FULL LENGTH	Iranian Biomedical Journal 28 (5 & 6): 282-296 September and November 2024

Optimization of Culture Conditions to Improve Follicle-Stimulating Hormone Production by CHO-DG44 Cells in Serum-Free Medium

Hanna Ghobadian[1,2], Khashayar Roshanzamir[2,3*], Mohammadhasan Kouhi Abdolabadi[2,3], Hadi Ostadi[2,3], Reza Zati Keikha[2,3], Mohammad Dolatkhah Baghan[2,3], Yeganeh Talebkhan[1*], Fatemeh Torkashvand[1]

[1]Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran; [2]CinnaGen Medical Biotechnology Research Center, Alborz University of Medical Sciences, Karaj, Iran; [3]CinnaGen Research and Production Co., Alborz, Iran

OPEN ACCESS

Received: March 11, 2024
Accepted: May 27, 2024
Published online: May 29, 2024

ABSTRACT

Background: In the present study, we attempted to adapt an adherent and serum-dependent Chinese hamster ovary DG44 cell line to a serum-free suspension culture and optimize the culture condition to achieve a higher yield of r-hFSH with acceptable quality. This approach helps to mitigate the risks associated with blood-borne pathogens, reduces lot-to-lot variability, and lowers costs, making it suitable for industrial processing and scale-up.

Methods: The cell adaptation was performed using different chemically defined SFM. This process was followed by optimization through statistical experimental design, focusing on selected physicochemical parameters, including chemical supplementation of the medium and temperature shift. Both small- and large-scale cultures were conducted to test the reproducibility of the optimized condition. The expressed protein was evaluated for comparability with the standard molecule according to the Pharmacopeia guidelines.

Results: RSM analysis indicated that supplementation of the culture medium with galactose and NaBu, along with a temperature downshift, were the main parameters leading to increased cell viability (10%), r-hFSH level (96%), and more importantly, the glycosylation content (49%) of r-hFSH compared to the control condition.

Conclusion: As r-hFSH isoforms generated during in vivo post-translational modifications typically exhibit different serum/plasma half-lives and bioactivity due to their incorporated sialic acid content/glycosylation, further optimizations of r-hFSH production are necessary to enhance its biological activity. In this study, following a primary screening of the studied parameters, optimization of culture conditions based on selected parameters resulted in enhanced quality and quantity of the produced r-hFSH. However, further examination is necessary before transitioning to industrial production. ***DOI: 10.61186/ibj.4160***

Citation:
Ghobadian H, Roshanzamir K, Kouhi Abdolabadi M, Ostadi H, Zati Keikha R, Dolatkhah Baghan M, Talebkhan Y, Torkashvand F. *Iranian biomedical journal* 2024; 28(5&6): 282-296

Keywords: CHO cells, Human follicle stimulating hormone, Serum-free culture medium

Corresponding Authors:
Khashayar Roshanzamir
CinnaGen Medical Biotechnology Research Center, Alborz University of Medical Sciences, Karaj, Iran; E-mail: Roshanzamirk@cinnagen.com

Yeganeh Talebkhan
Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran; E-mail: talebkhan@pasteur.ac.ir, ytgaroosi@gmail.com

List of Abbreviations:
CCD: central composite design; **CD-SFM:** chemically defined serum-free medium; **CHO:** Chinese hamster ovary; **DOE:** design of experiment; **FBS:** fetal bovine serum; **HPLC:** high-performance liquid chromatography; **NaBu:** sodium butyrate; **PBD:** Plackett-Burman design; **r-hFSH:** recombinant human follicle stimulating hormone; **RSM:** response surface methodology; **SDS:** sodium dodecyl sulfate; **SFM:** serum-free medium; **VCD:** viable cell density

[Downloaded from ibj1.pasteur.ac.ir on 2026-10-16]
[DOR: 20.1001.1.1028852.2024.28.5.7.0]
[DOI: 10.61186/ibj.4160]

[Downloaded from ibj1.pasteur.ac.ir on 2026-10-16]

[DOR: 20.1001.1.1028852.2024.28.5.7.0]

[DOI: 10.61186/ibj.4160]

INTRODUCTION

Mammalian cell culture is essential for biotechnological applications, particularly in the industrial production of recombinant glycoproteins[1,2]. This significance arises mainly from their ability to exert robust human-like post-translational modifications, thereby accelerating the development of biopharmaceuticals within drug development pipelines[3]. CHO cells are the preferred expression systems and predominant workhorses for producing recombinant therapeutic proteins[4].

CHO cell culture traditionally relies on FBS, providing essential components that facilitate cell attachment, proliferation, and maintenance[5,6]. However, using FBS can be problematic due to its undefined composition, lot-to-lot variations, and potential effects during downstream processes. In addition, FBS could increase the risk of contamination with bovine viruses and/or mycoplasma, which can adversely affect outcomes, particularly the reproducibility of cell culture systems[5,7-9]. Therefore, transitioning from adherent to suspension conditions requires adapting steps to reduce serum concentration[10-12]. Although several studies have aimed to establish suspension cultures by screening diverse commercial SFM and comparing cell growth and protein production efficiency in CHO[13,14], Vero[15], and Madin-Darby canine kidney[16] cells, robust adaptation and optimization protocols designed for specific recombinant proteins are still needed. In this regard, DOE is a systematic approach for efficient optimization of culture media through reduced number of experiments and identification of cost-effective critical components[17,18], followed by optimization of their concentrations, including PBD and RSM[19].

Based on the literature review, mild hypothermic conditions (33 or 31°C) and NaBu supplementation (0.5 to 3 mM) are crucial parameters to enhance CHO cell performance[18,20]. Galactose and uridine are also essential precursors in uridine diphosphate galactose production. Manganese has also been reported as a necessary cofactor for the activity of galacto-syltransferase[21]. The present study was conducted to elucidate the impact of the above-mentioned parameters on adapted CHO-DG44 cells expressing r-hFSH. This heterodimeric glycoprotein, secreted from pituitary gland, enhances the maturation of ovarian follicles and spermatogenesis. Due to the importance of its glycosylation pattern and sialyated isoforms/variants, CHO cells are the preferred expression platform representing a low specific productivity of about 0.32 pg/cell/day FSH, which may be due to the complexity of this heterodimeric glycoprotein. Figure S1 depicts a comprehensive scheme of the entire process from adaptation to optimization of culture steps.

MATERIALS AND METHODS

Adherent cell cultures

The adherent CHO-DG44 cell line expressing r-hFSH (Lonza, USA) was kindly provided by CinnaGen Company (Iran). The cells were cultured in T75 flasks in DMEM)/Ham's F-12 (Inoclon, Iran) medium, supplemented with 10% FBS (Gibco, UK), and incubated in 5% CO_2 at 37 °C to reach 80-90% confluency. The cells were then subcultured every 3-4 days.

Serum-free suspension adaptation

The cells grown in the FBS-supplemented DMEM/F-12 medium underwent a sequential adaptation in serum-free medium. Briefly, the cells were subcultured into a serum-containing and SFM (ProCHO5) mixture, initiating from 0% serum-free and gradually increasing to 10, 30, 50, 75, and 100%. Cell morphology was examined, and cell viability and VCD were measured daily. SFM adaptation was initiated with 0.3-0.5 × 10^5 cells/ml of exponentially growing cells, maintaining a viability rate of ≥95%. Subsequent passages were performed when the cell confluency decreased to 85%. The subsequent subcultures were performed with an initial cell density of 3-5 × 10^5 cells/ml in T175 and baffled 500 ml shake flasks containing a reduced level of FBS and an increased concentration of ProCHO5 medium, up to 100%. The ProCHO5-adapted cells underwent a minimum of five passages, consistently achieving high cell viability (>80%) and VCD (1.5-2 × 10^6 cells/ml). To examine the effect of the ProCHO5 medium on cell viability, VCD, glucose uptake, and r-hFSH level, a 10-day batch culture was run in ProCHO5 medium. The glucose concentration was monitored daily using a Colorimetric assay kit (Pars Azmun, Iran). The culture medium was centrifuged, and the supernatant was filtered using 0.45-0.22 µm filters. Quantitative analysis of r-hFSH level was carried out from day four to the last day of the culture using an ELISA kit (Pishtazteb, Iran). Afterwards, r-hFSH was purified through the Capture Select FSH-Affinity column (Thermo Fisher Scientific, USA). The purified protein was buffer exchanged, concentrated with10 kDa Amicon (Millipore, USA), and kept at 2-8 °C.

Medium selection

Fully adapted suspended cells were revived and tested for sequential adaptation to First CHOice (UGA, Germany) and Dynamis (Gibco) SFM by transferring them into a mixture of ProCHO5 and the specified CD-SFM. Cells/ml (3-5 × 10^5 cells/ml) were inoculated into 100% ProCHO5 medium (0% of the selected CD-SFM), gradually decreased to 0%. Two passages were performed every 3-4 days under each adaptation step.

After complete adaptation, a seven-day batch culture was conducted for all CD-SFM compared to ProCHO5 as the control condition. Cell viability, VCD, and r-hFSH level were examined as described in section "Serum-free suspension adaptation". Normal-phase and anion-exchange HPLC analyzed N-linked glycosylation patterns. In brief, 50-100 µg of protein was denatured with SDS and 1% 2-mercaptoethanol, then incubated with 25 mU of peptide N-glycosidase F at 37 °C for 24 h. The protein was precipitated by EB-10 cartridge (Ludger), and the glycan fraction was collected, lyophilized, labeled with Tag 2-AB Glycan Labeling Kit (Ludger), and incubated at 65 ± 3 °C for 3 h. After labeling, oligosaccharides were purified using a Clean S cartridge (Ludger) and analyzed by HPLC-Fluorescence Detector (Agilent Technologies, USA) on a weak anion exchange HPLC column using a gradient of 100% acetonitrile (eluent A), 0.5 M ammonium acetate (eluent B; pH 5.4), and 100% water (eluent C). The 2-AB labeled glycans were analyzed by fluorimeter at excitation and emission wavelengths of 330 and 420 nm, respectively. The Z-number was calculated using the formula: $Z=(A_0 \times 0)+(A_1 \times 1)+(A_2 \times 2)+(A_3 \times 3)+(A_4 \times 4)$, where A_0 to A_4 represent the peak area percentages related to the neutral, mono-, di-, tri, and tetra-sialylated forms of r-hFSH, respectively. The obtained Z-numbers were compared with the values reported in the European Pharmacopoeia monograph for FSH, which should be within the acceptance range of 177-233.

Plackett-Burman design

A nine-day fed-batch culture was conducted in the selected SF medium within a 2L baffled shake flask, and PBD was used to identify the crucial variables and assess their main effects. The selected parameters and their two tested levels (-1 and +1 as low and high levels, respectively) were galactose (5 and 25 mM), $MnCl_2$ (4 and 40 µM), deoxyuridine (10 and 100 mg/L), temperature shift (from 37 to 32 °C), and NaBu (0.5 and 2.5 mM), which were included in 12 experiments (Table S1). $MnCl_2$ and deoxyuridine were added to the medium on the first day of culture, while galactose and NaBu were added on day five. A temperature shift from 37 to 32 °C also occurred on day five. Cell viability, VCD, r-hFSH level, and glycosylation profile (Z-number) were then examined. r-hFSH level was monitored from day four until the last day of culture. Z-number was calculated during three last days of all experiments. An eleven-day fed-batch culture was also run to find the appropriate day for the temperature shift. The medium was divided into four parts, including the control condition (at 37 ºC) and those in which temperature shift occurred on days 5, 6, or 7. All groups were treated with selected galactose concentration on day five. Cell viability and VCD were monitored daily.

Response surface methodology

The parameters with the most significant effects were selected for further optimization using CCD, the most common fractional factorial design employed in RSM. This approach aims to identify the optimized conditions to achieve higher quality and quantity of the expressed r-hFSH. Cell viability, r-hFSH level, and Z-number were analyzed on the final day of a 500 ml fed-batch culture. A temperature shift was conducted on a selected day in all conditions. To optimize the two selected variables, 11 experiments were designed using a central composite (circumscribed) design (CCC) matrix in Design Expert® (v.13, Stat-Ease Inc., USA; Table S2) consisting of five levels (extreme high, higher, center, lower, and extreme low). The extreme high (+α) and low (-α) points of each variable were determined based on the maximum and minimum levels derived from the PBD, with $\alpha = 2$.

Reproducibility analysis

To test the reproducibility of the RSM-based results, supplementation of First CHOice medium with significant effective parameters was compared to the control condition, in which the cells did not receive any temperature shift or culture supplementation, except 4 g/L of glucose on day five. Samples were run in triplicate, and the responses were analyzed as described previously.

In vivo biological activity assay

The biological activity of r-hFSH samples was measured in accordance with FSH European Pharmacopeia 10th edition, 01/2020:2286. Immature female Wistar rats (aged 19-28 days, weights of 45-65g) were randomly divided into five groups of five rats. All animals, except the untreated ones, received 14 IU of pregnyl human chorionic gonadotropin (Merck, USA). Standard FSH and equivalent doses of r-hFSH (6, 3, and 1.5 IU) were administered subcutaneously. The first group received the NIBSC international standard. The test group received r-hFSH produced under RSM-optimized condition. The control group was administered r-hFSH, produced at CinnaGen Company, in the original adherent culture mode. The fourth and fifth groups received 14 IU of pregnyl human chorionic gonadotropin and phosphate buffer, respectively. Animals were kept at 20-25 °C for 24 hours, followed by injections on the first, second, and third days. The animals were decapitated 24 h after the last injection under CO_2/O_2 anesthesia. The ovaries were removed, weighed, and relative potency was calculated using PLA v.3.0 software. The estimated potency should be within the range of 64% to 156%, as specified in the 10th edition of the Pharmacopeia.

[Downloaded from ibj1.pasteur.ac.ir on 2026-10-16]
[DOR: 20.1001.1.1028852.2024.28.5.7.0]
[DOI: 10.61186/ibj.4160]

[Downloaded from ibj1.pasteur.ac.ir on 2026-10-16]

[DOR: 20.1001.1.1028852.2024.28.5.7.0]

[DOI: 10.61186/ibj.4160]

Bioreactor culture

Cells (0.5 × 10^6 cells/ml) were seeded in a 10 L bioreactor (Aroko, Iran) in a nine-day fed-batch mode. The pH, dissolved oxygen, and agitation rate were monitored daily. According to the monograph, the r-hFSH protein was characterized and compared to the standard molecule.

Statistical analysis

Data are presented as the mean of three independent experiments with standard deviation (SD). Normalization of data was evaluated using Shapiro-Wilk normality test. The student's t-test and two-way ANOVA were applied to compare the mean values using GraphPad Prism. The observed differences were statistically significant at a *p* value of 0.05. Figures were generated using Microsoft Power BI.

RESULTS

Establishment of serum-free suspension cultures

The sequential adaptation of adherent CHO-DG44 cells to serum-free suspension culture was performed at a constant temperature, CO_2 concentration, and shaking speed. The observed cell viability and VCD after each subculture indicated successful adaptation procedure, and gradual decrease in cell viability and VCD in response to the declining concentration of FBS was significantly recovered and gradually deceased cell viability and VCD, due to the declining FBS concentration, were significantly recovered (Fig. 1A). In the 10-day batch culture, cell viability, VCD, and r-hFSH levels were monitored on appropriate days of culture (Fig. 1B). VCD reached the highest level on day five, indicating the beginning of the stationary phase of the growth curve. A clear trend in decreased cell viability was observed from day 7 to 10, while the hFSH level showed no significant change. Additionally, glucose concentration gradually decreased during the culture period. The observed consistency of the first week of this batch culture was confirmed in the next seven-day batch (Fig. 1C).

Culture medium selection

Basal CD-SFM, First CHOice, and Dynamis were used for the sequential adaptation of fully ProCHO5-

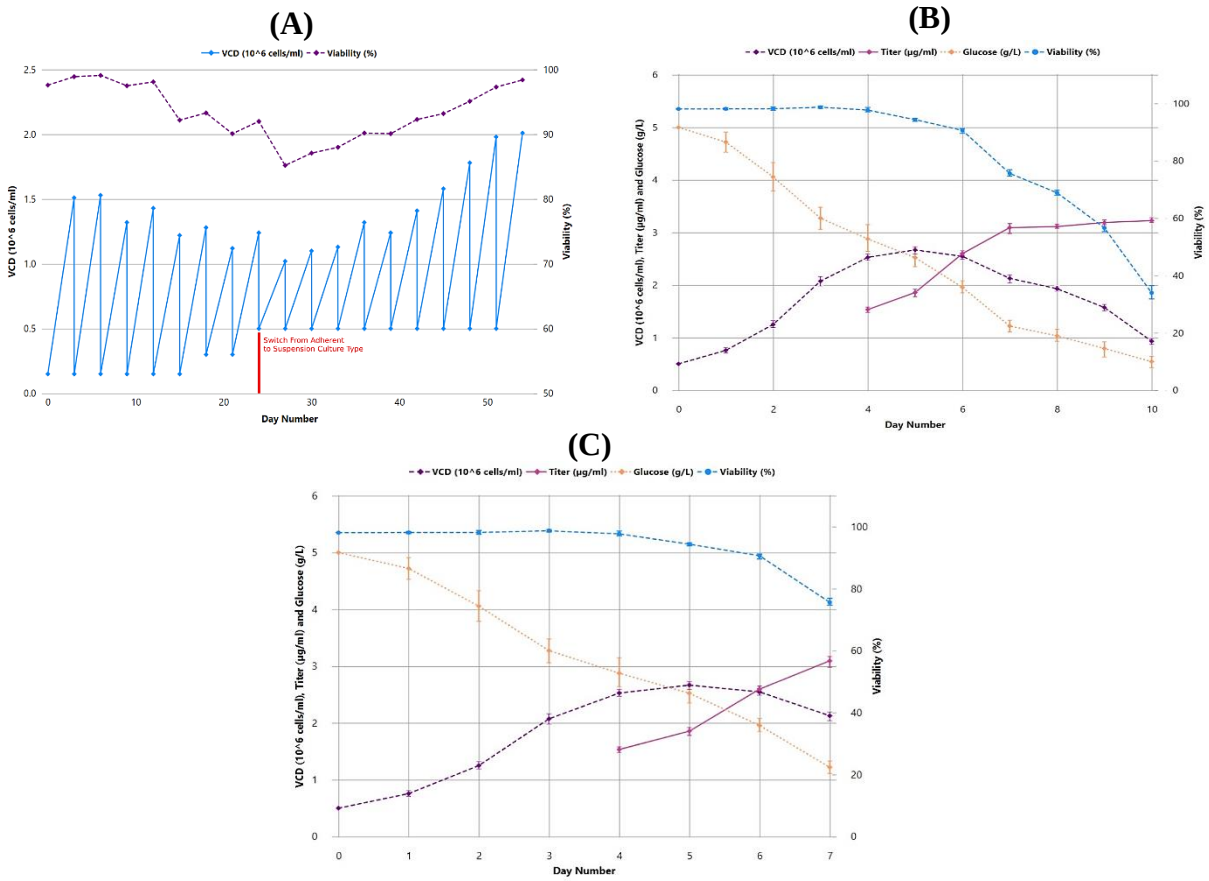

Fig. 1. Cell adaptation to serum-free medium. (A) Sequential adaptation of CHO-DG44 cells to ProCHO5 medium. Studied parameters in (B) a 10-day batch culture; (C) a 7-day batch culture. Error bars indicate the SD of three independent experiments.

adapted/suspended CHO-DG44 cells (Fig. 2A). These media were selected based on their specificity, efficiency, and availability for large-scale production of recombinant proteins. Following successful adaptation, seven-day batch cultures were run to compare the effect of these culture media on studied responses. On day seven, the Dynamis batch culture represented the highest VCD compared to the others (Fig. 2B). This medium also showed higher cell viability than the First CHOice (p<0.0001; Fig. 2C). Although the r-hFSH level in Dynamis medium was 39% and 30% higher than that in the First CHOice and ProCHO5 media, respectively (p<0.0001; Fig. 2D), it remarkably presented a lower Z-number compared to the other tested media (101 vs. 126 and 134, respectively; Fig. 2E). A significant decrease in glucose concentration was also observed from day five to six in seven-day First CHOice batch culture (Fig. 2F). Considering the importance of Z-number in potency of r-hFSH, as well as the cost-effectiveness and widespread availability of the First CHOice medium, an 11-day fed-batch culture was run. Supplementation of the First CHOice medium with 4 g/L of glucose on day five improved cell viability, and the Z-number of the produced r-hFSH, with no further progression after day nine (Fig. 2A-2F). Therefore, a nine-day fed-batch culture was carried out to examine the reproducibility of the results, which indicated no significant differences in the studied responses compared to the previously conducted 11-day batch culture (Fig. 2G). Therefore, next optimizations were conducted based on nine-day fed-batch First CHOice culture.

Screening of selected parameters

To optimize the culture condition, selected supplementary parameters and incubation temperature were targeted using the PBD through 12 experiments. The impact of galactose concentrations on the studied responses was compared to a control condition with 4 g/L glucose supplementation (Fig. S2). No statistically significant differences were observed regarding VCD and viability (P> 0.05). However, 10 mM of galactose increased the protein expression level and Z-number, making it a suitable choice for further analyses. According to Figures S3A and S3B, VCD and cell viability were significantly decreased at higher NaBu concentrations (p < 0.05). In contrast, r-hFSH levels increased 33, 45, 37, and 34% following the addition of 0.5, 1.5, 2.5, and 3.5 mM of NaBu, respectively (Fig. S3C). This outcome can be attributed to culture-specific productivity, as the 3.5 mM of NaBu exhibited the highest cell-specific productivity, up to 3.3 times that of the control culture (Fig. S3D). Figure 3 represents the positive and negative effects of the studied parameters on each response. Temperature shift and NaBu were the only significant parameters negatively influenced the cell viability. Galactose also exerted a statistically significant negative effect on protein levels, while NaBu and temperature shift contributed positively. Regarding the Z-number, NaBu indicated a significant positive effect, while galactose and temperature shift negatively influenced the glycan profile (Table 1). Temperature downshift on day five resulted in higher final VCD, cell viability, and r-hFSH level compared to cells maintained at 37 °C or those subjected to temperature shift on days six or seven (Fig. S4A-4C). It also indicated a gradual decrease in the Z-number from days 9 to 11, compared to the temperature shift on days 6 or 7 (Fig. S4D). Therefore, nine-day First CHOice fed-batch culture, with a temperature shift on day five, was selected for further experiments.

Optimization of culture condition by RSM

The effects of NaBu and galactose were further investigated by CCD (Table 2). The proposed models were expressed as empirical third-order polynomial equations 1 to 3, where A and B represent galactose and NaBu, respectively. The effects of the selected parameters are depicted by three-dimensional response surface and two-dimensional contour plots in Figure 4.

$$\text{Viability} = +65.26 - 6.51A + 6.65B + 3.49AB - 6.89A^2 - 7.12B^2 \quad \text{(Eq.1)}$$

$$\text{r-hFSH level } (\mu g/ml) = +6.81 - 1.22A + 1.28B + 1.06AB - 1.23A^2 - 1.27B^2 \quad \text{(Eq.2)}$$

$$\text{Z-number} = +197.95 - 20.56A + 22.21B + 16.67AB - 22.46A^2 - 22.63B^2 \quad \text{(Eq.3)}$$

The reproducibility of CCD results was confirmed by running a nine-day fed-batch First CHOice culture compared to the control culture. In this comparison, cell viability, r-hFSH level, and Z-number increased by 10, 96, and 49% compared to the control condition, respectively (Fig. 5).

Determination of the biological activity of r-hFSH

The calculated relative potency of r-hFSH produced in the control (adherent culture) and the optimized conditions were 92.2% and 103.3%, respectively, representing a 12% increase in potency of r-hFSH produced under optimized condition (Fig. 5F).

[Downloaded from ibj1.pasteur.ac.ir on 2026-10-16]
[DOR: 20.1001.1.1028852.2024.28.5.7.0]
[DOI: 10.61186/ibj.4160]

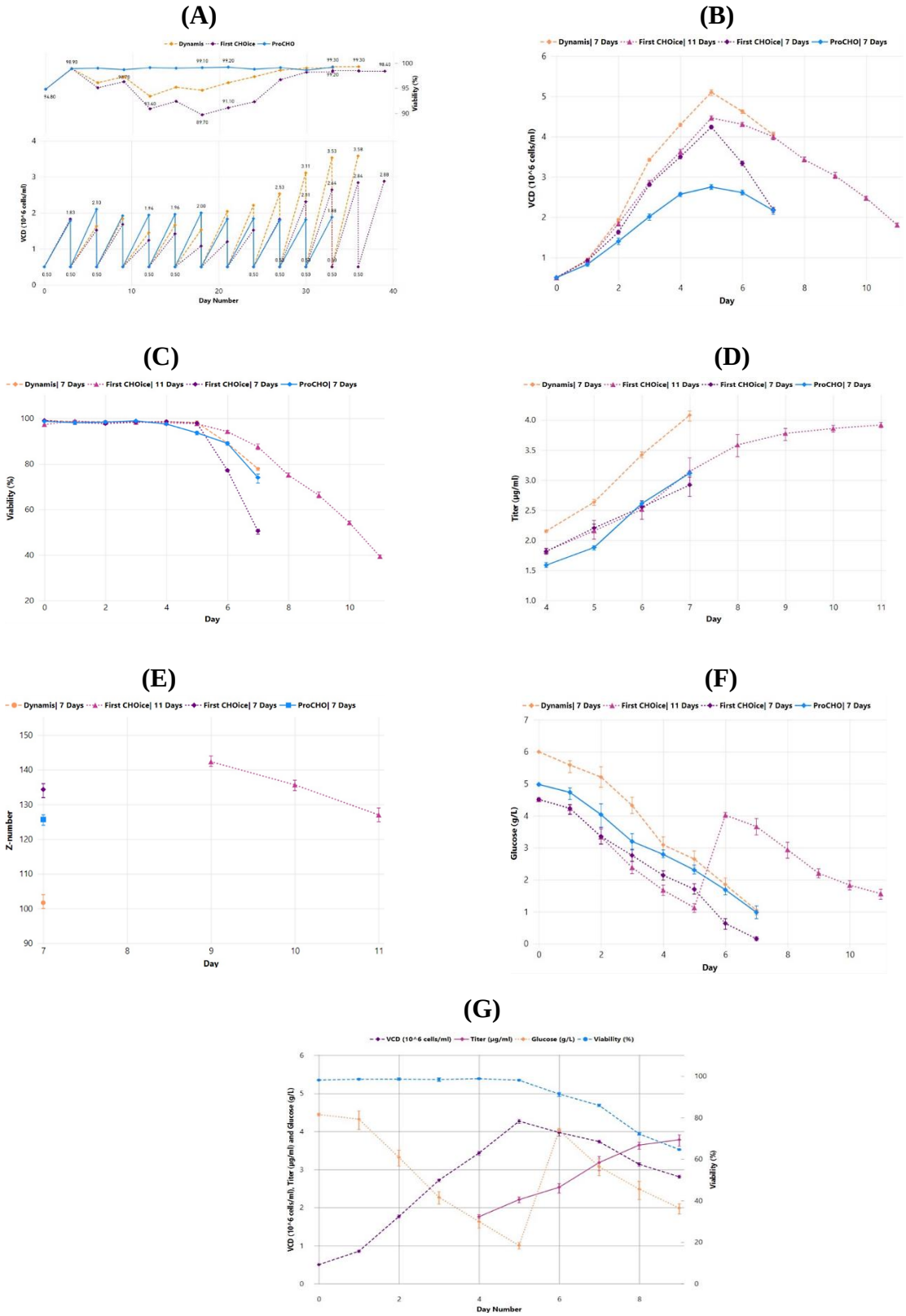

Fig. 2. Media selection of ProCHO5-adapted/suspended CHO-DG44 cells. (A) Studied cellular parameters within CD-SFM and ProCHO5; (B) VCD; (C) cell viability; (D) r-hFSH level; (E) Z-number; (F) glucose concentration during a seven-day batch culture of different CD-SFM (First CHOice, and Dynamis) under consistent conditions in comparison to an 11-day fed-batch First CHOice culture when ProCHO5 was set as control medium; (G) VCD, cell viability, glucose concentration, and r-hFSH level in a nine-day fed-batch First CHOice culture. Error bars indicate the SD of three independent experiments.

[Downloaded from ibj1.pasteur.ac.ir on 2026-10-16]
[DOR: 20.1001.1.1028852.2024.28.5.7.0]
[DOI: 10.61186/ibj.4160]

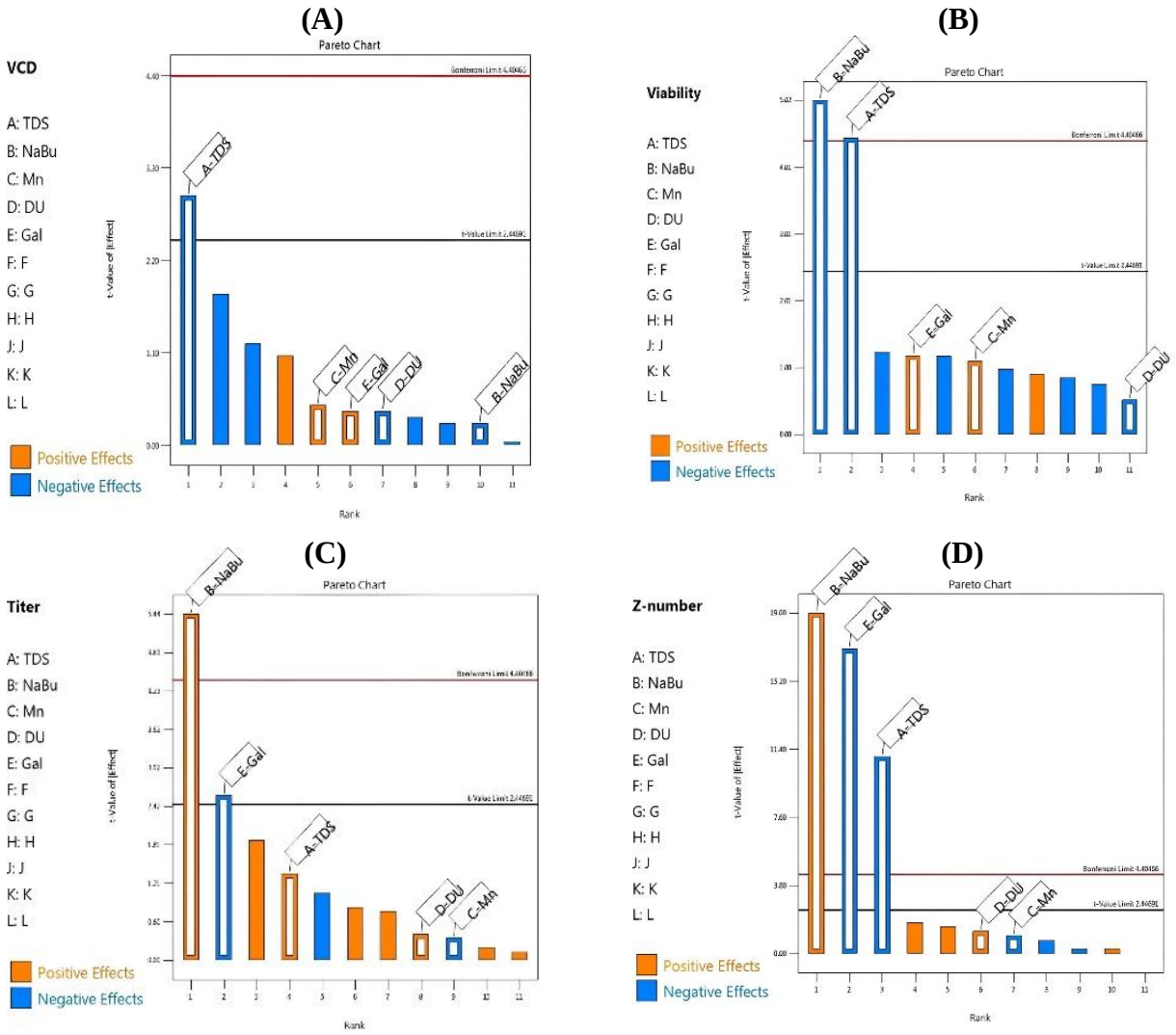

Fig. 3. Pareto charts for the PBD demonstrating the order and effect of each parameter on (A) VCD, (B) cell **viability**, (C) r-hFSH level, (D) Z-number. TDS: temperature downshift; NaBu: sodium butyrate; DU: deoxyuridine; Mn: manganese chloride; Gal: galactose. The remaining letters represent dummy variables in PB.

Bioreactor scale-up

Bioreactor characteristics such as temperature, along with initial and final cell densities, were previously determined within shake flask cultures. The studied responses confirmed the reproducibility of the obtained results and indicated approximately 4-5-fold increase in the r-hFSH production level compared to the original adherent cell culture (Fig. 5A-5F). Characterization studies represented remarkable consistency of the produced r-hFSH in the optimized and scale-up cultures (Table 3).

DISCUSSION

Advances in cell line development enable pharmaceutical companies to achieve higher titers of the protein drugs using appropriate genetic tools, including manipulated vectors and optimized integration/cloning strategies. Notably, these tools are not globally applicable solutions for all cell lines since each single cell requires specific optimizations based on the protein of interest, which may include clonal selection strategies and process optimization approaches, such as the culture medium and downstream steps. This study aimed to suspend and optimize the culture conditions of the previously developed adherent FSH-producing cell line to achieve higher productivity and sufficient quality of the drug substance. The quantity of this glycoprotein has consistently been reported to be significantly lower than that of monoclonal antibodies and other pharmaceutical proteins expressed in mammalian systems, which may be due to the complexity of this molecule. After adaptation to serum-free suspension culture, different media were compared for the expression of r-hFSH. To our knowledge, the optimization of physicochemical parameters affecting CHO-DG44 cellular growth, the

[Downloaded from ibj1.pasteur.ac.ir on 2026-10-16]
[DOR: 20.1001.1.1028852.2024.28.5.7.0]
[DOI: 10.61186/ibj.4160]

[Downloaded from ibj1.pasteur.ac.ir on 2026-10-16]

[DOR: 20.1001.1.1028852.2024.28.5.7.0]

[DOI: 10.61186/ibj.4160]

Table 1. Variance analysis of the effects of selected variables from PBD experiments on studies responses

Source	df	Viability (%)				VCD				r-hFSH level (µg/ml)				Z- number			
		Sum of square	**Mean square**	**F value**	***p* value**	**Sum of square**	**Mean square**	**F value**	***p* value**	**Sum of square**	**Mean square**	**F value**	***p* value**	**Sum of square**	**Mean square**	**F value**	***p* value**
Model	5	2071.88	414.38	9.56	**0.0080**	0.0585	0.0117	1.89	0.2306	3.55	0.7109	7.68	**0.0138**	4125.67	825.13	154.71	**<0.0001**
A, Temp shift	1	856.83	856.83	19.78	**0.0043**	0.0547	0.0547	8.82	**0.0249**	0.1704	0.1704	1.84	0.2237	645.33	645.33	121.00	**<0.0001**
B, NaBu	1	1090.61	1090.61	25.17	**0.0024**	0.0004	0.0004	0.0659	0.8060	2.74	2.74	29.55	**0.0016**	1925.33	1925.33	361.00	**<0.0001**
C, Mn2+	1	52.08	52.08	1.20	0.3150	0.0014	0.0014	0.2273	0.6504	0.0114	0.0114	0.1232	0.7376	5.33	5.33	1.00	0.3559
D, dU	1	11.60	11.60	0.2678	0.6233	0.0010	0.0010	0.1627	0.7007	0.0154	0.0154	0.1664	0.6975	8.33	8.33	1.56	0.2578
E, Gal	1	60.75	60.75	1.40	0.2811	0.0010	0.0010	0.1627	0.7007	0.6211	0.6211	6.71	0.0412	1541.33	1541.33	289.00	**<0.0001**
Residual error	6	259.97	43.33			0.0372	0.0062			0.5555	0.0926			32.00	5.33		
Cor. total	11	2331.85				0.11				4.11				4157.67			

Bolded values represent statistically significant associations.

quantity, and quality of r-hFSH protein has not been frequently reported. During the adaptation procedure, prolonger incubations in SFM effectively enhanced cell viability and VCD, similar to a recently published study using sequential serum-free cell adaption procedure[22]. A comparison of VCD and cell viability between 7- and 10-day batch cultures revealed that seven-day culture ensures reproducibility of the process, based on the measured VCD and high cell viability (>70%), which are critical in harvesting the culture medium to minimize the presence of host cell protein and DNA impurities[23]. In the media selection process, the lowest r-hFSH Z-number was obtained with the Dynamis medium compared to the other tested media, which made the First CHOice medium the most suitable SFM for the suspension culture of r-hFSH-producing cells.

Previous studies have reported that the synthesis of nucleotide sugars through feeding strategies could potentially increase protein glycosylation[24,25]. Herein, we observed a sharp glucose concentration reduction from day five in the seven-day First CHOice batch culture. Considering the metabolic similarity of glucose and galactose in extending cell culture duration, protein synthesis, sialic acid content, and sialylated glycans[26], supplementation of the medium with galactose was performed, in which a significantly increased Z-number was obtained. Although several studies have reported a significant positive role of dU and $MnCl_2$ in cell-feeding strategies for protein expression and galactosylation[18,20,27], PBD did not confirm these findings in the present study.

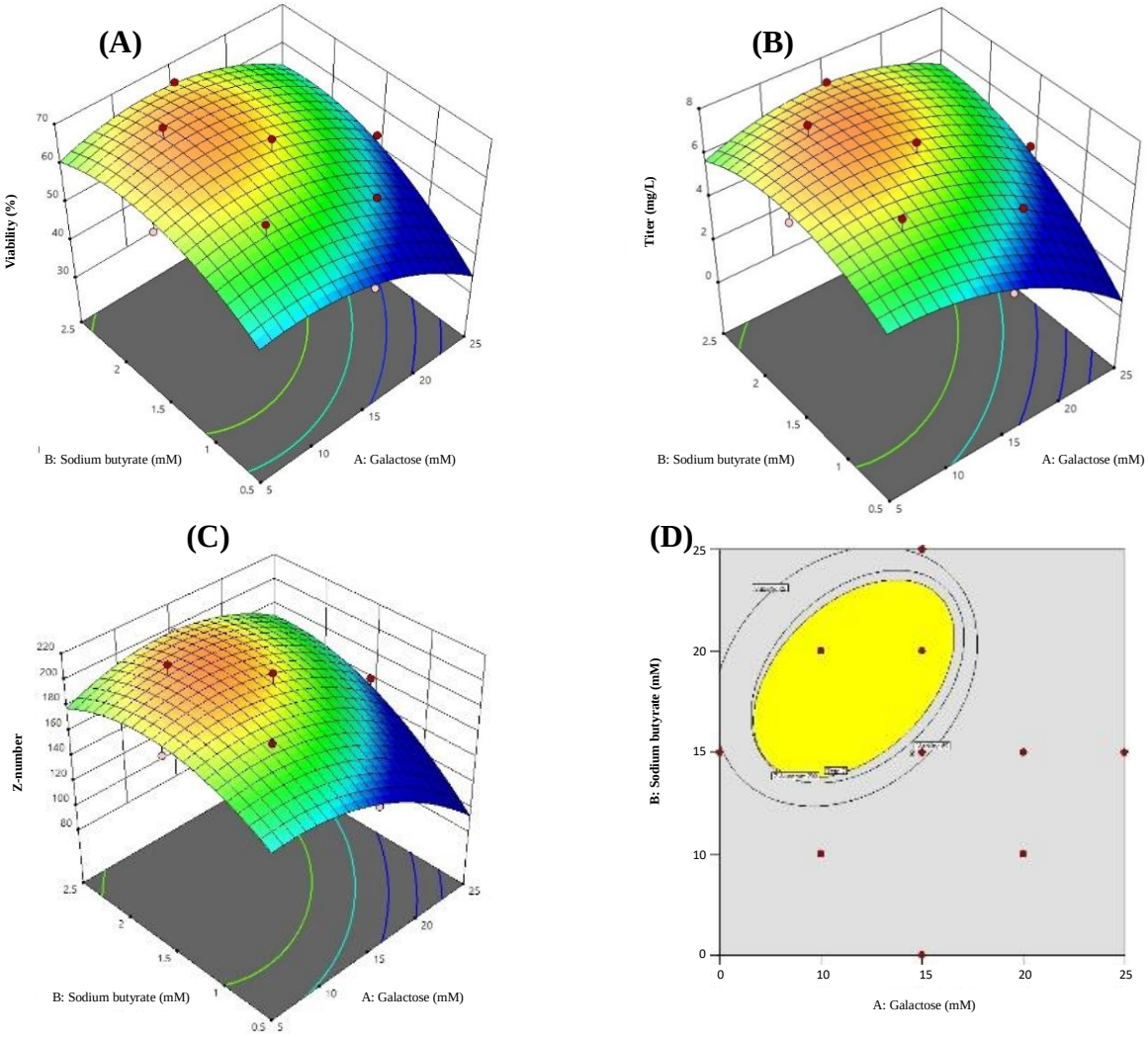

Fig. 4. Response surface three-dimensional plots showing the effects and interactions of galactose and NaBu on (A) Final cell viability, (B) r-hFSH level, and (C) Z-number. (D) The contour graph represents the effect of galactose and NaBu on all above-mentioned responses. Dots in the yellow zone indicate that increased NaBu and galactose concentrations to 2 and 10 mM may result in optimal levels of all responses.

[Downloaded from ibj1.pasteur.ac.ir on 2026-10-16]
[DOR: 20.1001.1.1028852.2024.28.5.7.0]
[DOI: 10.61186/ibj.4160]

[Downloaded from ibj1.pasteur.ac.ir on 2026-10-16]

[DOR: 20.1001.1.1028852.2024.28.5.7.0]

[DOI: 10.61186/ibj.4160]

Table 2. ANOVA analysis of the RSM (CCD) model for studied responses

Source	df	Viability				r-hFSH level				Z- number			
		Sum of square	Mean square	F value	*p* value	Sum of square	Mean square	F value	*p* value	Sum of square	Mean square	F value	*p* value
Model	5	361.66	72.33	7.58	**0.022**	12.76	2.55	9.52	**0.014**	3834.85	766.97	9.06	**0.015**
A, Gal	1	121.42	121.42	12.73	**0.016**	4.23	4.23	15.76	**0.010**	1210.15	1210.15	14.29	**0.013**
B, NaBu	1	126.51	126.51	13.26	**0.015**	4.67	4.67	17.40	**0.009**	1411.84	1411.84	16.68	**0.0095**
AB	1	2.08	2.08	0.22	0.66	0.19	0.19	0.72	0.435	47.35	47.35	0.56	0.49
A2	1	54.49	54.49	5.71	0.06	1.73	1.73	6.46	0.052	578.73	578.73	6.84	**0.047**
B2	1	58.16	58.16	6.10	0.06	1.84	1.84	6.87	**0.047**	587.43	587.43	6.94	**0.046**
Residual error	5	47.70	9.54			1.34	0.27			423.33	84.67		
Lack-of-fit	4	32.02	8.01	0.51	0.76	0.99	0.25	0.70	0.702	278.83	69.71	0.48	0.776
Pure error	1	15.68	15.68			0.35	0.35			144.50	144.50		
Cor. total	10	409.37				14.10				4258.18			

Bolded values represent statistically significant associations

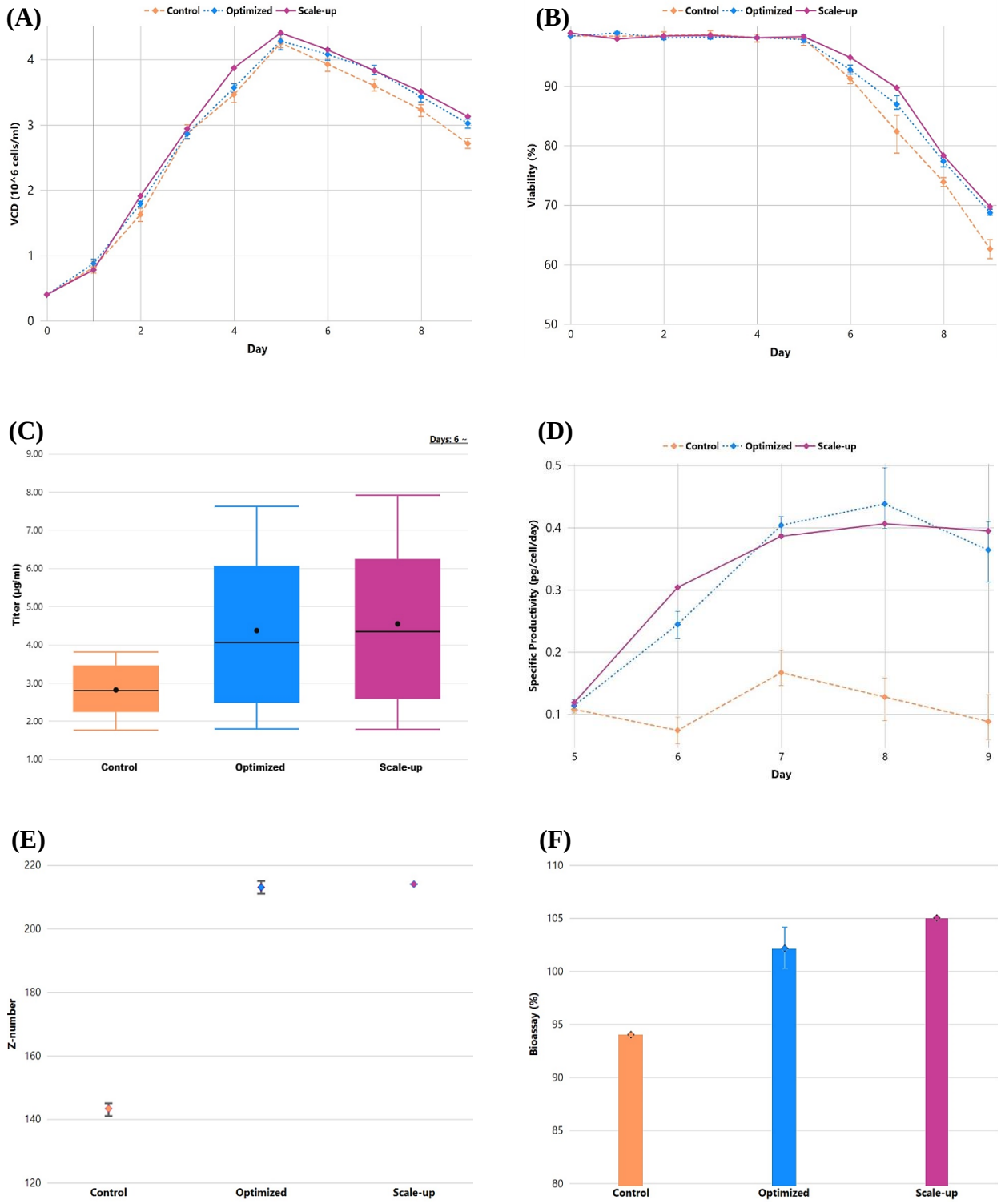

Fig. 5. Reproducibility of the optimized culture condition in 2 L shake flasks and 10 L bioreactor. (A) VCD, (B) cell viability, (C) r-hFSH level, (D) specific productivity from day 4 to 9, (E) Z-number, and (F) potency evaluation potency of r-hFSH produced under control (adherent culture) vs. optimized culture conditions. Control culture in all figures represents the production of r-hFSH from adherent cells. All experiments were run in triplicate except for the 10 L bioreactor. Bars represent the SD.

[Downloaded from ibj1.pasteur.ac.ir on 2026-10-16]
[DOR: 20.1001.1.1028852.2024.28.5.7.0]
[DOI: 10.61186/ibj.4160]

Table 3. Qualitative analysis of r-hFSH produced under optimized condition and its relevant scaled-up culture

Tests	Method	Acceptable range	Results	
			Optimized condition	Scaled-up culture
Concentration	SE-HPLC	0.40-0.80 mg/mL	0.66 ± 0.02	0.62
r-hFSH oligomers	SE-HPLC	Sum of the peaks with retention times less than of the main peak should not be more than 0.5%	0.0	0.0
Peptide mapping	RP-HPLC	Comparable with reference standard	Comparable	Comparable
Oxidization	RP-HPLC	NMT 6%	<1%	<1%
Free subunits	Non-reducing SDS-PAGE	NMT 3%	<3%	<3%
isoform distribution	Isoelectric focusing	Comparable with the reference standard	Comparable	Comparable

Optimized cultures were repeated three times.

Temperature shift is utilized extensively in manufacturing the mammalian-based therapeutic proteins to improve cell viability, protein productivity, and protein quality, which may differ based on the expression host cell and characteristics of the recombinant protein. McHugh et al. investigated the effect of different incubation temperatures and their shift day for simultaneous optimization of protein quality and productivity in CHO cells[28]. They demonstrated that temperature shift to 32 °C on day five significantly increased final mAb titer up to 25%, compared to 35 °C, even in the scaled-up bioreactor conditions. Our obtained results in both shake flask and bioreactor scales confirmed their findings. The other parameters examined in the optimization of culture conditions were NaBu supplementation and the introduction of mild hypothermic conditions on day five of the batch culture, which have frequently been used in optimization approaches of CHO cells[29]. Due to the cytotoxicity of NaBu[30], it is necessary to optimize its concentration, which is highly dependent on the cell line, target protein, and culture conditions, in order to achieve a balance between decreased cell viability and increased protein expression. Our findings indicated that while NaBu could increase the r-hFSH level, a dramatic reduction in VCD and viability happened at the same time. However, our CCD analysis revealed that NaBu and temperature shift significantly increased cell viability and VCD. Recent studies have reported that individual and combined effects of mild hypothermic shift (from 37 to 33-31°C) and NaBu supplementation (from 0.5 to 3 mM) on CHO cell culture had additive effects on increasing r-protein production in comparison to the control condition (37 °C, without NaBu supplementation), which is in agreement with our observations[30,31]. Studies have also demonstrated positive effects of 0.5-5 mM of NaBu administration on the expression of proteins such as r-htPA[30-32], mAbs[32,33], and erythropoietin[34,35] in CHO cells. The typical specific productivity of CHO cells in the expression of r-hFSH has been reported to be in the range of 0.5 to 1.5 pg/cell/day[36,37], and exceptionally high specific productivities achieved through cell engineering methods (up to 12.3 ± 1.7 pg/cell/day)[38]. In the present study, culture condition optimization yielded a final specific productivity of 0.43 ± 0.04 pg/cell/day. Although new strategies in cell engineering remain the most attractive and practical approaches for increasing protein yield, significant improvements can also be achieved through experimental optimization techniques utilizing previously developed cell lines.

Transitioning mammalian cell cultures from the laboratory to production scale is challenging. The established small-scale processes are typically scaled up to larger bioreactors to meet commercial demands and produce large quantities of the desired protein with consistent productivity and quality[39,40]. In this study, we performed a scale-up process from shake flasks to a 10 L bioreactor. It has been reported that IgG-expressing CHO cells exhibit different characteristics in shake flask and 5 L bioreactor cultures[41]. The bioreactors consistently demonstrated higher VCD and approximately three-fold higher productivity, while the viability profiles remained similar until day 11 when the shake flasks showed a decline in viability. This observation emphasizes optimizing process parameters for industrial scalability and reliability. The optimized process parameters can be extrapolated to bench-top

[Downloaded from ibj1.pasteur.ac.ir on 2026-10-16]
[DOR: 20.1001.1.1028852.2024.28.5.7.0]
[DOI: 10.61186/ibj.4160]

bioreactors, though there are known differences in biological responses between shake flasks and bioreactor cultures,. Conversely, refining shake flask parameters to small-scale bioreactor conditions facilitates comparative studies[39,41] as Ahleboot et al. provided additional support and revealed no significant difference in the critical parameters between 30 and 250 L bioreactors using DOE analysis through statistical optimization of selected variables[42]. This study also signified the direct effect of glycosylation pattern on biological activity of r-hFSH, where the recombinant protein produced under optimized condition represented an increase of Z-number (49%) yielding to approximately 12% increase in its potency compared to the protein produced under control conditions.

CONCLUSION

This study has outlined several key factors in serum-free suspension culture of r-hFSH-expressing CHO-DG44 cells, aimed to optimize the culture condition and develop scalable and cost-effective production process in compliance with the Pharmacopoeia requirements. First CHOice-adapted cells efficiently produced r-hFSH protein with high purity. Moreover, the adaptation strategy demonstrated an improved growth performance and r-hFSH protein production compared to the original adherent cell culture condition. The results confirmed the significant effects of the studied parameters (NaBu, galactose, and temperature shift) on cell responses, r-hFSH expression level and its Z-number, which could be beneficial for various industrial and research applications. Further optimizations should be conducted to achieve a desirable increase in r-hFSH productivity within the adapted CHO-DG44 cells.

DECLARATIONS

Acknowledgments

Authors gratefully acknowledge Manoucher Hosseini (DOE experiments), Zahra Azarniad (Analytical analysis), Pegah Molaei (HPLC experiments), and Narges Khazeh (ELISA assays) for their invaluable assistance. Special thanks are also extended to Dr. Haleh Hamedifar for financial sponsorship and Mahyar Abidi for Power BI analysis. Authors have not used artificial associated technologies in the production of the submitted work.

Ethical approval

Animal studies were approved by the Ethics Committee of Pasteur Institute of Iran (ethical code: IR.PII.AEC.1401.005) and performed according to the principles of the declaration of Helsinki.

Consent to participate

Not applicable.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

HGh: Conceived and designed the experiments, performed the experiments, analyzed the data, and wrote the paper; KhR: Conceived and designed the experiments and contributed reagents/materials/analysis tools; MKA: Conceived and designed the experiments and contributed reagents/materials/analysis tools; HO: Conceived and designed the experiments and contributed reagents/materials/analysis tools; RZK and MDB: Performed the experiments; YT: Conceived and designed the experiments, analyzed the data, and wrote the paper; FT: Conceived and designed the experiments and contributed reagents/materials/analysis tools.

Data availability

All relevant data can be found within the manuscript.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was funded by the CinnaGen Medical Biotechnology Research Center under Grant No. FNP0559017243.

Supplementary information

The online version contains supplementary material.

REFERENCES

1. Yanaka S, Yagi H, Yogo R, Onitsuka M, Kato K. Glutamine-free mammalian expression of recombinant glycoproteins with uniform isotope labeling: an application for NMR analysis of pharmaceutically relevant Fc glycoforms of human immunoglobulin G1. J Biomol NMR. 2022;76(1-2):17-22.
2. Arora P, Singh V, Kumar A. Mammalian Cell Culture: An Edge to Biopharmaceutical Industry. Biomanuf Sustainable Prod Biomol. 2023:297-313.
3. Zhu J. Mammalian cell protein expression for biopharmaceutical production. Biotechnol Adv. 2012;30(5):1158-70.
4. Glinšek K, Bozovičar K, Bratkovič T. CRISPR technologies in chinese hamster ovary cell line

[Downloaded from ibj1.pasteur.ac.ir on 2026-10-16]
[DOR: 20.1001.1.1028852.2024.28.5.7.0]
[DOI: 10.61186/ibj.4160]

engineering. Int J Mol Sci. 2023;24(9):8144.
5. Van Der Valk J, Bieback K, Buta C, Cochrane B, Dirks W, Fu J, et al. Fetal bovine serum (FBS): past–present–future. Altex. 2018;35(1):99-118.
6. Hesler M, Kohl Y, Wagner S, von Briesen H. Non-pooled human platelet lysate: A potential serum alternative for in vitro cell culture. Altern Lab Anim. 2019;47(3-4):116-27.
7. Kim YJ, Han SK, Yoon S, Kim CW. Rich production media as a platform for CHO cell line development. AMB Express. 2020;10(1):1-13.
8. Baker M. Reproducibility: Respect your cells! Nature. 2016;537(7620):433-5.
9. Chelladurai KS, Christyraj JDS, Rajagopalan K, Yesudhason BV, Venkatachalam S, Mohan M, et al. Alternative to FBS in animal cell culture-An overview and future perspective. Heliyon. 2021;7(8): e07686.
10. Beltran Paschoal JF, Patiño SS, Bernardino T, Rezende A, Lemos M, Pereira CA, Calil Jorge SA. Adaptation to serum-free culture of HEK 293T and Huh7.0 cells. BMC Proc. 2014;8:259.
11. Biaggio RT, Abreu-Neto MS, Covas DT, Swiech K. Serum-free suspension culturing of human cells: adaptation,growth,and cryopreservation. Bioprocess Biosyst Eng. 2015;38:1495-507.
12. Shridhar S, Klanert G, Auer N, Hernandez-Lopez I, Kańduła MM, Hackl M, et al. Transcriptomic changes in CHO cells after adaptation to suspension growth in protein-free medium analysed by a species-specific microarray. J Biotechnol. 2017;257:13-21.
13. Rodrigues ME, Costa AR, Henriques M, Cunnah P, Melton DW, Azeredo J, Oliveira R. Advances and drawbacks of the adaptation to serum-free culture of CHO-K1 cells for monoclonal antibody production. Appl Biochem Biotechnol. 2013;169(4):1279-91.
14. Jukić S, Bubenik D, Pavlović N, Tušek AJ, Srček VG. Adaptation of CHO cells in serum-free conditions for erythropoietin production: Application of EVOP technique for process optimization. Biotechnol Appl Biochem. 2016;63(5):633-41.
15. Rourou S, Ben Zakkour M, Kallel H. Adaptation of Vero cells to suspension growth for rabies virus production in different serum free media. Vaccine. 2019;37(47):6987-95.
16. Wang P, Huang S, Hao Ch, Wang Zh, Zhao H, Liu M, et al. Establishment of a suspension MDBK cell line in serum-free medium for production of bovine Alphaherpesvirus-1. Vaccine. 2021;9(9):1006.
17. Li F, Vijayasankaran N, Shen A, Kiss R, Amanullah A. Cell culture processes for monoclonal antibody production. MAbs. 2010;(5):466-77.
18. Torres M, Zuniga R, Gutierrez M, Vergara M, Collazo N, Reyes J, et al. Mild hypothermia upregulates myc and xbp1s expression and improves anti-TNFalpha production in CHO cells. Plos one. 2018;13(3):e0194510.
19. Torkashvand F, Vaziri B, Maleknia S, Heydari A, Vossoughi M, Davami F, Mahboudi F. Designed Amino Acid Feed in Improvement of Production and Quality Targets of a Therapeutic Monoclonal Antibody. Plos one. 2015;10(10):e0140597.
20. Torres M, Dickson AJ. Combined gene and environmental engineering offers a synergetic strategy to enhance r-protein production in Chinese hamster ovary cells. Biotechnol Bioeng. 2022;119(2):550-65.
21. Gyorgypal A, Fratz-Berilla E, Kohnhorst C, Powers DN, Chundawat SPS. Temporal effects of galactose and manganese supplementation on monoclonal antibody N-linked glycosylation infed-batch and perfusion bioreactor operation. Biorxiv. 2023; Doi: 10.1101/2023.04.15.535602.
22. Lomba ALO, Tirapelle MC, Biaggio RT, Abreu-Neto MS, Covas DT, Picanço-Castro V, et al. Serum-free suspension adaptation of HEK-293T cells: Basis for large-scale biopharmaceutical production. Braz Arch Biol Technol. 2021; DOI:10.1590/1678-4324-2021200817.
23. Li Y. Effective strategies for host cell protein clearance in downstream processing of monoclonal antibodies and Fc-fusion proteins. Protein Expr Purif. 2017;134:96-103.
24. Sha S, Yoon S. An investigation of nucleotide sugar dynamics under the galactose supplementation in CHO cell culture. Process Biochem. 2019;81:165-74.
25. Cha HM, Lim JH, Lee KS, Kim DI. Nucleotide sugar precursor feeding strategy to enhance sialylation of albumin-erythropoietin in CHO cell cultures. Process Biochem. 2018;66:197-204.
26. Liu J, Wang J, Fan L, Chen X, Hu D, Deng X, et al. Galactose supplementation enhance sialylation of recombinant Fc-fusion protein in CHO cell: an insight into the role of galactosylation in sialylation. World J Microbiol Biotechnol. 2015;31(7):1147-56.
27. Ehret J, Zimmermann M, Eichhorn T, Zimmer A. Impact of cell culture media additives on IgG glycosylation produced in Chinese hamster ovary cells. Biotechnol Bioeng. 2019;116(4):816-30.
28. McHugh KP, Xu J, Aron KL, Borys MC, Li ZJ. Effective temperature shift strategy development and scale confirmation for simultaneous optimization of protein productivity and quality in Chinese hamster ovary cells. Biotechnol Prog. 2020;36(3):e2959.
29. Toronjo-Urquiza L, Acosta-Martin AE, James DC, Nagy T, Falconer RJ. Resveratrol addition to Chinese hamster ovary cell culture media: The effect on cell growth, monoclonal antibody synthesis, and its chemical modification. Biotechnol Prog. 2020;36(3):e2940.
30. Avello V, Torres M, Vergara M, Berrios J, Valdez-Cruz NA, Acevedo C, et al. Enhanced recombinant protein production in CHO cell continuous cultures under growth-inhibiting conditions is associated with an arrested cell cycle in G1/G0 phase. Plos one. 2022;17(11):e0277620.
31. Avello V, Tapia B, Vergara M, Acevedo C, Berrios J, Reyes JG, Altamirano C. Impact of sodium butyrate and mild hypothermia on metabolic and physiological behaviour of CHO TF 70R cells. Electron J Biotechnol. 2017;27:55-62.
32. Chen F, Kou T, Fan L, Zhou Y, Ye Zh, Zhao L, Tan W-S. The combined effect of sodium butyrate and low culture temperature on the production, sialylation, and

[Downloaded from ibj1.pasteur.ac.ir on 2026-10-16]
[DOR: 20.1001.1.1028852.2024.28.5.7.0]
[DOI: 10.61186/ibj.4160]

biological activity of an antibody produced in CHO cells. Biotechnol Bioprocess Eng. 2011;16:1157-65.
33. Jiang Zh, Sharfstein ST. Sodium butyrate stimulates monoclonal antibody over-expression in CHO cells by improving gene accessibility. Biotechnol Bioeng. 2008;100(1):189-94.
34. Liu Y, Zhou X, Song Zh, Zhang Y. Sodium butyrate enhances the acidic isoform content of recombinant human erythropoietin produced by Chinese hamster ovary cells. Biotechnol Lett. 2014;36(5):907-11.
35. Ghafuri-Esfahani A, Shokri R, Sharifi A, Shafiee L, Khosravi R, Kaghazian H, Khalili M. Optimization of parameters affecting on CHO cell culture producing recombinant erythropoietin. Prep Biochem Biotechnol. 2020;50(8):834-41.
36. Arnold S, Jelinek N. FSH producing cell clone. Google Patents; 2012. Available from: https://patents.google.com/patent/AU2008267138B9/nl
37. Kim D-J, Seok S-H, Baek M-W, Lee H-Y, Juhn J-H, Lee S, et al. Highly expressed recombinant human follicle-stimulating hormone from Chinese hamster ovary cells grown in serum-free medium and its effect on induction of folliculogenesis and ovulation. Fertil Steril. 2010;93(8):2652-60.
38. Orlova NA, Kovnir SV, Khodak YA, Polzikov MA, Nikitina VA, Skryabin KG, Vorobiev II. High-level expression of biologically active human follicle stimulating hormone in the Chinese hamster ovary cell line by a pair of tricistronic and monocistronic vectors. Plos one. 2019;14(7):e0219434.
39. Xing Z, Kenty BM, Li ZJ, Lee SS. Scale-up analysis for a CHO cell culture process in large-scale bioreactors. Biotechnol Bioeng. 2009;103(4):733-46.
40. Goey CH, Bell Dj, Kontoravdi C. CHO cell cultures in shake flasks and bioreactors present different host cell protein profiles in the supernatant. Biology Eng J. 2019;144:185-92.
41. Qian Y, Xing Z, Lee Sh, Mackin NA, He A, Kayne PS, et al. Hypoxia influences protein transport and epigenetic repression of CHO cell cultures in shake flasks. Biotechnol J. 2014;9(11):1413-24.
42. Ahleboot Z, Khorshidtalab M, Motahari P, Mahboudi R, Arjmand R, Mokarizadeh A, Maleknia Sh. Designing a Strategy for pH Control to Improve CHO Cell Productivity in Bioreactor. Avicenna J Med Biotechnol. 2021;13(3):123-30.

[Downloaded from ibj1.pasteur.ac.ir on 2026-10-16]
[DOR: 20.1001.1.1028852.2024.28.5.7.0]
[DOI: 10.61186/ibj.4160]