OPTIMIZATION OF BIOSIMILAR MONOCLONAL ANTIBODY PRODUCTION IN CHO CELLS AND ITS CHARACTERIZATION

by
Berna Üstüner

Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in
Biotechnology

Yeditepe University
2021

OPTIMIZATION OF BIOSIMILAR MONOCLONAL ANTIBODY PRODUCTION IN CHO CELLS AND ITS CHARACTERIZATION

APPROVED BY:

Assist. Prof. Dr. Hüseyin Çimen ……
(Thesis Supervisor)
(Yeditepe University)

Assoc. Prof Dr. Ali Özhan Aytekin ……..
(Yeditepe University)

Assist. Prof Dr. Emrah Nikerel ……..
(Yeditepe University)

Prof. Dr. Meltem Müftüoğlu ……..
(Acıbadem Mehmet Ali Aydınlar University)

Assoc. Prof. Dr. Özge Can ……..
(Acıbadem Mehmet Ali Aydınlar University)

DATE OF APPROVAL: …./…./2021

I hereby declare that this thesis is my own work and that all information in this thesis has been obtained and presented in accordance with academic rules and ethical conduct. I have fully cited and referenced all material and results as required by these rules and conduct, and this thesis study does not contain any plagiarism. If any material used in the thesis requires copyright, the necessary permissions have been obtained. No material from this thesis has been used for the award of another degree.

I accept all kinds of legal liability that may arise in case contrary to these situations.

Name, Last Name ……..

Signature ……..

Dedicated to Bülent Metin Üstüner….

ACKNOWLEDGEMENTS

It is with immense gratitude that I acknowledge the continuous support, motivation, and endless patience of my advisor Assist. Prof. Dr. Hüseyin Çimen. It has been an honor to work with him as both his master's and Ph.D. student. I could not have imagined having a better advisor and mentor for my studies. I am thankful for all his efforts during my Ph.D., and his faith in me.

Besides my advisor, I would like to thank the rest of my thesis committee: Assoc. Prof. Dr. Özge Can and Assoc. Prof. Dr. Ali Özhan Aytekin, for all their support and efforts. With their insightful comments and questions, I was able to expand and improve my research.

I would like to express my gratitude to Turgut İlaçları A.Ş., for allowing me to proceed as a Ph.D. student and for all the financial support to conduct my experiments. I sincerely thank Dr. Ahmet E. Atik, Dr. Duygu Ayyıldız Tamiş, Dr. Deniz Demirhan and Seçil Dayankaç Ünver, my managers and co-workers in Turgut İlaçları A.Ş., for their contributions and moral supports.

Most importantly, I would like to thank my family for always being supportive and faithful during my Ph.D. studies. I thank my mother Yıldız Erdoğan for always being there for me and especially my father Bülent Üstüner, who always found a way to motivate me to proceed with my studies, and not to quit.

Last but not the least, I thank my soulmate Yiğit Erdemgil for his endless support and encouragement during my Ph.D.

ABSTRACT

OPTIMIZATION OF BIOSIMILAR MONOCLONAL ANTIBODY PRODUCTION IN CHO CELLS AND ITS CHARACTERIZATION

Biosimilar monoclonal antibody development is currently the major focus of the biopharmaceutical industry. Strategies to adjust the critical quality attributes early in the development stage saves time, labor, and costs considering the competitive global market. As a technologically and pharmaceutically developing country, particularly Turkey is currently expanding its biosimilars market and requires new developments.

This study focuses on generating optimal conditions for the development of a biosimilar of C5mAb, in Chinese Hamster Ovary (CHO) cells. C5mAb acts as a blocker to the complement component 5, an important target for certain rare diseases. The novelties of the study are set forth with the usage of CHO host cell expression system to produce a hybrid and humanized mAb, the incorporation of Design of Experiments (DoEs) to screen and investigate process parameters and culture feeding regimens, and also by the process parameters optimized for the production of a C5mAb biosimilar. The study involves glycan and potency analyses as key quality attributes, with further verification in bioreactor systems, aiming to contribute to the assessment of biosimilarity.

A robust 14 days fed-batch culture was developed with the E90-02 clone, under extended feed strategy. Applying temperature down-shift to 32°C from 37°C on the production day of 7, in stirred tank bioreactor system was confirmed to yield 2.9 g / L titer with 83.85 % binding potency, and matching glycosylation profiles, complying with the Originator molecule in terms of efficacy, safety, biological activity, immunogenicity, and stability.

The study put forward the reliability and applicability of experimental design strategies for manufacturing bioprocesses that ensure compliance of key quality attributes, and contribute to the knowledge in the field of biosimilars development.

ÖZET

CHO HÜCRELERİNDE MONOKLONAL ANTİKOR ÜRETİM OPTİMİZASYONU VE KARAKTERİZASYONU

Biyobenzer monoklonal antikor (mAb) geliştirilmesi, günümüz biyofarmasötik endüstrisinin odağı haline gelmiştir. Küresel market rekabeti göz önünde bulundurulduğunda, geliştirme sürecinin başında, kritik kalite özelliklerini ayarlamak için stratejilerin geliştirilmesi zaman, işgücü, ve maliyetler açısından büyük önem taşımaktadır. Özellikle teknolojik ve farmasötik olarak hızlı bir şekilde gelişmekte olan Türkiye, biyobenzer piyasasını genişletmekte olup, yeni buluşların ihtiyacını çekmektedir.

Bu çalışma, Çin Hemstır Yumurtalık (CHO) hücrelerinde, C5mAb'ın bir biyobenzerinin geliştirilmesi için optimum koşulların araştırılmasına odaklanmaktadır. C5mAb, kompleman sistemde C5'i bloke ederek bazı nadir hastalılar için önemli bir hedef oluşturmaktadır. Bu çalışmaya özgü yenilikler, CHO ekspresyon sisteminin kullanılarak hibrit ve insanlaştırılmış bir mAb üretiminin geliştirilmesi, Design of Experiments (DoEs) metodolojisi kullanılarak proses parametrelerinin ve kültür besleme stratejilerinin taranarak belirlenmesi, ve biyobenzer bir C5mAb üretimi için proses parametrelerinin optimize edilmesi ile ortaya konmuştur. Çalışma, biyobenzerliğin ortaya konması amacıyla, glikan yapıları ve potansi olmak üzere kritik kalite parametrelerinin analizini ve biyoreaktör sistemlerinde ileri doğrulamaları içerir.

E90-02 klonu ile, uzatmalı besleme stratejisi kullanılarak, dayanıklı bir 14 günlük kesikli-beslemeli kültür prosesi geliştirilmiştir. Karıştırmalı tank biyoreaktör sistemindeki prosesin, üretimin yedinci günü sıcaklığın 37°C 'den 32°C'ye düşürülmesi ile %83.85 bağlanma potansiyeline sahip, 2.9 g / L titre sağlayan, eşleşen glikozilasyon profilleri ile Orijinatör moleküle etkinlik, güvenlik, biyolojik aktivite, immünojensite, ve stabilite açısından gereken uygunluğu gösterdiği doğrulanmıştır. Sonuçlar, deneysel dizayn stratejilerinin üretime yönelik, kritik kalite özelliklerinin sağlandığı biyoproseslerde ve biyobenzer geliştirme alanında kullanımına ilişkin güvenilirliği, ve uygulanabilirliği açıkça ortaya koymaktadır.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

Ab	Antibody
AchR	Acetylcholine receptor
ACN	Acetonitrile
ADA	Anti-drug antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADCP	Antibody-dependent cell-mediated phagocytosis
aHUS	Atypical hemolytic uremic syndrome
anti-TNF	Anti-tumor necrosis factor
AOF	Animal origin-free
API	Active pharmaceutical ingredient
AQP4	Aquaporin-4 protein
Arg	Arginine
Asn	Asparagine
Bcl-2	Anti-apoptotic protein B-cell lymphoma 2
BEH	Ethylene bridged hybrid
BHK	Baby hamster kidney cells
BR	Bioreactor
CDC	Complement-dependent cytotoxicity
CDK	Cyclin-dependent kinase
CHO	Chinese hamster ovary

CPP	Critical process parameter
CQA	Critical quality attributes
CV	Coefficient of variation
Cys	Cysteine
C3	Complement component 3
C5	Complement component 5
C5mAb	Anti-complement component 5 monoclonal antibody
DHFR	Dihydrofolate reductase
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DoE	Design of experiment
D-PBS	Dulbecco’s phosphate-buffered saline
DSP	Downstream process
EC50	Half maximal effective concentration
E.coli	Escherichia coli
ELISA	Enzyme-linked immunosorbent assay
EMA	The European Medicines Agency
ER	Endoplasmic reticulum
EU	European Union
Fab	IgG antigen-binding region
Fc	IgG crystallizable region

FcɣR	Fc gamma receptor
FcRn	Neonatal Fc receptor
FDA	The United States Food and Drug Administration
Fuc	Fucose
Gal	Galactose
GalT	Galactosyltransferase
GlcNAc	*N*-acetylglucosamine
G_0	Growth phase resting state
G_1	Growth phase 1
gMG	Generalized myasthenia gravis
GMP	Good Manufacturing Practice
GnT-1	Glucose *N*-acetyltransferase 1
GOI	Gene of interest
GPI	Glycosylphosphatidylinositol
GS	Glutamine synthetase
HAMA	Human anti-murine antibody
HC	Heavy chain
HEK-293	Human embryonic kidney cells - 293
HILIC	Hydrophilic interaction chromatography
His	Histidine
HP	High-performance
hr	Hour

HSC	Hematopoietic stem cell
HTP	High-throughput
HUS	Hemolytic uremic syndrome
ICH	International Conference on Harmonisation
IgG	Immunoglobulin G
IPC	In-process control
K_D	Equilibrium dissociation constant
LC	Light chain
mAb	Monoclonal antibody
MAC	Membrane attack complex
Man	Mannose
MBL	Mannose-binding lectin
MG	Macroglobulin
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
NKC	Natural killer cell
NMOSD	Neuromyelitis optica spectrum disorder
NS0	Mouse myeloma cell line
OVAT	One-variable-at-a-time
PA-HPLC	Protein-A high-performance liquid chromatography
PD	Production day
PDL	Population doubling level

PK	Pharmacokinetics
PN	Passage number
PNH	Paroxysmal nocturnal hemoglobinuria
PTM	Post-translational modification
QbD	Quality by design
Q_p	Cell-specific productivity
Q-TOF	Quadripole-time of flight
Rb	Retinoblastoma protein
RH	Relative humidity
R&R	Repeatability and reproducibility
Rpm	Revolutions per minute
RWCB	Research working cell bank
S	Synthesis phase of growth
SD	Standard deviation
Ser	Serine
S/MAR	Scaffold/matrix attachment region
SPE	Solid-phase extraction
TCC	Terminal complement complex/cascade
Thr	Threonine
TL	Turkish lira
ToS	Time of shift
tPA	Tissue plasminogen activator

UCOE	Ubiquitous chromatin opening element
UDP	Uridine diphosphate
UPLC	Ultra performance liquid chromatography
US	United States
USP	Upstream process
V	Variable region
VC	Viable cell
VCD	Viable cell density
WHO	The World Health Organization

1. INTRODUCTION

1.1. BIOPHARMACEUTICAL DEVELOPMENT AND BIOSIMILARS

1.1.1. The Importance of Biosimilars

Biologics development began in the 1980s, to improve naturally occurring proteins, complex peptides, and glycoproteins through recombinant technology [1]. The evolving field of biotechnology paved the way for innovative strategies and the production of more complex biologics, such as monoclonal antibodies (mAbs), which gained importance in the purpose of developing therapeutic as well as diagnostic agents [1]. Although the mAb technology has 45 years of history, it was not until the recent advancements achieved in biotechnological techniques and knowledge that the importance of mAbs was appreciated, as they became promising therapeutics against autoimmune diseases, cancer, inflammation, and infectious diseases [2].

With the convergence of the human health segment of the biotechnology and biopharmaceutical industry, powerful pharmaceutical producers began to compete through ‘biosimilars’ [1]. Biosimilars are defined as “biological medicinal products that contain a version of the active substance of an already authorized original biological medicinal product” [3]. All biologics, as well as products derived from recombinant DNA technology, can serve for the development of a biosimilar, such as enzymes, vaccines, mAbs, human growth hormones, insulins, interleukins, tissue plasminogen activators [3,4].

The importance of biosimilars emerged with the expiry of exclusivities and patents of some biologics on market. Together with the fact that they bear the potential of significantly

reducing costs of already marketed biologics, the expired patents became an opportunity for biosimilar development and approval (Table 1.1) [3,5,6]. Guidelines defined by The European Medicines Agency (EMA), The United States Food and Drug Administration (FDA), and The World Health Organization (WHO) are considered as standards to be met, and all biosimilars undergo an evaluation procedure by these organizations prior to regulatory approval [5].

Table 1.1. Patent expiry dates in the EU and the US for important biologics between the years 2010-2020.

Biologics	**Patent Expiry in the EU**	**Patent Expiry in the US**
Humira® (adalimumab)	2018	2016
Avastin® (bevacizumab)	2022	2019
Enbrel® (etanercept)	2015	2028
Remicade® (infliximab)	2015	2018
NovoLog®/NovoRapid® (insulin aspart)	2011	2014
Lantus® (insulin glargine)	2014	2014
Avonex® (interferon beta-1a)	2015	2015
Tysabri® (natalizumab)	2015	2015
Xolair® (omalizumab)	2017	2017
Neulasta® (pegfilgrastim)	2017	2015
MabThera® (rituximab)	2013	2016
Lucentis® (ranibizumab)	2022	2020
Herceptin® (trastuzumab)	2014	2019

1.1.2. Biosimilars in World Market

Following rapid advancements in genetic engineering, biopharmaceutical, and biotechnology industries focused on developing new drugs, diagnostic tools, genetic analyses, and tests [7]. The cost of developing new biologics is much higher than producing chemical-based drugs, which augments their prices in the market, accordingly [8].

The first biologic drug approval took place in 1982, with the development of recombinant insulin [3]. The initial therapeutic application of a recombinant protein of animal origin became possible with the approval of human tissue plasminogen activator (tPA) in 1986, produced by Genentech [9]. Development of biologics being confined to relatively small populations with seriously high costs raised the overall drug expenditure, which cannot be considered as sustainable by national health services. This resulted in a downward trend in access of patients to safe and effective medicines, so the quality of health care began to decrease. Biosimilars recently emerged as a remedy for this situation [3]. Biosimilars make it possible to spare a budget for innovative medicines and reserve savings, while engaging patient access to costly medicines against immunological, metabolic, inflammatory diseases, and cancer [10,11].

Although countries worldwide are interested in this industry, the main markets for both the original biopharmaceuticals and for biosimilars are the EU and the US [1]. Regarding economic developments, the Asia-Pacific region is also expected to rapidly grow in market size in the upcoming years (Figure 1.1) [12].

Figure 1.1. Estimated growth in global biosimilar market size by region, 2019 – 24. Reprinted with permission from Mordor Intelligence, www.mordorintelligence.com [12].

The EU has the most developed biosimilars market, which makes it the global pioneer [13], and EMA is responsible for the marketing authorizations of biosimilars in Europe. EMA guidelines define principles regarding safety, efficacy, quality, clinical and non-clinical aspects of certain biosimilar products, such as mAbs and hormones [14]. The large, complex, less stable nature of biosimilar molecules, together with the challenges faced during research and development processes, makes their production much harder than that of chemical molecules. Moreover, the innovators - producers of the original molecule - never disclose the development, nor the production process of their molecule, so the biosimilar producers must track down the whole process and rediscover the steps leading to the development of the effective substance, and depending on the molecule they generate, might conduct clinical trials as well.

While the first biosimilar approval in the US by the FDA was in 2015 with Filgrastim-sndz, EMA had approved its first biosimilar as Somatropin in 2006. The first mAb approval was in 2013 with infliximab (Remsima, Inflectra) [14]. Turkey was one of the emerging countries to approve infliximab. The pharmaceutical market in Turkey is rapidly expanding and

currently, Turkey is one of the top 10 emerging countries in the biosimilars market (Figure 1.2) [15]. By the first quarter of 2017, the market showed an increase of 16.5 percent, reaching 6.15 billion TL [16]. Today, there are 261 reference biotechnological drugs under 116 brands in the Turkey market, and 86 of them are biosimilars under 25 brands [17].

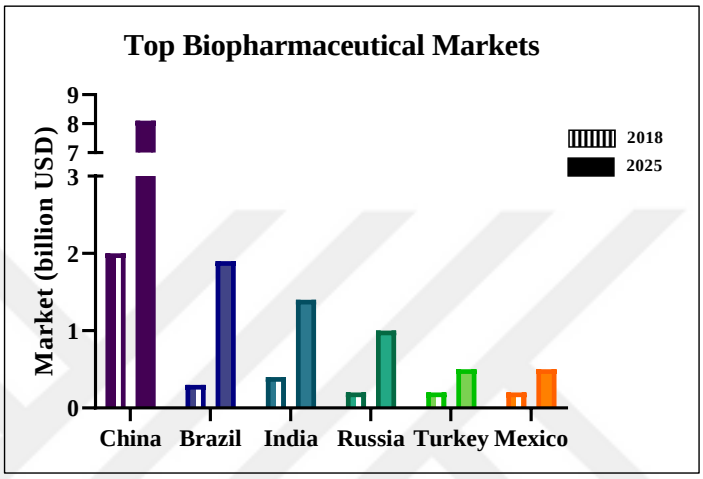

Figure 1.2. Estimated growth in global biosimilar market size, 2018 – 25 ($ billion, annual % increase).

Biosimilar industry concerns almost the whole world and the market experienced an annual growth rate of 35 percent from 2001 to 2012, and the market had reached $5 billion by 2013 [1]. Until 2017, 10 more biosimilars and 31 more mAbs appeared in the global market. With the rapid expansion, a total of 11 biosimilars and 57 mAbs became available for clinical use by the end of 2017 [18]. Between the years 2011-2016, the FDA approved 85 biologics while this number reached 109 for those approved by EMA. 12.8 percent of the biologics approved by EMA were of single or multiple indications to be used against diseases of allergic and immunologic origin [3,14]. By 2016, the global sales of biologics reached $210 billion and the patents of 11 biologics are expiring in 2020, accounting for over $60 billion of the global sales [19]. More specifically, the global market size for mAbs was reported as $37.13 billion in a report published in February 2020, which is estimated to reach $63.13 billion by 2025 [20].

1.2. BIOSIMILAR MONOCLONAL ANTIBODIES

1.2.1. Importance of Monoclonal Antibodies (mAbs) as Biosimilars

Therapeutic proteins bear the risk of immunogenicity, which may cause anti-drug antibody (ADA) responses that might, in turn, lead to hypersensitivity responses such as anaphylaxis and infusion reactions [21]. Immunogenicity can occur due to altered protein structures and when first discovered, mAbs raised concerns related to drug safety and efficacy, due to their potential of causing anti-drug antibody (ADA) and hypersensitivity responses [22]. Due to their heterogeneous nature, mAbs are highly susceptible to chemical modifications and degradation [23,24]. Post-translational modifications (PTMs), chemical modifications, and/or degradation may take place during either the preparation or storage processes [25–27]. However, the vast number of possible alterations that can be induced within mAb chemical structures make them invaluable, regarding therapeutics as well as biosimilars. The fact that natural immunoglobulins (IgGs) and recombinant therapeutic mAbs that have been approved so far are all glycosylated demonstrates their importance [22]. Plus, quality compliance and related approval of mAbs demand the controlled glycosylation to meet the required characteristics, thus biosimilarity.

1.2.2. Types of mAbs, Structure, and Modifications

When characterizing molecular structures of mAbs; size, aggregation, heterogeneity, and glycosylation are of critical importance [24]. Alterations in size are associated with enzymatic as well as non-enzymatic cleavage, or either mispaired or incomplete formation of disulfide bonds [28]. These alterations determine size distribution, which affects the safety and efficacy of mAb products [28]. Heterogeneities are important as well, as they are always encountered in purified products [28]. Two types of heterogeneity can be defined. Microheterogeneity is present if variations are in site-occupancy, while the term microheterogeneity is used when variations are in the structure of attached glycans [29]. Aggregation is a crucial heterogeneity that is mostly immunogenic in humans [30]. Types of antibody aggregates are dependent on several factors, such as interactions either covalent or non-covalent, solubility, reversibility, and also the denaturation of the monomer [31,32]. It is important to emphasize that during mAb production in living cells, protein folding and disulfide bond pairing do not always occur correctly. Plus, exposure to culture media or stress caused by factors such as temperature or pH can lead to further heterogeneities [33]. Complete understanding of causes and outcomes of alterations in mAb structures can be achieved through comprehending the mechanisms that lie within.

To begin with, antibody formation takes place in the endoplasmic reticulum (ER) (Figure 1.3) [34]. Human immunoglobulins consist of two light chains (LC) and two heavy chains (HC), which are identical. A heterodimer is formed through the pairing of one LC and one HC through disulfide bonds [35]. The folding and assembly of IgGs are supported when the ER machinery is enhanced, and the misfolded chains can be eliminated by the ER quality control mechanism [36–38].

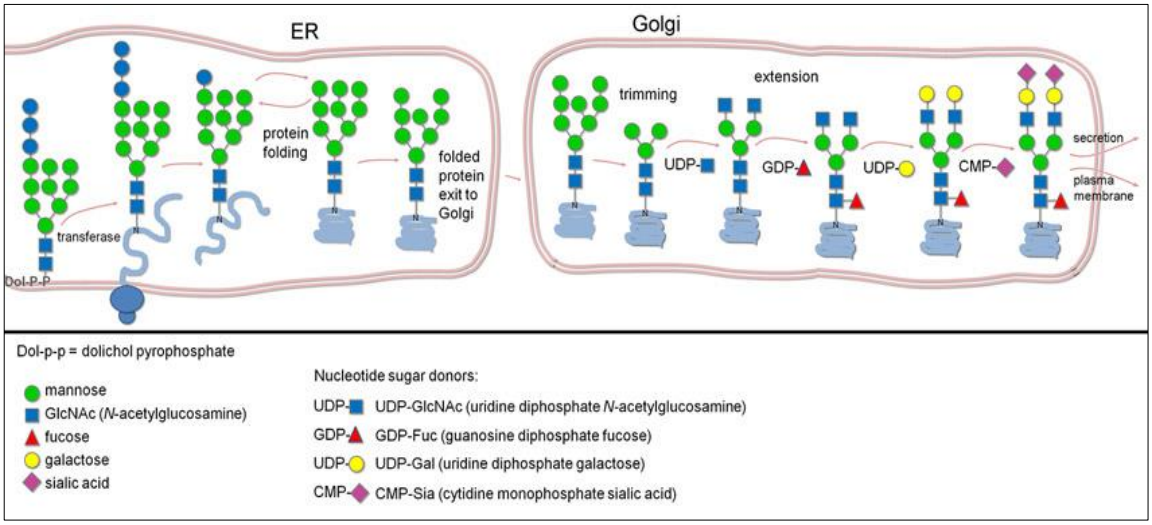

Figure 1.3. Synthesis of N-glycans in eukaryotic cells. Reprinted with permission from New England Biolabs, www.neb.com [34].

Glycoproteins consist of a single peptide backbone, but different glycoforms with varying glycan structures can be attached, changing glycosylation characteristics (Figure 1.4) [29]. Glycans gain heterogeneity due to binding of hundreds of possible glycoforms resulting from random pairing of HC glycans with different structures [39,40]. Glycosylation can be affected by cell line characteristics, process control parameters, and culture media components [41,42]. Alterations in glycan profiles impact the effector functions and PK profiles of antibodies, as well as clearance rate, stability, ADCC, and CDC activities [35].

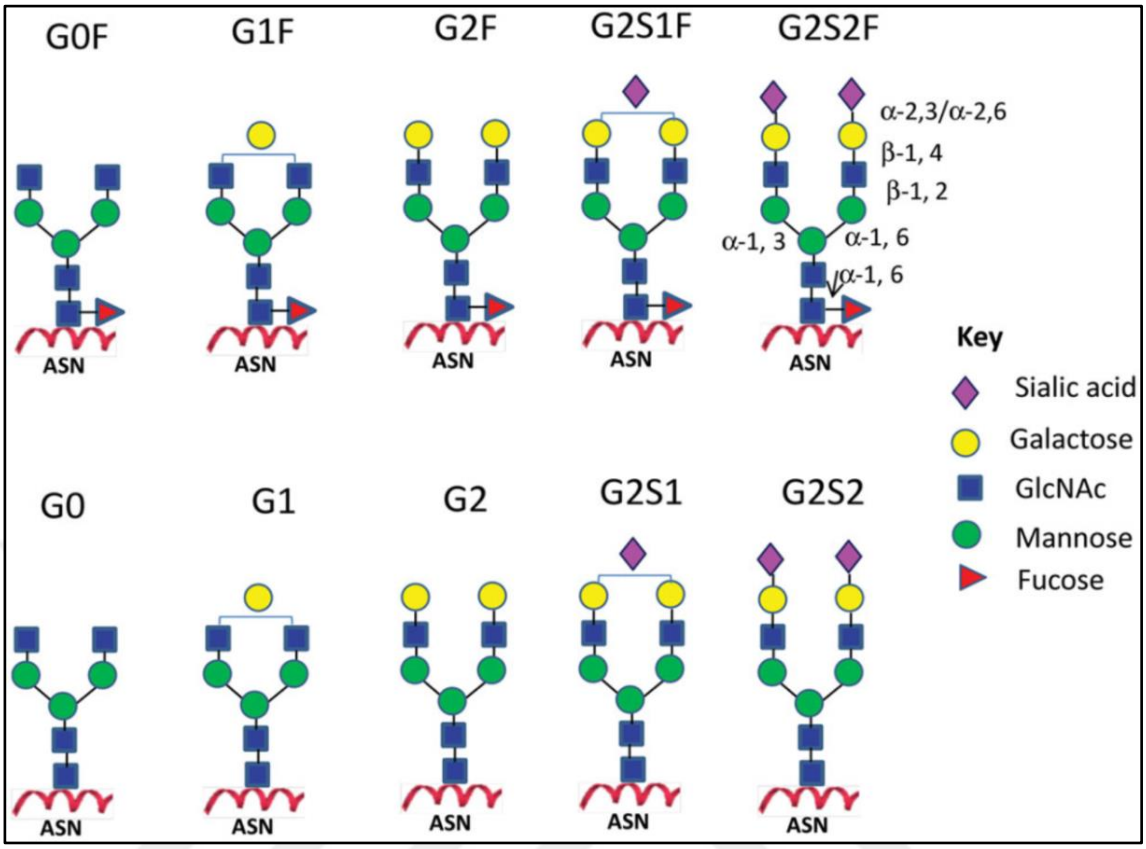

Figure 1.4. Variable structures of glycans [22].

IgGs have four subclasses, namely IgG1, IgG2, IgG3, and IgG4. Currently, all approved mAbs and their derivatives with therapeutic properties are of either IgG1, IgG2, or IgG4 [22]. The reason why IgG3 is not used is due to its unfavorable PK properties. While the terminal half-lives of IgGs 1, 2, 4 are about 21 days, IgG3 has a terminal half-life of seven days [22]. The major differences between IgG2, IgG3, and IgG4 are the length of their hinge region, their amino acid compositions, and the number of disulfide bridges between two HCs. These differences influence IgG Fc receptor (FcγR) binding, effector functions, and also PK properties [22].

IgGs have two main domains, the antigen-binding region Fab and the crystallizable region Fc [35]. These two domains perform distinct functions in the immune system, linking innate immunity to adaptive immunity [43]. Specific binding of antibodies to target antigens occurs through the Fab domain, whereas the Fc domain binds to receptor molecules, defining the effector function of antibodies [35].

Glycosylation characteristics are considered as one of the Critical Quality Attributes (CQAs) for most mAb-based products, especially for products against oncologic diseases, because tumor cell lysis is among the target mechanism of actions, and the Fc glycan structure directly impacts Fc effector functions [44].

Glycosylation takes place in the ER and Golgi apparatus, and only *N*-linked glycosylation occurs in IgGs, *O*-linked glycosylation is not present [22,45]. Glycoprotein processing happens from the ER towards the Golgi (Figure 1.5).

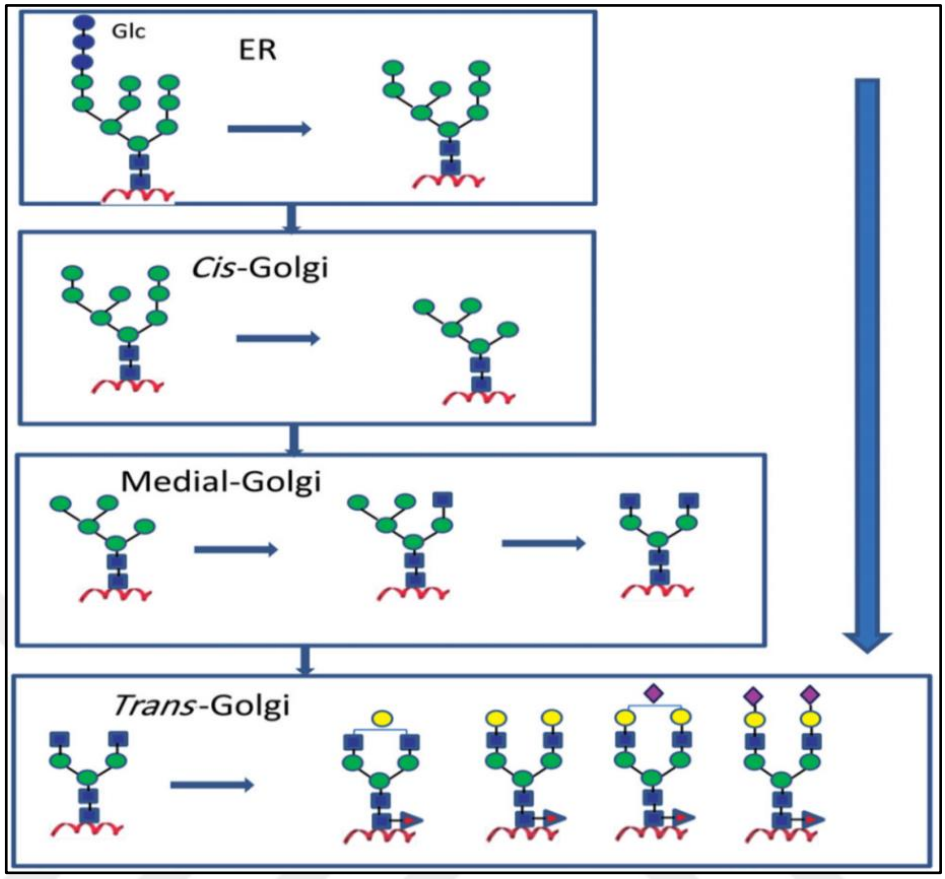

Figure 1.5. Glycoprotein processing from the endoplasmic reticulum towards the golgi apparatus [22].

As passing through the ER, the reducing ends of oligosaccharides with high mannose are cut by enzymes such as mannosidase-1, forming mannose-5 (Man5). Then, in the Golgi, a hybrid glycan is formed with the removal of α-1,3-mannose and the addition of *N*-acetylglucosamine (GlcNAc) (Figure 1.6.). The hybrid glycan then forms complex glycans [46]. The expressions of several glycosyltransferase enzymes found in the Golgi determine the pattern of glycosylation [29]. *N*-glycosylation is a co-translational process taking place in the ER. The *N*-glycan sequon, which is (Asn)-X-Ser/Thr, becomes exposed to the active site of the enzyme oligosaccharyltransferase (OST) [29]. Therapeutic mAbs have the (Asn)-X-Ser/Thr sequence for *N*-glycosylation at Asn^{297} in HC of CH2 constant domain [22]. X represents any amino acid except for proline. *N*-linked glycans of human IgGs are biantennary complex structures [22]. They have a conserved core structure which is

composed of two GlcNAc, three mannoses, two GlcNAc residues that are ß-1,2 linked to α-6 mannose and α-3 mannose, forming two antennae [22]. IgGs that are produced in mammalian cells usually contain high-mannose glycoforms, Man5-6-7-8-9, in levels less than five percent, and the most common glycoforms they contain are the complex glycoforms G0F, G1F, G2F. These complex glycoforms are considered as 'mature' glycoforms produced in Chinese hamster ovary (CHO) cells [47].

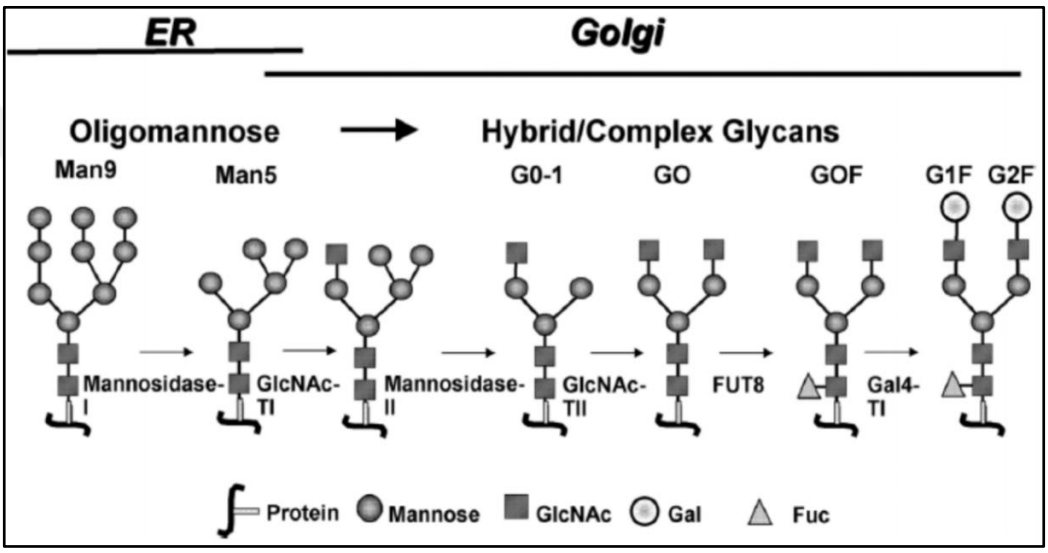

Figure 1.6. N-linked oligosaccharides processing pathway [46].

Fc glycans of human serum IgG are mainly in the form of a core fucosylated complex biantenna, with mostly G0F, G1F, and G2F glycoforms, and also trace amounts of sialylated glycoforms [39]. Additional fucose (Fuc), galactose (Gal), bisecting GlcNAc, or sialic acid might be added as well, depending on the host cell machinery [35,39]. Human serum IgGs are mostly less than 10 percent sialylated. To compare the recombinant mAbs generated in some common host cells, those generated in CHO cells have negligible amounts of sialylation, while mAbs produced in mouse hybridoma cells bear 50 percent sialylation [48].

Apart from the Fab and Fc regions, glycosylations that may occur in the variable region Fv also have the potential to impact antibody function. However, cetuximab being an exception, with *N*-glycosylation at Asn^{88} of the HC variable region, the majority of recombinant therapeutic mAbs are only glycosylated at the Fc region [39]. Binding activity, biological functions, and effector functions of mAbs are critical in terms of efficacy [49–51]. Fc receptor-mediated functions of IgG antibodies, for instance, rely on the presence of glycosylation [35]. Glycans located on the opposite Asn^{297} of each HC interact and maintain the conformation of the Fc domain. Thus, any changes in Fc glycosylation can alter the conformation of the Fc region. This may impact Fc receptor binding, which in turn, might affect mAb immunogenicity, pharmacokinetics (PK), stability, and safety [52].

IgG Fc receptors, denoted as 'FcγRs', are a family of molecules comprised of three activating (FcγRI, FcγRIII, FcγRIV in mice; FcγRI, FcγRIIa, FcγRIIc, FcγRIIIa in humans) and one inhibitory (FcγRIIb) receptor [53]. Humans have four types of Fc receptors for IgGs; FcγRI (CD64), FcγRII (CD32a,b,c), FcγRIII (CD16a,b,c), neonatal Fc receptor (FcRn). The FcRn-binding site is located at the CH2-CH3 domain which overlaps with the protein A-binding site. The glycosylation site of IgG Fc domains is, in general, relatively sequestered and buried in the CH2-CH3 domain, rendering any interactions with glycan-binding receptors sterically unfavored [35].

Antibody engagement of FcγR with the Fc region of an IgG mediates cell-based inflammatory responses ADCC, CDC, ADCP [35]. While FcγRI is a high-affinity receptor for monomeric IgGs, FcγRII and FcγRIII are of low-affinity for them [22]. The FcγRI family is the only Fcγ receptor class that appreciably binds monomeric IgG *in vivo* [54]. The affinity of human IgG to the human Fc receptor hFcγR is dependent on whether it is in a monomeric or an immune-complexed state [22]. ADCC is mediated by natural killer cells (NKCs) through FcγRIIIa bridging to the antibody bound on the target cell. This is an effector function for tumor death [55].

A therapeutic mAb with desired ADCC activity for its therapeutic effects requires a higher affinity to FcγRIIIa than endogenous IgG [56]. Fucosylation of the biantennary *N*-linked glycans on antibodies regulate binding to FcγRIII receptor and this can impact ADCC activity [57–59]. Core fucosylation requires GlcNAc addition by glucose *N*-acetyltransferase 1 (GnT-1); so, only hybrid and complex glycans can become core fucosylated. A unique carbohydrate-carbohydrate interface is involved in the interaction that takes place between afucosylated IgG1 and FcγRIIIa. This increases affinity for the afucosylated antibody through lack of steric hindrance, which is the case for fucosylated forms [60]. When the fucose content of IgG is reduced, ADCC increases [46]. On the other hand, increased sialylation reduces ADCC [61].

Alterations of some glycoforms can impact the PK of molecules as well [62]. For instance, high-mannose glycans have a negative effect. Human IgG contains low levels of Man5 [63]. When therapeutic mAbs are concerned, high levels of high-mannose glycoforms are risky regarding clearance, immunogenicity, and efficacy [46]. Also, plasma clearance rates of high-mannose glycoforms (Man5-9) are higher than mAbs with complex glycoforms, increasing the possibility of an impact on efficacy [62,64]. High-mannose contents in IgGs show higher binding affinity to FcγRIIIa, which in turn, result in higher ADCC activity [65–68]. The high ADCC activity might be attributed to the absence of fucosylation because high-mannose glycans do not bear core fucosylation [68]. Another possible glycan addition is galactosylation. It has been demonstrated that terminal galactosylation has no significant effect on the ADCC activity of IgGs, but it has an important role in CDC activity [69–71]. Reduction in terminal galactosylation also reduces CDC activity [69].

1.3. EFFECT OF BIOPROCESS PARAMETERS ON GLYCAN PROFILES

Cell culture parameters such as pH, temperature, and dissolved oxygen (DO) have an impact on quality attributes such as glycosylation [72–74]. Culture conditions are reported to impact both the localization and the activity of glycosylating proteins, and gene expressions of the Golgi resident proteins involved in glycosylation are variable during culture [46,75].

External pH variations may decrease the activities of key enzymes involved in glycosylation by altering the internal pH of the Golgi apparatus [29]. Another parameter, DO, is crucial for the growth of cells and the maintenance of optimal metabolism [76,77]. Temperature, on the other hand, is critical especially in terms of glycosylation. Culture temperatures below 32°C have been shown to reduce branched glycan structures and sialylation [46].

When cell culture processes are carried to bioreactor systems, additional parameters affecting glycan compositions arise. Besides pH, temperature, and DO; agitation, shear stress, sparging, dissolved carbon dioxide comes into account. These parameters may alter charge variants or glycosylation patterns [45,78–80], thus can impact product quality. Microheterogeneity is significantly affected by culture conditions. Glycoform heterogeneity might increase, batch-to-batch variations can occur, and therapeutic efficacy can be reduced accordingly [29].

Together with technological innovations, improvements in process development are increasing to enhance antibody quality, as well as to develop more efficient and economical processes.

Regarding current antibody manufacturing technologies, the development and optimization of both the Upstream Process (USP) and Downstream Process (DSP) steps are of major importance. In the last two decades, applying new media and process control strategies, while making use of the developing recombinant technologies, has proven to significantly increase process efficiency and product titers [81,82]. Starting from cell line selection, media optimization and harvest strategies set the basis for the development in USP.

The impact of process parameters poses a great challenge on the developmental stages of production. Apart from the host cell-line, fed-batch production parameters can alter final glycan patterns of antibodies, thus they shall be strictly controlled during the production phase of mAbs to monitor their impact on product quality attributes. The utilization of chemically defined media is mostly preferred in the manufacture of biosimilars, due to concerns related to batch-to-batch variation. However, it is possible to supplement the culture media with certain additives to adjust glycosylation profiles and charge variants [83,84]. Also, process conditions listed above may impact terminal galactosylation profiles of mAbs [85].

As an important parameter of process development, temperature optimization is relatively easier to control, compared to the levels of dissolved carbon dioxide, oxygen, or pH. Applying shifts during production to hypothermic temperatures – below 35°C – is known to hold potential benefits. Low-temperature cultivation aims to improve both cell culture longevity and recombinant protein production [74,86–88]. Depending on the cell line and production process, temperature down-shift can be a useful tool to optimize product quality and titer to meet certain quality attributes [89].

While the effects of shifting the temperature below physiological conditions are dependent on the cell line, production process, and media components; the improvements it serves for cell culture rely on cellular metabolism. Lowering the culture temperature usually leads to a resting state of cells towards the end of the G1 phase of cell division (Figure 1.7). Temperature down-shift is a widely applied strategy in CHO cell cultivation to improve protein productivity and quality [74,87,88,91].

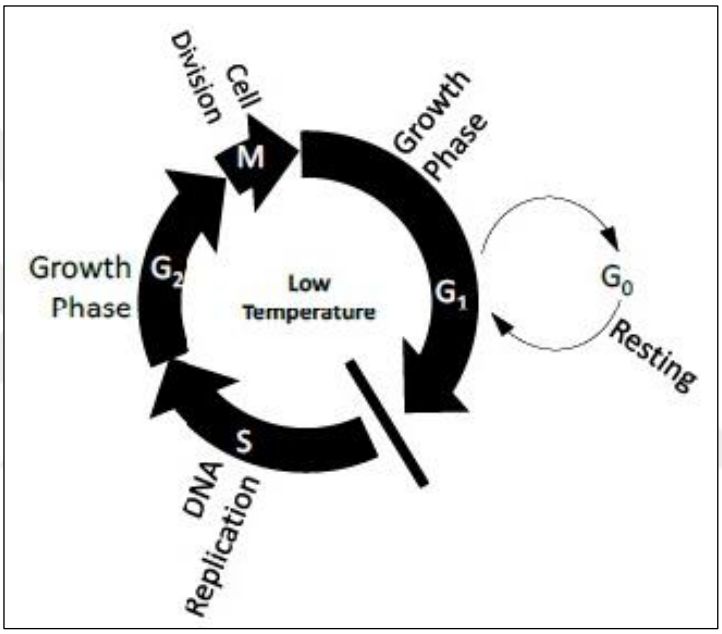

Figure 1.7. Mammalian cell cycle under mild hypothermia [90].

Temperature reduction from the optimal 37°C in mammalian cells results in a decreased growth rate, but cell viability increases under mild hypothermia [86,92,93]. Mild hypothermia refers to a few degrees of reduction in temperature from 37°C down until 28°C [94]. CHO cell cultivation within this temperature range can improve specific productivities of some recombinant proteins [74,87,91,95].

Table 1.2. Recombinant proteins with increased CHO cell-specific productivity under mild hypothermia.

Cell Line	Enhanced Production	Reference
CHO	Epo-Fc	[74]
	TNFR-Fc	[87]
	Anti-TNFα	[91]
	FSH	[95]

Effects of specific productivity and viable cell density (VCD) on recombinant protein production are directly proportional; however, if the optimal VCD is exceeded, the yield decreases [96]. Enhanced specific productivity at low cultivation temperatures is dependent on the recombinant protein to be produced [9], on the cell line, and it is valid for only certain CHO cell lines. In batch cultivation conducted at 30°C, IgG production was increased by two-folds [94]. Specific growth rates and specific productivity are affected by low-temperature conditions through different mechanisms [9].

Temperature reduction lowers metabolic rate, but recombinant protein production still might increase due to interactions of different mechanisms such as transcription, translation, cytoskeleton arrangement, and cell cycle [97,98]. Specific recombinant protein production rate increases during the G1 phase of the cell cycle [99,100]. Cyclin-dependent kinases (CDKs), a sub-class of Ser-Thr kinases, are cell cycle checkpoint regulators [96]. Checkpoint of G1 phase is regulated by cyclin D/CDK4/6 complex, which deactivates Retinoblastoma protein (Rb), a tumor suppressor, through phosphorylation [101]. Rb then activates the gene transcription of S phase cyclins and DNA replication begins. Manipulations such as applying lower temperatures during cultivation may arrest the cell cycle at the G1 phase and accordingly, may increase specific protein production while maintaining cell viability and

delaying apoptosis [100]. CHO cell cultivation processes at 30°C and 33°C resulted in the suppression of apoptosis-related cell death [100,102]. Temperature decrease enhanced the activity of an antioxidant enzyme glutathione peroxidase, which in turn increased the expression of anti-apoptotic protein Bcl-2. Low-temperature cultivation may be causing cell cycle arrest indirectly, by reducing the rates of global transcription/translation and accordingly resulting in lower cyclin levels [103].

Another advantage of temperature down-shift is the reduction of specific nutrient consumption. This way, energy utilization can be shifted towards product synthesis [74,86,104]. CHO cells manage biomass synthesis through glutaminolysis by metabolizing organic nitrogen that generates ammonium [105]. Nucleotide sugars are one of the key factors determining the final glycan structure of an Immunoglobulin G (IgG) mAb, as they promote sugar chain elongation reactions. During glycolysis, glucose is converted to glucose-6-phosphate (Glc-6P) and fructose-6-phosphate, from which Glc-6P is enzymatically reacted to generate UDP-glucose (UDP-Glc), UDP-galactose (UDP-Gal), and UDP-glucuronic acid (UDP-GlcA). These reactions then become the building blocks for galactosylation [106].

Several studies on temperature downshift in CHO cell cultivations resulted in cell growth inhibition, suppression of media consumption, maintenance of high VCD, suppression of impurity release, and cell cycle arrest at G1 phase [95,107]. An example can be given as a study conducted at mild hypothermic conditions, at 32°C, using CHO cells [108]. In this study, VCD was maintained for a longer period compared to standard 37°C condition, nutrient and nucleotide sugar donor metabolism rates decreased, and specific mAb productivity increased by 20 percent. Limitation in terminal galactosylation on the mAb Fc region was detected by glycan profiling. Plus, Galactosyltransferase (GalT) expression and nucleotide sugar donor production decreased.

Biphasic culture is frequently applied to improve the production process. This is a cultivation type where normal cell growth is initially achieved at 37°C and when the desired cell density is reached, the temperature is decreased [86,93]. Temperature shift on production day five (PD5) to either 33°C or 35°C was investigated by Kishishita *et al.* [109]. They observed no significant effect on mAb production through temperature downshift. However, temperature downshift suppressed the levels of acidic charge variants and the lowest levels were observed at 33°C, implying a 46 percent decrease compared to the control condition. Biphasic cultivation at 32°C was examined by Fox *et al*. [110] under multiple shift day conditions, at PD1, 2, 3, 4. Specific productivities increased at PD3 shift by 90 percent, compared to 37°C cultivation, and by 40 percent compared to 32°C cultivation, without any temperature shift days applied.

With all these studies that show the impact of temperature on mAb quality, it is worth to notify that the ideal temperature should be systematically analyzed, developed, and evaluated for each cell line and for the protein to be produced, individually.

1.4. RECOMBINANT CELLS FOR mAb PRODUCTION

Mammalian systems are preferred as expression systems for commercial therapeutic protein production because they exert efficient PTMs on the produced proteins [42,73,111]. Glycoforms produced in murine expression hosts may present immunogenicity [22], which generally induce ADA responses in humans. Orthoclone OKT3 anti-CD3 antibody is a good example. This was the first mAb to be approved for human use in 1986, against transplantation rejection [112]. Due to human anti-murine antibody (HAMA) responses, it induced cytokine release syndrome and caused accelerated clearance of the drug [113,114]. Thus, humanization greatly reduces immunogenicity in humans.

Most of the approved mAbs are produced in CHO cells. Other immortalized cell lines such as NS0, BHK, HEK-293 are used for a few individual products, but they are not considered as significant as CHO cell lines (Figure 1.8) [115].

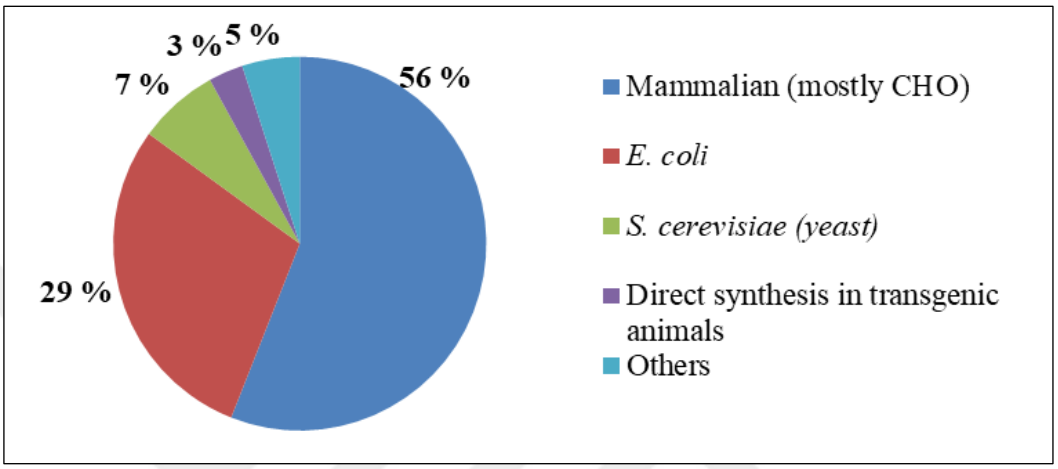

Figure 1.8. Expression systems employed manufacture approved biopharmaceuticals. Reprinted with permission from Foster Printing at Mossberg and Company Inc., www.mossbergco.com [116].

Stable cell lines were initially generated by non-viral gene delivery from immortalized cells, back in the early 1980s [117–119]. HEK-293, for instance, is a cell line transfected with sheared adenovirus DNA [120].

1.4.1. Recombinant CHO Cells and Their importance

Chinese hamster ovary (CHO) cells are considered as the gold standard due to the similar structure of glycans they produce to those naturally synthesized by human cells, their safety regarding human use, as well as their robust growth profile contributing to high mAb production [121]. They can produce Abs with complex glycosylation and PTM profiles in large amounts [122]. Compared to other host systems, the glycans they process are the most similar to human IgGs (Figure 1.9) [39,71].

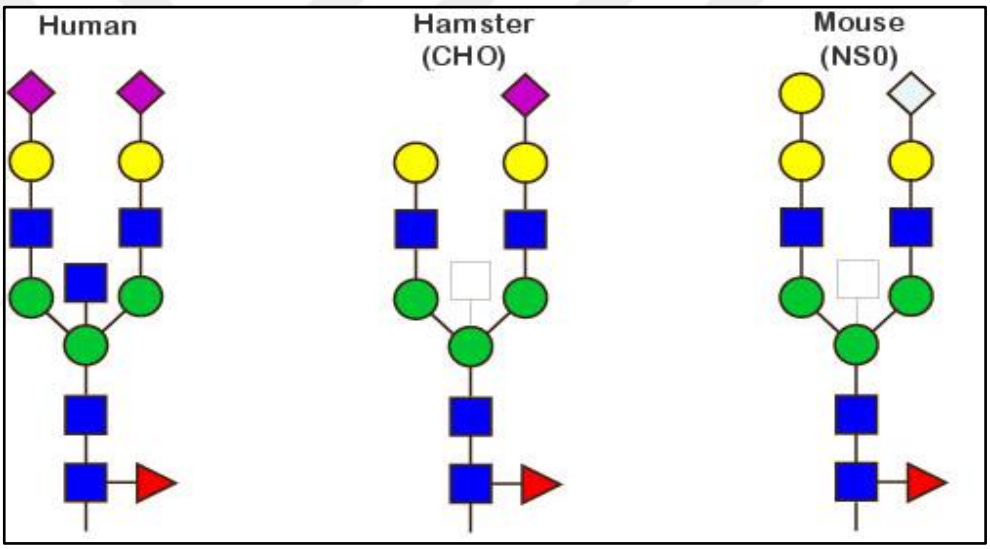

Figure 1.9. Glycan structures of Human, CHO cells, and NS0 cells. Reprinted with permission from IMGT®, www.imgt.org [123].

Mammalian expression systems for mAb production are preferred for over 92 percent of the commercial bioprocesses globally, and CHO cells are employed for 60 percent of these molecules [18].

CHO cells have a complex history including mutagenesis, adaptations to different growth conditions, and cloning. In 2011, Xu *et al.* [124] published the genomic sequence of an ancestral CHO genome, K1. The genome may be far from being representative of clonal cell lines cultivated in today's large-scale bioreactors. Cell culture process control is key to improved cell line characteristics.

Some published CHO karyotypes demonstrate that these cells are highly variable and that they are genetically complex. Cell line engineering is one of the major upstream development approaches to improve productivity while maintaining high product quality in CHO cells [125,126]. The ease of handling and rapid growth of CHO cells are the keys for the studies on gene expression and functioning. The availability of certain auxotrophic CHO mutant cell lines facilitated the generation and identification of recombinant CHO cells. Also, improvements in cell line characteristics achieved through cell culture process control in today's technology increased the importance of CHO cells in the pharmaceutical industry.

Protein manufacturing using CHO cells began in 1957, with cells 'spontaneously' immortalized from a primary culture of ovarian cells from a Chinese hamster, *Cricetulus griseus*. A proline-dependent clonal strain, CHO-K1, was derived from the original cell line. All the industrial cell lines in use today are proline-dependent [115]. The first CHO-derived recombinant cell lines produced interferons and tissue-type plasminogen activator (tPA) [119,127,128]. Activase, a recombinant tPA marketed by Genentech in 1987, was the first FDA-approved recombinant therapeutic protein made from animal cells cultivated in bioreactors. Suspension culture-adapted CHO cells were cultivated in large-scale stirred-tank bioreactors for the production. Epogen, marketed by Amgen, is the second approved protein to be produced in mammalian cells.

Stable cells, cells that are engineered by the insertion of the desired gene or genes of interest into their genome, are required for robust production. To obtain stable cells to be used in antibody production, the clonal selection is applied. Stable transfection differs from transient transfection by introducing DNA into desired cells for long-term, rather than several days.

A selectable marker is chosen for transfecting genes that provide resistance against certain antibiotics or genes that block enzymatic activities, regarding the selection system used. The stably transfected cells are determined by culturing the cells in a selective medium, as those which could not incorporate the selective gene marker will not survive.

Selection based on dihydrofolate reductase (DHFR) activity is a widely used method. DHFR is an enzyme ubiquitously expressed in all organisms. It has a key role in folate metabolism, cell growth, and proliferation. It also catalyzes a reaction for the *de novo* production of glycine and purine synthesis. Another selection marker used is the glutamine synthetase (gs) gene. GS is the enzyme that catalyzes glutamine synthesis from glutamate and ammonia. Selection based on GS activity was initially used for murine NS0-derived cell lines, but it is also used for CHO cells, mainly with CHO-K1 [115]. By supplementing the growth media with either of these, according to the selection system used, the probability of selecting cell lines with higher gene activity is increased, avoiding time-consuming gene amplification. A more specific selection methodology can be applied based on hygromycin and puromycin resistances of CHO clones, in which antibiotic resistance genes are used for transfection.

Growth rates and levels of recombinant protein production of each cell line vary due to the diversity in genotypes and phenotypes of cells, as well as the type and site of integration in the genome [115]. To obtain a few stably transfected cell lines with desired phenotypes, it is usually necessary to evaluate hundreds of clonal cell lines. Automated systems such as the ClonePix System are available for stable clone selection.

Once the clonal cell line selection is achieved, characterization for stability is required, because the expression of the integrated gene of interest (GOI) is not always maintained at a constant level over time. With reasons still not being clear, many clonal cell lines either lose or reduce the ability to synthesize mRNA driven from constitutive promoters. Thus, cell lines to be used in large-scale productions must undergo stability studies for several months before manufacturing processes. The major determinant of epigenetic gene silencing is the chromatin structure, the sequence composition or modifications of the histones and other

proteins, at the site of integration of the recombinant gene. The choice of the promoter or enhancer elements used to drive the GOI expression also influences the extent of gene silencing [115]. Scaffold/matrix attachment regions (S/MARs), insulators, antirepressor elements, ubiquitous chromatin opening elements (UCOEs) support more stable protein production in recombinant cell lines and increase the percentage of clonal cell lines with high expression levels [129–131]. These elements are small, so they can easily be cloned into the expression vector employed for GOI delivery.

1.5. C5 BLOCKER (C5mAb)

1.5.1. Importance of the C5 Blocker C5mAb in Market

C5mAb, a C5 blocker, is one of the most expensive drugs in the world, with net product sales of $3,946.4 million [132]. In Turkey, the contribution of novel drugs to the expansion in the drug market is 343 million TL in 2020 [17]. By the first quarter of 2020, the market value of biotechnological drugs reached 5.825,6 million TL, with an increase of 31.8 percent [17]. The current price of a single vial of 30 ml C5mAb in Turkey is 32,173.34 TL [17]. These statistics, together with the high price of C5mAb, show that the possible future market place of the biosimilar C5mAb is promising.

1.5.2. Clinical Importance of the C5 Blocker (C5mAb)

The humanized antibody C5mAb was approved by the FDA and the EMA to be used against Paroxysmal Nocturnal Hemoglobinuria (PNH) in 2007, and atypical Hemolytic Uremic Syndrome (aHUS) in 2011 [133]. Since last year, the usage of C5mAb has been expanded against Neuromyelitis Optica Spectrum Disorder (NMOSD) and Generalized Myasthenia Gravis (gMG). In November 2019, the C5mAb was approved for adults with NMOSD in Japan, where Phase 2 and Phase 3 studies in children and adolescents are ongoing. For gMG, a Phase 3 study in children and adolescents is underway. The humanized anti-complement component 5 (C5) monoclonal antibody binds to the α-chain of C5 and prevents C5 cleavage into C5a and C5b by the enzyme C5 convertase [134].

aHUS is a rare disease of complement dysregulation, leading to thrombotic microangiopathy and severe kidney damage [133]. Features related to the disease are hemolytic anemia, thrombocytopenia, acute renal insufficiency. There are two types of HUS diseases and the atypical form, which is the disease of interest regarding C5mAb, is unrelated to Shiga toxin.

Pathological conditions are caused by the insertion of the membrane attack complex (MAC) into renal capillary endothelial cells and by the recruitment of pro-inflammatory cells by C5a. Various types of antibodies and other compounds are being developed to inhibit C5, C5a as well as the antagonists of the C5a receptor [134]. C5a receptor antagonists target C5a alone, not C5b, so MAC can not be inhibited [134]. Thus, antagonists may lessen the risk of infection by pathogens. C5a receptor is expressed by hematopoietic stem cells, but also by non-immune cells such as vascular endothelial cells, liver cells, kidney cells, lung cells, spleen cells, and cells of the gastrointestinal tract [135]. Long-term studies are required to show whether blocking C5a has unanticipated effects.

PNH is a rare form of hemolytic anemia, caused due to an acquired genetic deficiency of endogenous complement inhibitors on the surface of blood cells. These cells are deficient in proteins that are normally linked to cell surfaces by a glycosylphosphatidylinositol (GPI) – anchor. PNH cells are clonal and mutations occur in multipotent hematopoietic stem cells (HSCs) [136]. PNH blood cells miss the wide array of GPI-anchored proteins from their surface but are normal regarding their physiological functions, which are fighting infection, clotting, and O_2 carriage [137].

NMOSD is a rare and severe inflammatory disease. It is a type of central nervous system disorder affecting optic nerves and the spinal cord through immune-mediated demyelination and axonal damage (NMOSD). The disease is caused by the selective binding of NMO-IgG antibody to the aquaporin-4 (AQP4) protein, leading to severe visual loss, limb weakness, sensory loss, and bladder dysfunction.

Also a rare disease, gMG is an autoimmune, chronic, and progressive disease in which antibody production against the acetylcholine receptor (AchR) leads to impaired electrical signal transmission from the nerves to the muscles (gMG). The primarily affected muscle groups are the head, limbs, spinal, eye, and respiratory muscles (gMG). The disease becomes life-threatening in case the respiratory muscles are affected, causing myasthenic crisis (gMG). The global gMG treatment market size was estimated as $1.16 billion in 2018 and

it is anticipated to grow at a compound annual growth rate of 7.5 percent over the forecast period until 2026 [138].

1.5.3. The Complement System

The complement system is activated upon any detection of pathogen-associated molecular patterns or danger-associated molecular patterns in the body. Over twenty serum proteins interact in a series of enzymatic cleavage and membrane binding events for the generation of products with immunoprotective, immunoregulatory, proinflammatory, cytolytic properties [139,140]. The activation of the complement system is crucial in the elimination of microbes, modulation of the adaptive immune system, clearance of immune complexes and apoptotic cells, inflammation, and tissue regeneration [141,142]. The complement system has two main parts with distinct roles, the proximal complement, and the terminal complement.

The proximal complement processes through three distinct pathways, namely the alternative, classical, and lectin pathways. All these pathways result in the generation of C3 convertase complexes, which mediate the cleavage of C3 to C3a and C3b [134,139]. The classical pathway is initiated by the interaction of C1q with the antigen-antibody complexes. The lectin pathway, on the other hand, is activated by the interaction of mannose-binding lectin (MBL) with specific mannose containing carbohydrate structures. Lastly, the alternative pathway is initiated by the deposition of preformed C3b on a variety of substrates such as bacteria or cell membranes.

C3 convertases consist of both one molecule of C3b and the serine protease Bb (C3bBb convertase) generated by the alternative pathway, or one molecule of C4b and serine protease C2a (C4bC2a convertase) generated by the classical pathway and the lectin pathway [143]. These enzymes cleave soluble, circulating C3 leading to the formation of membrane-targeted C3b and the release of anaphylatoxin C3a fragment [144,145]. Membrane-bound

C3b assembles into C3bBb and amplifies the activation signal. C3b and its proteolytic fragments trigger phagocytosis and modulate adaptive immune responses via B-cell stimulation [142]. C3b is continuously available due to the interaction of C3 with water. It is also necessary for the amplification and the progression of the complement cascade through all the pathways of activation, which makes it a key immunoprotective and immunoregulatory molecule [137]. Additional molecules of C3b associate with C3 convertases and form C5 convertases when complement activation levels are high [146–150]. The enzyme activity is in turn changed, so now it preferentially cleaves C5 rather than C3, generating C5b and C5a. This results in the attraction and activation of neutrophils, monocytes, and mast cells [151]. C5 cleavage leads to the formation of two fragments: C5a, the small fragment, and C5b, the large fragment [133]. C5a is a potent proinflammatory anaphylatoxin, which signals through C5aR1, triggering increased vascular permeability on endothelial cells, leukocyte chemotaxis, and oxidative burst on phagocytes. It also triggers the release of proinflammatory molecules, activation of the adaptive immune system, alters smooth muscle tone, and induces secondary inflammatory mediators such as hydrolytic enzymes, reactive oxygen species, arachidonic acid metabolites, cytokines [152,153]. C5b can associate with C6, C7, C8, and 12-18 copies of C9, leading to the formation of MAC, which then forms C5b-9, the terminal complement complex (TCC) [133,137]. MAC inserts into membranes of pathogens lacking a cell wall. As a result, lytic pores are formed [154,155]. Host cells are normally protected from MAC lysis by membrane-associated GPI-anchored regulators CD55 and CD59 [156,157].

All the pathways of complement activation converge at the cleavage point of C5 into C5a and C5b by C5 convertase enzyme complexes, C4b2a3b and C3bBb3b [150,158,159]. This way, the terminal complement cascade (TCC) becomes initiated [137]. C5a receptors are present on cell types that directly contribute to inflammation, namely monocytes, macrophages, and neutrophils [137]. The C5a receptor CD88 is a member of the N-formyl peptide receptor family. It transduces signals through a G protein-dependent pathway, resulting in biochemical proinflammatory responses [160]. Another C5a receptor, C5L2, has also been identified [161]. The non-hindered assembly of TCC on cell surfaces leads to cell lysis [137]. The deposition of TCC on erythrocytes, on the other hand, results in the destruction of erythrocytes in hemolytic diseases such as PNH [162].

C5 is an important target as it is common to all the pathways of complement activation. Blockade at this point stops the progression of the cascade regardless of the stimuli [137]. Prevention of C5 cleavage blocks the generation of potent pro-inflammatory C5a and cell lytic TCC [137]. C5 blockade preserves critical immunoprotective and immunoregulatory functions of upstream components that result in C3b-mediated opsonization and immune complex clearance. In case the complement system regulation fails, inflammation, autoimmunity, or tissue damage can arise due to infectious diseases [163]. Inhibition at the level of C5 prevents the formation of C5a and MAC via C5b but allows the generation of anaphylatoxin C3a and cellular opsonization by C4b and C3b [151,164].

1.5.4. The Engineering and Functionality of C5mAb

The discovery and development of the C5mAb humanized antibody were accomplished in 1996. To begin with, panels of murine anti-human C5 mAbs were generated and screened for their ability to inhibit TCC-mediated lysis of antibody-sensitized chicken erythrocytes by human complement, in a standard hemolytic assay [165]. Most potent candidates were cloned, purified, and their functional activities were further characterized to identify anti-C5 antibodies that can also effectively block the generation of C5a [137]. Almost 30.000 hybridomas were screened. Finally, one monoclonal antibody, the murine 5G1.1, was identified to achieve both. Murine 5G1.1 mAb binding to human C5 was then mapped to the N-terminal region of the α-chain [137]. In the following engineering studies, complementarity-determining regions of m5G1.1 mAb were cloned and grafted into human heavy and light chain antibody frameworks to reduce the potential for immunogenicity (Figure 1.10) [165,166]. The human heavy chain framework was derived from the H20C3H antibody variable region, which contained no amino acid changes relative to the original germline sequence [167]. On the other hand, the human light chain framework was derived from the I.23 antibody variable region [168], which contained only one amino acid difference from the original germline sequence.

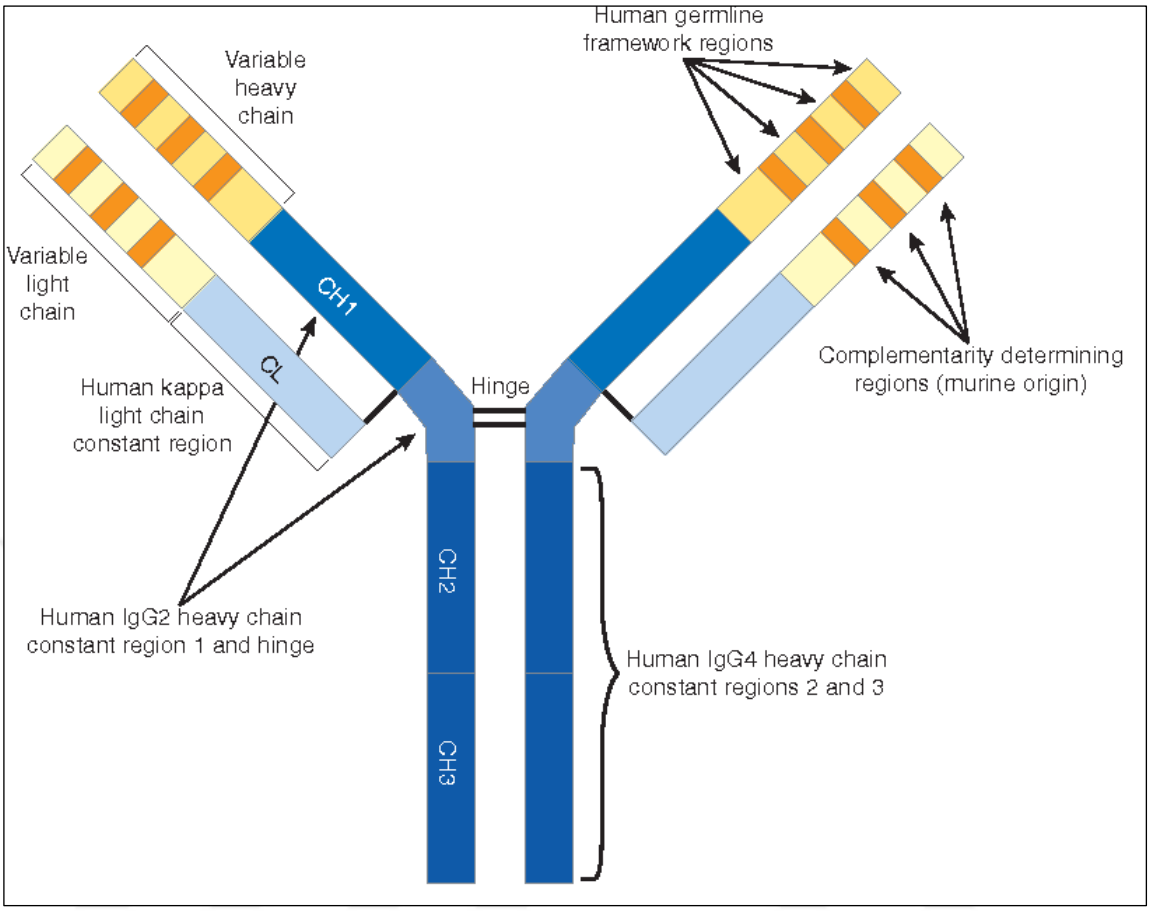

Figure 1.10. Engineering of C5mAb [137].

Sometimes, the use of antibodies in therapeutics becomes complicated by the effector functions of such antibodies that serve to activate the complement and/or to bind to antibody receptors (Fc receptors) on inflammatory cells after they bind to their target [137]. To reduce this risk of eliciting proinflammatory responses, the heavy chain constant region of the parental antibody was replaced with components of both human IgG2 and IgG4 constant regions [169]. The human IgG2 antibody isotype does not bind to Fc receptors and the human IgG4 isotype does not activate the complement cascade [170–172]. The IgG2 / IgG4 hybrid constant region of the C5mAb includes the CH1 and hinge regions of human IgG2, fused to the CH2 and CH3 regions of human IgG4, and cannot bind Fc receptor as well as to activate the complement cascade [169]. To avoid the generation of an antigenic site during fusion, a restriction endonuclease site common to both IgG2 and IgG4 was used [137].

C5mAb is highly species-restricted, showing minimal activity against any other primate or other mammalian C5. No non-specific binding to any human tissue was observed, regarding tissue cross-reactivity studies [137]. The affinity of C5mAb to human C5 has a K_D value of 120 pM, which is similar to that of parental m5G1.1 monoclonal antibody. This indicates that the binding properties were conserved during the humanization process [137]. The half-life of C5mAb was found to be 272 ± 82 hr, with a distribution primarily limited to the vascular space, according to intravenous administration studies. C5mAb serum concentration reached a steady state after 57 days [137].

In vivo pharmacological activities were investigated using anti-mouse C5 antibody, BB5.1, due to the species-restricted nature of C5mAb [137]. No mouse models exist for PNH, so *in vivo* studies targeted other therapeutic indications that involved terminal complement activation. These models are the collagen-induced model of arthritis, renal disease, lupus-like autoimmune model [173]. All models demonstrated that functionally blocking the anti-C5 antibody effectively and consistently blocked terminal complement activation. The same mouse antibody was used in mouse toxicity studies [137].

Initial human clinical studies of C5mAb were performed in individuals with rheumatoid arthritis and with systemic lupus erythematosus [174], to determine the dosing schedule and the safety of terminal complement inhibition in humans [137]. A dose range between 0.1 – 8 mg/kg was examined. The results demonstrated that all doses within the range were well-tolerated and safe. A dose of 8 mg/kg (600 mg total) provided complete complement blockade for 7-14 days [137]. C5mAb binds to the N-terminal region of the α-chain of human C5, providing complete inhibition of terminal complement activity in both PNH and aHUS [165]. It was also shown to bind C5-derived peptide fragments containing the KSSKC motif (residues 879-883) within the C5 macroglobulin (MG) 7 domain [175], distal to the scissile bond (R751-L752) that is cleaved by the convertase. This binding characteristic was observed in a small population of C5mAb resistant patients with PNH, who possessed Arg885His/Cys polymorphisms [176]. The study was conducted by preparing a Fab molecule with the same sequence as that of C5mAb in the V region. The structure of the complex between the Fab and the complement C5 was determined, accordingly. The results

suggested that C5mAb functions by sterically hindering the convertase from associating with C5, but not by directly preventing access to the scissile bond.

Cases of primary non-response to treatment in few patients were observed following C5mAb treatment [176,177], due to a point mutation in C5. This mutation lies in the macroglobulin 7 (MG7) domain, which is the location of the C5mAb epitope – the 'KSSKC' epitope [176]. As the C5mAb is highly specific to humans, it does not bind to the C5 of chimpanzees, baboons, rhesus, and cynomolgus monkeys [178]. Chimpanzee C5 differs from human C5 only by two amino acids in the MG7 domain located outside the KSSKC epitope, indicating that additional residues contribute to C5mAb binding [179]. Orthogonal techniques were carried out combining sequence analysis, protein-protein docking studies, and targeted peptide mapping of the C5mAb epitope. According to the results, 13 positions are involved in the binding of C5 convertases to the human C5 [179]. Two of these are subjected to variations in the KSSKC epitope, one at position 885, which is polymorphic in humans, and the other at position 917, which is specific to humans. R885 and W917 were found to be key species-specific residues. The results also demonstrated that the C5mAb conformational epitope composes of a ß-strand (884-890) and a ß-hairpin (913-922). The 'VRQKV' epitope on ß-strand overlaps with the 'KSSKC' linear epitope in its C-terminal region and it contains the polymorphic R885 residue (R885C or R885H), which is associated with primary non-response to C5mAb (Figure 1.11.).

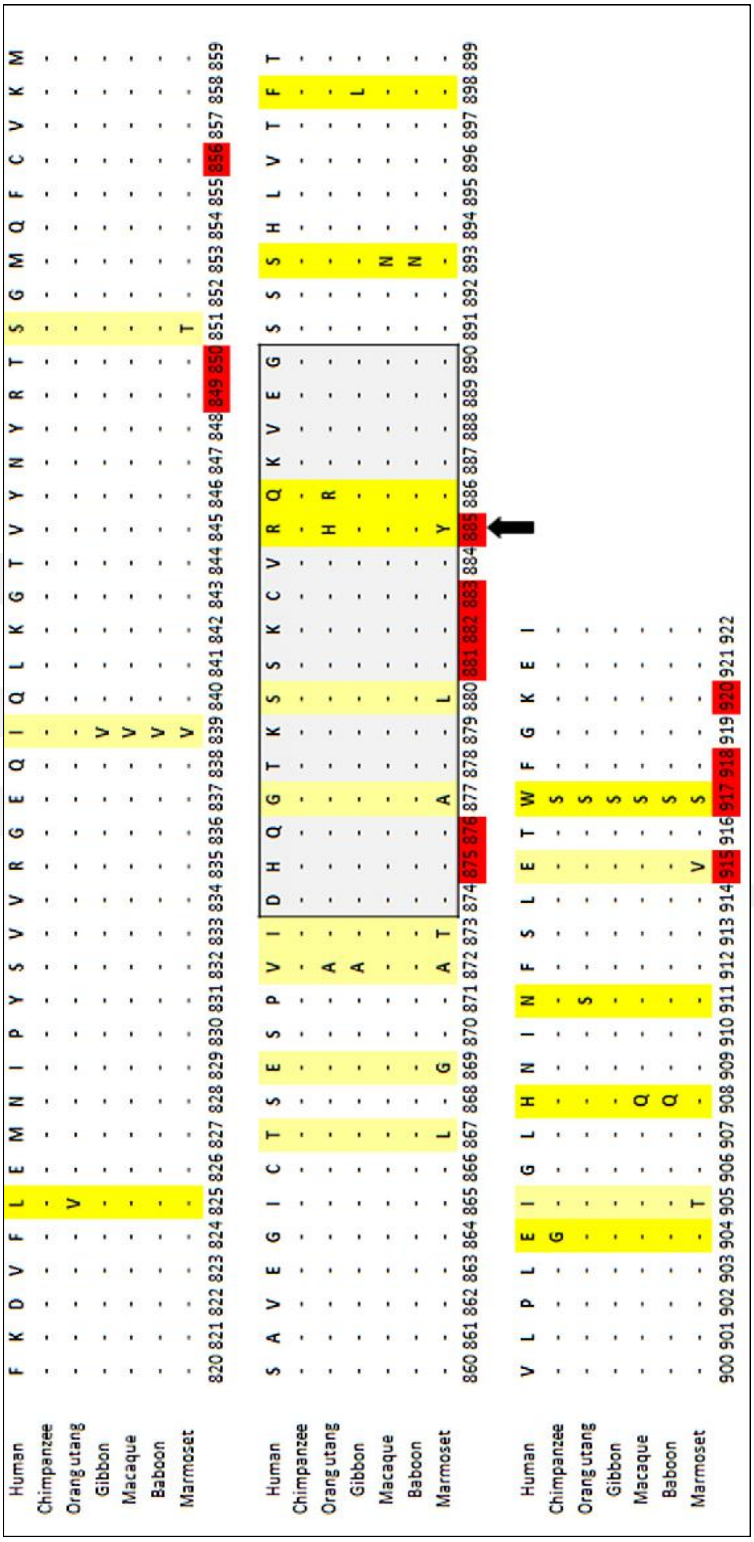

Figure 1.11. Alignment of MG7 domains of C5 from human and non-human primate species [180].

1.6. MULTIFACTORIAL STATISTICAL ANALYSES AND DESIGN OF EXPERIMENTS (DoEs)

1.6.1. Importance of Multi Factorial Statistical Analysis in Biologics Development

Quality, safety, and effectiveness need to be 'designed' and built into a product and its manufacturing process [181]. This brings up the term 'Quality by Design (QbD)'. QbD, as defined by the ICH, is a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding, and process control, based on sound science and quality risk management [182]. Bioprocesses aiming at the manufacture of active pharmaceutical ingredients (API) are influenced by complicated input and output parameters such as critical process parameters (CPPs) and critical quality attributes (CQAs) [181]. Thus, when designing quality parameters to build into the process, one must begin with the selection of process parameters and their ranges of investigation.

Parameters such as temperature, pH, reaction time can all be set at desired levels and finding the optimal settings and conditions for several factors is accomplished by optimization [183]. All the factors and their level ranges form an experimental domain [183]. These factors might interact with each other and the influence of one of the factors might be different at different levels of a second factor. This is defined as 2-factor interaction [183]. If the optimization of just one factor is the aim, a univariate procedure such as the one-variable-at-a-time (OVAT) approach is used [183]. However, the OVAT approach has some disadvantages. Interactions between factors, in this case, are not taken into account and many experiments become a requirement if the factor number increases. Plus, just a part of the experimental domain can be examined, which may not lead to the global optimum and the optimal conditions found may depend on starting conditions [184]. Multivariate procedures, on the other hand, provide wider range of analysis by varying several factors simultaneously.

1.6.2. DoEs – Definition, Importance, and Aim of Application

Considering the possibility of factors influencing the response of others, the Design of Experiments (DoEs) are crucial in statistical analyses. DoE is a structured, organized method for determining the relationships among factors affecting a process and its related outputs [182]. DoE applications provide companies with substantial benefits. Companies can gain the maximum number of information by conducting only a minimum number of experiments, can study the process effects individually by varying all the operating parameters at the same time, while taking into account the variability in experiments, processes, or raw materials as well [181]. By employing DoEs, companies can identify interactions among different process parameters, characterize acceptable ranges of key and critical process parameters contributing to the identification of a design space for the assurance of quality [181]. It should be pointed out that, working within a design space is not considered as a change, that is, execution of DoE within a design space is accepted to be safe under QbD in bioprocess industries [182].

For applications using the DoE approach, setting objectives that are specific, measurable, attainable, realistic, and time-based is crucial as an initial step. Knowledge and qualification in fields of process development, quality control, statistics, and engineering are required for robust analyses. Considering the design of a given process, DoE methods provide an understanding of the effects of possible multi-dimensional combinations and interactions of various parameters on the final drug quality [185], multiple process parameters, and raw material attributes on product CQAs, while leading to the establishment of a ‘design space’ and control strategies regarding manufacturing.

Selecting appropriate responses is an important step. Quantitative measurement is required to determine any changes that may have occurred due to varying levels of input process parameters [181]. Repeatability and reproducibility (R&R) errors are other factors to be considered. These are errors in measurement due to any variation in operators, assays, as well as their interactions. They are crucial to be able to address variability associated with

measuring a response variable before starting a DoE study. R&R errors can be partly compensated for by including replicates in a design [181]. Random variability is considered as noise and can be eliminated by applying three basic DoE principles: blocking, randomizing, and replication. Blocking takes into account known sources of variation that may affect a process, but are not a cause of great concern such as different flask locations in an incubator, which might cause slight differences in measurements due to heat transfer. Blocking is useful when conducting simultaneous experiments in different equipments by different operators [186]. Randomization satisfies the statistical assumption that observations are distributed independently and randomly among each other. These observations impact the processes in an uncontrolled way. The ambient temperature can be given as an example of such errors [186]. Lastly, replication accounts for random variation utilizing replicating all or selected experiments within the ranges of a DoE design matrix [186]. If robustness can be achieved in a DoE design, center-point runs at set-point conditions can be used to set specification limits for process parameters during validation [185], as well as to validate scale-up or scale-down models of a process [188]. Minimization of random variation and ensurance of repeatability are required to start a DoE study [181].

Experimental designs provide a simultaneous evaluation of several factors at given numbers of levels, in a pre-defined number of experiments. The choice of experimental design depends on the nature of the study, whether it aims at screening, optimization, or robustness. Also, the resources available to conduct the study - such as time, materials, labor, and cost – impact the design process.

Experimental designs are mainly divided into screening designs, response surface designs, and mixture designs. Screening designs allow screening a relatively large number of factors in a relatively small number of experiments. The factors and interactions to be studied are critical in this context. To be able to identify the most influencing factors, evaluations are mostly done at 2-levels. Full factorial, fractional factorial, Plackett-Burman designs are the screening designs frequently used.

Designs to be applied are dependent on the number and type of factors, the aim of their use, and the preference of the analyst [183]. In the scope of pharmaceutical manufacturing, optimization of formulations, products, and processes is critical and shall be handled in absolute sensitivity. For the optimization of formulations, products, and processes, mostly screening designs in 2-level are utilized, enabling the identification of the most important factors among those that are potentially affecting the responses. Fractional factorial or Plackett-Burman can be used for these optimizations [184,186,189].

DoE applications provide avoidance of experimental biases by reducing the number of experiments to be carried out, so they are very effective when planning factorial experiments to determine important experimental variables in a bioprocess, as well as when optimizing the variables in a biotechnical system [190–192]. DoEs are advantageous over methodologies that vary one variable at a time [193]. The factorial design provides reliable results with few experiments. Afterward, the following analyses can be estimated to find the desired optimum [193].

The desired approach to begin with a DoE methodology is to conduct screening experiments from selected corners in the experimental step. Using a fractional factorial design, a fraction of all the possible values can be analyzed [193]. Screening is then improved by including replicates in the center point of the experimental domain [191]. However, when the number of factor experiments is reduced, the statistical quality decreases [193]. Systematic error is avoided by performing the whole set of experiments at random [193]. A screening study provides the identification of a few crucial factors in bioprocessing. These factors are then used to set a new experimental design to evaluate the optimum values [193].

DoE methodology in bioprocesses provides an examination of defined input factors that are further subjected to a converting biosystem that generates common and well-defined output factors, the responses, such as product yield and productivity [193]. The advantage of applying DoE over other methodologies is that it also provides a determination of how interactions between input factors impact the output responses [193]. Biotechnology

processes are complicated due to shifts in metabolism and conversions of nutrient factors during the process. Also, not all input factors are settled from the beginning of the cultivation, some are added later in the process [193].

For a robust experimental design of a given bioprocess, clone selection is pivotal as all process parameters depend on the clone of choice, starting from the media and feed selection [194,195]. Production needs such as supporting cell growth, increasing productivity, or maintaining by-product formation under certain limits will be different for each cell line and clone as well. Thus, their nutritional requirements will also be unique and shall be screened and optimized, necessarily [196]. When cost and limited time factors are to be considered together with the significance of nutrient selection regarding cellular and product qualities, implementation of high-throughput (HTP) methods becomes a necessity in process development studies. Using HTP methods, a large number of screening experiments can be conducted at small scales, reducing analysis time and costs [85]. Furthermore, once HTP methods are combined with statistical experiments such as DoEs, it becomes highly advantageous and reliable to design and conduct process development studies.

Once screening experiments are conducted at small scales, the robustness and efficiency of processes should be confirmed and fine-tuned in pilot-scale for successful scale-up. The implementation of Single-Use (SU) systems has improved the reliability of processes – such as contamination-, introduced flexibility and reduced costs as they eliminate the need for sterilization and cleaning [197]. SU Bioreactors (SUB) have gained interest in the process development field, especially for companies aiming at Good Manufacturing Practice (GMP) productions. SU systems, which are available up to 2000 L, are well applicable for GMP manufacturing [9,197].

2. AIM OF STUDY

Conceiving the worldwide competition in the biosimilar market, it should not be surprising that Turkey, as an emerging country in terms of biotechnology and health services, will find itself in the middle of this expanding contest. Turkey is already involved in the biosimilars market with the approval of infliximab, to begin with.

As the patent expiry dates of certain important pharmaceutical drugs are approaching, it might be the perfect timing for Turkey to rise in the market, with the development of a biosimilar drug, which is one of the most expensive drugs in the world market. Apart from the economical point of view, this discovery and development process would ensure Turkey's force both in the biotechnology and health sectors, as well as pave the way for new advances in these fields.

According to all the literature data introduced above, the key step in the development of a biosimilar is the bioproduction process. Findings show that the expression system, the production conditions such as culture pH, temperature, DO levels, dissolved CO_2 levels, agitation in bioreactor systems are the most crucial factors affecting structural characteristics of a given monoclonal antibody, thus the level of its biosimilarity. So, the biosimilar pharmaceutical development of a desired monoclonal antibody shall begin with a robust, well-optimized bioprocess.

In this study, we aimed to develop the most optimal bioprocess conditions for a biosimilar of the C5 mAb. The clonal selection and the optimization strategy, using DoEs, are expected to add uniqueness and robustness to the development process.

3. MATERIALS AND METHODS

3.1. HOST CELL LINE GENERATION

Twelve stable CHO-M clones producing an IgG2 - IgG4 hybrid monoclonal antibody were generated through super transfection and the clonal selection was achieved based on hygromycin and puromycin resistance. Monoclonality was ensured using ClonePix 2 System (Molecular Devices), together with a high-throughput micro-well image cytometer.

3.2. CELL CULTURE AND MAINTENANCE

Cells maintained in liquid nitrogen stock were thawed rapidly, in a 37°C water-bath. Cells were sub-cultured in chemically defined basal 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).

3.3. IN-PROCESS CONTROL ANALYSIS

Samples of 1.5 ml volume were centrifuged at 200 × g, +4°C (Sigma 1-16K) to obtain supernatant and pellet samples to be used for in-process and quality analyses. mAb concentrations were analyzed using PA-HPLC (Waters Arc-Bio). Viability and viable cell density (VCD) were analyzed by Vi-CELL XR (Beckman Coulter). D-PBS (Gibco® Life Technologies) was used for sample dilutions, when necessary. Glucose, lactate, and pH

measurements were carried out using ABL90 FLEX (Radiometer). Osmolality measurements were done by Micro Osmometer 3320 (Advanced Instruments).

3.4. CELL GROWTH ANALYSIS

Cultured CHO cells were cultivated in vented spin tubes (Tubespin Bioreactor 50, TPP) to analyze two different inoculation densities in replicates, for the generation of a cell growth curve. Four batch cultures were initiated in 20 ml working volume, under 37°C, 5% CO_2, 80 - 85% RH, 300 rpm shaking conditions. The two spin tubes representing Condition 1 were initiated with 0.2×10^6 cells/ml and Condition 2 cultures were initiated with 0.3×10^6 cells/ml. Cells were counted every 24 hours until culture viability dropped below 60%. Sampling volumes were 250 µl between PD0-PD4, and 100 µl after PD5.

3.5. RESEARCH WORKING CELL BANK GENERATION

Research working cell banks (RWCB) were generated by expanding passage zero (PN: 0) cells until the population doubling level of 15 (PDL15). An initial clone elimination study was conducted with the 12 clones (E13-05, E13-08, E32-04, E32-14, E76-06, E76-08, E90-02, E90-22, E107-08, E107-19, E111-31, E111-36) to generate the cell banks and to launch a stability study. The eliminations were achieved regarding both the similarities of the major glycan compositions of these 12 clones to the originator molecule and the titers they produced. RWCB generation was conducted in parallel with the Stability-1 study, until PDL15.

To calculate the population doubling levels, the equation below was used where ‘n’ is the final PDL, ‘A’ is the final (reached) cell density, ‘B’ is the initial cell density, and ‘C’ is the doubling level of the inoculum used to initiate the subculture being passaged. Cell densities

were adjusted as 2×10^5 viable cells/ml (VC/ml) for a 3 - day passage, or as 3×10^5 VC/ml for a 2 - day passage, according to cell growth curve analysis.

$$n = 3.322 \text{ x } (log\ A\ -\ log\ B)\ +\ C \tag{3.1}$$

3.6. FED-BATCH CULTURES

Cells were fed with FeedA (HyClone Cell Boost 7a, Cytiva) and FeedB (HyClone Cell Boost 7b, Cytiva) as carbon and nitrogen sources. Culture glucose concentrations were maintained by glucose supplementation (D-(+)-Glucose, Sigma-Aldrich) from a stock solution of 350 g/L.

3.7. CLONE STABILITY

The stability-1 study was initiated with the six clones (E32-04, E76-06, E90-02, E90-22, E111-31, E111-36) selected according to the results of the initial clone elimination study. Cells with PN: 0 were thawed from stock for expansion in 125 ml vent-cap shake flasks. Once cells reached PDL15, PDL30, PDL45, and PDL60, in each of these steps, new cultures were initiated in replicates for 10 production days. Cells were fed with carbon and nitrogen sources under the standard feeding strategy, on production days (PD) of 3-6-7-8. Samplings were done on PDs of 0-3-6-7-8-9-10 for in-process control analyses, and both supernatant and pellet samples were taken after PD7 for quality analyses.

The Stability-2 study was initiated with two clones (E90-02 and E111-36) according to the results of the DoE-1 study. Culture supplementations, samplings, and analyses were conducted as done in the Stability-1 study. Clonal stability was assessed regarding the production yields and product qualities of cultures launched at different cell ages. The

experiments were conducted in replicates, using 50 ml spin tubes in 15 ml working volume, regarding a preliminary study performed in both 125 ml Erlenmeyer flasks and 50 ml spin tubes that compared both production conditions, and confirmed the reproducibility and robustness of the study in both culture containers.

3.8. DESIGN OF EXPERIMENTS AND STATISTICAL ANALYSIS

A total of 32 experimental runs for 16 possible combinations were carried out for the initial screening study, DoE-1. Four clones (E32-04, E90-02, E111-31, E111-36), two feeding strategies (‘extended’ and ‘distributed’), two temperature conditions (37°C and 35°C - PD7 shift) were screened in replicates. For the extended feed strategy, feeding was done on PD3-6-7-8-10-12 and for the distributed feed strategy, feeding was done each day between PD2 - PD12.

For the study, in-process control (IPC) parameters and production yields were evaluated. Viable cell density, per-cent viability, lactate concentration, pH, osmolality, and titer were analyzed, accordingly. For the experimental design, Design Expert (Dx7 software) program was used and General Factorial Design was chosen to identify the three best combinations based on production yields.

A second study, DoE-2, was conducted to scrutinize factors affecting process parameters. The study aimed to screen combinations of clone, temperature, and time-of-temperature shift (ToS) to produce a biosimilar C5 mAb, based on the production level and quality of the secreted protein. Twenty combinations were tested among 2 different clones under fed-batch culture conditions as one feeding strategy (extended feeding), 2 temperatures (35°C and 32°C), and 5 different ToS (PD3, PD5, PD6, PD7, PD8) platforms per clone. As the control group, 37°C cultures without temperature shift were analyzed. All the experiments were replicated.

Fed-batch cultures were carried out in 125 ml shake flasks, in 25 ml working volume for 14 days, and feedings were done on PD3-6-7-8-10-12 regarding extended feeding strategy.

Titer, cell-specific productivities (Qp), and major glycan compositions, namely G0, G0F-GN, G0F, G1F, G2F, and Man5 – were evaluated using the Design Expert (Dx7 software) Program.

The data were evaluated based on a confidence level of 95%, and $P \leq 0.05$ was considered to indicate a statistically significant difference.

3.9. PRODUCTIONS IN 3-L BIOREACTORS

The bioreactor productions were carried out in 3-L Single-Use Bioreactors (Mobius®, Merck), under fed-batch conditions for 14 days. Two bioreactors were run simultaneously, for each production step. pH, percent dissolved oxygen (DO), pCO_2 levels, and aeration were monitored in real-time, throughout the production processes. Both online and offline measurements of pH and pO_2 levels were checked daily to ensure probe calibrations. For offline pH measurements, both ABL90 Flex blood-gas analyzer (Radiometer) and pH meter (Seven Excellence, Mettler Toledo) were used to compare online-offline measurements. Foaming was controlled using FoamAway™ Irradiated AOF (animal origin-free) Antifoaming Agent (Gibco®) containing 3% simethicone. Antifoam was given on demand, provided that the amount did not exceed 5 ml/day. Production conditions are represented in Table 3.1. for BR-1, BR-2, BR-3, BR-4, and in Table 3.2., for BR-5, BR-6, BR-7, BR-8.

Table 3.1. Bioreactor production conditions of BR-1, BR-2, BR-3, and BR-4.

Bioreactor (BR)	**BR-1**	**BR-2**	**BR-3**	**BR-4**
Clone	E90-02	E90-02	E111-36	E111-36
Inoculum	0.25×10^6 cells/ml; >95% viability			
Production Media	BalanCD + 6 mM L-Gln			
Production volume	V_0: 1600 mL; V_{fin}: 2200 mL			
Feed Strategy	Extended Feeding			
Temperature	37°C	35°C – PD7 shift	37°C	35°C – PD7 shift
pH	6.9 ± 0.2			
pH Regulation	Base: 150 g/L Na_2CO_3 Acid: CO_2			
DO (%)	60%			
Agitation	180 rpm			
Glucose Target	PD6-14: 6.5 ± 0.5 g/L			
Antifoam	on demand			

Table 3.2. Bioreactor production conditions of BR-5, BR-6, BR-7, and BR-8.

Bioreactor (BR)	**BR-5**	**BR-6**	**BR-7**	**BR-8**
Clone	E90-02			
Inoculum	0.25 x 10^6 cells/ml; >95% viability			
Production Media	BalanCD + 6 mM L-Gln			
Production volume	V_0: 1600 mL; V_{fin}: 2200 mL			
Feed Strategy	Extended Feeding			
Temperature	32°C – PD7 shift	37°C	37°C	32°C – PD5 shift
pH	6.9 ± 0.2			
pH Regulation	Base: 150 g/L Na_2CO_3 Acid: CO_2			
DO (%)	60%			
Agitation	180 rpm			
Glucose Target	PD6-14: 6.5 ± 0.5 g/L			
Antifoam	on demand			

3.10. GALACTOSE SUPPLEMENTATION ASSAY

The galactose supplementation assay was conducted by feeding the cultures with two different galactose supplement concentrations under two different feed regimens. E90-02 cells were cultured in spin tubes of 15 ml working volume under 37°C, 5% CO_2, 300 rpm shaking conditions for 14 days of fed-batch production, with extended feed strategy. Cultures were supplemented with galactose (EX_CELL® Glycosylation Adjust (GAL+), Sigma-Aldrich), either as ‘Gal3’ or ‘Gal6’. Gal3 supplement was prepared by diluting the

stock galactose supplement with 1:10 D-PBS, whereas Gal6 was prepared by diluting it with 1:20 D-PBS. Each concentration was assayed by supplementing cultures under two different feed regimens, either on PDs of 2-4-6-8-10 or on PDs 3-6-7-8-10. Five different conditions, one being the control, were analyzed in replicates as represented in Table 3.3.

Table 3.3. Galactose supplementation scheme.

Conditions	
C1 (Control)	ActiCHO A (30%)/B (2.5%) with Extended Feed Strategy w/o Gal
C2	ActiCHO A (30%)/B (2.5%) Extended Feed Strategy **Gal 3** (PD2, PD4, PD6, PD8, PD10)
C3	ActiCHO A (30%)/B (2.5%) Extended Feed Strategy **Gal 3** (PD3, PD6, PD7, PD8, PD10)
C4	ActiCHO A (30%)/B (2.5%) Extended Feed Strategy **Gal 6** (PD2, PD4, PD6, PD8, PD10)
C5	ActiCHO A (30%)/B (2.5%) Extended Feed Strategy **Gal 6** (PD3, PD6, PD7, PD8, PD10)

3.11. TEMPERATURE REDUCTION ASSAY

E90-02 cells were cultured in spin tubes of 15 ml working volume under 37°C, 5% CO_2, 300 rpm shaking conditions for 14 days of fed-batch production with extended feed strategy. The cultures were initiated at 37°C and the temperatures were decreased to 30°C on either PD5 or PD7. Control conditions at 37°C were run simultaneously and all the conditions were replicated.

3.12. GLYCAN ANALYSIS

Rapi-Flour-MS™ (RFMS) kit (715004793EN, Waters, Milford) was used for *N*-Glycan analysis and all sample preparations were carried out as per the manufacturer's instructions. Samples were diluted to 2 mg/ml and denatured at 95°C for 5 min. Rapigest™ (Waters, Milford) was used to increase the unfolding of mAb and consequently enhance the enzyme activity. *N*-glycans were released from the mAb by incubating with PNGase F enzyme at 55°C for 5 min. Released glycans were labeled with RFMS dissolved in anhydrous dimethylformamide (DMF) for 5 minutes at ambient temperature. Derivatized *N*-glycans were separated from the mAbs by solid-phase extraction (SPE) micro elution plate. The *N*-glycans were eluted from the SPE plate with 200 mM ammonium acetate in 5% acetonitrile (ACN). Eluted samples were further diluted with 100 µl of DMF and 210 µl of ACN for injection to Acquity H-class Bio UPLC with fluorescence detector coupled to a XEVO G2-XS QToF-MS (Waters, Milford). Samples were prepared in replicates. Waters UPLC Glycan BEH Amide column (2.1 × 150 mm, 1.7 mm particle size, 130 Å pore size) was used for HILIC separation. 50 mM ammonium formate (pH 4.4) was used as Mobile Phase A and ACN was used as Mobile Phase B. The elution flow rate was 0.4 ml/min and gradient conditions ranged from 75 - 54% Mobile Phase B over 35 minutes. Excitation and emission wavelengths were 265 nm and 425 nm, respectively, for *N*-glycan detection. *N*-glycans were identified and quantified by utilizing a calibration curve derived from the RFMS labeled dextran ladder, and mass confirmation were done from Q-ToF MS data in UNIFI software v1.8 (Waters, Milford).

3.13. BINDING ASSAY

Wieslab® Complement system Classical pathway ELISA kit (COMPL CP 310, Svar Life Science AB) was used to investigate the binding potency of the developed C5-mAb candidates. Two different originator lots were included in the assay and one was chosen as a reference (Originator-1). All samples were run in replicates. The analysis was done with SpectraMax İ3x (Molecular Devices) using SoftMax Pro 7.1 GxP Software. End-point readings were done at 405 – 595 nm. Data analysis was achieved by fitting the mean absorbance values to a 4-parameter logistic curve with non-linear regression to obtain EC50 values, where EC50 values represent half-maximal effective concentration and %CV is the coefficient of variation. The EC50 values were then used to calculate the relative potency (%) for each sample, with 80 – 120% range as acceptance criteria.

4. RESULTS AND DISCUSSION

4.1. CELL GROWTH ANALYSIS

The growth phases of CHO cells under two different seeding densities were identified by counting the viable cell densities of cultured cells every 24 hours until cell viability dropped below 60%. The complete logarithmic phase of cell growth started on PD2 for cultures initiated with 0.3 x 10^6 cells/ml CHO cell density (Condition 2), while it was observed on PD3 for cultures with 0.2 x 10^6 cells/ml seeding density (Condition 1) (Figure 4.1.). As cells require a certain density for expansion, cell growth profile is dependent on the concentration of the cell population. Thus, more seeding density will result in earlier initiation of the logarithmic phase, compared to a culture seeded with a less concentration of cells. Once cells entered the logarithmic phase, growth started to proceed at a similar rate. Cells entered their death phase after PD5, with declining VCD trends observed in Figure 4.1. However, the viabilities of cultures initiated with 0.3 x 10^6 cells/ml density dropped below 60% earlier than that of cultures initiated with 0.2 x 10^6 cells/ml. This makes sense because initiating a culture with relatively more cells in the same volume of culture media will result in earlier exhaustion of nutrients and other vital media components (i.e. amino acids). Accordingly, while the viabilities of cultures initiated with 0.3 x 10^6 cells/ml density dropped below 60% on PD6, viabilities of cultures with 0.2 x 10^6 cells/ml initial density dropped below 60% on PD7 (Figure 4.2.).

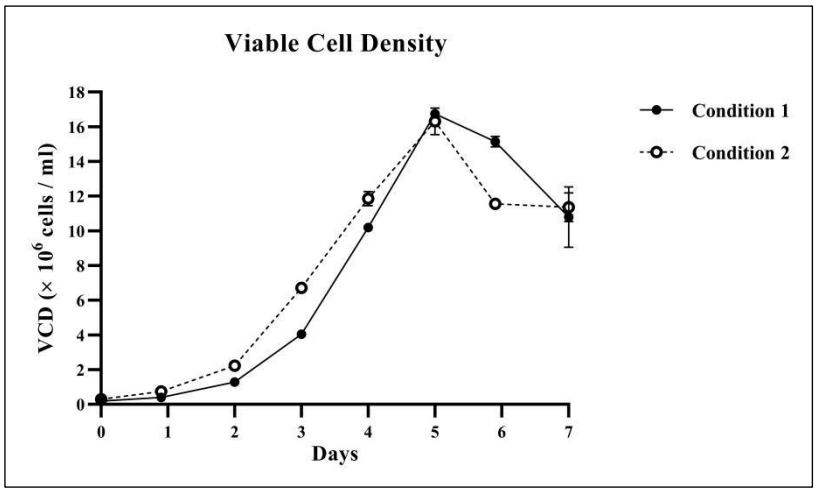

Figure 4.1. Cell growth curve representing the viable cell density results of cultured CHO cells under different seeding densities.

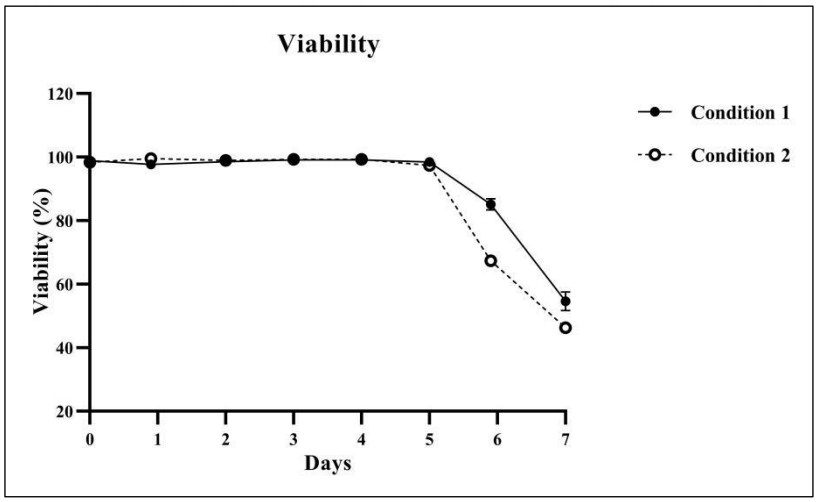

Figure 4.2. Cell growth curve representing the viability results of cultured CHO cells under different seeding densities.

4.2. CLONE ELIMINATION

The initial clone elimination was achieved among 12 different clones. The evaluation was based on the major glycan contents (percent) by the end of their production day of nine (PD9), regarding similarity to the originator molecule, as well as the protein concentrations that they produce.

Table 4.1. Major glycan compositions and titers of 12 clones.

Clone ID	% Glycan Contents					Titer (µg/ml)
	G0	Man5	G0F	G1F	G2F	
E13-05	0.7	3.3	73.8	13.9	1.7	410
E13-08	0.6	3.4	72.8	14.2	1.8	614
E32-04	0.7	3.1	75.4	15.2	1.8	1349
E32-14	0.8	2.9	75.6	15.7	1.8	882
E76-06	0.8	3.0	73.8	14.8	1.7	1240
E76-08	1.8	2.5	68.0	21.8	3.0	552
E90-02	0.6	3.4	72.6	15.9	2.1	1174
E90-22	0.7	2.7	73.2	16.5	2.1	1019
E107-08	0.7	3.3	68.5	17.8	2.6	1019
E107-19	0.8	2.7	68.4	20.0	3.1	994
E111-31	0.7	2.9	73.8	15.5	1.9	1172
E111-36	0.7	3.2	71.5	16.8	2.3	1218
ORIGINATOR	**0.2**	**3.6**	**72.2**	**16.3**	**2.0**	

There were no extreme differences among the major glycan compositions when the percent of contents represented in Table 4.1. were compared among clones. G0 contents of all 12 clones were higher, relative to that of the Originator. Man5 levels remained below 3.6% (Originator), which is a desired property regarding immunogenicity concerns. G0 represents the non-galactosylated structures in a total subclass of glycans, including G0F content, which is fucosylated. G1F and G2F contents, on the other hand, are generated by the addition of galactose structures. As these results were generated without conducting any optimization studies, and as glycan compositions can be altered with process conditions, titers produced by these 12 clones were critical in eliminating six of them.

While clones E13-05, E13-08, E32-14, E76-08, and E107-19 produced less than 1000 µg/ml titer, the titer concentrations of the clones E13-05, E13-08, and E76-08 were especially low. These five clones were immediately eliminated due to their undesired levels of production. Among the remaining seven clones, the lowest titer concentration was 1019 µg/ml, produced by both clones E90-22 and E107-08. In this case, the similarities of the glycan compositions of these two clones to those of the Originator were compared. As the Man5 level was lower, and as the percent G0F, G1F, G2F contents of E90-22 were closer to those of the Originator, the E107-08 clone was also eliminated.

Further studies were conducted with the selected six clones: E32-04, E76-06, E90-02, E90-22, E111-31, and E111-36.

4.3. IDENTIFICATION OF PDL15 AND RWCB GENERATION

RWCBs of the six clones (E32-04, E76-06, E90-02, E90-22, E111-31, E111-36) were selected according to the initial clone elimination study conducted with 12 clones, were generated by calculating their population doubling levels using the formula indicated in section 3.5. RWCB stocks were taken at PDL15 of each clone. During passaging, cell

viabilities of all the clones were between 98.7-99.5% at PDL15. The clones reached their PDL15 at PN: 4, after 266 hours of thawing (Table 4.2.).

Table 4.2. RWCB generation of clones (A) E32-04, (B) E76-06, (C) E90-02, (D) E90-22, (E) E111-31, (F) E111-36 at PDL15.

A	**E32-04**				
Passage Number	**Initial Cell Density (cells/ml)**	**Final Cell Density (cells/ml)**	**PDL**	**Culture Periods (days)**	**Doubling Time (hr)**
0	220000	2680000	3.61	3	19.96
1	300000	2290000	6.54	2	16.37
2	300000	2300000	9.48	2	16.33
3	200000	3010000	13.39	3	18.41
4	1000000	2710000	14.83	1	16.69
B	**E76-06**				
Passage Number	**Initial Cell Density (cells/ml)**	**Final Cell Density (cells/ml)**	**PDL**	**Culture Periods (days)**	**Doubling Time (hr)**
0	180000	1700000	3.24	3	22.23
1	300000	2570000	6.34	2	15.49
2	300000	2550000	9.43	2	15.55
3	200000	3400000	13.51	3	17.61
4	1000000	3070000	15.13	1	14.83
C	**E90-02**				
Passage Number	**Initial Cell Density (cells/ml)**	**Final Cell Density (cells/ml)**	**PDL**	**Culture Periods (days)**	**Doubling Time (hr)**
0	180000	2230000	3.63	3	19.83
1	300000	2730000	6.82	2	15.07

2	300000	2600000	9.93	2	15.41
3	200000	3910000	14.22	3	16.79
4	1000000	3140000	15.87	1	14.54
D	E90-22				
Passage Number	Initial Cell Density (cells/ml)	Final Cell Density (cells/ml)	PDL	Culture Periods (days)	Doubling Time (hr)
0	180000	2230000	3.63	3	19.83
1	300000	2280000	6.56	2	16.40
2	300000	2280000	9.48	2	16.40
3	200000	3330000	13.54	3	17.74
4	1000000	3030000	15.14	1	15.01
E	E111-31				
Passage Number	Initial Cell Density (cells/ml)	Final Cell Density (cells/ml)	PDL	Culture Periods (days)	Doubling Time (hr)
0	230000	2110000	3.20	3	22.52
1	300000	2100000	6.01	2	17.10
2	300000	2470000	9.05	2	15.78
3	200000	3610000	13.22	3	17.25
4	1000000	3290000	14.94	1	13.97
F	E111-36				
Passage Number	Initial Cell Density (cells/ml)	Final Cell Density (cells/ml)	PDL	Culture Periods (days)	Doubling Time (hr)
0	410000	1220000	1.57	2	26.09
1	300000	2520000	4.64	2	16.88
2	300000	5600000	9.45	3	15.38
3	200000	2310000	12.40	2	13.53
4	1000000	3610000	15.99	2	13.37

4.4. STABILITY-1

The Stability-1 study was conducted with the six clones selected regarding the initial clone elimination study, among 12 clones. The stability of a clone selected to produce a therapeutic recombinant protein should be demonstrated for a period covering a complete production process, from cell thawing to the end of the production. A clone is stable when copies of the genes of interest stay in the genome of the cells, and transcription and translation occur correctly while the culture ages. The stability of a clone is assessed by measuring the production yields and product qualities of cultures launched at different cell ages.

The stability-1 analysis also served to compare each clone to achieve the second step of clone elimination based on stabilities, for further studies.

The study proceeded in parallel with the RWCB generation, until PDL15. PDL30, PDL45, and PDL60 cultures were initiated with a similar methodology followed for PDL15 calculations, for the RWCB generation study. Stability was assessed by analyzing viable cell densities, viabilities, titer concentrations, and cell-specific productivities of each clone.

4.4.1. Viable Cell Density

Viable cell densities of clones E76-06, E90-02, E90-22, and E111-31 were relatively lower during PDL15 production, compared to the following productions. The second-lowest VCD profiles for these four clones were obtained during PDL30 productions. These results are meeting the expectation since it takes time for a clone to recover from a thawing process and reach stability. When cell densities represented in Figure 4.3. are compared among each production, it is observed that the highest VCD profiles were obtained during PDL60 productions for these four clones and PDL45 production profiles were similar. These results indicate that clones E76-06, E90-02, E90-22, and E111-31 recover and reach stable

conditions at nearly PDL45 and remain stable for 60 population doubling levels. This is desirable, because large-scale monoclonal antibody productions require high cell densities, thus longer periods of cell expansion. Cells already reach their population doubling levels of 45 until a bioproduction process is launched.

For clone E32-04, the highest VCD was observed during PDL15 production, contrary to other clones. VCDs proceeded similarly during the following productions, without any significant differences. However, a stable VCD profile was obtained at lower densities. This is not desirable, since VCD will be relatively lower for this clone, during large-scale production.

For clone E111-36, the lowest VCD profile was obtained during PDL30 production, followed by the PDL15 production. Higher standard deviations between the two replicates were observed during the PDL15 production, which can be attributed to instability following cell thawing. Viable cell densities increased at PDL45 and continued to increase at PDL60, reaching the highest-profile. This indicates improved cellular metabolism when cells are expanded for longer periods with the aim of large-scale production.

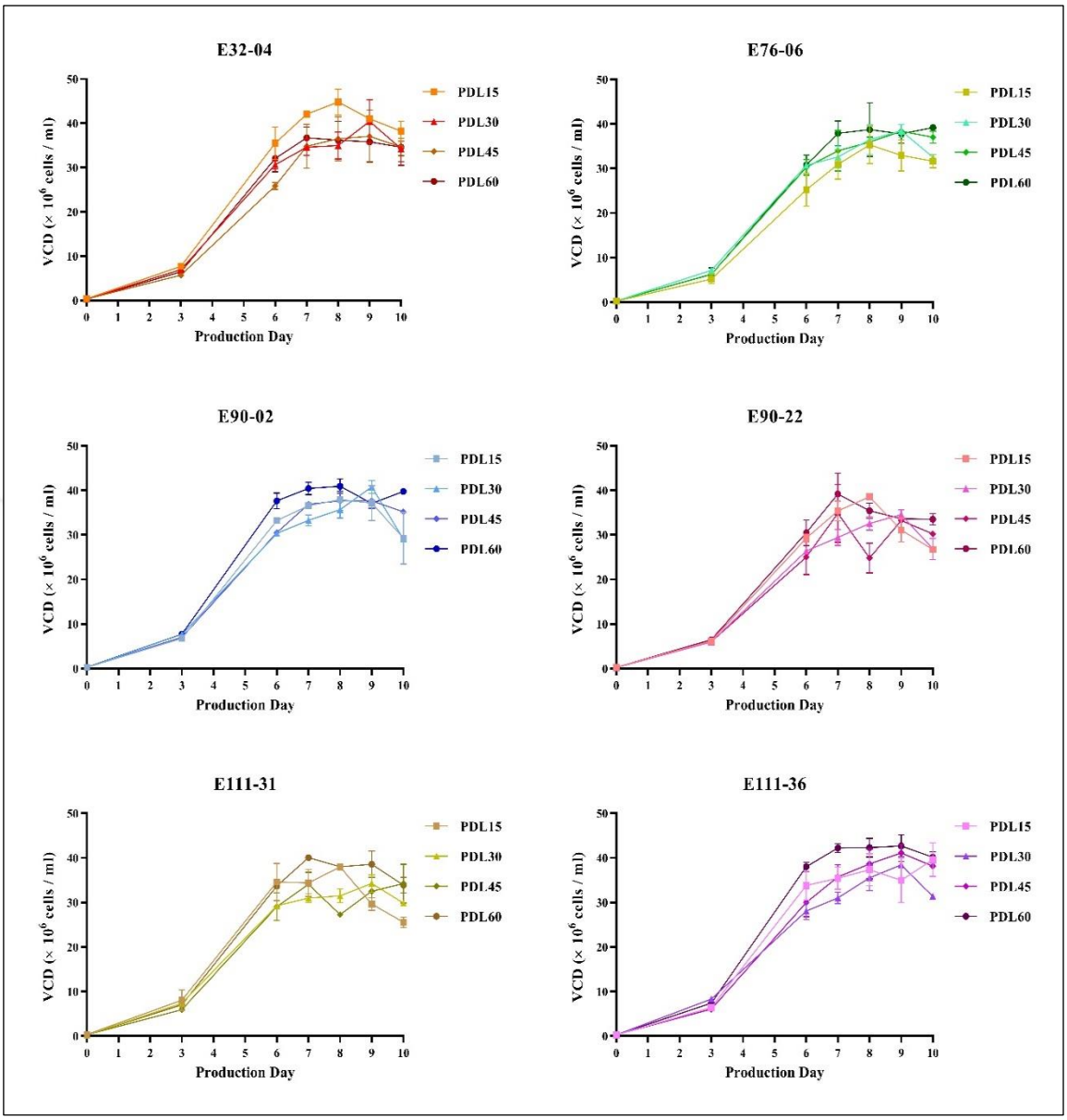

Figure 4.3. Viable cell density (VCD) profiles of the six clones representing the stability results of cultures initiated at four different population doubling levels (PDLs), until the end of PDL60.

4.4.2. Viability

Percent viabilities of clones E32-04 and E76-06 were very similar during their PDL15, PDL30, PDL45, and PDL60 production processes. No significant differences were observed.

Clones E90-02 and E111-36 reached similar and stable viability profiles, beginning from PDL30 productions, until the end of their PDL60 productions. Comparatively lower viabilities were observed during PDL15 in Figure 4.4. can be attributable to the cell recovery process after thawing. In terms of viability, these results show that these two clones are favorable for large-scale productions.

For clone E90-22, viability profiles were similar during PDL30, PDL45, and PDL60. However, they were lower, when compared to PDL15 production.

For clone E111-31, cellular viability profiles were similar during all four production processes, until PD8. Afterward, an apparent decrease in viability was observed during PDL45 production. This result is not desirable, since the cells are estimated to reach PDL45 until a large-scale production can be initiated.

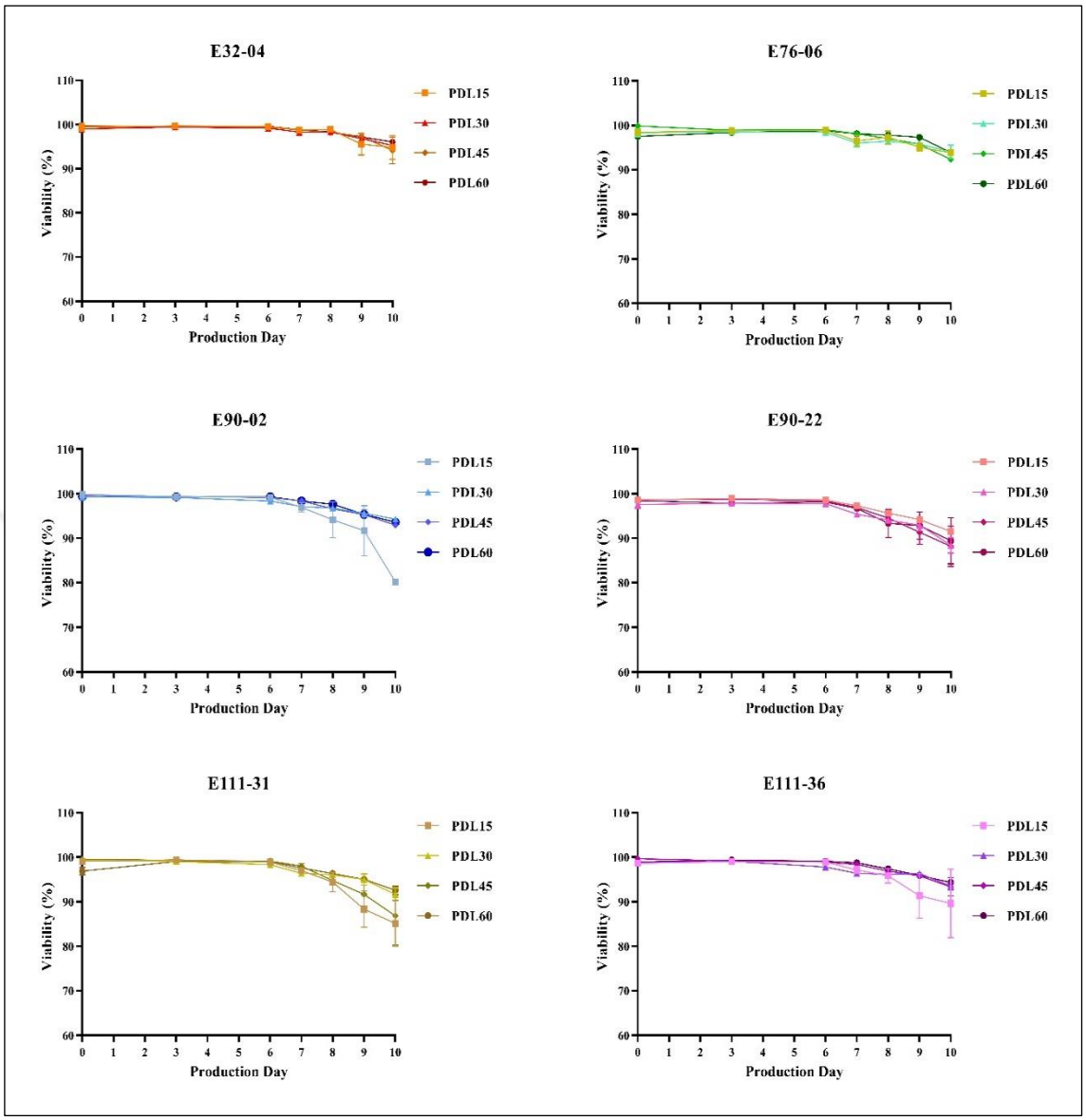

Figure 4.4. Percent viability (%) profiles of the six clones representing the stability results of cultures initiated at four different population doubling levels (PDLs), until the end of PDL60.

4.4.3. Titer

Regarding titer variabilities among cultures with different cell ages, variabilities lower than 30% are desired. According to the analysis, the reductions in titer amounts between PDL15-PDL60 of clones E90-02, E90-22, E111-31, and E111-36 were lower than 30% (Figure 4.5.). The minimum reductions in titers were observed in productions initiated with clones E90-02 and E111-36, by 16.3% and 16%, respectively. The most reductions were observed in productions initiated with clones E32-04 and E76-06, by 36.3% and 35.1%, respectively.

4.4.4. Cell-Specific Productivity

As with titers, variabilities lower than 30% are favorable in terms of cell-specific productivities. Accordingly, the only clone which showed reduced productivity over this limit was E76-06, by 45.2%. The minimum reduction was observed in productions initiated with the E90-02 clone, by 10.7%. Cell-specific productivities of the other four clones were very similar when the result bars in Figure 4.6. are analyzed. Reductions in productivities of clones E32-04, E90-22, E111-31 and E111-36 among all productions were by 26.7%, 27.1%, 28.4%, and 26.6%, respectively. Among these four clones, the least reductions were observed in productions initiated with clones E32-04 and E111-36, with the amounts being highly similar.

When all the quality results conducted to assess clonal stability are analyzed, it can be asserted that the clones E90-02 and E111-36 are the most stable clones among the six of them. E76-06, on the other hand, can be concluded to be the least stable one.

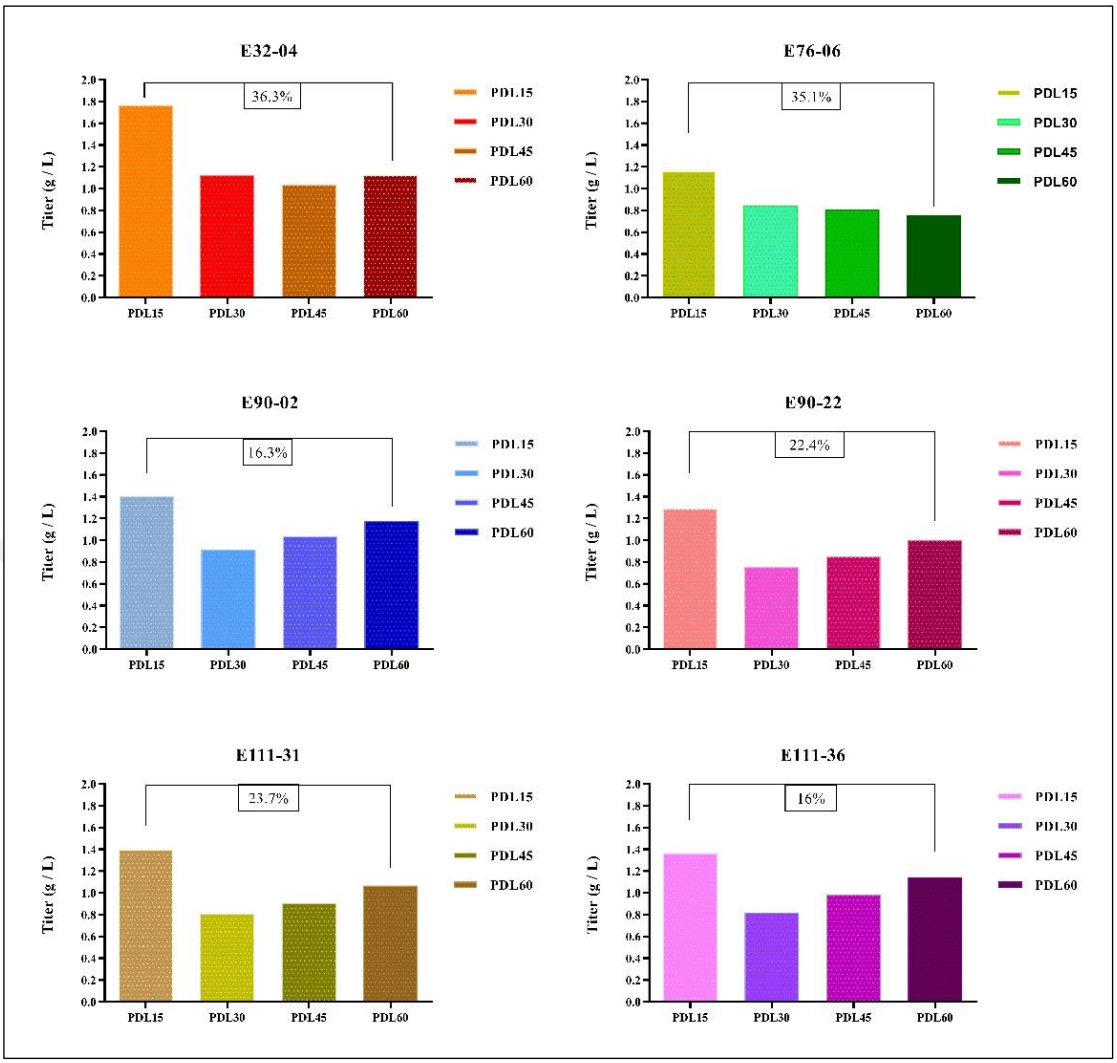

Figure 4.5. Titers of the six clones representing the stability results of cultures initiated at four different population doubling levels (PDLs), until the end of PDL60.

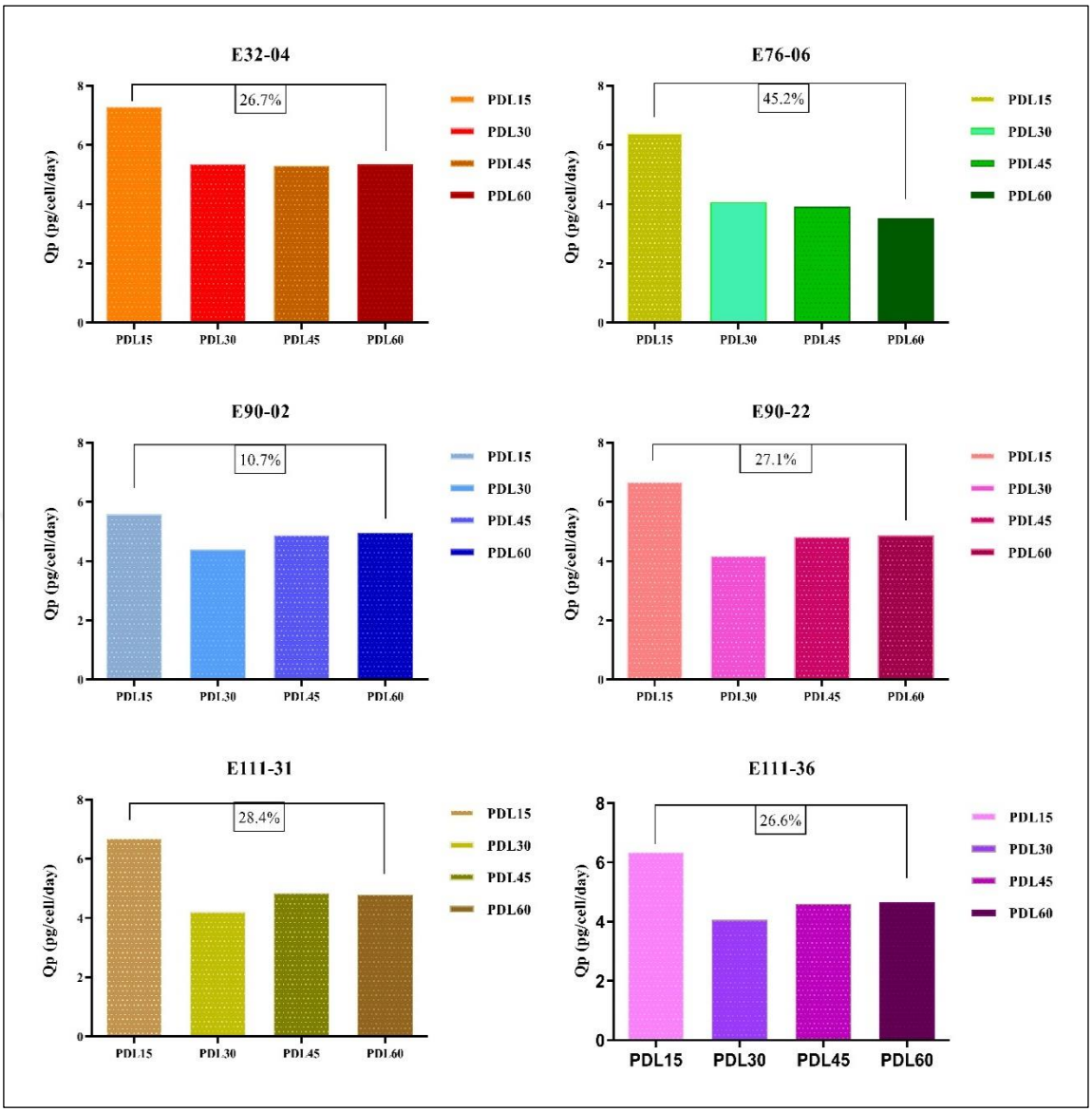

Figure 4.6. Cell-specific productivities (Qp) of the six clones representing the stability results of cultures initiated at four different population doubling levels (PDLs), until the end of PDL60.

4.5. DoE-1

The DoE-1 study was initiated according to the results of preliminary productivity tests and the Stability-1 study. To screen combinations of clone, feed strategy, and temperature for the expression of C5-mAb, General Factorial Design was used. Clone (1), feeding strategy (2), temperature (3) became the three factors to be analyzed. While the four clones generated four levels, different feed strategies and temperature conditions generated two levels each, for analysis. As each run was replicated, 32 experimental runs were carried out for 16 possible combinations. The study was divided into two parts as for extended feed strategy and for distributed feed strategy for handling concerns. Thus, the two experimental setups were launched at different time points and the PDLs of each clone were similar, yet different from each other (Table 4.3.). The design platform summarized in Table 4.4 was followed for the screening.

Table 4.3. Clone population doubling levels.

Clone	**PDL**	
	Distributed Feed Strategy	**Extended Feed Strategy**
E32-04	21	27
E90-02	17	28
E111-31	14	26
E111-36	22	28

Table 4.4. DoE-1 experimental design platform.

Run	Block	Factor 1 A: Clone	Factor 2 B: Feeding	Factor 3 C: Temperature
1	Block 1	E111-31	Extended	37ºC
2	Block 1	E90-02	Extended	37ºC
3	Block 1	E111-31	Extended	35ºC-PD7 shift
4	Block 1	E90-02	Extended	35ºC-PD7 shift
5	Block 1	E111-36	Distributed	37ºC
6	Block 1	E111-31	Extended	37ºC
7	Block 1	E90-02	Extended	35ºC-PD7 shift
8	Block 1	E111-36	Distributed	35ºC-PD7 shift
9	Block 1	E32-04	Distributed	35ºC-PD7 shift
10	Block 1	E32-04	Distributed	35ºC-PD7 shift
11	Block 1	E90-02	Distributed	37ºC
12	Block 1	E111-36	Extended	35ºC-PD7 shift
13	Block 1	E111-31	Distributed	35ºC-PD7 shift
14	Block 1	E111-31	Distributed	37ºC
15	Block 1	E32-04	Distributed	37ºC
16	Block 1	E111-36	Extended	37ºC
17	Block 1	E90-02	Distributed	35ºC-PD7 shift
18	Block 1	E90-02	Extended	37ºC
19	Block 1	E32-04	Distributed	37ºC
20	Block 1	E32-04	Extended	35ºC-PD7 shift

21	Block 1	E90-02	Distributed	37ºC
22	Block 1	E111-31	Extended	35ºC-PD7 shift
23	Block 1	E111-36	Distributed	35ºC-PD7 shift
24	Block 1	E111-36	Extended	35ºC-PD7 shift
25	Block 1	E32-04	Extended	37ºC
26	Block 1	E32-04	Extended	37ºC
27	Block 1	E111-36	Distributed	37ºC
28	Block 1	E111-31	Distributed	35ºC-PD7 shift
29	Block 1	E111-36	Extended	37ºC
30	Block 1	E90-02	Distributed	35ºC-PD7 shift
31	Block 1	E111-31	Distributed	37ºC
32	Block 1	E32-04	Extended	35ºC-PD7 shift

Clones were evaluated regarding their viable cell density (VCD), percent viability, lactate generation, pH, osmolality, and titer. Each parameter was analyzed both in terms of feed strategy and culture temperature condition, for each clone.

The highest peak VCD was obtained under the extended feed strategy for all four clones (Figure 4.7.). The enhancing effect of temperature down-shift on longevity was more distinctive under the distributed feed strategy. Although no significant differences were observed in viabilities among different feed strategies, the E90-02 clone showed the most stable process trends regarding VCD and viability profiles. E111-36 reached the highest cell density with 38.7×10^6 VC/ml on PD9, under 37°C, extended feeding condition. A significant difference was observed when the peak VCD values of E90-02 were compared among the two feed strategies at 37°C, which was the second clone to reach the highest cell density. A reduction of 16% was confirmed from 36.7×10^6 VC/ml to 30.84×10^6 VC/ml

when feeds were supplemented per distributed feeding strategy ($P < 0.05$). While E90-02 maintained over 90% viability under all conditions, E111-36 showed weak growth and lower viability among all clones. E111-31 clone showed the lowest VCD and viability trends among both feed strategies, and under both feed strategies. Plus, sharper drop in VCD and viability profiles were observed under the extended feed strategy.

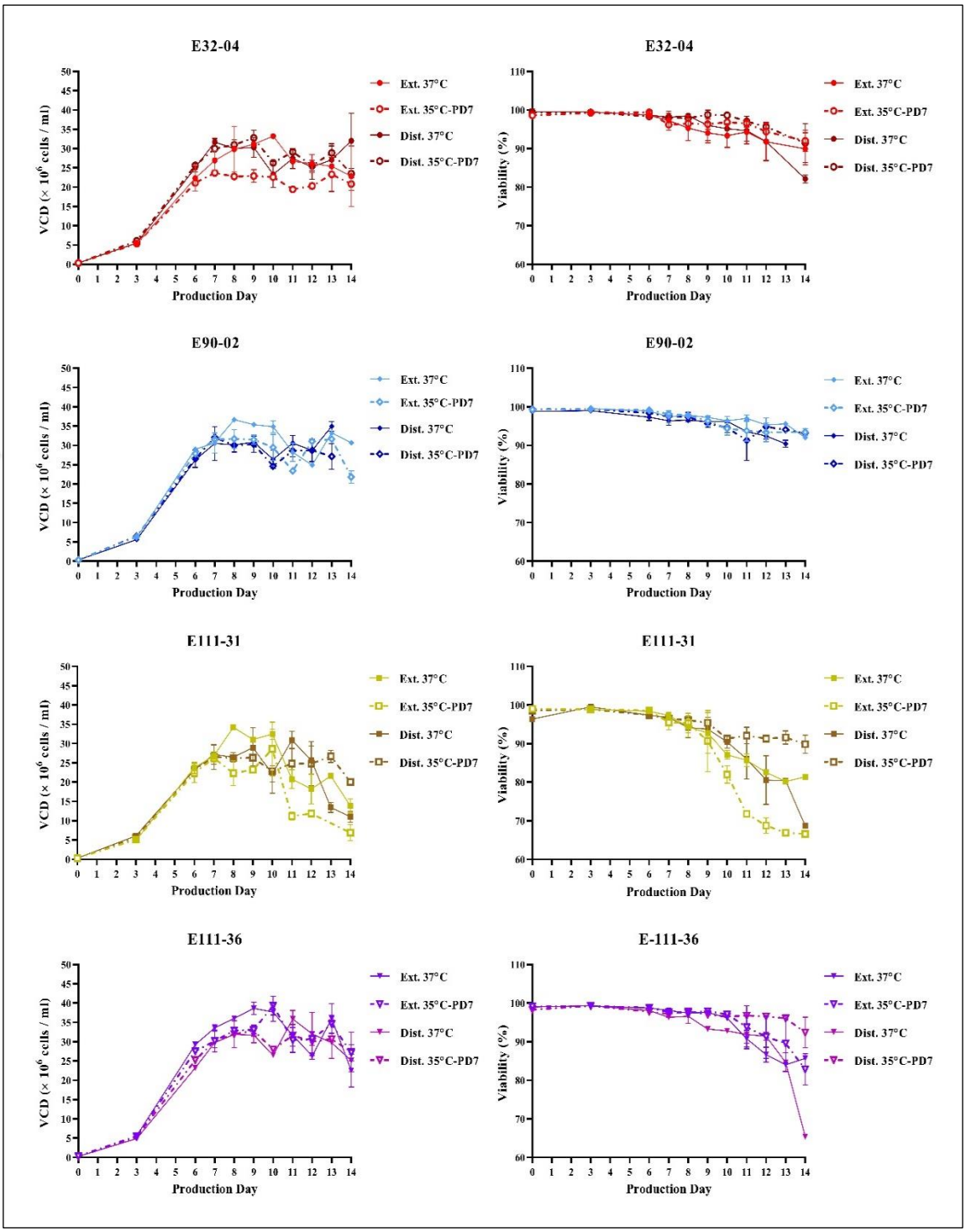

Figure 4.7. Viable cell density (VCD) and percent viability (%) of the four clones under different production conditions.

For all four clones, pH values were representative of respective lactate concentrations. Applying 35°C-PD7 shift was effective in reducing lactate generations, under both feed strategies (Figure 4.8.). For clones E32-04 and E111-31, 35ºC-PD7 shift conditions under distributed feeding provided the lowest lactate production during production. On the other hand, lactate concentrations remained lower with 35ºC-PD7 shift under extended feed strategy conditions for clones E90-02 and E111-36. Supplementations under distributed feeding resulted in significant increases by 84.6% and 77.7%, for E90-02 and E111-36 clones respectively, under 37°C. Also, for the E111-36 clone, the difference between the lactate generations among feed strategies under 35°C-PD7 shift condition was over 3.5 folds, with the distributed feed strategy resulting in an increased generation ($P < 0.05$).

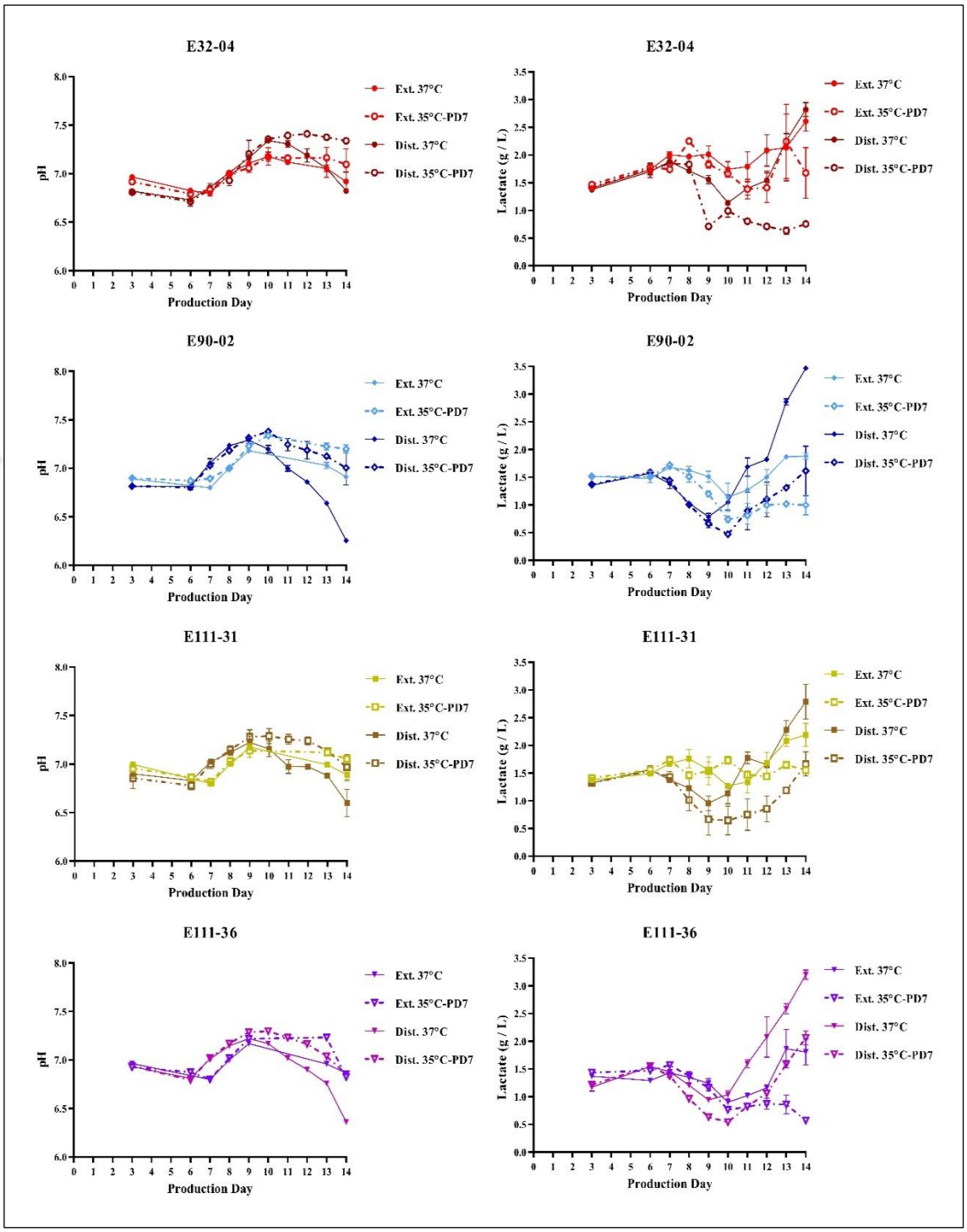

Figure 4.8. pH and lactate generations (g / L) of the four clones under different production conditions.

Osmolality profiles of clones E90-02 and E111-36 were very similar. When the osmolality trends represented in Figure 4.9. are compared among clones, it can be observed that the values and ranges were similar for both 37ºC and 35ºC-PD7 shift conditions under both feed strategies for E90-02, E111-31, E111-36 clones, but E32-04 showed a slightly different trend. For this clone, osmolalities remained lower with the distributed feed strategy, but under the extended feed strategy, osmolality proceeded higher with 35ºC-PD7 shift conditions compared to 37ºC.

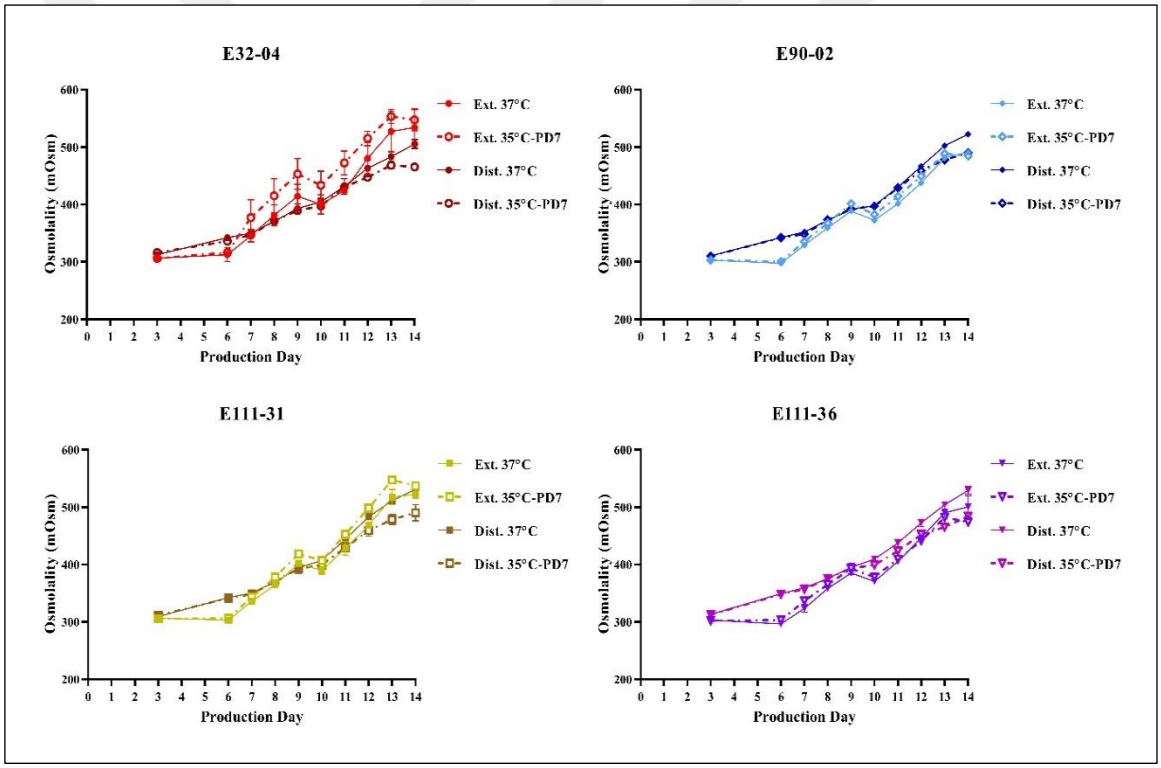

Figure 4.9. Osmolalities (mOsm) of the four clones under different production conditions.

Titer results were following the VCD and viability profiles of each clone. Both feed strategies resulted in a similar titer for the E32-04 clone, under 37ºC condition. However, under 35ºC-PD7 shift condition, the distributed feed strategy provided a higher titer. E90-02 and E111-36 clones produced the highest titers with the extended strategy, under both temperatures (Figure 4.10.). IgG productions under 37°C were 3.7 g/L, 3.88 g/L; and were 3.23 g/L, 3.27 g/L under 35°C for E90-02 and E111-36, respectively. While feed supplementation under distributed strategy reduced titers of E90-02 by 20% at 37°C, the difference was 33% for the E111-36 clone under the same conditions ($P < 0.05$). Lastly, titers were similar to 35ºC-PD7 shift conditions under both feed strategies, for the E111-31 clone. However, regarding 37ºC conditions, titers were higher under the extended feed strategy.

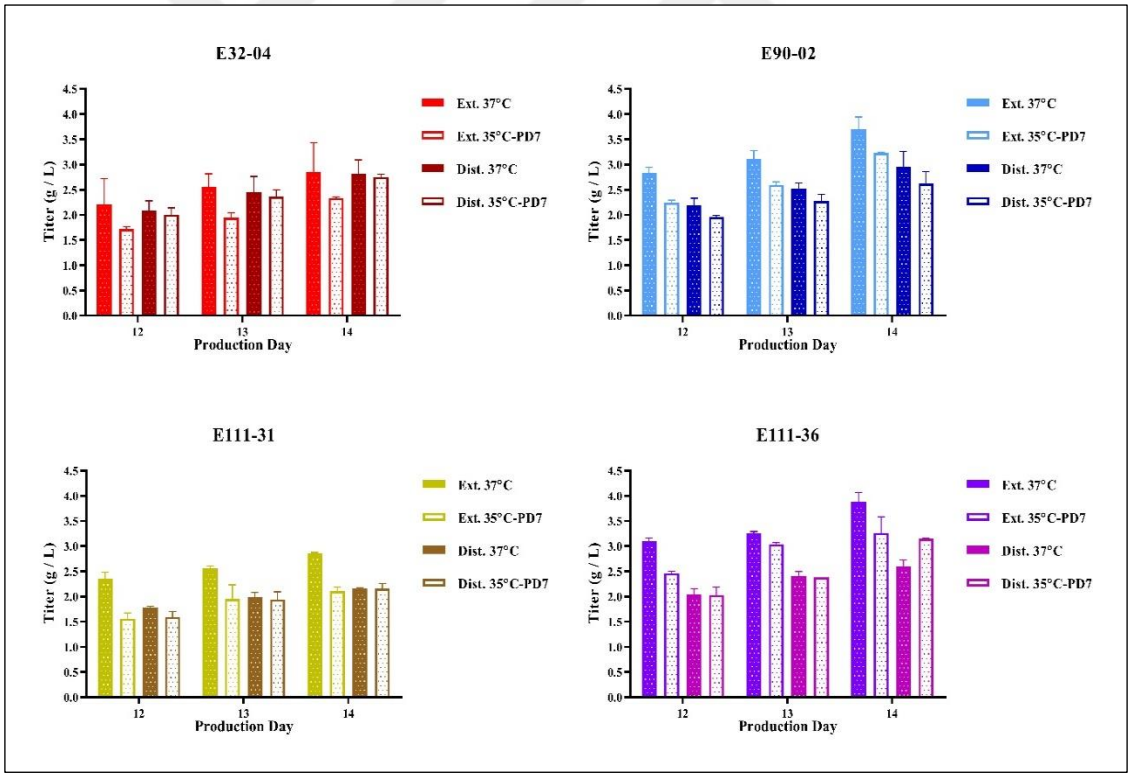

Figure 4.10. Titers (g / L) of the four clones under different production conditions.

4.6. STABILITY-2

This study intended to put forward the stability of the selected two clones, E90-02 and E111-36, over 60 population doubling levels (PDL) with the extended feed strategy. Stability cultures were initiated in vented spin tubes, with cells derived from the maintenance every 10 ± 1 day, corresponding to approximately PDL15, PDL30, PDL45, and PDL60 according to cell doubling times. No samplings were done when initiating the cultures, given the fact that the study was conducted in spin tubes with 15 ml working volume, as well as that the cell count and cell viabilities were being assessed while passaging and inoculating the cultures. All the cultures were initiated with 0.3×10^6 cells/ml. This measure was taken to eliminate the risk of decreasing the working volume prior to production.

For both clones, no significant differences were observed among PDL15, PDL30, PDL45, and PDL60 productions, regarding VCD values represented in Figure 4.11. The highest peak VCD was reached at PDL45 as 40×10^6 VC/ml for E90-02, and as 37×10^6 VC/ml for E111-36. Cell viabilities were relatively lower on PDL15 production, due to the time required for the cells to adapt to the culture environment, following cell thawing. After PDL15, viabilities were high, above 90 percent, until the end of PDL60 (Figure 4.11.).

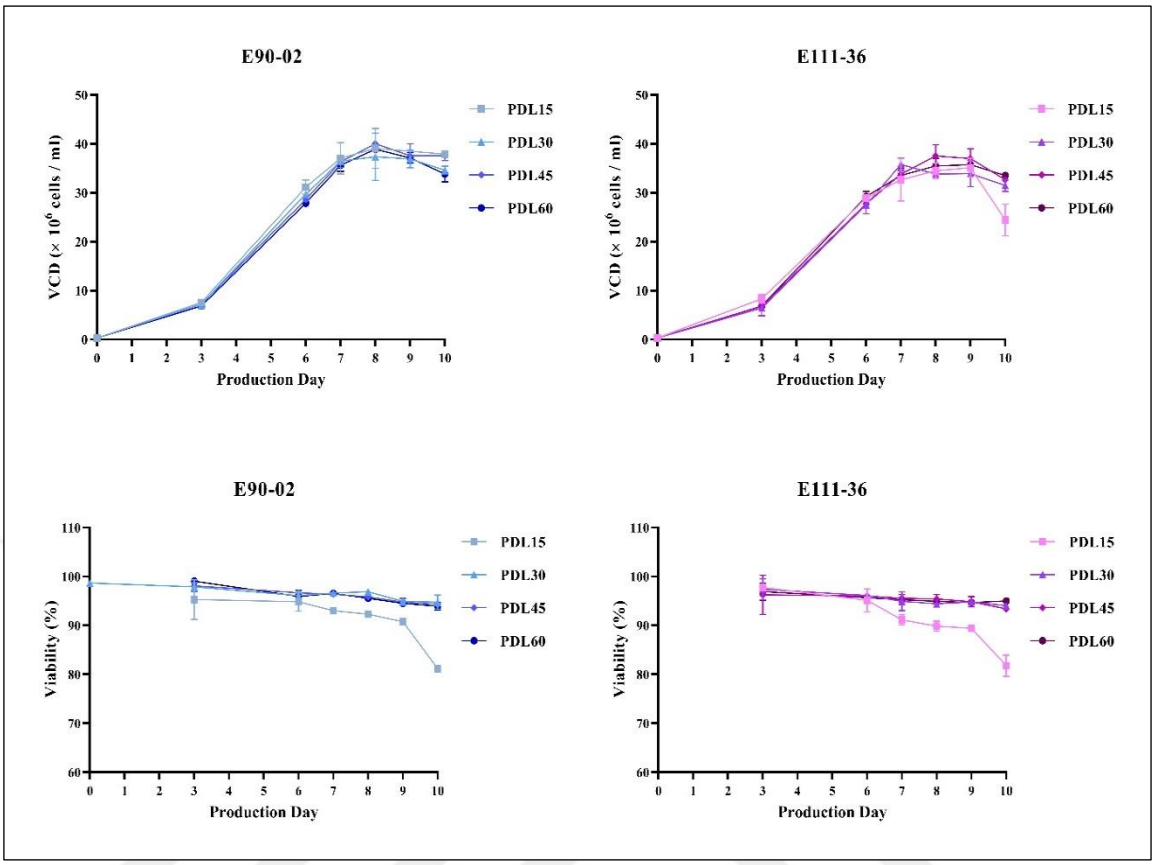

Figure 4.11. Viable cell density (VCD) and percent viability (%) profiles of the two clones representing the stability results of cultures initiated at four different population doubling levels (PDLs), until the end of PDL60.

For both clones, titers among PDL15, PDL30, PDL45, and PDL60 productions did not exceed 30 percent variability. The highest titers were obtained during PDL15 productions, as 2.3 g/L for E90-02 clone and 2.04 g/L for E111-36 clone (Figure 4.12.). The highest cell-specific productivities were achieved during PDL15 productions, being 10.1 pg/cell/day for E90-02 clone and 9.4 pg/cell/day for E111-36 clone (Figure 4.12.). The reduction in cell-specific productivities did not exceed 30%, for neither of the clones.

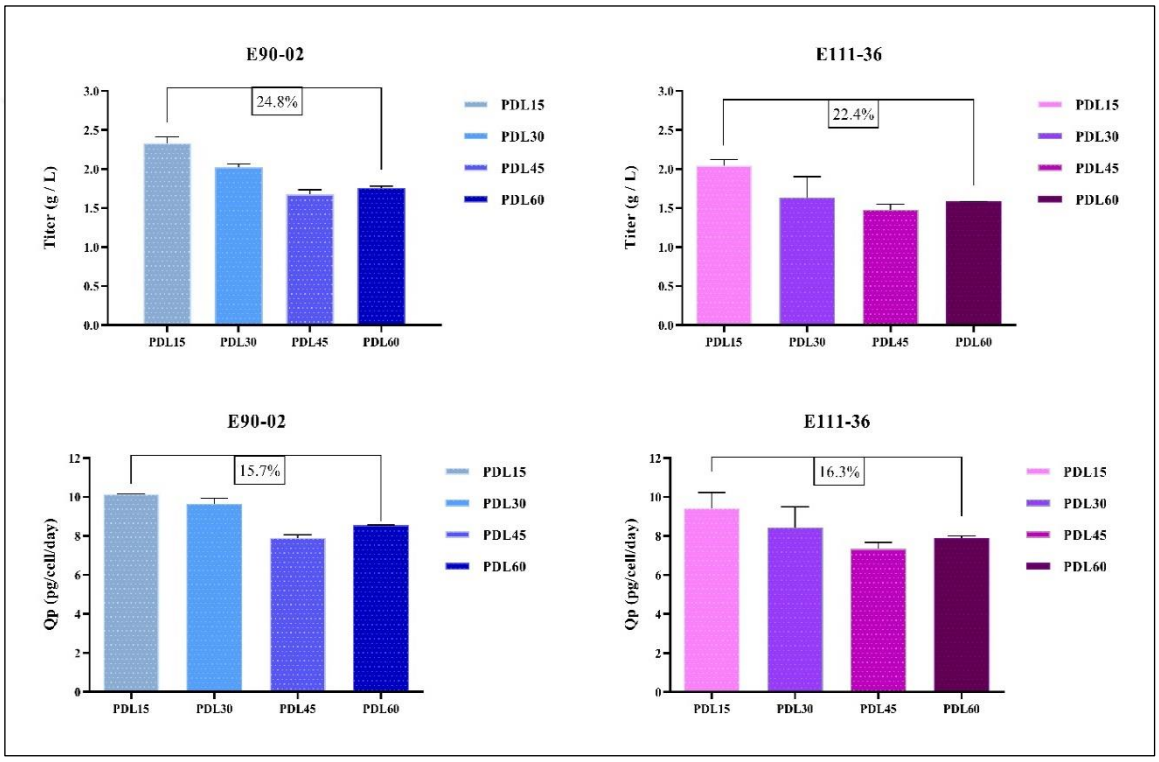

Figure 4.12. Titer (g / L) and cell-specific productivity (Qp) profiles of the two clones representing the stability results of cultures initiated at four different population doubling levels (PDLs), until the end of PDL60.

4.7. DoE-2

The DoE-2 study was initiated according to the results of the DoE-1 and the Stability-2 studies. Regarding the results of the DoE-1 study, two clones among four, namely E32-04 and E111-31 clones, were eliminated and a second stability study was conducted with the selected clones, E90-02 and E111-36.

To screen combinations of clone, temperature, and ToS for the expression of C5 mAb, General Factorial Design was used. Clone (1), temperature (2), ToS (3) became the three factors to be analyzed. While the two clones and different temperatures generated two levels each, ToS generated five levels, for analysis. As each run was replicated, 40 experimental runs were carried out for 20 possible combinations. The study was divided into two parts regarding temperatures to be shifted, for handling concerns. Thus, the two experimental setups were launched at different time points. Control conditions, at 37°C fixed temperature, were conducted once, due to limited availability in the incubators. These runs were evaluated for comparison following Design Expert analysis. Fed-batch cultures were initiated at PDL27, for both clones. The design platform summarized in Table 4.5 was followed for the screening.

Table 4.5. DoE-2 experimental design platform.

Run	Block	Factor 1 A: Clone	Factor 2 B: Temperature	Factor 3 C: Time of Shift
1	Block 1	E90-02	32	PD5
2	Block 1	E90-02	32	PD7
3	Block 1	E90-02	32	PD6
4	Block 1	E90-02	35	PD8
5	Block 1	E90-02	35	PD5
6	Block 1	E111-36	35	PD8
7	Block 1	E90-02	35	PD7
8	Block 1	E111-36	35	PD8
9	Block 1	E111-36	35	PD7
10	Block 1	E111-36	32	PD3
11	Block 1	E90-02	32	PD5
12	Block 1	E90-02	35	PD3
13	Block 1	E111-36	32	PD8
14	Block 1	E111-36	35	PD3
15	Block 1	E111-36	32	PD5
16	Block 1	E111-36	35	PD5
17	Block 1	E90-02	32	PD3
18	Block 1	E111-36	32	PD3
19	Block 1	E111-36	32	PD5
20	Block 1	E111-36	32	PD7
21	Block 1	E111-36	32	PD6

22	Block 1	E111-36	32	PD6
23	Block 1	E111-36	35	PD3
24	Block 1	E90-02	32	PD8
25	Block 1	E111-36	35	PD6
26	Block 1	E90-02	32	PD8
27	Block 1	E90-02	35	PD5
28	Block 1	E111-36	35	PD6
29	Block 1	E111-36	35	PD7
30	Block 1	E90-02	32	PD6
31	Block 1	E90-02	35	PD6
32	Block 1	E90-02	32	PD7
33	Block 1	E90-02	35	PD6
34	Block 1	E90-02	35	PD7
35	Block 1	E111-36	35	PD5
36	Block 1	E90-02	35	PD8
37	Block 1	E111-36	32	PD7
38	Block 1	E90-02	35	PD3
39	Block 1	E111-36	32	PD8
40	Block 1	E90-02	32	PD3

According to the results obtained through Design Expert, indicated factors have been implicated to have an impact both alone and also, in combination with other factors.

Table 4.6. DoE-2 results (Responses) of the analyzed factors.

Run	F. 1	F. 2	F. 3	R. 1	R. 2	R. 3	R. 4	R. 5	R. 6	R. 7	R. 8
	Clone	Temp. (°C)	Time of Shift	Titer (mg/L)	Qp (pg/ml-	G0F (%)	G1Fa (%)	G1Fb (%)	G2F (%)	G0 (%)	Man5 (%)
C-A1	E90-02	37	-	3520	11.461	60.02	2.69	3.21	0.96	0.67	11.05
C-A2	E90-02	37		3870	12.061	60.02	2.69	3.21	0.96	0.67	11.05
C-B1	E111-36	37	-	4010	11.288	58.71	2.36	2.67	0.82	0.73	12.00
C-B2	E111-36	37		3750	11.452	58.71	2.36	2.67	0.82	0.73	12.00
12	E90-02	35	3	3018	8.879	67.35	3.51	4.16	0.88	0.83	8.27
38	E90-02	35	3	3056	8.864	67.45	3.61	4.26	0.98	0.93	8.37
14	E111-36	35	3	3108	8.781	63.39	3.49	4.44	1.05	0.86	10.75
23	E111-36	35	3	2831	8.495	63.49	3.59	4.54	1.15	0.96	10.85
5	E90-02	35	5	3190	9.475	61.71	3.07	4.03	0.94	0.79	10.66
27	E90-02	35	5	2918	8.753	61.81	3.17	4.13	1.04	0.89	10.76
16	E111-36	35	5	3068	8.541	59.47	3.1	4.1	1.05	0.86	12.87
35	E111-36	35	5	3685	9.957	59.57	3.2	4.2	1.15	0.96	12.97
31	E90-02	35	6	2576	8.621	62.97	3.33	4.07	0.93	0.77	10.49
33	E90-02	35	6	2878	8.207	63.07	3.43	4.17	1.03	0.87	10.59
25	E111-36	35	6	3511	9.806	61.18	2.78	3.56	0.78	0.88	12.16
28	E111-36	35	6	3120	8.955	61.28	2.88	3.66	0.88	0.98	12.26
7	E90-02	35	7	3240	11.186	59.38	2.72	3.12	0.97	0.75	12.41
34	E90-02	35	7	3220	10.633	59.48	2.82	3.22	1.07	0.85	12.51
9	E111-36	35	7	3040	9.848	57.2	2.44	2.85	0.93	0.89	12.96
29	E111-36	35	7	3490	10.739	57.3	2.54	2.95	1.03	0.99	13.06
4	E90-02	35	8	2521	7.326	62.08	3.23	3.98	0.95	0.75	12.08
36	E90-02	35	8	2556	7.176	62.18	3.33	4.08	1.05	0.85	12.18
6	E111-36	35	8	2402	7.205	59.76	2.8	3.42	0.72	0.72	14.22

Run	F. 1	F. 2	F. 3	R. 1	R. 2	R. 3	R. 4	R. 5	R. 6	R. 7	R. 8
8	E111-36	35	8	2619	7.659	59.86	2.9	3.52	0.82	0.82	14.32
17	E90-02	32	3	1753	10.199	74.16	5.88	7.35	1.68	0.91	3.90
40	E90-02	32	3	1288	8.516	74.26	5.98	7.45	1.78	1.01	4.00
10	E111-36	32	3	1845	9.776	75.7	5.58	6.47	1.38	1.04	4.25
18	E111-36	32	3	1847	9.606	74.51	5.83	7.08	1.56	1.07	3.88
1	E90-02	32	5	2251	9.023	73.01	4.77	6.11	1.36	0.90	4.77
11	E90-02	32	5	2303	8.699	73.11	4.87	6.21	1.46	1.00	4.87
15	E111-36	32	5	2605	10.891	71.45	4.92	6.46	1.48	0.95	5.30
19	E111-36	32	5	2467	10.036	71.55	5.02	6.56	1.58	1.05	5.40
3	E90-02	32	6	2281	7.270	72.59	4.29	5.58	1.27	0.91	5.00
30	E90-02	32	6	2551	8.138	72.69	4.39	5.68	1.37	1.01	5.10
21	E111-36	32	6	2881	9.011	71.56	4.16	5.52	1.15	0.97	5.96
22	E111-36	32	6	2271	7.367	71.66	4.26	5.62	1.25	1.07	6.06
2	E90-02	32	7	2605	7.247	68.91	3.87	5.04	1.16	0.97	6.74
32	E90-02	32	7	2694	7.511	69.01	3.97	5.14	1.26	1.07	6.84
20	E111-36	32	7	2350	7.257	72.77	4.32	4.73	1.21	0.86	4.52
37	E111-36	32	7	2141	6.572	72.87	4.42	4.83	1.31	0.96	4.62
24	E90-02	32	8	2525	7.460	66.52	3.77	4.76	1.02	0.90	8.44
26	E90-02	32	8	2530	8.168	66.62	3.87	4.86	1.12	1.00	8.54
13	E111-36	32	8	2087	6.074	68.41	3.89	4.57	1.03	0.86	8.82
39	E111-36	32	8	2357	6.964	68.51	3.99	4.67	1.13	0.96	8.92

The temperature was shown to impact all parameters either alone or in combination with ToS. Titers increased with increasing temperature for both clones, and when ToS was delayed until PD8. The ToS-dependent increase in titer was apparent at 32°C especially on PD7 for E90-02 clone, and at 35°C on PD5 for E111-36 clone, which then remained constant

until PD8. However, productions under 32°C significantly lowered the IgG productions, for both clones ($P < 0.05$). Lowering the temperature to 32°C on PD5 decreased E90-02 titers by 25.4 % while lowering it on PD7 decreased E111-36 titers by 31.2 %. The reductions in IgG productions were reasonably more pronounced under the PD3-shift condition for both clones, as it represents the most stressful condition on cell metabolism, both in terms of temperature and the time of shift.

Cell-specific productivities were also affected due to temperature shift. Reducing the temperature to 32°C from 35°C on PD7 resulted in a 32.4% and 32.8% decrease in productivities of the E90-02 clone and E111-36 clone, respectively. While clonal difference did not result in high variations, temperature and ToS factors had a significant impact on titer and cell-specific productivities, both alone and with interaction ($P < 0.05$).

The screening study demonstrated that clone, temperature, and ToS have a significant impact on glycosylation ($P < 0.05$), both alone or as a combined effect through the interaction of either two or three of the factors.

G0F contents increased when the temperature was decreased to 32°C, for both clones. Temperature shift from 35°C to 32°C on PD5 resulted in an 18.3 % increase for the E90-02 clone, while shifting on PD7 increased G0F contents of the E111-36 clone by 27.2 %. This impact was also observed regarding G1F contents, noting that the increased ToS had a reducing effect.

Substantial increases in G1F contents of E90-02 were by 71.6 % and 52.5 %, with PD3 and PD5 shift conditions, respectively. PD5 shifting for E111-36, on the other hand, was by 57.3% and was increased with PD7 shifting, up to 69.8 %. While this result was valid for G2F contents with the E90-02 clone as well, ToS with the E90-02 clone did not follow a similar trend. G0 contents were observed to be affected only by temperature and they increased with decreasing temperature.

Man5 levels were observed to be affected by all three factors, including clones, either alone or in combination with each other. Decreasing the temperature to 32°C significantly reduced Man5 contents. However, delaying the ToS to PD8 was observed to increase them under this temperature, resulting in a 30% reduction for the E90-02 clone, and 37.8% for the E111-36 clone, which still represents a significant difference ($P < 0.05$). For the E90-02 clone, the highest reduction in Man5 contents was observed with PD5 shift by 55%, while it was with the PD7 shift condition for the E111-36 clone by 64.9%. These results indicate that glycosylation of the C5mAb is dependent on the clone, temperature, and the time of temperature shift.

When all the responses in Table 4.6 were introduced to the Design Expert program, the most ideal bioreactor condition to start with was suggested as the 35°C – PD7 shift condition.

4.8. PRODUCTIONS IN 3-L BIOREACTORS - PART 1

The initial bioreactor run conditions were chosen according to the results of the DoE-2 study. According to the results screened by the Design Expert program, the most ideal production condition to start with was suggested as 35°C – PD7 shift for both clones, in terms of titer and cell-specific productivity. Productions were analyzed by running two bioreactors simultaneously for each clone, with one 37°C no temperature shift control condition and one 35°C – PD7 shift condition. BR-1 and BR-2 bioreactor productions were achieved with the E90-02 clone, while BR-3 and BR-4 productions were conducted with the E111-36 clone.

When VCD and percent viabilities are analyzed, BR-4 reached the highest peak VCD and presented the highest viability trend. pH fluctuations were not observed, for neither of the bioreactor productions. Also, pH trends were similar among productions, even though the lactate concentrations were very different. Osmolality values remained within expected and reasonable limits, but it was observed that decreasing the temperature to 35°C also decreased osmolality values (Figure 4.13.).

Lactate generations were very different among productions. While the lowest concentrations were observed during production BR-2, the highest lactate production was observed with BR-3. The clonal difference was observed to affect lactate generations. Also, decreasing the temperature had an effect of lowering lactate concentrations (Figure 4.13.). Productions with E90-02 clone resulted in much lower lactate levels. pCO_2 levels were monitored throughout the processes to ensure that they remained below 200 mmHg, to avoid any harmful effects on cells and any impact on glycosylation.

While decreasing the temperature did not represent a major impact on the titer of the E90-02 clone, the titers increased with reduced temperature in bioreactor runs of the E111-36 clone (Figure 4.13.). Titers obtained from the E90-02 clone were slightly higher compared to those obtained from the E111-36 clone.

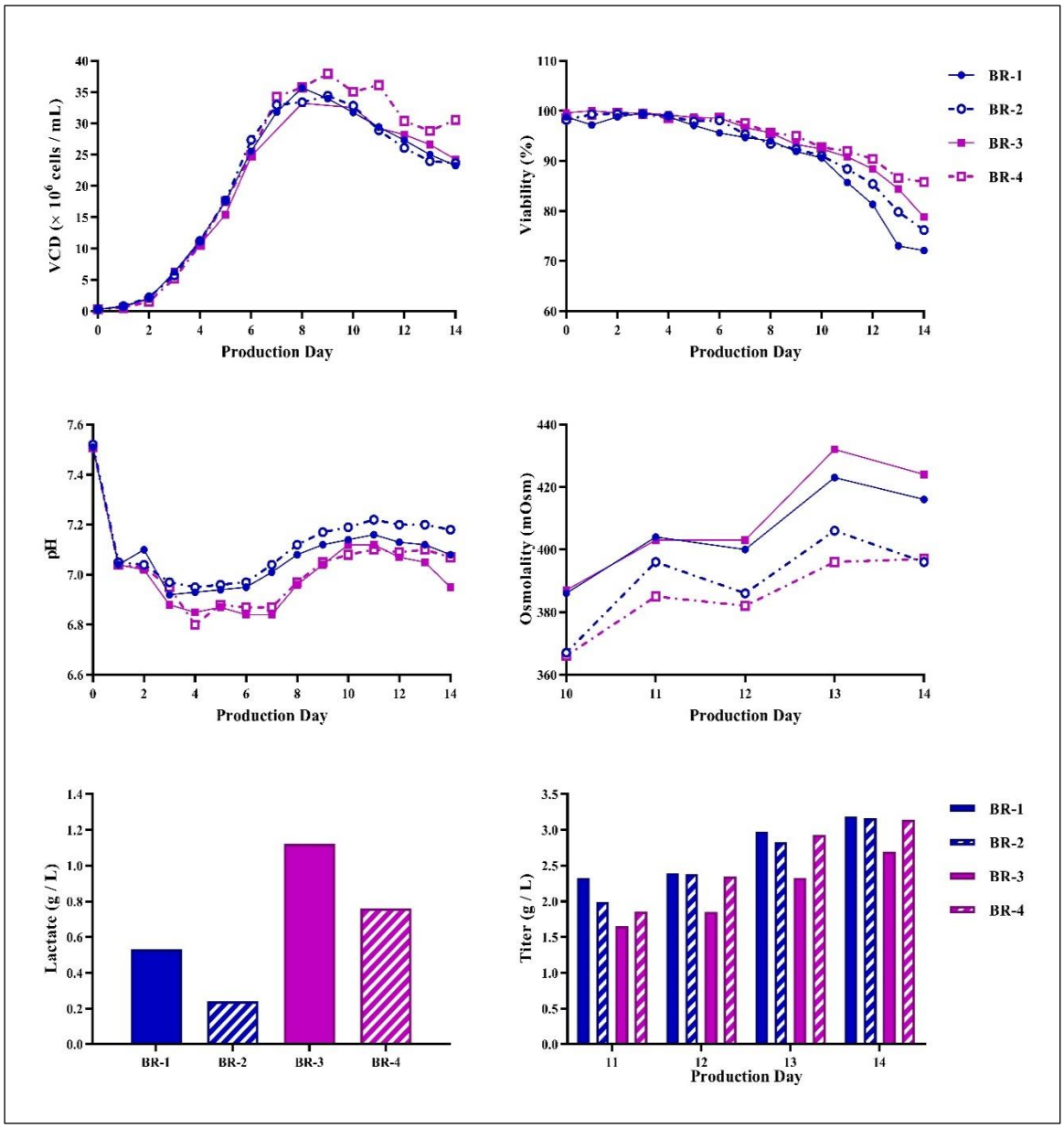

Figure 4.13. In-process control and titer results of the bioreactor productions BR-1, BR-2, BR-3, and BR-4.

Regarding the analyzed glycan compositions (Figure 4.14.), G0F contents were similar among bioreactors, also to that of the Originator when compared. Galactosylated glycoforms were observed to be very different, compared to the Originator. This indicated that optimization was required to enhance galactose contents, either through process parameters or by galactose supplementation if adjustments in process parameters did not suffice. Man5 levels were observed to be variable among productions, both in terms of clones and process conditions. While decreasing the temperature resulted in increased Man5 content with the E90-02 clone, it had a reducing effect with the E111-36 clone. Although the Man5 levels of BR-1, BR-2, and BR-3 were relatively higher than that of the Originator, it is possible to adjust Man5 contents through process optimization.

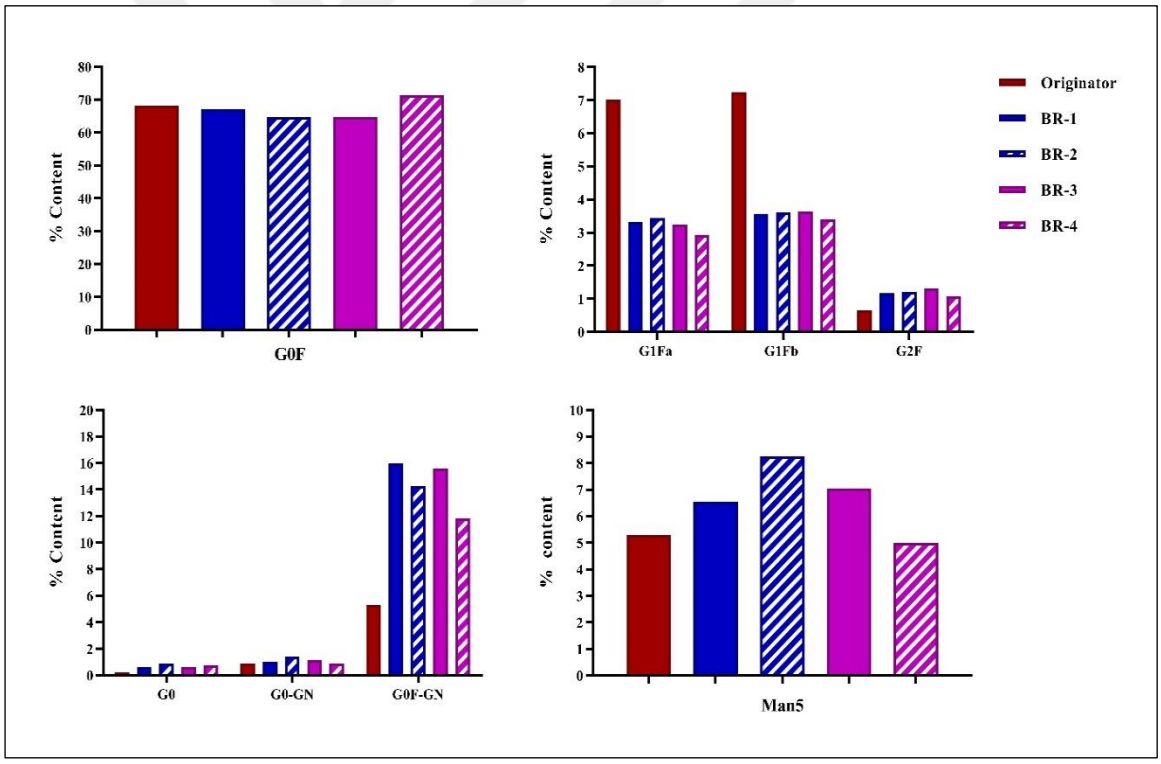

Figure 4.14. Glycan analysis results of the bioreactor productions BR-1, BR-2, BR-3, and BR-4 compared to the major glycan contents (%) of the originator.

4.9. GALACTOSE SUPPLEMENTATION ASSAY

Five different conditions were analyzed to understand the effects of galactose supplementation on glycan contents of the E90-02 clone, during fed-batch production under the extended feed strategy. Analyses were based on the results of VCD, viability, osmolality, pH, lactate and glucose concentrations, titer, cell-specific productivity (Qp), and glycan contents of the cultures. Qp assessment was done regarding the mean concentrations per day, between PD11-14.

Viable cell density, viability, pH, osmolality, and metabolite concentrations of the five different conditions were similar, throughout the processes.

Titer and cell-specific productivities of the different conditions were also very similar. No significant differences were observed.

Regarding the glycan profiles, galactose supplementation had a significant impact. The effects of different supplementation regimens were analyzed and compared to the control condition (C1) in Table 4.7.

Table 4.7. Alterations in glycan compositions of the galactose supplemented conditions relative to C1 control condition.

Cond.	Alterations in Glycan Compositions (%)							
	G0F	G0F-GN	G0	G0-GN	Man5	G1Fa	G1Fb	G2F
C2	1.6% ↓	45% ↓	76.2% ↑	9.2% ↓	4.3% ↓	102.5% ↑	85.2% ↑	187% ↑
C3	4% ↑	65% ↓	58.7% ↑	48.3% ↓	37.3% ↓	132.3% ↑	111.3% ↑	226% ↑
C4	5.6% ↑	37% ↓	30.3% ↑	28.7% ↓	23.3% ↓	57.4% ↑	46% ↑	102.9% ↑
C5	1% ↑	31.6% ↓	41.3% ↑	8% ↓	2.8% ↓	59.6% ↑	48.3% ↑	88.4% ↑

4.10. TEMPERATURE REDUCTION ASSAY

Reducing the temperature to 30°C provided insight into how the E90-02 clone would behave under sub-physiological temperatures during a fed-batch process.

Decreasing the temperature to 30°C on PD7 resulted in higher VCD, compared to the PD5-shift condition (Figure 4.15.). This can be attributed to the growth phases of the cultures. While PD5 is the early-exponential growth phase, PD7 is the middle of the growth phase. Thus, the cells were cultured under optimal temperature for a longer period.

Although temperature reduction is known to increase longevity, viabilities were higher under 37°C (Figure 4.15.). This is probably due to the reduced temperature being too low for the E90-02 clone. In a study analyzing the effect of culture temperature on TNFR-Fc productivity in CHO cells, it was monitored that the cell growth was suppressed at 30°C even though it remained over 90% until PD9 [87]. This assay generated similar results, as cell viability under 30°C conditions were above 90% until PD9, which started to decrease

afterwards. The results of this study being similar to these findings indicate the existence of a common metabolic response in terms of cell growth, that may be expressed by certain CHO clones that are engineered to produce biotherapeutics. However, this effect might not necessarily impact each monitored parameter in the same manner, as analyzing protein production might generate different outcomes. Yet, the findings support and clearly set forth the impact of 30°C shift as alow sub-physiological temperature, at which cells are prone to stress, regarding growth.

Lactate concentrations were significantly lower under 30°C (75 % for PD7-shift, 66 % for PD5 shift conditions), compared to 37°C condition. The higher pH profiles were representative of the reduction in lactate concentrations, as shown in Figure 4.15 for both parameters.

Protein productions were reduced by 60 % under 30°C-shift conditions, compared to 37°C (Figure 4.15.). This reduction is significant, showing that at a low sub-physiological temperature as 30°C, nutrients cannot be utilized effectively for mAb synthesis. This can also be observed from the glucose consumption profiles represented in Figure 4.15. Thus, this temperature is too low to maintain titers above 2 g/L.

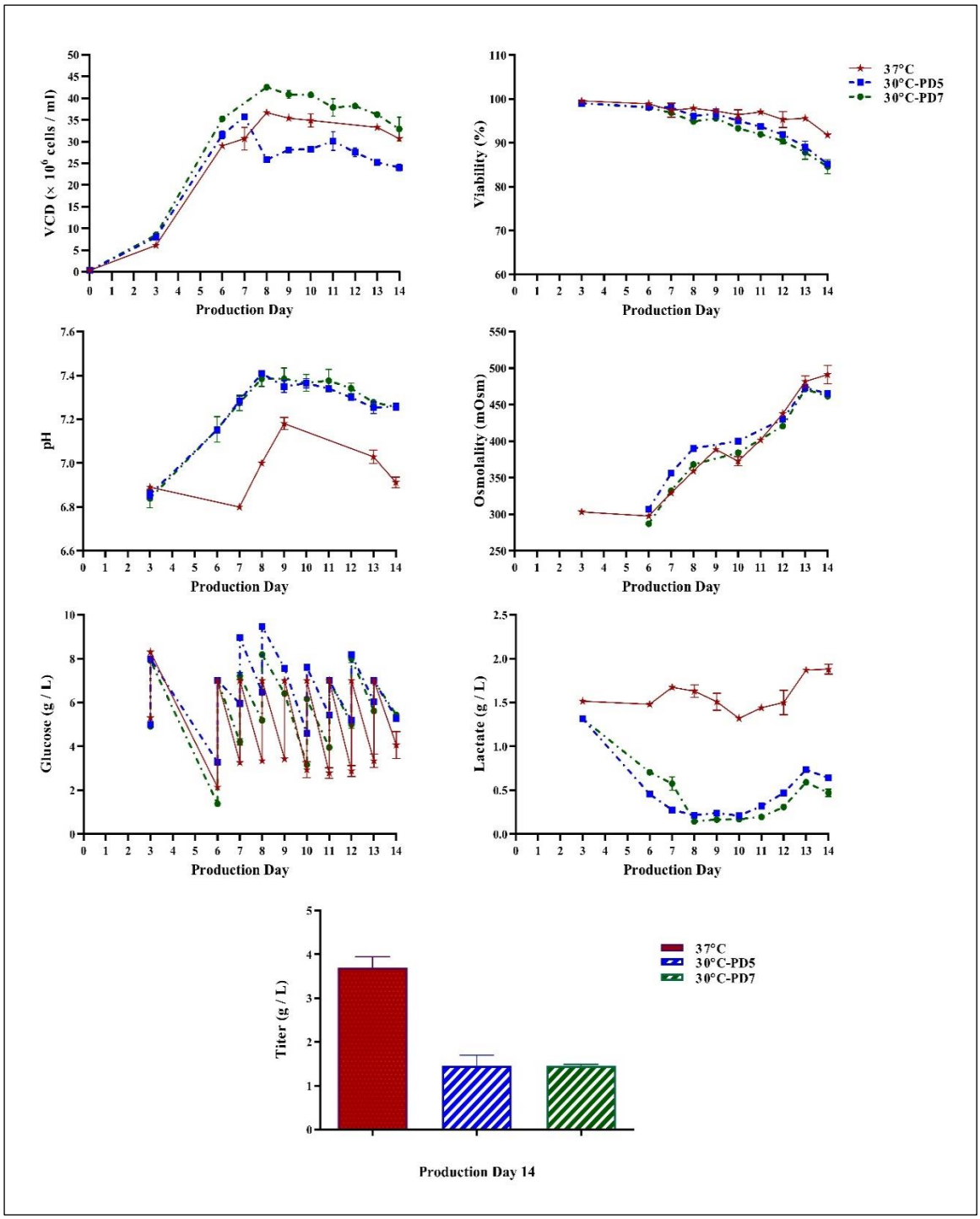

Figure 4.15. In-process control and titer results of the cultures under 30°C, shifted on different production days compared to the control condition maintained under 37°C.

Decreasing the temperature to 30°C significantly altered the glycan distributions, when compared to the 37°C condition. G0, G0F, G0F-GN, G1Fa, and G2F contents increased with reduced temperature, while G0-GN, G1Fb, and Man5 contents decreased. The impact on galactose compositions was over 40%. Shifting the temperature down to 30°C was effective in reducing Man5 levels over 2-folds, especially on PD5, which resulted in a reduced level of over 4-folds (Table 4.8.).

Table 4.8. Comparative glycan contents (%) of the originator and 37°C, 30°C-PD5 Shift, 30°C-PD7 shift conditions.

Glycan Structure	Originator	37°C	30°C-PD5	30°C-PD7
G0F	65.11	60.02	78.9	73.31
G1Fa	8.20	3.21	4.76	4.87
G1Fb	8.92	16.42	5.62	5.63
G2F	2.32	0.96	1.34	1.37
G0	0.18	0.67	0.76	0.86
G0-GN	0.79	1.28	0.43	0.77
G0F-GN	4.15	2.69	4.17	6.42
Man5	5.14	11.05	2.54	4.77

4.11. PRODUCTIONS IN 3-L BIOREACTORS - PART 2

4.11.1. Cellular and Metabolite Analyses

BR-5, BR-6, and BR-7 productions reached similar peak VCDs of 35.68 × 10^6 VC/ml, 35.15 × 10^6 VC/ml, and 34.31 × 10^6 VC/ml between PD8 - PD10, respectively. BR-8 had the lowest density of viable cells with 27.74 x 10^6 cells/ml observed on PD7. BR-5 production was terminated with the highest VCD of 29.44 x 10^6 cells/ml, as 32°C shift was applied on PD7, allowing the cells to grow through the exponential phase of growth. This also supports the viability results, where the highest levels of culture longevity were observed with BR-5 (87.5%) and BR-8 (91.7%) productions, with 32°C shifts on PD7 and PD5, respectively. Since PD5 is earlier in the exponential phase, even though the cells could not reach high densities as observed in BR-5, BR-8 viabilities were the highest at culture termination (Figure 4.16.). In a study conducted by Bollati-Fogolin *et al.* [93] on CHO cells producing recombinant human granulocyte macrophage colony-stimulating factor, by decreasing the temperature down to 33°C from 37°C after the lag phase of growth, they were able to maintain cell viability over 80% as seen in this study. While BR-5 and BR-8 end-culture viabilities were above 80%, bioreactors ran at 37°C showed decreased viability, below 80%. Regarding the results of the cell growth analysis, PD5 is the early-exponential phase that follows the lag-phase of the CHO clone used in this study. As 32°C and 33°C are higher temperatures compared to 30°C, the shifts applied are considered as mild temperature reductions. When both the results of the temperature reduction assay and this study are evaluated, it can be concluded that mild temperature reduction is an effective strategy to increase longevity in CHO cells, in processes that aim the manufacture of high yield proteins that require extended durations for production.

Lactate productions, pCO_2 levels, and pH trends were consistent. As the pCO_2 levels in BR-7 suddenly started to increase after PD7, lactate concentrations declined initially, but rapidly increased after PD9 as a result of the increased pCO_2 level. This was reflected in pH, with the lowest trend among production runs (Figure 4.16.). pCO_2 levels in BR-7 production

exceeded the high limit of 200 mmHg, reaching 317 mmHg and becoming harmful for cells. The decline in VCD started right after exceeding the pCO_2 limit. When analyzed, BR-7 was the only production in which the pH was managed to meet the set-point of 6.9. The pH trends in other productions were still within the set limits but proceeded at the upper limit of the dead-band. The extreme and sudden rise of pCO_2 level can be attributed to the fact that this pH level is low for the production and the process controls responded aggressively to support the high cell density reached on PD8, just before pCO_2 and lactate levels changed immediately.

When pH trends are analyzed, regarding the set-point of 6.9 ± 0.2, BR-5 production exceeded the limit by the end of production, due to low lactate generation by cells. The highest osmolality trend was observed in BR-7 production, which makes sense regarding lactate generation. Even though lactate concentrations rapidly increased in BR-8, this was reflected on neither the osmolality levels nor the protein production. This is probably due to the late increase of lactate, after PD11, compared to BR-7 production, in which this increase began on PD9 (Figure 4.16.).

4.11.2. Productivity

The highest titer was observed in BR-5 production, which is expected since temperature down-shift at exponential phase allows cells to reach a high density and then drives their metabolism towards production instead of growth. This is supported by a CHO-cell based Erythropoietin (EPO) production study, in which the 32°C shift condition produced higher EPO compared to the 37°C condition. Plus, as it was observed in the temperature reduction assay at 30°C, in this study as well, the EPO production was reduced, below the temperature 32°C [198]. The metabolic responses observed in CHO cells are highly dependent on the proteins they are intended to produce. However, common outcomes can be observed among most CHO clones, regarding their lower limits of the temperature shift. The 32°C temperature down-shift was effective in increasing both the cellular longevity and C5mAb productivity.

The 32°C shift in BR-8 was applied on PD5, which is the early exponential phase of growth. Thus, the cells were not allowed to grow to high density, resulting in an almost 8 x10^6 cells/ml difference compared to BR-5 production. The lowest production was observed for BR-7 production, due to high pCO_2 levels and increased lactate generation. If maintaining the pH at set-point had not posed these burdens on this production, the titer would be similar to that of BR-6, which ran under the same conditions. BR-6 production proceeded at a milder pH, remained at below-limit pCO_2 levels, and generated a very low concentration of lactate. As a result, the titer was comparable to BR-5 production as seen in Figure 4.16., in which temperature down-shift was applied to increase production, besides adjusting quality attributes. The temperature-shifting strategy was useful in boosting production, as supported by a study conducted by Pan X. *et al.* [196]. In this study, two CHO cell clones, one with high IgG producing and one with low IgG producing characteristics, were screened for different media and feed combinations to achieve high titers. Both clones, derived from the same parental cell line, were producing the same IgG1 antibody in 14 days of fed-batch cultures. The results showed that, when the feeding was initiated on PD3 with the same feed combinations as it was done with both the extended feeding strategy and the feed choice in this study, the highest titer was obtained as 1.2 g/L, with the high mAb producing clone. The screenings analyzed in this study initially focused on determining the most optimum feeding regimen, with the best performing clone. All the analyses of this study aimed to determine the process conditions that generate high titer, with complying key quality attributes. The findings of this study were obtained under comparable feeding regimens, as feedings were initiated on the exact same day of production, using the same feed combinations. Yet, the titer obtained with their highest producing clone was 41% lower than that obtained from the BR-5 production. As a conclusion, the developed BR-5 process, with 2.9 g / L C5mAb production, can be stated to yield high titer.

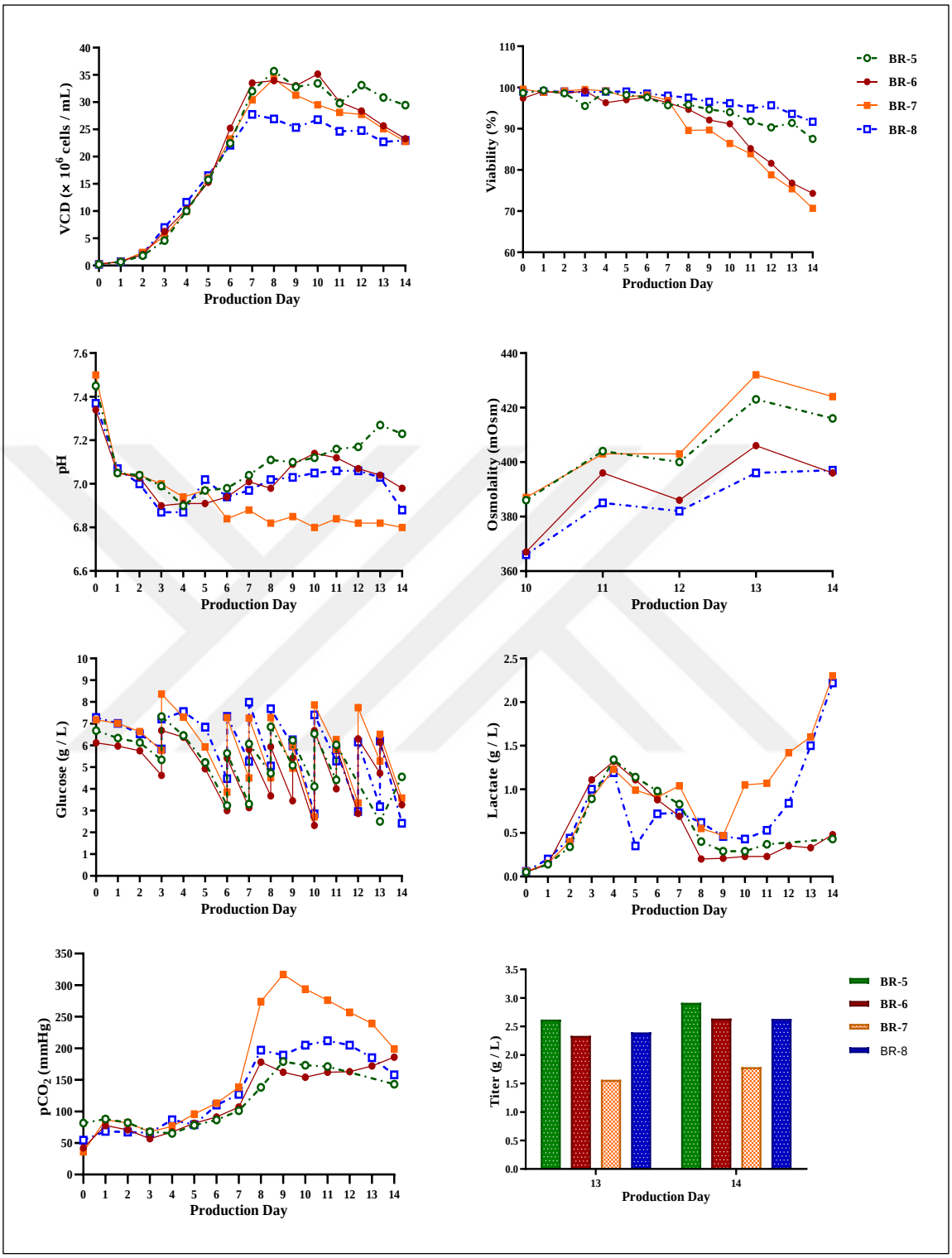

Figure 4.16. In-process control and titer results of the bioreactor productions BR-5, BR-6, BR-7, and BR-8.

4.11.3. Glycosylation

Four originator lots were analyzed together with the bioreactor production samples, for the evaluation of glycan contents. Compliance ranges for each glycan structure were defined by setting ± 3 SD as the limit. Total non-fucosylated, galactosylated, and high mannose structures were further calculated for better evaluation.

All productions yielded compliant structures of galactosylated and non-fucosylated glycans. When each content in Table 4.9 was investigated separately, all the productions seemed to exceed the G0 content limits, but as the glycans they generated had relatively lower amounts of G0-GN, the total non-fucosylated glycan limits were met. The Man5 contents of the productions BR-5, BR-7, and BR-8 remained below limits. Although low levels of high mannose structures are preferred due to immunogenicity concerns, the contents are expected to be within the originator limits, for the produced mAb to be considered as a biosimilar. However, it is worth noticing that only four originator lots were analyzed in this study to define the compliance limits. Increasing the number of the originator lots would, in turn, extend the range, and as the relative difference observed in Man5 contents of the productions is not high compared to that of the originator, this would help achieve the desired compliance.

Table 4.9. Major glycan contents (%) of bioreactor productions BR-5, BR-6, BR-7, BR-8 compared to the four different originator lots.

Glycan Structure	Originator Lot1	Originator Lot2	Originator Lot3	Originator Lot4	Originator AVG (n=4)	STD	Originator AVG ± 3SD	BR-5	BR-6	BR-7	BR-8
G0	0.21	0.24	0.19	0.2	0.21	0.022	0.15-0.27	0.97	0.56	0.35	0.89
G2F	0.45	0.24	1.30	1.48	0.87	0.614	0-2.71	1.10	1.06	1.35	1.02
G0-GN	0.75		1.005	1.16	0.97	0.207	0.35-1.59	0.64	0.98	0.26	0.65
G0F-GN	3.99	6.09	5.79	6.82	5.67	1.205	2.06-9.28	7.95	15.63	8.30	6.76
G0F	64.32	70.54	69.93	66.62	67.85	2.916	59.10-76.60	76.19	66.42	75.25	78.48
G1Fa	6.06	6.54	8.42	5.95	6.74	1.145	3.31-10.18	3.44	3.14	4.07	3.47
G1Fb	6.54	6.07	9.13	6.49	7.06	1.396	2.87-11.24	3.97	3.50	4.54	4.45
Man5	5.09	5.48	5.30	6.44	5.58	0.597	3.78-7.37	3.00	6.06	2.07	2.33
Man6	-	-	-	-	-	-	-	-	-	1.12	0.78
Man7	-	-	-	-	-	-	-	-	-		-
Man8	-	-	-		-	-	-	-	-	0.23	-
Non-fucosylated	0.96	0.24	1.20	1.36	1.18	0.23	0.50-1.86	1.61	1.54	0.61	1.54
Galactosylated	13.05	12.85	18.84	13.92	14.66	3.15	6.18-24.13	8.51	7.70	9.96	8.94
High mannose	5.09	5.48	5.30	6.44	5.58	0.60	3.78-7.37	3	6.06	3.42	3.11
Total non-fucosylated	6.05	5.72	6.49	7.8	6.76	0.83	4.28-9.23	4.61	7.6	4.03	4.65

4.11.4. Binding Assay

Regarding in-process control, production, and glycan analysis results, the most promising productions were BR-5 and BR-6 bioreactors. But, as production deviations were observed in BR-7 production leading to the lowest titer production, the binding of this production was also analyzed to investigate the potency of the developed C5-mAb, even with the least titer. The relative potency of all the samples remained within the 80 – 120 % acceptance range. When the results were compared among each other relative to that of the Originator -1 (reference), BR-7 production was assessed to generate the most optimum C5-mAb candidate in terms of binding, while BR-5 production represented the lowest potency among all (Table 4.10.).

Table 4.10. Relative binding potency (%) of BR-5, BR-6, and BR-7 productions compared to two different lots of originator.

C5-mAb	EC50	Relative Potency (%)	CV (%)
Originator-1	0.192	100	7.69
Originator-2	0.191	99.48	8.05
BR-5	0.161	83.85	8.36
BR-6	0.186	96.88	7.31
BR-7	0.191	99.48	9.90

5. CONCLUSION

This study intended to develop a robust production process strategy to achieve a biosimilar C5-mAb. The novelties of the study are set forth with clone selection to produce a hybrid, humanized monoclonal antibody, followed by the integration of design-of-experiments (DoE) to choose the ideal feed supplementation strategy and production temperature, which are pivotal for mammalian cell-based production processes seeking quality.

The well-designed substructure of the study led to progressive eliminations of variables to determine the crucial parameters that would impact the quality of the final product in terms of safety, efficacy, and potency. The initial four 3-L bioreactor productions were conducted to investigate the conditions which were not observed during small scale productions. The usage of shake flasks and spin tubes for the screening studies helped eliminate a considerable amount of experimental runs, saving time, labor, and costs. By conducting these screening studies, it was possible to screen and choose the clone, feeding regimen, temperature, and time of temperature shift through the DoE strategy, which made it possible to analyze over 40 experimental runs at once. However, it was necessary to analyze the determined process parameters in a more controlled environment. By running the selected conditions in 3-L bioreactors, it became possible to adjust and monitor process pH, as well as oxygenation. Plus, it became possible to analyze the interactions among parameters such as dissolved carbon-dioxide, pH, and lactate generation, and relate the outcomes accordingly, in terms of cell growth and protein production.

As a result of the initial four 3-L bioreactor productions, further analyses were considered necessary before conducting the fine-tuning production processes. This intermediate investigation step was critical, as it provided the data required to carry the study to the final step of process adjustment.

Regarding the results of these bioreactor productions, two strategies were considered: either supplementing galactose to fix the glycan structures or trying to adjust them by temperature reduction. The first strategy was to analyze the effects of galactose supplementation. According to the galactosylation results of the four bioreactor productions, it was necessary to increase the G1F contents, but the non-galactosylated structures were already close to those of the Originator. When supplementation was applied, instead of elevating mostly the levels of G1F structures, the galactosylation process was more driven towards the G2F contents, which increased to undesired levels. Accordingly, this strategy was eliminated and temperature reduction to low sub-physiological conditions was analyzed.

In certain CHO cell-based production systems, it becomes an advantage to decrease the temperature to low sub-physiological temperatures such as 30°C, as it results in both increased protein productivity and culture longevity. However, these potential outcomes are strictly dependent on the CHO clone being used as the expression system, since not every clone is expected to produce similar metabolic responses. In this study, it was demonstrated that 30°C temperature was not an ideal temperature for the E90-02 clone, in terms of both cell growth and production. The highest peak VCD of 42.51×10^6 VC/ml under 30°C – PD7 shift was achieved only with the sacrifice of C5mAb production and culture longevity. Both the titer and the culture viabilities were significantly decreased, compared to those at 37°C. In fact, the correlation of VCD and titer observed with this study matched the results of a study conducted by Fan L. *et al.* [87] on TNFR-Fc producing GS-CHO cells. According to their results, the highest TNFR-Fc productivity was obtained when culture temperature was decreased down to 30°C, but this effect was compromised once they increased culture VCD levels above a certain limit. When 30°C shift conditions at PD5 and PD7 in this study were compared, both productions generated low titers, even though the VCD levels under PD5 shift conditions were not as high as those observed under PD7 shift conditions. However, the reason behind this is most likely that PD5 is early in the exponential phase of cell growth, so the cell growth becomes inhibited, resulting in a lower titer. The similar outcomes of the 30°C – PD7 shift condition in this study and the findings of the study conducted with TNFR-Fc producing CHO cells suggests that, at 30°C, the beneficial effects of low-temperature production becomes dependent on the VCD levels. Thus, C5mAb

production under 30°C was concluded to result in increased cellular stress, lowering the culture viability and decreasing titers by 60 percent.

On the other hand, when the glycosylation profiles were analyzed, it was observed that decreasing the temperature had a significant impact on galactosylated and Man5 glycan structures. While protein productions were decreased down to undesired levels, G1F and Man5 levels approached those of the Originator. These results were critical in determining the strategy for the following experiments. The results indicated that temperature reduction is a promising strategy for adjusting the glycosylation profile of the E90-02 clone. Since 30°C was concluded to be a very low temperature, it was decided to conduct the next bioreactor productions by applying 32°C temperature shifts.

The final bioreactor productions demonstrated the advantages of the temperature reduction strategy, and finally, an ideal production temperature was determined for the E90-02 clone to produce the C5mAb. Two temperature shift days, as PD5 and PD7, were analyzed together with the 37°C control conditions. As PD5 was early in the exponential phase of growth, although over 90% end-culture viability was achieved, cell growth was decreased, lactate generation was increased, and C5mAb production was not enhanced compared to the control conditions. On the other hand, applying the temperature shift on PD7 provided the necessary culture duration under optimum growth conditions at 37°C, lowering lactate generation, and enhancing C5mAb production. The highest peak VCD level was achieved under this production and the end-culture viability was over 85%, while those of the control conditions remained under 80%. Besides cell growth, the highest titer of nearly 3 g / L was achieved under this production condition, while maintaining lactate concentration at significantly lower levels compared to the PD5 shift condition.

Compared to the results of the temperature reduction assay conducted at 30°C, shifting the temperature down to 32°C on PD7 provided the benefits of increased production and culture longevity, while still achieving high VCD levels. The results were considered to be related to the negative correlation of titer and VCD levels at 30°C, as similar outcomes were

observed with TNFR-Fc producing GS-CHO cells [87]. In fact, the findings of another CHO-cell based study that analyzed the effects of temperature on Erythropoietin production (EPO) [198] are supportive in this context as well. In the study, EPO production was observed to be the highest at 32°C culture temperature compared to the 25°C, 28°C, 30°C, and 37°C conditions, but specific EPO productivity at 30°C was found to be higher than that observed under 32°C. The conclusion drawn from the analyses of that study was also that the advantages of lowering culture temperature are lost at temperatures below 32°C, as the impacts on cell growth become detrimental.

The findings of these studies support the evaluations stated in this study, leading to the conclusion that for the E90-02 clone, 30°C production results with high cellular stress that negatively impacts culture longevity and C5mAb production. Instead, applying 32°C temperature shift in production results in the benefits of increased culture longevity and production, while maintaining comparable lactate generation.

When the glycosylation profiles were analyzed, it was observed that all the major glycan contents were within the acceptance ranges, defined by analyzing the Originator lots. Only high mannose levels were slightly below the lower limit, however, the difference observed was as small as to be related to the number of analysis. As only four Originator lots were analyzed together with the four bioreactor samples, the acceptance ranges to be met were narrow. If the number of Originator lots analyzed was to be increased, the compliance ranges would be extended as a result, making it not only possible but most likely to meet the acceptance criteria, in terms of high mannose levels.

The developed process was then carried to the next and final investigation step of this study, to verify its efficacy through conducting a binding assay. Two Originator lots and two 37°C control conditions were analyzed together with the developed process to assess binding. According to the results, although 37°C conditions showed higher potency, the developed BR-5 process was also confirmed to have comparable efficacy, by meeting the acceptance criteria in terms of binding potency. This final assay demonstrated the success of the

conducted study, aiming to develop an ideal production process to produce a highly biosimilar C5mAb.

As successful as it was, this study also had certain limitations and it still leaves behind important questions that remain to be answered.

Analyzing different originator lots is necessary to define acceptance ranges, regarding quality attributes. The fact that the quantitative values of the key quality attributes may differ among product lots in the market worldwide poses a risk to evaluate the biosimilarity unless a sufficient amount of lots are analyzed to define proper acceptance ranges. A reliable analysis would require the investigation of a minimum of ten different lots of the originator product. The limitation of the originator lots in this study, with four different lots for glycan analysis and two different lots for the binding assay, was a challenge. Nevertheless, it resulted in success.

According to the last 3-L productions and the binding assay, this study was successful in developing a biosimilar C5-mAb in terms of glycosylation and potency. Even though only four originator lots were included in the glycan analysis, adjusting the critical process parameters necessarily helped in achieving the acceptance criteria as galactosylation, total non-fucosylated structures, and binding potency met the product ranges. While the two 37°C productions yielded Man5 contents within ranges, the two temperature shift conditions were almost in ranges and would probably meet the criteria, assuming more originator data are to be included.

A thorough evaluation of this study would necessitate addressing future studies that would hopefully be established on these findings. As successful as it was in meeting the desired quality attributes, certain points of the study shall be highlighted to offer insight for further answers that still seek answers to.

When assessing biosimilarity, it should be ensured that all the critical quality attributes are met. The most striking advantage of developing biosimilars is that they significantly reduce costs. The reduction in costs is achieved by the reduced number of clinical studies that need to be conducted. Biosimilars offer an alternative to the original marketed product. As the therapeutic benefit of the original product is already identified, once the similarities of the developed product are exhibited, which means that the defined key quality attributes are verified to comply with those of the Originator, it becomes possible to manufacture the developed therapeutic by conducting seriously reduced amount of analysis, under Phase studies.

Although glycosylation and binding were analyzed in this study, another critical quality attribute, charge variants, was held beyond the scope of this study. Charge variants may alter mAb tissue penetration and distribution, binding properties, and PK. Degradation, sequence variants, post-translational modifications, production processes, and host cell line characteristics are all potential impacts on charge variation. It should be noted that their influence on antibodies is highly dependent on the amount, nature, and the site of post-translational modification, as glycosylation is a major post-translational modification for mAbs. They are also known to be influenced by culture media components and co-factor supplementations. To assume a product as a biosimilar candidate, it is necessary to confirm that both acidic and basic variants, as well as the main peak, comply with those of the originator. Considering the screening parameters focused in this study, charge variant analysis is critical for the final assessment of biosimilarity, as it would help carry the study to its final stage of success, qualifying the developed product as a high biosimilar.

As a final statement, adjusting the key quality attributes through well-designed optimization strategies is a powerful tool for achieving highly biosimilar mAbs, contributing to the world health segment by the development of therapeutics for rare diseases.

REFERENCES

1. Niosi J. Imitation and innovation new biologics, biosimilars and biobetters. *Technol Anal Strateg Manag*. 2016;29(3):251–62.

2. Del Val IJ, Kontoravdi C, Nagy JM. Towards the implementation of quality by design to the production of therapeutic monoclonal antibodies with desired glycosylation patterns. *Biotechnol Prog*. 2010;26(6):1505–27.

3. Bonini S, Bonini M. Biosimilars and drug development in allergic and immunologic diseases. *J Allergy Clin Immunol*. 2017;139(5):1461–4.

4. Ventola CL. Biosimilars: part 1: proposed regulatory criteria for FDA approval. *P T*. 2013;38(5):270–87.

5. Declerck P, Danesi R, Petersel D, Jacobs I. The Language of Biosimilars: Clarification, Definitions, and Regulatory Aspects. *Drugs*. 2017;77(6):671–7.

6. Derbyshire M. Patent expiry dates for best-selling biological. *Generics Biosimilars Initiat J*. 2015;4:178–9.

7. Niosi J. Biotechnology: A case of delayed international convergence? *Int J Biotechnol*. 2014;13(1–3):5–21.

8. Dimasi JA, Grabowski HG. The cost of biopharmaceutical R&D: Is biotech different? *Manag Decis Econ*. 2007;28(4–5):469–79.

9. Becerra S, Berrios J, Osses N, Altamirano C. Exploring the effect of mild hypothermia on CHO cell productivity. *Biochem Eng J*. 2012;60:1–8.

10. European Commission. What you need to know about Biosimilar Medicial Products. *Consensus Information Paper*. 2013.

11. European Commission. Pharmaceutical Sector Inquiry: Final Report. 2009.

12. Biosimilars market - Growth, Trends, and Forecast (2020-2025), Mordor Intelligence Pvt. Ltd.; [cited 2020 4 July]. Available from: https://www.mordorintelligence.com/industry-reports/global-biosimilars-market-

industry

13. Rémuzat C, Dorey J, Cristeau O, Ionescu D, Radière G, Toumi M. Key drivers for market penetration of biosimilars in Europe. *J Mark Access Heal Policy*. 2017;5(1):1272308.

14. FDA. 2017 Biological approvals. [cited 2017 28 November]. Available from: https://www.fda.gov/vaccines-blood-biologics/development-approval-process-cber/2017-biological-approvals

15. What's next for biosimilars in emerging markets?; [cited 2020 3 March]. Available from: https://www.mckinsey.com/industries/pharmaceuticals-and-medical-products/our-insights/whats-next-for-biosimilars-in-emerging-markets#

16. İlaç Endüstrisi İşverenler Sendikası. Türkiye İlaç Pazarı Ocak - Mart 2017; [cited 2017 3 March]. Available from: http://www.ieis.org.tr/ieis/assets/media/IPAR/Turkiye%20Ilac%20Pazari%20Ocak-Mart%202017.pdf

17. İlaç Endüstrisi İşverenler Sendikası. Türkiye İlaç Pazarı Ocak - Mart 2020; [cited 2020 19 March]. Available from: http://www.ieis.org.tr/ieis/assets/media/Yayinlar/TR_Ilac_Ocak_Mart2020.pdf

18. Grilo AL, Mantalaris A. The Increasingly Human and Profitable Monoclonal Antibody Market. *Trends Biotechnol*. 2018;37(1):9–16.

19. Emerging markets hold the potential for makers of biosimilars; [cited 2017 8 October]. Available from: https://www.beroeinc.com/whitepaper/biosimilars-emerging-markets/

20. Global Monoclonal Antibodies Market Size, Share, Trends & Growth Analysis Report - By Source, Indication, End-User, Application (Medical, Experimental, Western (Blot, ELISA, Radioimmune Assays, Immunofluorescence & others), By Region - Industry Forecast (2020 to 2025); [cited 2020 16 June]. Available from: https://www.marketdataforecast.com/market-reports/global-monoclonal-antibodies-market

21. Nechansky A, Kircheis R. Immunogenicity of therapeutics: a matter of efficacy and

safety. *Expert Opin Drug Discov*. 2010;5(11):1067–79.

22. Liu L. Antibody glycosylation and its impact on the pharmacokinetics and pharmacodynamics of monoclonal antibodies and Fc-fusion proteins. *J Pharm Sci*. 2015;104(6):1866–84.

23. Haberger M, Bomans K, Diepold K, Hook M, Gassner J, Schlothauer T, *et al.* Assessment of chemical modifications of sites in the CDRs of recombinant antibodies: Susceptibility vs. functionality of critical quality attributes. *MAbs*. 2014;6(2):327–39.

24. Rosati S, Yang Y, Barendregt A, Heck AJR. Detailed mass analysis of structural heterogeneity in monoclonal antibodies using native mass spectrometry. *Nat Protoc*. 2014;9(4):967–76.

25. Roque ACA, Lowe CR, Taipa MÂ. Antibodies and genetically engineered related molecules: Production and purification. *Biotechnology Progress.* 2004;20(3):639–54.

26. Cohen SL, Price C, Vlasak J. β-elimination and peptide bond hydrolysis: Two distinct mechanisms of human IgG1 hinge fragmentation upon storage. *J Am Chem Soc*.2007;129(22):6976–7.

27. Shukla AA, Hubbard B, Tressel T, Guhan S, Low D. Downstream processing of monoclonal antibodies-Application of platform approaches. *J Chrom B.* 2007;848(1):28–39.

28. Wang X, An Z, Luo W, Xia N, Zhao Q. Molecular and functional analysis of monoclonal antibodies in support of biologics development. *Protein Cell*. 2017;9(1):74-85.

29. Butler M. Optimisation of the cellular metabolism of glycosylation for recombinant proteins produced by mammalian cell systems. *Cytotechnology*. 2005;50(1–3):57–76.

30. Rosenberg AS. Effects of protein aggregates: An immunologic perspective. *AAPS J*. 2006;8(3):E501–7.

31. Cromwell MEM, Hilario E, Jacobson F. Protein aggregation and bioprocessing. *AAPS J*. 2006;8(3):E572–9.

32. Philo JS, Arakawa T. Mechanisms of protein aggregation. *Curr Pharm Biotechnol*. 2009;10(4):348–51.

33. Beck A, Wagner-Rousset E, Ayoub D, Van Dorsselaer A, Sanglier-Cianférani S. Characterization of therapeutic antibodies and related products. *Analytical Chemistry*. 2012;85(2):715–36.

34. Biosynthesis of N-Glycans; [cited 2018 19 October]. Available from: https://international.neb.com/applications/glycobiology-and-proteomics/recombinant-glycoprotein-expression/biosynthesis-of-n-glycans

35. Chiu ML, Goulet DR, Teplyakov A, Gilliland GL. Antibody Structure and Function: The Basis for Engineering Therapeutics. *Antibodies*. 2019;8(4):55.

36. Fagioli C, Mezghrani A, Sitia R. Reduction of Interchain Disulfide Bonds Precedes the Dislocation of Ig-μ Chains from the Endoplasmic Reticulum to the Cytosol for Proteasomal Degradation. *J Biol Chem*. 2001; 276(44):40962–7.

37. Kirk SJ, Cliff JM, Thomas JA, Ward TH. Biogenesis of secretory organelles during B cell differentiation. *J Leukoc Biol*. 2009;87(2):245–55.

38. Shaffer AL, Shapiro-Shelef M, Iwakoshi NN, Lee AH, Qian SB, Zhao H, *et al.* XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. *Immunity*. 2004;21(1):81–93.

39. Jefferis R. Glycosylation of recombinant antibody therapeutics. *Biotechnology Progress*. 2005;21(1):11–6.

40. Jefferis R. Recombinant antibody therapeutics: the impact of glycosylation on mechanisms of action. *Trends in Pharmacological Sciences*. 2009;30(7):356–62.

41. Andersen DC, Bridges T, Gawlitzek M, Hoy C. Multiple cell culture factors can affect the glycosylation of Asn-184 in CHO-produced tissue-type plasminogen activator. *Biotechnol Bioeng*. 2000;70(1):25–31.

42. Butler M. Animal cell cultures: Recent achievements and perspectives in the production of biopharmaceuticals. *Applied Microbiology and Biotechnology*.

2005;68(3):283–91.

43. Daëron M. Fc receptors as adaptive immunoreceptors. *Current Topics in Microbiology and Immunology*. 2014;382:131–64.

44. Hmiel LK, Brorson KA, Boyne MT. Post-translational structural modifications of immunoglobulin G and their effect on biological activity. *Analytical and Bioanalytical Chemistry*. 2015;407(1):79–94.

45. Ivarsson M, Villiger TK, Morbidelli M, Soos M. Evaluating the impact of cell culture process parameters on monoclonal antibody N-glycosylation. *J Biotechnol*. 2014;188:88–96.

46. Pacis E, Yu M, Autsen J, Bayer R, Li F. Effects of cell culture conditions on antibody N-linked glycosylation-what affects high mannose 5 glycoform. *Biotechnol Bioeng*. 2011;108(10):2348–58.

47. Van Berkel PHC, Gerritsen J, Perdok G, Valbjørn J, Vink T, Van De Winkel JGJ, *et al*. N-linked glycosylation is an important parameter for optimal selection of cell lines producing biopharmaceutical human IgG. *Biotechnol Prog*. 2009;25(1):244–51.

48. Raju TS. Glycosylation Variations with Expression Systems and Their Impact on Biological Activity of Therapeutic Immunoglobulins. *BioProcess Int*. 2003;1:44–53.

49. Kaneko E, Niwa R. Optimizing therapeutic antibody function: Progress with fc domain engineering. *BioDrugs*. 2011;25(1):1–11.

50. Overdijk MB, Verploegen S, Bögels M, Van Egmond M, Lammerts Van Bueren JJ, Mutis T, *et al*. Antibody-mediated phagocytosis contributes to the anti-tumor activity of the therapeutic antibody daratumumab in lymphoma and multiple myeloma. *MAbs*. 2015;7(2):311–20.

51. Kallewaard NL, Corti D, Collins PJ, Neu U, McAuliffe JM, Benjamin E, *et al*. Structure and Function Analysis of an Antibody Recognizing All Influenza A Subtypes. *Cell*. 2016;166(3):596–608.

52. Boune S, Hu P, Epstein AL, Khawli LA. Principles of N-Linked Glycosylation Variations of IgG-Based Therapeutics: Pharmacokinetic and Functional

Considerations. *Antibodies*. 2020;9(2):22.

53. Nimmerjahn F, Ravetch J V. Translating basic mechanisms of IgG effector activity into next generation cancer therapies. *Cancer immunity*. 2012;12:13.

54. Bruhns P, Iannascoli B, England P, Mancardi DA, Fernandez N, Jorieux S, *et al*. Specificity and affinity of human Fcgamma receptors and their polymorphic variants for human IgG subclasses. *Blood*. 2009;113(16):3716–25.

55. Suzuki E, Niwa R, Saji S, Muta M, Hirose M, Iida S, *et al*. A nonfucosylated anti-HER2 antibody augments antibody-dependent cellular cytotoxicity in breast cancer patients. *Clin Cancer Res*. 2007;13(6):1875–82.

56. Iida S, Misaka H, Inoue M, Shibata M, Nakano R, Yamane-Ohnuki N, *et al*. Nonfucosylated therapeutic IgG1 antibody can evade the inhibitory effect of serum immunoglobulin G on antibody-dependent cellular cytotoxicity through its high binding to FcγRIIIa. *Clin Cancer Res*. 2006;12(9):2879–87.

57. Matsumiya S, Yamaguchi Y, Saito J ichi, Nagano M, Sasakawa H, Otaki S, *et al*. Structural Comparison of Fucosylated and Nonfucosylated Fc Fragments of Human Immunoglobulin G1. *J Mol Biol*. 2007;368(3):767–79.

58. Masuda K, Kubota T, Kaneko E, Iida S, Wakitani M, Kobayashi-Natsume Y, *et al*. Enhanced binding affinity for FcgammaRIIIa of fucose-negative antibody is sufficient to induce maximal antibody-dependent cellular cytotoxicity. *Mol Immunol*. 2007;44(12):3122–31.

59. Shinkawa T, Nakamura K, Yamane N, Shoji-Hosaka E, Kanda Y, Sakurada M, *et al*. The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. *J Biol Chem*. 2003;278(5):3466–73.

60. Ferrara C, Grau S, Jager C, Sondermann P, Brunker P, Waldhauer I, *et al*. Unique carbohydrate-carbohydrate interactions are required for high affinity binding between Fc RIII and antibodies lacking core fucose. *Proc Natl Acad Sci*. 2011;108(31):12669–74.

61. Kaneko Y, Nimmerjahn F, Ravetch J V. Anti-Inflammatory Activity of Immunoglobulin G Resulting from Fc Sialylation. *Science*. 2006;313(5787):670–3.

62. Goetze AM, Liu YD, Zhang Z, Shah B, Lee E, Bondarenko P V, *et al.* High-mannose glycans on the Fc region of therapeutic IgG antibodies increase serum clearance in humans. *Glycobiology*. 2011;21(7):949–59.

63. Walsh G, Jefferis R. Post-translational modifications in the context of therapeutic proteins. *Nat Biotechnol*. 2006;24(10):1241–52.

64. Jones AJS, Papac DI, Chin EH, Keck R, Baughman SA, Lin YS, *et al.* Selective clearance of glycoforms of a complex glycoprotein pharmaceutical caused by terminal N-acetylglucosamine is similar in humans and cynomolgus monkeys. *Glycobiology*. 2007;17(5):529–40.

65. Kanda Y, Yamada T, Mori K, Okazaki A, Inoue M, Kitajima-Miyama K, *et al.* Comparison of biological activity among nonfucosylated therapeutic IgG1 antibodies with three different N-linked Fc oligosaccharides: The high-mannose, hybrid, and complex types. *Glycobiology*. 2007;17(1):104–18.

66. Yu M, Brown D, Reed C, Chung S, Lutman J, Stefanich E, *et al.* Production, characterization and pharmacokinetic properties of antibodies with N-linked Mannose-5 glycans. *MAbs*. 2012;4(4):475–87.

67. Kanda Y, Yamane-Ohnuki N, Sakai N, Yamano K, Nakano R, Inoue M, *et al.* Comparison of cell lines for stable production of fucose-negative antibodies with enhanced ADCC. *Biotechnol Bioeng*. 2006;94(4):680–8.

68. Zhou Q, Shankara S, Roy A, Qiu H, Estes S, McVie-Wylie A, *et al.* Development of a simple and rapid method for producing non-fucosylated oligomannose containing antibodies with increased effector function. *Biotechnol Bioeng*. 2008;99(3):652–65.

69. Hodoniczky J, Yuan ZZ, James DC. Control of recombinant monoclonal antibody effector functions by Fc N-glycan remodeling in vitro. *Biotechnol Prog*. 2005;21(6):1644–52.

70. Peipp M, Dechant M, Valerius T. Effector mechanisms of therapeutic antibodies against ErbB receptors. *Current Opinion in Immunology*. 2008;20(4):436–43.

71. Raju TS. Terminal sugars of Fc glycans influence antibody effector functions of IgGs. *Current Opinion in Immunology*. 2008;20(4):471–8.

72. Xu J, Tang P, Yongky A, Drew B, Borys MC, Liu S, *et al.* Systematic development of temperature shift strategies for Chinese hamster ovary cells based on short duration cultures and kinetic modeling. *MAbs*. 2019;11(1):191–204.

73. Sissolak B, Lingg N, Sommeregger W, Striedner G, Vorauer-Uhl K. Impact of mammalian cell culture conditions on monoclonal antibody charge heterogeneity: an accessory monitoring tool for process development. *J Ind Microbiol Biotechnol*. 2019;46(8):1167–78.

74. Kaisermayer C, Reinhart D, Gili A, Chang M, Aberg PM, Castan A, *et al.* Biphasic cultivation strategy to avoid Epo-Fc aggregation and optimize protein expression. *J Biotechnol*. 2016;227:3-9.

75. Wong DCF, Wong NSC, Goh JSY, May LM, Yap MGS. Profiling of N-glycosylation gene expression in CHO cell fed-batch cultures. *Biotechnol Bioeng*. 2010;107(3):516–28.

76. Jan DCH, Petch DA, Huzel N, Butler M. The effect of dissolved oxygen on the metabolic profile of a murine hybridoma grown in serum-free medium in continuous culture. *Biotechnol Bioeng*. 1997;54(2):153–64.

77. Heidemann R, Lütkemeyer D, Büntemeyer H, Lehmann J. Effects of dissolved oxygen levels and the role of extra- and intracellular amino acid concentrations upon the metabolism of mammalian cell lines during batch and continuous cultures. *Cytotechnology*. 1998;26(3):185–97.

78. Banerjee A. Designing in quality: Approaches to defining the design space for a monoclonal antibody process. *BioPharm International*. 2010;23:26–40.

79. Read EK, Bradley SA, Smitka TA, Agarabi CD, Lute SC, Brorson KA. Fermentanomics informed amino acid supplementation of an antibody producing mammalian cell culture. *Biotechnol Prog*. 2013;29(3):745–53.

80. Wong DCF, Wong KTK, Goh LT, Heng CK, Yap MGS. Impact of dynamic online fed-batch strategies on metabolism, productivity and N-glycosylation quality in CHO

cell cultures. *Biotechnol Bioeng*. 2005;89(2):164–77.

81. Jain E, Kumar A. Upstream processes in antibody production: Evaluation of critical parameters. *Biotechnol Adv*. 2008;26(1):46–72.

82. Shukla AA, Thömmes J. Recent advances in large-scale production of monoclonal antibodies and related proteins. *Trends Biotechnol*. 2010;28(5):253–61.

83. Gramer MJ, Eckblad JJ, Donahue R, Brown J, Shultz C, Vickerman K, *et al.* Modulation of antibody galactosylation through feeding of uridine, manganese chloride, and galactose. *Biotechnol Bioeng*. 2011;108(7):1591–602.

84. Luo J, Zhang J, Ren D, Tsai WL, Li F, Amanullah A, *et al*. Probing of C-terminal lysine variation in a recombinant monoclonal antibody production using Chinese hamster ovary cells with chemically defined media. *Biotechnol Bioeng*. 2012;109(9):2306–15.

85. Gramer MJ. Product quality considerations for mammalian cell culture process development and manufacturing. *Advances in biochemical engineering/biotechnology*. 2014;139:123–66.

86. Nam JH, Ermonval M, Sharfstein ST. The effects of microcarrier culture on recombinant CHO cells under biphasic hypothermic culture conditions. *Cytotechnology*. 2009;59(2).81-91.

87. Fan L, Zhao L, Ye Z, Sun Y, Kou T, Zhou Y, *et al.* Effect of culture temperature on TNFR-Fc productivity in recombinant glutamine synthetase-chinese hamster ovary cells. *Biotechnol Lett*. 2010;32(9):1239-1244.

88. Rodriguez J, Spearman M, Huzel N, Butler M. Enhanced production of monomeric interferon-β by CHO cells through the control of culture conditions. *Biotechnol Prog*. 2008;21(1):22-30.

89. Xu J, Tang P, Yongky A, Drew B, Borys MC, Liu S, *et al.* Systematic development of temperature shift strategies for Chinese hamster ovary cells based on short duration cultures and kinetic modeling. *MAbs*. 2019;11(1):191–204.

90. Lidman Olsson EO. Biphasic fermentation for enhanced production in a GS-CHO cell

line.*M.Sc. Thesis. Lund University, Faculty of Engineering, Department of Chemical Engineering*. 2014.

91. Torres M, Zúñiga R, Gutierrez M, Vergara M, Collazo N, Reyes J, *et al.* Mild hypothermia upregulates myc and xbp1s expression and improves anti-TNFα production in CHO cells. *PLoS One*. 2018;13(3):e0194510.

92. Yoon SK, Song JY, Lee GM. Effect of low culture temperature on specific productivity, transcription level, and heterogeneity of erythropoietin in Chinese hamster ovary cells. *Biotechnol Bioeng*. 2003;82(3):289–98.

93. Bollati-Fogolín M, Forno G, Nimtz M, Conradt HS, Etcheverrigaray M, Kratje R. Temperature reduction in cultures of hGM-CSF-expressing CHO cells: Effect on productivity and product quality. *Biotechnol Prog*. 2005;21(1):17–21.

94. Kantardjieff A, Jacob NM, Yee JC, Epstein E, Kok YJ, Philp R, *et al.* Transcriptome and proteome analysis of Chinese hamster ovary cells under low temperature and butyrate treatment. *J Biotechnol*. 2010;145(2):143–59.

95. Yoon SK, Ahn Y-H, Jeong MH. Effect of culture temperature on follicle-stimulating hormone production by Chinese hamster ovary cells in a perfusion bioreactor. *Appl Microbiol Biotechnol*. 2007;76(1):83–9.

96. Du Z, Treiber D, Mccarter JD, Fomina-Yadlin D, Saleem RA, Mccoy RE, *et al*. Use of a small molecule cell cycle inhibitor to control cell growth and improve specific productivity and product quality of recombinant proteins in CHO cell cultures. *Biotechnol Bioeng*. 2015;112(1):141–55.

97. Hendrick V, Winnepenninckx P, Abdelkafi C, Vandeputte O, Cherlet M, Marique T, *et al.* Increased productivity of recombinant tissular plasminogen activator (t-PA) by butyrate and shift of temperature: A cell cycle phases analysis. *Cytotechnology*. 2001;36(1-3):71–83.

98. Al-Fageeh MB, Marchant RJ, Carden MJ, Smales CM. The cold-shock response in cultured mammalian cells: Harnessing the response for the improvement of recombinant protein production. *Biotechnology and Bioengineering*. 2006;93:829–35.

99. Furukawa K, Ohsuye K. Effect of celture temperature on a recombinant CHO cell line producing a C-terminal alpha-amidating enzyme. *Cytotechnology*. 1998;26:153–64.

100. Moore A, Mercer J, Dutina G, Donahue CJ, Bauer KD, Mather JP, *et al.* Effects of temperature shift on cell cycle, apoptosis and nucleotide pools in CHO cell batch cultues. *Cytotechnology*. 1997;23(1–3):47–54.

101. Sherr CJ, Roberts JM. CDK inhibitors: Positive and negative regulators of G1-phase progression. *Genes and Development*. 1999;13:1501–12.

102. Moore A, Donahue CJ, Hooley J, Stocks DL, Bauer KD, Mather JP. Apoptosis in CHO cell batch cultures: examination by flow cytometry. *Cytotechnology*. 1997;17(1):1–11.

103. Kumar N, Gammell P, Clynes M. Proliferation control strategies to improve productivity and survival during CHO based production culture: A summary of recent methods employed and the effects of proliferation control in product secreting CHO cell lines. *Cytotechnology*. 2007;53(1-3):33–46.

104. Marchant RJ, Al-Fageeh MB, Underhill MF, Racher AJ, Smales CM. Metabolic rates, growth phase, and mRNA levels influence cell-specific antibody production levels from in vitro-cultured mammalian cells at sub-physiological temperatures. *Mol Biotechnol*. 2008;39(1):69–77.

105. Lu S, Sun X, Zhang Y. Insight into metabolism of CHO cells at low glucose concentration on the basis of the determination of intracellular metabolites. *Process Biochem*. 2005;40(5):1917–21.

106. Fan Y, Jimenez Del Val I, Müller C, Wagtberg Sen J, Rasmussen SK, Kontoravdi C, e*t al*. Amino acid and glucose metabolism in fed-batch CHO cell culture affects antibody production and glycosylation. *Biotechnol Bioeng*. 2015;112(3):521–35.

107. Gibbs RA, Weinstock GM, Metzker ML, Muzny DM, Sodergren EJ, Scherer S, *et al.* Genome sequence of the Brown Norway rat yields insights into mammalian evolution. *Nature*. 2004;428(6982):493–521.

108. Sou SN, Sellick C, Lee K, Mason A, Kyriakopoulos S, Polizzi KM, *et al.* Understanding the impact of different bioprocess conditions on monoclonal antibody

glycosylation in CHO cell cultures through experimental and computational analyses. *BMC Proc*. 2015;9(Suppl 9):O6.

109. Kishishita S, Nishikawa T, Shinoda Y, Nagashima H, Okamoto H, Takuma S, *et al.* Effect of temperature shift on levels of acidic charge variants in IgG monoclonal antibodies in Chinese hamster ovary cell culture. *J Biosci Bioeng*. 2015;119(6):700–5.

110. Fox SR, Patel UA, Yap MGS, Wang DIC. Maximizing Interferon-γ Production by Chinese Hamster Ovary Cells Through Temperature Shift Optimization: Experimental and Modeling. *Biotechnol Bioeng*. 2004;85(2):177–84.

111. Durocher Y, Butler M. Expression systems for therapeutic glycoprotein production. *Current Opinion in Biotechnology*. 2009;(20):700–7.

112. Smith SL. Ten years of Orthoclone OKT3 (muromonab-CD3): a review. *J Transpl Coord*. 1996;6(3):109–19.

113. Kimball JA, Norman DJ, Shield CF, Schroeder TJ, Lisi P, Garovoy M, *et al.* The OKT3 antibody response study: a multicentre study of human anti-mouse antibody (HAMA) production following OKT3 use in solid organ tranplantation. *Transpl Immunol*. 1995;3(3):212–21.

114. Sgro C. Side-effects of a monoclonal antibody, muromonab CD3/orthoclone OKT3: bibliographic review. *Toxicology*. 1995;105:23–9.

115. Wurm FM, Jesus M De. Manufacture of Recombinant Therapeutic Proteins Using Chinese Hamster Ovary Cells in Large - Scale Bioreactors. *Biosimilars of Monoclonal Antibodies*. 2016; ch.11:327–53.

116. Walsh G. Biopharmaceutical benchmarks 2010. *Nature Biotechnology*. 2010; 28(9):917-924.

117. Ringold G, Dieckmann B, Lee F. Co-expression and amplification of dihydrofolate reductase cDNA and the Escherichia coli XGPRT gene in Chinese hamster ovary cells. *J Mol Appl Genet*. 1981;1(3):165–75.

118. Kaufman RJ, Sharp PA. Amplification and expression of sequences cotransfected

with a modular dihydrofolate reductase complementary DNA gene. *J Mol Biol*. 1982;159(4):601–21.

119. Scahill SJ, Devos R, Van der Heyden J, Fiers W. Expression and characterization of the product of a human immune interferon cDNA gene in Chinese hamster ovary cells. *Proc Natl Acad Sci U S A*. 1983;80(15):4654–8.

120. Graham F, Smiley J, Russell W, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol*. 1977;36(1):59–74.

121. Takagi Y, Kikuchi T, Wada R, Omasa T. The enhancement of antibody concentration and achievement of high cell density CHO cell cultivation by adding nucleoside. *Cytotechnology*. 2017;69(3):511–21.

122. Horvat J, Narat M, Spadiut O. The effect of amino acid supplementation in an industrial Chinese Hamster Ovary process. *Biotechnol Prog*. 2020;36(5).

123. Fc glycosylation of monoclonal antibodies expressed in human cells, IMGT®; [cited 2017 16 December]. Available from: http://www.imgt.org/IMGTeducation/IMGTlexique/G/Glycosylation.html.

124. Xu X, Nagarajan H, Lewis NE, Pan S, Cai Z, Liu X, *et al*. The genomic sequence of the Chinese hamster ovary (CHO)-K1 cell line. *Nat Biotechnol*. 2011;29(8):735–41.

125. Xu J, Rehmann MS, Xu X, Huang C, Tian J, Qian NX, *et al.* Improving titer while maintaining quality of final formulated drug substance via optimization of CHO cell culture conditions in low-iron chemically defined media. *MAbs*. 2018;10(3):488–99.

126. Dangi AK, Sinha R, Dwivedi S, Gupta SK, Shukla P. Cell line techniques and gene editing tools for antibody production: A review. *Frontiers in Pharmacology*. 2018;9.

127. McCormick F, Trahey M, Innis M, Dieckmann B, Ringold G. Inducible expression of amplified human beta interferon genes in CHO cells. *Mol Cell Biol*. 1984;4(1):166–72.

128. Kaufman RJ, Wasley LC, Spiliotes AJ, Gossels SD, Latt SA, Larsen GR, *et al.* Coamplification and coexpression of human tissue-type plasminogen activator and murine dihydrofolate reductase sequences in Chinese hamster ovary cells. *Mol Cell*

Biol. 1985;5(7):1750–9.

129. Zahn-Zabal M, Kobr M, Girod P a, Imhof M, Chatellard P, de Jesus M, *et al.* Development of stable cell lines for production or regulated expression using matrix attachment regions. *J Biotechnol*. 2001;87(1):29–42.

130. Kwaks THJ, Barnett P, Hemrika W, Siersma T, Sewalt RGAB, Satijn DPE, *et al.* Identification of anti-repressor elements that confer high and stable protein production in mammalian cells. *Nat Biotechnol*. 2003;21(5):553–8.

131. Williams S, Mustoe T, Mulcahy T, Griffiths M, Simpson D, Antoniou M, *et al.* CpG-island fragments from the HNRPA2B1/CBX3 genomic locus reduce silencing and enhance transgene expression from the hCMV promoter/enhancer in mammalian cells. *BMC Biotechnol*. 2005;5(1):17.

132. Alexion Reports Fourth Quarter and Full Year 2019 Results; [cited 2020 6 July]. Available from: https://ir.alexion.com/news-releases/news-release-details/alexion-reports-fourth-quarter-and-full-year-2019-results

133. Schatz-Jakobsen JA, Zhang Y, Johnson K, Neill A, Sheridan D, Andersen GR. Structural Basis for Eculizumab-Mediated Inhibition of the Complement Terminal Pathway. *J Immunol*. 2016;197(1):337–44.

134. Horiuchi T, Tsukamoto H. Complement-targeted therapy : development of C5- and C5a-targeted inhibition. *Inflamm Regen*. 2016;36(1):1–5.

135. Klos A, Tenner AJ, Johswich KO, Ager RR, Reis ES, Köhl J. The role of the anaphylatoxins in health and disease. *Molecular Immunology*. 2009;46(14)2753–66.

136. Hu R, Mukhina GL, Piantadosi S, Barber JP, Jones RJ, Brodsky RA. PIG-A mutations in normal hematopoiesis. *Blood*. 2005;105(10):3848–54.

137. Rother RP, Rollins SA, Mojcik C, Brodsky RA, Bell L. Discovery and Development of the Complement Inhibitor Eculizumab for the Treatment of Paroxysmal Nocturnal Hemoglobinuria. *Nat Biotechnol*. 2007;25(11):1256–64.

138. Myasthenia Gravis Treatment Market Size, Share & Trends Analysis Report by Treatment (Medication, Surgery), by End Use (Hospitals, Clinics), and Segment

Forecasts, 2019-2026; [cited 2020 6 July]. Available from: https://www.grandviewresearch.com/industry-analysis/myasthenia-gravis-mg-treatment-market

139. Matis LA, Rollins SA. Complement-specific antibodies: designing novel anti-inflammatories. *Nat Med*. 1995;1(8):839–42.

140. Walport MJ. Complement. Second of two parts. *N Engl J Med*. 2001;344(15):1140–4.

141. Kemper C, Köhl J. Novel roles for complement receptors in T cell regulation and beyond. *Molecular Immunology*. 2013;56(3):181–90.

142. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. *Nat Immunol*. 2010;11(9):785–97.

143. Jore MM, Johnson S, Sheppard D, Barber NM, Li YI, Nunn MA, *et al*. Structural basis for therapeutic inhibition of complement C5. *Nat Struct Mol Biol*. 2016;23(5):378–86. Available from: http://www.nature.com/doifinder/10.1038/nsmb.3196

144. Law SKA, Dodds AW. The internal thioester and the covalent binding properties of the complement proteins C3 and C4. *Protein Sci*. 2008;6(2):263–74.

145. Coulthard LG, Woodruff TM. Is the Complement Activation Product C3a a Proinflammatory Molecule? Re-evaluating the Evidence and the Myth. *J Immunol*. 2015;194(8):3542–8.

146. Pangburn MK, Rawal N. Structure and function of complement C5 convertase enzymes. *Biochem Soc Trans*. 2002;30(6):1006–10.

147. Rawal N, Pangburn MK. Formation of High-Affinity C5 Convertases of the Alternative Pathway of Complement. *J Immunol*. 2001;166(4):2635–42.

148. Rawal N, Pangburn MK. Formation of high affinity C5 convertase of the classical pathway of complement. *J Biol Chem*. 2003;278(40):38476–83.

149. Daha MR, Fearon DT, Austen KF. C3 requirements for formation of alternative pathway C5 convertase. *J Immunol*. 1976;117(2):630–4.

150. Medicus RG, Schreiber RD, Gotze O, Mjller-Eberhard HJ, Mitler-Eberhard HJ. A molecular concept of the properdin pathway. *Proceedings of the National Academy of Sciences*. 1976;73(2):612–6.

151. Woodruff TM, Nandakumar KS, Tedesco F. Inhibiting the C5-C5a receptor axis. *Molecular Immunology*. 2011;48(14):1631–42.

152. Klos A, Wende E, Wareham KJ, Monk PN. International Union of Pharmacology. LXXXVII. Complement Peptide C5a, C4a, and C3a Receptors. *Pharmacol Rev*. 2013;65(1):500–43.

153. Muller-Eberhard HJ, Müller-Eberhard HJ. Molecular organization and function of the complement system. *Annu Rev Biochem*. 1988;57(1):321–47.

154. Götze O, Müller-Eberhard HJ. Lysis of Erythrocytes by Complement in the Absence of Antibody. *J Exp Med*. 1970;132(5):898–915.

155. Lachmann PJ, Thompson RA. Reactive lysis: the complement-mediated lysis of unsensitized cells. II. The characterization of activated reactor as C56 and the participation of C8 and C9. *J Exp Med*. 1970;131(4):643–57.

156. Podack ER, Kolb WP, Müller-Eberhard HJ. The C5b-6 Complex: Formation, Isolation, and Inhibition of Its Activity by Lipoprotein and the S-Protein of Human Serum. *J Immunol*. 1978;120(6):1841–8.

157. Rollins SA, Sims PJ. The complement-inhibitory activity of CD59 resides in its capacity to block incorporation of C9 into membrane C5b-9. *J Immunol*. 1990;144(9):3478–83.

158. Takata Y. Covalent association of C3b with C4b within C5 convertase of the classical complement pathway. *J Exp Med*. 1987;165(6):1494–507.

159. Kinoshita T, Takata Y, Kozono H, Takeda J, Hong KS, Inoue K. C5 convertase of the alternative complement pathway: covalent linkage between two C3b molecules within the trimolecular complex enzyme. *J Immunol*. 1988;141(11):3895–901.

160. Rabiet MJ, Huet E, Boulay F. The N-formyl peptide receptors and the anaphylatoxin C5a receptors: An overview. *Biochimie*. 2007;89(9):1089–106.

161. Chen N-J, Mirtsos C, Suh D, Lu Y-C, Lin W-J, McKerlie C, *et al*. C5L2 is critical for the biological activities of the anaphylatoxins C5a and C3a. *Nature*. 2007;446(7132):203–7.

162. Motoyama N, Okada N, Yamashina M, Okada H. Paroxysmal nocturnal hemoglobinuria due to hereditary nucleotide deletion in the HRF20 (CD59) gene. *Eur J Immunol*. 1992;22(10):2669–73.

163. Markiewski MM, Lambris JD. The Role of Complement in Inflammatory Diseases From Behind the Scenes into the Spotlight. *Am J Pathol*. 2007;171(3):715–27.

164. Mollnes TE, Kirschfink M. Strategies of therapeutic complement inhibition. *Molecular Immunology*. 2006;43(1-2):107–21.

165. Thomas TC, Rollins SA, Rother RP, Giannoni MA, Hartman SL, Elliott EA, *et al.* Inhibition of complement activity by humanized anti-C5 antibody and single-chain Fv. *Mol Immunol*. 1996;33(17–18):1389–401.

166. Riechmann L, Clark M, Waldmann H, Winter G. Reshaping human antibodies for therapy. *Nature*. 1988;332(6162):323–7.

167. Weng NP, Yu-Lee LY, Sanz I, Patten BM, Marcus DM. Structure and specificities of anti-ganglioside autoantibodies associated with motor neuropathies. *J Immunol*. 1992;149(7):2518-29.

168. Klein R, Jaenichen R, Zachau HG. Expressed human immunoglobulin kappa genes and their hypermutation. *Eur J Immunol*. 1993;23(12):3248–62.

169. Mueller JP, Giannoni MA, Hartman SL, Elliott EA, Squinto SP, Matis LA, *et al.* Humanized porcine VCAM-specific monoclonal antibodies with chimeric IgG2/G4 constant regions block human leukocyte binding to porcine endothelial cells. *Mol Immunol*. 1997;34(6):441–52.

170. Burton DR, Woof JM. Human antibody effector function. *Adv Immunol*. 1992;51:1–84.

171. Tao MH, Smith RI, Morrison SL. Structural features of human immunoglobulin G that determine isotype-specific differences in complement activation. *J Exp Med*.

1993;178(2):661–7.

172. Smith RI, Coloma MJ, Morrison SL. Addition of a mu-tailpiece to IgG results in polymeric antibodies with enhanced effector functions including complement-mediated cytolysis by IgG4. *J Immunol*. 1995;154(5):2226–36.

173. Wang Y, Hu Q, Madri JA, Rollins SA, Chodera A, Matis LA. Amelioration of lupus-like autoimmune disease in NZB/WF1 mice after treatment with a blocking monoclonal antibody specific for complement component C5. *Proc Natl Acad Sci U S A*. 1996;93(16):8563–8.

174. Rother RP, Mojcik CF, McCroskery EW. Inhibition of terminal complement: a novel therapeutic approach for the treatment of systemic lupus erythematosus. *Lupus*. 2004;13(5):328–34.

175. Evans M J, Matis LA, Mueller EE, Nye SH, Rollins S, Rother RP, *et al. C5-specific antibodies for the treatment of inflammatory diseases*. United States Patent application. 2002: US 08/487,283; US6355245 B1.

176. Nishimura J, Yamamoto M, Hayashi S, Ohyashiki K, Ando K, Brodsky AL, *et al.* Genetic Variants in C5 and Poor Response to Eculizumab. *N Engl J Med*. 2014;370(7):632–9.

177. Burwick RM, Burwick NR, Feinberg BB. Eculizumab fails to inhibit generation of C5a in vivo. *Blood*. 2014;124(23):3502–3.

178. EMEA. SOLIRIS. Scientific Discussion. [cited 2018 4 July]. Available from: http://www.ema.europa.eu/docs/en GB/document library/ EPAR - Scientific Discussion/human/000791/WC500054212.pdf.

179. Laursen NS, Andersen KR, Braren I, Spillner E, Sottrup-Jensen L, Andersen GR. Substrate recognition by complement convertases revealed in the C5-cobra venom factor complex. *EMBO J*. 2011;30(3):606–16.

180. Brachet G, Bourquard T, Gallay N, Reiter E, Gouilleux-Gruart V, Poupon A, *et al*. Eculizumab epitope on complement C%: Progress towards a better understanding of the mechanism of action. *Mol Immun*. 2016; 77:126-31.

181. Shivhare M, Mccreath G. Practical Considerations for DoE Implementation in Quality By Design. *Bio Process Int*. 2010:1–7.

182. EMA/CHMP/ICH/167068/2004. ICH guideline Q 8 (R2) Pharmaceutical Development, Step 5: Note for Guidance on Pharmaceutical Development; [cited 2017 24 July]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002872.pdf

183. Dejaegher B, Vander Heyden Y. Experimental designs and their recent advances in set-up, data interpretation, and analytical applications. *J Pharm Biomed Anal*. 2011;56(2):141–58.

184. Vander Heyden, Y., Perrin C *et al*. Optimization strategies for HPLC and CZE. *Separation Methods in Drug Synthesis and Purification*. 2000;1:163–212.

185. Rathore AS, Branning R, Cecchini D. Design space for biotech products. *BioPharm International*. 2007;20(4):36–40.

186. Montgomery DC. *Design and Analysis of Experiments*. John Wiley & Sons, Inc.; 2012.

187. Wang X, Germansderfer A, Harms J, Rathore AS. Using statistical analysis for setting process validation acceptance criteria for biotech products. *Biotechnology Progress*. 2007;23(1):55–60.

188. Kirdar AO, Conner JS, Baclaski J, Rathore AS. Application of multivariate analysis toward biotech processes: Case study of a cell-culture unit operation. *Biotechnology Progress*. 2007;23(1):61–7.

189. Dejaegher B, Vander Heyden Y. The use of experimental design in separation science. *Acta Chromatogr*. 2009;21(2):161–201.

190. Box GEP, Hunter J, Hunter WG. Statistics for Experimenters: Design, Innovation and Discovery. *Technometrics.* 2006;48(2):303–4.

191. Carlson R, Carlson J. *Design and optimization in organic synthesis.* Elsevier; 2005.

192. Brereton RG. Chemometrics: Data Analysis for the Laboratory and Chemical Plant.

Technometrics. 2004;46(1):110-110.

193. Mandenius C, Brundin A. Bioprocess optimization using using Design-of-experiments methodology. *Biotechnol Progr*. 2008;24(6):1191–203.

194. Rodrigues ME, Costa AR, Henriques M, Azeredo J, Oliveira R. Comparison of commercial serum-free media for CHO-K1 cell growth and monoclonal antibody production. *Int J Pharm*. 2012;437(1–2):303–5.

195. Reinhart D, Damjanovic L, Kaisermayer C, Kunert R. Benchmarking of commercially available CHO cell culture media for antibody production. *Appl Microbiol Biotechnol*. 2015;99(11):4645-4657.

196. Pan X, Streefland M, Dalm C, Wijffels RH, Martens DE. Selection of chemically defined media for CHO cell fed-batch culture processes. *Cytotechnology*. 2017;69(1):39–56.

197. Al-Fageeh MB, Smales CM. Control and regulation of the cellular responses to cold shock: The responses in yeast and mammalian systems. *Biochemical Journal*. 2006;397(2):247–59.

198. Woo SA, Jeon JJ, Jeong YR, Seung JL, Sung KY. Effect of culture temperature on erythropoietin production and glycosylation in a perfussion culture of recombinant CHO cells. *Biotechnol Bioeng.* 2008; 101(6):1234-44.