Acıbadem Univ. Sağlık Bilim. Derg. 2022; 13 (1): 6-15
https://doi.org/10.31067/acusaglik.934263

ORIGINAL ARTICLE / ARAŞTIRMA YAZISI

Biotechnology / Biyoteknoloji

Evaluation of Feed Strategy for High Quality Biosimilar IgG Production in CHO Cell Fed-batch Process

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Received : 7 May 2021
Accepted : 11 November 2021

ABSTRACT

Purpose: Chinese Hamster Ovary (CHO) cells are currently the leading hosts for biosimilar Immunoglobulin G (IgG) production in the biopharmaceutical industry. Most eukaryotic proteins are glycosylated, and charge variants affect both the in vivo and in vitro properties of monoclonal antibodies (mAb). Adjusting the N-glycosylation patterns and charge variants while achieving high antibody titer is a production challenge. In this study, the effects of feed type and strategy on cell growth, product titer, glycosylation and charge variation were investigated using different CHO clones producing different IgG mAbs.

Methods: Cultivated CHO cells were supplemented with different feeding schemes, under fed-batch productions of 14 days. Screenings were conducted in spin-tubes and further investigated in 3L bioreactor systems.

Results: Change in feed strategy decreased productivities by 10.4% ($P < 0.05$), while it increased non-fucosylated glycoforms by 33.3% and enhanced galactosylation up to 3-folds. Basic variants were observed to increase 2.5 folds.

Conclusion: These remarkable alterations are of great importance in terms of mAb quality, in a manufacturing point of view, as they provide modulation of efficacy and safety. This reveals that feed strategy is a major driving force that significantly impacts culture longevity, galactosylated glycoforms, high-mannose glycan contents and charge variants.

Keywords: CHO, Biosimilar, Monoclonal Antibody, Feed Strategy, Process Development

CHO Hücrelerinin Kesikli Beslemeli Prosesinde Besleme Stratejisinin Yüksek Kalitede Biyobenzer IgG Üretimi Üzerine Etkisinin İncelenmesi

ÖZET

Amaç: Çin Hemstır Yumurtalık (CHO) hücreleri, günümüzde biyofarmasötik endüstrisinde biyobenzer İmmünoglobulin G (IgG) üretimi için tercih edilmektedir. Ökaryotlar tarafından sentezlenen proteinlerin çoğu glikozillenmiş halde olup yük varyantları, monoklonal antikorların (mAb) in vivo ve in vitro özelliklerini etkilemektedir. Yüksek antikor titresini sağlayarak N-glikozil profilini ve yük varyantlarını ayarlamak, üretimde zorluk teşkil etmektedir. Bu çalışmada, farklı IgG mAb üreten farklı CHO klonları kullanılarak, besleme türünün ve stratejisinin hücre büyümesi, ürün titresi, glikozillenme ve yük varyantları üzerindeki etkileri araştırılmıştır.

Yöntem: Kültür edilen CHO hücreleri, 14 günlük kesikli-beslemeli üretimleri boyunca farklı besleme düzenleriyle takviye edilmiştir. Taramalar spin-tüplerde yapılıp ileri analizler 3L biyoreaktör sistemlerinde yürütülmüştür.

Bulgular: Besleme stratejisindeki değişim, fukozillenmemiş glikoyapıları 33.3% oranında, galaktozillenmeyi ise 3 kata kadar arttırırken, üretim verimliliğini 10.4% ($P<0.05$) düşürmüştür. Bazik varyantlar 2.5 kat artmıştır.

Sonuç: Bu değişimler, etkinlik ve güvenlik düzenlemelerini sağladıklarından, üretimde mAb'ların kalitesi açısından büyük önem taşımaktadır. Bu bulgular, besleme stratejisinin kültür ömrünü, galaktozillenmeyi, mannoz yapılarını ve yük varyantlarını önemli ölçüde etkilediğini göstermektedir.

Anahtar Kelimeler: CHO, biyobenzer, monoklonal antikor, besleme stratejisi, proses geliştirme

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In recent years, monoclonal antibodies (mAbs) have shown to be promising therapeutics against autoimmune diseases, cancer, inflammation, and infectious diseases (1). Quality compliance and related approval of mAbs demand controlled glycosylation to meet the required characteristics, thus biosimilarity. The conformational changes that cause altered glycan compositions, in result, impact their binding affinity to the Fcγ receptors, which affect mAb immunogenicity, pharmacokinetics (PK), stability and safety (2). Glycoprotein processing happens from the endoplasmic reticulum (ER) towards the Golgi (3). As passing through the ER, the reducing ends of oligosaccharides with high mannose are cut by certain enzymes and the process continues in the Golgi (4). Culture conditions are reported to impact the localization and the activity of the proteins involved in glycosylation, and gene expressions of the Golgi resident proteins involved are variable during culture (4,5). Any nutritional limitation may have the potential to reduce nucleotide sugar concentrations within the Golgi, resulting in poor glycosylation (6).

Charge variants are also affected by protein structural modifications, culture media components and co-factor supplementations. They may alter mAb tissue penetration and distribution, binding properties and PK (7).

Since biosimilar development requires the comparability to the reference product, the major quality attributes should be investigated in detail.

Chinese hamster ovary (CHO) cells are known for producing highly similar structures of glycans to those naturally synthesized by humans, while showing reproducible growth that contributes to high mAb production, reducing biomanufacturing costs (8). As with all the expression systems, CHO presents possible drawbacks regarding stability to maintain product quality and uniformity that causes phenotypic drift (9,10). The current industry is rapidly investing on chemically defined media and feed developments that support clonal stability, aiming to boost titers while meeting the quality demands (11).

Clone and product specific media-feed screening requires an in-depth cell culture study, that would mimic the conditions of a production process. Fed-batch culture is a widely used strategy to screen for the optimum supplements regarding clone and product of interest.

Here, we emphasize the criticality of the feed choice and regimen, as the major driving forces compromising cell growth and longevity, production, glycosylation and mAb charge distribution. We performed a comparative study using different CHO clones cultured under varying feed regimens and concentrations. Two different CHO clones were used to investigate the clonal dependence of alterations in quality attributes related to feed strategy, such as titer, glycosylation and charge variance. Three chemically defined feeds, developed for CHO fed-batch cultures were investigated under two different feeding regimens, as Feed A – Feed B combination or solely Feed Y. Feed A is a supplement containing various nutrients with a glucose concentration of 80 g / L, designed to be used in combination with an amino acid supplement Feed B, to boost mAb production. Feed Y is a single part high nutritional supplement that contains 20 g / L glucose. Feed analysis under different Feed Y concentrations ensures that the outcomes are only related to the feed and supplementation schedule, minimizing the experimental errors related to nutrient shortage. We conducted a detailed screening study to analyze the effects of the stated parameters on cell growth, viability, lactate generation, culture pH, osmolality, mAb production and scrutinized the impact of feed selection on quality attributes, by conducting fed-batch bioreactor productions with the optimum supplementation. Titers were analyzed using HLPC, glycosylation and charge variations as key quality attributes were investigated with mass spectrometry and iCEF, respectively. The study clearly reveals the product related clonal dependency of feed supplementation.

MATERIALS AND METHODS

Cell lines

Two stable CHO-M clones, producing either an IgG1 (CHO-02 clone) or an IgG2-IgG4 (CHO-03 clone) hybrid mAb were sub-cultured in chemically defined media (BalanCD CHO Growth A, Irvine Scientific) supplemented with 6 mM L-glutamine (Gibco®, Life Technologies). Cells were cultivated in vent-cap shake flasks (Corning®, Sigma-Aldrich) under 37 °C, 5% CO_2, 80 - 85% RH, 150 rpm shaking conditions, in CO_2 -controlled incubator (Thermo Scientific).

Fed-batch culture

Cultured CHO cells were cultivated in vented spin tubes (Tubespin Bioreactor 50, TPP) with 15 mL working volume at 3 (± 0.5) × 10^5 VC / mL inoculation density for fed-batch experiments of 14 days, at 37 °C, 5% CO_2, 80 - 85% RH, 300 rpm shaking conditions. Cells were fed with FeedA (HyClone Cell Boost 7a, Cytiva), FeedB (HyClone Cell Boost 7b, Cytiva) as control condition (C-1), and FeedY (BalanCD CHO Feed4, Irvine Scientific) for six experimental conditions (C-2 to C-7), as carbon and nitrogen sources (Table.1).

Table.1 Experimental design for fed-batch feed strategy screening of CHO-03 and CHO-02 clones in 50 mL vented spin tubes. All conditions were replicated (n=2)

FS-1 (Standard Feed Strategy)	PD3, PD6, PD7, PD8, PD10 and PD12 (6 days)
FS-2 (Test Feed Strategy)	PD3, PD5, PD7, PD9, and PD11 (5 days)
Conditions (% v/v) for CHO-03 clone	
C-1 (Control)	FeedA (30%) + FeedB (2.5%) with FS-1 Strategy
C-2	FeedY (30%) - with FS-1 Strategy
C-3	FeedY (25%) - with FS-1 Strategy
C-4	FeedY (20%) - with FS-1 Strategy
C-5	FeedY (30%) - with FS-2 Strategy
C-6	FeedY (25%) - with FS-2 Strategy
C-7	FeedY (20%) – with FS-2 Strategy
Conditions (% v/v) for CHO-02 clone	
C-1 (Control)	FeedA (30%) + FeedB (2.5%) - with FS-1 Strategy
C-5	FeedY (30%) - with FS-2 Strategy

Bioreactor production

CHO-02 Fed-batch productions were conducted in 3L Single-Use Bioreactors (Mobius® Cell Ready), with a starting cell density of 3 (± 0.5) × 10^5 VC / mL , under FS-1 strategy either using FeedA (30% v/v) – FeedB (2.5% v/v) combination (BR-AB Bioreactor, Control) or using FeedY (30% v/v) (BR-Y Bioreactor). Productions were conducted at moderate pH of 6.9, 60% dissolved oxygen conditions. Temperature was shifted to 35 °C on PD5 and glucose concentrations were targeted to be 5 g / L starting from PD6. The control reactors represent our standard production.

Analyses

Samples were centrifuged at 200 × g, + 4 °C (Sigma 1-16K). Titers were analyzed using PA-HPLC (Waters Arc-Bio). Viability and viable cell density (VCD) were analyzed by Vi-CELL XR (Beckman Coulter). Metabolite and pH measurements were conducted using ABL90 FLEX (Radiometer). Osmolality measurements were done by Micro Osmometer 3320 (Advanced Instruments). Glycosylation and charge variant analyses were performed on supernatant samples after purification with Amicon® Pro Purification System (Merck).

Glycosylation

Rapi-Flour-MSTM (RFMS) kit (Waters, Milford) was used for N-Glycan analysis regarding the manufacturer's instructions. Released N-glycans were injected to Acquity H-class Bio UPLC with FLR detector coupled to XEVO G2-XS QToF-MS. BEH Amide column (2.1 × 150 mm, 1.7 mm particle size, 130 Å pore size) (Waters, Milford) was used for HILIC separation. N-glycans were identified and quantified by utilizing calibration curve derived from RFMS labeled dextran ladder. Mass confirmation was achieved with Q-ToF MS data, via UNIFI software v1.8 (Waters, Milford).

Charge variants

The iCE3 detector with PrinCE autosampler (Protein Simple, San Jose, CA) and flouro-carbon coated (FC-coated) capillary (100 µm ID X 50 mm, Protein Simple) was used for imaged capillary isoelectric focusing analysis (icIEF). Methyl cellulose (MC) solutions, pI markers, anolyte and catholyte solutions were obtained from ProteinSimple. Ampholite mixture consisting of Servalyte 7-9 (Serva) and Pharmalyte 3-10 (GE Healthcare Life Sciences) at 3 : 1 ratio respectively, was used for sample preparation. IEF was performed with 0.08 M phosphoric acid in 0.1% MC as anolyte and 0.1 M sodium hydroxide in 0.1% MC as catholyte. Electropherograms were captured at 280 nm with iCE 21 CFR Software (Protein Simple). Data were analyzed with Empower 3 Software (Waters, Milford).

Statistical analysis

Significance of the CHO-03 screening results were evaluated using either one-way (titer) or two-way variance (VCD and viability) analysis (one-way ANOVA, two-way ANOVA), with ± 95% confidence interval. CHO-02 bioreactor production results were evaluated using t-test ($P < 0.05$). GraphPad Prism 9.0.0 software was used for all the statistical analyses.

Results and discussion

Feed strategy, cell growth and metabolism

The effect of feed type on the production clone is highly dependent on the feeding strategy. Supplementation of 30% feed using FeedA+B versus FeedY, either with FS-1 or FS-2 strategies significantly impacted clonal performance in terms of VCD, viability and titer ($P < 0.05$). C-1 (control) cultures of CHO-03 clone reached a peak VCD of 42.91 × 10^6 cells / mL, while it remained at 38.60 × 10^6 cells / mL and 34.76 × 10^6 cells / mL for C-2 and C-5 cultures, respectively (Fig.1a). C-2 cultures could not survive, with culture termination on PD13 (48.9% viability) (Fig.1b). Actually, none of the cultures supplemented with FeedY under FS-1 strategy managed to survive and were all terminated on PD13. Cultures supplied with FeedY only survived under FS-2 strategy.

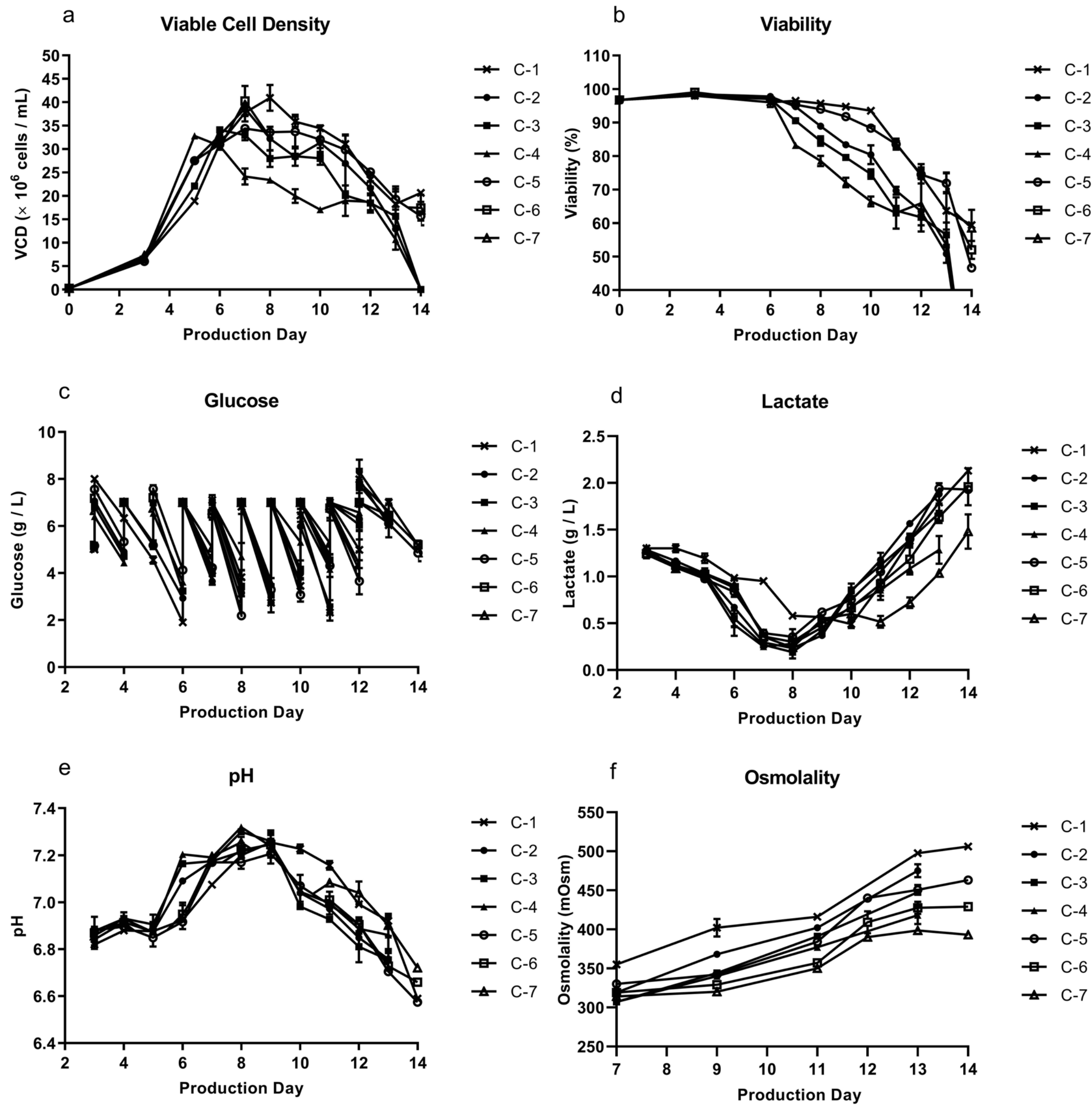


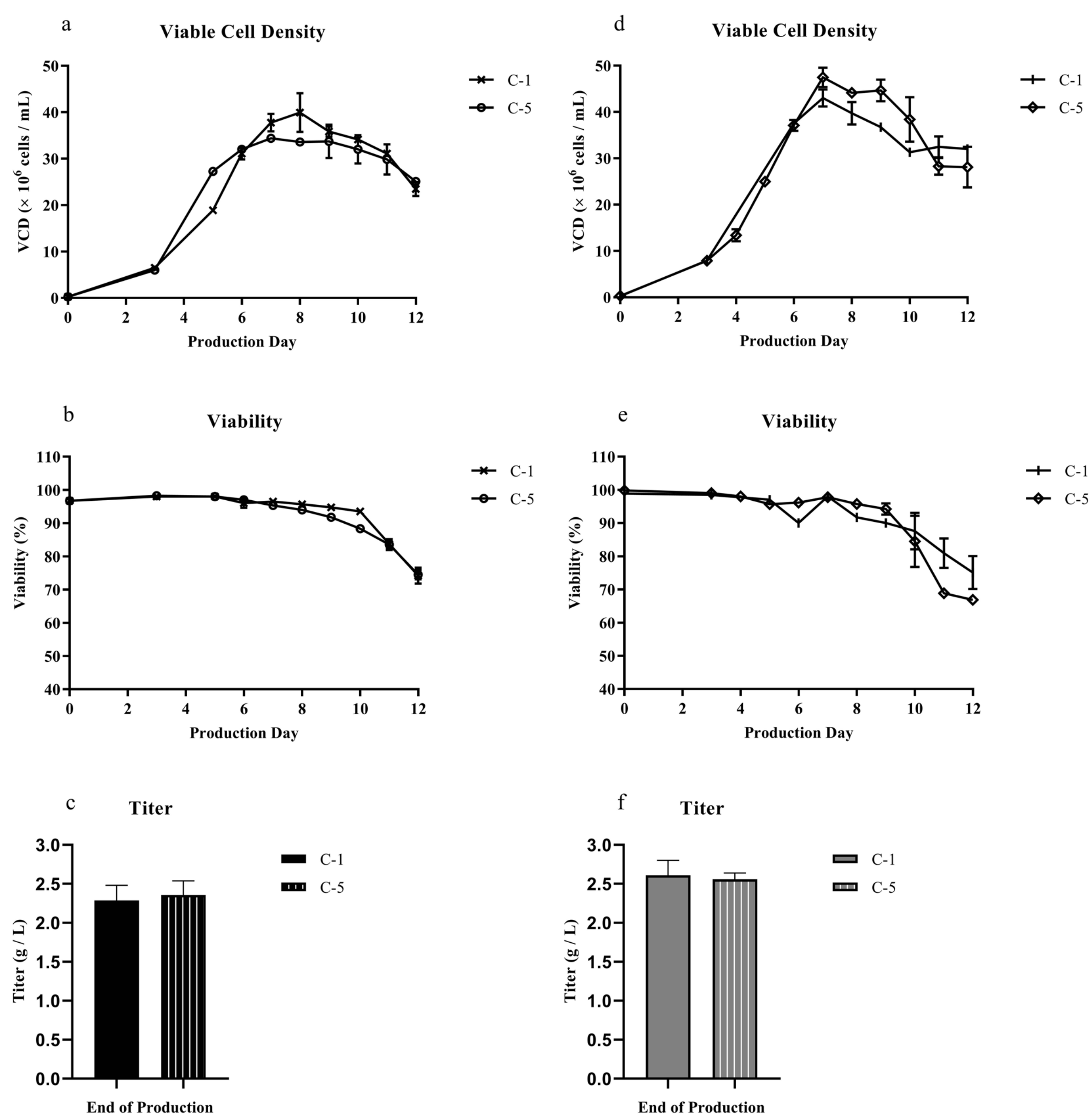
Fig.1 Influence of the seven different feed strategies on CHO-03 cultures monitored during 14 days of fed-batch productions (Fig. 1a – Fig. 1f). The results are presented according to their respective production day measurements. All results are expressed as the mean value ± standard deviation (SD) of two independent samples and error bars are based on ± 5% analytical accuracy of measurement

A study conducted by Rekena et al. in 2019 shows that viable cell densities and viabilities are dependent on feed compositions, regardless of glucose concentrations (12). Different outcomes were observed between FS-1 and FS-2 strategies, which can indicate that feeding regimen is also critical for the proper delivery of nutrients, at required stages of culture growth, maintenance and production (Fig.1c).

CHO-02 spin tube productions had similar VCD, viability and titer with CHO-03 from C-1 to C-5 (data not shown). The best clonal performances were observed with C-1 and C-5, represented in Supplementary Information. The production results showed comparable performance in terms of cell growth and viability, under both feed strategies (Fig.3a, 3b), suggesting similar cellular responses to Feed A (30%) + Feed B (2.5 %) under FS-1 strategy, and to Feed Y (30%) under FS-2 strategy. During the bioreactor

productions, peak VCD were observed on PD9 for both conditions, but BR-AB cells reached higher VCD of 39.18 × 10^6 cells / mL, compared to cells in BR-Y with 33.56 × 10^6 cells / mL. On the other hand, higher end-of-culture viability was observed with the BR-Y (79.9%), compared to the control bioreactor (72.4%).

Lactate productions in CHO-03 spin tube cultures (Fig.1d) were similar and remained below 2.2 g / L among different conditions, with C-4 and C-7 resulting with the lowest concentrations of 1.29 g / L and 1.48 g / L, respectively. This makes sense, since these were the conditions with lowest feed concentrations of 20% v/v. The highest generation of lactate was observed as 2.13 g / L under C-1 condition, resulting with the highest osmolality level of 506 mOsm (Fig.1f).



Supplementary Information Comparative VCD, viability and mAb production results of CHO-03 (left, a – c) and CHO-02 (right,d - f) clones under control condition (30% v/v FeedA + FeedB supplementation with FS-1) and test condition (30% v/v FeedY supplementation with FS-2) monitored during 12 days of fed-batch productions in spin tubes. The results a - f are expressed as the mean value ± standard deviation (SD) of two independent samples and error bars are based on ± 5% analytical accuracy of measurement

FeedY was very effective in lowering lactate generation in bioreactor productions with the CHO-02 clone (Fig.3d). Lactate concentrations were significantly lower with FeedY feeding under FS-2 strategy throughout the process and although they were low and harmless under both conditions, BR-Y production had 66.3% lower final lactate concentration of 0.35 g / L, while the control production was finalized with 1.04 g / L ($P < 0.05$). The osmolality levels observed in the bioreactors, with the set-point pH values successfully maintained, also demonstrates the differences in lactate generations (Fig.3c, 3e).

Lactate accumulation is known to lower culture viability and protein production (13). However, in this study, the viability drops and culture terminations were not related to lactate accumulation, but to the interrelation of the feed type and feeding regimen, as the lactate concentrations did not reach the level to trigger any metabolic effect (14,15). Conditions C-1 and C-4 are supportive in this context (Fig.1a, 1b and 1d). While the viability trend of C-1 culture was the highest throughout the process, C-4 condition could not manage to survive until the end of production.

In a study conducted by Pan et al.(16) on mAb producing CHO cells, following transition from the growth phase to the stationary phase, specific amino acid consumption rates were different than that of glucose. They were decreased during the stationary phase, while the rate for glucose increased. So, for enhanced longevity and productivity, feeding regimen might also be changed during process, to meet the amino acid requirements. These findings also emphasize the importance of glucose addition separately from the feeds, as was done in our study.

Osmolality levels can be affirmative of pH changes during process. As enzymatic activities are dependent on pH, the effects of feed strategy on glycans observed in this study can be attributed to the potential alterations of glycosylation-related enzymes localized in Golgi, according to the results given in Fig.1e and Table.2 (17).

Feed strategy and productivity

Feed type had no significant impact on titers. The C-5 condition produced comparatively similar concentrations of mAb (2.36 g / L) as the control condition (2.29 g / L) with CHO-03 clone (Fig.2a). However, as culture viabilities and VCDs were affected by the feeding regimen, titers were also impacted. The delivery of certain nutrient components on particular production days to support in vitro cell metabolism is necessary to achieve mAb production (18). As FeedY supplementation under FS-1 strategy was not suitable for the cultures metabolically; conditions C-2, C-3, C-4 were terminated earlier in the production process, resulting in lower titers. This finding is supportive of the fact that the timing and concentration is critical, as feed supplements are more concentrated than basal media and that they can contain up to 10 - 15 times higher concentrations of nutrients, when compared to basal media (19). Cultures under C-5 condition exhibited 10.4% lower level of cell specific productivity with 6.9 pg/cell/day, compared to the C-1 condition (Fig.2b), with 7.7 pg/cell/day ($P < 0.05$). A study conducted by Fan et al. (20) on IgG1 producing CHO cells has demonstrated that a balanced amino acid concentration in culture is determinant for enhanced growth to promote IgG production.

Bioreactor production of the control condition yielded higher titer of 3.2 g / L compared to the production with FeedY as 2.8 g / L (Fig.3f).

Table.2 Results of major N-Glycan contents (%) of CHO-03 fed-batch cultures under different feed strategies

N-Glycan Structures	Content (%)							
	Originator (n=4) AVG ± 3SD	C-1	C-2	C-3	C-4	C-5	C-6	C-7
G0	0.15 - 0.27	0.54	1.45	1.32	1.36	1.99	1.54	1.42
G0F	59.10 – 76.60	63.09	59.01	57.51	57.52	59.85	61.45	62.21
G0-GN	0.35 – 1.59	1.19	0.51	0.42	0.37	0.74	0.55	0.40
G0F-GN	2.06 – 9.28	16.43	2.11	1.70	1.66	2.84	2.40	2.26
G1F	6.18 – 21.42	7.30	25.35	27.32	27.73	22.97	23.29	24.00
G2F	0 - 2.71	0.91	5.95	6.43	6.25	4.99	5.10	4.92
Man5	3.78 – 7.37	7.89	4.03	3.75	3.55	4.76	4.08	3.34
Man6	Not Detected	0.89	0.83	0.83	0.90	0.94	0.86	0.81

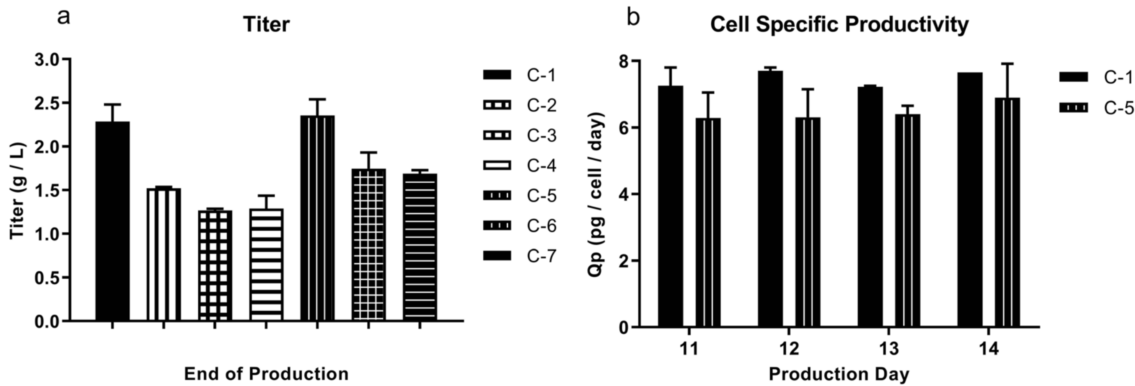


Fig.2 Influence of the different feed strategies on mAb production (left, Fig. 2a) and cell specific productivities (right, Fig. 2b) of CHO-03 cultures monitored during the 14 days of fed-batch productions. Produced mAb concentrations (g / L) are given for all conditions according to the end of production measurements, whereas cell specific productivities (pg / cell / day) are given only for the condition with the highest titer and the control condition, starting from the production day of 11. All results are expressed as the mean value ± standard deviation (SD) of two independent samples and error bars are based on ± 5% analytical accuracy of measurement

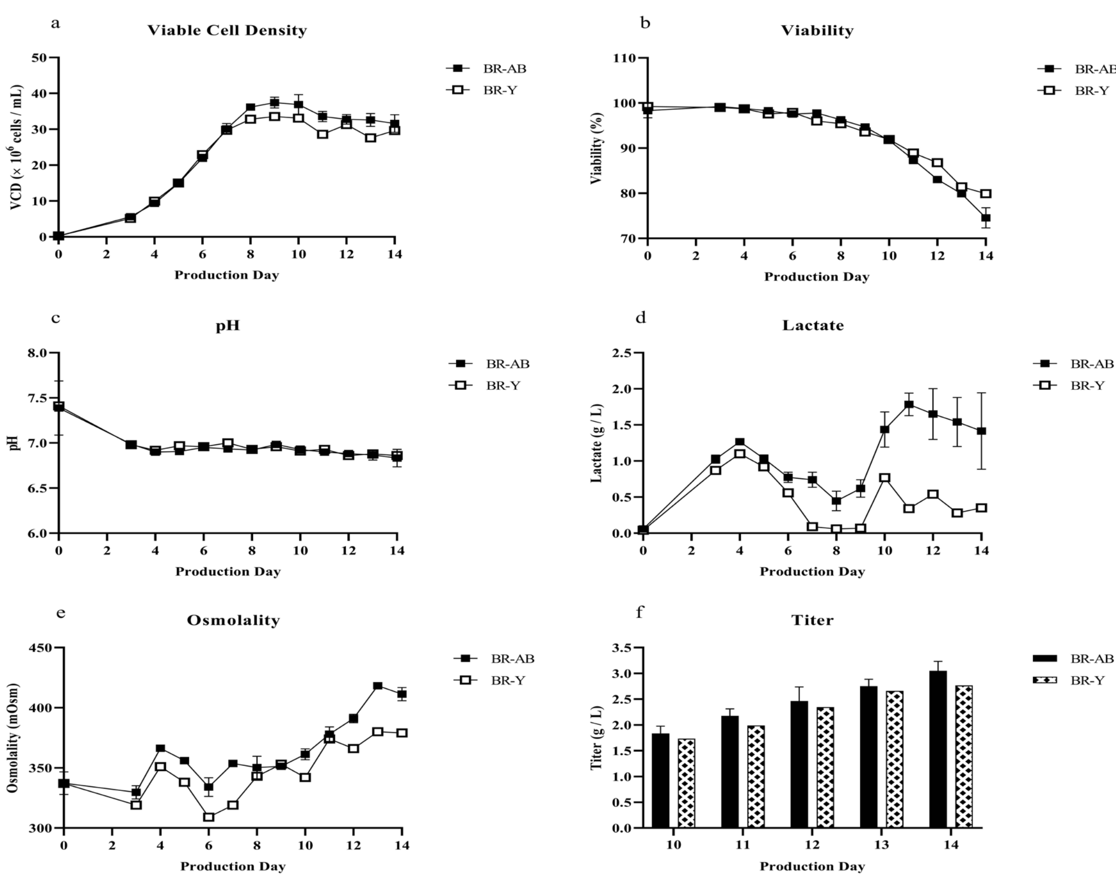


Fig.3 Comparative process monitoring (Fig. 3a – Fig. 3e) and mAb production results (Fig. 3f) of 14 days fed-batch bioreactor productions conducted with CHO-02 clone, under control condition (30% v/v FeedA + FeedB supplementation with FS-1) and test condition (30% v/v FeedY supplementation with FS-2). Statistical significance is based on t-test, with P < 0.05

Feed strategy and glycosylation

Feed type was found to impact glycosylation both alone and in correlation with the feeding regimen (Table.2). With the use of FeedY, G0 and galactosylated glycoform contents increased in spin tubes and bioreactor productions, for both clones. C-2 condition of spin tube CHO-03 culture resulted in 2.7-fold, 3.5-fold and 6.5-fold increases in G0, G1F and G2F contents, respectively compared to the control condition. These ratios were 3.7 - folds, 3.2 - folds and 5.5 - folds under FS-2 strategy (C-5). G0 contents were higher under FS-2 strategy and were observed to decline with reducing feed concentrations under this strategy. Galactosylated glycan contents were higher under FS-1 strategy and G1F levels were found to increase with reduced feed concentrations, under both feeding strategies. FeedY supplementation resulted in significant reduction of 39.7% - 57.7% in Man5 levels among the spin tube conditions. While reduced feed concentrations decreased Man5 contents under both feed strategies, the impact was more pronounced under C-7 condition, with a 57.7% reduction compared to the control condition.

The decrease in Man5 levels, together with increasing galactosylated glycan contents observed for C-3 and C-4 conditions with respect to C-2, and C-6 and C-7 conditions with respect to C-5, can be attributed to cell cycle arrest. In a study conducted with CHO cells, by targeting the G1-checkpoint of the cell-cycle, high mannose levels were reduced while galactosylated glycan levels increased (21). However, the reduction in Man5 levels was not observed in bioreactor productions conducted with CHO-02 clone (Table.3).

This finding supports that the key quality attributes of a given protein is highly dependent on process conditions that is specifically adjusted for a certain clone.

Non-fucosylated glycan contents were found to increase with the use of FeedY in bioreactor productions (Table.3). Although the levels exceed the originator ranges for this mAb, this feature can be beneficial for other mAb developments, since reduced fucosylation is one approach to enhance antibody-dependent cellular cytotoxicity (ADCC) activity, as it provides higher antibody efficacy (22). Thus, feed strategy is a successful tool for optimizing mAb glycosylation patterns.

Feed strategy and charge variation

FeedY increased both the acidic and the basic charge variants, the latter being more pronounced, while reducing the main fraction (Table.4). Among processes that contribute to charge heterogeneity such as deamidation, oxidation, sialylation, C-terminal lysine clipping, isomerization, N-terminal pyroglutamic acid and succinimide formation; lysine variants, are generally responsible for the increase in basic variants and may occur either due to product inhibitory effects of Arg and Lys on carboxypeptidase enzymes or due to temperature-dependence of these enzymes responsible for C-terminal lysine cleavage (23,24). Also, high proline amidation linked to high copper and reduced zinc concentrations result in increased lysine variants (25,26). Regarding the impact of temperature, both productions were down-shifted to 35 °C on PD5; however, BR-Y yielded higher basic peaks. Accordingly, the increase in basic variants can be attributed to the difference in amino acid and metal ion concentrations among feeds.

Table.3 Results of N-Glycan contents (%) and calculated structural compositions (%) of CHO-02 fed-batch production bioreactors

N-Glycan Structures	Content (%)					
	Originator (n=10)		3-L CellReady Single-Use Bioreactor			
	– 3 STD	+ 3 STD	BR-AB (control)			BR-Y
G0	0.95	1.93	1.63	1.9	1.81	2.9
G0F	80.69	89.39	77.71	75.93	77.28	68.47
G1F	1.18	13.8	6.69	6.23	6.9	18.97
G2F	0.05	1.34	0.5	0.47	0.52	2.57
G0-GN	0.06	0.39	0.75	0.91	0.74	0.54
G0F-GN	0.14	6.64	8.12	9.54	7.83	2.16
Man5	0.35	1.09	2.03	2.38	2.18	2.2
Man6	0	0.30	0.71	0.57	0.54	0.61
Non-fucosylated	1.01	2.32	2.38	2.81	2.55	3.44
Galactosylated	1.18	15.32	7.19	6.7	7.42	21.54
High Mannose	0.35	1.39	2.82	3.05	2.72	2.81
Total non-fucosylated	1.36	3.71	5.2	5.86	5.27	6.25

Table.4 Results of charge distributions (%) of CHO-02 fed-batch production bioreactors

cIEF	Originator ± 3 STD	BR-AB	BR-Y
Acidic Peaks	19.67-25.46	19.49	22.65
Main Peaks	69.10-76.98	75.71	63.9
Basic Peaks	2.95-5.84	4.81	12.33

CONCLUSION

The abundance of possibilities regarding post-translational modifications necessitates to approach each protein as a unique case during development. As each cell line differs in terms of production needs, their nutritional requirements will be unique and shall be screened and optimized, necessarily. The results of this study clearly put forward the impact of feed and feed strategy on cell growth, viability, glycosylation and charge variants. In the light of the current goal in cell-culture processes towards biosimilar development, adjusting the protein quality attributes from the beginning of the process is pivotal, making media and feed optimization an essential part of process development. Finally, feed strategy has the power to determine culture longevity and modulate protein function through glycosylation and charge variation.

DECLARATIONS

Funding

This research was supported by Turgut İlaçları A.Ş.

Conflict of interest statement

There are no conflicts of interest among the authors.

Ethics approval

Not Applicable.

Availability of data and material

All data is available.

Authors' contributions

All the designated authors meet the ICMJE criteria for authorship.

Acknowledgements

This research was supported and funded by Turgut İlaçları A.Ş. The authors wish to thank Dr. Ahmet Emin Atik (Head of Protein Analytics, Turgut Biotechnology Group) and Yiğit ERDEMGİL, MSc (Protein Analysis Specialist, Turgut Biotechnology Group) for their contributions regarding analytical assays.

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