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Technology development and medium optimization for perfusion cultures of a mAb producing CHO cell line

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Abstract

Continuous production streams of therapeutic proteins provide many advantages compared to batch-based processes and attract increasing importance in the biopharmaceutical industry. Within this work a 'pseudo perfusion' method based on a semi-continuous perfusion mode feasible in tubes and shaking flasks was established for a monoclonal antibody producing CHO (Chinese hamster ovary) cell line. Optimal shaking parameters regarding shaking speed, setting angle and vessel were determined. The optimal settings were used for different combinations of Cell Boosts, spiked into CDM4NS0 basal medium to determine the impact on cell metabolism and antibody productivity. This enabled the optimization of a perfusion medium using a novel approach based on Design of Experiments (DoE). For the selected CHO perfusion cultures it is suggested to spike the CDM4NS0 basal medium with 11.06% Cell Boost 1 and 19.90% Cell Boost 3. The Cell Boost spiked medium showed in a WAVE perfusion experiment at a perfusion rate of 1 RV/d a 1.8-fold higher peak cell concentration of 13.4×10^7 c/mL as well as a 2.0-fold higher peak titer of 1.4 g/L compared to the plain medium. By increasing the perfusion rate of the spiked medium to 2 RV/d the peak cell concentration was further increased to 22.5×10^7 c/mL reaching peak titers of up to 3.4 g/L in perfusion mode.

Keywords

Chinese hamster ovary (CHO) cells, recombinant protein expression, monoclonal antibody, media optimization, perfusion process

Zusammenfassung

Kontinuierliche Produktion von therapeutischen Proteinen bietet viele Vorteile gegenüber Batch Verfahren und gewinnt in der biopharmazeutischen Industrie zunehmend an Bedeutung. Im Rahmen dieser Arbeit wurde eine "Pseudoperfusions"-Methode auf Basis eines diskontinuierlichen Perfusionsmodus, der in Tubes und Schüttelkolben durchführbar ist, für eine Antikörper exprimierende CHO (Chinese hamster ovary) Zelllinie entwickelt. Optimale Schüttelparameter bezüglich Schüttelgeschwindigkeit, Einstellwinkel und Kultivierungsgefäß wurden ermittelt. Die optimalen Einstellungen wurden für das mit verschiedenen Kombinationen von Cell Boosts angereicherte CDM4NS0-Basalmedium verwendet, um den Einfluss auf den Zellmetabolismus und die Antikörperproduktivität zu bestimmen. Dies ermöglichte die Optimierung eines Perfusionsmediums unter Verwendung eines neuartigen Ansatzes basierend auf statistischer Versuchsplanung. Für die ausgewählten CHO-Perfusionskulturen wird vorgeschlagen, das CDM4NS0-Basalmedium mit 11,06% Cell Boost 1 und 19,90% Cell Boost 3 anzureichern. Das mit Cell Boost angereicherte Medium zeigte in einem WAVE-Perfusionsexperiment bei einer Perfusionsrate von 1 RV/d sowohl eine 1,8-fach höhere maximale Zellkonzentration von $13{,}4\times10^7$ c/mL als auch einen 2,0-fach höheren maximalen Titer von 1,4 g/L im Vergleich zum gewöhnlichen Medium. Durch Erhöhung der Perfusionsrate des angereicherten Mediums auf 2 RV/d wurde die maximale Zellkonzentration auf $22{,}5\times10^7$ c/mL weiter erhöht und maximale Titer von bis zu 3,4 g/L im Perfusionsmodus erreicht.

Schlagworte

Chinese hamster ovary (CHO) Zellen, rekombinante Proteinexpression, monoklonaler Antikörper, Medienoptimierung, Perfusionsprozess

Abbreviations

Abbreviations	Explanations
CB	Cell Boost
CC	Cell concentration
CHO	Chinese hamster ovary
CSPR	Cell specific perfusion rate
DHFR	Dihydrofolate reductase
DO	Dissolved oxygen
DoE	Design of Experiments
Gluc	Glucose
IgG	Immunoglobulin G
MSX	Methionine sulfoximine
MTX	Methotrexate
μ	Specific growth rate
mAb	Monoclonal antibody
PBS	Phosphate buffered saline
q_P	Specific productivity
RV	Reactor volume
STR	Stirred tank reactor
VCCD	Viable cumulative cell days
VCD	Viable cell days

Table of Contents

1. Introduction

1.1 Role of mammalian cells in biopharmaceutical industry

Mammalian expression systems are the preferred choice in the biopharmaceutical industry to manufacture complex molecules, such as monoclonal antibodies (mAbs), vaccines, hormones, growth factors and blood factors [1]. Compared to bacteria, fungi or yeasts, animal cells are more attractive for producing protein pharmaceuticals with post-translational modifications, especially glycosylation, similar to those produced in humans [2]. Most marketed biopharmaceutical products have been produced in Chinese hamster ovary (CHO) cells, murine myeloma cells (NS0 and Sp2/0), Human Embryonic Kidney 293 (HEK 293) cells, and baby hamster kidney (BHK) cells [3]. However, CHO cells are the most widely used mammalian host for the production of recombinant biopharmaceutical proteins, most of them being mAbs [4].

First a high-producing cell line has to be established by introducing DNA containing the gene of interest into the host cell and selecting stable transfected cells with the integrated DNA in the chromosome. After identification of the best clone, medium optimization and fine-tuning of cultivation parameters such as dissolved oxygen, pH or temperature can be performed [5].

Especially for the production of therapeutic proteins that are applied in high dosages, such as mAbs, the enhancement of productivity is very important to make the biotechnological process profitable. Besides new strategies in cell engineering or vector optimization, adapting mammalian cells to serum-free medium was a big improvement in the development of production cell lines. The use of serum-free medium reduces the operating costs and process variability. Furthermore a potential source of infectious agents can be eliminated [6]. Due to the use of serum-free medium, it is possible to grow mammalian cells in suspension cultures. This offers many advantages compared to adherent cell cultures, for example increasing the simplicity and homogeneity of the culture and facilitating scale-up [7].

1.2 Recombinant monoclonal antibody production with CHO cells

Compared to other biopharmaceutical products therapeutic mAbs have become the dominant product class within the biopharmaceutical market [8]. The market demand for therapeutic mAbs keeps increasing and CHO cells are the predominant host used to produce them [9].

Since the isolation of the first Chinese hamster ovary (CHO) cells from a Chinese hamster by Theodor Puck in 1957 [10], they have become the most commonly used

mammalian cells for the production of therapeutic proteins in biopharmaceutical industry. There are several reasons for the popularity of CHO cells. First of all, due to the long term usage of these cells they have been demonstrated as safe hosts free of human viruses. Therefore the approval to market of a new therapeutic protein may be easier [11]. Furthermore CHO cells grow as suspension culture in serum free medium which is preferred for a large-scale culture in bioreactors. Another important issue that makes these cells beneficial expression hosts is their ability to yield human compatible post-translational modification (PTM) of secreted proteins. Proper folding including the formation of disulphide bonds and correct PTMs, like glycosylation patterns, are required for biological activity, protein longevity and to reduce safety concerns [12].

Mammalian production platforms show often a low specific productivity (qP), but this problem can be overcome by gene amplification. Two powerful gene amplification systems used in CHO cells are the dihydrofolate reductase (DHFR) system and the glutamine synthetase (GS) system [13]. For example, DHFR-negative CHO strains (e.g. CHO-DG44), are transfected with recombinant DNA consisting of the gene of interest closely linked to the gene for DHFR. Growing these transfected cells under selective conditions using methotrexate (MTX) in the medium, which binds to DHFR and thereby inhibiting the enzyme, enables the generation of high-expressing cells. The GS system uses a GS-knockout CHO-K1 strain and has a similar principle as the DHFR system. In this setting, methionine sulphoximine (MSX), which binds to the GS enzyme and prevents the production of glutamine, is used as selective reagent instead of MTX [14].

1.3 Bioprocess engineering techniques in animal cell culture

The demand for biopharmaceutical products increased significantly over the last decades [15, 16]. Therefore the development of production processes that can deliver sufficient amounts of product to meet market demands at acceptable prices is absolutely essential. Beside improvements in the formulation of the culture media or the optimization of process parameters such as pO_2, pH and temperature [17], the choice of a suitable bioreactor system as well as the selected operation mode is of major importance. There exist a couple of different bioreactor technologies for animal cell culture with their pros and cons.

Several different systems have been invented to cultivate cells. The most commonly applied bioreactors are stirred tank reactors (STRs), airlift bioreactors and wave-mixed bag bioreactors. Among the different available systems, the STR is still one of the most prominent systems in the large-scale cell culture production of recombinant proteins and antibodies due to its simplicity, and the ease of monitoring and scale-up [18]. One drawback are high shear forces caused by agitation and gas sparging which provoke cell

stress, resulting in decreasing viabilities. This problem can be overcome in an airlift bioreactor due to the lack of an impeller and reduced exposure of cells to bubble burst. Although these two systems are commonly used for suspension cell culture, the same bioreactors can also be operated with anchorage-dependent cells on microcarriers. However for strictly adherent cell lines cultivated on microcarriers high-density/low-volume systems like fixed or fluidized-bed bioreactor are even more beneficial [19]. In both systems the immobilized cells are retained in a cylindrical vessel through which the medium is recirculated, thus agitation and aeration are separated from the carriers. Therefore lower shear forces are present in these systems, resulting in higher cell densities with a lower concentration of free cells in suspension compared to microcarrier culture in stirred tanks.

An alternative to stainless-steel bioreactors is the use of disposable bioreactors which are gaining increasing popularity for industrial applications and have become widely used in mammalian cell bioprocesses [20]. They offer many advantages, like low initial investment costs, high flexibility, reduced validation, low risk of cross-contamination and eliminating time and costs for cleaning and sterilization. But there are also critical issues concerning limited material strength, secretion of leachables and extractables, higher running costs, increase of solid waste as environmental burden, restricted availability of reliable, single-use sensors and extensive training of staff with rising culture volume to guarantee process safety. There are wave-mixed, orbitally shaken or stirred disposable bioreactors from milliliter up to cubic meter scale available. One of the most famous single-use bioreactors is the wave-mixed bag bioreactor. First described in the late 1990s [21], it has become a common devices in modern biotechnological processes. An inflated disposable plastic bag containing the cell suspension is placed on a rocking platform, causes a wave-induced agitation to accomplish oxygen transfer and mixing. The rocking mechanism is defined as rocking rate and rocking angle. The process control of temperature, pH and DO is performed by optical sensors. Low shear stress, high cell density and adequate productivity up to pilot scale are only some of the reasons for the popularity of this disposable bioreactor [22].

1.4 Operation modes in animal cell culture

Several operation modes are available for the cultivation of mammalian cells, known as batch, fed-batch and continuous fermentation.

1.4.1 Batch process

In a batch process a certain volume of medium and inoculum is added into the reactor at the starting point. During the process no additional supplements are added, which means that the reactor volume remains constant.

1.4.2 Fed-batch process

The fed-batch is characterized by periodical or continuous addition of fresh medium or specific feed concentrates, which provide the required nutrients for cell growth. With a fed-batch culture, higher cell and product concentrations can be achieved due to the fact that feed strategies replenish depleted nutrients and optimize the growth and productivity of the cells. In a fed-batch process the duration is prolonged resulting in accumulation of more product and mAb titers beyond 10 g/L are becoming reality [23].

1.4.3 Continuous process

A continuous process is defined by a flow of medium in and a flow of harvest out of the bioreactor. Usually the rates of mass input and output are equal to keep the volume in the system constant. While in a chemostat the harvest contains cells, in a perfusion process the cells are retained. A chemostat allows to control the specific growth rate of the cells through the dilution rate, which is defined as the feed rate per reactor volume. An important risk is the washout of the cells if the dilution rate exceeds the maximum growth rate of the culture [24]. However the main advantage of this process mode is to reach a steady state after an initial start-up period, where all culture parameters such as culture volume, cell density, nutrient and metabolite concentrations, pH or dissolved oxygen remain constant over time. Thus, chemostat culture offers constant physicochemical conditions and therefore emerges as relevant tool for studying the effect of certain parameter shifts and determine kinetics [25]. Nevertheless, the application of a chemostat for the production of biopharmaceuticals in industry is limited, as the main goal here is reaching high cell densities coupled with high product titers. Thus a more commonly used continuous approach is the application of perfusion cultures, where the cells are retained or recycled back into the bioreactor.

The main benefits of using perfusion systems in cell culture are to increase culture viability by constantly feeding fresh medium and removing toxic by-products, enhance volumetric productivity by increasing the cell concentration in the bioreactor and improve product quality by reducing the residence time of the product under cultivation. Therefore perfusion processes are more convenient for the production of unstable glycoproteins compared to fed-batch processes. It has been shown that a semicontinuous perfusion mode promotes more fully glycosylated proteins than fed-batch cultures [26].

Even though, perfusion cultures are technical more complex, labour intensive and more sensitive to contaminations compared to batch or fed-batch mode, there is a growing interest in implementing such processes in biopharmaceutical industry. One challenge here is the interface to downstream processing, which consists usually of multiple batch

unit operations. Therefore a shift to an integrated continuous processing in biologics manufacturing is recommended to solve this problem. In recent years several studies for implementing fully continuous manufacturing in biopharmaceutical industry had been carried out [27-30]. By changing large-scale batch facilities to smaller, cost-efficient multipurpose plants designed for continuous bioprocessing will save high capital investment costs and enables highly standardized platforms for the production of all kinds of drugs, regardless of the volumetric demand of the product. An increase in the production capacity can be achieved by the addition of several parallel production lines instead of volumetric scale-up [31]. Due to the elimination of single batch processes in continuous manufacturing, a new lot definition need to be considered, which can be simply implemented through the production time period.

A challenging task is to implement a robust cell separation technique in perfusion cultures. There are several methods for the cell retention available, which are based on different physical principles like filtration or enhanced sedimentation [32]. Filtration devices, such as external tangential flow filtration (TFF) or alternating tangential flow (ATF) and internal perfusion floating filter are used frequently, but share the common risk of membrane fouling and clogging. This problem can be overcome by using sedimentation devices as they have the advantage of no physical barriers. Achieving enhanced sedimentation by centrifugation has the drawback of moving parts and potential mechanical problems during long periods of operation. Acoustic filtration, where the cells are trapped in an acoustic field, might be an alternative, but shows a limited maximum cell concentration of about 6×10^7 c/mL [33]. Beside the cell-separation devices, a limitation of oxygen and nutrient supply can restrict the maximal achievable cell density.

Most current perfusion systems pump cells through an external separation device and back to the bioreactor and thereby subject them to damaging shear forces and oxygen depletion. By using an internal perfusion floating filter, as it was done in this study, this problems can be overcome and maintaining sterility becomes easier. Depending on the cell line and separation device, cell densities of $20\text{-}200\times10^6$ c/mL have been described in perfusions performed in a Wave Bioreactor [34, 35]. A strategy to improve the overall cell culture viability and remove dead cells from the system is the implementation of cell bleeds [36].

2. Aim of the study

First, a platform is developed to mimic perfusion cultures in small scale by taking advantage of shake tubes, which are operated in a batch-refeed mode. Such batch-refeed or 'pseudo perfusion' cultures rely on a daily medium exchange to supply the cultures with nutrients and remove toxic by-products. Shaking parameters, such as the speed and setting angle, are adapted to this semi-continuous production of a recombinant antibody in a perfusion-like mode.

Second, the established pseudo perfusion model is used in a Design of Experiment (DoE) screening of a basal medium mixed with various combinations of Cell Boosts (CBs) in order to select the best performing CBs at optimal ratios for perfusion cultivation.

Third, the best combination and ratio of CBs is compared to the non-spiked basal medium by running them as perfusion cultures under controlled conditions in a WAVE Bioreactor.

Figure 1 shows a flow chart to illustrate the purpose of this study.

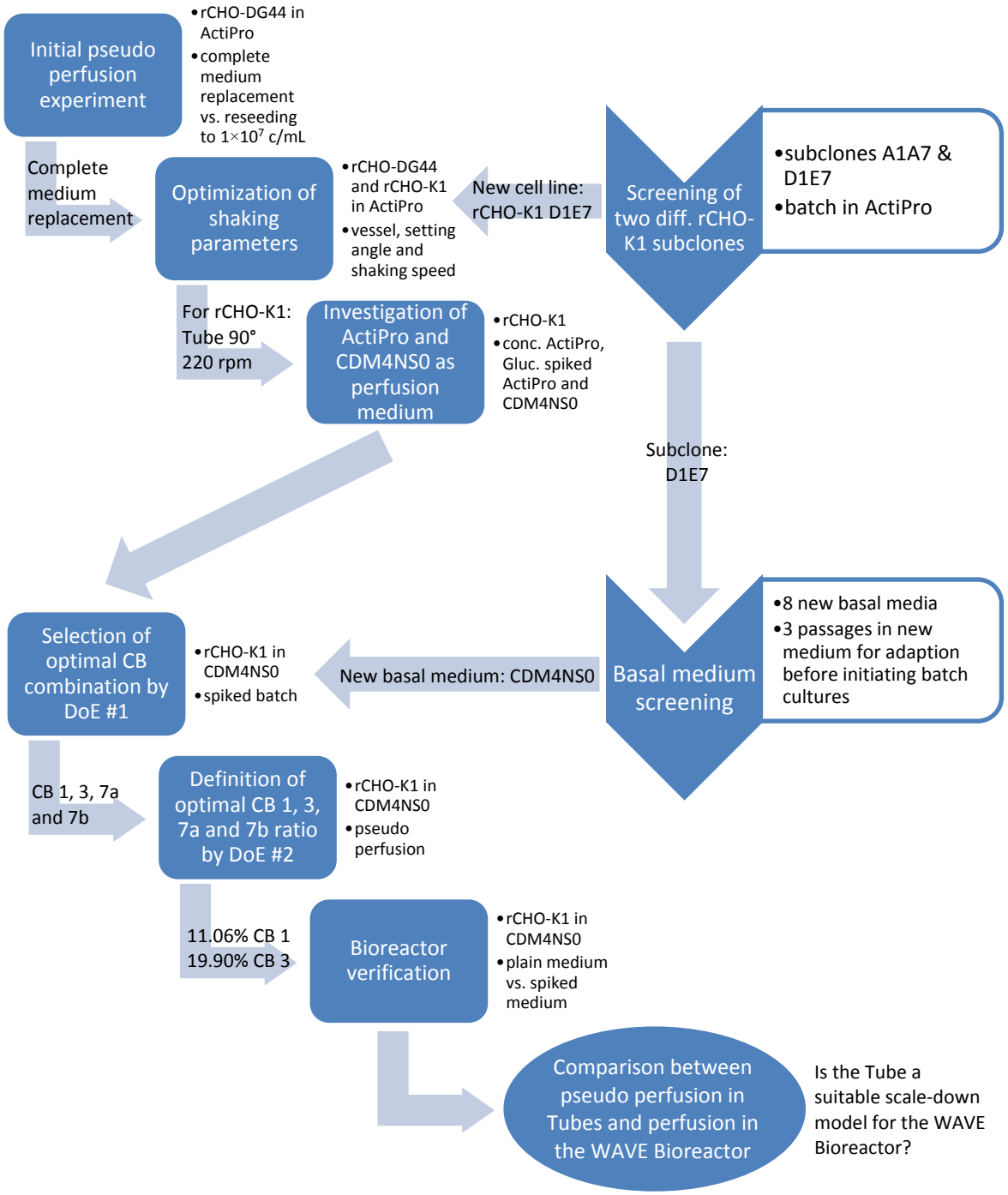


Figure 1: Flow chart of the single steps during the perfusion development in this study

3. Material and Methods

3.1 Material

3.1.1 Cell lines

Two different cell lines were used for the herein described experiments.
The first one was a recombinant CHO-DG44 cell line expressing an IgG antibody (rCHO-DG44). Basal medium was ActiPro (GE Healthcare), a chemically defined cell culture medium rich in nutrients. Routine culture was in basal medium supplemented with 6 mM L-glutamine and 30 nM methotrexate (MTX). During batch or perfusion cultivation, the basal medium was supplemented with 6 mM L-glutamine and Cell Boost (CB) 7a and 7b, when indicated.
The second cell line was a recombinant CHO-K1 cell line expressing also an IgG antibody (rCHO-K1). Basal media were ActiPro and CDM4NS0 (GE Healthcare), both media being rich in nutrients and chemically defined. Cells were routinely cultured in the basal media supplemented with 37.5 µM methionine sulphoximine (MSX). During batch or perfusion cultivation, the basal media were supplemented with CB 1, 2, 3, 4, 5, 6, 7a or 7b only if applicable (Table 1).

Table 1: Summary of cell lines, basal media, selective reagents and supplements

Cell line	Basal media	Selective reagent	Supplements
rCHO-DG44	ActiPro	MTX (30 nM)	L-glutamine (6 mM) CB 7a and 7b
rCHO-K1	ActiPro CDM4NS0	MSX (37.5 µM)	CB 1, 2, 3, 4, 5, 6, 7a or 7b

3.1.2 Instruments and consumables

All glassware was depyrogenated and sterilised. All plastic ware was purchased sterile and cell culture grade. Reagents were sterile-filtered, if desired, using a 0.2 µm pore sized filter, autoclaved or purchased sterile. The lab ware used in the experiments are described in Table 2: Instrumentation, Table 3: Software packages, Table 4: Materials, Table 5: Chemicals and cell culture media and Table 6: Prepared buffers.

Table 2: Instrumentation

Instrument	Specification	Manufacturer
Analytical scale	Max: 4200 g d: 0.01 g	Sartorius
BioProfile 100 Plus	Quantification of pH, glucose, lactate, glutamine, glutamate, ammonium	Nova Biomedical
DOOPT-Probe	optical DO detector with miniature fiber-optic microprobes	GE Life Science

Instrument	Specification	Manufacturer
Hareus oven	Inside: 610/672/514 (BxHxT, mm)	Thermo scientific
Laboratory Centrifuge	Heraeus Megafuge 16	ThermoFisher Scientific
Laminar flow hood	aseptic work area	Thermo Scientific MSC-Advantage™
Microscope DM IL LED	S40/0,45 + 10x/0,22 lens	Leica Microsystems
Octet QK System	Quantification of mAb titer	Pall FortéBio
Osmomat 030	Quantification of medium, CB and cell culture supernatant osmolality	Gonotec GmbH
pH meter	inoLab pH7110	WTW
Shaking incubator	80% humidity, 7% CO_2, 37°C, 50 mm orbital throw *	Kuhner Shaker GmbH
Steam Sterilizer	Type 500E	Thermo scientific
Tubing pump	Ecoline VC-360	ISMATEC
Ultrapure water System	Ultra Clear UV	SG
Vi-CELL XR	Quantification of cell concentration and viability	Beckman Coulter
Vortex mixer	Vortex-Genie 2	Scientific Industries, U.S.
Waterbath	1003	GFL
WAVE Bioreactor 20/50	24 rpm, angle 9°, 37°C	GE Life Science
WAVEPOD II	controlled gas-supply, pH = 7	GE Life Science

* For rpm specification see description of the different experiments in 3.2 Methods

Table 3: Software packages

Instrument	Software packages
Vi-CELL XR	ViCELL XR 2.04
Octét QK System	Octét Data Acquisition 6.4 Octét Data Analysis 6.4

Table 4: Materials

Material	Specification	Manufacturer
0.22 µm sterile filter	Rotilabo®-syringe filters, PES, sterile	Carl Roth GmbH + CO.KG
1.5 mL micro centrifuge tubes	Disposable, non-sterile	VWR International
10 mL centrifuge tubes	Polystyrene, sterile	Thermo scientific
10 mL, 20 mL, 30 mL, 50 mL syringe	Omnifix® Solo, sterile	B Braun
2.0 mL screw cap micro vial	Conical bottom, with support skirt, sterile	Sarstedt
50 mL centrifuge tube	Conical bottom, sterile	Greiner Bio – One
50 mL centrifuge tube	Conical bottom, with support skirt, sterile	Greiner Bio – One
96 MicroWell plate	Black	Nunc™
Aluminium foil	-	-

Material	Specification	Manufacturer
Cellbag bioreactor (2 L; pHOPT and perfusion version)	CB0002L10-14	GE Life Science
Dip and Read Biosensors	Protein A, For quantitation	fortéBIO
Erlenmeyer shake flask (1 L)	200 – 300 mL working volume	Corning
Erlenmeyer shake flask (125 mL)	15 mL working volume	Corning
Gloves	Latex disposable gloves	VWR
Measuring vessels for osmolality	For OSMOMAT 3000/030/010	Gonotec GmbH
Millipore® Stericup™ filter units	150 mL or 500 mL; 0.22 µm PES; Fast flow, low protein binding	Millipore, U.S.
Mini bioreactor tube (50 mL)	10 mL (perfusion), 30 mL (batch) working volume	Corning
Pipet boy	CellMate II	Matrix Technologies Corporation
Pipette tips (200 µL, 1000 µL)	graduated	Gilson Inc.
Pipettes	Gilson pipetman® neo P1000N 10-1000 µL Gilson pipetman® neo P200N 20-200 µL Gilson pipetman® neo P100N 10-100 µL Gilson pipetman® neo P20N 2-20 µL	Gilson Inc.
Schott bottle (100mL, 250 mL, 500 mL, 1 L, 10 L)	Glass, depyrogenated	Duran
Serological pipettes (2 mL, 5 mL, 10 mL, 25 mL, 50 mL)	Sterile, disposable	Corning® Costar®
Vi-CELL Sample vails	For all Vi-CELL models	Beckman Coulter

Table 5: Chemicals and cell culture media

Substance	Specification	Manufacturer
Antifoam C Emulsion	Diluted 1:100 with ultrapure H_2O	Sigma-Aldrich
Coulter Clenz cleaning Agent	-	Beckman Coulter
D-(+)-Glucose monohydrate	250 g/L stock solution Dissolved in ultrapure H_2O	Sigma-Aldrich
Di-Sodium hydrogen phosphate dehydrate	Cat. No. 4984.3	Carl Roth GmbH
HyClone ActiPro	Cat. No. SH31037.02 with Poloxamer 188 without L-Glutamine	GE Life Science
HyClone CDM4NS0	Cat. No. SH30578.02 Recommended additions: + 3.2 g/L Sodium Bicarbonate + 0.5 g/L Poloxamer 188	GE Life Science

Substance	Specification	Manufacturer
	without L-Glutamine	
HyClone CDM4CHO	Cat. No. SH30558.02 without L-Glutamine	GE Life Science
HyClone HyCell CHO	Cat. No. SH30934.01 with Poloxamer 188 without L-Glutamine	GE Life Science
HyClone CDM4PerMAb	Cat. No. SH30871.02 without L-Glutamine	GE Life Science
HyClone SFM4CHO	Cat. No. SH30548.02 + 2.2 g/L Sodium Bicarbonate without L-Glutamine	GE Life Science
HyClone CDM4HEK293	Cat. No. SH30858.02 without L-Glutamine	GE Life Science
HyClone CDM4MAb	Cat. No. SH30801.02 + 6.0mM L-Glutamine	GE Life Science
HyClone SFM4MAb	Cat. No. SH30513.02 + 6.0mM L-Glutamine	GE Life Science
HyClone Cell Boost 1	Cat. No. SH30584.02 without L-Glutamine	GE Life Science
HyClone Cell Boost 2	Cat. No. SH30596.01 without L-Glutamine	GE Life Science
HyClone Cell Boost 3	Cat. No. SH30825.01 without L-Glutamine	GE Life Science
HyClone Cell Boost 4	Cat. No. SH30857.01 without L-Glutamine	GE Life Science
HyClone Cell Boost 5	Cat. No. SH30865.01 without L-Glutamine	GE Life Science
HyClone Cell Boost 6	Cat. No. SH30866.01 without L-Glutamine	GE Life Science
HyClone Cell Boost 7a	Cat. No. SH31026.07 without L-Glutamine	GE Life Science
HyClone Cell Boost 7b	Cat. No. SH31027.01 without L-Glutamine	GE Life Science
Hypochloric acid 25%	Cat. No. 1.00316.1000	Merck
Isopropanol	Purity ≥ 99.5%	Carl Roth GmbH
Kolliphor P188	Cat. No. K4894 Synonym: Poloxamer 188 10% solution Dissolved in ultrapure H_2O	Sigma Aldrich
L-Glutamine	250 mM stock solution Dissolved in ultrapure H_2O	Merck
MSX	Methionine sulfoximine Stock: 100 mM	Sigma-Aldrich
MTX	Methotrexate Stock: 960 µM	Sigma-Aldrich
Polyphosphate buffered solution (PBS) Dulbecco	w/o Ca^{2+} w/o Mg^{2+} low endotoxin, sterile	Biochrom GmbH
Potassium chloride	Cat. No. HN02.3	Carl Roth GmbH
Potassium dihydrogen phosphate	Cat. No. 104873	Merck
Sodium bicarbonate	Cat. No. S5761 Synonym: Sodium hydrogen carbonate	Sigma-Aldrich

Substance	Specification	Manufacturer
	4% and 8% solution Dissolved in ultrapure H_2O	
Sodium chloride	Cat. No. P029.3	Carl Roth GmbH
Sodium hydroxide pellets	Cat. No. 28245.367	VWR International
Trypan blue 0.4% solution	0.2 µm filtrated, liquid	Sigma Aldrich
Tween 20	Cat. No. 9127.1	Carl Roth GmbH

Table 6: Prepared buffers

Buffer	Preparation
10x PBS (5 Liter)	57.5 g $Na_2HPO_4 \cdot 2\ H_2O$ 10 g KH_2PO_4 10 g KCl 400 g NaCl Fill up to 5000 mL with RO-H_2O
PBST (PBS with 0.1% Tween) for Octét (100 mL)	10 mL PBS 10x Fill up to 100 mL with RO-H_2O 100 µL TWEEN 20 0.2 µm filtrated

3.2 Methods

3.2.1 Routine cell culture

Cells were seeded at 2×10^5 c/mL in either ActiPro or CDM4NS0 supplemented with 30 nM MTX (rCHO-DG44) or 37.5 µM MSX (rCHO-K1). The working volume was 15 mL in a 125 mL shake flask incubated in a Kuhner shaker at 37°C, 80% relative humidity, 7% CO_2 and 150 rpm at 50 mm orbital shaking amplitude. The cells were split every 3-4 days, twice per week.

The calculation for the cell splitting step was done with equation (1) and (2).

$$V_{cells} = \frac{V_{intended} * X_{intended}}{X_{sample}} \quad (1)$$

$$V_{medium} = V_{intended} - V_{cells} \quad (2)$$

V_{cells} volume of cell suspension [mL]
$V_{intended}$ intended volume after dilution [mL]
$X_{intended}$ intended cell density [c/mL]
X_{sample} cell density of parental cell suspension [c/mL]
V_{medium} added medium for dilution [mL]

3.2.2 Medium and Cell Boost preparation

The different HyClone media except ActiPro and CDM4NS0 were purchased as liquid medium ready for use. ActiPro and CDM4NS0 media were purchased as powder and were reconstituted according to the recommendation of the manufacturer. A description of the preparation can be found in Table 7 and Table 8.

Table 7: Preparation protocol for HyClone ActiPro powder medium

ActiPro	
1)	Use a depyrogenated flask, stirrer and lid.
2)	Determine empty weight.
3)	Fill the vessel to 90% of the final volume with ultrapure water.
4)	While stirring, add **22.36 g/L ActiPro powder**.
5)	Stir on a magnetic stirrer until no clumps of powder remain. Note: At this step the solution will remain cloudy.
6)	Add **6.5 mL/L** of a **5N NaOH** solution Stir for 5 minutes. Note: At this point the solution should become clear.
7)	Add **1.8 g/L sodium bicarbonate** ($NaHCO_3$) into the vessel and mix until dissolved.
8)	Check pH: If necessary: adjust to pH 6.90 – 7.35
9)	Stir for 20 minutes to solubilize all components.
10)	Fill up to the final volume with ultrapure water and mix for additional 10 min.
11)	**Final pH: 6.90 – 7.55**
12)	**Final osmolality: 270 – 330 mOsmol/kg**
13)	Filter the solution with a 0.2 µm filter unit
14)	Store in a dark place at 4°C.

Table 8: Preparation protocol for HyClone CDM4NS0 powder medium

CDM4NS0	
1)	Use a depyrogenated flask, stirrer and lid.
2)	Determine empty weight.
3)	Fill the vessel to 90% of the final volume with ultrapure water.
4)	While stirring, add **21.5 g/L CDM4NS0 powder**.
5)	Stir on a magnetic stirrer for 20 min until dissolved.
6)	Add **0.5 g/L poloxamer 188** and **3.2 g/L sodium bicarbonate** ($NaHCO_3$) into the vessel and mix for at least 20 min.
7)	Check pH: If necessary: adjust to pH 7.00 – 7.40
8)	Fill up to the final volume with ultrapure water and mix for additional 10 min.
9)	**Final pH: 7.00 – 7.40**
10)	**Final osmolality: 280 – 310 mOsmolol/kg**
11)	Filter the solution with a 0.2 µm filter unit
12)	Store in a dark place at 4°C.

All CBs were provided as powder by the manufacturer. CB 1 to 6 were prepared according to protocols developed in the working group.

CB 1, 2 and 4 were hydrated as 10% stock solutions in deionized water and subsequently the pH was adjusted to 9.5 – 10.0 with 5N NaOH to solubilize the formulation. To ensure the solubilisation of all components the solution was mixed for 20 minutes. Afterwards the pH was adjusted to 7.2 – 7.4 with 5N HCl.
CB 3, 5 and 6 were solubilized as 5% stock solutions directly in deionized water at 28°C. The pH was adjusted to 7.2 – 7.4 with 5N NaOH.
The reconstitution of CB 7a and 7b powder was done according to the recommendation of the manufacturer. A description of the preparation can be found in Table 9 and Table 10.

Table 9: Preparation protocol for HyClone Cell Boost 7a powder supplements

Cell Boost 7a	
1)	Use a depyrogenated flask, stirrer and lid.
2)	Determine empty weight.
3)	Fill the vessel to 80% of the final volume with ultrapure water.
4)	While stirring, add **181.04 g/L Cell Boost 7a powder**.
5)	Stir on a magnetic stirrer for 30 min until no clumps of powder remain. Note: At this step the solution will remain cloudy.
6)	Add **18.6 mL/L** of a **5N NaOH** solution. Stir for 60 minutes. Note: At this point the solution should become clear.
7)	Check pH: If necessary: adjust to pH 6.60 – 6.80 Stir for additional 60 minutes to solubilize all components.
8)	Fill up to the final volume with ultrapure water and mix for additional 10 min.
9)	**Final pH: 6.60 – 6.80**
10)	**Final osmolality: 247 – 303 mOsmol/kg (diluted 1:5)**
11)	Filter the solution with a 0.2 µm filter unit
12)	Store in a dark place at 4°C.

Table 10: Preparation protocol for HyClone Cell Boost 7b powder supplements

Cell Boost 7b	
1)	Use a depyrogenated flask, stirrer and lid.
2)	Determine empty weight.
3)	Fill the vessel to 75% of the final volume with ultrapure water.
4)	While stirring, add **94.6 g/L Cell Boost 7b powder**.
5)	Stir on a magnetic stirrer for 30 min until no clumps of powder remain. Note: At this step the solution will remain cloudy.
6)	Add **160.5 mL/L** of a **5N NaOH** solution. Stir for 60 minutes. Note: At this point the solution should become clear.
7)	Check pH: If necessary: adjust to pH 11.0 – 11.4 Stir for additional 60 minutes to solubilize all components.
8)	Fill up to the final volume with ultrapure water and mix for additional 10 min.
9)	**Final pH: 11.0 – 11.4**
10)	**Final osmolality: 218 – 266 mOsmol/kg (diluted 1:5)**
11)	Filter the solution with a 0.2 µm filter unit
12)	Store in a dark place at 4°C.

3.2.3 Cell cultivation

3.2.3.1 Batch experiments

All batches were performed in 50 mL SpinTube Bioreactors incubated in a Kuhner shaker at 37°C, 80% relative humidity, 7% CO_2 and 220 rpm at 50 mm orbital shaking amplitude. Shake tubes were inoculated on a Friday (day 0) at the conditions defined in Table 11. Sampling of cell concentration, viability, antibody concentration and metabolites (i.e. glucose, lactate, glutamine, glutamate and ammonium) were performed directly after inoculation, on day 3 and then every day onwards. Batches were terminated once the viability dropped below 60%. At all times, glucose concentrations were monitored throughout the batch culture. If the glucose concentration dropped below 3 g/L it was increased to 6 g/L by addition of a concentrated (250 g/L) glucose solution.

Table 11: Settings for the batch experiments

Parameter	Settings
Seeding cell concentration	3×10^5 c/mL
Cell line	rCHO-K1
Medium	ActiPro or CDM4NS0
Supplements	CB 1–6, 7a and 7b
Vessel and working volume	SpinTube bioreactors (30 mL)
Angle of tubes	90°
Rotation rate	220 rpm
Orbital shaking amplitude	50 mm

3.2.3.2 Pseudo perfusion experiments

Pseudo perfusion was performed in 50 mL SpinTube Bioreactors or 125 mL Erlenmeyer shake flasks incubated in a Kuhner shaker at 37°C, 80% relative humidity, 7% CO_2 and different rotation rates (Table 12) at 50 mm orbital shaking amplitude. Shake tubes and flasks were inoculated on a Monday (day 0) at the conditions defined in Table 12 and sampled daily for 11 days. To daily exchange the medium, the vessels were centrifuged at 188×g for 10 min. While the shake tubes were directly centrifuged, the shake flasks suspension was transferred to a fresh centrifuge tube each time. The supernatant was then discarded, the pellet gently re-suspended by tipping the tube, and filled up with the respective amount of fresh medium, pre-warmed to 37°C. Thus, 100% of the reactor volume was exchanged per day (1 RV/d). The removed spent medium was used to measure the metabolites (BioProfile 100Plus) as well as antibody concentration (Octét).

To avoid large volume losses, only 0.5 mL cell suspension was sampled before centrifugation to determine cell size, concentrations and viability with the Vi-CELL.
The centrifugation for medium exchange was performed only if there was no excessive accumulation of cell debris at the vessel wall. In order to avoid dead cells from the vessel wall to enter the culture, the vessel was changed if needed.
The cultures were terminated after 11 days or earlier if the viability dropped below 60%.

Table 12: Settings for the pseudo perfusion experiments

Parameter	Settings
Seeding cell concentration	1×10^7 c/mL
Cell lines	rCHO-K1 or rCHO-DG44
Medium	ActiPro or CDM4NS0
Supplements	CB 1–6, 7a and 7b
Vessel and working volume	SpinTube bioreactors (10 mL) Erlenmeyer shake flasks (15 mL)
Angle of tubes	90° or 45°
Rotation rate [rpm]	150, 200, 220, 250 or 300
Orbital shaking amplitude	50 mm

3.2.3.3 Perfusion in a WAVE Bioreactor

The best performing pseudo perfusion strategy was investigated in a bioreactor under controlled conditions. For this purpose, the rCHO-K1 cell line was perfused in plain CDM4NS0 and compared to the optimized CDM4NS0 perfusion medium (CDM4NS0 spiked with 11.06% CB1 and 19.90% CB3).
Since the feed-spiked CDM4NS0 contains a higher glucose concentration, the glucose concentration of plain CDM4NS0 was adjusted accordingly to 16.5 g/L by addition of a 250 g/L stock solution.
The WAVE Bioreactor 20/50 and a WAVEPOD II combined with a 2 L Cellbag compatible for perfusion (CB0002L10-14, pHOPT and perfusion version) was applied as bioreactor. Figure 2 shows an illustration of the complete experimental setup and Table 13 summarizes a detailed description of the ports of the used cell bag. The standard settings were 37°C, 24 rpm at an angle of 9°, pH 7.00 and a DO of 30%.
The pH was controlled by CO_2 concentration in the gas phase of the headspace and by addition of a 4% $NaHCO_3$ solution. Since there was a high consumption of the base in the first run, an 8% $NaHCO_3$ solution was used as base afterwards.
Cells were inoculated at a starting cell concentration of 1×10^6 c/mL using a working volume of 600 mL plain CDM4NS0. Therefore the required amount of cells were centrifuged and re-suspended in completely fresh CDM4NS0 medium. The current cell specific growth rate was used to predict the future cell concentration for the next day. Based on the predicted cell concentration and the consumption rate of glucose the

demand of glucose for the next day was determined. One day before glucose level would drop to zero, the working volume was adjusted to 500 mL by an appropriate perfundate sample and perfusion was initiated applying a rate of 1 RV/d. When the cells reached their maximum cell concentration of 7.5×10^7 c/mL with plain CDM4NS0 medium and 13.4×10^7 c/mL with CB-spiked medium, respectively (indicated by a plateau phase), cells were removed to 85% of the peak cell concentration daily by manual bleeds. A steady-state was maintained for 5 days. Afterwards the perfusion rate was increased to 2 RV/d.

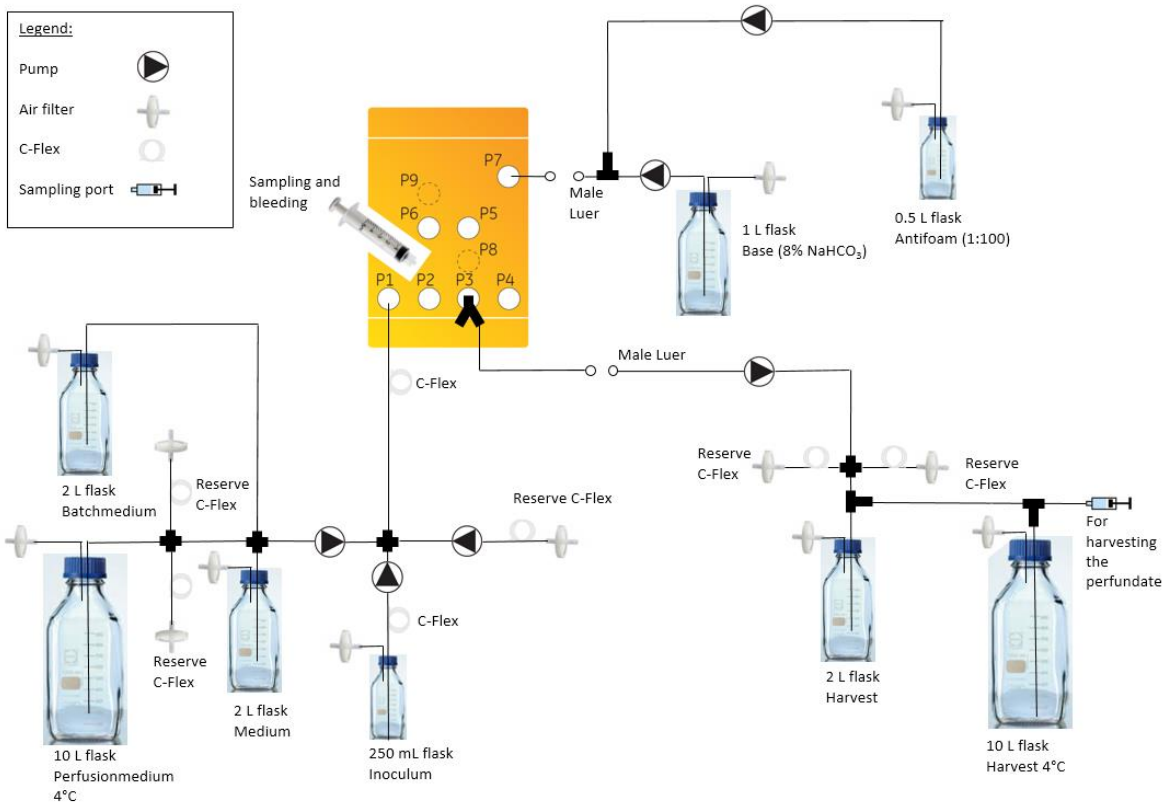


Figure 2: Experimental setup of the perfusion in the WAVE Bioreactor

Table 13: Description of the ports of the cellbag used for perfusion

CB0002L10-14, pHOPT and perfusion version	
P1	1/8 in × 1/4 in × 39 in C-Flex, female Luer
P2	3/16 in × 3/8 in × 2 in silicone, needleless sampling
P3	Y-connection attached to perfusion filter
P4	Oxywell2, for DOOPT probe
P5	Air inlet filter
P6	Air outlet filter
P7	3/16 in × 3/8 in × 2 in silicone, female Luer
P8	pHOPT sensor body - bottom of bag
P9	N/A

3.2.4 Analyses during cell cultivation

3.2.4.1 Quantification of cell concentration

Cell concentration and viability were determined using a Vi-CELL XR (Beckman Coulter) instrument. This Cell Viability Analyser is a video imaging system for analysing yeast, insect and mammalian cells in culture media. It automates the widely accepted trypan blue dye exclusion protocol. The collection of settings that control the sample preparation and analysis is summarized in a so called 'cell type'. The used cell type "CHO-K1" settings are described in Table 14. The instrument has a measurable cell concentration range between 4×10^4 c/mL and 1×10^7 c/mL. Therefore samples with a higher cell concentration had to be diluted with PBS. After entering the sample ID and the dilution factor the auto sampler queue was started. Each measurement consisted of 50 pictures, which were then averaged. After each sample, an automatic decontamination and cleaning cycle with 70% Isopropanol, Coulter Clenz and PBS Dulbecco was carried out. To prevent cell sedimentation, the sample vials were filled only shortly before each measurement. Besides the viability and the cell growth, the average diameter and circularity were also documented for possible trend analysis.

Table 14: Settings that control the sample preparation and analysis during Vi-CELL XR analysis

Cell size Thresholds	
Minimum diameter [microns]	6
Maximum diameter [microns]	50
Sample preparation	
Aspirate cycles	1
Trypan blue mixing cycles	3
Image analysis settings	
Cell brightness [%]	85
Cell sharpness	100
Viable cell spot brightness [%]	75
Viable cell spot area [%]	5
Minimum circularity	0
Decluster degree	Medium

3.2.4.2 Quantification of key metabolites and by-products

Key metabolites and metabolic by-products (i.e. glucose, lactate, glutamine, glutamate and ammonium) were quantified with a BioProfile 100 Plus Analyzer (Nova Biomedicals). Additionally the instrument also measures the pH which was used for off-set analysis and re-calibration of the internal pH probe of the WAVE Bioreactor. The different testing methods are shown in Table 15. Metabolite analysis was done fully automated according to the instrument's manual using at least 750 µL of culture supernatant or cell

suspension in case of bioreactor experiments to simultaneously recheck the measured pH of the pH sensor.

Table 15: Overview of parameters tested with the BioProfile 100 Plus instrument

Parameters	Test Parameters Methodology	Measuring Range	Imprecision +/-
Glutamine	Enzyme/Amperometric	0.2-6.0 mmol/L	5.0%
Glutamate	Enzyme/Amperometric	0.2-6.0 mmol/L	5.0%
Glucose	Enzyme/Amperometric	0.2-15.0 g/L	5.0%
Lactate	Enzyme/Amperometric	0.2-5.0 g/L	5.0%
Ammonium	Ion Selective Electrode	0.2-25.0 mmol/L	5.0%
pH	Ion Selective Electrode	5.00-8.00 pH units	0.01
Na^+	Ion Selective Electrode	40-220 mmol/L	1.5%
K^+	Ion Selective Electrode	1.0-25.0 mmol/L	3.0%

3.2.4.3 Quantification of antibody titer

The antibody concentration in the culture supernatant was determined using an Octet QK (FortéBio). The technology is based on the interaction of light waves, called optical interferometry. Figure 3 shows on the left side a single biosensor-tip followed by an illustration of the analytical technique. White light is reflected from two different biosensor tip surfaces, namely a layer of immobilized molecules on the biosensor tip and an internal reference layer. The two reflections are measured in real-time for different wavelengths and an interference pattern is generated. Any change in the number of bounded molecules on the surface of the biosensor causes a shift in the interference pattern. Therefore the wavelength shift (Δλ) corresponds to the change in thickness of the biological layer. The interference pattern is not affected by unbound molecules and changes in the refractive index of the surrounding medium [37].

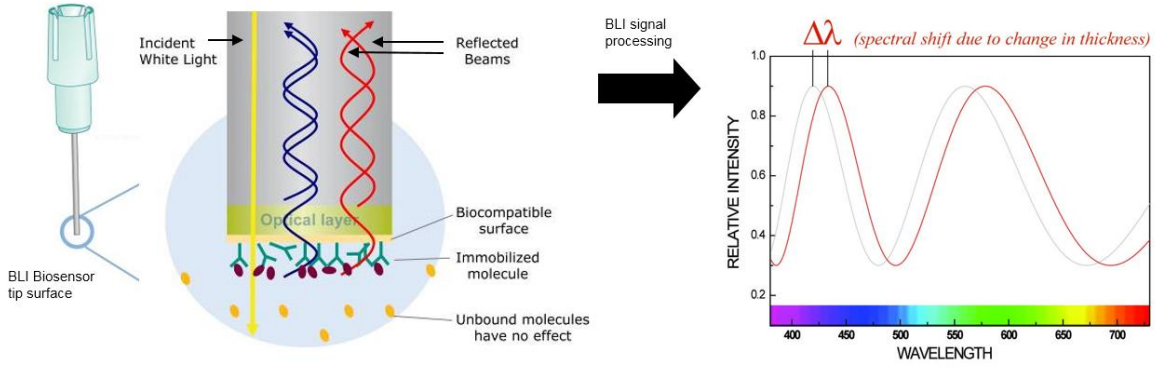


Figure 3: Principle of the FortéBio Bio-Layer Interferometry technique (Figure adapted from [37])

Supernatants were diluted with PBST to a maximal antibody concentration of 25 µg/mL and 200 µL were transferred to a 96 well black nucleon plate (air bubble-free). Column 11 was filled with glycine buffer and column 12 was filled with PBST, 200 µL of

each. This was done to enable a regeneration followed by a neutralisation and reuse of the used protein A biosensors. For at least 10 minutes before starting the measurement, the biosensors were activated in 200 µL of PBST. After the sample plate and the plate containing the biosensors were put into the instrument, the "Data Acquisition" software (FortéBio) was started. A quantification with regeneration measurement was done with the setup parameters showed in Table 16. The location of the samples, their IDs and the dilution factor were entered and once the location of the biosensors was also noted, the measurement was started. The evaluation was done using the "Data Analysis" software (FortéBio) and a previously measured protein standard curve.

Table 16: Setup Parameters for the Octét measurement

Name	Value	Unit
Assay time	120	s
Flow rate	1000	rpm
Cycles per regeneration	3	n/a
Regeneration flow rate	1000	rpm
Neutralization flow rate	1000	rpm
Regeneration time	5	s
Neutralization time	5	s
Preconditioning	1	n/a
Postconditioning	0	n/a

3.2.4.4 Determination of osmolality

The osmolality of the culture supernatant was determined using an Osmomat 030 (Gonotec GmbH) device. This instrument is a cryoscopic osmometer, which determines the total osmolality of an aqueous solution by comparative measurements of the freezing points of pure water and test solutions. The freezing point of a solution decreases as the concentration of ionic components in solution increases. Whereas water has a freezing point of 0°C, a solution with saline concentration of 1 osmol/kg has a freezing point of -1.858°C. That means that one mol of a given non-dissociated substance (6.023×10^{23} parts diluted in one kilogram of water) lowers the freezing point of a solution by 1.858°C [38]. The following definitions are used to calculate osmolality (Equation (3)):

$$c_{osm} = \frac{\Delta T}{K} \tag{3}$$

c_{osm} osmolality [osmol/kg]
T Freezing point depression [°C]
K 1.858°C kg/osmol freezing point constant

The osmolality indicates the concentration of all osmotically active dissolved parts in the solvent. Since the freezing point depression is directly proportional to the dissolved parts, the Osmomat measures the osmolality directly.
Prior to analysis, the instrument was calibrated by means of a two-point-calibration, using RO-H_2O to determine the intercept and a 300 mOsmol/kg standard to determine the slope, respectively. Analysis was performed according to the instrument's manual using 50 µL culture supernatant.

3.2.5 Calculations of growth characteristics and cell specific rates

For assessment of the cultivation performance of the CHO cell lines in different media or cultivation settings the following calculations were performed.

The cell specific growth rate (µ) [1/d] was calculated according to equation (4).

$$\mu = \frac{\ln(X_2) - \ln(X_1)}{\Delta t} \quad (4)$$

For calculation of the viable cell specific growth rate μ_v X_i was replaced by the concentration of viable cells X_{vi}
The viable cell days (VCD) [cells×d/mL] were calculated using equation (5).

$$VCD = \frac{X_{v2} - X_{v1}}{\mu_v} \quad (5)$$

For a better comparison, the viable cumulative cell days (VCCD) [cells×d/mL] were calculated with equation (6).

$$VCCD_2 = VCD_2 + VCCD_1 \quad (6)$$

Using equation (7), the cell specific productivity of mAb (q_p) [pg/(cell×d)]was calculated.

$$qP = \frac{T_2 - T_1}{VCD_2} * 10^6 \quad (7)$$

- X_1 cell density [cell/mL] on the previous day
- X_2 cell density [cell/mL] on the present day
- Δt time interval [days] between two samplings (~ 1 day)
- T_1 mAb concentration [mg/L] on the previous day*
- T_2 mAb concentration [mg/L] on the present day

*In pseudo perfusion experiments the mAb concentration was zero due to the daily complete medium exchange.

During the perfusion in the WAVE Bioreactor cell specific rates were calculated according to following equations:

The cell specific growth rate μ [1/day] was calculated according to equation (8).

$$\frac{\ln(X_2) - \ln\left(X_1 * \frac{V_1 - V_{Bleed}}{V_2}\right)}{\Delta t} \quad (8)$$

The calculation for the cell specific consumption rates of glucose, glutamate and ammonium q_{met} [pg/(cell×d)] were done with equation (9).

$$q_{met} = \left(\frac{Met_1 - Met_2}{\Delta t} + D * Met_{Feed} - D * \frac{Met_1 + Met_2}{2}\right) * 10^6 / \left(\frac{X_1 + X_2}{2}\right) \quad (9)$$

The cell specific production rates of lactate and glutamine q_{met} [pg/(cell×d)] were calculated using equation (10).

$$q_{met} = -\left(\frac{Met_1 - Met_2}{\Delta t} + D * Met_{Feed} - D * \frac{Met_1 + Met_2}{2}\right) * 10^6 / \left(\frac{X_1 + X_2}{2}\right) \quad (10)$$

The cell specific perfusion rate (CSPR) [nL/(cell×day)] was derived from equation (11).

$$CSPR = \frac{D}{X} * 10^6 \quad (11)$$

The cell specific productivity of mAb (q_P) [pg/(cell*day)] was calculated according to equation (12):

$$q_P = \left(\frac{T_2 - T_1}{\Delta t} + D * \frac{T_1 + T_2}{2}\right) * 10^6 / \left(\frac{X_1 + X_2}{2}\right) \quad (12)$$

- X_1 cell density [cell/mL] on the previous day
- X_2 cell density [cell/mL] on the present day
- Δt time interval [days] between two samplings (~ 1 day)
- V_1 bioreactor volume [mL] on the previous day
- V_2 bioreactor volume [mL] on the present day
- V_{Bleed} bleed volume [mL]
- Met_1 metabolite concentration [mg/L] in the bioreactor on the previous day
- Met_2 metabolite concentration [mg/L] in the bioreactor on the present day
- Met_{Feed} metabolite concentration [mg/L] in the feed medium
- T_1 mAb concentration [mg/L] in the bioreactor on the previous day
- T_2 mAb concentration [mg/L] in the bioreactor on the present day
- D perfusion rate [1/day]

3.2.6 Design of experiments (DoE) approach

A DoE approach was applied to spike different combinations of CBs to the new basal medium CDM4NS0 to explore the best performing CBs in respect to growth and

productivity of the rCHO-K1 cell line. This approach reduced the number of different CB combinations to pre-select only those that are beneficial for the culture performance.

All experiments were conducted using CB 1, 2 or 4 as 10% stock solutions (prepared according to predetermined methods), CB 3, 5 or 6 as 5% stock solutions (prepared according to predetermined methods), CB 7a and 7b as 18.1% and 9.5% stock solutions (prepared according to manufacturer's recommendations Table 9 and Table 10), respectively. Since all CBs contained different amounts of amino acids, all CBs were leveraged by calculating the molar ratio of the contained amino acids setting CB1 as reference (Table 17). The column "Molar ratio" indicates the amino acid content of a single CB as proportion to CB1.

Table 17: Molar ratio of amino acids in stock solutions of CBs.

Cell Boost #	Stock solution [%]	Molarity of total amino acid content [mM]	Molar ratio
1	10	208.0	1.00
2	10	351.6	0.59
3	5	115.6	1.80
4	10	365.5	0.57
5	5	131.3	1.58
6	5	135.6	1.53
7a	18.1	603.0	0.34
7b	9.5	313.3	0.03*

*Note: For CB7b 10% of the amount of CB7a is used.

DoE level -1 was defined as no (0%) CB addition.

For definition of DoE level +1, following empirical procedure was applied:

1. For each CB a molar ratio was defined according to the total molar amino acid content using CB1 as reference (Table 17).
2. All CBs were mixed according to their molar ratio and added to the basal medium CDM4NS0 to reach a final osmolality of 400 mOsmol/kg (Table 18). Therefore, each CB contributes with equal amounts of (total) amino acids to the final spiked basal medium. Basal medium with maximum addition of all CBs should not surpass the critical osmolality threshold of 400 mOsmol/kg, above which negative effects on cell growth were expected [39].

DoE Level 0 is the half-maximum CB addition.

The spiked medium combinations were used to perform batch cultures.

Table 18: Definition of the CB addition for DoE #1 levels -1, 0 and +1 of rCHO-K1 in CDM4NS0 spiked batches. CB7b was defined as 10% of CB7a (red). For maximum

DoE #1 levels (+1) each CB (except CB7b) adds a total of 15 mM amino acids to the basal medium.

	criteria	CB1 [%]	CB2 [%]	CB3 [%]	CB4 [%]	CB5 [%]	CB6 [%]	CB7a [%]	CB7b [%]
-1	No Cell Boosts	0	0	0	0	0	0	0	0
0	Half-max. Cell Boosts	3.61	2.13	6.49	2.05	5.71	5.53	1.24	0.12
+1	Max. Cell Boosts for 400 mOsmol/kg	7.21	4.27	12.98	4.10	11.43	11.06	2.49	0.25

Results of the first DoE (DoE #1) were used to select a reduced number of CBs that were beneficial to the batch performance. Using the statistical software Modde, models were predicted to further optimize the concentration of the selected CBs and a second DoE experiment (DoE #2) for pseudo perfusion was designed. These pseudo perfusion experiments were conducted as described above. Again, all selected CBs were combined according to the molar ratio and added to the basal medium CDM4NS0 to a maximum concentration in order not to surpass the critical osmolality threshold of 400 mOsmol/kg above which negative effects on cell growth were expected (Table 19). Since a potential positive effect of CB7b was observed in the previous experiment, the ratio of CB7b was doubled from ten to twenty percent of the amount of CB7a.

In contrast to the first DoE where the spiked medium combinations were used to perform batch cultures, in the second DoE the spiked medium combinations were exchanged daily (pseudo perfusion mode).

Table 19: Definition of the CB addition for DoE #2 levels -1, 0 and +1 of rCHO-K1 in CDM4NS0 pseudo perfusion. CB7b was defined as 20% of CB7a (red). For maximum DoE #2 levels (+1) each CB (except CB7b) adds a total of 23 mM amino acids to the basal medium.

	criteria	CB1 [%]	CB3 [%]	CB7a [%]	CB7b [%]
-1	No Cell Boosts	0	0	0	0
0	Half-max. Cell Boosts	5.53	9.95	1.91	0.38
+1	Max. Cell Boosts for 400 mOsmol/kg	11.06	19.90	3.81	0.76

4. Results

4.1 Screening of two different rCHO-K1 subclones

Two rCHO-K1 subclones (A1A7 and D1E7) were received from GE and cultivated in 125 mL shake flasks in ActiPro + 37.5 µM MSX. Established master cell banks (MCB) showed the same growth properties during routine cultures as the obtained parental cells (data not shown). Both A1A7 and D1E7 subclones showed similar growth behaviour in routine cultures reaching cell specific growth rates of 0.75 1/d. Additionally, batch cultures in duplicate were performed in 50 mL shake tubes (220 rpm) in ActiPro to further characterize the performance of the two cell lines (Figure 4). Subclone A1A7 showed slightly higher µ until day 5 resulting in higher peak cell concentration of 21×10^6 c/mL. Similar final max. titers of 1.4 g/L were obtained for both cell lines.

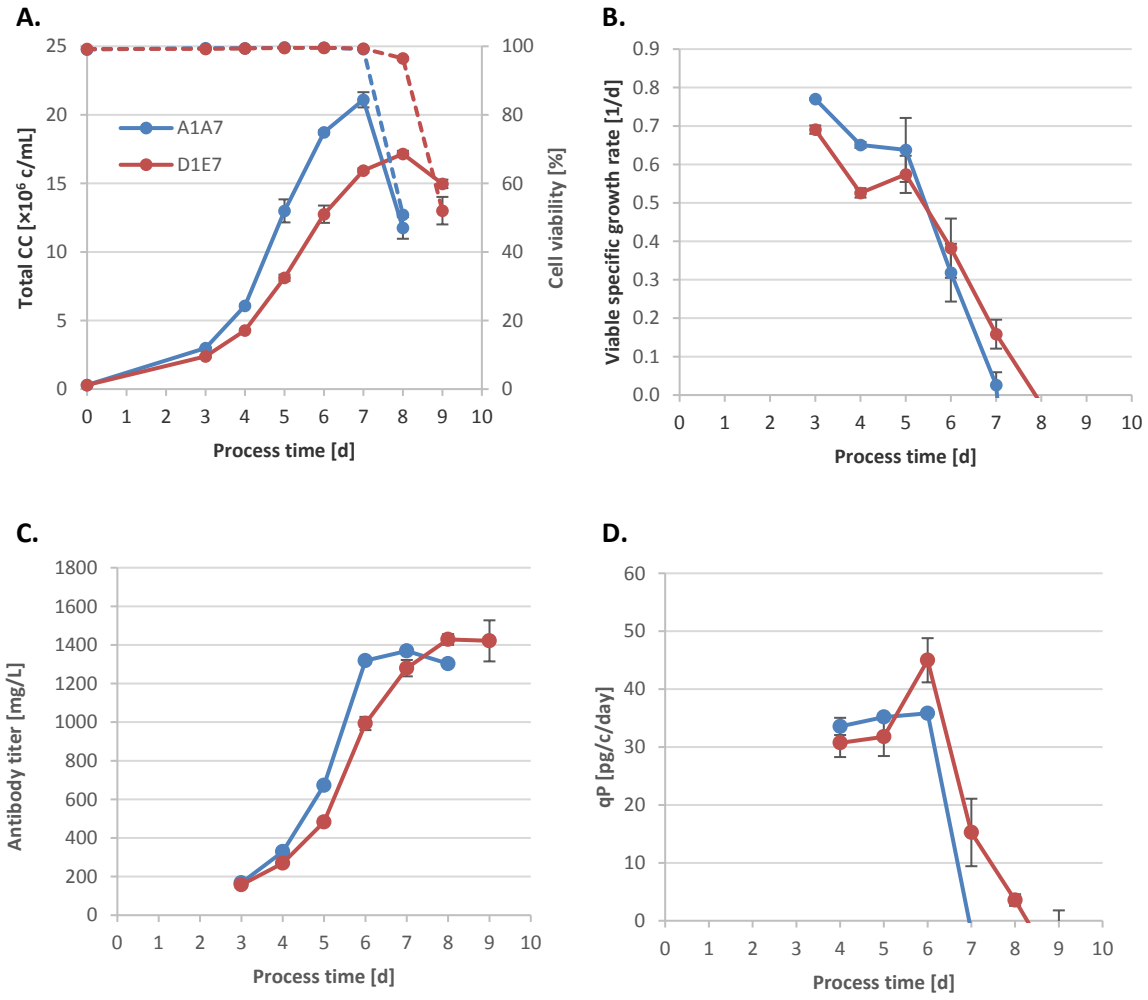
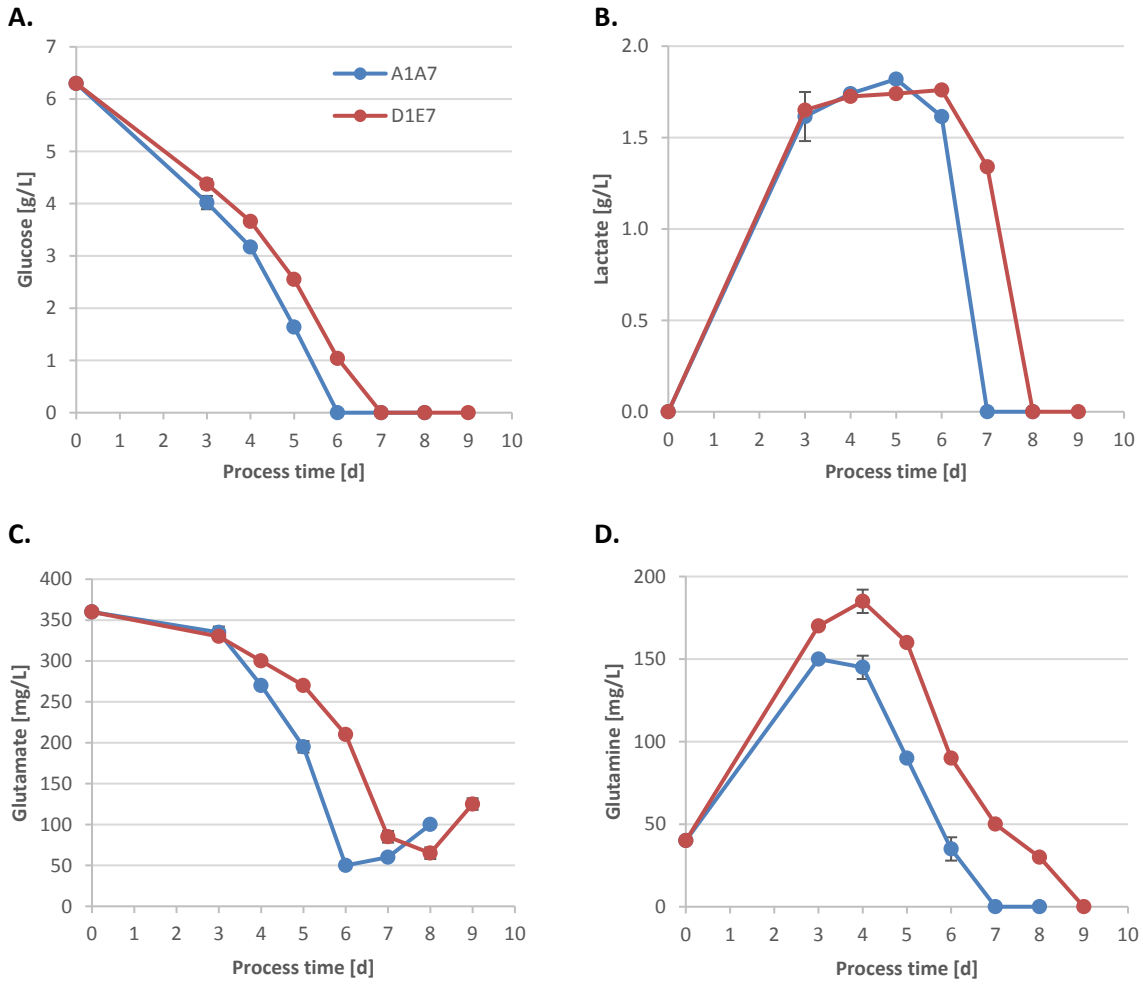


Figure 4: Cell concentration (straight line) and viability (dashed line) (A.), viable specific growth rate (B.), antibody concentration (C.) and and qP (D.) of a batch verification of the two rCHO-K1 subclones A1A7 (blue) and D1E7 (red) in ActiPro.

Due to faster growth of A1A7 also the glucose was consumed faster resulting in max. lactate levels of 1.7 g/L (Figure 5). Cell viability of the batch culture of A1A7 dropped one day earlier compared to D1E7 as a result of the critically low glucose concentration. Glutamate was consumed down to 50 mg/L on day 6 for A1A7 and day 8 for D1E7. Glutamine first produced by both cell lines in the first 3-4 days but then completely consumed. Ammonia was consumed giving a similar profile as for glutamate consumption. Starting from day 6 or 7 a strong increase in ammonia was observed.



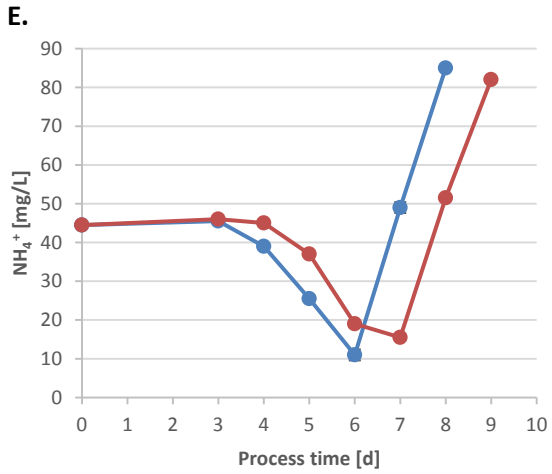


Figure 5: Concentrations of key metabolites of a batch verification of the two rCHO-K1 subclones A1A7 (blue) and D1E7 (red) in ActiPro, showing glucose (A.), lactate (B.), glutamate (C.), glutamine (D.) and ammonium (E.).

For all following experiments the rCHO-K1 subclone D1E7 was chosen as the preferred cell line because of good growth, longer batch process duration and slightly higher final product titers.

4.2 Basal medium screening for rCHO-K1 using HyClone media systems

The rCHO-K1 cell line was originally grown in ActiPro medium. Eight other basal cell culture media were screened to select the medium that supports the highest cell and antibody concentrations to be used for subsequent perfusion medium development. A list of cell culture media to be screened is shown in Table 20. For medium adaptation, the rCHO-K1 cell line was directly inoculated at a cell concentration of 4×10^5 c/mL in the respective medium and grown for at least 3 passages before initiating the batch cultures.

Table 20: Hyclone media tested for rCHO-K1 cultivation

HyClone media
ActiPro
CDM4CHO
HyCell CHO
CDM4NS0
CDM4PerMAb
SFM4CHO
CDM4HEK293
CDM4MAb
SFM4MAb

rCHO-K1 D1E7 showed a viable specific growth rate above 0.4 1/d in eight out of nine tested media (Figure 6). Only in SFM4MAb, cell viability dropped below 90%. In all

tested media no cell aggregation was observed. Highest specific productivities were achieved with CDM4NS0 (60 p/c/d) resulting also in the highest observed titers during routine cultivation (1 g/L). In CDM4NS0 culture remained in homogenous suspension without extensive clumping.

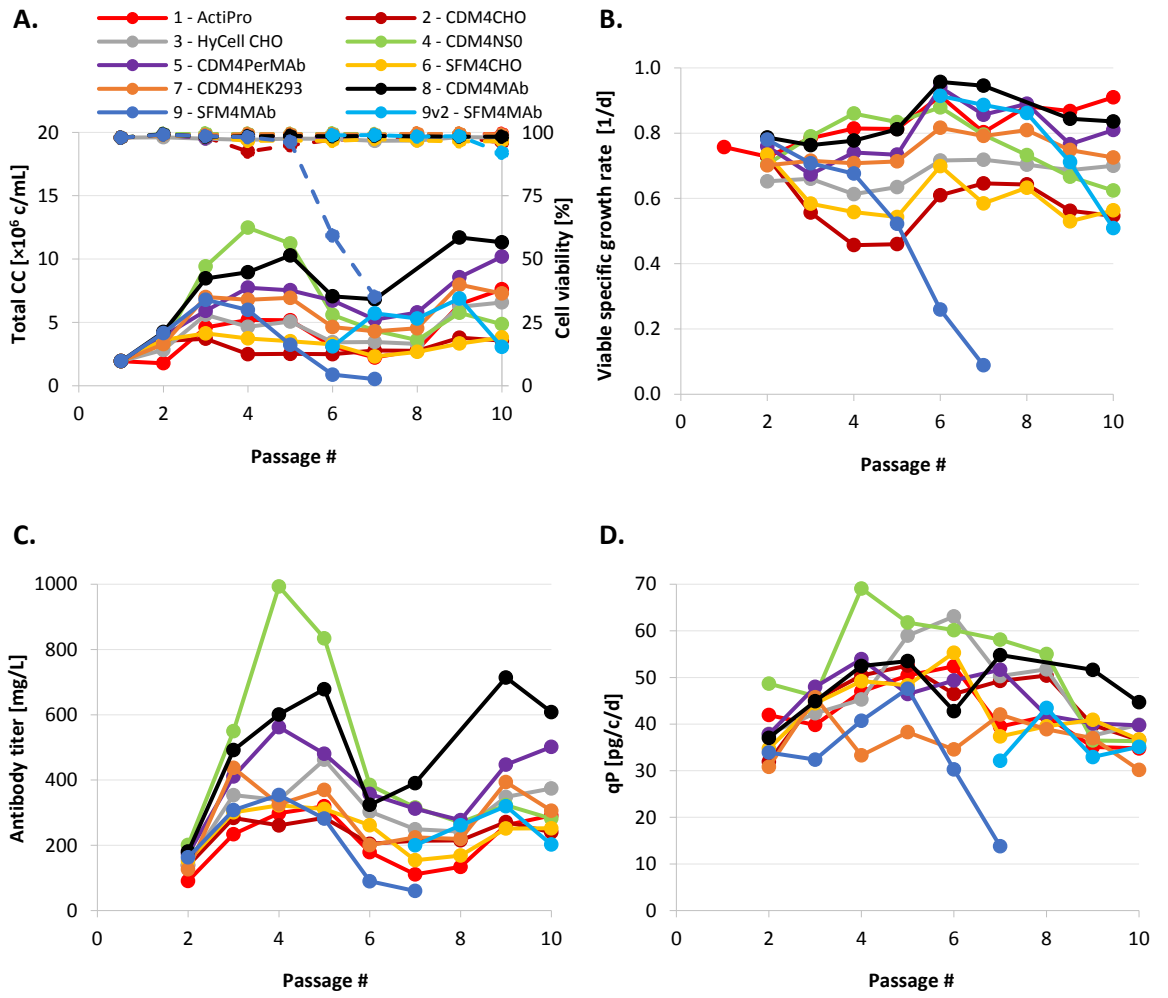


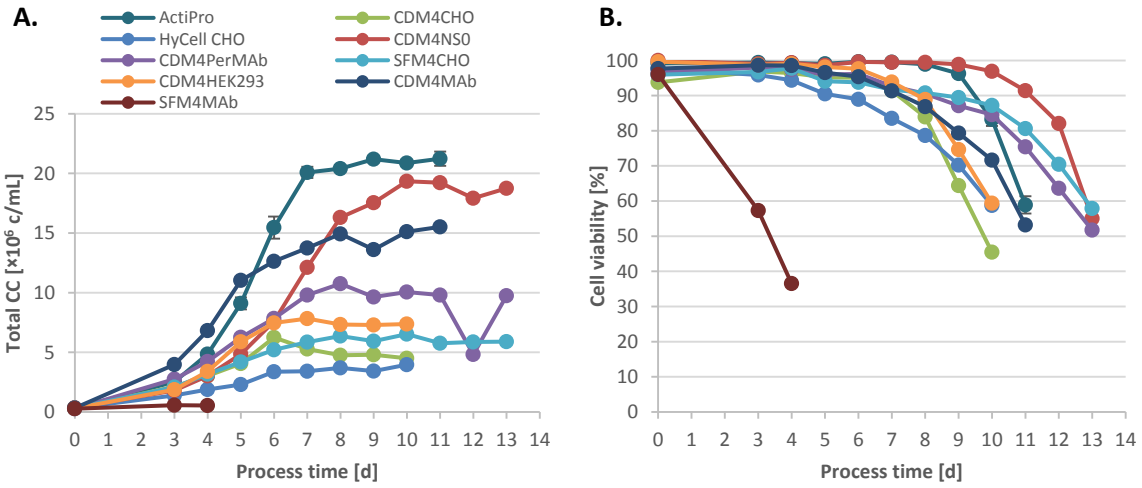
Figure 6: Cell concentration (straight line) and viability (dashed line) (A.), viable specific growth rate (B.), antibody concentrations (C.) and specific productivity (D.) of rCHO-K1 routine cultures in nine different HyClone media (ActiPro, CDM4CHO, HyCell CHO, CDM4NS0, CDM4PerMAb, SFM4CHO, CDM4HEK293, CDM4MAb and SFM4MAb).

Table 21: Summary of rCHO-K1 routine cultures in nine different HyClone media (ActiPro, CDM4CHO, HyCell CHO, CDM4NS0, CDM4PerMAb, SFM4CHO, CDM4HEK293, CDM4MAb and SFM4MAb).

Basal medium	Total CC [×10⁶ c/mL]	mAb [mg/L]	qP [pg/c/d]
1 - ActiPro	4.1±2.0	210±80	43±6
2 - CDM4CHO	3.0±0.6	230±50	45±7
3 - HyCell CHO	4.3±1.6	310±90	47±9
4 - CDM4NS0	4.9±2.2	330±100	49±9

Basal medium	Total CC [×10⁶ c/mL]	mAb [mg/L]	qP [pg/c/d]
5 - CDM4PerMAb	6.4±2.3	390±110	45±6
6 - SFM4CHO	3.2±0.7	240±70	43±7
7 - CDM4HEK293	5.5±2.0	290±100	37±5
8 - CDM4MAb	7.8±3.2	500±180	48±6
9 - SFM4MAb	3.3±2.4	210±120	33±11

Batch cultures with the nine selected HyClone media were started using rCHO-K1 cultures adapted at least for three passages in the respective media. 3×10^5 c/mL were seeded in a 50 mL SpinTube bioreactor (220 rpm). Cultures were fed with Glucose to 6 g/L when concentration levels fell below 3 g/L. Highest peak cell concentrations were reached with ActiPro, CDM4NS0 and CDM4MAb achieving 21, 19 and 16×10^6 c/mL on day 7, 10 and 8, respectively (Figure 7A). For these cultures, viability was maintained above 60% for 10 (CDM4MAb and ActiPro) or 12 days (CDM4NS0) (Figure 7B). Only for SFM4MAb a rapid decline in viability was observed. For ActiPro and CDM4NS0 similar max. titers of 1.5 g/L were achieved (Figure 7C). A good correlation between cumulative viable cell days and maximum titers was observed (Figure 7D). CDM4NS0 and ActiPro showed exceedingly good mAb accumulation per viable cell days.



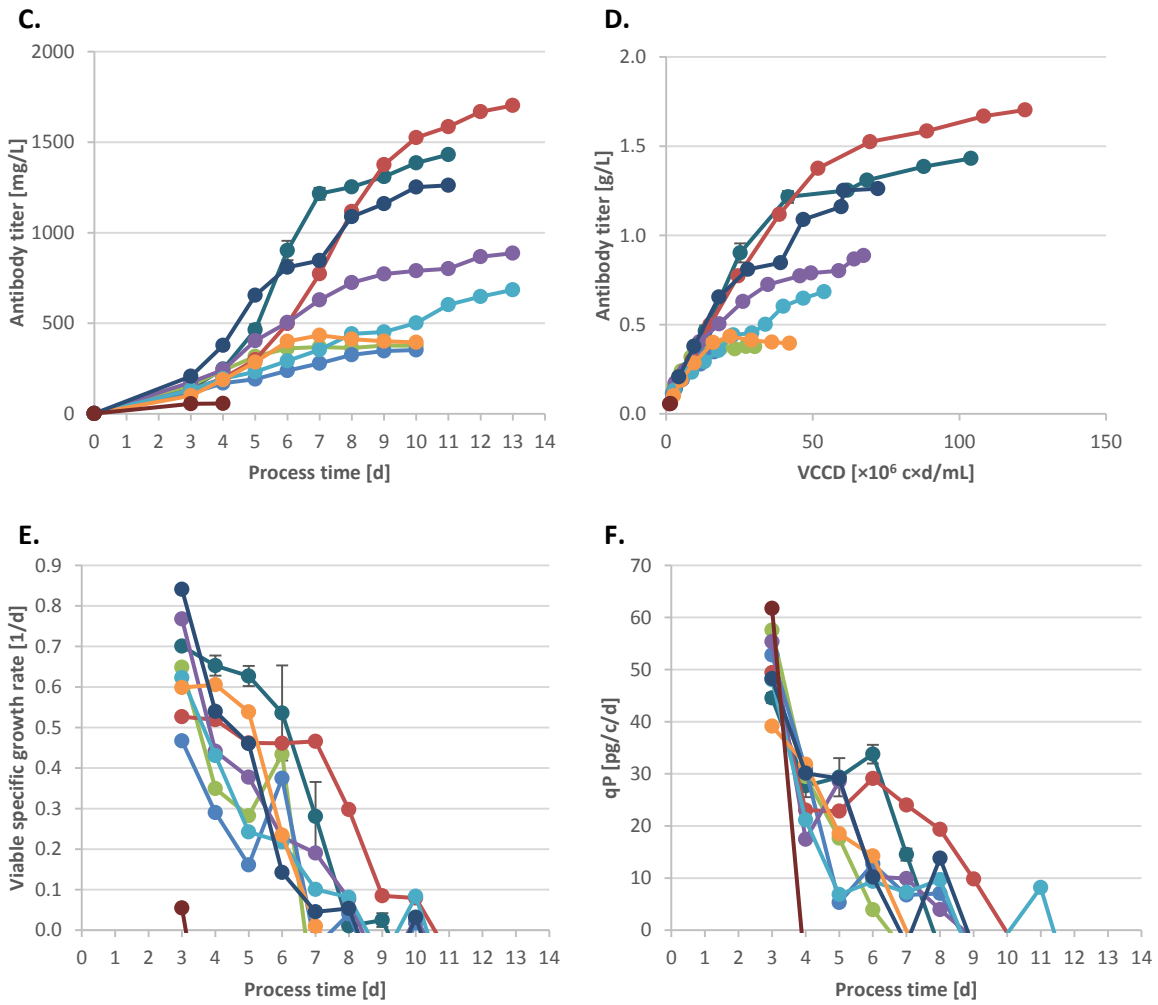
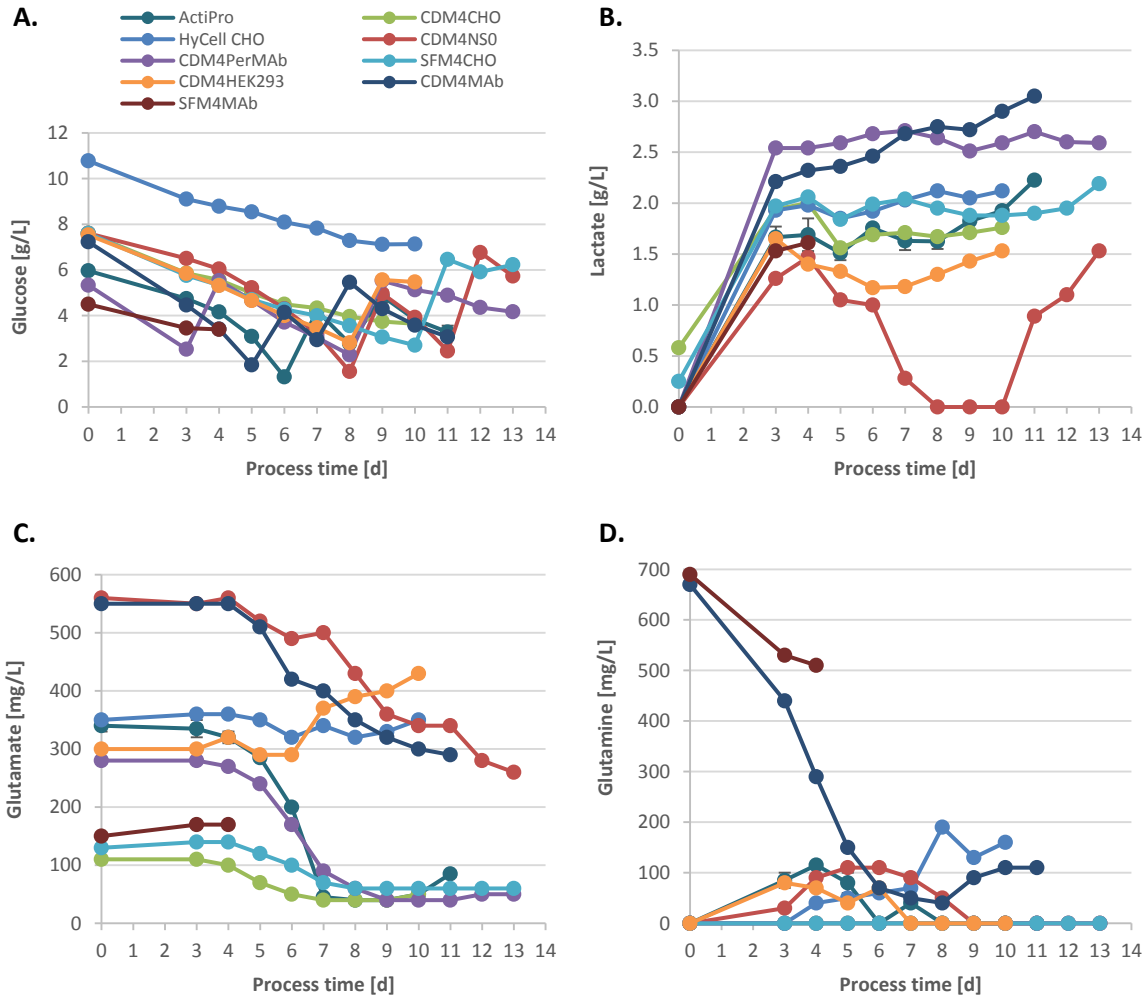


Figure 7: Cell concentrations (A.), viabilities (B.), antibody concentrations (C.), titer vs. VCCD (D.), viable specific growth rate (E.) and qP (F.) of batch cultures of medium adapted rCHO-K1 cell lines in nine different HyClone media (ActiPro, CDM4CHO, HyCell CHO, CDM4NS0, CDM4PerMAb, SFM4CHO, CDM4HEK293, CDM4MAb and SFM4MAb).

Table 22: Summary of batch cultures of medium adapted rCHO-K1 cell lines in nine different HyClone media (ActiPro, CDM4CHO, HyCell CHO, CDM4NS0, CDM4PerMAb, SFM4CHO, CDM4HEK293, CDM4MAb and SFM4MAb). Best performances are highlighted in green.

Basal medium	Peak CC [×10⁶ c/mL]	Peak mAb [mg/L]
1 - ActiPro	21.2	1430
2 - CDM4CHO	6.2	380
3 - HyCell CHO	4.0	350
4 - CDM4NS0	19.3	1700
5 - CDM4PerMAb	10.8	890
6 - SFM4CHO	6.5	680
7 - CDM4HEK293	7.8	430
8 - CDM4MAb	15.5	1260
9 - SFM4MAb	5.7	60

ActiPro showed lowest glucose level on day 7 at which a glucose feed was applied (Figure 8A). CDM4NS0 contains higher glucose concentrations at around 8 g/L resulting in a later requirement for glucose feed two days later. For CDM4NS0 intensive lactate consumption was observed starting from day 5, when Glucose levels were still high (Figure 8B). In contrast, ActiPro batch cultures never switched to lactate consumption. Glutamate never reached limiting concentrations in CDM4NS0 because of quite high starting concentrations of the basal media of 550 mg/L (Figure 8C). However, ActiPro reached low levels on day 8, accompanied with a decrease in viability.CDM4MAb with L-glutamine showed a strong increase in ammonia levels up to 130 mg/L (Figure 8D and E). All cultures did not surpass the critical 400 mOsmol/kg threshold (Figure 8F).



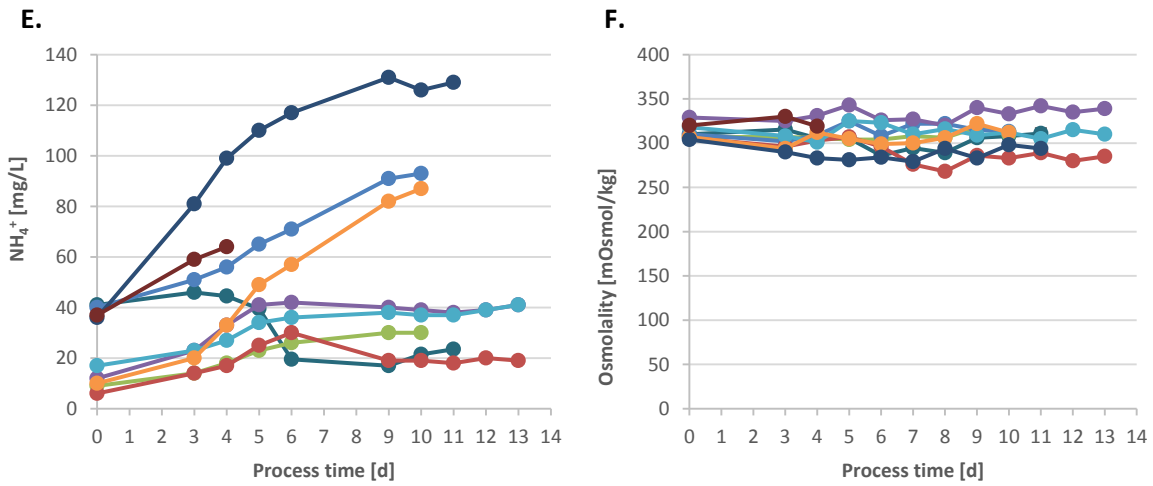


Figure 8: Concentrations of key metabolites of batch cultures of medium adapted rCHO-K1 cell lines in nine different HyClone media, showing glucose (A.), lactate (B.), glutamate (C.), glutamine (D.), ammonium (E.) and measured osmolalities (F.).

4.3 Initial rCHO-DG44 pseudo perfusion experiment in ActiPro

Two cultures of rCHO-DG44 in ActiPro medium were compared in a batch-refeed (pseudo perfusion) mode. A SpinTube bioreactor was used at a rotation rate of 220 rpm. In a first experiment, medium was replenished daily and all cells were retained by centrifugation and resuspension in the same culture volume . In the second run, the cell suspension was diluted with fresh media daily to constantly remain a cell density of 1×10^7 c/mL.

The daily complete medium exchange enabled a constant high cell concentration (CC) of up to 29×10^6 c/mL and viability above 85% was maintained for 11 days (Figure 9). Initially high specific growth rates of 0.65 d^{-1} gradually decreased to reach a stationary phase on day three to four.

Maintaining cell density at 1×10^7 c/mL did not result in healthy cultures for prolonged time-periods, presumably due to depletion of nutrients. Starting from day 2 a drop in specific growth was observed, accompanied with a drop in viability starting from day 3. The highest cell concentration was 17×10^6 c/mL.

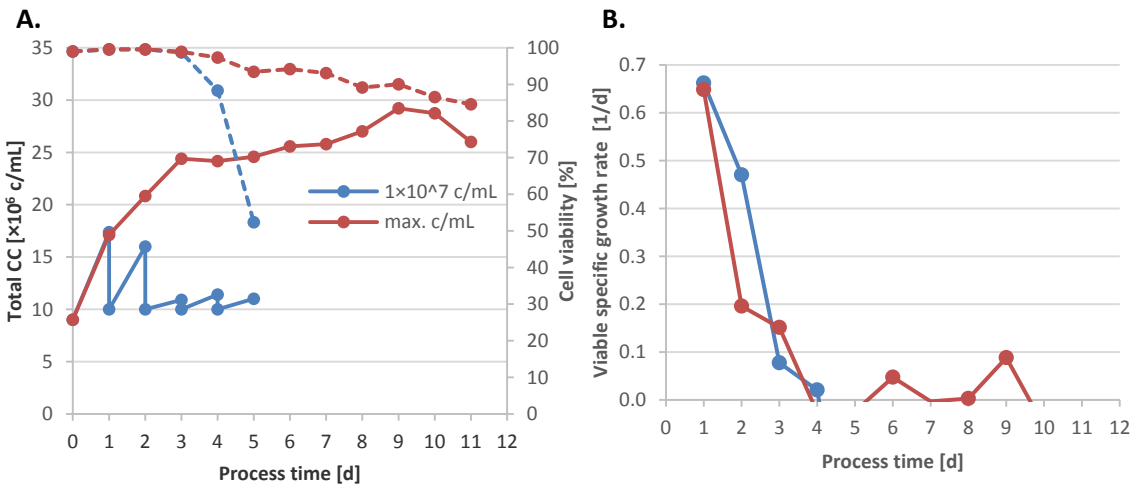


Figure 9: Cell concentration (straight line) and viability (dashed line) (A.) and viable specific growth rate or μ_v (B.) of two different pseudo perfusion conditions of rCHO-DG44 in ActiPro by daily complete medium replacement (red) and daily re-seeding to 1×10⁷ c/mL by splitting (blue).

The pseudo perfusion setup resulted in constant high mAb titers of about 750 mg/L (Figure 10). However, mAb production dropped on day 6 and resulted in a decrease of 30% in qP at the end of the experiment.

For split cultures a similar same titer was observed, though this did not represent the accumulated titer because residual mAb was measured from previous days also.

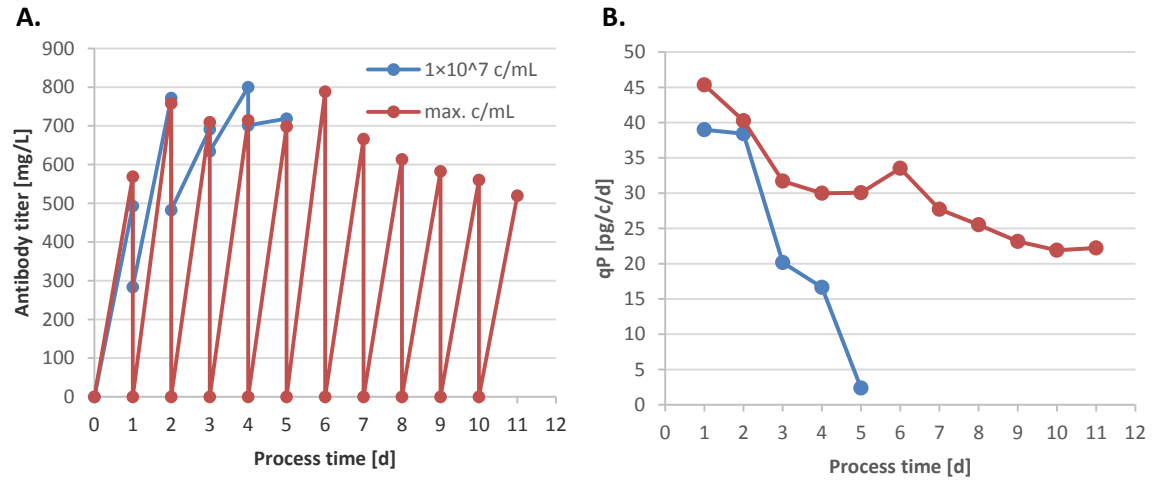


Figure 10: : Antibody concentrations (A.) and cell-specific antibody production rate or qP (B.) of two different pseudo perfusion conditions of rCHO-DG44 in ActiPro by daily complete medium replacement (red) and daily re-seeding to 1×10⁷ c/mL by splitting (blue).

After reaching the stationary phase in the pseudo perfusion setup, glucose was consumed within one day (Figure 11). For media-exchanged cultures the glucose limitation started at day 3, which was also the first time point where no lactate

production was observed. This glucose limitation was accompanied with the start of declining viability. Glutamine was consumed by the cells down to a concentration of 80 mg/L. Ammonium accumulated up to 230 mg/L on day 3.

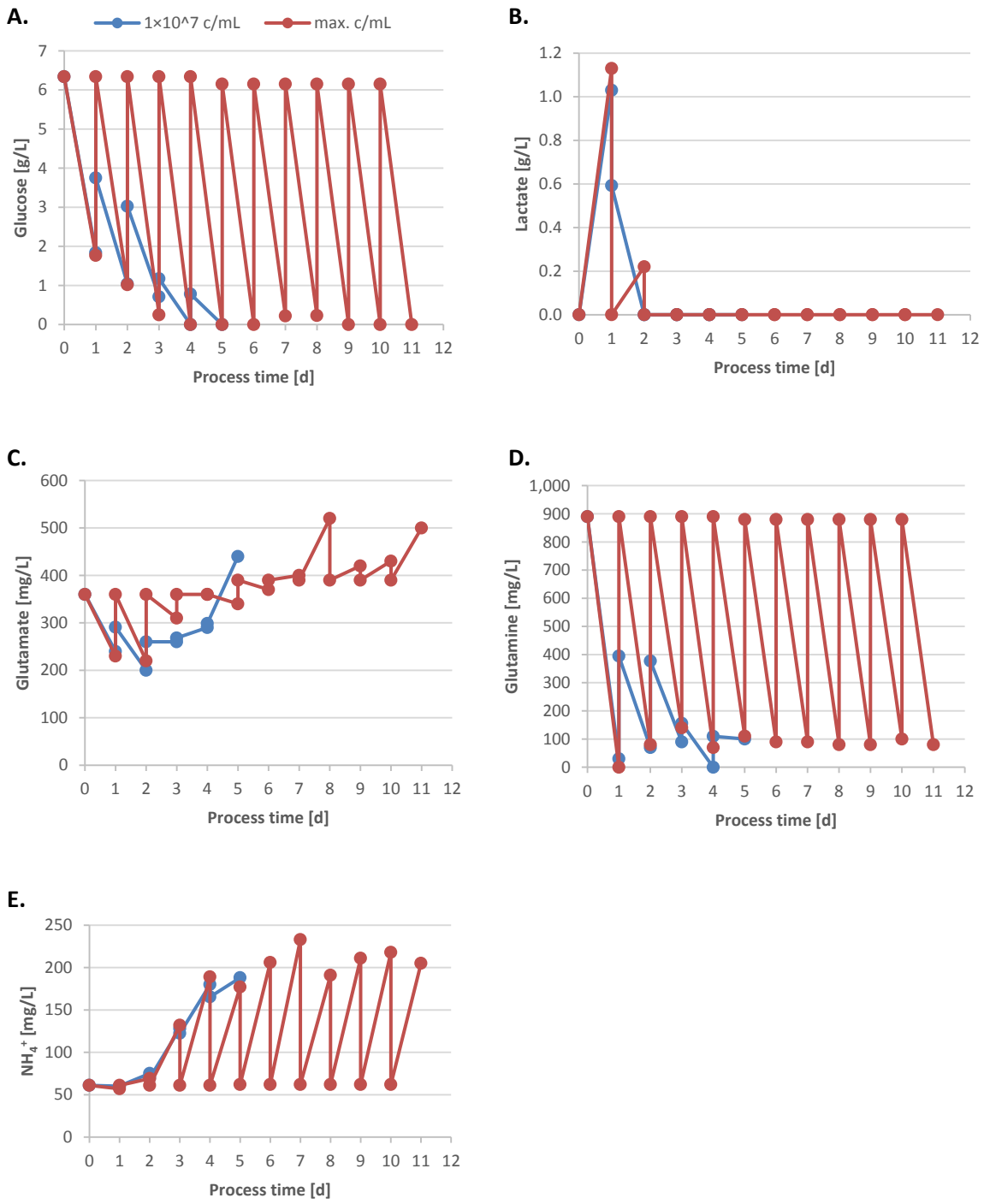


Figure 11: Concentrations of key metabolites of two different pseudo perfusion conditions of rCHO-DG44 in ActiPro by daily complete medium replacement (red) and daily re-seeding to 1×10^7 c/mL by splitting (blue), showing glucose (A.), lactate (B.), glutamate (C.), glutamine (D.) and ammonium (E.).

4.4 Optimization of shaking parameters for pseudo perfusion experiments

As only the pseudo perfusion setup enabled constantly high cell density and viability, this mode was selected as method of choice for the following experiments. Different shaker settings (angle, rotation rate and vessel) were compared to define conditions resulting in the highest cell concentrations and titers and to identify possible limitations. At first, optimization of shaking parameters was performed for the rCHO-DG44 cell line and afterwards for the rCHO-K1 cell line with the plain medium ActiPro and ActiPro spiked with 7% CB7a and 0.7% CB7b.

4.4.1 rCHO-DG44

Cell sedimentation occurred in the 90° Tube at 150 rpm (Figure 12), but adequate mixing was enabled at 150 rpm when cultured at 45° (Figure 13). The 45° Tubes above 200 rpm showed strong foaming and the cell suspension contacted the cap, which led to cell deposition on the membrane and therefore blocked the exchange of gases (Figure 14). At 300 rpm fewer cell deposits accumulated on the membrane due to higher shear forces.

Due to the massive ring-structured cell deposits at the vessel walls of the 90° Tubes and the 45° Tube at 150 rpm, these tubes had to be changed after 3 to 4 days. This was done before centrifugation to prevent accumulation of cell deposits in the cell pellet.

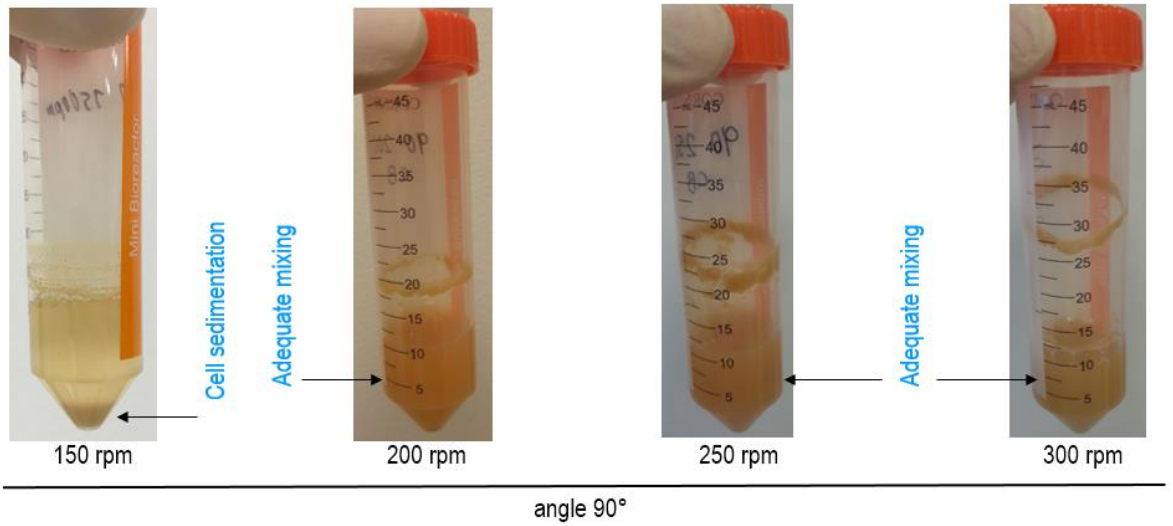


Figure 12: SpinTube bioreactors of rCHO-DG44 pseudo perfusion cultures incubated with an angle of 90° at different rotation rates

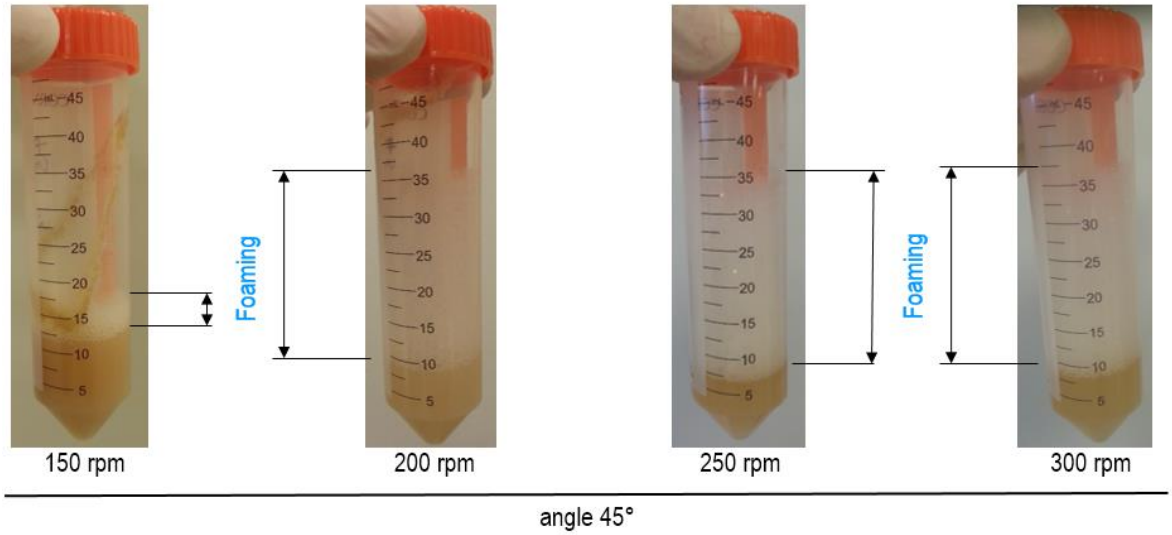


Figure 13: SpinTube bioreactors of rCHO-DG44 pseudo perfusion cultures incubated with an angle of 45° at different rotation rates

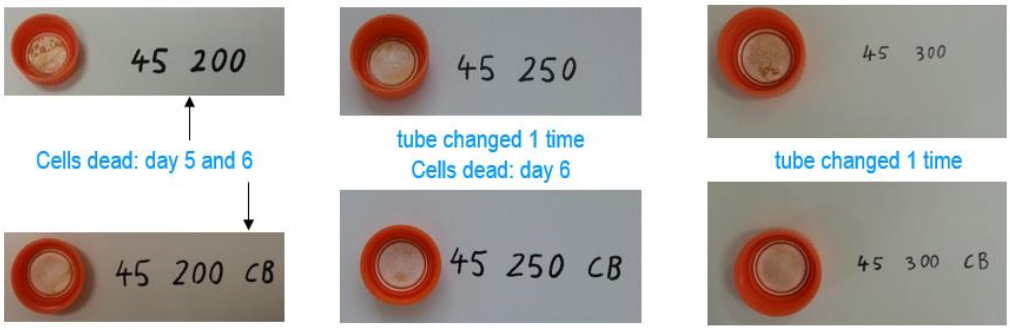


Figure 14: Cap of the SpinTube bioreactors of rCHO-DG44 pseudo perfusion cultures incubated with an angle of 45° at different rotation rates

Cell deposits from vessel walls of the shake flasks were removed by washing with 10 mL sterile PBS (Figure 15). Therefore no shake flask change was necessary. Most cell deposits occurred at 150 rpm because of the lower shear forces. The amount of removed cell deposits is shown in the 50 mL Tube. At 300 rpm cell deposit also occurred on the bottom of the shake flask due to the higher shaking speed.

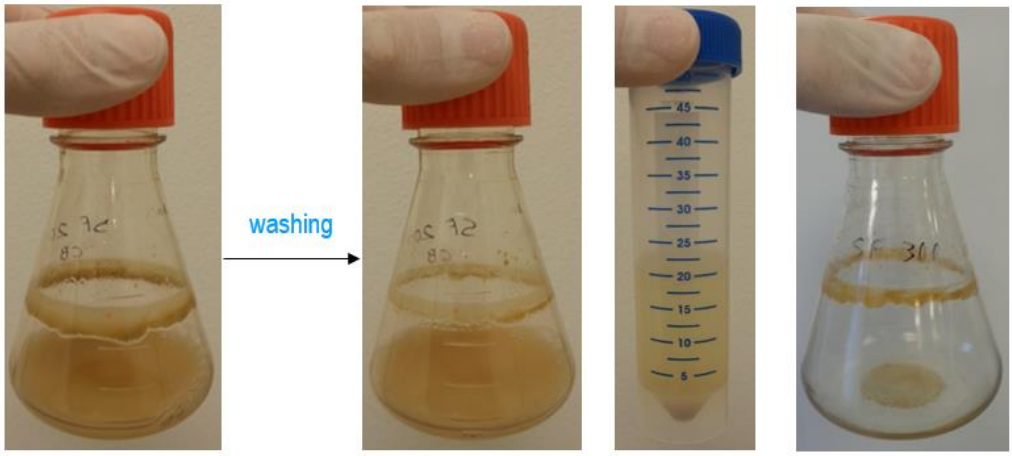


Figure 15: Washing Erlenmeyer shake flasks of rCHO-DG44 pseudo perfusion cultures with 10 mL sterile PBS, collected PBS in a 50 mL Tube after washing contained removed cell deposits and empty Erlenmeyer shake flasks cultivated at 300 rpm

Table 23 shows the average total cell concentration and titer in the pseudo steady state of rCHO-DG44 cultures in ActiPro. The highest cell concentration of 3.4±0.7×10^7 c/mL and titer of 802±90 mg/L was reached in the 45° tube at 150 rpm with a viability of 85% after 11 days. The 45° tubes at 200 rpm and 300 rpm and the shaking flask at 150 rpm showed titers above 750 mg/L as well.

Table 23: Summary of rCHO-DG44 pseudo perfusion cultures with ActiPro at different rotation rates in SpinTube bioreactors at 90°, 45° or in Erlenmeyer shake flasks. Best performances are highlighted in green.

w/o CBs	Sample ID	Total CC [×10^7 c/mL]	Titer [mg/L]	Process time (% viab.)	Comments
Shaking tube	90° 150 rpm	1.6±0.3	576±120	3 days (<60%)	Cell sedimentation
	90° 200 rpm	3.0±0.2	607±110	11 days (87%)	
	90° 250 rpm	3.0±0.3	533±60	11days (70%)	
	90° 300 rpm	3.3±0.4	640±100	11 days (76%)	
	45° 150 rpm	3.4±0.7	802±90	11 days (85%)	
	45° 200 rpm	2.6±0.3	762±80	5 days (<60%)	Membrane blocked
	45° 250 rpm	3.1±0.5	648±210	11 days (98%)	Tube changed
	45° 300 rpm	3.0±0.3	814±150	11 days (98%)	Tube changed
Shaking flask	SF 150 rpm	2.4±0.4	763±120	11 days (91%)	
	SF 200 rpm	3.3±0.2	607±60	11 days (79%)	
	SF 250 rpm	2.6±0.4	494±40	11 days (92%)	
	SF 300 rpm	2.7±0.4	653±50	11 days (97%)	

Table 24 shows the average total cell concentration and titer in the pseudo steady state of rCHO-DG44 cultures in ActiPro spiked with 7% CB7a and 0.7% CB7b. The highest cell concentration of 5.2±0.5×10^7 c/mL and titer of 2264±480 µg/mL connected with a

high viability of 91% after 11 days was reached in the shaking flask at 150 rpm. The 45° tube at 150 rpm showed a comparable titer, but a decrease in viability. The 90° tubes at 200 rpm and 250 rpm showed viabilities over 85%, but only half of the titer compared to the shaking flask at 150 rpm. In some cultures it wasn't possible to maintain a high viability over 11 days and therefore it dropped below 60%. A reason might be a nutrient depletion 1 day before they died.

The addition of CBs to ActiPro increased the cell concentration from ~30×10^6 c/mL to 35–65×10^6 c/mL and the mAb titer from 600–800 mg/L to 1000–2300 mg/L.

Table 24: Summary of rCHO-DG44 pseudo perfusion cultures with spiked ActiPro (7% CB7a and 0.7% CB7b) at different rotation rates in SpinTube bioreactors at 90°, 45° or in Erlenmeyer shake flasks. Shaking parameters with an unknown reason for the drop in viability are highlighted in red, good performances are highlighted in orange and the best one in green.

With CBs	Sample ID	Total CC [×10^7 c/mL]	Titer [mg/L]	Process time (% viab.)	Comments
Shaking tube	90° 150 rpm	1.2±0.1	378±350	2 days (<60%)	Cell sedimentation
	90° 200 rpm	3.5±0.2	934±270	11 days (96%)	
	90° 250 rpm	4.4±0.2	1105±400	11 days (88%)	
	90° 300 rpm	6.0±1.4	1885±420	8 days (<60%)	perhaps nutrient depletion 1 day before
	45° 150 rpm	6.4±0.9	2336±600	11 days (78%)	
	45° 200 rpm	3.1±0.7	599±390	6 days (<60%)	Membrane blocked
	45° 250 rpm	3.1±0.4	766±420	6 days (<60%)	Membrane blocked
	45° 300 rpm	3.4±0.8	1496±300	11 days (97%)	Tube changed
Shaking flask	SF 150 rpm	5.2±0.5	2264±480	11 days (91%)	
	SF 200 rpm	5.6±0.6	1663±560	11 days (69%)	
	SF 250 rpm	4.7±1.2	2079±230	5 days (<60%)	perhaps nutrient depletion 1 day before
	SF 300 rpm	5.9±1.0	1936±450	8 days (<60%)	perhaps nutrient depletion 1 day before

4.4.2 rCHO-K1 D1E7

Cell sedimentation occurred in the 90° Tube at 150 rpm (Figure 16), but adequate mixing was enabled at 150 rpm when cultured at 45° (Figure 17). The 45° Tubes above 200 rpm showed strong foaming and the cell suspension contacted the cap, which led

to cell deposits on the membrane and therefore blocked it (Figure 18).At 300 rpm fewer cell deposits accumulated on the membrane due to higher shear forces.

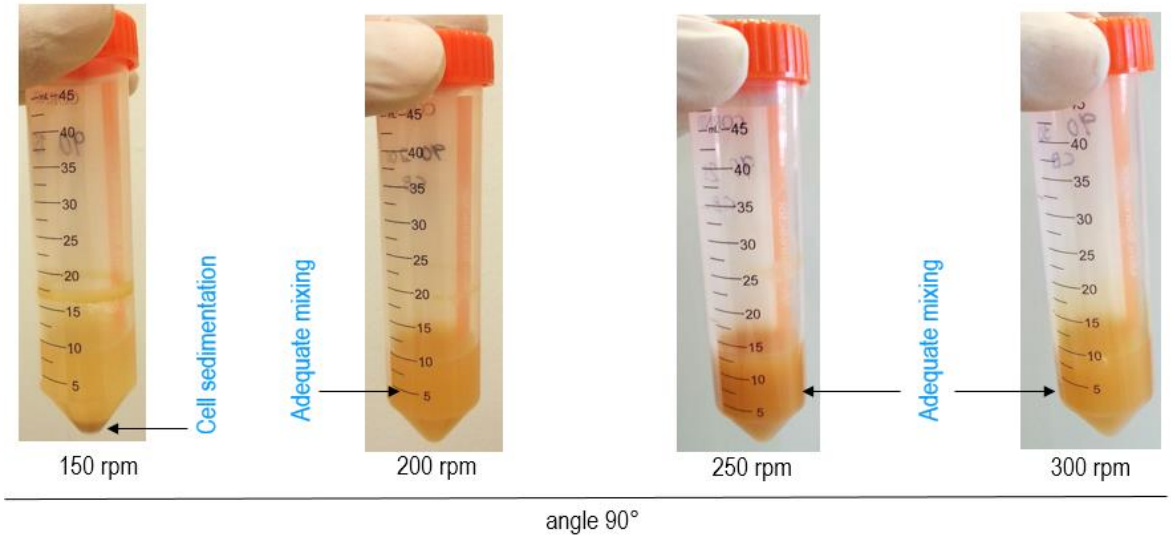


Figure 16: SpinTube bioreactors of rCHO-K1 pseudo perfusion cultures incubated with an angle of 90° at different rotation rates

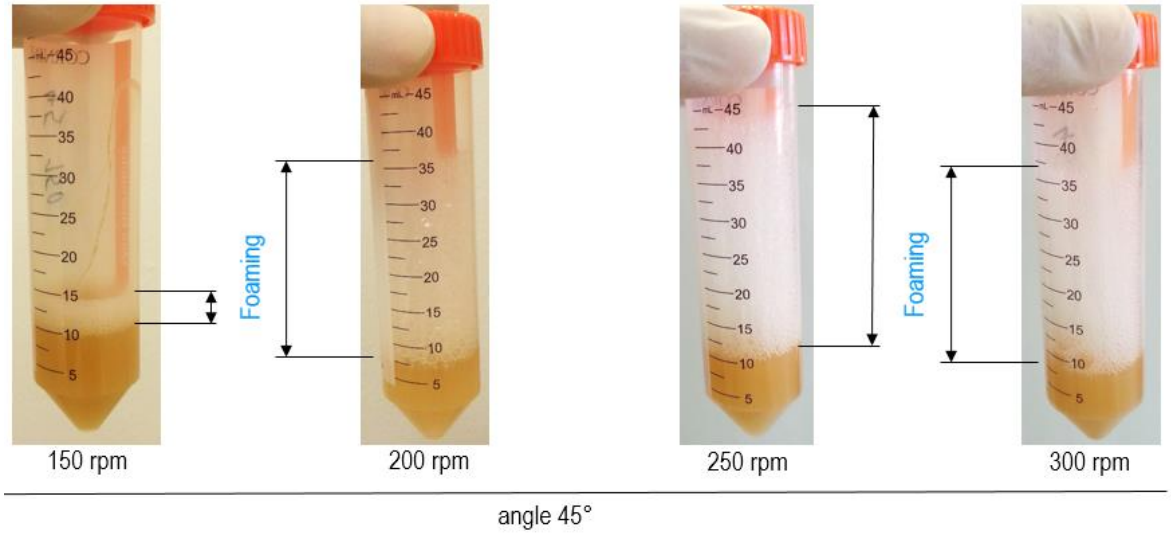


Figure 17: SpinTube bioreactors of rCHO-K1 pseudo perfusion cultures incubated with an angle of 45° at different rotation rates

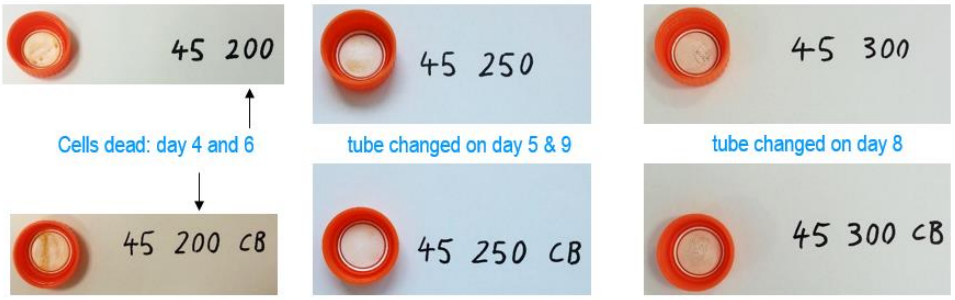


Figure 18: Cap of the SpinTube bioreactors of rCHO-K1 pseudo perfusion cultures incubated with an angle of 45° at different rotation rates

The cell deposits from vessel walls of the shake flasks were removed by washing with 10 mL sterile PBS (Figure 19). Therefore no shake flask change was necessary. Most cell deposits occurred at 150 rpm because of the lower shear forces.

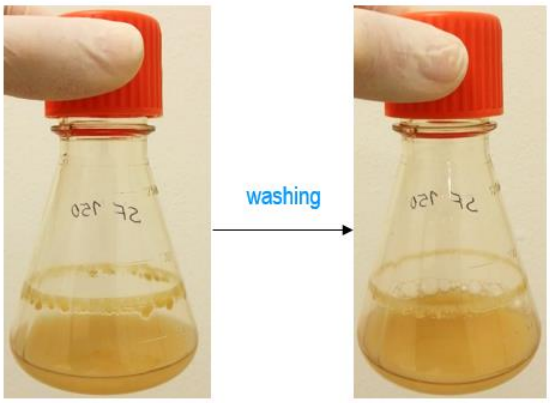


Figure 19: Washing Erlenmeyer shake flasks of rCHO-K1 pseudo perfusion cultures with 10 mL sterile PBS

Table 25 shows the average total cell concentration and titer in the pseudo steady state of rCHO-K1 cultures in ActiPro. Since only two pseudo perfusion cultures showed a viability above 80% after 11 days, it is obvious that ActiPro alone is not a suitable perfusion media for rCHO-K1.

Table 25: Summary of rCHO-K1 pseudo perfusion cultures with ActiPro at different rotation rates in SpinTube bioreactors at 90°, 45° or in Erlenmeyer shake flasks.

w/o CBs	Sample ID	Total CC [$\times 10^7$ c/mL]	Titer [mg/L]	Process time (% viab.)	Comment
Shaking tube	90° 150 rpm	3.5±0.1	520±160	5 days (<60%)	Cell sedimentation
	90° 200 rpm	5.4±0.6	890±70	8 days (<60%)	Shaker off
	90° 250 rpm	5.5±0.6	1230±50	6 days (<60%)	perhaps nutrient depletion 1 day before
	90° 300 rpm	5.1±0.9	1290±0	5 days (<60%)	perhaps nutrient depletion 1 day before

w/o CBs	Sample ID	Total CC [×10⁷ c/mL]	Titer [mg/L]	Process time (% viab.)	Comment
	45° 150 rpm	6.4±0.8	1180±70	10 days (<60%)	perhaps nutrient depletion 1 day before
	45° 200 rpm	5.0±0.7	1260±160	6 days (<60%)	Membrane blocked
	45° 250 rpm	6.2±0.5	1010±170	11 days (93%)	Tube changed
	45° 300 rpm	6.5±0.3	930±60	11 days (85%)	Tube changed
Shaking flask	**SF 150 rpm**	6.8±0.8	1170±200	11 days (72%)	
	SF 200 rpm	5.7±0.8	1170±60	8 days (<60%)	Shaker off
	SF 250 rpm	6.5±0.8	1120±280	11 days (66%)	
	SF 300 rpm	6.6±1.2	1090±190	11 days (<60%)	perhaps nutrient depletion 1 day before

Table 26 shows the average total cell concentration and titer in the pseudo steady state phase of rCHO-K1 cultures in ActiPro spiked with 7% CB7a and 0.7% CB7b. The best performance in cell concentration and titer was achieved with tubes at 90° / 250 rpm and the shaking flask at 150 rpm with a viability above 90%. The 45° Tube at 150 rpm had also a good performance, but the cells died on day 7. All other 45° tubes with strong foaming had lower titers compared to the 90° tubes and shaking flasks.

Without addition of CBs to ActiPro the viability decreased in all cultivations. This might be because of nutrient depletion. Spiking of ActiPro with CBs increased cell concentrations from 5.0–6.8×10^7 c/mL to 6.5–8.7×10^7 c/mL, mAb titer from 1000–1200 mg/L to 1700–2600 mg/L. Exceptions were the 45° tubes with strong foaming and the 90° Tube at 150 rpm, showing cell sedimentation.

Table 26: Summary of rCHO-K1 pseudo perfusion cultures with spiked ActiPro (7% CB7a and 0.7% CB7b) at different rotation rates in SpinTube bioreactors at 90°, 45° or in Erlenmeyer shake flasks. Shaking parameters with an unknown reason for the drop in viability are highlighted in red and the best performances are highlighted in green.

With CBs	Sample ID	Total CC [×10⁷ c/mL]	Titer [mg/L]	Process time (% viab.)	Comment
Shaking tube	**90° 150 rpm**	1.5±0.1	320±130	2 days (<60%)	Cell sedimentation
	90° 200 rpm	4.6±0.3	1110±220	8 days (<60%)	Shaker off
	90° 250 rpm	7.9±0.5	2210±410	11 days (92%)	
	90° 300 rpm	7.0±1.1	2020±10	8 days (<60%)	perhaps nutrient depletion 1 day before
	45° 150 rpm	6.8±1.1	2190±460	7 days (<60%)	perhaps nutrient depletion 1 day before
	45° 200 rpm	2.4±0.7	860±300	4 days (<60%)	Membrane blocked

With CBs	Sample ID	Total CC [×10⁷ c/mL]	Titer [mg/L]	Process time (% viab.)	Comment
	45° 250 rpm	6.1±0.6	1060±270	11 days (82%)	Tube changed
	45° 300 rpm	6.5±0.4	1090±90	11 days (78%)	Tube changed
Shaking flask	SF 150 rpm	8.7±0.2	1780±160	11 days (91%)	
	SF 200 rpm	6.5±1.2	2250±410	7 days (<60%)	perhaps nutrient depletion 1 day before
	SF 250 rpm	6.9±1.3	2640±350	7 days (<60%)	perhaps nutrient depletion 1 day before
	SF 300 rpm	7.7±1.1	1890±600	11 days (67%)	

4.5 Investigation of ActiPro and CDM4NS0 as perfusion medium for rCHO-K1

The best performing shaker settings for rCHO-K1 pseudo perfusion cultures were used from now on i.e. a 50 mL SpinTube bioreactor with 10 mL working volume cultivated at an angle of 90°. Since no difference was observed regarding cell growth and mAb production between 200 rpm and 250 rpm, the standard settings of 220 rpm were applied from now on.

It was known from previous studies that ActiPro can be prepared as a 3-fold concentrate. No concentration of the basal medium was possible for CDM4NS0. A medium concentrate of ActiPro was diluted to an osmolality of 380, 400 and 420 mOsmol/kg and then applied for daily media exchange. Basal media (ActiPro and CDM4NS0) were applied in normal (1×) concentrations and additional the glucose concentration was increased to 12 g/L in both basal media (Table 27).

Table 27: Summary of the experimental setup for the investigation of ActiPro and CDM4NS0 as perfusion medium

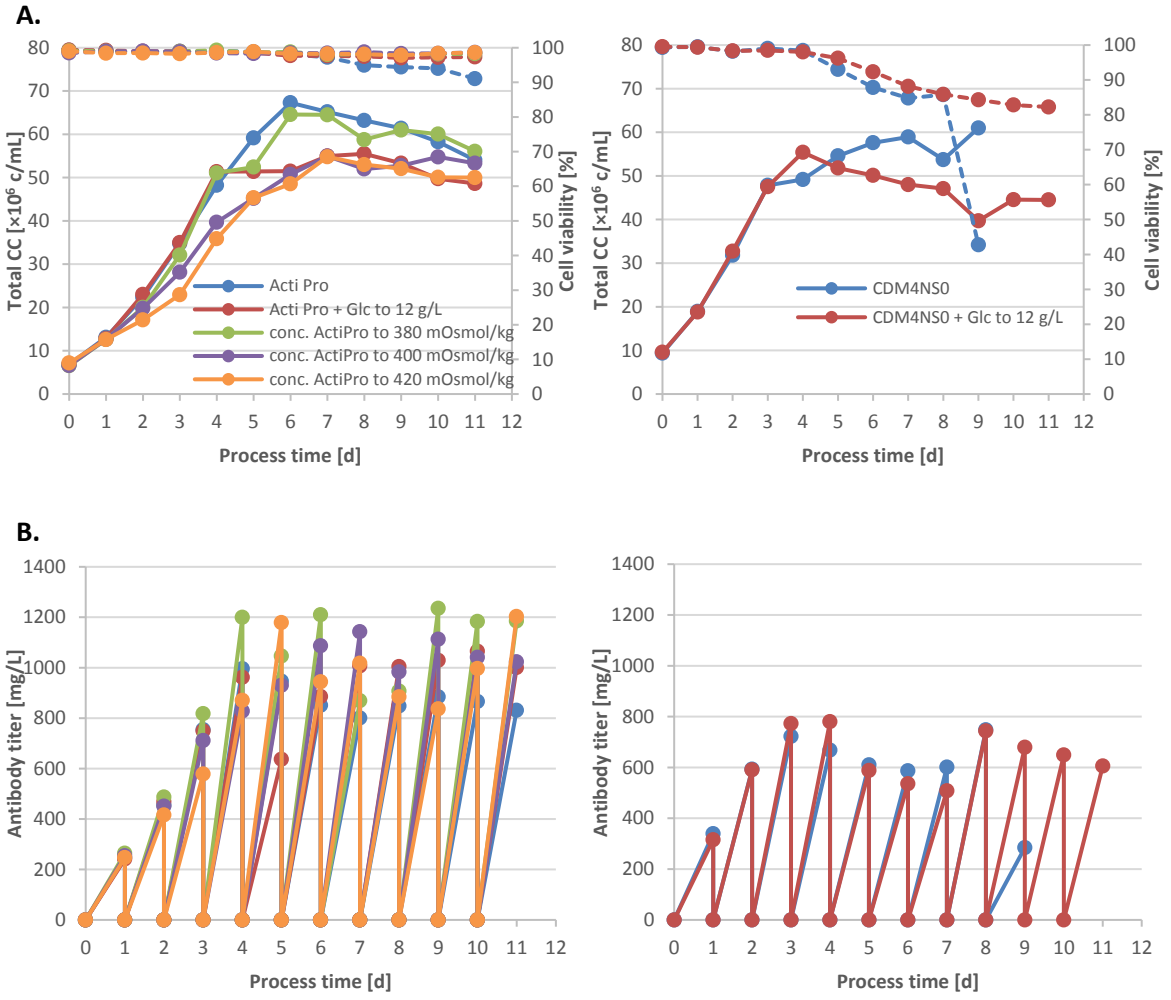
Basal medium	Concentration factor [×]	Glucose concentration [g/L]	Osmolality [mOsmol/kg]
ActiPro	1.00	6.6	300
	1.00	12.0	330
	1.24	8.6	380
	1.31	9.0	400
	1.37	9.7	420
CDM4NS0	1.00	8.0	300
	1.00	12.0	320

1× and 1.2× (380 mOsmol) ActiPro showed the highest peak cell concentrations of 6.7×10^7 c/mL and 6.5×10^7 c/mL (Figure 20). While the glucose addition to ActiPro yielded a slightly lower cell count of 5.6×10^7 c/mL and a slightly higher viability on

day 11. Glucose addition to CDM4NS0 yielded significant higher viabilities on day 9 and a prolonged process time.

The highest titers of 1100mg/L were reached with 1.2× ActiPro, Compared to 1× ActiPro with a titer of 880 mg/L this is an increase of more than 20%. The specific productivity increased from 19 pg/c/d to 24 pg/c/d with the 1.2× ActiPro. Glucose addition to ActiPro and CDM4NS0 increased the titer by 8%, from 880 mg/L to 950 mg/L in ActiPro and from 600mg/L to 650 mg/L in CDM4NS0.

Table 28 summarize cell concentration and mAb production of the different media preparations.



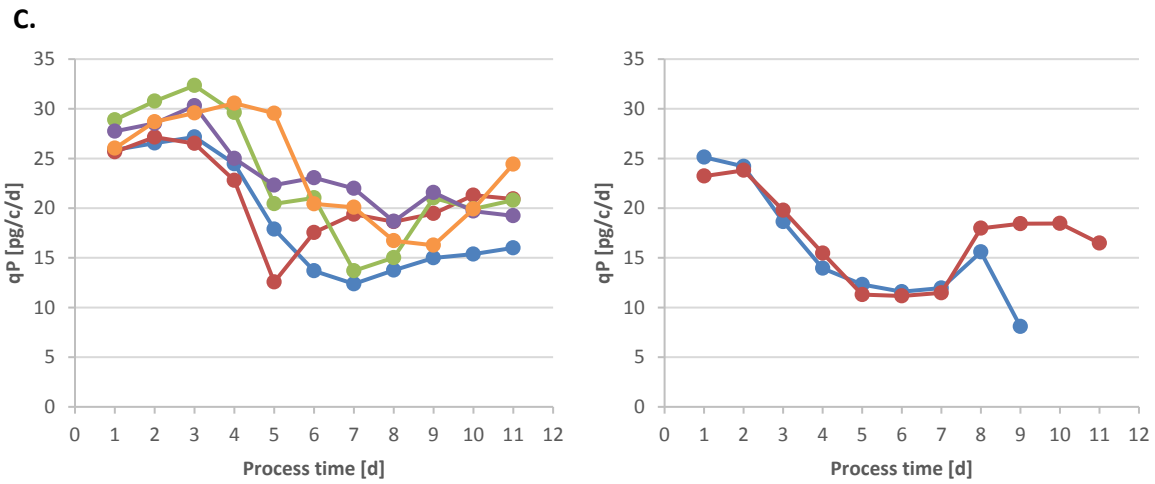
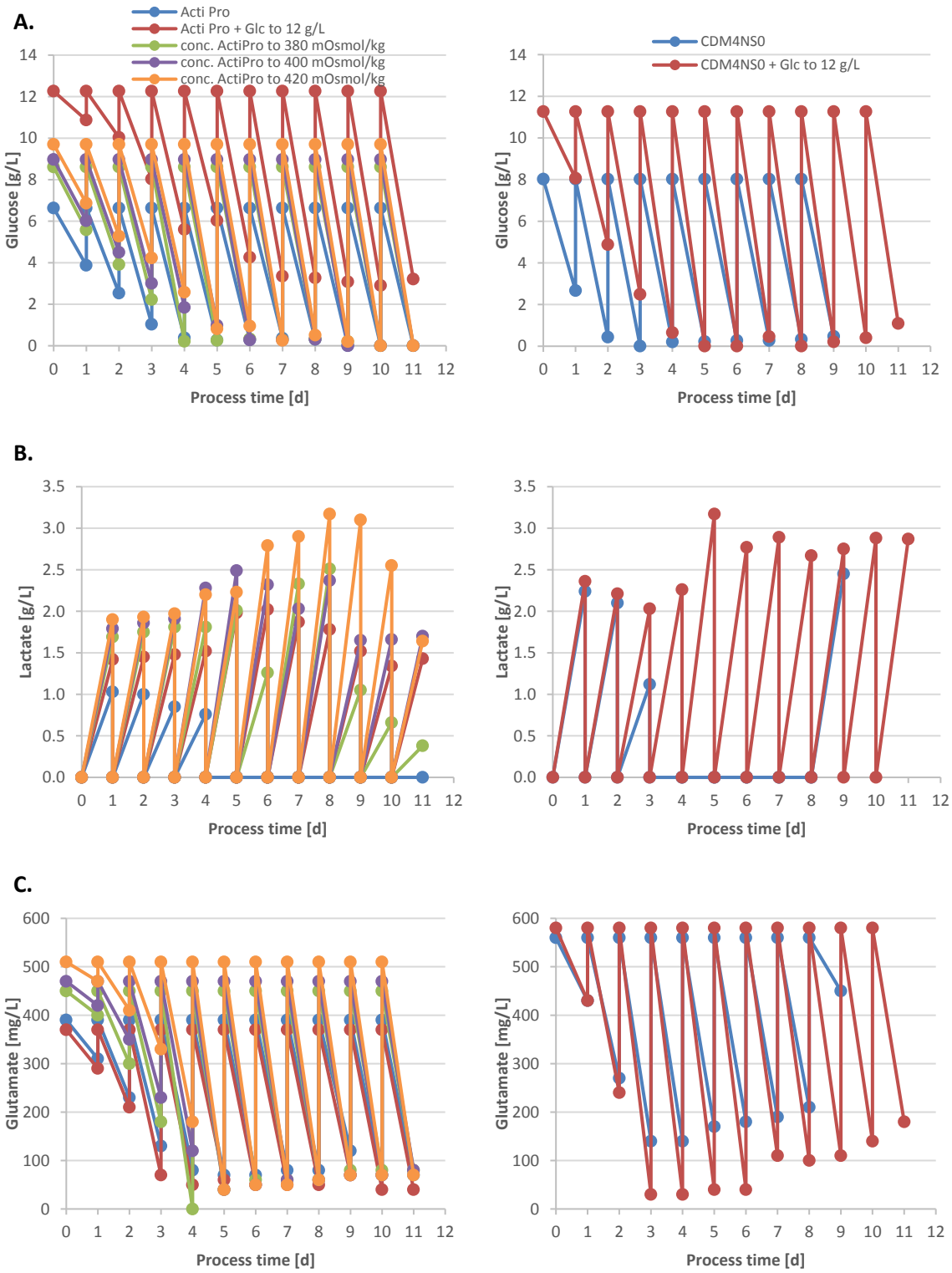


Figure 20: Cell concentration (straight line) and viability (dashed line) (A.), antibody concentrations (B.) and specific productivity (C.) of pseudo perfusion experiments of rCHO-K1 in ActiPro, 12 g/L Gluc-spiked ActiPro, ActiPro concentrated to 380, 400, or 420 mOsmol/kg, CDM4NS0 and 12 g/L Gluc-spiked CDM4NS0.

At high cell concentrations glucose was consumed within one day in ActiPro and 1.2× ActiPro starting from day 4, in 1.3× ActiPro from day 6 and in 1.4× ActiPro from day 7 (Figure 21). Glucose levels could be kept above 3 g/L in ActiPro cultures spiked with 12 g/L glucose. The complete consumption of glucose in CDM4NS0 started on day 3 and in CDM4NS0 spiked with glucose to 12 g/L on day 5. In plain ActiPro and CDM4NS0 lactate was produced until day 3 or 4. With the glucose spiked and concentrated media lactate was produced up to 3 g/L. In all ActiPro cultures glutamate was consumed to 50 mg/L, in CDM4NS0 to 150 mg/L and in Gluc-spiked CDM4NS0 to 30–100 mg/L. No glutamine was secreted after day 3 in ActiPro and after day 2 in CDM4NS0. Also in the Gluc-spiked CDM4NS0 no glutamine was secreted after day 2, but the production started again on day 7 to 100–200 mg/L. Glutamine levels between 100 and 500 mg/L where observed for the glucose spiked and concentrated ActiPro cultures. The ammonium consumption in ActiPro shifted to production after day 4 to reach a final peak ammonium concentration of 100–180 mg /L. In CDM4NS0 the peak ammonium concentration of 120 mg/L was higher compared to the Gluc-spiked CDM4NS0 with 80 mg/L ammonium.


A.
Acti Pro
Acti Pro + Glc to 12 g/L
conc. ActiPro to 380 mOsmol/kg
conc. ActiPro to 400 mOsmol/kg
conc. ActiPro to 420 mOsmol/kg
Glucose [g/L]
Process time [d]
CDM4NS0
CDM4NS0 + Glc to 12 g/L
Glucose [g/L]
Process time [d]
B.
Lactate [g/L]
Process time [d]
Lactate [g/L]
Process time [d]
C.
Glutamate [mg/L]
Process time [d]
Glutamate [mg/L]
Process time [d]

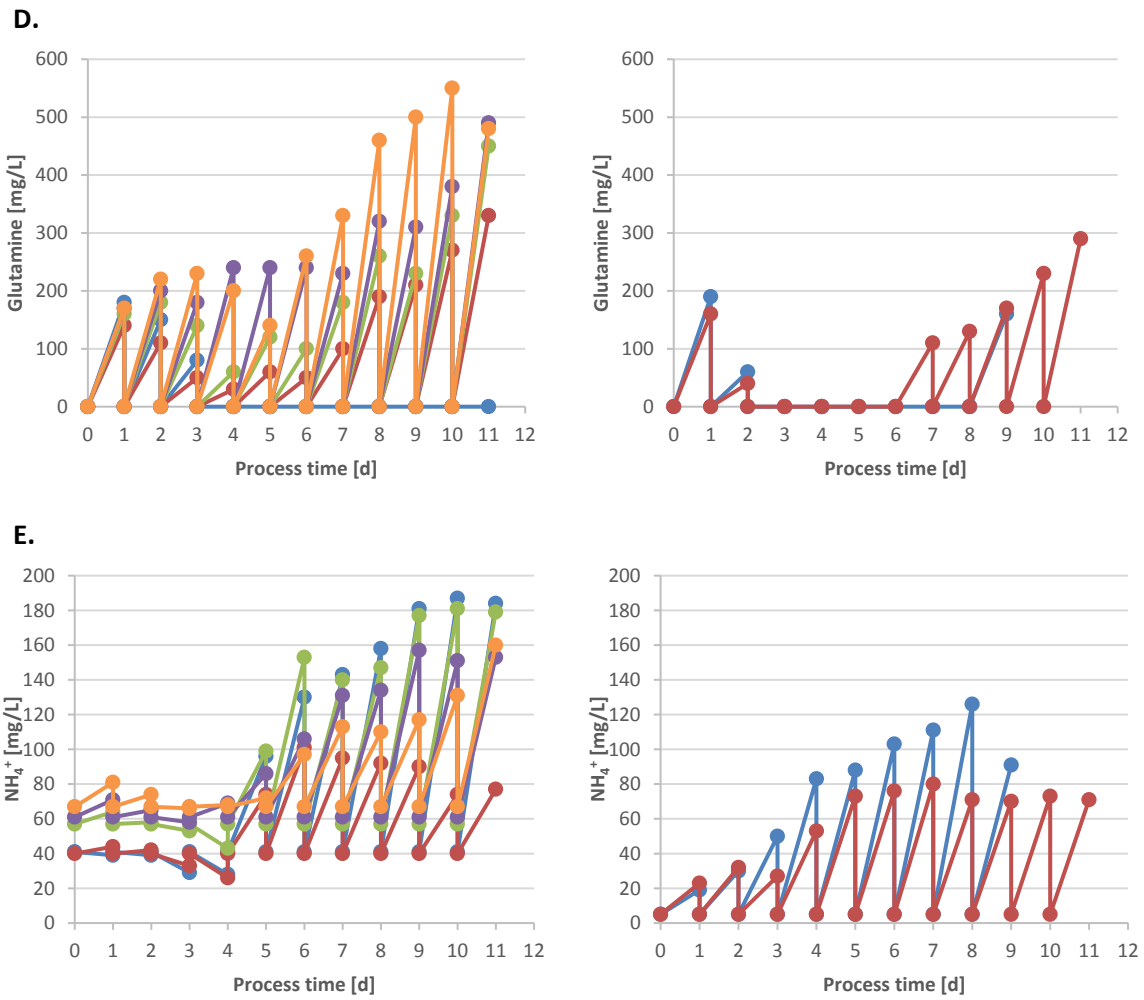


Figure 21: Concentrations of key metabolites of pseudo perfusion experiments of rCHO-K1 in ActiPro, 12 g/L Gluc-spiked ActiPro, ActiPro concentrated to 380, 400, or 420 mOsmol/kg, CDM4NS0 and 12 g/L Gluc-spiked CDM4NS0, showing glucose (A.), lactate (B.), glutamate (C.), glutamine (D.) and ammonium (E.).

Table 28: Summary of pseudo perfusion experiments of rCHO-K1 in ActiPro, 12 g/L Gluc-spiked ActiPro, ActiPro concentrated to 380, 400, or 420 mOsmol/kg, CDM4NS0 and 12 g/L Gluc-spiked CDM4NS0. Best performances are highlighted in green.

Basal medium	Conc. factor [×]	Gluc. [g/L]	Total CC [$\times 10^7$ c/mL]	VCCD [10^9 c×d/mL]	mAb [mg/L]	qP [pg/c/d]
ActiPro	1.00	6.6	6.0±0.6	5.0	880±60	19±6
	1.00	12.0	5.2±0.2	4.6	950±140	21±4
	1.24	8.6	5.9±0.5	5.0	1100±150	24±6
	1.31	9.0	5.0±0.5	4.3	1020±100	23±4
	1.37	9.7	4.9±0.6	4.1	990±140	24±5
CDM4NS0	1.00	8.0	5.5±0.5	3.6	600±150	16±6
	1.00	12.0	4.8±0.5	4.2	650±100	17±5

4.6 Perfusion medium development of rCHO-K1 in CDM4NS0

4.6.1 Selection of optimal Cell Boost combination by DoE #1 for rCHO-K1 in CDM4NS0 spiked-batches

In the basal medium screening CDM4NS0 was identified as a suitable alternative medium to ActiPro and was chosen as basis for the development of a perfusion medium. The performance of the CDM4NS0 spiked with different combinations of CBs was tested with batch experiments while keeping the glucose concentration above 3 g/L.
A DoE #1 design table was established to screen the basal medium CDM4NS0 spiked with different combinations of CBs in three different amounts according to DoE levels -1, 0 and +1 (Table 29).

Table 29: DoE #1 design to pre-select optimal CBs for rCHO-K1 in spiked CDM4NS0 batches

Exp. No.	CB1	CB2	CB3	CB4	CB5	CB6	CB7a	CB7b
control - 1	-1	-1	-1	-1	-1	-1	-1	-1
2	1	-1	-1	-1	-1	1	1	1
3	-1	1	-1	-1	1	-1	1	1
4	1	1	-1	-1	1	1	-1	-1
5	-1	-1	1	-1	1	1	1	-1
6	1	-1	1	-1	1	-1	-1	1
7	-1	1	1	-1	-1	1	-1	1
8	1	1	1	-1	-1	-1	1	-1
9	-1	-1	-1	1	1	1	-1	1
10	1	-1	-1	1	1	-1	1	-1
11	-1	1	-1	1	-1	1	1	-1
12	1	1	-1	1	-1	-1	-1	1
13	-1	-1	1	1	-1	-1	1	1
14	1	-1	1	1	-1	1	-1	-1
15	-1	1	1	1	1	-1	-1	-1
all CBs - 16	1	1	1	1	1	1	1	1
triplicate - 17	0	0	0	0	0	0	0	0
triplicate - 18	0	0	0	0	0	0	0	0
triplicate - 19	0	0	0	0	0	0	0	0

Measurement of osmolalities indicated that only for the "all CBs" condition the critical osmolality of 400 mOsmol/kg was slightly surpassed (Figure 22).

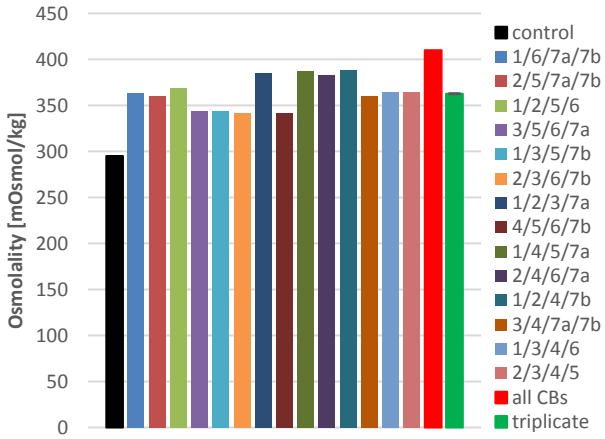
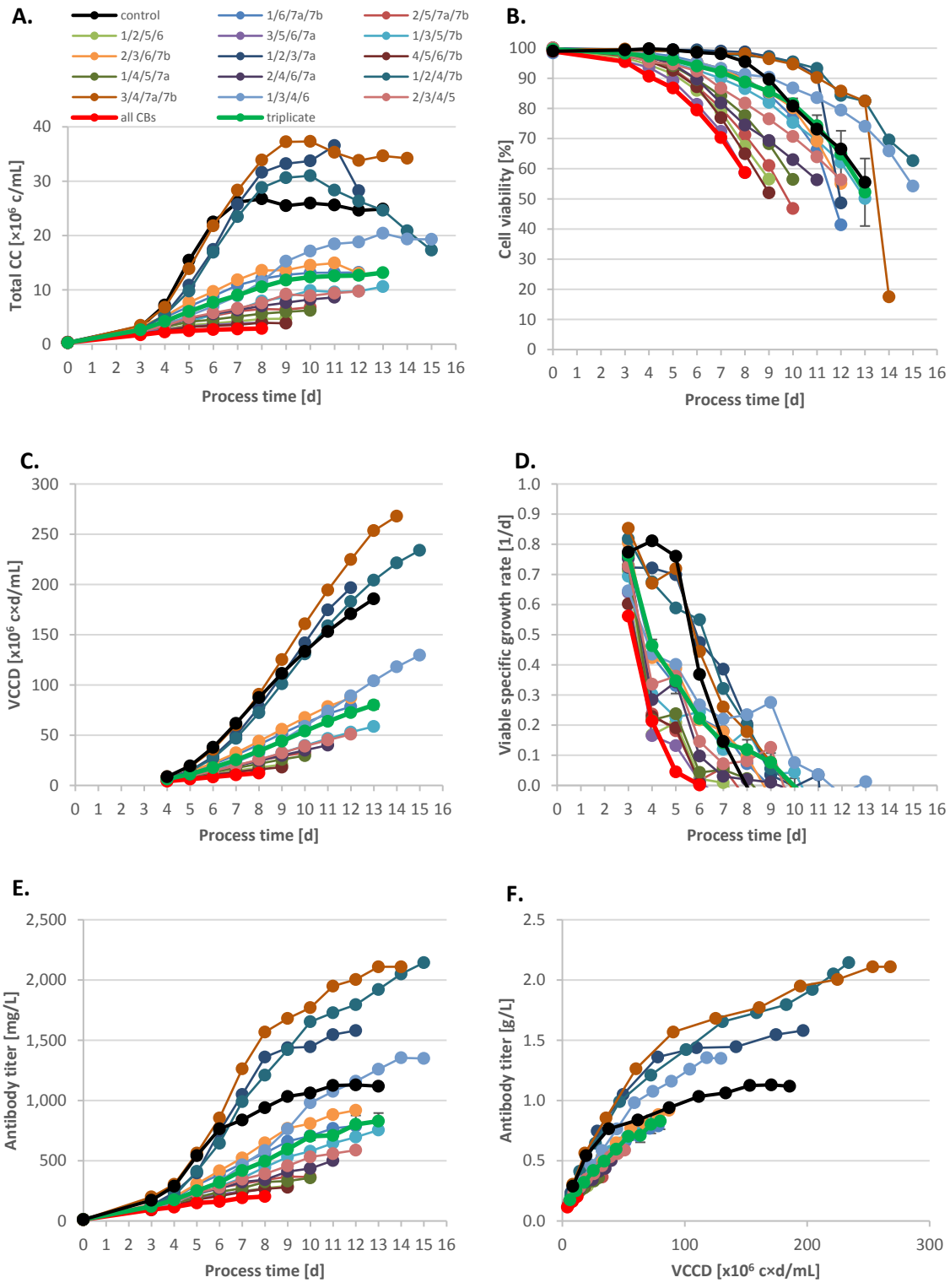


Figure 22: Measured osmolalities of CDM4NS0 basal medium spiked with different combinations of CBs for DoE #1 for the rCHO-K1 cell line.

Figure 23 and Table 30 summarizes the rCHO-K1 batch experiments, to finally calculate the optimal CB combination.

Comparison of the control CDM4NS0 basal medium batch without any CB (DoE levels -1), triplicate cultures spiked with half-maximum CB addition (DoE levels 0) and spiked-batches with all CBs at maximum concentrations (DoE levels +1) showed peak cell densities of 26.7×10^6 c/mL, 13.2×10^6 c/mL and 2.9×10^6 c/mL with maximum titers of 1.1 g/L, 0.8 g/L and 0.2 g/L, respectively (Figure 23). The control CDM4NS0 batch experiment gave similar results as in the experiment for comparison of different basal media batches (Figure 7). Only three CB combinations improved the peak cell densities and viable cumulative cell days (VCCD) compared to the control experiment, namely #8-1/2/3/7a, #12-1/2/4/7b and #13-3/4/7a/7b, which also increased the total process time promoted by higher viabilities at later time points. Also these three experiments increased final product titer up to 2.1 g/L, which together with #14-1/3/4/6 lie higher than the control batch (1.1 g/L). Overall, CB addition increased the specific productivities in all spiked-batches, however, only four conditions increased final titers where viabilities and cell densities (or cell days) could be kept at a maximum.


A.
control
1/2/5/6
2/3/6/7b
1/4/5/7a
3/4/7a/7b
all CBs
1/6/7a/7b
3/5/6/7a
1/2/3/7a
2/4/6/7a
1/3/4/6
triplicate
2/5/7a/7b
1/3/5/7b
4/5/6/7b
1/2/4/7b
2/3/4/5
Total CC [x10⁶ c/mL]
Process time [d]
B.
Cell viability [%]
Process time [d]
C.
VCCD [x10⁶ c×d/mL]
Process time [d]
D.
Viable specific growth rate [1/d]
Process time [d]
E.
Antibody titer [mg/L]
Process time [d]
F.
Antibody titer [g/L]
VCCD [x10⁶ c×d/mL]

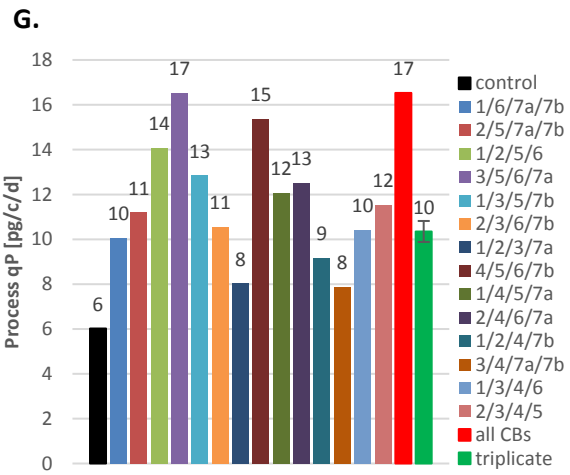


Figure 23: Cell concentrations (A.), viabilities (B.), viable cumulative cell days VCCD (C.), viable specific growth rate (D.), antibody concentrations (E.), titer vs. VCCD (F.) and specific productivity (G.) of the first DoE-based spiked-batch experiments of rCHO-K1 in CDM4NS0 basal medium spiked with different combinations of CBs. qP values were calculated as specific productivities until culture harvest.

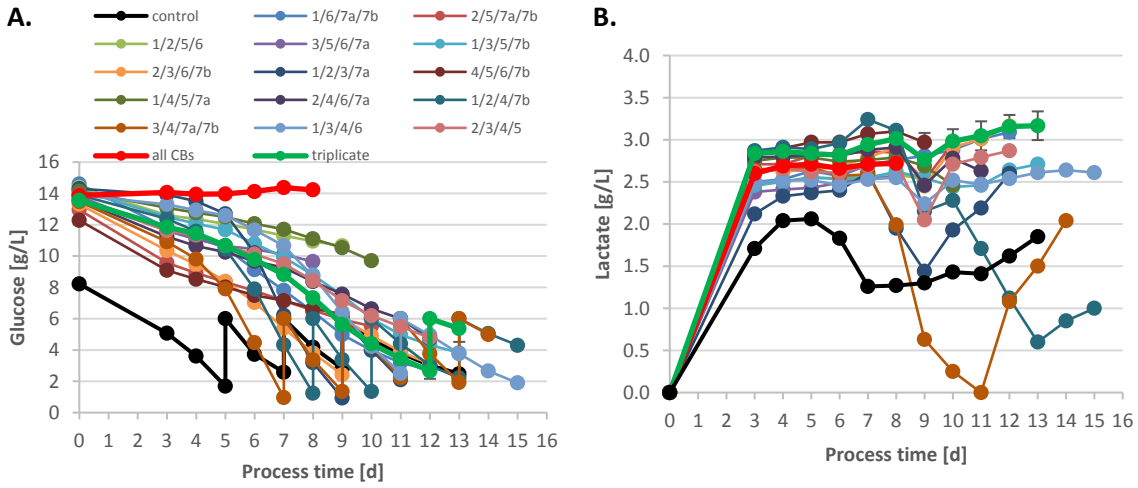
The generated cell culture data was summarized (Table 30) and used to perform regression analysis and generation of models using the statistical software Modde (results and details are not shown).

Table 30: DoE #1 input table summarizing batch results for optimal CB selection for rCHO-K1 in CDM4NS0. Conditions better than the control experiment (no CBs) are highlighted in green.

Exp. No.	Cell Boosts	Peak titer [mg/L]	VCCD [×10^7 c×d/mL]	Peak CC [×10^6 c/mL]	qp D0-X [pg/c/d]
1	control (no CBs)	1129	18.6	26.7	6.0
2	1/6/7a/7b	789	7.8	13.2	10.1
3	2/5/7a/7b	368	3.3	6.8	11.2
4	1/2/5/6	291	2.1	4.7	14.1
5	3/5/6/7a	266	1.6	4.0	16.5
6	1/3/5/7b	754	5.9	10.6	12.9
7	2/3/6/7b	919	8.7	14.9	10.6
8	1/2/3/7a	1580	19.7	33.7	8.0
9	4/5/6/7b	280	1.8	3.9	15.4
10	1/4/5/7a	358	3.0	6.2	12.1
11	2/4/6/7a	502	4.0	8.6	12.5
12	1/2/4/7b	2144	23.4	31.0	9.2
13	3/4/7a/7b	2110	26.8	37.3	7.9
14	1/3/4/6	1354	13.0	20.4	10.4
15	2/3/4/5	588	5.1	9.7	11.6
16	all CBs	203	1.2	2.9	16.5
17	all CBs 0.5	826	7.8	12.8	10.6

Exp. No.	Cell Boosts	Peak titer [mg/L]	VCCD [×10⁷ c×d/mL]	Peak CC [×10⁶ c/mL]	qp D0-X [pg/c/d]
18	all CBs 0.5	763	7.8	13.0	9.8
19	all CBs 0.5	897	8.5	13.6	10.6

Besides the critical cell culture parameters used for DoE analysis (Figure 23 and Table 30), also metabolites where measured (Figure 24). The control batch without any CB reached the chosen 3 g/L glucose threshold at first on day 5 and a continuous increase in lactate formation up to peak concentrations of 2 g/L. Following day 5, this culture switched to lactate consumption reducing the accumulated 2 g/L lactate over time until the second glucose feed was added on day 7, after which lactate levels remained relatively constant with only a slight increase over time. Because no CB was added, this batch culture had the lowest initial glucose concentration and therefore showed the lowest lactate concentrations. In contrast, by adding CBs glucose concentrations of the spiked media increased to 14 g/L causing higher lactate concentrations up to 3 g/L. Interestingly, the optimal conditions mentioned above (#8-1/2/3/7a, #12-1/2/4/7b, #13-3/4/7a/7b) concerning cell growth, where the only cultures that switched to lactate consumption besides the no CB control. This switch usually started when glucose reached around 1 g/L. The unspiked control experiment was also the first culture that reached critical glutamate concentrations at day 7, which is the starting point of declining viability. Only the four mentioned conditions with excellent growth showed fast consumption of the available glutamate. After an initial rise in glutamine concentrations, the peak vanished as a result of glutamine consumption. Ammonium levels increased in all cultures, but the three batches with the optimal conditions mentioned above showed an ammonium consumption starting on day 6 or 7. The lowest peak ammonium concentration of 27 mg/L was observed in the control batch without any CB.



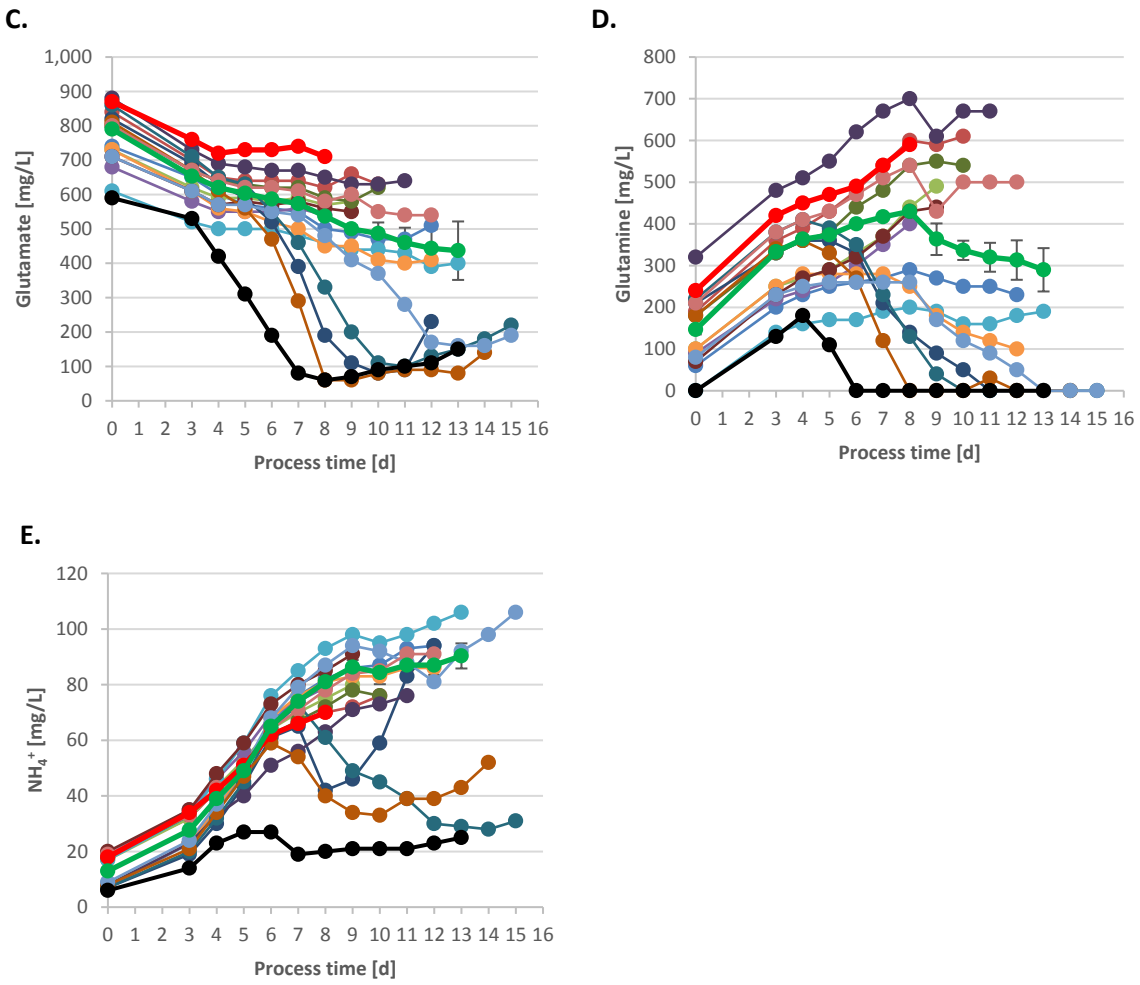


Figure 24: Concentrations of key metabolites of the first DoE-based spiked-batch experiments of rCHO-K1 in CDM4NS0 basal medium spiked with different combinations of CBs, showing glucose (A.), lactate (B.), glutamate (C.), glutamine (D.) and ammonium (E.).

4.6.2 Definition of optimal Cell Boost 1, 3, 7a and 7b ratio by DoE #2 for rCHO-K1 in CDM4NS0 pseudo-perfusions

A DoE #2 design table was established to screen the basal medium CDM4NS0 spiked with different combinations of CB 1, 3, 7a and 7b in three different amounts according to DoE levels -1, 0 and +1 (Table 31).

Table 31: DoE #2 design for choosing the optimal CB 1, 3, 7a and 7b ratio for rCHO-K1 in spiked CDM4NS0 pseudo-perfusions

Exp. No.	CB1	CB3	CB7a	CB7b
control - 1	-1	-1	-1	-1
2	1	-1	-1	-1
3	-1	1	-1	-1
4	1	-1	1	-1

Exp. No.	CB1	CB3	CB7a	CB7b
5	-1	1	1	-1
6	1	1	1	-1
7	-1	-1	-1	1
8	-1	1	-1	1
9	1	1	-1	1
10	-1	-1	1	1
11	1	-1	1	1
all CBs - 12	1	1	1	1
13	-1	0	0	0
14	1	0	0	0
15	0	-1	0	0
16	0	1	0	0
17	0	0	-1	0
18	0	0	1	0
19	0	0	0	-1
20	0	0	0	1
triplicate - 21	0	0	0	0
triplicate - 22	0	0	0	0
triplicate - 23	0	0	0	0

Figure 25 shows that the osmolality of all perfusion media remained below the critical threshold of 400 mOsmol/kg.

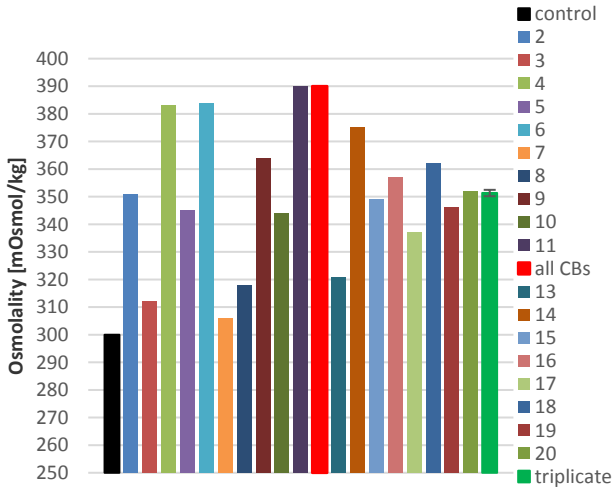


Figure 25: Measured osmolalities of CDM4NS0 basal medium spiked with different combinations of CB 1, 3, 7a and 7b for DoE #2 for the rCHO-K1 cell line

The performance of the CDM4NS0 spiked with different combinations of CBs 1, 3, 7a and 7b was tested by using these media for the daily complete medium exchange in pseudo perfusion experiments. Figure 26 and Table 32 summarizes the rCHO-K1 pseudo perfusion experiments, to finally calculate the optimal CB ratio.

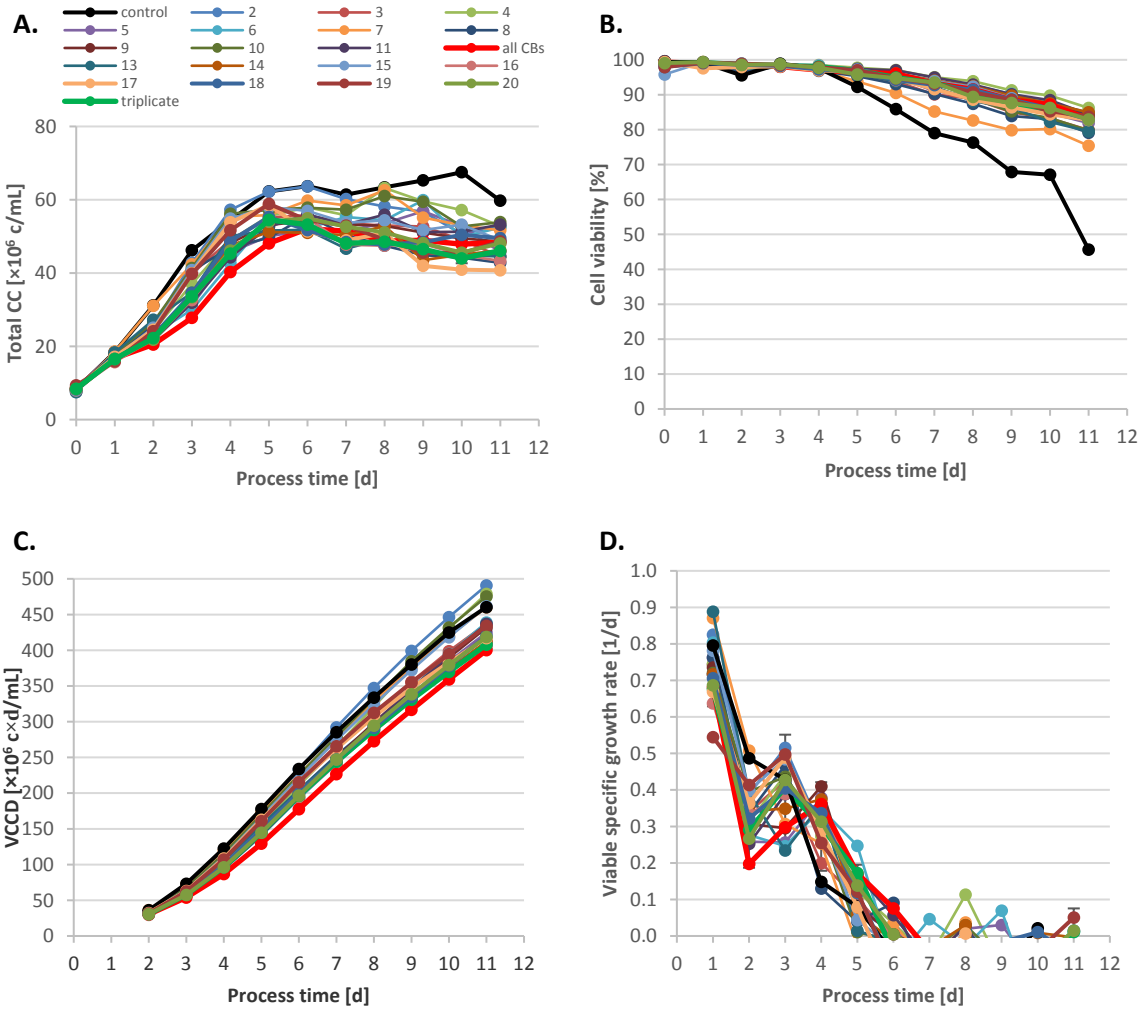
Comparison of the control CDM4NS0 basal medium without any CB (levels -1), triplicate cultures spiked with half-maximum CB addition (levels 0) and perfusion medium spiked with all CBs at maximum concentrations (levels +1) showed peak cell concentrations of

6.8×10^7 c/mL, 5.6×10^7 c/mL and 5.2×10^7 c/mL, respectively. The mean peak cell concentration of all cultures was 5.7±0.4×10^7 c/mL, indicating similar good performance of all tested media. The viability of the control decreased significantly and dropped below 60% on day 11.

The control showed a high VCCD (46×10^7 c×d/mL) while the triplicate showed a low VCCD (41×10^7 c×d/mL) and the max. spiked showed the lowest VCCD (40×10^7 c×d/mL).

The maximum titers of control, triplicate and max. spiked cultures were 718 mg/L, 862 mg/L and 940 mg/L, respectively. The mean peak titer of all cultures was 893±73 mg/L.

Comparison of the control, triplicate and max. spiked cultures showed specific productivities of 13.8 pg/c/d, 19.1 pg/c/d and 19.7 pg/c/d, respectively. The mean qP of all cultures was 18.2±1.6 pg/c/d. No substantial difference due to media is observed, but there was a trend of higher specific productivities and final titers for higher spiked perfusion media.



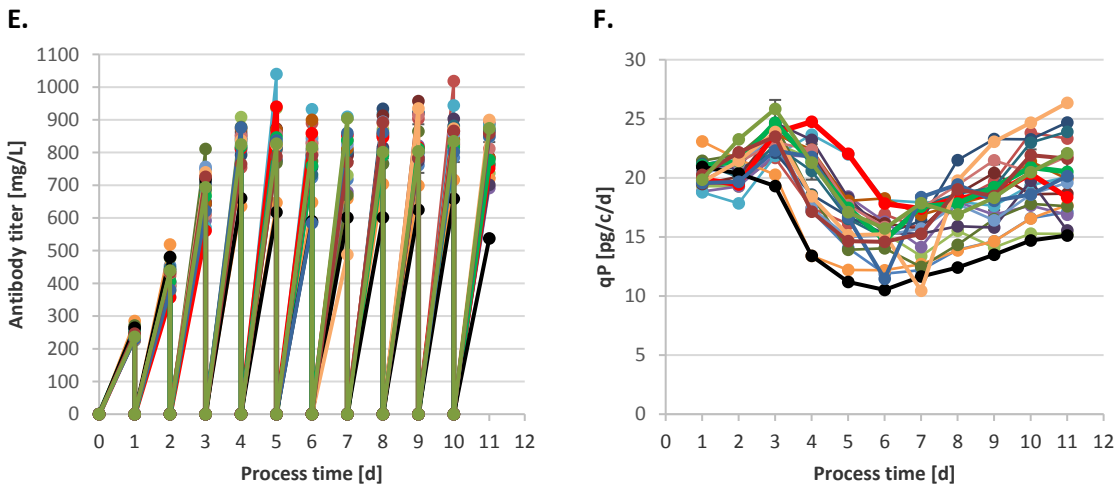


Figure 26: Cell concentrations (A.), viabilities (B.), viable cumulative cell days VCCD (C.), viable specific growth rate (D.), antibody concentrations (E.) and specific productivity (F.) of the second DoE-based pseudo perfusion experiments of rCHO-K1 in CDM4NS0 basal medium spiked with different combinations of CB1, CB3, CB7a and CB7b.

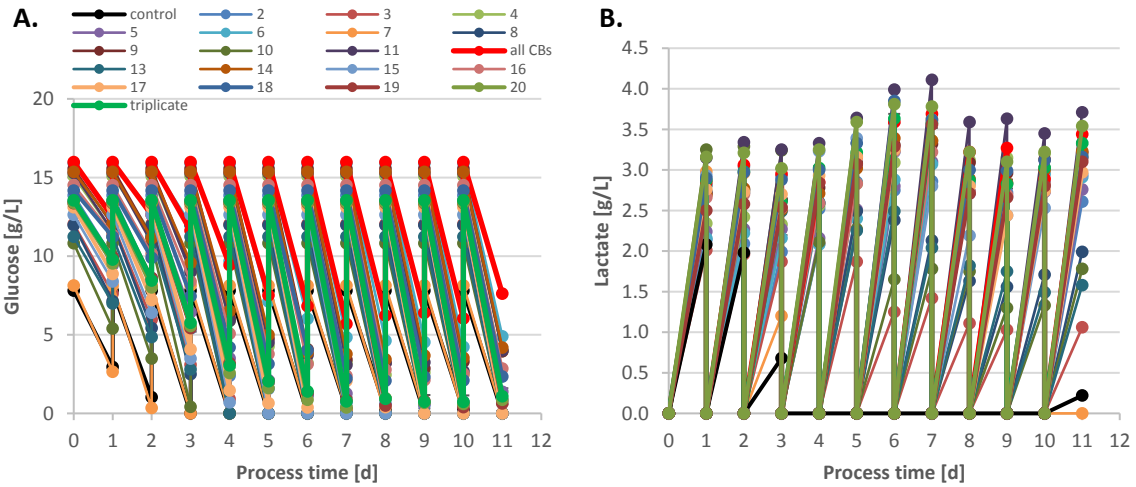
The generated cell culture data was summarized (Table 32) and used to perform regression analysis and generation of models using the statistical software Modde (results and details are not shown). The mean values in Table 32 are calculated starting from day 4, when the cells reached their maximal cell concentration, which is indicated by a plateau phase.

Table 32: DoE #2 input table summarizing pseudo perfusion results for optimal CB ratio for rCHO-K1 in CDM4NS0. Conditions better than the control experiment (no CBs) are highlighted in green.

Exp. No.	DoE levels for Cell Boosts 1/3/7A/7b	Peak titer [mg/L]	Mean titer D4-11 [mg/L]	VCCD D0-11 [×10⁷ cxd/mL]	Peak CC [×10⁷ c/mL]	Mean CC D4-11 [×10⁷ c/mL]	Mean qP D4-11 [pg/c/d]
1	control -1/-1-/-1/-1	718	611	46.1	6.8	6.2	12.8
2	1/-1/-1/-1	839	772	49.1	6.4	5.8	14.7
3	-1/1/-1/-1	1018	864	43.7	5.6	5.2	18.9
4	1/-1/1/-1	936	830	47.9	6.3	5.6	16.1
5	-1/1/1/-1	856	792	42.5	5.7	5.1	17.5
6	1/1/1/-1	1040	923	43.9	6.0	5.3	19.6
7	-1/-1/-1/1	728	679	46.0	6.3	5.6	14.2
8	-1/1/-1/1	934	838	40.8	5.6	4.8	20.0
9	1/1/-1/1	957	853	43.2	5.4	5.1	18.5
10	-1/-1/1/1	878	791	47.5	6.1	5.7	15.6
11	1/-1/1/1	903	816	43.7	5.6	5.2	17.4
12	all CBs 1/1/1/1	940	843	40.0	5.2	4.8	19.7

Exp. No.	DoE levels for Cell Boosts 1/3/7A/7b	Peak titer [mg/L]	Mean titer D4-11 [mg/L]	VCCD D0-11 [×10^7 cxd/mL]	Peak CC [×10^7 c/mL]	Mean CC D4-11 [×10^7 c/mL]	Mean qP D4-11 [pg/c/d]
13	-1/0.5/0.5/0.5	882	819	40.9	5.2	4.8	19.3
14	1/0.5/0.5/0.5	900	843	41.3	5.3	4.8	19.2
15	0.5/-1/0.5/0.5	894	837	46.2	5.8	5.4	17.0
16	0.5/1/0.5/0.5	916	846	40.7	5.5	4.8	19.6
17	0.5/0.5/-1/0.5	935	818	41.7	5.9	4.8	19.1
18	0.5/0.5/1/0.5	877	807	42.0	5.5	5.0	18.1
19	0.5/0.5/0.5/-1	892	814	43.4	5.9	5.1	17.8
20	0.5/0.5/0.5/1	904	836	41.9	5.5	5.0	18.7
21	0.5/0.5/0.5/0.5	896	842	41.3	5.6	4.8	19.3
22	0.5/0.5/0.5/0.5	848	796	40.9	5.6	4.9	18.4
23	0.5/0.5/0.5/0.5	842	785	40.2	5.4	4.8	18.3

The glucose was not consumed within one day in the perfusion media spiked with all CBs at maximum concentrations (#12) and in cultures #4, 6, 9, 11, 14, 16 and 18 (Figure 27). In all these combinations a lot of CB1 and CB7a was added. There was no production of lactate in the control and #7, which was spiked with CB7b only. These two perfusion media had the lowest glucose concentration. In all other cultures plenty of Glucose was in the media and lactate levels reached up to 4 g/L. The glutamate never dropped below 70 mg/L and ammonia continuously increased up to 140 mg/L. Therefore enough precursors for de novo glutamine synthesis were available at each time point. Low Glutamine concentrations were observed in the control and #2, 3, 7, 8 and 17, which coincides with the absence of CB1 and/or CB7a. This effect remains elusive since glutamate and ammonia were not limiting in those cultures.



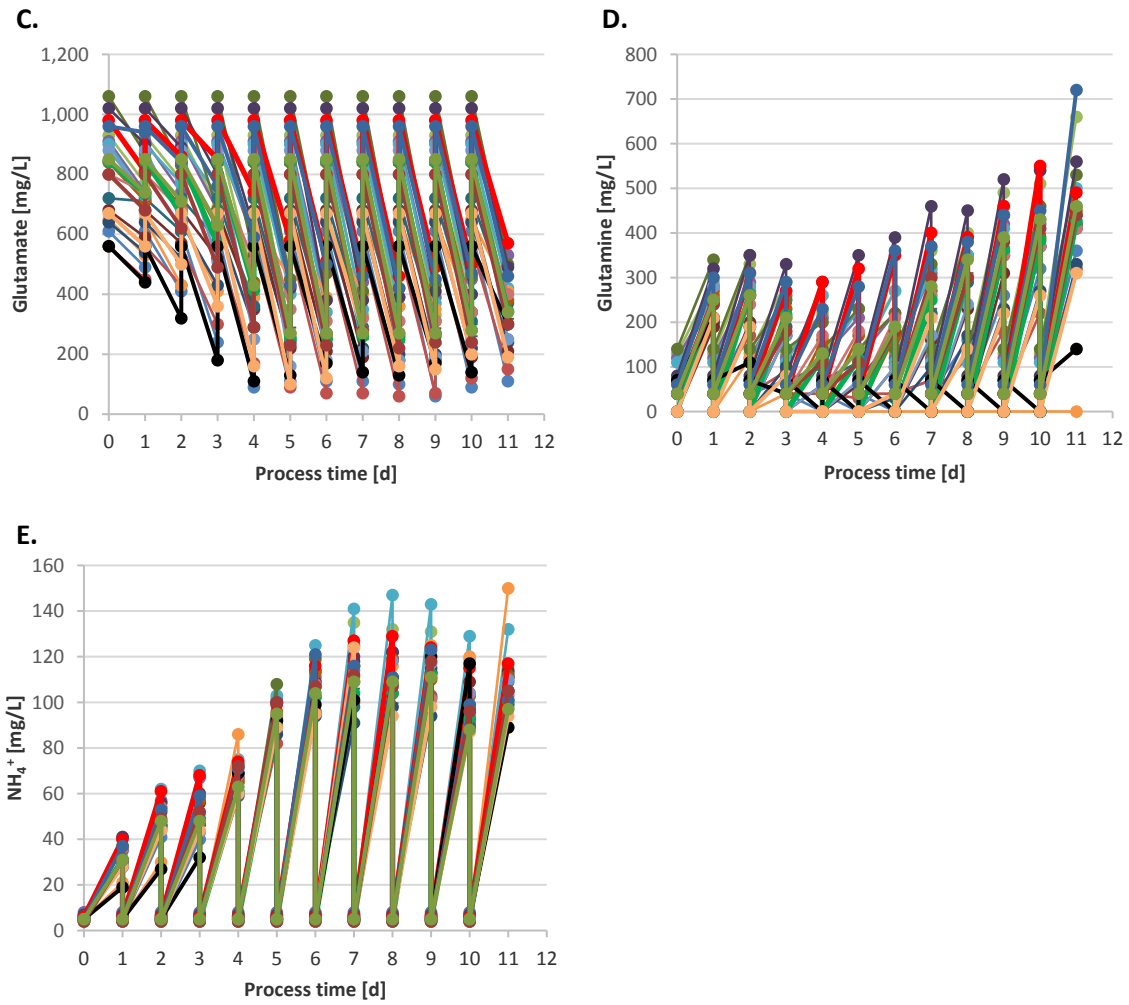


Figure 27: Concentrations of key metabolites of the second DoE-based pseudo perfusion experiments of rCHO-K1 in CDM4NS0 basal medium spiked with different combinations of CB1, CB3, CB7a and CB7b, showing glucose (A.), lactate (B.), glutamate (C.), glutamine (D.) and ammonium (E.).

4.6.3 Bioreactor verification of best performing Cell Boost combination for rCHO-K1 in CDM4NS0 perfusions

For verification of the optimized perfusion media two different compositions were investigated in perfusion cultivation by applying a WAVE Bioreactor. The two media were plain CDM4NS0 and the optimized CDM4NS0 perfusion medium (CDM4NS0 spiked with 11.06% CB1 and 19.90% CB3). Since the feed-spiked CDM4NS0 contains a higher glucose concentration, the glucose concentration of plain CDM4NS0 was adjusted accordingly.

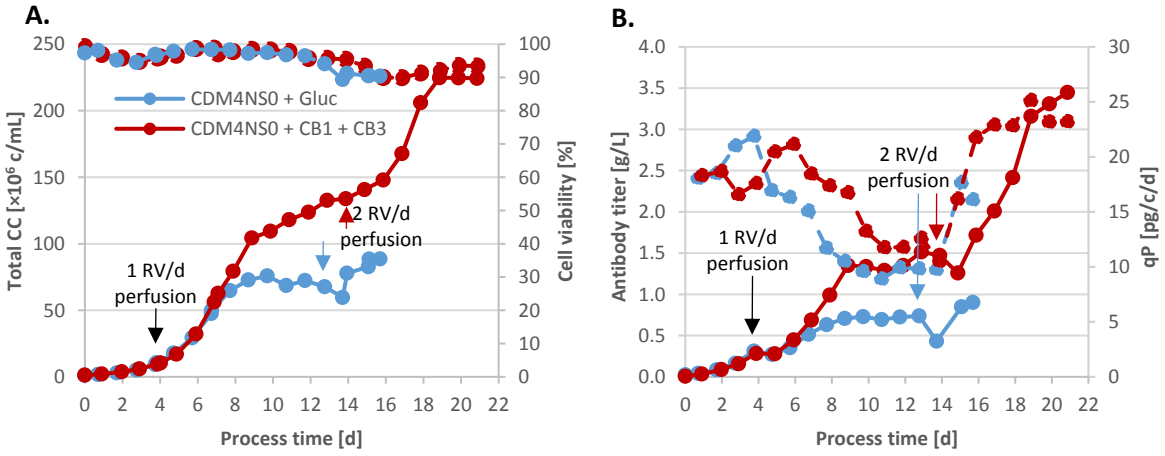
In both reactor runs, cells grew from 1.0×10^6 c/mL up to 9.4×10^6 c/mL in batch mode for four days in plain CDM4NS0 medium (Figure 28). Glucose concentration dropped from 8 g/L to 3.3 g/L. The cell specific consumption rate of glucose was 330 pg/c/d and therefore by assuming a similar growth rate for the next day, the glucose concentration

would be limited. So the perfusion was initiated applying a rate of 1 RV/d. During the next four days the cells grew up to ~7×10^7 c/mL in the glucose spiked CDM4NS0 medium. Starting from day 9 the cells reached their maximal cell concentration, which is indicated by a plateau phase at 7.5×10^7 c/mL. The titer increased from 0.3 g/L when the perfusion was initiated up to 0.7 g/L on day 8 reaching also a constant level for the following days. This steady state was kept for 5 days and afterwards on day 13 the perfusion rate was raised to 2 RV/d to reach 8.8×10^7 c/mL. The titer slightly increased to 0.9 g/L at 2 RV/d.

The CDM4NS0 medium spiked with 11.06% CB1 and 19.90% CB3 showed a similar cell growth until day 7. Afterwards the cell concentration increased from 6.9×10^7 c/mL up to 12.4×10^7 c/mL. This was a boost of 70% compared to the glucose spiked CDM4NS0. The titer was doubled to 1.4 g/L with the optimized medium leading to a 20% higher qP of 12 pg/c/day compared to the medium without CBs (i.e. 10 pg/c/d). In contrast to the CDM4NS0 glucose spiked culture an increase of the perfusion rate to 2 RV/d led again to even higher cell numbers and antibody titers. The cell concentration showed an increase of 80% resulting in a cell concentration of 22.5×10^7 c/mL and the titer increased to 3.4 g/L which is a boost by a factor of 2.4, translating into an increase of qP from 12 pg/c/d to 23 pg/c/d.

The viability could be maintained above 90% for three weeks in both reactor runs.

Based on the fact that with the CB-spiked medium the cell concentration showed always a slight increase, no real plateau phase was reached and therefore the μ of 0.15 1/d was higher compared to 0.05 1/d with the glucose spiked medium.



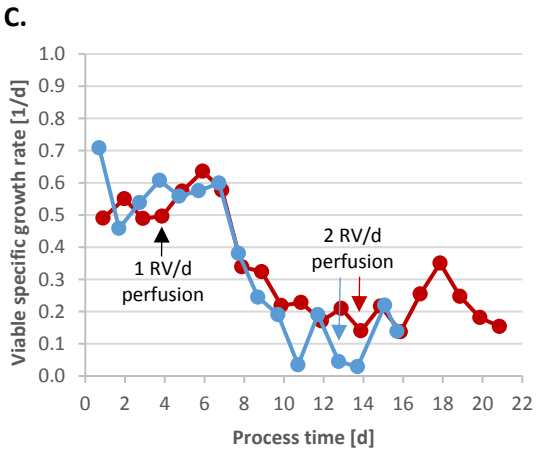


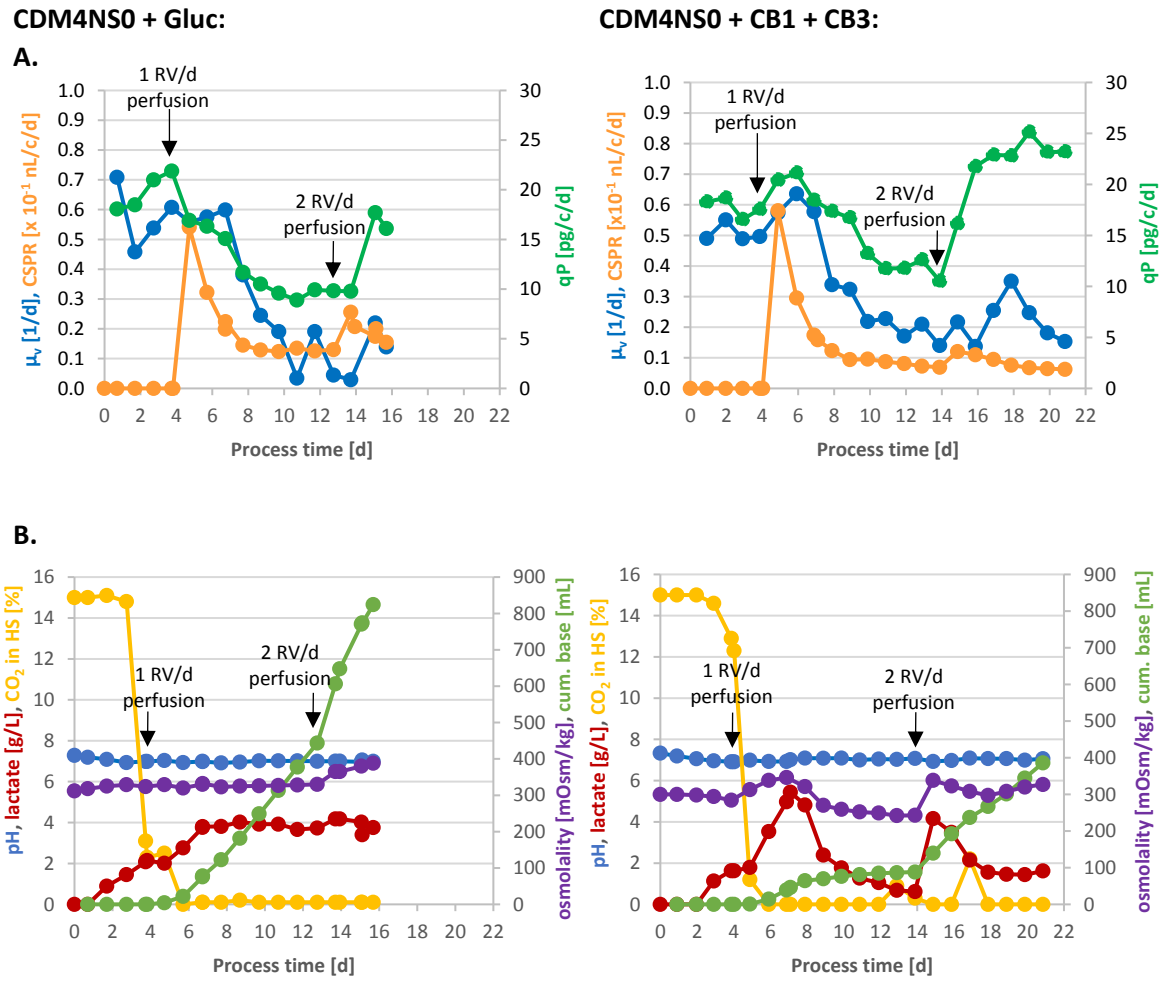
Figure 28: Cell concentration (straight line) and viability (dashed line) (A.), Antibody concentration (straight line) and qP (dashed line) (B.) and viable specific growth rate (C.) of a perfusion in a WAVE Bioreactor of rCHO-K1 in CDM4NS0 spiked with glucose (blue) and CDM4NS0 spiked with CB1 and CB3 (red).

Figure 29A shows the correlation between the viable specific growth rate μ and the cell-specific antibody production rate qP. During the batch phase the μ was around 0.6 1/d with a qP of 20 pg/c/d. During the perfusion a plateau of the cell concentration was reached characterized by a decline in μ and qP. The cell specific perfusion rate (CSPR) at steady state ranged from 10 pL/c/d for the CB-spiked medium to 15 pL/c/d for the glucose-spiked medium.

At the beginning of the fermentation the pH was controlled by the CO_2 concentration in the gas phase of the headspace and brought into liquid by the rocking motion of the WAVE Bioreactor (Figure 29B). Due to the lactate production the pH decreased and therefore the requested pH level of 7.0 was reached by lowering the CO_2 level and afterwards by base addition. During the perfusion with the glucose spiked CDM4NS0 medium high lactate production up to 4 g/L was observed. To control pH at 7.0 the system added 825 mL base into a reactor volume of 500 mL over 12 days. With the CB-spiked medium the lactate concentration first increased up to 5.4 g/L and then decreased to 0.6 g/L which reduced the cumulative base addition over the whole process to ~390 mL. The reduced lactate concentration and therefore the decreased base consumption showed an impact on the osmolality. In the perfusion with the glucose spiked medium the osmolality was ~330 mOsmol/kg and by increasing the perfusion rate to 2 RV/d the osmolality increased to 390 mOsmol/kg. With the CB-spiked medium the osmolality was ~240 mOsmol/kg and by applying a rate of 2 RV/d it increased to 330 mOsmol/kg.

In the first perfusion experiment the air flow was 0.33 L/min (Figure 29C). The DO was kept over the hole process above 30% with an O_2 concentration of up to 25% in the

head space. In the second experiment the air flow was reduced to 0.10 L/min to have lower evaporation effects. To keep the DO above 30% it was necessary to raise the air flow to 0.20 L/min on day 7 and the O_2 concentration in the headspace increased up to 50% which is the upper limited of the control system. Since the DO drop down to 6% on day 16, an external gas supply was installed by adding pure oxygen to the headspace. Nevertheless it wasn't possible to maintain the DO at 30% for the remained process time. Therefore after a short increase the DO dropped down to a level of 3%.



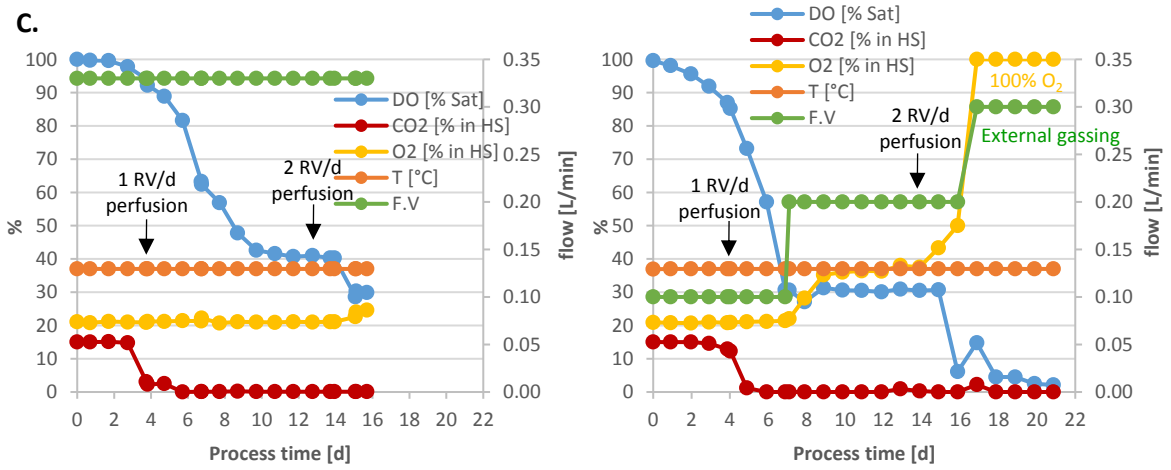


Figure 29: Correlation between viable specific growth rate μv (blue), qP (green) and CSPR (orange) (A.), influence of lactate production on the pH control and osmolality (B.) and process control parameter (C.) of a perfusion in a WAVE Bioreactor of rCHO-K1 in CDM4NS0 spiked with glucose (left) and CDM4NS0 spiked with CB1 and CB3 (right).

During the batch phase in the first 4 days glucose and glutamate were consumed by the cells to 3.3 g/L and 520 mg/L, while lactate, glutamine and ammonium were produced to a concentration of 2 g/L, 250 mg/L and 45 mg/L (Figure 30). Due to the start of the perfusion on day 4 the glucose concentration increased to 9 g/L in the reactor on the next day and also an increased lactate production was observed. Afterwards the glucose concentration decreased again and was completely consumed by using the CB-spiked medium on day 8 respectively with the glucose spiked medium on day 12. In the first perfusion experiment the lactate concentration increased up to 4 g/L, while with the CB-spiked CDM4NS0 medium the lactate increased up to 5.4 g/L on day 7 and afterwards decreased to a level of 0.6 g/L on day 14. By applying a rate of 2 RV/d it increased again to 4.2 g/L on day 15 and subsequently dropped to 1.4 g/L. The glutamate was consumed but never dropped below 40 mg/L. Starting on day 7 no glutamine secretion was observed in both fermentations. The ammonium concentration in the perfusion with the glucose spiked medium was with 14 mg/L a little bit lower compared to 20 mg/L with the CB-spiked medium.

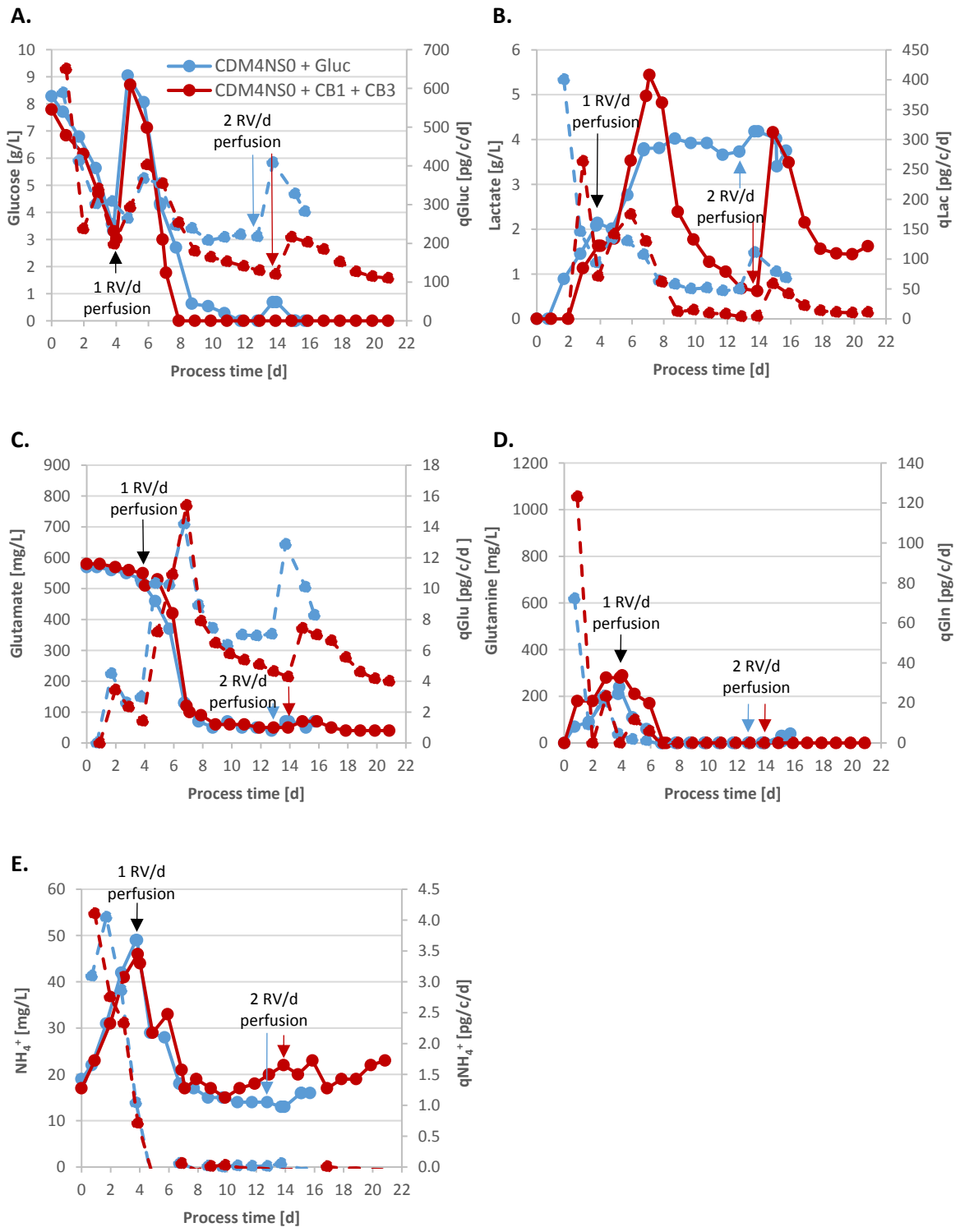


Figure 30: Concentrations of key metabolites (straight line) and their cell specific rates (dashed line) of a perfusion in a WAVE Bioreactor of rCHO-K1 in CDM4NS0 spiked with glucose (blue) and CDM4NS0 spiked with CB1 and CB3 (red), showing glucose (A.), lactate (B.), glutamate (C.), glutamine (D.) and ammonium (E.).

Table 33: Summary of a perfusion in a WAVE Bioreactor of rCHO-K1 in CDM4NS0 spiked with glucose and CDM4NS0 spiked with CB1 and CB3.

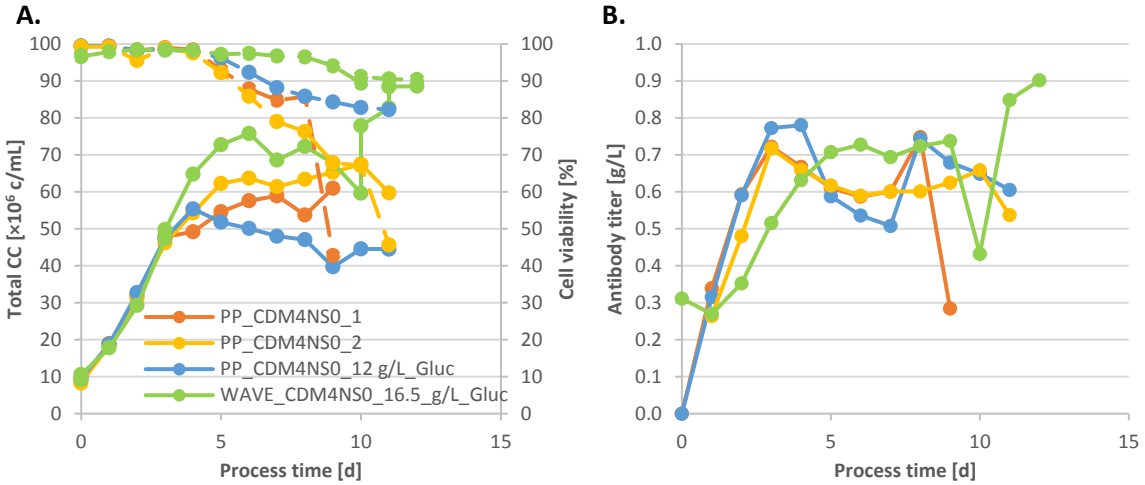
	CDM4NS0 + Gluc		CDM4NS0 + CB1 + CB3	
Perfusion rate [RV/d]	**1**	**2**	**1**	**2**
Total CC [×10⁷ c/mL]	7.5±0.4	8.8±0.3	12.4±0.9	22.5±0.6
mAb [g/L]	0.70±0.04	0.90±0.04	1.40±0.09	3.40±0.11

4.7 Comparison between pseudo perfusion in Tubes and perfusion in the WAVE Bioreactor

The perfusion in the WAVE Bioreactor showed the highest peak cell concentration of 7.5×10^7 c/mL and the highest viability of 90% over the whole process duration using CDM4NS0 spiked to 16.5 g/L glucose (Figure 31). The pseudo perfusions in the Tubes showed peak cell concentration of 5.9×10^7 c/mL for the plain CDM4NS0 and 5.5×10^7 c/mL for the CDM4NS0 spiked to 12 g/L glucose. The viability dropped below 60% on day 9 respectively on day 11 in a second experiment using the plain CDM4NS0 medium for pseudo perfusion.

Addition of glucose showed an increase of the titer of about 8% and 20%, from 0.60±0.15 g/L to 0.65±0.10 g/L for the pseudo perfusion and to 0.72±0.02 g/L for the WAVE perfusion.

The viable specific growth rate μ and the cell-specific antibody production rate qP seemed to be comparable for all experiments.



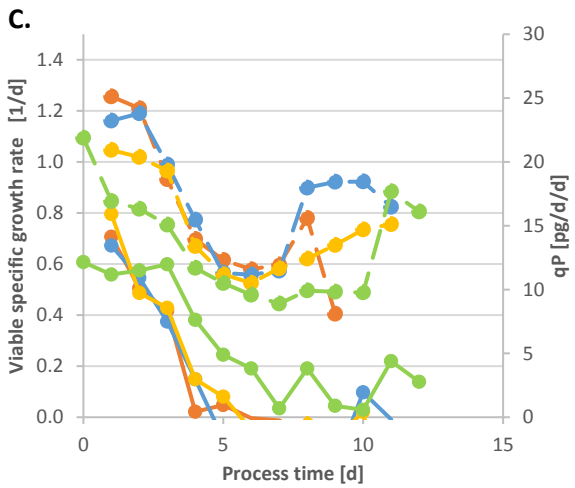
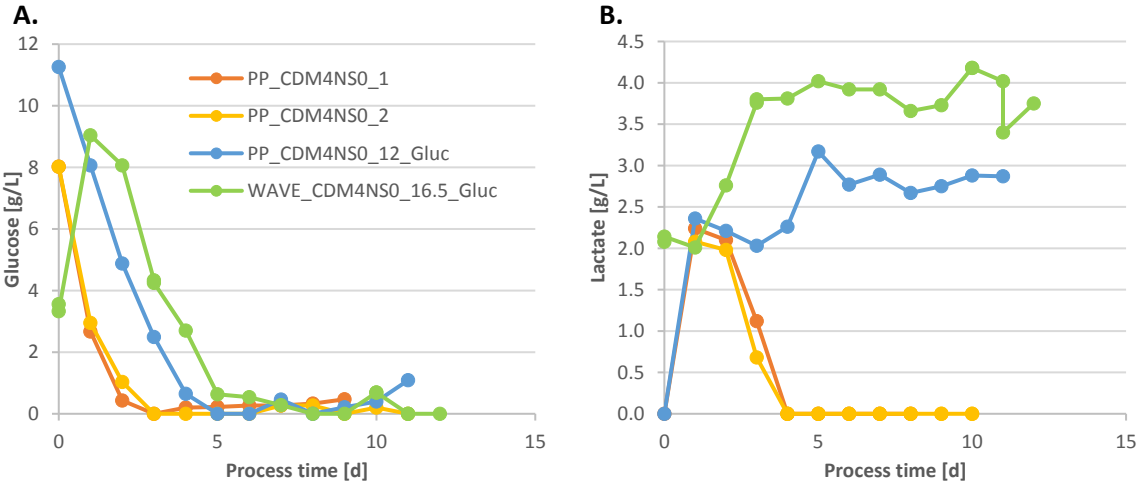


Figure 31: Cell concentration (straight line) and viability (dashed line) (A.), Antibody concentration (B.) and viable specific growth rate (straight line) and qP (dashed line) (C.) of a pseudo perfusion in a tube using CDM4NS0 (orange and yellow), using 12 g/L Gluc-spiked CDM4NS0 (blue) and of a perfusion in a WAVE Bioreactor using 16.5 g/L Gluc-spiked CDM4NS0 (green) of rCHO-K1.

In all perfusion modes the glucose was completely consumed starting on day 5 (Figure 32). Spiking with glucose resulted in a lactate production in the Tube up to 3 g/L as well as in the WAVE up to 4 g/L whereas with plain CDM4NS0 the lactate was consumed. The glutamate was consumed to ~40 mg/L in the pseudo perfusion as well as in the perfusion with the glucose spiked medium, but increased to 110 mg/L in the pseudo perfusion on day 7, correlated with a glutamine secretion on the same day. The ammonium concentration increased up to 80 – 120 mg/L in the pseudo perfusions as in the perfusion the concentration was around 14 mg/L.



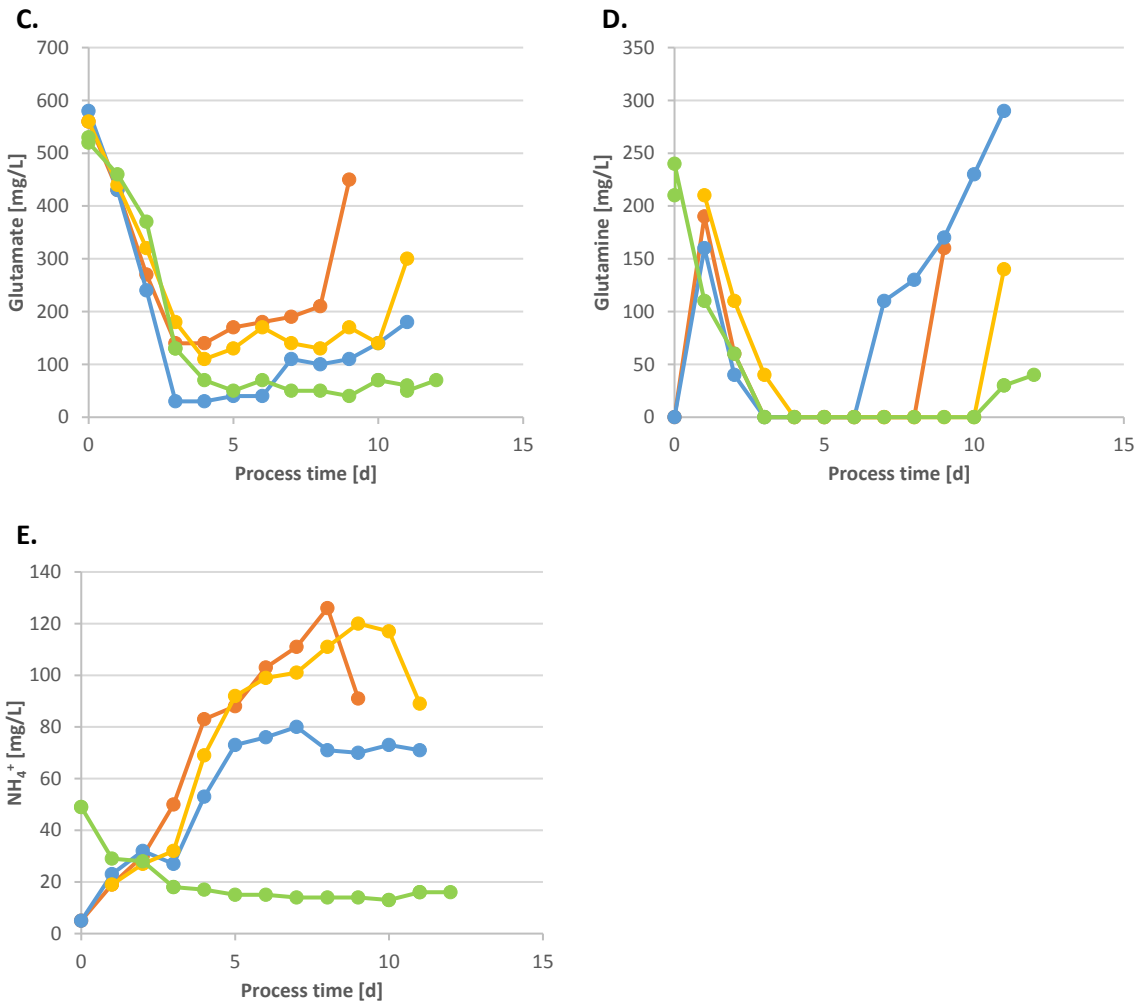


Figure 32: Concentrations of key metabolites of a pseudo perfusion in a tube using CDM4NS0 (orange and yellow), using 12 g/L Gluc-spiked CDM4NS0 (blue) and of a perfusion in a WAVE Bioreactor using 16.5 g/L Gluc-spiked CDM4NS0 (green) of rCHO-K1, showing glucose (A.), lactate (B.), glutamate (C.), glutamine (D.) and ammonium (E.).

5. Discussion

5.1 Selection of a new cell line subclone and a new basal medium

The study was started by comparing the two rCHO-K1 subclones A1A7 and D1E7 in ActiPro batch cultures. A1A7 achieved slightly higher peak cell densities of 21×10^6 c/mL, however, the same titer (1.4 g/L) was achieved with D1E7 with only 15×10^6 c/mL, indicating higher specific productivities. D1E7 was chosen as the preferred clone for this study since better production properties along with lower cell concentrations were reasoned beneficial during high cell density cultivation.

Different HyClone media were then compared in routine and batch cultures. CDM4NS0 was identified as a suitable alternative medium to ActiPro with excellent batch performance reaching titers up to 1.7 g/L compared to 1.6 g/L in ActiPro. All other media supported titers ranging from 0.4 to 1.2 g/L. Potential reasons for the diverse performance of the investigated cell line in different media were assumed to be due to the different media formulations. Different CHO cell types have different nutrient requirements and therefore the optimal composition of a basal medium is highly dependent upon the basic type of CHO cell used [40, 41]. It was shown that high amino acid concentrations alone are not sufficient to support high cell growth and antibody production. Instead a balanced glucose and key amino acids composition that meets the needs of the used cell line is much more important [42]. One major by-product during cell growth is lactate which causes acidification of the medium. It is known that high glucose concentrations accelerate glycolysis and suppress the respiration and oxidative phosphorylation, called Crabtree effect. This leads to a high lactate production which can be overcome by a low initial glucose concentration in the medium.

5.2 Development of a pseudo perfusion method

In the initial pseudo perfusion experiment of rCHO-DG44 in ActiPro it was shown that only the daily complete medium replacement enabled constantly high viabilities and cell concentrations of up to 29×10^6 c/mL, while daily splitting of cells to 1×10^7 c/mL was not able to maintain cultures in a healthy state. A continuous decline in specific growth was observed and therefore at low μ the split cultures did not receive enough fresh media, consequently glucose and glutamine was limited starting from day 4. The daily complete medium replacement yielded similar mAb titers of about 700 mg/L daily comparable to batch culture after 8 days. While in the stationary phase of a batch culture the qP declines significantly to zero with the pseudo perfusion high qP values could be maintained at 25 pg/c/d at late culture times (day 11).

In a next step an optimization of the shaking parameters regarding the vessel, speed, setting angle and medium was performed to find the best performing pseudo perfusion model. The setup of this approach was based on an already established cell culture model used for mimicking perfusion processes [43]. Media spiked with 7% CB7a and 0.7% CB7b led to an increase of cell concentrations and titers for both cell lines. Nevertheless the optimized shaking parameters differed between rCHO-DG44 and rCHO-K1. The best results for the rCHO-DG44 cell line were achieved with spiked ActiPro in a shaking flask at 150 rpm showing a cell concentration of 5.2×10^7 c/mL and a titer of 2.3 g/L. With the rCHO-K1 cell line the spiked ActiPro in the 90° tube at 250 rpm performed best yielding a cell concentration of 7.9×10^7 c/mL and a titer of 2.2 g/L.
Furthermore the rCHO-K1 cell line showed less cell deposits at vessel walls compared to rCHO-DG44 cultures. The extent of accumulating such deposits seems to be cell line dependant. However as these cell deposits consists out of dead cells a correlation with the cell viability is reasonable.
Summarized in Table 34 for the rCHO-K1 cell line in ActiPro spiked with 7% CB7a and 0.7% CB7b the shaking tube showed a 9% lower cell concentration, a 24% higher titer and a 36% higher qP compared to the shaking flask. Therefore the overall performance of the tube was found to be better compared to the flask. Another benefit of using a shaking tube instead of a shaking flask for further pseudo perfusion screenings was the easier handling during the centrifugation step by removing a process step to transfer the cell suspension into a centrifugation tube.

Table 34: Comparison of the two best perfoming rCHO-K1 pseudo perfusion cultures with spiked ActiPro (7% CB7a and 0.7% CB7b)

	Shake flask 150 rpm	Shake tube 250 rpm, 90°
Cell concentration	8.7×10^7 c/mL	7.9×10^7 c/mL
Antibody concentrations	1780 mg/L	2210 mg/L
qP	22 pg/cell/day	30 pg/cell/day

No difference was observed regarding cell growth and mAb production between 200 rpm and 250 rpm. Therefore, 50 mL SpinTube bioreactor with 10 mL working volume cultivated at an angle of 90° and 220 rpm were defined as standard settings for rCHO-K1 pseudo perfusion cultivation.

5.3 Investigation of two different basal media as perfusion medium

ActiPro and CDM4NS0 were investigated to determine their perfusion performance regarding cell growth and mAb production. 1.24× concentrated ActiPro which correspond to an osmolality of 380 mOsmol/kg showed an increase of 25% in mAb concentration (1.10 g/L) and qP (24 pg/c/d), compared to ActiPro in normal (1×)

concentration (0.88 g/L and 19 pg/c/d). Glucose addition to 12 g/L resulted in slightly lower cell counts and the mAb concentration increased by 7% to 0.95 g/L. Basal CDM4NS0 medium could not be prepared as higher concentrated versions. CDM4NS0 media spiked with glucose resulted in higher viabilities and therefore a longer process time was observed. Furthermore, the mAb concentration increased by 8% to 0.65g/L translating into similar qP values.

In summary, concentrated ActiPro as well as glucose addition showed beneficial effects on cell growth and antibody production. Glucose functions as the main carbon source for nucleosides, amino sugars, and for some amino acids and is beside glutamine the major source of energy. Furthermore it was shown that glucose concentration in the medium has an impact on the glycosylation pattern of the produced protein [44]. However a glucose feed alone is not sufficient enough to accomplish a significant boost in antibody production. A better approach is to ensure a balanced glucose and amino acid concentration in the culture to achieve high productivity.

5.4 Definition of optimal Cell Boost combination and ratio by a DoE approach

Best performing cell culture processes provide nutrients to the growing and producing cultures at optimal levels over the entire process period. Neither a limitation of any substrate, nor an accumulation of toxic by-products or overfeeding by media substrates may occur in order to force mammalian cells to highest performances. It was shown in literature that above a critical osmolality threshold of 400 mOsm/kg usually impaired cell growth was observed [39]. For the DoE approach this osmolality level was chosen to define the maximum amount (level +1) of CBs added to the CDM4NS0 basal medium. Optimized CB combinations could be defined by spiked-batches in CDM4NS0 using a first DoE (DoE #1) approach. The basal medium had an initial osmolality of around 300 mOsm/kg. Upon addition of different combination of CBs for DoE #1 the osmolality increased to reach final levels of 340-410 mOsm/kg.

Lower peak cell concentrations, viabilities and shorter process duration compared to the unspiked culture was observed for the majority of CB combinations added to basal CDM4NS0 in DoE #1. Peak cell densities gradually decreased from 27×10^6 c/mL of unspiked cultured down to 13×10^6 c/mL and 3×10^6 c/mL when basal CDM4NS0 was spiked with half-maximal and maximal CB amounts, respectively, leading to a decrease in final titers from 1.1 g/L to 0.2 g/L. The worst performance therefore was observed when all CBs were added at the maximal DoE #1-defined amounts (level +1). Such a spiked medium showed highest osmolality above 400 mOsm/kg, probably inhibiting cell growth. This demonstrates the requirement for optimal selection of individual CBs and

critical fine-tuning of feed ratios to get beneficial effects promoted by these CBs. Although the 'all CBs' experiment showed the highest specific productivity, the lowest titer was caused by a rapid decline in viability at lowest cell densities, probably resulting from the highest osmolality compared to other conditions.

Generally CB addition delayed cell growth. However, CB addition improved specific productivities in all tested experiments. Specific productivity improved from 6 pg/c/d of the unspiked CDM4NS0 batch up to 17 pg/c/d. Four conditions showed superior performance compared to the unspiked control. The specific growth rate of these conditions started lower compared to the control, but remained higher from day six, which resulted in higher VCCD starting from day eight or ten.

Using this DoE #1 approach, conditions could be found, leading to a substantial 2-fold increase in product titer from 1.1 g/L (unspiked control) on day 13 up to 2.1 g/L on day 15 by spiking CDM4NS0 with selected combinations of CBs. Furthermore, the peak cell concentration could be increased from 27 to 37×10^6 c/mL, representing an 1.4-fold increase using optimal combinations of CBs.

No glucose limitation was observed for any of the tested cultures. However, glutamate was consumed to critical levels for the top-performers indicating a need for adjusted CB ratio. Interestingly, top-performing cultures in CDM4NS0 clearly separate from other cultures in their lactate profile. At low glucose concentrations, these cultures switch to lactate consumption. This seems to be beneficial for the cell performance, as high lactate concentrations as well as increased ammonium levels are described to have adverse effects on the cell growth and protein productivity [45, 46]. Additionally, ammonium concentrations were lower for the two top-performers and the unspiked control at batch end.

Summing up, as the distinct formulation of cell culture media and feeds is not disclosed by manufacturers it is hard to say which components had a positive effect on the cell culture process. Cell culture medium is a complex mixture of nutrients like amino acids, vitamins, inorganic salts, and a carbon source for energy such as glucose [47]. Therefore additional analytical methods, such as a high-performance liquid chromatography to determine amino acid concentrations, are necessary to see nutritional bottle necks. However in this study a DoE approach was used to find out an optimized medium.

Regression analysis in the DoE evaluation of the generated cell culture data pointed out that CB 5 and 6 showed negative effects on titers, VCCD and peak viable cell days (VCD). CB 4 was removed since it is the only CB that contains growth factors. CB 1, 3 and 7b showed a positive effect on titer, VCCD and peak VCD. These CBs were considered for further optimization together with CB7a. Although CB7a did not show any

obvious positive effect it is also included in the optimized CB selection since it is usually combined with CB7b and because it would be possible that negative effects of CB 5 and 6 obscured the positive effect of CB7a. Based on the obtained cell culture data, summarized in Table 30, the established DoE was analyzed highlighting CB 1, 3, 7a and 7b as optimal output for beneficial performance for rCHO-K1 in CDM4NS0.
To optimize the ratio of those selected CBs a second DoE (DoE #2) approach for pseudo perfusion experiments was applied. Upon addition of different combination of the selected CBs the osmolality increased to reach final levels of 306-390 mOsmol/kg.
All combinations of CB1, 3, 7a and 7b in CDM4NS0 for DoE #2 showed good and comparable performances suitable as perfusion media. Only with the plain CDM4NS0 as control culture the viability declined below 60%. The mean peak cell concentration of all cultures was 5.7×10^7 c/mL with a peak titer of 0.89 g/L and a qP of 18.2 pg/c/d. No substantial difference due to the perfusion medium composition could be observed, although there was a trend towards higher titers and qP values for higher spiked media. A reason for comparable values might be the limited cell growth due to oxygen supply. A limit of nutrient supply is less likely due to the metabolic analysis, which showed that glucose was not completely consumed within one day in 8 different perfusion media and glutamate never drop below 70 mg/L.
Again the generated cell culture data, summarized in Table 32, was used to perform regression analysis, which pointed out that CB 7a and 7b showed no effects on titer, VCCD and peak CC. CB 1 and 3 showed a positive effect on titer, VCCD and peak CC. From the DoE calculation it was determined that DoE levels 1, 1, -1 and -1 for CB 1, 3, 7a and 7b would be beneficial for rCHO-K1 in CDM4NS0 pseudo-perfusions translating into CB additions of 11.06%, 19.90%, 0.00% and 0.00%, respectively.

5.5 Bioreactor verification

The best performing CB combination was investigated in a WAVE Bioreactor perfusion run under controlled conditions and compared to plain CDM4NS0 medium with a glucose concentration adjusted to the feed-spiked medium (16.5 g/L). Viabilities above 90% were achieved over the total process duration of 21 days. Plain CDM4NS0 medium showed a peak cell concentration of 7.5×10^7 c/mL on day 10 and the CB-spiked culture at 13.4×10^7 c/mL on day 14 which is an increase by a factor of 1.8. By applying a perfusion rate of 2 RV/d the cell concentration was boosted to 22.5×10^7 c/mL with the optimized perfusion medium. This value fits together with a reported perfusion process of CHO cells in a WAVE Bioreactor using external hollow fiber filter as cell separation device, where maximal total cell densities of 22.4×10^7 c/mL had been achieved for the first time [35]. A steady decline in viable specific growth rate was observed especially

from day 8 on, but remained higher for the spiked medium. Due to the optimized medium the peak titer was increased from 0.7 g/L to 1.4 g/L and boosted up to 3.4 g/L at 2 RV/d. Similar to the μ, also a steady decline in qP, especially on day 7 or 9, was noticed, but generally qP remained higher for the spiked medium. This indicates a correlation of μ and qP and promotes a potential for testing a chemostat process by continuously harvesting cell suspension. A reactivation of the qP and μ was possible by increasing the perfusion rate of the spiked medium to 2 RV/d.

In literature perfusion processes of CHO cells are often described with a cell specific perfusion rate (CSPR) in a range of 0.04–0.06 nL/c/d [35, 48, 49]. Increasing the titer can be achieved through a reduction of the dilution rate while retaining high cell density. Therefore operating at a low CSPR is recommended [50], thus the here accomplished CSPR of 0.01–0.02 nL/c/d seems to be beneficial for the efficiency of the perfusion process. However as this parameter depends on the used medium and cell line, the identification of the optimal CSPR is a time-consuming task in process development.

For the rCHO-K1 cell line, the WAVE system supported a peak cell concentration of 13.4×10^7 c/mL with a DO above 30% with O_2 enrichment in the gas of the headspace below 50%. However the DO dropped to 5% with further increased cell concentrations. Nevertheless, the viability remained above 90% which indicates there was still sufficient oxygen supply. High pressure during permeate sampling was required, which is an indication for filter clogging, but continuous permeate harvest was still possible. At later process times the harvest was getting more turbid over time though without containing any cells. The turbidity might came from cell fragments of dead cells, which were able to pass the filter and were identified under the microscope.

The glucose level dropped to zero on day 12 (plain medium) or day 8 (spiked medium) with a constant specific consumption rate of 200 pg/c/d. An increase of qGluc was observed at 2 RV/d. The plain CDM4NS0 medium showed lactate levels of 4 g/L and production rates of 50 pg/c/d. Therefore the pH was stabilized by daily addition of up to 65 mL of 4% NaHCO3. In contrast, the spiked medium showed a decrease in lactate production starting on day 7 and no base addition. Glutamate was consumed to critical near 0 mg/L levels at day 7 and the ammonium concentration remained low at around 17 mg/L. Xing et al. [39] found that ammonium levels above 5.1 mM (92 mg/L) have inhibitory effects on cell growth. This suggests a limitation for *de novo* glutamine synthesis is more reasonable than a toxic ammonium concentration and therefore reaching the maximal cell concentration, indicated by a plateau phase.

5.6 Shake tubes in a batch-refeed mode as perfusion scale-down model for the WAVE Bioreactor

As bioreactor systems are more controllable, but also more complex than spin tubes and shake flasks, it is very difficult to develop a small-scale model which truly represents a bioreactor process [51]. It was shown that productivity and product quality attributes like glycosylation or charge distribution can be influenced during the scale-up from small scale systems to bioreactors. An alternative is the usage of controllable miniaturized bioreactors which have a better scalability [52, 53], but are much more expensive compared to spin tubes and shake flasks. Comparison between the pseudo perfusion in tube format and the perfusion in the WAVE Bioreactor showed peak cell concentrations of 5.5×10^7 c/mL and 7.5×10^7 c/mL, titers of 0.65 g/L and 0.72 g/L, respectively. The metabolic analysis showed differences regarding glutamine secretion and high ammonium levels in the tube. However, the glucose concentration in the used CDM4NS0 medium was increased to two different levels. Therefore further experiments are necessary to clarify if the pseudo perfusion in the tube is a suitable scale-down model for a continuous perfusion in a WAVE Bioreactor.

6. Conclusion

Within this work a pseudo perfusion method was developed based on a daily centrifugation of the cells, removal of the supernatant and resuspending the cell pellet in the same amount of fresh medium. Thus, 100% of the reactor volume were exchanged per day (1 RV/d) similar to the tested WAVE perfusion experiment. Furthermore, an optimization of the shaking parameters regarding the shaking speed, setting angle and vessel was performed resulting to define optimal standard settings for pseudo perfusion in tubes at 90° and 220 rpm for the CHO-K1 cell line rCHO-K1.

An perfusion medium with the optimal CB combination and ratio was developed using a sophisticated DoE approach. CB 1 and 3 are recommended as 11.06% and 19.90% spike into CDM4NS0 medium for rCHO-K1 perfusion cultures.

This optimized perfusion medium was compared to the unspiked basal medium as perfusion cultures under controlled conditions in a WAVE Bioreactor. The CB-spiked medium showed highest peak cell concentration of 13.4×10^7 c/mL as well as the highest peak titer of 1.4 g/L. By increasing the perfusion rate from 1 RV/d to 2 RV/d the peak cell concentration was further increased to 22.5×10^7 c/mL and the peak titer up to 3.4 g/L.

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8. List of figures

9. List of tables

DECLARATION OF AUTHORSHIP

I hereby affirm that this thesis was written solely by me and that I have not previously submitted this work on another educational institution for the purpose of receiving an academic degree. In particular, contributions by other persons in this work have been appropriately cited and the data gathered through the methods described have been accurately reproduced.

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Date Signature Florian Wiederstein