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Master Thesis

Optimization of an In-House Developed Cell Culture Medium for the Cultivation of CHO Cells

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1 Abstract

Production of recombinant proteins using Chinese hamster ovary (CHO) cells is the most successful platform for large-scale production in the biopharmaceutical industry worldwide (Walsh, 2014). This has been mainly achieved by establishment of high producer cell lines and process optimization approaches such as optimization of cell culture media. The best performing and stable producer cell lines would never achieve titers in the g/L range, if they were cultivated in poor media which do not satisfy the cells' nutritional needs. Further, all process steps of recombinant protein production, from cell line development to the final product, have to be considered as a whole, since each step may have detrimental effects on protein yield and product quality. Nonetheless, the choice of a properly defined and balanced cultivation medium is one of the most critical steps with respect to therapeutic protein products.

In this project an in-house developed chemically defined cell culture medium formulation (MV3-2) was benchmarked against a commercially available formulation in order to identify critical aspects for further optimization of the current formulations (basal medium + feed media). Moreover, various types of media supplements were evaluated towards their potential to improve general process performance and to boost product titers. The results show that the performance of the basal medium could be improved by 70 % in regard of product titers. However, the in-house developed feeding medium still has some room for improvement, since product titers and general culture performance were lower compared to a commercial feeding medium. Supplementation with media additives (D(+)-trehalose, D(+)-mannose, taurine, glutathione, lipids, valproic acid and glycerol) showed quite promising results for some additives, concerning increased product titers and applicability.

2 Zusammenfassung

Die Produktion rekombinanter biopharmazeutischer Produkte, mit Ovarienzellen des chinesischen Hamsters (CHO Zellen) ist der heute am häufigsten angewendete Prozess in der biopharmazeutischen Industrie weltweit. Dieser Umstand ist vor allem der Einführung von stabilen Produktionszelllinien und ständiger Prozessoptimierung (z.B. Optimierung von Zellkulturmedien) zu verdanken. Auch die stabilste und ertragreichste Zelllinie könnte niemals die heute üblichen Produktkonzentrationen im Bereich von g/L erreichen, wenn die Zusammensetzung des Kulturmediums nicht auf die Ansprüche der entsprechenden Zellen abgestimmt wurde. Des Weiteren ist es unabdingbar alle Prozessschritte eines biopharmazeutischen Produktionsprozesses als Einheit zu erfassen, da jeder einzelne Schritt äußerst gravierende Auswirkungen auf den Produktertrag oder die Produktqualität haben kann. Jedoch stellt die Wahl entsprechend definierter und optimierter Medien einen der einflussreichsten Schritte in der Produktion therapeutischer Proteine dar.

In dieser Arbeit wurde ein nicht kommerziell entwickeltes, chemisch definiertes Medium (MV3-2) mit einer aktuell am Markt erhältlichen Medienformulierung hinsichtlich möglicher Verbesserungen während der Kultivierung von CHO Zellen verglichen (Basal Medienformulierung + Feed Medienformulierungen). Des Weiteren wurden verschiedene Substanzen auf ihre Anwendbarkeit als Medienzusätze für die Verbesserung der Kulturbedingungen sowie zur Erhöhung der resultierenden Produktkonzentration getestet. Die Ergebnisse dieser Arbeit zeigen, dass die Produkterträge des Basalmediums um 70% gesteigert werden konnten. Andererseits zeigt die Medienformulierung des Feed Mediums noch beträchtliche Schwächen hinsichtlich der Gesamtdauer des Prozesses sowie der finalen Produktkonzentration. Dies wurde im Vergleich zu einem aktuell erhältlichen Feed Medium getestet. Bei den Medienzusätzen (Trehalose, Mannose, Taurin, Glutathion, Lipid Mix, Valproat, Glycerin) zeigten die Ergebnisse für einige der verwendeten Zusätze ihre gute Anwendbarkeit für die Verbesserung der finalen Produktkonzentrationen.

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3 Introduction

Advances in medical and scientific research and an increasing market for recombinant protein pharmaceuticals has made mammalian cell culture an inevitable part of today's biotechnology industry (Walsh, 2010). Escherichia coli, which has been the laboratory workhorse for decades, has limited power when protein pharmaceuticals with sophisticated needs for post-translational modifications (e.g. disulfide bonds and glycosylation patterns) are the product of interest. Today, quite a number of mammalian cell lines such as Baby Hamster Kidney (BHK), mouse myeloma-derived NS0 or Human Embryonic Kidney (HEK-293) and numerous other cell lines are available for production of relevant protein biopharmaceuticals. However, one specific cell line, namely Chinese Hamster Ovary (CHO) cells have demonstrated an extraordinary ability for production of biopharmaceuticals (Dalton and Barton, 2014). With the successful approval of tissue plasminogen activator (tPA, Activase®) by Genentech in 1987 and 35% of global cumulative approved (1982-2014) recombinant therapeutic proteins (Walsh, 2014), the Chinese Hamster Ovary cell line is the most successful mammalian production cell line world-wide (Jayapal et al., 2007).

3.1 Chinese Hamster Ovary Cells (CHO)

Although, tPA was approved almost 30 years ago CHO cells are still the most prominent production workhorse for therapeutic proteins. This can be mainly attributed to the available knowledge from years of CHO research, their ease of handling, and the ability to grow CHOs in suspension culture. Further, since CHO cells are of non-human origin they are less prone to human viruses and (Boeger et al., 2005). Another important issue that makes CHOs attractive expression hosts is their ability to yield native like and bioactive post-translational modifications of secreted mammalian proteins (Dalton and Barton, 2014).

CHO cells have a widely distributed family tree that goes back into 1957 when CHO cells were first established (Puck et al., 1958). There are a number of CHO cell line phenotypes that refer to names such as CHO-K1, CHO-DG44, CHO-DX B11, CHO-Toronto, CHOpro3- or CHO-S. All these CHO cell lines are immortalized and can be considered a "quasispecies" as proposed by Eigen and Schuster in the 1970s (Wurm, 2013).

Since the 1980s, product titers generated from CHO bioprocesses have drastically increased from milligrams to grams per liter, at least for antibody products, and reported product titers even continue to rise (Hauser and Wagner, 2014). Enhanced recombinant protein product formation is strongly influence by the ability of a certain cell line to grow to high viable cell densities during fed-batch cultivation, as well as to maintain a high specific productivity (q_p) over the whole process.

First, a suitable cell line with the desired growth and protein production properties has to be established before the fine-tuning of physiological process parameters such as pH, dissolved oxygen, osmolality, temperature and chemical conditions through cellular metabolism control and medium optimization can be performed (Yu et al., 2011). Optimization of media (basal and feed media) is, besides others such as host cell engineering or vector optimization, a suitable and practicable approach to enhance both, growth performance and specific productivity in an industrial environment.

3.2 Media in Cell Culture

The history of media development is directly connected with the development of cell culture techniques. Henry Eagle was first to develop a defined medium, Eagle's Minimal Essential Medium (MEM) (Eagle, 1959). His medium formulation was mainly based on a balanced salt solution (BSS) supplemented with major amino acids, some vitamins, glucose and serum of horse or calve origin. Eagle's medium was then further modified by Dulbecco (Dulbecco's Minimal Essential Medium – DMEM). For successful proliferation, cells require hormones and growth factors that are usually provided via the addition of serum. Further advances in cell culture, e.g. the development of auxotrophic host cell lines and generation of recombinant clones from those made the development of more complex media formulations necessary. In the 1980s, important research was done towards the development for replacing serum since serum can be considered as a complex and 'non-defined' medium component that poses the risk for virus and prion infections as well as high lot-to-lot inconsistency (Barnes and Sato, 1980).

Today, quite a number of serum-free media formulations are available. This has been mainly driven by the development of more effective techniques to identify inevitable serum components and research towards possible chemically defined alternatives. However, serum is still extensively used in research since supplementation of serum to a basic medium formulation (MEM, DMEM) is an effective way to provide cells with a favorable growth environment (Gstraunthaler and Lindl, 2013).

3.2.1 Classification of cell culture media

Media supplemented with serum	Basic basal media formulations are supplemented with serum to fulfill the required needs for proteins, adhesion factors, enzymes, hormones, growth factors, fatty acids, lipids, vitamins, carbohydrates. (Gstraunthaler and Lindl, 2013) (Freshney, 2005).
Serum-free media formulations	In these media serum is replaced by purified serum components such as hormones and growth factors. Sato et al. (1980) were first to describe such a medium composition.
Protein-free media formulations &	High molecular protein fractions are replaced by peptides and protein hydrolysates.
Media formulations free of animal derived components	Peptides or protein fractions of animal origin can be replaced by plant protein hydrolysates or yeast extract.
Chemically defined media formulations	The medium formulation is free of protein and protein components of unknown composition and origin. Hormones and growth factors for supplementation (if needed) are highly purified and are mainly of recombinant origin. (Gstraunthaler and Lindl, 2013)

3.2.2 Overview of selected cell culture media

The following paragraph gives an overview of a selection of historically important cell culture media. Complexity of media formulations increases from top to the bottom. Media formulations containing serum will be discussed only briefly since this thesis deals with the development of a chemically defined medium formulation.

Minimal Essential Medium (MEM) (Eagle, 1959)	• Based on Henry Eagle's BME (Basal Medium Eagle) MEM contains increased concentrations of amino acids to match the protein composition of mammalian cells. • Serum is added to supplement for hormones, growth factors, etc.
Glasgow Minimum Essential Medium (MacPherson and Stoker, 1961)	• Modification of BME with a doubled concentration of amino acids, vitamins, glucose and Na_2CO_3. • Prior application: 10% tryptose phosphate will be added. • Supplemented with serum.
Dulbecco's Modified Eagles Medium (DMEM) (Dulbecco and Freeman, 1959)	• Four-fold concentrated version of Eagle's MEM supplemented with: glycine, serine, pyruvate, Fe^{3+} and linoleic acid. • Supplemented with serum.
Ham F-10, Ham F-12 Hams Nutrient Mixtures (Ham, 1963 and 1965)	• Ham F-10 was developed for the cultivation of CHO cells and made a cultivation with only 2% serum added possible. • Ham F-12 a modified version of F-10 with increased concentrations of some amino acids and $ZnSO_4$ and putrescine and linoleic acid added. • Ham F-12/DMEM (1:1) for CHO cultivation without serum
Leibowitz Medium L15 (Leibowitz 1963)	• Medium formulation for fast growing cell lines for cultivation without CO_2. The buffering capacity of the medium is achieved by the basic amino acids l-arginine, l-histidine and l-cysteine together with Na_2HPO_4. • D(+)-glucose is replaced by D(+)-galactose, sodium pyruvate, and l-alanine.

Medium 199 (M199) (Morgan, Morton and Parker, 1950)	• Medium 199 was designed for the cultivation of chick-embryo-fibroblast and was the first defined medium formulation. It contains more than 60 synthetic components.
McCoys 5A (Mc. Coy et al. 1959)	• Salt concentrations are based on BME (Basal Medium Eagle) but amino acid and vitamins are added according to the formulation of M 199 • Standard medium for growth of hepatoma cell lines.
RPMI-1640 (developed at Roswell Park Memorial Institute Buffalo, N.Y., U.S.) (Moore et al., 1967)	• Part of a series of related media formulations (RPMI series) • Mainly for cultivation of lymphocytes of animal or human origin. • Applied in hybridoma technology together with HAT or HT supplementation. • Supplementation with serum.
MCDB-series (developed at the University of Colorado)	• Cultivation under serum-free like conditions with low amount of proteins present in the medium. Hormones, growth factors and trace elements or very low amounts of fetal calve serum are added to the medium. • Numerous variations are available for a number of cell lines.

3.2.3 Components of cell culture media with regards to chemically defined media

Balanced salt solutions

All cell culture media formulations are based on a balanced salt solution (BSS) to mimic the extracellular electrolyte composition of the organism of origin. The most important ions of these salt solutions are Na^+, Cl^-, K^+, Ca^{2+}, Mg^{2+}, HCO_3^- and PO_4^{3-} which are important for osmotic pressure, cell adhesion (adhesion cultures) and buffer capacity of media formulations (Gstraunthaler and Lindl, 2013). Osmolality in cell culture media should be usually maintained in a range of 300 to 320 mOsm/kg. However, cells have a wide tolerance for osmotic pressure (Xing et al., 2008).

The buffer capacity of a cell culture medium is directly connected to its basic salt components. For mammalian cell culture media, often a buffer strategy with regards to natural systems (physiological pH 7.4) is applied. Therefore the HCO_3^-/CO_2 system, as it is known from human blood, is mostly applied. In cell culture the physiological pH is maintained by addition of $NaHCO_3$ to the medium where the vessel is incubated within a controlled CO_2 atmosphere. According to the equation of Henderson-Hasselbalch, the buffer capacity of cell culture media is defined as the concentration ratio of $[HCO_3^-]/[CO_2]$ (Gstraunthaler and Lindl, 2013).

However, there are other possibilities to maintain a physiological pH. HEPES, an organic buffering substance is much stronger in the pH range of 7.2-7.4 than the $[HCO_3^-]/[CO_2]$ system (pKa = 6.1). Unfortunately, HEPES is toxic in high concentrations which makes it less attractive for industrial application.

Glucose as major carbon source

Glucose functions as the main carbon source and is usually converted to pyruvate via glycolysis pathway and further metabolized in pentose phosphate pathway or further oxidized to CO_2 and water in the citric acid cycle (TCA). However, glucose metabolism is heavily linked to lactic acid production as a major by-product that causes acidification of the medium. The inefficient way of lactic acid formation results in a lack of energy that has to be replenished via glutamine (Hauser and Wagner, 2014). Alternative substrates such as mannose and galactose have been investigated to serve as glucose replacements (Berrios et al., 2011).

Amino Acids

Glutamine as major nitrogen source

Glutamine is usually applied in higher amounts to cell culture media than all other amino acids (2-8 mM) as it serves for energy replenishment due to a lack of energy caused by inefficient glycolytic conversion of glucose into lactate. Moreover, glutamine itself is a precursor for the synthesis of ribonucleotides. However, glutamine metabolism causes increased levels of toxic ammonia since glutamine is converted to glutamate and further to alpha keto acid accompanied by the release of ammonia. Further, glutamine is not stable in media formulations and has to be stored at -20 °C as stock solutions (e.g. 250 mM) prior to use (Gstraunthaler and Lindl, 2013).

Other amino acids

Concentrations of amino acids and their balance, as they are applied in chemically defined media, is an important issue that has to be stressed for the design of new media formulations. Furthermore, there are essential and non-essential amino acids. Essential amino acids (Arg, Cys, Gln, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Tyr, and Val) are those which cannot be synthesized by the cells themselves. The amino acids alanine, asparagine, aspartic acid, glutamic acid, glycine, proline and serine are non-essential since most cell lines are able to synthesize those (Stryer, 1999). As proposed by Landauer a new medium should be able to propagate cell growth to at least 5×10^6 cells/mL and overall to more than 3×10^7 cell-days/mL (IVCD – Integral of viable cell density). He also suggested to consider the abundance of amino acids in the final protein product produced.

Vitamins

Complex cell culture media mostly contain the group of B vitamins such as biotin (Vit B_7), folic acid (Vit B_9), nicotinamide (Vit B_3), panthothenic acid (Vit B_5), pyridoxine/pyridoxal (Vit B_6), riboflavin (Vit B_2) and thiamine (Vit B_1). Additionally, cobalamin (B_{12}), choline and myo-inositol can be added. Some media also contain ascorbate (Vit C) and tocopherole (Vit E) as anti-oxidants (Gstraunthaler and Lindl, 2013)(Freshney, 2005). For the development of media formulations it has been suggested to copy an already published vitamin formulation. As a first step the whole group of vitamins should be increased and as a second step fine-tuning of concentrations should be done (Landauer, 2014). However, supplementation of vitamins and their stability in combination with other media ingredients poses serious risks to encounter undesired effects by oxidation due to temperature, light exposure and the presence of metal ions (Cu^{2+}, Fe^{2+}). Especially, lipophilic vitamins, vitamin C, thiamin, riboflavin, and biotin are prone to oxidation. (Combs, 2008).

Trace elements

Trace elements are besides the already mentioned salts an inevitable source of metal ions that are required for cultivation of mammalian cells in serum-free culture medium. The most important trace metals are iron, copper, zinc, cobalt, chrome, manganese, molybdenum, and vanadium (Gstraunthaler and Lindl, 2013). Especially selenium plays an important role since it was demonstrated that selenium acts as an important cofactor for detoxification of free radicals (Freshney, 2005).

Lipids

Lipids have a major function as building blocks for cell membranes as well as for energy storage. Lipids are obligatory components of serum-free media formulations. The most important media components of the group of lipids are linoleic acid, lipoic acid, ethanolamine, ethanolamine HCl, phosphoethanolamine and cholesterol (Landauer, 2014).

Other components

Hormones

Some cell lines demand for growth factors and hormones supplemented to the media formulation. Estrogens, gestagens, prostaglandins and growth factors such as EGF, NGF, FGF, PDGF are some examples for possible candidates.

Pyruvate

Pyruvate is often supplemented as sodium pyruvate to media formulations in concentrations of 100-400 mg/L. Pyruvate has an essential role in the regulation of the carbohydrate metabolism due to its function as key intermediate at the transition point from glycolysis towards the citric acid cycle (TCA) (Gstraunthaler and Lindl, 2013).

Putrescine

Putrescine, a polyamine, was found to be involved in the cellular metabolism of eukaryotic cells. Kim et al., (2005) showed that putrescin had a positive influence on cell growth and recombinant protein product formation.

3.2.4 General considerations for the optimization of defined media

It is obvious that chemically defined media (CDM) compositions are of increased complexity compared to serum supplemented media, where only a small number of defined components are used. Media optimization for CDM requires all components of the medium to be considered as well as possibly occurring component interactions that might negatively affect the quality of the final protein product. Different cell lines have distinct requirements on the exact medium composition in order to develop their full potential towards growth and protein production. Another important question prior to medium development to be addressed, deals with the type of production process. A fed-batch process strategy where concentrated feed medium is added, demands for a basal medium that is able to propagate conditions for cell growth in the early phase of cultivation, whereas the feeding phase is marked by elongation of process time and product formation. Therefore, feeding strategies have to be planned with exceptional consideration to the cellular metabolism and the nutritional needs required for high-quality product formation. During fed-batch cultivation nutrients are added to the fermentation vessel either in regular time intervals or according to the cells' needs via online monitoring of cultivation conditions. Various strategies are available such as control of glucose concentration in the vessel, pH, flow cytometry to measure cessation of cells and oxygen uptake. All these control strategies have their up and downsides (e.g. by-product formation, alterations of glycosylation patterns) which have to be evaluated before a new process is planned, but always in consideration with the quality of the final protein product.

3.3 Aim of Study

The aim of this study was to benchmark an in-house developed medium formulation (MV3-2) against a commercially available production medium formulation in order to assess overall culture performance as well as to identify critical aspects for further optimization of the current formulation. This was done for the basal medium (MV3-2) formulation and for feed media formulations (Feed A(h.m) / Feed B(h.m). The following gives an overview of the experiments that were performed for this thesis:

Improvement of MV3-2 medium formulation

- Supplementation of MV3-2 with various iron salts in order to find the best performing iron source (combination of salts and concentration) for MV3-2 supplementation.
- Supplementation of MV3-2 with the amino acids l-asparagine, l-proline and l-serine to overcome limitations which were experienced during batch cultivation of a previous experiment.
- Enhancement of the overall compound formulation of MV3-2 in order to make it competitive to a regular production medium (ActiCHO P).

Improvement of feed media formulations

- Evaluation of feed media (Feed A(h.m) / Feed B(h.m)) in a feed media cross-comparison experiment with a commercially available feed media system. This experiment was performed to identify critical aspects for improvement of feed media formulations.

Media supplements

- Supplementation experiments with various medium additives (D(+)-trehalose, D(+)-mannose, taurine, glutathione, lipids) and titer enhancers (valproic acid and glycerol) to evaluate their potential towards improvement of culture performance (growth and process time) and enhancement of product concentrations.

4 Material and Methods

4.1 Material

4.1.1 Equipment, disposables and reagents

Table 1. List of lab equipment

Equipment	Manufacturer
Balance Lab scale	Sartorius, Germany
Vortex Genie 2	Scientific Industries, U.S.
MSC-Advantage™ Class II Biological Safety Cabinet	Thermo Scientific, U.S.
Climo Shaker ISF1-XC	Kuhner, Switzerland
Hemocytometer Neubauer Improved	Marienfeld, Germany
Z2 Coulter®Particle Count and Size Analyzer	Beckman-Coulter, U.S.
BioProfile® 100 Plus	Nova Biomedical, U.S.
Osmomat 030D	Gonotec GmbH, Germany
Inverted Lab Microscope with LED Illuminiation **Leica DM IL LED**	Leica, Germany
Hereaus Megafuge 16 Centrifuge	Thermo Scientific, U.S.
Heracell™ 240i CO2 Incubator	Thermo Scientific, U.S.
Heracell™ 150i CO2 Incubator	Thermo Scientific, U.S.
WTW inoLab pH720 pH meter	WTW, Germany
IKA C-MAG H54 magnetic stirrer	IKA, Germany

Equipment	Manufacturer
Incubating Microplate Shaker	VWR, U.S.
Plate Washer (96 well)	Tecan, Switzerland
Infinite M1000 Reader (96 well)	Tecan, Switzerland
Finnpipette® F2 Multi Channel pipette	Thermo Scientific, U.S.

Table 2. List of disposables

Disposable		Manufacturer
Erlenmeyer flasks	125 mL, 250 mL 2μm vent cap Polycarbonate, non-pyrogenic	Corning, U.S.
Tissue culture flasks (T25)	25 cm^2, with plug seal cap	VWR, Intl.
Micro centrifuge tubes for high G-force	1.5 mL	VWR, Intl.
Beaker filter 'Stericup® 500mL Millipore Express® PLUS'	150 mL, 500 mL 0.22 μm PES Fast flow, low protein binding	Millipore, U.S.
Omnifix® Syringes	30 mL Non-toxic, non-pyrogenic	Millipore, Irland
Costar® Stripette®	2 mL, 5 mL, 10 mL, 25 mL, 50 mL Non-toxic, non-pyrogenic	Thermo Scientific, U.S.
Coulter Counter sample container		VWR, Intl.
F96 Maxisorp Nunc Immuno Plates	96 well plate	Thermo Scientific, U.S.
F96 Without Lid SH Microwell Plates	96 well plate	Thermo Scientific, U.S.
Falcon™ 50 mL conical centrifuge tube		Fisher Scientific, U.S.

Table 3. List of software packages

Software packages	Instrument
Coulter®Z2 AccuComp®	Coulter®Z2 AccuComp®
Magellan (V6.6)	Infinite M1000 Reader (96 well)

Table 4. List of reagents

Reagents	Chemical formula	Description; MW	Manufacturer
Trypan blue	-	0.4 % solution	Sigma-Aldrich, Germany
Phenol red solution	-	0.5 % in DPBS; cell culture tested	Sigma-Aldrich, Germany
Citric acid	$C_6H_8O_7$	≥99.5 % Ph. Eur. MW (210.14 g/mol)	C.Roth, Germany
Sodium bicarbonate	$NaHCO_3$	MW (84.01 g/mol)	Merck, Germany
Sodium carbonate	Na_2CO_3	MW (105.99 g/mol)	Merck, Germany
Disodium phosphate dihydrate	$Na_2HPO_4 \cdot 2H_2O$	> 99.5 % p.a. MW (177.99 g/mol)	C. Roth, Germany
Monopotassium phosphate	KH_2PO_4	≥ 99 % Cellpure® MW (136.09 g/mol)	Merck, Germany
Potassium chloride	KCl	MW (74.56 g/mol)	C. Roth, Germany
Sodium chloride	NaCl	≥99.5 % Ph. Eur MW (58.44 g/mol)	C. Roth, Germany
L-alanyl-l-glutamine (Ala-Gln)	-	200 mM solution	Biochrom, Germany
G418-BC (25,000 U/mL)	-	Genetecin, antibiotic	Biochrom, Germany
Tween20	-	Ph. Eur.	C. Roth, Germany
Triton X100	-	-	Merck, Germany

Reagents	Chemical formula	Description; MW	Manufacturer
Tetramethylbenzidine – (TMP) stabilized chromogen	-	-	Invitrogen, U.S.
2.5M Sulfuric acid	H_2SO_4	MW (98.08 g/mol)	C. Roth, Germany
Lectin (G.nivalis) in RO-H_2O	-	c = 5 mg/mL	Sigma, Germany
D(+)-trehalose · $2H_2O$	$C_{12}H_{22}O_{11} \cdot 2H_2O$	MW (378.33 g/mol)	Sigma, Germany
D(+)-mannose	$C_6H_{12}O_6$	MW (180.16 g/mol)	Sigma, Germany
Sodium valproate	$C_8H_{15}NaO_2$	MW (166.19 g/mol)	Sigma, Germany
Glycerin	$C_3H_8O_3$	MW (92.09 g/mol)	C.Roth, Germany
Ammonium iron(III) citrate	$C_6H_8O \cdot xFe \cdot xH_3N$	-	Merck, Germany
Sodium hydroxide	NaOH	MW (40.00 g/mol)	Merck, Germany
<u>Antibodies</u>			
hm Ab 5F3 IgG1 (anti-gp41 HIV-1) **LOT #T74/5F3/B1**	-	c = 1.25mg/mL	Polymun Scientific GmbH, Austria
HRP-Goat Anti Human IgG (Gamma), LOT #925503A	-	-	Invitrogen, U.S.
<u>Standards</u>			
gp140 Standard **LOT #140114_A**	-	in PBST + 1 % BSA; c = 100 ng/mL	Polymun Scientific GmbH, Austria)

Table 5. List of freshly prepared solutions

Solution	Preparation
CD-Lipid Mix (GIBCO® Life Technologies, U.S)	-
Phosphate Buffered Saline (PBS) (10X) pH 7.2 – 7.4	100 mM $Na_2HPO_4 \cdot 2H_2O$ 20 mM KH_2PO_4 27mM KCl 1370 mM NaCl
PBS (w/o Mg^{2+}, Ca^{2+}) (ready-to-use) Biochrom, Germany	-

4.1.2 Cell culture media

Table 6 gives an overview of all ready-to-use media (liquid and powder based), which were used for the experimental part of this thesis. All media were supplemented with l-glutamine, G418 and phenol red according to the respective experimental setup. Further information, including medium preparation and specific alterations of media compositions, are described in section 4.2.2. All media used were serum-free and chemically defined (CD).

Table 6. Cell culture media and feed media

Cell culture medium	Manufacturer
CD CHO (liquid)	GIBCO® Life Technologies, U.S.
ActiCHO P (powder-based)	GE, U.S.
ActiCHO SM (powder-based)	GE, U.S.
MV3-2 cell culture medium	Merck, Germany (according to an in-house developed medium formulation)
Feed media	**Manufacturer**
ActiCHO Feed-A CD (powder-based)	GE, U.S.
ActiCHO Feed-B CD (powder-based)	GE, U.S.
Feed A (in-house formulation)	In-house at Polymun Scientific, Austria
Feed B (in-house formulation)	In-house at Polymun Scientific, Austria

4.2 Methods

4.2.1 Maintenance cultivation of routine cultures

Maintenance of mammalian cells in culture and their cultivation in general can be laborious and time consuming. Compared to bacteria or yeast cultures, mammalian cells have longer doubling times and need to be cultured in media with complex nutrient compositions. Maintenance procedures involve the monitoring of cell vitality and cell growth as well as the change of the culture medium every third to fourth day. In batch culture, mammalian cells follow a typical growth profile starting with a phase of reduced growth at the beginning where the cells adapt to the cultivation conditions (lag-phase). Following adaptation, the cells continuously grow at their maximum growth rate (μ_{max}) according to nutrient and oxygen supply as well as pH. This phase is called exponential phase. Nutrient depletion and toxic by-product formation (lactate, ammonium) causes unfavorable growth conditions for mammalian cells. Hence, cell growth and cell vitality are reduced and as one result stagnation of cell growth can be observed (plateau phase; $\mu = 0$). The lack of nutrients and further production of by-products causes a shift in the equilibrium of cell division and cell death. As a result cell numbers decline and cell viability drops heavily.

Since it is abundant for experiments in cell culture to work with cells of high viability, cells must be cultured in a way to keep them in the exponential growth phase. This may be achieved by reducing total cell numbers and change of cultivation medium every third to fourth day. Cells that provided the starting material for the experiments presented in this thesis, named routine culture from now, were cultivated in 125 mL shaking flasks with a 2µm vent cap (Corning, U.S) in suspension. The starting cell density was 300,000 cells/mL in a total volume of 30 mL of CD CHO medium (chemically defined, Gibco®, Life Technologies). An aliquot of 2.5 mL cell suspension was taken every third to fourth day for determination of cell concentrations and viability as it will be described later. The sample taken was centrifuged at 100-200 x g for 10 min and 1 mL of the supernatant was frozen for product analysis (gp140 ELISA). After determination of cell concentrations the culture solution was transferred into a 30 mL universal tube, and the shaking flask was rinsed with PBS (w/o Mg^{2+}, Ca^{2+}) to remove remaining cells from the shaking flask. The medium CD CHO (+ 6 mM l-alanyl-l-glutamine, 0.5 mg/mL G418, 15 mg/mL phenol red) was pre-warmed to 37°C in a water bath and the respective amount was transferred back into the shaking flask under sterile conditions. Then, an aliquot was drawn from the culture solution (30 mL universal tube has to be inverted before aliquotation) that would be equivalent to 900,000 cells (300,000 cells/mL in 30 mL medium). This aliquot was transferred back to the pre-warmed medium in the shaking flask. The remaining culture solution was discarded. The procedure of constantly re-culturing cells is called passaging. Thereby the passage number is equivalent to the number of re-culturing steps since the culture was first inoculated.

4.2.2 Medium preparation

Preparation of medium for maintenance of routine cultures

Medium	**CD CHO**
Supplements	+ 6 mM l-alanyl-l-glutamine (Biochrom) + 2 mg/mL G418 (Biochrom) + 15 mg/L phenol red (Sigma-Aldrich)

Medium for the maintenance of routine cultures (CD CHO with 6 mM L-alanyl-l-glutamine, 2 mg/mL G418, 15 mg/L phenol red) was prepared by adding the above-mentioned supplements under sterile conditions to the liquid medium. Prepared medium was stored between 2 to 8°C.

Preparation of media for experiments

The following paragraphs show the standard formulations of all cell culture media as they were used for various experiments in this thesis.

ActiCHO Media System

The ActiCHO Media System of GE Healthcare is chemically defined, protein-free, and free of hydrolysates and animal derived components. (GE Healthcare, 2013)

Medium	**ActiCHO SM** **ActiCHO P**
Supplements	+8 mM l-glutamine +15 mg/L phenol red

The media of the ActiCHO series, used for this thesis were provided as powder. According to the manufacturer's instructions "Protocol for Use: ActiCHO Media System (Version 1.0 06/2013)", media solutions were freshly prepared prior to the experiments.

Medium	**ActiCHO Feed-A CD** **ActiCHO Feed-B CD**
Supplements	No additional medium supplements added.

Feed media solutions of the ActiCHO series were provided powder-based. According to the manufacturer's instruction "Protocol for Use: ActiCHO Media System (Version 1.0 06/2013)", media solutions were freshly prepared prior to experiments.

MV3-2 Medium

The MV3-2 medium formulation was developed by Polymun Scientific GmbH (Klosterneuburg, Austria) and is chemically defined, protein free, and free of hydrolysates and animal derived components. The medium formulation was ordered as powder from Merck, Germany. Since further improvement of the MV3-2's original formulation was part of this thesis, the listing below shows all variations of the original MV3-2 medium.

Medium	**MV3-2**
Description	Standard medium formulation as developed by Polymun Scientific GmbH, Austria.

Medium	**MV3-2/6**
Description	Improved medium formulation with ammonium iron(III) citrate as iron source.

Medium	**MV3-2/6$_{(+30\%)}$**
Description	Improved medium formulation with increased compound concentrations.

Supplements (for the 3 mentioned media)	+ 8 mM l-glutamine + 15 mg/L phenol red

Aliquots (200 mL) of the freshly prepared liquid medium were stored between +2°C to 8°C. Prior to application, l-glutamine and phenol red were added.

Preparation of in-house feed media

Due to proprietary reasons there is no component listing of in-house developed feed media formulations included in this thesis. Feed media were prepared as two independent feed solutions due to major differences in solubility (pH dependency) of various compounds. The formulation of Feed A, which had a rather neutral pH, contained all well soluble components as well as components that had to be added as stock solutions due to their low abundance in Feed A. Feed B only contained a short list of components that were not soluble at neutral pH at their respective concentrations. Following preparation, feed solutions were filtered (0.22 µm beaker filter) and then stored light protected, between 2-8°C.

4.2.3 Cell line

The cell clone (CHO-K1/L-CN54/LL1/2E4/N13) used for the presented experiments in this thesis was derived from a CHO-K1 (ATCC CCL-61) host cell line that was adapted to serum-free cultivation conditions and was transfected with the Caggs:BAC^{Rosa26} vector hosting the HIV-1 envelop glycoprotein CN54gp140 as model protein (Zboray et al., 2015). The HIV-1 envelope glycoprotein CN54gp140 is a highly N-glycosylated, homotrimeric and soluble protein which has a key function for target cell recognition and fusion of the virus membrane with the cell membrane of target cells. Thus, recombinant soluble envelope glycoprotein trimers of gp140 have been targets for intensive research towards an HIV-1 vaccination strategy since native-like gp140 envelope protein may induce a neutralizing antibody response that is strong enough to block HIV-1 from entering the target cells (Ringe et al., 2015).

4.2.4 Adaptation of routine cultures to various experimental setups

Cell culture experiments demand for detailed planning prior to the start of the actual experiment. For the experiments presented in this work it was crucial to adapt the cells to various types of media in order to ensure comparability as well as reliability of results. For medium adaptation, all instructions apply according to the passaging procedure for routine cultures, but with the exception of the type of culture medium used for passaging. The cells were cultured for 3 consecutive passages in the new medium to ensure that the cells had enough time to adopt their metabolism to the changed nutrient conditions. Thus, ELISA and growth monitoring were performed to check for a constant growth rate (μ) and product formation (q_p) behavior.

4.2.5 Determination of cell viability using Trypan blue exclusion

Today several methods for determination of viable cell numbers are commonly used. These methods are usually based on the fact that the intact cell wall of living cells acts as a barrier to withstand the uptake of a coloring agent such as Trypan blue. Trypan blue is an acidic dye which when present in its anionic form easily binds to proteins. When cells reach the end of their lifespan intracellular processes cause apoptosis and finally, dissociation of cell wall components causes the cell to lose its integrity. Once this happens, coloring agents such as Trypan blue can pass the cell boundaries and are instantly bound to intracellular proteins. Thereby, the cells get colored in a typical blue color, which indicates that the cells have lost membrane integrity (Gstraunthaler, 2013). For the actual determination of vital and dead cells some alternative systems are on the market. All systems have in common that they use coloring agents to discriminate between viable and dead cells although they are distinct in how result evaluation is done. For the work presented in here, a hemocytometer (Neubauer improved) was used to discriminate between viable and dead cells as well as to evaluate the overall viability value of the tested culture. The hemocytometer used for this work provided two counting chambers with a respective volume of $1mm^3$.

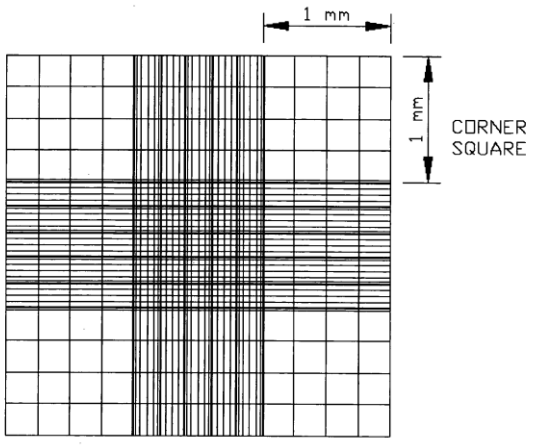

Figure 1. Counting chamber (Neubauer improved).

The four counting fields (big squares on the edges) are separated by a narrow grid-like pattern. The counting fields have an area of $1mm^2$. Cells are counted row-wise, from top to the bottom of each counting field. Cells shall not be too dense in order to omit inaccurate measurements. Approximately 100 cells per counting field are appropriate to obtain reliable results. (U.S Patentnr. US20040145805 A1, 2004)

Each counting chamber provides a grid engraved on top of its surface as it is depicted in Figure 1. The grid consists of four big squares and narrow lines in between. When preparing the chamber the counting chamber, will be covered by a cover slid that is fixed by pressing it on the glass support bridges left and right to the counting chamber until the typical Newtonian Rings appear. Before sample application the sample has to be diluted. Proper dilution is necessary in order to ensure an optimal number of cells evenly distributed throughout the counting chamber (practicable cell concentrations: 10^5-10^6 cells/mL). As a rule of thumb, one big square should approximately held 100 cells in order to obtain statistically reasonable data. Sample dilution was performed using PBS (w/o Mg^{2+}, Ca^{2+}) (Biochrom), and the respective amount of Trypan blue was added (40 µL Trypan blue solution for 200 µL sample). The sample was transferred into the counting chamber by simply pipetting a drop of solution on the edge of the cover slip and the solution was immediately soak in the counting chamber by capillary force. Then the total cell number per big square (non-colored + blue-colored cells) was determined as well as the number of colored cells (dead cells). Evaluation of overall sample viability was then done according to Eq.1.

Eq. 1. Calculation of culture viability from determination with a hemocytometer.

$$Viability\ [\%] = \frac{number\ of\ dead\ cells}{number\ of\ total\ cells} \times \frac{100}{1}$$

4.2.6 Determination of total cell numbers (Z2™ Coulter Counter®)

The Coulter Counter particle count technology is a non-optical measurement, which determines the number and size distribution of particles by impedance change of a conductive electrolyte solution. A tube with an aperture of a defined diameter is immersed in a container with an electrolyte that contains the particles to be measured. During the measurement a vacuum is applied, which causes the particles to pass through the aperture inside the tube. As the particle passes the cross-section of the aperture a volume equivalent to the size of the particle is displaced from the aperture. The drop in aperture volume, owing to the displacement by the particle's volume, causes an immediate change in impedance that is sensed by two electrodes, one inside and another one outside the tube. The change in impedance is proportional to the volume of the sensed particle and the number of pulses is proportional to the number of particles. The instrument's electronics further evaluate the data, resulting in a particle count to size distribution. As this method is non-optical, particle shape and granularity of the surface as well as color does not have any negative influence on the result. Hence enabling high resolution and accuracy compared to optical methods. (www.beckmancoulter.com, 2015)

Table 7. Solutions for the determination of total cell numbers using the Z2™ Coulter Counter®

Coulter lysis buffer	0.1 M Citric Acid 2 $\%_{(w/w)}$ Triton R X100 dissolved in RO-H_2O
Physiological NaCl solution	0.9 % NaCl dissolved in RO-H_2O 0.22 µm filtered

2 mL of sample were drawn from the culture using a 10 mL universal tube and were followed by a centrifugation step for 10 min with 100-200 g (Hereaus Megafuge 16 Centrifuge). The supernatant was discarded and remaining medium was removed. To avoid clumping, the cell pellet was disaggregated by flicking, and subsequently 1 mL of Coulter lysis buffer was added to lyse the cell membrane and free the cell nuclei. The lysis step was performed for a minimum of 1 hour. However, samples can be stored for a couple of hours at room temperature. Since cell numbers are usually too high to perform accurate and reliable measurements, sample dilutions were performed. 200-400 µL of sample were diluted in a sampling container with 9 mL of physiological saline solution in order to obtain between 10,000 and 20,000 particle counts per measurement. The measurement was done in duplicates or even triplicates to avoid misinterpretation caused by inhomogeneous sample dilutions. Further, evaluation was done for particles within a range of 3.013 µm – 10.54 µm as it is shown in Figure 2. According to the respective dilution performed, the final cell concentration was then calculated using equation two (Eq. 2).

Eq. 2 – Calculation of cell concentrations from data obtained with the Z2™ Coulter Counter®

$$Cell\ Concentration\ \left[\frac{cells}{mL}\right] = \left[\frac{particles}{mL}\right] \times \frac{Vol(a)}{Vol(b)} \times \frac{Vol(c)}{Vol(d)} \times \frac{Vol(a)}{Vol(e)}$$

Index for equation 2:

Vol.(a) sample volume
Vol.(b) aliquot taken by the Z2 Coulter® Particle Counter
Vol.(c) total volume of diluted sample
Vol.(d) sample aliquot for dilution
Vol.(e) original sample volume

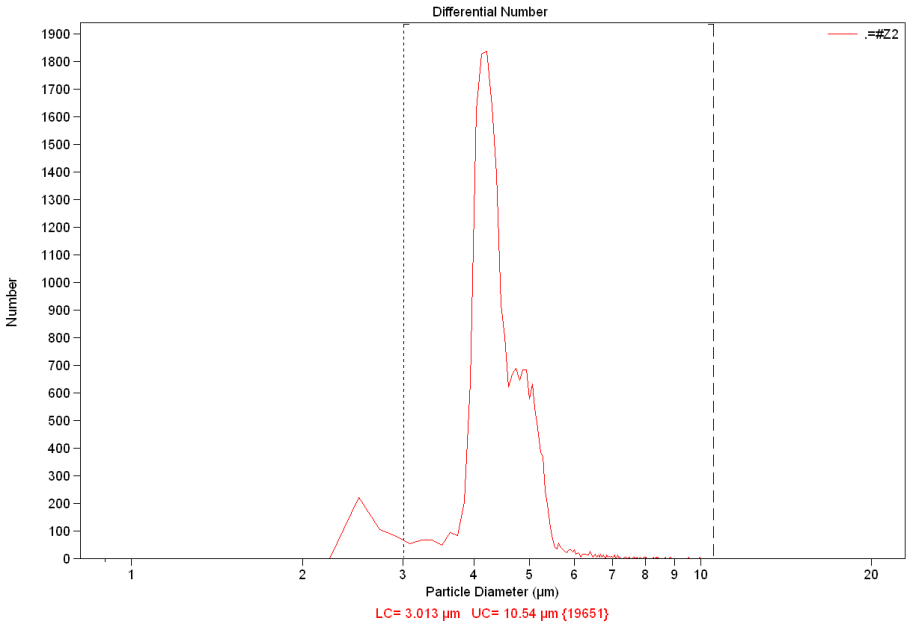

Figure 2. Particle count to diameter distribution obtained from the Z2 Coulter Counter®

On the y-axis the number of particles for a given diameter range is shown. The x-axis gives the cell diameter range (µm). The differential number for particles counted in the range from 3.013-10.54 is shown in red below the x-axis.

4.2.7 Metabolite and by-product analysis - BioProfile® 100 Plus

Monitoring of key metabolites and metabolic by-products during cultivation of mammalian cells using the BioProfile® 100 Plus instrument is an efficient tool to get an insight into the cellular metabolism. Metabolite analysis was done fully automated according to the instrument's manual. The different testing methods are shown in Figure 3. For metabolite and by-product profiling, an aliquot of 1 mL culture supernatant was drawn from samples following the centrifugation step during determination of cell concentrations. Samples were measured subsequently upon sampling. Samples that were retained for future measurements were stored at -20°C.

BioProfile 100 Plus

Parameter	Test Methodology	Measuring Range	Imprecision Resolution†
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%

†All imprecision values are within run

Figure 3. Overview of parameters tested with the BioProfile® 100 Plus instrument

(Source: http://www.novabiomedical.com/responsive/bioprofile_analyzer.php)

4.2.8 Determination of osmolality - OSMOMAT 030D

The OSMOMAT 030D is an automated osmolality analyzer, which determines the osmolality of a solution by comparative analyses of a solution's freezing point. The freezing point of a solution decreases as the concentration of ionic components in solution increases. Prior to analysis, the instrument was calibrated by means of a two-point-calibration. For calibration purposes, RO-H_2O was used to determine the intercept and a 300 mOsm/kg standard was used to determine the slope. For each measurement 50 µL sample (centrifuged culture supernatant) were drawn and analysis was performed according to the instrument's manual. Further measurements, which were performed for the purpose of osmolality adjustment of media, were evaluated using Eq. 3.

Eq. 3. Approximation for the adjustment of osmolality during media preparation

$$NaCl\,[g] = \left[\frac{\frac{\Delta mOsm}{kg}}{2}\right] \times M(NaCl)\frac{g}{mol} \times \frac{Vol(x)}{1000}$$

$M_{(NaCl)}$ … 58.44 g/mol
$Vol_{(x)}$ … Volume of medium solution

4.2.9 Enzyme linked immunosorbent assay (ELISA)

Enzyme linked immunosorbent assays (ELISA) and immune assays in general are based on the ability of strong and selective binding of antibodies to their specific antigen. Selectivity, relatively low costs and a high sample throughput are major points that make ELISA a widely used method. Lectin from snowdrop (Galanthus nivalis) was used for coating of a 96-well plate. Since lectins are highly specific for glyco-structures on proteins, they can be used to un-specifically bind glycoproteins. Gp140, the protein expressed by the cell line used in this work, is a highly glycosylated protein carrying α(1-3) and α(1-6) high-mannose structures, protruding from the molecule's surface. These glyco-structures bind to the lectin that was coated on the surface of the 96-well plate. The human IgG variant 5F3, which specifically targets the gp41 subunit of gp140, was used as conjugation antibody. For quantification, a horse-radish-peroxidase (HRP) labeled goat anti human IgG was used, which specifically binds the gamma Fc-part of 5F3. Horse-radish peroxidase together with the substrate TMB (3,3', 5,5;-tetramethylbenzidine) results in a blue color reaction with an absorbance maximum at 650 nm which can be photometrically measured and used for gp140 quantification.

Table 8. Solutions for gp140 ELISA and their respective concentrations

10X PBS solution	100 mM Na_2HPO_4 20 mM KH_2PO_4 27mM KCl 1370 mM NaCl pH 7.2 – 7.4
Coating buffer	100 mM $NaHCO_3$ 39.6 mM Na_2CO_3 pH 9.5 – 9.8
Washing buffer	1X PBS 0.1 $\%_{(v/v)}$ Tween20
Dilution buffer	Washing buffer 1 $\%_{(w/w)}$ BSA

Table 9. Coating- and antibody solutions for gp140 ELISA

Coating	Lectin (G.nivalis) (Sigma-Aldrich) + dilution buffer (working conc. = 2.5 µg/mL)
Conjugation	hm Ab 5F3 IgG_1 (anti-$gp41^{HIV}$) (Polymun Scientific, Austria) + dilution buffer (c = 1 µg/mL)
Detection	HRP-Goat Anti Human IgG (γ-chain specific) (Invitrogen) + dilution buffer (1:5,000 dilution)

Coating and plate saturation

For the purpose of coating, an aliquot of the lectin [c = 5mg/mL] (*G.nivalis*; Sigma-Aldrich) was diluted in dilution buffer to a final concentration of 2.5 µg/mL (coating solution). 100 µL of coating solution were applied to each well of a 96-well MaxiSorp™ plate (Nunc, Thermo Scientific) and stored overnight at 4°C. Following overnight coating, the plate was washed three times with washing buffer in an automated 96-well plate washer (Tecan, Switzerland) to remove excess and non-bound lectin molecules. In order to reduce the background signal, 100 µL dilution buffer were applied to saturate all non-occupied spots with BSA on the wells' surface. The plate was incubated for another 60min at RT and 200 rpm.

Sample dilutions and serial 1:2 dilutions

Pre-dilution of samples is crucial for ELISA analysis since sample concentrations should be in the linear range of the resulting sigmoidal graph. For quantification, purified gp140 (c = 100 ng/mL) was used. In order to find a suitable sample dilution some experience is needed. As a rule of thumb, a three-days culture harvest may be diluted approximately 1:250 to be in the linear range of the standard curve. In order to obtain a standard curve a serial dilution by the means of 1:2 was performed. Therefore, the respective well position of row H, of a 96-microwell plate (Nunc, Thermo Scientific), was set up with 240 µL of sample/standard/blank per well. Then, row A – G were filled with 120 µL dilution buffer each and a 1:2 serial dilution was performed by transferring 120 µL from row H into row G; stepwise continued. As a next step, 50 µL of each well were transferred into the respective well of the previously coated and saturated 96-well MaxiSorp™ plate, followed by an incubation step of 60min at RT and 200 rpm.

Application of the conjugation antibody

Following sample incubation, the plate was washed; 5F3 conjugation antibody was diluted to a final concentration of 2.5 µg/mL in dilution buffer and was then applied to the plate (50 µL per well). Another incubation step followed with 60min at RT and 200 rpm.

Application of the detection antibody

HRP-Goat anti human IgG (γ-chain specific) was diluted in dilution buffer (1:5,000), an aliquot of 50 µL was transferred to each well of the previously washed (3x) 96-well MaxiSorp™ plate and further incubation followed for another 60min at RT and 200 rpm.

Color reaction and evaluation

TMB (3,3', 5,5;-tetramethylbenzidine) was used as substrate for the coloring reaction. 100 µL TMB (at RT) were applied to each well from the lowest to the highest sample concentration. As the coloring reaction proceeded and the blue color developed, the reaction was stopped by adding 100 µL 2.5M H_2SO_4 immediately after the wells with the lowest concentrations turned light blue. The measurement was done with a 96-well plate reader (Tecan, Switzerland) at 650nm.

4.2.10 Batch cultivation

Per definition, the process of batch cultivation is defined as a closed vessel with neither input nor output or any other manipulation to the vessel's interior during cultivation except pH control and oxygen supply. Batch cultivation as it will be presented in this work was even more simplified since it was performed in shaking flasks with limited oxygen supply and pH adjustment by means of CO_2 exchange via the surface area of the culture solution.

Batch cultures follow a typical growth curve as it is shown in figure 4. At the beginning of batch cultivation the cells need to adopt their metabolism to the conditions essentially imposed by the culture medium and the culture vessel. This phase of reduce growth is called lag-phase ($\mu < \mu_{max}$). As the cells adopt to the cultivation conditions they start to proliferate and continue to grow with a more or less constant maximum specific growth rate ($\mu \sim \mu_{max}$) with respect to the supply of nutrients and oxygen as well as pH. This phase is called exponential growth phase since the cell number increases exponentially. At the end of exponential growth, nutrient supply runs out as well as metabolic by-products such as lactate and ammonium accumulate and thereby slow down cell growth. This phase is called plateau phase or stationary phase, since dying cells and proliferating cells keep cell numbers constant. While in plateau phase the specific growth rate is approximately zero ($\mu \sim 0$) until all key nutrients, which have been provided by the medium, are depleted and metabolic by-products create a non-favorable environment for cell proliferation. Hence, the equilibrium of dying and proliferating cells is shifted towards a decline of total cells, resulting in a theoretically negative specific growth rate ($\mu < 0$).

The exact setup for batch experiments in this thesis was as follows. Prior to seeding of batch cultures the cells were adapted to the particular medium used for the respective experiment. Media adaptation was performed as it is described in the section 4.2.4. Seeding was performed with a cell concentration of 300,000 cells/mL in a total culture volume of 45 mL. Prior transferring the cells into fresh medium, the culture was centrifuged (Hereaus Megafuge 16 Centrifuge, Thermo Scientific) using a 50 mL Falcon tube at 188 x g (1000 rpm) for 10min. The remaining medium was discarded and the pellet was re-suspended in 45 mL of pre-warmed (37°C), freshly prepared medium according to the experimental setup. The cell suspension was transferred into a 125 mL shaking flask and incubated in a shaking incubator (Climo Shaker ISF1-XC, Kuhner, Switzerland) at 37°C and 140 rpm in a controlled atmosphere of 6.8 % CO_2 for control of pH. Sampling started on day 3 of batch cultivation and was followed by subsequent daily sampling of 2.5 mL for determination of cell viability, cell number and product concentration (ELISA), and metabolite formation. The critical viability value for culture termination was set to 80 %.

4.2.11 Fed batch cultivation

Fed Batch cultivation in mammalian cell culture compared to batch cultivation describes a process where nutrients are added as concentrated feed solutions on a regular basis. Feeding of balanced 'feed-cocktails' containing nutrients such as glucose, amino acids, trace elements and vitamins at high concentrations may result in a prolonged cultivation time and increased cell concentrations as well as increased product titers.

The growth behavior describes an altered version of what has been described for batch cultivation. In fed-batch cultivation the exponential growth phase is usually prolonged as well as the plateau phase. This is due to the regular input of nutrients since thereby nutrient depletion is avoided and the cells grow to higher densities. However, feeding strategies and feed media compositions enormously affect the previously mentioned process parameters. Therefore, various feeding strategies and strategies for feed media balancing have been developed over the last years.

The setup for fed-batch experiments in this thesis was as it was described for batch cultivation with exception to the addition of feed media as follows. Feed media used for the experiments were a two-feed-solution system (Feed A / Feed B) where Feed A was added to in order to adjust the glucose level to 6.5 g/L. Feed B was added as a constant volume feed of 0.28 $\%_{(v/v)}$ of the actual culture volume. Both feed media were added daily after sampling. Samples of 2.5 mL were drawn from day 3 on, for the determination of cell viability, cell number, product concentration and by-product concentrations. The viability criterion applied for culture termination was 80 % viable cells in culture.

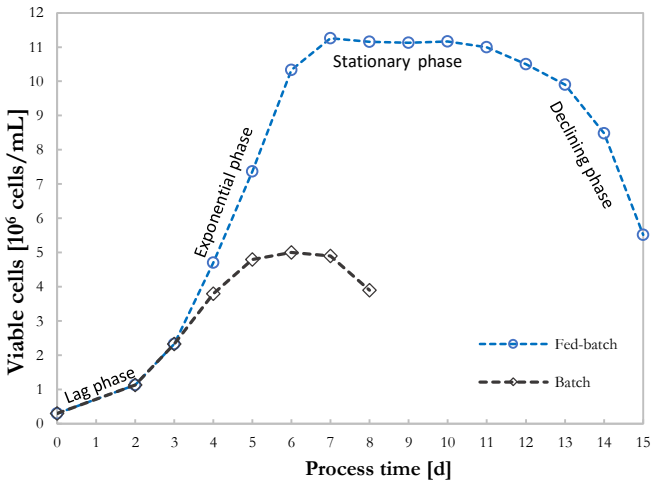

Figure 4. Typical growth profiles of mammalian cells during batch and fed-batch cultivation.

During lag-phase, cells adopt to conditions imposed by the media. Cells in lag-phase show reduced growth ($\mu < \mu_{max}$). As cells have successfully adapted to cultivation conditions, they start to grow with a maximum growth rate with respect to the cultivation conditions ($\mu = \mu_{max}$). At higher cell densities nutrient depletion and toxic by-product formation cause a phase of reduced growth (stationary phase) where apoptosis and cell growth are in equilibrium ($\mu = 0$). Towards the end of cultivation, viable cell numbers decline since nutrient depletion (only for batch cultivation) and by-product formation cause a toxic, non-favorable environment.

4.2.12 Calculations

Specific growth rate

$$\mu\ [1/h] = \frac{(lnX_i - lnX_{i-1})}{(t_i - t_{i-1})}$$ **Eq. 4 – Specific growth rate [μ]**

X_i … cell concentration [cells/mL]
t_i … time [h]

For calculation of $\mu_{(viable)}$ X_i was replaced by the concentration of viable cells (X_{vi})

Viable Cell Days (VCD) or Integral of Viable Cell Density

$$VCD = \frac{VCC_{ti+1} - VCC_{ti}}{\mu v}$$ **Eq. 5 – Viable Cell Days (VCD) [cell×days/mL]**

VCC … Viable cell concentration [viable cells/ml]
μ_v … specific growth rate of viable cells [1/h]

Viable Cumulative Cell Days (VCCD)

$$\mathbf{VCCD_i = VCD_i + VCD_{i-1}}$$ **Eq. 6 – Viable Cumulative Cell Days (VCCD)**
[cell×days/mL]

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

Specific productivity

$$q_p = \frac{c_{i+1} - c_i}{VCC_{i+1} - VCC_i}$$ **Eq. 7 – Specific productivity (q_p)**

c_i … product concentration of a specific sample [μg/mL]
q_p … specific productivity [pg/cell×days]

5 Results

5.1 Pre-experiments

5.1.1 Applicability and solubility of different iron salt candidates for supplementation of MV3-2

The original formulation of MV3-2 medium did not contain any iron source since the question of which iron salts would work best considering handling, solubility, pH and growth performance as well as product formation rates was part of this thesis. Table 10 lists a selection of iron salts which were subject to testing. To evaluate the salts' applicability in terms of solubility and pH, 50-fold and 100-fold solutions were prepared for supplementation of MV3-2 medium. As it is shown in Table 11, 50-fold and 100-fold solutions of ferric citrate (Merck) were only soluble at a temperature of 90°C and stirring for 60 min; additionally, both solutions had a rather low pH. Concentrated solutions of ammonium iron(III) citrate (Merck) and iron(III) pyrophosphate (Sigma-Aldrich) were well soluble at room temperature and the pH remained almost neutral. In contrast, iron(III) pyrophosphate (Merck) was not soluble at all under the rather harsh conditions that were applied for solubility testing.

Table 10. Iron salt candidates used for supplementation of MV3-2 medium

Salt	Formula	Manufacturer
Ferric citrate anhydrous micronized	$C_6H_8O_7 \cdot xFe_3^+ \cdot yH2O$	Merck
Ammonium iron(III) citrate	$C_6H_8O_7 \cdot xFe_3^+ \cdot yNH_3$	Merck
Iron(III) pyrophosphate	$Fe_4(P_2O_7)_3$	Merck
Iron(III) pyrophosphate	$Fe_4(P_2O_7)_3$	Sigma-Aldrich

Table 11. Solubility conditions and pH values of concentrated (50-fold and 100-fold) solutions of iron salts used for supplementation of MV3-2 medium

Salt	Solubility conditions (temp.)	pH of solution	
		50X	100X
Ferric citrate anhydrous micronized (Merck)	90°C for appr. 60 min	2.24	1.98
Ammonium iron(III) citrate (Merck)	Soluble at RT	6.86	6.88
Iron (III) pyrophosphate (Merck)	< 100°C – insoluble	-	-
Iron (III) pyrophosphate (Sigma)	Soluble at RT	6.74	6.76

5.2 Testing of iron salts for medium supplementation

Experiments under section 5.1.1 revealed that three out of four possible iron containing salts seemed applicable for medium supplementation in terms of solubility and pH. These three iron containing salt variants are listed below.

Iron citrate (micronized) (Merck)

Ammonium iron(III) citrate (Merck)

Iron(III) pyrophosphate (Sigma-Aldrich)

5.2.1 Experimental setup

Table 12. Experimental setup: Testing of iron salts for medium supplementation

Process type	Batch in 125 mL shaking flasks (duplicates) Working volume: 45mL
Initial cell concentration	300,000 cells/mL
Media	MV3-2 Acti CHO SM ActiCHO P CD CHO All media were supplemented with 8mM l-glutamine and 15 mg/mL phenol red prior to the experiment.
Medium adaptation	3 consecutive passages
Sampling	Cell number Viability Product concentration (gp140) Off-line monitoring of: L-glutamine L-glutamic acid Lactic acid NH_4^+

Culture-to-medium adaptation (section 4.2.4) and setting up of batch cultures (section 0) as well as sampling were performed as it has already been described. As reference media, duplicates of ActiCHO P, ActiCHO SM and CD CHO were seeded, applying the same conditions as previously described. Iron supplementation was done for the respective MV3-2 media setups (duplicates) using 100-fold solutions of the respective iron salts to reach the final concentrations given in Figure 5. Figure 5 illustrates the six different MV3-2 media setups with their respective absolute iron concentrations in mg per liter medium solution.

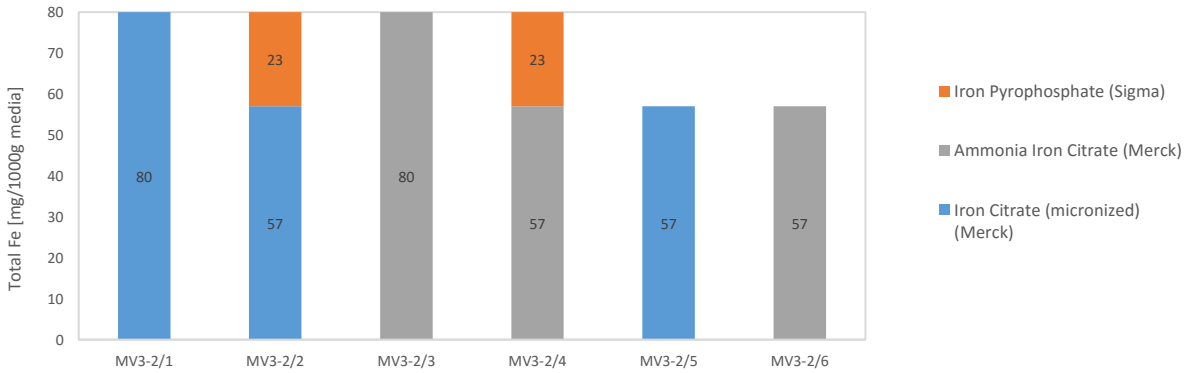

Figure 5. Total amount of iron supplemented for various MV3-2 medium setups

Iron was supplemented by adding different iron salts and combinations of these salts to evaluate the effects on culture growth, viability and product formation.

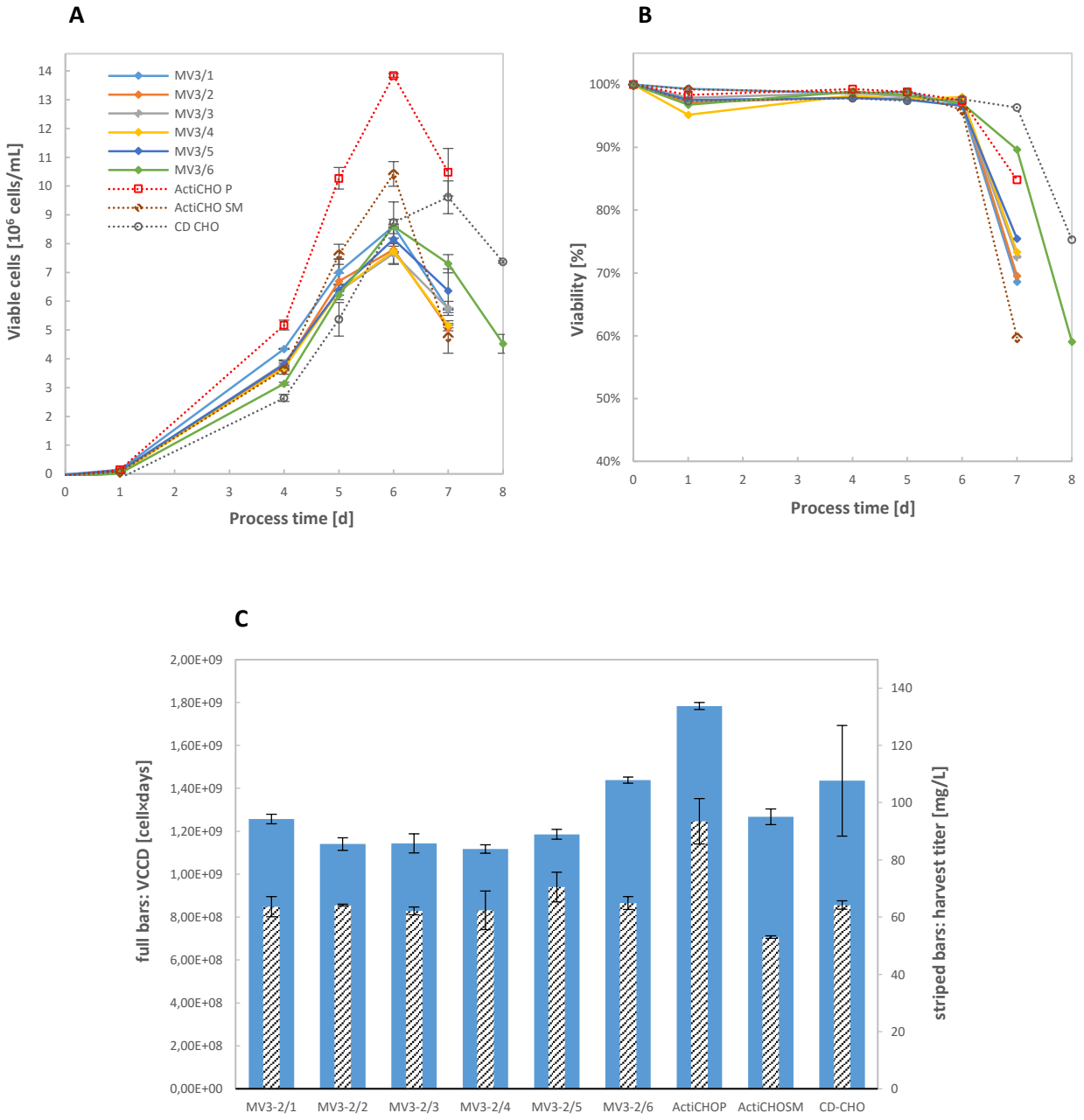

Figure 6. Effects of various iron salts on cell culture performance

(A) Viable cell concentration, (B) viability, (C) harvest titer vs. VCCD [cell×days]. 6 variants of MV3-2 were set up by supplementation with 100-fold stock solutions of the respective iron salts (Figure 5). (A) The media variants of MV3-2 are depicted as full lines whereas the reference media ActiCHO P, ActiCHO SM and CD CHO are shown as dashed lines. (C) The full bars account for viable cumulative cell days (VCCD) of cultures that were cultivated in one of the following media: MV3-2 media variants (1-6) and reference media ActiCHO P, ActiCHO SM, CD-CHO. The striped bars illustrate respective titers on day of harvest.

5.2.2 Experimental results

The supplementation of MV3-2 medium with 3 different iron salts in a combination of a total of 6 media variants resulted in the range of 8.06-9.00×10^6 viable cells/mL in terms of peak cell concentrations. MV3-2 variants 1 and 6 both showed outstanding peak cell numbers of 9.00×10^6 cells/mL (Table 13, Figure 6) throughout the selection of MV3-2 medium variants. ActiCHO SM (10.8×10^6 viable cells/mL) and CD CHO (10.0×10^6 viable cells/mL) media both of which were reference media showed viable peak cell concentrations in the range of the MV3-2 medium variants. ActiCHO P, a nutrient rich production medium with a nutrient content that is widely known to be superior over ActiCHO SM and CD CHO, showed viable peak cell concentrations of 14.2×10^6 viable cells/mL. In terms of process time MV3-2/6 and CD CHO showed a prolonged cultivation time of 8 days instead of 7 days as it was for the other cultures (Figure 6B, Figure 1). Although MV3-2/6 and CD CHO showed a prolonged cultivation time they did not seem to be superior in terms of product formation. Cultures which were cultivated in ActiCHO P resulted in a harvest titer of 93.5 mg/L whereas cultivation in CD-CHO (64.2 mg/L) and ActiCHO SM (53 mg/L) (Figure 6C, Table 13). Cultures that were cultivated with one of the MV3-2 medium variants 1-6 showed similar titers within a close range of 62.2-70.0 mg/L with no significant differences (Figure 6C, Table 13). Figure 6C also suggests that although there are no major differences in product titers between the MV3-2 medium variants the media variants MV3-2/1 and MV3-2/6 seem to be superior in terms of cell growth. This is illustrated as viable cumulative cell days (VCCD) with a total of 1.26×10^9 cell×days for MV3-2/1 and 1.44×10^9 cell×days for MV3-2/6. The reference media ActiCHO SM and CD CHO were both in the range of the MV3-2 medium variants whereas cultivation in ActiCHO P revealed an outstanding value of 1.78×10^9 cell×days (Figure 6C) which is mainly responsible for the previously described higher product titer of cultures that were cultivated in ActiCHO P.

Table 13. Process relevant data of batch experiment: Testing of iron salts for medium supplementation

	MV3-2/1	MV3-2/2	MV3-2/3	MV3-2/4	MV3-2/5	MV3-2/6	ActiCHO P	ActiCHO SM	CD CHO
Viable peak cell conc. (VPCC) [10^6 cells/mL]	9.00	8.19	8.06	8.14	8.56	9.00	14.2	10.8	10.0
Process time [d]	7	7	7	7	7	8	7	7	8
Harvest titer [mg/L]	63.6	64.2	62.2	62.4	70.5	64.9	93.5	53.0	64.2
VCCD [10^9 cell×days]	1.26	1.14	1.14	1.12	1.19	1.44	1.78	1.27	1.44

5.3 Supplementation of Critical Amino Acids

5.3.1 Experimental setup

Table 14. Experimental setup: Supplementation of critical amino acids

Process type	Fed-batch in 125 mL shaking flasks (singlets)
Initial cell concentration	300,000 cells/mL
Media	MV3-2/1 MV3-2/6 ActiCHO P (All media were supplemented with 8mM l-glutamine and 15 mg/mL phenol red.)
Medium adaptation	3 consecutive passages
Feed media	ActiCHO Feed A Powder Base CD ActiCHO Feed B Powder Base CD
Feeding strategy	Feed Start: day 4 of fed-batch Feed A: adjusted to 6.5 g/L glucose in the shaking flask Feed B: 0.28 $\%_{(v/v)}$ of the actual culture volume
Sampling	Cell number Viability Product concentration (gp140) Off-line monitoring of: L-glutamine L-glutamic acid Lactic acid NH_4^+

On the one hand, the aim of this experiment was to evaluate the performance of the two best performing iron-supplemented media (experiment 5.2) during fed-batch cultivation. The second aim of this experiment was to find out whether supplementation of the culture medium, supplemented with limiting amino acids (l-asparagine, l-proline and l-serine) would improve culture performance (cell growth, product formation and process time) in a fed-batch setup. A previous work revealed that these key amino acids were already found depleted in an early phase of batch cultivation. Table 15 illustrates the supplementation scheme according to which the respective setups were supplemented. Amino acids were added as stock solutions to give a final concentration that would be double-fold of the original concentration of MV3-2. To ensure equivalent starting volumes for all flasks, single amino acid supplemented flask and controls, were supplemented with the equivalent volume of sterile water.

Table 15. Amino acid supplementation regime for MV3-2 media supplementation

Concentrations given in the table represent the double-fold of concentrations of the original MV3-2 formulation.

Medium	l-asparagine $C_{(final)}$ = 1205 mg/L	l-proline $C_{(final)}$ = 1155 mg/L	l-serine $C_{(final)}$ = 968.8 mg/L)	no amino acid supplementation
MV3-2/1 MV3-2/6	×			
MV3-2/1 MV3-2/6		×		
MV3-2/1 MV3-2/6			×	
MV3-2/1 MV3-2/6	×	×	×	
MV3-2/1 MV3-2/6				×
ActiCHO P				×

5.3.2 Experimental results

Data from previous experiments revealed that the amino acids l-asparagine, l-proline and l-serine were limiting during batch cultivation with MV3-2 medium. These amino acids were supplemented to the double-fold concentration in order to evaluate whether further balancing of MV3-2's amino acid content is necessary. Another aim of the experiment was to gain a closer insight into which of the two medium variants (MV3-2/1, MV3-2/6; supplemented with either iron citrate or ammonium iron(III) citrate) is favorable to be used as cell culture medium.

Supplementation of the respective medium variant with the amino acids l-asparagine, l-proline and l-serine did not show to improve overall culture performance compared with ActiCHO P (reference medium). Viable peak cell concentrations (VPCC) for MV3-2/1 were in the range of 11.0×10^6 cells/mL (V1+Asn) to 12.2×10^6 cells/mL (V1+Asn, Pro, Ser). Although VPCC for MV3-2/6 were marginally increased from 12.7×10^6 cells/mL for V6+Ser to 13.4×10^6 cells/mL for V6+Asn/Pro/Ser, VPCC for ActiCHO P (17.0×10^6 cells/mL) outperformed both best performing MV3-2 variants (Table 16, Figure 7). Besides the marginally increased viable peak cell numbers of MV3-2/6 over MV3-2/1, MV3-2/1 showed to have lower viability values at the day of culture termination (Figure 8). ELISA analysis of the culture supernatants of MV3-2/1 gave peak product titers ranging from 230 mg/L (V1+Asn) to 269 mg/L (V1 control) and for MV3-2/6 ranging from 224 mg/L (V6 Asn+Pro+Ser) to 302 mg/L (V6+Pro) on day 9 of cultivation (Figure 9).

Although cultivation with ActiCHO P resulted in a higher VPCC (17.0×10^6 cells/mL) the peak titer of cultivation with ActiCHO P (274 mg/L) was in the same range as product titers generated by either MV3-2 variant (Figure 9). Interestingly, gp140 titers on day 10 were found to be lower than on day 9. A possible explanation might be that this phenomenon occurred due to product degradation effects as well as ELISA inaccuracy due to increased levels of intracellular components released as the cells died.

Figure 10 shows absolute numbers of viable cumulative cell days (VCCD) and gp140 titers calculated as averages of the respective medium variants (MV3-2/V1 or MV3-2/V6 supplemented with amino acids). This was done to gain insight into which of the 2 iron supplemented medium variants might be appropriate as basis for further experiments. Cultivation in MV3-2/1 (2.89×10^9 cell×days ± 0.14) resulted in a lower number of $VCCD_{(absolute)}$ than cultivation in MV3-2/6 (3.28×10^9 cell×days ± 0.06) (Figure 10,Table 16). However, product titers have not shown to be different (Figure 10).

Table 16. Process relevant data of fed-batch experiment: Supplementation of critical amino acids

Comment: Average VCCD on day 9 was calculated from VCCD values of the single and triple amino acid supplemented media of the respective MV3-2 medium variant (V1, V6).

	V1	V1 Asn	V1 Pro	V1 Ser	V1 Asn Pro Ser	V6	V6 Asn	V6 Pro	V6 Ser	V6 Asn Pro Ser	Acti CHO P
Viable peak cell conc. (VPCC) [10^6 cells/mL]	11.0	11.3	11.8	11.4	12.2	13.4	13.6	13.4	12.7	13.4	17.0
peak titer (Day 9) [mg/L]	269	230	252	246	236	253	260	302	283	224	274
absolute titer (Day 9) [mg] (calc. Value)	15.3	13.1	14.4	14.1	13.5	14.4	15.0	17.2	16.1	12.9	16.1
VCCD [10^9cell×days]	2.76	2.97	2.83	2.78	3.13	3.11	3.38	3.28	3.24	3.39	4.19
VCCD [10^9cell×days] (Average)	2.89 ± 0.14					3.28 ± 0.06					4.19

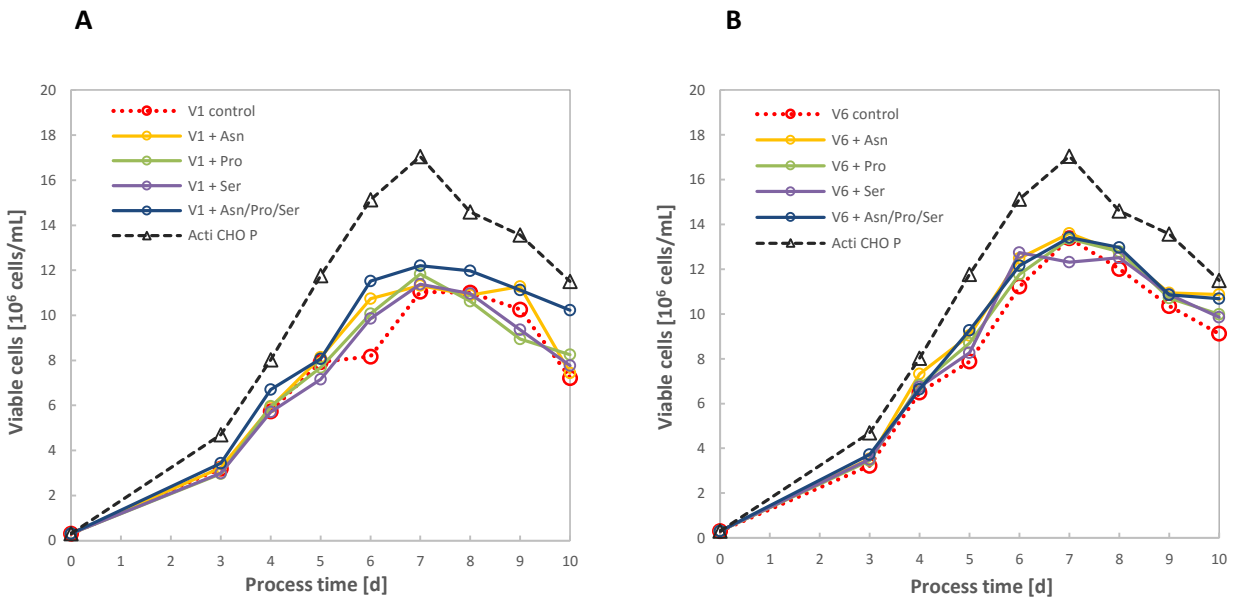

Figure 7. Viable cell concentrations of fed-batch experiment: Supplementation of critical amino acids

Figures (A) MV3-2/1 and (B) MV3-2/6 illustrate viable cell concentrations during fed-batch cultivation of MV3-2 medium variants (V1 & V6) and ActiCHO P as reference medium. MV3-2 medium variants were supplemented to the double-fold concentrations of the original MV3-2 formulation.

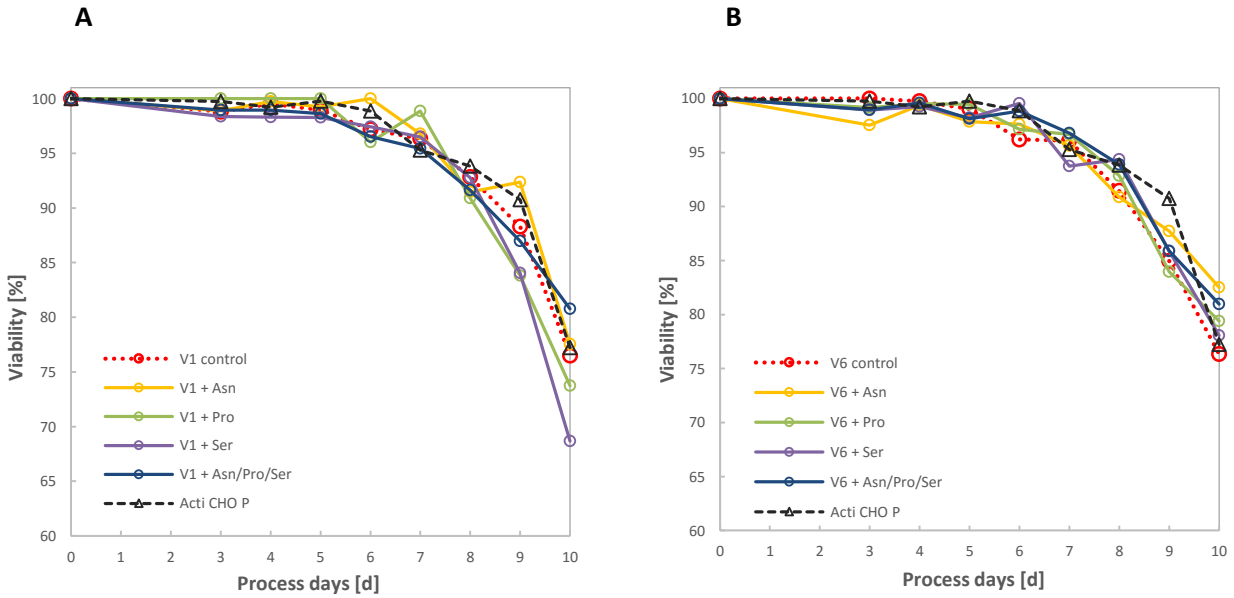

Figure 8. Viability of fed-batch experiment: Supplementation of critical amino acids

Figures (A) MV3-2/1 and (B) MV3-2/6 illustrate the viability profiles during fed-batch cultivation of MV3-2 medium variants (V1 & V6) and ActiCHO P as reference medium. MV3-2 medium variants were supplemented to the double-fold concentrations of the original MV3-2 formulation.

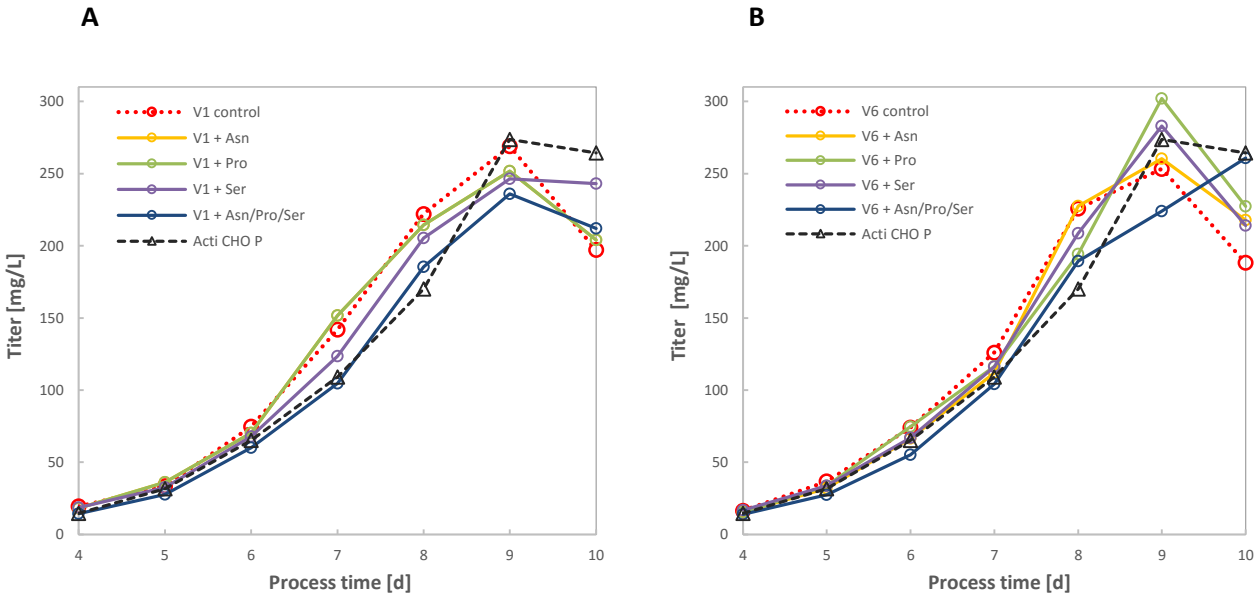

Figure 9. Gp140 titers of fed-batch experiment: Supplementation of critical amino acids

Figures (A) MV3-2/1 and (B) MV3-2/6 illustrate the gp140 titer profiles during fed-batch cultivation of MV3-2 medium variants (V1 & V6) and ActiCHO P as reference medium. MV3-2 medium variants were supplemented to the double-fold concentrations of the original MV3-2 formulation.

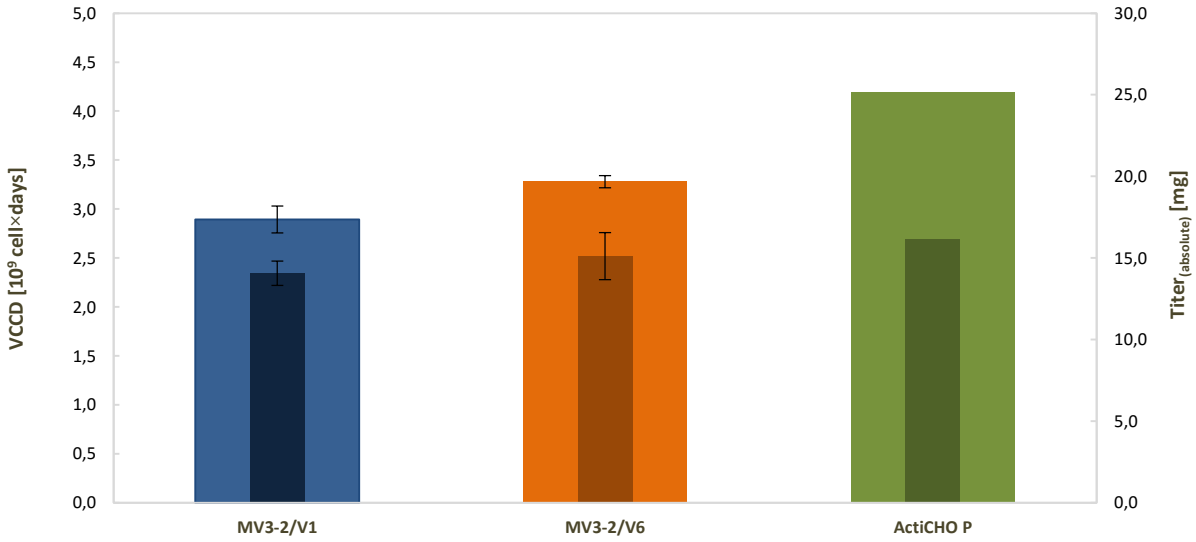

Figure 10. Absolute values of viable cumulative cell days (VCCD) [cell×days] and gp140 titers calculated as averages of the respective media setups

As there were no major differences between the amino acid supplemented MV3-2 setups, the respective data of MV3-2/V1 and MV3-2/V6 setups were combined to calculate the averages. Values for VCCD are shown as light colored bars. Titers are shown as dark colored bars.

Monitoring of key metabolites and metabolic by-products.

L-glutamine monitoring showed that l-glutamine concentrations decreased until day 5 where almost all the l-glutamine present was exhausted. However, as feeding started on day 4 a steady increase in l-glutamine concentration was observed. Concentrations of l-glutamic acid remained relatively constant until day 4 of fed-batch cultivation. With the start of feeding (day 4), concentrations of l-glutamic acid started to increase until culture termination.

Monitoring of metabolic by-products such as lactate and ammonium revealed major differences between amino acid supplemented MV3-2 medium variants and ActiCHO P as reference medium. Lactate accumulation for cultivation in ActiCHO P peaked at a concentration of 2.55 g/L at day 7. This was not the case for the MV3-2 medium variants, which showed peak lactate concentrations at day 4 (highest: MV3-2/V6 Asn/Pro/Ser; 1.80 g/L and lowest: MV3-2/V1 Ser; 1.47 g/L). Overall, accumulation of lactic acid was higher for cultures in ActiCHO P compared to all amino acid supplemented MV3-2 setups.

Ammonium accumulation behaved contrary to lactate accumulation. Two groups of media could be identified according to their initial ammonium concentration. As assumed, variants of MV3-2/1 and ActiCHO P did not show an initial ammonium concentration. In contrast, MV3-2/6 variants showed to contain some initial ammonium which was most likely due to the supplementation with ammonium iron(III) citrate. This specific difference in concentration could be observed over the whole process for all MV3-2 variants (Figure 11D). In general, ammonium concentrations peaked at day 4, then dropped at day 5 and further remained more or less constant until day 10 (Figure 11D). Interestingly, MV3-2 variants supplemented with either only l-asparagine or the triple combination l-asparagine/l-proline/l-serine showed to have higher ammonium concentrations over the whole process compared to MV3-2 variants containing l-proline or l-serine or neither of both. In contrast, ActiCHO P showed lower ammonium concentrations throughout the process compared to any of the MV3-2/1 and MV3-2/6 medium variants (Figure 11D).

Monitoring of glucose concentration during fed-batch cultivation for calculation of feeding volumes for Feed A (according to the feeding strategy described in 0) revealed a continuous decrease in glucose concentrations until day 4. At day 4, feeding started and glucose was thereby elevated to a concentration of 6.5 g/L (not shown in figure 11, since glucose was again consumed between sampling). From day 5 to day 7 glucose levels of ActiCHO P were lower compared to supplemented MV3-2 setups. Interestingly, almost no glucose was consumed between day 7 and 8 by some of the cultures (Figure 12) whereas for V1 control, V1+Pro, V1+Ser the glucose consumption remained relatively constant.

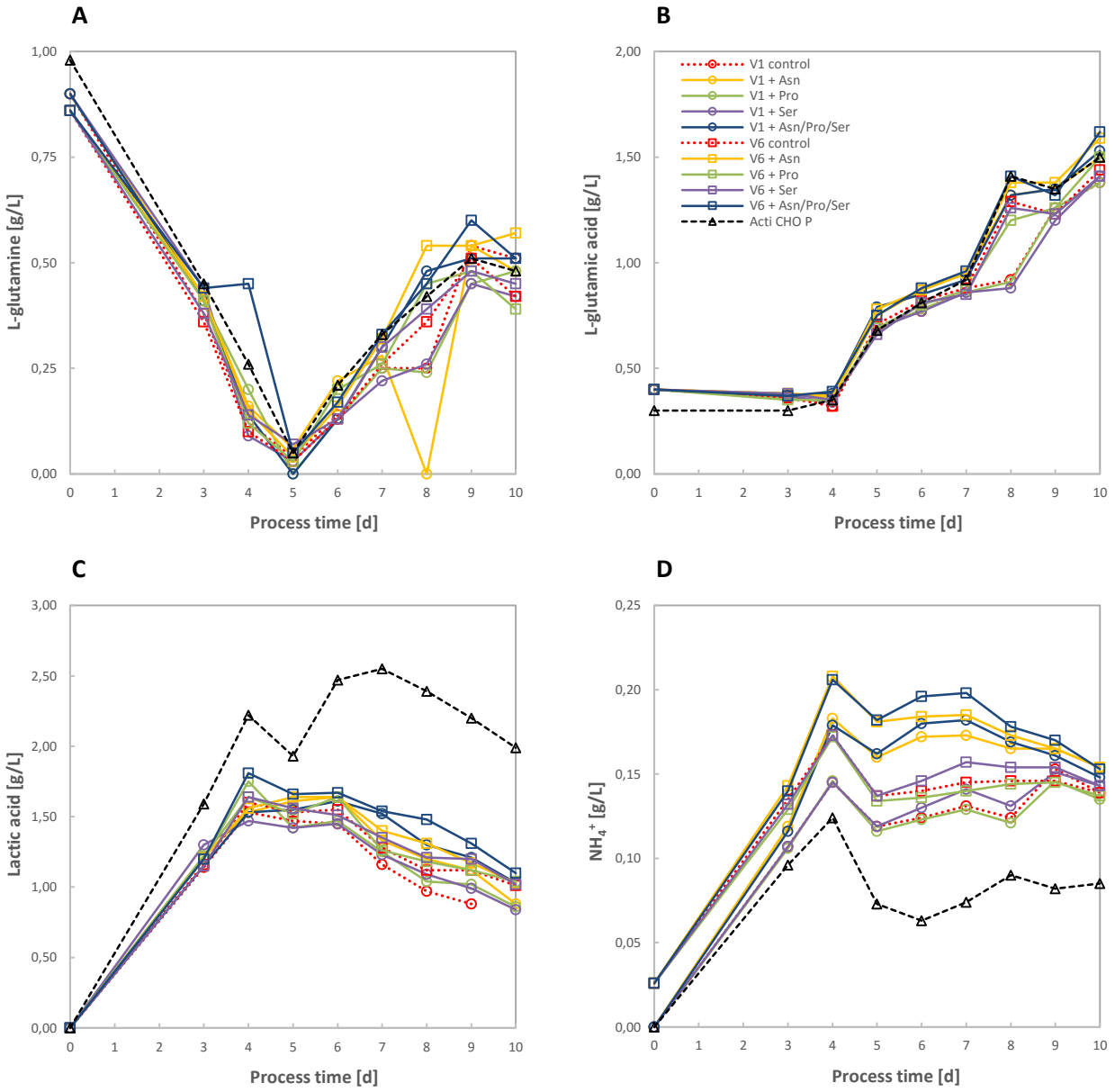

Figure 11. Metabolite and by-product profiles of fed-batch experiment: Supplementation of critical amino acids

Concentration graphs show incline/decline of the following key metabolites and by-products as they were routinely monitored from culture supernatants. (A) L-glutamine, (B) L-glutamic acid, (C) lactic acid (lactate), (D) ammonium (NH_4^+).

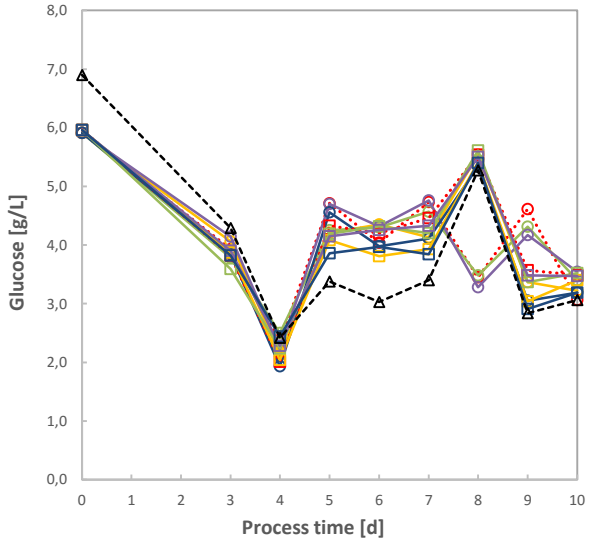

Figure 12. Glucose profile of fed-batch profile: Supplementation of critical amino acids

The graph illustrates the concentration profile of glucose during fed-batch cultivation. Feeding started on day 4 and was continued daily, where Feed A (GE) was added in order to adjust the glucose levels to 6.5 g/L.

5.4 Testing of an enhanced medium formulation of MV3-2

The original medium formulation of MV3-2 was based on a less compound-rich medium formulation than ActiCHO P (reference medium). On average, the major fraction of component concentrations are approximately 30% lower in MV3-2 compared to ActiCHO P. Therefore, MV3-2 compound concentrations were increased to reach growth performance levels as well as product formation levels of the reference medium.

5.4.1 Experimental setup

Table 17. Experimental setup: Testing of an enhanced medium formulation of MV3-2

Process type	Fed-batch in 125 mL shaking flasks (singlets)
Initial cell concentration	300,000 cells/mL
Media	MV3-2/6 MV3-2/6$_{(+30\%)}$ ActiCHO P (All media were supplemented with 8mM L-glutamine and 15 mg/mL phenol red.)
Medium adaptation	3 consecutive passages
Feed media	ActiCHO Feed A Powder Base CD ActiCHO Feed B Powder Base CD
Feeding strategy	Feed Start: day 4 of fed-batch Feed A: adjusted to 6.5 g/L glucose Feed B: 0.28 %$_{(v/v)}$ of the actual culture volume
Sampling	Cell number Viability Product concentration (gp140) Off-line monitoring of: L-glutamine L-glutamic acid Lactic acid NH_4^+

Prior to the experiment the media solutions of medium variant MV3-2/6, a variant of MV3-2/6 with increased compound concentrations and ActiCHO P as reference medium were prepared according to the respective preparation protocols. The variant of MV3-2/6 with increased compound concentrations was named MV3-2/6$_{(+30\%)}$ as the major fraction of compound concentrations was increased by simply increasing the amount of medium powder used by a factor of 1.3-fold of the original MV3-2 formulation.
Compounds that were identified to exceed the 30 % difference were supplemented to MV3-2/6$_{(+30\%)}$ in order to reach the actual concentration levels of the reference medium (ActiCHO P).

Culture-to-medium adaptation (section 4.2.4) and setting up of fed-batch cultures (section 0) as well as sampling was performed as it is described in the respective section. As reference medium, a shaking flask with ActiCHO P was seeded, applying the same conditions as previously described. Feeding was performed according to the described feeding strategy, with feeds of the ActiCHO system.

5.4.2 Experimental results

The increase in compound concentrations of the original MV3-2/6 medium formulation resulted in increased cell growth and product titer, prolonged cultivation time as well as an increased overall productivity of the cultures. Cultivation in MV3-2/6 medium resulted in viable peak cell concentrations (VPCC) of 1.45×10^7 cells/mL whereas cultivation with the improved variant MV3-2/6$_{(+30\%)}$ resulted in a VPCC of 1.90×10^7 cells/mL, which accounts for 31 % more viable cells compared to what was obtained with MV3-2/6 (Figure 13A). However, cultivation with ActiCHO P (reference medium) resulted in a VPCC of 2.40×10^7 cells/mL. Unfortunately, this rather high value seems to be an outlier according to Figure 13A. Although this value might not be reliable, the overall growth performance of ActiCHO P was better than for MV3-2/6 or MV3-2/6$_{(+30\%)}$. Regarding gp140 titers, cultivation in MV3-2/6$_{(+30\%)}$ showed a 70 % increase in harvest titer (512 mg/L) compared to MV3-2/6 (300 mg/L) (Figure 13C). Although, a 70 % improvement in titer is quite impressive, cultivation in ActiCHO P resulted in a harvest titer of 562 mg/L, which is still 10 % higher compared to the titer obtained by cultivation with MV3-2/6$_{(+30\%)}$ (Figure 13C). Interestingly, as it is shown in Figure 13D, overall productivity was higher for cells when they were cultivated in MV3-2/6$_{(+30\%)}$ compared to cultivation in MV3-2/6 or ActiCHO P.

Key metabolite concentrations were monitored daily for the following parameters: l-glutamine, l-glutamic acid, lactic acid and ammonium (NH_4^+). For l-glutamine and l-glutamic acid, no major differences could be identified between MV3-2/6 and MV3-2/6$_{(+30\%)}$, and ActiCHO P (Figure 14A/B). Concentrations of lactic acid peaked at day 4 for all three media and were again consumed in shaking flask with either MV3-2/6 or MV3-2/6$_{(+30\%)}$ medium. In contrast, supernatants derived from shaking flasks with ActiCHO P showed a more or less steady lactic acid levels from days 4 to 6, followed by a slow decrease until day 10 (Figure 14C).

Levels of ammonium were higher in the beginning for cultivation with MV3-2/6 and MV3-2/6$_{(+30\%)}$ compared to ActiCHO P, which was due to supplementation of MV3-2/6 with ammonium iron(III) citrate. Metabolic production of ammonium first peaked on day 4 for all media and was subsequently consumed until day 6. Interestingly, supernatants from cultivation with MV3-2/6 showed a reduced consumption of ammonium after day 4 and ammonium levels were highest on the last day of cultivation (day 9). On day 9 ammonium concentrations from cultivation in MV3-2/6 medium were 35 % higher compared to ammonium levels from cultivation in MV3-2/6$_{(+30\%)}$ and 58 % higher than levels resulting from cultivation with ActiCHO P.

Glucose profiling revealed decreasing glucose levels until day 4 (feed start) with glucose concentrations of 2.71 g/L glucose for ActiCHO P, 2.54 g/L for MV3-2/6 and 4.19 g/L for MV3-2/6$_{(+30\%)}$ (Figure 15A). After feeding started, ActiCHO P and MV3-2/6 showed increased glucose levels on day 5 (both 3.92 g/L). However glucose concentration of cultivation with ActiCHO P was again lower on day 6 and remained relatively constant in a range of lowest 2.49 g/L and highest 2.88 g/L until culture termination at day 10. In contrast the glucose level of MV3-2/6 was relatively constant from day 5 on, in a range of lowest 3.87 g/L (day 6) and highest 4.16 g/L (day 8). MV3-2/6$_{(+30\%)}$ glucose concentration decreased until day 6 (3.31 g/L glucose) and then remained rather constant until culture termination.

Additionally, osmolality values were determined (Figure 15B) from sample supernatants to identify effects of high osmolality on culture performance. All media used in this experiment had initial osmolality values in the range of 290-298 mOsm/kg. Osmolality values remained rather constant until day 4 and increased after feed addition. The terminal osmolality values were 0.459 Osm/kg for ActiCHO P, 0.421 Osm/kg for MV3-2/6$_{(+30\%)}$, and 0.418 Osm/kg for MV3-2/6.

Table 18. Process relevant data from fed-batch experiment: Testing of an enhanced media formulation of MV3-2

	MV3-2/6	**MV3-2/6$_{(+30\%)}$**	**ActiCHO P**
Viable peak cell concentrations (VPCC) [10^6 viable cells/mL]	14.5	19.0	24.4
µ(exp. phase) [1/d]	0.57	0.62	0.64
Process time [d]	8	9	9
Harvest titer [mg/L]	300	512	562
VCCD [10^7cell×days/mL]	6.44	8.48	10.6

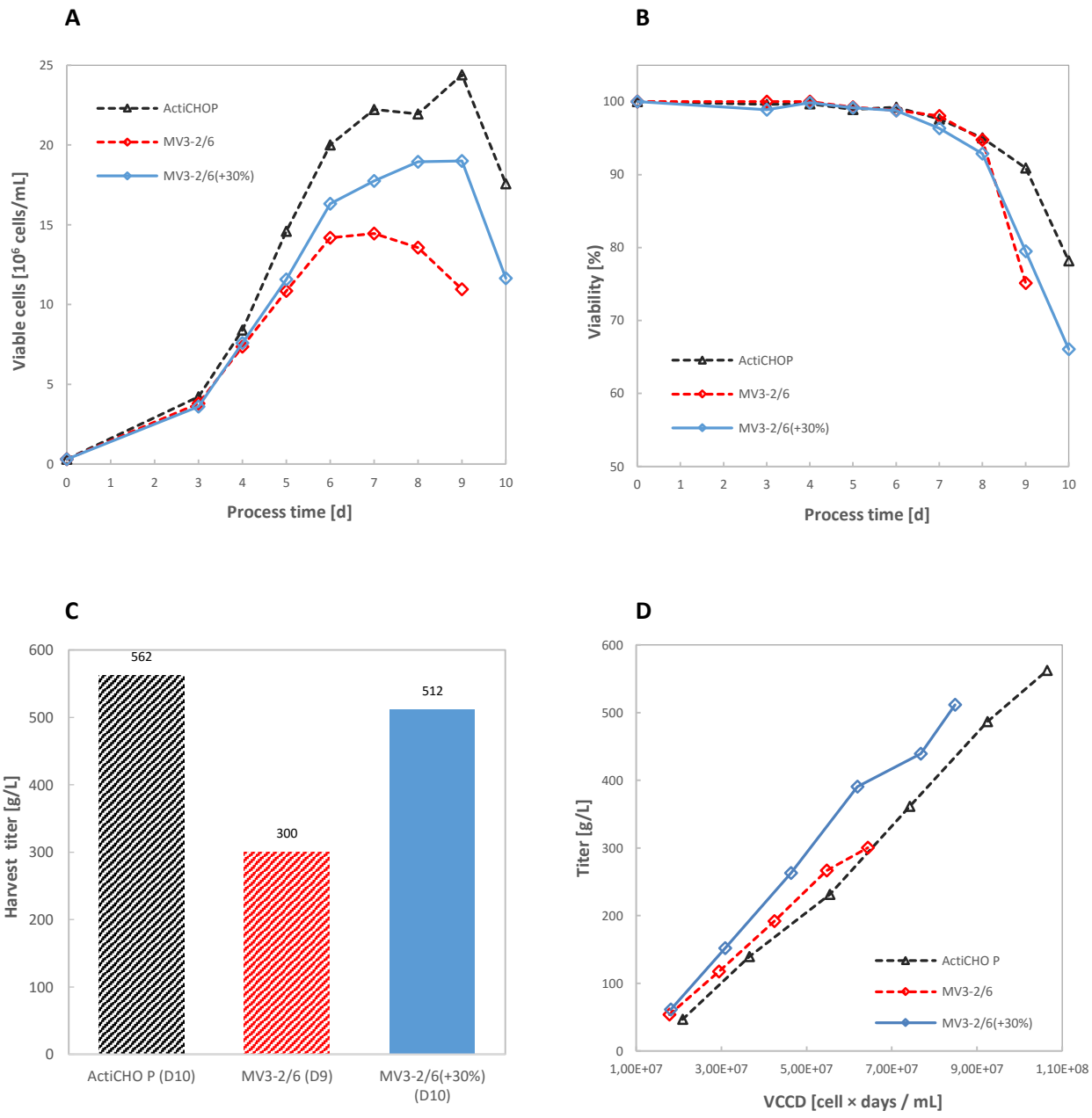

Figure 13. Effects of increased compound concentrations of MV3-2 on fed-batch culture performance

Cell growth (A), viability (B), gp140 product titer (C), and productivity (D) as titer vs. VCCD during fed-batch cultivation. Cultivation was performed in 125mL shaking flasks, and feeding was performed using a commercially available feeding system (ActiCHO Feed A, ActiCHO Feed B; GE HC). Increased compound concentrations of MV3-2/6 resulted in improved growth behavior, increased product formation as well as an increased overall productivity.

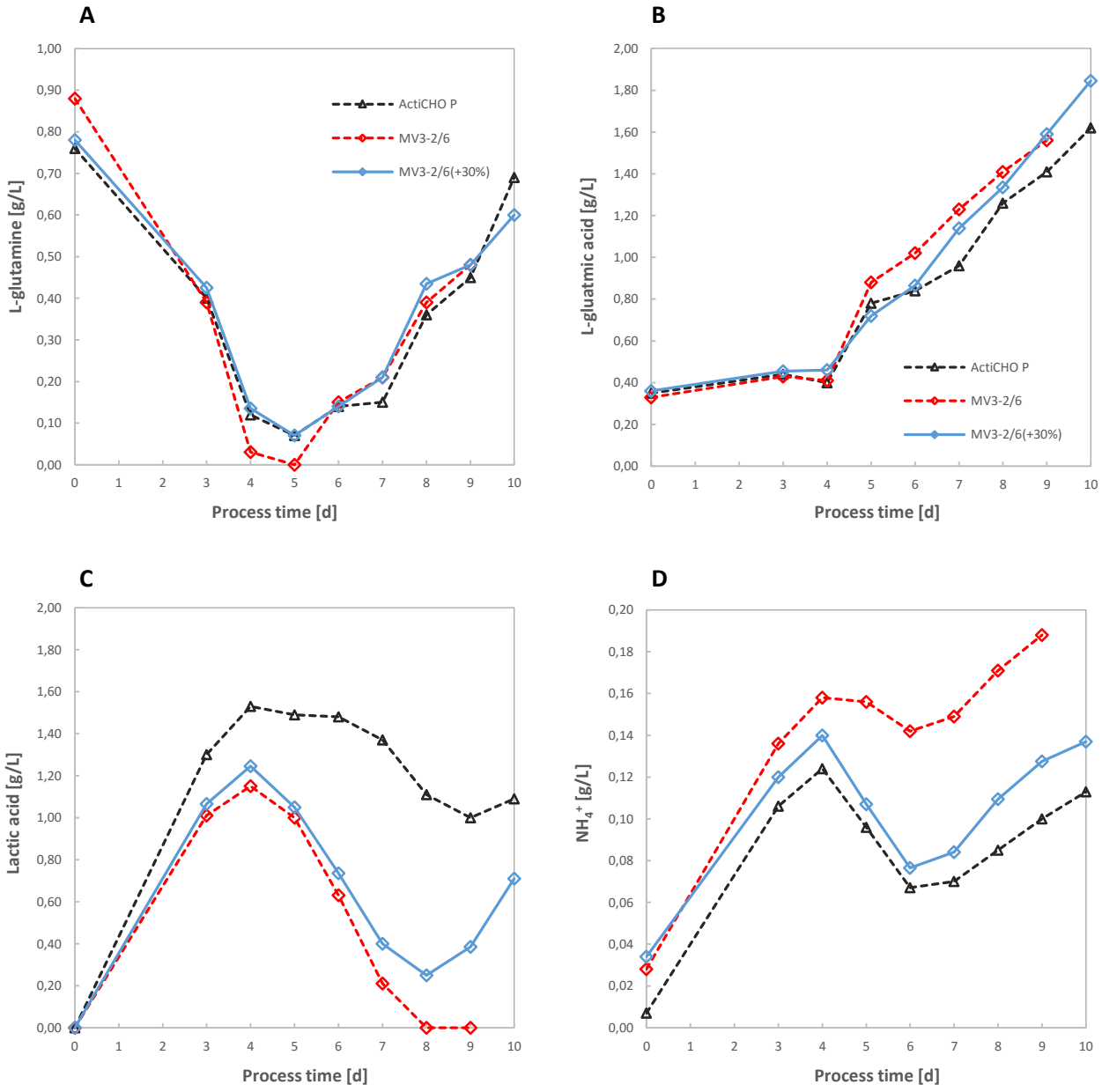

Figure 14. Metabolite and by-product profiles of fed-batch experiment: Testing of an enhanced medium formulation of MV3-2

Concentration graphs show incline/decline of the following key metabolites as they were routinely monitored from culture supernatants. (A) L-glutamine, (B) L-glutamic acid, (C) lactic acid (lactate), (D) ammonium (NH_4^+).

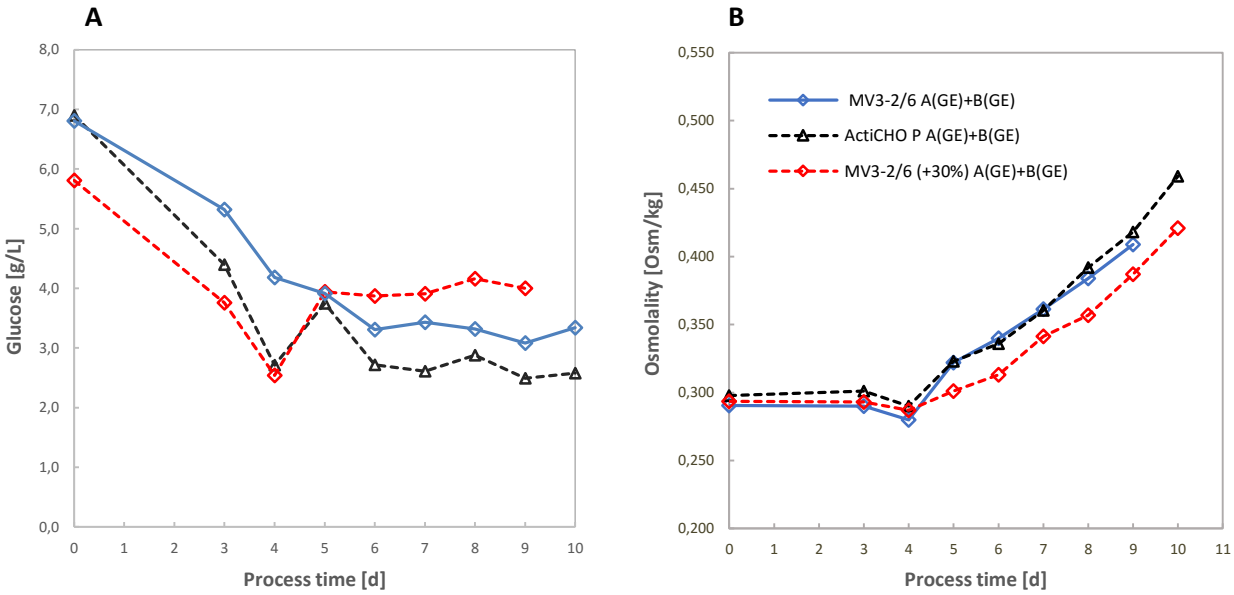

Figure 15. Glucose and Osmolality profile of fed-batch experiment: Testing of an enhanced medium formulation of MV3-2

(A) Glucose concentration profile and (B) osmolality profile during fed-batch cultivation. Glucose levels decrease until day 4 when feeding started. Osmolality values were in the same range for all media until day 4. Feeding resulted in continuously increasing osmolality with final osmolality values as follows: ActiCHO P (0.459 Osm/kg, MV3-2/6$_{(+30\%)}$ (0.421 Osm/kg), MV3-2/6 (0.418 Osm/kg).

5.5 Comparison of in-house developed feed media formulations and commercially available feed media formulations

Feeding strategies and feed media formulations are major obstacles when fed-batch processes are set up. Commercially available feed media are often considered a black box since customers most often lack information regarding the formulation. Thus, finding a suitable feeding strategy is mostly a 'trial-and-error' process, which can be laborious and time consuming. Additionally, mostly unknown media formulations results in less flexible processes which can be critical for scientific research, because the feeding input is simply unknown.

In this experiment two in-house developed feed media formulations were compared to a commercially available feed media system (ActiCHO powder base Feed A; Feed B by GE). The aim of this experiment was to gain information about, whether any of the two in-house developed feed media formulations or both would need further improvement. Additionally, overall culture performance of the applied feed media (commercially, in-house) in combination with two distinct basal media (MV3-2/6 and ActiCHO P) was an interesting aspect of this experiment.

5.5.1 Experimental setup

Table 19. Experimental setup: Comparison of in-house developed feed media formulations and commercially available feed media formulations

Process type	Fed-batch in 125 mL shaking flasks (singlets)
Initial cell concentration	300,000 cells/mL
Media	MV3-2/6 ActiCHO P (All media were supplemented with 8mM L-glutamine and 15 mg/mL phenol red.)
Medium adaptation	3 consecutive passages
Feed media	ActiCHO Feed A Powder Base CD(GE) ActiCHO Feed B Powder Base CD (GE) In-house Feed A (Polymun Scientific, Austria) In-house Feed B (Polymun Scientific, Austria)

Feeding strategy	Feed Start: day 4 of fed-batch Feed A: adjusted to 6.5 g/L glucose in the shaking flask Feed B: 0.28 $\%_{(v/v)}$ of the actual culture volume
Sampling	Cell number Viability Product concentration (gp140) Off-line monitoring of: L-glutamine L-glutamic acid Lactic acid NH_4^+

Table 20. Basal and feed media combinations of MV3-2/6 and ActiCHO P

Basal medium	Feed media combinations
MV3-2/6	Feed A (GE) + Feed B (GE) Feed A (GE) + Feed B (h.m) Feed A (h.m) + Feed B (GE) Feed A (h.m) + Feed B (h.m)
ActiCHO P	Feed A (GE) + Feed B (GE) Feed A (GE) + Feed B (h.m) Feed A (h.m) + Feed B (GE) Feed A (h.m) + Feed B (h.m)

5.5.2 Experimental results

A feed media cross-comparison of the ActiCHO Feed A / Feed B feeding system and an in-house developed feeding system was performed to identify aspects for further improvement of the in-house developed feed media system. In general, the evaluation of fed-batch results revealed that in-house developed Feed B showed a similar performance compared to ActiCHO Feed B when applied with either basal medium (MV3-2/6 or ActiCHO P). However, in-house developed Feed A showed an overall weaker performance compared to its competitor medium ActiCHO P Feed A.

The following description can be considered with reference to Figure 16A. In general, monitoring of process time showed that cultivation, using ActiCHO P with the commercially feeding system (Feed A(GE)/Feed B(GE)) allowed a total cultivation time of 10 days. Also when MV3-2/6 was used in combination with Feed A(GE) and Feed B(GE), a process time of 9 days was possible. Replacing Feed B(GE) by in-house Feed B(h.m), but still in combination with Feed A(GE), resulted in a similar process time of 10 days for ActiCHO P and 9 days for MV3-2/6.

Viable cell concentrations peaked on day 9, for ActiCHO P + Feed A(GE)/Feed B(GE) at 2.44×10^7 cells/mL and for ActiCHO P + Feed A(GE)/Feed B(h.m) at 2.50×10^7 cells/mL. Combinations of ActiCHO P + Feed A(h.m)/Feed B(GE) and ActiCHO P + Feed A(h.m)/Feed B(h.m) resulted both in a shortened process time of 9 days and viable peak cell concentrations (VPCC) of 2.25×10^7 cells/mL and 2.14×10^7 cells/mL, respectively. MV3-2/6 + Feed A(GE)/Feed B(GE) and MV3-2/6 + Feed A (GE)/Feed B(h.m) resulted in an overall process time of 9 days with VPCC of 1.45×10^7 cells/mL and 1.59×10^7 cells/mL, at day 7. Interestingly, feed media combinations with Feed A (GE)/Feed B(h.m) showed higher VPCCs than combinations with both commercially available feeds. In contrast to feeding combinations with Feed A(GE), combinations of MV3-2/6 + Feed A (h.m)/Feed B (GE) and Feed A (h.m)/Feed B (h.m) resulted in VPCCs of 1.36×10^7 cells/mL and 1.25×10^7 cells/mL, already on day 6.

Determination of gp140 concentrations of culture supernatants by ELISA revealed a similar pattern as was described for VPCCs. On average, harvest titers were 47 % lower for MV3-2/6 compared to ActiCHO P when MV3-2/6 was in combination with Feed A(GE). Titer reduction was even higher when MV3-2/6 was used in combination with Feed A(h.m) (54 % lower harvest titer compared to ActiCHO P). Combinations of Feed A(GE)/Feed B(GE) with either MV3-2/6 or ActiCHO P resulted in titers of 300 mg/L and 562 mg/L respectively. In contrast, combinations of MV3-2/6 (312 mg/L) and ActiCHO P (590 mg/L) with Feed A(GE)/Feed B(h.m) resulted in slightly higher product titers compared to the combination of Feed A (GE)/Feed B (GE). However, a major difference in titer could be observed as Feed A(GE) was replaced by Feed A(h.m). Combinations of MV3-2/6 with Feed A(h.m) + Feed B(GE) and Feed A(h.m) + Feed B(h.m) resulted in a titer of 212 mg/L (-29 % comp. to Feed A (GE)/Feed B (GE)) and 208 mg/L (-30 % comp. to Feed A (GE)/Feed B (GE)) respectively. ActiCHO P in combination with Feed A(h.m) + Feed B(GE) and Feed A(h.m) + Feed B(h.m) resulted in titers of 465 mg/L (-17% comp. to Feed A(GE)/Feed B(GE)) and 454 mg/L (-19 % comp. to Feed A (GE)/Feed B (GE)) (Figure 16C, Table 21).

Figure 16D is a plot of gp140 titers and their according viable cumulative cell days (VCCD), depicting that specific productivity was similar between MV3-2/6 and ActiCHO P feed media combinations as all slopes, show a similar incline.

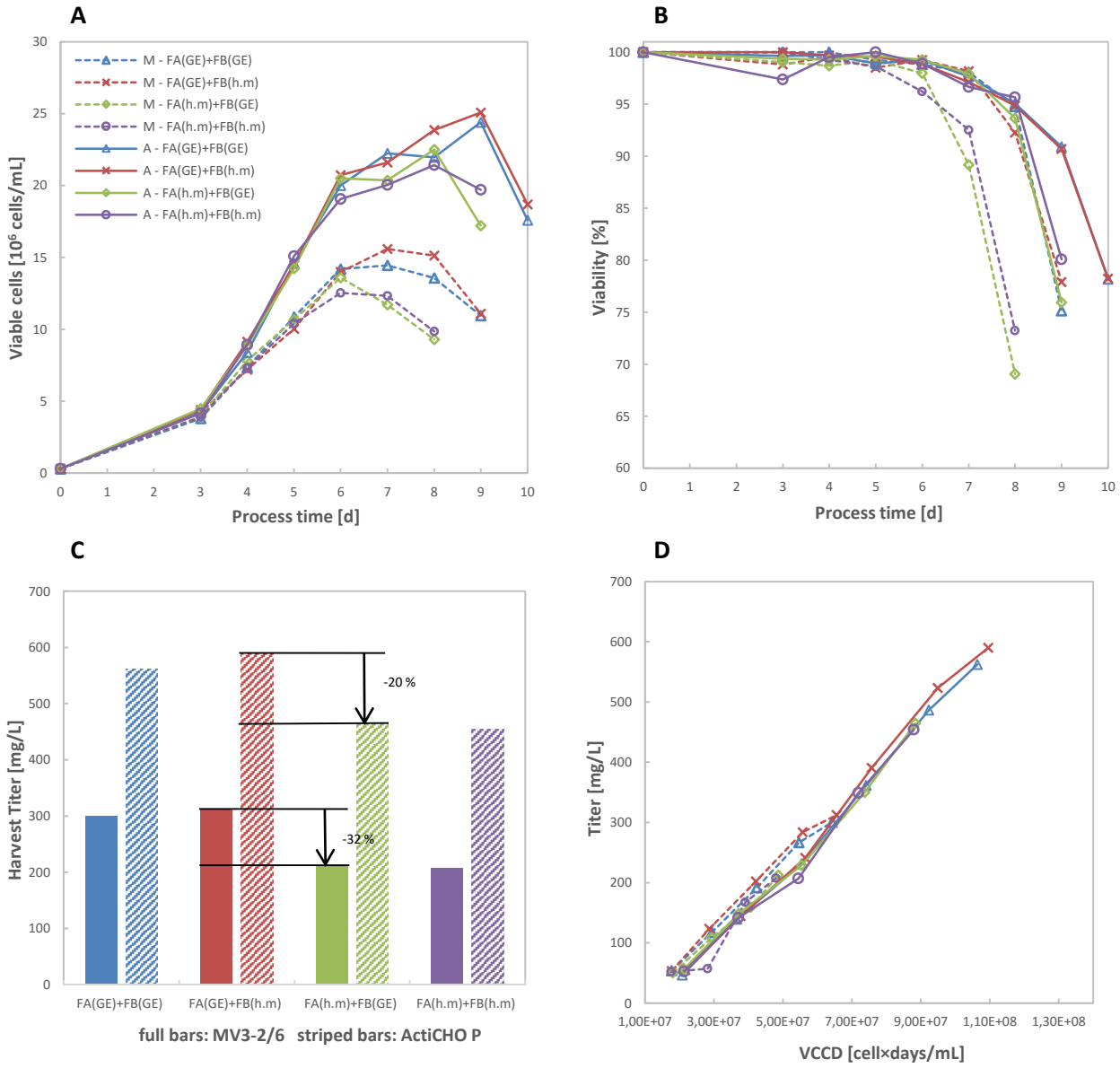

Figure 16. Feed media cross-comparison of the ActiCHO Feed A / Feed B System (GE) versus an in-house developed feed media system (Feed A (h.m) / Feed B (h.m))

(A) Viable cell concentrations, (B) viability graph, (C) harvest titers and (D) specific productivity of fed-batch cultivation of various feed-media combinations applied with MV3-2/6 and ActiCHO P as basal media. Besides the overall influence of the basal media (MV3-2/6 and ActiCHO P) feed media combinations with Feed A (GE) showed an improved overall performance compared to feed media combinations with Feed A (h.m). In contrast, combinations with Feed B(h.m) showed a similar performance compared to combinations were only Feed B(GE) was used.

Table 21. Process relevant data from fed-batch experiment: Comparison of in-house developed feed media formulations and commercially available feed media formulations

Table Legend: BOLD numbers: ActiCHO P; REGULAR numbers: MV3-2/6

	FA(GE)+FB(GE)	FA(GE)+FB(h.m)	FA(h.m)+FB(GE)	FA(h.m)+FB(h.m)
VPCC [10^6 viable cells/mL]	15.6 **24.4**	14.5 **25.1**	13.6 **22.5**	12.5 **21.4**
μ(exp. phase) [1/d]	0.57 **0.64**	0.55 **0.64**	0.56 **0.64**	0.54 **0.64**
Process time [d]	9 **10**	9 **10**	8 **9**	8 **9**
Harvest titer [mg/L]	300 **562**	312 **590**	212 **465**	208 **454**
VCCD [10^7cells×days/mL]	6.44 **10.6**	6.56 **11.0**	4.87 **8.85**	4.80 **8.81**

Monitoring of key metabolites and metabolic by-products.

L-glutamine, l-glutamic acid, lactic acid and ammonium were monitored over the period of fed-batch cultivation. L-glutamine was consumed until day 5 for all feed media combinations and was even depleted at day 5 for combinations with MV3-2/6. From day 5 onward, l-glutamine concentrations increased again, mainly due to feeding of cultures. Interestingly, l-glutamine concentrations remained relatively low for cultures which were fed with Feed A(h.m), in either combination with MV3-2/6 or ActiCHO P. On the other hand, combinations that had Feed A(GE) added showed a sharp increase of l-glutamine concentrations starting at day 6 until culture termination at day 9 (MV3-2/6), day 10 (ActiCHO P) (Figure 17A). Concentrations of l-glutamic acid remained constant until day 4 (start of feeding) and increased the following days. As for l-glutamine, combinations which used Feed A(h.m) had a less steep incline of l-glutamic acid concentrations than combinations with Feed A(GE) (Figure 17B).

Monitoring of lactic acid revealed two clearly distinct groups of graphs mirroring that two distinct basal media (MV3-2/6, ActiCHO P) were used for this experiment. Lactic acid concentrations peaked on day 4 for all feed media combinations and were followed by a subsequent consumption of lactic acid culture termination (Figure 17C). Feed media combinations with ActiCHO P showed peak lactic acid concentrations in the range of 1.6 g/L, whereas combinations with MV3-2/6 had lower concentrations in the range of 1.2 g/L. Although lactic acid peak concentrations were reached at the same day the following consumption behavior was distinct. As described earlier, feed media combinations of MV3-2/6 + Feed A(GE)/Feed B(GE) had lactic acid entirely consumed at day 8. Replacing Feed B(GE) with Feed B(h.m) changed the results only slightly. ActiCHO P showed a similar consumption profile for all combinations with roughly 1.2 g/L lactic acid at day 10. As for lactic acid, two distinct groups of graphs could be observed for monitoring of ammonium. Combinations of feed media with either MV3-2/6 or ActiCHO P showed almost identical concentration profiles according to the feed medium used (Figure 17D).

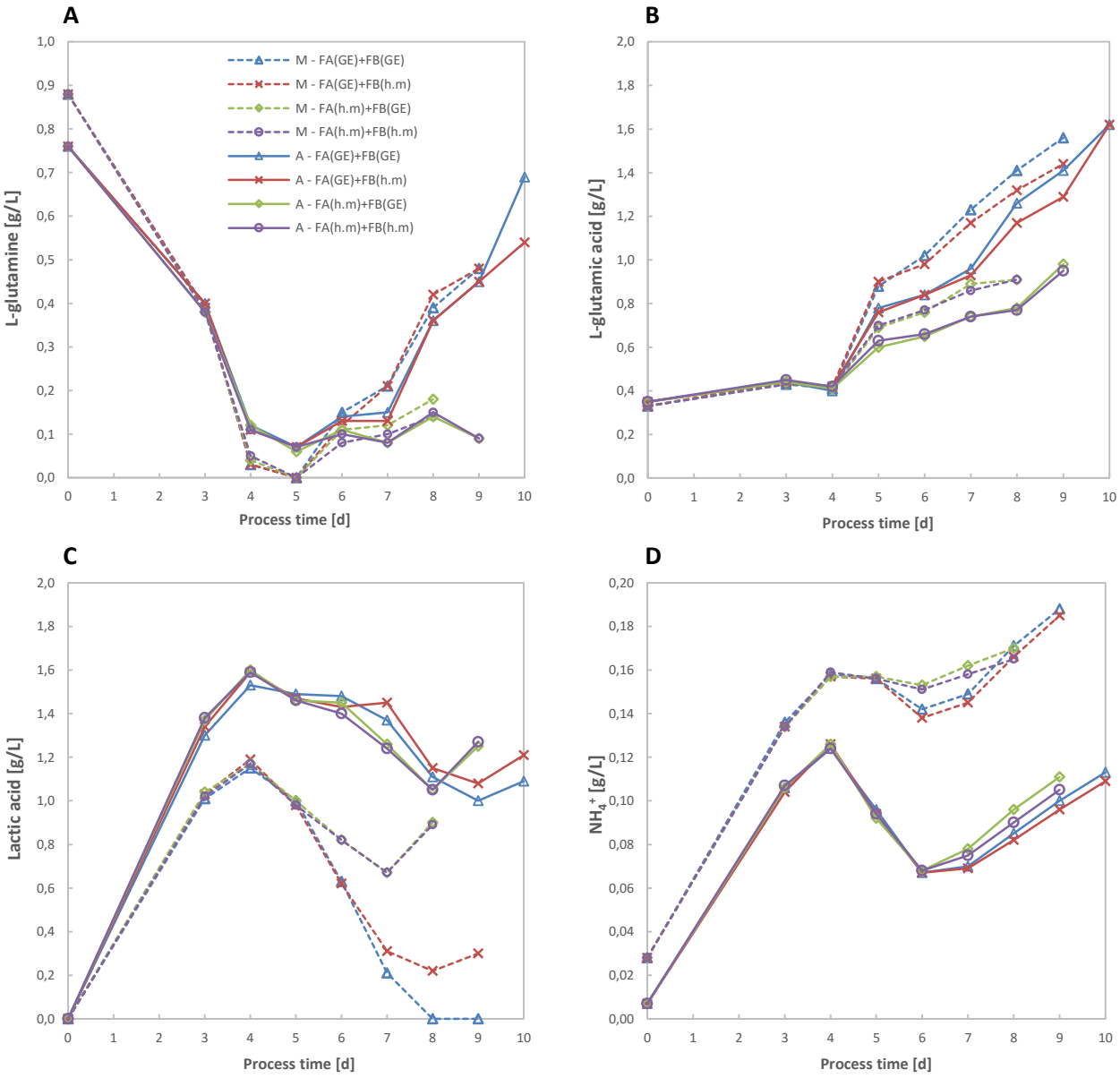

Figure 17. Metabolite and by-product profiles of fed-batch experiment: Comparison of in-house developed feed media formulations and commercially available feed media formulations

Concentration graphs for incline/decline of the following key metabolites as they were routinely monitored from culture supernatants during fed-batch cultivation. (A) L-glutamine, (B) L-glutamic acid, (C) lactic acid (lactate), (D) ammonium (NH_4^+).

Monitoring of glucose levels showed a continuous decline of glucose levels until day 4 (in the range of 2.70 g/L glucose). At day 5 glucose levels were higher for all combinations, due to start of feeding at day 4. However, combinations of feed media with MV3-2/6 showed relatively constant glucose levels from day 5 until culture termination. In contrast, glucose levels for feed combinations with ActiCHO P were again decreased at day 6 and further remained constant until culture termination.

Profiling of supernatant osmolality showed a similar trend as it has already been described earlier. Osmolality values remained constant until day 4 (feed start) and constantly increased until culture termination (final osmolality values: 0.459 Osm/kg for ActiCHO P + Feed A(GE)/Feed B(GE), 0.445 Osm/kg for ActiCHO P + Feed A(GE)/Feed B(h.m) and values of 0.401-0.417 Osm/kg for all other combinations.

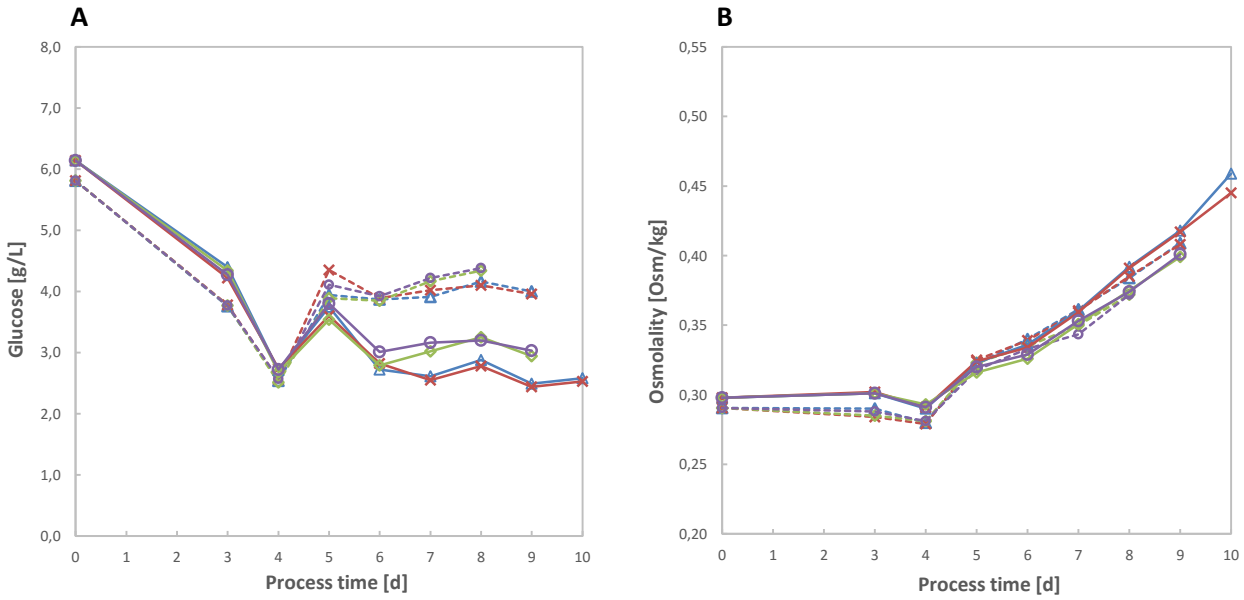

Figure 18. Glucose concentration and osmolality profile of fed-batch experiment: Comparison of in-house developed feed media formulations and commercially available feed media formulations

(A) Glucose concentration profile and (B) osmolality profile during fed-batch cultivation. Glucose levels decrease until day 4 when feeding started. Osmolality values were in the same range for all media until day 4. Feeding resulted in continuously increasing osmolality was similar for all tested combinations of MV3-2 and ActiCHO P with feed media.

5.6 Testing of an altered feeding strategy for Feed B

The effects of a changed feeding regime for Feed B was tested against the standard feeding regime as explained in section 0. According to 0, the standard feeding regime for feed solution B was a 0.28 $\%_{(v/v)}$ feed of the actual culture volume, as it was applied for fed-batch experiments presented in this thesis. As feed solution A was added in order to add glucose up to a level of 6.5 g/L, the feeding-volume of feed solution B was then adjusted accordingly.

The experimental set up was again the same as described in section 5.4.1, except that a different feeding strategy for feed B was applied. The following table shows the original and altered feeding strategies in combination with the respective media.

Table 22. Overview of feeding regimes of Feed B solutions and their alterations, as applied in the experiment

	ActiCHO Feed A **Feed A (GE)**	**ActiCHO Feed B** **Feed B (GE)**
MV3-2/6 + ActiCHO P **Feed B - Standard**	Feeding volume adjusted to add up to 6.5 g/L glucose	0.28% (v/v) of the actual culture volume
MV3-2/6 + ActiCHO P **Feed B - Altered**	Feeding volume adjusted to add up to 6.5 g/L glucose	Feeding volume adjusted to Feed A (Ratio: 1:10)

5.6.1 Experimental results

In general, the alternative feeding strategy for feed solution B resulted in a prolonged cultivation time (+1 day; viability criterion: 80%) but did not show to have any effects on maximum viable peak cell numbers (VPCC) or gp140 titers.

In more detail, viable peak cell concentrations (VPCC) of the combination ActiCHO P + FA(GE)/FB(GE)(linked) reached 2.22×10^7 cells/mL, and for ActiCHO P + FA(GE)/(GE) 2.16×10^7 cells/mL were obtained (Figure 16A). Combinations of MV3-2/6 + FA(GE)/FB(GE)(linked) showed a VPCC of 1.53×10^7 cells/mL and for FA(GE)/FB(GE) 1.45×10^7 cells/mL. The gap in VPCCs between MV3-2/6 and ActiCHO P can be mainly attributed to effects of the respective medium itself, than effects caused by a different feeding strategy for feed solution B. As for VPCCs, also harvest titers were generally higher when ActiCHO P was used in combination with either feeding strategy. However, an inter-media comparison of the two different feeding strategies for MV3-2/6 or ActiCHO P gave no homogenous results for effects caused by the alternative feeding strategy.

Combinations with FA(GE)/FB(GE)(linked) showed a 15 % increase in harvest titer for MV3-2/6 and a 7 % decline for ActiCHO P (Figure 16C). Regarding overall productivity, no differences between the two different feeding regimes for feed solution B could be observed as the slopes of the graphs inclined similarly. Metabolite monitoring and the corresponding concentration profiles did not reveal any differences compared to what was already known from previous experiments.

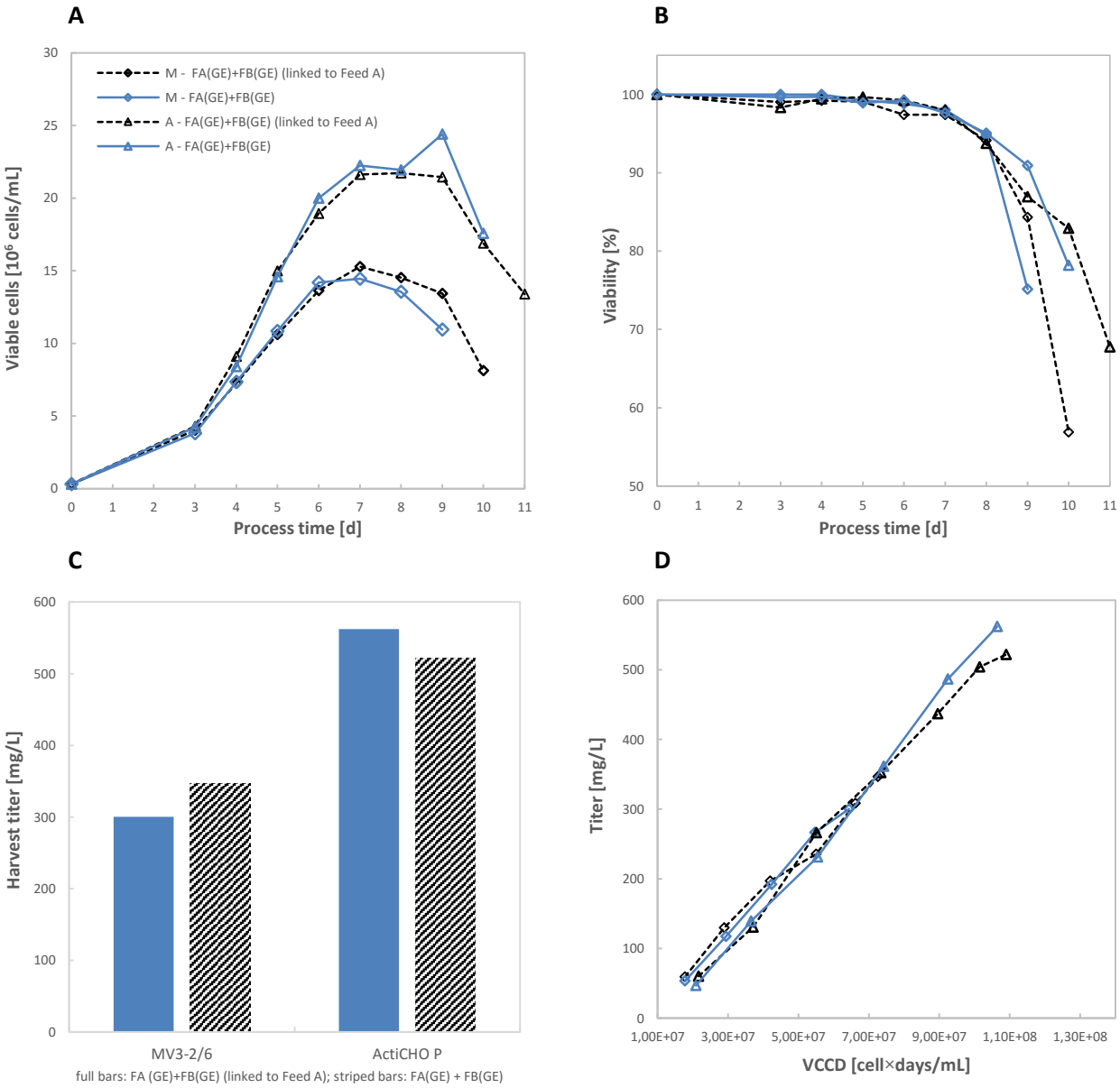

Figure 19. Comparison of different feeding regimes for Feed B

(A) Viable cell concentrations, (B) viability graph (C) harvest titers (D) specific productivity of fed-batch cultivation of the tested feeding setups. The feeding strategy for feed-solution B was altered in order to meet the ratio of feed-solution A to the actual culture volume. An altered feeding strategy for feed-solution B resulted in a prolonged cultivation time (+1 day) (A; B) but no improvement in product formation (C) or specific productivity (D).

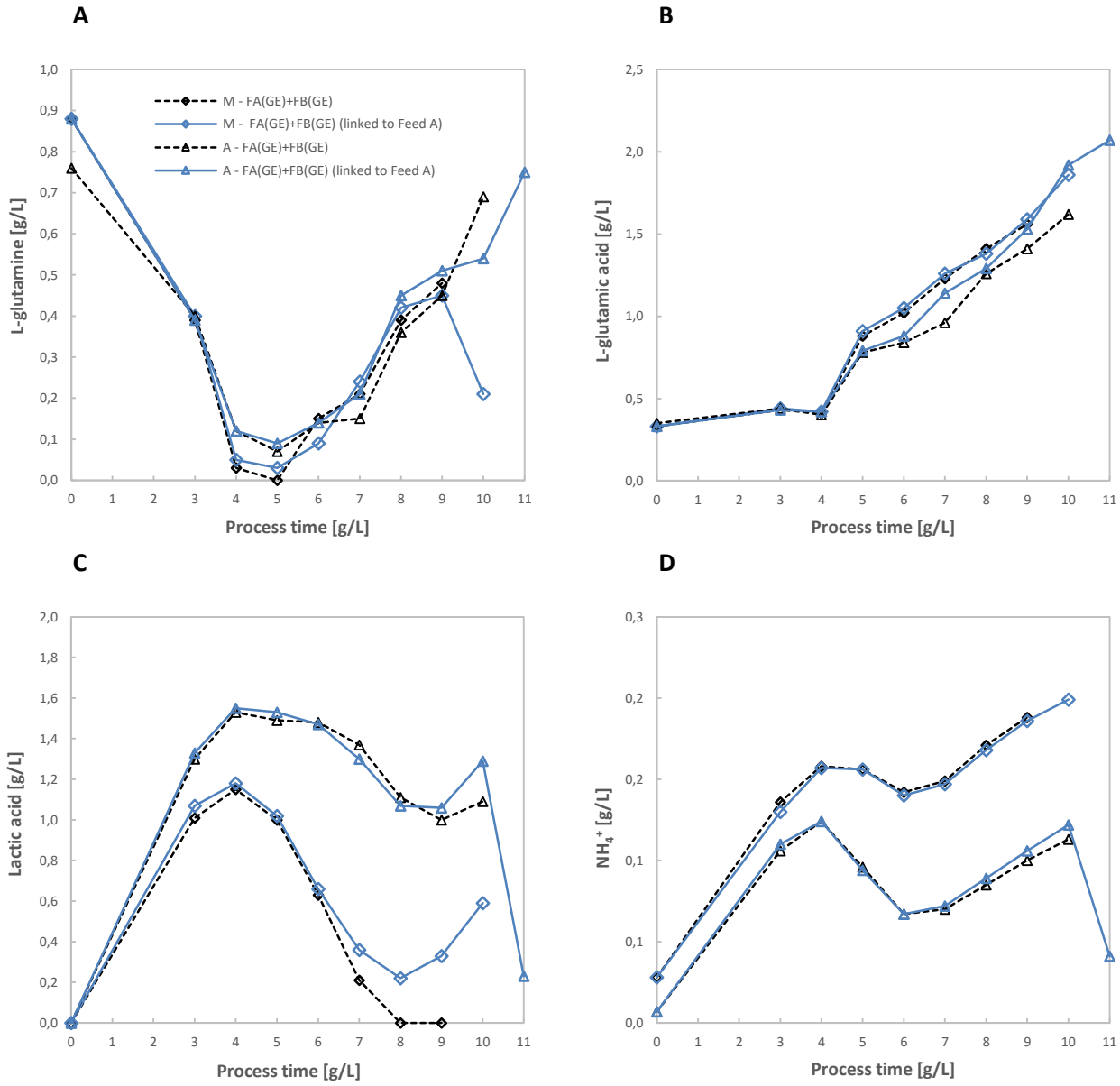

Figure 20. Metabolite and by-product profiles of fed-batch experiment: Testing of an altered feeding strategy for Feed B

Concentration graphs for incline/decline of the following key metabolites as they were routinely monitored from culture supernatants during fed-batch cultivation. (A) L-glutamine, (B) L-glutamic acid, (C) lactic acid (lactate), (D) ammonium (NH_4^+).

5.7 Effects of medium supplementation with D(+)-trehalose, D(+)-mannose, taurine, glutathione and a chemically defined lipid mix on Fed-batch culture performance

Based on results of previous batch experiments, culture supplementation with various supplements was performed in order to evaluate effects on culture performance. The following supplements were used for this experiment: the sugars D(+)-trehalose and D(+)-mannose, the organic acid taurine, the well-known redox mediator glutathione and a commercially available and chemically defined lipid mix (Gibco®, Life Technologies) since the original MV3-2 formulation does not contain lipids.

5.7.1 Experimental setup

Table 23. Experimental setup: Effects of medium supplementation with various supplements

Process type	Fed-batch in 125 mL shaking flasks (duplicates) Working volume: 46.5 mL
Initial cell concentration	300,000 cells/mL
Media	MV3-2/6$_{(+30\%)}$ ActiCHO P (All media were supplemented with 8mM L-glutamine and 15 mg/mL phenol red.)
Medium adaptation	3 consecutive passages
Feed media	ActiCHO Feed A Powder Base CD(GE) ActiCHO Feed B Powder Base CD (GE) In-house Feed A (Polymun Scientific, Austria) In-house Feed B (Polymun Scientific, Austria)
Feeding strategy	Feed Start: day 4 of fed-batch Feed A: adjusted to 6.5 g/L glucose in the shaking flask Feed B: 0.28 %$_{(v/v)}$ of the actual culture volume On day 7, the feeding strategy was changed to a 2.8% volumetric feed for Feed A due to difficulties with the BioProfile®100Plus instrument

Sampling	Cell number	
	Viability	
	Product concentration (gp140)	
	Off-line monitoring of:	L-glutamine
		L-glutamic acid
		Lactic acid
		NH_4^+
Supplements	0.5 mg/mL D(+)-trehalose	$C_{(stocksol.)}$= 30 mg/mL
	1.5 mg/mL D(+)-Mannose	$C_{(stocksol.)}$= 50 mg/mL
	0.1 mg/mL Taurine	$C_{(stocksol.)}$= 20 mg/mL
	100 µM Glutathione	$C_{(stocksol.)}$= 10 mg/mL
	CD-Lipid Mix - 1:1,000 diluted (Gibco®, Life Technologies)	

Prior to the experiment media solutions of MV3-2/6$_{(+30\%)}$ and ActiCHO P were prepared as described under 4.2.2. Cells were adapted to the respective medium over 3 consecutive passages according to 4.2.4. After inoculation, medium supplement stock solutions (Table 23) were added to the respective flasks under sterile conditions.

5.7.2 Experimental results

Supplementation of MV3-2/6$_{(+30\%)}$ medium with the previously mentioned supplements resulted in a slightly increased titer but no major difference in viable peak cell concentrations (VPCC).

The un-supplemented control of MV3-2/6$_{(+30\%)}$ (with Feed A/B (h.m)) showed a VPCC of 1.07×10^7 cells/mL, whereas cells cultivated in ActiCHO P (with Feed A/B (GE)) resulted in 1.65×10^7 cells/mL (Figure 21A). It was already shown that this difference can be mainly described by influences of the basal medium itself as well as the different feed media used. This behavior could also be observed for harvest titers. Cultivation in un-supplemented MV3-2/6$_{(+30\%)}$ medium showed a harvest titer of 204 mg/L in comparison with ActiCHO P, which resulted in 300 mg/L.

Supplementation of MV3-2/6$_{(+30\%)}$ with trehalose (0.5 mg/mL) resulted in 1.03×10^7 cells/mL, mannose (1.5 mg/mL) resulted in 1.07×10^7 cells/mL and taurine (0.1 mg/mL) resulted in 1.07×10^7 cells/mL (Figure 21A, Table 24). Supplementation of 100 µM glutathione resulted in 9.79×10^6 cells/mL and the addition of a chemically defined lipid mix resulted in 1.06×10^7 cells/mL (Figure 21A, Table 24). As figure 16B shows, supplementation of glutathione resulted in a 1 day shortened cultivation time. In terms of cell growth, comparison of data for VPCCs showed that there could be no major differences identified between the supplemented medium setups and the MV3-2/6$_{(+30\%)}$ control (Figure 21A, Table 24).

Evaluation of ELISA data gave detailed in-sight to product formation (Figure 21C, Table 24). Cultivation in MV3-2/6$_{(+30\%)}$ medium when supplemented with trehalose (0.5 mg/mL) resulted in a harvest titer of 220 mg/L which was 8 % higher compared to the control (Figure 21C, Table 24). Supplementation of mannose (1.5 mg/mL) and taurine (0.1 mg/mL) resulted in similar results of 224 mg/L and 221 mg/L, which accounts for a titer increase of 10 % for mannose and 9 % for taurine compared to the un-supplemented control (Figure 21C, Table 24). In contrast, supplementation of 100 µM glutathione showed a 21 % decrease in harvest titer of the respective medium setup, to 160 mg/L in comparison to the control (Figure 21C, Table 24). In general, as it is shown in figure 16D, no increase in specific productivity could be observed due to supplementation of the mentioned medium supplements.

Table 24. Process relevant data from fed-batch experiment: Effects of medium supplementation with various supplements

	0.5 mg/mL Trehalose MV3-2/6$_{(+30\%)}$	1.5 mg/mL Mannose MV3-2/6$_{(+30\%)}$	0.1 mg/mL Taurine MV3-2/6$_{(+30\%)}$	100 µM Glutathione MV3-2/6$_{(+30\%)}$	Lipid Mix (CD) MV3-2/6$_{(+30\%)}$	Control MV3-2/6$_{(+30\%)}$	ActiCHO P
VPCC [10^6 viable cells/mL]	10.31 (± 0.35)	10.65 (± 0.16)	10.72 (± 0.08)	9.79 (± 0.21)	10.57 (± 0.14)	10.69 (± 0.09)	16.47
µ(exp. phase) [1/d]	0.51	0.52	0.53	0.48	0.56	0.57	0.67
Process time [d]	9	9	9	8	9	9	9
Harvest titer [mg/L]	220 (± 3)	224 (± 7)	221 (± 4)	160 (± 4)	205 (± 5)	204 (± 4)	300
VCCD [10^7cell×days/mL]	2.55 (± 0,07)	2.29 (± 0.01)	2.74 (± 0.03)	2.16 (± 0.02)	2.70 (± 0.02)	2.71 (± 0.05)	3.93

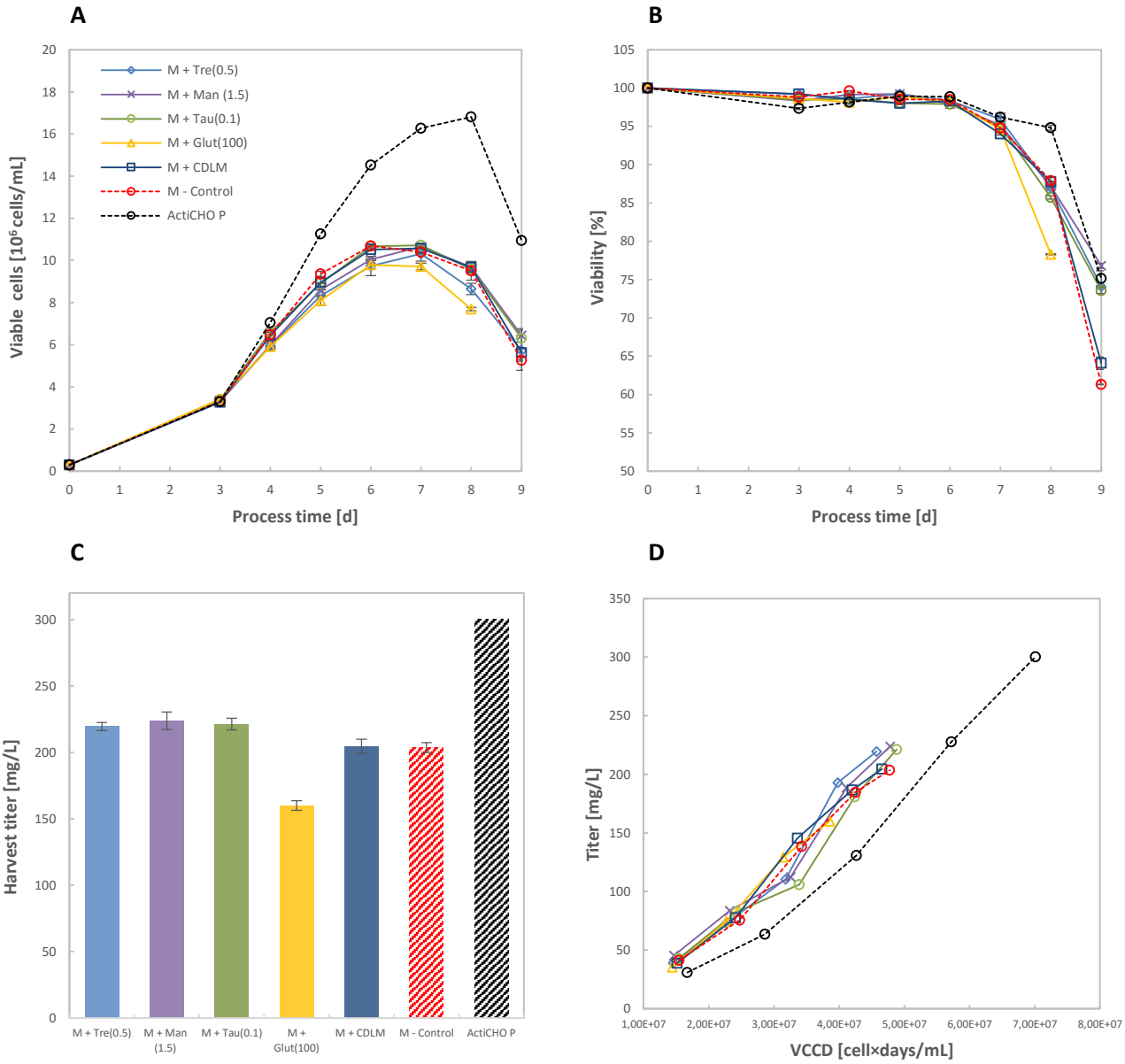

Figure 21. Comparison of trehalose, mannose, taurine, glutathion and a chemically defined (CD) lipid mix as supplements for MV3-2/6$_{(+30\%)}$ medium formulation.

MV3-2/6$_{(+30\%)}$ medium was supplemented with the following additives: 0.5 mg/mL D(+)-trehalose (Tre0.5), 1.5 mg/mL D(+)-mannose(Man1.5), 0.1 mg/mL traurine(Tau0.1), 100 mM glutathione(Glut100), lipid mix (CD) (diluted 1:1000)(CDLM). Fed-batch cultivation was performed in duplicates. Feeding of cultures started on day 4 using Feed A/B (in-house) for cultures in MV3-2/6$_{(+30\%)}$, and Feed A/B (GE) for ActiCHO P reference cultures. Growth behavior (A) and the corresponding culture viabilities (B) of cultures in supplemented MV3-2/6$_{(+30\%)}$ are shown above. Figure C shows gp140 product titers on the day of harvest and figure D illustrates the overall productivity of supplemented cultures in MV3-2/6$_{(+30\%)}$, and MV3-2/6 and ActiCHO P as references. Data illustrated in the figures above show the averages of duplicates, except data for ActiCHO P which represents only one flask due to cultivation difficulties on days 8 and 9.

5.8 Effects of valproic acid (VPA) on fed-batch culture performance

Valproic acid (VPA), a branched-chain carboxylic acid, is widely known as small molecular titer enhancer in mammalian cell culture. Although, VPA and other small molecular titer enhancers have been extensively used for transient expression experiments, the goal of this experiment was to successfully implement VPA in a fed-batch setup in order to boost product formation. VPA acts as an inhibitor of histone deacetylase (HDAC) which causes cellular stress by de-regulation of histone packing. As a consequence, the optimal time point as well as concentration of VPA added to cell cultures, is crucial in order to boost product titers instead of inducing apoptotic effects.

5.8.1 Experimental setup

Table 25. Experimental setup: Effects of valproic acid (VPA) on fed-batch culture performance

Process type	Fed-batch in 125 mL shaking flasks (duplicates) Working volume: 45 mL
Initial cell concentration	300,000 cells/mL
Medium	MV3-2/6$_{(+30\%)}$ (Medium was supplemented with 8mM l-glutamine and 15 mg/mL phenol red.)
Medium adaptation	3 consecutive passages
Feed media	Feed A (in-house, Polymun Scientific) Feed B (in-house, Polymun Scientific)
Feeding strategy	Feed Start: day 4 of fed-batch Feed A: adjusted to 6.5 g/L glucose in the shaking flask Feed B: 0.28 %$_{(v/v)}$ of the actual culture volume
Sampling	Cell number Viability Product concentration (gp140) Off-line monitoring of: L-glutamine, L-glutamic acid, Lactic acid, NH_4^+
Supplement	Valproic Acid (VPA) ($c_{[stock\ solution]}$ = 505.4 mM) (Sigma-Aldrich)

Medium preparation and adaptation of cells to MV3-2/6$_{(+30\%)}$ cultivation conditions was done according to 4.2.4. VPA ($c_{[stock\ solution]}$ = 505.4 mM) was alternatively added at days 5, 6 or 7 of cultivation in order to reach a final concentration of 3.5 mM based on the actual culture volume of the respective day. As control, a duplicate of MV3-2/6(+30%) without VPA supplementation was performed in parallel to the supplemented setup.

5.8.2 Experimental results

Valproic acid (VPA) was added at 3 different time points of fed-batch cultivation and showed to increase harvest titers when VPA was supplemented during later stages of fed-batch cultivation.
Evaluation of viable peak cell concentrations (VPCC) resulted in 1.12×10^7 cells/mL for the control setup (MV3-2/$6_{(+30\%)}$ without VPA), whereas cultivation with VPA revealed a trend for increasing VPCCs, the later VPA was added (Figure 22A, Table 26). Addition of VPA on day 5 resulted in 9.66×10^6 cells/mL, day 6 showed a total of 1.04×10^7 cells/mL and addition on day 7 showed 1.09×10^7 cells/mL (Figure 22A, Table 26).

Viabilities were slightly decreased for VPA supplemented setups compared to the control, however VPCC for addition of VPA on day 5 peaked already at day 6 (Figure 22A/B). ELISA analysis of culture supernatants showed increasing product titers with later addition of VPA (Figure 22C, Table 26). The un-supplemented control setup reached 247 mg/mL gp140, whereas early VPA addition (day 5) resulted in a 5 % decreased harvest titer compared to the control. On the other hand, VPA addition at day 6 (264 mg/mL gp140) and day 7 (271 mg/mL gp140) showed increased titers by 7 % and 10 % respectively (Figure 22C, Table 26). Figure 22D, a graph of titer vs. viable cumulative cell days (VCCD) illustrates overall specific productivity. Interestingly, addition of VPA at day 5 resulted in a higher specific productivity early on, but still did not exceed the harvest titers of day 6 and day 7 (Figure 22D, Table 26). Data for key-metabolite analysis and metabolic by-product formation is not shown since no different phenomena were observed compared to what was presented earlier.

Table 26. Process relevant data from fed-batch experiment: Effects of valproic acid (VPA) on fed-batch culture performance

	Day 5	Day 6	Day 7	Control
VPCC [10^6cells/mL]	9.66 (±0.04)	10.60 (±0.19)	11.06 (±0.21)	11.16 (±0.09)
μ(exp. Phase) [1/d]	0.57	0.58	0.59	0.59
Process time [d]	9	9	9	9
Harvest titer [mg/L]	235 (±6.6)	264 (±2.3)	271(±0.2)	247 (±3.5)
VCCD [10^7cell×days/mL]	3.82 (±0.13)	4.08 (±0.40)	4.12 (±0.05)	4.15 (±0.05)

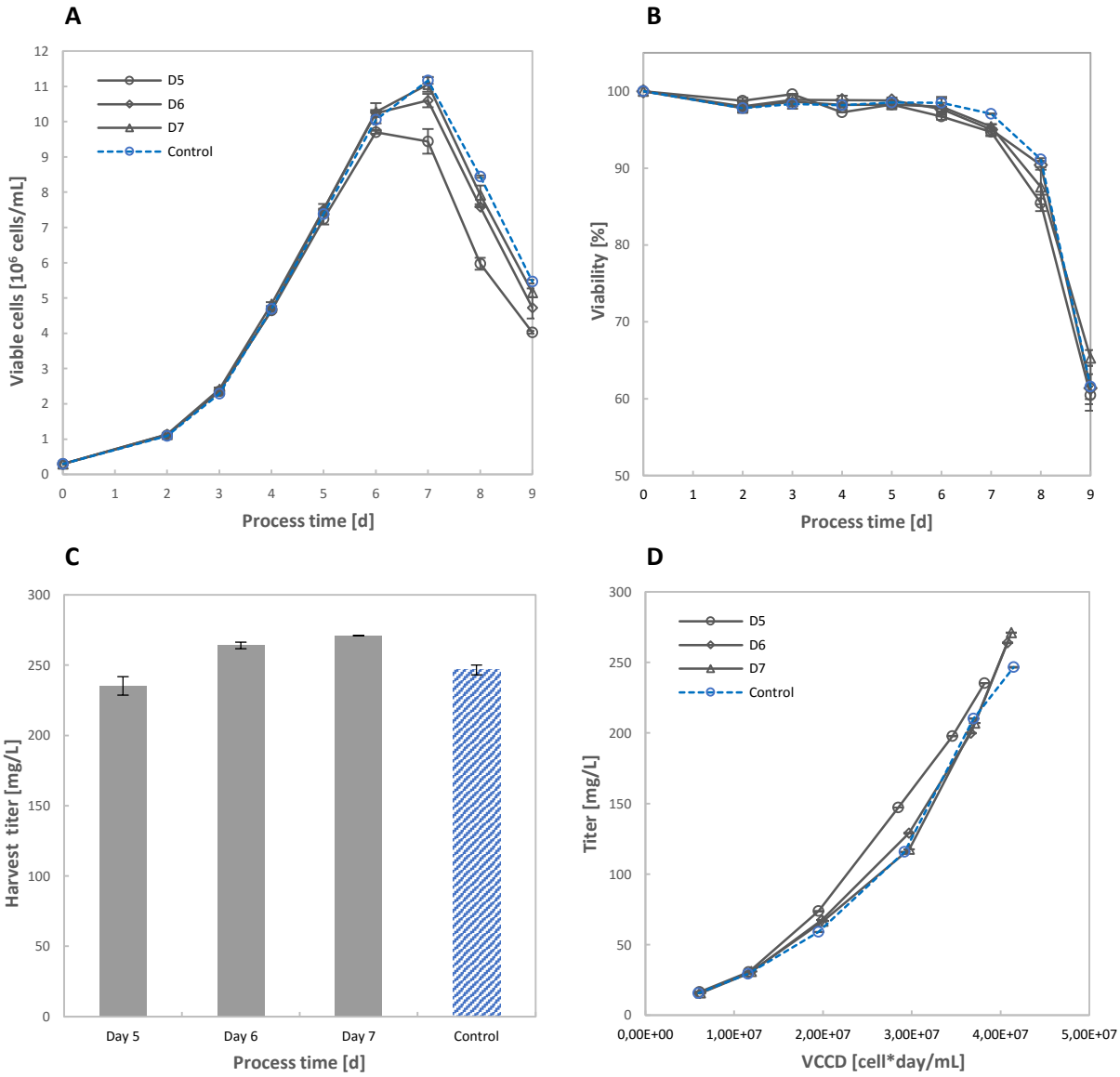

Figure 22. Effects of supplementation with 3.5 mM valproic acid (VPA) at different days of fed-batch cultivation

(A) Viable cell concentrations, (B) viability, (C) harvest titers, (D) graph of titer vs. VCCD. Later addition of VPA during fed-batch cultivation increased product yield when VPA was added on day 7. VPA was added on days 5, 6 and 7 of fed-batch cultivation to a final VPA concentration of 3.5 mM. For reference purposes a control flasks of MV3-2/6$_{(+30\%)}$ + Feed A/B (in-house) without VPA addition was setup.

5.9 Effects of glycerol on fed-batch culture performance

Glycerol was shown to slow down cell growth while increasing productivity (Liu and Chen, 2007)(Rezaei et al., 2007). Thus, the aim of this experiment was to evaluate the effects of adding glycerol, on cell growth and product formation during fed-batch cultivation. Glycerol was alternatively added on days 2, 3, 4 to investigate time dependent effects of glycerol supplementation.

5.9.1 Experimental setup

Table 27. Experimental setup: Effects of glycerol on fed-batch culture performance

Process type	Fed-batch in 125 mL shaking flasks (duplicates) Working volume: 45 mL
Initial cell concentration	300,000 cells/mL
Medium	MV3-2/6$_{(+30\%)}$ (Medium was supplemented with 8mM L-glutamine and 15 mg/mL phenol red.)
Medium adaptation	3 consecutive passages
Feed media	Feed A (in-house, Polymun Scientific) Feed B (in-house, Polymun Scientific)
Feeding strategy	Feed Start: day 4 of fed-batch Feed A: adjusted to 6.5 g/L glucose in the shaking flask Feed B: 0.28 %$_{(v/v)}$ of the actual culture volume
Sampling	Cell number Viability Product concentration (gp140) Off-line monitoring of: L-glutamine L-glutamic acid Lactic acid NH_4^+
Supplement	Glycerol (VWR, Germany) $c_{[final]}$ = 1 % $_{(v/v)}$ of actual culture volume

5.9.2 Experimental results

Adding glycerol to fed-batch cultures showed to reduce cell growth, but increased product formation when added at different time points in fed-batch cultivation. The non-glycerol supplemented control obtained a viable peak cell concentration (VPCC) of 1.12×10^7 cells/mL. In contrast, all setups where glycerol was added on day 2, day 3 or day 4 had lower VPCCs than an un-supplemented control (Figure 23A, Table 28). Figure 23B illustrates the corresponding viability profiles. It is obvious, that adding glycerol in early stages of fed-batch cultivation supports cells to maintain higher viability later on. This is illustrated in figure 22B as the control had a much lower viability than all glycerol-supplemented setups. ELISA analysis of culture supernatants showed that cultivation without glycerol resulted in a gp140 concentration of 247 mg/L. Glycerol addition at day 4 (260 mg/L gp140) increased the product titer by 5 %, addition at day 3 (255 mg/L) showed an increase of 3 % and adding glycerol at day 2 showed not to improve product formation (Figure 23C, Table 28). Interestingly, glycerol addition in general showed to increase specific productivity from day 5 to day 7 as the slopes of the graphs incline differently compared to the control (Figure 23D).

Data for key metabolite analysis is not shown as no different phenomena were observed compared to data that has already been presented previously in this thesis.

Table 28. Process relevant data from fed-batch experiment: Effects of glycerol on fed-batch culture performance

	Day 2	Day 3	Day 4	Control
VPCC [10^6cells/mL]	9.70 (±0.37)	10.48 (±0.13)	9.32 (±0.34)	11.16 (±0.09)
μ(exp. Phase) [1/d]	0.54	0.56	0.56	0.59
Process time [d]	9	9	9	9
Harvest titer [mg/L]	248 (±8.4)	255 (±3.8)	260 (±0.5)	247 (±3.5)
VCCD [10^7 cell×days/mL]	3.69 (±0.10)	3.93 (±0.08)	3.96 (±0.09)	4,15 (±0.05)

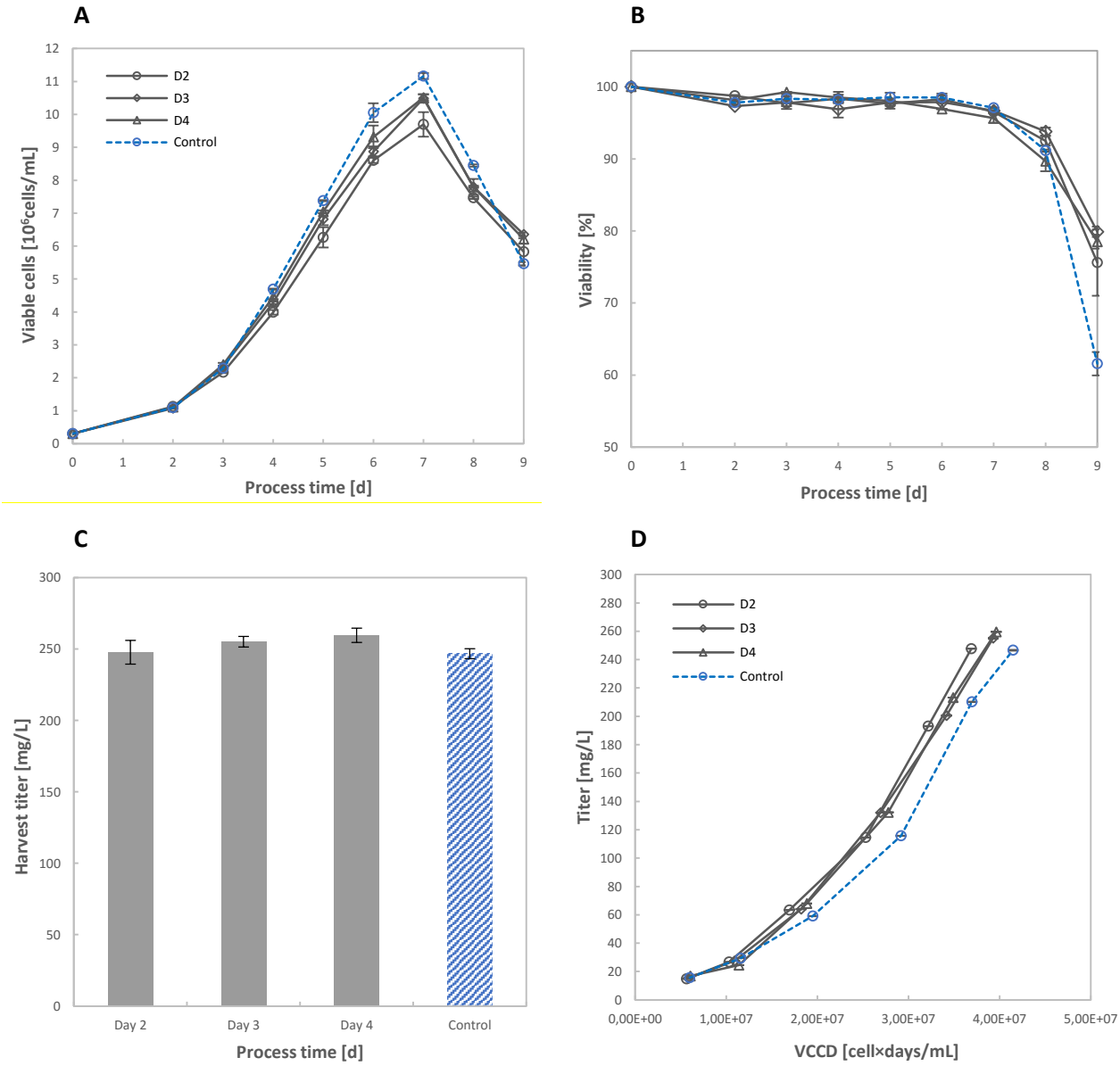

Figure 23. Effects of supplementation with 1 % Glycerol at different days of fed-batch cultivation

Glycerol addition alternatively on day 2, 3 or 4 during fed-batch cultivation resulted in less viable cells but increased productivity compared to a non-glycerol supplemented control. (A) viable cell concentrations, (B) viability, (C) harvest titers, (D) graph of titer vs. VCCD.

6 Discussion

Overall, the aim of this thesis was to evaluate the cultivation performance of an in-house developed cell culture medium (basal medium) and in-house developed feed media in comparison to commercially available media formulations. This was done to reveal possible weaknesses of the in-house developed media formulations (basal medium and feed media) in order to obtain information for follow-up studies on possible improvement of these media formulations.

First, the original medium formulation (MV3-2) did not contain the trace metal iron since the best performing iron source for supplementation of MV3-2 was to be evaluated during this thesis (sections: 5.1, 5.2). In another experiment, concentrations of critical amino acids (l-asparagine, l-serine and l-proline) were elevated in order to overcome suggested limitations imposed by these specific amino acids (section: 5.3). Further, general elevation of basal medium compound concentrations was done in order to reach common concentrations that are usually applied for production media. Then, cross-comparison experiments of feed media formulations followed. In a last series of experiments the effects of various substances (trehalose, mannose, taurine, glutathione, concentrated lipid mix, valproic acid and glycerol) as media supplements on culture performance were evaluated.

6.1 Identification of the best performing iron source for MV3-2 supplementation

Iron, a major trace element of serum-free cell culture media, was supplemented in form of various iron salts (iron(III) citrate (micronized powder), ammonium iron(III) citrate, and iron pyrophosphate) to an in-house developed basal medium formulation (MV3-2 medium by Polymun Scientific GmbH, Austria). Excess iron and undesired forms of iron may negatively influence culture growth and product formation. It was shown that iron transfer into the cell is a critical issue that has to be addressed since chemically-defined media (free of serum components and undefined protein hydrolysates) lack transferrin, the main iron importer together with transferrin receptor. Replacing transferrin some small molecule delivery systems such as tropolone, selenite and citrate salts of iron were found suitable for sufficient transfer of iron into the cell. Experimental results with ferrous citrate and ferrous ammonium citrate showed to improve titers by 40 % compared to ferrous sulfate and ferrous gluconate only (Bai et al., 2011). Hence, the experiment presented in this thesis was performed with iron salts containing citrate. Further, the combination of the mentioned iron salts with iron pyrophosphate was evaluated. However, supplementation with iron(III) pyrophosphate as additional iron source did not seem to have any positive effects on culture growth and product formation compared to the single supplemented experiments with either iron citrate (micronized powder) or ammonium iron(III) citrate. Furthermore, one can assume that the presence of citrate, which causes an improved uptake of iron into the cells, had a superior effect over presence of two different iron sources with a total higher iron concentration.

Further, handling and applicability of the presented iron salt candidates were tested in order to find the best candidate in terms of medium preparation. Iron(III) citrate (micronized powder, Merck) were only soluble when prepared as stock solutions (50-fold, 100-fold), under rather harsh conditions when heat was applied. In contrast ammonium iron(III) citrate (Merck) was well soluble at RT. Since easy handling and applicability of media components has been a pre-requisite for the development of MV3-2 medium, ammonium iron(III) citrate (Merck) is favorable over iron(III) citrate (micronized powder, Merck). According to the results it can be suggested, to favorably use iron in the form of ammonia iron(III) citrate(Merck) over iron(III)citrate (micronized powder, Merck). Ammonia iron(III) citrate (Merck) can be directly supplemented as powder, whereas iron(III)citrate (micronized powder, Merck) would need harsh treatment and preparation as stock solution prior to medium preparation.

6.2 Supplementation of l-asparagine, l-proline and l-serine alone did not improve media performance of MV3-2/1 and MV3-2/6 basal media formulations

Analytics of amino acid concentrations of previous batch experiments (data not shown in this work) revealed that the amino acids l-asparagine, l-proline and l-serine were consumed to a greater extend already at day 4 of batch cultivation compared to all other amino acids. Later (day 7) during batch cultivation these amino acids were even exhausted (Hüller, 2014). These results suggested to supplement MV3-2 medium with the amino acids l-asparagine, l-proline and l-serine in order to provide sufficient amounts for prolongation of cell growth and product formation. However, experimental data suggests, that the effect of supplementation was outperformed by sufficient feeding, since the cultivation process was performed in fed-batch mode (ActiCHO Feed A/B system) contained l-asparagine, l-proline and l-serine). Hence, no differences were observed for cell growth or product formation that could be linked to supplementation of the mentioned amino acids.

Another aspect of this experiment resulted in the final decision for iron supplementation. Since this experiment was performed with MV3-2 basal medium which was supplemented with either, iron(III) citrate (micronized powder)(MV3-2/1) or ammonium iron(III) citrate (MV3-2/6) and when we take into consideration that amino acid supplementation gave comparable results for cell concentrations and product titers, we can get a closer insight into which of the two iron salt variants favorably influenced culture performance (Figure 10). The results further suggest that ammonia iron(III) citrate (MV3-2/6) is favorable over iron(III) citrate (micronized powder)(MV3-2/1). Stating this, we were able to confirm what has already been proposed for the experiment in section 6.1 (Identification of the best performing iron source for MV3-2 supplementation).

To conclude, gaining a better insight into the metabolism and consumption of amino acid is a critical step when searching for improvement of cell culture media formulations. The supplemented amino acids were already added in sufficient amounts to the original medium formulation in accordance with concentrations proposed by Landauer, 2014. A spent media analysis together with knowledge of the most abundant amino acids present in the protein product (Fan et al., 2015) can be used as inevitable tools for further optimization of the MV3-2 medium formulation.

6.3 An overall increase in compound concentrations resulted in an improved medium formulation: MV3-2/6$_{(+30\%)}$

The improved basal medium formulation (MV3-2/6$_{(+30\%)}$) was compared with its progenitor medium MV3-2/6 and ActiCHO P production medium (reference) in a fed-batch setup using the ActiCHO feeding system (ActiCHO Feed A/B, GE, U.S.). Since the original formulation of MV3-2/6 medium was based on a less compound rich formulation, concentrations of

MV3-2/6$_{(+30\%)}$ were increased by approximately 30 % in order to reach growth performance levels and product formation levels of the reference medium.

The increase of compound concentrations resulted in a 31 % higher viable peak cell concentration compared to the original formulation of MV3-2/6, but this was still less than what was obtained from cultivation with ActiCHO P. The increase in viable peak cell concentration can be mainly attributed to a sufficient nutrient supply until day 4 when feeding was started. For cultivation in MV3-2/6 l-glutamine levels were almost consumed at day 4 and depleted at day 5. Glucose as carbon source and l-glutamine as primary nitrogen source are the major energy providing components of the cell metabolism (Quek et al., 2010). Results from another study postulate that l-glutamine and l-asparagine play a major role in TCA replenishment during exponential growth phase (Dean and Reddy, 2013). These findings suggest that the depletion of l-glutamine (MV3-2/6) at day 5 resulted in a reduced TCA activity with a reduced growth rate for cultivation in MV3-2/6 medium compared to MV3-2/6$_{(+30\%)}$ or ActiCHO P. Interestingly, specific productivity, as a criterion for metabolic effectiveness towards protein product formation, was higher in MV3-2/6$_{(+30\%)}$ cultures compared to MV3-2/6 or ActiCHO P.

Lactate and glucose concentration profiles reveal insights into glycolysis behavior of cultivation in MV3-2/6, MV3-2/6$_{(+30\%)}$ and ActiCHO P. Although, viable peak cell concentrations at day 4 were comparable between the tested media variants, higher lactic acid levels were observed for cultivation in ActiCHO P (appr. +22 %) compared to MV3-2/6 and MV3-2/6$_{(+30\%)}$. In general, lactic acid peak concentrations at day 4 coincided well with the findings of Fan et al. (2015) who described a rapid increase of lactic acid during exponential growth phase and further consumption during stationary phase (Fan et al., 2015). Another study even revealed that this drastic increase in lactic acid is mainly caused by excessive consumption of l-glutamine rather than glucose (Dean and Reddy, 2013). These findings coincide well with the results obtained from this experiment. With start of feeding, l-glutamic acid accumulated but subsequently served, together with accumulated ammonium, as a precursor for l-glutamine formation due to the activity of the enzyme glutamine synthetase (feed media contained l-glutamic acid). This finding was confirmed by the fact that l-glutamine was exhausted at day 5, but further increased although none of the feed media formulations contained l-glutamine. Furthermore, lactic acid profiles showed that over the course of fed-batch cultivation lactic acid levels for cultivation in ActiCHO P remained relatively high in stationary phase. This can be mainly explained by higher cell numbers obtained from cultivation with ActiCHO P and lactate levels remained above the critical levels given in literature with 1 g/L (11.1 mM) (Landauer, 2014). This may as well contribute to the explanation of an increased specific productivity observed with cells cultivated in MV3-2/6$_{(+30\%)}$ compared to ActiCHO P.

Ammonium concentration for media that were previously supplemented with ammonium iron(III) citrate had higher initial ammonium concentrations (0.03 g/L) compared to ActiCHO P (0.007 g/L). For all media the highest ammonium concentrations at day 4 coincided well with lowest l-glutamine concentrations at days 4 and 5. Interestingly, cultivation in MV3-2/6 showed

the highest ammonium concentrations of all cultures with remaining high values above the growth inhibiting levels of 90.2 mg/L (5 mM) proposed in literature (Ha and Lee, 2014). This further suggests that the reduced growth observed with cultures from cultivation in MV3-2/6 was caused to some extent by high ammonium levels.

High osmolality was reported to effect viability of cell cultures negatively when osmolality values exceed 380 mOsm/kg (Xing et al., 2008). Although, this threshold was exceeded by day 8 for all cultures, viability values were still above 90 %.

6.4 A feed media cross comparison revealed potential for improvement of in-house developed feed media

Besides improvement of basal medium, improved feed media formulations together with balanced nutrient compositions are crucial bottlenecks in recombinant protein production. Depletion of key nutrients such as amino acids, vitamins and trace elements together with unbalanced formulations of those, can have detrimental negative effects on fed-batch performance (Yu et al., 2011). In this specific experiment an in-house developed feeding system (Feed A(h.m) / Feed B (h.m)) was compared to the commercially available ActiCHO P feeding system (Feed A (GE) / Feed B (G)). All four feed media were evaluated for culture performance and product formation behavior during fed-batch cultivation in combination with MV3-2/6 cell culture medium (in-house developed) and ActiCHO P production medium.

6.4.1 Evaluation of in-house Feed A for further feed medium improvement

According to results that were presented earlier in this thesis (section: 5.5), culture performance (process time, cell growth, viability, and final product titer) of fed-batch experiments was significantly lower when Feed A(GE) was replaced by in-house developed Feed A(h.m). Besides the effects which could be directly associated to the basal medium (section: 6.3), some distinct observations could be directly linked to Feed A(h.m). When Feed A(h.m) was applied for either medium, process time was shortened by 1 day, and also viability values were significantly decreased. Additionally, product titers were 20 % lower when Feed A(GE) was combined with ActiCHO P, and 32 % lower in combination with MV3-2/6. Although, feeding with Feed A(h.m) had a major influence on overall cell growth, specific productivities were not influenced over the course of fed-batch cultivation (Figure 16). The results suggest that Feed A(h.m) significantly lacks the ability to prolong process time and hence, high protein product concentrations. Another interesting detail that was found to be distinct from data obtained with Feed A(GE) is that after l-glutamine was depleted at day 5 (see section: 6.3) the concentration again increased until day 6 and then remained rather constant. This behavior was also found for the concentration profile of lactic acid since lactic acid re-consumption, which was found to be typical for cell cultures in stationary phase (Fan et al., 2015), showed a distinct course of consumption compared to that presented earlier for cultivation in MV3-2/6 medium in combination with Feed A (GE). Lactic acid in general

was less fast re-consumed, which can be linked to lower viability values and an overall lower metabolic activity due to feeding with Feed A (h.m).

The following section will present some aspects that might have caused poor culture performance for cultivation with Feed A:

Combination of a non-production medium with nutrient rich feeding media

MV3-2/6 medium, by means of its formulation, cannot be considered an optimal production medium, since compound concentrations are in general up to 30 % lower compared to ActiCHO P production medium. Results from fed-batch cultivation with either basal medium in combination with in-house developed Feed A(h.m) showed that both basal media were affected by poor cultivation performance in a similar manner. However, cultivation of MV3-2/6 + Feed A(h.m) showed a higher percentage decrease of product titers compared with AcitCHO P. This might have been caused by cellular stress, induced by nutrient addition in form of high concentrated feed solutions in combination with cells that were poorly adopted to a 'high production situation' as it is usually favored by highly concentrated feed media formulations.

Preparation of feed media

In-house Feed A(h.m) was prepared partially from highly concentrated stock solutions (e.g. vitamins and trace elements) since the addition in form of salts was not possible either due to their low abundance in the final formulation or for solubility reasons. Thus, vitamin and trace element solutions for supplementation of in-house Feed A were prepared as aqueous or basic solutions, which might not have been favorable in terms of stability. An issue that needs to be specifically addressed is to find an applicable strategy under which vitamins are stable and can be applied in a reliable manner in order to ensure reproducible medium performance (feed and basal media). Balancing of vitamin concentrations with considerations to interactions with other medium components will be inevitable for further improvement of media formulations.

Formation of reactive oxygen species (ROS)

However, stability and shelf life assessment is especially crucial for in-house developed feed media since the amino acids l-tryptophan and l-tyrosine are photoactive and were reported to form H_20_2 and reactive oxygen species (ROS) under light exposure when they were applied in combination with riboflavin (Stoien and Wang, 1974)(Wang and Nixon, 1978). Riboflavin or vitamin B_2 is the precursor of FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide) and undergoes degradation when exposed to light which comes along with formation of H_20_2 formation (Combs, 2008). Experiments with PBS supplemented with various compounds revealed that only in setups where riboflavin was added ROS were formed. However, l-tryptophan, l-tyrosine, pyridoxine and folic acid seemed to enhance the effect of ROS formation. The most probable dismutation products of riboflavin therefore could be determined as super oxides,

hydrogen peroxide and singlet oxygen. Singlet oxygen species were especially found to play a role in the riboflavin-sensitized-photodegradation of guanine base of DNA and RNA (Grzelak et al., 2001). The effects of photodegradation not exclusively affect the feeding media when applied in a fed-batch setup, but also the basal medium formulation which as well contains components that might be affected by photodegradation.

6.4.2 Evaluation of cell culture performance of in-house developed Feed B

In-house developed Feed B(h.m) was prepared as a 'stand-alone' feed solution since some components were only soluble at rather basic pH. Feeding of high pH solutions to cell cultures is not favorable since changes in pH (acidic or basic) will negatively affect cell culture performance. Feed B(h.m) in combination with Feed A(GE), applied with either basal medium (MV3-2/6 or ActiCHO P) showed similar results for viable cell concentrations, process time and titer compared to MV3-2/6 or ActiCHO P + Feed A(GE) / Feed B(GE). The results suggest that in-house developed Feed B does not need further improvement.

6.5 Media supplements are valuable tools for fine-tuning of cell culture medium performance

Two series of fed-batch experiments were performed in order to evaluate the following media supplements for applicability in fed-batch setups and their effects on process performance: mannose, trehalose, taurine, glutathione and a chemically defined lipid mix. In a second fed-batch experiment the effects of valproic acid and glycerol were evaluated during fed-batch cultivation.

6.5.1 D(+)-Mannose

Other hexose sugars than glucose such as galactose (Altamirano et al., 2004) and mannose (Berrios et al., 2011) have been studied as substitutes for glucose in order to decreased by-product formation (lactate, ammonium) and increased protein product formation due to a more energy efficient metabolism. Berrios et al. (2011) could show that with mannose (10 mM) as only carbon source, a 30 % increase of volumetric protein production in combination with lower hexose uptake rates and by-product formation (lactate, ammonium) compared to their glucose control was observed. The experiment in this thesis was conducted with mannose as a media supplement instead of replacing glucose, since the MV3-2/$6_{(+30\%)}$ medium formulation and in-house developed feed medium (Feed A) already contained glucose. Based on previous experiments (Hüller, 2014), mannose was supplemented in a concentration of 1.5 g/L (8.3 mM) and showed to increase the resulting titer by 10 %. Interestingly, data for toxic by-product formation showed no differences compared to the un-supplemented control which was most likely due to the dominance of glucose metabolism.

6.5.2 D(+)-Trehalose

The disaccharide trehalose was supplemented at the beginning of fed-batch cultivation at a concentration of 0.5 mg/mL (1.46 mM), since this specific concentration seemed to be most promising according to results obtained from previous experiments in a batch setup (Hüller, 2014). Trehalose is a well-known and already approved protective additive for protein and antibody products such as Herceptin, Avastin, Lucentis, and Advateto (Ohtake and Wang, 2011) due to its anti-aggregation behavior. Onitsuka et al. (2014) found out that protein aggregation was significantly reduced during cultivation of an antibody producing CHO cell line with trehalose added. Furthermore, they experienced increased product titers which they were able to link to the protective ability of trehalose. It is believed that protein products are kept in a partially unfolded state (e.g. due to cellular stress) from which they can be easily recovered into their native state by cellular factors (e.g. chaperons). We were able to confirm these findings at least for product titers. Gp140 product titers were increased by 8 % with trehalose added compared to an un-supplemented control. The function of how trehalose affects productivity is still unknown. However, it was reported that trehalose supplementation might protect mRNAs from intracellular degradation and thereby increasing the available amount of mRNA present for translation into protein products (Onitsuka et al., 2014).

6.5.3 Taurine

Taurine, an aminosulfonic acid, is currently being heavily investigated towards its numerous physiological functions. For cell culture purposes anti oxidative mechanisms, membrane stabilization and detoxification are interesting attributes of taurine (Mankovskaya et al., 2000), since photo oxidation of media components (riboflavin, l-tyrosine, l-tryptophan) can be the reason for ROS (reactive oxygen species) formation. For instance, hypochlorite, a strong oxidizing substance was found to react with taurine to N-chlorotaurine. Further, N-chlorotaurine is reduced intracellularly to taurine and chloride causing that by this reaction cascade hypochlorite is detoxified (Huxtable, 1992). Based on previous experiments (Hüller, 2014) 0.1 mg/mL (0.7 mM) taurine was supplemented to the basal medium before fed-batch start and resulted in an 9 % increase of product gp140 titer. According to what has been suggested in literature, the results coincide well since a higher titer compared to the control was achieved. This also suggests taurine as a promising media supplement for future media formulations.

6.5.4 Glutathione

Glutathione is important to protect cells from oxidative stress, for regeneration of antioxidants (e.g. ascorbate) and conversion of peroxides into water and CO_2 (Bannai et al., 1977). For the experiment presented here we supplemented 100 μM glutathione at the beginning of fed-batch cultivation. Unfortunately, resulting titers for glutathione supplemented shaking flask had 21 % less harvest titer compared to the un-supplemented control. Additionally, viability values were

lower which resulted in an overall shortened process time by 1 day. Under normal physiological conditions very low concentrations of oxidized glutathione are present due to the enzyme glutathione reductase. However, it has been reported that in media, where selenium was added together with molecules that have reduced sulfhydryl groups such as cysteine or glutathione, super oxides were formed and oxidative stress occurred (Media Expert by Sigma Aldrich, 11-07-2015). This effect together with an assumed high concentration of oxidized glutathione may have caused reduced viability and decrease in gp140 harvest titers.

6.5.5 Chemically defined lipid mix

Lipids are essential components for cells when cultured in serum-free medium. Lipids are major building blocks for membrane synthesis and signal transduction (Gstraunthaler and Lindl, 2013). A chemically defined lipid mix was used to provide the cells sufficiently with lipids and fatty acids. The chemically defined lipid mix was supplemented according to the manufacturer's instruction as a 1:1,000 dilution for CHOs. However no improvement in growth behavior or product formation could be observed due to supplementation with lipids. A possible reason to explain this result might be that the lipid mix used for this experiment was already expired and oxidation processes may have occurred. Another reason might be that the dilution of 1:1,000 was too high and therefore lipid concentrations were too low to have growth promoting effects. Data from literature suggests to have concentrations for e.g. linoleic acid of 56 μg/L (Hammond et al. 1984) or even 100-940 μg/L as proposed by Landauer, (2014). The proposed concentrations from literature are in heavy conflict to 10 μg/L as suggested by the manufacturer.

6.5.6 Valproic acid

Small molecule titer enhancers (e.g. valproic acid and sodium butyrate) for recombinant protein production are a valuable tools to boost protein titers (Allen et al., 2008). In this experiment, the hydroxamic acid valproic acid (VPA) was evaluated regarding its ability to increase harvest titers during fed-batch cultivation of CHO cells. VPA was added as sodium valproate (Sigma Aldrich, U.S.) at days 5, 6 or 7 of fed-batch since investigation for the optimal time point of VPA addition was part of this experiment. Yang et al. (2014) found out that the effect of VPA is a function of concentration and time. For simplicity of the experimental setup we adopted their optimal concentration found for VPA supplementation which was 3.5 mM.

Later addition of 3.5 mM VPA during fed-batch cultivation shows enhanced titers.

VPA is toxic to the cells and therefore the optimal time point of addition and the optimal concentration is crucial for titer enhancement. This was confirmed by viable peak cell concentrations since cell concentration for VPA supplementation were lowest when VPA was added at day 5 (-24 % cells/mL) and were increased the later VPA was added (day 6: -6 % cells/mL and day 7: no decrease). Regarding the harvest titers, the proposed effects were confirmed as well. Early supplementation of VPA during fed-batch cultivation (day 5) seemed to heavily effect cell

growth but specific productivity was enhanced. However, cultures that were supplemented at day 5 did not outperform in terms of product formation since overall viable cell concentrations were lower. In contrast, VPA supplementation on days 6 and 7 outperformed the un-supplemented control by 7 % and 10 % respectively.

Effect of VPA depends on the chromosomal location of the gene of interest.

VPA and its function as histone deacetylase inhibitor is heavily dependent on the chromosomal environment of the gene of interest (GOI) (Sarkar et al., 2011). Insertion of a GOI in the host genome is mostly random and chromosomal environments can be tightly packed (downregulated) or easily accessible (highly active regions). Yang et al. (2014) reported a 15 % increase of resulting harvest titer which is higher compared to results presented in here. At this point it has to be mentioned that the cell line which was used for experiments presented in this thesis was generated by transfection of a CHO-K1 (ATCC CCL-61) cell line with a Rosa26 based BAC vector (Caggs:BAC^{Rosa26}) which can be considered as a highly transcriptionally active element (Zboray et al., 2015). This may be a possible explanation to why the genetic environment of the GOI was less prone to be affected by VPA.

6.5.7 Glycerol

The sugar alcohol glycerol (1,2,3-propanetriole) is well known to prevent recombinant proteins from aggregation and against thermal degradation due to its shielding effect. Rodriguez et al. (2008) could show that in a bioreactor set up β-IFN (β-interferon) aggregation was reduced by more than 50 % due to the addition of glycerol in concentrations higher than 1 % and they additionally found out that glycerol inhibits cell proliferation by inhibition of cytokinesis. Another study proposed that glycerol acts as a chemical chaperon without affecting specific productivity (Brown et al. 1996) and Liu and Chen (2007) presented a 38 % increased titer of rM-CSF (recombinant macrophage colony stimulating factor) produced in CHO with 1 % glycerol supplemented to the culture. In this experiment we supplemented fed-batch cultures with 1 %$_{(v/v)}$ glycerol either on days 2, 3 or 4 of cultivation. We could observe that all cultures which had glycerol added showed reduced growth and therefore lower viable peak cell concentrations compared to the un-supplemented control. Interestingly, cultures with glycerol added showed higher viability levels than the control. It can be assumed that glycerol addition and thereby reduced proliferation rates contribute to an overall higher cell viability. Considering product titers, day 4 was found to be optimal time point for glycerol addition since gp140 protein product titers were increased by 5 %. The optimal day of glycerol can be most likely defined by a cell concentration dense enough to overcome limitations impaired by inhibition of proliferation and enhanced specific productivity.

7 Conclusion and Outlook

Overall, the results of this work suggest that chemically defined MV3-2 cell culture media in form of its improved version MV3-2/6$_{(30\%)}$, with ammonia iron(III) citrate as appropriate iron source and increased compound concentrations can be considered a decent medium alternative to commercially available media formulations. Moreover, first attempts to formulate two complex and chemically defined feed media formulations were made with moderate success in terms of cell growth and viability, as well as product titers and elongation of process time. Additionally, various media supplements were successfully evaluated towards their potential as being possibly added to a future MV3-2 medium formulation or as titer enhancers for process supplementation.

Supplementation of various iron salt candidates (iron(III) citrate (micronized), ammonium iron(III) citrate and iron(III) pyrophosphate) for MV3-2 medium supplementation, and different concentration of these iron salts, did not show clear distinctive results. However, ammonium iron(III) citrate seems to positively affect cell growth, whereas product titers were similar throughout the tested iron salts.

A follow up experiment with the MV3-2 + ammonia iron(III) citrate (MV3-2/6) medium variant supplemented with some amino acids (l-asparagine, l-serine and l-proline) was further performed to overcome proposed limitations of these amino acids. Supplementation to the twofold concentration of these amino acids suggested, that when added during fed-batch cultivation, these amino acids were no more limiting due to a sufficient nutrient supply by addition of feed media. Hence, the following experiment was performed in order to focus on general improvement of MV3-2/6 medium compound concentrations.

Since amino acid supplementation alone has not been the key to improved overall process performance for MV3-2/6 medium, the whole of compound concentrations was increased for the majority of components by a factor of 1.3. Due to the increase of compound concentrations an increased viable peak cell density (+31 % VPCC/mL), as well as an increase of final product titer by 70 % (mg/L gp140), could be achieved. Although an increase of final product by 70 % is quite impressive the reference production medium (ActiCHO P by GE, U.S) still resulted in a 10 % higher harvest titer.

As most industrial high-protein-yield processes are performed in fed-batch mode, two chemically defined feed media were prepared (Feed A(h.m) and Feed B(h.m)). This was done to assess the feed media's ability towards prolongation of cultivation time and thereby further increase of resulting titers. However, it could be shown that Feed A(h.m) was not able to prolong cultivation time and meet the benchmarks set by commercial Feed A(GE) (ActiCHO Feed A, GE, U.S.) for VPCCs and harvest titers. In contrast, in-house Feed B(h.m) showed similar culture performance as achieved with Feed B(GE) (ActiCHO Feed B, GE, U.S.). According to the results it can be suggested to revise Feed A(h.m) feed formulation for component interactions and stability as well

as for specific demands of the cultured cells for nutrients such as amino acids, vitamins and trace elements.

In the last two experiments the potential of the sugars D(+)-trehalose and D(+)-mannose, taurine and glutathione as well as a chemically defined lipid mix were evaluated for product titer enhancement. It was shown that product titers were elevated due to supplementation with D(+)-trehalose (+8 %), D(+)-mannose (+10 %) and taurine (+9 %). In contrast, supplementation of glutathione, which acts as redox mediator for cellular stress, surprisingly resulted in 21 % less protein product compared to an un-supplemented control. Further, the addition of a chemically defined lipid mix did not show to enhance product titer, which can be possibly attributed the applied concentration that was too low to influence culture performance positively.

The second experiment of this series dealt with substances as titer enhancers which were applied at certain time points during cultivation. The potential for titer enhancing abilities of valproic acid, a histone deacetylase inhibitor, and glycerol, a sugar alcohol, were investigated. Addition of valproic acid at day 7 of fed-batch cultivation resulted in a 10 % improved product titer. Addition of glycerol at day 4, according to the suggestion of adding glycerol in an early phase of cultivation, showed to increase product titers by 5 % together with enhanced overall specific productivity. The tested supplements, with the exception of glutathione, seem to be valuable tools for further improvement of the tested medium formulation as well as to enhance product formation in a fed-batch process.

In order to evaluate the overall performance and applicability of findings of this work it is suggested to combine all successful findings into a single fed-batch experiment. This experiment shall be conducted with MV3-2/$6_{(+30\%)}$ as basal medium and in-house developed feed media for feeding purposes. General fed-batch settings shall be applied as described in section 5.4.1. Prior to the start of fed-batch cultivation, MV3-2/$6_{(+30\%)}$ medium shall be supplemented with 1.5 mg/mL D(+)-mannose, 0.5 mg/mL D(+)-trehalose and 0.1 mg/mL taurine. The feeding strategy shall be applied as for fed-batch experiments performed in this thesis (Feed A: adjusted to 6.5 g/L glucose by daily feeding, Feed B: 0.28 % (v/v) of actual culture volume; feed start: day 4). Additionally to the start of feeding at day 4, glycerol shall be added to a final concentration of 1 $\%_{(v/v)}$. Finally at day 7, valproic acid shall be supplemented to the cultures to a final concentration of 3.5 mM. For reference purposes, it is suggested to run fed-batch experiments with ActiCHO P production medium together with its appropriate feeding system and un-altered setups with MV3-2/$6_{(+30\%)}$ and in-house developed feed media. All experimental setups shall be carried out in duplicates or even triplicates.

8 List of Figures

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10 Bibliography

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