Development and Customization of Protein-Free, Animal Component-Free Media for the Enhanced Expression of Recombinant Proteins in Chinese Hamster Ovary (CHO) Cells

By James Ross, Ken Kao, Alison Albee, Dana Goodnight and Mathew Caple
Sigma-Aldrich Corporation, St. Louis, MO USA

Introduction

The expression of recombinant proteins has increased in importance for both research and biopharmaceutical manufacturing applications. Chinese Hamster Ovary (CHO) cell lines are one of the most frequently used cell lines for the expression of recombinant proteins that require post-translational modification to exhibit full biological function. CHO cells used for large-scale production of recombinant proteins are often grown as suspension cultures using traditional serum-supplemented medium. For biopharmaceutical manufacturers the use of serum-supplemented medium poses numerous difficulties. If possible, most therapeutic producers would prefer to eliminate all animal-derived components from their culture systems.

Sigma has devoted a major part of its cell culture development efforts in the past 2-3 years to the development of media for biopharmaceutical production that are completely free of animal components. We have employed a factorial matrix approach to medium development that allows us to rapidly replace the functionality provided by serum- and animal-derived proteins.[1] Application of this approach to CHO systems led to the development of Sigma's CHO Protein-free, Animal Component-free Medium (CHO PF-AF; Product Code: C 5467). We have successfully optimized and modified this medium to further enhance the expression of a variety of recombinant proteins by different CHO cell lines. This "second generation" medium supports better, or at least comparable, cell growth and recombinant protein expression than that seen with serum-supplemented medium. Performance results of this optimized CHO PF-AF versus serum-containing medium and competitor formulations are presented in this report.

Materials and Methods

All materials were supplied by Sigma-Aldrich Corporation (St. Louis, MO) unless otherwise stated.

Cell Lines

In this study, three genetically engineered CHO cell lines were used for media development and were evaluated for cell growth and recombinant protein expression. CHO cell line 1, submitted by a customer for custom medium development, produces a proprietary recombinant IgG antibody. CHO cell line 2, producing recombinant human alkaline phosphatase, was created by Sigma R&D for cell culture media development. CHO cell line 3, submitted by a customer for custom medium development, produces a proprietary recombinant enzyme for potential use as a therapeutic agent.

Culture Media

The media used in this study are CHO Protein-Free Animal Component-Free Medium (Product Code: C 5467), DME/F-12 (Product Code: D 8900) with 10% Fetal Bovine Serum (FBS; Product Code: F 2442) and other CHO protein-free media from competitors.

Cell Growth Assays

All media were purchased fresh and appropriate amounts of supplements were added as indicated by the manufacturers' product inserts. Small-scale, stirred, suspension cultures were grown in spinner flasks (Techne Inc. Princeton, NJ) containing 250 ml of medium on a magnetic stirrer platform (Thermolyne, Dubuque, IA) at 80 rpm. All cell cultures were incubated at 37 °C with humidified air and 5% CO_2. Cells were harvested from a stock culture growing in protein-free medium with appropriate selective agents and having greater than 95% cell viability. Harvested cells were centrifuged for five minutes at 200 xg. Cells were then resuspended in trypsin-EDTA solution and incubated at 37 °C for 5 minutes. After incubation, an equal volume of trypsin inhibitor solution was added to the cell suspension, which was then centrifuged. Cell pellets were resuspended in Hanks' Balanced Salt Solution (HBSS). Stock cell densities were determined and culture flasks were inoculated with a low concentration of 5×10^4 viable cells/ml. Samples were taken each day for counting. The total cell counts were determined using a Casy®1 Particle Cell Analyzer (Scharfe Systems, Reutlingen, Germany). Cell viability was determined by trypan blue dye exclusion using a hemacytometer and calculated as percent viability times total cells/ml. Spinner cultures were removed from the study once they reached less than 30% cell viability. Duplicate spinner flasks were set up for each treatment.

General Procedure for Cell Adaptation

Cells grown in serum-supplemented medium were adapted to serum-free medium by initially inoculating cells into a 1:1 mixture of serum-supplemented and serum-free medium at 2×10^5 cells/ml. Cells were allowed to reach 1×10^6 cells/ml and then passaged into medium containing increasing proportions of serum-free medium (1:3 ratio followed by 1:7 ratio) before being transferred to 100% serum-free medium. Cells were considered adapted to serum-free medium when they achieved greater than 5.0 population doublings within seven days of culture in serum-free medium. CHO cultures in protein-free medium may form 2 to 10 cell clusters in suspension; therefore it is necessary to break up clumps at each subculture to achieve single cell uniformity. Cell growth experiments used cells that had been previously adapted to the appropriate medium for up to 15 days prior to use in experiments.

Quantitation of Recombinant Protein Production

All small-scale experimental data are representative of results obtained over multiple independent experiments with a given cell line. Samples were taken daily for determination of the amount of expressed protein. The IgG secreted into the medium by CHO cell line 1 was measured by HPLC (Waters 2690 HPLC; Milford, MA) using a Protein-A affinity column. Absorbance values were read at 280 nm. A standard curve was used to determine IgG concentration. CHO cell line 3 productivity was measured by a proprietary HPLC enzymatic assay provided by the customer.

Alkaline Phosphatase Assay

Activity of the recombinant alkaline phosphatase produced by the CHO cell line 2 is assayed by using a Sigma Diagnostic Kit

(Product Code: 104-LS). Absorbance values were read at 405 nm. Absorbance readings were compared with a standard curve to determine alkaline phosphatase activity levels.

Analysis of Spent Media

Glucose, lactate, and L-glutamine concentrations were measured in spent medium samples using a YSI 2700 Select Analyzer (YSI Incorporated, Yellow Springs, Ohio) for some experiments.

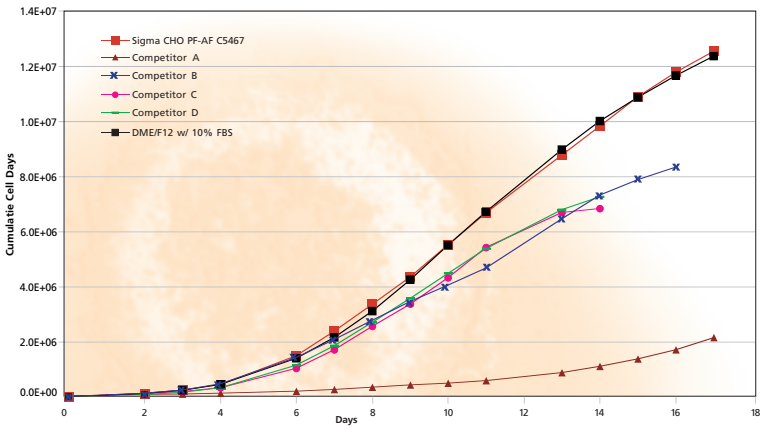

Figure 1. Comparison of cell growth of CHO cell line 1 in Sigma CHO PF-AF medium (Product Code: C 5467) and various competitor's protein-free media. *IgG producing CHO cells were seeded at 50,000 cells/ml in spinner flasks with CHO PF-AF medium and competitors' media. Samples were taken every day to analyze cell growth. Each data point represents the average viable cell number from duplicate spinner flasks. CHO cell line 1 growing in Sigma's CHO PF-AF reached a maximum cumulative cell days of 1.27 x 10^7, at day 17 of culture, whereas the cell growth in competitors' media showed less maximum cell density or longevity.*

Amino Acid Analysis of Spent Media

Amino acid analysis in spent media was done using a pre-column derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ-Tag™ amino acid labeling system; Waters Corporation, Milford, MA). The HPLC separation was on an AccQ-Tag™ C18 column (4.6 x 15 cm).

Results and Discussion

Comparison Study

A comparison study between Sigma's CHO PF-AF Medium and other major manufacturers of protein-free media was performed. Cells were prepared as described in the Materials and Methods and then seeded into spinner flasks containing CHO PF-AF medium (Product Code: C 5467) and media from different competitors. The results shown in Figure 1 indicate that cell growth of CHO cell line 1 in Sigma's CHO PF-AF media was comparable to growth in DME/F12 (Product Code: D 8900) with 10% FBS throughout 17 days of culture, reaching more than 1.27 x 10^7 cumulative cells-days. All days were measured as means of expressing the contribution of both cell density and culture longevity in the medium. Sigma's CHO PF-AF medium outperforms most major competitors as measured in cell days (Figure 1). Cell growth in CHO-PF-AF was increased by 30% over its nearest competitor. Figure 2 depicts the antibody production of CHO cell line 1. The data is expressed as cumulative antibody expression over the 17 days of the assay. Initially, up to day 12 of the assay, the productivity of CHO cell line 1 growing in the serum-containing medium was outperforming all other media. However, as the cultures continued to grow, the production of antibody in the cultures with protein-free media began to increase. This is a typical phenomenon of recombinant protein expression in cultures with protein-free media. As cells begin to adapt to the deficiency of growth factors and other nutrients that serum brings to culture media, protein-free media begin to approach equivalent levels of productivity. In comparison to all leading market competitors, a much higher antibody production level was observed in the CHO cell line 1 grown in CHO-PF-AF.

In order to confirm the performance of Sigma's medium, CHO cell line 2, producing human recombinant alkaline phosphatase, was employed. As shown in Figure 3, the cumulative cell-days of CHO cell line 2 reaches 1.47 x 10^7 with Sigma's CHO PF-AF medium on day 17 of the assay. In this assay, CHO PF-AF medium gave the best performance in comparison with the serum-containing control medium and the entire set of other commercially available media tested. The other aspect of using this cell line was the ability to easily quantitate productivity of the enzyme in the supernatant. Figure 4 shows that the human recombinant alkaline phosphatase productivity of CHO cell line 2 in Sigma's CHO PF-AF medium far exceeded the serum control and all other commercially available media. The cumulative expression of 231 enzyme units for Sigma's CHO PF-AF media as compared to 125 enzyme units of the nearest competitor medium (competitor C), translates to more than a 46% increase in productivity. This improvement represents savings for a biopharmaceutical manufacturing facility trying to maximize its production system.

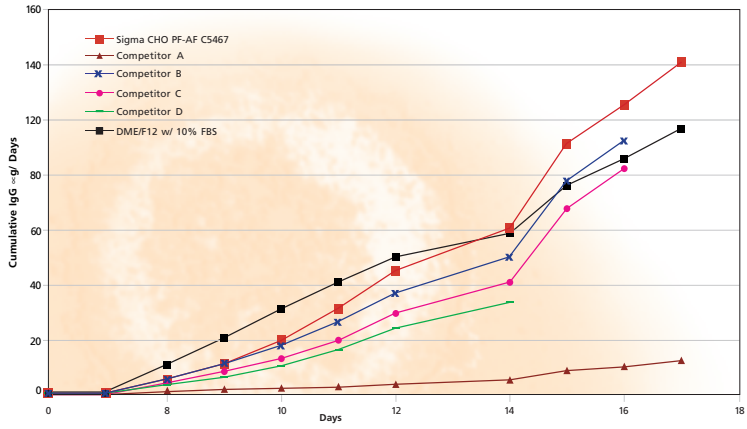

Figure 2. Comparison of IgG production of CHO cell line 1 in Sigma CHO PF-AF medium (Product Code: C 5467) and various competitors' protein-free media. *Spent media from the samples as described in Figure 1 were analyzed for the production of IgG by using HPLC with a Protein-A affinity column. Sigma CHO PF-AF supported the highest cumulative IgG production in CHO cell line 1 when compared with competitors' media. The maximum cumulative IgG production, 140 µg/days, was found at day 17 of culture in CHO PF-AF medium.*

Customization

It is clear that the behavior of different CHO cell lines varies in terms of cell growth and productivity in the same medium. These differences are largely due to the specific medium requirements of the cells based on the recombinant protein being produced. Moreover, a given cell line's growth rate, maximum cell densities, and recombinant

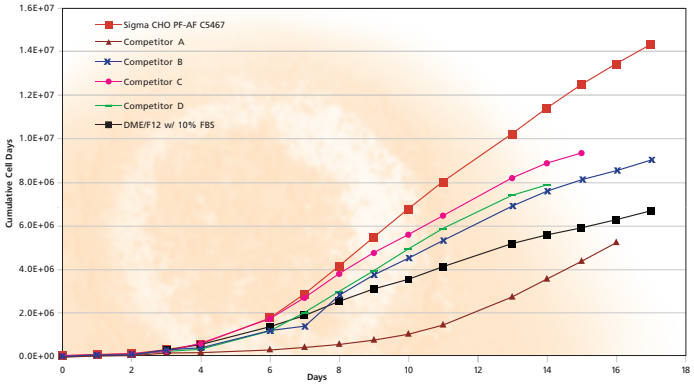

Figure 3. Comparison of cell growth of CHO cell line 2 in Sigma CHO PF-AF medium (Product Code: C 5467) and various competitors' protein-free media. *CHO cells, producing a proprietary enzyme, were seeded at 50,000 cells/ml in spinner flasks with CHO PF-AF medium and competitors' media. Samples were taken every day to analyze cell growth. Each data point represents the average viable cell number from duplicate spinner flasks. CHO cell line 2 showed the best cell growth in CHO PF-AF medium as compared with competitor's media. CHO cell line 2 reached the maximum cell days of 1.43 x 10⁷ at day 17 of culture, whereas the cell growth in competitor's media showed less cell density or longevity.*

protein productivity might be totally different in spinner flasks and in stirred-tank bioreactors operating in batch mode with the same protein-free medium. Therefore, we have developed a systematic protocol for the optimization of any given CHO cell line based on our experience with CHO PF-AF medium. This procedure also includes scale up from spinner flask culture to stirred-tank bioreactors. Significant increases in productivity have been achieved by modifications identified through this optimization procedure.

We have identified medium modifications that generally have been proven to be cell line-specific in their ability to enhance productivity. Spent medium analysis of amino acids proves to be a powerful tool in narrowing down some of the individual nutrient requirements of different proteins produced in a CHO expression system. This information can then be utilized for the addition of rate limiting amino acids to achieve higher levels of protein expression. Figure 5 illustrates a different amino acid consumption rates per cell for CHO clone. The amino acids consumed in this assay, including asparagine, serine, tyrosine, tryptophan, isoleucine, and lysine are typical amino acids that are utilized during cell growth. The consumption of amino acids alone is not enough to warrant adding components back to the medium. To understand the complexity of amino acid consumption, it is necessary to look at the total amount and ratio of amino acids available at the time cells begin to slow in growth and productivity. In this particular experiment, the amino acids that were >50% consumed were selected as targets for additional amino acid supplementation. The amounts of supplementation were determined by further experimental analysis of the individual amino acids. This customization process has allowed quick improvement of CHO PF-AF medium (Product Code: C 5467) for any given CHO recombinant protein expression clone.

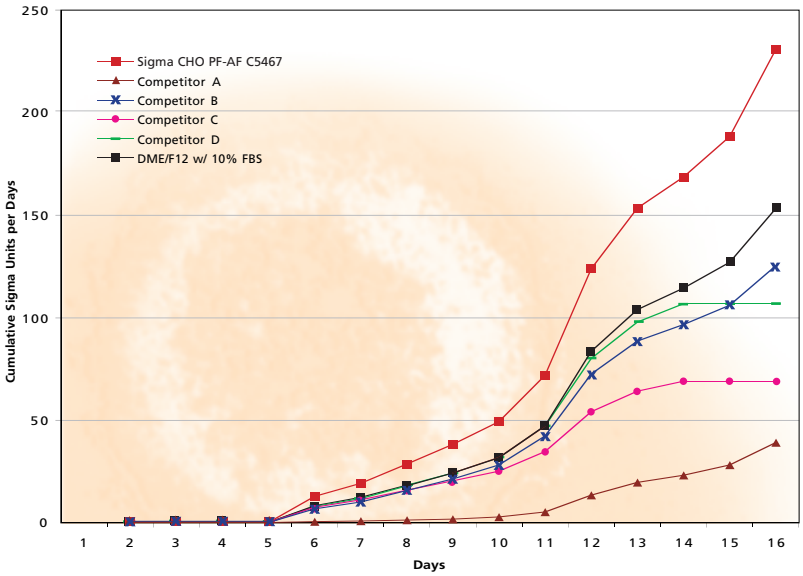

Figure 4. Comparison of recombinant enzyme production of CHO cell line 2 in Sigma CHO PF-AF medium (Product Code: C 5467) and various competitors' protein-free media. *Enzyme activity was measured in spent media samples described in Figure 3. The highest production of recombinant enzyme by CHO cell line 2 is seen in the culture grown in CHO PF-AF medium. The maximum cumulative enzyme production was 231 Sigma enzyme units/days.*

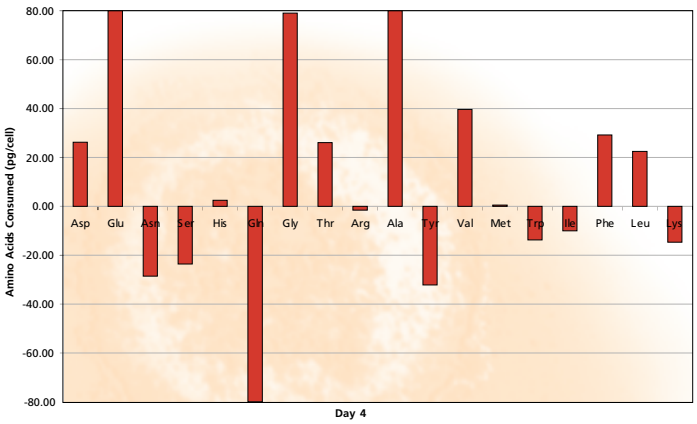

Figure 5. Amino acid consumption analysis in Sigma CHO PF-AF medium (Product Code: C 5467) CHO PF-AF medium was analyzed to determine the amino acid consumption/cell after 4 days of culture. *Negative bars indicate amino acid consumption and positive bars indicate increasing concentration. Individual amino acids with quantities greater than 80 pg (± neutral point) were truncated for lesser amounts to be visualized.*

Using this medium optimization procedure previously mentioned, we have successfully developed a set of medium formulations that have been modified for the CHO cell lines of several customers. Figures 6 and 7 illustrate the data from a study that was performed for one client. The client was using a CHO formulation developed by another manufacturer for the expression of the proprietary enzyme, which was being expressed at 25,000 units per ml. We were asked to increase the expression level. CHO cells (CHO cell line 3) were then transferred to our laboratory and banked in the original medium. We were able to replicate the results using a 5 L stirred-tank bioreactor.

Using iterative testing, CHO PF-AF medium was modified by changing the amino acid levels and adding sugars and other components to optimize cell growth as well as the quantity and quality of expressed protein. Figure 6 depicts cell densities that could be achieved using the original medium, CHO PF-AF medium, and the newly optimized CHO PF-AF custom formulation with supplements. The results clearly indicate that the modifications made to the protein-free medium allowed the customer's CHO cell cultures to reach cell densities of approximately 1.4 x 10^6 cells per ml compared to approximately 0.7 x 10^6 per ml in the original medium. Additionally, Figure 7 depicts the levels of protein expression achieved using the original medium, CHO PF-AF medium, and the customized CHO PF-AF medium. The customized CHO formulation supports expression of enzyme levels in excess of 150,000 units per ml, whereas the expression levels achieved with the competitor's formulation remained at 25,000 units per ml. This was a convincing 6-fold increase in productivity.

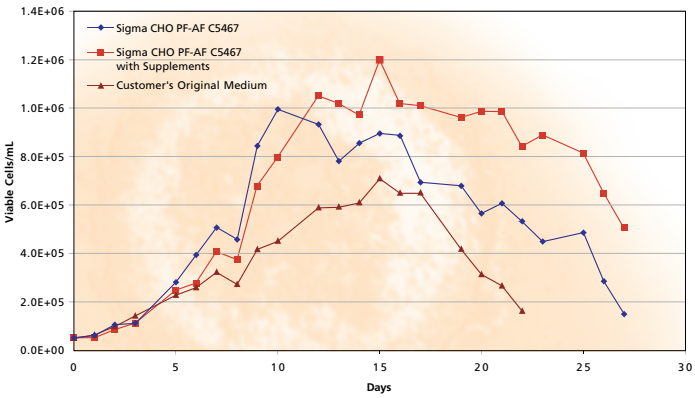

Figure 6. Enhanced cell growth of CHO cell line 3 in Sigma CHO PF-AF medium (Product Code: C 5467) by supplementation. *CHO cell line 3 was cultured in a 5-liter B. Braun stirred-tank bioreactor with a customer's original medium and CHO PF-AF medium with and without supplements. A higher cell density was observed in the cultures growing in CHO PF-AF- based medium. Addition of supplements of amino acids and sugars to CHO PF-AF medium further improved the growth of this cell line by supporting higher cell density and cell viability.*

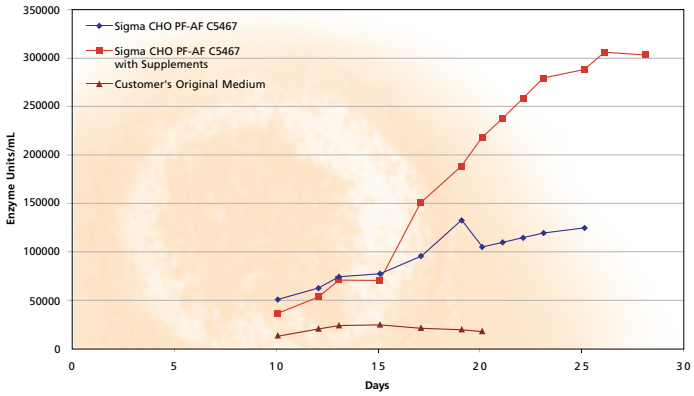

Figure 7. Increased recombinant enzyme productivity of CHO cell line 3 cultured in Sigma CHO PF-AF medium (Product Code: C 5467) by supplementation. *Recombinant enzyme productivity was increased in CHO cell line 3 with CHO PF-AF medium as compared to that in the customer's original medium. Moreover, the addition of supplements to CHO PF-AF medium dramatically increased the production of recombinant enzyme by CHO cell line 3.*

Conclusions

The development of new media to meet the needs of biotechnology manufacturers must often focus on two distinct and sometimes divergent goals. First, the elimination of animal-derived components to meet current and anticipated regulatory concerns regarding raw materials used in the manufacture of biotherapeutic agents. And second, the maintenance of cell growth and protein production characteristic of serum-supplemented medium. With these objectives in mind, we have developed a CHO medium that contains no protein and is free of all components that are isolated from animal sources or synthesized using animal-derived materials. With our CHO PF-AF medium, cells require little or no adaptation.They exhibit similar or superior growth when compared to serum-supplemented medium. Additionally, our medium supports rapid initial cell growth and high levels of protein expression in suspension cultures for extended periods of time. Compared to serum-supplemented media, this results in equivalent or superior productivity at a reduced cost. The success in scaling up to 5-L stirred-tank bioreactors directly points to the potential application of this medium for large-scale bioproduction. In fact, this medium and its custom siblings are being used in large-scale phase II and phase III applications. Additionally, CHO Protein-free Animal Component-free medium can be further optimized to yield improved performance for any specific CHO cell line expressing recombinant protein.

Acknowledgements

We would like to thank all who contributed to the research and development of the CHO media product line at the Sigma-Aldrich R&D, St. Louis, MO. The customization of CHO Protein-free media for customers has led to collaborations and scientific interactions at many institutions around the world.

Reference

1. Peppers, S. et al, Performance-optimized hybridoma medium: replacing serum and other animal-derived components. LifeScience Quarterly, **2(2)**, 6-10 (2001).

About the Authors

James Scott Ross, B.S., and Ken Kao, Ph.D., are senior scientists and Alison Albee, B.S., and Dana Goodnight, B.S. are associate scientists in Cell Culture R&D, Sigma-Aldrich, St. Louis, MO. Mathew Caple is the manager of Cell Culture R&D, Sigma-Aldrich, St. Louis,MO.

ORDERING INFORMATION

Product Code	Product name	Unit size
C 5467	CHO Protein-free Medium	1 L

See detailed listing in the Life Science Catalog 2000-2001, page 347.

SUPPORTING LITERATURE

Media for Biotechnology brochure (DOJ) pages 4-5.

WEB SITE LINKS

http://www.sigma-aldrich.com/saws.nsf/cellculture?OpenFrameset