9. The cell growth profile for each of the feed media is shown in Figure 11. The results indicate that 1.5%:0.1% Cell Boost 7a/7b gave the highest cell density with 5.5 ± 0.3 $\times 10^6$ viable cells/mL and extended the culture until day 10. Also, the cells reached the stationary phase between day 7 and 8 instead of day 5.

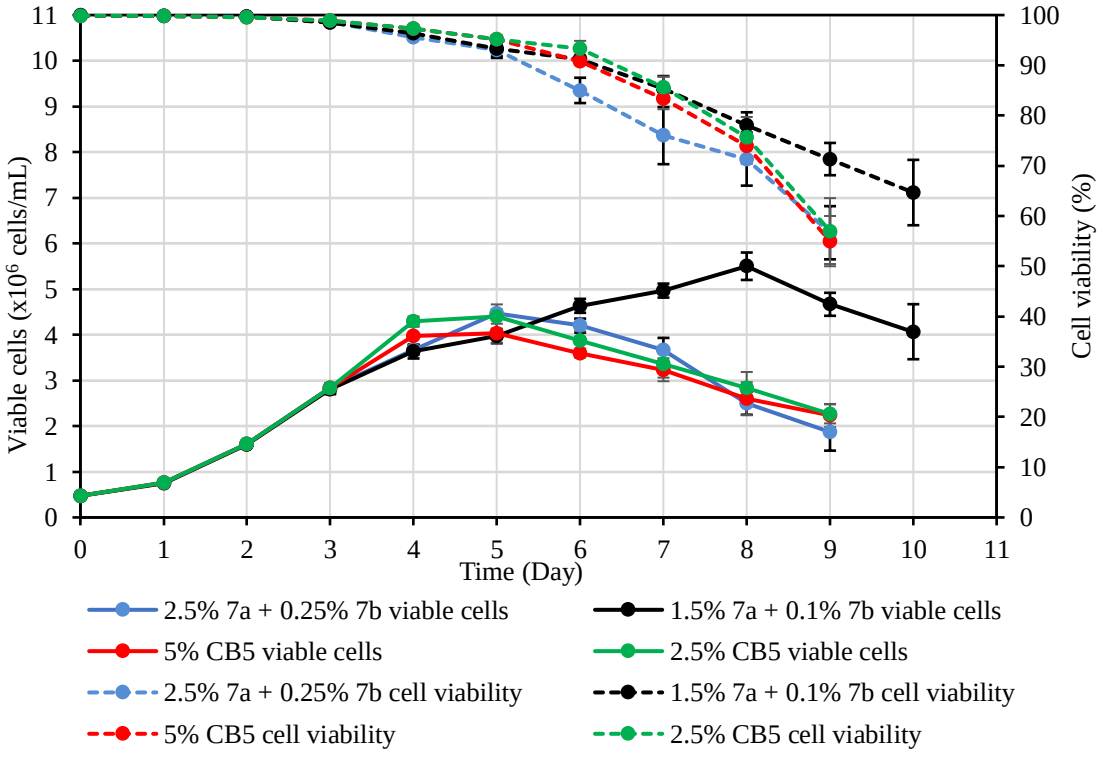

Figure 11. Growth profile for fed-batch culture using cell supplements 7a/7b or cell boost 5 every other day.

Figure 12 presents the profile of glucose and lactate through the fed-batch culture using the tested feed media. The results show a significant decrease of glucose levels with all tested feeds. Fed-batch culture with 1.5%:0.1% Cell boost 7a/7b gave the lowest accumulation of glucose through the culture period and the lowest glucose final concentration at 3.8 g/L. Also, the concentration of glucose reached levels below the threshold concentration (*i.e.*, 4 g/L) at day 7 and day 9 in the culture supplemented with 1.5%:0.1% Cell boost 7a/7b, which indicates low accumulation of glucose above the consumption rate of cells at the late stage of the fed-batch culture.

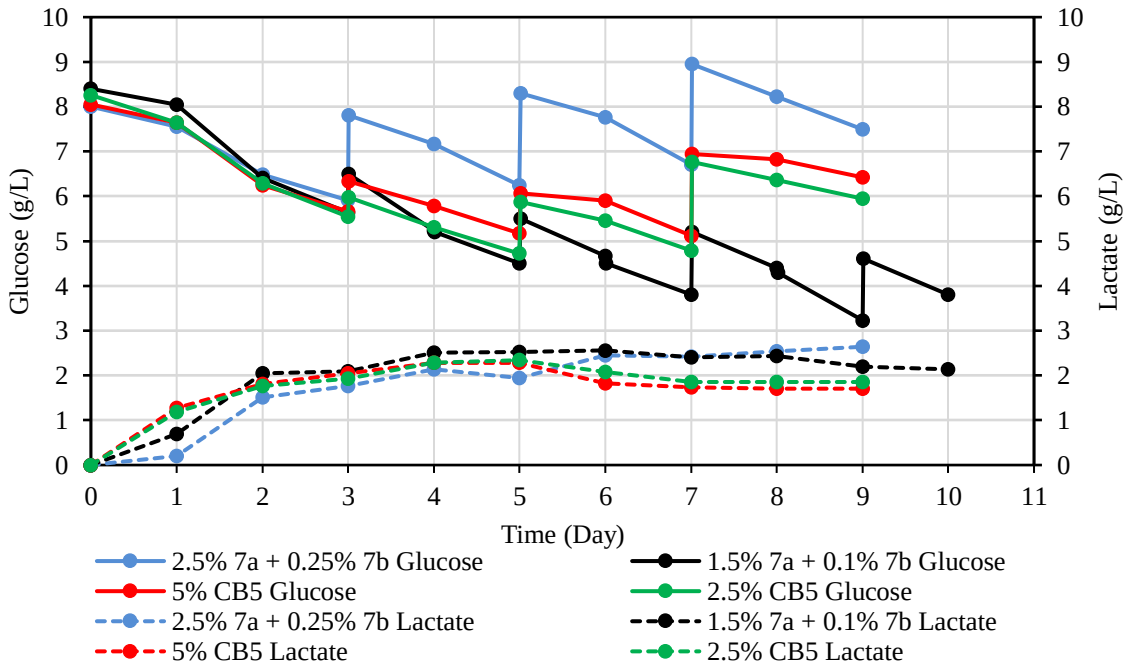

Figure 12. Glucose and lactate concentration profile for fed-batch culture using cell supplements 7a/7b or CB5 every-other-day.

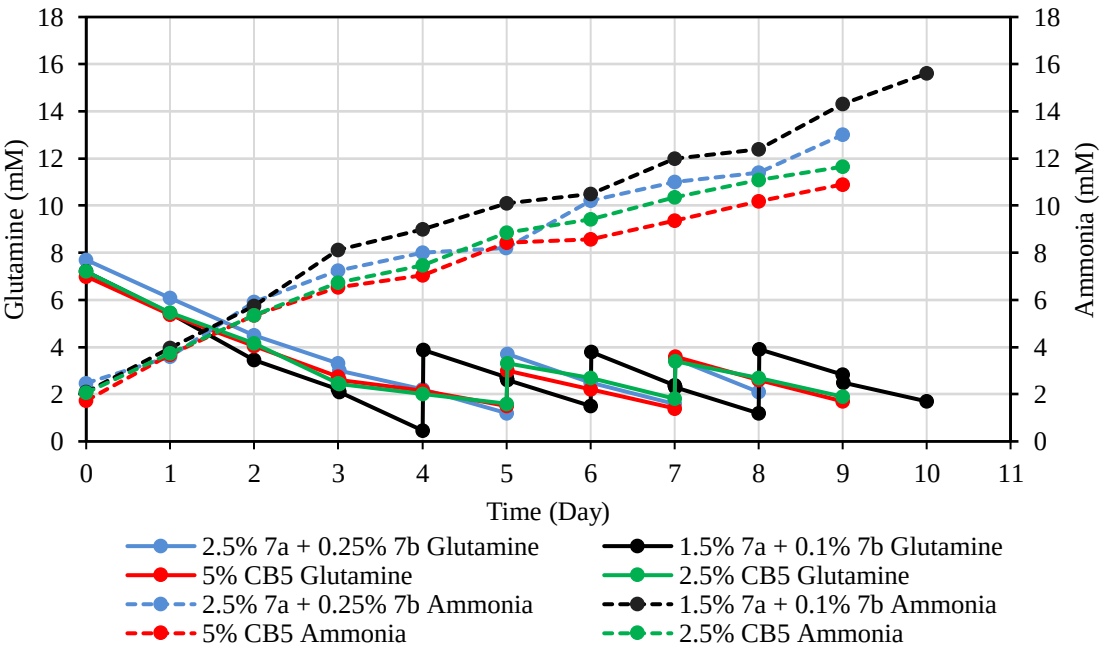

Figure 13. Glutamine and ammonia concentration profile for fed-batch culture using cell supplements 7a/7b or CB5 every other day.

Although the every other day feed regimen with a low concentration of Cell Boost 7a/7b decreased the accumulation of glucose, the ammonia level reached an inhibitory level (*i.e.*, >10 mM) at day 6 (Figure 13). Table 6 shows a significant difference between the calculated osmolality resulting from the feed media 2.5%:0.25% and 1.5%:0.1% Cell boost 7a/7b. Using the higher concentration of Cell Boost 7a/7b increased the osmolality to inhibitory levels on day 4, while the lower concentration of Cell Boost 7a/7b did not reach high osmolality levels until day 7. The high levels of ammonia and osmolality on day 6 and 7, respectively, led to the cells entering the stationary phase between day 7 and 8.

Table 6. Osmolality for fed-batch culture using Cell boost 7a/7b every other day.

Day	2.50/0.25 % Cell boost 7a/7b fed-batch culture osmolality (mOsm/Kg)	1.5/0.1 % Cell boost 7a/7b fed-batch culture osmolality (mOsm/Kg)
1	395	396
2	388	383
3	399	395
4	429	388
5	430	399
6	432	395
7	438	417
8	442	415
9	438	411
10	NA	423

In summary, the every other day feed regimen shows an increase in the growth rate performance of CHO-3440 cells. The maximum cell density reached $5.5 \pm 0.3 \times 10^6$ viable cells/mL and the culture was extended to 10 days. The feeding regimen of Cell Boost 7a/7b decreased the accumulation of glucose, especially during the late stage of the culture.

2.3.5. SFM4CHO (80%, 15.9 g/L) medium with Cell Boost 7a/7b

Although the every other day feed regimen decreased the accumulation of glucose during the late stage of fed-batch culture, the high initial concentration of glucose in the SFM4CHO medium combined with the addition of glucose from Cell Boost 7a led to high levels of glucose

during the early stage of the cell culture. To study the high initial concentration of glucose, and the negative effects of this on the cell culture, it is proposed that a lower initial concentration of SFM4CHO basal medium was prepared at a concentration 80% (15.9 g/L) of the supplier recommended concentration and the feeding regimen was carried out while monitoring the cell culture growth parameters. The diluted SFM4CHO medium had an initial concentration of glucose of ~6 g/L. Shake flask cultures with 80% SFM4CHO basal medium were supplemented with 1.5%:0.1% Cell Boost 7a/7b on day 3 and every other day until day 9. Additionally, supplemental glutamine and glucose were added daily, as needed, to restore the concentrations to 2 – 4 mM and 4 g/L, respectively.

Figure 14 shows a comparison between the every other day feed regimen with 100% and 80% SFM4CHO medium. The results showed an increase in the maximum cell density to 9.2 ± 0.1 $\times 10^6$ viable cells/mL when the concentration of SFM4CHO was decreased to 80%. Also, the longevity of the culture increased to 11 days instead of 10 days. Both the higher cell density and extended culture time reflected the better performance of the culture with 80% diluted SFM4CHO medium.

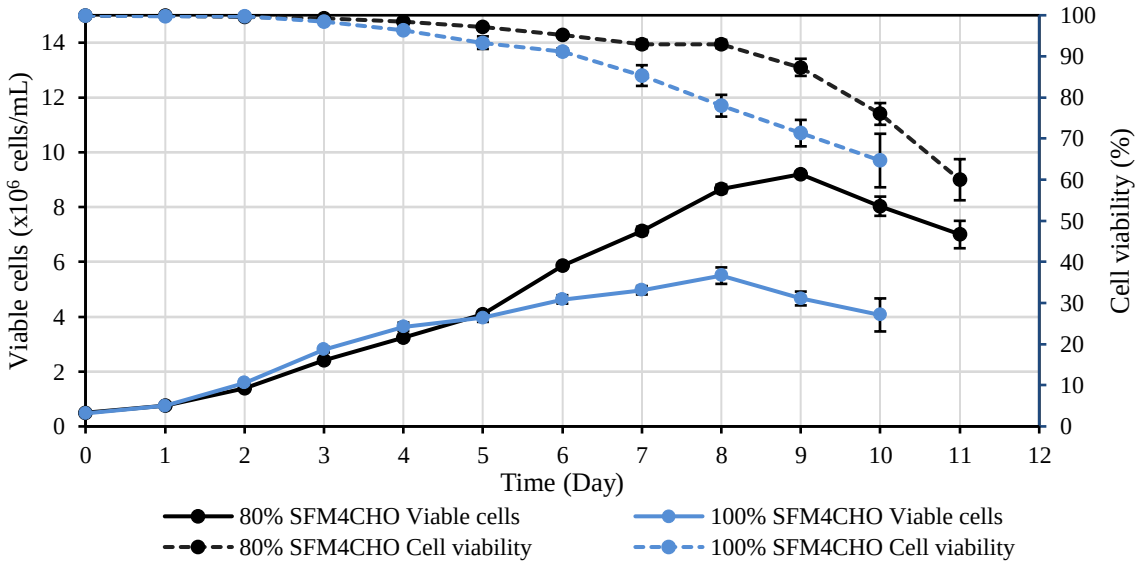

Figure 14. Growth profile for fed-batch culture using 1.5%:0.1% Cell Boost 7a/7b every other day with 100% and 80% SFM4CHO medium

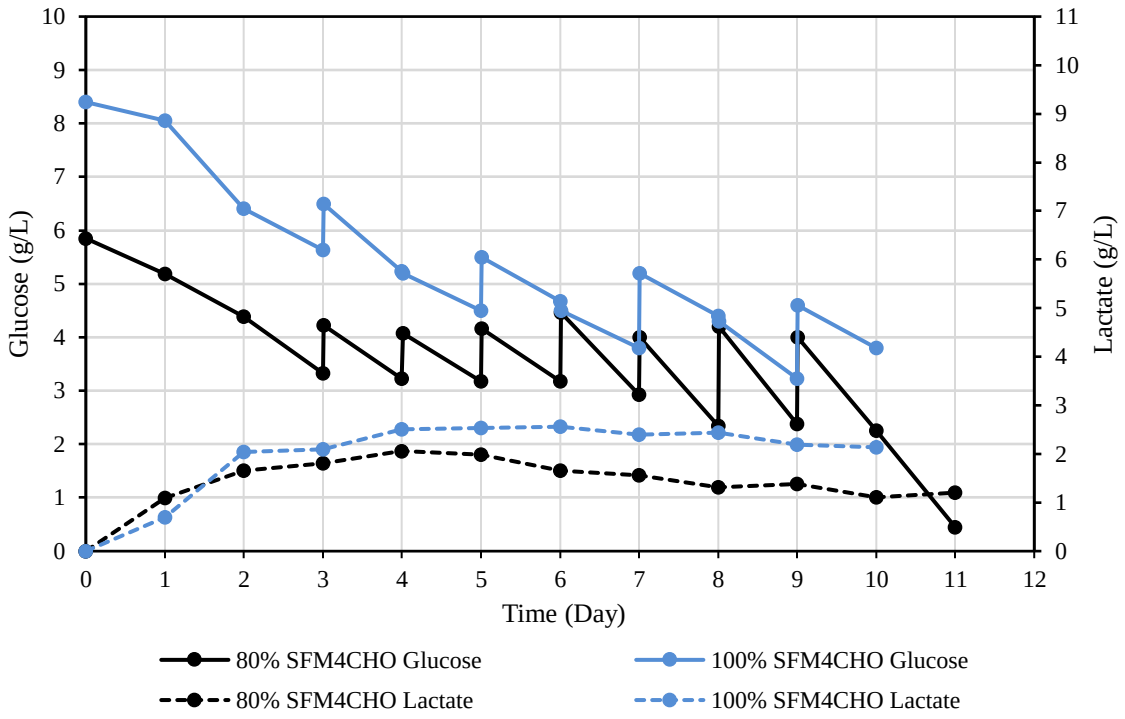

Figure 15. Glucose and lactate concentration profile for fed-batch culture using 1.5%:0.1% Cell Boost 7a/7b every other day with 100% and 80% SFM4CHO medium.

The lower concentration (15.9 g/L) of SFM4CHO medium led to a lower initial concentration of glucose to ~6 g/L. This lower initial concentration significantly decreased the accumulation of glucose, especially in the early stage of the culture (Figure 15). In fact, it was necessary to add supplement glucose on days when Cell Boost 7a/7b was not added in order to restore the concentration of glucose to minimum of 4 g/L. The need for supplemental glucose shows that the addition of glucose contained in Cell Boost 7a/7b to the 80% SFM4CHO culture did not provide glucose in excess of what the cells could consume. Figure 15 shows a decrease in lactate concentration when using 80% SFM4CHO medium due to the decrease of the initial concentration of glucose. The maximum lactate concentration was 2.56 and 2.06 g/L for fed-batch culture using 100% and 80% SFM4CHO medium, respectively.

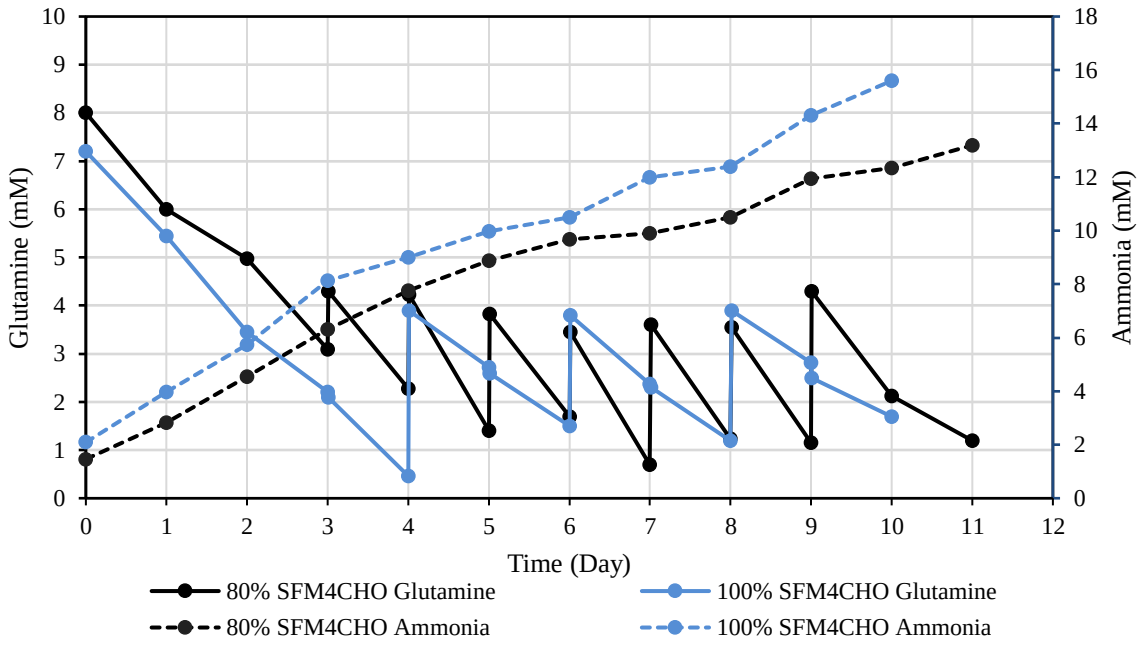

Figure 16. Glutamine and ammonia concentration profile for fed-batch culture using 1.5%:0.1% Cell Boost 7a/7b every other day with 100% and 80% SFM4CHO medium.

Figure 16 shows the concentration profile of glutamine and ammonia for the fed-batch culture using the same feed regimen with different SFM4CHO medium concentrations, 100% and 80%. While the glutamine profile was the same for both concentrations of the basal medium, the ammonia level for fed-batch using 80% SFM4CHO medium was lower than the one with 100% SFM4CHO. While ammonia reached an inhibitory level (*i.e.*, >10 mM) on day 6 in the fed-batch culture using 100% SFM4CHO, the inhibitory level of ammonia was delayed until day 8 using the 80% SFM4CHO medium. Also, the maximum concentration of ammonia in the fed-batch using 80% SFM4CHO medium was 13.19 mM compared to 15.60 mM in the fed-batch culture using 100% SFM4CHO medium. Similarly, Table 7 shows a significant decrease in osmolality with fed-batch culture using 80% SFM4CHO medium. While the fed-batch culture with 100% SFM4CHO medium reached an inhibitory osmolality level (*i.e.*, >400 mOsm/Kg) on day 7, the culture with 80% SFM4CHO medium remained below this level throughout the entire culture period.

Table 7. Osmolality for fed-batch culture using 1.5%:0.1% Cell Boost 7a/7b every other day with 100% or 80% SFM4CHO medium.

Day	1.5/0.1 % Cell boost 7a/7b fed-batch culture using 100% SFM4CHO osmolality (mOsm/Kg)	1.5/0.1 % Cell boost 7a/7b fed-batch culture using 80% SFM4CHO osmolality (mOsm/Kg)
1	396	315
2	383	306
3	395	303
4	388	330
5	399	313
6	395	303
7	417	317
8	415	316
9	411	322
10	423	330
11	NA	314

To summarize the impact of diluted basal medium, decreasing the concentration of SFM4CHO basal medium led to a decrease in the initial concentration of glucose. The lower initial concentration of glucose led to a sharp decrease in the accumulation of glucose and allowed for better control of the concentration of glucose, especially in the early stage of the culture. Also, decreasing the accumulation of glucose decreased the concentrations of lactate and ammonia, and subsequently decreased the osmolality, delayed reaching the stationary phase, and extended the culture longevity to 11 days. Consequently, the maximum cell density achieved using 80% SFM4CHO medium with 1.5%:0.1% Cell Boost 7a/7b every other day reached more than 1.6 times the maximum cell density with the same feed regimen but using 100% SFM4CHO medium.

2.3.5.1. SFM4CHO (80%, 15.9 g/L) medium with the anti-clumping agent

While using 80% SFM4CHO as a basal medium with Cell Boost 7a/7b as a feed media increased the maximum cell density and the longevity of the culture, cell clumps appeared when cells reached high density. Figure 17 shows the cell clumping in a shake flask on day 9. Cell clumps hinders accurate cell counting, monitoring, and impairs the transport of nutrients and

products to and from the cells. Also, the aggregation of cells reduces their growth by up to 50% and increases cell death rates [133, 135]. GIBCO® anti-clumping agent is a concentrated liquid reagent that reduces cell clumping to attain higher viable cell densities in suspension culture. The anti-clumping agent is an animal origin-free, chemically defined formulation that contains no protein, enzymes, hydrolysates, or components of unknown composition. GIBCO® anti-clumping agent was added to 80% SFM4CHO medium with a 1:1000 and 1:500 dilution to find the better of the two concentrations to prevent aggregation.

Figure 17. Cell clumps at day 9 of fed-batch culture using 80% SFM4CHO and Cell Boost 7a/7b.

Using GIBCO® anti-clumping agent with 80% SFM4CHO medium with 1:1000 dilution decreased the cell clumping; however, it did not completely prevent aggregation. Therefore, a 1:500 ratio of the anti-clumping agent was added to 80% SFM4CHO medium and was shown to prevent cell clumps entirely. Figure 18 shows a decrease in the aggregation of cells with the anti-clumping agent used at a 1:1000 dilution and complete disappearance of cell clumps with a 1:500 dilution.

(A) (B) (C)

Figure 18. The effect of using GIBCO® Anti-Clumping Agent with SFM4CHO medium on cell aggregation. (A) SFM4CHO without Anti-clumping agent (B) SFM4CHO with 1:1000 Anti-clumping agent dilution (C) SFM4CHO with 1:500 Anti-clumping agent dilution.

The growth profile for the fed-batch culture using 80% SFM4CHO medium with and without 1:500 anti-clumping agent and 1.5%:0.1% Cell Boost 7a/7b as feed supplements are shown in Figure 19. The results show that the use of the anti-clumping agent increased the maximum cell density to 13.03 ± 0.25 $\times 10^6$ viable cells/mL and extended the culture to 12 days. While using the anti-clumping agent decreased the tendency of the cells to aggregate and prevented cell clumps, it increased the nutrient transport performance to the cells, leading to an increase in the maximum cell density and the longevity of the culture.

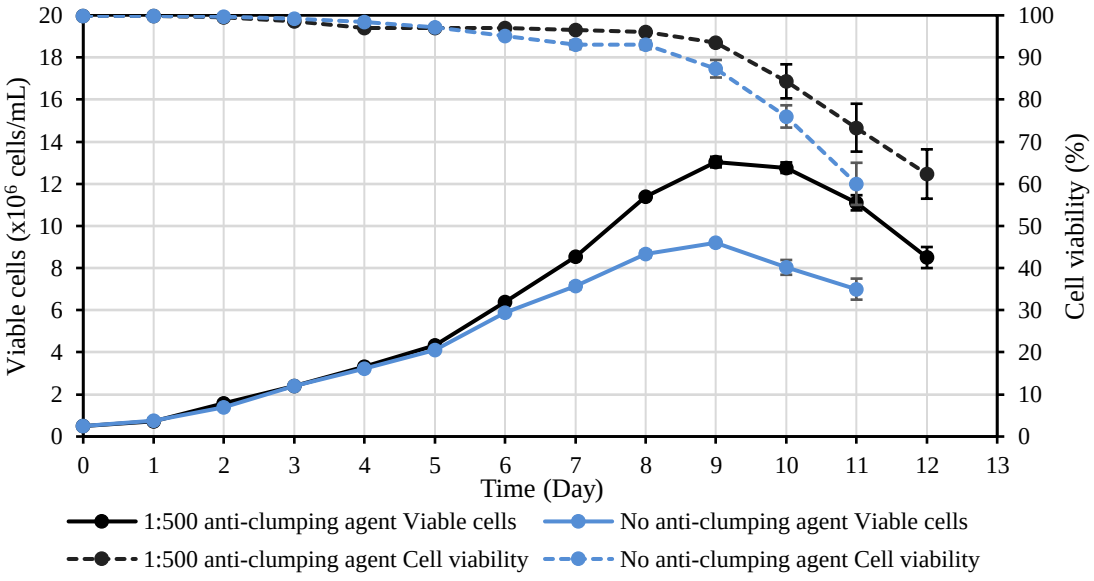

Figure 19. Growth profile for fed-batch culture using 1.5%:0.1% Cell Boost 7a/7b every other day and 80% SFM4CHO medium with/without Anti-clumping agent.

2.4 Conclusion

The Cell Boost 7a/7b combination is an effective feed medium and when used with the SFM4CHO fed-batch culture led to an increase in the maximum cell density and the longevity of the culture. The high glucose content in SFM4CHO medium and Cell Boost 7a, however, resulted in glucose being added at a rate greater than what the cells could consume. Decreasing the initial concentration of glucose in the basal medium, consequently, led to a decrease in the accumulation of glucose and a more optimized fed-batch culture based on cell growth and the profile of metabolites. The optimized feed strategy using 80% SFM4CHO medium with addition of 1.5%:0.1% Cell Boost 7a/7b on day 3 and every other day led to a maximum cell density 9.2 ± 0.1 $\times 10^6$ viable cells/mL. This approach, however, increased the aggregation of cells and slowed down the cell growth. GIBCO® anti-clumping agent is an excellent reagent to decrease the aggregation of cells and was added to the basal medium at a 1:500 dilution ratio. The optimized fed-batch culture using 80% SFM4CHO with 1:500 dilution of the anti-clumping agent increased the maximum cell density to 13.03 ± 0.25 $\times 10^6$ viable cells/mL and extended the culture to 12 days. Compared to the batch culture this represent an increase in the maximum cell density of 3.5-fold and doubled the longevity of the culture. This work improves our understanding the effect of using glucose-containing feeds with basal medium that has a high glucose content while simultaneously maintaining glutamine levels between 2 – 4 mM. Decreasing the concentration of SFM4CHO decreased the initial concentration of glucose, but it can also affect the concentration of other essential nutrients. Further work, therefore, needs to be performed to understand this impact and compare 100% SFM4CHO with an alternate feed that contains no glucose.

CHAPTER III

EFFECTS OF FEEDING STRATEGY ON THE PERFORMANCE OF CHO-3440 CELLS IN FED-BATCH CULTURE USING HYCLONE™ SFM4CHO™ MEDIUM WITH CELL BOOST 7A-WITHOUT GLUCOSE AND 7B SUPPLEMENTS AND ALANYL-L-GLUTAMINE

3.1 Introduction

The fed-batch culture is the most widely used operation mode for the large-scale manufacturing of biotherapeutics as it increases the longevity of the culture, maximum cell density, and overall productivity [55]. As the fed-batch operation mode is considered a partly open system, this mode sustains cell growth by preventing the depletion of essential nutrients using a concentrated feed medium. The addition of the essential nutrients extends the culture time and increases the maximum density, however, adding nutrients leads to an increase in the accumulation of by-products. When the by-products reach certain inhibitory levels, this condition inhibits the cell growth and alters the glycosylation pattern of the recombinant proteins [61]. Among the essential nutrients in the fed-batch culture, glucose and glutamine are the most important energy sources for CHO cells. While maintaining the levels of glucose and glutamine above the critical concentrations support the cell growth, the high levels of nutrients increase the ammonia and

lactate concentrations that led to reaching the stationary phase and shortens the culture period [62]

In the previous chapter, we discussed the effect of the initial glucose concentration on the accumulation of glucose and lactate through the fed-batch culture while adding feed media containing glucose. Additionally, the concentration of the basal medium was optimized to extend the culture period and increase the maximum cell density while using Cell Boost 7a/7b feed media. The use of 80% (15.9 g/L) SFM4CHO basal medium led to optimizing the initial concentration of glucose, however, the dilution of medium could affect the concentration of other nutrients in the basal medium. Here, another approach is proposed to use the recommended concentration (100%) of SFM4CHO basal medium with a feed medium lacking glucose while controlling the concentration of glucose separately. Cell Boost 7a-without glucose is a suitable feed supplement for the proposed approach as this feed has the same composition of Cell Boost 7a except for the glucose content.

In addition to the glucose, glutamine is a significant energy source for CHO cells. The addition of glutamine to the feed media leads to achieving high growth rates, cell densities, and productivities, however, the levels of glutamine results in high levels of ammonia that inhibit cellular growth and alter the post-translational modification [143, 144]. Therefore, this work will also focus on minimizing the generation of ammonia by limiting the concentration of glutamine. In this study, stabilized glutamine-containing dipeptides is used in the feed media while retaining the L-glutamine in the basal medium with a concentration of 4mM. The replacement of L-glutamine in feed medium only leads to shorten the lag phase and allows the cells to reach higher densities before adapting them to the glutamine substitute [92-94]. Also, the decrease of the initial glutamine concentration from 8 mM to 4 mM leads to decrease the ammonia production through the early stage of the culture.

3.2 Materials and methods

3.2.1. Cell line

CHO-3440 cells that has been adapted to grow in SFM4CHO medium were used for this process development study. The adapted cells were stored in cryopreservation medium, SFM4CHO + 5% DMSO, with a concentration of 1 – 2 $\times 10^7$ viable cells/mL. The L-glutamine concentration was 8 mM in the initial medium, however, the SFM4CHO medium, in this study, was supplemented with 4 mM L-glutamine to limit the concentration of glutamine in the early stage of the culture and decrease the ammonia production. The working conditions were kept as before in the previous study by using a shaker flask at a rocking speed of 140 rpm at 37 °C in a 5% CO_2 humidified incubator.

A 1 mL vial thawed rapidly in 15 mL SFM4CHO medium supplemented with 4 mM L-glutamine, 1 g/L P-F68, 2.2 g/L sodium bicarbonate, and 1:500 GIBCO® anti-clumping agent in a 75 cm^2 T-Flask. The cells were maintained at 37 °C in a 5% CO_2 humidified incubator. After 2 days, the cells were transferred to a 125 mL shake flask containing 30 mL complete SFM4CHO medium supplemented with 4 mM L-glutamine at seeding density 0.45 – 0.55 $\times 10^6$ viable cells/mL at 37 °C in a 5% CO_2 humidified incubator at a rocking speed of 140 rpm. Every 2 – 3 days, when the cells reach ~2 $\times 10^6$ viable cells/mL, the cells were subcultured to 0.45 – 0.55 $\times 10^6$ viable cells/mL. The used cells in fed-batch studies were passaged at least two times with SFM4CHO medium supplemented with 4 mM L-glutamine to ensure the growth rate and viability of the cells before using in any further studies.

3.2.2. Cell Boost 7a-without glucose and 7b

Cell Boost 7a-without glucose has the exact composition of the standard Cell Boost 7a, which has been used in the previous study, except that the new supplement lacks the glucose

content. Cell Boost 7a-without glucose has a pH close to neutral and contains a mixture of amino acids, vitamins, and trace elements, while Cell Boost 7b has an alkaline pH and is a concentrated solution of amino acids. Cell Boost 7a-without glucose is used in combination with Cell Boost 7b to provide the essential nutrient through the fed-batch culture. Cell Boost 7a-without glucose is a prototype feed media supplement and was provided as a free sample from Cytiva. The use of Cell Boost 7a-without glucose in the fed-batch culture gives a privilege of controlling the concentration of glucose separately. Using Cell Boost 7a-without glucose, therefore, allows trying our optimized feed strategy with 100% SFM4CHO instead of the 80% (15.9 g/L) concentration. Through this study, 1.5%:0.1% Cell Boost 7a-without glucose/7b of the culture working volume was added on day 3 and every other day until day 9 of the fed-batch culture. Meanwhile, the level of glucose was maintained at the critical concentration (*i.e.*, 4 g/L).

3.2.3. UltraGlutamine supplement

UltraGlutamine supplement is a 200 mM alanyl-L-glutamine in normal saline. Alanyl-L-glutamine is dipeptide glutamine and is considered stable glutamine which can be used as L-glutamine substitution. Using stabilized glutamine dipeptides reduce ammonia production by 60 – 90% [92] as the cells do not break all of it at one time using the cytosolic peptidase [93]. Also, dipeptide glutamine has the advantage of good stability even at 37 °C. Currently, in the glutamine replacement studies, two strategies are used; the first strategy is a complete replacement, where the glutamine supplement is replaced in both the basal medium and in the feed media. The second approach is feed media glutamine replacement while retaining the L-glutamine in the basal medium. The second approach helps to shorten the lag phase and allow the cells to reach higher densities before adapting them to the glutamine substitute [94]. In this study, glutamine feed was replaced with UltraGlutamine supplement. The equivalent volume of 2 mM UltraGlutamine was added on day 3 and every other day until day 9 of the fed-batch culture while retaining the L-glutamine in the basal medium with an initial concentration of 4 mM.

3.2.4. Fed-batch culture

Fed-batch culture in shake flasks were conducted to study the effect of using Cell Boost 7a-without glucose and UltraGlutamine on the optimized feeding strategy with 100% SFM4CHO medium. The study was performed in 125 mL shake flasks with starting volumes of 30 mL SFM4CHO basal medium. Cells were inoculated at 0.45 – 0.55 $\times 10^6$ viable cells/mL and maintained at 37 °C in a 5% CO_2 humidified incubator at a rocking speed of 140 rpm. The feed addition began on day 3 with 1.5%:0.1% Cell Boost 7a-without glucose/7b and 2 mM UltraGlutamine every other day until day 9. The concentration of glucose was maintained at the critical level (*i.e.*, 4 g/L) by adding the required amount from the glucose stock solution with a concentration of 450 g/L. For the preparation of glucose stock solution, 450 g/L D-glucose was dissolved in ultrapure water under low heating conditions; the solution was then sterilized using a 0.22 µm filter and stored at room temperature.

Table 8 shows the feed volumes for a 30 mL fed-batch culture in a 125 mL shake flask. Samples were taken daily and cell density, viability, and metabolite content were measured. The fed-batch culture was maintained until the cell viability reach <70%. All experiments were performed in duplicate.

Table 8. Setup of shake flask experiment using Cell Boost 7a-without glucose/7b with UltraGlutamine every other day.

Shake flask volume (mL)	**SFM4CHO starting working volume (mL)**	**Cell Boost 7a/7b (% of working volume)**	**Cell Boost 7a (µL)**	**Cell Boost 7b (µL)**	**UltraGlutamine 200 mM (µL)**
125 mL	30 mL	1.5/0.1	450	30	300

3.2.5. Analytical methods

3.2.5.1. Cell density and viability

A hemocytometer-based trypan blue dye exclusion assay was used to count the number of viable and non-viable cells. After mixing the culture to ensure the uniformity of the sample, 200 µL of culture was aseptically transferred to a 1.5 mL clear Eppendorf tube as a representative sample. The sample (50 µL) was mixed with an equal volume of 0.4% trypan blue in isotonic salt solution. Viable (seen as bright cells) and non-viable (stained blue) cells were counted using a dual-chamber hemocytometer and a light microscope. Ideally, 100 – 200 cells should be counted in order to increase the accuracy of the measurement. If the number of cells was above 200, the sample was diluted with medium before adding the trypan blue. The concentration of viable and non-viable cells was calculated using Equation 2, where viability is the percentage of viable cells relative to the total cell count (viable and non-viable). Each sample was repeated three times and the results were used to calculate the average and standard deviation (SD). The error bars on the cell growth profile graphs show ± 1 SD of the average.

$$Cell\ Count\ (Cells/mL) = \frac{Number\ of\ cells\ counted}{4} \times Dilution\ factor \times 10^4 \qquad \text{(Equation 2)}$$

3.2.5.2. Metabolite analysis

A Bioprofile 400 automated chemistry analyzer was used to measure the concentrations of glucose, lactate, glutamine, glutamate, and ammonia, as well as the concentration of potassium and sodium cations in cell culture supernatant. This multi-channel analyzer also offers the option to measure pH, PO_2, and PCO_2, plus calculating the osmolality. Some of the cell culture (1 mL) was aseptically transferred to a 1.5 mL Eppendorf tube; then, the cell suspension was centrifuged for 4 minutes at 10,000 rpm at room temperature. The supernatant was used for the Bioprofile 400 analyzer test.

Although Bioprofile 400 analyzer can accurately measure the concentration of glutamine in the cell culture supernatant, the analyzer cannot accurately measure the dipeptide glutamine. Alanyl-L-glutamine addition through this study does not depend on the glutamine concentration while depends on the addition of a fixed amount, 2mM, every other day.

3.3 Results and discussion

As discussed in the previous chapter, the optimized fed-batch culture was based on using a lower concentration (80%, 15.9 g/L) of SFM4CHO medium with the addition of 1.5%:0.1% Cell Boost 7a/7b on day 3 and every other day until day 9. Meanwhile, the concentrations of glucose and glutamine were maintained at 4 g/L and 2 – 4 mM, respectively. The optimized feeding strategy led to an increase in the maximum cell density to $13.03 \pm 0.25 \times 10^6$ viable cells/mL and the longevity of the culture to 12 days. On the metabolite level, the concentration of ammonia reached an inhibitory level of >10 mM at day 8 and the maximum concentration of lactate was 2.06 g/L. As a result of limiting the accumulation of by-products, the osmolality was significantly decreased and did not reach the inhibitory level of >400 mOsm/Kg. The lower concentration of SFM4CHO medium, however, could decrease the concentration of other nutrients which may affect the behavior of cells, especially in the early stage of the cell culture.

Here, the proposed feeding strategy depends on using 100% SFM4CHO basal medium with Cell Boost 7a-without glucose and Cell Boost 7b and replace L-glutamine in the feed with alanyl-L-glutamine. On day 3 and every other day until day 9, 1.5%:0.1% Cell Boost 7a-without glucose/7b and 2 mM UltraGlutamine was added to the cell culture while maintaining the concentration of glucose at 4g/L. The proposed strategy gives better controlling on the concentration of glucose and tries to minimize the accumulation of ammonia by using feed media without glucose and replacing L-glutamine in the feed media.

3.3.1. Growth profile

The cell growth profile for fed-batch culture grown in 100% SFM4CHO basal medium with the proposed feeding strategy and the optimized fed-batch culture using 80% SFM4CHO medium are displayed in Figure 20. The comparison between the two fed-batch cultures shows a shorten in the lag phase for fed-batch culture with 100% SFM4CHO medium. The short lag phase led to an increase in the cell density through the culture period beginning on day 3. The maximum cell density, therefore, increased to 14.0 ± 0.2 $\times 10^6$ viable cells/mL and the longevity of the culture extended to 14 days.

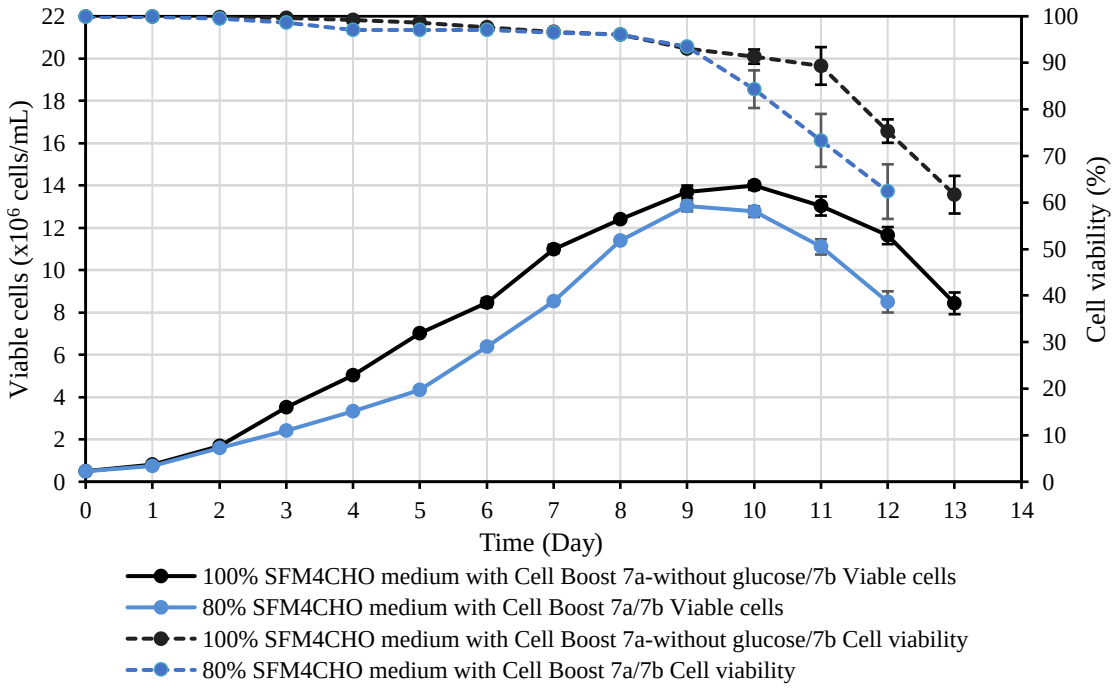

Figure 20. Growth profile for fed-batch cultures using 100% SFM4CHO medium with Cell Boost 7a-without glucose/7b and 80% SFM4CHO medium with Cell Boost 7a/7b.

The effect of the concentration of basal medium appeared on day 3 of the culture. The culture that used 100% SFM4CHO medium reached cell density 3.52 ± 0.08 $\times 10^6$ viable cells/mL on day 3 while the culture with 80% SFM4CHO medium reached cell density 2.4 ± 0.1 $\times 10^6$ viable cells/mL on the same day. The difference between the cell density at day 3 shows that

100% SFM4CHO medium provides the required nutrients to support the cell culture especially in the early phase of the culture. Lowering the concentration of the basal medium, however, decreases the concentrations of some essential nutrients that led to an extended lag phase.

3.3.2. Metabolite analysis

Figure 21 shows the profile of glucose and lactate for the proposed feeding strategy using Cell Boost 7a- without glucose with 100% SFM4CHO medium and the optimized fed-batch culture using Cell Boost 7a with 80% SFM4CHO medium. The optimized fed-batch culture using a lower concentration of SFM4CHO led to a decrease in the initial concentration of glucose to reach a concentration ~ 6 g/L. The lower concentration of glucose decreased the accumulation of glucose and led to a better controlling of glucose levels while adding Cell Boost 7a that contains glucose. Also, the glucose level reached the threshold concentration every day after the addition of feeds, indicating that the addition of the feed to the 80% SFM4CHO culture did not exceed the glucose consumption of cells.

While using 100% SFM4CHO medium increased the initial concentration of glucose, the lack of glucose in the feed medium led to controlling the concentration of glucose in the same manner as the optimized fed-batch using 80% SFM4CHO medium. Also, the lactate profile of the proposed feeding strategy shows a similar behavior to the optimized fed-batch culture. The similar lactate profile indicates that the higher initial concentration of glucose did not affect the accumulation of lactate.

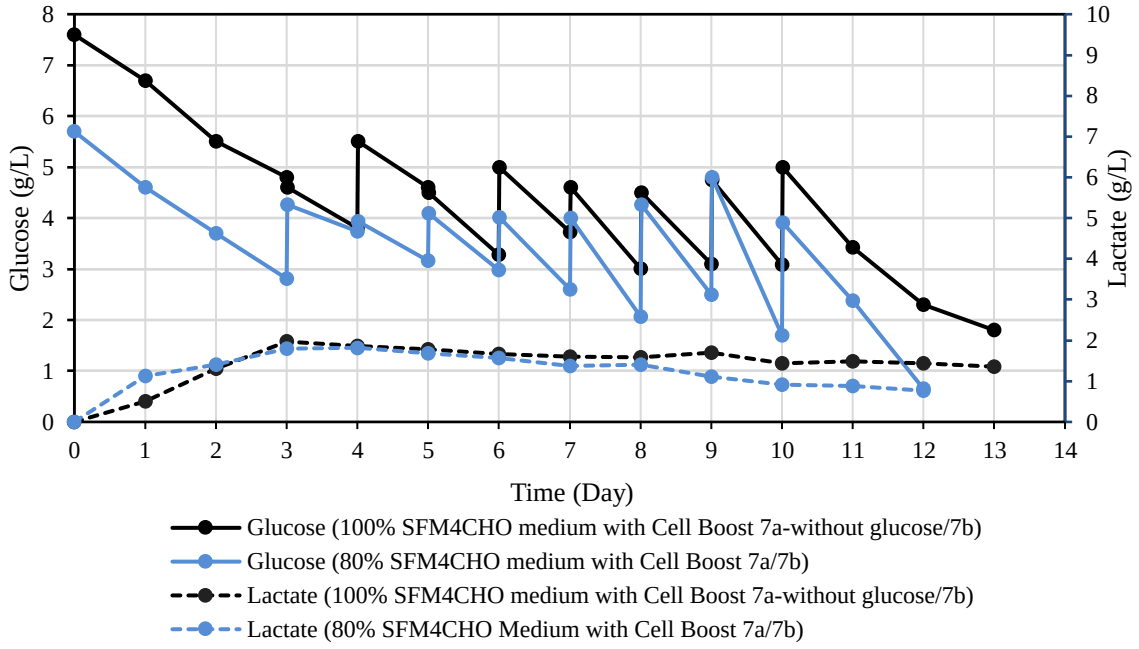

Figure 21. Glucose and lactate profile for fed-batch cultures using 100% SFM4CHO medium with Cell Boost 7a-without glucose/7b and 80% SFM4CHO medium with Cell Boost 7a/7b.

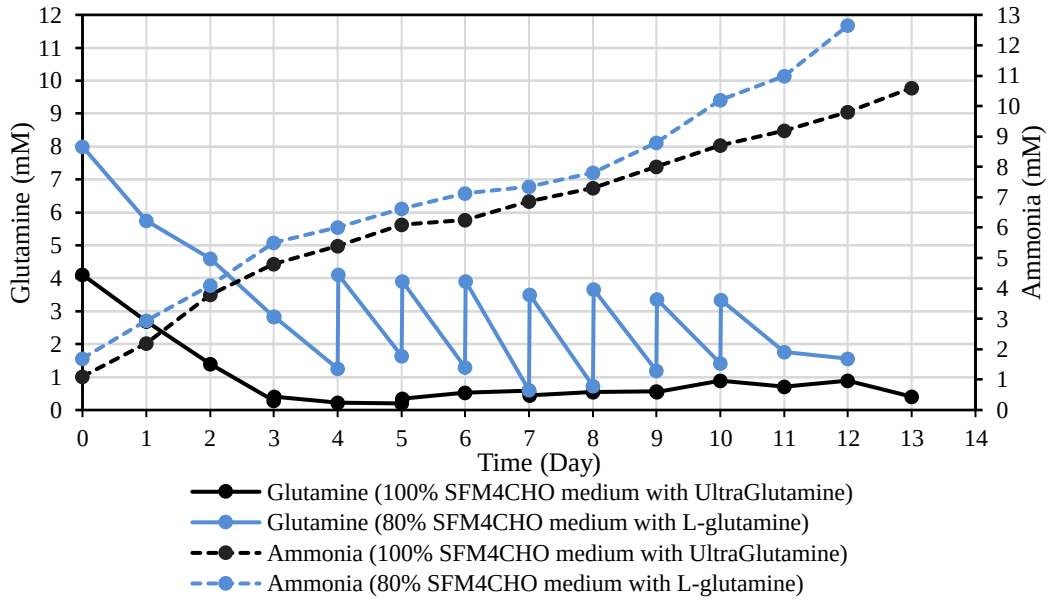

Figure 22. Glutamine and ammonia profile for fed-batch cultures using 100% SFM4CHO medium with UltraGlutamine and 80% SFM4CHO medium with L-glutamine.

In addition to the difference of glucose content, the proposed feeding strategy with 100% SFM4CHO medium differs from the optimized fed-batch culture that use 80% SFM4CHO in the type and concentration of glutamine content. The proposed feeding strategy depends on using 4

mM L-glutamine in the basal medium and adding 2 mM UltaGlutamine on day 3 and every other day until day 9. On the other hand, the optimized fed-batch culture depends on using 8 mM L-glutamine in the basal media and maintaining L-glutamine within the range 2 – 4 mM using 200 mM L-glutamine stock solution. Figure 22 shows the glutamine and ammonia profile for the proposed feeding strategy and the optimized fed-batch culture. The results show a decrease in the accumulation of ammonia in both early and late stages of the cell culture that use the proposed feeding strategy. Decreasing the initial concentration of L-glutamine led to decreasing the ammonia concentration at day 3 while substitution of L-glutamine in the feed medium with UltraGlutamine decreased the accumulation of ammonia through the culture period. Consequently, the concentration of ammonia at the end of culture decreased from 12.65 mM to 10.60 mM. While ammonia reached an inhibitory level (*i.e.*, >10 mM) on day 10 in the optimized fed-batch culture, the inhibitory level of ammonia was delayed until day 13 using the proposed fed-batch culture.

Table 9. Osmolality for fed-batch cultures using 100% SFM4CHO medium with Cell Boost 7a-without glucose/7b and 80% SFM4CHO medium with Cell Boost 7a/7b.

Day	1.5%:0.1 % Cell boost 7a-without glucose/7b fed-batch culture using 100% SFM4CHO osmolality (mOsm/Kg)	1.5%:0.1 % Cell boost 7a/7b fed-batch culture using 80% SFM4CHO osmolality (mOsm/Kg)
1	395	313
2	398	310
3	393	305
4	386	328
5	386	310
6	390	308
7	374	320
8	390	315
9	385	322
10	388	308
11	390	324
12	396	310
13	398	NA

Table 9 shows the levels of osmolality in the optimized fed-batch and using the proposed feeding strategy. The results indicate an increase in osmolality when using 100% SFM4CHO basal media, which refer to a higher concentration of the salts and nutrients in the basal medium. The use of recommended concentration of basal medium, 100% SFMCHO medium, provided the culture with the optimum concentration of essential nutrients and salts that led to a shorten lag phase and higher cell density especially in phase where the culture was supported by the basal medium only without the feeds. Although the use of 100% SFM4CHO basal medium led to an increase in osmolality through the culture period, the level of osmolality was not increased to inhibitory levels, >400 mOsmo/Kg, in the early or late stages of the culture. The combination of 100% SFM4CHO basal medium with Cell Boost 7a-without glucose fed the culture with the optimum nutrients in the early phase of cell culture while provided a good control on the concentration of glucose to prevent the accumulation of by-products and the elevation of osmolality.

3.4 Conclusion

SFM4CHO is a highly enriched medium with glucose and decreasing the concentration of the initial medium led to controlling the accumulation of glucose and by-products. Dilute the medium to 80%, however, can decrease the concentration of essential nutrients, other than glucose, to their critical levels, especially in the early stage of fed-batch cell culture. The importance of using the recommended concentration of the basal medium appeared in the difference of the lag phase and the cell density at day 3 of the fed-batch cultures using 80% and 100% SFM4CHO basal medium. Cell Boost 7a-without glucose afford a good combination with SFM4CHO medium as lacking glucose in the feed supplement provides a separate controlling on the concentration of glucose in the feed media whereas delivers the essential nutrients to the cell culture. On the other hand, a relatively high concentration of L-glutamine in the initial medium and maintain the glutamine within range of 2 – 4 mM through the culture increase the ammonia

to inhibitory concentration at day 10. Decreasing the initial concentration of glutamine in the basal medium to 4 mM and replacing the L-glutamine with UltraGlutamine in the feed media led to a decrease in the concentration of ammonia, especially in the late stage of the culture. The proposed feeding strategy using Cell Boost 7a-without glucose and UltraGlutamine with 100% SFM4CHO medium led to increase the maximum cell density to $14.0 \pm 0.2 \times 10^6$ viable cells/mL and the longevity of the culture extended to 14 days instead of $13.03 \pm 0.25 \times 10^6$ viable cells/mL and 13 days using the optimized fed-batch using Cell Boost 7a and L-glutamine with 80% SFM4CHO medium.

CHAPTER IV

CONCLUSIONS AND FUTURE WORK

Mammalian cells, such as CHO cells, are the most widely used systems to produce biopharmaceutical products. Currently, CHO cells are the gold standard in the biopharmaceutical industry due to their human-like post-translational modifications and a long history of regulatory approval. CHO cells, however, suffer from slow growth, high nutrient consumption rates, and accumulation of toxic metabolites. These factors can result in low cell growth rates, productivity, and product quality. Therefore, the monitoring and control of nutrients consumption rate and accumulation of by-products that affecting the cell growth is essential to improve the productivity of CHO cells. Although CHO cells have a long successful history in the production of recombinant proteins, the process optimization and up-scaling process are still cell line dependent and differs from one product to another. Additionally, the biopharmaceutical companies have a big challenge to produce enough amounts of recombinant proteins for clinical trials and establish a large-scale manufacturing process to meet the market demand. A high cell density in a large culture volume is required to make the production process economically efficient. The advancement of single-use technology, especially using single-use bioreactor, helps to decrease the product cycle from lab to market by eliminating the required time for the cleaning and sterilization after every trial or production batch.

The aim of the research was to develop a feeding strategy for a fed-batch culture of CHO-3440 cells with SFM4CHO basal medium using Cell Boost 7a/7b feed medium to be used in the future for a validation process of an innovative multi-chamber single-use bioreactor. This was determined by trying different feeding strategy with different concentrations of the feed medium while measuring the cell growth rate and monitoring the concentration of nutrients and by-products.

4.1 Conclusions

The results demonstrated the importance of determining an appropriate feeding regimen based on the nutritional requirements of a specific cell line so as to avoid overfeeding the culture. Using feed media containing glucose with the high glucose content SFM4CHO basal medium resulted in an increase in the levels of glucose above the amounts that cells could consume, especially in the early stage of the culture. Consequently, the accumulation of by-products increased above the inhibitory levels and led to a decrease in the maximum cell density and the longevity of the culture.

The Cell boost 7a-without glucose/7b provides a suitable combination with SFM4CHO basal medium. The lack of glucose within Cell Boost 7a-without glucose offers good control over the concentration of glucose through the culture as the addition of glucose would happen only when the glucose reached the critical concentration (*i.e.*, 4 g/L). This approach resulted in a decrease in the accumulation of by-products, especially lactate. Also, the combination of using UltraGlutamine with Cell Boost 7a-without glucose/7b showed a decrease in the accumulation of ammonia.

Table 10 shows a summary of the different feeding strategies that we tried with the maximum cell density and the longevity of the culture as the outcome. The results show that the feeding strategy using 1.5%:0.1% Cell Boost 7a-without glucose/7b and 2 mM UltraGlutamine

with every other day feeding regimen gave the highest maximum cell density and the longest culture period.

Table 10. Feeding strategy trials summary

Basal medium	Feed medium	Glutamine	Anti-clumping agent	Outcome	
				Maximum cell density ($x10^6$ viable cells/mL)	Culture longevity (days)
100% SFM4CHO	Cell Boost 7a/7b, 2.5%:0.25%, every day	Maintain L-glutamine (2 – 4 mM)	–	4.47	9
	Cell Boost 7a/7b, 2.0%:0.2%, every day		–	4.60	9
	Cell Boost 7a/7b, 2.5%:0.25%, every other day		–	4.40	9
	Cell Boost 7a/7b, 1.5%:0.1%, every other day		–	5.50	10
	Cell Boost 5, 5%, every other day		–	4.03	9
	Cell Boost 5, 2.5%, every other day		–	4.40	9
	Cell Boost 7a-without glucose/7b, 1.5%:0.1%, every other day	UltraGlutamine (2 mM) every other day	1:500	14.00	13
80% SFM4CHO	Cell Boost 7a/7b, 1.5%:0.1%, every other day	Maintain L-glutamine (2 – 4 mM)	–	9.20	11
	Cell Boost 7a/7b, 1.5%:0.1%, every other day		1:500	13.03	12

Figure 23 shows the growth profile of the batch culture and the optimized fed-batch culture using Cell Boost 7a-without glucose/7b. The results show an increase in the maximum cell density to $14.0 \pm 0.2 \times 10^6$ viable cells/mL and extended the culture to 13 days. Compared to the batch culture this represent an increase in the maximum cell density of 4-fold and more than 2-fold of the longevity of the culture.

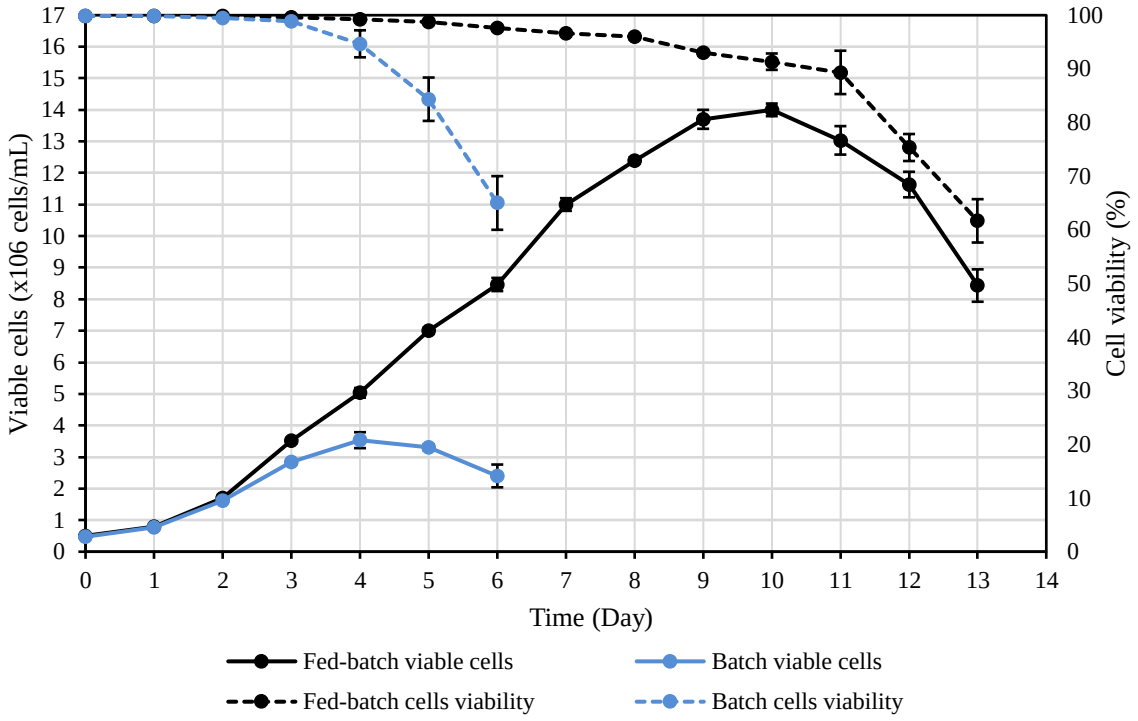

Figure 23. Growth profile for fed-batch culture using 100% SFM4CHO medium with Cell Boost 7a-without glucose/7b and batch culture using 100% SFM4CHO medium.

4.2 Future work

Shake flasks are easy-to-use, inexpensive choice, and suitable for early process development applications, such as screening, media optimization and feed regimen design. The use of shake flask, however, do not provide detailed insight into culture performance or opportunity for online measurement of DO or pH. On the other side, bioreactors are close systems equipped with multiple sensors, motor-driven impellers for agitation, and instruments for temperature control, gassing and supply of liquids for pH and DO control.

Testing our developed fed-batch culture for the cell line CHO-3440 using different scale bioreactors will give us the opportunity to validate our feeding regimen under controlled conditions of DO and pH. Our multi-chamber single-use bioreactor afford two different volume scale, 10 L and 200 L, with a controlled conditions of temperature, agitation, and gassing. Also, the multi-chamber bioreactor is provided with optical sensors that are connected to PID control

system for pH and DO online observation and controlling. The multi-chamber single-use bioreactor is still a prototype, however, the extensive engineering characterization and computational fluid dynamics (CFD) simulations that were carried out on this prototype are particularly valuable to minimize the risk of unsuccessful cell culture process inside the proposed design. Additionally, the large volume culture using the multi-chamber single-use bioreactor will provide enough sample volumes for the chimeric IgG 1 quantification and quality tests, such as glycan analysis. On the other hand, perform a cell culture process using the gold standard CHO cells within our prototype bioreactor will help to validate our design and lead to the identification of some area for potential improvement in the bioreactor and the fed-batch culture process.

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APPENDICES

Table 11. Bioprofile results for Fed-batch culture using 2.5%:0.25% Cell Boost 7a/7b every day

Time (Day)	pH	PO_2 (mmHg)	PCO_2 (mmHg)	Glutamine (mM)	Glutamate (mM)	Glucose (g/L)	Lactate (g/L)	Ammonia (mM)	Osmolality (mOsm/Kg)
0	7.74	172.7	25.8	7.80	1.16	8.00	0	2.47	NA
1	7.30	172.5	22.0	6.09	1.54	7.55	0.50	5.45	395
2	7.10	154.2	22.5	4.51	1.54	6.48	1.51	5.90	388
3	6.90	167.8	23.0	3.30	2.12	5.91	1.76	7.25	399
3.01	7.16	140.5	22.7	2.64	3.73	7.66	1.85	6.98	415
4	6.73	150.8	23.3	2.00	3.90	7.08	2.04	9.80	424
4.01	7.15	167.0	8.7	3.50	5.60	9.12	1.96	9.60	432
5	6.73	155.6	18.8	2.30	5.08	8.08	1.96	11.50	440
5.01	6.96	150.7	13.1	2.10	6.24	10.03	2.26	11.20	445
6	6.71	143.8	19.0	1.70	8.50	9.25	1.94	11.90	430
6.01	6.92	167.9	20.0	3.00	8.30	11.67	2.45	11.50	442
7	6.67	145.0	20.1	2.50	10.24	9.80	2.84	12.60	441
7.01	6.81	176.9	11.9	2.20	11.65	12.39	2.60	12.50	443
8	6.47	160.3	19.9	1.70	16.20	11.75	2.50	13.70	448
8.01	6.50	179.3	18.0	3.00	12.40	13.73	2.00	13.70	446
9	6.43	173.5	17.7	2.50	13.98	13.35	1.89	15.20	440

Table 12. Bioprofile results for Fed-batch culture using 2%:0.2% Cell Boost 7a/7b every day

Time (Day)	pH	PO_2 (mmHg)	PCO_2 (mmHg)	Glutamine (mM)	Glutamate (mM)	Glucose (g/L)	Lactate (g/L)	Ammonia (mM)	Osmolality (mOsm/Kg)
0	7.74	172.7	25.8	7.40	1.16	8.00	0	2.47	N/A
1	7.30	172.5	24.2	6.09	1.54	7.55	0.60	5.45	396
2	7.10	154.2	23.8	4.51	1.54	6.48	1.51	5.90	400
3	6.90	167.8	22.0	3.30	2.12	5.91	1.76	7.25	395
3.01	7.10	159.6	20.7	2.93	3.41	7.34	1.86	7.42	422
4	6.80	158.9	18.7	2.12	3.22	6.81	2.08	9.20	414
4.01	7.15	171.0	8.50	2.01	4.55	8.11	1.85	9.10	425
5	6.74	157.2	21.3	1.08	5.15	7.50	1.92	10.70	415
5.01	6.98	149.1	14.0	3.00	5.35	9.25	1.90	10.60	430
6	6.80	161.8	17.0	2.50	7.20	8.70	2.10	11.40	432
6.01	6.99	159.1	17.5	2.20	6.90	10.7	2.11	11.30	434
7	6.68	148.4	21.3	1.70	9.70	9.88	2.49	11.80	440
7.01	6.92	141.8	13.0	3.00	9.53	11.13	2.39	11.80	438
8	6.65	155.1	20.5	2.50	11.04	10.36	2.70	12.70	444
8.01	6.70	145.2	21.0	2.40	13.48	12.19	2.60	12.60	442
9	6.61	144.6	23.0	2.00	12.48	11.70	2.20	14.00	448

Table 13. Bioprofile results for Fed-batch culture using 5% Cell Boost 5 every other day

Time (Day)	pH	PO_2 (mmHg)	PCO_2 (mmHg)	Glutamine (mM)	Glutamate (mM)	Glucose (g/L)	Lactate (g/L)	Ammonia (mM)	Osmolality (mOsm/Kg)
0	7.58	180.9	30.4	7	1.46	8.05	0	1.73	N/A
1	7.54	179.2	19.9	5.38	1.5	7.65	1.28	3.68	386.7
2	7.11	168.4	17.4	4.04	1.69	6.24	1.81	5.35	396.2
3	6.87	162.8	16.8	2.76	1.7	5.67	2.04	6.54	400.6
3.01	7.05	170.6	9.1	2.6	2.51	6.33	1.9	6.22	388.9
4	6.82	174.3	19	2.15	2.53	5.79	2.28	7.04	390
5	6.8	162.7	21	1.5	2.64	5.18	2.28	8.43	381.8
5.01	6.9	158	15	3	3.65	6.06	1.91	7.76	384.8
6	6.73	159.8	21.1	2.2	4.07	5.9	1.82	8.58	389.5
7	6.83	155.5	15.4	1.4	3.8	5.12	1.73	9.37	387.7
7.01	6.9	169.9	10.9	3.6	4.72	6.94	1.97	9.5	382.5
8	6.75	162.1	20.4	2.6	4.71	6.83	1.7	10.18	377.1
9	6.71	159.1	20.7	1.7	4.53	6.42	1.71	10.9	384.1

Table 14. Bioprofile results for Fed-batch culture using 2.5% Cell Boost 5 every other day

Time (Day)	pH	PO_2 (mmH)	PCO_2 (mmHg)	Glutamine (mM)	Glutamate (mM)	Glucose (g/L)	Lactate (g/L)	Ammonia (mM)	Osmolality (mOsm/Kg)
0	7.59	179.9	29.4	7.20	1.18	8.26	0	2.06	N/A
1	7.52	177.7	20.6	5.47	1.53	7.65	1.19	3.74	397
2	7.08	164.9	18.6	4.17	1.55	6.29	1.76	5.35	392
3	6.88	168.6	17.6	2.46	2.10	5.54	1.93	6.73	398
3.01	6.98	161.3	12.3	2.44	2.18	5.98	2.05	6.52	395
4	6.80	155.7	17.3	2.00	2.52	5.30	2.29	7.47	391
5	6.72	152.9	19.0	1.60	2.38	4.72	2.34	8.85	394
5.01	6.82	151.2	22.0	3.30	2.88	5.88	2.20	8.28	394
6	6.79	155.0	20.2	2.70	3.65	5.45	2.08	9.42	389
7	6.91	164.4	14.8	1.80	2.91	4.78	1.86	10.36	386
7.01	6.93	159.2	12.4	3.40	3.68	6.77	2.14	10.44	410
8	6.79	157.6	21.8	2.70	4.17	6.37	1.86	11.08	392
9	6.76	150.9	22.5	1.90	3.88	5.94	1.85	11.65	394

Table 15. Bioprofile results for Fed-batch culture using 2.5%:0.25% Cell Boost 7a/7b every other day

Time (Day)	pH	PO_2 (mmHg)	PCO_2 (mmHg)	Glutamine (mM)	Glutamate (mM)	Glucose (g/L)	Lactate (g/L)	Ammonia (mM)	Osmolality (mOsm/Kg)
0	7.74	172.7	25.8	7.70	1.16	8.00	0	2.47	N/A
1	7.30	172.5	24.0	6.09	1.54	7.55	0.20	3.60	395
2	7.10	154.2	23.0	4.51	1.54	6.48	1.51	5.90	388
3	6.90	167.8	23.5	3.30	2.12	5.91	1.76	7.25	399
3.01	7.16	162.7	22.7	3.00	4.20	7.81	1.98	7.58	416
4	6.74	156.1	22.5	2.20	3.46	7.17	2.13	8.00	429
5	6.75	158.5	18.9	1.20	3.67	6.25	1.94	8.20	430
5.01	6.99	146.3	12.9	3.70	5.08	8.30	1.93	9.64	434
6	6.71	163.3	18.5	2.50	5.88	7.77	2.45	10.20	432
7	6.64	144.7	17.6	1.60	6.28	6.70	2.42	11.00	438
7.01	6.97	173.2	9.5	3.50	8.00	8.95	2.44	10.80	440
8	6.61	146.4	19.2	2.10	9.40	8.23	2.54	11.40	442
9	6.55	136.0	21.0	1.50	9.48	7.49	2.64	13.00	438

Table 16. Bioprofile results for Fed-batch culture using 1.5%:0.10% Cell Boost 7a/7b every other day

Time (Day)	pH	PO_2 (mmHg)	PCO_2 (mmHg)	Glutamine (mM)	Glutamate (mM)	Glucose (g/L)	Lactate (g/L)	Ammonia (mM)	Osmolality (mOsm/Kg)
0	7.78	188.1	22.4	7.20	1.13	8.79	0	2.09	N/A
1	7.54	175.8	22.4	5.44	1.41	8.05	0.70	3.96	396
2	7.07	181.2	16.9	3.45	1.67	6.41	2.04	5.75	383
3	6.78	157.2	20.8	2.20	1.71	5.63	2.09	8.12	395
3.01	6.85	161.5	17.0	2.10	2.50	6.50	2.00	8.10	405
4	6.77	146.9	19.3	0.46	1.66	5.24	2.51	9.00	388
4.01	6.88	161.1	13.4	3.89	3.57	5.20	2.14	9.36	406
5	6.80	163.6	21.1	2.72	3.52	4.50	2.53	9.98	399
5.01	6.82	128.0	19.3	2.60	3.55	5.50	2.53	9.80	398
6	6.87	181.3	20.6	1.50	3.75	4.67	2.56	10.50	395
6.01	6.99	154.0	13.8	3.80	4.62	4.50	2.40	10.30	405
7	6.86	119.0	19.9	2.37	5.15	3.80	2.40	12.00	417
7.01	6.95	138.0	21.0	2.30	5.50	5.20	2.38	11.80	420
8	6.83	139.9	21.4	1.20	6.00	4.40	2.44	12.40	415
8.01	6.91	135.7	16.4	3.90	6.08	4.30	2.32	12.20	411
9	6.77	79.8	31.0	2.82	6.64	3.23	2.19	14.30	411
9.01	6.86	107.3	22.2	2.50	6.28	4.60	2.20	14.20	420
10	6.80	120.9	27.0	1.70	7.32	3.80	2.13	15.60	423

Table 17. Bioprofile results for Fed-batch culture using 80% SFM4CHO medium with 1.5%:0.10% Cell Boost 7a/7b every other day

Time (Day)	pH	PO_2 (mmHg)	PCO_2 (mmHg)	Glutamine (mM)	Glutamate (mM)	Glucose (g/L)	Lactate (g/L)	Ammonia (mM)	Osmolality (mOsm/Kg)
0	7.75	179.4	21.5	8.00	0.93	5.85	0	1.46	NA
1	7.50	180.2	20.1	6.00	0.95	5.18	1.09	2.82	315
2	7.10	169.7	26.6	4.97	1.07	4.39	1.65	4.54	306
3	6.95	163.9	28.1	3.09	1.33	3.32	1.81	6.30	303
3.01	7.00	147.7	26.3	4.29	2.46	4.23	1.77	6.10	314
4	6.91	176.1	18.6	2.28	2.63	3.23	2.06	7.76	330
4.01	6.91	164.4	18.2	4.22	2.57	4.08	2.07	7.77	318
5	6.92	164.3	23.0	1.40	2.93	3.18	1.99	8.87	313
5.01	7.04	151.6	19.1	3.82	4.07	4.16	1.93	8.93	314
6	6.88	175.7	14.2	1.70	4.00	3.18	1.66	9.67	303
6.01	6.97	147.3	24.3	3.45	4.26	4.46	1.71	9.50	310
7	6.90	161.5	16.5	0.70	4.53	2.93	1.56	9.89	317
7.01	7.03	150.0	23.5	3.60	5.45	4.00	1.51	9.58	321
8	6.85	140.0	25.0	1.24	5.05	2.34	1.31	10.49	316
8.01	7.00	138.5	26.9	3.55	5.24	4.20	1.35	10.40	320
9	6.80	140.0	28.0	1.15	6.08	2.38	1.38	11.95	322
9.01	7.10	120.0	22.6	4.30	6.80	4.00	1.22	11.87	321
10	7.20	120.0	30.2	2.12	7.30	2.25	1.11	12.35	330
11	7.30	60.1	14.2	1.20	7.00	0.45	1.20	13.19	314

Table 18. Bioprofile results for Fed-batch culture using 80% SFM4CHO medium with 1:500 anti-clumping agent and 1.5%:0.10% Cell Boost 7a/7b every other day

Time (Day)	pH	PO_2 (mmHg)	PCO_2 (mmHg)	Glutamine (mM)	Glutamate (mM)	Glucose (g/L)	Lactate (g/L)	Ammonia (mM)	Osmolality (mOsm/Kg)
0	7.90	164.4	16.0	8.00	0.87	5.70	0	1.70	N/A
1	7.58	170.8	19.8	5.75	0.95	4.60	1.13	2.95	313
2	7.30	160.0	19.4	4.60	1.20	3.70	1.40	4.10	310
3	6.91	154.1	20.7	2.83	1.68	2.81	1.80	5.50	305
3.01	7.14	161.1	14.1	2.83	2.73	4.27	1.72	5.40	296
4	6.90	150.9	18.7	1.26	2.67	3.74	1.82	6.00	328
4.01	6.93	152.4	16.5	4.12	2.58	3.94	1.75	5.84	313
5	6.95	156.7	20.4	1.65	2.70	3.17	1.68	6.62	310
5.01	7.09	144.5	16.3	3.90	3.80	4.10	1.62	6.41	314
6	6.83	120.0	28.1	1.30	3.91	2.98	1.57	7.12	308
6.01	6.94	146.7	21.9	3.90	3.93	4.02	1.55	6.95	318
7	6.92	141.0	24.9	0.60	4.18	2.60	1.38	7.34	320
7.01	7.08	145.4	18.0	3.50	5.12	4.00	1.32	6.91	313
8	6.86	103.4	30.3	0.73	5.07	2.06	1.41	7.80	315
8.01	6.98	139.6	22.7	3.66	4.06	4.26	1.34	7.53	320
9	7.02	118.0	28.1	1.20	4.55	2.50	1.11	8.80	322
9.01	7.20	145.8	16.1	3.36	5.82	4.80	1.43	8.50	329
10	7.02	135.6	28.2	1.42	5.43	1.70	0.91	10.20	308
10.01	7.03	97.2	25.1	3.35	5.11	3.91	0.95	10.00	316
11	7.18	140.3	27.3	1.77	5.70	2.38	0.88	10.99	324
12	7.18	148.5	23.7	1.56	5.44	0.65	0.77	12.65	310

Table 19. Bioprofile results for Fed-batch culture using 100% SFM4CHO medium with 1:500 anti-clumping agent and 1.5%:0.10% Cell Boost 7a-without glucose/7b every other day

Time (Day)	pH	PO_2 (mmHg)	PCO_2 (mmHg)	Glutamine (mM)	Glutamate (mM)	Glucose (g/L)	Lactate (g/L)	Ammonia (mM)	Osmolality (mOsm/Kg)
0	7.70	180.0	26.5	4.11	1.30	7.60	0	1.10	N/A
1	7.40	171.0	22.0	2.70	1.33	6.70	0.50	2.20	395
2	7.20	168.0	23.0	1.40	1.35	5.50	1.30	3.80	398
3	6.96	164.0	15.9	0.29	1.39	4.80	1.97	4.80	393
3.01	6.94	167.0	20.3	0.40	2.70	4.60	1.82	5.02	391
4	6.90	168.2	21.6	0.22	3.19	3.80	1.86	5.40	386
4.01	6.95	170.0	20.6	0.23	3.15	5.50	1.83	5.30	392
5	7.05	184.4	16.7	0.20	3.07	4.60	1.78	6.10	386
5.01	6.96	154.0	20.7	0.35	4.20	4.50	1.74	6.08	386
6	6.97	150.6	23.3	0.52	4.22	3.28	1.66	6.26	390
6.01	6.98	155.0	22.3	0.45	4.25	5.00	1.64	6.23	392
7	7.05	168.7	19.2	0.59	3.66	3.73	1.60	6.87	374
7.01	7.20	146.8	17.4	0.45	5.21	4.60	1.50	6.54	388
8	7.08	146.1	24.0	0.55	5.60	3.01	1.59	7.30	390
8.01	7.09	147.1	24.6	0.56	5.50	4.50	1.56	7.24	394
9	6.95	149.5	23.4	0.56	5.33	3.10	1.70	8.00	385
9.01	7.00	153.4	21.5	0.55	6.00	4.75	1.65	8.20	387
10	7.20	157.9	19.4	0.90	5.90	3.08	1.44	8.70	388
10.01	7.21	160.3	22.0	0.85	5.87	5.00	1.46	8.67	391
11	7.09	179.3	19.4	0.70	6.10	3.42	1.49	9.20	390
12	7.05	167.7	21.4	0.90	6.20	2.30	1.43	9.80	396
13	7.15	171.0	22.0	0.40	6.70	1.80	1.35	10.60	398

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