Development of an Efficient Medium Optimization Kit for Factorial Matrix Design—a statistical approach to increase cell growth and productivity of recombinant CHO cells

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Abstract

Chinese Hamster Ovary (CHO) cells have been used for large-scale recombinant protein expression in the majority of pharmaceutical companies worldwide. The most challenging aspect of culturing recombinant CHO cell clones is providing for the diverse nutritional requirements that are unique to every transfected cell line. Therefore, using factorial matrix statistical assays to accelerate the optimization of cell culture medium has received great attention in many pharmaceutical companies. Based on the format of factorial matrix we have recently developed a component medium optimization kit. This kit which consists of one basal medium and 6 concentrated grouped media supplements (including Amino Acids, Vitamins, Iron chelator, Lipids, Hydrolysates and Metals) will provide an easy access to perform various factorial matrix assays. In previous work, it was found that the supplemental components included in this kit have a significant effect on the growth and production levels of CHO cells either alone or as a group. The statistical program predicts the most optimal levels of the components and additional matrix assays can be set up to confirm these results or narrow the search window for the components. Once the critical components have been optimized or identified they can be added directly to the medium to promote optimal growth and/or productivity. By using this medium optimization kit, we have demonstrated that this kit can be used successfully to achieve optimal growth and productivity in multiple CHO cell lines with unique nutritional requirements. Taken together, our data strongly suggests that a combination of grouped components and the use of a statistical approach can facilitate the timely development of the "best" medium for any given recombinant CHO clone.

Introduction

Chinese Hamster Ovary (CHO) cells have been of great interest to pharmaceutical companies because of these cells' ability to express human recombinant proteins on a large scale. However, developing cell culture media is very challenging because there are several CHO cell clones, each having unique nutritional requirements. When developing an optimized medium for a specific CHO clone, pharmaceutical companies typically follow a traditional approach of testing one medium component at a time in order to determine the most optimal level of supplementation. Several issues arise when using this complex method of medium optimization: it can be labor-intensive, costly, and can take years to design an optimized medium. In order to reduce costs and decrease new product cycle time, the pharmaceutical industry has shown a recent interest in factorial matrix design. A benefit of factorial matrix design is the ability to recognize interactions between components early in the development process, which accelerates medium optimization. Additionally, with the factorial matrix design method, experiments are more simplistic and have fewer test conditions without eliminating significant interactions between components.

In order to assist pharmaceutical companies in improving their medium optimization process, Sigma-Aldrich has developed a medium component optimization kit that allows the customer to easily perform factorial matrix experiments. This kit consists of a concentrated basal medium and six concentrated grouped media supplements, which have been previously determined to have a significant effect on cell growth and recombinant protein production in CHO cells. We have tested this kit using two cell lines to demonstrate how the statistical program predicts the most optimal levels of each component for unique medium supplementation. Taken together, our results indicate that this medium optimization kit is a powerful tool for timely medium development for any CHO clone.

Materials and Methods

Cell Lines: Two CHO cell lines were used for this development. CHO K1 cell (ATCC #CRL-61) is a non-producing CHO clone. CHO recombinant clone 1 is engineered to produce human recombinant IgG.

Chemicals: All materials used in this work were obtained from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise stated.

Design-Expert Software: Factorial matrix software used to generate data analysis was Design-Expert®, version 6.0.2 (Stat-Ease, Inc., Minneapolis, MN).

Cell Stocks: Stock cultures were grown in C 5467 + 4 mM Gln. The cells were passaged twice a week and seeded at 2.0 x 10^5 cells/mL. Cells were maintained in 1 L spinner flasks (Techne, Inc., Princeton, New Jersey) on magnetic stir plates (Thermolyne Corp., Dubuque, Iowa) and incubated at 37 °C and 5% CO_2.

Base Medium Formulation: The Base Medium was developed based on classical, serum-free media formulations already in use by many pharmaceutical companies, with a target performance value of 1e^6 cells/mL by day 6. The Base Medium consists of low levels of amino acids, metals, iron, vitamins, lipids, and hydrolysates so that each component can be titrated. It does not contain insulin, glucose or glutamine.

Determining Supplements: Components included in the kit were determined from previous work developing a custom medium for a particular CHO cell clone (Table 1). All components were found to have a significant effect on cell growth and production. The first step in matrix design was to determine a base level for each component as well as a higher, non-toxic concentration level to test. Initial experiments titrated one component at several low and high levels while leaving the other components constant at their base levels. This particular method was done in order to determine the appropriate levels of low and high titrations to suggest for customer use.

Matrix Design: Once all component titration values had been determined, two full factorial matrix assays per cell line were set up testing three components at their two pre-determined levels (2^3 factorial matrix). Each assay consisted of a centerpoint which was 100% of the three components being titrated (the average amount between the low and high values) while the other components were kept constant at their base levels. Data used in Design-Expert® analysis consisted of cell days and average IgG production (µg/mL) at equal importance levels. The Base Medium is used only as a control in each experiment and therefore is left out of data analysis.

Matrix Assay: The media for each condition was formulated by adding back the appropriate amounts of each supplement being tested to the Base Medium. Iron 2, Insulin and Glucose were added back at a 100% level. Osmolarity and pH were adjusted for each condition by using 5 M NaCl and 1 N HCl solutions, respectively. Each condition was filtered using a 0.2 µm Millipore filter flask. All assays were run in duplicate in 125 mL spinner flasks stored at 37 °C, 5% CO_2, and stirring at 80 rpm. Assays using CHO K1 cells were inoculated at 9e4 cells/mL, while assays testing CHO recombinant clone 1 cells were inoculated at 5e4 cells/mL on day zero and counted on a CASY®-1 cell counter (Scharfe Systems, Reutlingen, Germany) and by the trypan blue exclusion method. Cell counts continued until cell viability fell below 50%. IgG concentrations were determined by HPLC using a Protein-G binding column. Data was then input into Design-Expert® for analysis.

Stability Studies: Stability studies were completed for days 1, 5 (data not shown), and 14 (Fig. 1) using CHO recombinant clone 1 cells. On day zero, each supplement was incubated separately at 37 °C, room temperature (RT) and 4 °C. Also on day zero a complete medium was formulated and incubated at the same three temperatures (represented as 'Medium stored' in Fig. 1). Both individual supplements and the complete medium were observed for precipitation. On day 14, the individual supplements stored at each temperature were used to formulate another complete medium (represented as 'Supplements stored' in Fig. 1). The two medium formulations for the three temperatures were compared for cell growth and IgG production.

Results and Discussion

Table I

Component	Fold Concentrate	Low Titration	High Titration
Base Medium	2X	NA	NA
Metals	5000X	25%	175%
Amino Acids	25X	50%	150%
Vitamins	20X	50%	150%
Iron Chelator 1	1666X	70%	130%
Iron Chelator 2	2000X	NA	NA
Lipids	2325X	50%	150%
Hydrolysates	100X	50%	150%
Insulin	3333X	NA	NA
Glucose	100X	NA	NA
NaCl		NA	NA

*Supplements included in CHO kit and their concentration levels. Low and high titration levels are suggested levels, which is related to 1X complete medium, to test for initial matrix experiment.

Stability Studies: Fig. 1 shows the cell growth on day 14 of the stability study. Theoretically, 'Supplements stored' and 'Medium stored' should equal each other because the medium was formulated in the same manner, just at different time points in the assay. All conditions showed basically equivalent growth, especially on day 10, with no precipitation seen. Both the supplements and the complete medium are stable under the tested conditions.

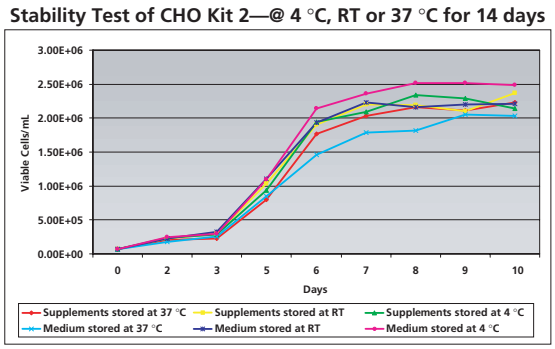

Figure 1. Stability test of CHO Kit 2 using CHO recombinant clone 1. CHO Kit 2 was stored at 4 °C, room temperature (RT) or 37 °C for 14 days. 'Supplements stored' represents supplements stored individually at each temperature and then used to formulate medium on day 14. 'Medium stored' represents a complete medium formulated on day 0 and stored at each temperature. The data showed that CHO Kit 2 was stable when stored at 37 °C for at least 14 days.

CHO K1:

First Matrix Assay. In this matrix assay Amino Acids (AA), Metals, and Iron 1 were tested at two levels each. As shown in Fig. 2A, the Base Medium grew to 1.5e^6 cells/mL by day 7 while all conditions reached cell densities of about 3e^6 cells/mL. On day 12, the top conditions all have a low level of AA as the common factor. The benefit of AA can also be seen in the cube graph (Fig. 2B).

Cube graphs demonstrate how interactions of the three components effect the cell growth results. With these graphs, use the highest number in each of the corners of a cube graph to determine the levels of each supplement that should be added back to the base medium. The cube graph for the first experiment only shows two results for growth–2.58e^7 and 2.0e^7–which change on the X-axis only. There can be more results shown in a cube graph, but the fact that there are two numbers that differ on the X-axis alone shows that only AA effect the cell growth.

The Metals and the Iron 1 supplements appear to show no effect, since altering their low and high levels still result in the same cell growth number on the cube graph. These results are confirmed in the Normal Plot (Fig. 2C), which shows the positive or negative effect of the supplements on cell growth. In a Normal Plot, components to the left of the line have a negative effect, components to the right of the line have a positive effect and components on the line are not significant. The Normal Plot for this experiment shows that only AA have a negative effect, but the Metals and the Iron 1 are on the line representing no effect on growth. Because Metals and Iron 1 do not appear to affect growth, their levels were maintained at the centerpoint value of 100%.

The predicted optimal levels of the components tested for CHO K1 cells are:

50% Amino Acids, 100% Metals, and 100% Iron 1

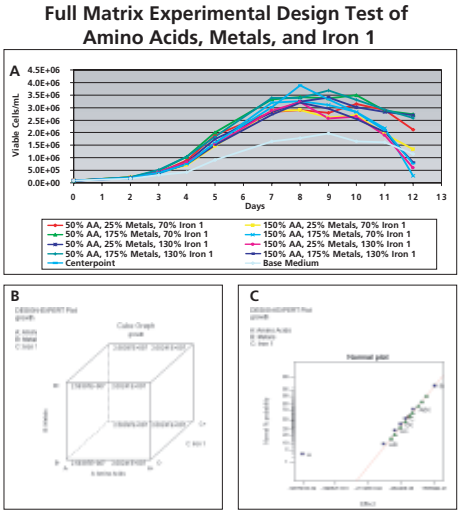

Figure 2. Full matrix experimental design titrating Amino Acids (AA), Metals, and Iron 1 using CHO K1 cells. (A) Viable cell growth/mL, each data point represents an average of two spinners. Centerpoint equals 100% level of AA, Metals and Iron 1 with base levels of Vitamins, Lipids and Hydrolysates. Base Medium was used as control. **(B)** Design-Expert® graph depicts cell growth results in cube graph format. Amino Acids shown on X-axis, Metals on Y-axis, Iron 1 on Z-axis. Numbers represent cell days (10^7). Data shows that only AA have a significant effect. **(C)** Design-Expert® Normal plot shows amount of effect supplements have on CHO K1 cells. A (Amino Acids) shows a significantly negative effect. Both B (Metals) and C (Iron 1) show no effect.

Second Matrix Assay. In this matrix assay Vitamins, Lipids and Hydrolysates were tested at two levels each. As shown in Fig. 3A, each tested condition has higher cell densities than the Base Medium control. The cube graph (Fig. 3B) depicts that Vitamins have a relatively small effect, but they are more beneficial at the low level. The Normal Plot (Fig. 3C) confirms this result by showing the Vitamin supplement having a slightly negative effect. The Normal Plot also shows Hydrolysates have a significantly positive effect and conditions combining negative Vitamins and positive Hydrolysates can result in slightly positive interactions. Since the Normal Plot shows that Lipids do not have a significant effect, the level was maintained at the centerpoint value of 100%.

The predicted optimal levels of the components tested for CHO K1 cells are:

50% Vitamins, 100% Lipids, and 150% Hydrolysates

Full Matrix Experimental Design Testing of Vitamins, Lipids, Hydrolysates

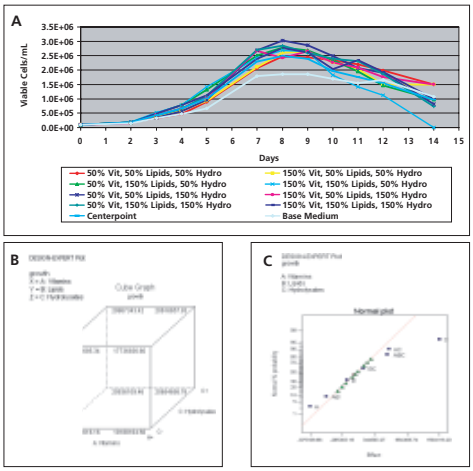

Fig. 3. Full matrix experimental design titrating Vitamins, Lipids and Hydrolysates using CHO K1 cells. (A) Viable cell growth/mL, each data point represents an average of two spinners. Centerpoint equals 100% level of Vitamins, Lipids, and Hydrolysates with base levels of Amino Acids, Metals and Iron 1. Base medium was used as a control. **(B)** Design-Expert growth plot represents interactions of supplements and depicts cell growth results in cube graph format, with Vitamins shown on X-axis, Lipids on Y-axis, and Hydrolysates on Z-axis. Numbers represent cell days (10^7). **(C)** Design-Expert Normal Plot shows the amount of effect supplements have on CHO K1 cells. C (Hydrolysates) shows the most significantly positive effect, while A (Vitamins) shows a slightly negative effect. B (Lipids) does not demonstrate a significant effect.

CHO Recombinant Clone 1:

First Matrix Assay. In this matrix assay Amino Acids (AA), Metals, and Iron 1 were tested at two levels each. Cell growth and production of CHO recombinant clone 1 are shown in Figures 4 and 5. The cell growth graph (Fig. 4A) shows that all the tested conditions have only a slight increase over the centerpoint. The condition with the highest cell density has a high level of AA, a low level of Metals, and a low level of Iron 1. The high cell density for this condition is confirmed in the cube graph (Fig. 4B). The differences in cell densities seen in the cube graph are significantly different as shown in the Normal Plot (Fig. 4C). The Normal Plot shows that Metals and Iron 1 are slightly negative, while AA have a positive effect. Also, because AA has more of an effect than Metals or Iron 1, interactions with AA can either be equaled out to be non-significant or slightly positive.

Full Matrix Experimental Design Test of Amino Acids, Metals, Iron 1—CHO Recombinant Clone 1 (Assay for Cell Growth)

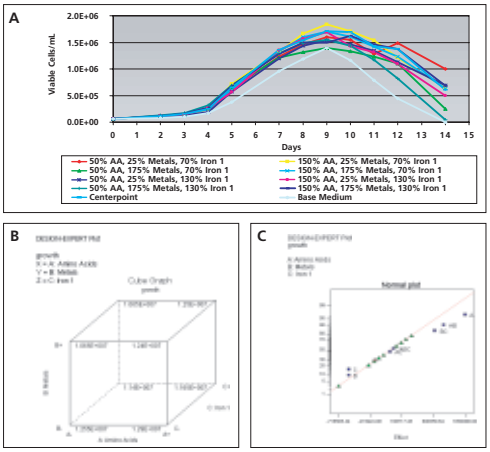

Figure 4. Full matrix experimental design test titrating Amino Acids (AA), Metals and Iron 1 using CHO recombinant IgG producing clone 1. Cell growth data was analyzed by Design-Expert®. **(A)** Viable cells/mL, average two spinners. Centerpoint equals 100% of AA, Metals and Iron 1 with the base levels of Vitamins, Lipids, and Hydrolysates. Base Medium was used as a control. **(B)** Design-Expert growth plot depicts interactions between supplements in cube graph format. Amino Acids are shown on X-axis, Metals on Y-axis, and Iron 1 on Z-axis. Numbers represent cell days (10^7). **(C)** Design-Expert Normal graph shows amount of effect supplements have on cell growth. A (Amino Acids) shows a positive effect. Both B (Metals) and C (Iron 1) show a slightly negative effect.

The average IgG production (Fig. 5A) shows a difference in supplementation as well. The cube graph (Fig. 5B) coincides with the data in that the biggest increase in IgG production comes from a high AA level. Since there are only two data results in the cube graph it shows that only AA have an effect on production. This phenomenon is also seen in the Normal Plot (Fig. 5C), which shows that AA have a significantly positive effect while the other two supplements' effects appear insignificant.

The predicted optimal levels of the components tested in this assay based on equal importance of cell growth and production are:

150% Amino Acids, 25% Metals, and 70% Iron 1

Full Matrix Experimental Design Test of Amino Acids, Metals, Iron 1—CHO Recombinant Clone 1 (Assay for rIgG Productivity)

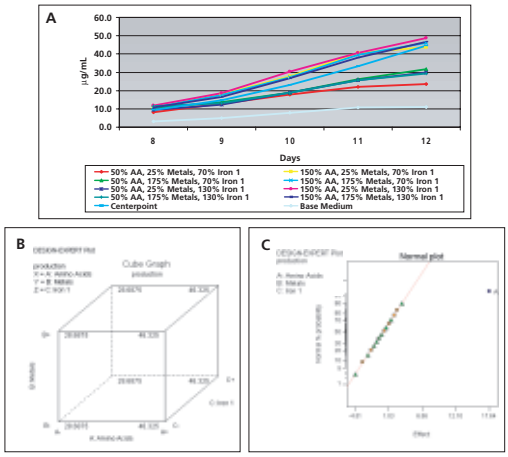

Figure 5. Full matrix experimental design test titrating Amino Acids (AA), Metals and Iron 1 using CHO recombinant IgG producing clone 1. Recombinant IgG production data was analyzed by Design-Expert. **(A)** Average IgG production (µg/mL). Centerpoint equals 100% of AA, Metals and Iron 1 with the base levels of Vitamins, Lipids, and Hydrolysates. Base Medium was used as a control. **(B)** Design-Expert graph for rIgG production levels in cube graph format. Amino Acids shown on X-axis, Metals on Y-axis, and Iron 1 on Z-axis. Numbers represent µg/mL. **(C)** Design-Expert Normal plot shows the amount of effect supplements have on rIgG production. Only Amino Acids show a significantly positive effect.

Second Matrix Assay. In this matrix assay Vitamins, Lipids and Hydrolysates were tested at two levels each. The second matrix experiment (Fig. 6A) shows lower cell densities overall than in the first matrix experiment. For the first time, some tested conditions produced lower cell densities than the Base Medium. On day 8 of the assay, the conditions which are lower than the Base Medium all have a high level of Vitamins, but by day 12 the conditions which are lower all have a low level of Hydrolysates in common. The beneficial conditions, which have high levels of Hydrolysates, are portrayed in the cube graph (Fig. 6B). On this graph, of the two highest data points one has a low level of Lipids, while the other highest data point has a high level of Lipids. Because of this, it appears that the level of Lipids does not play a significant role. However, note that both of these two highest cell densities have a low level of Vitamins and a high level of Hydrolysates. These relationships are further demonstrated in the Normal Plot (Fig. 6C), which shows Vitamins having a negative effect, Hydrolysates having a positive effect, and Lipids having no significant effect on growth.

Full Matrix Experimental Design Test of Vitamins, Lipids, Hydrolysates—CHO Recombinant Clone 1 (Assay for Cell Growth)

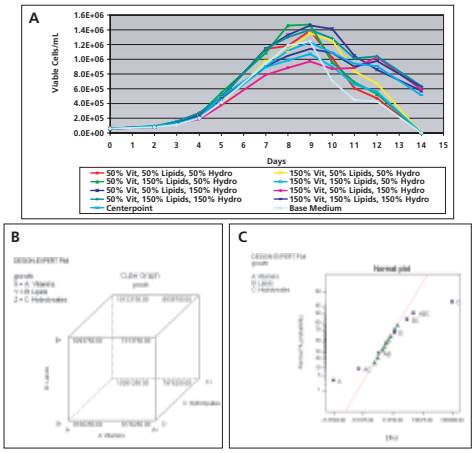

Figure 6. Full matrix experimental design test titrating Vitamins, Lipids and Hydrolysates using CHO recombinant IgG producing clone 1. Cell growth data was analyzed by Design-Expert. **(A)** Cell growth plot shows viable cells/mL, average two spinners. Centerpoint equals 100% level of Vitamins, Lipids and Hydrolysates with base levels of Amino Acids, Metals and Iron 1. Base Medium was used as a control. **(B)** Design-Expert cell growth graph depicts interactions between supplements in cube graph format. Vitamins are shown on X-axis, Lipids on Y-axis, and Hydrolysates on Z-axis. Numbers represent cell days (10^7). **(C)** Design-Expert Normal plot shows the amount of effect supplements have on cell growth. A (Vitamins) shows negative effect, B (Lipids) shows no effect and C (Hydrolysates) shows a positive effect.

Even though some conditions were lower cell densities than the Base Medium, all conditions generated higher IgG production (Fig. 7A). The cube graph representing production (Fig. 7B) shows higher IgG production whenever there is a high level of Hydrolysates. However, there is a relationship between the Vitamins and the Lipids– if there is a low level of Vitamins there should be a high level of Lipids and if there is a high level of Vitamins there should be a low level of Lipids. This inverse relationship is shown in the Normal Plot (Fig. 7C) as having a negative effect, meaning Vitamins at a high level and Lipids at a low level will result in the highest productivity. It also shows that Hydrolysates have a positive effect.

The predicted optimal levels of the components tested in this assay based on equal importance of cell growth and production are:

50% Vitamins, 150% Lipids, and 150% Hydrolysates

Full Matrix Experimental Design Test of Vitamins, Lipids, Hydrolysates—CHO Recombinant Clone 1 (Assay for rIgG Production)

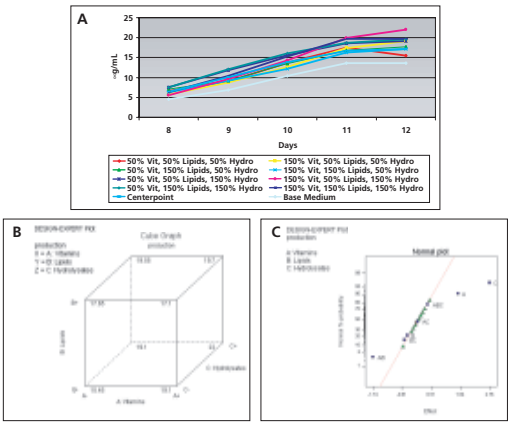

Figure 7. Full matrix experimental design test titrating Vitamins, Lipids and Hydrolysates using CHO recombinant IgG producing clone. Recombinant IgG production data was analyzed by Design-Expert. **(A)** Average IgG production (µg/mL). Centerpoint equals 100% level of Vitamins, Lipids and Hydrolysates with base levels of Amino Acids, Metals and Iron 1. Base Medium was used as a control. **(B)** Design-Expert graph shows production results in cube graph format. Vitamins are shown on X-axis, Lipids on Y-axis and Hydrolysates on Z-axis. Numbers represent µg/mL. **(C)** Design-Expert Normal plot shows amount of effect supplements have on production. Both A (Vitamins) and C (Hydrolysates) show a positive effect, but B (Lipids) shows no effect.

Test of Predicted New Formulation:

From the two factorial matrix assays using CHO K1 cells (as shown in Figures 2 and 3), we obtained two predicted optimal growth formulations. Combining data from the two assays, a new medium was prepared: **50% Amino Acids, 100% Metals, 100% Iron, 50% Vitamins, 100% Lipids, and 150% Hydrolysates.** The cell growth performance of this new medium was tested on CHO K1 cells (Fig. 8). The results clearly showed that CHO K1 cells grew to a 1.5-fold higher cell density in this new medium than in the original Base Medium or in the two competitors' media tested.

Comparison of Cell Growth with The Best Predicted Medium, Original Medium and Competitor Media

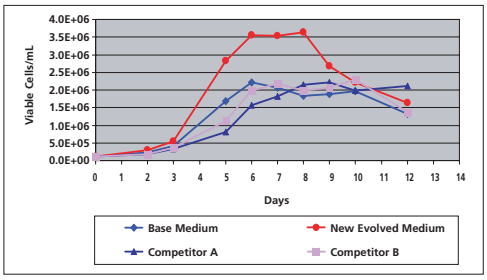

Figure 8. Test of cell growth with the best predicted medium using CHO K1 cells. New evolved medium was prepared according to the predicted best formulations obtained from two factorial matrix assays using CHO K1 cells (Figures 2 and 3). CHO K1 cells growing in this newly evolved medium gave a 1.5-fold higher cell density as compared with the original medium (Base Medium) and two competitors' media.

Cell Line Comparisons:

These results clearly demonstrate that using this medium optimization kit and Design-Expert® software on diverse CHO cell lines can generate a different optimized media formulation. In the cell lines tested there were some similarities observed, such as the requirement for low levels of Vitamins and high levels of Hydrolysates for optimal growth, but there were also many differences seen between the two cell lines. CHO K1 cells grew to higher cell densities than did CHO recombinant clone 1 cells, which can represent different capabilities present in each cell line. Secondly, the concentrations of Metals and Iron 1 had no significant effect on the cell growth of CHO K1 cells, while with CHO recombinant clone 1 cells these supplements were found to be more beneficial with low levels. Furthermore, CHO K1 cells had higher growth when Amino Acids were at the lower level, but CHO recombinant clone 1 cells required Amino Acids at a high level for optimal growth. Also, CHO K1 cells needed a lower level of Lipids for optimal growth, while CHO recombinant clone 1 cells needed a higher level of Lipids.

Conclusions

- Factorial matrix design, a statistical approach, has greatly enhanced the process of cell culture medium development and optimization.
- Sigma-Aldrich's CHO Medium Component Optimization Kit is formatted to facilitate easy medium formula manipulation through factorial matrix assays.
- Using factorial matrix design and this medium component optimization kit, an optimized medium formulation can be generated rapidly.
- By streamlining the process of medium optimization for any given recombinant CHO clone, Sigma-Aldrich's CHO Medium Component Optimization Kit can provide great benefits to the pharmaceutical industry.

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