Research Article

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Development of a serum free medium for HUMIRA® biosimilar by design of experiment approaches

Deney tasarımı yaklaşımlarını kullanarak HUMIRA® biyobenzeri için serumsuz ortam tasarımı

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Abstract

Background: Serum have been traditionally used to support growth of animal cell cultures. However, the increasing growth of therapeutic biopharmaceuticals market, accelerated the high demand for the serum-free medium (SFM).

Objective: The main objective is to design a SFM for a stable rCHO cell line that produces a fully anti-human TNF- α monoclonal antibody (mAb) corresponding to HUMIRA® biosimilar.

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Materials and methods: Design of Experiment (DoE) approaches were used to determine the key factors due to their effect on specific growth rate and mAb production. The production was carried out in T-flasks at different initial cell concentrations and then in Erlenmeyers with the developed SFM. mAb production was compared with commercial SFMs in terms of yield and productivity.

Results: Regarding to our findings, when the developed SFM-adapted cells were compared with the cells produced in commercial SFMs, the mAb productivity in developed SFM were higher (1.3–1.6 times) depending on higher mAb concentration and less (3–5 times) cell concentration. Additionally, the produced mAb in the developed SFM provided high conformational similarity with its originator HUMIRA®.

Conclusion: DoE approaches could be used to reduce cost and time in designing SFM for any commercially important cell line to produce high value biologics.

Keywords: Biosimilar; Biopharmaceutical production; Design of Experiment; Recombinant Chinese Hamster Ovary cell line; Serum-free medium design; HUMIRA.

Öz

Genel Bilgiler: Serum, hayvan hücre kültürlerinin büyümesini desteklemek için geleneksel olarak kullanılmaktadır. Ancak, terapötik biyofarmasötik pazarının hızla büyümesi, serumsuz ortam (SFM) için talebi arttırmıştır.

Amaç: Bu çalışmanın amacı, tamamen anti-insan tümör TNF-alfa monoklonal antikoru (mAb) olan HUMIRA® biyobenzerini üreten stabil rCHO hücre hattı için SFM'nin tasarlanmasıdır.

Gereç ve Yöntemler: Design of Experiment (DoE) yaklaşımları, anahtar faktörlerin spesifik büyüme hızı ve mAb üretimi üzerindeki etkilerini belirlemek için kullanılmıştır. Geliştirilen ortam ile, farklı başlangıç hücre konsantrasyonlarında T-flasklarda ve ardından erlenlerde üretim gerçekleştirilmiştir. mAb üretimi verim ve verimlik bakımından ticari SFM'ler ile karşılaştırılmıştır.

Bulgular: Bulgularımıza göre, geliştirilen SFM'e adapte edilmiş hücreler ile ticari ortamda üretilen hücreler karşılaştırıldığında, SFM'de üretilen hücrelerin mAb verimliliğinin daha yüksek mAb konsantrasyonuna ve daha düşük hücre konsantrasyonuna (3–5 kat) bağlı olarak daha yüksek (1,3–1,6 kat) olduğu belirlenmiştir. Ayrıca, geliştirilen SFM'de üretilen mAb, orjinatorü HUMIRA® ile yüksek yapısal benzerlik sağlamaktadır.

Sonuç: DoE yaklaşımlarının, yüksek katma değerli biyolojik ürün üretimi için ticari açıdan önemli herhangi bir hücre hattında SFM'nin tasarlanmasında maliyet ve zamanı azaltmak amacıyla kullanılabilir.

Anahtar kelimeler: Biyobenzer; Biyofarmasötik üretimi; Design of Experiment; Rekombinant Çin Hamster Yumurtalık hücresi; Serumsuz ortam tasarımı; HUMIRA.

Introduction

Mammalian cell lines are the preferred host cells for biopharmaceutical production. For the reason that, they allow correct folding by forming correct disulphide bonds in the complicated quaternary structures of the proteins and make “posttranslational modifications” such as glycosylation, phosphorylation and deamination [1, 2]. Hence, especially for complex biopharmaceuticals like monoclonal antibodies (mAbs), enzymes, growth factors, hormones, and interferons mammalian cell lines are used [1, 3, 4]. Historically, the murine mAbs produced by classical hybridoma technology caused an human anti-mouse antibody (HAMA) response in humans, so production of humanized or fully human mAbs became popular over time to be used clinically [5]. To meet this, Chinese Hamster Ovary (CHO) have been used as a cell line for mAb production [6, 7].

After the patent expiration, the ‘biosimilars’ of the mAbs came to the market [8, 9]. Adalimumab (HUMIRA®) is recombinant immunoglobulin G₁ (IgG₁) mAb, contains completely human sequences and targets tumor necrosis factor-alpha (TNF- α) in the treatment of rheumatoid arthritis (RA) which is produced by a CHO mammalian cell line [10].

Historically, serum is one of the most important components in cell culture media and source of many nutrients including amino acids, proteins, vitamins, carbohydrates, lipids, hormones, growth factors, minerals and trace elements [11, 12]. It supports cell growth and protects cells against large scale production problems like shear stress. However, it is a potential source for contaminants in addition downstream process especially the purification step becomes very difficult because of the high protein content of the serum [13–15]. Animal derived components and serum do not allowed for industrial biopharmaceuticals production for human use by the licensing agencies such as American Food and Drug Administration (FDA) and the European Medicine Agency (EMA) [11, 16]. For this reason, “serum-free media (SFM)” is used to reduce contamination from adventive and infective agents by the biopharmaceutical industry. SFM are often developed specifically for a single cell type, and the components in this media are also known as well-defined media [13].

The main objective of this study is to develop SFM for commercial production of biopharmaceuticals for therapeutic use by rCHO cells. Design of Experiment (DoE) is the most widely used statistics program for this purpose [17]. It is aimed both to increase the product yield and the productivity while reducing the dependence on high cost chemically defined commercial media. For this purpose, a fully anti-TNF- α mAb producing stable CHO cell line which produces a biosimilar of HUMIRA® was used as a model. DMEM-Ham's/F12 was used as a basal media, recombinant human insulin, plant peptone, linoleic acid-albumin, dextran sulphate, Pluronic F68, yeast extract, glutamine, non-essential amino acids (NEAA) mix, recombinant human transferrin, bovine serum albumin (BSA), hypoxanthine and thymidine supplements were added at concentrations given by the Plackett-Burman design to determine the main effects on the overall system by the 12 experiments as means of specific growth rate and mAb production. After that, the interactions of the components between each other were determined by fractional factorial design through 35 different experiments due to their effect on specific growth rate and mAb production. After the optimization of the components that can replace serum, with the developed SFM, the effects of different initial cell concentrations on mAb production yields and productivity were determined. In addition, the conformational similarity of the mAb produced with the developed SFM with its originator HUMIRA® has been determined by enzyme linked immunosorbent assay (ELISA).

Materials and methods

Cell lines and cell culture

Humanized recombinant anti-TNF- α mAb producing stable rCHO cells with a glutamine synthetase gene expression system, were purchased from Creative Dynamic Inc., USA as HUMIRA® biosimilar. Cultures were performed at 37°C and in a 5% CO₂ humidified incubator.

Media and reagents

All media including commercial serum-free medium for static culture (SFS), commercial serum-free medium for dynamic culture 1 (SFD1) and commercial serum-free medium for dynamic culture 2 (SFD2) were purchased from Gibco, Gaithersburg, MD, USA and DMEM-Ham's/F12 was from Sigma- Aldrich, St Louis, MO, USA. All chemical supplements including 100X concentrated hypoxanthine-thymidine (HT) supplement was obtained from Gibco, USA, 100 mM L-methionine sulfoximine (MSX) was from Millipore, Burlington, MA, USA recombinant human insulin and NEAA mixture were from Biochrome, Berlin, Germany plant peptone, linoleic acid-albumin, yeast extract, L-glutamine, recombinant human transferrin and BSA were from Sigma, USA and dextran sulphate and Pluronic F68 were from Applichem, Darmstadt, Germany. Bambanker™ serum-free type cell freezing medium was obtained from Lymphotec Inc., Tokyo, Japan. After this section, medium with 1% (v/v) HT supplement and 0.05% (v/v) MSX is called as ready-to-use medium for this study.

Serum free medium development

The “Design of Expert 7.0.0 (DoE, Stat-Ease Inc., Minneapolis, MN, USA)” program was used for SFM development. Experiments were performed in 25 cm² flasks at 4 mL working volume with an initial cell concentration of 3×10⁵ cells/mL. Ready-to-use DMEM-Ham's/F12 medium was used as a basal medium for developed SFM in our study. Ready-to-use SFS and DMEM-Ham's/F12 media were used as a positive and a negative control, respectively. The growth kinetics of the controls and experiments were calculated by daily cell count and, the mAb concentrations on last day of the cultures were determined by using a modified indirect ELISA method as described previously [18]. In ELISA, TNF- α protein was used as covered protein, diluted samples and controls were used as primary antibody and anti-human immunoglobulin G (IgG) conjugated with

horseradish peroxidase (HRP) was used as conjugated secondary antibody. Absorbance was recorded at 450 nm on a spectrophotometer (SpectraMax 190, VersaMax, USA). mAb titres were calculated by using a standard curve (Figure 1) (D. Ayyildiz-Tamis, Unpublished data).

Plackett-Burman design

Eleven factors and their concentrations stated in Table 1 were selected for SFM development, according to the literature which are recombinant human insulin, plant peptone, linoleic acid-albumin, dextran sulphate, Pluronic F68, yeast extract, glutamine, NEAA mix, recombinant human transferrin, BSA and hypoxanthine-thymidine supplement [6, 15, 19–23]. These factors were introduced into the Plackett-Burman design in the DoE program for determining the main effects on the overall system as means of specific growth rate and mAb production.

Figure 1: The standard curve used to determine the amount of anti-TNF α .

Table 1: Plackett-Burman design factors and minimum and maximum concentrations used in the experiments.

Factors	Unit	−1	+1	Reference
A – Recombinant human insulin	mg/L	0	5	[15]
B – Plant peptone	mg/mL	0	2.5	[15]
C – Linoleic acid-albumin	mg/L	0	0.08	[19]
D – Dextran sulphate	mg/mL	0	0.5	[15]
E – Pluronic F68	mg/L	0	1	[20]
F – Yeast extract	mg/mL	0	2.5	[15]
G – L-glutamine	mM	0	8	[21]
H – NEAA mixture	mM	0	1	[21]
J – Recombinant human transferrin	mg/mL	0	0.1	[21]
K – BSA	mg/mL	0	1	[22]
L – HT supplement	mL/L	0	20	[6]

BSA, Bovine serum albumin; HT, hypoxanthine-thymidine; NEAA, non-essential amino acid; −1, minimum values; +1, maximum values.

2⁶⁻² Fractional factorial design

The 2⁶⁻² fractional factorial design was used to determine the optimum concentrations of the significant factors which were already evaluated in the Plackett-Burman design. Six significant factors and their 2 level interactions at the minimum and maximum concentrations (Table 2) gave 35 experiments with 2 repetitions and 3 central points. After 35 experiments were performed, the best concentrations due to their effect on specific growth rate and mAb production were determined.

The different initial cell concentration trials

After SFM optimization studies, cells were cultivated in T-25 flasks in triplicates with initial cell concentrations of 1×10⁵ cells/mL, 3×10⁵ cells/mL and 5×10⁵ cells/mL. The cells were also cultivated in ready-to-use SFS medium was used as a positive control. The growth kinetics of the cells were monitored by cell counts made with trypan blue staining from the samples taken daily and maximum specific growth rate ($\mu_{\max}$) and doubling time (t_d) were calculated according to Eqs. 1 and 2, respectively [18]. Also, the mAb concentrations in the supernatants on the last day of the cultures were determined by indirect ELISA.

$$\mu_{\max} = \frac{\ln(x_2) - \ln(x_1)}{t_2 - t_1} \quad (1)$$

$$t_d = \frac{\ln(2)}{\mu} \quad (2)$$

where x_1 was the cell concentration of the first day of logarithmic phase, x_2 was the cell concentration of the last day of logarithmic phase, t_1 was the first day of logarithmic phase and t_2 was the last day of logarithmic phase.

Table 2: 2⁶⁻² Fractional factorial design factors and their values.

Factors	Unit	−1	0	+1
A – Recombinant human insulin	mg/L	0.5	1.25	2
B – Plant peptone	mg/mL	2.5	3.75	5
C – Dextran sulphate	mg/mL	0.1	0.3	0.5
D – Linoleic acid-albumin	mg/mL	0.1	0.55	1
E – NEAA mixture	mM	5	7.5	10
F – Recombinant human transferrin	mg/mL	0.05	0.08	0.1

NEAA, Non-essential amino acid; −1, minimum values; 0, centre values; +1, maximum values.

mAb production in batch mode in shake flasks

After the cells were adapted to the developed SFM at least three passages in static T-flasks and without adaptation, the batch culture in shake flasks were performed. Batch CHO cell cultures were grown with an initial cell concentration of 3×10⁵ cells/mL in 25 mL working volume shake flasks with agitation at 125 rpm. Because of the protecting cells against shear stress, 0.1% (w/v) Pluronic-F68 was added. SFM and ready-to-use SFD1 and SFD2 media were compared according to the cell growth kinetics and mAb yield. pH and partial pressure of oxygen (pO₂) measurements were done online by Sensolux® tray device (Sartorius AG, Germany). Glucose consumption and lactic acid productions were quantified by a high performance liquid chromatography (HPLC) system with Thermo Scientific Finnigan Surveyor RI plus detector. Samples were separated on a Phenomenex Rezex Monosaccharide RHM, (H+) maintained at 65°C. The mobile phase was 5 mM sulfuric acid with a flow rate of 0.8 mL/min. The injection volume was 15 µL [24].

Conformational characterization

The Adalimumab conformational characterization kit (BIOLIKE® ENZ-KIT122, EnzoLifeSciences AG, Switzerland) was used to determine the structural similarity of the mAbs obtained from shake flask batch productions. Supernatants from the cultures, performed in developed SFM, SFD1 and SFD2 were tested in three times according to manufacturer's instructions, and the absorbance values were determined at 450 nm by using spectrophotometer.

Statistical analysis

Statistical analysis was carried out by using one-way and two-way variance analysis (one-way ANOVA with Dunnett's multiple comparisons test, Tukey's multiple comparisons test and two-way ANOVA with Dunnett's multiple comparisons test, Sidak's multiple comparisons test) with ±95% confidence interval and Student's t-test (p < 0.05) in GraphPad Prism 6.0e.

Results

SFM development

When Plackett-Burman design was conducted with 11 factors, it was determined that recombinant human

Table 3: ANOVA results on the specific growth rate of the Plackett-Burman design.

Source	SS	DF	MS	F-value	p>F value
Model	1.02	6	0.17	10.37	0.0107
A – Recombinant human insulin	9.577×10^{-3}	1	9.577×10^{-3}	0.58	0.4800
B – Plant peptone	0.12	1	0.12	7.28	0.0429
C – Linoleic acid-albumin	0.016	1	0.016	0.95	0.3750
D – Dextran sulphate	0.19	1	0.19	11.67	0.0189
H – NEAA mix	0.22	1	0.22	13.25	0.0149
J – Recombinant human transferrin	0.47	1	0.47	28.48	0.0031
Residual	0.082	5	0.016		
Cor total	1.11	11			
Standard deviation	0.13		R-squared		0.9256
Mean	0.24		Adjusted R-squared		0.8363
C.V.%	53.23		Predicted R-squared		0.5715
(PRESS)	0.47		Adequate precision		9.402

C.V., Coefficient of variation; DF, degrees of freedom; MS, mean square; NEAA, non-essential amino acid; PRESS, predicted residual sum of squares; SS, sum of squares.

insulin, plant peptone, linoleic acid-albumin, dextran sulphate, NEAA mixture and recombinant human transferrin had a positive effect on the specific growth rate of rCHO cells, but Pluronic F68, yeast extract, L-glutamine, BSA and HT supplement have no effect on the specific growth rate. As a result of ANOVA with these 6 factors which were showing positive effects (Table 3), it was concluded that the model was “meaningful” when $p > F$ value (0.0107) was lower than 0.05. Although the p values of recombinant human insulin and linoleic acid-albumin were higher than 0.05, it was decided to make fractional factorial design with these 6 factors because of the decrease of R-square value (from 0.9256 to 0.7974) as a result of removing these two factors from the model (Table 3).

In fractional factorial design (Figure 2), because of 6 significant factors and their 2 level interactions gave 35 experiments with 2 repetitions and 3 central points, all repetitions experiments were shown as a number which was given by DoE program. mAb production of positive control was 272.080 mg/L. mAb production of experimental repetitions trial 12 and 21 was 192.731 mg/L, trial 4 and 7 was 236.810 and trial 18 and 19 was 271.203, respectively. Since there was no statistically significant difference between positive control and these three trials ($p > 0.05$), and also the highest mAb concentration was 18 and 19. It was decided to use the media composition of runs 18 and 19 instead of the commercial SFS medium. This media composition is also very similar to the composition proposed in the numerical optimization section of the DoE program. The content of the developed SFM was adding 0.5 mg/L recombinant human insulin, 5 mg/mL plant peptone, 0.1 mg/mL dextran sulphate, 1 mg/mL linoleic

Figure 2: mAb concentrations of controls and experimental repetitions in fractional factorial design; as (+) control: ready-to-use SFS (commercial serum-free medium for static culture) was used, as (–) control: ready-to-use DMEM-Hams’s F12 was used.

ns, $p > 0.05$. As explanation of the column titles, first column numbered with 14 and 17 were experimental repetitions. Also, number 10, 25 and 34 were experimental repetitions and central point of design on seventeenth column.

acid-albumin, 10 mM NEAA mix and 0.05 mg/mL recombinant human transferrin to ready-to-use DMEM/Ham’s-F12 basal medium (Table 4).

The effect of different initial cell concentrations

1×10^5 cells/mL, 3×10^5 cells/mL and 5×10^5 cells/mL initial cell concentrations were tested in developed SFM. Results were shown that the highest number of cells from

Table 4: Components and concentrations of the developed serum-free medium.

	Recombinant human insulin (mg/L)	Plant peptone (mg/mL)	Dextran sulphate (mg/mL)	Linoleic acid-albumin (mg/mL)	NEAA mix (mM)	Recombinant human transferrin (mg/mL)
Trial 18 and 19	0.5	5.0	0.1	1.0	10.0	0.05
Numerical optimization of DoE	0.51	5.0	0.1	1.0	10.0	0.05

DoE, Design of expert; NEAA, non-essential amino acids.

all experiments reached much less than the commercial medium, however there was no statistically significant difference ($p > 0.05$) between the mAb concentrations produced (Figure 3). When the initial cell concentration was 3×10^5 cells/mL, doubling time (t_d) was the shortest

(1.245 ± 0.036 day) and specific mAb production productivity (q_{mab}) ($10.25 \pm 0.700 \times 10^9$ mg mAb/cells-day) was the highest at day 6 in developed SFM (Table 5). Therefore, this initial cell concentration was decided for later Erlenmeyer flask productions.

Figure 3: (A) Growth kinetics and (B) mAb concentrations of developed SFM (serum-free medium) and SFS (commercial serum-free medium for static culture) with different initial cell concentrations (C_0).

$p < 0.05$; no statistically significant difference between the mAb concentrations produced.

Table 5: $\mu_{\max}$, t_d , q_{mab} and total mAb productivity of the CHO cells for different initial cell concentrations (C_0) in SFM and SFS media in T- flasks.

Media and initial cell concentration (cells/mL)	Maximum specific growth rate ($\mu_{\max}$) (day ⁻¹)	Doubling time (t_d) (day)	Specific mAb production productivity (q_{mAb}) (mg mAb/cells-day)*10 ⁹		Total mAb productivity (mg mAb/L. day)
			t_6	t_8	
SFM C_0 : 1×10^5	0.448 ± 0.054	1.546 ± 0.190	9.12 ± 0.896	7.45 ± 0.898	30 ± 1.043
SFS C_0 : 1×10^5	0.517 ± 0.058	1.341 ± 0.062	4.70 ± 0.269	3.65 ± 0.083	22 ± 0.940
SFM C_0 : 3×10^5	0.581 ± 0.013	1.245 ± 0.036	10.25 ± 0.700	9.88 ± 0.720	25 ± 0.915
SFS C_0 : 3×10^5	0.657 ± 0.022	1.056 ± 0.031	4.53 ± 0.676	3.57 ± 0.183	26 ± 0.975
SFM C_0 : 5×10^5	0.259 ± 0.083	2.674 ± 0.167	9.79 ± 0.570	11.80 ± 0.658	21 ± 0.867
SFS C_0 : 5×10^5	0.480 ± 0.016	1.444 ± 0.044	3.75 ± 0.220	5.06 ± 0.768	27 ± 0.897

mAb production in batch mode in shake flasks

The batch cultures in shake flasks with SFM were performed with 3×10^5 cells/mL initial cell concentration. Figure 4 show that, the rCHO cells cultured in batch mode were reached approximately 9×10^6 cells/mL in SFD1 medium and 1.5×10^7 cells/mL in SFD2 medium, while the developed SFM medium reached only 3.2×10^6 cells/mL. Depending on the growth rate of the cells pH was decreased, however, after exponential phase pH was increased, again. Also, it was observed that the amount of pO₂ in the medium was decreased with time due to the generation of cells, and the amount of pO₂ was increased with the cells entering the death phase. The glucose content of SFM medium was 3 g/L; 5 g/L of SFD1 medium and 6 g/L of SFD2 medium were determined by HPLC method. On day 8, cell deaths were accelerated due to a significant reduction of glucose in the medium. It was found that the pH was decreased due to the accumulation of lactic acid and the pH change throughout the culture and the glucose utilization of the cells were compatible with cell proliferation. Accumulation of mAb concentration on day 13 was reached 388.055 mg/L in developed SFM, 350.00 mg/L in SFD1 and 412.907 mg/L in SFD2, and there was significant difference ($p < 0.0001$) between developed SFM and two commercial media. Figure 5 and Table 6 show that, adaptation of cells to the new developed SFM at least three passages in static T-flasks decreased lag phase and t_d (from 2.332 ± 0.293 to 1.398 ± 0.149 day), and closed commercial media. Also, with adaptation, mAb concentration was increased from 388.055 to 544.977 mg/L and there was significant difference ($p < 0.0001$). In addition, total mAb productivity was increased from 30 ± 1.587 to 42 ± 1.058 mg mAb/L.day with adaptation thus total mAb productivity of rCHO cells in developed SFM was overpassed in commercial SFD1 and SFD2 and there was significant difference ($p < 0.0001$ and $p < 0.001$).

Conformational characterization analysis

The Adalimumab conformational characterization kit was used to determine the structural similarity of the mAbs obtained from shake flask batch productions. In Figure 6, the supernatant samples were not statistically different from the control ($p > 0.05$). According to results, optical density (OD) of Adalimumab was 0.059, SFM was 0.063, SFD1 was 0.189 and SFD2 was 0.201. For this reason, conformational similarity between Adalimumab and mAb from developed SFM was 99.6%, SFD1 was 87% and SFD2 was 85.5%, basically. As a result, structure of the mAb from the developed media was closer to the control HUMIRA® more than from two commercially available media.

Discussion

The development of serum free media is inevitable while maintaining the cell line productivity for industrial scale for production of human biopharmaceuticals [2, 11, 12]. Depending on the metabolism and nutrient consumption of each cell line, the optimization of cell culture media is specific to cell lines [25]. Although the best-known method for SFM development is one factor at a time (OFAT) media optimization, it quite time-consuming and also does not allow the interaction between factors to be examined. Therefore, using of high-throughput statistical programs such as “Design of Experiment (DoE)” used widely [17, 26]. This program allows to analyze larger number of components to determine the most important ones at a shorter time frame [27, 28].

SFM are divided into three groups including non-serum media, animal component-free media and protein-free or chemical defined (CD) media [12, 27, 29]. DMEM [7], DMEM/Ham's-F12 [15], RPMI-1640 Medium and IMDM [30] are generally used as basal media in designing of

Figure 4: Erlenmeyer flask productions in developed SFM (serum-free medium) and commercial SFD1 (serum-free medium for dynamic culture-1) and SFD2 (serum-free medium for dynamic culture-2) (A) growth kinetics, (B) mAb concentrations, (C) media pH changes, (D) pO₂ changes, (E) glucose consumption and (F) lactic acid production according to the culture period.

****, $p < 0.0001$; pO₂, dissolved oxygen concentration.

non-serum and animal component-free SFM. During the development of the SFM, DMEM/Ham's-F12 was used and non-animal origin media components were selected. Therefore, the developed SFM is classified as animal component-free media.

Recombinant human insulin concentration was 0.5 mg/L in the final SFM. It promotes cell growth, regulates glucose and lipid metabolism, and enhances the biosynthesis of fatty acids and nucleic acids [21, 31]. Liu and Chang developed a SFM for macrophage

Figure 5: Adaptation effect on (A) cell growth, (B) mAb production and (C) cell viability in SFM, (D) total mAb productivity between SFM, SFD1 and SFD2.

ns, $p > 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Table 6: $\mu_{\max}$, t_d , $q_{\max}$ and total mAb productivity of the CHO cells with and without adaptation in SMF and commercial SFD1 and SFD2 media in shake flasks with 3×10^5 cells/mL initial cell concentration (C_0).

Media and initial cell concentration (cells/mL)	Maximum specific growth rate ($\mu_{\max}$) (day ⁻¹)	Doubling time (t_d) (day)	Specific mAb production productivity ($q_{\max}$) (mg mAb/cells-day)*10 ⁹		Total mAb productivity (mg mAb/L.day)
			t_6	t_8	
SFM without adaptation	0.297 ± 0.037	2.332 ± 0.293	19.40 ± 1.703	7.63 ± 0.856	30 ± 1.587
SFM with adaptation	0.496 ± 0.046	1.398 ± 0.149	18.45 ± 1.664	13.33 ± 1.014	42 ± 1.058
SFD1	0.600 ± 0.038	1.125 ± 0.092	6.07 ± 0.711	4.30 ± 0.975	26 ± 1.175
SFD2	0.672 ± 0.042	1.042 ± 0.078	2.55 ± 0.379	3.12 ± 0.973	32 ± 1.807

colony-stimulating factor producing CHO cells by fractional factorial design. Seven factors were tried, one of them was 5 mg/L insulin. According to cell growth and M-CSF production, two factors were significant for CCD and developed SFM had 0.093 mg/L insulin [15].

Plant peptone is another important nutrient in the cell culture media that increases cell growth without consuming carbon source and increasing production of lactic acid, but also promotes the production of specific

antibodies [32, 33]. In our study, it was determined that the plant peptone concentration was 5 mg/mL. Liu and Chang also tried 2.5 mg/mL meat peptone in fractional factorial design. Although it had stimulatory effects on cell growth, on M-CSF production it has inhibitory effects so it was removed from the final SFM formulation [15].

Dextran sulphate prevents cell clustering and allows individual cells in the suspension. However, increasing

Figure 6: Conformational similarity of Adalimumab and mAbs from different media. SFM, serum-free medium; SFD1, commercial serum-free medium form dynamic culture-1; SFD2, commercial serum-free medium form dynamic culture-2; L, light chain; H, heavy chain; ns, $p > 0.05$.

concentrations have adverse effects on cell growth and production [19, 34]. Kim et al. tried 30–50–100 mg/L dextran sulphate, the final SFM had 30 mg/L dextran sulphate [19]. However, in our study, it was determined that the dextran sulphate concentration was 0.1 mg/mL due to its adverse effects.

Linoleic acid-albumin is another ingredient which is a lipid source and is required for cell growth [15]. Kim et al. also tried linoleic acid at concentrations of 0.1, 0.5 and 1 mg/L to stimulate cell proliferation and recombinant antibody production, the final developed SFM had 0.5 mg/L linoleic acid [19]. In our study, in the same manner it was determined that the linoleic acid-albumin concentration was 1 mg/mL.

Parampalli et al. developed a serum free medium for CHO-DG44 cells against recombinant antibody against Botulinum A in which 5 factors were used in the experimental design adopted Box's CCD. One of them was NEAA mix at 0.25, 0.5, 1, 1.75, 2.5, 3.25X concentrations [21]. 0.5X NEAA mix were used in the final SFM developed, in our study, the developed SFM has 10 mM NEAA mix. NEAA mix were used because, amino acids are the primary source of nitrogen that protects cells from nutrient deprivation and also protects the cells from osmolarity and dissolved carbon dioxide increase and serves as a buffer in the stabilization of intracellular pH [21, 35].

The other important component promoting cell growth in SFM is transferrin. As an iron-binding glycoprotein, the

interaction of transferrin with surface receptors allows the passage of iron through the cell membrane [21, 36]. As the recombinant human transferrin is relatively costly and cannot be used in CD media, iron ions can be used as an alternative in the growth of CHO cells [27]. Lee et al. developed SFM for erythropoietin producing rCHO cells by Plackett-Burman design in which one of 21 factors was transferrin at concentrations of 10 mg/L and 20 mg/L which was relatively high concentrations compared to our study at 0.05 mg/mL.

In the shake flask experiments, Pluronic F-68 was added to protect cells from shear stress resulting from aeration and mixing. It is suggested that 0.1% (w/v) Pluronic F-68 is generally used in animal cell culture media and it should be added in the range of 0.05–0.2% (w/v) [37]. Similarly, in our study, 0.1% (w/v) Pluronic F-68 was added to SFM.

Liu et al. used 0.8×10^5 cells/mL of initial cell concentration, Schröder et al. used 1×10^5 cells/mL, Castro et al. and Lee et al. used 1.5×10^5 cells/mL and Kim et al. and Parampalli et al. used 2×10^5 cells/mL for optimizing of serum-free culture media for CHO cells [7, 19–22, 36]. It was determined that all initial cell concentrations (1×10^5 , 3×10^5 and 5×10^5 cells/mL) tested in developed SFM could not reached same concentrations with SFD1 and SFD2. However, there was no statistically significant difference ($p > 0.05$) between the produced mAb concentrations (Figure 3).

Enhanced production of mAb in shake flasks is due to increasing transfer of oxygen resulting from shaking as literature [38]. Adaptation of cells to the SFM at least three passages in static T-flasks prior to the Erlenmeyer experiments, decreased lag phase and doubling time (from 2.332 ± 0.293 to 1.398 ± 0.149 day), while the overall cell concentration was increased according to the culture period. Also, mAb concentration (from 388.055 to 544.977 mg/L) and total mAb productivity (from 30 ± 1.587 to 42 ± 1.058 mg mAb/L.day) were increased.

The quality of the product determines the glycosylation profile which also depends on the cell clone, culture conditions and medium components [39]. Although there was no similar study has been found in the literature, it was determined that while adalimumab production from shake flasks in our developed SFM has high conformational similarity (99.6%) with HUMIRA®, similarity of commercial SFD1 was 8% and SFD2 was 85.5% in this study (Figure 6). Within these results, it was concluded that total adalimumab productivity and conformational similarity to HUMIRA® of rCHO cells were increased with our newly developed SFM.

Conclusion

SFM development is an inevitable step in therapeutic biopharmaceutical industry for licensing the final product. Compared to classical one dimensional one factor at a time approach in SFM development, different multifactorial approaches are being used to produce high yield of mAbs at a relatively shorter time period. For this reason, in this study, as a design of experiment approach Plackett-Burman and fractional factorial design were used consequently to develop SFM for rCHO cells producing anti-TNF- α mAb as a model. Due to the results of this study, it can be concluded that a sequential modification of SFM components could provide efficiently optimize the media and this approach could be used to design SFM for other commercially important cell lines.

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