Diploma thesis

Comparison of CHO EpoFc producer's growth characteristics during bioprocesses after adaptation to higher cell densities and different glutamine concentrations by assisted evolution

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by

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Declaration

I hereby certify that,

- I performed this thesis without external assistance, and that no other than the listed references have been used as sources of information.

- no other persons than the authors have significantly contributed to this work.

Statement on gender-neutral formulations

The author is aware of the problem of the correct usage of gender conscious notations and has largely abandoned those for the sake of undisturbed readability of complex scientific matters. Female forms are explicitly meant to be included.

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Submitted to Metabolic Engineering

Growth behaviour of CHO EpoFc producer cell lines adapted to higher cell densities and absence of glutamine by assisted evolution

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Abstract

A primary objective of biotechnologically used cell culture is the increase of culture performance, measured by desirable properties such as high cell concentration, viability, productivity and product quality. A major limitation of mammalian cells in culture is their inefficient energy metabolism which results in high uptake rates of glucose and glutamine and the concomitant accumulation of waste products which in turn limits final cell concentrations and growth. One approach to reduce this effect is the limitation of available substrate using fed batch processes. Recently, a CHO host cell line was established which is able to grow in completely glutamine free medium [1]. To determine the influence of this adaptation on productivity and product quality, the same procedure was repeated with an erythropoietin (EpoFc) producing CHO (CHO-EpoFc) cell line. After adaptation to higher cell densities and glutamine free medium, culture performance was monitored in batch fermentation processes and revealed comparable growth properties and EpoFc product formation in both cell lines. The physiological state of the cells was investigated by monitoring nucleotides and sugar nucleotides revealing elevated UDP-sugars in cells grown in the absence of glutamine. Both the level of reactive oxygen species and the level of the oxido-protective glutathione were elevated in these cells, reflecting a higher level of oxidative stress, coupled with adequate response. Furthermore the glycosylation of EpoFc produced by both cell lines was determined and revealed higher antennarity of sialylation in N-glycans on the Epo part of the product obtained from the adapted cell line, but overall very comparable product quality.

Keywords: CHO; Erythropoietin; high density; glutamine free; glycosylation; sialylation; cell sorting; nucleotides; oxidative stress

1 Introduction

CHO cells have been a major source for recombinant therapeutic proteins for over 20 years which is, to some extend, due to their readiness to adapt to different culture conditions [2]. Different approaches have shown that their adaptation can lead to higher cell density [3] which in turn results in improved productivity [4]. Their heterogeneity is part of the reason for their common use, as they can be easily adapted to protein free media and it is easy to isolate new phenotypes with specific or improved properties. On the other hand, the same heterogeneity is a major source for the elaborate and long development times required to establish a new cell line or process [5]. Due to the characteristic energy metabolism observed in mammalian cells in culture, the optimisation of media composition has been an important topic for the enhancement of cell lines and processes [6-10]. Mammalian cells in culture usually need high concentrations of glucose and glutamine which result in the accumulation of the by-products lactate and ammonia, which in turn can cause a lower final cell concentration and product titre [11]. Cultivation of cells at lower nutrient concentrations results in a metabolic shift to a more efficient utilization of substrates and thus decreases waste product levels [12, 13]. Typically, cells convert ~80 % of the available glucose to lactate, which was first described as the Warburg effect [14]. Although this has a negative impact on culture growth and viability, the major problem comes from the influence that ammonia has on product quality [15]. McKeehan [16] defined the term glutaminolysis for the metabolic pathway that oxidises glutamine as an alternative energy source, which results in a higher energy output than that of the inefficiently used

glucose [17]. However, despite its beneficial effect on growth and viability, two molecules of ammonia are released from each glutamine before its entry into the TCA cycle as α-ketoglutarate [8], resulting in concentrations of up to 10 mM ammonium [18, 19]. Unfortunately, two important aspects of protein quality are directly affected by the concentration of ammonium in the cells: the increased synthesis of UDP activated N-acetylhexosamine (UDP-HexNAc) impairs the transport of Cytidine monophosphospho-N-acetylneuraminic acid (CMP–NANA), a precursor for sialic acid [20], into the Golgi, causing a decrease in terminal sialylation and an increase in antennarity of the oligosaccharides of glycoproteins [15, 21, 22]. These properties, however, are critically important for accurate functionality and in-vivo half life [23-25].

As they serve as precursors for glycan chain synthesis, intracellular nucleotide and nucleotide sugar levels have become of major interest [26, 27]. It has been demonstrated that increases in UDP-sugar levels negatively affect sialylation and are accompanied by elevated levels of UTP and CTP but decreased levels of ATP in rat hepatocytes treated with Urd [21]. Nucleotide sugar precursor feeding had diverse effects on glycan structures in other studies [28]. Therefore, intracellular ribonucleotide pools are used as meaningful indicators for cell behaviour and protein synthesis [29]. Several methods using capillary electrophoresis or anion-exchange chromatography are available for the determination of nucleotides and sugar nucleotides [30, 31]. During this investigation a liquid chromatography-electrospray ionization-mass spectrometry method that uses surface-conditioned porous graphitic carbon (LC-ESI-MS) was used [32].

Furthermore, connections between the oxidative stress of cells and their viability and productivity are described in literature. Reactive oxygen species (ROS) and superoxides (SOx), which are held responsible for the onset of apoptosis, are reported to be detoxified by the cell´s glutathione system. Higher levels of the antioxidant glutathione (GSH) are directly correlated with inhibition of oxidative stress, resulting in enhanced culture performance [33-35]. Due to the direct connection between the generation of energy from glucose and glutamine and the generation of oxidative species, the intracellular levels of both GSH as the protectant and ROS and SOx as the toxic agents were determined by flow cytometry.

Bort et al. [1] have presented a method to quickly generate CHO-K1 host cell lines that are able to grow without glutamine with comparable growth and viability. In the present work, the process was repeated using a CHO cell line producing an erythropoietin-Fc fusion protein to further examine the effect of glutamine free growth on productivity and product quality. As an evaluation of product quality was not possible in the previous work performed with a host cell line, in the present paper we present an in depth evaluation of the effects of this adaptation on productivity and product quality, both of which were shown to be affected only to a minor degree.

2 Materials and methods

2.1 Cell line and media

The CHO-EpoFc cell line was originally established by Lattenmayer [25] and was later adapted to growth in serum free CD CHO medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 4 mM L-glutamine,

0,096 µM MTX and 1 ml anti clumping agent (Gibco, Invitrogen, Carlsbad, CA, USA) per 500 ml medium.

2.2 Adaptation to high density and glutamine free media

Cells were cultivated in spinner flasks with a working volume of 60 ml at 37 °C (7 % CO_2, 50 rpm magnetic stirring). After seeding at a concentration of $1x10^7$ cells/ml in T25 roux flasks, surviving cells were isolated using the dead cell removal kit Dead-Cert (ImmunoSolv Limited, Edinburgh, UK) before the viability of the culture dropped below 20 %, which usually occurred within 20 hours. Dead Cert removes both early apoptotic cells and dead cell debris, which lead to higher survival rates of the remaining population. For $5x10^6$ cells in 0,5 ml 30 µl magnetic beads were used for incubation (1 hour). The remaining cell suspension with a concentration of $1–2x10^5$ cells/ml was seeded in 24 well plates in 1ml medium consisting of 50 % fresh CD CHO and 50 % conditioned medium taken from the former spinner flask culture. After one day the culture was passaged into T25 roux flasks with 4 ml fresh CD CHO. After doubling of the cell number, 6 ml fresh medium were added and after one week the cells were seeded into 60 ml spinner flasks. This procedure started with cells adapted to 4 mM glutamine. After recovery cells were seeded in medium with 2 mM glutamine. After 1-2 passages the viability of the culture was better than 90 % and the next sorting cycle was processed (Fig. 1). The final cell line was able to grow without glutamine addition.

2.3 Bioreactor system and fermentation settings

A DASGIP bioreactor system (DASGIP AG, Jülich, Germany) controlled by DASGIP Control 4.0 was used for repeated batch cultures. Oxygen concentration (Broadley James Oxyprobe) was held constant at 30 % air saturation via a cascade with variable airflow (0-9 l/h) and variable oxygen content (21–100 %). Neutral pH (Mettler Toledo) was regulated with 0,5 M NaOH and CO_2, respectively. Vessels used for the cultivation were advanced spinners (300 ml – 800 ml) with a magnetic drive and pitch blade impellers at constant stirring (80 rpm). Temperature was measured by a PT100 and held steady at 37 °C. Controllers were TC4/SC4 (temperature/drive), MX4/4 (gas mixing), OD4 (optical density) and MP8 (pumps).

2.4 Measurement of culture performance

The growth curves of the spinner flask batches were determined by daily measurement of cell viability using a Hemocytometer (Neubauer) and 0,5 % trypan blue solution. Total cell counts were measured using a Coulter Counter (Beckman Coulter, Fullerton, CA, USA) after lysis of cells in 0,1 M citric acid (2 % Triton X-100) for at least 30 min.

2.5 Measurement of metabolites, conditions and productivity

Culture metabolites including glutamine, glucose, lactate and ammonia were determined by a YSI 7100 MBS Bioanalytical System (YSI Life Sciences, USA). For the fermentation culture, parameters such as pH, dissolved oxygen, air saturation and TCO_2 were measured by online monitoring of the

bioreactor system and simultaneously by pHOx (MuT, Berlin, Germany) directly after sampling.

The EpoFc concentration was quantified by ELISA. Polyclonal goat anti human IgG (γ-chain specific) antibodies (Sigma, I-7883) were used for coating and blocked with Tween 20 (Sigma, P7949). An internal standard and the supernatant of the culture samples were diluted with washing buffer containing 1 % bovine serum albumin and loaded onto the plates. A horseradish peroxidase conjugated polyclonal goat anti human IgG (γ-chain specific) antibody (Zymed, 62-8420) was used for detection of EpoFc. The labelling reagent consisted of 100 µl OPD (Ortho-phenylenediamine dihydrochloride, Fluka, Switzerland) and 6 µl H_2O_2 in 10 ml dyeing buffer. 2,5 M sulphuric acid was used as stop solution. The product titre was measured using an ELISA reader (Sunrise, TECAN) at 492 nm and 620 nm as reference wavelength and the software Magellan according to the instruction manual.

2.6 Analysis of ROS, SOx and GSH

1-3x10^6 cells/mL were stained with 50 µM of monochlorobimane (mCBI; Invitrogen, M-1381MP) for analysis of intracellular GSH; or 10 µM of dichlorodihydrofluorescein-diacetate (H_2DCFDA; Invitrogen, C2938) to determine intracellular ROS; or 25 µM of dihydroethidine (DHE; Invitrogen, D23107) for staining of intracellular SOx. The fluorescent signals for ROS (ex. 488 nm, em. filter BP 530/30) and SOx (ex. 488 nm, em. 585 nm) were measured using a BD FACSCalibur™ flow cytometer (Becton Dickinson, USA) with a 488 nm Argon laser; and for GSH (ex.

multiline UV, em. filter 424/44), using a BD FACSVantage™ flow cytometer (Becton Dickinson, USA) with two argon lasers tuned to 488 nm and multiline UV.

2.7 Analysis of sugar nucleotides, nucleotides and GSSG

For extraction of sugar nucleotides and nucleotides from CHO cells, $1x10^7$ cells were washed twice with ice cold PBS buffer by gentle shaking. The buffer was removed after centrifugation at 1500 rpm (1 min). For quenching and lysis of the cells, 1 ml NaF buffer (40 mM) was added to the pellet (10 s). The lysed cells were vortexed (1 min) and centrifuged at 4 °C (max. speed, 5 min). After introduction of a spike (10 nmol, ATP ^{13}C10, Sigma Aldrich Nr 710695), $1x10^6$ cells were applied to a 10 mg Hypercarb solid-phase extraction (SPE) cartridge (Thermo Scientific, 96 well plate, 60302-606). The cartridge was placed into a 2 ml vial and centrifuged. Preconditioning was accomplished by washing the cartridge with 0,5 ml of elution buffer, consisting of 60 % acetonitrile and 40 % formate buffer (0,3 % formic acid adjusted to pH 9,0 with ammonia) at 2000 rpm for 1 min and a second washing step with deionized water (1 ml, 2000 rpm, 1 min). After a final washing step with 1 ml of deionized water, nucleotide sugars were eluted by 250 µl of elution buffer. The sample was freeze dried in a speed-vac-concentrator without heating or light. Complete dryness was reached after approximately 45 min. The samples were dissolved in 10 µl TRIS buffer (5 mM) and subjected to LC-ESI-MS using a Hypercarb PGC column (0,32x100 mm, GE Healthcare) mounted with polyetherketone capillaries (25 µm i.d.). The formate buffer from the sample extraction was used as a

starting buffer and a 20 min gradient from 2 to 15 % acetonitrile (8 µl/min flow rate) was build up by an Ultimate 3000 (Dionex). For elution of the nucleotides a 1 min ramp was used with 50 % acetonitrile for 5 min. Q-TOF settings (Ultima, Waters-Micromass) were -2.7 kV capillary voltage, 80 V cone voltage, 75 and 150 L/h cone and desolvation gas flows and 100 and 120 °C source and cone temperatures using negative mode ESI-MS as well as 20 % dissociation energy and argon. Software used was MassMap (Vs. 2010-06-13, MassMap, Wolfratshausen, Germany).

2.8 Analysis of glycosylation pattern

The highly glycosylated Epo-Fc, a fusion protein of recombinant human erythropoietin N-terminally linked with the Fc part of human IgG, was analysed using LC-ESI-MS. The required sample volumes were taken from the 3rd batch at the times shown in Fig.3, containing 5 µg or more of Epo-Fc, and purified by affinity chromatography using protein A for binding to the Fc-region. The samples were subjected to S-carbamidomethylation for blocking re-formation of disulfide bonds and proteolytic digestion with trypsin after standard SDS-PAGE [36, 37]. Glycopeptides and oligosaccharides were extracted and purified in additional steps using a PT C18 cartridge (Varian, Paolo Alto, CA) and a 10 mg HyperSep Hypercarb SPE cartridge (Thermo Scientific, Waltham, MA, USA). An analysis was performed by RPC, using 65 mM ammonium formate buffer as LC solvent, coupled to an ESI Q-TOF MS. Extraction and MS protocols were applied as described elsewhere [38].

3 Results

3.1 Establishment of the cell line

The goal of this work was to establish and characterize a CHO producer cell line growing to satisfactory cell densities in the absence of glutamine. This was achieved by stressing the cells with high density culture conditions and selection of survivors, followed by reduction of glutamine supplementation and another round of selection. During the first adaptation attempts it was observed that few cells survived the treatment as most of the still viable cells had already entered into apoptosis. Applying a sorting method with nanoparticles, that effectively bind also to early apoptotic cells, led to successful isolation of adapted cells. After sorting, the cells showed a prolongued lag-phase which typically lasted one week before the cell concentration doubled for the first time. Reducing glutamine supplementation after the full recovery of the cells did not decrease the peak cell density significantly, rather, in spinner or T flask culture, the cells with 4 mM glutamine had a slower growth rate than the cell lines with 2 mM and 0 mM glutamine (data not shown). Similar behaviour was observed in previous such adaptations [1].

3.2 Growth characteristics in repeated batches

For detailed characterization of cell physiology, the cells were seeded into 4 independent reactors in duplicates and analysed over 3 repeated batches (Fig.2). All batches lasted at least 200 h before the viability dropped below 90 %. At this point, the batches were restarted with an initial cell concentration of approx. $2x10^5$ cells/ml. Cells adapted to 0 mM glutamine

were faster in adapting to the fermentation conditions during the 1st batch and reached a peak cell density of $6,5x10^6$ cells/ml, whereas cells grown in 4 mM glutamine had a delayed start of exponential phase and only reached $4,7x10^6$ cells/ml. In the following batches the 4 mM cells showed earlier starting points of exponential phase and grew to slightly increased final cell concentration compared to the 0 mM cell line (+17 % in batch 2 and +11 % in batch 3). Overall, performance of each population in the second and third batch was comparable, the only difference being a 10 % reduced maximum growth rate of the 0 mM glutamine cultures, followed by a slightly prolongued stationary phase at high viability. Samples for detailed analysis were taken from the 3rd batch to be able to also include samples from the late stationary and decline phase (Fig.3).

3.3 Culture metabolites and productivity

The nutrient consumption of glucose and glutamine and the by-product accumulation of lactate and ammonia were monitored in batch 3 (Fig.4). Values were determined twofold for each cell line in two independent reactors. Due to the slightly lower starting cell concentration in the 0 mM glutamine culture, glucose consumption and lactate accumulation started later but showed comparable rates to cells grown in 4 mM glutamine. The ratio of lactate production to glucose consumption was comparable. In the 4 mM cultures all energy substrates (glucose and glutamine) were depleted on day 7, resulting in a significant decrease in viability on day 8, while the depletion of glucose was complete in the 0 mM glutamine culture on day 8, followed by a delayed onset of cell death on day 10. Due to the absence of

glutamine, the 0 mM cells produced 40 % less ammonia compared to 4 mM cells. During the late exponential phase both cell lines showed a decline in ammonia accumulation which again reversed after attaining the plateau of the stationary phase. This dip in the ammonium curve coincided with the start of lactate consumption and with a sudden increase in the production of alanine (data not shown). It was not observed in any of the batches run in spinner flasks before. Total ammonia concentrations at the end of the 3rd batch reached 7,5 mmol/l in cultures with 4 mM glutamine and 4,7 mmol/l in cultures without glutamine.

3.4 Oxidative stress level – ROS, SOx and GSH, GSSG

Oxidative stress whose main source is oxidative phosphorylation in the mitochondria producing ROS and SOx, has influence on the regulation of cell growth in culture. Intracellular ROS and SOx were high in early exponential phase of culture in both cell lines (Fig.7). However, high amounts of intracellular GSH during this culture phase were also present. During middle exponential phase, ROS, SOx and GSH sank to their lowest points. ROS and SOx levels started to increase again during the late-exponential phase, while GSH remained low, thus providing no protection against oxidative damage as during the exponential growth phase. This behaviour is routinely observed in CHO cell cultures (data not shown). Both indicators of oxidative stress, as well as the protective glutathione and its reduced equivalent GSSG were observed to be higher in 0 mM cell lines compared to 4 mM cells.

3.5 Intracellular sugar nucleotide and nucleotide contents

The intracellular sugar nucleotide and nucleotide precursor molecules were analysed during the 3rd batch. Data are shown for those that indicated differences between the two cell lines: UDP-HexNAc, UDP-Glc, UDP-Gal and GDP-Man (Fig. 7). While the 4 mM cells showed a continuous, linear decrease in their UDP sugar pools, the 0 mM cells had an upswing of UDP-HexNAc during exponential phase, followed by a decrease to similar levels as the 4 mM glutamine cultures, while the concentrations of UDP-Glc, UDP-Gal and GDP-Man remained at higher concentrations until the end of the batch. The CMP-NANA pool was not significantly different in cells with or without glutamine (data not shown). Concomitant with the higher levels of UDP-sugars, all uracil nucleotides (UMP, UDP and UTP) showed higher intracellular concentrations throughout the batch in the cells grown without glutamine (data not shown).

3.6 Glycosylation pattern of EpoFc

The EpoFc fusion protein, consisting of two Epo polypeptides linked to the two chains of the Fc part of human IgG_1, includes 3 N-glycan and one O-glycan residues in the Epo part and one N-glycan in the Fc part of the protein (Fig.8). Samples were analysed from Batch 3 at the time points indicated in Fig. 3.

Interestingly, all possible N-glycosylation sites were occupied throughout the batch and for all samples (Fig. 9). There was a significant difference however in the structures that were found on the Fc-derived peptides (Fig. 10) and those that came from the Epo-part (Fig. 9A). The N-glycans on the

Fc-fragment consisted exclusively of bi-antennary structures, while the N-glycans on the Epo fragment also contained large proportions of tri- and tetra-antennary glycans. The N-glycans on the Fc-fragment remained unchanged throughout the batch culture.

On the Epo-derived fragments the level of undersialylated N-glycoforms slightly increased during the batch, but was not significantly different between the two cell lines. While the abundance of tri-antennary N-glycans remained the same in both cell lines and over the course of the batch, the main variation was observed in the percentage of bi- and tetra-antennary structures. Whereas bi-antennary N-glycans increased, tetra-antennary N-glycans decreased during the batch. Cultures with glutamine showed this effect to a higher degree than cultures without glutamine, so that at the end of the batch the product derived from 0 mM cells had a significantly higher antennarity compared to that produced by 4 mM cells.

The O-glycosylation site occupancy was lower as shown in Fig. 9B, reaching levels of up to 50 % in 0 mM and up to 45 % in 4 mM cells. This remained on the same level throughout the batch culture. Di- and mono-sialylated O-glycans also maintained their relative abundances over the batch course. The 0 mM cell line showed 5 % more mono-sialylated and 5 % less di-sialyled O-glycans compared to the parental cell line.

4 Discussion

Our attempt to obtain a well growing cell line in glutamine free medium yielded a physiologically altered cell line that was able to grow without glutamine supplementation within 10 weeks. Previous reports [1] have

shown that the simple removal of glutamine without adaptation led to a decrease in growth rate and viable cell density, becoming worse with every passage. In the present work we could again demonstrate that using the procedure described it is possible to maintain growth at a comparable rate to cells grown with glutamine. A slight decrease in growth rate is again observed, and also a prolongued viable stationary phase, as in the previous report. The metabolic analysis revealed no drastic changes in the glucose consumption or lactate production, so that it appears that no shift towards oxidative phosphorylation occurred. Nevertheless, intracellular reactive oxygen and superoxide levels were increased. At the same time the oxido-protective glutathione and its reduced equivalent GSSG were also increased in cells adapted to 0 mM glutamine, which is the more surprising, as glutamine is an important precursor for GSH synthesis. It seems that the little endogenously produced glutamine was efficiently used to maintain the GSH and GSSG levels in the cells.

In the literature a direct connection between the intracellular pool of UDP-N-acetylhexosamine and ammonia is described, as well as its relevance for glycosylation. Elevated ammonium levels resulted in an increased intracellular UDP-HexNAc pool and resulted in higher antennarity of glycostructures [15, 22, 26, 27]. As shown in Fig. 4B, the ammonia level was decreased in cultures grown without glutamine as expected but surprisingly still led to an elevated UDP-HexNAc pool (Fig. 7A) compared to cells grown with glutamine. This result contradicts other reports. There may be several explanations for this behaviour which currently are only speculation. One possible explanation could be that the absolute

concentration of ammonia in both cell lines was significantly lower than the concentrations used in previous studies targeting at evaluating the effect of ammonium on glycosylation (4,7 and 7,5 mM in the present report vs. 30 mM in [15]). Another explanation may be linked to the fact that ammonia was reported to push cellular metabolism into excretion of alanine in order to achieve ammonia detoxification [8]. This effect was also observed in our study where twice the amount of alanine was secreted in the 4 mM culture (data not shown). Thus potentially the intracellular concentration of ammonium was maintained constant in spite of the observed extracellular ammonium concentrations. This does not explain the observed differences in the intracellular UDP-sugar pools. It seems that there must be other, additional regulatory mechanisms at work.

Irrespective of the reason, the effect of the elevated UDP-HexNAc pool itself was similar to that observed in previous studies [41]: an increase in antennarity was observed as a result of a higher UDP-HexNAc level, with tetra-antennary glycans by 6 % more abundant in 0 mM cells at the end of the batch, which was matched by 6 % less bi-antennary structures. In our work no drastic differences in O-glycans of Epo were detectable since non-glycosylation occurred to comparable levels in both cell lines. Additionally, the antennarity of N-glycans in the Fc derived peptide was found as consistently different from the N-Glycans on the Epo fragment and as not influenced by the presence or absence of glutamine, as only bi-antennary glycoforms occurred in both cultures.

The second major issue with ammonia described in other studies [42] is its impact on intracellular pH influencing the sialylation state of N- and O-

glycans. It is not yet clearly defined whether the level of UDP-sugars or the pH or both together have more impact on sialylation of glycans. It was assumed in one study that UDP-HexNAc affects antennarity, whereas the ammonium dependent intracellular pH affects sialylation [41]. In other studies it was hypothesised that the effect on sialylation also strongly depends on the extracellular pH [19, 43]. As suggested, higher pH causes ammonia being present as NH_3 which is able to diffuse into the cell affecting intracellular pH. During our fermentation the pH value of the cultures was held constant at 7.0, thus the concentration of NH_3 was low. In our experiments a slight increase in non-sialylated termini was observed in the culture without glutamine, which correlated to the increased antennarity observed. Overall, despite the lower ammonia concentration, the glycans in the 0 mM culture were slightly less sialylated, contrary to previous reports. As the intracellular content of CMP-NANA, the precursor for sialylation, was unchanged, it is likely that the higher abundance of UDP-sugars interfered with uptake of CMP-NANA into the ER and Golgi, as described by Ryll et al. [26]. All in all, no substantial difference in O-glycosylation of these two cell lines over the course of the batch was detected.

5 Conclusion

The present work extends a previous report of adaptation to glutamine free media to a production cell line and presents our findings on the effect on productivity and product quality in such adapted cells. It was shown that the adaptation to glutamine free medium can be reliably achieved within 10 weeks using the previously described protocol. In a controlled bioprocess

both cell lines behaved similarly with respect to growth and medium consumption, however, cells adapted to 0mM glutamine had an improved robustness against oxidative stress as well as a prolonged life span. Intracellular levels of UDP-sugars resulted in increased antennarity of the produced protein. Productivity itself was only slightly affected.

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Figure 1

Genesis of CHO-EpoFc cell line adapted to high density and glutamine free media.

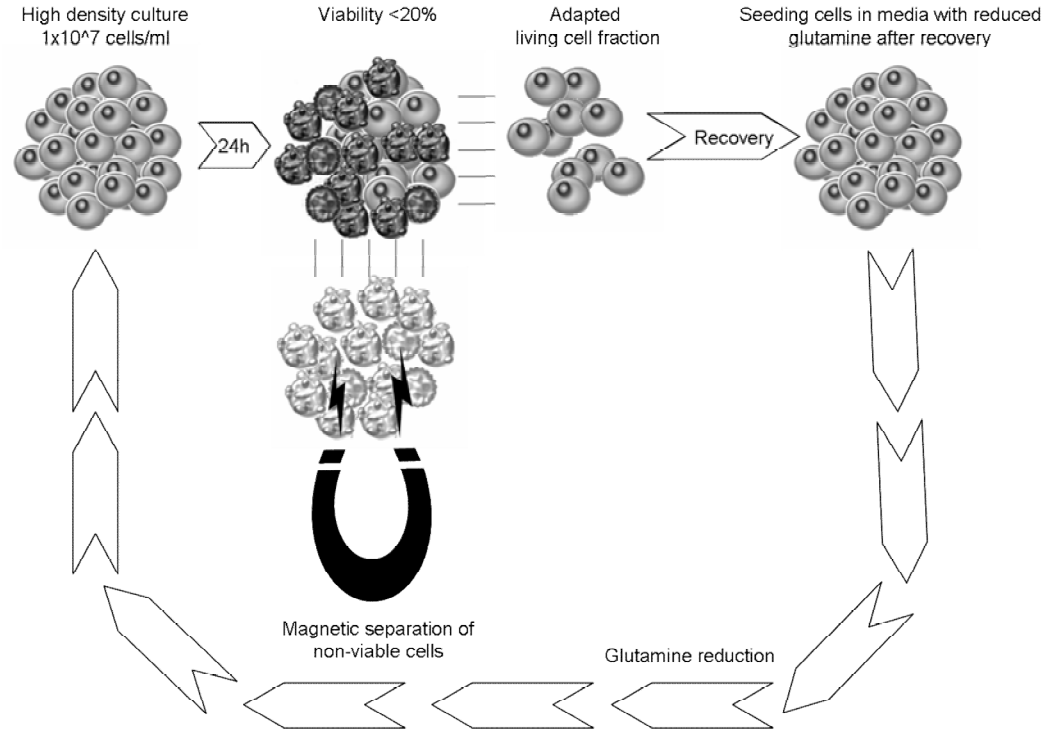


Figure 2

Growth curves of CHO-EpoFc cells during 3 repeated batches in 4 bioreactors. Reactor types: T1/T3 = 4 mM parental cells, T2/T4 = 0 mM adapted cells.

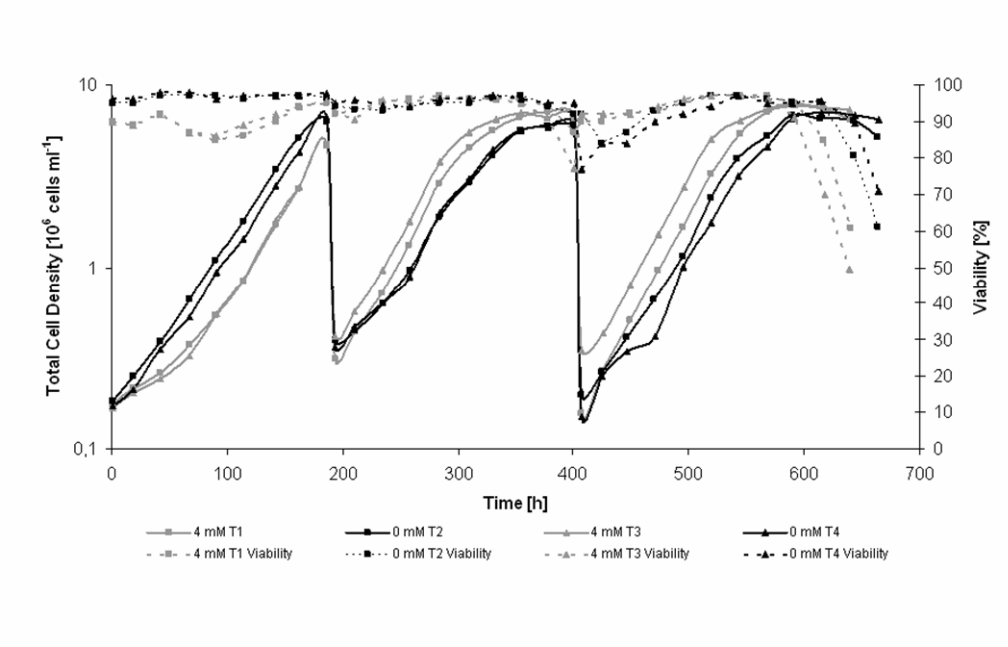


Figure 3

Total and viable cell densities of 3rd batch. Phases labeled are time points of taking samples for glycosylation analysis.

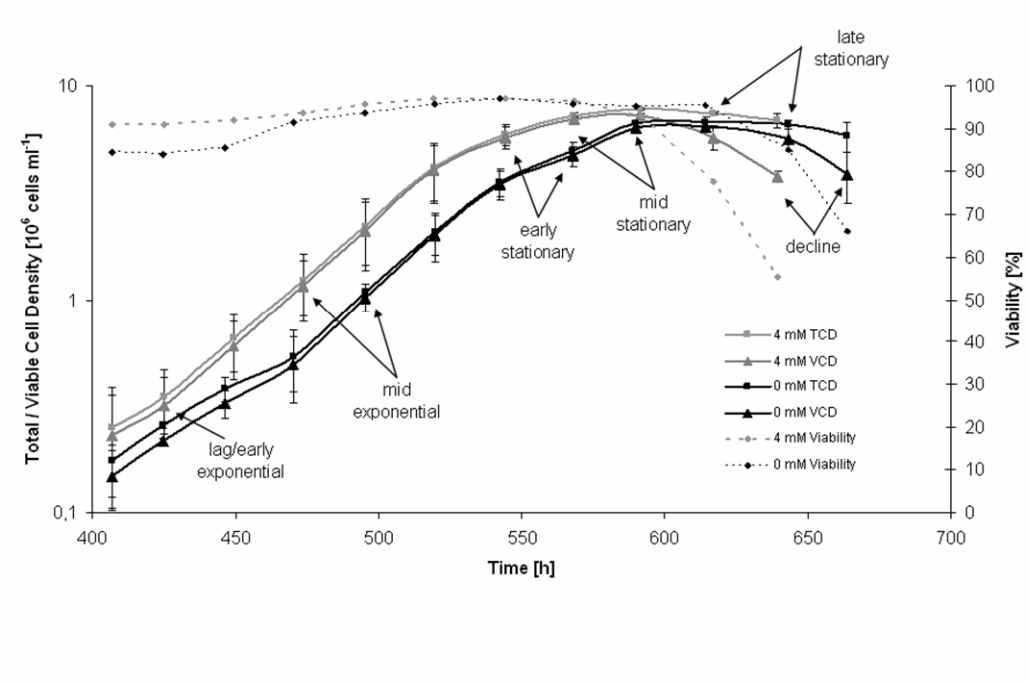


Figure 4

Means of glucose consumption and lactate accumulation (A) and glutamine consumption and ammonia accumulation (B), respectively. (3rd batch)

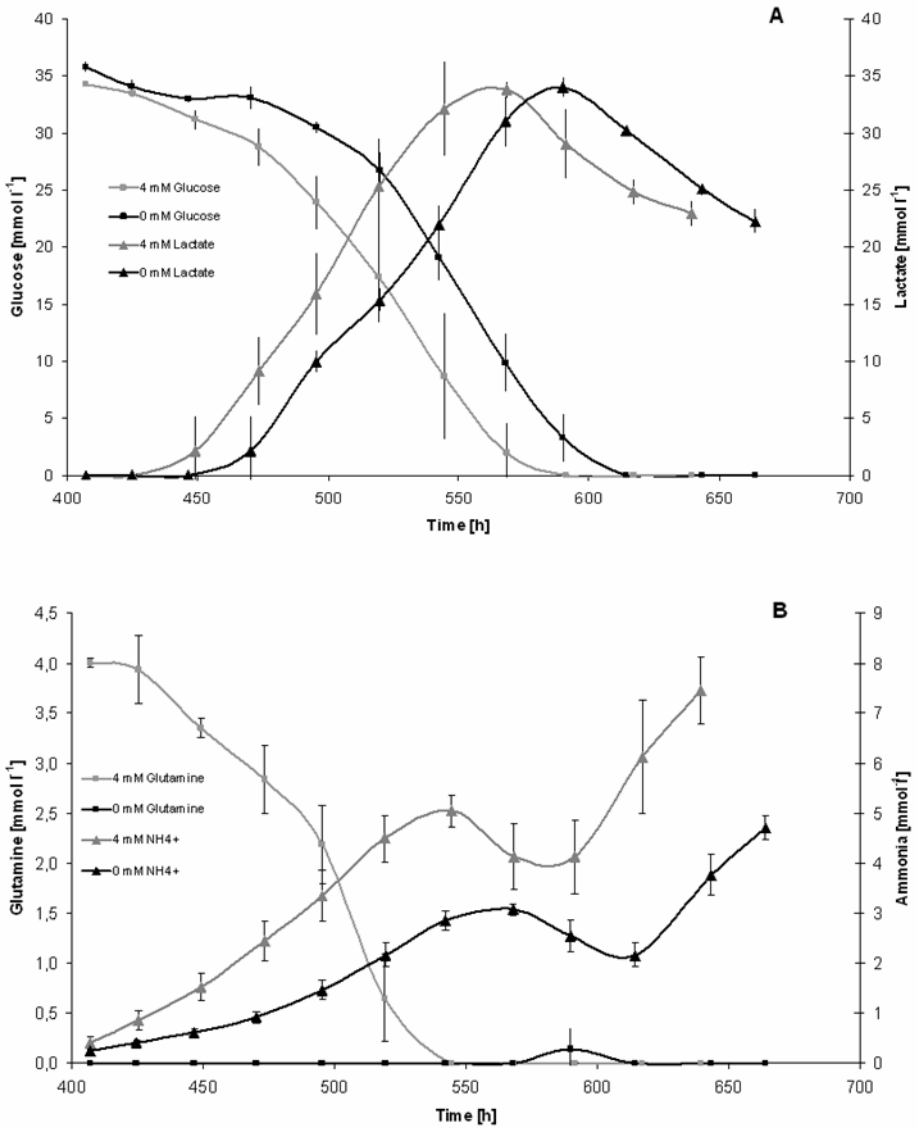


Figure 5

Product concentrations of EpoFc are presented as means of two 0 mM batches and two 4 mM batches respectively. Specific productivity is calculated from means of product titre and viable cumulative cell days based on means of cell concentrations. (3rd batch)

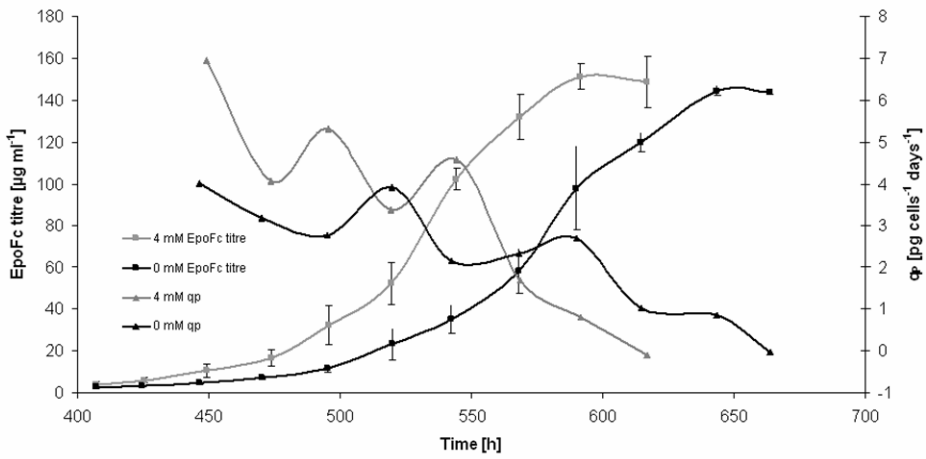


Figure 6

Fluorescent signals for reactive oxygen species (A), superoxides (B) and glutathione (C) and intracellular content of GSSG measured by mass spectrometry (D). Values are means of two 0 mM batches and two 4 mM batches respectively. (2nd batch)

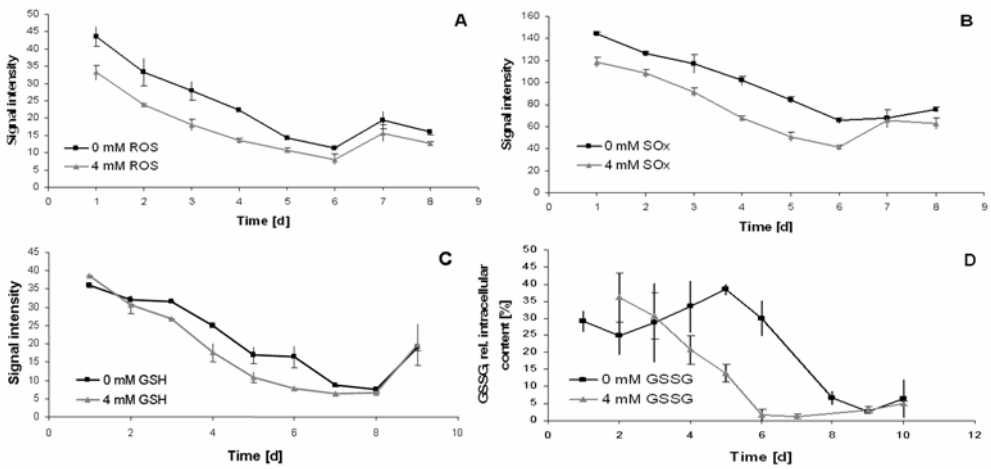


Figure 7

Sugar nucleotides in % of internal standard $^{13}C^{15}N$_AMP/_ADP/_ATP. Values are means of two 0 mM batches and two 4 mM batches respectively. (3rd batch)

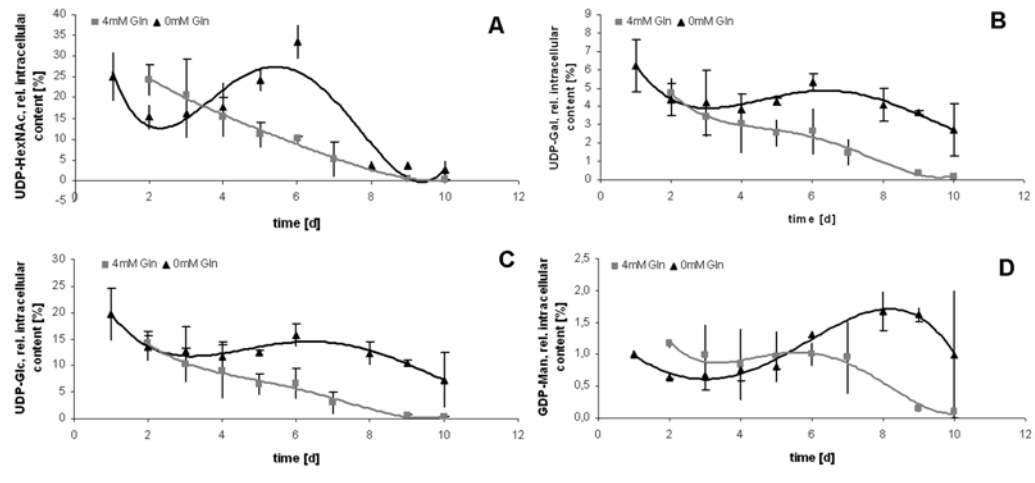


Figure 8

The structure of EpoFc. An Erythropoietin peptide is N-terminally linked to the hinge region of the Fc part of a human IgG molecule. The final molecule is a homodimer.

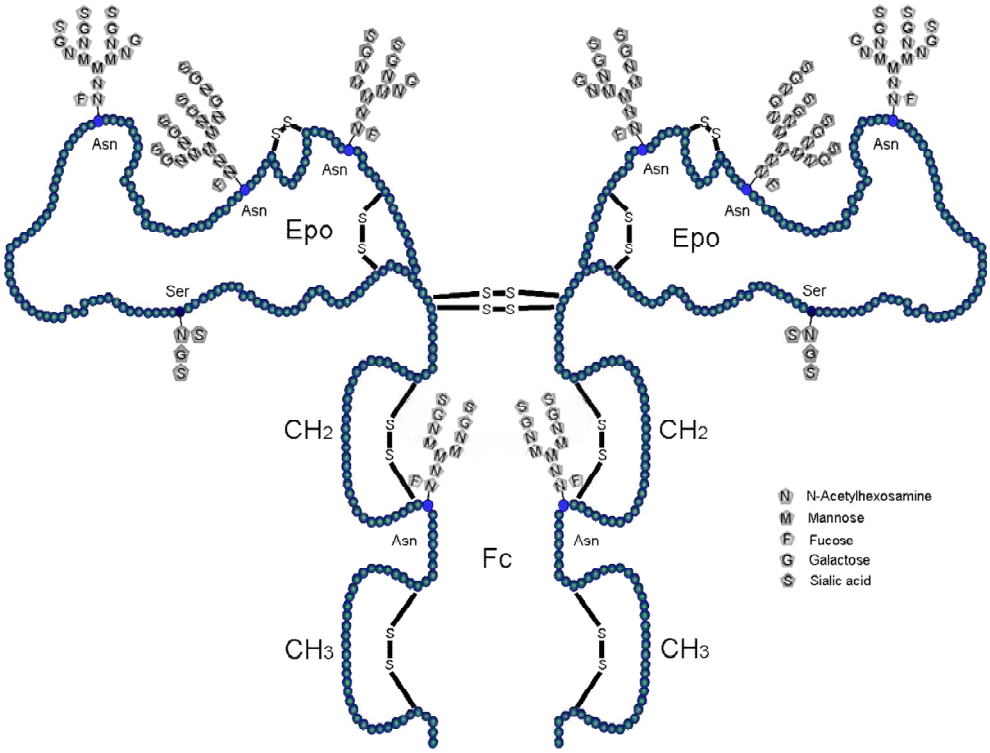


Figure 9

Relative abundances of N- (A) and O-glycosylation (B) in Epo derived tryptic peptides of 0 mM and 4 mM cell lines. (3rd batch) A: Sialylation and antennarity of N-glycans. Site occupancy was 100% throughout. B: Unglycosylated, HexNAc+Hex+NeuAc, HexNAc+Hex+2xNeuAc of O-glycans. (L/EEXP: lag/early exponential, MEXP: mid exponential, ESTAT: early stationary, MSTAT: mid stationary, LSTAT: late stationary, DEC: decline)

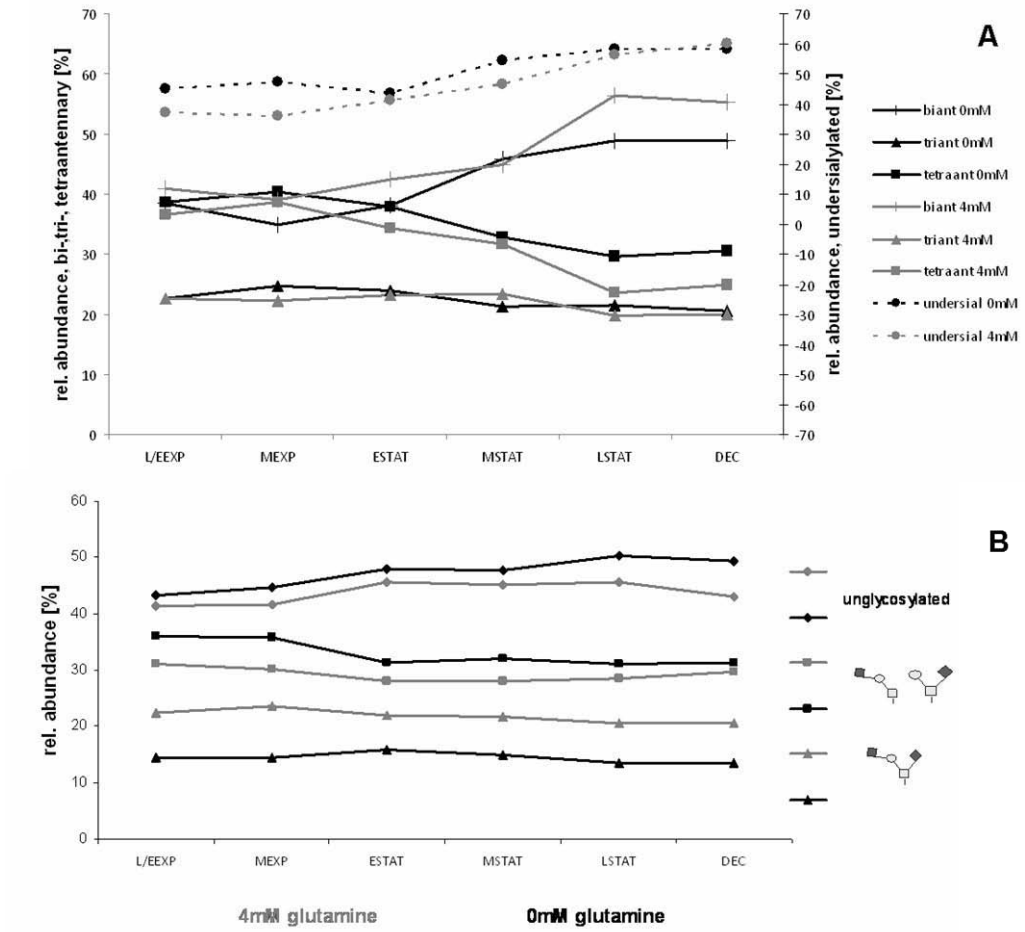
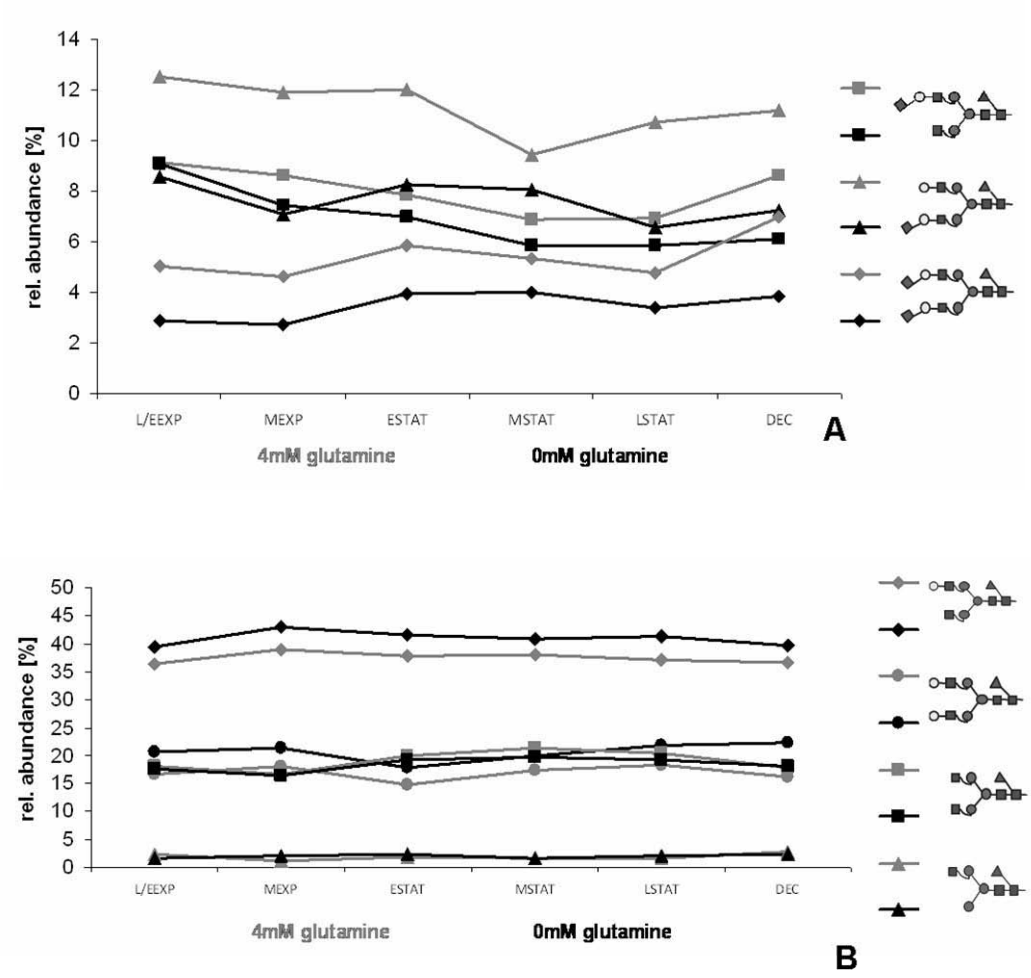


Figure 10

Relative abundances of N-glycosylation in Fc derived tryptic peptides of 0 mM and 4 mM cell lines. (3rd batch) A: Glycans containing sialic acid (NaGnF, NaAF, NaNaF). B: Glycans without sialic acid (AGnF, AAF, GnGnF, GnMF). [according to Consortium of Functional Glycomics]



Appendix

Additional experiments performed

1. Establishment of cell lines by MACS Dead Cell Removal Kit

Objective:

In order to obtain a stress-resistant cell line, a so called "assisted evolution approach" was applied. The main goal was to enhance final cell density in a glutamine free medium compared to the parental cell line in glutamine supplemented medium by cell sorting methods. For the first approaches, the MACS Dead Cell Removal Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) was used.

Execution:

Based on an EpoFc producing cell line originally cultivated in CD-CHO medium with 4 mM glutamine a successive adaptation process was executed. The first step was to adapt the cell line to grow under high density conditions. The second step was to decrease the glutamine supplementation with every adaptation round provided that the cells survive the first step. At the same time CHO-K1 cell lines already adapted to glutamine concentrations of 8, 4, 2 and 0 mM were used for adaptation to high density. Cells were cultivated in spinner flasks with a working volume of 60 ml in a humidified atmosphere containing 7 % CO2 (37 °C, 50 rpm constant stirring). For CHO-EpoFc cells 0,096 mM MTX were added to the medium. Seeding cell concentration was $2x10^5$ cells/ml. After reaching a cell density of $2x10^6$ cells/ml, 50 ml of the culture were centrifuged to gain a total cell concentration of $1x10^8$ cells. Seeded in a T25 roux flask in a volume of 10 ml an initial cells density of $1x10^7$ cells/ml was used to pressurize the cells to adjust their physiology to the new culture conditions. After 24 hours the viability decreased below 20 % and the survivors were tried to be rescued by MACS, assuming that they have been adapted to high density. For the

MACS procedure, a MS selection column was used. According to the instruction manual, an 1x binding buffer was prepared. 1 ml of the binding buffer was used for conditioning of the column. 1 ml of the cell culture was used for cell separation. The cells were centrifuged (300 g, 10 min) and washed with PBS. The supernatant was removed completely and 1x10^7 cells were incubated (30 min) by resuspending them in 100 µl magnetic microbeads. The suspension was applied onto the column placed in the MACS separator and rinsed two times with 500 µl binding buffer. The effluent was collected as live cell fraction with an expected viable cell concentration of approximately $2x10^6$ cells/ml. The cells were seeded in a T25 roux flask in 20 ml volume to gain an initial cell concentration of $1x10^5$ cells/ml.

Results:

The MACS cell sorting was applied four times on every cell line according to the procedure described above. After seeding the living cell fraction the viability was expected to increase every day until the cultures became ready to be passaged into spinner flasks. However, within one week almost every culture died or showed increasing abundance of apoptotic cells.

Conclusion:

Considering the fact that the method was not established well enough the handling with two different cell lines in several batches was rejected for being to expensive. Therefore the approach was reduced to CHO-EpoFc cells. The reason for no culture surviving the sorting was believed to be the method not being able to bind enough early apoptotic cells and cell debris. Apoptotic signals avoiding the survival of the cells could have been the main factor influencing the viability of the cultures. Also the absence of growth factors in fresh medium used for seeding after sorting was believed to have negative effects. A third impact on survival may have been an unfavourable cell concentration to volume ratio when seeding the living cell fraction in T25 roux flasks.

It was decided to choose another sorting method from Immunosolv which was described as being able to capture early apoptotic cells and cell debris very effectively. Additionally, the medium for seeding sorted cells was altered to consist of 50 % fresh medium and 50 % conditioned medium from the former spinner flask cultures. The initial volume for inoculation was altered too. Instead of 20 ml in T25 roux flasks, 24 well plates were used with a volume of 1 ml.

2. Analysis Batch 1

Objective:

After the CHO-EpoFc 4 mM cell line was adapted to high density as well as to glutamine concentrations of 2 mM and 0 mM a spinner flask batch was performed to determine the growth curves of the different cell lines. The objective was to investigate if the adaptation to culture at high density has any effect on final cell density.

Execution:

The batches were performed in spinner flask batches with a working volume of 60 ml in a humidified atmosphere containing 7 % CO2 (37 °C, 50 rpm constant stirring). The media consisted of CD-CHO with glutamine concentrations of 4, 2 and 0 mM respectively. Additional 0,096 mM MTX and 1 ml anti clumping agent per 500 ml media were added. The batches started with an initial cell density of $2x10^5$ cells/ml. Total cell concentrations were measured by using Coulter Counter (Beckman Coulter, Fullerton, CA, USA), and viable cell concentration and viability by using a Neubauer hemocytometer and trypan blue.

Results:

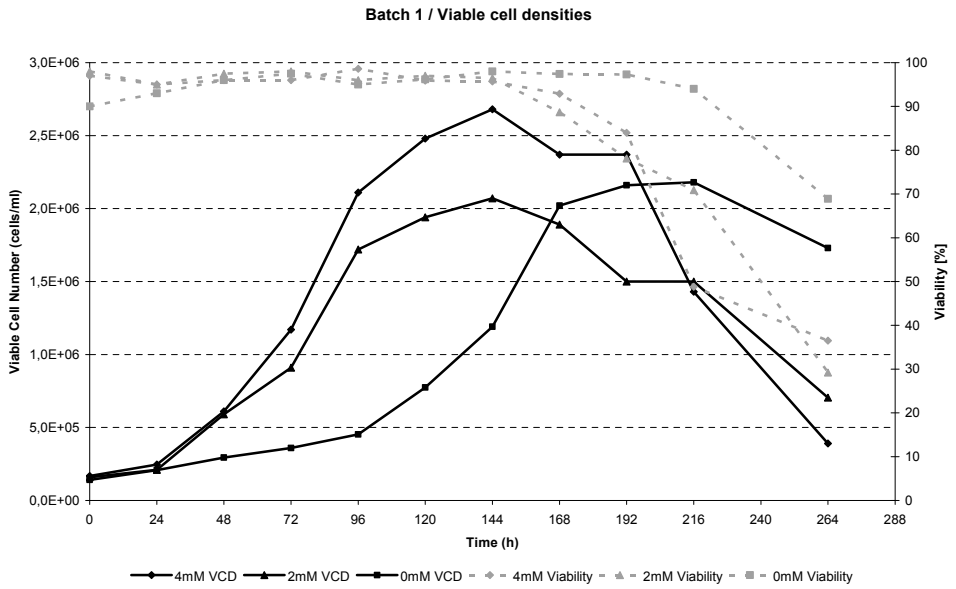


Figure 1: Viable cell densities of CHO-EpoFc with 4 mM, 2 mM and 0 mM glutamine

Conclusion:

The cell lines with 4 mM and 2 mM glutamine did not show a difference in their growth rates (Fig.1). The 0 mM cell line was directly passaged from T25 roux flasks and had a longer lag phase according to its adaptation to spinner flask conditions. Anyway it showed the same growth rate after a delay of 72 hours. Maximum cell density reached $3{,}5x10^6$ cells/ml with the 4 mM and $3{,}0x10^6$ cells/ml with the 2 mM cells. The 0 mM cells reached at least a maximum cell density of $3{,}0x10^5$ cells/ml. For this reason we decided that the high density adaptation did have an effect because a lower cell density was expected without the supplementation of glutamine.

3. Analysis Batch 2 and 3

Objective:

For further verification two additional spinner flask batches were performed with all sustained cell lines. For closer examination the 0 mM and the 4 mM as reference cell line were chosen. The objective was to determine if the cell lines showed growth behaviour comparable to the first batch. Additionally the metabolites in the supernatant and the EpoFc titre have been under investigation.

Execution:

The batches were performed under the same conditions as the first one. The cell concentrations and viability were examined according to those of the first batch.

Metabolites were determined by everyday measurement using a Bioprofiler (YSI 7100 MBS Bioanalytical System, YSI Life Sciences, USA). The EpoFc productivity was quantified by ELISA using polyclonal goat anti human IgG (γ-chain specific) antibody (Sigma, I-7883) for coating and a horseradish peroxidase conjugated polyclonal goat anti human IgG (γ-chain specific) antibody (Zymed, 62-8420) for binding to EpoFc. The ELISA was performed according to the internal protocol of Polymun.

Results:

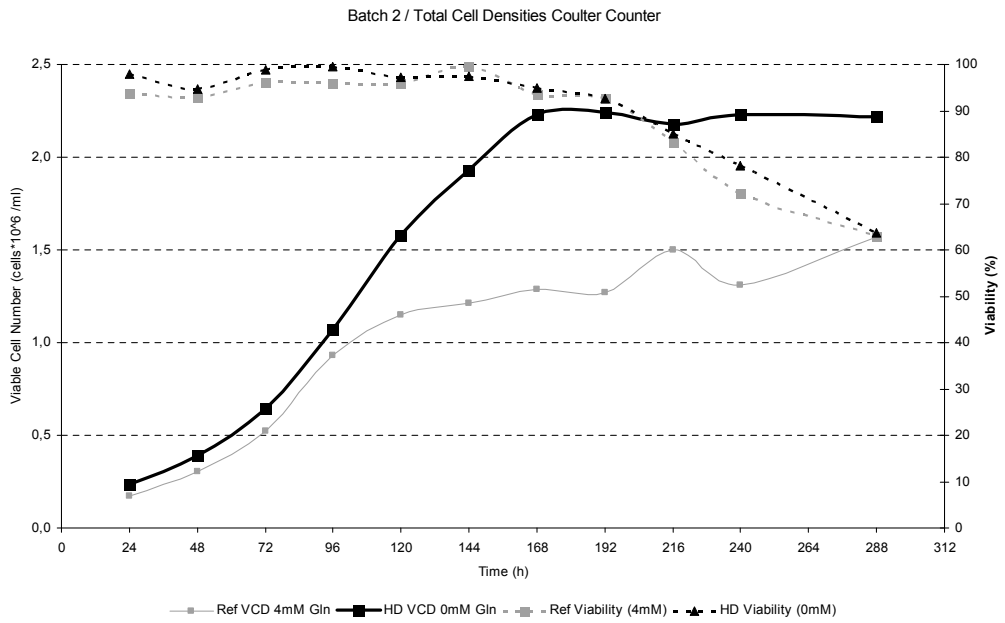


Figure 2: Total cell densities and viability of 4 mM and 0 mM cell lines of batch 2

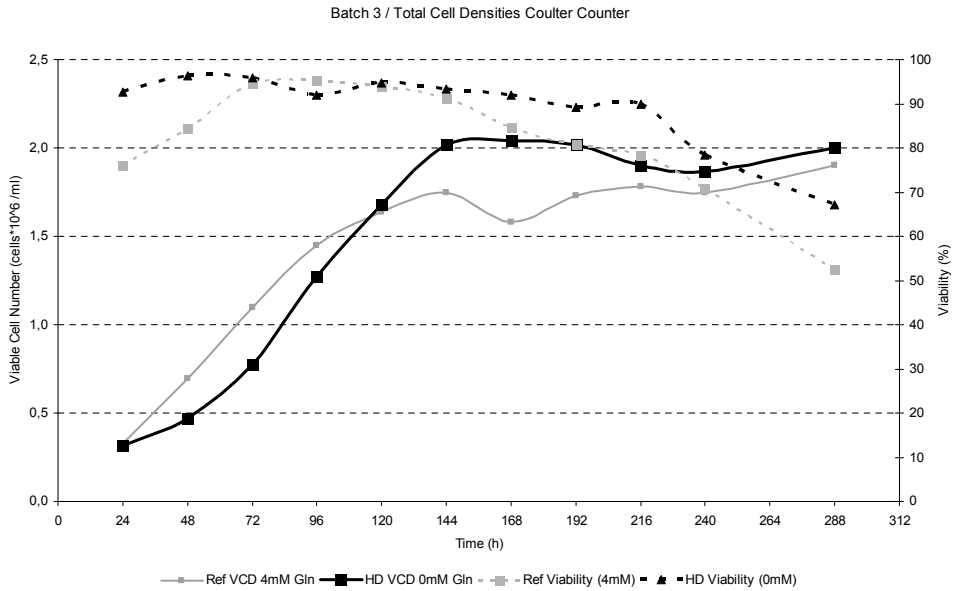


Figure 3: Total cell densities and viability of 4 mM and 0 mM cell lines of batch 3

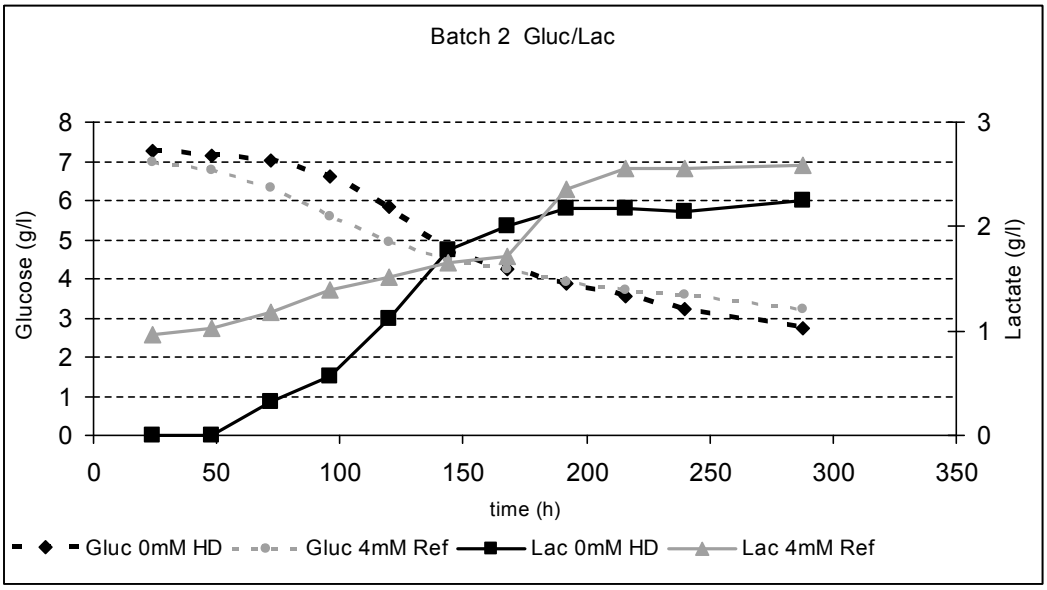


Figure 4: Total concentrations of glucose and lactate of batch 2

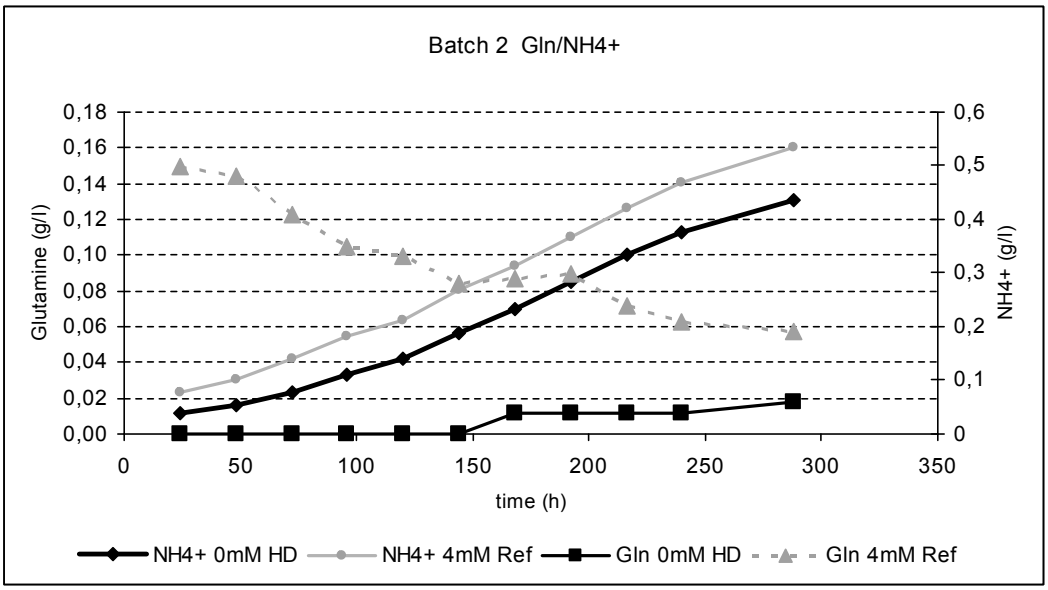


Figure 5: Total concentrations of glutamine and NH4+ of batch 2

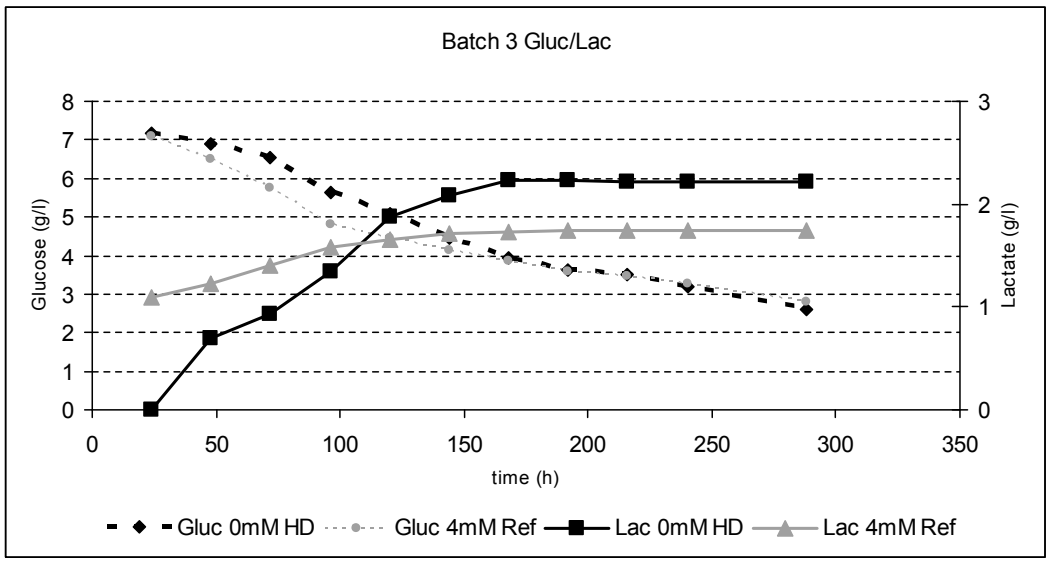


Figure 6: Total concentrations of glucose and lactate of batch 3

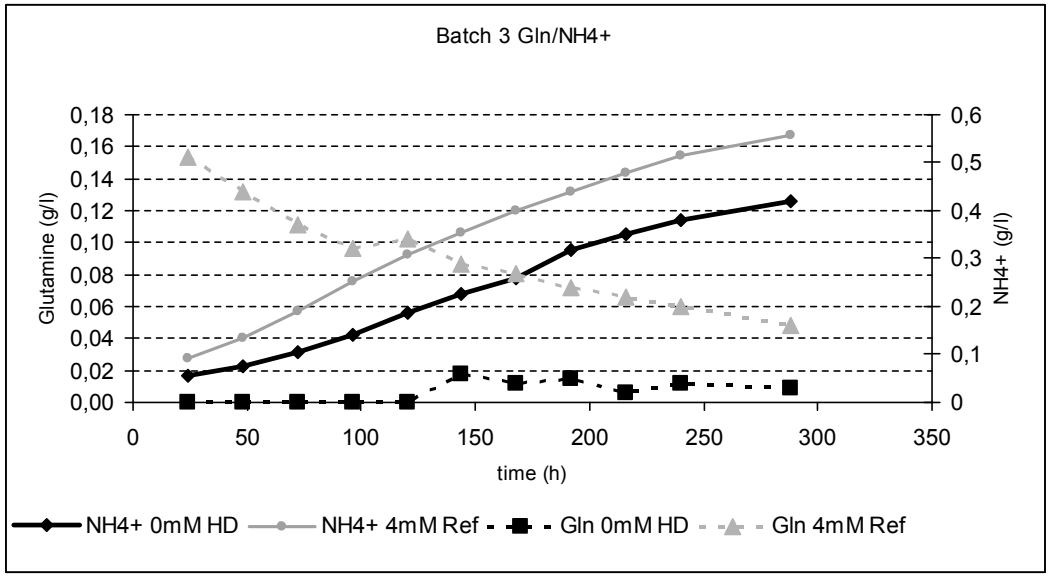


Figure 7: Total concentrations of glutamine and NH4+ of batch 3

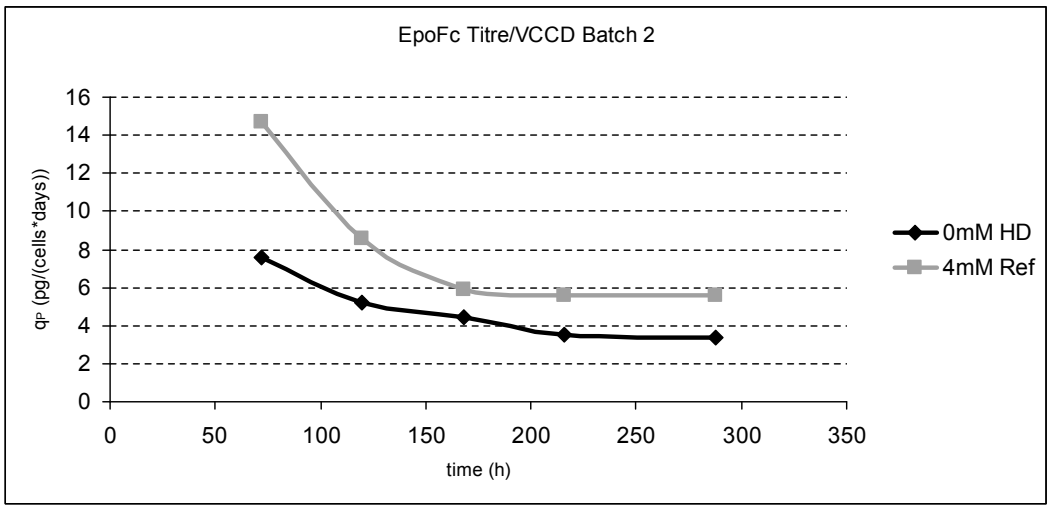


Figure 8: EpoFc product titre divided by cumulative integral of viable cell days of 0 mM and 4 mM cells of batch 2.

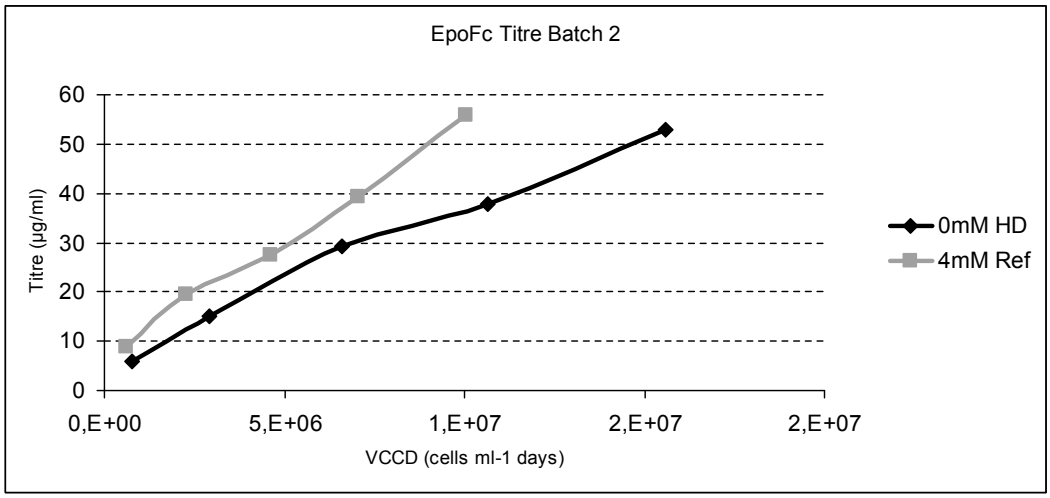


Figure 9: EpoFc product titre over cumulative integral of viable cell days of batch 2.

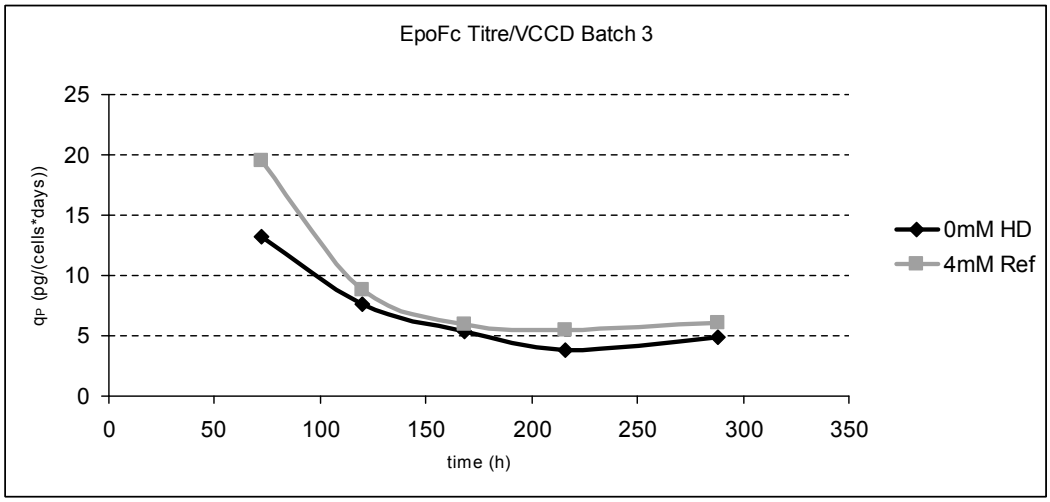


Figure 10: EpoFc product titre divided by cumulative integral of viable cell days of 0 mM and 4 mM cells of batch 3.

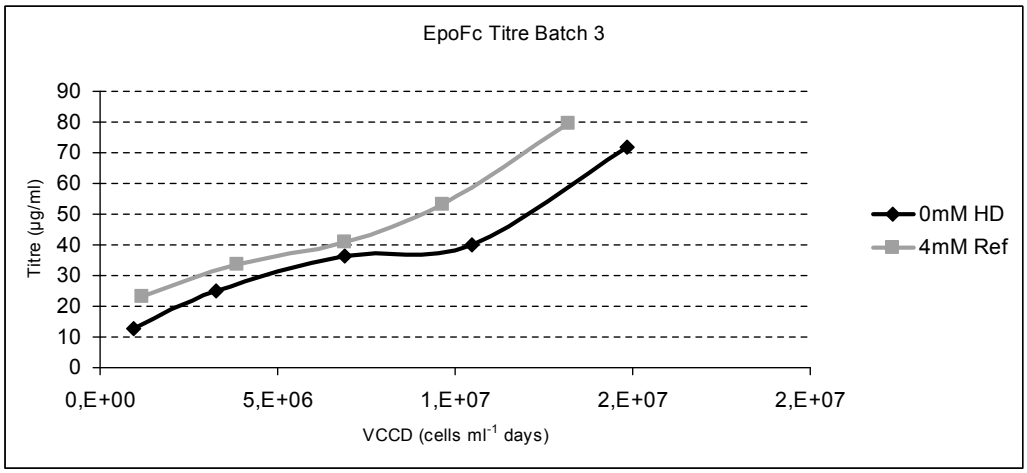


Figure 11: EpoFc product titre over cumulative integral of viable cell days of batch 2.

Conclusion:

It can be stated that the two cell lines almost had the same growth rate at the beginning of the culture (Fig.2). At a cell concentration of $1x10^6$ cells/ml the 4 mM cells started to decline and reached a final cell concentration of $1,5x10^6$ cells/ml. The same cell line had a peak cell density of $1,7x10^6$ cells/ml (Fig. 3). Due to the 0 mM cells reaching a cell concentration above $2x10^6$ cells/ml in both figures the positive effect of the high density adaptation could be proofed. Culture life time was the same within both cultures.

Glucose consumption was approximately 15 % lower with the 0 mM cells during exponential phase but became the same for both cell lines during stationary phase (Fig. 4/6). Lactate accumulation in the 0 mM cultures started later and reached a level of 50 % of that of the
4 mM cultures after 75-100 hours. At the end of the culture the final lactate accumulation was 2,3 g/l with the 0 mM in both batches. The final lactate concentration with the 4 mM cell line varied between 1,75 and 2,5 g/l. Considering the higher performance in cell density during batch 2 (Fig.2) the overall lactate production of the 0 mM cells was significant lower than that of the 4 mM cells.

Considering the production of ammonia (Fig. 5/7) it can be stated that its accumulation during both batches was constantly 30 % lower with the 0 mM cells.

The titre of EpoFc was divided by the integral of viable cell days in Figures 8 and 10 which gives a better conclusion about the productivity of the cells. In batch 2 the productivity of the 0 mM culture was only 60 % of that of the 4 mM whereas in batch 3 the productivity reached a better grade. Nevertheless, the total product concentration in batch 2 got as high as 52 µg/ml in the 0 mM culture and 55 µg/ml in the 4 mM culture. In batch 3 it got as high as 72 µg/ml in the 0 mM culture and 79 µg/ml in the 4 mM culture (Fig. 9/11).
Those results indicate that due to the fact that every cell line has the same starting conditions in terms of glucose, media volume and initial cell concentrations and despite the fact that the productivity of the 0 mM cell culture is lower, an adequate final product concentration could be reached without the supplementation of glutamine.

4. Analysis of glycosylation pattern of batch 3

Objective:
The hypothesis was that due to growth without glutamine an expected lower ammonia level should have a positive effect on the glycosylation pattern of EpoFc. The sialylation and the complexity of the the sugar residues have been of interest therefore.

Execution:
Samples of the culture supernatant from batch 3 were taken from day 4, 8 and 12 for analysis by the Department of Biochemistry. The glycosylation was determined by Friedrich Altmann and Martin Papst using an internal protocol for mass spectrometry. The N-Glycosylation site in the tryptic peptide "GQALLVNSSQPWEPLQLHVDK" and the O-Glycosylation site in EPO-derived tryptic peptide "EAISPPDAASAAPLR" were received in relative abundance.

Results:

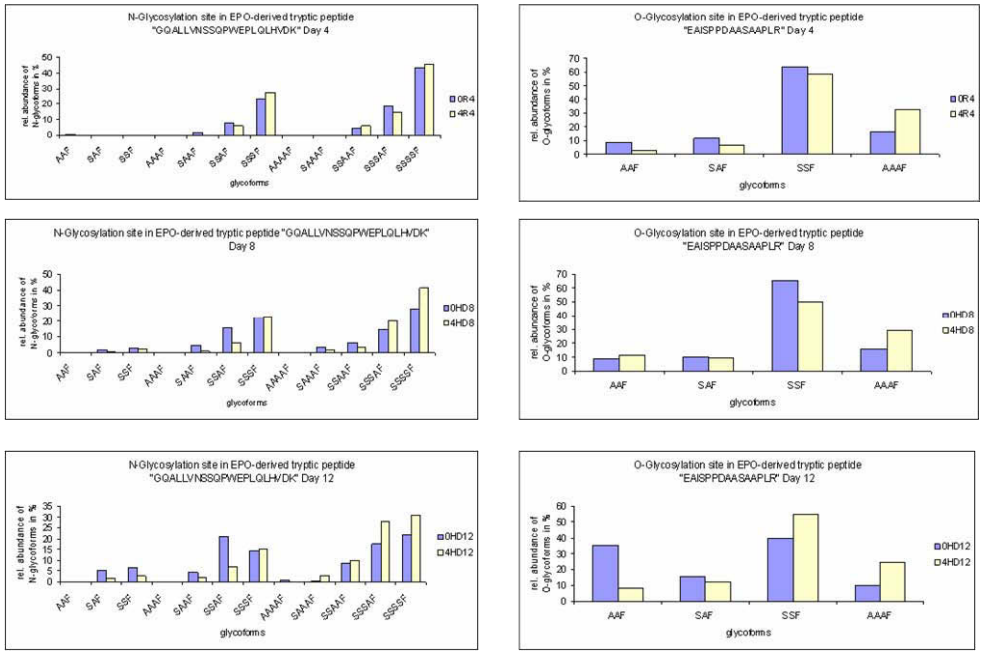


Figure 12: Relative abundance of N- and O-Glycosylation in Epo derived tryptic peptides. Cell lines are 0 mM and 4 mM high density adapted (HD) and their parental cell lines (R). S=Sialic acid, A=Galactose, F=Fucose

Conclusion:

Comparing the high density adapted cell line and the corresponding parental cell line of the 0 mM and the 4 mM cells were chosen (Fig. 12). Due to problems with the analytical method results were gained just for the parental cell lines from day 4 and high density cell lines from day 8 and 12. However, the results do not indicate significant tendencies and are difficult to interpret. Considering the N-Glycosylation it can be seen that the oligosaccharides SSSAF and SSSSF show a slightly higher abundance in the 4 mM cell line. On the other hand there is a tendency of a shift from longer glycoforms to shorter glycoforms from day 4 to day 12 with the 0 mM cells. It may be stated that more sialylated glycoforms have higher abundances in the 4 mM cells and single or double sialylated glycoforms have higher abundances in the 0 mM cells. Considering the O-Glycosylation the results do not indicate a concrete behaviour in distribution of the different glycoforms. Generally the 0 mM cell line shows lower sialylation grades. The glycoform SSF on day 12 is the only one with a higher abundance in the 0 mM cell line.

5. Analysis of aminoacid contents of fermentation batch 3

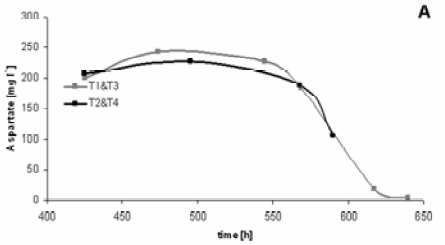
Objective:

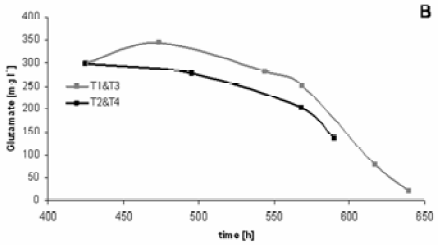
To gain additional indications for potential metabolic changes, the aminoacid concentrations during batch 3 have been determined.

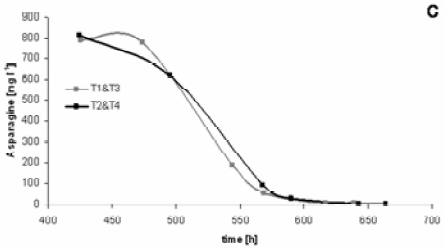
Execution:

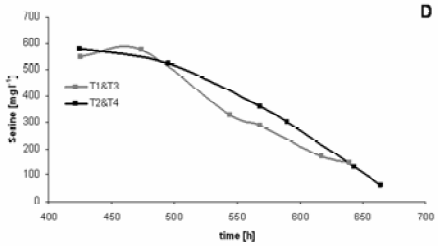
Samples were taken from 6 time points over the course of batch 3 from every bioreactor and applied on HPLC. 18 aminoacids were analysed from 4 mM and 0 mM cell lines.

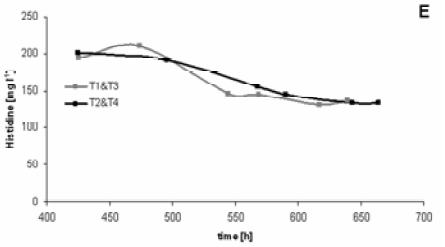
Results:

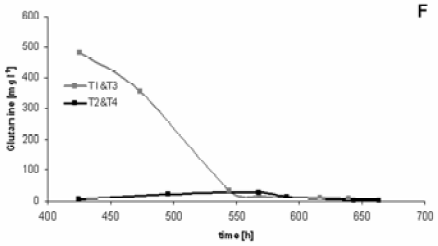

A
Aspartate [mg l-1]
T1&T3
T2&T4
time [h]

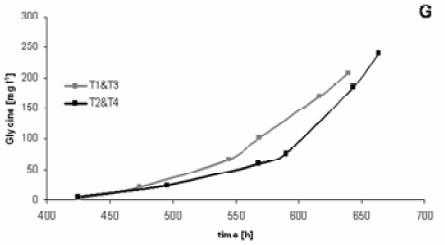

B
Glutamate [mg l-1]
T1&T3
T2&T4
time [h]

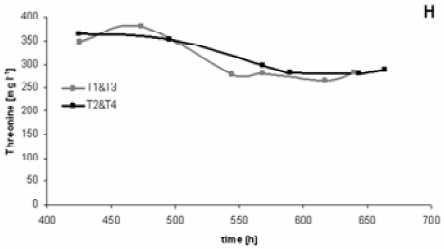

C
Asparagine [mg l-1]
T1&T3
T2&T4
time [h]

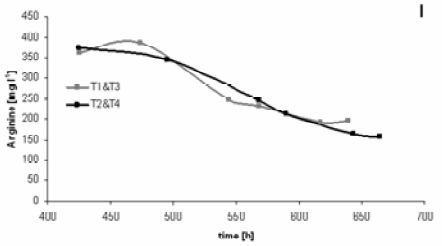

D
Serine [mg l-1]
T1&T3
T2&T4
time [h]

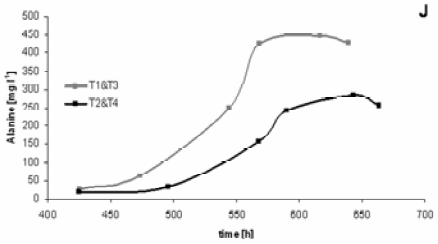

E
Histidine [mg l-1]
T1&T3
T2&T4
time [h]

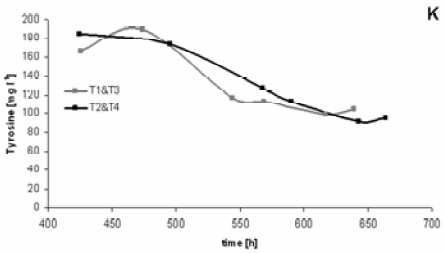

F
Glutamine [mg l-1]
T1&T3
T2&T4
time [h]

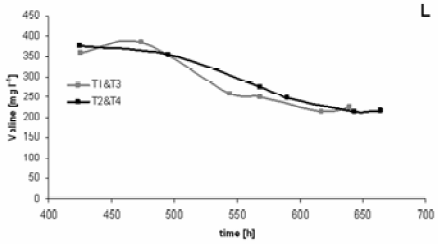

G
Glycine [mg l-1]
T1&T3
T2&T4
time [h]


H
Threonine [mg l-1]
T1&T3
T2&T4
time [h]


I
Arginine [mg l-1]
T1&T3
T2&T4
time [h]


J
Alanine [mg l-1]
T1&T3
T2&T4
time [h]


K
Tyrosine [mg l-1]
T1&T3
T2&T4
time [h]


L
Valine [mg l-1]
T1&T3
T2&T4
time [h]

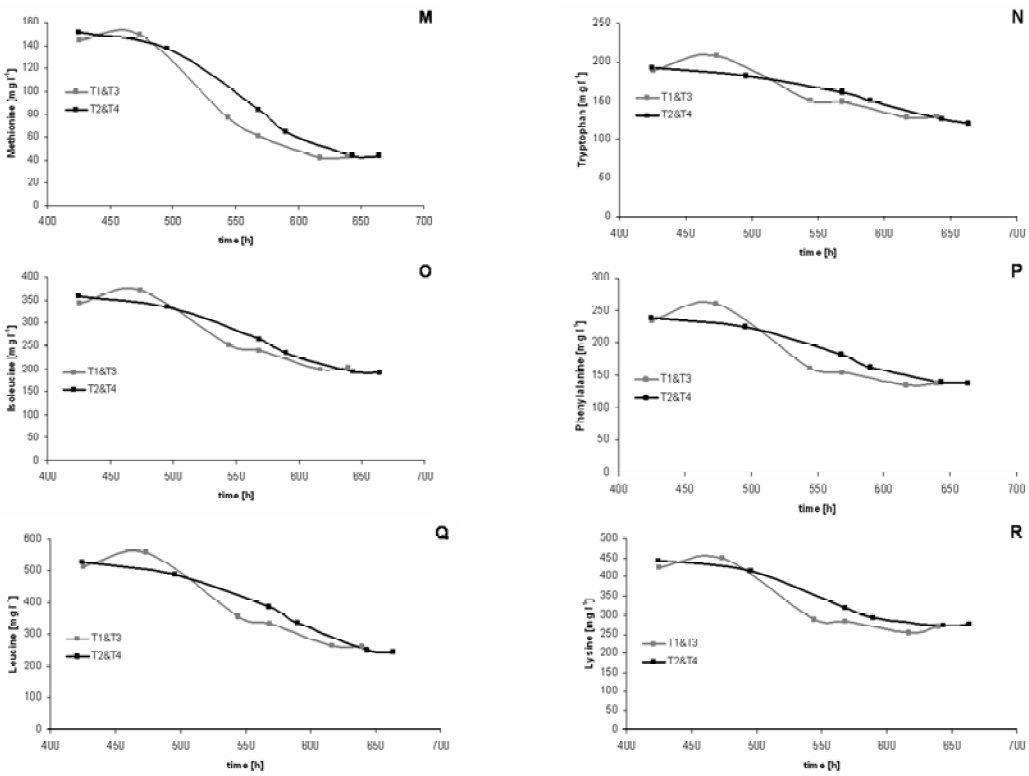


Figure 13: Diagrams A-R represent the concentrations of 18 aminoacids. T1&T3 are 4 mM cells, T2&T4 are 0 mM cells.

Conclusion:

No significant unexpected differences were observed between cells growing with or without glutamine. Almost every aminoacid was consumed at comparable rates in both cell lines except glycine, which accumulated over the batch but without differences between the cultures. The glutamate level was slightly lower in the 0 mM cell line as a result of the absent glutamine. The only variation occurred in alanine production of the 0 mM cultures which reached the twofold level of the 4 mM cultures. This indicated that as a response to the intracellular ammonia level in 4 mM cells, which was also twice as high compared to the glutamine free cells, alanine production could have been used as ammonia detoxifying pathway. Considering the delay of the exponential phase of the 0 mM cells, this cell line has consumed most of the amino acids to a higher degree during the first two culture days balancing the lack of glutamine.

6. Analysis of nucleotides and sugar nucleotides of fermentation batch 3 (additional data)

Objective:

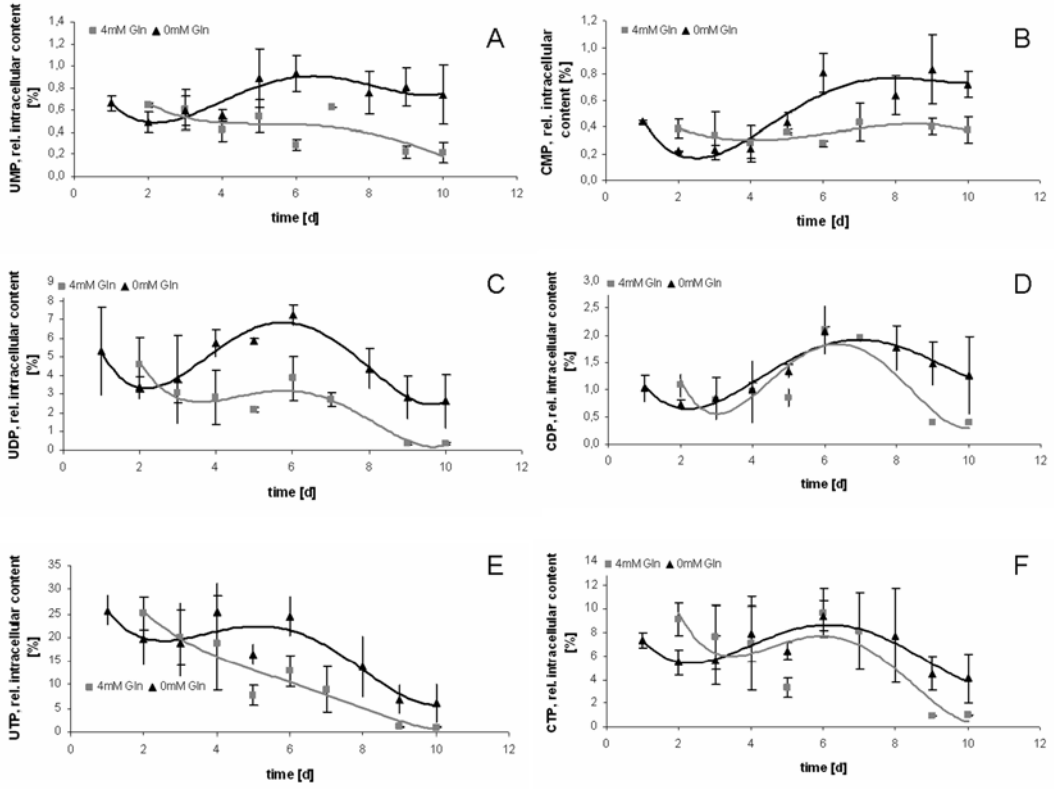
For determination of the physiological state and energy level of the cells, the nucleotides and sugar nucleotides, which act as glycosylation precursors, were analysed.

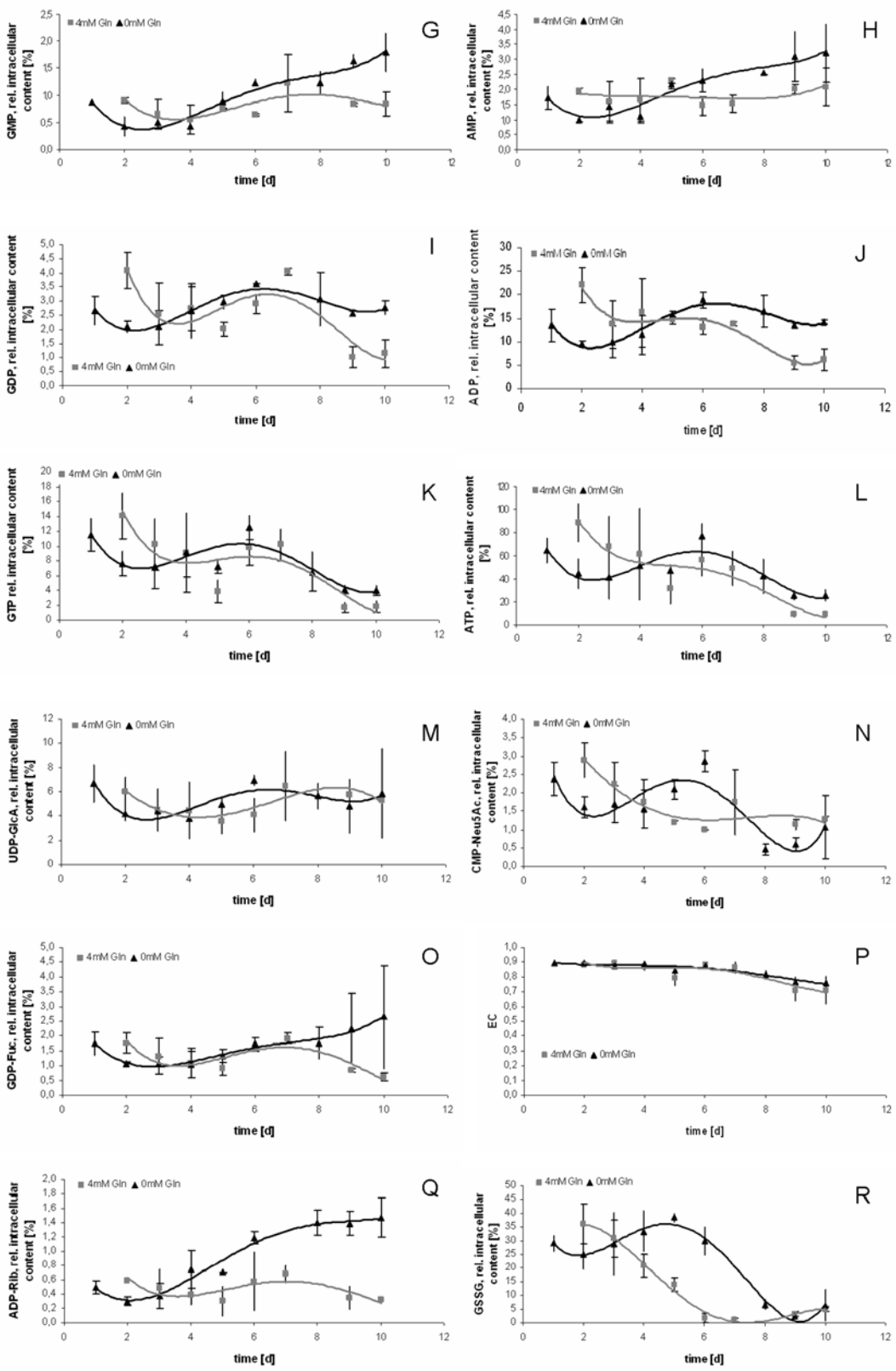
UDP-HexNAc, UDP-Gal, UDP-Glc and GDP-Man were chosen as representative data for the paper article. Additional results are annexed here.

Execution and conclusion:

Reference is made at this point to the analytical procedures and discussions described in the paper article.

Results:




G
GMP, rel. intracellular content [%]
H
AMP, rel. intracellular content [%]
I
GDP, rel. intracellular content [%]
J
ADP, rel. intracellular content [%]
K
GTP rel. intracellular content [%]
L
ATP, rel. intracellular content [%]
M
UDP-GlcA, rel. intracellular content [%]
N
CMP-Neu5Ac, rel. intracellular content [%]
O
GDP-Fuc, rel. intracellular content [%]
P
EC
Q
ADP-Rib, rel. intracellular content [%]
R
GSSG, rel. intracellular content [%]
4mM Gln
0mM Gln
time [d]

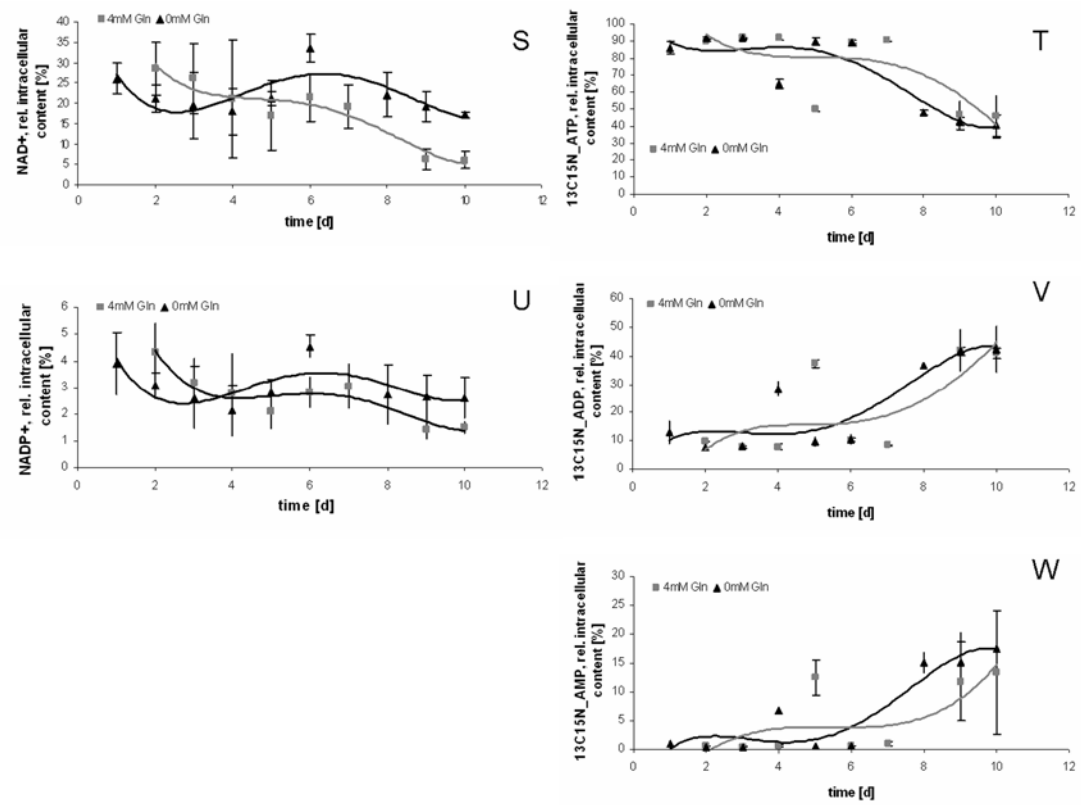


Figure 14: A-W, Nucleotides and sugar nucleotides of fermentation batch 3.