Impact of Process Parameters on Cell Growth, Metabolism and Antibody Glycosylation

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“The process is the product.”
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Abstract

Bioreactor process parameters influence the growth behavior and metabolism of mammalian cell culture, and the quality of the produced monoclonal antibodies (mAbs). A systematic assessment of their impact allows for a better link between process-related parameters and cellular processes, such as N-linked glycosylation, lactate metabolism and cell cycle transition. Thus, understanding the effect of process parameters is a prerequisite for providing better controllability over cell growth, productivity and mAb critical product quality.

Initially, we investigated the effect of single and combined process parameters on the glycan microheterogeneity of an IgG1 antibody using a shift-experiment procedure in batch culture of a hybridoma cell line. The N-linked glycosylation profile of the murine IgG1 was found to be highly complex since it included terminal galactosylation and sialylation, as well as variable core-fucosylation. Within a pH range of 6.8 to 8.0, differences in galactosylation and sialylation of approximately 50% were obtained. Variation of dissolved O2 (10 – 90% air saturation) resulted in a maximum variability of 20% in galactosylation and 30% in sialylation. In contrast, no significant effect on the glycosylation profile was observed when osmolarity increased from 320 to 420 mOsm/kg and sparging from 0.05 to 0.2 vvm.

Apart from its role in glycosylation, pH alteration during the early exponential growth phase was found to control lactate formation and consumption. In particular, lactate consumption was induced even at high glucose concentrations at pH 6.8, whereas highly increased production of lactate was obtained at pH 7.8. Lactate accumulation in mammalian cell culture is known to impede cellular growth and productivity. Consequently, constraint-based metabolic flux analysis was used to examine pH-induced metabolic states in the same growth state. We demonstrated that lactate influx at pH 6.8 led cells to maintain high fluxes in the TCA cycle and malate-aspartate shuttle resulting in a high ATP production rate. In contrast, under increased pH conditions, less ATP was generated and different ATP sources were utilized. Gene expression analysis led to the conclusion that lactate formation at high pH was enabled by gluconeogenic pathways in addition to facilitated glucose uptake. The obtained
results provide new insights into the influence of pH on cellular metabolism, and are of importance when considering pH heterogeneities typically present in large-scale industrial bioreactors.

On industrial scale, fed-batch processes are mainly used for mammalian cell cultivation and protein production. Cell productivity in fed-batch processes can be increased by cell cycle arrest through mild hypothermia. Since hypothermia can simultaneously reduce cell growth, which is regulated by the cell cycle, temperature shifts have to be considered on the cell cycle level. Consequently, the time point for the temperature shift is important and requires optimization. An unstructured cell cycle model including the distribution of proliferating (G1, S, G2/M) and arrested cells (G0) has been proposed in order to predict the time point of temperature shift in fed-batch culture. The mathematical model is generally applicable and a procedure on how to evaluate the required model parameters is described. The parameters are estimated from batch and fed-batch cultivations, each carried out once at 37 °C and once at 33 °C in order to characterize temperature dependency. The batch cultures are also used to evaluate substrate depletion and fed-batch cultures are used to study the impact of metabolite accumulation on the cell cycle, in particular on the quiescent cell cycle phase. The reliability of the proposed procedure for parameter estimation is validated using a mAb-producing hybridoma cell culture and the model predicts hypothermic transitions within the cell population at different shift time points. Thus, this framework can be used to optimize the time point of the temperature shift, which is commonly adjusted in industrial fed-batch processes in order to obtain a good balance between temperature induced growth limitation and cell cycle specific enhanced productivity.

In this work, a better understanding of bioprocess-related parameters affecting growth, metabolism and critical quality attributes under the scope of quality by design (QbD) is provided and can bring us one step closer towards stable process performance while ensuring desired glycosylation for therapeutic proteins.
Zusammenfassung


In dieser Arbeit wurden HFN 7.1 Hybridomazellen zunächst im Batch-Modus kultiviert und während der exponentiellen Wachstumsphase die Veränderung einzelner sowie kombinierter Prozessparameter vorgenommen. Auf diese Weise konnten die Parameterinflüsse auf die Verteilung der Glykanstrukturen eines IgG1 Moleküles untersucht werden. Aufgrund terminaler Galaktosylierung und Sialylierung, sowie variabler Fukosylierung, stellte sich das N-Glykosylierungprofil als äussert komplex dar. Die Unterschiede in terminaler Galaktosylierung und Sialylierung innerhalb eines pH-Bereiches von 6.8 bis 8.0 lagen bei ungefähr 50%, wohingegen die Variation des Gelöstsauerstoffes (10 – 90% Luftsättigung) in einer Änderung von 20% in Galaktosylierung und 30% in Sialylierung resultierte. Im Gegensatz dazu, führte eine abrupte Veränderung der Osmolarität in einem Bereich zwischen 320 und 420 mOsm/kg, sowie eine Änderung der Begasungsraten von 0.05 bis 0.2 vvm zu keinem Unterschied im Glykosylierungsprofil.


eine Optimierung des Zeitpunktes, an dem eine Temperaturänderung vorzunehmen ist um ein gutes Gleichgewicht zwischen temperaturbedingter Wachstumslimitation und erhöhter zellzyklusspezifischer Produktivität zu erhalten.

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1 Introduction

Around 30% of all drugs approved by the European Medical Agency (EMA) up to 2012 were biologically-derived. Among them glycoproteins are the largest and fastest growing group [1]. Mammalian cells are the predominant production system of glycoproteins due to their ability to express large proteins and perform post-translational modifications, such as glycosylation [2]. Therapeutic glycoproteins require glycosylation for their biological function, and mammalian cells possess the machinery to provide proper glycosylation that is human-like and therefore shows a reduced risk of immunogenicity [3]. Only recent advances in yeast and plant technology have led to the first approvals of a \textit{P. pastoris}-derived glycoprotein [4], and a genetically modified plant cell-derived glycoprotein [5] by the US Food and Drug Administration (FDA). Nonetheless, the use of mammalian cell culture for the production of complex, large, recombinant proteins for therapeutic or diagnostic purpose remains unparalleled to date.

1.1 Mammalian Cell Culture Technology

Mammalian cell culture technology has evolved dramatically since its first use for large-scale vaccine production in the 1960s [6]. The biggest advances in mammalian cell culture technology included the establishment of continuous, immortal cell lines that could be grown in suspension in contrast to earlier adherent primary cell lines [7]. Thus, immortal cells, such as baby hamster kidney cells (BHK) for veterinary use, and later Chinese hamster ovary (CHO), myeloma (Sp2/0, NS0) and human embryonic kidney (HEK) cells, facilitated the production in large-scale bioreactor systems. Due to the simpler scale up by volume instead of surface, reactor scales could be extended to 10 000 L systems, such as for the production of interferon alpha in the 1980s [8]. In addition, immortal cell lines could be genetically modified with emerging recombinant DNA technology. As a result, tissue-type plasminogen activator protein was the first commercial recombinant protein produced in a CHO cell line [9].

Over the years a variety of recombinant proteins was generated from mammalian cell culture such as cytokines, hormones, growth factors, clotting factors, vaccines, fusion
proteins and monoclonal antibodies (mAbs) [10], [11]. Since the first approval of a monoclonal antibody drug in 1987 [12], therapeutic mAbs mainly used for the treatment of cancer, anti-immune and inflammatory diseases, have witnessed an incredible growth. Nowadays, they represent the most important drug product class with currently 40 FDA-approved mAbs, and annual sales of around $50 billion [13], which represents around 40% of the total biologically-derived drug market [11]. The increase in monoclonal antibody drugs also fostered a corresponding improvement of the production processes to meet the high product demand of several tons/year [14]. Such a development was achieved by the generation of high-producer cell lines, mostly CHO cells, using optimal gene amplification systems [15] and by including growth-promoting genes [16]. Such genetic engineering approaches resulted in a 2-fold increase in specific productivity [17]. More interestingly, a 20-fold increase in volumetric productivity could be obtained in the same time frame through optimization of bioprocess conditions including the control of the culture environment and the development of chemically defined media and optimized feeding techniques [7], [17]. As a consequence, newly developed fed-batch production processes are reported to produce mAb titers of more than 10 g/L [15], [18].

Considering the recent emergence of biosimilar drugs [10], the focus in mammalian cell culture technology and bioprocess development is shifting increasingly towards matching given product attributes, rather than aiming exclusively at increased growth and productivity. Small differences in the three-dimensional structure, charge variants profile or post-translational modifications of the protein can arise from minor variations in the production process and can result in differences in drug efficacy in comparison to the originator drug [19], [20]. Considering post-translational modifications, different levels of core-fucosylation in the glycosylation pattern have been shown to have severe consequences on the effector function of a mAb [21]. Thus the process as a whole has to be put into focus in order to qualify for biosimilarity [10]. Moreover, methods that can link individual process steps to product quality require development.
1.2 Operating Conditions in Bioreactor Systems

Mammalian cells for the production of recombinant proteins are typically cultured in stirred tank bioreactors in a scale from 10 L to 20 000 L. Such reactors require a suitable design to provide proper mixing and mass transfer of oxygen and carbon dioxide (CO₂) [22]. Physical control parameters are the inlet gas flow rate, stirring speed and temperature, whereas pH, osmolarity, dissolved oxygen (DO) and dissolved CO₂ are considered chemical control parameters. Process parameters are used to define the optimal culture environment that is intended to provide homogenous conditions to the cultured cells. Sensors and control systems are used to monitor and adjust the culture environment. pH is controlled through CO₂ sparging and base addition, while constant dissolved oxygen levels are provided by sparging air or oxygen into the culture at constant gas flow rates. While initial limitations regarding shear stress sensitivity of mammalian cells by agitation and aeration have been mostly overcome [23–25], other process parameters are still known to cause variations in growth, productivity and product quality, in particular during scale-up. pH heterogeneity can arise from base addition in poorly mixed bioreactors [26]. Poor mixing can possibly also lead to gradients in temperature and osmolarity due to the addition of base and feed close to the surface [27]. Insufficient CO₂ removal in large-scale bioreactors is likewise a common problem and is connected to low agitation rates, insufficient gas transfer and small bubble size [28]. Since high levels of dissolved CO₂ negatively influence cell growth and product quality [27], [29], [30], the presence of different dissolved CO₂ levels in different reactor scales has to be avoided. In this context, variability in the culture environment and thus in single process parameters should be linked to cellular processes that are related to growth, productivity and product quality. By understanding the impact of relevant parameter ranges it should be possible to better provide optimal operating conditions at all bioreactor scales.

Apart from process parameter control, improvement of the culture media played an important role in the development of cell culture processes. The adaptation of mammalian cells to serum-free conditions and the replacement of serum proteins by chemically defined, protein- and peptide-free media increased process reproducibility, facilitated downstream processing, excluded the risk of contamination by animal-
derived components and led to a general cost reduction [22], [28]. The main components of chemically defined, commercial or proprietary media are glucose, amino acids, vitamins, trace elements, inorganic salts and lipids. Determining the optimal ratio of components requires systematic studies using different available techniques [31] and is often dependent on the cell line. Likewise the composition of feed solutions and the feeding strategies need to be investigated with the additional aim of reducing by-product accumulation (lactate and ammonia). Optimization of media and feeding strategies has proved to have a dramatic benefit on culture longevity and specific productivity. More recently, control of glycosylation has also been achieved by the addition of certain media components [32–37].

1.3 Glycosylation of Monoclonal Antibodies

Monoclonal antibodies of the immunoglobulin G (IgG) isotype are the most common therapeutic antibodies [38]. An IgG molecule consists of 4 polypeptide chains (2 heavy chains and 2 light chains) that are bound covalently through disulfide bridges. The target specificity, and thus the antigen binding, of IgG arises from the hypervariable region formed by the variable domains of the heavy and the light chain. Two constant domains of the heavy chains form the crystallizable fragment (Fc)-region that is responsible for the immune effector functions [39]. Activation of immune effector functions such as antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) is mostly dependent on the binding to the FcγRIII receptor and to the C1q component of complement, and triggers a cascade that ultimately leads to the death of target cells [40]. This mode of action is used by monoclonal antibodies for treatment of cancer and infectious diseases [41]. Instead, binding of IgG-Fc to the FcγRIIb receptor is exploited for the treatment of autoimmune diseases as it leads to a modulation of the immune response [42], [43]. The binding of the Fc region to Fc receptors or the C1q component of complement is critically dependent on N-linked glycosylation [3], [44], [45]. N-linked glycosylation of therapeutic monoclonal antibodies implies the presence of oligosaccharide structures, so called glycans, in the Fc region of the IgG molecule [3]. Glycosylation of the Fab-fragment which has an involvement in antigen binding affinity [46] and
antibody half-life [47], is less common for therapeutic antibodies but has also been reported [48].

Monoclonal antibodies were initially produced in hybridoma cells formed by the fusion of a mouse B cell and a human cancer cell [49]. The resulting murine antibodies are specific to a single antigen, but have the disadvantage of carrying murine-type glycosylation, which can induce immune reactions in humans. This stimulated further developments including the generation of chimeric mouse-human antibodies and humanized antibodies that could reduce immunogenicity [50]. Later, the development of transgenic mice [51], [52] as well as the use of phage display technology [53] allowed the production of fully human antibodies, which have replaced hybridoma cells for monoclonal antibody generation. Nowadays, monoclonal antibodies are further engineered to overcome immunogenicity, improve their biological function and their biophysical properties such as solubility and half-life [54–58]. At the same time bispecific antibodies, as well as antibody-drug conjugates have been developed and are reviewed elsewhere [59], [60].

In this work, we have cultivated HFN 7.1 hybridoma cells in batch and fed-batch culture. HFN 7.1 cells produce a murine IgG₁ antibody against human fibronectin, commonly used in diagnostics. The antibody is characterized by high oligosaccharide content and represents a good model protein for the study of antibody glycosylation. In Chapter 2, we present a methodology to study the influence of different bioreactor process parameter ranges on glycan microheterogeneity, in particular on terminal galactosylation, sialylation and core-fucosylation. The degrees of glycosylation obtained within relevant process parameter ranges are compared to simultaneous variations of multiple process parameters and to literature. The study revealed the importance of pH for IgG glycosylation.

In Chapter 3, the impact of pH alterations on the cellular metabolism was further investigated. We have identified high pH to cause overexpression of metabolic enzymes, which results in changes in cell metabolism, in particular the lactate metabolism. Additionally, high pH showed negative effects on cell growth and antibody production, whereas low pH improved antibody productivity. At the same
time, low pH resulted in reduced lactate accumulation. Flux balance analysis was applied to give more insight into the redistribution of the main energy metabolism at different pH values that mimic heterogeneities in large-scale bioreactor systems.

Apart from the implication of process parameters in scale-up, they can be actively used to optimize process conditions. In this context, hypothermia is known to prolong culture longevity through its action on the cell cycle. In Chapter 4, an unstructured model based on cell cycle transition and quiescence that was developed and employed to predict temperature-shifts in fed-batch culture is presented. At the same time, metabolic cell cycle arrest, induced by nutrient depletion and production of toxic metabolites was evaluated and included in the model. The model was able to capture growth, basic metabolism and mAb production characteristics. Following the described procedure it can be employed to optimize the temperature shift time point in order to obtain a good balance between cell growth and productivity.
2 Evaluating the Impact of Cell Culture Process Parameters on Monoclonal Antibody N-Glycosylation

2.1 Introduction

The relevance of monoclonal antibodies as therapeutic agents against cancer, autoimmune and inflammatory diseases has been widely recognized. Most of the mAbs approved to date are based on the immunoglobulin (IgG) isotype, carrying a consensus N-linked glycosylation site on the CH2 domain of each heavy chain [61] and further possible, but less common, N-linked glycosylation sites on the antigen-binding fragment (Fab) [48]. Glycosylation is a complex process of oligosaccharide attachment to the polypeptide backbone of a protein taking place in the endoplasmic reticulum (ER) and Golgi apparatus. Presence or absence of certain oligosaccharides can critically impact mAb stability [62], effector functions [38], immunogenicity [63], [64], and clearance rate[65], [66], through a clearly defined structure/function relationship. More precisely, reduced terminal galactosylation decreases complement-dependent cytotoxicity (CDC) [67–69], absence of core-fucosylation results in increased antibody-dependent cytotoxicity (ADCC) [70–72], and high sialylation levels reduce ADCC activity and impact inflammatory responses [73], [74]. Furthermore, the use of CHO cells, mouse NS0 cells or Sp2/0 mouse myeloma cells for mAb production can introduce non-human epitopes such as galactose-alpha-1,3-galactose (α-gal) and N-glycolyneuraminic acid (NGNA) residues that act immunogenically [75], [76].

Moreover, it is understood that variations in the N-linked glycan profile (microheterogeneity) can occur during the mAb production process and therefore glycosylation is considered a critical quality attribute of the end product. Studying the impact of various environmental factors and process conditions can enlarge the understanding of the glycosylation machinery and at the same time provide potential to control and target desired mAb glycoforms. Multiple cell culture conditions during
upstream processing such as different feeding regimes [32–35], media supplementation [36], [37], as well as waste metabolite accumulation [77–79] can affect glycosylation. Bo et al. (2013) recently showed a reduction in terminal galactosylation for a chimeric heavy chain antibody at limiting glucose concentrations in fed-batch culture. Moreover, supplementation with manganese chloride, galactose and uridine can provide control over galactosylation during fed-batch cultivation [80], [81]. Apart from metabolic control, bioreactor process parameters can lead to variability in the glycoform profile and thus, play an important role in manipulating the product quality. pH, temperature, dissolved O₂ and CO₂ levels, and shear stress through agitation and sparging are classical process parameters. On the one hand they can be applied to actively control the glycan microheterogeneity; on the other hand such parameters are partially bioreactor scale-dependent and can inadvertently lead to inconsistent glycosylation during scale-up. The impact of process parameters on N-linked glycosylation has been summarized in literature [82], [83], however the effects shown are often incoherent and incomplete, and therefore do not allow general conclusions. This is partly due to the fact that glycosylation is dependent on the cell line [84], [85] and the structure of the glycoprotein [86], but it is also influenced by the experimental methodology that is used to study process-related impact factors.

In this work, we systematically investigated multiple process parameters using shift-experiments in batch culture which allowed us to study each process parameter individually. We focused on pH, dissolved O₂ (DO) and osmolarity as chemical stress parameters and sparging as a mechanical stress parameter assessing their impact on cellular level and protein quality level. In addition, we compared the impact of single parameters to combined effects (pH/osmolarity). Using hydrophilic interaction chromatography (HILIC) and MALDI TOF mass spectrometry we were able to identify the impact of process parameters on N-linked glycosylation of a murine IgG₁. The present study provides a systematic procedure carried out in controlled bioreactors, thus minimizing interaction effects, which can help to identify most profound process-related factors affecting mAb glycosylation. Apart from commonly assessed variations in terminal galactosylation, sialylation and core-fucosylation, less common structures carrying α-gal and NGNA residues were investigated.
2.2 Material and Methods

2.2.1 Cell Line and Cell Culture

The murine hybridoma cell line HFN 7.1 [87], producing an immunoglobulin G1 (IgG1) antibody against human fibronectin was obtained from the American Type Culture Collection (ATCC CRL-1606) and adapted to the protein- and peptide-free culture media Turbodoma® TP6 (Cell Culture Technologies) supplemented with 4.5 g/L D-glucose, 4 mmol/L L-glutamine and 0.1% (w/v) pluronic F-68.

Cells were expanded for 14 days in suspension in a humidified atmosphere (5% CO₂) at 37 °C and cultivated in a DasGip bioreactor system (DasGip) equipped with a pitched-blade impeller and a porous sparger (10 µm). Exponentially growing cells from the expansion were inoculated into the reactor at a seeding concentration of 0.6 x 10⁶ cells/mL. Standard batch cultures were carried out at a working volume of 1 L, temperature equal to 37 °C, dissolved O₂ (DO) set to 50% air saturation, stirring speed of 150 rpm and an aeration rate of 0.05 vvm. pH was controlled at 7.2 with CO₂ sparging.

A shift in one of the process parameters pH, DO, sparging or osmolarity was performed during batch cultivation once the viable cell concentration reached 1.5 x 10⁶ cells/mL. After the process parameter was shifted to the new setpoint it was kept constant until the end of the cultivation. pH was shifted by base (1 M sodium hydroxide) or acid (1 M hydrochloric acid) addition. During the shift, the pH controller was turned off and only after the new setpoint was reached the pH was again controlled by base addition and CO₂ sparging. The increase in osmolarity due to acid or base addition was below 5% and this was considered to have a negligible effect on cells. The DO shift was performed by changing the inlet gas composition through the DO controller. The time needed for the adjustment of the new pH and DO setpoints was in the range of a few minutes. The osmolarity shift was performed by addition of 6 M sodium chloride (NaCl) and monitored by offline measurements using an OsmoLab One osmometer (LLA Instruments GmbH). The sparging shift was performed by changing the gas flow rate in the controlled bioreactor system. Perturbations of the system, in particular DO and pH, due to the new gas flow rate
were negligible. In this manner an operating range of pH from 6.5 to 8.5, DO from 10% to 90% air saturation, sparging from 0.05vvm to 0.2 vvm and osmolarity from 320 mOsm/kg to 450 mOsm/kg was investigated. Moreover, the combined effect of pH and osmolarity was studied in cross experiments by performing a shift of both parameters at the same time point following the same procedure as described above.

Cell culture samples were taken twice a day and in addition right after the parameter shift. Cell number and viability were determined by the trypan blue exclusion method [88] using a CedeX cell counter (Innovatis). Glucose and lactate concentrations were determined enzymatically using a Super GL compact instrument (Hitado). Ammonium and glutamine concentrations were determined using the L-Glutamine/Ammonia (Rapid) Assay Kit (Megazyme). Samples were spun down and supernatants were stored at -20 °C and later used for measurement of antibody concentration (PA ImmunoDetection® Sensor Cartridge, Applied Biosystems) and N-linked glycosylation analysis.

Profiles of the viable cell concentration were calculated using a generalized logistic fitting equation containing 4 non-negative model parameters A, B, C and D (Eq. 2-1) [89]. The specific growth rate $\mu$ was calculated by differentiating the fitted viable cell concentration profile $X$ according to Eq. 2-2.

$$X = \frac{A}{\exp(Bt) + C \exp(-Dt)}$$  \hspace{1cm} (2-1)

$$\mu = \frac{1}{X} \frac{dX}{dt}$$  \hspace{1cm} (2-2)

2.2.2 Monoclonal Antibody Purification

200 µg of mAb was purified from the supernatant of each batch cultivation using the Vivapure® miniprepG purification kit (Sartorius Stedim Biotech) according to the manufacturer’s protocol. The kit contains pre-packed Protein G resin plugs and utilizes affinity purification to separate the monoclonal antibody from the supernatant. Protein
G affinity purification was used for the mouse IgG1, as it only showed weak binding affinities to Protein A. The purified monoclonal antibody was concentrated to 20 µL with a Vivaspin® 500 (30 kDa) centrifugation filter (Sartorius Stedim Biotech) at 1800 g (4 °C) and used for analysis of the glycosylation profile as described in the Appendix (6.1).

2.2.3 Calculations

Monoclonal antibody samples were taken from each batch culture at the end of cultivation (t_{end}) and prior to the parameter shift (t_{shift}). Glycosylation profiles were determined at both time points by HPLC. The relative peak area (%Area) of the detected glycoforms was extracted from the HPLC analysis and was equal to the mole fraction $x_i$ of each species. The aim was to identify the change in glycosylation of each species $x_i$ produced between the shift time point (t_{shift}) and the end time point (t_{end}). In order to account for differences in cell productivity under different operating conditions, $x_i$ was normalized to the difference in mAb concentration ($c_{mAb}$) between the two time points, calculated using the molar mass of IgG1 of 150 kDa. Assuming a constant macroheterogeneity during the culture, the change in each glycoform fraction $\Delta F_i$ was calculated according to Eq. 2-3.

$$\Delta F_i = \frac{c_{mAb, end} \times x_{i, end} - c_{mAb, shift} \times x_{i, shift}}{c_{mAb, end} - c_{mAb, shift}}$$

(2-3)

The degree of galactosylation (GI) [90] was calculated according to Eq. 2-4 as the fraction of tri-galactosylated (G3), di-galactosylated (G2), mono-galactosylated (G1) and non-galactosylated (G0) structures:

$$GI = \frac{3 \times G3 + 2 \times G2 + G1}{(G0 + G1 + G2 + G3) \times 3}$$

(2-4)

In the same manner the sialylation (SI) [90] and fucosylation (FI) indices were calculated (Eq. 2-5 and Eq. 2-6), where only di-sialylated (S2), mono-sialylated (S1)
and non-sialylated (S0) structures, and mono-fucosylated (F1) and non-fucosylated (F0) structures respectively, were observed:

\[ SI = \frac{2 \times S2 + S1}{(S0 + S1 + S2) \times 2} \]  \hspace{1cm} (2-5)

\[ FI = \frac{F1}{F0 + F1} \]  \hspace{1cm} (2-6)
2.3 Results and Discussion

2.3.1 Process Parameter Shift Experiments

Murine hybridoma cells (ATCC CRL 1606) were adapted to a commercial, protein- and peptide-free media (Turbodoma® TP6) and showed a similar growth and metabolic behavior as when cultivated with zinc supplemented IMDM media as reported elsewhere [91]. In Figure 2-1 a typical batch culture averaged from four independent experiments is shown. The maximum viable cell concentration of $3.1 \pm 0.2 \times 10^6$ cells/mL was reached after 54 hours (Figure 2-1A) coinciding with glutamine depletion (Figure 2-1D), which led to a stop in glucose consumption (Figure 2-1B). The culture was continued until 80 hours and reached a final monoclonal antibody concentration of $98.9 \pm 7.2$ mg/L (Figure 2-1C). The production of by-products (lactate and ammonia) did not exceed growth-inhibiting concentrations of 40 mM for lactate and 4 mM for ammonia [92] (Figure 2-1B, D).

A shift in one of the investigated process parameters (pH, osmolarity, dissolved O$_2$ and sparging) was performed during the early exponential growth phase of a standard batch culture in order to systematically study the impact of chemical and mechanical stress parameters on cell growth, productivity and N-linked glycosylation. Due to the controlled bioreactor environment, one parameter at a time could be shifted and subsequently kept constant until the end of the cultivation.

For illustration, Figure 2-2 shows the influence of pH on viable cell concentration, viability and monoclonal antibody concentration in 7 shift-experiments. Each experiment was started at standard pH 7.2 and shifted at the indicated time point (dashed line). While under standard conditions the culture reached the highest viable cell concentration, all pH shifts led to reduced growth profiles (Figure 2-2A). In the case of pH 6.5 and 8.5 the culture immediately entered death phase, as evident from the viability profiles (Figure 2-2B). In comparison, a shift to pH 6.8 resulted in a beneficial behavior regarding antibody concentration, with an 11% increase in final mAb concentration compared to standard conditions (Figure 2-2C).
Due to the complexity of the shift experiments described above, in the following we will analyze the corresponding results in a more synthetic way.

**Figure 2-1**: Standard batch cultivation of murine hybridoma cells in a controlled bioreactor system (n=4). Profile of (A) viable cell concentration, (B) glucose (■) and lactate (□), (C) monoclonal antibody and (D) glutamine (●) and ammonia (○) concentrations.

### 2.3.2 Growth Rate Dependency on Bioreactor Process Parameters

The specific growth rate $\mu$ was calculated during the exponential growth phase of the batch culture 10 hours after the respective parameter shift according to Eq.2-2. As evident from Figure 2-3, the highest growth rate was reached under standard conditions. Consequently, this value was used for normalizing all other specific growth rate values. The most severe reduction of the specific growth rate was induced with a shift in pH. A drop to pH 6.8 resulted in 32% reduction of the specific growth rate whereas an increase to pH 8.0 led to a reduction of 53% (Figure 2-3).
Figure 2-2: pH shift experiments during batch cultivation of murine hybridoma cells. Starting at standard pH condition 7.2 a pH shift was performed in the early exponential phase indicated by a dashed line and covered a pH range from 6.5 – 8.5. Profile of (A) viable cell concentration, (B) viability and (C) mAb concentration at different pH setpoints.
Exceeding these limits by further decreasing or increasing pH (pH 6.5 and pH 8.5) led to immediate cell death (Figure 2-2A) and is therefore excluded from the analysis.

Furthermore, an increase in osmolarity to 420 mOsm/kg resulted in 45% reduction in \( \mu \) and is therefore ranked as the second most important parameter affecting cell growth. Similarly to the effect with pH shifts, a further increase in osmolarity to 450 mOsm/kg resulted in immediate cell death (data not shown).

Figure 2-3. Impact of process parameter shifts (pH, osmolarity, dissolved \( \text{O}_2 \) and sparging) on the specific growth rate 10 hours after the parameter shift. The specific growth rate was calculated for each condition according to Eq. 2-2 and normalized to the specific growth rate obtained under standard conditions. The standard deviation was calculated from four independent standard cultivations.

The two process parameters dissolved \( \text{O}_2 \) (DO) and sparging gave a maximum growth rate reduction of 27% and 15% (Figure 2-3), therefore ranking 3\(^{\text{rd}}\) and 4\(^{\text{th}}\) respectively. Since the investigated range of DO and sparging already accounted for the entire
operating range in common mammalian cell bioprocesses [93], a further extension was not considered.

Growth rate dependencies were determined in order to define the parameter ranges in which the change in glycosylation should be investigated. In these parameter ranges, cells are in the growth state and produce IgG₁, crucial for the subsequent analysis of glycosylation which focuses on glycoforms produced between the time of the parameter shift and the end of the cultivation. The parameter range could vary depending on the cell line and the experimental procedure used to introduce environmental perturbations. It is worth noting that the obtained specific growth rate dependency was comparable with previous literature reports for pH [93–96], osmolarity [93], [97], hyperoxia [98] as well as sparging [96], [99].

2.3.3 Analysis of N-linked Glycan Microheterogeneity

In order to evaluate the differences in the glycosylation profiles (microheterogeneity) induced by process parameter shifts, oligosaccharides were enzymatically released by PNGase F from affinity-purified IgG₁ antibody, fluorescently labeled with 2-AB and characterized by MALDI TOF mass spectrometry. All determined oligosaccharide masses obtained by MALDI-TOF MS in reflectron positive and negative ion mode using DHB matrix are shown in the Appendix (Figure A 1). In positive ion mode, oligosaccharides formed sodium or potassium adducts with sodium adducts having the higher intensity. Structures of oligosaccharides were assigned either through database comparison (GlycoWorkBench2) [100] or by comparison to the glycosylation profile of Cetuximab, a commercially available antibody with known glycosylation profile, obtained by hydrophilic interaction chromatography (HILIC) [48]. In some cases, structures were further determined from the fragmentation pattern obtained by MALDI-TOF MS/MS, as shown in the Appendix (Figure A 1C) for a core-fucosylated, bi-antennary structure carrying one terminal galactose (G1F). All clearly identifiable glycan structures are of the complex type containing terminal galactose, α-linked galactose and/or sialic acid residues. Both types of terminal sialic acid residues (NGNA and NANA) were detected, whereas terminal NGNA residues were predominant. The proposed glycoforms are given in the Appendix (Table A 1).
In addition, fluorescently labeled glycoforms were separated by HILIC chromatography and the mass of each well separated fraction was likewise determined by MALDI-TOF MS and compared to the characterized oligosaccharide structures given in the Appendix (Table A1). The glycan structures could subsequently be assigned to each well separated fraction in the HILIC glycosylation profile (Figure 2-4). The glycosylation profile demonstrated the separation of oligosaccharides between 35 and 84 minutes. The profile is characterized by three major structures, one eluting after 39 and two structures co-eluting after 46 minutes. These three glycan structures contributed to 57% of the whole glycosylation profile. Through MALDI-TOF MS/MS, it was found that the major glycans represent core-fucosylated, bi-antennary structures with none or one terminal galactose, G0F and G1F respectively, where the two co-eluting glycans are assumed to be G1F carrying the galactose either on the $\alpha(1\rightarrow6)$ or the $\alpha(1\rightarrow3)$ arm. All characterized glycan structures that could be identified through MALDI-TOF MS and assigned to the HILIC profile are shown in Figure 2-4. They represent 90% of the whole glycosylation profile of the HFN 7.1 antibody.

2.3.4 Variation in N-linked Glycan Microheterogeneity

Under standard cultivation conditions, the N-linked glycosylation profile of HFN 7.1 is dominated by a bi-antennary, fucosylated structure with one terminal galactose (G1F), which comprised 31% ($\pm$ 2%) of the overall oligosaccharides, followed by the G0F structure, which accounted for 26% ($\pm$ 2%). The fully galactosylated and fucosylated bi-antennary structure G2F only contributed to 8% of the total glycan pool. Other oligosaccharide structures released from HFN 7.1, apart from the non-fucosylated and non-galactosylated G0 structure, showed higher complexity, carrying mostly terminal N-glycolylneuraminic acid residues and/or $\alpha$-linked galactose residues (Figure 2-4), both typical for murine-derived IgG proteins [48], [64], [101].
Figure 2-4: N-linked glycosylation profile of HFN 7.1 antibody (IgG1). The antibody was purified at the end of a standard batch culture, glycans were released by PNGase F, labeled with 2-AB and fluorescently detected by HILIC. The predominant peaks were fractionated and the masses were measured by MALDI TOF MS and compared to characterized oligosaccharide structures. Sugar residues are as reported in the Appendix (Table A 1).

Before investigating the impact of bioprocess parameters on N-linked glycosylation of IgG1, variations in the glycosylation profile over the course of a standard batch culture were considered [102] in order to rule out the presence of degradative enzymes in the supernatant. Released glycosidases and sialidases are known to cleave terminal sugar residues (galactose and sialic acids) particularly towards the end of the culture due to cytolysis [103–105]. Simultaneously, nutrient limitations, such as glucose and glutamine towards the late-stage of the culture, can potentially affect nucleotide sugar substrate availability [106–108]. Furthermore, the accumulation of ammonia can lead to variations in the glycoform profile [78]. To check whether post-secretory degradation, substrate limitation or waste metabolite accumulation affect glycosylation of HFN 7.1 over time, the secreted monoclonal antibody was purified from three repeated batch cultures at three different time points and the glycosylation profile was determined (Figure 2-5A). The relative peak area of nine glycoforms was compared after 26 hours corresponding to early exponential growth phase $t_{shift}$, after 54 hours...
when maximum viable cell concentration was typically reached $t_{\text{Xv}}$ and at the end of the culture after 80 hours $t_{\text{end}}$.

Figure 2-5: N-linked glycosylation profile of purified monoclonal antibody analyzed at three different time points throughout a batch culture [$t_{\text{shift}} = 26\text{h}$, $t_{\text{Xv}} = 54\text{ hours}$, $t_{\text{end}} = 80\text{ hours}$]. Relative peak areas of (A) a standard batch culture without process parameter shift were compared to (B) pH-shifted culture to pH 7.8.

Throughout the course of standard batch cultures the relative peak area remained constant (Figure 2-5A) and therefore we concluded that N-linked glycosylation did not vary within the time frame of a standard batch culture. In contrast, when pH was shifted to 7.8, the glycosylation profile changed over the time course of the
exponential growth phase, as is most evident from the decrease of terminal galactosylation (G1F and G2F) and the increase of the relative peak area of G0F by 6% from $t_{shift}$ to $t_{Xv}$ (Figure 2-5B). However, the glycosylation profile remained unchanged within the last 26 culture hours from $t_{Xv}$ to $t_{end}$ indicating that post-secretory degradation was not observed in the shifted culture even though culture viability was low (see Figure 2-2B). To conclude, changes in the glycan microheterogeneity evident from the relative peak area of the most abundant glycoforms were induced solely by process parameter shifts.

2.3.5 Impact of Chemical and Mechanical Stress Parameters on N-linked Glycan Microheterogeneity

In order to evaluate the effect of multiple process parameters on glycosylation of a monoclonal antibody, the glycoform profile of the investigated IgG1 protein was determined prior to the parameter shift and at the end of the culture. Since cell productivity was influenced differently by each process parameter shift, changes in the glycan profile are further expressed relative to changes in mAb concentration produced within the same time frame, in order to provide better comparability. In this way the fraction of each glycoform could be determined as a function of process parameter and mAb concentration according to Eq. 2-3. Figure 2-6 shows the impact of pH and DO, Figure 2-7 the impact of sparging and osmolarity on the proportion of the most abundant glycoforms. In order to summarize the observed effects the galactosylation (GI), sialylation (SI) and fucosylation (FI) indices [90] were calculated according to Eq. 2-4, Eq. 2-5 and Eq. 2-6.

A shift in pH in the early exponential growth phase led to most profound changes in glycan microheterogeneity of the IgG1 antibody. Terminal galactosylation and sialylation (NGNA) decreased steadily with increased pH (see Figure 2-6A), which is further reflected in the decrease of the GI from 0.43 to 0.24 and of the SI from 0.15 to 0.08 between pH 6.8 and 8.0 (Table 2-1), leading to a gradual increase in the G0F fraction from 16 to 35% (Figure 2-6A). Simultaneously, the afucosylated structure G0 increased with increasing pH, resulting in a reduction of the FI from 0.99 to 0.94 (see Table 2-1). It is worth noting that the range of variability in galactosylation reached in the studied pH region is significant, but remarkably smaller compared to a GI range
reported for glucose limited cultures (GI = 0.35 – 0.72) [32]. Nevertheless, both process conditions could be applied to control galactosylation in the determined range.

The effect of culture pH on glycosylation has been investigated earlier for different glycoproteins produced in a variety of cell lines. While Muthing et al. (2003) reported an increase in galactosylation (G2F) with increased culture pH for an IgG3 protein produced in a hybridoma cell line, Seo et al. (2013) showed the opposite behavior for a mAb produced in a human cell line. At the same time, Muthing et al. (2003) observed an increase in afucosylation. In terms of sialylation, Yoon et al. (2005) reported decreased sialic acid content in Epo with increasing culture pH (pH 7.8). Other reports showed differences in the NANA/NGNA ratio at different pH values [109], as well as no effect of pH 6.8–7.3 on the NANA content [95]. A juxtaposition of the obtained results to earlier literature reports revealed that, as a process parameter, pH can dramatically affect glycosylation of multiple glycoproteins produced in various cell lines, including HFN 7.1 produced in murine hybridoma cells. Nevertheless, a generally valid conclusion on the degree and direction of pH-induced variation of N-linked glycosylation cannot be drawn. Indeed, the impact of pH as well as other process parameters has to be assessed case-by-case depending both on the cell line and the expressed protein. The mechanism of glycosylation is in fact at the same time influenced by cell line-dependent differences, such as the expression levels of glycosylation genes [82], and by the accessibility of the glycosylation site which is protein-specific [110]. In addition, different experimental procedures were previously applied to study the effect of culture pH on glycosylation and it has been shown that the procedure itself can influence glycosylation [111]. Nevertheless, the obtained results extend the knowledge of pH-induced variations in N-glycosylation, using a model system that is characterized by a complex glycosylation profile.

Osmolarity shifts, while ranked second in the cell growth analysis, did not affect glycosylation of the investigated IgG1 protein (see Figure 2-7B), as likewise reported for β-IFN [112]. However, increased osmolarity was shown to increase the antibody Man5 level [113], a glycoform not detected in the HFN 7.1 glycosylation profile.
Figure 2-6: Change in each glycoform fraction $\Delta F_i$ induced by process parameter (A) pH and (B) dissolved O$_2$ (DO). The change in glycoform fraction is expressed as difference of the produced glycoform between the parameter shift time and the end of the culture, normalized to the change in antibody concentration and was calculated according to Eq. 2-3. ($\blacksquare$) G0F, ($\vee$) G1F, ($\circ$) G2F, ($\triangleleft$) G3F, ($\bullet$) G0, ($\blacktriangle$) G2F-NGNA, ($\square$) G2F-NGNA2, ($\times$) G2F-GalNGNA, ($\blacklozenge$) G0F-GalNGNA. Sugar residues are as reported in the Appendix (Table A 1).

Apart from single parameter changes, osmolarity and pH were investigated in cross experiments in order to evaluate their combined effect on glycosylation. pH and osmolarity are important during fed-batch cultivation where increases in osmolarity can be related to pH control by base addition or to feed addition. At the same time it has been shown that low pH and high osmolarity can increase the mAb production rate in hybridoma cell culture [114]. Thus, both parameters were shifted simultaneously in the early exponential growth phase combining low pH with high osmolarity as well as high pH with high osmolarity. For comparison an intermediate pH and osmolarity cross-condition was included (Table 2-1). The combined effect on glycosylation was compared to the GI, SI and FI reached from single process parameter shifts. As evident
from Table 2-1, in the low pH 6.8 and high osmolarity (420 mOsm/kg) cross experiment the effect of pH was predominant and the degree of galactosylation, sialylation and fucosylation was comparable to a pH-shift to pH 6.8 alone. In the other two cross conditions where pH and osmolarity were increased (pH 7.5/380 mOsm/kg; pH 7.8/420 mOsm/kg) the GI, SI and FI followed the osmolarity behavior with consistent glycosylation.

Table 2-1: Degree of galactosylation (GI), sialylation (SI) and fucosylation (FI) as a function of the single shift parameter pH or osmolarity (mOsm/kg), and as a function of the combined effect of pH and osmolarity.

<table>
<thead>
<tr>
<th>pH</th>
<th>Osmolarity</th>
<th>GI</th>
<th>SI</th>
<th>FI</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.8</td>
<td>320</td>
<td>0.43</td>
<td>0.15</td>
<td>0.99</td>
</tr>
<tr>
<td>7.0</td>
<td>320</td>
<td>0.37</td>
<td>0.13</td>
<td>0.99</td>
</tr>
<tr>
<td>7.2</td>
<td>320</td>
<td>0.35</td>
<td>0.11</td>
<td>0.98</td>
</tr>
<tr>
<td>7.5</td>
<td>320</td>
<td>0.31</td>
<td>0.10</td>
<td>0.97</td>
</tr>
<tr>
<td>7.8</td>
<td>320</td>
<td>0.25</td>
<td>0.10</td>
<td>0.95</td>
</tr>
<tr>
<td>8.0</td>
<td>320</td>
<td>0.24</td>
<td>0.08</td>
<td>0.94</td>
</tr>
<tr>
<td>7.2</td>
<td>350</td>
<td>0.34</td>
<td>0.11</td>
<td>0.99</td>
</tr>
<tr>
<td>7.2</td>
<td>380</td>
<td>0.32</td>
<td>0.10</td>
<td>0.98</td>
</tr>
<tr>
<td>7.2</td>
<td>420</td>
<td>0.34</td>
<td>0.09</td>
<td>0.99</td>
</tr>
<tr>
<td>6.8</td>
<td>420</td>
<td>0.43</td>
<td>0.13</td>
<td>1.00</td>
</tr>
<tr>
<td>7.5</td>
<td>380</td>
<td>0.38</td>
<td>0.14</td>
<td>0.99</td>
</tr>
<tr>
<td>7.8</td>
<td>420</td>
<td>0.37</td>
<td>0.13</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Regarding other chemical stress parameters, DO shifts resulted in slight changes of the glycoform fractions (Figure 2-6B). In particular, with DO of 10% and 90% air saturation the GI increased to 0.44 and 0.42 and the SI increased to 0.16 and 0.13 respectively, whereas at 50% air saturation, both indices remained slightly lower (GI = 0.35; SI = 0.11). Earlier literature reports demonstrate rather consistent glycosylation
at DO levels from 10 – 100% air saturation with small increases in sialylation of the follicle-stimulating hormone (FSH) [115] and in terminal galactosylation (G2) of an IgG [116], both at increased DO levels (90 – 100 % air saturation). Therefore, when assessing chemical stress parameters and their effect on glycosylation, DO is ranked as second most important single parameter that causes variations in HFN 7.1 glycosylation.

Finally, the mechanical stress parameter of sparging (in a range of 0.05 – 0.2 vvm) was studied as an impact parameter of glycosylation. Our results suggest no significant difference in any of the glycoforms within this sparging range (Figure 2-7A), whereas hydrodynamic stress induced by sparging was earlier reported to result in a minor increase of the G0F fraction [25].

![Figure 2-7: Change in each glycoform fraction \( \Delta F_i \) induced by process parameter (A) sparging and (B) osmolarity. The change in glycoform fraction is expressed as difference of the produced glycoform between the parameter shift time and the end of the culture, normalized to the change in antibody concentration and was calculated according to Eq. 2-3. (■) G0F, (∇) G1F, (○) G2F, (△) G3F, (◆) G0, (▲) G2F-NGNA, (□) G2F-NGNA2, (×) G2F-GalNGNA, (●) G0F-GalNGNA.](image-url)
2.4 Conclusions

We propose a shift-experiment methodology that can be applied to systematically investigate the effect of varying chemical and mechanical stress parameters that constitute common bioreactor process parameters, on cellular growth and glycosylation of a monoclonal antibody. Applying this methodology, we could show a comprehensive picture of variations in complex N-linked glycosylation of one particular glycoprotein, HFN 7.1, characterized in this study. An initial assessment of the cell growth rate dependency on process parameters was used to define an operating range in which cells were viable and produced monoclonal antibodies. As a result, pH and osmolarity were considered as critical parameters in terms of growth, whereas sparging and DO did not impact the specific growth rate in the relevant range. Regarding the effect of process parameters on glycosylation, pH showed the most profound impact on the variation of glycan microheterogeneity, followed by DO, which had a slight impact on galactosylation and sialylation. Osmolarity and sparging did not affect glycan microheterogeneity significantly. However, high osmolarity prevailed regarding GI, SI and FI in combination with high pH, whereas the combined effect of low pH with high osmolarity showed pH predominance on glycan microheterogeneity.

Monitoring of process parameters and their impact on N-linked glycosylation is crucial to provide desired as well as consistent product quality throughout a bioprocess. The methodology we have presented here can provide an initial ranking of the impact of environmental parameters that have to be considered under the Quality by Design (QbD) scope. Furthermore, data obtained can be used for validation of recently available mathematical models of mAb glycosylation [117], [118] and improve their ability to capture the effect of process conditions. Ideally, this methodology can be further applied in a high-throughput setting and connected to high-throughput analytical techniques [119].

2.5 Remark

The work presented in this chapter has been partially submitted for publication to the Journal of Biotechnology.
3 Insights into pH-induced Metabolic Switch by Flux Balance Analysis

3.1 Introduction

Mammalian cells are used for the production of recombinant proteins that are large, complex and require post-translational modifications [7]. With the increased medical and economic demand for recombinant proteins, primarily mAbs, particular attention has been paid to mammalian cell culture processes with the aim to consistently produce high quality material. Over the past 25 years, bioprocesses have been prolonged through process modifications and media optimization as well as through genetic engineering, resulting in substantial increases of maximum viable cell concentrations and product concentrations [17].

Nevertheless, mammalian cells are characterized by an inefficient metabolism [120]. With the increase in glycolytic rate observed under growth conditions, and with excess glucose [121], pyruvate is preferentially reduced to lactate by lactate dehydrogenase A (LDH A), instead of entering the mitochondria and being further metabolized in the tricarboxylic acid (TCA) cycle. This has a negative impact on the energy yield which can be achieved from glucose. Furthermore, it has been shown that lactate itself can downregulate enzymes in the glycolytic process, namely hexokinase (HK) and phosphofructokinase (PFK), thereby altering the cellular metabolism during fed-batch cultivation [122], [123]. In addition, lactate accumulation results in reduced cell growth in batch and fed-batch cultures [124], [125] and has a negative impact on cell productivity [126]. Different strategies have been developed in an attempt to limit the secretion of lactate and the associated adverse effects. Apart from approaches where metabolic pathways were genetically modified [127], [128], several strategies for operating the bioreactor were proposed that aim to reduce lactate accumulation and often even result in lactate consumption. The latter are centered on the optimization of the media and feeding strategies by reducing the initial glucose concentration [129], [130] or feeding galactose [131] or even lactate directly [132] as alternative carbon sources. Finally, lactate secretion can also be affected externally by pH alteration. As
demonstrated by Osman et al. (2001), lactate consumption can be induced in GS-NS0 cells by reducing pH below 7.5, with concomitant decreases in cell growth and glucose uptake. They also showed that the specific lactate consumption rate increased as the pH value was further reduced, while the specific lactate production rate increased when pH was set above 8.0. A similar behavior was observed in a different study using a CHO cell line [95].

Efforts to characterize the cell metabolism have included $^{13}$C metabolic flux analysis (MFA) as well as constraint-based flux balance analysis (FBA). $^{13}$C-MFA is a powerful method which can be used to identify the split ratio of fluxes at branch points or in cyclic pathways, such as the diversion of glucose to the partially cyclic pentose phosphate pathway [134]. However, this technique relies on isotoptomer measurements and requires the use of $^{13}$C-labelled substrates in order to investigate intracellular flux distributions. Despite the accuracy of MFA estimations, this method is experimentally tedious and expensive, in particular when working on bioreactor scale. FBA on the other hand, fully depends on the mass balance and can be applied effectively to underdetermined systems by including thermodynamic and regulatory constraints. These constraints play a key role in reducing the solution space [135]. Thus, the intracellular flux distribution can be obtained from optimization towards a given objective. FBA has been used to calculate the flow of metabolites through primary metabolic pathways [136], [137]. The accuracy of FBA estimations using the objective function ‘maximize ATP’ has been successfully validated in hybridoma cell culture using experimental $^{13}$C-determined flux data [138]. In recent studies, both $^{13}$C-MFA and FBA have been applied as tools to study lactate metabolism under glucose depletion [139], and to compare different growth phases during fed-batch cultivation [121], [123], [140], and different cell lines [141].

In this work, flux balance analysis was used to investigate the effect of pH on the lactate metabolism of a murine hybridoma cell line during the exponential growth phase of the culture. Lactate consumption was induced even at high glucose concentrations by altering the pH from standard pH 7.2 to a lower value (pH 6.8), whereas lactate production was highly increased once pH was set to a higher value (pH 7.8). While in other studies metabolic states were compared before and after the lactate
switch [123], [139], we altered the pH in parallel controlled batch cultures and analyzed the metabolic states considering the effect of culture time and thus the growth phase of the cells. Furthermore, gene expression analysis by real-time PCR was implemented to capture the pH-induced changes on the enzymes involved in the central metabolism of murine hybridoma cells at the transcriptional level. The information obtained thereby led to the identification of a regeneration pathway that was activated at pH 7.8, and on this basis the metabolic network was enlarged. Finally, simulation results were used to identify the consequences of pH alterations on energy metabolism.

3.2 Material and Methods

3.2.1 Cell Line and Media

The hybridoma cell line HFN 7.1 (ATCC CRL-1606) [87], producing an immunoglobulin G₁ (IgG₁) antibody against human fibronectin was used in this study. The initially serum-dependent cells were sequentially adapted to the chemically defined protein- and peptide-free culture media Turbodoma® TP6 (Cell Culture Technologies) supplemented with 4.5 g/L D-glucose, 4 mM L-glutamine and 0.1% (w/v) pluronic F-68. Cells were expanded as suspension cultures for 2 weeks in 50 mL spinner flasks and 2 L roller bottles in a humidified atmosphere containing 5% carbon dioxide (CO₂) at constant temperature of 37 °C.

3.2.2 Bioreactor Setup and pH Shift Experiments

A DasGip bioreactor system (DasGip) was used for the cultivation of HFN 7.1 cells under controlled culture conditions. The exponentially growing cells were transferred to the bioreactor at a concentration of 2 x 10⁶ cells/mL and diluted to a working volume of 1 L to result in a seeding cell concentration of 0.6 x 10⁶ cells/mL. Batch cultivation was performed under standard conditions with temperature equal to 37 °C, dissolved oxygen set to 50% air saturation and stirrer speed equal to 150 rpm. The pH was controlled at 7.2 with CO₂ sparging. Once viable cell concentration reached 1 x 10⁶ cells/mL, a single pH shift was performed by the addition of a defined volume of 1 M sodium hydroxide (NaOH) or 1 M hydrochloric acid (HCl). Due to the small base or acid amount used, the time needed to shift the pH to the new setpoint was in
the range of a few minutes. During the shift, the pH controller was turned off and only after the new setpoint was reached the pH was again controlled by base and CO₂ sparging in order to keep the pH constant at the new setpoint until the end of cultivation. The osmolarity of each culture was measured with an OsmoLab One osmometer (LLA Instruments). The increase in osmolarity due to acid or base addition was below 5% and this was considered to have a negligible effect on the cells.

Cell culture samples were taken twice a day and additionally before and after the pH shift. Cell number and viability were determined using a CedeX cell counter (Innovatis). Glucose and lactate concentrations were determined using a Hitado Super GL compact instrument (Hitado). Samples were spun down and supernatants were stored at -20 °C and later used for measurement of antibody concentration (PA ImmunoDetection® Sensor Cartridge, Applied Biosystems) and amino acid concentration (ZORBAX Eclipse Plus C18 4.6mm x 150mm, Agilent Technologies) by HPLC. Prior to amino acid measurement, supernatant samples were filtrated using a Vivaspin® 500 (5kDa) centrifugation filter (Satorius Stedim Biotech) at 4000 rcf at 4 °C for 25 minutes. Derivatization of amino acids with ortho-phthalaldehyde (OPA) was performed with an online injector program according to Agilent’s application note [142].

### 3.2.3 TaqMan® Gene Expression Assay

Cell samples lysed in TRIzol® reagent (Life Technologies) at day 1.1 and 1.9 from each culture were stored at -80 °C. RNA was extracted from cell samples according to the manufacturer’s recommendations. Quantity and quality of isolated RNA was assessed by spectrophotometry with a NanoDrop 1000 (Thermo Scientific). All RNA samples had an A₂₆₀/A₂₈₀ ratio within 2.00 to 2.03, which represents high purity of RNA.

For each reaction, cDNA was prepared from 2 µg of RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s recommendations. A TaqMan® gene expression assay containing a predesigned array of murine oligonucleotide primers and probes for target genes involved in murine central metabolism (Appendix Table A 2) was purchased from Life Technologies. Quantitative PCR reactions were performed in triplicates according to
supplier’s protocol using TaqMan® Gene Expression Master Mix (2x) (Rox; Life Technologies) in an AB 7900 HT Real-Time PCR System (Life Technologies, USA). The results were analyzed by applying the comparative $C_T$ method ($2^{-\Delta\Delta C_T}$ method) [143] with glyceraldehyde-3-phosphate dehydrogenase *Gapdh* as an internal control gene for the normalization of mRNA expression levels. The expression of each target gene relative to the internal control gene after pH-shift (at day 1.9) was compared to the relative expression before pH-shift (at day 1.1), for both pH-shifted conditions respectively.

3.2.4 Flux Balance Analysis (FBA)

Flux balance analysis as described previously [144] was applied to the central metabolic network based on Mulukutla *et al.* (2012). The network is compartmentalized into mitochondria and cytosol and contains reactions of the main energy metabolism of glycolysis, glutaminolysis and TCA cycle, as well as biomass and antibody synthesis. The carbon shunt into the pentose phosphate pathway (PPP) cannot be resolved and was assumed to only result in DNA and RNA synthesis. Amino acid degradation and synthesis pathways have been adjusted according to the KEGG database for *Mus musculus* [145]. Apart from the main energy metabolism, the malate-aspartate shuttle has been included in the metabolic network to capture the regeneration of the cofactor NAD$^+$ in the cytosol. Since the cofactor requirement of some enzymatic reactions is still under investigation and due to possible mitochondrial transhydrogenase action, the cofactors NADH and NADPH were lumped together. The total production of NAD(P)H and FADH$_2$ was calculated from the network and a theoretical P/O value of 2.5 and 1.5 respectively was considered for ATP production in oxidative phosphorylation. Further, reactions that can restore the phosphoenolpyruvate and therefore pyruvate level in the cytosol have been added to the metabolic network after identification of high expression levels of the mitochondrial phosphoenolpyruvate carboxykinase (PEPCK2) at culture pH 7.8 from gene expression data. The reactions and metabolites considered in the metabolic network are listed in the Appendix (Table A 3), together with the scheme of the metabolic network (Figure A 2).
Metabolite consumption and production profiles were fitted using a logistic decline equation (Eq. 3-1) to describe metabolite consumption and a logistic growth function (Eq. 3-2) to describe metabolite production. Viable cell concentration data can be fitted using a generalized logistic equation containing non-negative model parameters A, B, C, D and E (Eq. 3-3).[89] The parameter E used in all equations has been added to account for data that do not start from zero or converge to zero, as follows:

\[ N = \frac{A}{\exp(Bt) + C} + E \]  
\[ P = \frac{A}{1 + C \exp(-Dt)} + E \]  
\[ X = \frac{A}{\exp(Bt) + C \exp(-Dt)} + E \]

Specific metabolite consumption rates \( q_N \) and specific production rates \( q_P \) were calculated by differentiating the fitted logistic equations and consequently used as an input in FBA:

\[ q_N = \frac{1}{X} \frac{dN}{dt} \]  
\[ q_P = \frac{1}{X} \frac{dP}{dt} \]

The biomass composition for hybridoma cell lines has been described previously [146–148] and is provided in the Appendix 6.2.

Flux balance analysis using maximization of ATP production as an objective function was employed to estimate intracellular fluxes. ‘Maximization of ATP’ has proven to be a valid objective function for hybridoma cells due to their hyperactive, cancer-like metabolic behavior [138], [149]. The reactions in the network were programmed in Matlab using the SimBiology® model object and a sequential quadratic programming
algorithm was utilized to solve the optimization problem. The 95% confidence interval was calculated as two times the estimated standard error. Finally, the reaction network and the validity of the objective function were verified using literature data from $^{13}$C-tracer studies [121], [150]. Comparison of the calculated intracellular fluxes with the experimentally determined values gave an acceptable agreement within the main energy metabolism with expected discrepancies in the pentose phosphate pathway and in the anaplerotic reaction of pyruvate to oxaloacetate (data not shown).

3.3 Results and Discussion

3.3.1 Characteristics of Cell Growth and Metabolism

pH-shift experiments to high (pH 7.8) and low pH (pH 6.8) were performed in duplicates during HFN 7.1 batch cultivation in controlled bioreactors once cells were growing exponentially and had reached a cell concentration of $1 \times 10^6$ cells/mL. Profiles of viable cell, antibody, glucose and lactate concentrations were compared to an averaged standard cultivation from four independent experiments with constant pH 7.2 (Figure 3-1). Under standard conditions the peak viable cell concentration of $3.3 \pm 0.3 \times 10^6$ cells/mL as well as a maximum growth rate of $1.1 \ \text{d}^{-1}$ were reached during the exponential growth phase. In contrast, the highest mAb concentration was observed in the pH 6.8 shifted culture, even though growth was reduced compared to standard cultivation conditions.

The lowest growth profile was obtained at pH 7.8, where a growth rate equal to $0.70 \ \text{d}^{-1}$ was measured during the exponential phase, resulting in a maximum viable cell concentration of $2.1 \times 10^6$ cells/mL with a final mAb concentration 27% lower compared to standard conditions (see Figure 3-1A, B). It is worth noting that the observed variations in growth rate and productivity are in agreement with previously published pH-shift experiments using a GS-NS0 mouse myeloma cell line [133].
Figure 3-1: Profiles of (A) viable cell concentration, (B) antibody concentration, (C) lactate concentration and (D) glucose concentration of a standard cultivation pH 7.2 (n=4) and two pH-shifted cultivations (pH 6.8 and pH 7.8) performed in duplicates. pH shift was performed once cells reached 1 x 10^6 cells/mL and is indicated by dashed line. Measured data points were fitted using logistic equations (3-1), (3-2) and (3-3).
More interestingly, lactate and glucose metabolism was instantly affected by pH shift (Figure 3-1C, D), also as reported earlier [93], [94], [133]. Once pH was reduced to 6.8, the lactate concentration decreased, indicating a lactate consumption state that was triggered by pH and remained present throughout the cultivation. Concurrently, glucose uptake was substantially reduced. In contrast, the lactate concentration increased dramatically as soon as pH was set to 7.8 resulting in a final lactate concentration of 39.3 (±3.3) mmol/L compared to 15.6 (±3.3) mmol/L under standard conditions. The increase in lactate concentration was connected to an increase in glucose consumption (see Figure 3-1 C, D).

Specific consumption and production rates of all measured metabolites were calculated over the time of the exponential growth phase (WD 1.0, WD 1.5, WD 1.9) for the averaged standard cultivation, and compared in order to assess the effect of culture time on specific rates. As can be seen from Figure 3-2A, specific production and consumption rates of basic metabolites, amino acids, as well as biomass and mAb decreased over time. Therefore, a fair comparison between different conditions can only be made at the same time point, so as to exclude metabolic alteration due to culture time. Consequently, specific consumption and production rates for the pH-shifted conditions were calculated at working day 1.9 and compared to standard conditions at the same time point (see Figure 3-2B, C). Apart from changes in glucose and lactate metabolism, consumption and production of several other amino acids was clearly altered, e.g. the metabolism of glycine switched from consumption to production in the high lactate production state and at the same time glutamate production was significantly increased. Specific rates from Figure 3-2 were used as input fluxes $v_m$ in the subsequent flux balance analysis.

The HFN 7.1 batch cultivation is characterized by glutamine depletion after 2.1 days. Therefore, data after 2.1 days were not considered in flux balance and gene expression analysis.
Figure 3-2: Specific consumption and production rates of glucose, lactate, various amino acids (nmol/10^6 cells/h), biomass and mAb (µg/10^6 cells/h) under different pH conditions. (A) Standard batch cultivation (pH 7.2) at three time points (WD 1.0, WD 1.5 and WD 1.9) during exponential growth phase. (B) Comparison of two metabolic states at pH 7.2 and at pH 6.8 evaluated 17 hours after pH-shift (WD 1.9). (C) Comparison of two metabolic states at pH 7.2 and at pH 7.8 evaluated 17 hours after pH-shift (WD 1.9). Indicated time points were considered in FBA.
3.3.2 Metabolic Flux Distribution of Standard Cultivation during Exponential Growth Phase

The metabolic model based on Mulukutla et al. (2012) contains 4 degrees of freedom coming from the pyruvate carboxylation reaction as an alternative entry to the TCA cycle, from two malate to pyruvate pathways located in the cytosol and in the mitochondria respectively, and the last one from an amino acid synthesis pathway. The flux distribution in the central metabolic network was investigated using a flux balance approach with ‘maximization of ATP production’ as the objective function as suggested and validated for hybridoma cells [138].

The average of the calculated fluxes is shown in Figure 3-3 with error bars representing the 95% confidence interval corresponding to the variation of input fluxes calculated from 4 repeated standard cultivations. For convenience, amino acid synthesis and degradation pathways are not shown. A representation of all intracellular fluxes is presented in the Appendix (Figure A 3). As shown in Figure 3-3, intracellular fluxes decrease over time during exponential growth at pH 7.2, which is most apparent in the glycolytic pathway. Nevertheless, TCA fluxes remain high over the first 12 hours by reducing the lactate flux and therefore increasing the pyruvate influx. Consequently, the malate-aspartate flux is retained at high rates in order to maintain the NAD⁺/NADH balance in the cytosol. At working day 1.9 intracellular fluxes were significantly reduced compared to the early exponential phase, corresponding to a reduction of the growth rate by 36% to 0.70 d⁻¹.

The distribution of intracellular fluxes during the short time frame of the exponential growth phase furthermore clearly demonstrated the effect of culture time, which should consequently be considered when comparing distinct metabolic states, especially under the impact of additional external parameters such as pH. This is also relevant, but often neglected, when differentiating between lactate consumption states in the stationary growth phase and lactate production states during exponential growth of fed-batch cultures.
Figure 3-3: Intracellular flux distribution within glycolysis, TCA cycle, malate-aspartate shuttle, glutaminolysis, biomass and mAb synthesis of standard batch cultivation at three time points during exponential growth phase. The pathways of amino acid metabolism are not shown. Values of all calculated fluxes are reported in the Appendix (Figure A3).

3.3.3 Metabolic Flux Distribution in Lactate Production and Lactate Consumption State

The two metabolic states of lactate production under standard conditions and lactate consumption triggered by pH 6.8 are compared in Figure 3-4. While the glucose uptake rate at low pH (32 nmol/10⁶ cells/h) was reduced compared to standard conditions (88 nmol/10⁶ cells/h), pH-induced lactate consumption contributed to a comparable influx into the TCA cycle through pyruvate and triggered a 37% increase in glutamine uptake. Similarly, high fluxes through the malate-aspartate shuttle were maintained, presumably due to the requirement of cofactor balancing in the cytosol [123], [151]. While under standard conditions cytosolic NADH produced by glyceraldehyde phosphate dehydrogenase (GAPDH) in the glycolytic process is
primarily regenerated by lactate dehydrogenase A (LDH-A) reducing pyruvate to lactate, a switch to lactate consumption causes an additional generation of NADH, and therefore requires an increased activation of the malate-aspartate shuttle to regenerate the cofactor NAD⁺. Thus, the influx into mitochondria was preserved through the pyruvate node, glutaminolysis and the malate-aspartate shuttle, and resulted only in an insignificant reduction of the final TCA flux. Therefore, pH-triggered lactate consumption had no severe consequences on the flux distribution within the main metabolic network compared to pH 7.2. Nevertheless a 35% increased mAb synthesis rate was obtained at pH 6.8 mainly due to altered amino acid utilization (Appendix Figure A 4).

Figure 3-4: Intracellular flux distribution within glycolysis, TCA cycle, malate-aspartate shuttle, glutaminolysis, biomass and mAb synthesis in lactate consumption state (pH 6.8) compared to lactate production state at standard conditions (pH 7.2) at working day 1.9. The pathways of amino acid metabolism are not shown. Values of all calculated fluxes are reported in the Appendix (Figure A 4).
Mechanistic explanations of the pH effect on cell metabolism, in particular glycolysis and lactate production/consumption, have been proposed earlier [93], [133]. As discussed by these authors, lactate consumption might be triggered by a self-regulation mechanism of the cell trying to counteract the extracellular pH by importing lactate through the monocarboxylate transporter (MCT), which requires the symport of H\(^+\) ions [152]. The authors also argue that pH might alter the activity of glycolytic enzymes or the membrane potential, which affects the glucose uptake rate and therefore lactate production and consumption. Even though gene expression of glycolytic enzymes at pH 6.8 and pH 7.2 does not differ (see Figure 3-5), enzyme activity can still be affected by pH and is not reflected within gene expression analysis. At this point, we do not provide any further explanation of the mechanism of action of pH reduction on the cell metabolism. However, we were able to reveal the consequences of pH reduction on the intracellular metabolism by FBA.

### 3.3.4 Metabolic Flux Distribution in Unfavorable Lactate Over-Production State

The metabolic state of cells at pH 6.8 was the opposite to that observed in cultures at pH 7.8. Lactate was overproduced, again being triggered by either a self-regulation mechanism of the cell, by altered membrane potential or by higher enzyme activity in glycolysis. However, the latter cause of lactate overproduction is yet again not apparent from gene expression analysis (see Figure 3-5).

Figure 3-5: Gene expression of 32 genes involved in the central murine metabolism. Data were measured in triplicates and mRNA expression of the two pH-shifted conditions was compared relative to mRNA expression at the time of pH shift. 50% over- or under-expression is considered as not significant.
Together with the high lactate production, an increase in the glucose uptake rate was observed (196 nmol/10^6 cells/h compared to 88 nmol/10^6 cells/h) which was necessary to keep up with the carbon requirements to produce lactate. A closer look further revealed that the ratio of total carbon channeled to lactate could not be met solely by the measured glucose uptake rate (ΔL/ΔG=2.2) and therefore an additional pathway had to be active, which could provide more pyruvate for lactate formation. Two PEP regeneration pathways (OAA → PEP, OAA_m → PEP_m), that are part of the gluconeogenic pathway, were considered to fulfill the pyruvate balance. However, due to the presence of two cyclic pathways and the thereby increased complexity around the pyruvate node, a unique optimized solution could not be obtained. To identify the active pathway, mRNA expression of the involved enzymes has hence been taken into consideration (see Figure 3-5). Considering the conversion of oxaloacetate to phosphoenolpyruvate, *Pepck1* and *Pepck2* are the two genes encoding either the cytosolic or mitochondrial enzyme phosphoenolpyruvate carboxykinase. According to gene expression analysis, mRNA level of the cytosolic form *Pepck1* did not exceed the threshold value in the qPCR reaction after 36 PCR cycles and therefore was considered as not expressed. This led to the conclusion that the cytosolic reaction of oxaloacetate to phosphoenolpyruvate was inactive under all pH conditions and was therefore not considered in the metabolic network. In contrast, the mitochondrial form *Pepck2* showed a 1.2-fold higher expression at pH 7.8. Thus, under the assumption that gene expression correlates with enzyme activity, the mitochondrial reaction OAA_m → PEP_m has been included in the metabolic network. Furthermore, the phosphoenolpyruvate transport flux from mitochondria to cytosol was unbound. Other theoretically possible pathways that could replenish the pyruvate requirements [141], including the malate to pyruvate conversion by malic enzymes (ME1, ME2 or ME3) and the cytosolic malate to oxaloacetate conversion by malate dehydrogenase (MDH1) were excluded after consideration of mRNA expression data (see Figure 3-5). Hence, a validation of the metabolic network was performed based on gene expression data. Finally, the identified PEP regeneration reactions were constrained to zero at all pH conditions other than pH 7.8. However, even without constraining them, this pathway was not chosen under standard and low pH conditions due to the objective function requirement ‘maximization of ATP production’, as the mitochondrial PEP
regeneration requires ATP and additionally takes away flux from the TCA cycle (data not shown). Even though this metabolic state appears highly energy-inefficient, it is further confirmed by a 1.2 fold increase in \(Pdk2\) expression. The \(Pdk2\)-encoded protein phosphorylates pyruvate dehydrogenase, down-regulating the activity of the mitochondrial pyruvate dehydrogenase complex that drives pyruvate decarboxylation \((\text{PYR}_m \rightarrow \text{AcCoA}_m)\). The calculated flux distribution is presented in Figure 3-6. By considering the above described regulatory constraints, the tremendous lactate formation was fulfilled using PEP regeneration in addition to increased glycolytic fluxes. As a result, the TCA cycle and malate-aspartate shuttle were almost shut off. Only an increased glutamate flux by amino acid degradation pathways being converted to \(\alpha\)-ketoglutarate (\(\text{AKG}_m\)) resulted in minor fluxes in the lower part of TCA.

To conclude, the unfavorable pH of 7.8 led to the expression of enzymes that are traditionally considered to be involved in gluconeogenesis but were recently found to play an important role in the transition of CHO cells from growth to stationary phase of fed-batch production [141]. Further, such genes are involved in the regulation of the TCA cycle activity and concurrently the energy metabolism in different cell types [153], [154].

Finally, a detailed understanding of the pH effect on intracellular metabolism was provided, even though the signaling pathway on regulatory level remained unknown. It is assumed that pH-induced changes were guided by the combination of proton gradient maintenance, redox balancing and pathway regulation, \(e.g.\) inhibition of glycolytic enzymes. In order to gain a mechanistic understanding of the effect of pH on cell metabolism, activities of glycolytic enzymes and expression of signaling and regulatory factors as well as transporters should be further investigated. Likewise, the intracellular flux redistribution through phosphoenolpyruvate should be confirmed by isotope measurements using \(^{13}\text{C-MFA}\) in an appropriate experimental setup in order to provide additional evidence to transcript data.
Figure 3-6: Intracellular flux distribution including additional mitochondrial PEP recycle pathway of high pH condition (pH 7.8) compared to lactate production state at standard conditions (pH 7.2) at working day 1.9. The pathways of amino acid metabolism are not shown. Values of all calculated fluxes are reported in the Appendix (Figure A 5).

### 3.3.5 Consequences of pH-Shifts on the Energy Metabolism

In order to gain more insight into the energy metabolism at different pH conditions, the ATP production rates were evaluated from the calculated fluxes by applying the theoretical P/O ratio of 2.5 for NAD(P)H and 1.5 for FADH₂ respectively, and are represented in Figure 3-7. Throughout the exponential growth phase of the standard batch culture (WD 1.0, WD 1.5 and WD 1.9), the total ATP production \( q_{\text{ATP}} \) decreased from 5530 (±574) nmol/10⁶ cells/h to 2168 (±702) nmol/10⁶ cells/h within almost 24 hours and thereby followed the same trend as the intracellular flux distribution (Figure 3-3). Even though the total ATP production rate decreased over time, the ratio between the different originating pathways remained equal between working day 1.0 and working day 1.9. The main substance contributing to energy generation was
NAD(P)H, which, together with FADH$_2$, was utilized to produce up to 84-87% of the total ATP by oxidative phosphorylation. The redox cofactors, i.e. NAD(P)H and FADH$_2$, involved in oxidative phosphorylation can originate either from the TCA cycle (65-72%), or from other reactions such as the pyruvate decarboxylation reaction (PYR$_m$ → AcCoA$_m$) and reactions within the amino acid metabolism (14-19%) (see Figure 3-7). Glycolysis accounted for 7-9% of the total ATP production rate and succinyl CoA synthetase in the TCA cycle contributed to 7% of the ATP production (Figure 3-7: WD 1.0, WD 1.5, WD 1.9). Throughout the exponential growth phase of HFN 7.1 cells, the majority of the energy (72% to 79%) was generated in the TCA cycle either directly in the form of ATP or through oxidative phosphorylation. ATP utilization for biomass formation, mAb synthesis, amino acid synthesis and degradation was not considered in this analysis.

**Figure 3-7:** Comparison of energy metabolism of lactate consumption state (pH 6.8) and lactate overproduction state (pH 7.8) with lactate production state during the exponential growth phase at standard conditions (pH 7.2). Total ATP production rate by ATP, NADH and FADH$_2$ was calculated considering different originating pathways. A P/O ratio of 2.5 for NADH and 1.5 for FADH$_2$ was assumed.

Comparison of the ATP production rates and the originating pathways was made between the standard culture on working day 1.9 and pH-shifted conditions (Figure 3-7: pH 6.8, pH 7.8). The metabolic state at pH 6.8 resulted in a slightly lower $q_{ATP}$ of
1752 (±770) nmol/10^6 cells/h. Energy in the form of ATP was likewise primarily generated by oxidative phosphorylation (TCA: 73%, others: 17%) and further by succinyl CoA synthetase in the lower part of the TCA cycle (7%). Furthermore, energy efficiency of the lactate production and consumption state was compared by calculating the total ATP production per total carbon moles of substrate. The pH-triggered lactate consumption state corresponded to an energy efficiency of 4.8 mol ATP/C-mol, whereas under standard conditions the energy equivalent was equal to 4.1 mol ATP/C-mol. Hence, pH reduction induced a slightly more energy efficient metabolic state and should therefore be considered similar to glucose depletion in CHO fed-batch cultures [139], as a trigger for the initiation of energy-efficient metabolic states.

In the lactate overproduction state at pH 7.8 the low fluxes in TCA and malate-aspartate shuttle led to an overall decrease in total ATP production of 71% (634 (±172) nmol/10^6 cells/h ATP) compared to standard conditions. At the same time NAD(P)H was no longer the main contributor to the overall ATP production (Figure 3-7). Instead, more than two-thirds of the produced energy came from ATP directly generated in glycolysis (68% of total ATP production).

### 3.3.6 Relevance of pH-induced Metabolic States for the Design and Scale-up of Industrial Bioprocesses

It is well known that pH has a significant influence on cell performance [97], [155] and product quality [82], [156] during mammalian cell cultivation. Therefore, pH is commonly investigated in design of experiments (DOE) approaches and tightly controlled in an optimal range during cultivation. However, it has been shown that pH heterogeneities within the bioreactor are present due to carbon dioxide accumulation or base addition [26]. This is particularly significant when comparing small-scale bioreactors used for process development and large-scale reactors applied in production. Therefore, pH heterogeneities should be kept in mind when alterations in cellular metabolism, particularly in lactate, appear throughout process development and scale-up. The present study showed that a pH reduction to pH 6.8, which in a large-scale bioreactor could be caused by CO_2 accumulation, instantly induced lactate consumption and a metabolic state that was energetically more efficient with increased
cell productivity. In contrast, at pH 7.8, a pH region that cells could easily experience locally during alkali addition in large-scale bioreactors, less ATP was generated due to the fact that glucose was used for lactate formation instead of entering the TCA cycle. Furthermore, the unusually high lactate formation was afforded not only by increased glucose uptake, but also by a PEP regeneration pathway. The presented approach can be applied to increase large-scale process understanding (e.g. heterogeneous culture conditions), as well as to deduce recommendations for bioprocess control strategies, such as avoiding the use of base for pH control and rather accept shifts to lower pH values (up to pH 6.8). According to our results, low pH showed no negative effect on the culture in terms of energy metabolism but, in contrast, resulted in increased mAb production.

Hence, by focusing on the whole cellular metabolism, and in particular on the related energy requirements, rather than just on purely macroscopic variables, we became aware of additional possibilities for process optimization in murine hybridoma cell culture.

3.4 Conclusions

This work provides the first example of using a flux balance model containing the major metabolic pathways of glycolysis, glutaminolysis and the TCA cycle, as well as the malate-aspartate shuttle for cofactor balancing, to analyze the influence of pH on cell metabolism during mammalian cell cultivation. Due to a residual degree of freedom in the model, ‘maximization of ATP’ has been chosen as objective function and the calculated fluxes were sufficiently tight to describe the metabolic behavior of murine hybridoma cells. Furthermore, gene expression analysis of metabolic enzymes was employed for validation of the proposed metabolic network. The analysis additionally revealed the presence of a pathway that was only activated under high pH conditions.

Before applying FBA to pH-shifted cultures, the intracellular flux distribution of a standard cultivation with constant pH was calculated at different time points to emphasize the often neglected effect of time on cell metabolism. Thus, a comparative analysis of the two distinct pH-induced metabolic states of lactate consumption and
lactate overproduction was performed at the same time point. The presented results demonstrate the ability of mammalian cells to switch to more energy-efficient states at low pH and additionally led to the discovery of undesirable changes in metabolic pathways required to fulfill an increased lactate production at high pH. Finally, implications of such metabolic states in process control and optimization are provided.

### 3.5 Remark

The work presented in this chapter has been partially submitted for publication to Biotechnology Progress.
4 An Unstructured Model of Metabolite and Temperature Dependent Cell Cycle Arrest in Hybridoma Batch and Fed-Batch Cultures

4.1 Introduction

Fed-batch processes for the production of monoclonal antibodies are well established at industrial scale. The complexity of these proteins, in particular the requirement of post-translational modifications, necessitates the use of mammalian cell culture for their expression. Since the initial use of cell culture technology for mAb production [12], the biggest achievement in mammalian cell culture over the last three decades has been the increase in cell productivity [17]. This has on the one hand been achieved by the use of advanced production cell lines, originating from CHO cells [157]. On the other hand, optimized culture media [16] and a better comprehension of fed-batch processes have fostered this improvement. Regarding the latter, process parameters used to provide control over the culture environment can be tuned to maximize cell growth as well as productivity [95]. A decrease in the bioreactor pH setpoint, for instance, increased productivity in multiple mAb producing cell lines [95], [158], [159].

Temperature, a process parameter that can be rather easily controlled, has been reported to similarly influence the mAb production rate, particularly when applying mild hypothermic conditions. NS0 cells, when grown at 34 °C accumulated in the G₁ phase and achieved higher antibody production rates, while reaching higher maximal viable cell concentration with prolonged cell viability [160]. Similarly, CHO cells showed cell arrest in G₁ with a 1.7-fold increase in specific productivity at 30 °C accompanied by a reduced cell growth rate [161]. The reported effect of temperature is not limited to the G₁ phase, as others have reported cell blockage in the G₂/M phase post temperature shift [162], or even a rapid decrease in the S phase [163]. Moreover, Ducommun et al. [164] reported a 6-fold increase in the specific production rate and a stabilization of the viable cell concentration in continuous CHO cell culture with
temperature decrease to 32 °C. Similarly, Schatz et al. [165] showed a 38-fold increase in product yield with a temperature shift from 37 °C to 28 °C. Nevertheless, temperature shifts do not always result in an increased product yield. Some reports showed a prolonged cell viability [166] or higher maximum viable cell concentration [167] at lower temperatures, accompanied by a lower antibody yield. Others yet have reported a longer lag phase at 33 °C but achieved comparable cell densities to 37 °C, together with reduced productivity [168]. Moreover, temperature influenced the cell cycle, as evident from an increased G1 phase fraction [169], though this was not linked to a positive effect on mAb titer.

Literature reports are variable and the results seem to depend on the cell lines, the expression systems, and the expressed recombinant product [170], [171]. To some extent, the differences can be attributed to the complex link between the cell cycle being at the center of cellular growth, death, and productivity [172], [173]. In order to find a good balance between temperature-induced growth limitation (and consequently the integral viable cell density – IVCD [174]) and cell-specific productivity [171], temperature dependency has to be investigated and the time point of the temperature shift needs to be optimized with the aim to increase the final antibody titer.

A quantitative understanding of the effect of temperature can aid bioprocess development to improve product titer and product quality. Different temperature conditions and shift strategies are investigated during industrial bioprocess development. However, these investigations are empirical and lack a systematic, model-based approach. The development of relevant mathematical models can help define optimal temperature profiles and is therefore of practical importance. Early contributions [175], [176], initiated the study and analysis of the temperature effect on the cell cycle by employing and formulating mathematical models. Further developments of cell cycle models to study and analyze conditions that enhanced antibody production have been reported [177–180], although without accounting for temperature effects. Temperature was however considered in the study of Fox et al. [181] with the aim to optimize temperature shift times, albeit without considering the cell cycle. Recently, Karra et al. [96] proposed a detailed mass-based population balance model with cell cycle segregation and linked it to an unstructured model.
including the temperature effect. Unfortunately, the model was validated only during batch culture, which lacks industrial relevance.

Herein we formulate a temperature-dependent cell cycle model for a mAb producing mammalian cell line. The model is developed for cultures in batch and fed-batch mode – covering a range of nutrient conditions – while capturing the distribution of cells in different cell cycle phases, including the quiescent state (G0). We validate the model experimentally and use it to predict the time points for temperature shifts in fed-batch culture. The overall methodology maps the path to systematically develop industrially relevant mathematical models with the aim to optimize cultivation temperature profiles.

4.2 Materials and Methods

4.2.1 Cell line and Media

HFN 7.1 murine hybridoma cells (ATCC CRL-1606) [87] adapted to chemically defined, protein- and peptide-free culture media Turbodoma® TP6 (Cell Culture Technologies) were used in this study. The media was prepared following the vendor’s instruction and supplemented with 4.5 g/L D-glucose, 4 mM L-glutamine and 0.1% (w/v) pluronic F-68. Cells were sub-cultured in suspension for 14 days at 37 °C in a humidified incubator containing 5% CO2 and subsequently used for batch and fed-batch cultivations.

4.2.2 Shake Flask Batch Culture

HFN 7.1 cells were cultured in triplicates in 1 L Erlenmeyer flasks (Corning) with 240 mL working volume at a seeding cell concentration of 0.6 x 10^6 cells/mL. At the start of each batch experiment the temperature was set to either 37 °C or 33 °C. Mixing was accomplished using a Stuart SSL1 orbital shaker (Bibby Scientific) at 125 rpm. Viable cell concentration and viability were evaluated manually by a dye-exclusion method using Erythrosin-B stain solution. Samples were counted using a haemocytometer under the Leica DM-IL inverted phase microscope (Leica). Samples were taken twice a day and centrifuged at 660rpm for 3 minutes. The supernatant was stored at -20 °C for metabolic profiling and antibody titer determination. 1 x 10^6 cells were fixed by
drop wise addition of 1 mL of 75% v/v ice-cold ethanol for DNA analysis. The fixed cells were stored at -20 °C prior to cell cycle analysis.

4.2.3 Fed-Batch Cell Culture

Fed-batch cultures were performed in a parallel bioreactor system (DasGip) equipped with online temperature, pH and dissolved oxygen measurement probes (Mettler-Toledo), a pitched-blade impeller and a porous sparger (10 µm). Cells were seeded into the bioreactor at a cell concentration of 0.6 x 10^6 cells/mL in a working volume of 1 L. Dissolved oxygen was controlled at 50% air saturation with a constant airflow of 3 L/h and an oxygen on demand strategy. pH setpoint was set equal to 7.2 over the whole culture and controlled initially by CO₂ sparging and in some cases by base addition (2 mol/L sodium hydroxide) depending on the lactate production of the growing cells. Temperature was either set to 37 °C and kept constant throughout the culture or shifted to mild hypothermia (33 °C) at three different time points (t = 0 h, t = 30 h, t = 76 h) by changing the controller setpoint. The time required for the reactor to reach the new temperature setpoint was negligible short.

A continuous feed of amino acids (RPMI-1640 50x, Sigma), vitamins (RPMI-1640 100x, Sigma), trace elements (trace element mix 1000x, Bioconcept) and L-glutamine (64 mmol/L) was initiated 30 hours after inoculation with a constant pump rate of 2.33 mL/h. D-glucose feeding solution (1 mol/L) was fed from the beginning of the culture in order to keep the glucose concentration in the bioreactor at a setpoint of 20 mM. The pump rate of the glucose solution was adjusted every 12 hours according to the measured viable cell concentration and specific glucose consumption rate resulting in a semi-continuous feeding mode.

Samples were taken twice a day and the viable cell concentration was determined by a CedeX cell counter (Innovatis) using the trypan blue exclusion method [88]. Concentrations of glucose and lactate were measured immediately using a Super GL compact instrument (Hitado). Samples were stored at -20 °C for later analysis of metabolites and IgG₁ concentration.

Once a day 2 x 10^6 cells were fixed by drop wise addition of 2 mL of 75% v/v ice-cold ethanol. The fixed cells were stored at -20 °C prior to cell cycle analysis.
4.2.4 Cell Cycle Analysis

Prior to flow cytometric analysis, fixed cell samples from the batch culture were washed with a rinsing solution with 1% w/v bovine serum albumin (Sigma) and 0.01% w/v sodium azide (Sigma Aldrich) in PBS [182]. DNA staining was performed for 30 minutes in the dark, followed by the addition of 1mL of 50 μg/mL propidium iodide (PI; Sigma, P4170) and of 100 μg/mL ribonuclease A (Sigma, R4875) in PBS at room temperature. Fixed fed-batch cell samples were washed twice with 1% w/v fetal bovine serum (PAN Biotech GmbH) in PBS and re-suspended in 200 µL washing solution. 100 µL of the cell suspension was incubated with 5 µL FITC conjugated Mouse Anti-Human Ki-67 antibody (556026, BD Pharmingen) for 30 minutes in the dark at room temperature. Ki-67 staining is used to determine the proliferating cell population (G1, S, G2/M). After incubation with Ki-67 antibody, the excess stain was washed with 1% w/v fetal bovine serum (PAN Biotech GmbH) in PBS. DNA was stained by the addition of 0.5 mL propidium iodide (1 μg/ml) (P4864, Sigma) and 100 μg/ml ribonuclease A (EN053, Thermo Scientific) in PBS and incubated for 30 minutes in the dark at room temperature.

Flow cytometry was performed on a LSRFortessa (BD) with an excitation wavelength of 561 nm, or on a Calibur flow cytometer (BD) with an excitation wavelength of 488 nm. The data were collected with a 582/15 filter for PI on FACS LSRFortessa, or a 585/42 filter for PI on FACS Calibur and a 530/30 filter for FITC on FACS Calibur. To reduce the spillover of FITC into the PI channel, compensation was performed using a FITC single color control sample. Between 10,000-20,000 events/sample were collected. The data were analyzed using FlowJo software (Tree Star Inc).

4.2.5 Metabolic Profiling

The extracellular concentrations of ammonium, lactate, glutamine and glucose in the batch culture were measured from the supernatant samples using a Nova BioProfile 400 Analyzer (Nova Biomedical).

Glutamine concentrations in the fed-batch culture were measured from the supernatant by HPLC (ZORBAX Eclipse Plus C18 4.6 mm x 150 mm, Agilent Technologies) according to Agilent’s application note (5990-3283EN). Prior to the HPLC
measurement, supernatant samples were filtered at 4000 rcf, 4 °C for 25 minutes using a Vivaspin500 (5 kDa) centrifugation filter (Satorius Stedim). Ammonium concentrations were determined using the L-Glutamine/Ammonia (Rapid) Assay Kit (K-GLNAM, Megazyme) following the vendor’s protocol.

4.2.6 Extracellular Antibody Quantification

Monoclonal antibody concentrations were determined from the supernatant by standard affinity chromatography using a PA ImmunoDetection® Sensor Cartridge (Applied Biosystems) containing immobilized protein G.

4.3 Modeling Aspects

4.3.1 Model Structure

The model was developed on basis of previous models [183–185] that included the description of the cell cycle phases ($X_{G0}$, $X_{G1}$, $X_S$, $X_{G2M}$). The model is stated in the form of ordinary differential equations (Eq.4-1 – Eq. 4-4). The transition between the phases is schematically presented in Figure 4-1.

\[
\frac{d(X_{G1}V)}{dt} = 2k_{G2/M-G1}X_{G2/M}V - k_{G1-S}X_{G1}V - k_{G1-G0}X_{G1}V - k_dX_{G1}V - F_{OUT}X_{G1}
\]  
(4-1)

\[
\frac{d(X_SV)}{dt} = k_{G1-S}m_{stress}X_{G1}V - k_{S-G2/M}X_{S}V - k_dX_{S}V - F_{OUT}X_{S}
\]  
(4-2)

\[
\frac{d(X_{G2/M}V)}{dt} = k_{S-G2/M}X_{S}V - k_{G2/M-G1}X_{G2/M}V - k_dX_{G2/M}V - F_{OUT}X_{G2/M}
\]  
(4-3)

\[
\frac{d(X_{G0}V)}{dt} = k_{G1-S}(1 - m_{stress})X_{G1}V + k_{G1-G0}X_{G1}V - k_dX_{G0}V - F_{OUT}X_{G0}
\]  
(4-4)

\[
\frac{d(X_{V}V)}{dt} = \frac{d(X_{G0}V)}{dt} + \frac{d(X_{G1}V)}{dt} + \frac{d(X_{S}V)}{dt} + \frac{d(X_{G2/M}V)}{dt}
\]  
(4-5)

Viable cells:
Dead cells: \[
\frac{d(VX_D)}{dt} = X_V k_d V - X_D k_{lys} V
\] (4-6)

Cell Cycle fractions: \[
f_i = \frac{X_i}{X_V}
\] (4-7)

The viable cell population \((X_V)\) is calculated as the sum of all cell cycle phases (Eq. 4-5). The dead cell population \((X_D)\) can be estimated from the viable cells (Eq. 4-6). Thus, the cell cycle fractions \((f_i)\) can be calculated according to equation 4-7.

The transition rates between the subpopulations \(X_{G1}-X_S\) \((k_{G1-S})\) (Eq. 4-8), \(X_S-X_{G2/M}\) \((k_{S-G2M})\) (Eq. 4-9), and \(X_{G2/M}-X_{G1}\) \((k_{G2/M-G1})\) (Eq. 4-10) are stated as previously derived [183]. The transition to the \(G_0\) phase is triggered by two types of stresses: temperature-dependent stress \((k_{G1-G0})\) (Eq. 4-11), which is expected to have a constant rate \(r_T\) when lowered from \(37 ^\circ C\) to \(33 ^\circ C\) and/or metabolic stresses \((m_{stress})\) (Eq. 4-12). The metabolic stress is defined as the product of the substrate limitation, toxic metabolite inhibition, and a certain resistance to such stresses \((f_{res})\).

\[G_1 \text{ to } S: \quad k_{G1-S} = \left(\mu + \frac{F_{OUT}}{V}\right) \left(\frac{2 - f_{G1}}{f_{G1}}\right)
\] (4-8)

\[S \text{ to } G2/M: \quad k_{S-G2/M} = \left(\mu + \frac{F_{OUT}}{V}\right) \left(\frac{1 + f_{G2/M}}{f_S}\right)
\] (4-9)

\[G2/M \text{ to } G1: \quad k_{G2/M-G1} = \left(\mu + \frac{F_{OUT}}{V}\right) \left(\frac{1}{f_{G2/M}}\right)
\] (4-10)

\[G1 \text{ to } G0: \quad k_{G1-G0} = \begin{cases} T = 33 ^\circ C & = r_T \\ T = 37 ^\circ C & = 0 \end{cases}
\] (4-11)

Metabolic stresses:
\[m_{stress} = \begin{cases} \text{if } f_{inh1}f_{inh2}f_{lim1}f_{res} > 1 & = 1 \\ \text{else} & = f_{inh1}f_{inh2}f_{lim1}f_{res} \end{cases}
\] (4-12)

As can be seen from equations 4-8 – 4-10, the growth-associated transitions are dependent on the growth rate \((\mu)\) (Eq. 4-13), which is formulated as a function of the characteristic times that are required to complete every cell cycle phase \((t_{G1}, t_S, t_{G2/M})\) (Eq. 4-14). Several biological contributions were considered to affect these
characteristic times including temperature \((k_T)\) (Eq. 4-15) [186], the availability of substrate \(f_{\text{lim1}}\) (Eq. 4-16), and the toxic effect of metabolites \(f_{\text{inh1/inh2}}\) (Eq. 4-17) [125]. In the case of the last two, a Monod kinetics form was assumed [187]. The death rate \((k_d)\) (Eq. 4-18) is formulated based on previous reports [187], [188], including glutamine limitation \(f_{\text{lim1}}\), and it is considered that death can occur at any of the cell cycle phases.

Growth rate:
\[
\mu = \frac{\ln(2)}{t_{G1} + t_s + t_{G2/M}} \tag{4-13}
\]

Cell cycle times:
\[
t_{G1,G2/M} = \frac{t_{G1,G2/M\,\text{initial}} k_T}{f_{\text{lim1}} f_{\text{inh2}} f_{\text{inh1}} n} \quad t_s = \frac{t_{S\,\text{initial}} + k_T}{f_{\text{lim1}} f_{\text{inh2}} f_{\text{inh1}}^2} \tag{4-14}
\]

Cell cycle temperature factor
\[
k_T = \begin{cases} T = 33^\circ C & k_T \\ T = 37^\circ C & 1 \end{cases} \tag{4-15}
\]

Limiting functions:
\[
f_{\text{lim1}} = \frac{[\text{Gln}]}{K_{\text{Gln}} + [\text{Gln}]} \tag{4-16}
\]

Inhibition functions:
\[
f_{\text{inh1}} = \frac{K_{\text{Lac}}}{K_{\text{Lac}} + [\text{Lac}]} \quad f_{\text{inh2}} = \frac{K_{\text{Amn}}}{K_{\text{Amn}} + [\text{Amn}]} \tag{4-17}
\]

Death rate:
\[
k_d = \frac{k_{d\,\text{MAX}}}{1 + f_{\text{lim1}} \frac{K_{d\,\text{Amn}}}{[\text{AMM}]} \tag{4-18}
\]

The changes in substrate and metabolite concentrations are calculated by performing a mass balance around the bioreactor while accounting for the cell metabolism [187], [188]. The bioreactor functions under the assumptions of perfect mixing and operates under batch and fed-batch mode. The change in volume \((V)\) is therefore related to feeding \((F_{\text{Glc}}, F_{\text{Gln}})\) or sampling \((F_{\text{out}})\) (Eq. 4-19). The change in glutamine concentration \([\text{Gln}]\) is dependent on the glutamine uptake \((Q_{\text{Gln}})\), glutamine degradation into ammonia \(K_{\text{deg}}\), and the presence of glutamine in the feed \([\text{Gln}_{\text{feed}}]\) (Eq. 4-20). The glutamine uptake depends on the cell growth rate considering a specific yield on glutamine \(Y_{\text{Gln}}\), an uptake limitation function following Monod kinetics \(f_{\text{upt}}\), and a maintenance term \(m_{\text{Gln}}\) [189] (Eq. 4-21). The ammonia
concentration \([\text{Amm}]\) is closely linked to the glutamine changes by the yield of ammonia on the glutamine consumption \((Y_{\text{Amm}})\) and the degradation of glutamine (Eq. 4-22), as described above.

Reactor volume:
\[
\frac{dV}{dt} = F_{\text{Glc}} + F_{\text{Gln}} - F_{\text{OUT}} \tag{4-19}
\]

Glutamine:
\[
\frac{d([\text{Gln}]V)}{dt} = -Q_{\text{Gln}}X_{V}V - K_{\text{deg}}[\text{Gln}]V + F_{\text{Gln}}[\text{Gln}_{\text{Feed}}] \\
- F_{\text{OUT}}[\text{Gln}] \tag{4-20}
\]
\[
Q_{\text{Gln}} = \left( \frac{\mu}{Y_{\text{Gln}}} \right) f_{\text{upt}} + m_{\text{Gln}} \\
f_{\text{upt}} = \frac{[\text{Gln}]}{K_{\text{Gln}} + [\text{Gln}]} \tag{4-21}
\]
\[
m_{\text{Gln}} = \frac{\alpha_{1}[\text{Gln}]}{\alpha_{2} + [\text{Gln}]} \]

Ammonium:
\[
\frac{d([\text{Amm}]V)}{dt} = Q_{\text{Gln}}Y_{\text{Amm}}X_{V}V + K_{\text{deg}}[\text{Gln}]V - F_{\text{OUT}}[\text{Amm}] \tag{4-22}
\]

The glucose concentration \([\text{Glc}]\) (Eq. 4-23) depends on the glucose uptake rate \((Q_{\text{Glc}})\), the glucose maintenance consumption \((m_{\text{Glc}})\), and the glucose concentration in the feed \([\text{Glc}_{\text{Feed}}]\). \(Q_{\text{Glc}}\) has been formulated considering glutamine being an essential biosynthetic precursor for cells [190], and glucose consumption being temperature-dependent [166], as well as inhibited by lactate [151]. Equation 4-24 combines those three effects on glucose uptake (Eq. 4-24). Moreover, it is assumed that cells in the quiescent state \(G_{0}\) will consume glucose at a maintenance rate, as in this phase there is minimal cell growth [191]. The lactate balance \([\text{Lac}]\) (Eq. 4-25) is connected to glucose consumption \((Q_{\text{Glc}}, m_{\text{Glc}})\) by a variable yield of lactate on glucose \((Y_{\text{Lac}})\) induced by a feedback inhibition as previously reported [125] (Eq. 4-26).

Glucose:
\[
\frac{d([\text{Glc}]V)}{dt} = -Q_{\text{Glc}}X_{V}(1 - f_{G0})V - m_{\text{Glc}}X_{V}f_{G0}V \\
+ F_{\text{Glc}}[\text{Glc}_{\text{Feed}}] - F_{\text{OUT}}[\text{Glc}] \tag{4-23}
\]
\[
Q_{\text{Glc}} = \left( \frac{\mu}{Y_{\text{Glc}}} \right) f_{\text{upt}}f_{\text{inh1}}^{k_{T}} \tag{4-24}
\]
\[
\frac{d([Lac]V)}{dt} = Y_{Lac} Q_{Glc} X_V (1 - f_{G0}) V - Y_{Lac} m_{Glc} X_V f_{G0} V - F_{OUT}[Lac]
\]

Lactate:

\[
Y_{Lac} = Y_{Lac \text{ initial}} f_{\text{inh1}}^{(m+k_T)}
\]

The change in monoclonal antibody concentration ([mAb]) (Eq. 4-27) is growth associated and separated in antibody specific cell cycle productivity rates (\(q_i\)).

\[
\frac{d([mAb]V)}{dt} = \mu [q_{G1/G0}(X_{G1} + X_{G0}) + q_S X_S + q_{G2/M} X_{G2/M}] - F_{OUT}[mAb]
\]

\textbf{4.3.2 Temperature Dependent Population Transition}

An important aspect of the proposed population modelling is the cell behavior at the time of temperature transition. Given that the cell growth (\(\mu\)) has been formulated as being temperature-dependent, cell growth transition takes place when the reactor temperature is switched (i.e. from normal conditions at 37 °C to mild hypothermia at 33 °C). This formulation reflects the dynamic and adaptive process of cell growth. Therefore, the temperature shift is not expected to be reflected instantaneously in the cell growth. Such behavior has been previously reported after a temperature shift as a gradual decrease in the S phase [163], a partial blockage in the G\(_2/M\) [162] and an eventual increase in the G\(_1\) cell phase [160]. In order to capture such dynamics, it is herein assumed that after a temperature shift the existing cell population will proliferate at a transitory rate, where the G\(_1\) and S phase will remain at a fast rate (i.e. 37 °C) and G\(_2/M\) will be affected immediately (i.e. 33 °C). However, every new born cell of the existing population will proliferate under new reactor temperature (Figure 4-1). Therefore, the existing population will be metabolically active during transition until this population disappears either because it completes the cell cycle, gets arrested at G\(_0\) or dies. The cells in G\(_0\) will continue to consume glutamine at the transition conditions until the end of the culture. Thereby cells in G\(_0\) that are still active in biosynthesis – since glutamine plays a key role in cell metabolism [192–194] – but do not grow or commit to apoptosis (an energy intensive process) [195], are considered.
On contrary, the metabolism of all newly born cells will be immediately affected and is therefore calculated considering the new reactor temperature.

Figure 4-1: Schematic representation of the cell cycle model.

4.3.3 Global Sensitivity Analysis and Parameter Estimation

After defining the model type and structure, the uncertainty of the parameters in the model output was studied as outlined in the mathematical modelling development framework [196–198]. In order to systematically evaluate the output variability, global sensitivity analysis (GSA) was performed. The full range of 27 parameters was varied ±50% from their nominal value and evaluated over 40,000 scenarios generated using the sobolset function in Matlab. Each scenario was evaluated using the go:MATLAB tool from gPROMs® providing the link between both softwares. The sensitivity indexes (SIs) were calculated using the GUI-HDMR software [199].

Six outputs were evaluated ($X_V, f_{G1}, f_S, f_{G2M}, f_{G0}$ and $mAb$) at five time points (12, 24, 36, 48, 96 and 144h) of a fed-batch culture. The SIs are the result of the GSA and vary between 0 and 1, with 0 being not significant. The cut-off value to determine the significant parameters was set at 0.1 since 10% can be attributed to experimental errors [200]. Therefore, parameters that are not identified as significant can be fixed at their nominal value and the significant parameters should be re-estimated using experimental data to minimize the output uncertainty.
4.4 Results

4.4.1 Global Sensitivity Analysis and Parameter Estimation

Global sensitivity analysis allows studying the output sensitivity to the variation of the model input parameters. This process can aid the model development as the most significant parameters are identified (and re-estimated), whereas the non-significant parameters can be fixed at their initial value (from literature or assumed). Herein, the majority of the nominal values were set to literature values as previously reported [201]. The time points for the GSA were selected in order to evaluate different phases of a fed-batch process including a temperature shift during the exponential growth phase at 40 hours. Specifically the time points from the lag phase (day 0.5), early exponential (day 1), exponential phase (day 1.5), exponential after the temperature shift (day 2), stationary phase (day 4) and decline phase (day 6) were evaluated. GSA results in Figure 4-2 are represented at 3 time points to illustrate the time evolution of the sensitivity index (SI) using six output parameters (\(X_V\), \(mAb\), \(f_{G0}\), \(f_{G1}\), \(f_S\) and \(f_{G2/M}\)).

The analysis resulted in the identification of 9 significant parameters (i.e. SI above 0.1 as defined). The SIs for the output of viable cell concentration (\(X_V\)) for the different time points are shown (Figure 4-2A). \(X_V\) was sensitive to the variation of the cell cycle times (\(t_{Sinitial}\), \(t_{G2/Minitial}\)) at early stages of the culture and its sensitivity decreased in time (though remaining significant). This can be attributed to the connection of characteristic cell cycle times to the cell growth rate. In contrast, the significance of the resistance to the metabolic stresses (\(f_{res}\)) increased along the culture time, reaching significance at the stationary growth phase. The sensitivity towards the yield of biomass on glutamine (\(Y_{Gln}\)) increased at early stages, peaked at day 1.5 (data point not shown), and decreased towards the end of the culture. Comparable results were observed for the output considering \(mAb\) concentration (Figure 4-2B). In addition to the cell cycle times, the \(mAb\)-specific productivity for the G1/G0 phase (\(q_{G1/G0}\)) and S phase (\(q_S\)) were found to be significant at early stages of the culture, thereby showing the importance of defining cell cycle specific production rates. The cell cycle temperature factor (\(k_T\)) and the sensitivity of cell cycle times to lactate inhibition (\(n\)) were significant during the stationary and decline growth phase. In these phases lactate is likely to reach inhibitory concentrations. The G0 cell fraction (\(f_{G0}\)) output was highly
sensitive to the resistance to metabolic stresses throughout the culture (Figure 4-2C). Similarly, other cell cycle fractions showed an increasing sensitivity towards this parameter along the culture. The $G_1$ cell fraction ($f_{G1}$) was sensitive at early stages to the initial S phase cell cycle time and at late stages to the $G_2/M$ cell cycle time, as well as to the initial $G_1$ cell cycle time ($t_{G1\text{initial}}$) throughout culture time (Figure 4-2D). The S phase cell fraction ($f_S$) was sensitive to the initial S phase cell cycle time and to the $G_1$ cell cycle time (Figure 4-2E), and the $G_2/M$ fraction ($f_{G2/M}$) was sensitive to the initial S phase cell cycle time and to the $G_2/M$ cell cycle time (Figure 4-2F). The three cell cycle fractions ($f_{G1}, f_S, f_{G2/M}$) associated to proliferation showed sensitivity to the cell cycle temperature factor ($k_T$) at the time points after the temperature shift.

The association of cell cycle time estimates to all studied GSA outputs stresses the importance of considering the cell cycle heterogeneity. Similarly, the cell cycle temperature factor was identified significant for the outputs of proliferating cell cycle phases, $X_T$ and the antibody production. Such a dependency supports the importance and critical aspect of finding a balance between growth and productivity when using temperature to optimize fed-batch processes. Particularly, if one or more of the cell cycle phases are associated to productivity, as obtained from GSA.

Based on these results, significant parameters were re-estimated with the gPROMS parameter estimator (based on the maximum likelihood formulation), using experimental data from the 37 °C and 33 °C batch and 37 °C and 33 °C fed-batch cultivations. The chosen experimental data sets assess apart from two temperature setpoints (37 °C and 33 °C), the effect of metabolic stresses, which are partially also identified as sensitive parameters. Batch cultures cover nutrient depletion, that is responsible for triggering changes in the cell behavior [202]. Similarly, fed-batch cultures capture the dynamics of toxic metabolite accumulation due to feeding strategies which are of industrial relevance. The new parameter set, estimated from 4 experimental data sets is reported with the 95% confidence intervals in the Appendix (Table A 6). The largest confidence intervals are reported for cell cycle specific productivities ($q_{G1/G0}, q_S$) and reflect a weak estimation of these parameters which is due to the growth-associated antibody production of HFN 7.1 cells.
Figure 4-2: Sensitivity indices for parameters at simulation times (day 1, 4 and 6). 
(A) viable cell concentration (Xv), (B) mAb titer, (C) G₀ cell fraction (f_G₀), (D) G₁ cell fraction (f_G₁) (E) S cell fraction (f_S) (F) G₂/M cell fraction (f_G₂/M). The nomenclature is equal as in Eq. 4-1 – 4-27.
4.4.2 Temperature Shifted Batch Cultures

The 37 °C batch culture performed in shake flasks under uncontrolled pH conditions shows the typical batch operation profiles (Figure 4-3). Briefly, the viable cell concentration ($X_v$) increases exponentially after a short period of adaption (lag phase), and peaks around the time when one of the key substrates (in this case glutamine $GLN$) is exhausted. After glutamine depletion, the proliferation slows down and a significant death rate is observed (decline phase), coinciding with the increase of the lumped $G_1/G_0$ population and the corresponding decrease of the S and G2/M fractions. The toxic metabolite production ($LAC$, $AMM$) is correlated to the substrate consumption ($GLC$, $GLN$), as well as the antibody concentration ($mAb$) to the viable cell concentration.

The experimental data of the 37 °C batch culture are used for parameter estimation in the formulated model. The fit of the 37 °C batch culture is shown in Figure 4-3. The model describes the viable cell concentration and viability trends, although there are small deviations between absolute values of viability in the model and the experiment. Regarding cell cycle fractions, the change in the cell cycle trend after the exhaustion of glutamine was captured. At the same time, an initial discrepancy in the model description for the S and G2/M cell cycle fractions is observed at early stages (~0.5 days). Substrate profiles and lactate production are well predicted by the model, whereas the ammonia profile is consistently over-estimated. The yield of ammonia on glutamine ($Y_{amm}$) is considered constant in the model. However, it has been shown that $Y_{amm}$ depends on temperature and pH [93], [203]. This can serve as a reason for the observed discrepancies in the ammonia profile particularly in pH-uncontrolled shake-flask cultures. Nevertheless, the overall trend describing ammonia production is correct. The antibody titer is satisfactorily described up to around 2 days of culture. After this point the model starts to slightly deviate from the experimental data.
Figure 4-3: Shake flask batch culture at 37 °C. Experimental results of viable cell concentration ($X_v$), viability, antibody concentration (mAb), glucose (GLC), lactate (LAC), glutamine (GLN) and ammonia (AMM) concentrations, and cell cycle fractions $G_0/G_1$, $S$, $G_2/M$ compared to model results (solid line: $X_v$, LAC, AMM, $G_0/G_1$, $S$, $G_2/M$ fractions; dashed lines: viability, mAb, GLC, GLN).

The 33 °C batch cultivation includes the effect of temperature reduction at the beginning of the culture (0 days) (Figure 4-4). The trends observed for viable cell concentration $X_v$, viability, substrates (GLC, GLN) and toxic metabolites (LAC, AMM) are comparable to the 37 °C culture. However, a significantly lower maximum $X_v$ is achieved (1.8-fold smaller compared to the 37 °C batch), with a corresponding lower final antibody concentration (1.4-fold lower compared to the 37 °C batch) despite
having a 1.5-fold longer culture time. The substrate/toxic metabolite profiles still have a good correlation, although the lactate production was further increased at late stages of the culture even at low glucose consumption. The cell cycle fractions showed minor changes during the first 0.5 day of culture. Around day 1 a significant change in the cell cycle distribution was observed, which coincided with the initiation of exponential growth, and was followed by rather constant cell cycle fractions until the end of the culture. After glutamine depletion, an increased dispersion in the G1/G0 and G2/M fractions was observed.

The experimental trends of the 33 °C batch culture that were likewise used for parameter estimation are fitted by the proposed model (Figure 4-4). Maximum $X_V$ and the timing at which it was achieved (day 3) are properly captured. However, some differences are observed at early stages corresponding to a pronounced lag phase, as well as during the decline phase. The latter is also reflected in the overestimation of viability compared to the experimental data. The substrate profiles ($GLC$, $GLN$) together with the $AMM$ and antibody profiles are satisfactorily modeled throughout the culture. The lactate profile shows a good fit up to 3 days, even though the experimentally observed constant increase cannot be captured by the model. It is assumed that uncontrolled pH-conditions in the shake flask system are responsible for the observed differences. The sharp changes in the cell cycle distribution observed at day 1 are reflected in the model simulations, however appear earlier compared to the experimental cell cycle fractions. The model formulation is unable to capture the prolonged lag phase that seemed to take place while the cells were adapting to the new temperature condition. This is due to the fact that cells are considered homogenous within a cell cycle phase. Such differences can be attributed to specific cell cycle transitions of cohorts or partially synchronized populations. In order to model these particular cell cycle changes, another internal coordinate needs to be employed in a segregated formulation such as population balance models. Additional discrepancies appear in the simulations of lumped G1/G0 and G2/M fractions at the late stage of the cell culture.
Figure 4-4: Shake flask batch culture at 33 °C. Experimental results of viable cell concentration (Xv), viability, antibody concentration (mAb), glucose (GLC), lactate (LAC), glutamine (GLN) and ammonia (AMM) concentrations, and cell cycle fractions G₀/G₁, S, G₂/M compared to model results (solid line: Xv, LAC, AMM, G₀/G₁, S, G₂/M fractions; dashed lines: viability, mAb, GLC, GLN).

4.4.3 Temperature Shifted Fed-Batch Cultures

Standard 37 °C fed-batch cultures were carried out as duplicates in controlled bioreactors, and were used for parameter estimation in addition to batch cultures (Figure 4-5). The increase in viable cell concentration by 2.1-fold compared to a standard batch culture is attributed to a continuous feed of glucose, amino acids
(including glutamine), vitamins and trace elements. Avoidance of key substrate exhaustion \((\text{GLC, GLN})\) results in a prolonged stationary phase for several days at maintained high viability. Glucose and glutamine are consumed throughout the culture, although at a slower rate towards the end. The feeding strategy results in high toxic metabolite \((\text{LAC, AMM})\) levels [125]. In particular, ammonia reaches 5.5-fold higher concentrations compared to the 37 °C batch. Around day 3, ammonia exceeds toxic concentrations and simultaneously lactate concentration plateaus. As a result, an arrest in the \(\text{G}_0\) cell cycle phase is triggered. The \(\text{G}_0\) fraction increases steadily until the end of the cultivation with the accumulation of lactate and ammonia over this period. The observed experimental variation in the lactate profile is attributed to pH control by \(\text{CO}_2\) sparging and varying amount of base (sodium hydroxide) added in the bioreactor control strategy. Similarly the relationship of lactate production and base addition in pH-controlled bioreactors accompanied by high osmolarity, has been shown in industrial fed-batch processes to result in big differences in the final lactate profile, without affecting the growth profile [204].

The model fit for the fed-batch culture at 37 °C is provided in Figure 4-5. The increase, peak and decline of \(X_V\) are closely fitted. While the viability decline in the late stage of the culture is underestimated in batch, it is better captured in fed-batch. This difference in the model performance can be explained by the different experimental setups used for batch and fed-batch cultures. Initial comparative 37 °C batch cultures performed in both cultivation systems showed good agreement, except for cell viability profiles (data not shown). In general, viability remains higher in the controlled bioreactor. This observation is attributed to the different cell counting methods used to determine viable cell concentrations. In batch culture, viable cells were counted manually, whereas in fed-batch cells they were determined automatically with a lower cell cut-off size set at 9 µm. Moreover, hydromechanical stress rates are considerably smaller in shake flask cultures [205] and therefore the variations in viability can also be attributed to differences in cell lysis within the two experimental setups, which are not considered in the model.
Figure 4-5: Controlled fed-batch culture at 37 °C. Experimental results of viable cell concentration (Xv), viability, antibody concentration (mAb), glucose (GLC), lactate (LAC), glutamine (GLN) and ammonia (AMM) concentrations, and cell cycle fractions G₀, G₁, S, G₂/M compared to model results (solid line: Xv, LAC, AMM, G₁, S, G₂/M fractions; dashed lines: viability, mAb, GLC, GLN, G₀ fraction).

Regarding the trend of cell cycle fractions \( f_{G0}, f_{G1}, f_S, f_{G2/M} \) including the timing of G₀ increase triggered by metabolite accumulation \( (LAC, AMM) \), it is well described in the model. Taking decreased substrate uptake rates along the culture into consideration, a good description of the glutamine profile was achieved, whereas the glucose
consumption seems to be even further decreased in the last two days of culture. Profiles of AMM and antibody show a good fit, with some differences in antibody concentration on the last culture day. The decrease in titer can be attributed to protein degradation due to increased proteolytic activity towards the end of the culture [206]. The averaged lactate profile is also adequately modeled. Since experimentally observed differences in the lactate profile did not significantly affect other model outputs (i.e. viable cells, viability, mAb titer, cell cycle distribution), it was sufficient to use the averaged lactate concentration as a triggering range.

A 33 °C fed-batch culture with initial temperature shift (at time point 0) represents a further experimental data set for parameter estimation (Figure 4-6). A significantly lower cell concentration \(X_v\) is reached (2.6-fold lower when compared to the 37 °C fed-batch culture). Due to decreased glucose consumption rates, especially towards the end of the culture, a lower lactate concentration is obtained. However, ammonia concentrations reach levels comparable to those at 37 °C, while less glutamine is consumed. Most importantly, a cell arrest towards the G_0 cell cycle fraction is evident from the beginning of the culture with a steady increase over the remaining cultivation time. Thus, cell cycle arrest is, in addition to metabolic control, also triggered by hypothermia.

The fit of the 33 °C fed-batch culture is shown in Figure 4-6. \(X_v\) is accurately fitted and only slightly over-estimated during the decline phase. Cell viability, antibody concentration and cell cycle fractions are well described. Substrates (GLC, GLN) as well as the LAC profile show acceptable agreement, while the ammonia simulation follows the data trend but is highly under-estimated. Ammonia production was assumed to be solely dependent on glutamine. However, ammonia is also a product of asparagine consumption [37], an amino acid present in high concentration in the feed. In addition, amino acid consumption has been shown to be affected by temperature [207], thus providing an explanation for the discrepancies.
Figure 4-6: Controlled fed-batch culture shifted to 33 °C at culture start. Experimental results of viable cell concentration ($X_v$), viability, antibody concentration (mAb), glucose (GLC), lactate (LAC), glutamine (GLN) and ammonia (AMM) concentrations, and cell cycle fractions $G_0$, $G_1$, $S$, $G_2/M$ compared to model results (solid line: $X_v$, LAC, AMM, $G_1$, $S$, $G_2/M$ fractions; dashed lines: viability, mAb, GLC, GLN, $G_0$ fraction).

4.4.4 Prediction of Temperature Shift Time in Fed-Batch Cultures

The model was used to predict two fed-batch experiments with temperature shifts from 37 °C to 33 °C during the exponential growth phase (30 hours) and in the early stationary phase (76 hours).
Figure 4-7: Controlled fed-batch culture with temperature shift to 33 °C after 30 h. Shift is indicated by dotted line. Experimental results of viable cell concentration (Xv), viability, antibody concentration (mAb), glucose (GLC), lactate (LAC), glutamine (GLN) and ammonia (AMM) concentrations, and cell cycle fractions G0, G1, S, G2/M compared to model prediction (solid line: Xv, LAC, AMM, G1, S, G2/M fractions; dashed lines: viability, mAb, GLC, GLN, G0).

Temperature reduction after 30 hours is overall well predicted by the model (Figure 4-7). The profile of Xv follows the trend with minor discrepancies. The exponential phase, during which the temperature shift is performed, shows an over-estimation, whereas the prolonged stationary/decline phase is slightly under-estimated. In fact, the resulting viable cell concentration is comparable to the 37 °C fed-batch culture (85%).
The antibody concentration is slightly underestimated after day 4.5, but the prediction of decreased final mAb concentration compared to 37 °C (90%) is met. Likewise, the decrease in GLC consumption accompanied by a lower LAC production, as well as ammonia production expected to remain high as in previous fed-batch cultures, is well predicted. Glutamine consumption properly predicted until day 2, shows a systematic difference between the model and the data suggesting that glutamine is consumed at a higher rate than predicted. The increase in quiescent cells due to toxic metabolite accumulation and temperature shift is represented by the increase of the G0 fraction at the time of the temperature shift and further rise throughout the culture. Furthermore, the shift in temperature is followed by changes in the proliferating cell cycle fractions such as an increase in the G1 cell fraction. Initial discrepancies in the S and G2/M fraction are in the same range as the ones observed in the 37 °C standard fed-batch culture, even though the general trend is captured.

The second model prediction considers a fed-batch culture with temperature shift from 37 °C to 33 °C at 76 hours (Figure 4-8). A further delay of the temperature shift time is expected to result in higher cell concentrations compared to the earlier shift time points. Experimental data show that the viable cell concentration is affected instantly by the temperature shift, reaching 95% of \(X_V\) at standard 37 °C, and the model matches the profile during all growth phases to satisfaction. Viability and antibody concentration profiles show good agreement between the simulation and the data. Final antibody titer remains at 90% of the 37 °C standard fed-batch culture and does not show further improvement. The glucose consumption trend is well predicted, even though after the temperature shift glucose consumption seems to be systematically under-predicted. On the contrary, the glutamine profile shows systematic differences by under-estimating glutamine consumption after the temperature shift. Toxic metabolite profiles followed the prediction with similar differences as observed before. The G0 fraction starts to increase at day 2 due to metabolic effectors and is further triggered by the temperature shift. The model is able to predict the behavior of cell arrest. Solely an experimentally observed decrease in G0 during the last 2 day of the cultivation accompanied by an increase in G1 fraction is not represented in the model.
As in the previous cases, soon after the temperature shift the sharp change in the cell cycle fractions is in good agreement to the model prediction.

Figure 4-8: Temperature shifted controlled fed-batch culture to 33 °C after 76 h. Shift in indicated by a dotted line. Experimental results of viable cell concentration (Xv), viability, antibody concentration (mAb), glucose (GLC), lactate (LAC), glutamine (GLN) and ammonia (AMM) concentrations, and cell cycle fractions G₀, G₁, S, G₂/M compared to model prediction (solid line: Xv, LAC, AMM, G₁, S, G₂/M fractions; dashed lines: viability, mAb, GLC, GLN, G₀ fraction).
4.5 Discussion

The presented model serves as a tool to study the temperature effect in mammalian cell culture systems. The impact of temperature shifts is addressed by considering an unstructured cell cycle model that captures the basic heterogeneity of the system. In addition to temperature effects, metabolic stresses, such as substrate depletion or toxic metabolite accumulation, are considered on the cell cycle level to account for different process modes (batch and fed-batch). This could be accomplished by providing experimental measurements of the quiescent cell cycle phase in addition to commonly measured proliferating cell cycle phases for model development. Thus, the model was formulated using first principles and further developed by using the existing model development framework [196–198]. The framework describes a procedure that includes the identification of sensitive parameters and their re-estimation using experimental data from batch and fed-batch cultures at two different temperature setpoints. When applying this model to other cell lines, such parameters can easily be estimated from small-scale cultures used for pre-studies in bioprocess development.

The proposed procedure, validated in HFN 7.1 hybridoma cell culture was able to capture the experimental trends of batch and fed-batch cultures, especially considering the relationship between temperature, growth and productivity as the main focus. Minor discrepancies are mostly observed on metabolic level. In particular, the glucose uptake rate seemed to be even further decreased along the culture than predicted by the model. Similarly, ammonia production was considered to only depend on glutamine consumption and degradation, which resulted in an underestimation of experimental results. In order to achieve a better representation of substrate and metabolite profiles, metabolic relations need to be further redefined. Nonetheless, the model was able to successfully predict two temperature shift scenarios and to capture most of the experimentally observed trends, not only on growth and metabolism, but also at the cell cycle level. Most importantly, the model correctly captured the G0 cell cycle phase arrest. The importance of the cell arrest is reflected in a number of studies [95], [161], [163], [173], [208] that have used this phase to increase productivity in commercially relevant cell lines. Moreover, the relevance of considering the cell cycle segregation
and the temperature growth regulation is of industrial interest as this is often the method of choice for improving productivity [186].

HFN 7.1 cells are characterized by growth-associated antibody production reflected in a weak estimation of cell cycle specific productivities \( q_{G1/G0}, q_S \). On the contrary, productivity in other cell lines has been associated to specific cell cycle phases [172], [173], [209]. In such cases, the developed model is readily applicable and provides a means to systematically optimize the cell cycle-to-productivity-relationship. However, the formulation of an industrially significant cell cycle model is not limited to temperature dependency, but also to the inclusion of biologically relevant events (e.g. apoptosis, product quality).

This model approach can be employed to systematically study and optimize temperature shift strategies. Particularly, the use of computational modeling is a complementary tool that needs to be adopted in biochemical processes [210]. The advantage of the presented approach over the commonly adopted design of experiment (DoE) approach relies on the ability to capture the complex dynamic relations of the mammalian culture system.

4.6 Conclusions

The importance of the results presented herein lies in the development of relevant modeling approaches and a procedure to assist mammalian cell culture process development. In particular, time points of temperature shifts can be predicted in fed-batch culture in order to optimize productivity. The development of an unstructured model of metabolic- and temperature-dependent cell cycle arrest is validated in hybridoma batch and fed-batch cultures. Through sensitivity analysis and parameter estimation using two batch and two fed-batch experimental data sets 9 sensitive parameters are re-estimated and used to predict different temperature shift time points in fed-batch culture. This model formulation allows studying complex processes of biological systems with the experimental tools currently available.
4.7 Remark

The work presented in this chapter has been performed in collaboration with Prof. Mantalaris’ group from Imperial College London. The model has been developed in the Mantalaris group and batch cultivations have been performed in their group. Fed-batch experiments including cell cycle analysis have been performed in the Morbidelli group at ETH Zurich.
5 Concluding Remarks

Through this work, a better understanding of the influence of process parameters on cultured cells and their expressed proteins has been obtained.

The first two studies focused on experimental approaches that can be applied to optimize process conditions with the aim of obtaining a comparable cell behavior regardless of bioreactor scale or aiming in desired product quality. In particular, pH was identified as a crucial parameter, as it can be used to modify mAb glycosylation and control lactate production and consumption. The effect of pH on glycosylation was evaluated in controlled batch culture using MALDI-TOF and HPLC-based glycosylation analysis. The redistribution of the cell metabolism due to pH alteration was identified by flux balance analysis and validated by gene expression analysis. The calculated flux distribution provides an insight into pathway utilization and energy generation in cell metabolism. Due to the difficulties regarding pH control in large-scale bioreactors, the findings of both studies explain observed changes in glycosylation and metabolism during scale-up and can be further exploited for process control and optimization.

In addition to experimental investigations, mathematical models can support process optimization by linking complex biological system to process conditions such as hypothermia. The last study described a mathematical model that predicts the time point of temperature shifts on the level of cell proliferation and arrest. In addition to temperature-dependent cell cycle arrest, the model is validated against metabolite-dependent arrest and is applicable to different culture modes. The aim of such a model is to achieve a good balance of cell growth and productivity in fed-batch culture.

The presented studies fit well in the scope of Quality by Design (QbD) principles, where process parameters are investigated in order to identify their impact on critical quality attributes. In this context, the first study assessed ranges of different physical and chemical stress parameters representing common bioprocess conditions and defined their ability to maintain a given glycosylation pattern. Through this analysis, certain parameters such as sparging rate could be excluded from being qualified as
critical, whereas osmolarity only became important once combined with pH. The presented methodology could in future be further applied in a high-throughput setting for studying additional parameters such as CO₂, as well as for experiments where multiple process parameters are simultaneously changed. In this way a broad range of process parameters could be defined as truly critical for mAb glycosylation.

The use of a QbD-based approach to assess environmental conditions is gaining increasing importance due to the emergence of biosimilar drugs. In the case of biosimilars, the originator drug defines the reference quality attributes such as the glycosylation pattern. Experimental or model-based approaches such as the ones presented here are therefore becoming an integral part of bioprocess development.
6 Outlook

The experimental and model-based approaches that have been presented in this thesis were tested with one cellular system, a murine hybridoma cell line producing a monoclonal antibody. In this way the impact of multiple process parameters on mAb glycosylation, cellular growth and metabolism could be compared among each other. In order to test for the generality of the approaches, the developed methodologies require further application on multiple cellular systems, as well as on one defined cell line producing different glycoproteins. In addition to hybridoma cells, it would be beneficial to apply the presented procedures to CHO cells, as the most commonly used mammalian host cells for monoclonal antibody production in industry.

In this way more general conclusions on process parameters affecting glycosylation and on the degree of the observed changes in glycosylation, as presented in chapter 2 could be drawn. In addition, an extension of the study could include a separate investigation of glycosylation control by nutrients and feeds using the same cellular system. The outcome of this study could then be compared to the impact of process parameters. This would help defining general and complete process conditions and process parameters that can be applied as control variables to obtain the desired glycosylation profile following the QbD approach.

The methodology used to study pH alterations on cellular metabolism, as described in chapter 3, would also benefit from a general approach including different cell lines. This would provide an insight into whether the flux balance model together with the chosen objective function hold for the cultivation of multiple mammalian cell lines in bioreactor systems. In order to confirm the calculated pathway utilization at different pH conditions, the flux distribution requires experimental validation by isotope measurements using $^{13}$C-metabolic flux analysis. Such measurements should however be performed in a different, more appropriate experimental setup such as a shake flask system, since they are not feasible in bioreactors.

The cell cycle model framework together with the described procedure to re-estimate model parameters presented in chapter 4 will ideally be tested using a cell line that
shows growth-independent productivity. Such cell lines are characterized by a production phase that appears during stationary growth and would therefore be highly related to specific cell cycle phases. Due to the different intrinsic cellular characteristics, such a system would provide an ideal validation of the developed model framework.

To conclude, QbD-based studies profit from high-throughput and automatization allowing the simultaneous investigation of multiple process conditions with high reproducibility. Thus, it will be beneficial to perform future studies in novel high-throughput bioreactor systems.
7 Appendix

7.1 Appendix for “Evaluating the Impact of Cell Culture Process Parameters on Monoclonal Antibody N-Glycosylation”

7.1.1 Deglycosylation by PNGase F and 2-AB Labeling

N-linked oligosaccharides of each mAb preparation were enzymatically released by addition of 2 µL of N-glycosidase F (PNGase F glycerol free) (New England Biolabs) in the supplied buffer for 14 hours at 37 °C. Prior to deglycosylation each mAb preparation was denatured with the supplied glycoprotein denaturing buffer at 37 °C for 10 minutes. After deglycosylation the oligosaccharides were bound to a Supelclean ENVI-Carb column tube (Sigma Aldrich) in 2% acetonitrile / 0.1 mol/L ammonium acetate solution, washed and eluted with 50% acetonitrile in H₂O. The eluent was collected and evaporated to dryness in a Vacufuge® plus (Eppendorf).

N-linked oligosaccharides were fluorescently labeled with 20 µL of 0.35 mol/L 2-aminobenzamide (2-AB) (Sigma Aldrich) in a solution of 70% DMSO, 30% glacial acetic acid, 1 mol/L sodium cyanoborohydride according to Bigge et al. (1995) for 2 hours at 65 °C. The reaction mixture was cooled down and diluted with 380 µL acetonitrile. Labeled oligosaccharides were purified with Whatman 3MM filter paper disks according to Merry et al. (2002). Briefly, the reaction mixture was loaded on the paper disk and excess 2-AB dye was washed 3 times with 95% acetonitrile. The 2-AB labeled oligosaccharides were eluted 3 times with Milli-Q water (Millipore) and stored at -20 °C.

7.1.2 HPLC Separation of 2-AB Labeled Oligosaccharides

30 µL of the 2-AB labeled oligosaccharides sample was diluted in 70 µL acetonitrile. 90 µL of the prepared sample was injected at a flow rate of 0.6 mL/min on an Agilent 1200 HPLC system (Agilent Technologies) equipped with a fluorescence detector for analysis by hydrophilic interaction chromatography (HILIC) using a GlykoSep N-Plus column (4.6 mm x 150 mm, Prozyme). N-linked oligosaccharides were separated based on their hydrodynamic volume using a gradient system of buffer A (10 mmol/L
formic acid, 80% acetonitrile) and buffer B (30 mmol/L formic acid, 40% acetonitrile) at pH 4.4 with a linear increase from 30 – 90% of buffer B over 90 min. The separated oligosaccharides were fluorescently detected with an excitation wavelength of 250 nm and an emission wavelength of 410 nm. The peak fractions were collected every minute with a fraction collector FC 203B (Gilson), concentrated to approximately 10 µL in a Vacufuge® plus (Eppendorf), and stored at -20 °C for mass spectrometry (MS) analysis.

7.1.3 N-linked Oligosaccharide Analysis by Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) Mass Spectrometry (MS)

MS analysis was performed at the Functional Genomic Center Zurich using a 4800 Plus MALDI TOF/TOF system (AB SCIEX) equipped with a 200 Hz Nd:YAG laser. 2-AB labeled N-linked oligosaccharides were desalted by ZipTip µ-C18 pipette tips (Millipore). ZipTips were wetted with 80% acetonitrile and twice equilibrated with Milli-Q water (Millipore), 10 µL of sample was bound by 5 times pipetting up and down and washed twice with 0.1% formic acid. 0.9 µL of 2,5-dihydroxybenzoic acid (DHB) matrix (10 g/L) in 70% acetonitrile was pipetted 3 times up and down with the previously prepared ZipTip µ-C18, spotted onto a 384 Opti-TOF MALDI target plate and dried at room temperature. The MALDI-TOF mass spectrometer was operated in reflectron positive and negative ion mode with a laser intensity of 5400 – 5600 arb. Mass spectra were acquired by accumulating 1000 – 4000 subspectra in a mass range from 750 m/z – 4000 m/z. Fragment ions of oligosaccharides were detected by MS/MS with collision-induced dissociation (CID) in positive and negative ion mode. The following parameter settings were applied in the MS/MS mode: Laser intensity: 5700 – 6000 arb, collision gas pressure: 2.5 x 10⁻⁶ Torr, timed ion selector (TIS) resolution: 150, accumulation of 2000 – 4000 subspectra.

Proposed glycan structures were either deduced from the fragmentation pattern obtained from MS/MS analysis, by comparison of obtained masses with databases provided in GlycoWorkBench2 [100] or by comparison of HPLC profiles with commercial Cetuximab antibody with a known glycan profile [48].
Figure A1: MALDI-TOF MS spectra of the 2-AB labeled oligosaccharide released from HFN 7.1 antibody by PNGase F in (A) positive ion mode and (B) negative ion mode. Peaks in positive ion mode are either detected as Na$^+$ or K$^+$ adducts. (C) Fragmentation pattern of G1F ($m/z$ 1767.70) obtained by MALDI-TOF MS/MS in positive ion mode. Sugar residues are N-acetylglucosamine (GlcNAc, □), fucose (F, ▲), mannose (M, ●), galactose (G, ○).
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Table A 1: Measured masses of reductively aminated oligosaccharides determined by MALDI-TOF MS and compared to theoretical masses. Proposed glycoforms were assigned to the glycosylation profile in Figure 2-4.
### Measured m/z values

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<td>(HexNAc2Hex3)Fuc(HexNAcHexNeuGc) (HexNAcHex2)HexNAcHex2</td>
<td></td>
<td>2903.05</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*a* Hex, HexNAc, Fuc, NeuGc, NeuAc indicate hexose, N-acetylhexosamine, fucose, N-glycolylneuraminic acid and N-acetylneuraminic acid, respectively.

*b* Sugar residues are N-acetylglucosamine (GlcNAc, ■), fucose (F, △), mannose (M, ●), galactose (G, ○) and N-glycolylneuraminic acid (NGNA, ◦)
7.2 Appendix for “Insights into pH induced Metabolic Switch by Flux Balance Analysis”

7.2.1 Gene Expression Analysis

Table A 2: List of target genes investigated by gene expression analysis.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hk1 hexokinase 1</td>
</tr>
<tr>
<td>2</td>
<td>Hk2 hexokinase 2</td>
</tr>
<tr>
<td>3</td>
<td>Pfkl phosphofructokinase, liver</td>
</tr>
<tr>
<td>4</td>
<td>Pgam2 phosphoglycerate mutase 2</td>
</tr>
<tr>
<td>5</td>
<td>Pkm2 pyruvate kinase muscle</td>
</tr>
<tr>
<td>6</td>
<td>Pepck1 phosphoenolpyruvate carboxykinase 1, cytosolic</td>
</tr>
<tr>
<td>7</td>
<td>Pepck2 phosphoenolpyruvate carboxykinase 2, mitochondrial</td>
</tr>
<tr>
<td>8</td>
<td>Ldha lactate dehydrogenase A</td>
</tr>
<tr>
<td>9</td>
<td>Pcx pyruvate carboxylase</td>
</tr>
<tr>
<td>10</td>
<td>Pdha1 pyruvate dehydrogenase E1 alpha 1</td>
</tr>
<tr>
<td>11</td>
<td>Pdha2 pyruvate dehydrogenase E1 alpha 2</td>
</tr>
<tr>
<td>12</td>
<td>Dlat dihydrolipoamide S-acetyltransferase</td>
</tr>
<tr>
<td>13</td>
<td>Pdk1 pyruvate dehydrogenase kinase, isoenzyme 1</td>
</tr>
<tr>
<td>14</td>
<td>Pdk2 pyruvate dehydrogenase kinase, isoenzyme 2</td>
</tr>
<tr>
<td>15</td>
<td>Pdk3 pyruvate dehydrogenase kinase, isoenzyme 3</td>
</tr>
<tr>
<td>16</td>
<td>Cs citrate synthase</td>
</tr>
<tr>
<td>17</td>
<td>Idh3a isocitrate dehydrogenase 3 alpha</td>
</tr>
<tr>
<td>18</td>
<td>Ogdh oxoglutarate dehydrogenase</td>
</tr>
<tr>
<td>19</td>
<td>Sdha succinate dehydrogenase complex, subunit A</td>
</tr>
<tr>
<td>20</td>
<td>Fhl fumarate hydratase 1</td>
</tr>
<tr>
<td>21</td>
<td>Mdh1 malate dehydrogenase 1, NAD</td>
</tr>
<tr>
<td>22</td>
<td>Mdh2 malate dehydrogenase 2, NAD, mitochondrial</td>
</tr>
<tr>
<td>23</td>
<td>Acly ATP citrate lyase</td>
</tr>
<tr>
<td>24</td>
<td>Me1 malic enzyme 1, cytosolic</td>
</tr>
<tr>
<td>25</td>
<td>Me2 malic enzyme 2, mitochondrial NAD dependent</td>
</tr>
<tr>
<td>26</td>
<td>Me3 malic enzyme 3, mitochondrial NAD dependent</td>
</tr>
<tr>
<td>27</td>
<td>$Gpt2$</td>
</tr>
<tr>
<td>28</td>
<td>$Got1$</td>
</tr>
<tr>
<td>29</td>
<td>$Got2$</td>
</tr>
<tr>
<td>30</td>
<td>$Glud1$</td>
</tr>
<tr>
<td>31</td>
<td>$Gls$</td>
</tr>
<tr>
<td>32</td>
<td>$G6pdh$</td>
</tr>
</tbody>
</table>
### 7.2.2 Metabolic Network for Flux Balance Analysis

Table A 3: Reactions in the metabolic network used for flux balance analysis.

<table>
<thead>
<tr>
<th>Glycolysis</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 GLC6P $\rightarrow$ F6P</td>
<td></td>
</tr>
<tr>
<td>2 F6P + ATP $\rightarrow$ ADP + 2 GAP</td>
<td></td>
</tr>
<tr>
<td>3 GAP + NAD + ADP $\rightarrow$ [3PG] + NADH + ATP</td>
<td></td>
</tr>
<tr>
<td>4 3PG $\rightarrow$ PEP</td>
<td></td>
</tr>
<tr>
<td>5 PEP + ADP + H $\rightarrow$ PYR + ATP</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pentose Phosphate Pathway</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>6 GLC6P + 2 NADP + H2O $\rightarrow$ RBL5P + 2 NADPH + CO2</td>
<td></td>
</tr>
<tr>
<td>7 RBL5P $\rightarrow$ R5P</td>
<td></td>
</tr>
<tr>
<td>8 RBL5P $\rightarrow$ X5P</td>
<td></td>
</tr>
<tr>
<td>9 2 X5P + R5P $\rightarrow$ 2 F6P + G3P</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pyruvate Metabolism</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>10 PYR + H $\rightarrow$ PYR$_m$ + H$_m$</td>
<td></td>
</tr>
<tr>
<td>11 PYR$_m$ + CoA$_m$ + NAD$_m$ $\rightarrow$ CO2 + AcCoA$_m$ + NADH$_m$ + H$_m$</td>
<td></td>
</tr>
<tr>
<td>12 PYR$_m$ + HCO3$_m$ + ATP$_m$ $\rightarrow$ OAA$_m$ + ADP$_m$ + Pi</td>
<td></td>
</tr>
<tr>
<td>13 MAL$_m$ + NAD$_m$ $\rightarrow$ PYR$_m$ + NADH$_m$ + H$_m$ + CO2</td>
<td></td>
</tr>
<tr>
<td>14 MAL + NADP $\rightarrow$ PYR + NADPH + H + CO2</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TCA Cycle</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>15 OAA$_m$ + AcCoA$_m$ + H2O $\rightarrow$ CIT$_m$ + CoA$_m$</td>
<td></td>
</tr>
<tr>
<td>16 CIT$_m$ + NAD$_m$ + H2O $\rightarrow$ AKG$_m$ + NADH$_m$ + CO2 + H$_m$</td>
<td></td>
</tr>
<tr>
<td>17 AKG$_m$ + NAD$_m$ + CoA$_m$ $\rightarrow$ SUCCoA$_m$ + NADH$_m$ + H$_m$ + CO2</td>
<td></td>
</tr>
<tr>
<td>18 SUCCoA$_m$ + ADP$_m$ + Pi$_m$ + FAD$_m$ $\rightarrow$ FUM$_m$ + ATP$_m$ + CoA$_m$ + FADH2$_m$</td>
<td></td>
</tr>
<tr>
<td>19 FUM$_m$ + H2O $\rightarrow$ MAL$_m$</td>
<td></td>
</tr>
<tr>
<td>20 MAL$_m$ + NAD$_m$ $\rightarrow$ OAA$_m$ + NADH$_m$ + H$_m$</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Malate Aspartate Shuttle</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>21 OAA$_m$ + GLU$_m$ $\rightarrow$ AKG$_m$ + ASP$_m$</td>
<td></td>
</tr>
<tr>
<td>22 AKG + ASP $\rightarrow$ OAA + GLU</td>
<td></td>
</tr>
<tr>
<td>23 ASP$_m$ + GLU $\rightarrow$ ASP + GLU$_m$</td>
<td></td>
</tr>
<tr>
<td>24 OAA + NADH + H $\rightarrow$ MAL + NAD</td>
<td></td>
</tr>
<tr>
<td>25 MAL + AKG$_m$ $\rightarrow$ MAL$_m$ + AKG</td>
<td></td>
</tr>
</tbody>
</table>
### Glutaminolysis

26 \( \text{GLN} + \text{H}_2\text{O} \rightarrow \text{GLU} + \text{NH}_4 \)
27 \( \text{GLU}_m + \text{H}_2\text{O} + \text{NAD}_m \leftrightarrow \text{AKG}_m + \text{NADH}_m + \text{NH}_4 \)
28 \( \text{GLU} \leftrightarrow \text{GLU}_m \)

### Acetyl CoA Formation

29 \( \text{CIT} + \text{CoA} + \text{ATP} + \text{H}_2\text{O}\rightarrow \text{AcCoA} + \text{OAA} + \text{ADP} + \text{Pi} \)

### Amino Acid Synthesis

30 \( \text{GLU} + \text{PYR} \leftrightarrow \text{AKG} + \text{ALA} \)
31 \( \text{3PG} + \text{NAD} + \text{GLU} + \text{H}_2\text{O}\rightarrow \text{NADH} + \text{H} + \text{AKG} + \text{Pi} + \text{SER} \)
   \( \text{GLU} + \text{ATP} + \text{H} + \text{NADPH} + \text{H} + \text{NADH} \rightarrow \text{ADP} + \text{NADP} + \text{Pi} + \text{H}_2\text{O} + \text{NAD} + \text{PRO} \)
32 \( \text{SER} + \text{THF} \leftrightarrow \text{GLY} + \text{MTHF} + \text{H}_2\text{O} \)
33 \( \text{GLN} + \text{ASP} + \text{ATP} + \text{H}_2\text{O} \leftrightarrow \text{GLU} + \text{ASN} + \text{AMP} + 2 \text{Pi} + \text{H} \)

### Amino Acid Degradation

35 \( \text{SER} \leftrightarrow \text{PYR} + \text{NH}_4 \)
36 \( \text{THR} + \text{NAD} + \text{CoA} \rightarrow \text{GLY} + \text{AcCoA} + \text{NADH} + \text{H} \)
   \( \text{ARG}_m + \text{H}_2\text{O} + \text{AKG}_m + \text{NAD}_m \leftrightarrow 2 \text{GLU}_m + \text{UREA}_m + \text{NADH}_m + \text{H}_m \)
37 \( \text{H}_m \)
38 \( \text{PRO}_m + 2 \text{H}_2\text{O} + \text{NAD}_m \rightarrow \text{GLU}_m + \text{NADH}_m + \text{H}_m \)
39 \( \text{HIS}_m + 3 \text{H}_2\text{O} \rightarrow \text{GLU}_m + \text{Formamide}_m + \text{NH}_4 \)
   \( \text{LYS}_m + \text{NADPH} + 2 \text{AKG}_m + 4 \text{NAD}_m + 2 \text{H}_2\text{O} + 2 \text{CoA}_m \rightarrow \text{NADP} + 2 \text{H}_2\text{O} + 2 \text{AcCoA}_m \)
40 \( \text{H}_m + 4 \text{NADH}_m + 2 \text{GLU}_m + 2 \text{CO}_2 + 2 \text{AcCoA}_m \)
41 \( \text{PHE} + \text{NADPH} + \text{H} + \text{O}_2 \rightarrow \text{TYR} + \text{NADP} \)
   \( \text{TYR} + \text{AKG} + 2 \text{O}_2 + \text{CoA} + \text{H}_2\text{O} \leftrightarrow \text{GLU} + \text{CO}_2 + 2 \text{H} + \text{FUM}_m + \text{AcCoA} + \text{Acetate} \)
42 \( \text{VAL}_m + \text{AKG}_m + \text{CoA}_m + 3 \text{NAD}_m + \text{FAD}_m + 3 \text{H}_2\text{O} + \text{ATP}_m \rightarrow 2 \text{H}_m + \text{GLU}_m + \text{SUCCoA}_m + \text{CO}_2 + 3 \text{NADH}_m + \text{FADH2}_m + \text{ADP}_m + \text{Pi}_m \)
43 \( \text{ILE}_m + \text{AKG}_m + 2 \text{NAD}_m + 2 \text{CoA}_m + \text{FAD}_m + \text{H}_2\text{O} + \text{HCO}_3\text{m} + \text{ATP}_m \rightarrow \text{GLU}_m + \text{SUCCoA}_m + \text{AcCoA}_m + 2 \text{NADH}_m + \text{CO}_2 + 2 \text{FADH2}_m + \text{ADP}_m + \text{Pi}_m \)
44 \( \text{LEU}_m + \text{AKG}_m + \text{NAD}_m + 2 \text{CoA}_m + \text{ATP}_m + \text{HCO}_3\text{m} + \text{H}_2\text{O} \rightarrow \text{GLU}_m + \text{NADH}_m + \text{CO}_2 + \text{H}_m + \text{Pi}_m + \text{ADP}_m + \text{Acetate} + 2 \text{AcCoA}_m \)
45 \( \text{GLU}_m + \text{NADH}_m + \text{CO}_2 + \text{H}_m + \text{Pi}_m + \text{ADP}_m + \text{Acetate} + 2 \text{AcCoA}_m \)
46 \( \text{CYS} + \text{AKG} + \text{SO}_3 + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{GLU} + \text{PYR} + \text{S}_2\text{O}_3 + 2 \text{H} \)
   \( \text{MET} + 2 \text{ATP} + \text{H}_2\text{O} + \text{SER} + \text{CoA} + \text{NAD} + \text{HCO}_3 \rightarrow \text{NH}_4 + \text{CYS} + \text{CO}_2 + \text{NADH} + \text{SUCCoA}_m + \text{ADP} + \text{Pi} \)
Transport

<table>
<thead>
<tr>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>48  CIT$_m$ + MAL $\leftrightarrow$ CIT + MAL$_m$</td>
</tr>
<tr>
<td>49  PRO $\leftrightarrow$ PRO$_m$</td>
</tr>
<tr>
<td>50  ARG $\leftrightarrow$ ARG$_m$</td>
</tr>
</tbody>
</table>

PEP Regeneration

<table>
<thead>
<tr>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>51  OAA$_m$ + ATP$_m$ $\rightarrow$ ADP$_m$ + PEP$_m$ + CO2</td>
</tr>
<tr>
<td>52  PEP $\leftrightarrow$ PEP$_m$</td>
</tr>
</tbody>
</table>

Oxidative Phosphorylation

<table>
<thead>
<tr>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>53  NADH$_m$ + H$_m$ + 0.5 O2 + 2.5 ADP$_m$ + 2.5 Pi$_m$ $\rightarrow$ NAD$_m$ + H$_2$O + 2.5 ATP$_m$</td>
</tr>
<tr>
<td>54  FADH$_2$m + 0.5 O2 + 1.5 ADP$_m$ + 1.5 Pi$_m$ $\rightarrow$ FAD$_m$ + H$_2$O + 1.5 ATP$_m$</td>
</tr>
</tbody>
</table>

Nucleotide Synthesis

<table>
<thead>
<tr>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>55  R5P + ATP $\rightarrow$ PRPP + AMP</td>
</tr>
<tr>
<td>56  PRPP + 2 GLN + GLY + ASP + 4 ATP + CO2 $\rightarrow$ IMP + 2 GLU + FUM + 4 ADP + 2 H$_2$O</td>
</tr>
<tr>
<td>57  IMP + ASP + 2 ATP + GTP $\rightarrow$ dATP + FUM + 2 ADP + GDP</td>
</tr>
<tr>
<td>58  IMP + GLN + 3 ATP + NAD + 2 H$_2$O $\rightarrow$ dGTP + GLU + 2 ADP + AMP + NADH</td>
</tr>
<tr>
<td>59  HCO$_3$ + NH$_4$ + ASP + PRPP + 3 ATP + NAD $\rightarrow$ dTTP + 3 ADP + CO$_2$ + NADH</td>
</tr>
<tr>
<td>60  dTTP + GLN + ATP $\rightarrow$ dCTP + GLU + ADP</td>
</tr>
</tbody>
</table>

Uptake or Production of Substrates (Measured Fluxes)

<table>
<thead>
<tr>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>61  GLC$<em>6$P + ADP + H $\rightarrow$ GLC$</em>{ex}$ + ATP</td>
</tr>
<tr>
<td>62  PYR + NADH + 2 H $\rightarrow$ LAC$_{ex}$ + NAD + H</td>
</tr>
<tr>
<td>63  GLN $\leftrightarrow$ GLN$_{ex}$</td>
</tr>
<tr>
<td>64  GLU $\leftrightarrow$ GLU$_{ex}$</td>
</tr>
<tr>
<td>65  ALA $\rightarrow$ ALA$_{ex}$</td>
</tr>
<tr>
<td>66  SER $\rightarrow$ SER$_{ex}$</td>
</tr>
<tr>
<td>67  GLY $\rightarrow$ GLY$_{ex}$</td>
</tr>
<tr>
<td>68  ASP $\rightarrow$ ASP$_{ex}$</td>
</tr>
<tr>
<td>69  ARG$<em>m$ $\rightarrow$ ARG$</em>{ex}$</td>
</tr>
<tr>
<td>70  HIS$<em>m$ $\rightarrow$ HIS$</em>{ex}$</td>
</tr>
<tr>
<td>71  CYS $\rightarrow$ CYS$_{ex}$</td>
</tr>
<tr>
<td>72  THR $\rightarrow$ THR$_{ex}$</td>
</tr>
<tr>
<td>73  ILE$<em>m$ $\rightarrow$ ILE$</em>{ex}$</td>
</tr>
</tbody>
</table>
74  VAL_m -> VAL_ex
75  PHE -> PHE_ex
76  TYR -> TYR_ex
77  LYS_m -> LYS_ex
78  LEU_m -> LEU_ex
79  PRO -> PRO_ex
80  MET -> MET_ex
81  ASN -> ASN_ex

\[
\begin{align*}
&0.0148 \text{ dATP} + 0.0099 \text{ dCTP} + 0.0099 \text{ dGTP} + 0.0148 \text{ dTTP} + 0.07 \text{ ATP} + 0.033 \\
&\text{ dATP} + 0.0551 \text{ dCTP} + 0.0624 \text{ dGTP} + 0.033 \text{ dTTP} + 0.07 \text{ ATP} + 0.6 \text{ ALA} + \\
&0.377 \text{ ARG м} + 0.359 \text{ ASP} + 0.288 \text{ ASN} + 0.145 \text{ CYS} + 0.322 \text{ GLN} + 0.386 \\
&\text{ GLU} + 0.538 \text{ GLY} + 0.143 \text{ HIS м} + 0.324 \text{ ILE м} + 0.564 \text{ LEU м} + 0.57 \\
&\text{ LYS м} + 0.138 \text{ MET} + 0.219 \text{ PHE} + 0.313 \text{ PRO} + 0.43 \text{ SER} + 0.386 \text{ THR} + \\
&0.044 \text{ TRP} + 0.182 \text{ TYR} + 0.416 \text{ VAL м} + 29.04 \text{ ATP} + 0.38 \text{ GLC6P} + 1.3148 \\
&\text{ ATP} + 0.109 \text{ GAP} + 1.9184 \text{ AcCoA} + 1.70476 \text{ ATP} + 3.40952 \text{ NADH} + 3.51852 \\
&\text{ H} + 0.109 \text{ NADH} + 0.008 \text{ GAP} + 0.0704 \text{ AcCoA} + 0.06256 \text{ ATP} + 0.12512 \\
&\text{ NADH} + 0.13312 \text{ H} + 0.008 \text{ NADH} + 0.324 \text{ AcCoA} + 0.324 \text{ ATP} + 0.288 \text{ NADH} + \\
&0.288 \text{ H} -> \text{ Biomass} + 0.07 \text{ ADP} + 0.07 \text{ Pi} + 0.07 \text{ ADP} + 0.07 \text{ Pi} + 29.04 \text{ ADP} + \\
&29.04 \text{ Pi} + 1.3148 \text{ ADP} + 1.3148 \text{ Pi} + 1.9184 \text{ CoA} + 1.70476 \text{ ADP} + 1.70476 \text{ Pi} + \\
&3.40952 \text{ NAD} + 1.49112 \text{ H2O} + 0.109 \text{ NAD} + 0.0704 \text{ CoA} + 0.06256 \text{ ADP} + \\
&0.06256 \text{ Pi} + 0.12512 \text{ NAD} + 0.05472 \text{ H2O} + 0.008 \text{ NAD} + 0.162 \text{ CoA} + 0.324 \\
&\text{ ADP} + 0.324 \text{ Pi} + 0.288 \text{ NAD} + 0.162 \text{ CO2} \\
&0.5768 \text{ ALA} + 0.411919 \text{ ASN} + 0.330176 \text{ ASP} + 0.35901 \text{ GLN} + 0.464684 \text{ GLU} + \\
&0.683611 \text{ GLY} + 0.669275 \text{ PRO} + 1.010565 \text{ SER} + 0.198476 \text{ ARG м} + \\
&0.232693 \text{ CYS} + 0.204171 \text{ HIS м} + 0.30046 \text{ ILE м} + 0.574408 \text{ LEU м} + \\
&0.54615 \text{ LYS м} + 0.114338 \text{ MET} + 0.366898 \text{ PHE} + 0.979228 \text{ THR} + 0.19333 \\
&\text{ TRP} + 0.251256 \text{ TYR} + 0.796933 \text{ VAL м} + 39.89 \text{ ATP} -> \text{ MAB} + 39.89 \text{ ADP} + \\
&39.89 \text{ Pi}
\end{align*}
\]
The biomass composition of murine hybridoma cells averaged in previous work by Altamirano et al. (2001), Selvarasu et al. (2010) and Xie and Wang (1994) was obtained using the relative compositions from Sheikh et al. (2005) (Appendix Table A 4). The mole of each cellular component can be calculated from the biomass weight. Dry cell weight of ATCC-CRL 1606 cells was considered as 250 pg/cell [215].

Furthermore, diphosphatidylglycerol was not taken into consideration because of its negligible proportion in cellular composition. Therefore, protein, DNA/RNA, carbohydrate, cholesterol, and phospholipids (phosphoglycerides and sphingomyelin) were included in the equations of biomass formation.

In addition to biomass synthesis, monoclonal antibody synthesis was also considered in our network by utilizing the amino acids composition of IgG1 from Quek et al. (2010) (Appendix Table A 5). The theoretical amount of 4.3 ATP per mol amino acid is taken into account for protein synthesis.
Table A 4: Averaged dry cell composition (protein, carbohydrates, nucleotides, lipids) of murine hybridoma cells derived from literature [148].

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>nmol /μg</th>
<th>Metabolite</th>
<th>nmol/μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALA</td>
<td>0.6</td>
<td>Glycogen</td>
<td>0.279</td>
</tr>
<tr>
<td>ARG</td>
<td>0.377</td>
<td>dAMP</td>
<td>0.0148</td>
</tr>
<tr>
<td>ASP</td>
<td>0.359</td>
<td>dCMP</td>
<td>0.0099</td>
</tr>
<tr>
<td>ASN</td>
<td>0.288</td>
<td>dGMP</td>
<td>0.0099</td>
</tr>
<tr>
<td>CYS</td>
<td>0.145</td>
<td>dTMP</td>
<td>0.0148</td>
</tr>
<tr>
<td>GLN</td>
<td>0.322</td>
<td>AMP</td>
<td>0.033</td>
</tr>
<tr>
<td>GLU</td>
<td>0.386</td>
<td>CMP</td>
<td>0.0551</td>
</tr>
<tr>
<td>GLY</td>
<td>0.538</td>
<td>GMP</td>
<td>0.0624</td>
</tr>
<tr>
<td>HIS</td>
<td>0.143</td>
<td>UMP</td>
<td>0.033</td>
</tr>
<tr>
<td>ILE</td>
<td>0.324</td>
<td>Cholesterol</td>
<td>0.018</td>
</tr>
<tr>
<td>LEU</td>
<td>0.564</td>
<td>Phosphatidylcholine</td>
<td>0.069</td>
</tr>
<tr>
<td>LYS</td>
<td>0.57</td>
<td>Phosphatidylethanolamine</td>
<td>0.026</td>
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Table A 5: Amino acid composition of IgG\textsubscript{1} derived from an average IgG\textsubscript{1} protein sequence [137].

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<tr>
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<th>Mass fraction g AA/g mAB</th>
<th>Molar fraction nmol AA/\mu g mAB</th>
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sum (nmol AA/\mu g mAB) | 9.264
Figure A 2: Flux balance model scheme. PEP regeneration pathway was only active at pH 7.8 and therefore the fluxes OAAm→PEPm and PEPm→PEP were constrained under all other conditions.
### 7.2.3 Calculated Intracellular Fluxes from Flux Balance Model

![Figure A 3: Intracellular fluxes calculated by flux balance analysis at three different time points (WD 1.0, WD 1.5, WD 1.9) for standard pH 7.2 culture. Numbers correspond to reactions specified below.](image-url)

1. GLC6P -> F6P
2. F6P -> 2GAP
3. GAP -> 3PG
4. 3PG -> PEP
5. PEP -> PYR
6. GLC6P -> RBL5P
7. PYR -> PYRm
8. PYRm -> AcCoAm
9. PYRm -> OAAm
10. MALm -> PYRm
11. MAL -> PYR
12. OAAm -> CTtm
13. CTtm -> AKGm
14. AKGm -> SUCCoAm
15. SUCCoAm -> FUMm
16. FUMm -> MALm
17. MALm -> OAAm
18. OAAm+GLUm -> AKGm+ASPm
19. AKG+ASP -> OAA+GLU
20. ASPm+GLU -> ASP+GLUm
21. OAA -> MAL
22. MAL+AKGm -> MALm+AKG
23. GLN -> GLU
24. GLUm -> AKGm
25. GLU -> GLUm
26. CIT -> AcCoA+OAA
27. GLU+PYR -> AKG+ALA
28. 3PG+GLU -> AKG+SER
29. GLU -> PRO
30. SER -> GLY
31. GLN+ASP -> GLU+ASN
32. SER -> PYR
33. THR -> GLY+AcCoAm
34. ARGm+AKGm -> 2GLUm
35. PROm -> GLUm
36. HISm -> GLUm
37. LYSm+2AKG_m -> 2GLUm+2AcCoAm
38. PHE -> TYR
39. TYR+AKG -> GLU+AcCoA
40. VALm+AKGm -> GLUm+SUCCoAm
41. ILEm+AKGm -> GLUm+SUCCoAm+AcCoAm
42. LEUm+AKGm -> GLUm+2 AcCoAm
43. CYS+AKG -> GLU+PYR
44. MET+SER -> CYS+SUCCoAm
45. CITm+MAL -> CIT+MALm
46. PRO -> PROm
47. ARG -> ARG_m
Figure A 4: Comparison between intracellular fluxes at culture pH 7.2 and pH 6.8 calculated by flux balance analysis at WD 1.9. Numbers correspond to reactions specified below.

1. GLC6P -> F6P  17. MALm -> OAAm  33. THR -> GLY+AcCoAm
2. F6P -> 2GAP  18. OAAm+GLUm -> AKGm+ASPm  34. ARGm+AKGm -> 2GLUm
3. GAP -> 3PG  19. AKG+ASP -> OAA+GLU  35. PROm -> GLUm
4. 3PG -> PEP  20. ASPm+GLU -> ASP+GLUm  36. HISm -> GLUm
5. PEP -> PYR  21. OAA -> MAL  37. LYSm+2AKG_m -> 2GLUm+2AcCoAm
6. GLC6P ->RBL5P  22. MAL+AKGm -> MALm+AKG  38. PHE -> TYR
7. PYR -> PYRm  23. GLN -> GLU  39. TYR+AKG -> GLU+AcCoA
8. PYRm -> AcCoAm  24. GLUm -> AKGm  40. VALm+AKGm -> GLUm+SUCCoAm
9. PYRm -> OAAm  25. GLU -> GLUm  41. ILEm+AKGm -> GLUm+SUCCoAm+
10. MALm -> PYRm  26. CIT -> AcCoA+OAA  42. LEUm+AKGm -> GLUm+2 AcCoAm
11. MAL -> PYR  27. GLU+PYR -> AKG+ALA  43. CYS+AKG -> GLU+PYR
12. OAAm -> CITm  28. 3PG+GLU -> AKG+SER  44. MET+SER -> CYS+SUCCoAm
13. CITm -> AKGm  29. GLU -> PRO  45. CITm+MAL -> CIT+MALm
14. AKGm -> SUCCoAm  30. SER -> GLY  46. PRO -> PROm
15. SUCCoAm -> FUMm  31. GLN+ASP -> GLU+ASN  47. ARG -> ARG_m
16. FUMm -> MALm  32. SER -> PYR
Figure A 5: Comparison between intracellular fluxes at culture pH 7.2 and pH 7.8 calculated by flux balance analysis at WD 1.9. Numbers correspond to reactions specified below. Reactions 48 - 50 included to metabolic network as additional constraints according to gene expression data.
7.3 Appendix for “An Unstructured Model of Metabolite and Temperature Dependent Cell Cycle Arrest in Hybridoma Batch and Fed-Batch Cultures”

7.3.1 Model Parameter Estimation

Table A 6: Model parameter values.

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<th>Value</th>
<th>Re-estimated value</th>
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9 Figure and Table Index

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