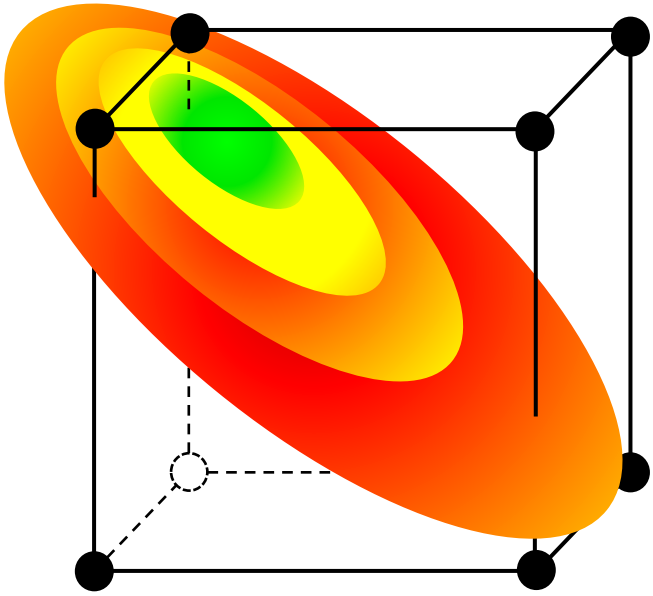
University of Natural Resources
and Life Sciences, Vienna
Department of Biotechnology

Utilizing experimental design to evaluate and optimize extrinsic production factors in cultivation media regarding CHO cell performance



Submitted by

Marcus Kirschner, BSc

Vienna, November 2019

Supervisor: Univ.Prof. Dipl.-Ing. Dr. Renate Kunert

Co-Supervisor: Dipl.-Ing. Patrick Mayrhofer, PhD

"Erst wenn es regnet, spann den Schirm auf."

My Mother

ACKNOWLEDGMENTS

First of all, I would like to thank Prof. Renate Kunert for giving me the opportunity to work within her working group at the Institute of Animal Cell Culture Technology and Systems Biology as well as for being a passionate teacher and the resulting exciting and inspiring discussions.

In addition, I want to thank Patrick Mayrhofer for his special supervision and input throughout the practical as well as theoretical part of my master thesis.

Furthermore, I would like to deeply thank Elisabeth Lobner for her extraordinary encouragement and support during this time both inside and outside the lab.

I want to thank Willibald Steinfellner and Lukas Damjanovic for their great support, guidance and patience while teaching me all the laboratory work required to conduct this thesis.

A further special thanks go to all the PhD students, Julia Hennicke, Linda Schwaigerlehner and Philipp Mundsperger for their valuable input and interesting discussions which greatly contributed to this thesis. I highly value the warm welcome from all of you which made the time in this working group exceptional from the very beginning.

Moreover, I am extremely grateful for the time I spend with my dear master colleagues Anna Sieber, Anna Wachernig and Simon Anders. Thank you for the great support and contribution to this thesis. I especially appreciated all the countless times that we were losing ourselves in conversations both engaging and serious.

Finally, I would like to thank my family and friends for their loving encouragement and endless support during my entire study. Thank you all for always having an open ear in times of doubts and uncertainty. None of this would have been possible without all of you.

STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used other than the declared sources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

Marcus Kirschner, BSc Vienna, November 2019

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre eidesstattlich, dass ich die Arbeit selbständig angefertigt habe. Es wurden keine anderen als die angegebenen Hilfsmittel benutzt. Die aus fremden Quellen direkt oder indirekt übernommenen Formulierungen und Gedanken sind als solche kenntlich gemacht. Diese schriftliche Arbeit wurde noch an keiner Stelle vorgelegt.

Marcus Kirschner, BSc Vienna, November 2019

ABSTRACT

Recombinant mammalian cells are representing one of the major workhorses in biopharmaceutical industry nowadays. In particular, Chinese hamster ovary (CHO) cells are the most prevalent expression platform for the recombinant production of antibodies in scientific as well as industrial applications. Beside other bioprocess considerations, the development of high-performing cell culture media significantly contributed to enormous success in antibody production, yielding in titer of up to 10 g/L.

Since the field of establishing perfectly adapted chemically defined media is strongly emerging, this thesis focused on the optimization of a high-performing cell culture media. At first, a panel of feed stock solutions was created which are representing media component classes typically found in commercially available media. Based on the utilization of experimental design spaces, impact and synergies of the feed stock solutions were investigated using a monoclonal antibody expressing CHO cell line in regards to essential screening parameters. After a combinatorial application of screening and optimization experimental design as well as traditional one-factor-at-a-time experiments, a formulation comprised of various media components was realized. Utilization of the in-house developed formulation as a supplement in batch cultivations as well as a feed-media in fed-batch bioprocesses revealed a significantly improved cell performance in both applications. Absolute molecular mass and protein size analyses of purified protein samples from bioreactor fed-batch cultures revealed no alteration compared to an antibody reference sample. Additionally, Raman spectroscopy was performed to identify certain media components and highlighted the potential usage as promising tool for media analysis.

ZUSAMMENFASSUNG

Rekombinante tierische Zellen repräsentieren heutzutage eine der meist verwendeten Produktionsplattformen in der biopharmazeutischen Industrie. Chinese hamster ovary (CHO) Zellen sind eine zentrale Zellfabrik in industriellen als auch wissenschaftlichen Fermentationsverfahren zur Gewinnung von rekombinanten Antikörpern. Neben anderen Optimierungsansätzen in der Bioprozesstechnik, hat die Entwicklung von qualitativ hochwertigen Nähr- und Kulturmedien einen wesentlichen Beitrag zur Gewinnung von Antikörperkonzentration von bis zu 10 g/L geleistet.

Durch den stetig steigenden Bedarf an optimal angepassten Kulturmedien für die jeweilige Zelllinie bzw. Prozessführung, wurde in dieser Studie das Augenmerkt auf die Optimierung eines kommerziellen Hochleistungsmediums gelegt. Als Grundlage wurden verschiedene chemisch definierte Substanzlösungen hergestellt, welche Hauptkomponenten einer generellen Medienformulierung repräsentieren. Durch die Anwendung von statistischen Versuchsplänen, wurden Einflüsse und Interkation dieser Substanzlösungen in Bezug auf Kultivierungsparameter einer CHO DG44 Zell Line evaluiert. Basierend auf der Auswertung von diversen experimentellen Versuchsmethoden konnte eine Medienformulierung entwickelt werden, welche als Supplementierung von Basalmedium in Batch sowie als Feed Medium in Fed-Batch Kultivierungen erhöhte Produkterträge erzielen konnte. Hinsichtlich der absoluten molaren Masse sowie Proteinform konnte keine Veränderung des aufgereinigten Produktes in direkten Vergleich zu einer Referenzprobe nachgewiesen werden. Weiters konnte durch die Anwendung von Raman Spektroskopie eine Identifizierung von definierten Medienkomponenten nachgewiesen und dessen potenzielle Anwendbarkeit im Bereich der Medienanalytik aufgezeigt werden.

TABLE OF CONTENT

1. INTRODUCTION

In the past quarter century, mammalian cells have become major workhorses in the field of biopharmaceuticals production, correlating with an immeasurable impact on human society nowadays (Walsh 2018a). While industrial application of various cell factories has a long history, it was not until the arise of eukaryotic cells that it became possible to produce multiple biopharmaceuticals meeting most stringent requirements in terms of protein properties and quality (Davy, Kildegaard, and Andersen 2017). Production hosts originated from bacterial, yeast or fungal strains are widely used in various biotechnological fields with huge success but still encounter recurrent problems of recovering substantial yields of correctly folded and biological active recombinantly expressed proteins (Zelena, Eisele, and Berger 2014). Consequently, the investigation and further development of mammalian cell factories gave rise to multiple cell lines derived from various organisms and tissues, such as human embryonic kidney (HEK 293), mouse myeloma (NS0, Sp2/0), monkey kidney epithelial (Vero) and Chinese hamster ovary (CHO) cells (Bulletin and Khan 2013; Wurm and Hacker 2011). Especially derivates (CHO-K1, DUK-XB11 and DG44) of the latter are extensively used as major production hosts in the field of recombinant protein expression, given the many clinical approved products (Walsh 2018).

1.1. A short history of the Chinese hamster ovary (CHO) cell line

Since the first approval of recombinant produced proteins in the early 1980s, production processes and the requirement to meet high standards in terms of biopharmaceutical product quality are still critical points in the field of biotechnological industrial as well as scientific applications. Advances in cell line engineering have given rise to enormous achievements to encounter these crucial challenges in which CHO cells are representing the nowadays gold standard for the manufacturing of therapeutic proteins (Walsh 2018; Wurm 2013). The interest of CHO cells dates back to 1957 when this cell line was established from spontaneously immortalized cells out of ovary tissue for genetic studies due to their low chromosome number (Puck, Cieciura, and Robinson 1958). Since then, the extent approach of various cellular and genetic engineering methods resulted in the generation of diverse cell lines proven to be robust and reliable in culture (Bandaranayake and Almo 2014). Subcloning from the originator CHO

cells, resulted in the establishment of the very first widely used cell line, namely CHO-K1 (Wurm 2013). Exploiting the CHO-K1 cell line as reliable expression platform and subsequent genetic modification of the host population, resulted in the establishment of further cell lines differing in their phenotype such as the CHO-DXB11, CHO-S and DG44 cell line (Bandaranayake and Almo 2014; Graf and Chasin 1982; Urlaub and Chasin 1980; F. Wurm 2013). An important characteristic of CHO cells and reason for their widespread usage in biotechnological application is their adaptability to a broad range of bioprocesses, enabling cells to grow at high densities in suspension culture correlating with enhanced volumetric production. Next to their immense adaptive ability, expression of therapeutic proteins in their biologically active forms, which requires proper posttranslational modification of carbohydrate moieties and folding of the protein of interest, qualified CHO cells to be the industry's eminent workhorse (Jayapal et al. 2007; Kunert and Reinhart 2016).

1.1.1. Characteristics of PG9 and its expression platform CHO DG44

As previously mentioned, the original CHO cell line gave rise to various derivates differing in their respective phenotype. In this thesis the CHO DG44 cell line was cultivated to observe growth and production deviations of various environmental influences as a model expression platform. In the early 1980s the CHO DG44 line was created by ionizing radiation to obtain a cell line to be dihydrofolate reductase (DHFR) negative in both alleles (Urlaub et al. 1983). Due to this double knock out in both alleles, CHO DG44 exploited to be a highly applicable expression platform. Since DHFR catalases reduction of dihydrofolic acid to tetrahydrofolic acid, essential for the *de novo* synthesis of purines, the DHFR deficiency enabled selection of highly productive recombinant clones. Therefore, cells are transfected with a vector harboring the protein of interest gene and endogenous *Dhfr* genes. This selection pressure is based on cultivating the recombinant cell in media lacking hypoxanthine and thymidine (Chromikova, Zaragoza, and Krammer 2017). Thus, enabling the successfully transfected cells to proliferate whereas cells lacking the vector are not able to synthesize essential building blocks out of media precursors. Nowadays, CHO DG44 and CHO DUXB11 are the most widely used CHO cell lines, and CHO cells in general, have become major workhorses in the field of recombinant production platforms. These cell lines are reported to deliver up to 10g product per litre culture in highly optimized processes (Chromikova et al. 2017; Reinhart et al. 2015; Walsh 2018; Wurm and Hacker 2011).

The monoclonal antibody PG9 (mAb), which was used in this thesis as a model protein, is a broadly neutralizing anti-HIV 1 immunoglobin G subtype 1 (Phogat et al. 2011). Due to the high affinity of the glycosylated Fab region to the variable regions (V1 and V2) of the human immunodeficiency virus-1 (HIV-1) gp120 envelope glycoprotein, this antibody is a promising tool to overcome critical obstacles in the field of HIV treatment. In particular, PG9 and the somatically related PG12 monoclonal antibody neutralize 70-80% of the circulating HIV-1 variants (Euler et al. 2011; Phogat et al. 2011). A fundamental feature to achieve these high affinities to the heavily glycosylated V1 and V2 regions of the envelope, is the extraordinary glycan decoration within the variable region of the PG9 mAb. By directly engineering the specific glycosylation and tyrosine sulfatation, found within the complementary determining region, modified PG9 derivates has been reported to demonstrate even superior performance in terms of mAb effector functions (Loos et al. 2015).

1.2. The theory of cell culture media

Animal cell culture technology has advanced significantly over the past two decades and has given rise to a broad range of clinical approved biopharmaceuticals. Beside bioprocess and cell engineering considerations, the adaptation and optimization of cell media to the respective culture, especially in mammalian cells, is one major factor to enhance the overall cell performance. It is essential to provide an optimal balanced media to ensure and support cell survival and proliferation as well as the full maintenance of cellular function (Yao and Asayama 2017). Within the time period of first trails to cultivate animal cells and the establishment of state of the art chemically defined media cell culture, the investigation and advance of cell culture media entered various stages of development (Jedrzejczak-Silicka 2017). However, in 1959s the first animal cell culture media, namely BME (Basal Medium, Eagle), was established based on the objective to investigate essential components for cell survival and proliferation (Eagle 1959; Eagle 1955). Further endeavors yielded in modified and improved synthetic media based on the understanding of essential integral parts found in serum, plasma or tissue extracts but researchers did not succeed to develop defined cell culture media till the late 1950s. Henry Eagle's pioneer work led to the first developed media, namely Eagle's Minimal Essential Medium (EMEM), which could support the growth and proliferation of two different mammalian cells (Eagle 1959). Although this media still contained serum, the basic formulation

gave rise for several other promising media formulations such as the Dulbecco and Freeman's DMEM - Dulbecco's Minimal Essential Medium (Dulbecco and Freeman 1959). Serum is a highly complex biological liquid composed out of large and various numbers of essential nutrients, growth factors, salts and protein carrier ensuring a successful proliferation and maintenance of various mammalian cell lines (van der Valk et al. 2018). However, the use of serum is associated with a number of problematic issues such as unknown composition and concentration of the respective constituents as well as geographical and seasonal variabilities (Baker 2016). Furthermore, severe safety concerns regarding unexpected and undesired interaction with test substances as well as for laboratory personnel can occur due to e.g. endotoxins and prions found within the serum (Hawkes 2015). Consequently, the development of a completely defined cell culture media was driven to overcome these obstacles. Richard G. Ham was one of the first to develop a fully synthetic, defined and serum-free nutrient mixture which was able to support clonal proliferation of CHO cells (HAM 1965).

Since then, various chemically defined media were developed and optimized to meet the most stringent requirements for a successful clonal growth and maintenance of various cell lines. Beside the general consideration to develop basal media, great efforts have been made to advance formulations for different requirements in terms of scientific and industrial production processes. Hence, media development is an integral step towards optimizing cell culture process enabling high recombinant protein yields.

1.2.1. Overview of media classifications

Table 1. Overview of different media classes and their respective characteristics.

Serum containing media	Fetal bovine serum is the most common supplement and embedded supplementation practice in animal cell culture media. It is used as a low-cost supplement to provide indispensable nutrients, fatty acids, growth factors, salts, trace elements and protein carrier (van der Valk et al. 2018).
Serum-free media	These media are composed of defined media constituents and are mostly optimized for the growth requirements of cell-specific applications. Added serum components have been purified to remove e.g. inhibitors and growth factors (Mariani et al. 1991).
Chemically defined media	Chemically defined media are considered to contain contamination-free highly pure inorganic and organic ingredients. Presence of pure protein additives in the media which are most likely recombinantly produced (Nagle and Jr. 1968).
Protein-free media	The media formulation is free of any animal derived proteins or peptides and only contain non-protein constituents. Application of protein-free media promotes superior cell growth and protein expression and mostly facilitates downstream purification processes of expressed products (Gregory Hamilton and Ham 1977).

1.2.2. Overview of selected cell culture media constituents typically found in chemically defined media

The following paragraphs give an overview of selected cell culture media component classes which have been tested within experimental design spaces over the course of this thesis.

Carbohydrates

Carbohydrates, mostly found in the form of sugars, are representing a major energy source. In particular, glucose functions as the primary energy source in most media since e.g. CHO cells transform glucose into pyruvate via glycolysis. Pyruvate is then further oxidized within the Krebs cycle to generate energy of up to 36 mol ATP per 1 mol glucose (Ahn and Antoniewicz 2012). Although glucose is the main energy source, correlating accumulation of pyruvate and the direct coupled TCA cycle leads to an increase in lactate concentration resulting in an inhibitory effect of cell growth (Cruz et al. 2000). Next to glucose, hexoses such as Galactose, fructose, and mannose can serve as an important alternative carbon source which are also partially converted to pyruvate in glycolysis and were shown to enable increased volumetric productivity (Altamirano et al. 2013; Berrios et al. 2011).

Amino acids

Overall, amino acids can be classified as nonessential and essential amino acids and have been identified as indispensable components in cell culture media since early ages of cell culture processes (EAGLE 1955). Although nonessential amino acids are not crucial or are requisite for a successful cell growth maintenance, studies have shown a general improved cell performance in media containing these nonessential amino acids (Huang et al. 2010; Xing et al. 2011). In contrast, essential amino acids are crucial molecules which cells are unable to synthesize and must therefore be available as supplements within every cell culture media. Moreover, a balanced amino acid composition for the respective cell line is an important factor of success which must be considered. Metabolic flux analysis highlighted the importance of well-balanced amino acid composition within the media, since impaired cell proliferation and expressed product formation, respectively, were shown due to adverse amino acid composition within cell culture processes (González-Leal et al. 2011). Furthermore, amino acid supplementation

has to be designed in regard to the molecular structure of each product, since the amino acid demand for protein expression is product specific (Carrillo-Cocom et al. 2015).

In a previous study, a well-adapted supply of asparagine and valine yielded in a significantly improved final mAb titer whereas arginine supplementation was associated with highest viable cell counts. In contrast, unfavourable leucine concentration has a been reported to have a prominent negative effect on cellular growth rate (González-Leal et al. 2011). Moreover, amino acids can be applied in media optimization as an alternative energy source to glucose. Next to glycolysis, the glutaminolysis is an important metabolic pathway for the replenishment of the TCA cycle and the energy metabolism. Since glutamate is the main intermediate of glutaminolysis, replacement of glutamine with the same concentration of glutamate, resulted in reduced ammonium and lactate accumulation within CHO cell culture processes (Altamirano et al. 2000). Asparagine and aspartate are major ammonium donors and acceptors within the biochemical transition of glutamine and glutamate, which can generate both oxaloacetate and glutamate (Ritacco, Wu, and Khetan 2018). Absence of asparagine in CHO culture media was reported to result in higher conversion of arginine, aspartate, glutamine, serine and glutamate and may even result into an overall increased consumption of the whole available amino acid pool at higher rates. Consequently, the resulting amino acid deficit can impair product and overall gene expression (Ludwig, Tomeczkowski, and Kretzmer 1992). Thus, the understanding of a cell needs in raged to the amino acid availability as well as the specific consumption and transition rates, respectively, is of great importance.

Vitamins

Although vitamins are represented in relatively low amounts within cell culture media compared to carbohydrates and amino acids, respectively, they are mandatory key components, especially in chemically defined media. Vitamins serve as cofactors in signal cascades, prosthetic groups as well as inhibitors and activators of certain enzymatic reactions and even hormones. Overall, vitamins can be classified as either water soluble (Vit. B and C) or fat-soluble (Vit, A, D, E and K). However, in regard to their chemically or metabolic function, the classes themselves have little in common (Bühler 2001). B vitamins such as riboflavin (B_2), folic acid (B_9), cyanocobalamin (B_{12}), thiamine (B_1), pyridoxine (B_6), nicotinamide (B_3) and biotin (B_7) are considered to be the most essential group for chemically defined media. One crucial step

towards the development of defined media, is the implementation of a balanced vitamin pool to overcome most common degradation pathways accruing due acid/base-catalysed reactions, UV light initiated decomposition and thermal degradation (Schnellbaecher et al. 2019). Next to B vitamins, supplementation strategies of ascorbic acid (Vit. C) and tocopherol (Vit. E), commonly found in CHO cell culture media, are highly vulnerable to air oxidation. Therefore, protection from light as well as heat is crucial during storage to ensure unchanged media and correlating product quality (Ritacco et al. 2018).

Pyruvate

Pyruvate, mostly supplemented in form of sodium pyruvate, is representing another major energy source next to carbohydrates, which may be used to reduce an ammonium accumulation following glutamine reduction in CHO cell culture media. Moreover, pyruvate is an intermediate within the TCA cycle and therefore directly coupled to lactate generation and can limit the lactate depletion rate. Since, high lactate concentration directly lowers the pH and has been shown to negatively affect culture processes, pyruvate supplementation strategies is strongly dependent on the respective cell line (Li et al. 2012). Consequently, it can be suggested that pyruvate supplementation can even be unfavourable within cultivation of high lactate producing cell lines.

Membrane precursor

Membrane precursor, also generally considered as lipids, are major components of biological membranes and can also serve as signalling molecules as well as an energy source. Although it has been reported that CHO cells are capable to adapt and proliferate in lipid free defined media, a supplementation of media components related to the group of lipids such as ethanolamine, putrescine, choline chloride, myo-inositol, pyridoxine HCl and thiamine HCl has been shown to significantly improve product formation and enhanced cell growth (Jenkins et al. 1994; Kim et al. 2005).

Trace Elements

Trace elements are representing an indispensable media component class within every cell culture media. The effective concentration of various metal elements are generally rather low but do play a critical role in the regulation of metabolic pathways and the activity of various enzymes and signal transduction related molecules (Ritacco et al. 2018a). In particular, inorganic trace elements such as copper, manganese, cobalt, molybdenum, selenium and iron are present in commercial media. The knowledge about the respective metal ion uptake within the respective cell line is of great importance, since high concentrations can have a toxic effect and ultimately result in a strong negative impact on cell growth (Keenan et al. 2018). Copper deficiency can result in reduced lactate dehydrogenase and further mitochondrial oxidative enzymes. In contrast to a copper deficiency, it has been reported that high copper concentration can strongly increase the amount of antibodies in their basic form (Yuk et al. 2015). Furthermore, zinc was reported to be one of the most influencing trace elements within CHO culture processes. Addition of $ZnSO_4 \cdot 7H_2O$ to several commercial chemically defined media yielded in a 1.2-fold increase of mAb production. This effect was only attained up to a certain amount of zinc within the media, concentration higher than 90 µM ultimately resulted in a gradual decrease of cell density (Kim and Park 2016).

1.3. Experimental design spaces in a nutshell

In contrast to traditional one factor at a time, statistical experimental design methods are widely used to develop and optimize biopharmaceutical processes when it comes to numerous factors which can interfere within an experiment (Kalil, Maugeri, and Rodrigues 2000). Different experimental design spaces, also known as design of experiment (DoE), have been implemented and adapted to increase understanding of complex process and their relating variables. A major benefit of these statistical applications is the enhanced robustness and reproducibility within experimental approaches as well as the aid to optimized troubleshooting based on profound process understanding. Furthermore, the design space can be applied to the experimental demands regarding the interaction between certain factors. Thus, enabling the simultaneous testing of various variables and the respective interaction within one experimental approach. Consequently, a high throughput of various process variables and the

possibility to identify the effect of different factors by reduced number of experiments can be achieved. In contrast to traditional “trial and error” experimental approaches, a profound and comprehensive understanding, based on statistical significance, within shorter amount of time can be accomplished (Möller and Pörtner 2017).

1.3.1. Overview of experimental design terminology

System

The system describes the experimental basis of each design space and needs to comprise defined boundaries. In this thesis, the system is represented by the cell cultivation process with controllable defined factors which are included within the system boundaries. Parameter or factors which are classified as uncontrollable are not considered within the statistical evaluation (Siebertz, Bebber, and Hochkirchen 2010).

Factor & Level

All different parameter which can influence the system are classified as a factor. Such factors directly impact the system and can either be controllable or uncontrollable. Uncontrollable factors are for example the humidity, temperature or CO_2 control unit within an incubator. Although these factors can theoretically be manually manipulated, an unnoticed malfunction would directly yield to a falsified outcome and interpretation, respectively, correlating with a loss of reproducibility. Controllable factors, such as the type and concentration of any applied feed stock solution, are considered to be part of the system boundaries and are directly linked to the experimental analysis. The foundation of each design of experiment is the setting of factors to different levels. Each factor has to be set at least to two different levels within a defined range. For first evaluation or screening experiments, it is suggested to set rather high intervals between the levels to investigate distinct effects of each level. Consequently, higher numbers of levels and smaller ranges between the levels are advised for optimization approaches, since small deviation within the factors and possibly correlating responses can be investigated (Siebertz, Bebber, and Hochkirchen 2010; Tye 2004).

Response & Effect

Responses are defined as the investigated outcome and should be in general well-defined in advance of each experimental approach. The final antibody titer, specific cell growth or maximal viable cell concentration are typical responses within biological systems. Factors, either controllable or uncontrollable, typically influence the system and the correlating response. This change of response between the various systems, due to different factors and their respective level settings, is considered as a main effect. Since only controllable factors are within the system boundaries, the effect on the system of exclusively these factors can be used for a sensible statistical evaluation (Siebertz, Bebber, and Hochkirchen 2010).

Resolution & Interaction

The term resolution describes the overall quality of the applied experimental design space and provides information about the statistical power to properly interpret whether two or more factors are dependent on each other. These factor dependencies are classified as interactions between the defined input factors on the observed effect within the system. The choice of design resolution should be in dependency of the desired data and possible experimental numbers. If main effects cannot be separated from adverse interaction effects in the statistical model, the interaction is classified as confounded. To overcome undesired confounding interaction and provide various solutions in dependency of the research topic, four different resolutions (III to V+) are defined (Siebertz, Bebber, and Hochkirchen 2010; Tye 2004).

Table 2. Overview of selcetd resultion classes which are implemented to define the overall quality of an experimental desgin space.

III	This experimental design is considered to be the most economical design but no possibility to determine any interaction effects. Resolution III is favourable for pre-experimental screening approaches (Siebertz et al. 2010; Tye 2004).
IV	Less severe confounding design, due to higher order interactions which are less likely to be significant compared to resolution III. Useful to determine main effects without considering interaction effects (Siebertz, Bebber, and Hochkirchen 2010).
V	Main effects can be confounded in dependency of the factor number. Interaction effect are generally clear of each other based on a high experimental order. Due to the high experimental number, resolution V experiments are considered cost and time consuming (Siebertz, Bebber, and Hochkirchen 2010).
V+	The possibility of confounded interactions is strongly reduced. However, no significantly improved outcome and understanding, respectively, is suggested within resolution V+ designs compared to resolution V (Siebertz et al. 2010; Tye 2004).

1.3.2. Application of experimental design spaces

As previously mentioned, the choice of experimental design is a compromise between required information and the number of conducted experiments. To apply Design of Experiment in the most efficient way, it is suggested to design different design spaces regarding the research phase. These can either be conducted as full factorial or fractional experimental designs. Independent of the design space, the response and interaction evaluations is realized via linear regression analyses (Siebertz et al. 2010).

Since full factorial experiments are limited to the amount of factors, the most common screening strategies are fractional designs in early research phases. The latter design enables the simultaneous evaluation of a large number of various variables on a system, since these approaches are harboring only a subset number of experiments compared to full factorial designs. However, since only five defined media constituent classes were defined as factors within this thesis, it was possible to perform full factorial screening and optimization approaches. Furthermore, both full factorial design spaces were performed with resolution V and additional center points. These center points are representing a further level setting and enabling a stronger statistical analyzation power within the linear regression method as well as an enhancement on the overall reproducibility of an experiment (Bowden, Pichler, and Maurer 2019; Siebertz et al. 2010). Therefore, application of an appropriate experimental design space within different research stages in combination with advanced statistical software packages, highlights the powerful advantages of using DoE in broad field of scientific subjects.

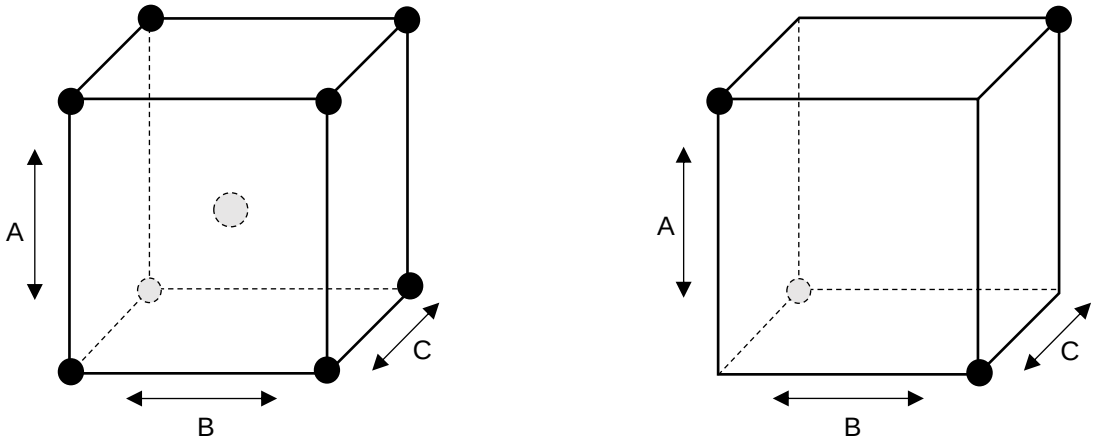


Figure 1. Schematic representation of full factorial experimental design with 3 factors at two levels including one center point (left) and factorial experimental design with 3 factors at two levels without any center point (right).

2. AIM OF THE STUDY

Manufacturing of recombinantly expressed products using mammalian expression platforms made significant progress in the last decades. Chinese hamster ovary (CHO) cells are representing nowadays one major expression host in the field of biopharmaceutical production, especially monoclonal antibodies. Process parameter and conditions such as pH, temperature, dissolved oxygen and CO_2, vessel form, stirrer speed and the overall nutrient supply over the course of a process are described to influence the growth and production properties of the host cells (Huang et al. 2010). Next to different process control strategies, cell culture media optimization is considered as one of the key contributors to increase productivity and prolong culture longevity (Kunert and Reinhart 2016).

Thus, the aim of this study was to develop a media supplement formulation to enhance the overall productivity of a recombinant CHO DG44 cell line producing the human anti-HIV-1 IgG1 antibody PG9. The formulation was aimed to comprise only media constituents commonly found in commercially available media to reduce the probability of inferior product quality and toxic effects on the cells. A panel of defined feed stock solutions, representing major media component classes, were realized to evaluate interactions and influence on the respective cell line. Full factorial screening experiments were conducted to investigate effects of these feed stock solutions on defined process responses. Statistical analyzes have been used to develop a formulation in order to assess the application as a media supplement in batch processes. Full factorial optimization experiments with resolution V were conducted to enhance specific productivity of CHO cells by varying the working concentration as well as overall formulation with additional media constituents. To benchmark the potential of the in-house developed formulation, batch and fed-batch processes with commercially available supplements from the same culture media system were performed and tested against each other. In addition, the expressed antibody within supplemented cultures was monitored via size exclusion chromatography coupled to multi-angle light scattering to verify unchanged absolute molecular mass and size of the recombinant protein.

3. MATERIAL & METHODS

3.1. Material

3.1.1. Equipment

Table 3. Summary of laboratory equipment

Vi-CELL XR Cell Viability Analyzer	Beckman Coulter, U.S.
DS-11 Series Spectrophotometer	DeNovix, Germany
DASGIP Parallel Bioreactor Systems	Eppendorf, Germany
Gilson Pipeteman Classic P100	Fisher Scientific, U.S.
Gilson Pipeteman Classic P1000	Fisher Scientific, U.S.
Gilson Pipeteman Classic P20	Fisher Scientific, U.S.
Gilson Pipeteman Classic P200	Fisher Scientific, U.S.
ÄKTA Pure protein purification system	GE Healthcare, U.S.
HiLoad 16/600 Superdex 200 pg	GE Healthcare, U.S.
HP Spin Trap Protein A HP 5 mL	GE Healthcare, U.S.
Superdex 200 10/300 GL	GE Healthcare, U.S.
Sterile Tube Fuser-Dry	GE Healthcare, U.S.
XploRATM INV Raman microscope	Horiba, Japan
Osmomat 030	Gonotec, Germany
IKA C-MAG H54	IKA, Germany
PIPETBOY acu 2	Integra Bioscience, Switzerland
ISF1-XC (Climo Shaker)	Kuhner, Switzerland
BioProfile 100 Plus	Nova Biomedical, U.S.
FortéBio Octet RED96e	PALL, U.S.
Balance Lab scale	Sartorius, Germany
Vortex Genie 2	Scientific Industries, U.S.
Reinstwassersystem Ultra Clear	SGwater, Germany
Heraeus Megafuge 16	Thermo Scientific, U.S.
Heraeus oven	Thermo Scientific, U.S.

MSC-Advantage (Laminar flow hood)	Thermo Scientific, U.S.
pH7110	WTW, Austria

3.1.2. Disposables

Table 4. List of disposables

Luer Lock Solo, 4617304F	B.Braun, Germany
Sample cup, 08229NT	Beckman Coulter, U.S.
Serological pipettes 2 mL, 4486	Corning, U.S.
Serological pipettes 5 mL, 4487	Corning, U.S.
Serological pipettes 10 mL, 4488	Corning, U.S.
Serological pipettes 50 mL, 4490	Corning, U.S.
Erlenmeyer cell culture flasks 125 mL, S430421	Corning, U.S.
Erlenmeyer cell culture flasks 250 mL, S431144	Corning, U.S.
Erlenmeyer cell culture flasks 500 mL, S431145	Corning, U.S.
Protein A HP SpinTrap, 28903132	GE Healthcare, U.S.
CELLSTAR 50 mL, 27261	Greiner Bio-One, Germany/Austria
Ultratip 200 µL, 739290	Greiner Bio-One, Germany/Austria
Ultratip 1000 µL, 740290	Greiner Bio-One, Germany/Austria
µ-Slide 8 Well Glass Bottom, 80827	ibidi, Germany
UltrafreeR MC-VV Centrifugal Filters, Durapore, UFC30VV00	Merck, Germany
Amicon Ultra 15 mL Centrifugal Filters 10K, UFC901024	Merck, Germany
Stericup - 022 µm/500 mL, MPSCGPU05RE	Millipore, Irland
ProA Dip and Read Biosensors, 1806191	PALL, U.S.
Rotilabo - Syringefilter, P668.1	Roth, Germany

C-Flex laboratory tubing, T7288	Saint-Gobain Life Sciences, Int.
SpectraPor Dialysis Membrane; 132720	Spectrum Laboratories,U.S.
Nunclon Delta Surface , 163371	Thermo Scientific, U.S.
Nunclon Delta Surface , 153732	Thermo Scientific, U.S.
Nunc-Immuno™ MicroWell™ 96 well, P8741	Thermo Scientific, U.S.
Falcon 50mL Centrifuge Tubes, 1786.51 997.69	Thermo Scientific, U.S.
Falcon 15mL Centrifuge Tubes, 1200.73 1828.96	Thermo Scientific, U.S.
TubeSpin Bioreactor 50 mL, 87050	TPP, Switzerland
Microcentrifuge tubes, 211-0015	VWR, Int.

3.1.3. Chemicals, Reagents & Media

Table 5. List of chemicals and reagents

Osmolality standard, 0.300 Osm/kg, 30.9.0020	Gonotec, Germany
L-glutamine, 1.00286.1000	Merck, Germany
Sodium dihydrogen phosphate, 13472-35-0	Merck, Germany
Glycine, 1.04201.5000	Merck, Germany
Bioprofile 100 plus Reagent Pack, 38291	Nova Biomedical, U.S.
Bioprofile Control Level 1, 22600	Nova Biomedical, U.S.
Bioprofile Control Level 2, 22601	Nova Biomedical, U.S.
Bioprofile Control Level 3, 22602	Nova Biomedical, U.S.
Bioprofile Control Level 4, 24617	Nova Biomedical, U.S.
Bioprofile Control Level 5, 24619	Nova Biomedical, U.S.
Dulbecco's Phosphate Buffered Saline, D8537	Sigma-Aldrich, Germany
Phenol red, P0290	Sigma-Aldrich, Germany
Trypan blue, T8154	Sigma-Aldrich, Germany
Tween20, 9000-5-64-5	Sigma-Aldrich, Germany

AF C Emulsion, A80011	Sigma-Aldrich, Germany
D-Glucose monohydrate,14431-43-7	Sigma-Aldrich, Germany
Glycerol, 24386298	VWR, Int.

Table 6. List of applied commercially available media

CD CHO, 10743-029	Gibco, U.S.
HyClone ActiPro, SH31039.02	GE Healthcare, U.S.
HyClone Cell Boost 7a supplement, SH31026	GE Healthcare, U.S.
Hyclone Cell Boost 7b Supplement, SH31027	GE Healthcare, U.S.

3.2. Methods

3.2.1. Cell line

A CHO DG44 cell line producing a recombinant human anti-HIV-1 IgG1 antibody PG9 was used as a model cell line within all cultures. Since this cell line comprises characteristic genetical modifications (see section 1.1.1), the cells were transfected with the gene of interest along with a functional copy of the DHFR gene. To obtain an efficient cell performance in terms of constant productivity, the genes were integrated into the host genome via a stable transfection using a modified bacterial artificial chromosome (BAC). This specific CHO DG44 clone was previously established within the Animal Cell Factory Design working group of Univ. Prof. Renate Kunert at the Department of Biotechnology (University of Natural Resources and Life Sciences, Vienna).

3.2.2. Thawing of the cells

Since it is reported that long term cultivation and passaging of mammalian cells is expected to impair the respective cell lines in terms of viability as well as specific productivity, cryopreservation in liquid nitrogen is the most common method to properly store established cell lines. Storage of cells at -196°C significantly lessens the probability of contamination and increasing variability through sub cultivation and instabilities regarding the phenotype and

genotype. Typically, cryoprotective agents like DMSO and glycerol are applied within the storage medium to prevent the intra and extracellular formation of adverse ice crystals. After proper temperature reduction to the desired freezing point, a long-term storage of the given cell without any negative effect is enabled due to a significant reduction of all biological reactions.

After removing the cryo-vial from the liquid nitrogen storage tank, thawing of the recombinant CHO DG44/PG9 cell line was conducted under sterile conditions in the laminar hood. The vial, containing 5×10^6 cells in 1 mL of the given cell line, was placed into 70% ethanol for disinfection. After thawing, the cell suspension was transferred into pre-cooled culture media (ActiPro™) and centrifuged for 10 minutes at 200 g. The supernatant, containing toxic cryoprotective agents, was removed and the cell pellet resuspended in 5 mL fresh and pre-warmed cell culture media. Subsequently, the cell suspension was transferred into a 125 mL shaking flask (Corning), containing additional 5 mL pre warmed fresh media, and cultivated at 37°C with 220 rpm (Kuhner shaker). Humidity and CO_2 in the incubator were maintained at 70% and 7%, respectively.

3.2.3. Maintenance of routine culture

Ensuring high viability and contamination free cell culture is of great importance in every field of cell culture process. Consequently, the given cell line has to remain in the exponential growth phase by a reduction of the cell concentration within sterile media every 3 or 4 days. Passaging typically describes the process in which cells are transferred within a defined ratio to freshly provided culture media.

To achieve this, the given recombinant CHO DG44 cell line was cultivated in batch process modes using 125 ml shaking flasks (Corning) with a venting cap, enabling a contamination free cultivation without any additional gassing periphery. The starting concentration of each passage was 0.3×10^6 cells/mL in a total volume of 30 mL of the respective media (ActiPro™ + 15 mg/L phenol rad + 8 mM L-Gln). To enable a continuous exponential phase without undergoing into the adverse stationary or death phase, the cell concentration and viability of the routine culture was determined ever 3rd or 4th day using the Vi-Cell XR Cell Counter (Beckman Coulter). Subsequently, the desired total volume of cell suspension (Eq. 1) were transferred into fresh media to maintain the exponential growth phase without any nutrient deficiency.

3.2.4. Media preparation

All commercially available media (Table 6) were either available as ready-to-use liquid media or in powder form. Preparation of the latter was conducted according to the vendor's provided instructions. To ensure optimal growth and proliferation conditions, L-glutamine (8 mM) was added to each culture media. All media for the experimental part in this thesis were chemically defined, animal component free without serum and antibiotic supplementation. Medium for the maintenance was supplemented with 15 mg/L phenol red to observe a pH reduction based on the buffer specific color change following a possible contamination. Prepared media were sterile filtered by using 0.22 µm filter units (Millipore) and stored at 4°C.

3.2.5. Batch cultivation

Batch fermentations are described to be the simplest models for the investigation of specific growth and production parameters in cell culture. Typically, a batch process is characterized as a closed system, whereby the respective media and producing cell line is added to the system at day 0 without any further addition of supplements or manipulation regarding the cell concentration. Optimization and direct adjustment of the process cultivation parameters is only realized by controlled temperature, pH or dissolved oxygen settings via internal or external process control elements. Therefore, batch cultures follow a typical growth curve as shown in Figure 2 due to limited nutrient and oxygen supply as well as accumulation of metabolic by-products over the course of the process.

In this thesis, batch experiments were performed by cultivating the CHO cells in a total working volume of 30 mL using 50 mL TubeSpin bioreactor tubes (Corning) with a venting cap at 37°C, 7% CO_2, 80% humidity and 220 rpm (Kuhner shaker). CD CHO (Gibco) media was used only within the first batch culture (Section 4.1), all other batch as well as fed-batch cultivations were performed by using ActiPro™ (GE Healthcare) as a basal media. Cultures were inoculated on day 0 by resuspending the cell pellet in fresh media with an initial concentration of 0.3×10^6 cells/mL and cultivated till day 3 without sampling. Afterwards, 24h sampling intervals were performed to determine cell specific process parameters as described in the following sections. Therefore, 2mL were drawn from the cell suspension with subsequent cell concentration-, size distribution- and viability determination using the Vi-Cell XR Cell Counter (Beckman Coulter). The supernatant was obtained by centrifugation of the remaining cell suspension for 7 min at

317 g and stored at -20°C for further antibody titer-, metabolite-, by-product- and osmolality analysis (see sections below). Based on Equation 3, the total volume of each batch culture for the respective supplement media was calculated and added prior to inoculation. Table 7 gives a representative overview of the applied total working volumes of the basal and two supplement media, respectively, for an applied supplementation ratio of 5.5% used within the experimental part of this thesis.

Table 7. Composition of a batch culture in regarding the level of basal and supplement media within a total working volume of 30 mL with 5.5% supplementation ratio.

	Basal media	Supplement A	Supplement B
Supplementation ratio [%]	-	5	0.5
Volume [mL]	30	1.5	0.15

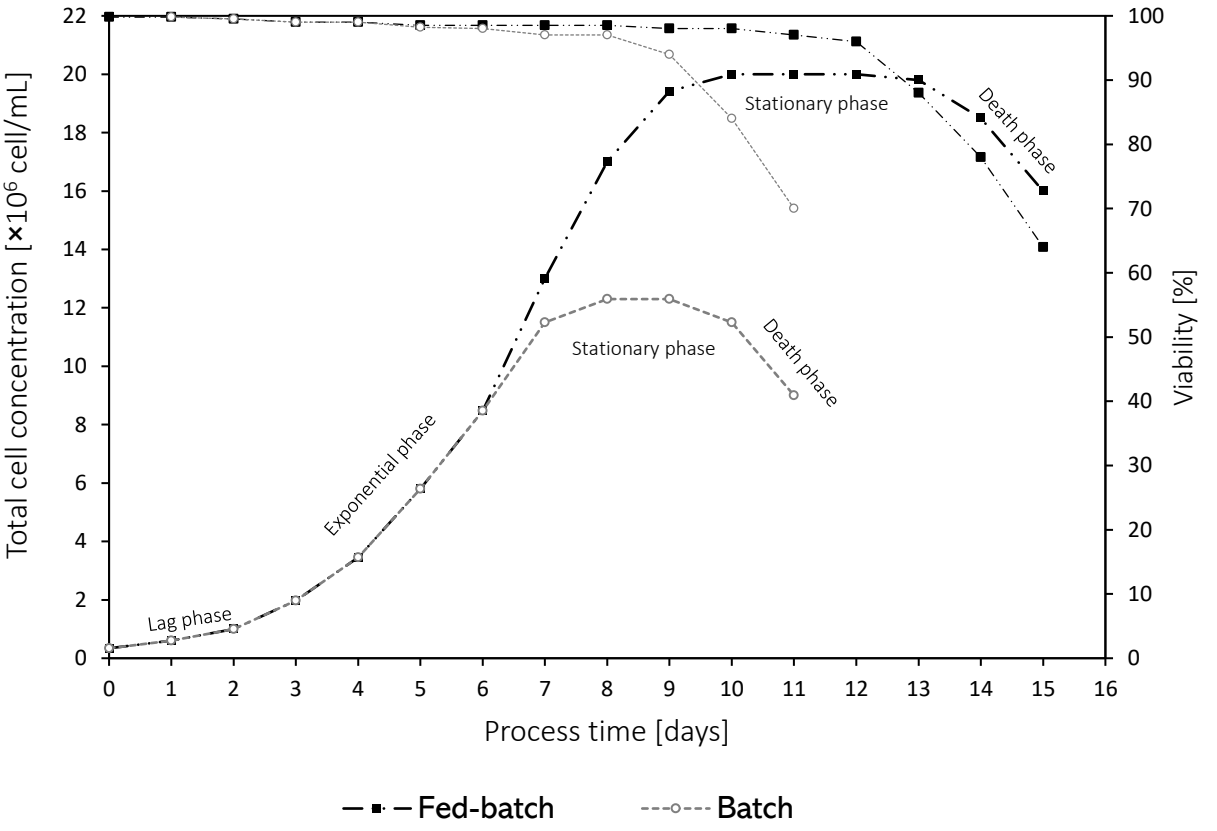


Figure 2. Typical growth phase profile of mammalian cells in batch and fed-batch process mode.

3.2.6. Fed-batch cultivation

In contrast to a batch cultivation, in which growth and process cultivation time is limited due nutrient depletion, fed-batch cultivations are describing the process of a regular supply of fresh nutrients to the cultivation. Hence, the process time and cell densities are increased and only limited due to metabolic by-product formation and vessel size. To overcome the latter and avoid correlating high dilution rates, feed media are mostly highly concentrated formulations of essential media components such as carbohydrates, amino acids, vitamins, membrane precursors and trace elements. Due to the regular input of fresh nutrients, the growth behaviour varies from a batch cultivation in terms of higher cell concentration, prolonged exponential as well as stationary phase and may results in higher product formation (Figure 2). However, applied feeding regime and media composition highly influence the respective growth behaviour and needs to be adapted to the given cell line and process conditions.

In order to obtain optimal bioprocess performance and enable online monitoring of the process parameters, the fed-batch cultivations were performed using DASGIP Bioreactors (Eppendorf). To ensure the absence of pyrogens, heat sensitive system components were immersed in 1 M NaOH for at least 24 hours. All other system relevant components were dry heated at 220°C for de-pyrogenization overnight. Afterwards, the bioreactors were assembled according to the standard operation procedure with respect to the defined feeding regime. This included the basal media, feed media, waste, base and anti-foam adaptations as well as air in- and outlets, the dissolved oxygen (DO) sensor (Hamilton) and pH sensor (Mettler Toledo). All open port and tubing ends were closed with 0.2 µm hydrophobic filter (Millipore) to prevent a possible contamination after autoclaving. Subsequently, the assembled bioreactor system was sterilized by autoclaving it at 121°C, 1.0 bar for 30 min. Before and after the autoclaving procedure, pressure tests were performed to ensure a completely closed and tight system. Prior to inoculation, sterility and overall functionality of the bioreactor system was assessed by incubating 300 mL plain basal media at 37° C for 24 hours.

To obtain the necessary amount of cell number for inoculation, the precultures were propagated in 250 mL and 500 mL Erlenmeyer flasks (Corning) at 37° C, 7% CO_2, 80% humidity and 220 rpm (Kuhner Shaker). The bioreactors were inoculated with exponentially growing cells at an initial concentration of 0.4×10^6 cells/mL in a final working volume of 400 mL and cultivated in batch process for 3 days. To enable an aseptic inoculation, cell suspension was

transferred via a tube fusing system (Sterile Tube Fuser-Dry, GE Healthcare). For the batch as well as fed-batch cultivation, the cells were grown at 37° C, pH 7.0, 30% DO, 3 L/h volumetric gas flow rate and 80 rpm stirrer speed. A constant set point control for the stated process conditions was realized via the internal DASGIP software. To reduce and hinder the formation of foam, antifoam C Emulsion (Sigma Aldrich) was added manually on demand. Figure 3 illustrates a reactor build up for a representative fed-batch process.

Fed-batch cultivation was initiated after 3 days of cultivation with a daily feed-media volume addition of 1.1% of the actual working volume. Adaptation of the feed-media periphery was also performed as described for the inoculum via a sterile tube fusing system (Sterile Tube Fuser-Dry, GE Healthcare). Since the feeding-strategy was directly linked to the actual working volume in the vessel, calculation of the feed media volume was based on Equation 5. To avoid any falsified parameter analysis, 3 mL sample port dead volume were drawn from the sample port and discarded prior to sampling. Subsequently, 3 mL sample of the cell suspension was drawn for analysis as described in the following sections at 24 h intervals. Table 8 gives an overview of process relevant parameter of the fed-batch process.

Table 8. Default parameter settings and used media/reagents for fed-batch cultivation

Cell line	CHO DG44/PG9
Basal media	ActiPro™ + 8 mM L-Gln
Feed media	HyClone CellBoost 7a HyClone CellBoost 7b Supplementation media A Supplementation media B
Working volume	400 mL
Feeding strategy	Feed start at day 3 Daily 1.1% feed of the actual culture volume
Seeding (total)	0.4×10^6 cells/mL (160×10^6 cells)
Reactor	DASGIP Bioreactor (Eppendorf)
Incubation temperature	37.0°C
pH-Setpoint	7.00
Dissolved oxygen setpoint	30%
Volumetric flow rate	3 L/h

Stirrer speed	80 rpm
Dissolved oxygen (DO) setpoint	30%
Base	8% $NaHCO_3$
Anti-foam	1:100 diluted AF C Emulsion
Termination criterion	Viability < 60%

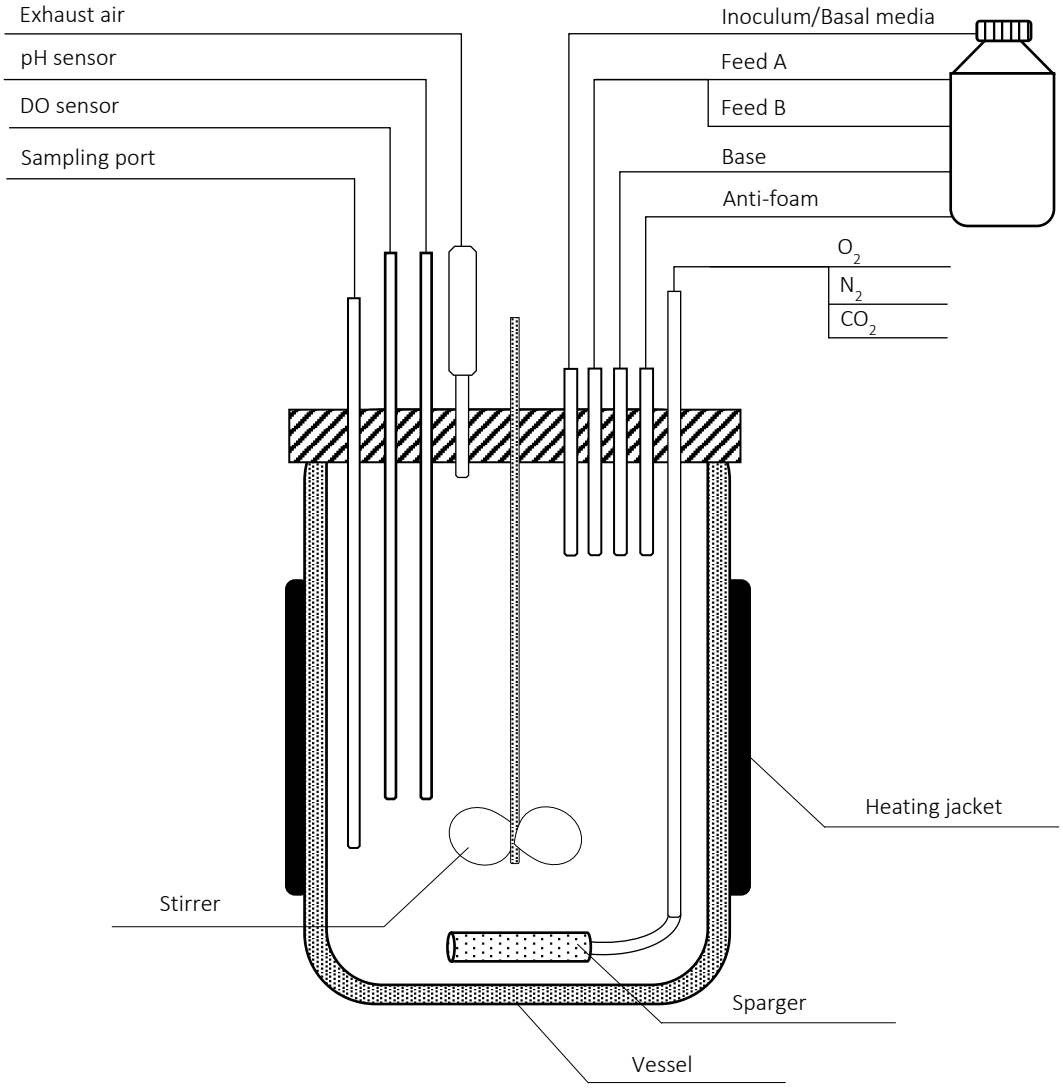


Figure 3. Schematic illustration of a controlled bioreactor set up including main reactor periphery.

3.2.7. Determination of bioprocess parameters

Gathering information about the current state of a cell cultivation process in representative sampling intervals is of great importance in order to monitor specific cell growth and productivity performance.

3.2.8. Evaluation of cell growth and viability

Nowadays there are several methods for determination of cell number and viability available which are based on different cell properties such as size distribution and membrane integrity. For the thesis represented here, a fully automatic ViCell XR - Cell analyser (Beckman Coulter) was used to determine cell concentration, viability, size and circularity of the respective suspension. The utilized method is based on trypan blue dye exclusions. Trypan blue can only pass a cell's membrane following a dissociation of cell membrane components. It is based on the principle that living cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas in dead cells the dye can surpass the membrane and bind to intracellular proteins. Therefore, the blue rendered dead cells can be distinguished from living cells via optical based determination (Strober 2001).

As previously described, samples were drawn from the respective cultivation for further analysis. Determination of the cell culture parameters via ViCell XR - Cell analyser (Beckman Coulter) required approx. 700 µL cell suspension, the remaining culture was centrifuged at 317 g for 7 min and the supernatant collected for further analysis. Since an accurate analysis is limited in terms of high cell concentrations, the cell suspension was diluted to expected concentration below 10×10^6 cells/mL. Analysis was performed by transferring the 700 µL sample into sampling cups (Beckman Coulter) and placing it into the auto sampler unit. Prior to the analysis, default parameters such as dilution rate and cell line were set within the analysis software. Subsequently, the determination of the cell culture parameters was initiated and automatically performed.

3.2.9. Determination of metabolites and by-products

In general, all cell culture media formulations are based on a balanced nutrient solution to ensure optimal growth conditions. Occurring depletion of essential nutrients and accumulation of metabolic by-products over the process time course can have a severe effect on cell growth and product formation. Therefore, the determination of key-metabolites is essential in every cell culture process to acquire knowledge about general cultivation requirements for every cell line. Thus, metabolite analysis over the whole process time is fundamental for further media optimization approaches.

To monitor metabolites and by-products, 1 mL of the respective cultivation supernatants or cell suspension from fed-batch cultures was analysed using the Bioprofile 100 Plus device (Nova Biomedical). The subsequent analysis was done fully automated. In case of the fed-batch cultivation, in which the cell suspension was used for the analyses, the obtained pH values were used as a reference to the online measurement within the bioreactors. In case of a set point deviation of ± 0.05, the pH sensor from the DASGIP module was manually adjusted. All analysed parameter and the referring principle measurement methods are summarized in Table 9.

Table 9. BioProfile 100 Plus test parameter and referring methodology[1].

Parameter	Principal measurement methodology
Ammonium	Enzyme/Amperometric
Glucose	Enzyme/Amperometric
Glutamate	Enzyme/Amperometric
Glutamine	Enzyme/Amperometric
Lactate	Enzyme/Amperometric
pH	Ion selective electrode

[1] https://novabio.us/bioprofile-400/

3.2.10. Determination of osmolality

The osmolality of a solution refers to concentration of soluble components in a given weight or volume of water. Therefore, it is either expressed as osmolality [Osm/kg] or osmolarity [Osm/L]. The Osmomat 030 (Genotec) determines the osmolality of a liquid, based on a comparable analysis to its freezing point. Prior to the measurement, the Osmomat 030 (Genotec) was calibrated by using a 300 mOsm/kg reference standard and desalted water (0 mOsm/kg). Subsequently, 50 µL of sample was placed into the analysis chamber. Determination of osmolality was afterwards executed based on the standard operation procedure and automatically performed.

3.2.11. Determination of antibody titer via biolayer interferometry

Biolayer interferometry is based on the optical determination of specific wavelength shifts within a remitted white light between two surfaces. These two surfaces are represented as an internal reference layer and a layer of immobilized ligands on the biosensor tip. In case of a binding event between the immobilized ligand and the target protein, the resulting change in thickness of the biosensor layer leads to a wavelength shift of the reemitted light. These optical characteristics can further be used to determine binding specificity, rates of association and dissociation as well as concentration of the target protein (FORTÉBio n.d.). Since the model protein PG9 belongs to the IgG antibody isotype, biosensors with immobilized protein A were applied for the antibody titer determination. Protein A is the surface protein of *Staphylococcus aureus* and typically found in biotechnological application to specifically bind to the Fc regions of IgG antibodies between the CH2 and CH3 domains (Kobayashi and DeLeo 2013).

Determination of the antibody titer was performed in cell free supernatant within 96 well plates (Thermo Fisher) using the Octet RED96e (FortéBio-PALL). Since the applied standard curve showed linearity between a concentration of 0.1 - 50 µg/mL, the respective supernatants were diluted to an expected concentration between those thresholds with a 0.1% Tween20/PBS solution. Afterwards, 200 µL of the diluted samples as well as 200 µl of glycine-HCl buffer (pH 2.5) and 0.1% Tween20/PBS buffer for regeneration and neutralization, respectively, were transferred into the well plate according to the illustrated sample pattern in Figure 4. To avoid adverse matrix effects, the samples were diluted with a minimum dilution rate of 1:4. Prior to the measurement, the respective default settings were configurated in the Octet data

acquisition software (Version 11.0, FortéBio-PALL) according to Table 10. Subsequently, the prepared 96 well plate was placed into the measurement chamber. To obtain a consistent measurement, the protein A coated tips were equilibrated by dipping the tips into a 0.1% Tween20/PBS buffer within the defined neutralization wells at 25°C for 10 minutes. Initiation of the fully automated binding rate determination was performed according to the standard procedure operation. After the measurement run, obtained binding rates and the corresponding protein concentration were calculated based on the data evaluation software (Version 11.0, FortéBio-PALL).

Table 10. Overview of default settings for the antibody titer determination using the Octed RED96e system.

Process step	Temperature [°C]	Cycles	Duration [sec]	Plate rotation [rpm]
Quantification	25	1	120	1000
Regeneration	25	3	5	1000
Neutralization	25	3	5	1000

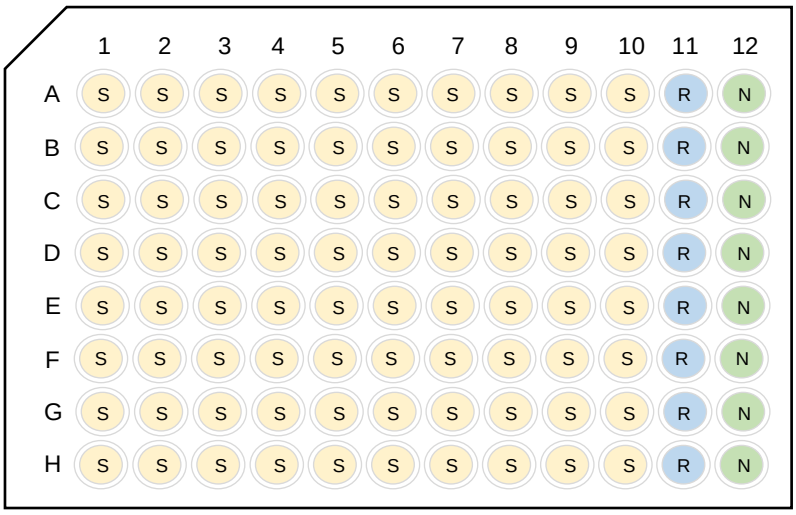


Figure 4. Schematic representation of the pipetting scheme in a 96 well plate using the OctetRED96e system: Sample (S), Neutralization buffer (N), Regeneration buffer (R)

3.2.12. Media component analysis via Raman spectroscopy

Raman spectroscopy is a spectroscopic measurement based on inelastic scattering of monochromatic light. In most applications, the light source is realized via high performance laser units. Inelastic scattering describes the frequency of photons in monochromatic light, emitted from the light source, upon interaction with the specimen. The frequency of reemitted photons following absorption by the sample are detected and referred to the original frequency of the emitted monochromatic light. These up and down shifts are generally known as Raman effects and can be utilized to identify specific vibrational, rotational and other low frequency transitions in molecules. Application of Raman spectroscopy is of great interest in various scientific and industrial fields due the possibility to obtain specific qualitative and quantitative information about solid, liquid and gaseous samples (Buckley and Ryder 2017; Princeton Instruments n.d.). Furthermore, this analytical spectroscopy provides effective technical possibilities to overcome complex burden in bioprocess and cell culture media analysis. As described in section 1.2, cell culture media represent highly complex formulations and comprise different concentration ranges of analytes. Consequently, it is of great importance to identify the presence and absence of a specific component within cell culture media. In particular, utilization of Raman spectroscopy and integration of computational based analysis methods such as principal component analysis (PCA) and soft independent modelling of class analogy (SIMCA) gave rise to highly effective qualitative and quantitative analyses (Li et al. 2010). In general, the evaluation of obtained spectral data set is based on the same principle, which aims to compare and match specific pattern shifts within the spectra out of a data library. One major problem with Raman spectroscopy is the fact that obtained data spectra of complex samples are difficult to unscramble due to the contribution of every molecular component within the sample. Although single substance within a media can show specific shifts, various chemicals or proteins can extensively overlap with analyte bands from the media and increases the difficulty of the analysis (Buckley and Ryder 2017). However, extensive research and improvement of computational based analysis methods has proven the applicability of Raman spectroscopy as a powerful tool for qualitative and quantitative determination of certain media components. Furthermore, Whelan J. et al. reported the successful application of Raman spectroscopy, to in-line monitor glucose, glutamine, lactate, ammonia, glutamate and even total cell density within bioprocesses (Whelan, Craven, and Glennon 2012).

In this thesis, Raman spectroscopy was applied to identify the presence of feed stock solutions within different supplemented basal media based on the experimental design matrix. To obtain a representative data library, Raman spectra of various combinatorial solution of basal media, feed stock solution and CellBoost 7a/b were generated. Afterwards, the obtained spectra were evaluated via principal component analysis to gather information about the possibility to separately identify the presence of the respective solutions. All system relevant units such as the microscope stand, light source, control unit and the Spectroscope (horiba) itself was turned on according to the standard operation procedure. Prior to the spectroscopy, 300 µL of the samples were transferred into 8 well glass slides (ibidi) and placed on the motorized stage. Furthermore, the measurement rating was set to 1800 and a shift range between 300 – 1800 cm^{-1} was defined. Afterwards, each single sample was measured 2 times to remove possible cosmic ray distortion within the spectrum and 5 times at different position within the specimen chamber via the internal 532 nm laser. Generated spectra where then exported to obtain a practical data set for subsequent PCA analysis.

3.2.13. Evaluation of process parameter based on experimental design

Evaluation of various screening parameters by utilizing a reliable experimental design can provide an effective approach to understand and optimize the respective process. In contrast to traditional OFAT trials, such design of experiment approaches offer opportunity for improved statistical analysis while saving time and costs at the same time. A general introduction and further explanation regarding experimental design approaches are described in section 1.3.

In this thesis, the design and configuration of the general experimental set up was realized by using the MODDE 12.1 software package (Umetrics). The principal evaluation of screening as well as optimization approaches remained unchanged, since they only differ in the experimental level setting. Table 11 illustrates a full factorial 2^2 experimental design matrix with two factors and their corresponding level setting (-1, 0, 1) as well as three center points (Experiment #5, #6 and #7). Afterwards, representative responses for each design space were defined to cover a broad range of specific process parameter (see section 4.2.3).

Table 11. Representative experimental design matrix of a 2^2 full factorial experiment including three center points and defined responses (X and Y). Colours are indicating the level setting of each experiment.

Experiment No.	Factor A	Factor B	Response X	Response Y
1	1	1	X_1	Y_1
2	1	-1	X_2	Y_2
3	-1	1	X_3	Y_3
4	-1	-1	X_4	Y_4
5	0	0	X_5	Y_5
6	0	0	X_6	Y_6
7	0	0	X_7	Y_7

Based on the above stated design of experimental matrix, Figure 5 illustrates a representative sample preparation. Exemplary, the set up for experiment #2 would have been performed as followed. This experiment was supplemented with Factor A solution, represented by the +1 level, and the corresponding osmolality adjusted HQ-H_2O solutions of the Factor B solution (-1 level). The center point experiments (Experiment #5, #6 and #7), which are performed to improve the statistical power, are supplemented with half of the working concentration (0 level) of Factor A and B as well as 50% of the corresponding osmolality adjusted HQ-H_2O solutions. Osmolality adjustment was performed by dissolving a calculated amount of NaCl (Equation 10) in HQ-H_2O.

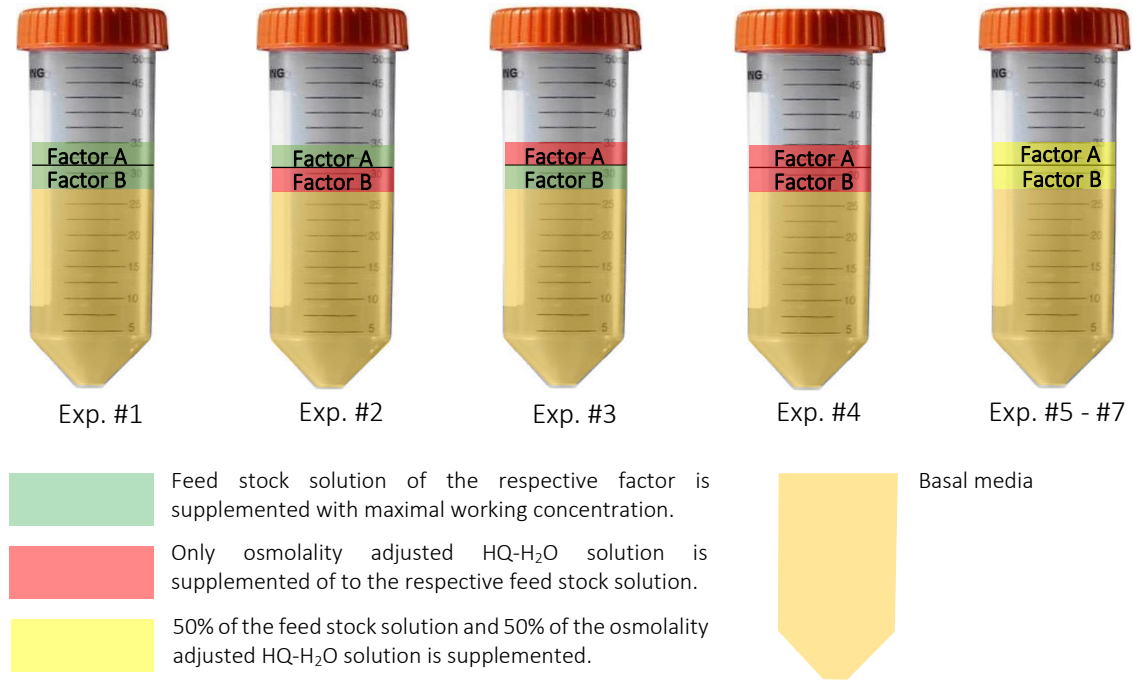


Figure 5. Representative sample preparation of a 2^2 experimental design. Four different sample set up and their corresponding supplementation are illustrated based on the experimental design matrix. +1 level (green), 0 level (yellow), -1 level (red), basal media (orange). Source of tube picture: https://www.thomassci.com/scientific-supplies/50-Ml-Sterile-Centrifuge-Tube.

The statistical evaluation of defined responses was based on a linear regression analysis to quantify the effect of each factor on the response. The principal regression analysis (Equation 13) is based on the effect evaluation (Equation 12) following the mean value determination of each response (Equation 11) regarding the level setting. As already mentioned above, the overall effect evaluation was conducted by using the statistical analysis MODDE 12.1 software package (Umetrics). Application of such statistical tools enables an advanced and convenient method to interpret rather large numbers of various input factors. Since the main goal of this thesis was the effect-based optimization of a supplement media, the software was utilized to obtain effect plots for every defined response. Furthermore, the model quality was assessed by the R^2 and Q^2 values. The R^2 values describes the so-called goodness of fit and is suggested within the software to be above a threshold of 0.8, which states that over 80% of the obtained response data can be described with the applied model. In contrast, Q^2 describes the power of prediction which indicates that a certain percentage of the obtained response data can be predicted. According to MODDE 12.1 (Umetrics), the Q^2 value should be $\geq$ 0.6 (60%). Figure 6 illustrates two representative effect plots on two different responses with the corresponding error bars to indicate significance. The effect blot on the left side in Figure 6, illustrates a significant positive effect of factor A on response X whereas factor B seems to have negative effect which is not significant. Contrary to the right effect plot, Factor A shows a slightly positive effect on response Y which is again not significant since the error bars cross the zero line of the plot. Factor B highlights a strong negative impact on response Y with great significance. Based on such obtained effect plots, a general influence, either significant or not, of the performed experimental design could be stated to develop and potentially optimize the developed in-house formulations.

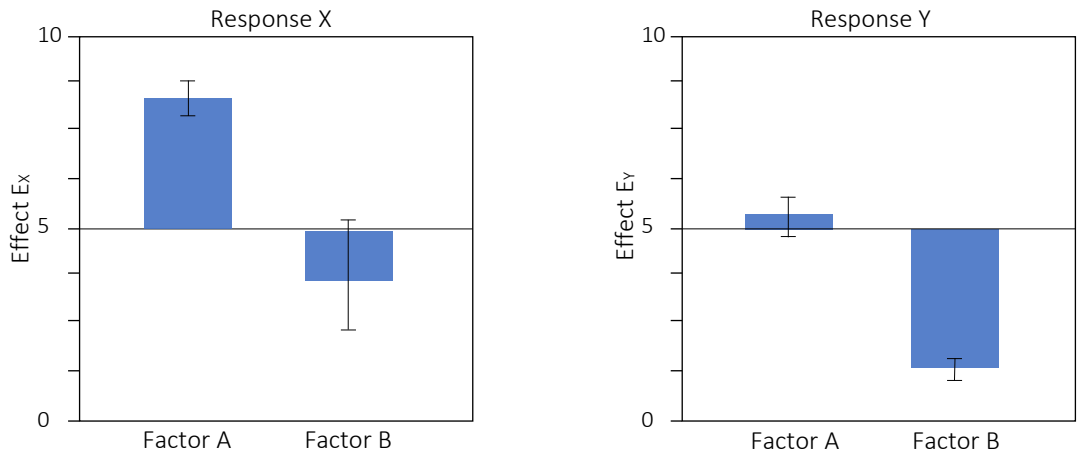


Figure 6. Representative effect plots on response X (left) and response Y (right) obtained via regression analysis of factor A and factor B.

3.2.14. Protein purification

During downstream processes the protein of interest has to be separated from complex solutions, comprising various types and concentrations of different compounds. Therefore, purification steps within almost every biotechnological production are crucial to obtain a highly pure product. Furthermore, one major goal of each downstream process is the volume reduction correlating with concentrating of the protein of interest within the solution.

To ensure high purity of the expressed monoclonal antibody, two chromatography methods were applied which are based on different separation principles. The performed Protein A and size exclusion chromatography using the ÄKTA™ pure system (GE Healthcare) are described in the following sections. Prior to column loading, the harvested supernatants from each DASGIP Bioreactor (Eppendorf) were filtrated through 0.22 µm membrane filter units (Millipore). Subsequently, approximately 150 mL of each filtrated supernatant was used for the following purification steps.

3.2.14.1.1. Protein A purification

The extracellular expressed PG9 antibody was purified based on the binding properties to protein A (see section3.2.11) within the first capture step using the ÄKTA™ pure system (GE Healthcare) and a HiTrap™ 5 mL Protein A HP column (GE Healthcare). Furthermore, binding and dissociation events are based on a pH change within the environment of the loaded protein of interest. Typically, this change in pH is obtained by flushing the respective system with a combinatorial use of different buffers varying in their pH.

The system as well as the column were stored in 20% EtOH for disinfection. Prior to the capture step, the column was washed with 10 column volumes (CV) and subsequently equilibrated with the running buffer till a constant UV and conductivity signal was obtained. To ensure a proper purification run, the system alarm pressure and maximal flow rate were set to 0.5 MPA and 3 mL/min, respectively (Unicorn, GE Healthcare). The filtrated supernatant was then loaded onto the column via the sample loop (flow through fraction), followed by flushing the system with appx. 10 CV running buffer till a constant UV signal was reached (wash fraction). The bound antibody was eluted by applying the elution buffer onto the column. Fractionation of the purified protein was initiated immediately after a rise of the UV signal was observable. Since exposure of the antibody to the low pH of the elution buffer (pH 3.5) could possible result in

adverse conformational changes, all fractions were immediately neutralized by adding 6 µL 2 M TRIS-buffer (pH 12) to the collected fractions. Fractionation of the mAb was performed within the elution step till a drop of the UV signal was observable. Afterwards, the system was purged with 0.1 M glycine buffer (pH 2.5) to remove any retained proteins within the column (clean fraction). Prior to the start of the next purification run, the column was washed with 10 CV running buffer. After all purification runs, the whole system was purged with 20% ethanol for disinfection. Aliquots from the respective fractions, except of the eluate, were again purified by using Protein A HP SpinTraps (GE Healthcare) according to the vendors instruction. For a subsequent protein quantification (see 3.2.15), the purified aliquots were stored at 4°C. The collected elution fractions were pooled and subsequently dialyzed using the SpectraPor Dialysis Membrane by stirring the dialysis tube in 1 × PBS buffer (pH 7.4) at 4°C for at least 12 hours with two dialysis buffer exchanges.

Running buffer	20 mM Sodium phosphate, 200 mM NaCl pH 7.4
Elution buffer	0.1 M Glycine pH 3.5
Regeneration buffer	0.1 M Glycine pH 2.5
Dialysis buffer	1 x PBS pH 7.4
Disinfection solution	20% EtOH

3.2.14.2. Size exclusion chromatography (SEC)

The second step within the purification procedure was a size exclusion chromatography (SEC). This separation method is based on different size and conformation distribution between proteins and aggregates, respectively, as they pass through the column. Therefore, different molecules can be isocratically separated from each other in a simply and harmless way as this process can be performed with this use of buffers varied to suit the sample needs without directly affecting it. The stationary phase of SEC consists of a small-porous polymer resin with various pore diameters. In dependency of the analyte's size, it can either penetrate the pore of the polymer matrix, or, if they exceed the largest pore diameter, will not be hold back and eluate first. Consequently, analytes being smaller than the smallest pore diameter, penetrate the whole pore volume and will eluate last. Comparable to the detection method in the

previous protein A chromatography, the obtained analyte fractions are detected by photometric analysis (Hong, Koza, and Bouvier 2012).

To remove possible remained impurities and formed aggregates, the dialyzed PG9 fractions were purified by utilizing a HiLoad 16/600 Superdex 200 pg column (GE Healthcare) within the ÄKTA pure system (GE Healthcare). Since the loading volume within a SEC run is limited, the protein A purified PG9 sample were concentrated using centrifugal filters (10 kDa MWCO, Merck). Prior to sample loading, the column was flushed with appx. 2 CV HQ-H_2O and subsequently equilibrated by flushing 2 CV of the running buffer through the column till a constant UV signal was obtained. Afterwards, 1 mL of the sample were loaded onto the column following injection into the sample loop. Purification was initiated by setting the valves to flush 1 x PBS through the loaded column. Elution of the protein of interest could be monitored by a alteration in UV signal and was immediately collected in 1 mL fractions. Disinfection with 20% EtOH of the whole system was performed with the same procedure as for the protein A chromatography.

Running buffer	1 x PBS pH 7.4
Disinfection solution	20% EtOH

3.2.15. Photometric quantification of purified protein

Next to the biolayer quantification technique described in section 3.2.11, UV-Vis spectrophotometry represents another convenient way for total protein concentration analysis. One major disadvantage of this method is the need of a highly pure sample compared to the OCTETRed96e system (FortéBio - Pall), which is capable to specifically detect and quantify the protein of interest within an inhomogeneous solution such as a culture supernatant. However, once the protein of interest is purified, UV-Vis spectrophotometry represents an effective and easy to use method for various applications regarding protein characterisation. In general, protein quantification is based on the UV absorbance of a wavelength at 280 nm. A prerequisite for this UV absorbance determination is a reliably known or calculated extinction coefficient (Ɛ). This extinction coefficient varies between different

proteins, since it is calculated from the amino acid composition of the respective protein of interest (Raynal et al. 2014).

Therefore, the online tool ProtParam[2] was utilized to evaluate the specific extinction coefficient of the PG9 antibody based on the provided amino acid sequence (ε_{PG9} = 235320 $M^{-1} \times cm^{-1}$). In this thesis, protein quantification of the purified samples was realized by using the DS-11 Spectrophotometer (DeNovix). Prior to the quantification, the applied wavelength of 280 nm as well as the evaluated extinction coefficient were set within the device user interface. Afterwards, the lens was cleaned using HQ-H_2O and the blank intensity determined with a buffer with the same composition and pH as that of the sample. Afterwards, the blank adjustment was verified by measuring the same blank buffer a second time. Then 1 µL of the respective sample was directly placed on the measurement lens, the cantilever closed, and the fully automated quantification started. Each measurement was performed at least two times to avoid any outliers or falsified measurements.

3.2.16. Absolute molecular mass and protein size verification via SEC MALS

Since it is indispensable for experiments in cell culture to characterize and verify specific properties of the protein of interest, the absolute molecular mass as well as the protein size was evaluated by utilizing size exclusion chromatography coupled with multi-angle light scattering (SEC-MALS). As described in section 3.2.14.2, SEC provides an efficient method to separate the protein of interest into fraction of the desired mono or oligomer form as well as a removal of other impurities. However, the molecular mass of the analytes eluted in each SEC peak cannot be obtained through column calibration approaches, in which refence samples are separated according to protein radius and not their molecular mass (Raynal et al. 2014). In order to ensure the accuracy of the analysis, scattering-based approaches, such as MALS and small angle X-ray scattering (SAXS) can be utilized for an improved protein characterization (Fekete et al. 2014). MALS is based on the illumination of a solution containing the molecule of interest with a polarized light beam and the following determination of the scattered light. Intensity detection of the reemitted light provides information about the molar mass whereas the relative angular dependence of the light beam carries information about the size of the molecule (Wyatt 1993). Thus, these combinatorial approaches are highly suitable for the

[2] https://web.expasy.org/protparam/

analysis and validation of antibody drugs, since aggregates and fragments may potentially affect immunogenicity and potency (Lu et al. 2013).

All molecular mass measurements of the respective samples were performed with a Prominence Modular LC 20 HPLC (Shimadzu) equipped with a refractive index detector (RID-10A, Shimadzu), a refractive a diode array detector (SPD-M20A, Shimadzu) and Heleos Dawn8 plus QELS MALS unit (WyattTechnology). Equilibration of the column (Superdex 200 10/300 GL, GE Healthcare) was performed by flushing the column with the running buffer (pH 7.4). To avoid blocking of the column system, the sample was centrifuged at maximal rpm settings (VWR) and filtrated through 0.1 µm filter units (Millipore) to remove impurities. In order to assess the molecular mass of the purified PG9 antibody, a PG9 reference sample (Polymun) was implemented into the experimental set up. Since a minimum of 20 µg total protein is required for a reliable measurement, the protein quantification was conducted as described in section 3.2.15 and the respective amount of sample transferred into 0.3 mL micro vials. The measurement was initiated by placing the vials into the auto sampler chamber and setting the flow rate and internal unit temperature to 0.75 mL/min and 25°C, respectively. Evaluation of the obtained results was realized by applying the ASTRA 6 software (WyattTechnology) with subsequent export into a proper data format.

Running buffer	1 x PBS. 200 mM NaCl pH 7.4
Disinfection solution	20% EtOH

3.2.17. Calculations

<u>Seeding and basal media volume for routine culture maintenance and fed- batch processes</u>

$$\text{Seeding volume [mL]} = \frac{\text{Cell number}_{\text{target}}}{\text{CC}_{\text{initial}}}$$ *Eq. 1 - Amount of seeding volume*

$$\text{Amount of basal media [mL]} = \text{WV} - \text{Seeding volume}$$ *Eq. 2 - Amount for basal media*

$CC_{initial}$ … Total cell concentration of inoculum [cells/mL]

$Cell\ number_{target}$ … Total cell number in final cultivation [cells]

WV … Working volume [mL]

Media supplementation for batch processes

$$\text{Supplement volume [mL]} = \text{WV} \times \text{SR}$$ *Eq. 3 - Amount of supplement volume*

$$\text{Amount of basal media [mL]} = \text{WV} - \text{Supplement volume}$$ *Eq. 4 - Amount of basal media for supplemented batch processes*

WV … Working volume [mL]

SR … Supplementation ratio [% × 100]

Feed media for fed-batch processes

$$\text{Feed volume [mL]} = \text{WV} \times \text{SR}$$ *Eq. 5 - Amount of feed volume*

SR … Feed ratio [% × 100]

WV … Working volume [mL]

Specific growth rate μ

$$\mu\,[1/\text{day}] = \frac{\ln(\text{CC}_i) - \ln(\text{CC}_{i-1})}{t_i - t_{i-1}}$$ *Eq. 6 - Specific growth rate μ*

CC_i … Cell concentration [cells/mL]

t_i … time [days]

Viable cell days VCD

$$\text{VCD [cells} \times \text{day/mL]} = \frac{\text{VCC}_i - \text{VCC}_{i-1}}{\mu_V}$$ *Eq. 7 - Viable cell days VCD*

VCC … Viable cell concentration [viable cells/ml]

μv … specific growth rate of viable cells [1/day]

Viable cumulative cell days VCCD

$$\text{VCCD [cells} \times \text{day/mL]} = \text{VCCD}_{i-1} + \text{VCD}_i$$ *Eq. 8 - Viable cumulative days VCCD*

VCCD … Viable cumulative cell days [viable cells × day/mL]

Specific productivity q_p

$$q_p[\text{pg/cell} \times \text{day}] = \frac{\text{titer}_i - \text{titer}_{i-1}}{\text{VCD}_i} \times 10^6$$ *Eq. 9 - Specific productivity q_p*

Antibody titer … Product titer in [μg/mL]

VCD … Viable cell days [viable cells × day/mL]

Osmolality adjusted HQ – H_2O solution

$$m_{NaCl}[g] = \left(\frac{\Delta mOsm}{2}\right) \times M_{NaCl} \times \frac{V_{HQ-H_2O}}{1000}$$ *Eq. 10 – Osmolality calculation*

m_{NaCl} … Mass of NaCl in solution [g]

ΔmOsm … Osmolality of solution [mOsm/L]

M_{NaCl} … Molecular weight of NaCl [g/mol]

V_{HQ-H2O} … Volume of HQ – H_2O [L]

Overall mean of the responses X at the corresponding level i

$$\bar{y} = \frac{1}{n}\sum_{i=+1}^{n} X_i \text{ and } \bar{y} = \frac{1}{n}\sum_{i=-1}^{n} X_i$$ *Eq. 11 - Overall response mean*

$\bar{y}$ … Overall response mean

n … Number of experiments

X … Defined response

i … Level

Factor effect E

$$E = \frac{1}{n}\sum_{i=+1}^{n} X_i - \frac{1}{n}\sum_{i=-1}^{n} X_i$$ *Eq. 12 – Factor effect*

E … Factor effect

n … Number of experiments

X … Defined response

i … Level

Linear regression model

$$\hat{y} = \bar{y} + \frac{E_A}{2}X_A + \frac{E_B}{2}X_B + \frac{E_C}{2}X_C + \frac{E_D}{2}X_D$$ *Eq. 13 – Linear regression model*

$\hat{y}$ … Prediction of quality feature

$\bar{y}$ … Overall response mean

E … Factor effect

X … Defined response

A-D … Factor

4. RESULTS

4.1. Definition of the best performing basal media in batch cultivation

Since cell culture media are a significant factor for the success of every bioprocess, the aim of this experiment was to evaluate the best performing culture media in terms of cell performance in dependency of the plain basal media. Based on the observed outcome, the superior performing media for subsequent optimization approaches should be selected.

4.1.1. Experimental setup

Duplicate batch experiments were performed in two different chemically-defined, protein- and animal component free commercial basal media, namely HyClone ActiPro™ and CD CHO. Setting up of the batch cultivation as well as sampling was performed as already described in section 3.2.5. The principal experimental setup of this experiment is summarized in Table 12.

Table 12. Experimental setup: Identification of the best performing basal media

Operation mode	Batch
Cell line	CHO DG44/PG9
Basal media	CD CHO + 8mM L-Gln ActiPro™ + 8mM L-Gln
Working volume	30 mL
Seeding (total)	0.3×10^6 cells/mL (9×10^6 cells)
Reactor	50 mL reactor tubes with venting membrane
Incubation temperature	37.0°C
Humidity	80%
CO_2 level	7.0%
Rotation shaker speed	220 rpm
Termination criterion	Viability < 60%

4.1.2. Performance of clone CHO DG44/PG9 in batch cultivation

In this experiment, the performed batch cultivation using ActiPro™ showed an approximately 43% higher maximal total cell density of 18.0 x 10^6 cells/mL after the 9th day compared to 12.6 10^6 cells/mL after the 8th day of cultivation in the one using CD CHO as basal media. Although, a slightly higher specific productivity within the first days of cultivation could be observed as shown in Figure 7 within the CD CHO media, a significant higher final antibody titer of 113.4 µg/mL after the 10th day of inoculation could be achieved when the cells were cultivated in ActiPro™. Both experiments showed a typical batch mode associated growth rate, characterized by a continuous decrease over the process time. The obtained maximal values are summarized in Table 13.

Table 13. Overview of obtained relevant cell perfromance parameter.

	Max. viable cell concentration [× 10^6 c/mL]	Max. antibody titer [µg/mL]	µ (exp. phase) [1/d]	max. spec. Prod. [pg/cell/day]	max. VCCD [cell x day/mL]	Process duration [d]
CD CHO	10.84	96.55	**0.58**	**3.65**	4.57E+07	9
ActiPro™	**16.89**	**113.40**	0.54	2.71	**7.58E+07**	**10**

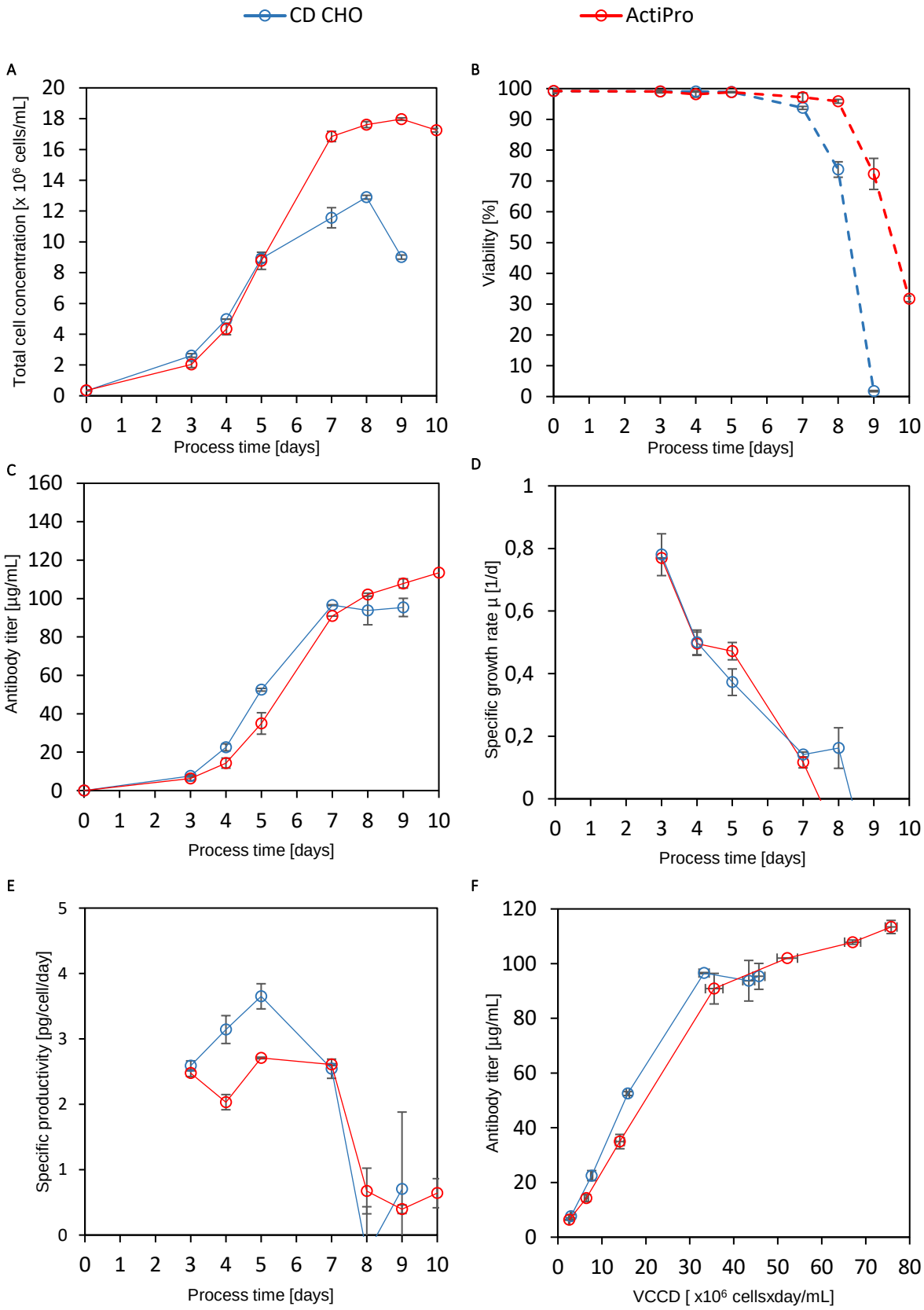


Figure 7. Obtained cell culture profiles comparing two different basal media (A) total cell concentration (B) Viability (C) Antibody titer (D) Specific growth rate (E) Specific productivity (F) Antibody titer plotted over the course of the viable cumulative cell days.

4.2. Characterization of media constituents by full factorial screening experimental design

The aim of this experiment was to gain information about, whether a combinatorial supplementation of four defined media constituent formulations, defined as feed stock solution, can improve the overall culture performance or would impair the obtained cell performance. Furthermore, Raman spectroscopy of the plain feed stock solutions as well as of the respective supplemented basal media coupled with principal component analysis was conducted to find inter-correlated quantitative dependent variables between the obtained spectra.

4.2.1. Experimental setup

As previously described in section4.1, batch experiments were performed to evaluate the best performing media out of two. Within this experiment, the cultivation conducted with ActiPro™ as basal media, showed superior cell performance especially in terms of cell concentration and final antibody titer (Table 12). Therefore, this media was selected as a basis to conduct a full factorial screening experiment of resolution V, to investigate potential effects of organic substance constituents supplemented to the basal media on the cell performance. The general experimental design set up consists of 19 experiment, comprised out of 16 individual experiments and a center point represented by biological triplicates. The triplicate center point is used to estimate significant effects and to detect curvature in the factor-response relationship. Additionally, a 20^{th} experiment was performed were a commercially available media supplementation was applied with 5.5% of the working concentration, namely HyClone Cell Boost 7a and Cell Boost 7b (CB 7a/b). These first 16 experiments are performed as a 2^4 full factorial design approach and tested against four different supplement solutions, classified as factors. All screening experiments were set up, according to the experimental design matrix shown in Table 15. Consequently, these factors are tested against each other based on different level settings including every possible combination to exclude potential confounding events. Based on various responses, a statistical evaluation was performed to determine certain main and interaction effects of different media substances, namely amino acids, vitamins, membrane precursor and pyruvate.

Exemplary, the set up for experiment #7 was performed as followed. This experiment was supplemented with a pyruvate and vitamin feed stock solution represented by the +1 level and the corresponding osmolality adjusted HQ-H_2O solutions of the amino acid and membrane precursor solution represented by the -1 level.

The basal media for all batch experiments, except of experiment #20, were supplemented with 3.5 g/L glucose and 0.26 g/L L-glutamate to mimic the expected higher nutrient concentration of the CB 7a/b supplemented batch cultivation. All experiments were supplemented with the respective feed stock solution prior to inoculation.

Table 14. Experimental setup: Characterization of media constituents by full factorial screening experimental design.

Operation mode	Batch
Cell line	CHO DG44/PG9
Basal media for CB 7a/b supplementation	ActiPro™ + 8mM L-Gln
Basal media for DoE experiments	ActiProTM + 8mM L-Gln + 3.5 g/L Gluc + 0.26 g/L+ L-Glu
Media Supplements	HyClone Cell Boost 7a HyCLone Cell Boost 7b Amino acid solution Membrane precursor solution Pyruvate solution Vitamin solution Osmolality adjusted factor solutions
Working volume	30 mL
Supplementation strategy	Prior to inoculation
Seeding (total)	0.3×10^6 cells/mL (9×10^6 cells)
Reactor	50 mL reactor tubes with venting membrane
Incubation temperature	37.0°C
Humidity	80%
CO_2 level	7.0%
Rotation shaker speed	220 rpm
Termination criterion	Viability < 60%

4.2.2. Factor impacts of defined feed stock solutions within experimental screening design

For this first screening experiment, maximal viable cell concentrations between 20.43 × 10^6 cells/mL (Experiment # 17 at day 13) to 10.62 × 10^6 cells/mL with peak antibody titers between 162.85 µg/mL (Experiment #2) at day 10 and 108.09 µg/mL within experiment #9 at day 11. Process duration ranged between 13 and 10 days before dropping below the viable termination criterion of 60%. The principal experimental design set up as well as referring maximal process parameter of each performed experiment are listed in Table 15.

Table 15. Full factorial experimental matrix including cell perfromance relevant parameter. Colours are indicatig the level setting of each experiment.

Experiment No.	Amino acid	Pyruvate	Vitamins	Membrane precursor	max. Viable cell concentration [× 106 c/mL]	Max. antibody titer [µg/mL]	µ (exp. phase) [1/day]	max. spec. Prod. [pg/cell/day]	max. VCCD [cell x day/mL]	Process duration [days]
1	-1	-1	-1	-1	15.71	126.30	0.47	5.19	8.13E+07	11
2	1	-1	-1	-1	18.75	**162.85**	0.47	3.73	8.58E+07	10
3	-1	1	-1	-1	13.92	131.53	0.47	4.04	7.80E+07	11
4	1	1	-1	-1	17.65	142.77	0.48	4.08	9.42E+07	11
5	-1	-1	1	-1	15.10	126.09	0.49	4.40	8.32E+07	11
6	1	-1	1	-1	20.43	144.72	0.47	4.21	9.73E+07	11
7	-1	1	1	-1	15.21	132.00	0.48	3.45	6.99E+07	10
8	1	1	1	-1	19.59	128.72	0.48	4.52	9.76E+07	11
9	-1	-1	-1	1	14.01	108.09	0.49	**5.69**	8.09E+07	11
10	1	-1	-1	1	18.32	141.66	0.49	4.00	9.69E+07	11
11	-1	1	-1	1	15.43	136.30	0.47	4.85	8.53E+07	11
12	1	1	-1	1	17.79	128.72	**0.52**	5.26	8.51E+07	11
13	-1	-1	1	1	14.90	126.09	0.47	4.77	8.22E+07	11
14	1	-1	1	1	19.16	139.70	0.48	5.41	1.01E+08	11
15	-1	1	1	1	13.18	111.02	0.47	4.21	8.03E+07	**12**
16	1	1	1	1	19.15	133.36	0.49	4.60	1.01E+08	11
17	0	0	0	0	**19.93**	155.91	0.48	5.17	8.95E+07	11
18	0	0	0	0	18.70	142.26	0.48	4.46	8.64E+07	11
19	0	0	0	0	18.84	149.96	0.51	4.73	**1.00E+08**	11
20	Hyclone CellBoost 7a/b				10.62	128.51	0.39	4.35	**7.53E+07**	**13**

Figure 8 illustrates the obtained cell culture profiles graphically. Only the negative control experiment (#1), experiment # 16 including all supplements, the center point generated out of the mean values from experiment #17 to #19 as well as the HyClone CellBoost 7a/b supplemented cultivation are highlighted due to simplified depiction. Each tested media constitute within the course of the experimental factorial design is highlighted in respect to level setting subsequently in this section. In the final analysis, only supplementation strategies which showed a significant influence on the respectively defined cell performance parameter are statistically evaluated. In general, it can be stated that cultivations supplemented with either factor level setting 0 or +1 for all constituents performed superior to the negative control. Interestingly, the HyClone CellBoost 7a/b supplemented cultivation showed the longest cultivation time of 13 days and an overall better specific productivity over the whole batch process.

A significant positive impact within all cultivation which got supplemented with the amino acid solution, as shown in Table 16, could be achieved regarding an average viable cell concentration of 18.85×10^6 cells/ml ± 0.93 compared to the one without the amino acid supplementation (14.68×10^6 cells/ml ± 0.88). Consequently, a further significant positive impact on the obtained maximal viable cell days, 9.48×10^7 cells×day/mL ± 6.19×10^6 compared to 8.01×10^7 cells×day/mL ± 4.67×10^6 was obtained. Furthermore, a superior effect on the final antibody titer (140.31 µg/mL ± 11.07) over the course of the process within an amino acid supplementation compared to 124.68 µg/mL ± 10.03 without the respective constituents was revealed.

A moderate beneficial influence on the maximal specific productivity (4.71 pg/cell/day ± 0.47) of membrane precursor supplemented cultivations compared to 4.20 pg/cell/day ± 0.53 without membrane precursor was also obtained (Table 16). However, no further significant main and interaction effects could be determined.

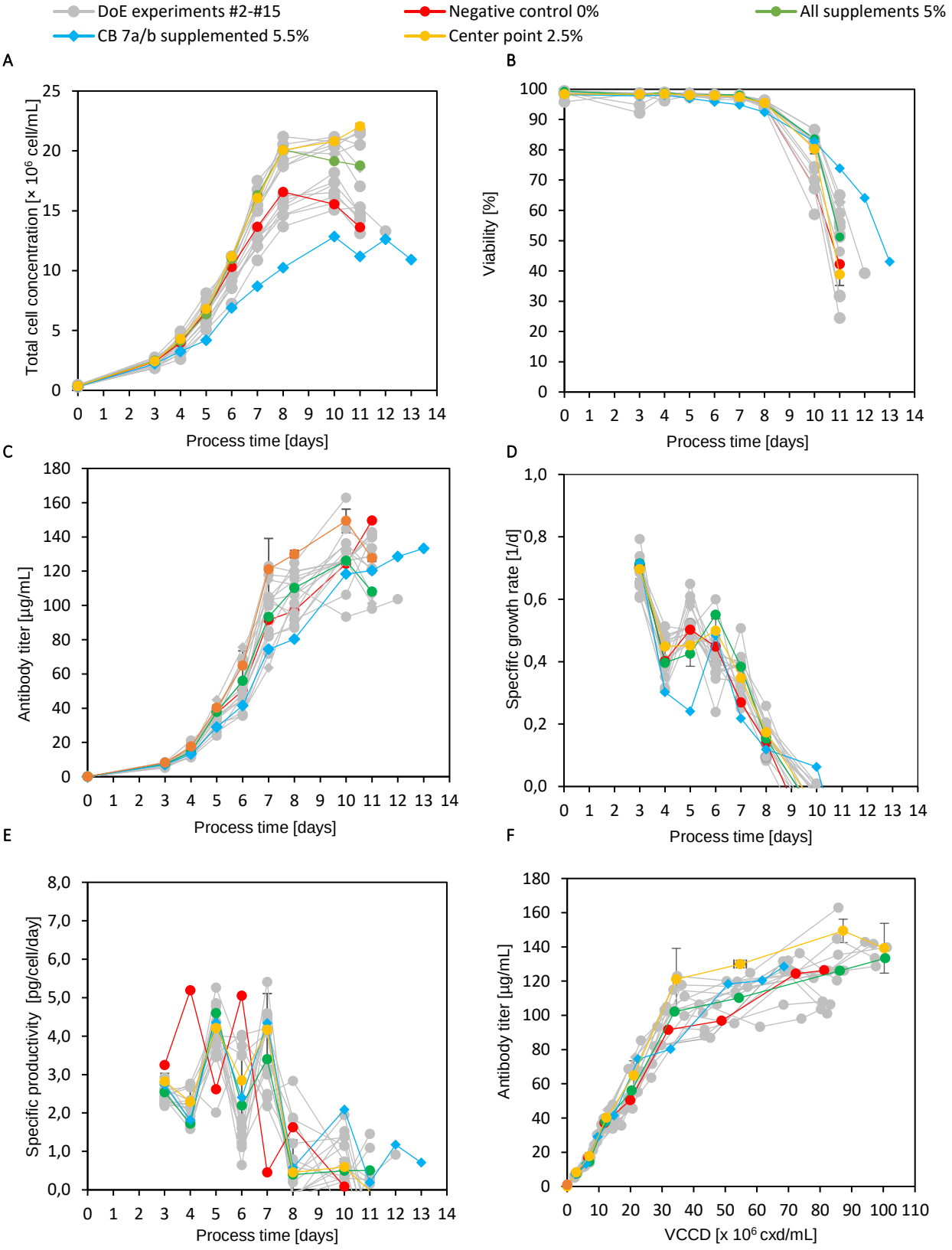


Figure 8. Obtained cell culture profiles of the full factorial screening experiment: (A) Total cell concentration, (B) viability, (C) antibody titer, (D) viable specific growth rate, (E) specific productivity, (F) antibody titer plotted over the course of the viable cumulative cell days. Experiment No. 1 represents the negative control which was only volume adjusted by the respective osmolality solutions by setting the factor level to -1. Experiment #16 included all four tested media constitutes at level 1. Center points experiments were obtained by a half-maximum factor setting of level 0 and are displayed as the mean value including the corresponding standard deviation. All experiments were supplemented prior to inoculation.

4.2.2.1. *Vitamin supplementation*

Experiments supplemented with the vitamin solution, consisting of 9 different integral parts as previously described, did not show any significant impact on defined cell performance parameter. Furthermore, no obvious trend can be stated out of the graphically illustrated cell parameter profiles in Figure 9 nor from the obtained maximal process parameter in the dot plot (Figure 10) extracted from Table 15 since all representative experiments covered a broad deviation of the observed parameter throughout the whole process.

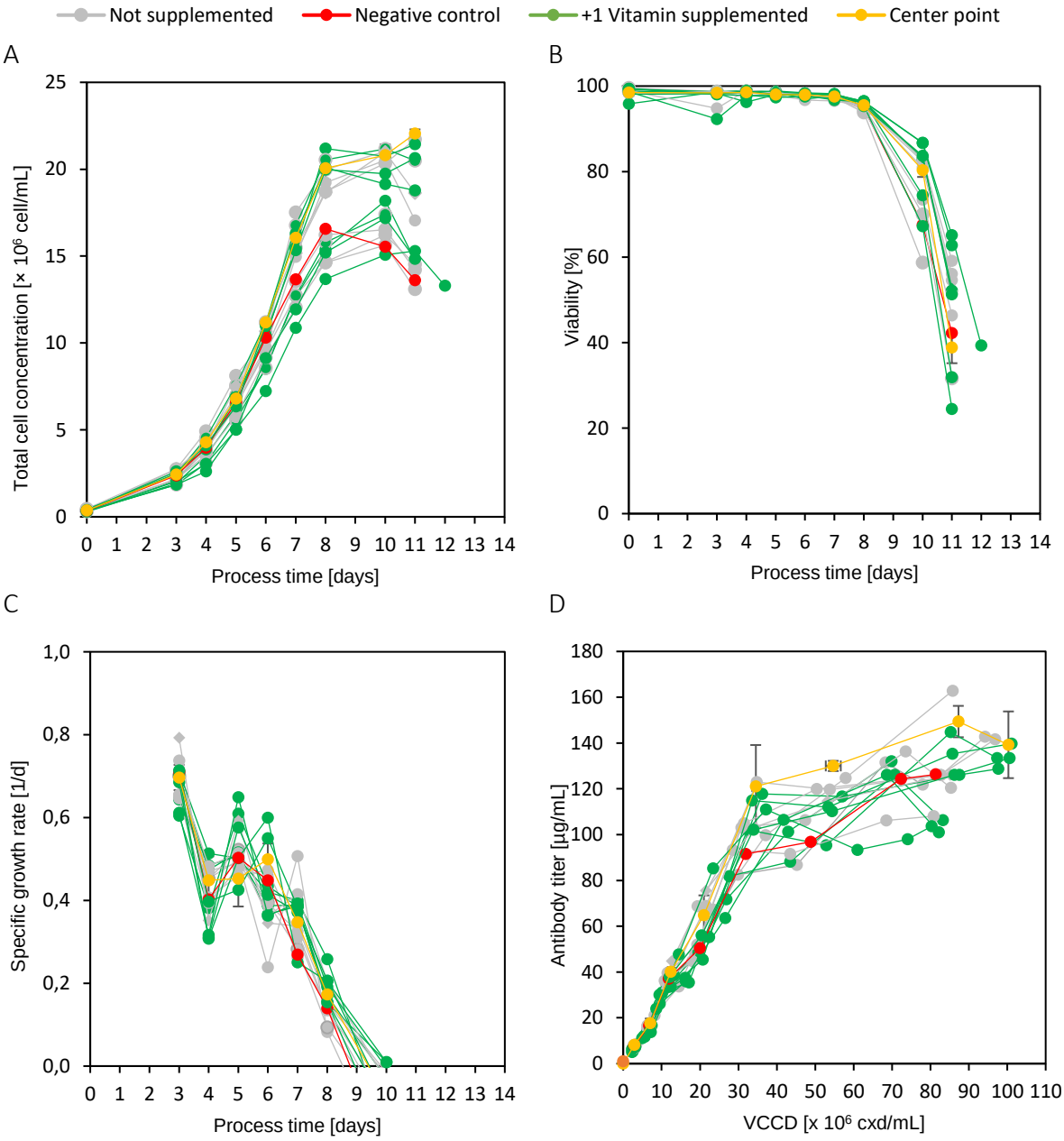


Figure 9. Overview of vitamin supplemented cell culture profiles of the full factorial screening experiment: (A) Total cell concentration, (B) viability, (C) viable specific growth rate, (D) antibody titer plotted over the course of the viable cumulative cell days

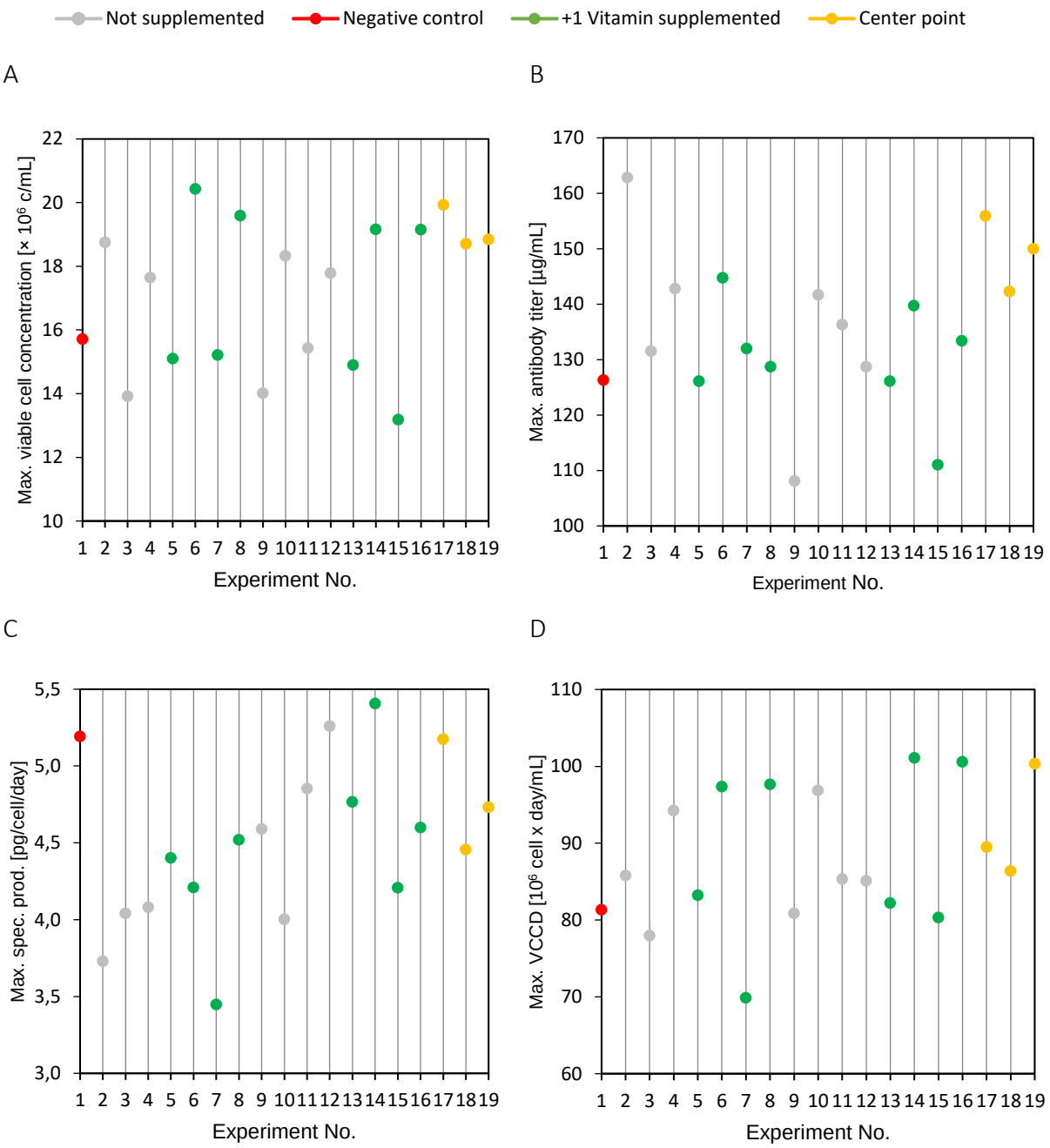


Figure 10. Obtained maximal cell performance parameter of vitamin supplemented cell culture represneted as a dot plot graphles: (A) maximal viable cell concentration, (B) maximal antibody titer, (C) maximal specfifc productivity, (D) maximal viable cumulative cell days.

4.2.2.2. *Membrane precursor supplementation*

Determination of the cell performance relating to a supplementation with membrane precursors revealed rather low total cell concentrations along with higher viabilities over the course of the process time in Figure 11 A and B. Furthermore, considerable high titers could be observed within these cultures correlating with an enhanced maximal specific productivity (Figure 12 C).

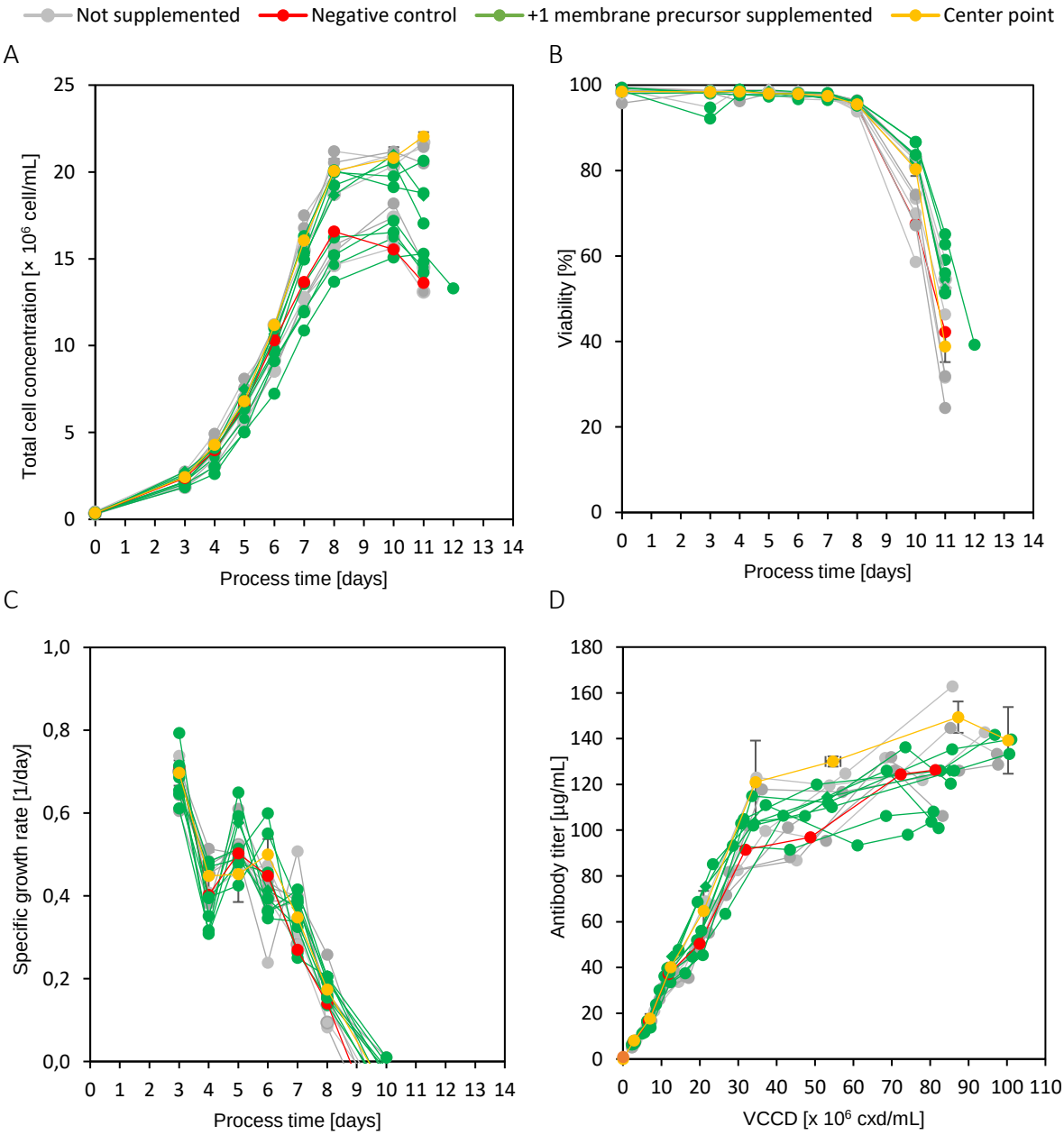


Figure 11. Overview of membrane precursor supplemented cell culture profiles of the full factorial screening experiment: (A) Total cell concentration, (B) viability, (C) viable specific growth rate, (D) antibody titer plotted over the course of the viable cumulative cell days.

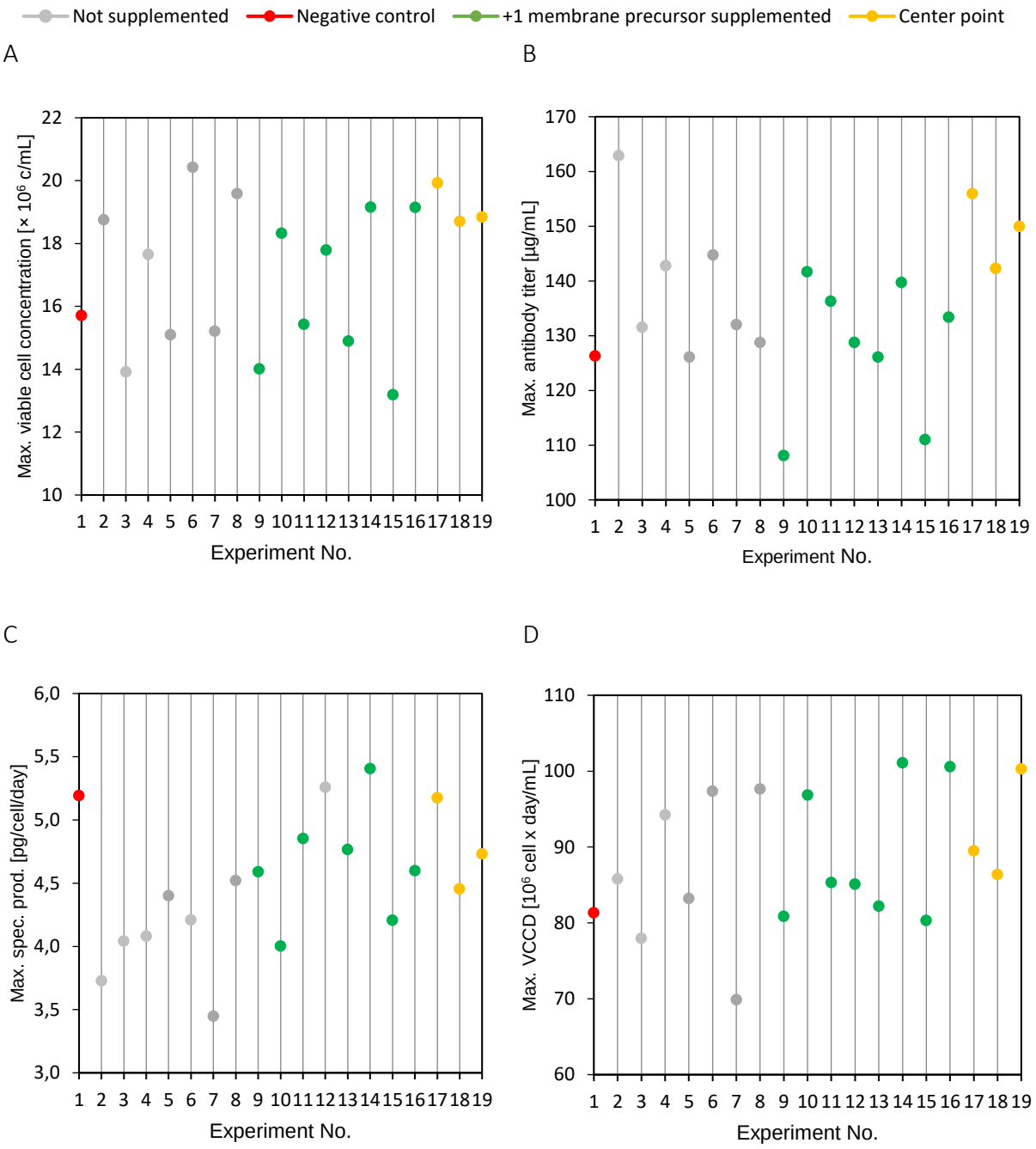


Figure 12. Obtained maximal cell performance parameter of membrane precursor supplemented cell culture represneted as a dot plot graphles: (A) maximal viable cell concentration, (B) maximal antibody titer, (C) maximal specfifc productivity, (D) maximal viable cumulative cell days.

4.2.2.3. *Amino acid supplementation*

By complementing the respective basal media with a feed stock solution, consisting out of 17 essential amino acids, a significant enhancement in terms of cell proliferation could be achieved. The maximal obtained cell concentration was significantly increased as shown in Figure 13 A and within the maximal process parameter dot plot (Figure 14 A). Furthermore, an obvious separation regarding the total cell concentration profile after day 6 between cultivations set to level -1 and +1 can be seen in Figure 13 A. Although, a relatively high standard deviation between the center points can be seen within Figure 14 D and B, a significant maximal antibody titer outcome can be stated. Figure 13 D shows an improved antibody production due to higher numbers of viable cumulated cell days. Although, the antibody titer was improved comparable rather low in terms of a significance, no significant decline of the specific productivity over the process time course can be stated. Experiment #2, where only the amino acid stock solution was set to level +1, revealed the highest maximal antibody titer (162.85 µg/mL) at day 10 compared to all other experiments. Furthermore, Figure 14 A suggests a quadratic curvature correlation between the center point and the respective level settings between -1 and +1. However, this relation could not be stated within the applied statistical model.

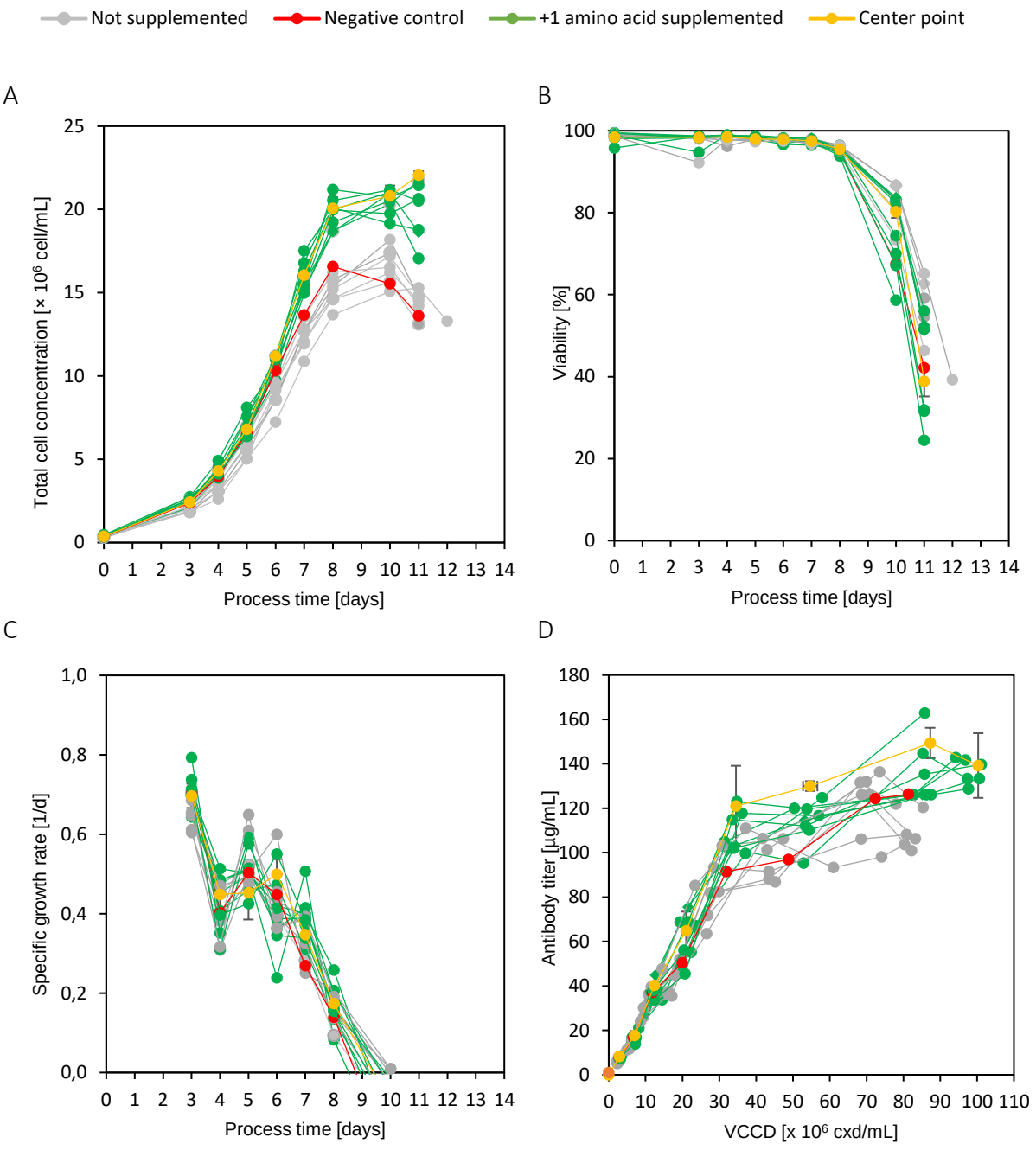


Figure 13. Overview of amino acid supplemented cell culture profiles of the full factorial screening experiment: (A) Total cell concentration, (B) viability, (C) viable specific growth rate, (D) antibody titer plotted over the course of the viable cumulative cell days.

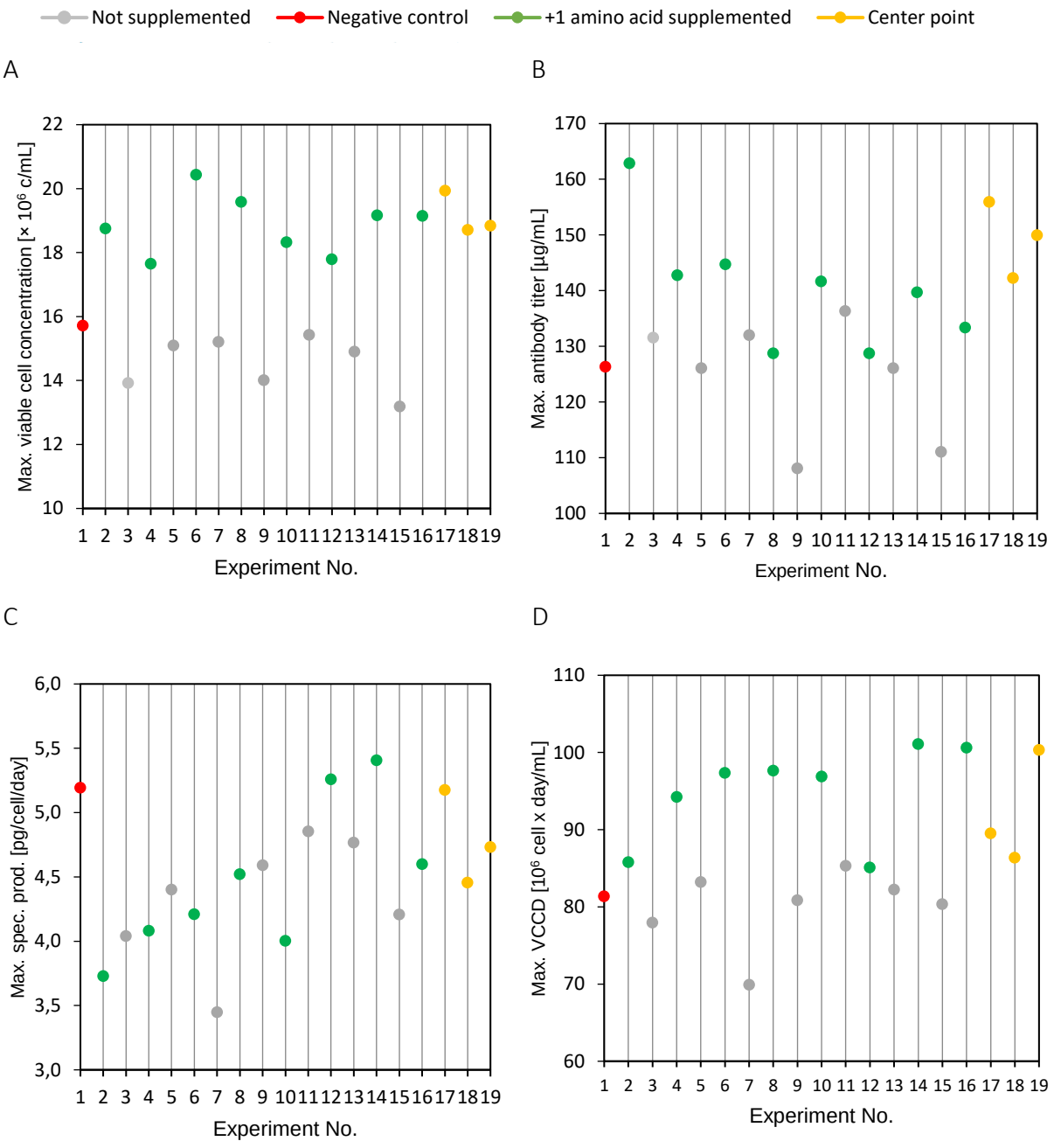


Figure 14. Obtained maximal cell performance parameter of amino acid supplemented cell culture represneted as a dot plot graphles: (A) maximal viable cell concentration, (B) maximal antibody titer, (C) maximal specfifc productivity, (D) maximal viable cumulative cell days.

4.2.2.4. *Pyruvate supplementation*

By identifying the cell performance of batch cultivations supplemented with the respective pyruvate solution, no significant impact on defined evaluation performance parameter could be obtained. No discrete trend can be stated out of the graphically illustrated cell parameter profiles in Figure 15 nor from the obtained maximal process parameter within the dot plot (Figure 16) due to a rather high distribution of the respective process parameter over the cultivation time course.

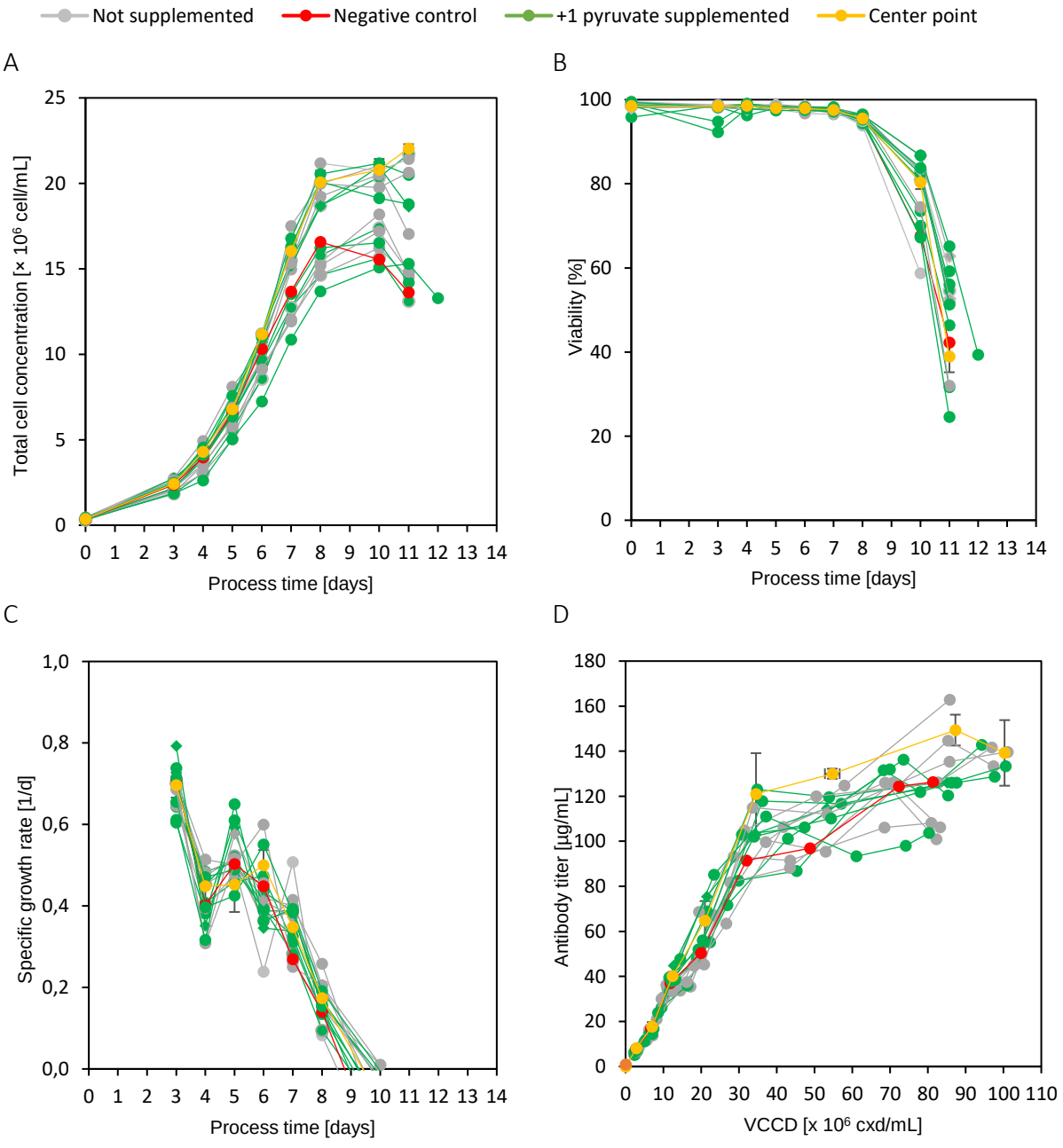


Figure 15. Overview of pyruvate supplemented cell culture profiles of the full factorial screening experiment: (A) Total cell concentration, (B) viability, (C) viable specific growth rate, (D) antibody titer plotted over the course of the viable cumulative cell days.

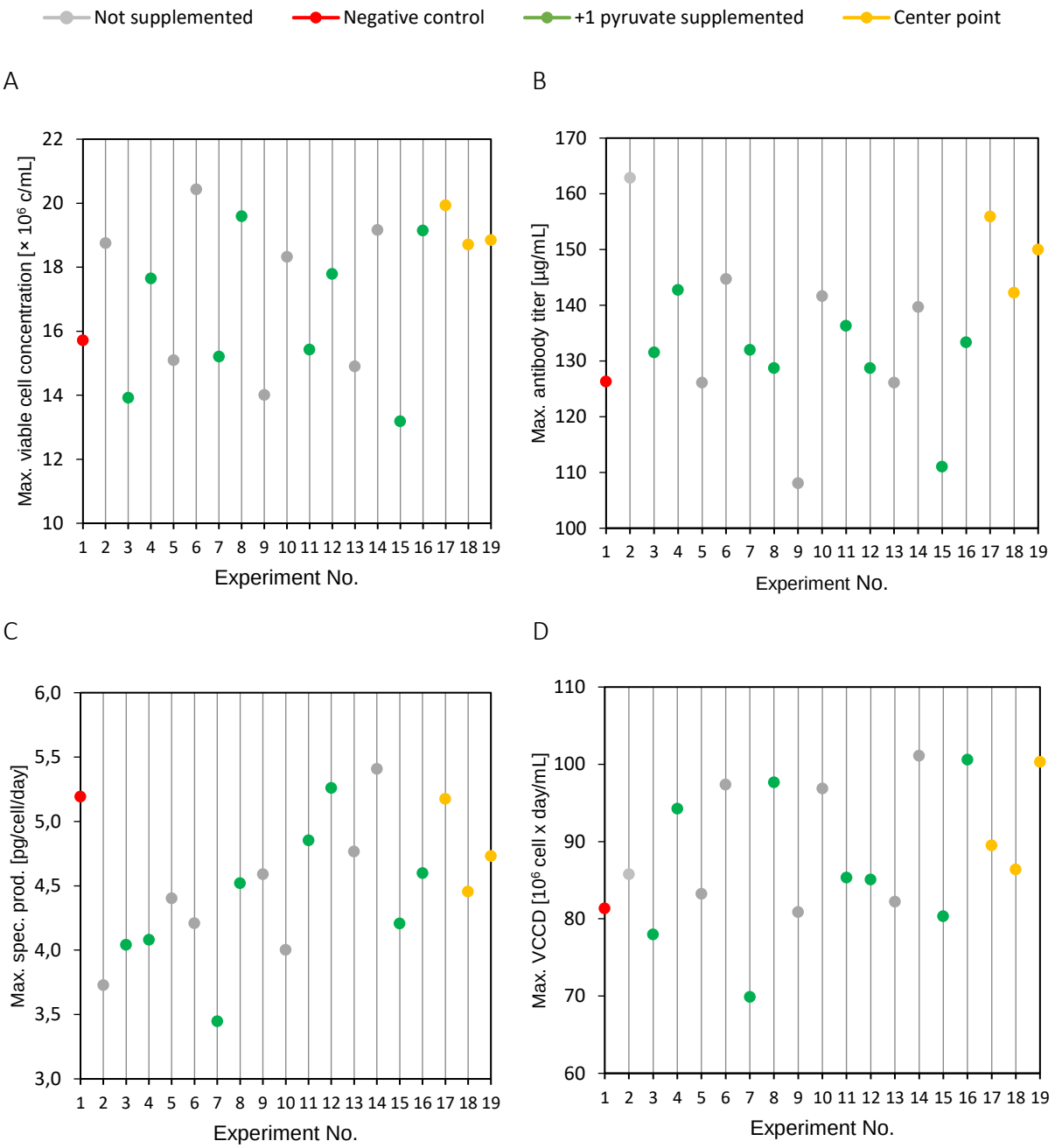


Figure 16. Obtained maximal cell performance parameter of pyruvate supplemented cell culture represneted as a dot plot graphles: (A) maximal viable cell concentration, (B) maximal antibody titer, (C) maximal specfifc productivity, (D) maximal viable cumulative cell days.

4.2.3. Statistical evaluation of the main effects

This experimental set up was conducted as an orthogonal design comprising all level combinations of the respective factors. By performing this 2^4 full factorial design space with a resolution of V, main effects and all factor interactions, like squared and cross over, are clear of each other. In other words, the responses can be stated as confounded and are not influenced in dependency of any other factor correlation or association. As responses the maximal viable cell concentration (mVCC), the maximal viable cumulative cell days (mVCCD), the obtained maximal antibody titer (mTiter) and maximal specific productivity were defined to evaluate the impact of the respective constituents on the cell performance. However, only two factors showed a significant impact on defined screening responses, namely amino acids and membrane precursor. Furthermore, supplementing the cultivations with the respective amino acid solution resulted in the only adequate model quality within the suggested threshold of R^2 and Q^2 for a proper evaluation of main effects.

The effect plots in Figure 17 of the maximal viable cell concentration ($R^2 = 0.76$; $Q^2 = 0.67$), the maximal viable cumulative cell days ($R^2 = 0.67$; $Q^2 = 0.57$) as well as the maximal antibody titer ($R^2 = 0.43$; $Q^2 = 0$) showed a significant positive impact when the respective batch cultivation is supplemented with amino acids. Unfortunately, due to a rather high standard deviation (Figure 17 B) within the mTiter values for the center point experiments, the model quality and prediction power is downgraded. Enhancement of mVCC and therefore a statistical correlation of mVCCD is not surprisingly as all cultivations showed a similar viability profile but still highlights the overall effect of amino acid supplementation as there are no main outliners within the experimental design space. Membrane precursor positively affected the maximal specific productivity, although the statistical model quality is quite low as both parameter ($R^2 = 0.21$; $Q^2 = 0$) did not reach the relevant threshold as suggested in the literature. No further significant impacts could be obtained regarding other screening parameters. Consequently, no significant factor interaction nor squared terms could be obtained according to the applied linear regression model.

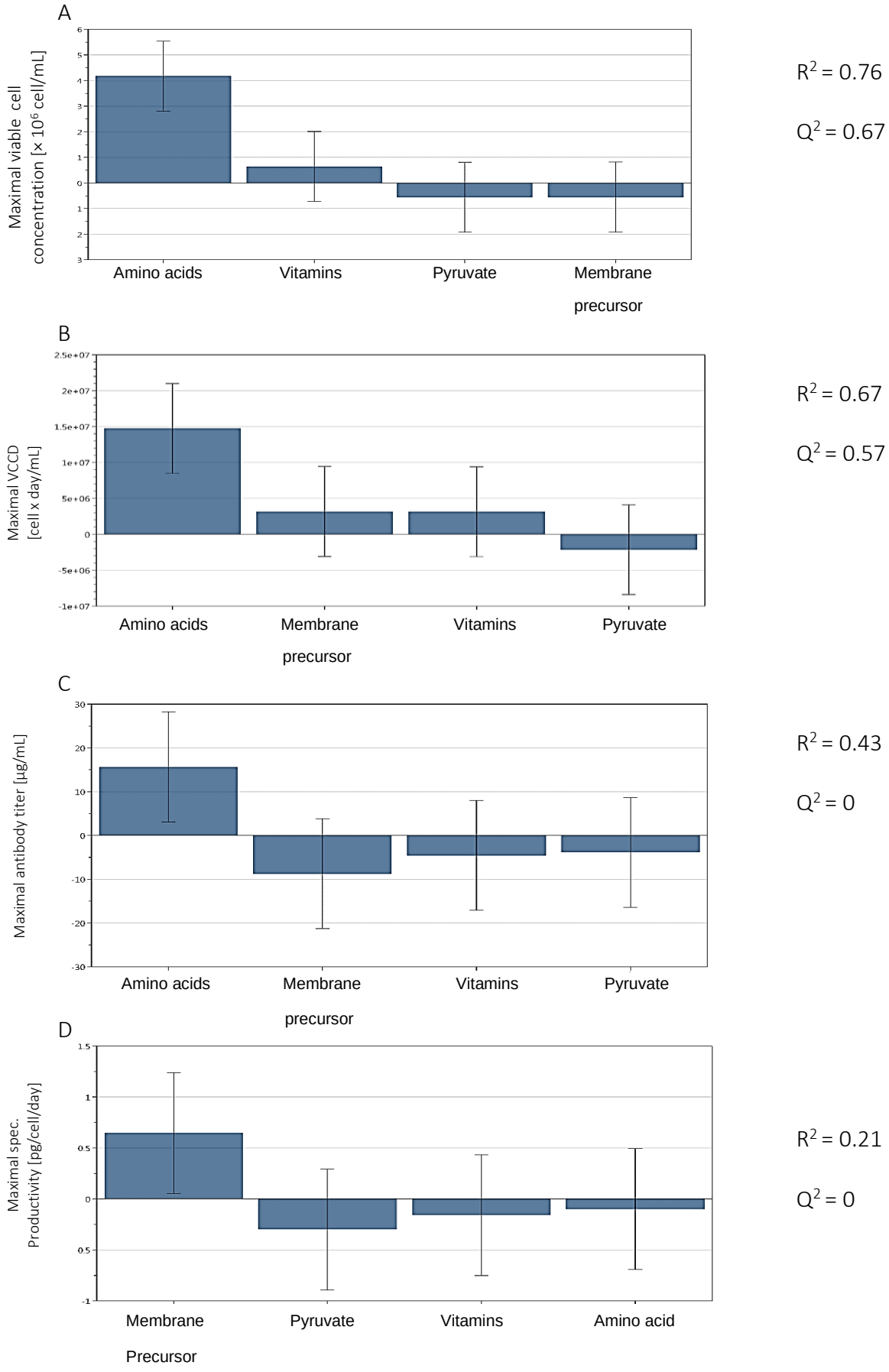


Figure 17. Effect plot of defined screening parameters obtained by linear regression analysis the full factorial screeningexperiment: (A) maximal viable cell concentration, (B) maximal viable cumulative cell days. (C) maximal antibody titer, (D) maximal specific productivity.

All obtained average values and corresponding standard deviations of the respective factor responses for the statistical evaluation are summarized in Table 16.

Table 16. Overview of observed factor responses including mean and standard deviation values of all +1 and -1 level.

			max. Viable cell concentration [x 10^6 c/mL]	Max. antibody titer [µg/mL]	µ (exp. phase) [1/d]	max. spec. Prod. [pg/cell/day]	max. VCCD [cell x day/mL]	Process duration [days]
Amino acid	- 1	Mean	14.68	124.68	0.48	4.44	8.01E+07	11
		Std. deviation	0.88	10.03	0.02	0.54	4.67E+06	0
	1	Mean	18.85	140.31	0.49	4.48	9.48E+07	11
		Std. deviation	0.93	11.04	0.02	0.60	6.19E+06	0
Pyruvate	- 1	Mean	17.05	134.44	0.48	4.54	8.86E+07	11
		Std. deviation	2.39	16.45	0.02	0.57	8.38E+06	1
	1	Mean	16.49	130.55	0.48	4.38	8.64E+07	11
		Std. deviation	2.39	9.11	0.02	0.56	1.05E+07	1
Vitamins	- 1	Mean	9.68	74.23	0.25	**4.47**	4.93E+07	11
		Std. deviation	9.29	72.97	0.25	**0.59**	4.68E+07	0
	1	Mean	11.14	86.56	0.30	**4.44**	5.69E+07	11
		Std. deviation	8.72	66.59	0.25	**0.56**	4.54E+07	1
Membrane precursor	- 1	Mean	8.84	71.51	0.25	**4.20**	4.65E+07	11
		Std. deviation	8.98	69.08	0.19	**0.53**	4.63E+07	1
	1	Mean	9.46	71.72	0.25	**4.71**	4.82E+07	11
		Std. deviation	5.76	49.63	0.19	**0.48**	3.10E+07	0
	0	Mean	19.16	149.38	0.49	4.79	9.21E+07	11
		Std. deviation	0.67	6.85	0.02	0.36	7.32E+06	0

In general, no significant negative main as well as interaction effect of any applied supplementation strategy could be obtained. Although Figure 8 shows that some cultivations performed slightly negative compared to the negative control, an overall positive effect on the cell performance within the triplicate center point experiments was seen. Consequently, it was suggested to develop a chemically defined in house supplementation media (SM) compiled out of the respective media constituents. Due to proprietary reasons, there is no concentration listed of the developed media formulation.

4.3. Principal component analysis following Raman spectroscopy to quantitatively analyze feed stock solutions within basal media

To evaluate the possibility to separately identify the presence of feed stock solutions within different supplemented basal media, Raman measurements were conducted to obtain spectra representing the different solutions. Since Raman spectra, obtained from similar specimen, are rather complex and can therefore not easily be distinguished from each other in terms of their profile, the spectra had to be analyzed by multivariate data analysis using principal component analysis (PCA) to determine variations and highlight strong patterns in the obtained dataset. Consequently, an orthogonal transformation of data points was applied to find variances, represented by predominant Raman peaks, via principal component analysis. Therefore, Raman measurements of the plain feed stock solutions as well as of the respective supplemented media (Section 4.2) was conducted to evaluate inter-correlating dependent variables between the obtained spectra.

4.3.1. Raman spectroscopy of representative solutions applied in the screening experimental design

To create a representative data library, Raman spectra of various combinatorial solution of basal media, feed stock solution and CellBoost 7a/b were generated. Firstly, spectroscopic measurements of plain feed stock solutions were performed to obtain a basis for subsequent principal component analysis. Additional to the all amino acids feed stock solution (amino acid solution A) a separate solution named amino acid solution B was included to the measurement. Each single sample was measured 2 times to remove possible cosmic ray distortion within the spectrum and 5 times at different position within the specimen to obtain representative data. Spectroscopic measurements, illustrated in Figure 18, revealed unique spectra for all observed feed stock solutions. However, these spectra were only measured to obtain characteristic pattern of the feed stock solution and were not utilized within the principal component analysis nor used for any further analysis methods. Due to better illustration, the spectra of the respective feed solutions in Figure 18 are separated with a shift of 5000 AU between each other.

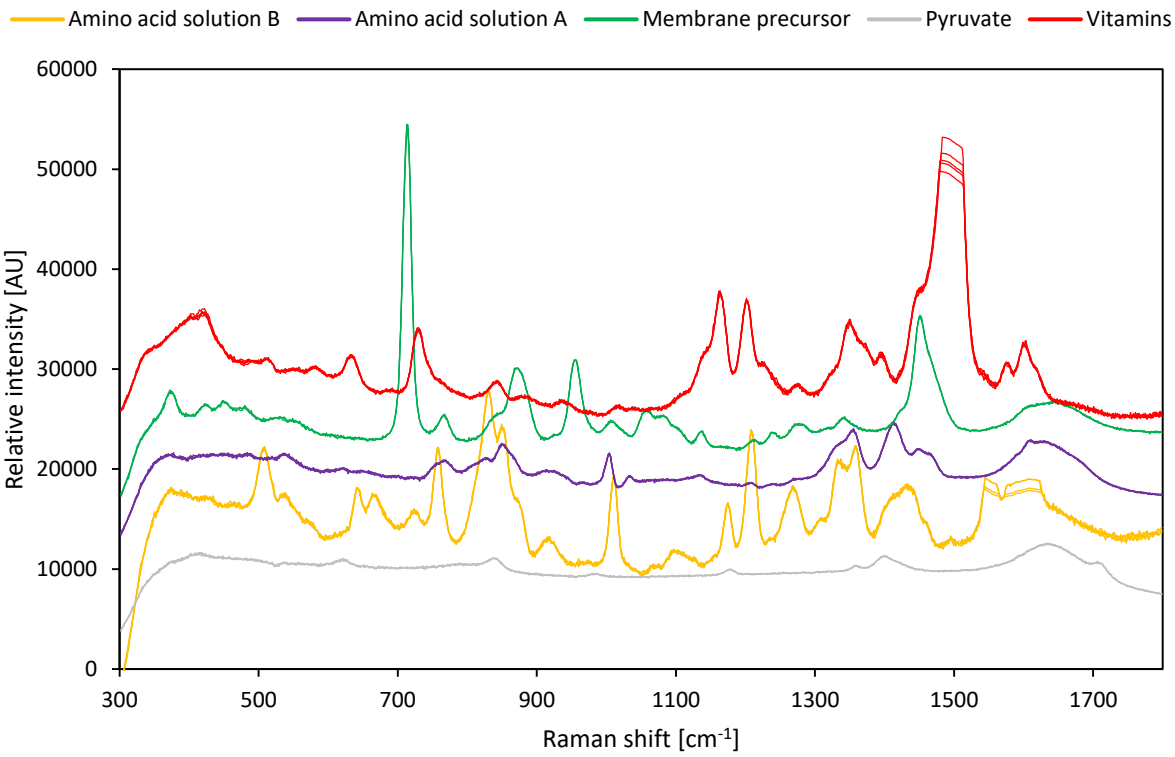


Figure 18. Obtained Raman spectra of applied feed stock solutions within the experimental screening design. An offset of 5000 AU was applied to the Y-Axis for better readability.

Subsequently, samples of all feed stock solution supplemented basal media combinations, according to the experimental design matrix, with addition to the CellBoost 7a/b supplemented basal media were measured using Raman spectroscopy. Obtained experiment (#1 to #19) spectra are illustrated in Figure 19 and colored to their respective setup level as mentioned in4.2.1. By observing the colored spectra according to their supplementation level, only one apparent common profile correlating with the level set up of vitamin feed stock solution can be seen in Figure 19 A at approx. 1500 cm^{-1}. Furthermore, a smaller peak at the exact same Raman shift can be seen within all center point profiles in Figure 19, which contain half of the vitamin concentration compared to a +1 level setting. However, no further unique profile characteristics correlating with any other level setting can be stated. Due to better illustration, the spectra of the respective media combinations in Figure 19 are separated with a shift of 1000 AU between each other.

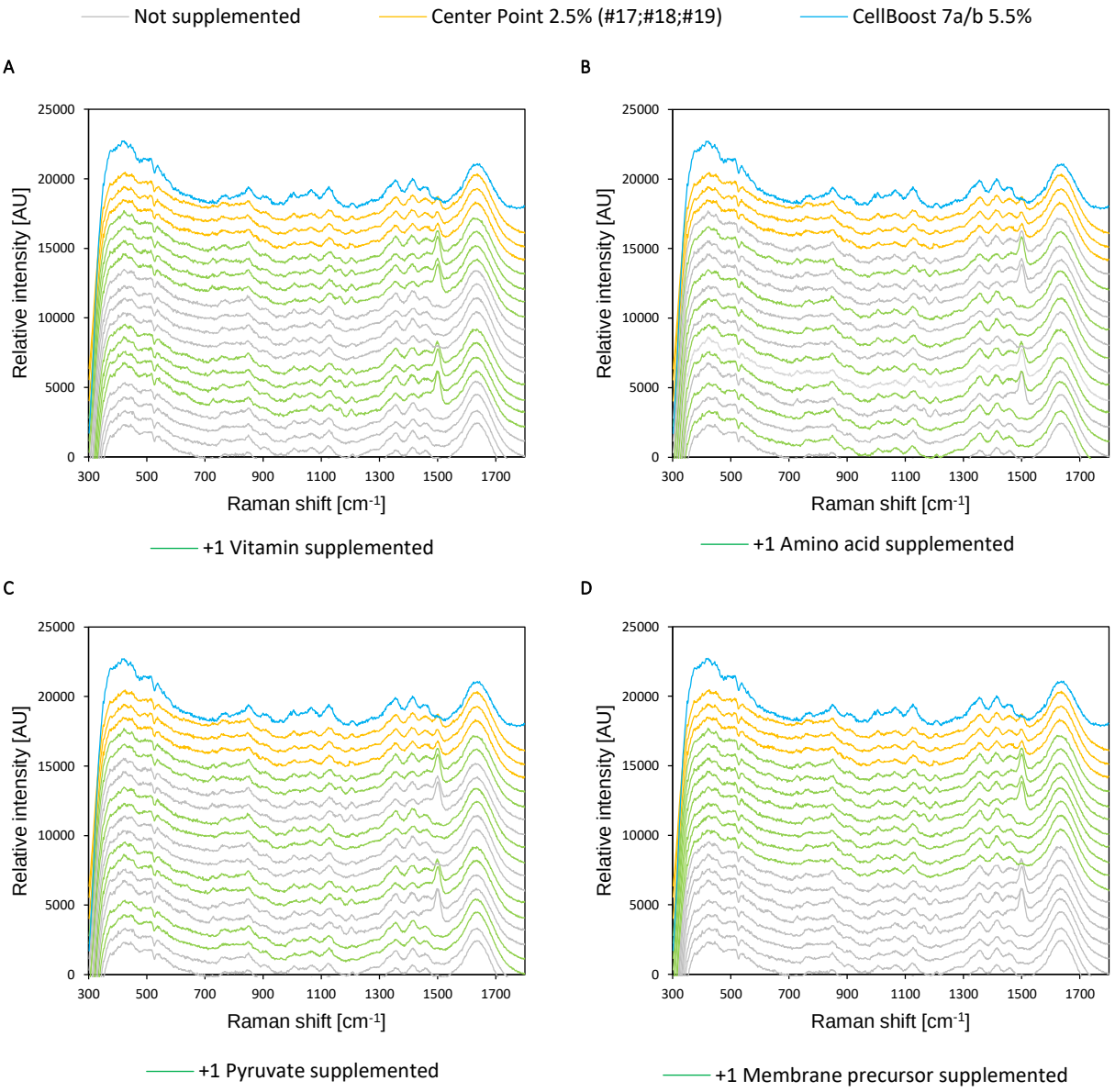


Figure 19. Obtained Raman spectra of supplemented basal media combination within the experimental screening design. An offset of 1000 AU between each experiemnt was applied to the Y-Axis for better readability.

4.3.2. Entity observation of basal media supplemented with feed stock solutions via principal component analysis (PCA)

To investigate unique profile characteristics within the obtained Raman spectra and data sets, an orthogonal linear transformation was conducted to convert main similarities within the data points into normalized component scores. Application of this exploratory data analysis can be used to compile defined evaluation parameters into fitted ellipsoids based on their eigenvalue decomposition. Figure 20 shows the application of a principal component analysis according to the component score of each single Raman measurements of the respective experiment within the experimental design. As seen within the data point deviation in Figure 20, no clear trend and separated clusters between the obtained proportions of eigenvalues can be stated for all measurements. Only for experiment #5, which was only supplemented with the vitamin feed stock solution, a clear separation in terms of variance can be seen between PC 2 (10%) and PC1 (51%).

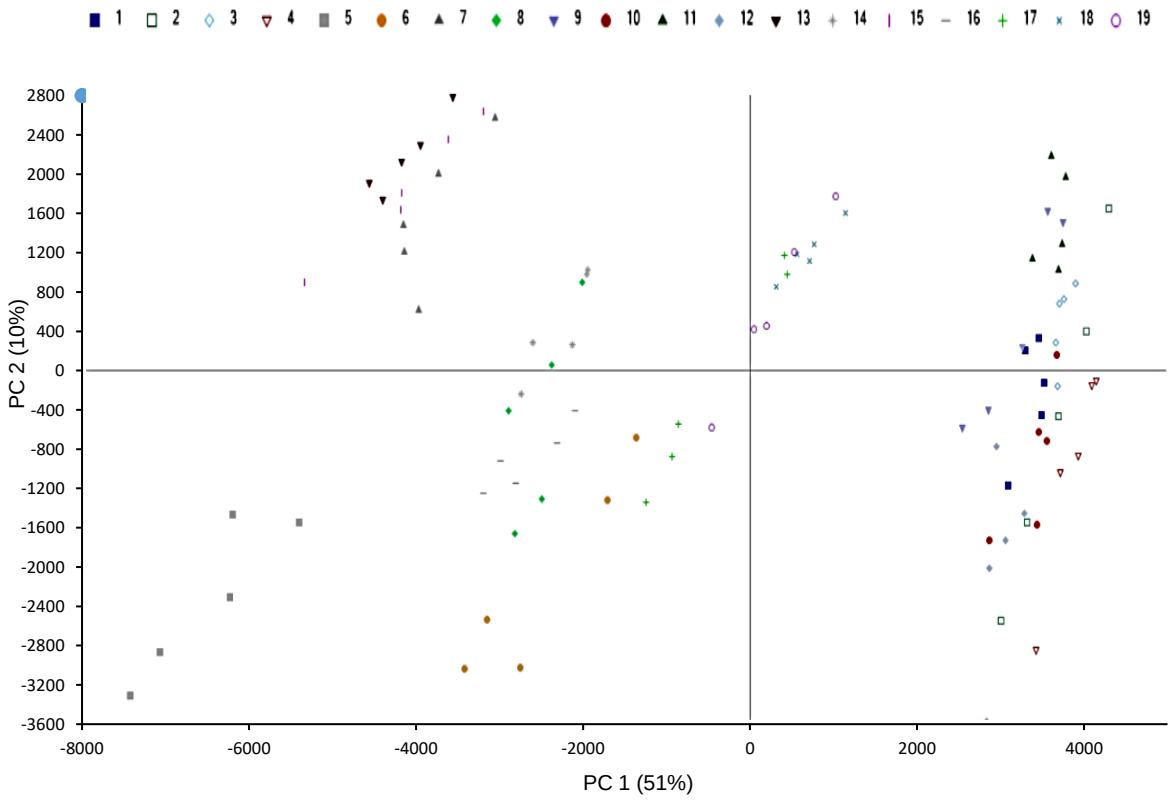


Figure 20. Principl component analysis of all media combinations perfromed within the experimental screening design.

By defining new classification of the transformation, a clustering of principal components could be found within amino acid supplemented solutions. Although PC1 describes 51% of the data variance, only a partial separation of the respective data points can be seen in Figure 21. The overlay of the ellipsoids revealed that the whole data set cannot be completely described by plotting PC 2 (10%) against PC 1 (51%) and PC 3 (4%), respectively.

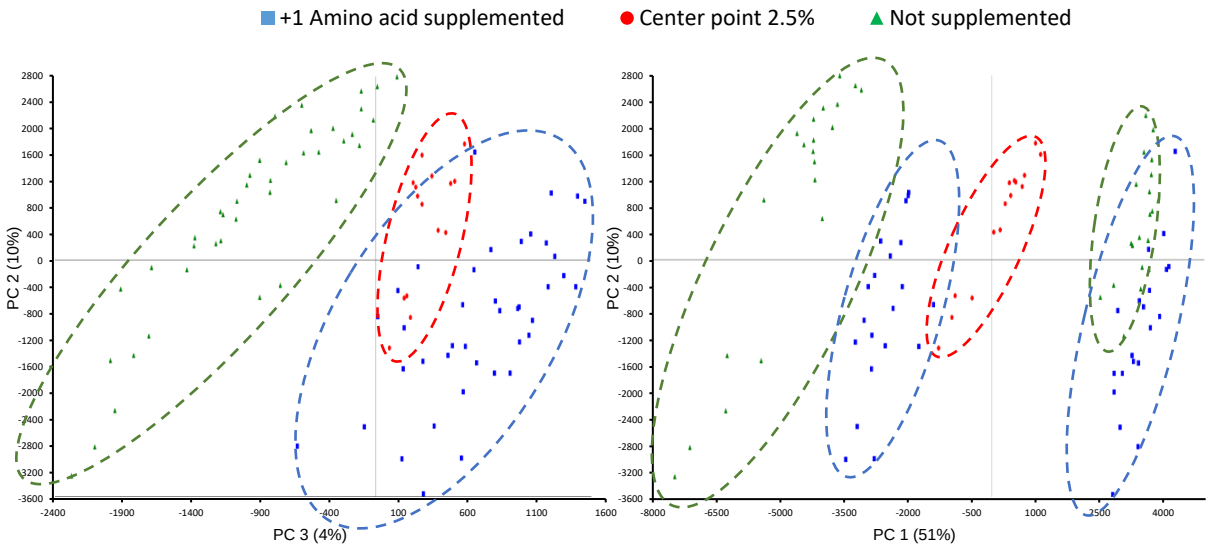


Figure 21. PCA of amino acid supplemented screening experiments. Ellipsoids are the respective clusters according to the level setting.

Only a complete separation of the data points was observed when the determined principal components were evaluated in regard to vitamin supplemented solutions (Figure 22). By plotting the PC 2 (10%) against PC1 (51%), a clear qualitative and quantitative separation between the respective level settings of vitamin supplemented experiments within the experimental design could be stated.

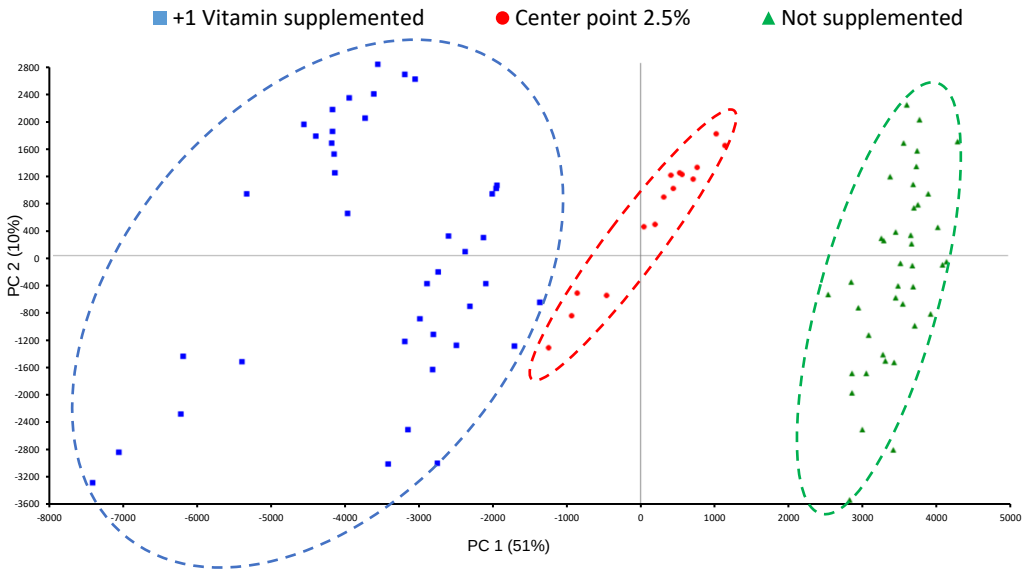


Figure 22. PCA of vitamin supplemented screening experiments. Ellipsoids are representing the of the resepctive clusters according to the level setting.

4.4. Comparison of the in-house developed supplementation media to commercially available media supplements

General chemically defined media formulation, prior to inoculation supplementation approaches as well as subsequent feeding strategies of the respective formulation are major obstacles to provide high yields of recombinant proteins in cell culture processes. Beside bioprocess considerations like stirrer speed, different vessel and stirrer designs, agitation techniques or temperature and pH shifts etc., challenges regarding the cell culture media are derived mainly from two factors. One the one hand, different cell lines must be provided with different growth and production correlating constituents over the whole process time course. On the other hand, there is a lack of knowledge regarding the comprised media constituents and their particular composition within commercially available media. Therefore, finding the optimal media formulation as well as supplementation strategy to ensure an optimal cell performance is often laborious, time consuming as well as critical in terms of scientific research. Thus, the aim of this experiment was to compare in house developed supplement media (SM) to a commercially available media (ActiPro^{TM}) with and without their related commercial supplement formulations, namely HyClone CellBoost 7a and CellBoost 7b. Furthermore, it was suggested to perform batch experiments prior to fed-batch processes due to their relatively simple set up and are therefore less error prone.

4.4.1. Experimental set-up

The within this experiment applied in-house supplement media (SM) was developed based on the outcome from the first full factorial screening experiment, which is also comprised out of two separate formulations. As the optimal applied working concentration of the in-house formulations were unknown regarding the given cell line performance, three different working concentrations were suggested which are summarized in Table 17. The overall composition of both supplementation media is stated in Table 18 and Table 19. All experiments were performed as duplicates.

Table 17. Experimental setup: Comparison of the in-house developed supplementation media to commercially available mediasupplements

Process type	Batch mode
Cell line	CHO DG44/PG9
Basal media	ActiPro^{TM} + 8mM L-Gln
Media Supplements	HyClone CellBoost 7a (5%) HyClone CellBoost 7b (0.5%) Feed supplement media A (5%;7%:9%) Feed supplement media B (0.5%;0.7%:0.9%)
Working volume	30 mL
Supplementation strategy	Prior to inoculation
Seeding (total)	0.3×10^6 cells/mL (9×10^6 cells)
Reactor	50 mL reactor tubes with venting membrane
Incubation temperature	37.0°C
Humidity	80%
CO_2 level	7.0%
Rotation shaker speed	220 rpm
Termination criterion	Viability < 60%

The general formulation of feed supplement media A was composed out of five different media component classes, which were based on the previous performed experiment in section 4.2. In contrast to formulation A, the supplementation media B was composed out of one single substance class. These defined classes, representing essential media constitutes generally found in commercially available media, are either comprised out of several or out of one single essential substance. The overall composition of the respective classes for supplementation media A and B are summarized in Table 18 and Table 19, respectively.

Table 18. Overview of the 5 substance classes and their respective composition of feed supplement A

1 Amino acid solution A	Comprised out of 15 selected essential amino acids
2 Membrane precursor	Comprised out of 4 selected membrane precursors
3 Vitamins	Comprised out of 9 selected vitamins
4 Pyruvate	
5 Glucose	

Table 19. Overview of the substance class and the respective composition of feed supplement media B.

Amino acid solution B	Comprised out of additional amino acids readily soluble at alkaline pH

4.4.2. Performance of clone CHO DG44/PG9 in differently supplemented basal media

Based on the obtained data out of the full factorial screening design, as described in in section4.2, a subsequent development of in-house supplementation media was conducted. This media, consisting out of two separate formulations, were tested against plain ActiPro™ and ActiPro™ supplemented with a working concentration of 5% HyClone CellBoost 7a and 0.5% HyClone CellBoost 7b (CB 7a/b 5.5%). Furthermore, the basal media was supplemented with a combinatorial working concentration of 5.5%, 7.7% and 9.9% with supplementation media A and B (SM 5.5%, SM 7.7%, SM 9.9%,). The maximal obtained values are summarized inTable 20.

Table 20. Overview of obtained relevant cell performance parameter.

	max. total cell concentration [× 10^6 c/mL]	max. viable cell concentration [× 10^6 c/mL]	Max. antibody titer [µg/mL]	µ (exp. phase) [1/d]	max. spec. Prod. [pg/cell/day]	max. VCCD [cell × day/mL]	Process duration [days]
Control 0%	17.97	16.89	113.40	**0.54**	2.71	7.58E+07	10
CB 7a/b 5.5%	13.78	12.05	137.60	0.46	3.11	7.05E+07	11
SM 5.5%	**18.95**	**17.80**	144.55	0.53	2.94	9.84E+07	11
SM 7.7%	18.04	16.79	**146.40**	0.51	**3.23**	9.41E+07	**12**
SM 9.9%	15.12	13.75	140.70	0.46	3.22	8.07E+07	**12**

Monitoring of the obtained cell performance outcomes in Table 20 and in Figure 23, revealed an overall significant impact of the basal media modification approaches. A maximal total cell concentration of 18.95 × 10^6 cells/mL could be achieved using a 5.5% SM supplementation strategy on the 8^{th} and 10^{th} day compared to the plain basal media were the total cell concentration of 17.97 × 10^6 cells/mL peaked after 9 days of cultivation. The highest titer accumulation of 146.40 µg/mL was shown within the 7.7% SM supplemented experiment at day 12 which was slightly higher when compared to the cultivations supplemented with 5.5% SM (144.55 µg/mL) at day 10 and 9.9% SM (140.70 µg/mL) at day 12. Antibody titer analysis of plain ActiPro™ revealed the lowest accumulation of 113.40 µg/mL at day 10. CellBoost 7a/b

showed the lowest overall cell concentration with a maximal total cell concentration of 13.78 × 10^6 cells/mL cells/mL at day 10 but still outperformed plain ActiPro™ in terms of antibody production (137.60 µg/mL) at day 12. Consequently, a higher specific productivity of CB 5.5% over the process time course compared to the other competitors could be revealed (Figure 23 E). However, each cultivation which got supplemented either with the in-house developed media or the commercially available CellBoost 7a/b outperformed plain ActiPro™ as shown in Figure 23 F. Regarding the cell viability, a prolonged cultivation process time of 12 days (SM 7.7% and SM 9.9%) and 11 days (SM 5.5% and CB 7a/b) before undergoing the viability threshold of 60% in comparison to 10 days of cultivation in ActiPro™ was revealed.

Although cultivations with SM 5.5% and SM 7.7% resulted in significantly improved higher maximal cell concentrations, antibody titers of cultivations which got supplemented with either CB 7a/b 5.5% or SM 9.9% were within a marginal lower range. These outcomes are highlighting the typically desired effect of media supplementation strategies, which mostly aims to achieve high yields of protein while keeping the cell proliferation rate at reasonable levels. By supplementing the commercial ActiPro™ media with a working concentration of 9.9% of the respective developed supplementation formulations, this desired profile could be achieved. However, highest antibody titer within this experimental set-up correlated with higher cell concentrations using the in-house formulation. Obtained cell performance profiles are summarized in Figure 23.

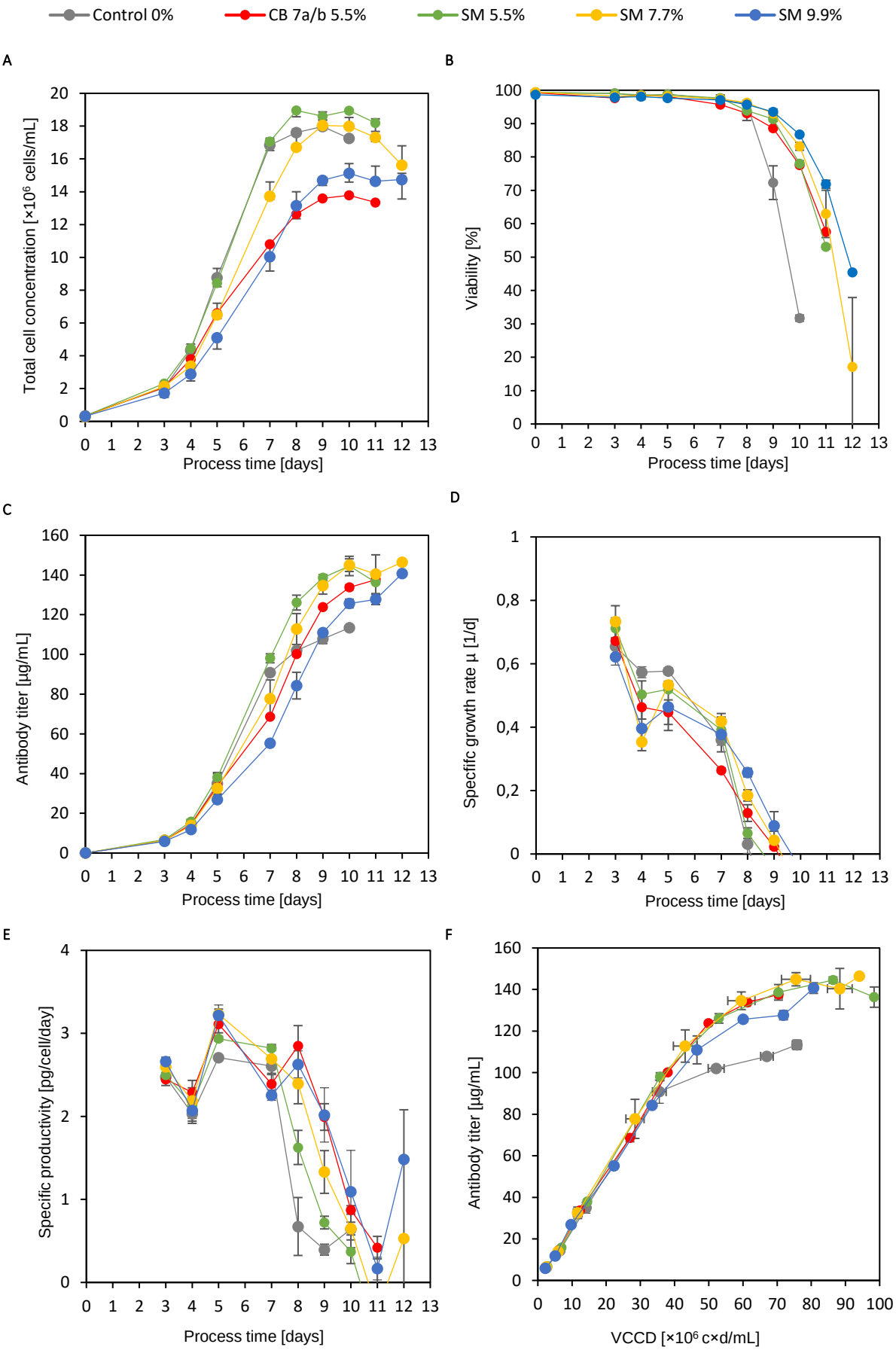


Figure 23. Obtained cell culture profiles of in-house SM supplemented cultivation with different working concentration, CellBoost 7a7b supplemented cultivations and plain ActiPro™ batch experiments : (A) Total cell concentration, (B) viability, (C) antibody titer, (D) viable specific growth rate, (E) specific productivity, (F) antibody titer plotted over the course of the viable cumulative cell days. Respective experiments were supplemented prior to inoculation.

In addition to the already described cell performance parameter, the osmolality profile of the respective cultivations was observed which is represented over the time course in Figure 23. Furthermore, the average cell diameter was also taken into account (Figure 25) to determine whether a direct influence of the osmolality regarding the cell size can be stated or not.

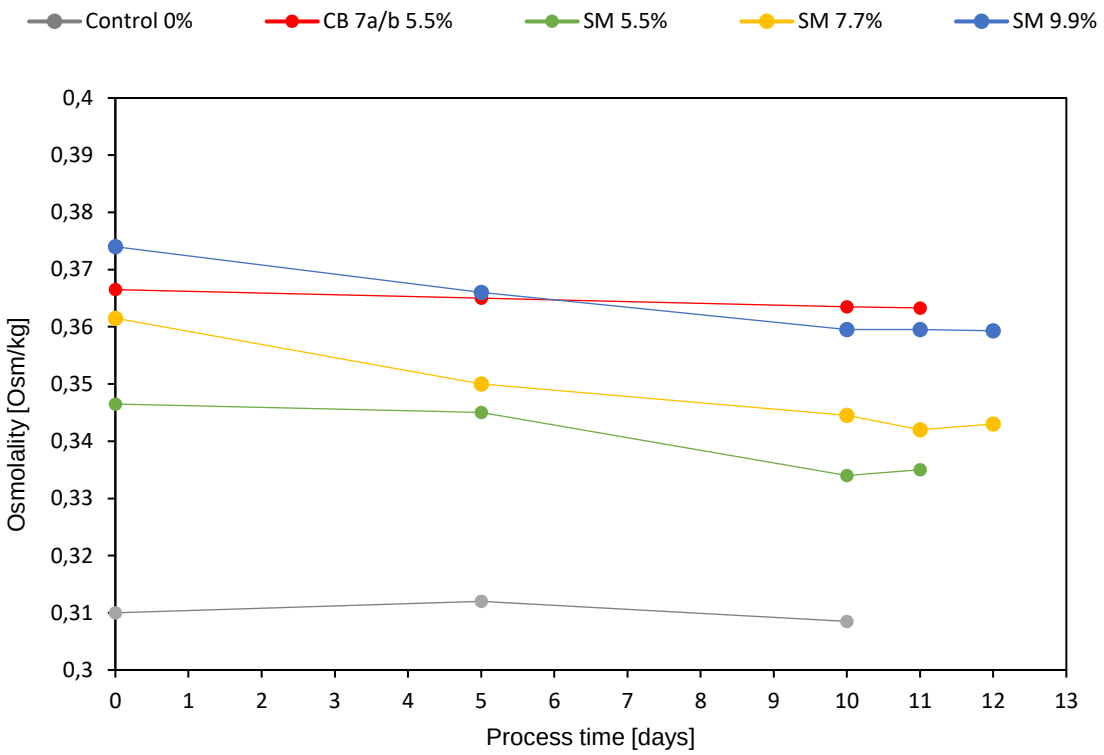


Figure 24. Osmolality effect of tested supplementation media and plain basal media over the process time course.

A direct comparison between the supplemented cultures in terms of the average cell diameter, revealed an overall similar profile trend over the process time course as shown in Figure 25. This trend, seen within all cultivations, can be described as a slight diameter increase ranging between 13.25 µm and 13.49 µm at day 0 to 13.81µm and 14.27µm till day 5. After the 5th day of inoculation the diameter profiles start to separate into two sections, those are cultivations where ActiPro^{TM} got modified and the one using only plain ActiPro^{TM} as a media. Supplemented experiments continue with an overall marginal diameter increase within a small range of 14.87 µm and 14.90 µm till day 9. In contrast to the cell diameter within ActPro^{TM}, which starts to significantly decline over the time course between day 5 (13.81 µm) and day 10 (11.70 µm). After the 9th day of inoculation, the SM 5.5% profile starts to decrease until a cell diameter reduction to 12.68 µm could be observed till day 11. Although there can be a significant

osmolality difference between SM 9.9% and CB 7a/b 5.5% to SM 7.7% stated, cell diameter profiles of these experiments followed an almost identical trend till the termination criteria was reached which can be seen at day 11 and day 12, respectively, in Figure 25.

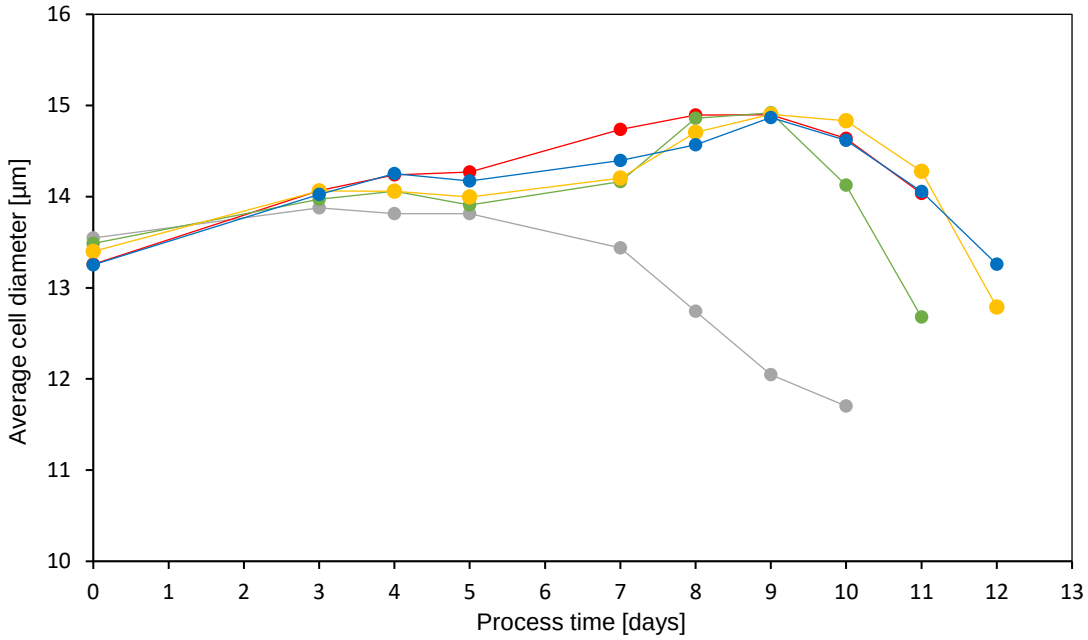


Figure 25. Effect of tested supplementation media and plain basal media on the average cell diamter over the process time course.

Summing up, a general impact of the cell culture media osmolality on cell diameter could be observed but the difference within the supplemented experiments can not only be explained by a change in osmolality. Consequently, it was assumed that either a higher constituent concentration, derived from a higher working concentration within SM 9.9%, or a higher osmolality, derived from other supplement constituents, is in direct correlation to obtain a cell performance seen in SB 9.9% and CB 7a/b 5.5%.

4.5. First crossover comparison of the original and trace element supplemented in-house supplement media against to commercially available media supplements

Based on the outcome under section 3.3., which revealed that higher working concentrations and the respective correlating higher osmolalities within a supplemented basal media seem beneficial in terms of an improved cell productivity, a new supplementation approach was proposed. As seen in Figure 26, a working concentration of 5.5% revealed the highest cell concentration whereas a supplementation ratio of 9.9% revealed the highest overall specific productivity when the basal in-house supplementation media was applied. These experiments were compared to the observed experimental result where HyClone CellBoost 7a/b was supplemented at 5.5% of the working volume (Figure 26). In that case it could be stated that, with an equal working concentration a significant decrease of the total cell concentration was revealed. Furthermore, a higher osmolality and higher antibody titer was determined compared to the higher working concentration within the SM 9.9% batch cultivation.

To obtain information whether a higher constituent concentration or a higher osmolality, derived from additional media constituents enhance the cell performance, an altered composition of the original in-house supplementation media was developed. Within the original formulation of the developed supplementation media, only 5 defined constituent classes were considered. Therefore, an additional class of essential media constituents was implemented in addition to the initial formulation, namely, trace elements.

4.5.1. Experimental set up

As previously described, a supplementation media was developed out of the full factorial experimental design approach. Within this experiment an additional class, generally described as trace elements, was supplemented to the initial supplementation media formulation to further characterize effects of major components found in chemically defined media. Based on the outcome described above, a crossover comparison between the in-house supplementation media either with (SMTE) or without (SM) trace elements as well as to CellBoost 7a/b supplements with same working concentration was conducted. Supplementation of the respective formulations prior to inoculation as well as the general batch cultivation was performed as previously described in section 3.2.5. The overall experimental set up is summarized in Table 21.

Table 21. Experimental setup: Crossover comparison of the original and altered formulation of the in-house supplementation media against to commercially available media supplements.

Operation mode	Batch
Cell line	CHO DG44/PG9
Basal media	ActiPro™ + 8mM L-Gln
Media Supplements	HyClone CellBoost 7a (5%; 9%) HyClone CellBoost 7b (0.5% 0.9%) Supplement media A (5%:9%) Supplement media A + trace elements (5%; 9%) Supplement media B (0.5%; 0.9%)
Working volume	30 mL
Supplementation strategy	Prior to inoculation
Seeding (total)	0.3×10^6 cells/mL (9×10^6 cells)
Reactor	50 mL reactor tubes with venting membrane
Incubation temperature	37.0°C
Humidity	80%
CO_2 level	7.0%
Rotation shaker speed	220 rpm
Termination criterion	Viability < 60%

Overall constituent formulation of the newly adapted supplementation media A with the additional trace elements is shown in Table 22. Supplementation media B within the SMTE was not adapted. The formulation of both initial formulations used in the previous section was unchanged (Table 18).

Table 22. Overview of the altered supplementation meda A including an addiotonal substance class

1 Amino acids	Comprised out of 15 selected amino acids
2 Membrane precursor	Comprised out of 4 selected membrane precursor
3 Vitamins	Comprised out of 9 selected vitamins
4 Pyruvate	
5 Glucose	
6 Trace elements	Comprised out of 9 trace elements

4.5.2. Experimental results

Adding various trace elements to the supplementation media showed an overall decrease in cell growth and product formation when compared to the same working concentration of the original in-house formulation without additional trace elements (Figure 26). Furthermore, a significant increase of osmolality within cell culture media compared to those with no additional trace element could be observed (Figure 26).

The graph in Figure 26 A shows the highest overall cell concentration of 17.02 × 10^6 cells/mL at day 9 with SM 5.5% compared to 15.60 × 10^6 cells/mL at day 10 with additional trace elements (SMTE) and an equal working concentration. Therefore, an appx. 10% higher total cell concentration compared to the control experiment (15.39 × 10^6 cells/mL) at day 8 and even a 47% increase compared to CB 5.5% where the total cell concentration (11.61 × 10^6 cells/mL) peaked at day 9 could be achieved. Again, highest antibody titer of 130.60 µg/mL could be obtained with SM 5.5%, although SMTE 5.5% also peaked at day 11 with an accumulated antibody titer of 128.45 µg/mL. The same working concentration of 5.5% CB 7a/b revealed a maximal antibody titer of 113.75 µg/mL after the 11^{th} day of inoculation. The plain basal media (control 0%) showed a further decrease of product formation (105.70 µg/mL) which peaked after 10 days of inoculation. The overall longest cultivation time of 13 days before undergoing a viability threshold of 60% was shown in SM 9.9% whereas CB 9.9% and the control experiment dropped below the termination criterion already at day 10 (Figure 26 B). Interestingly, addition of 9.9% CB 7a/b revealed an extremely weak cell proliferation and viability profile as shown in Figure 26 A and B. In general, it can be stated that within this experiment a working concentration of 9.9%, all applied supplements revealed a decreased cell performance compared to a 5.5% supplementation ratio of the working volume (Figure 26). However, an overall increased specific productivity over the time course after day 8 was determined (Figure 26 E). Especially, the specific productivities profiles of SM 9.9% and SMTE 9.9% illustrate an untypical stabilization behavior between day 7 and 9. Maximal obtained values are summarized in Table 23.

Table 23. Overview of obtained relevant cell perfromance parameter.

	max. total cell concentration [× 10^6 c/mL]	max. viable cell concentration [× 10^6 c/mL]	Max. antibody titer [µg/mL]	µ (exp. phase) [1/d]	max. spec. Prod. [pg/cell/day]	max. VCCD [cell x day/mL]	Process duration [days]
Control 0%	15.39	14.87	105.70	0.50	2.84	7.05E+07	10
CB 5.5%	11.61	10.16	113.75	0.43	3.72	5.98E+07	11
CB 9.9%	3.60	2.96	35.25	0.26	2.77	1.85E+07	10
SM 5.5%	**17.02**	**15.61**	**130.60**	**0.52**	**4.34**	**8.72E+07**	11
SM 9.9%	11.84	10.47	106.75	0.46	4.19	6.63E+07	**13**
SMTE 9.9%	10.09	8.76	98.65	0.44	3.70	5.41E+07	12
SMTE 5.5%	15.60	14.27	128.45	**0.52**	**4.34**	8.17E+07	11

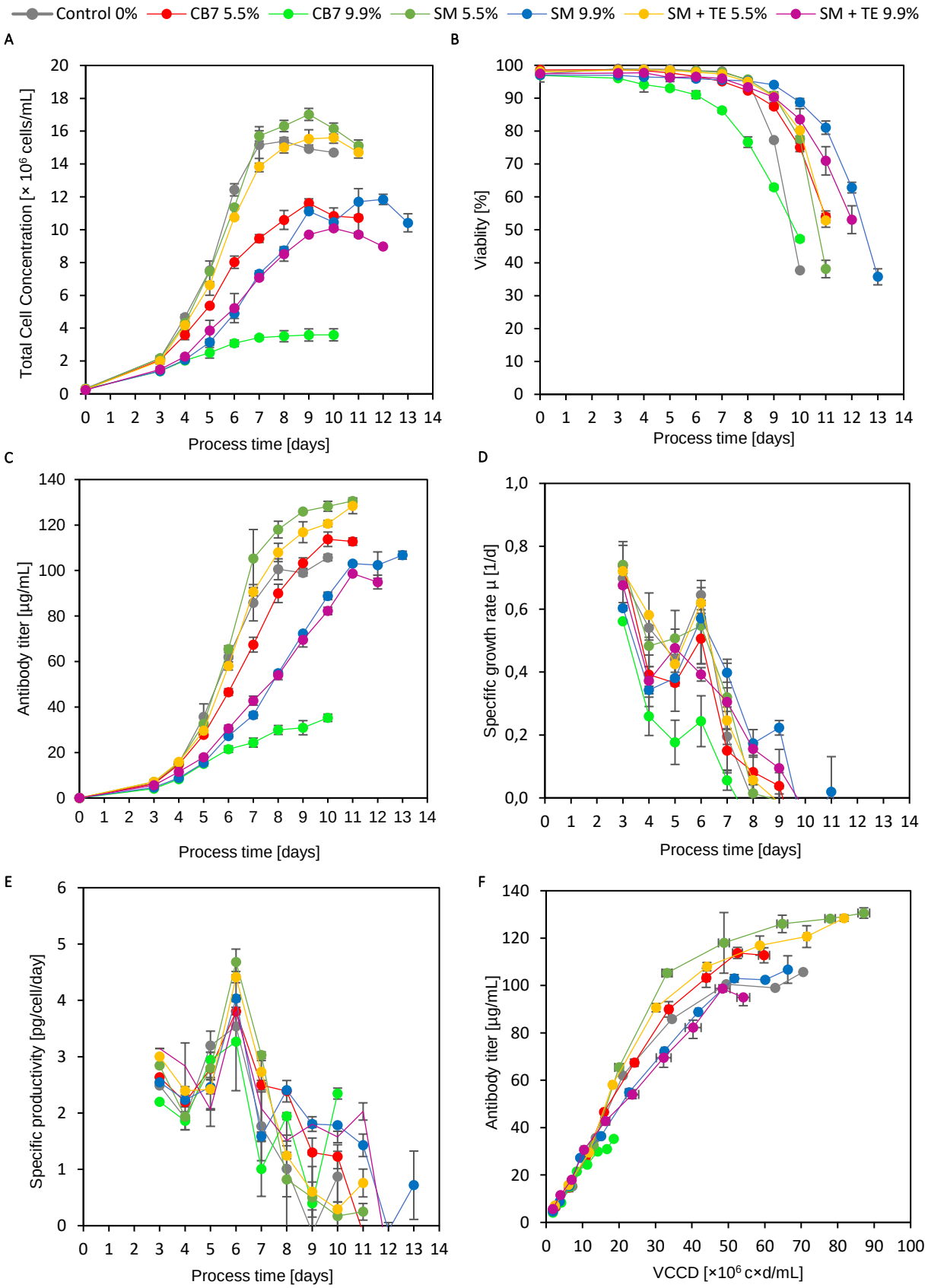


Figure 26. Obtained cell culture profiles of in-house media supplemented cultivation with different working concentration, CellBoost 7a7b supplemented cultivations and plain ActiPro™ batch experiments : (A) Total cell concentration, (B) viability, (C) antibody titer, (D) viable specific growth rate, (E) specific productivity, (F) antibody titer plotted over the course of the viable cumulative cell days. Respective experiments were supplemented prior to inoculation.

A clear separation between osmolality profiles of the respective cultivations is highlighted over the process time course in Figure 32. Although a slight aberration in the previously observed osmolality profiles in **Figure 24** can be seen within the already performed experiments (Control 0%, CB 7a/b 5.5%, SM 5.5% and SM 9.9%), the overall outcome stayed identical. Addition of trace elements showed significantly higher osmolalities for SMTE 5.5% (0.348 Osm/kg ± 0.002) and SMTE 9.9% (0.387 Osm/kg ± 0.006) when compared to SM5.5% (0.335 Osm/kg ± 0.002) and SM9.9% (0.372 Osm/kg ± 0.006). Overall higher osmolalities within CellBoost 7a/b supplemented cultivation media were obtained to those supplemented with the in-house developed media. CB 7a/b 9.9% revealed the highest osmolality in contrast to plain ActiPro™ (0.303 Osm/kg ± 0.003) over the process time course (0.387 Osm/kg ± 0.011).

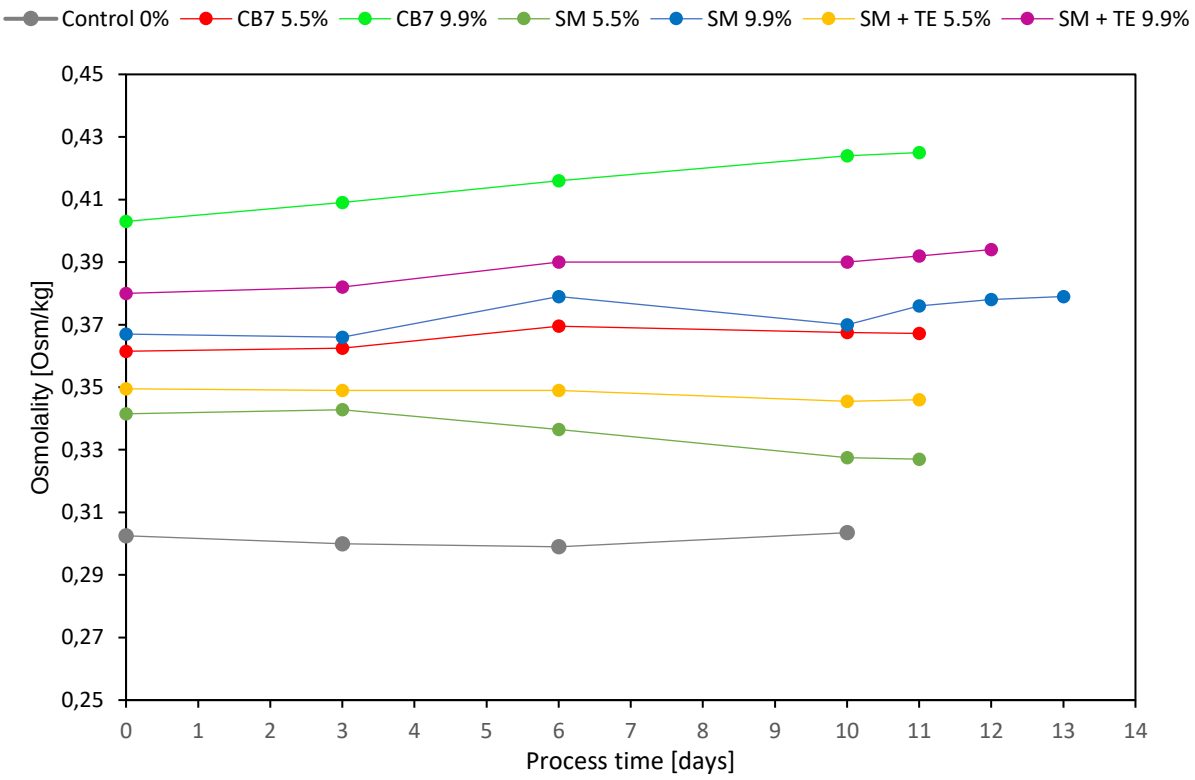


Figure 27. Osmolality effect of original and altered formulation, CB 7a/b supplement and plain basal media over the process time course.

Except of the cultivation supplemented with 9.9% CellBoost 7a/b of the working concentration, observation of the average cell diameter profiles (Figure 28) only showed slightly higher separation in respect to the supplementation effect compared to the obtained time profiles illustrated in the previous section 4.4 (Figure 25). The light green curves (CB 7a/b 9.9%) in Figure **28** are indicating an average cell diameter increase from 13.33 µm at the day of inoculation up

to 15.51 µm within 7 days of cultivation, accompanied by a comparable diameter reduction profile to the other experiments. Comparing this result to other obtained cell average in regard to respective media osmolality, a general correlation effect of higher osmolarities resulting in higher cell diameters can be stated.

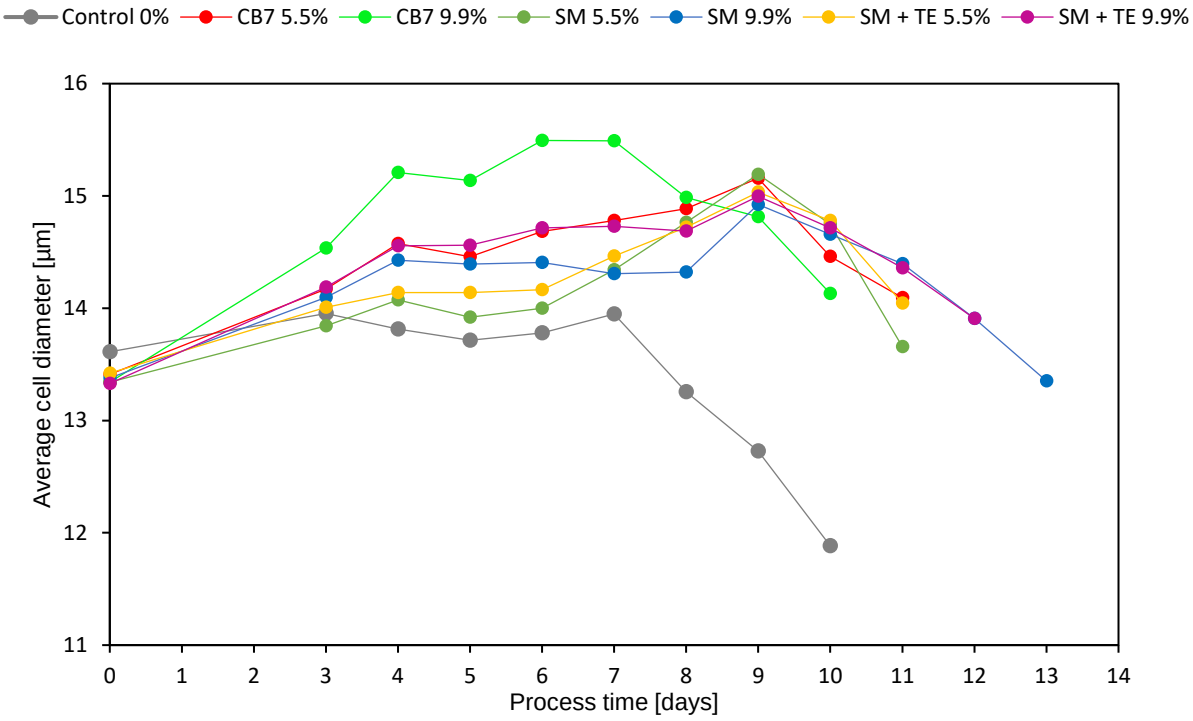


Figure 28. Effect of original and altered formulation, CB 7a/b supplement and plain basal media on the average cell diamter over the process time course

4.5.3. Monitoring of key metabolites

By analyzing the respective key metabolite profile of a given cell line over the process time course, the metabolic status can be revealed by an observed depletion or abundance of essential metabolites at certain time points. Therefore, general requirements for an enhanced cell growth can possibly be drawn and used for further media optimization approaches.

Monitoring of the illustrated glucose levels in Figure 29 A revealed an overall similar concentration decline over the time course. Experiments supplemented with CB 7a/b showed a general higher glucose concentration compared to the experiment supplemented with the in-house supplements. Furthermore, no effect of added trace elements between the respective cultivation supplemented with the initial developed supplementation media was shown. Within

the control experiment and both 5.5% in-house supplements, a depletion of glucose at day 9 and 10, respectively, resulted in a drop of viability under the termination criterion one day after the observed depletion (Figure 29 A and Figure 29 B). This glucose dependent viability profile could not be observed in any other experiments.

Contrary to a declining glucose concentration profile, a static increase of the lactate concentration was revealed (Figure 29 B). Highest lactate concentrations over 4 g/l could be observed for SM 5.5%, SMTE 5.5.% and SM 9.9% after 11 and 13 days of inoculation, respectively. Supplementation with 5.5% of the working concentration with CellBoost 7a/b showed a slightly lower lactate concentration (3.69 g/L) at end of the process at day 11. Although lowest glucose concentration within the SM 5.5% and SMTE 5.5% cultivation was observed, a steeper increase of the lactate concentration profile was revealed. This phenomenon can be explained by the higher obtained cell concentrations as seen in Figure 26 A. In contrast, cells cultivated in plain basal media only showed a maximal lactate concentration of 2.8 g/L at day 7 which stayed moderately stable till day 10 after inoculation. However, no significant difference in the overall profile trend between supplemented ones with or without additional trace elements is illustrated in Figure 29 B.

Monitoring of the L-glutamine concentration profile revealed a similar decline which could be seen in the lactate accumulation over the process time, except for the control experiment. The L-glutamine profiles shown in Figure 29 illustrated the faster L-glutamine decrease in cultivation with higher cell concentrations. Interestingly, although no additional L-glutamine was added to the in-house supplementation media, a complete depletion of this metabolite at day 7 could be revealed only in the cultivation using plain ActiPro^{TM} (Figure 29 D).

In Figure 29 the supplementation effect of L-glutamate containing formulations is clearly shown. However, no observable consumption nor accumulation of L-glutamate could be revealed as the overall trend showed a constant profile over the course of the process time.

Monitoring of the by- product ammonium revealed a similar trend as the one observed in the lactate concentration profile. Again, maximal determined concentration was shown for both 5.5% supplemented in house-formulation media (0.335 g/L) and SMTE 9.9% at day 9 and 13, respectively. In contrast to the obtained lactate profile, a slight decrease of ammonium within SM 5.5% and SMTE 5.5% after day 9 till the 11th day (0.314 g/L) of inoculation. Furthermore, no difference regarding the overall profile trends between the cultivations could be stated.

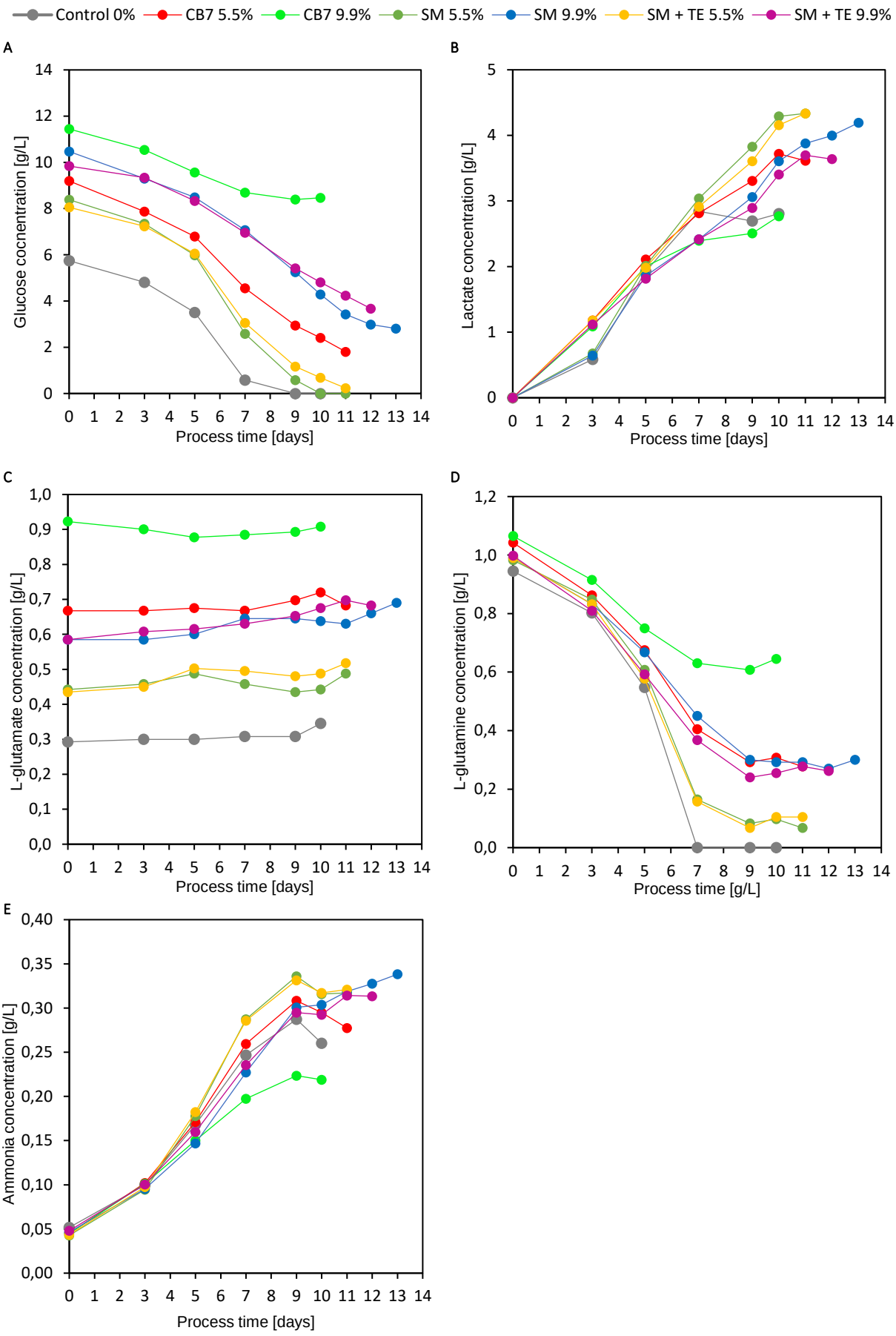


Figure 29. Metabolite and by-product profiles of-batch experiments in plain basal media and supplemented with original and altered formulation, CB 7a/b supplement and plain basal media

4.6. Full factorial optimization experiment for an enhanced in-house supplementation media

The first experimental design space, described in section3.2.13, was set up as a full factorial 2^4 screening experiment, to determine significant main and interaction effects of the respective factors at defined levels. Subsequently, an in-house supplementation media was developed and further traditional one-factor-at-a-time (OFAT) experiments were conducted. OFAT trials in section 4.4 and 4.5 were based on one factor setting and traditional comparison methods between two approaches. Based on the outcome in section 4.5, no significant influence of the added trace element nor of the correlating osmolality on cell performance could be stated. Consequently, an adapted three-level full factorial optimization experimental design was conducted to investigate possible influences of osmolality on the cell performance. Therefore, trace elements were separately tested against the original in-house formulation to analyze the cell performance at different decoupled working concentration of the defined factors. As such a 3^2 experimental design plus center point triplicate approaches, comprises 12 experiments referred to low, intermediate and high level setting of the defined factors, the low level setting (-1) was adapted to a biological system comparable to section 4.2.1.

4.6.1. Experimental set up

As previously mentioned, the aim of this experiment was to determine correlating influences on the cell performance based on different applied working concentrations of trace elements and supplementation media with respect to cell culture media osmolality. This utilized optimization design space was adapted to a biological system, by setting the -1 level of the supplementation media factor to 5.5% of the working concentration whereas the –1 level of the trace element formulation was realized by an osmolality adjusted salt solution. Subsequent levels were set to obtain a linear interval between the respective factors. Overall, the volume related ratio between in-house supplementation media and added trace elements is not stated in this thesis due to proprietary reasons. To ensure the application of a linear regression analysis between the conducted batch cultivations, a biological triplicate center point was performed. In contrast to a full factorial screening design space, one 0-0 level experiment is also included within optimization design space, resulting in 4 identical experiments within one experimental set up. As a reference, which is not suggested and included within the design space, additional duplicate experiments with plain ActiPro™, with no CellBoost 7a/b supplements, was

performed. Supplementation of the respective formulations prior to inoculation as well as the general batch cultivation was performed as previously described in section 3.2.5 The overall experimental setup of all 13 experiment is summarized in Table 24. Based on the working matrix seen in Table 25, the cultivations were supplemented either with the in-house formulation, trace element solution or an osmolality adjusted solution. Subsequent statistical evaluation was realized by applying a liner regression analysis. Due to the already obtained metabolite profiles, no metabolite monitoring was conducted. As this experimental set up is based on an optimization approach, no analogous "negative control" and "all supplements" experiments can be illustrated as in the first full factorial screening experiment in section 4.2.1.

Table 24. Experimental setup: Full factorial optimization experiment for an enhanced in-house supplementation media.

Operation mode	Batch
Cell line	CHO DG44/PG9
Basal media	ActiPro™ + 8mM L-Gln
Media Supplements	Feed supplement media A Feed supplement media B Trace element solution Osmolality adjusted solution
Working volume	30 mL
Supplementation strategy	Prior to inoculation
Seeding (total)	0.3×10^6 cells/mL (9×10^6 cells)
Reactor	50 mL reactor tubes with venting membrane
Incubation temperature	37.0°C
Humidity	80%
CO_2 level	7.0%
Rotation shaker speed	220 rpm
Termination criterion	Viability < 60%

4.6.2. Effect of combinatorial variants of feed stock and trace element solution in batch cultivations

The obtained cell performance profiles are illustrated in Figure 30 in which the colored curves are representing the level setting of the applied trace elements and the brightness of the curves the corresponding level of supplementation media, e.g. the dark red curves (Exp.3) represents the -1 level for trace elements with corresponding +1 level of supplementation media whereas the bright red curves (#1) represent the -1 level for trace elements with corresponding -1 level of supplementation media.

A deviation within almost every determined process parameter between the respective experiments could already be seen after the 3rd day of inoculation. Monitoring of the obtained cell performance parameter (Table 25 and Figure 30) revealed the effect of the multi combinatorial supplementation effect of the defined factors. This approach resulted in an overall stepwise increase as well as decrease of the observed parameter in comparison to the control experiment. Experiment #9, in which the highest overall trace element supplementation was applied, dropped below the viability criterion already after the first measurement at day 3 of inoculation (Figure 30 B). The longest process cultivation time of 15 days could be achieved within experiment #8 illustrated as the green line in Figure 30. Plain ActiPro^{TM} resulted in a maximal total cell concentration of 15.20 × 10^6 cells/mL at day 7 compared to 1.59 × 10^6 cells/mL at day 11 in experiment #8. Although, experiment #3 revealed only a maximal total cell concentration of 11.58 × 10^6 cells/mL at day 12, the highest overall antibody titer accumulation of 139.10 µg/mL could be determined via biolayer interferometry analysis after 12 days of inoculation. In other words, the maximal total concentration was reduced by 24% compared to the plain basal media but a 142% higher antibody titer was revealed within experiment #3 compared to the plain commercially available basal media (97.65 µg/mL). Although, no cultivation with CellBoost 7a/b was performed within this experiment, the obtained cell parameter profiles of experiment #13 are quite similar to the one of CellBoost 7a/b seen in Figure 26, if not even better. Combination of lower concentration of the in-house formulation and osmolality adjusted solution in experiment #1 (129.80 µg/mL) and experiment #2 (130.50 µg/mL) also resulted in 33% higher accumulated titers but lower reduction in the maximal total cell concentration (Table 25). Furthermore, the triplicate CenterPoint and experiment #5, which can be seen together as a biological quadruplet, significantly outperformed the plain ActiPro^{TM} with a maximal accumulated antibody titer of

119.43 µg/mL ± 1.89 at day 11. All obtained maximal values for the respective cell performance parameter are summarized in Table 25.

Table 25. Overview of obtained relevant cell perfromance parameter including the experimental data. Levels are coloured to their respective setting.

Experiment No.	Supplementation media	Trace elements	max. total cell concentration [× 10^6 c/mL]	max. viable cell concentration [× 10^6 c/mL]	Max. antibody titer [µg/mL]	µ (exp. phase) [1/d]	max. spec. Prod. [pg/cell/day]	max. VCCD [cell x day/mL]	Process duration [days]
1	-1	-1	14.86	14.21	129.80	**0.65**	4.84	8.05E+07	12
2	0	-1	14.28	13.55	130.50	0.60	3.46	**8.23E+07**	12
3	1	-1	11.58	10.24	**139.10**	0.54	4.87	6.45E+07	12
4	-1	0	14.13	13.49	115.70	0.55	3.50	7.53E+07	12
5	0	0	13.03	11.94	119.40	0.58	4.51	7.05E+07	12
6	1	0	9.76	8.95	98.70	0.46	3.09	5.67E+07	13
7	-1	1	6.18	4.78	89.20	0.41	**6.20**	3.93E+07	14
8	0	1	1.59	1.01	26.10	0.29	6.19	1.11E+07	**15**
9	1	1	0.33	0.33	1.54	0.00	3.31	4.65E+05	3
10	0	0	13.33	12.76	122.10	0.55	3.38	7.25E+07	12
11	0	0	12.97	12.36	118.30	0.52	3.63	4.98E+07	12
12	0	0	13.55	12.53	117.90	0.59	3.49	5.27E+07	10
ActiPro™ control			**15.20**	**14.90**	97.65	0.52	3.07	6.81E+07	10

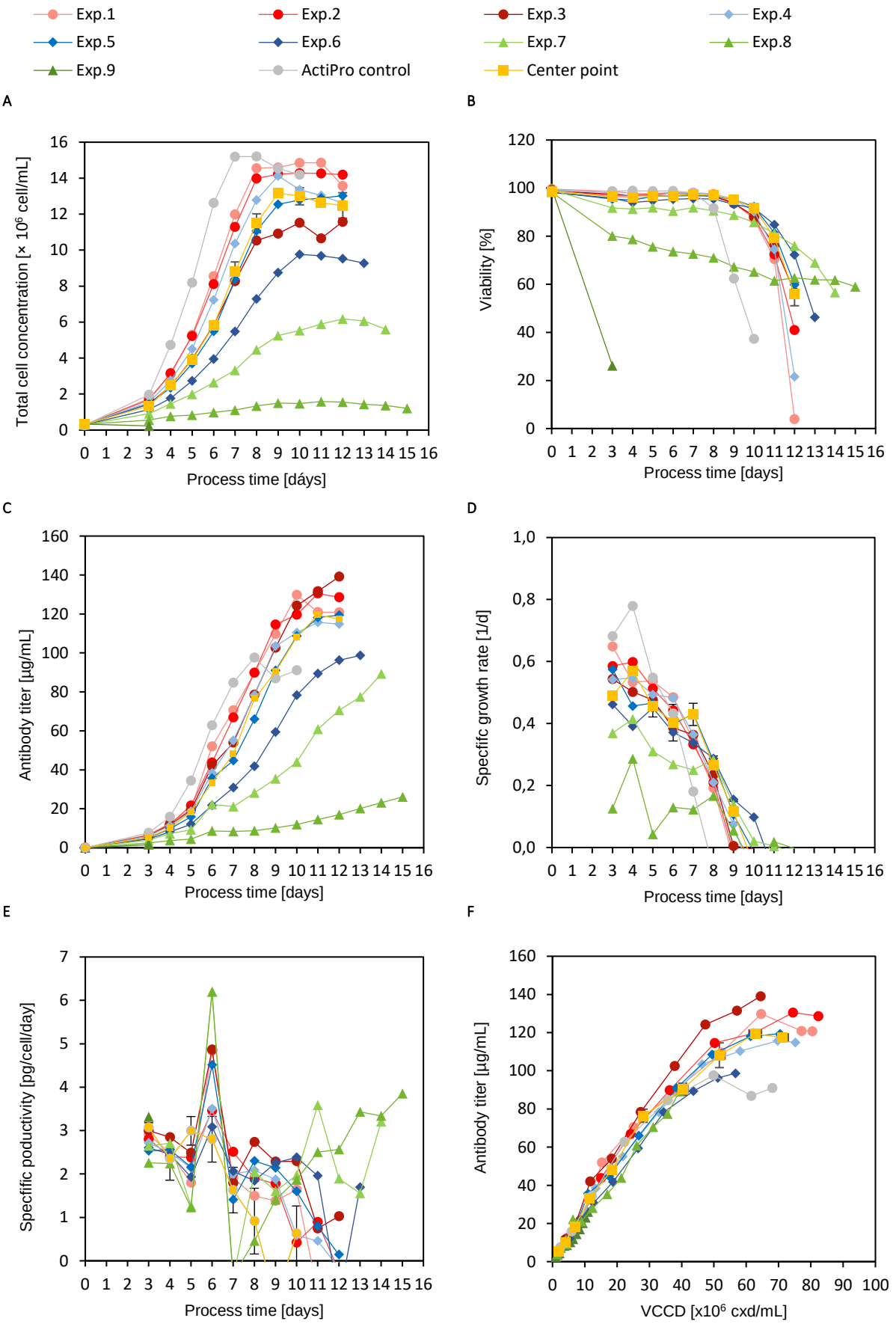


Figure 30. Obtained cell culture profiles supplemented with the original in-house supplement media and tarce elements according to the experimental matrix: (A) Total cell concentration, (B) viability, (C) antibody titer, (D) viable specific growth rate, (E) specific productivity, (F) antibody titer plotted over the course of the viable cumulative cell days. Respective experiments were supplemented prior to inoculation.

Monitoring of the cultivation media osmolality revealed the aimed stepwise separation between the different combinatorial supplementation approaches within the experimental design space. All osmolality profiles illustrated in Figure 31 showed an almost constant trend over the process time. Although, the -1 level setting of the trace element factor were realized by an osmolality adjusted solution without trace elements, a slightly higher osmolality within experiment #1, #2 and #3 in direct comparison to the trace element supplemented counterparts (Experiment #4, #5 and #6) is illustrated in Figure 31. Osmolality determination by freezing point technique revealed a range between 0.304 Osm/kg ±0 .003 within the control cultivation up to 0.542 Osm/kg ± 0.007 in experiment #9. As previously mentioned, the longest cultivation time, before undergoing the viability criterion of 60%, could be obtained within experiment #8 where the second highest media osmolality of 0.427 Osm/kg ± 0.004 was determined.

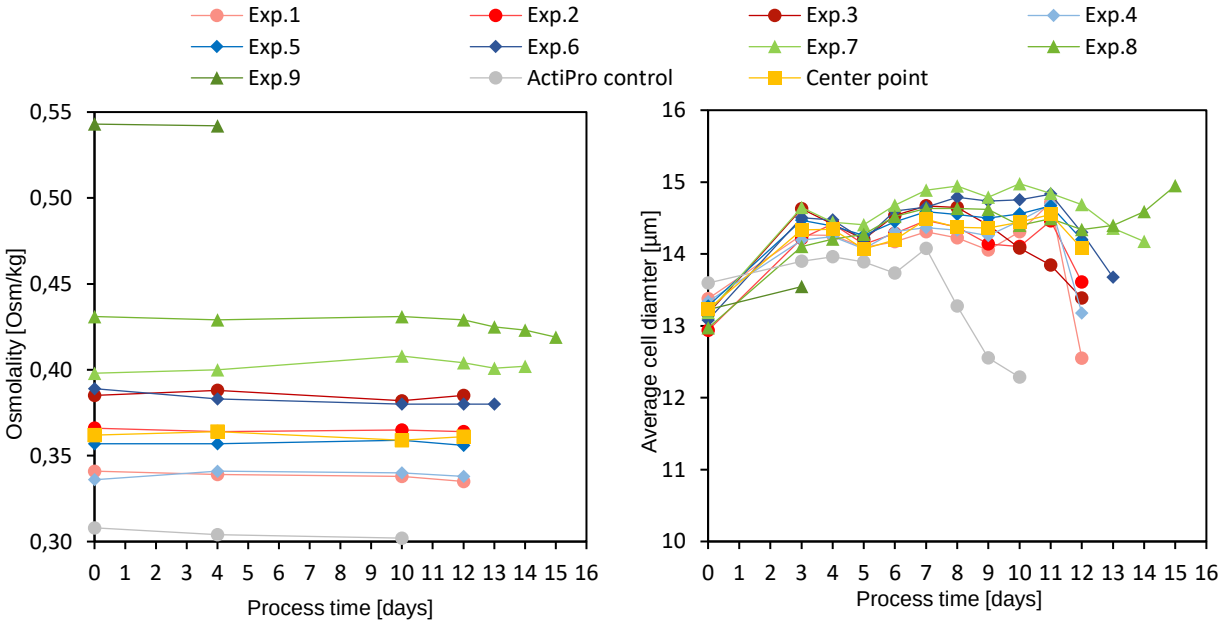


Figure 31. Osmolality effect of full factorial optimization experimental design over the process time course (left); Average cell diamter profile over the process time course obtained from the full factorial optimization experimental design (right)

Determination of the average cell diameter profile of the course of the process time in Figure 31 showed no difference in comparison to previous obtained cell diameter data, except of experiment #8. While this batch cultivation showed the highest cultivation media osmolality and longest cultivation time, the average cell diameter starts to increase after day 11 in contrast to all other cultivation experiments where a constant decline can be stated after a certain time period. Contrary to the other performed cultivations within the experimental design where the cell diameter stayed almost constant till day 11, experiment #3 revealed a constant decrease in cell diameter after day 7.

4.6.3. Statistical evaluation of the main effect

This experimental set up was performed based on a quadratic optimization 3^2 full factorial design model, describing a linear regression analysis of defined responses out of 2 factors varied at 3 different linear levels. By applying this response surface methodology adapted to a biological system, the goal was to find an experimental setpoint of the original in-house supplement media for an enhanced cell performance out of 12 cultivation runs. As within the first 2^4 full factorial screening experiment, the maximal viable cell concentration (mVCC), the maximal viable cumulative cell days (mVCCD), the obtained maximal antibody titer (mTiter) and maximal specific productivity were defined as responses to evaluate the impact of possible correlating combination effects. Contrary to the previous design space, 2 factors (supplementation media and trace elements) at 3 levels instead of 4 factors at 2 levels were tested. Within this approach several significant main effects and interaction could be stated which can be seen in the effect plots.

The effect plots Figure 32 of the observed responses show all significant main and interaction effects. As the obtained values within the triplicate center point as well as experiment #4 showed small standard deviations, resulting in a high statistical model quality, the overall outcome out of this optimization experimental design is well suited for a reliable interpretation of the effects. Within this experimental design a general higher statistical model quality compared to the one previously performed can be seen as the responses for the maximal viable cell concentration (R^2 = 0.98; Q^2 = 0.95), the maximal viable cumulative cell days (R^2 = 0.93; Q^2 = 0.84) as well as the maximal antibody titer (R^2 = 0.96; Q^2 =0.83) reach the relevant threshold as suggested in literature. Except for the maximal specific productivity (R^2 = 0.54; Q^2 = 0.39) where a significant positive impact of squared trace elements (Figure 32 D) can be seen, all other main and interaction effects seem to have an overall negative impact on the monitored responses. However, the value for R^2 is rather low (0.54), meaning a general poor statistical model quality. This can be explained due to the general error prone maximal specific productivity. Furthermore, highest significant negative main and squared effects can be seen for the mVCC, mVCCD as well as for the maximal antibody titer when the respective batch cultivation is supplemented with trace elements.

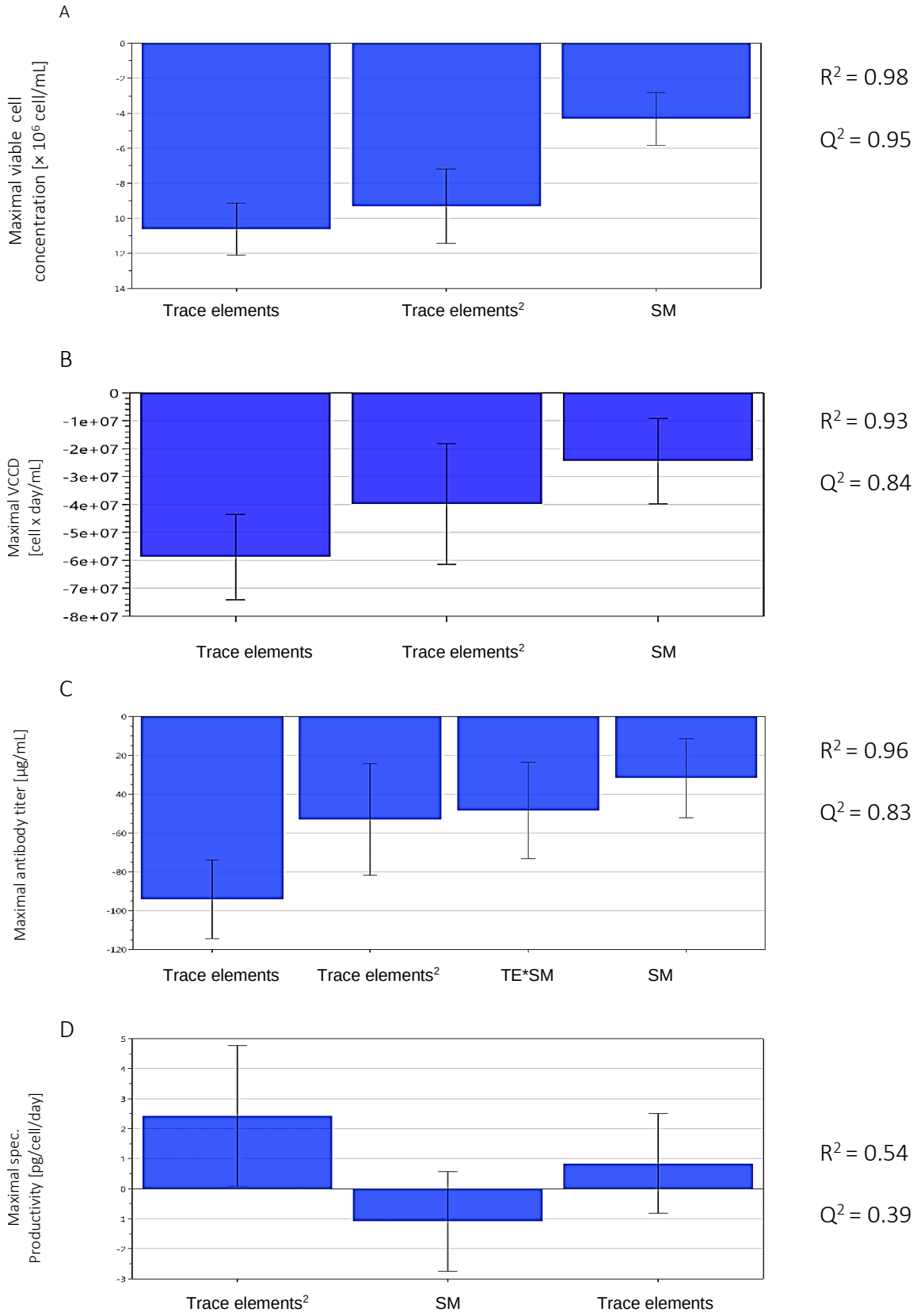


Figure 32. Effect plot of defined screening parameters obtained by linear regression analysis of the full factorial optimization experimental design experiment: (A) maximal viable cell concentration, (B) maximal viable cumulative cell days. (C) maximal antibody titer, (D) maximal specific productivity.

4.7. Testing the in-house supplementation media against commercially available supplements in bioreactor fed-batch process mode

Experimental data obtained from previously performed experiments yielded information about the positive impact of the in-house developed supplementation media in batch experiments. Furthermore, it was shown that by varying the constituent as well as working concertation of the formulation, a completely altered cell growth and antibody production was revealed. Within these batch experiments, the cultivation media was always supplemented with the respective formulation prior to inoculation without any subsequent manipulation of the cultivation media. Consequently, fed-batch experiments were designed to determine the applicability of the developed formulation as a feed medium. Since HyClone CellBoost 7a and 7b is developed as a supplement for a high-yield protein production within fed-batch processes, the aim of this experiment was a direct comparison of the in-house developed supplementation media against CellBoost 7a/b under controlled process conditions. Out of this cell cultivations, subsequent purification of the recombinant protein by stepwise application of protein A and size exclusion chromatography followed by a quality verification via SEC- MALS should be performed. Since molecules and proteins, respectively, of the same size differ in their molecular weight and can therefore not be properly determined only on the elution position, the SEC-MALS was chosen as a final verification of the absolute molar mass (Ogawa and Hirokawa 2018). Next to the CellBoost 7a/b standard feeding application, three different feeding regimes of the in-house media were designed. Unfortunately, only one fed-batch experiment using the original supplementation media will be interpreted within this thesis due to technical issues within the two other reactors where no reliable interpretation of the obtained results was possible.

4.7.1. Experimental set up

As previously mentioned, fed-batch experiments using the DASGIP benchtop system were designed to directly compare the application of the in-house supplementation media against the combinatorial application of CellBoost 7a and 7b within a feeding regime. Based on obtained results from previously performed fed-batch experiments within the working group, a feeding strategy was developed. Feed media were added after 3 days of batch cultivation with a daily constant volume feed of 1.1% of the present culture volume. Furthermore, a constant increase of up to 3.3% volume feed was planned in case of a glucose depletion, if the

lactate concentration (6 g/L) and media osmolality (0.400 Osm/kg) do not reach previously defined thresholds. Since the stated thresholds were reached within 7 and 8 days after inoculation (Figure 35), the bolus feed of 1.1% was never changed over the process time. Daily sampling and subsequent evaluation of the osmolality as well as key-metabolites was performed to adjust the feeding strategy of the culture in respect of their growth need and defined parameter. The overall experimental set up is summarized in Table 26.

Table 26. Experimental setup: Testing the in-house supplementation media against commercially available supplements in fed batch process

Operation mode	Fed-batch
Cell line	CHO DG44/PG9
Basal media	ActiPro™ + 8mM L-Gln
Media Supplements	HyClone CellBoost 7a HyClone CellBoost 7b Supplementation media A Supplementation media B
Working volume	400 mL
Feeding strategy	Feed start at day 3 Daily 1.1% feed of the culture volume (adaptable to present culture parameter)
Seeding (total)	0.4×10^6 cells/mL (160×10^6 cells)
Reactor	DASGIP System
Incubation temperature	37.0°C
pH-Setpoint	7.00
Stirrer speed	80 rpm
Dissolved O_2 setpoint	30%
Termination criterion	Viability < 60%

4.7.2. Performance of clone CHO DG44/PG9 in fed-batch cultivation

Performing a fed-batch process by using the in-house supplementation media and HyClone CellBoost 7a and 7b revealed significant differences within the monitored process parameter. Although, the cell performance in both reactors did not reveal the expected outcome in terms of higher cell concentrations, longer process time and improved protein yields, the obtained data proofed the possible application of the in-house developed formulation as a suitable feed media. Maximal total cell concentration, as shown in Figure 33 was strongly increased in the experiment within the in-house media culture (18.04 × 10^6 cells/mL at day 7) in direct comparison to the fed-batch process in which CellBoost 7a/b was applied as feed medium (15.01 × 10^6 cells/mL). Figure 33 B illustrates, that the cultivation time could also be prolonged for one additional day by feeding the culture with the supplementation media, resulting in an overall process time of 10 days before undergoing the viability termination criterion of 60%. Monitoring the maximal antibody concentration via biolayer interferometry revealed a 22% higher product titer in the supplementation media (122.40 µg/mL at day 10) compared to the CellBoost 7a/b (100.10 µg/mL at day 9). However, maximal harvest titer within batch experiments could not be obtained. Calculation of the specific productivity showed in both cultivations a contrary behavior when compared to previously performed batch experiments. While a constant decline of the specific productivity profile was shown in all previous cultivations over the process time course, Figure 33 E illustrates a constant trend till day 7 with a subsequent sharp increase in both fed-batch experiments till the end of the process. Obtained maximal cell performances are listed down in below in Table 27.

Table 27. Overview of obtained relevant cell perfromance parameter of perfromed fed-batch process.

Experiment.	max. total cell concentration [× 10^6 c/mL]	max. viable cell concentration [× 10^6 c/mL]	Max. antibody titer [µg/mL]	µ (exp. phase) [1/d]	max. spec. Prod. [pg/cell/day]	max. VCCD [cell x day/mL]	Process duration [days]
CB 7 a/b feed	15.01	14.42	100.10	**0.65**	4.41	2.48E+10	9
SM feed	**18.04**	**17.22**	**122.40**	0.60	**5.43**	**3.41E+10**	**10**

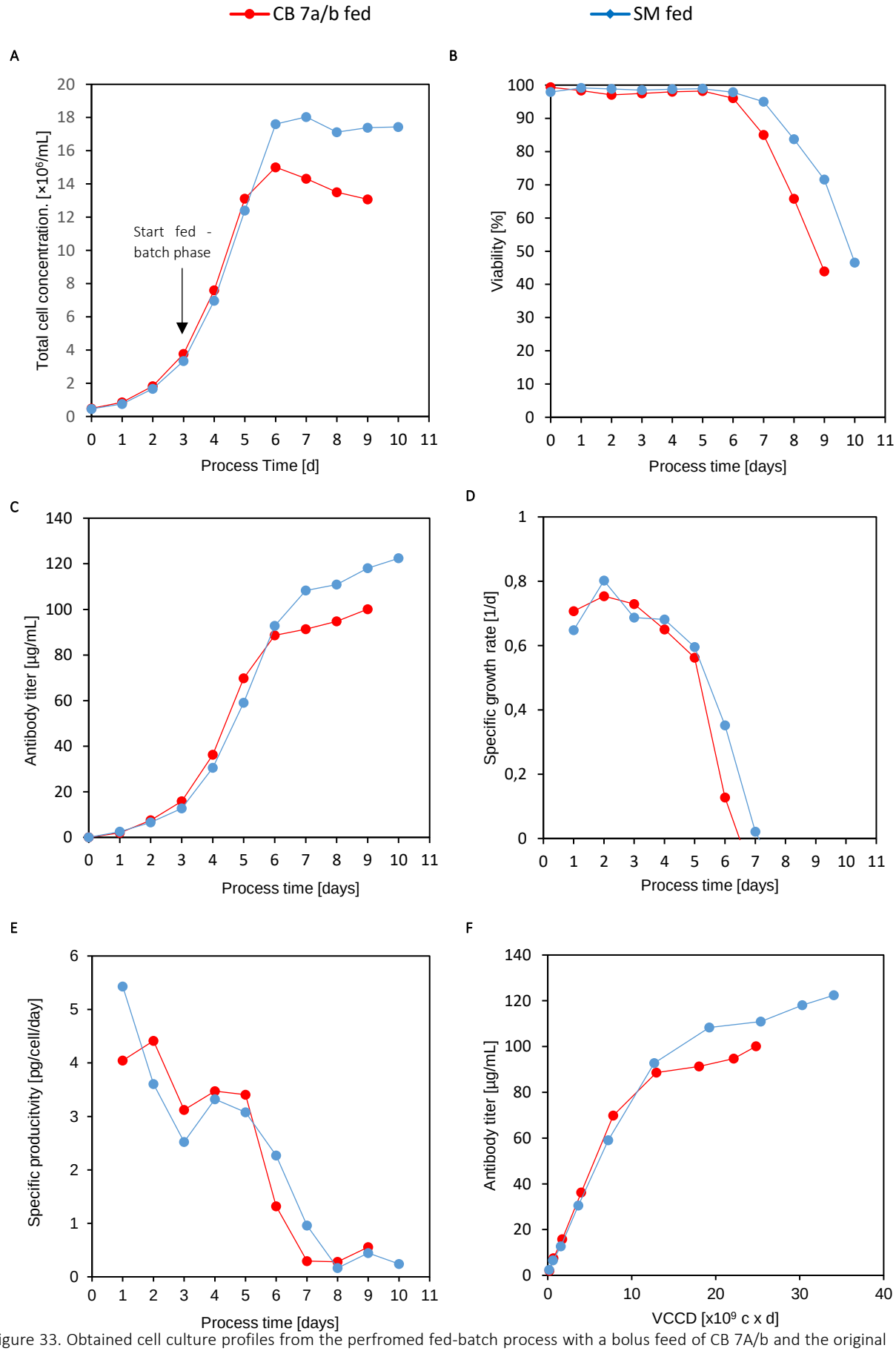


Figure 33. Obtained cell culture profiles from the perfromed fed-batch process with a bolus feed of CB 7A/b and the original in-house fromulation: (A) Total cell concentration, (B) viability, (C) antibody titer, (D) viable specific growth rate, (E) specific productivity, (F) antibody titer plotted over the course of the viable cumulative cell days. Respective experiments were supplemented prior to inoculation.

Monitoring of the key metabolite concentration in Figure 34 revealed similar profiles to already obtained ones in section4.5.3, except for L-glutamate and L-glutamine. Since both feed media contain the amino acid L-glutamate, a continuous increase over the process time course can be seen in Figure 34 C in which a maximal L-glutamate concentrations of 0.57 g/L at day 10 in the SM fed batch and 0.72 g/L at day 9 in the CellBoost 7a/b fed experiment was obtained. Although the in-house supplement media did not contain any additional L-glutamine, determination of the profiles in Figure 34 D revealed a constant concentration decrease till day 5 followed by a slow decline without an observable depletion of the amino acid till the end of the process in contrast to other performed batch cultivations. Furthermore, a sharp increase of lactate accumulation within both culture media up to 5.63g/L (CB 7a/b) and 6.04 g/L within 9 and10 days, respectively, was observed.

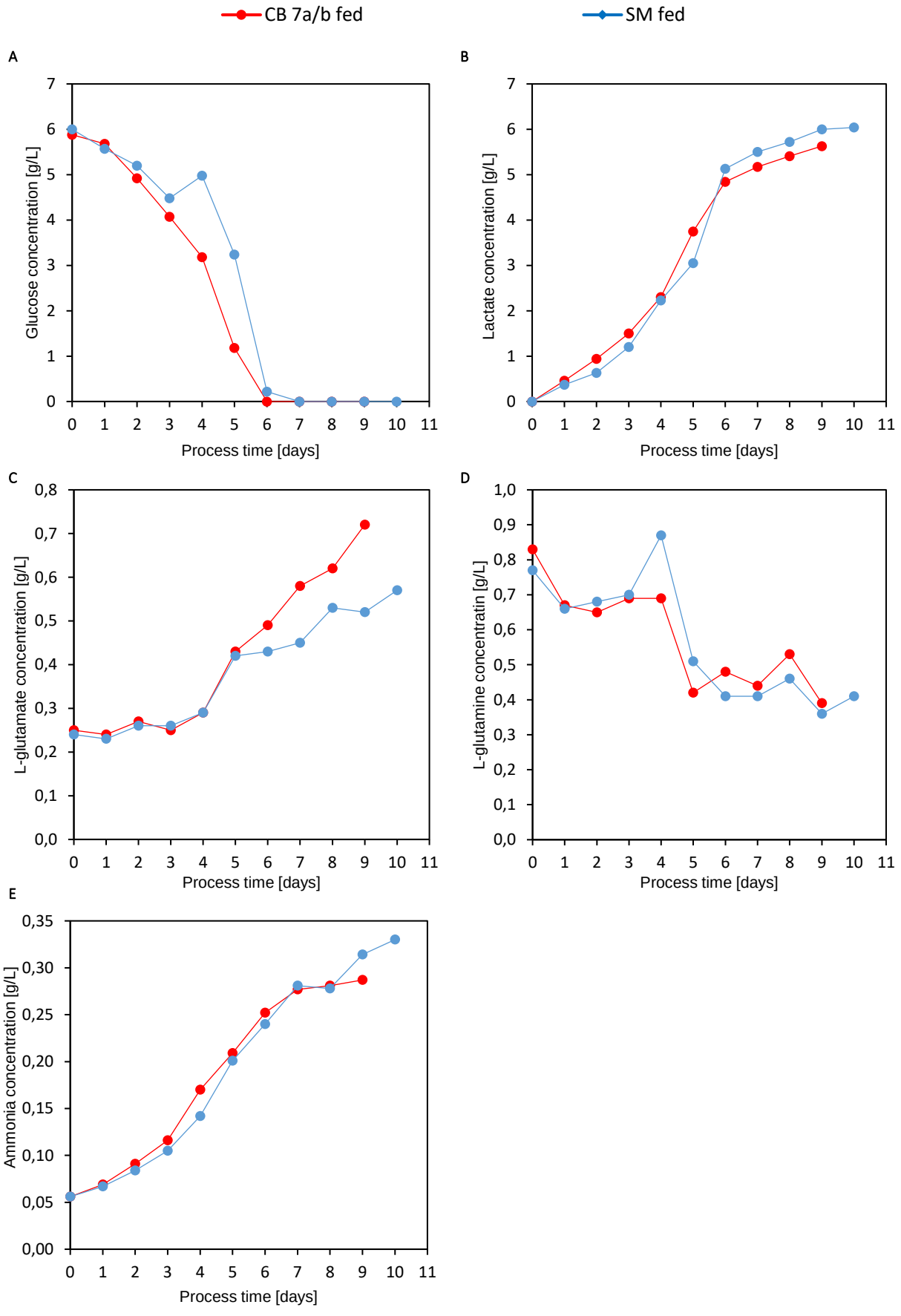


Figure 34. Metabolite and by-product profiles of-fed-batch experiments using a bolus feed of CB 7a/b and the original in-house supplement media.

As previously mentioned in the experimental set up section, the phase transition from a batch to fed-batch regime was executed after 3 days of inoculation. Figure 35 A illustrates the feeding start of the respective supplements as an increase of osmolality within the cell culture media. As both experiments were conducted under equivalent starting conditions, the osmolality from day 0 till day 3 stayed constant till day 3. After the phase transition a constant osmolality increase of the time course up to 0.411 Osm/kg within the CellBoost 7a/b and 0.380 Osm/kg in the in-house media fed-batch was observed.

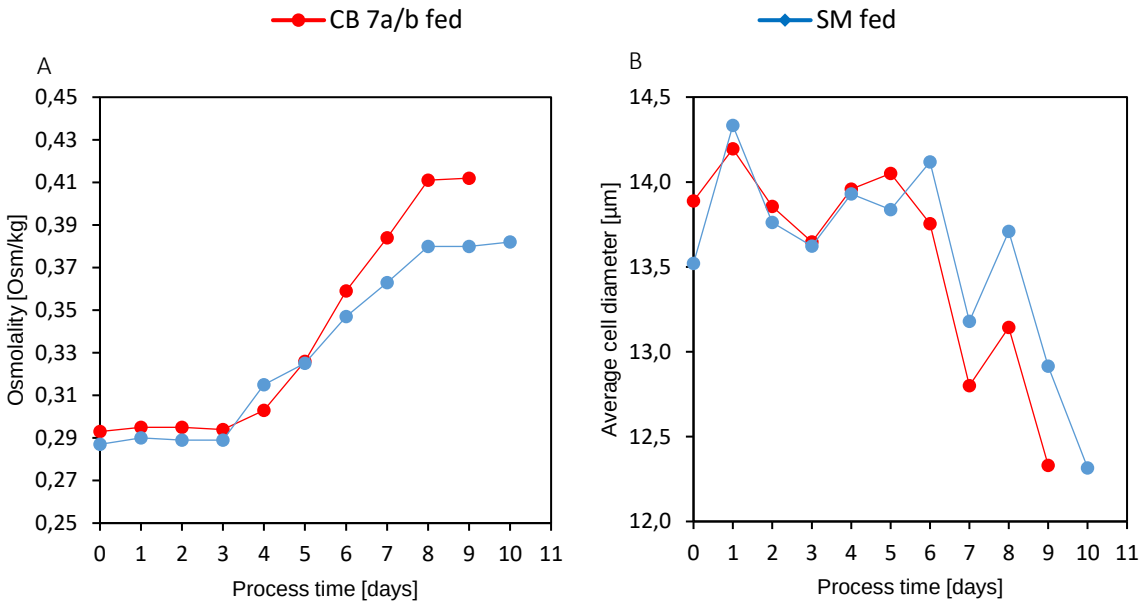


Figure 35. Osmolality effect (left) and average cell diamter (reight) profile over the process time course obtained from the fed-batch process.

Monitoring the cell diameter adaptation over the process time course in Figure 35 B, did not reveal any diverging phenomena compared to batch experiments with a comparable supplementation set up.

4.7.3. Absolute molecular mass and protein size verification of the recombinantly expressed protein

In order to evaluate the applicability of the in-house developed formulation as a potential supplementation and feed media, the quality of the expressed recombinant protein had to be analyzed. To ensure unchanged protein quality, the purified monoclonal antibodies were characterized with respect to correct protein folding and absolute molecular mass using multiangle light scattering following fractionation (SEC-MALS).

Therefore, filtrated supernatants obtained from the previously performed fed batch processes were purified using two separate chromatographic methods. Based on the binding properties of the Fc region, the harvested supernatants were applied on a protein A HP column to selectively capture and purify antibodies with correctly expressed binding domains and their corresponding consensus binding site. Protein capture and elution was performed according to the buffer system described in section 3.2.14. Afterwards, the obtained eluates were pooled and re-buffered into a phosphate buffer system via dialysis. As a final polishing step, the collected fractions were purified by performing a size-exclusion chromatography to remove remained impurities and formed aggregates. To verify correct protein properties and molar size of the expressed and purified protein, a final SEC-MALS measurement was performed. Thus, the purified proteins from both fed-batch processes as well as a commercially available monoclonal antibody standard got applied onto a Superdex^{TM} 200 column (GE Healthcare). Figure 36 illustrates the obtained profiles of the analyzed protein samples.

Table 28. Protein A mass balance obtained from SM fed-batch supernatant.

	Volume [mL]	Antibody titer [mg/mL]	Total antibody [mg]	Yield [%]
Supernatant	145	0.13	19.00	100%
Flow through	153	-	-	-
Wash	34	0.04	1.48	8%
Eluate	14	1.20	16.99	89%
Clean	48	0.03	1.34	7%

Table 29. Protein A mass balance obtained from CB 7a/b fed-batch supernatant.

	Volume [mL]	Antibody titer [mg/mL]	Total antibody [mg]	Yield [%]
Supernatant	149	0.11	16.39	100%
Flow through	157	-	-	-
Wash	58	0.02	1.10	7%
Eluate	13	1.14	14.74	90%
Clean	52	0.01	0.47	3%

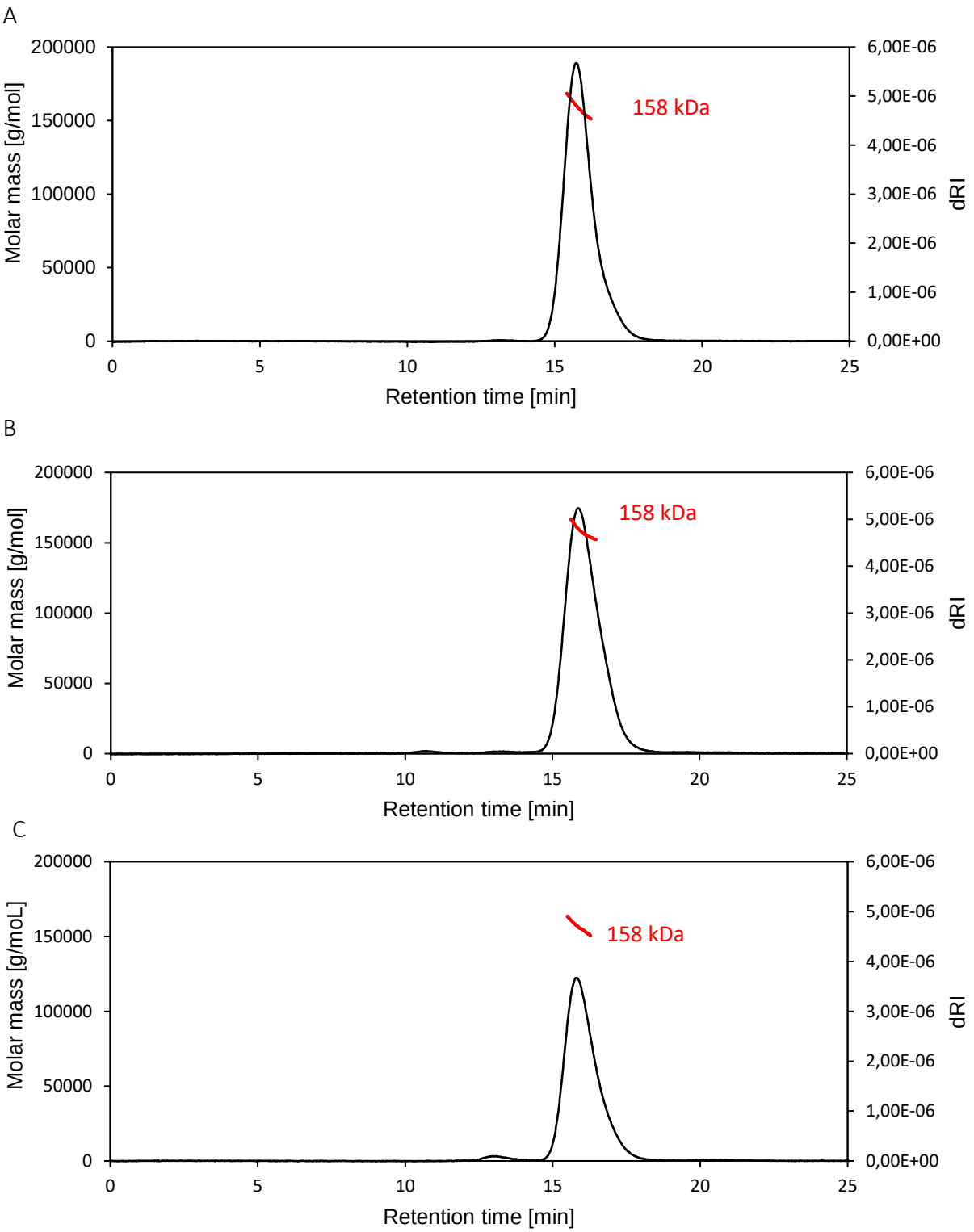


Figure 36. SEC-MALS profiles of PG9 obtained from: (A) in-house media fed-batch, (B) CB 7a/b fed-batch, (C) PG9 reference sample

Monitoring of obtained SEC-MALS outcome in Figure 36 revealed an overall identical profile of the observed monoclonal antibodies. Since, no remarkable peak after the expected void volume at a retention time of approximately 10 min prior nor after the exhibited peak at 17

min was revealed, no aggregate or antibody fragments within the purified samples could be detected. Furthermore, determination of the molar masses by multi-angle static light scattering revealed the exact same protein mass of 158 kDa within the measured product from fed-batch cultivation as well as of the PG9 reference sample provided by Polymun Scientific GmbH. Consequently, it can be stated, that the supplemented media within the feed regime (Figure 26) did not show any negative impact on the protein quality under the observed quality parameter.

5. DISCUSSION

In this study a commercially available, chemically defined and animal component free cell culture media was supplemented by an in-house developed feed media formulation to evaluate the bioprocess performance under batch and fed-batch cultivations using an IgG-producing CHO DG44 cell line. Application of different media constitute ratios and feed volumes were tested against plain basal media as well as in combination with a commercial supplement system. Development of the in-house formulation was based on the utilization of full factorial screening and optimization experimental designs in combination with traditional one-factor-at-a-time (OFAT) approaches. In order to meet stringent requirements on chemically defined media, only protein free and animal component free substances were used in this thesis.

Monitoring of the obtained cell performance parameters revealed significantly improved recombinant product yields in batch cultivations under optimized media adjustments compared to the plain basal media. Fed-batch experiments highlighted the potential use of the developed formulation as a feed-media which outperformed commercially available media supplements in terms of higher cell concentrations and antibody titer. Depending on the applied feed concentration within the supplementation media, a ratio depending cell performance could be shown in order to enhance cell proliferation, specific productivity or prolong the cultivation time. By characterizing the expressed monoclonal antibody, no impairment on the overall protein quality within the applied analysis methods could be observed.

5.1. Cell performance comparison in two commercially available basal media

Since the development of serum free, chemically defined media is a challenging field from early stages in mammalian cell culture technology (Glassy, Tharakan, and Chau 1988), the need to supply an adequate amount of essential growth and production related media constituents is still critical in cell culture medium development (Ritacco, Wu, and Khetan 2018b). A direct comparison between HyClone ActiPro™ (GE) and CD CHO (Gibco) in a batch setup was performed to evaluate the best performing commercially available media for the respective cell line. Although both formulations are supposed to be optimized for CHO cell cultivation, an overall superior cell performance in ActiPro™ was shown which yielded in a 140% higher total

cell concentration and 19% higher maximal antibody titer compared to CD CHO. Hereby, it has to be mentioned that the cell line was not adapted to CD CHO which could impair the observed cell performance. However, due to pre-experimental data (Reinhart et al. 2015) and the observed cell performance parameter, ActiPro™ was chosen as a basis for subsequent optimization approaches in this thesis.

5.2. Utilizing experimental design as powerful tool to identify best performing media composition

Application of statistical methods to simultaneously reduce the number of required experiments and enhance statistical power gave several important insights to improve overall critical factors in mammalian cell culture bioprocesses as well as media optimization approaches (Möller and Pörtner 2017). In this thesis, two full factorial experimental designs, differing in their analysis objective, were applied to determine the effect of five different feed substance classes at two and three levels.

5.2.1. Full factorial screening experimental design

To evaluate the impact of four different feed stock solutions, vitamins, pyruvate, amino acids and membrane precursors, a full factorial screening experiment at resolution V design was conducted. Although there is no beneficial reduction of required experiments compared to a fractional design, a full factorial experiment is especially reasonable in early stages of an experimental approach (Antony and Antony 2014). This 2^4 experimental design space comprised 16 unique screening experiments including a negative control and additional biological triplicates as center point to verify a linear correlation between the distinct level settings. Since all four defined factors (vitamins, pyruvate, amino acids and membrane precursor) were considered to be in a linear range based on the level settings, a statistically significant evaluation of main and interaction effects could be performed to exclude potential confounding events.

Amino acids are key media constituents which have to be provided in cell culture media at sufficient amounts to support biomass synthesis and productive protein expression (Fan et al. 2015). Therefore, a balanced composition of amino acids is crucial to provide a high performing cell culture media. Determination of the statistical evaluation revealed a 24% significantly

higher maximal viable cell concentration and a 13% higher antibody titer within cultures supplemented with the respective amino acid solution compared to non-supplemented batch cultivations. Furthermore, only a slightly positive but significant main effect on specific productivity could be revealed within membrane precursor supplemented cultures. Thus, amino acids were verified to be an indispensable substance class to develop a suitable supplementation media. Since the maximal specific productivity is more error prone compared to the mean specific productivity during the exponential phase, it is suggested to define the latter as a further response parameter in future data evaluation but this is not shown here.

For pyruvate and vitamins no significant main or interaction effects could be stated for the given cell line. Monitoring of the best performing culture (experiment #2) only supplemented with amino acids showed an 29% higher antibody titer associated with 19% improved maximal total cell concentrations. However, since the triplicate as well as the culture which got supplemented with all factors at +1 level setting showed highest cell numbers and titers, the firstly developed feed media comprised all four evaluated substance classes.

5.2.2. Full factorial optimization experimental design

To evaluate the effect of additional trace elements and the change in osmolality, a full factorial 3^2 optimization experiment was designed. Additionally, biological triplicates were performed within this experimental design as center points to improve the statistical power. Since this approach comprised each factor at three levels, the aim was to find the optimal factor level setting (i.e. concentration) for the feed media in regard to an improved cell performance. In order to obtain a sensible interpretation regarding possible osmolality effects, the -1 level of this factor was realized by an osmolality adjusted solution.

By interpreting the statistical analysis obtained in 4.6.3, an overall significant negative impact of the factors was shown. Especially main and quadratic effects of trace elements revealed a significant impairment on maximal VCD and recombinant product titer. A stronger impact of the respective trace element concentration on the cell performance compared to only osmolality adjusted cultures could be stated. Since modifications of the media osmolality are well reported (Zhang et al. 2010), the combinatorial application of the original formulation and the respective -1 level factors showed an overall improved specific productivity. This enhancement yielded in up to 44% higher product yields and a 31% reduction of the observed total cell concentration in direct comparison to the batch control cultivated in basal medium

only. Furthermore, the combinatorial variation of the respective factors at different levels, revealed an overall contrary cell performance. Experimental data showed, that these cultivation adjustments can significantly influence the expressed product yield, cell proliferation as well as process time. One single different factor setting revealed a difference in cultivation time of 12 days until the termination criteria of 60% viability was reached. Moreover, an up to 10-fold stepwise separation of the determined cell concentration within the experiments was shown, suggesting a major interference in the biomass synthesis in dependency of the media formulation. Interestingly, no distinct correlation between the cell proliferation and product titer. Because of higher specific production, lower cell concentrations yielded in higher antibody titer and vice versa.

5.3. Effect of varying working concentrations on the cell performance

In order to verify the effect of different applied working concentrations (Reinhart et al. 2018), traditional one-factor-at-a-time (OFAT) approaches were performed. By varying the supplementation ratio, the cell performance was investigated by supplementing the basal with the in-house formulation with and without additional trace elements. As an additional benchmark, batch experiments with commercially available supplements from the same culture media system were performed. In the latter experiments, the applied concentration variation showed significant divergent cell performance outcomes. Furthermore, an increase of the average cell diameter correlating with higher osmolarities was observed as already reported in the literature (Ozturk and Palsson 1991). However, since rather low size deviation within the supplemented cultivation were shown, no distinct impact of media constituents and osmolality could be stated. Resulting data suggested an overall improvement in terms of specific productivity when the media was supplemented with commercial supplements. However, improved maximal cell concentrations and antibody titer could be obtained when plain basal media was supplemented with both respective in-house formulations. Furthermore, all cultivations in which supplements were applied at 5.5% of the working volume, outperformed the batch controls cultivated in basal medium only. Next to other bioprocess consideration like a shift in the pH and temperature (Hogiri et al. 2018; Kaisermayer et al. 2016; Trummer et al. 2006), varying media compositions have been reported to directly affect metabolic pathways. These changes can either promote desired product formation or can lead to drastic impairments of cell proliferation, product yield and quality (Crowell et al. 2007; Xie

and Wang 1994, 1997). In this study, a direct influence on the observed L-glutamine concentration profile over the time course could be observed in dependency of the supplementation ratio. Since, no L-glutamine was added in the in-house supplementation media, this effect can be directly linked to other media constituents found in the developed formulation. Possible candidates influencing the glutamine synthetase such as glutamate, arginine and glucose are reported by Noh et al. 2018. Contrary to the observed profile of plain basal cultivated cells, Figure 29 D revealed no L-glutamine depletion at any time point over process course when the culture media was supplemented. This observation was independent of the applied concentration and formulation, although higher working concentration revealed lower L-glutamine consumption because of lower cell numbers.

5.4. Evaluation of the in-house formulation as a promising feed medium

The increasing demand for recombinantly expressed products in recent decades has led to the development of various process approaches and process establishments. Batch cultivation is generally known as the most favorable process strategy for early experimental stages due to comparably marginal efforts and easy reproducibility. However, batch processes are inadequate due to their inherent process-related limitation and fail to comply with the need for an increased efficiency and economics of production processes. Therefore, a fed-batch strategy was designed to evaluate the applicability of the in-house formulation as a feed medium under controlled process parameters. As in previous batch-experiments, the commercially available two component supplement was used as benchmark. A bolus feeding strategy was designed based on pre-experimental data from the working group and no variation of the original in-house formulation and different feeding regimes were tested due to time and technical issues.

While the exponential growth rate within both fed-batch culture was rather equal, the maximal obtained cell concentrations differed significantly after undergoing into the stationary phase. In particular, feeding the CHO cell line with the in-house formulation yielded in up to 20% higher total cell concentrations and an increase of 22% in terms of the final antibody titer. Furthermore, the process time could be prolonged for one day when developed supplementation media was used as a feed. While other observed cell parameter and key metabolites profiles only differed marginally, a clear separation of the cell culture media

osmolality was observed after 6 days of cultivation. Observed media osmolalities resulted in a range between 0.382 Osm/kg and 0.412 Osm/kg before the cells dropped below the viability criterion. Since increased osmolality and even hyperosmolalities are reported to have a positive impact on the cell performance, high media osmolalities within this project resulted in a cultivation time of up to 15 days (Ozturk and Palsson 1991; Shen and Sharfstein 2006; Zhang et al. 2010).However, cells within the fed-batch regime dropped below termination criterion of 60% viability after 9 and 10 days, respectively. While, fed-batch processes are supposed to enhance the cultivation time due a continuous supply of fresh nutrients, in this experiment no improvement in terms of a longer cultivation time could be shown (Pörtner, Schwabe, and Frahm 2004; Wlaschin and Hu 2006). Possible reasons for this outcome could have been the high accumulation of lactate and the correlating high addition of base. However, the in-house formulation outperformed the commercial supplement system, which has already been reported in the working group to be highly efficient for fed-batch processes, in terms of higher achieved biomass and product yield. Furthermore, no observable impairment of the protein quality could be stated when the expressed antibody was directly compared to a PG9 reference sample standard as described in section 4.7.3. However, this is not surprisingly since it can only be stated that the already SEC purified sample did not show aggregate formation after the purification run. To proof comparable stable properties of the expressed antibody in comparison to the reference mAb, a long-term stability observation has to be performed. This outcome proofs the possible application for the developed formulation to be used as an overall improvement for basal media and can be considered as an effective feed-media for different types of bioprocess strategies.

6. CONCLUSION & OUTLOOK

Since the field of establishing perfectly adjusted chemically defined media to the requirements of cells in culture is strongly emerging (Kuwae, Miyakawa, and Doi 2018), this thesis focused on the optimization of a high-performing cell culture media. By utilizing full factorial experimental designs and traditional one-factor-at-a-time experiments to understand the impact of defined feed stock solutions on growth and productivity of a recombinant CHO cell line, an in-house developed supplement media was realized to aim an improved cell performance for a recombinant DG44 CHO cell line. A panel of defined substance solutions, composed of components typically found in commercial cell culture media, were compiled and tested at various working concentrations in batch processes. As an additional benchmark, batch and fed-batch experiments with commercially available supplements from the same culture media system were performed. By varying the formulation composition as well as the overall working concentration, significant improvement on growth and product yield could be achieved in direct comparison to batch controls cultivated in basal medium only. It could be shown that application of the in-house media increased cell growth and product yield in batch and fed-batch experiments even when compared to the commercial supplement system. Observation of the recombinantly expressed product revealed no influence on protein size and molecular mass when cells were grown in the modified basal media. Moreover, all constituents within the developed supplementation media are typically found in commercially available cell culture media, the overall formulation can therefore be considered as uncritical in terms of process and product formation.

Since major responses in terms of cell performance on varied media composition and working concentration were revealed, it is suggested to design different media modification strategies for future experiments. Such can be based on the single shot supplementation method prior to inoculation, after a defined time period or designed as a continuous single or multiple component feed. More precise evaluation of the different media constituent classes could also reveal important insights on the cell performance. This understanding could further be applied to design an optimal media composition, customized to the needs of different cell lines. As supplements are generally considered to be highly concentrated formulations, the in-house developed composition could possible give rise to the development of a complete in-house cell culture media system.

7. REFERENCES

Ahn, Woo Suk and Maciek R. Antoniewicz. 2012. "Towards Dynamic Metabolic Flux Analysis in CHO Cell Cultures." *Biotechnology Journal* 7(1):61–74.

Altamirano, C., C. Paredes, J. J. Cairo, and F. Godia. 2000. "Improvement of CHO Cell Culture Medium Formulation: Simultaneous Substitution of Glucose and Glutamine." *Biotechnology Progress* 16(1):69–75.

Altamirano, Claudia, Julio Berrios, Mauricio Vergara, and Silvana Becerra. 2013. "Advances in Improving Mammalian Cells Metabolism for Recombinant Protein Production." *Electronic Journal of Biotechnology* 16(3).

Antony, Jiju and Jiju Antony. 2014. "Full Factorial Designs." *Design of Experiments for Engineers and Scientists* 63–85.

Baker, Monya. 2016. "Reproducibility: Respect Your Cells!" *Nature* 537(7620):433–35.

Bandaranayake, Ashok D. and Steven C. Almo. 2014. "Recent Advances in Mammalian Protein Production." *FEBS Letters* 588(2):253–60.

Berrios, Julio, Claudia Altamirano, Nelson Osses, and Ramon Gonzalez. 2011. "Continuous CHO Cell Cultures with Improved Recombinant Protein Productivity by Using Mannose as Carbon Source: Metabolic Analysis and Scale-up Simulation." *Chemical Engineering Science* 66(11):2431–39.

Bowden, Gregory D., Bernd J. Pichler, and Andreas Maurer. 2019. "A Design of Experiments (DoE) Approach Accelerates the Optimization of Copper-Mediated 18F-Fluorination Reactions of Arylstannanes." *Scientific Reports* 9(1):11370.

Buckley, Kevin and Alan G. Ryder. 2017. "Applications of Raman Spectroscopy in Biopharmaceutical Manufacturing: A Short Review." *Applied Spectroscopy* 71(6):1085–1116.

Bühler, Volker. 2001. *Vademecum for Vitamin Formulations*. Wissenschaftliche Verlagsgesellschaft.

Bulletin, Advanced Pharmaceutical and Kishwar Hayat Khan. 2013. "Gene Expression in Mammalian Cells and Its Applications." 3(2):257–63.

Carrillo-Cocom, L. M., T. Genel-Rey, D. Araíz-Hernández, F. López-Pacheco, J. López-Meza, M. R. Rocha-Pizaña, A. Ramírez-Medrano, and M. M. Alvarez. 2015. "Amino Acid Consumption in Naïve and Recombinant CHO Cell Cultures: Producers of a Monoclonal Antibody." *Cytotechnology* 67(5):809–20.

Chromikova, Veronika, Maria A. Zaragoza, and Florian Krammer. 2017. "Generation of a Serum Free CHO DG44 Cell Line Stably Producing a Broadly Protective Anti-Influenza Virus Monoclonal Antibody" edited by H. Wanjin. *PLOS ONE* 12(9):e0183315.

Crowell, Christopher K., Gustavo E. Grampp, Gary N. Rogers, Janis Miller, and Robert I. Scheinman. 2007. "Amino Acid and Manganese Supplementation Modulates the Glycosylation State of Erythropoietin in a CHO Culture System." *Biotechnology and Bioengineering* 96(3):538–49.

Cruz, Freitas, Alves, Moreira, and Carrondo. 2000. "Effects of Ammonia and Lactate on Growth, Metabolism, and Productivity of BHK Cells." *Enzyme and Microbial Technology* 27(1–2):43–52.

Davy, Anne Mathilde, Helene Faustrup Kildegaard, and Mikael Rørdam Andersen. 2017. "Cell Factory Engineering." *Cell Systems* 4(3):262–75.

Dulbecco, R. and G. Freeman. 1959. "Plaque Production by the Polyoma Virus." *Virology* 8(3):396–97.

Eagle, H. 1959. "Amino Acid Metabolism in Mammalian Cell Cultures." *Science* 130(3373):432–37.

EAGLE, H. 1955. "Nutrition Needs of Mammalian Cells in Tissue Culture." *Science (New York, N.Y.)* 122(3168):501–14.

Euler, Zelda, Evelien M. Bunnik, Judith A. Burger, Brigitte D. M. Boeser-Nunnink, Marlous L. Grijsen, Jan M. Prins, and Hanneke Schuitemaker. 2011. "Activity of Broadly Neutralizing Antibodies, Including PG9, PG16, and VRC01, against Recently Transmitted Subtype B HIV-1 Variants from Early and Late in the Epidemic." *Journal of Virology* 85(14):7236–45.

Fan, Yuzhou, Ioscani Jimenez Del Val, Christian Müller, Jette Wagtberg Sen, Søren Kofoed Rasmussen, Cleo Kontoravdi, Dietmar Weilguny, and Mikael Rørdam Andersen. 2015. "Amino Acid and Glucose Metabolism in Fed-Batch CHO Cell Culture Affects Antibody

Production and Glycosylation." *Biotechnology and Bioengineering* 112(3):521–35.

Fekete, Szabolcs, Alain Beck, Jean-Luc Veuthey, and Davy Guillarme. 2014. "Theory and Practice of Size Exclusion Chromatography for the Analysis of Protein Aggregates." *Journal of Pharmaceutical and Biomedical Analysis* 101:161–73.

FORTÉBio. n.d. "BLI Technology." Retrieved October 6, 2019 (https://www.moleculardevices.com/applications/biologics/bli-technology#gref).

Glassy, Mark C., John P. Tharakan, and Pao C. Chau. 1988. "Serum-Free Media in Hybridoma Culture and Monoclonal Antibody Production." *Biotechnology and Bioengineering* 32(8):1015–28.

González-Leal, I. J., L. M. Carrillo-Cocom, A. Ramírez-Medrano, F. López-Pacheco, D. Bulnes-Abundis, Y. Webb-Vargas, and M. M. Alvarez. 2011. "Use of a Plackett-Burman Statistical Design to Determine the Effect of Selected Amino Acids on Monoclonal Antibody Production in CHO Cells." *Biotechnology Progress* 27(6):1709–17.

Graf, L. H. and L. A. Chasin. 1982. "Direct Demonstration of Genetic Alterations at the Dihydrofolate Reductase Locus after Gamma Irradiation." *Molecular and Cellular Biology* 2(1):93–96.

Gregory Hamilton, W. and Richard G. Ham. 1977. "Clonal Growth of Chinese Hamster Cell Lines in Protein-Free Media." *In Vitro* 13(9):537–47.

HAM, R. G. 1965. "CLONAL GROWTH OF MAMMALIAN CELLS IN A CHEMICALLY DEFINED, SYNTHETIC MEDIUM." *Proceedings of the National Academy of Sciences of the United States of America* 53(2):288–93.

Hawkes, Percy William. 2015. "Fetal Bovine Serum: Geographic Origin and Regulatory Relevance of Viral Contamination." *Bioresources and Bioprocessing* 2(1):34.

Hogiri, Tomoharu, Hiroshi Tamashima, Akitoshi Nishizawa, and Masahiro Okamoto. 2018. "Optimization of a PH-Shift Control Strategy for Producing Monoclonal Antibodies in Chinese Hamster Ovary Cell Cultures Using a PH-Dependent Dynamic Model." *Journal of Bioscience and Bioengineering* 125(2):245–50.

Hong, Paula, Stephan Koza, and Edouard S. P. Bouvier. 2012. “Size-Exclusion Chromatography for the Analysis of Protein Biotherapeutics and Their Aggregates.” *Journal of Liquid Chromatography & Related Technologies* 35(20):2923–50.

Huang, Yao-Ming, WeiWei Hu, Eddie Rustandi, Kevin Chang, Helena Yusuf-Makagiansar, and Thomas Ryll. 2010. “Maximizing Productivity of CHO Cell-Based Fed-Batch Culture Using Chemically Defined Media Conditions and Typical Manufacturing Equipment.” *Biotechnology Progress* 26(5):1400–1410.

Jayapal, Karthik Pennadam, Katie Fraass Wlaschin, Wei-Shou Hu, and Miranda Gek-Sim Yap. 2007. “Recombinant Protein Therapeutics from CHO Cells - 20 Years and Counting.”

Jedrzejczak-Silicka, Magdalena. 2017. “History of Cell Culture.” in *New Insights into Cell Culture Technology*. InTech.

Jenkins, N., P. Castro, S. Menon, A. Ison, and A. Bull. 1994. “Effect of Lipid Supplements on the Production and Glycosylation of Recombinant Interferon-Gamma Expressed in CHO Cells.” *Cytotechnology* 15(1–3):209–15.

Kaisermayer, Christian, David Reinhart, Andreas Gili, Martina Chang, Per-Mikael Aberg, Andreas Castan, and Renate Kunert. 2016. “Biphasic Cultivation Strategy to Avoid Epo-Fc Aggregation and Optimize Protein Expression.” *Journal of Biotechnology* 227:3–9.

Kalil, S. J., F. Maugeri, and M. I. Rodrigues. 2000. *Process Biochemistry.* Vol. 35. Elsevier Science Pub. Co.

Keenan, Joanne, Karina Horgan, Martin Clynes, Indre Sinkunaite, Patrick Ward, Richard Murphy, and Finbarr O’Sullivan. 2018. “Unexpected Fluctuations of Trace Element Levels in Cell Culture Medium in Vitro: Caveat Emptor.” *In Vitro Cellular & Developmental Biology - Animal* 54(8):555–58.

Kim, Bong Gyun and Hong Woo Park. 2016. “High Zinc Ion Supplementation of More than 30 MM Can Increase Monoclonal Antibody Production in Recombinant Chinese Hamster Ovary DG44 Cell Culture.” *Applied Microbiology and Biotechnology* 100(5):2163–70.

Kim, Do Yun, Joon Chul Lee, Ho Nam Chang, and Duk Jae Oh. 2005. “Effects of Supplementation of Various Medium Components on Chinese Hamster Ovary Cell Cultures Producing Recombinant Antibody.” *Cytotechnology* 47(1–3):37–49.

Kobayashi, Scott D. and Frank R. DeLeo. 2013. “Staphylococcus Aureus Protein A Promotes Immune Suppression.” *MBio* 4(5):e00764-13.

Kunert, Renate and David Reinhart. 2016. “Advances in Recombinant Antibody Manufacturing.” *Applied Microbiology and Biotechnology* 100(8):3451–61.

Kuwae, Shinobu, Ichiko Miyakawa, and Tomohiro Doi. 2018. “Development of a Chemically Defined Platform Fed-Batch Culture Media for Monoclonal Antibody-Producing CHO Cell Lines with Optimized Choline Content.” *Cytotechnology* 70(3):939–48.

Li, Boyan, Paul W. Ryan, Bryan H. Ray, Kirk J. Leister, Narayana M. S. Sirimuthu, and Alan G. Ryder. 2010. “Rapid Characterization and Quality Control of Complex Cell Culture Media Solutions Using Raman Spectroscopy and Chemometrics.” *Biotechnology and Bioengineering* 107(2):290–301.

Li, Jincai, Chun Loong Wong, Natarajan Vijayasankaran, Terry Hudson, and Ashraf Amanullah. 2012. “Feeding Lactate for CHO Cell Culture Processes: Impact on Culture Metabolism and Performance.” *Biotechnology and Bioengineering* 109(5):1173–86.

Loos, Andreas, Johannes S. Gach, Thomas Hackl, Daniel Maresch, Theresa Henkel, Andreas Porodko, Duc Bui-Minh, Wolfgang Sommeregger, Gordana Wozniak-Knopp, Donald N. Forthal, Friedrich Altmann, Herta Steinkellner, and Lukas Mach. 2015. “Glycan Modulation and Sulfoengineering of Anti–HIV-1 Monoclonal Antibody PG9 in Plants.” *Proceedings of the National Academy of Sciences* 112(41):12675–80.

Lu, Connie, Dandan Liu, Hongbin Liu, and Paul Motchnik. 2013. “Characterization of Monoclonal Antibody Size Variants Containing Extra Light Chains.” *MAbs* 5(1):102–13.

Ludwig, Andreas, Jörg Tomeczkowski, and Gerlinde Kretzmer. 1992. “Influence of the Temperature on the Shear Stress Sensitivity of Adherent BHK 21 Cells.” *Applied Microbiology and Biotechnology* 38(3).

Mariani, E., A. R. Mariani, M. C. G. Monaco, E. Lalli, M. Vitale, and A. Facchini. 1991. “Commercial Serum-Free Media: Hybridoma Growth and Monoclonal Antibody Production.” *Journal of Immunological Methods* 145(1–2):175–83.

Möller, Johannes and Ralf Pörtner. 2017. “Model-Based Design of Process Strategies for Cell Culture Bioprocesses: State of the Art and New Perspectives.” in *New Insights into Cell Culture Technology*. InTech.

Nagle, S. C. and Jr. 1968. “Heat-Stable Chemically Defined Medium for Growth of Animal Cells in Suspension.” *Applied Microbiology* 16(1):53–55.

Noh, Soo Min, Seunghyeon Shin, and Gyun Min Lee. 2018. “Comprehensive Characterization of Glutamine Synthetase-Mediated Selection for the Establishment of Recombinant CHO Cells Producing Monoclonal Antibodies OPEN.” 8:5361.

Ogawa, Tadayuki and Nobutaka Hirokawa. 2018. “Multiple Analyses of Protein Dynamics in Solution.” *Biophysical Reviews* 10(2):299–306.

Ozturk, Sadettin S. and Bernhard O. Palsson. 1991. “Effect of Medium Osmolarity on Hybridoma Growth, Metabolism, and Antibody Production.” *Biotechnology and Bioengineering* 37(10):989–93.

Phogat, Sanjay, Baoshan Zhang, Ian A. Wilson, Gwo-Yu Chuang, Jiang Zhu, Laura M. Walker, Saidi H. Kapiga, Andrew B. Ward, Jason S. McLellan, Jean-Philippe Julien, Jason Gorman, Devan Diwanji, Zhi-Yong Yang, Marie Pancera, Sijy O’Dell, John R. Mascola, Peter D. Kwong, Reza Khayat, Ivelin Georgiev, Yongping Yang, Carole A. Bewley, William R. Schief, Jeffrey C. Boyington, Richard Wyatt, Doyung Lee, Robert Pejchal, Nikita Patel, Robert Louder, Chris Carrico, Stephanie Moquin, Barton F. Haynes, Jared Orwenyo, Kaifan Dai, Mallika Sastry, Noel E. Sam, Mark K. Louder, Mattia Bonsignori, Wayne C. Koff, James Arthos, Syed Shahzad-ul-Hussan, Dennis R. Burton, Young Do Kwon, Tongqing Zhou, Lai-Xi Wang, Gary J. Nabel, Stephen D. Schmidt, and John A. Crump. 2011. “Structure of HIV-1 Gp120 V1/V2 Domain with Broadly Neutralizing Antibody PG9.” *Nature* 480(7377):336–43.

Pörtner, Ralf, Jan-Oliver Schwabe, and Björn Frahm. 2004. “Evaluation of Selected Control Strategies for Fed-Batch Cultures of a Hybridoma Cell Line.” *Biotechnology and Applied Biochemistry* 40(1):47.

Princeton Instruments. n.d. *Raman Spectroscopy Basics*.

Puck, E. T., Steven J. Cieciura, and R. Robinson. n.d. *GENETICS OF SOMATIC MAMMALIAN CELLS HI. LONG-TERM CULTIVATION OF EUPLOID CELLS FROM HUMAN AND ANIMAL SUBJECTS*".

Raynal, Bertrand, Pascal Lenormand, Bruno Baron, Sylviane Hoos, and Patrick England. 2014. "Quality Assessment and Optimization of Purified Protein Samples: Why and How?" *Microbial Cell Factories* 13(1):180.

Reinhart, David, Lukas Damjanovic, Andreas Castan, Wolfgang Ernst, and Renate Kunert. 2018. "Differential Gene Expression of a Feed-Spiked Super-Producing CHO Cell Line." *Journal of Biotechnology* 285(May):23–37.

Reinhart, David, Lukas Damjanovic, Christian Kaisermayer, and Renate Kunert. 2015. "Benchmarking of Commercially Available CHO Cell Culture Media for Antibody Production." *Applied Microbiology and Biotechnology* 99(11):4645–57.

Ritacco, Frank V., Yongqi Wu, and Anurag Khetan. 2018a. "Cell Culture Media for Recombinant Protein Expression in Chinese Hamster Ovary (CHO) Cells: History, Key Components, and Optimization Strategies." *Biotechnology Progress* 34(6):1407–26.

Ritacco, Frank V., Yongqi Wu, and Anurag Khetan. 2018b. "Cell Culture Media for Recombinant Protein Expression in Chinese Hamster Ovary (CHO) Cells: History, Key Components, and Optimization Strategies." *Biotechnology Progress*.

Schnellbaecher, Alisa, Dennis Binder, Stephanie Bellmaine, and Aline Zimmer. 2019. "Vitamins in Cell Culture Media: Stability and Stabilization Strategies." *Biotechnology and Bioengineering* 116(6):1537–55.

Shen, Duan and Susan T. Sharfstein. 2006. "Genome-Wide Analysis of the Transcriptional Response of Murine Hybridomas to Osmotic Shock." *Biotechnology and Bioengineering* 93(1):132–45.

Siebertz, Karl, David van Bebber, and Thomas Hochkirchen. 2010a. *Statistische Versuchsplanung*.

Strober, Warren. 2001. "Trypan Blue Exclusion Test of Cell Viability." P. Appendix 3B in *Current*

Protocols in Immunology. Vol. Appendix 3. Hoboken, NJ, USA: John Wiley & Sons, Inc.

Trummer, Evelyn, Katharina Fauland, Silke Seidinger, Kornelia Schriebl, Christine Lattenmayer, Renate Kunert, Karola Vorauer-Uhl, Robert Weik, Nicole Borth, Hermann Katinger, and Dethardt Müller. 2006. "Process Parameter Shifting: Part II. Biphasic Cultivation—A Tool for Enhancing the Volumetric Productivity of Batch Processes Using Epo-Fc Expressing CHO Cells." *Biotechnology and Bioengineering* 94(6):1045–52.

Tye, Heather. 2004. "Application of Statistical 'Design of Experiments' Methods in Drug Discovery." *Drug Discovery Today* 9(11):485–91.

Urlaub, Gail and Lawrence A. Chasin. 1980. *Isolation of Chinese Hamster Cell Mutants Deficient in Dihydrofolate Reductase Activity*. Vol. 77.

Urlaub, Gail, Emmanuel K&s, Adelaide M. Carothers, and Lawerence A. Chasin. 1983. *Deletion of the Diploid Dihydrofolate Reductase Locus from Cultured Mammalian Cells*. Vol. 33.

van der Valk, Jan, Karen Bieback, Christiane Buta, Brett Cochrane, Wilhelm G. Dirks, Jianan Fu, James J. Hickman, Christiane Hohensee, Roman Kolar, Manfred Liebsch, Francesca Pistollato, Markus Schulz, Daniel Thieme, Tilo Weber, Joachim Wiest, Stefan Winkler, and Gerhard Gstraunthaler. 2018. "Fetal Bovine Serum (FBS): Past – Present – Future." *ALTEX* 35(1):99–118.

Walsh, Gary. 2018a. "Biopharmaceutical Benchmarks 2018." *Nature Biotechnology* 28(9):917–24.

Walsh, Gary. 2018b. "Biopharmaceutical Benchmarks 2018." *Nature Biotechnology* 36(12):1136–45.

Whelan, Jessica, Stephen Craven, and Brian Glennon. 2012. "In Situ Raman Spectroscopy for Simultaneous Monitoring of Multiple Process Parameters in Mammalian Cell Culture Bioreactors." *Biotechnology Progress* 28(5):1355–62.

Wlaschin, Katie F. and Wei-Shou Hu. 2006. "Fedbatch Culture and Dynamic Nutrient Feeding." *Advances in Biochemical Engineering/Biotechnology* 101:43–74.

Wurm, Florian. 2013. "CHO Quasispecies—Implications for Manufacturing Processes."

Processes 1(3):296–311.

Wurm, Florian M. and David Hacker. 2011. “First CHO Genome.” *Nature Biotechnology* 29(8):718–20.

Wyatt, Philip J. 1993. “Light Scattering and the Absolute Characterization of Macromolecules.” *Analytica Chimica Acta* 272(1):1–40.

Xie, Liangzhi and Daniel I. C. Wang. 1994. “Applications of Improved Stoichiometric Model in Medium Design and Fed-Batch Cultivation of Animal Cells in Bioreactor.” *Cytotechnology* 15(1–3):17–29.

Xie, Liangzhi and Daniel I. C. Wang. 1997. “Integrated Approaches to the Design of Media and Feeding Strategies for Fed-Batch Cultures of Animal Cells.” *Trends in Biotechnology* 15(3):109–13.

Xing, Zizhuo, Brian Kenty, Inna Koyrakh, Michael Borys, Shih Hsie Pan, and Zheng Jian Li. 2011. “Optimizing Amino Acid Composition of CHO Cell Culture Media for a Fusion Protein Production.” *Process Biochemistry* 46(7):1423–29.

Yao, Tatsuma and Yuta Asayama. 2017. “Animal-Cell Culture Media: History, Characteristics, and Current Issues.” *Reprod Med Biol* 16:99–117.

Yuk, Inn H., Stephen Russell, Yun Tang, Wei-Ting Hsu, Jacob B. Mauger, Rigzen P. S. Aulakh, Jun Luo, Martin Gawlitzek, and John C. Joly. 2015. “Effects of Copper on CHO Cells: Cellular Requirements and Product Quality Considerations.” *Biotechnology Progress* 31(1):226–38.

Zelena, Katerina, Nadine Eisele, and Ralf G. Berger. 2014. “Escherichia Coli as a Production Host for Novel Enzymes from Basidiomycota.” *Biotechnology Advances* 32(8):1382–95.

Zhang, Xiaowei, Isabel Fernandez Garcia, Lucia Baldi, David L. Hacker, and Florian M. Wurm. 2010. “Hyperosmolarity Enhances Transient Recombinant Protein Yield in Chinese Hamster Ovary Cells.” *Biotechnology Letters* 32(11):1587–92.

8. APPENDIX

8.1. List of Figures

8.2. List of Tables