Tobias Habicher, BSc

Evaluating the Effect of Temperature and Osmolarity on Cell Cycle Distribution in Hybridoma Fed-batch Culture with Controlled Glucose Feeding

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Ass.Prof. Dipl.-Ing. Dr.nat.techn. Univ.-Doz. Mario Klimacek

Institut für Biotechnologie und Bioprozesstechnik

Dr. Miroslav Soos
Institut für Chemie- und Bio-Ingenieurwissenschaften, ETH Zürich

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Evaluating the Effect of Temperature and Osmolarity on Cell Cycle Distribution in Hybridoma Fed-batch Culture with Controlled Glucose Feeding

By

Tobias Habicher

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ABSTRACT

In fed-batch cultures glucose is often controlled at low level to limit lactate accumulation and improve productivity of mammalian cell cultures. However, this triggers the risk of unfavorable growth conditions if glucose is depleted. In order to avoid glucose depletion a controlled glucose feeding strategy was implemented. Based on this strategy different glucose profiles in the range of 2 and 30 mM have been investigated in hybridoma CRL-1606 fed-batch culture. As a result, growth was limited when glucose was controlled at 2 mM. In contrast to this, the specific antibody production was increased temporarily by 122%. Nevertheless, lactate was not reduced and accumulated to 29 mM. In experiments where initial glucose concentration was above 2 mM no effect on growth and antibody production was observed. Although in late exponential growth phase cells exhibited lactate consumption. However, lactate consumption was decreased when glucose was fed within the initial growth phase. In addition to the glucose study, the effect of temperature and osmolarity on the cell cycle distribution, productivity and apoptosis have been studied in cultures with decreased temperature (33 ^{o}C), shifted temperature (37-33 ^{o}C) and osmolarity (337-455 mOsm/kg). Temperature reduction to 33 ^{o}C caused an accumulation of cells in G0 and G1 phase, respectively, and a reduction of the cycling fraction, especially the S phase. Nevertheless, culture longevity was not extended even though the increase of the apoptotic fraction was delayed. Shifting temperature from 37 to 33 ^{o}C at 30 and 76 hours after inoculation had negligible effect on the population dynamics. Increasing the osmolarity 76 hours after inoculation initiated abrupt cell death accompanied by impulsive rise of apoptotic and G0 fractions. However, neither for temperature nor for osmolarity shifted conditions specific productivity was enhanced. Finally, it was shown that hybridoma cells CRL-1606 were characterized by a growth associated antibody production.

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1. INTRODUCTION

The increasing demand for monoclonal antibodies for research and therapeutic use has led to a sustained interest in optimized production using hybridoma cell culture. The intensification of the antibody production process primarily requires knowledge of the influence of bioreactor operating parameters on hybridoma growth and productivity. Certainly, viable cell concentrations and culture longevity are crucial parameters to obtain increased antibody concentrations (Xie and Wang, 1997). Due to their operational simplicity, reliability and flexibility, fed-batch cultures represent the most attractive choice to maximize these two parameters. However, in most of the hybridoma fed-batch cultures, cell density and product concentration is low due to the accumulation of toxic end-products such as lactate and ammonia (Ozturk *et al.*, 1992).

High concentration of glucose is accompanied by increased lactate production, whereas glucose is the major carbon and energy source for hybridoma cells (Xie and Wang, 1994). Due to this important role and high consumption rates, it is usually assumed to be one of the nutrients limiting cell growth. Therefore, in a fed-batch process effective control of the supplemental glucose feed is required to overcome depletion and reduce lactate accumulation. Apart from the toxicity of lactate, metabolizing glucose into lactate has a negative effect on the energy yield. When pyruvate is reduced to lactate by lactate dehydrogenase -A 2 ATP per glucose are generated, instead of 36 ATP when being further metabolized in the tricarboxylic acid (TCA) cycle. Chen *et al.* (2001) were able to reduce lactate formation in hybridoma CRL-1606 cells by partially disrupting the LDH-A gene. In contrast to the genetic engineering approach, Chang, Grodzinsky, and Wang (1995) removed lactate in-situ using electrophoresis and showed increased growth and antibody titer. Although in both techniques

lactate was decreased successfully, genetic manipulation and electrophoretic mechanisms are not widely implemented in hybridoma fed-batch cultures.

Recent efforts focus on automated dynamic feeding strategies based on online nutrient measurements such as online glucose measurements (Wong *et al.*, 2005; Lu *et al.*, 2013). Through dynamic feeding, especially of glucose and glutamine, real time nutrient demands of cells are met using a variety of feeding algorithms (Zhang *et al.*, 2004) and this further enables the control of nutrients at relatively constant and low concentrations.

Motivated by these promising results we developed a semi-continuously operating controlled glucose feeding strategy for fed-batch cultivation of murine hybridoma cells HFN 7.1. The applied strategy was able to discharge glucose demand, making constant glucose concentrations feasible. Hence a highly reproducible lactate profile was obtained. Applying this strategy the impact of different glucose concentrations and varying feeding profiles on the lactate metabolism, as well as cellular growth and productivity could be studied.

The impact of media and feeding on productivity can be explained by the relationship between productivity and cell cycle. It was claimed by Dutton, Scharer, and Moo-Young (2006) that one of the most important characteristics determining specific productivity is the linkage between protein expression and the cell cycle phase distribution. It has been shown that in hybridoma cells the specific production rate is increased in the G1 phase of the cell cycle (Hayter *et al.*, 1992; Kromenaker and Srienc, 1991). Based on this fact investigations have been made to arrest cells at this particular point in the cell cycle to improve antibody productivity. Addition of media components such as thymidine, lactate, rapamycin and butyrate has been attempted in order to increase the G1 subpopulation (Balcarcel and Stephanopoulos, 2001; Hayter *et al.*, 1992; Hendrick *et al.*, 2001; Kromenaker and Srienc, 1994). Apart from this, the environment itself can have an effect on the cell cycle phases.

Thus, altering operating parameters as for example temperature gives the possibility to influence population dynamics (Hendrick *et al.*, 2001). Besides temperature osmolarity is another environmental factor which has an influence on cell growth and productivity. It has been shown for GS-NS0 cells, that under hyperosmotic conditions growth was decreased and specific antibody production rate increased (Wu *et al.*, 2004).

Our aim was to give a more profound insight into the cell cycle distribution of the HFN 7.1 hybridoma cell by separating the G1/0 phase in two distinct physiological phases namely G1 and G0 using Ki-67 staining. We studied the cell cycle phase distribution as a function of temperature and osmolarity in a fed-batch culture of hybridoma cells. Finally the distribution pattern was used to allocate in which phase the antibody production occurs primarily.

2. THEORETICAL BACKGROUND

2.1. The cell cycle

The cell cycle is a series of events which a cell undergoes for its division and duplication. Under the presumption that hybridoma cells show the same cell kinetics than other mammalian cells, a murine hybridoma cell passes through four distinct phases: the gap 1 phase (G1-phase), the DNA synthesizing phase (S-phase), the gap 2 phase (G2-phase) and the mitotic phase (M-phase) (Suzuki and Ollis, 1989). The first phase after the previous cell division is the so called G1- phase. Within this phase the cell metabolism is reactivated and the cell size increases. Late in the G1-phase cells reach a point called the restriction point. If DNA damage or unfavorable growth conditions are present cells go into a quiescent state (G0-phase). Cells in G0 are metabolically active but they do not undergo the cell cycle (Morgan, 2007). However, in the case of ameliorating culture conditions cells can exit from the G0 phase and re-enter the cycling fraction. After completion of the G1-phase the cells start to replicate their DNA. The outcome of the S-phase is an identical copy of the DNA within one single cell. G2-phase is characterized by further growth and preparation for the cell division. During the M-phase the chromosomes are separated into two identical sets and the mother cell divides into two daughter cells.

2.2. Principle of flow cytometry

Flow cytometry is a technology that enables simultaneous measurement and analysis of multiple physical and chemical characteristics of single cells and other particles within a heterogeneous suspension. It is used for a wide range of applications including cell cycle analysis, cell sorting, cancer diagnosis, ploidy analysis and cell counting. A flow cytometer consists of three main systems: fluidic system, optic system (including lasers) and electronics

as schematized in Figure 1. Briefly, the cells or particles are drawn up the fluidics system and are pumped to the flow chamber. Within the flow chamber a hydrodynamically focused stream aligns cells/particles to pass single file through a beam of light. At this point numerous detectors capture the spectrally filtered, scattered or fluorescent light and convert it into electrical signals. The resulting voltage pulse is assigned a digital value which is analyzed to quantitate the characteristics of the cells and particles (Figure 1).

The scattered light is collected at two different angles which distinguish the forward (FSC) and side scatter (SSC). Forward scattered light is measured in the direction of the laser beam and is proportional to cell surface area or size. Side scattered light is collected at approximately 90 degrees from the lasers path and is proportional to granularity or internal complexity (Rowley, 2013).

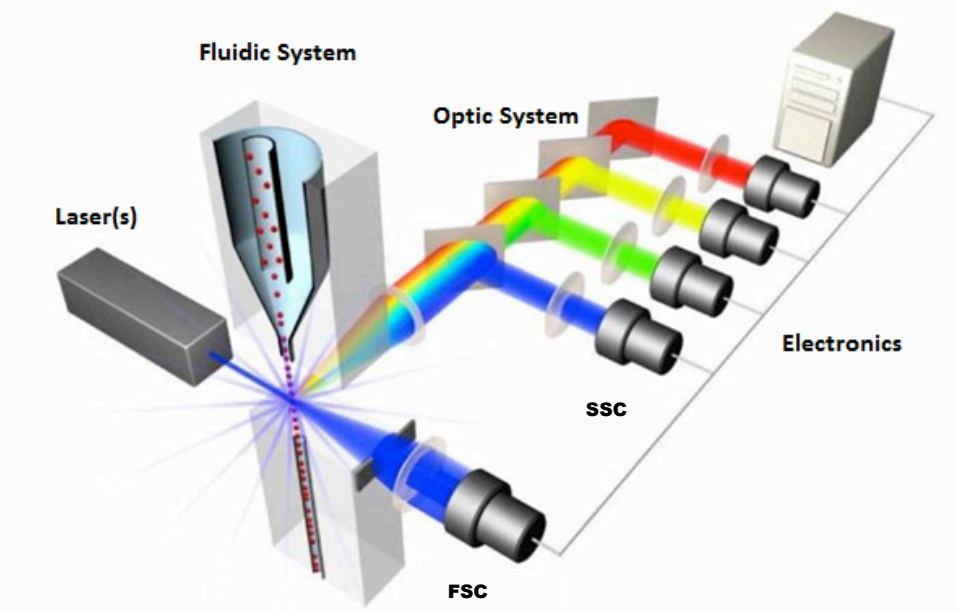

Figure 1. Basic set up of a flow cytometer. Within the fluidic system a hydrodynamically focused stream aligns cells and particles to pass single file through the laser. Scattered and fluorescent light is spectrally filtered (optic system), captured by detectors and converted into a digital value (electronics).

2.3. Cell cycle determination using propidium iodide

Propidium Iodide (PI) is used as DNA stain to evaluate the DNA content in cell cycle analysis. Before cells are stained, the cells are fixed and permeabilized with 75% ethanol. Fixation is needed because living cells actively pump out PI whereas a permeable membrane allows easy access of PI to the DNA. With the binding of PI to nucleic acids such as DNA or RNA, the fluorescence increases roughly 50 times. Due to the unspecific binding of PI, treatment with nucleases is necessary to distinguish between DNA and RNA. Addition of RNase results in RNA fragmentation and allows specific determination of the DNA amount within the cell. The principle of the cell cycle determination is based on a typical content of DNA in each cell cycle phase. Quiescent and G1 cells have one copy of DNA whereas G2 and M cells have two copies of DNA. Cells in the S-phase display an intermediate amount of DNA. The DNA content is proportional to the fluorescent intensity thus resulting in a histogram. A modeling program is required to de-convolute the populations and assign percentage values to each population (Figure 2).

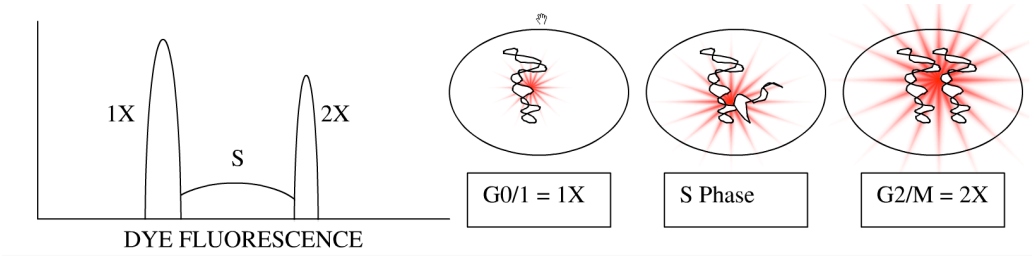

Figure 2. Principle of cell cycle determination using propidium iodide (PI) staining. Cell cycle phases are de-convoluted by means of a typical amount of DNA in each cell cycle phase using a modeling program.

PI can also be used in combination with fluorescently labeled antibodies. Immunostaining of cellular antigens allows a simultaneous determination of the expression of specific proteins during the cell cycle in a two color fluorescent plot (Michele Pagano, 1995).

2.3.1. Distinction between G0/G1 using the monoclonal antibody Ki-67

The monoclonal human antibody Ki-67 recognizes a nuclear antigen that is present in proliferating cells and absent in quiescent cells regardless from the lineage from which the cells are derived. Thus, a fast and accurate discrimination of cells undergoing the cell cycle (G1, S, G2, M) and resting cells (G0) is feasible in a two-color fluorescent plot (Baisch and Gerdes, 1990).

2.4. Development of a controlled feeding strategy

A fed-batch process is characterized by the addition of one or more nutrients during the cultivation thereby enhancing cell concentration, culture longevity and antibody yield. Fed-batch processes have been widely applied due to their ease of operation, flexibility and robustness and are currently state of the art for most industrial cell culture processes. Efforts to enhance the performance of the culture ranging from medium design through application of stoichiometric analysis of animal cell growth (Xie and Wang, 1994) to automated dynamic online nutrient feeding (Lu *et al.*, 2013; Maranga and Goochee, 2006; Selvarasu *et al.*, 2009) have been reported previously. However, effective control of nutrient addition is crucial to decrease waste product accumulation hence achieving optimal growth conditions (Bibila and Robinson, 2000). In the pursuit of the optimal feeding strategy both feed composition and the method of feed delivery are crucial (Babel *et al.*, 2007). With a feeding strategy for amino acids, vitamins and trace elements in hand, we focused in this work on the addition of glucose, implementing a strategy with which a constant glucose environment with decreased oscillation and low concentration was feasible.

2.4.1. Description of feeding strategy

The feeding strategy was based on off-line measured parameters such as viable cell concentration (*Xv*) and glucose concentration (*Glc*). Furthermore the reactor volume (*Vr*) was recorded by monitoring the feed volume and the sample volume. At predetermined time intervals, the actual viable cell concentration (Xv_i) and the previous viable cell concentration (Xv_{i-1}) were used to calculate the volumetric cell growth rate *G*

$$G = \frac{\Delta Xv}{\Delta t} = \frac{Xv_i - Xv_{i-1}}{t_i - t_{i-1}} \tag{1}$$

where t_i is the current and t_{i-1} the previous sampling point. Glucose consumption is characterized similarly to cell growth and results in volumetric glucose consumption Q_{Glc}

$$Q_{Glc} = \frac{\Delta Glc}{\Delta t} = \frac{Glc_i - Glc_{i-1}}{t_i - t_{i-1}} \tag{2}$$

Constitutive on equation (2) the specific glucose consumption was calculated assuming the reactor volume to be constant

$$q_{Glc} = \frac{Q_{Glc}}{\overline{Xv}} \tag{3}$$

q_{Glc} is the glucose consumption per cell and time where $\overline{Xv}$ is the mean viable cell concentration of the previous and current measurement. Once glucose enters into the system, the term in equation (2) is not sufficient to calculate the specific glucose consumption rate accurately. The glucose consumption is affected by the amount of glucose entering into the system, giving

$$Q_{Glc(mole)} = \frac{Glc_i Vr_i - (Glc_{i-1} Vr_{i-1} + F_{Glc}(t_i - t_{i-1}))}{t_i - t_{i-1}} \tag{4}$$

where F_{Glc} is the glucose flow in moles per time and $(t_i\text{-}t_{i-1})$ is the time interval in which the glucose flow was kept constant. At this point it is worth to mention that glucose flow adjustment is always accompanied by offline measurement of glucose and viable cell concentration. Thus, the span between measurement and flow adjustment was minimized as far as possible. Due to low nutrient consumption and growth rate in hybridoma cell culture, a time interval of 30 minutes was considered to be negligible within calculations.

As arising from equation (4), $Q_{Glc(mole)}$ results in the consumption of glucose (mole) within a defined time interval. In order to get the volumetric glucose consumption, the mean reactor volume $\overline{Vr}$, calculated according to the previous (Vr_{i-1}) and current (Vr_i) reactor volume, has to be included into equation (4).

$$Q_{Glc\,+\,Feed} = \frac{Q_{Glc(mole)}}{\overline{Vr}} \tag{5}$$

Based on the volumetric glucose consumption $Q_{Glc+Feed}$, the specific glucose consumption can be easily calculated by dividing equation (5) with the mean viable cell concentration.

$$q_{Glc\,+\,Feed} = \frac{Q_{Glc\,+\,Feed}}{\overline{Xv}} \tag{6}$$

Based on historical data, the specific glucose consumption rate $q_{Glc+Feed}$ calculated at a certain time point was decreased by 20% over the next time interval. The reduction of $q_{Glc+Feed}$ was performed within the first 2 days after inoculation. From then on the glucose consumption remained constant, thus the calculated $q_{Glc+Feed}$ was not adjusted further. However, dynamic change of the parameter Xv requires accurate prevision to compensate consumption, allowing us to keep a constant glucose concentration in the system. For prediction of Xv the time interval between the current (t_i) and the following (t_{i+1}) measurement is needed. This was simply done by fixing the time point t_{i+1} of the next measurement.

$$Xv_{i+1} = G(t_{i+1} - t_i) \tag{7}$$

In the exponential growth phase the volumetric cell growth rate G is increased and thus the deviation between the predicted and the measured value for X_v was substantial at the point where transition from exponential to stationary phase occurred. In order to decrease the error at the transition phase, the maximal value for predicted Xv was limited at 7×10^6 cells per milliliter. This threshold value was based on historical data and thus the implementation was reasonable in order to optimize glucose control at the transition phase.

Having the predicted Xv_{i+1} from equation (7), predicted Vr_{i+1} (volume flows in and out of the system are known) and $q_{Glc+Feed}$, the glucose flow for the compensation of the consumption can be calculated.

$$F_{Glc_{cons.}} = \frac{(Xv_i + Xv_{i+1})}{2} \frac{(Vr_i + Vr_{i+1})}{2} q_{Glc+Feed} \tag{8}$$

In case of a situation where the measured glucose concentration Glc_i was above (overfeeding) or below (underfeeding) the desired setpoint, we implemented an adjustment flow

$$F_{Glc_{adj.}} = \frac{(Glc_{SP} - Glc_i)Vr_{i+1}}{t_{i+1} - t_i} \tag{9}$$

where Glc_{SP} is the glucose setpoint concentration. Making the sum of equation (8) and (9) gives the total glucose flow for the next time interval.

$$F_{Glc_{tot}} = F_{Glc_{cons.}} + F_{Glc_{adj.}} \tag{10}$$

Overfeeding can lead to a situation where $F_{Glcadj.} > F_{Glccons.}$ which is resulting in a negative F_{Glctot}. If this was the case, the total flow was automatically set to 0.

3. MATERIAL AND METHODS

3.1. Cell line and media

The cell line used in all experiments was the murine hybridoma cell line HFN 7.1 (ATCC® CRL-1606™) producing an immunoglobulin G1 (IgG1) antibody against human fibronectin. Hybridoma CRL-1606 cells have been formed by fusing spleen cells (extracted from a mouse immunized with purified human fibronectin) with the mouse myeloma cell P3-X63-Ag8 (Schoen *et al.*, 1982). The initially serum-dependent cells were sequentially adapted to the chemically defined protein- and peptide-free culture media Turbodoma® TP6 (Cell Culture Technologies, Switzerland) containing 4.5g/L glucose, 4mM L-glutamine and 0.1% (w/v) Pluronic F-68 (Sigma-Aldrich®, USA).

3.2. Cell Expansion

Cells were expanded in suspension culture for 16 days in a humidified atmosphere containing 5% carbon dioxide (CO_2) at constant temperature of 37 °C. Expansion started with a viable cell density of 0.6×10^6 cells/ml in spin flasks (TPP, Tubespin®Bioreaktor 50). Once the volume exceeded 100 mL, the cells were transferred to roller bottles (PS, Corning® Surface). Cultures were maintained by addition of fresh media in intervals of 2-2-3 days, thus resulting in a seeding cell concentration of 0.6×10^6cells/ml for the 2 day interval. For the 3 day interval the seeding density was 0.3×10^6 cells/ml. Dilution on the corresponding day was performed when cultures reached concentrations between 2×10^6 and 2.4×10^6 cells/ml.

3.3. Bioreactor Culture Conditions

For the fed batch cultivation of the hybridoma CRL-1606 cell line a parallel bioreactor system (DASGIP Inc., Germany) was used. Four overhead driven vessels (working volume 400 – 1600 ml) featuring a stainless steel head plate, one pitched blade impeller (angle 45°), a porous sparger (10 µm), exhaust gas cooling device and 7 ports for probes, feeds, pH control and sampling were connected to the DASGIP control software. Temperature and stirring were kept constantly at 37 °C and 150 rpm via the TC4/SC4 control system. The pH was controlled at 7.2 with CO2 sparging and by addition of 2 M NaOH (pH4/pO4 unit). Gas mixing was done by the MX4/4 unit and dissolved oxygen (DO) was set equal to 50% air saturation. Two separate feeds were continuously added with individually controllable pumps (MP8 unit). Feed 1 was a 1 M glucose solution where the flow rate was calculated according to the desired glucose setpoint using the strategy described above. Feed 2 was a concentrated mixture of RPMI 1640 amino acid solution (50x, Sigma-Aldrich®), RPMI 1640 vitamine solution (100x, Sigma-Aldrich®), trace elements (1000x, BioConcept®) and additional L-glutamine (Fluka, Sigma-Aldrich®). The final L-glutamine concentration was 32 mM. The feed composition has been studied beforehand in our group (Talluri, 2012). Addition of feeding solution 2 was initiated after 30 hours and the flow rate was calculated in order to get 1x concentration of amino acids, vitamins and trace elements in the bioreactor. The targeted glutamine concentration was 1 mM. In order to prevent flow rate adjustment during the cultivation, the reactor volume was controlled at a constant level.

3.3.1. Feeding Process

Cell culture samples were taken approximately every 12 hours and the viable cell concentration and glucose concentration were measured and used together with the reactor volume to calculate the flow rate for the glucose feeding solution 1 for the next time interval. The flow rate was manually adjusted in the pump control software. This process was repeated at every time point resulting in a semi-continuous feeding mode.

3.4. Viable cell concentration and viability measurement

Suspended cells were counted using a CedeX cell counter (Roche, Switzerland). Cell viability was determined with Trypan Blue (Fluka Analytical, Switzerland) solution which selectively colors dead cells. Therefore 50 µL of cell suspension was mixed with 50 µL of Trypan Blue solution. 10 µL of the mixture was pipetted on the measurement slide giving the corresponding viable cell concentration and viability (%). Further characteristics such as average compactness, average diameter and aggregation rates were recorded.

3.5. Analysis of glucose and lactate concentration

Glucose and lactate concentrations were determined with the electrochemical measuring device Hitado Super GL compact (Hitado, Germany). The analysis was based on an enzymatic sensor that allows measuring glucose and lactate in a range between 0.6 – 50 mM and 0.5 – 30 mM, respectively, with an error of < 1.5% for glucose and < 2% for lactate. For the measurement 10 µL of cell culture supernatant was mixed with 500 µL of buffer solution (BSL) provided from the supplier.

3.6. mRNA sample

The cell suspension containing 1 – 3 x 10^6 cells per milliliter was transferred to RNase free tubes and centrifuged at 300g for 3 minutes. The supernatant was discarded and to the remaining cell pellet 1 ml of TRIzol® reagent (Life Technologies, USA) was added. Cells were lysed by pipetting up and down and stored at -80 °C.

3.7. Titer determination

The concentration of the monoclonal antibody IgG1 in the culture supernatant was determined by HPLC (Agilent Technologies, USA). The method was based on the binding of the Fc region of IgG with recombinant protein G immobilized on the stationary phase of the PA ImmunoDetection® Sensor Cartridge column (Applied Biosystems, USA). Together with the loading buffer (10mM phosphate, 150mM NaCl, pH 7.5) 20 µL of filtered sample was injected at a flow rate of 1 mL/min whereby IgG is strongly bound to the stationary phase and impurities flow through. IgG was eluted by a 10 mM HCl, 150 mM NaCl buffer with pH 2.0 and the signal was recorded with a UV detector at a wavelength of 280 nm. At the end of the cycle for each sample a cleaning in place procedure with 20% acetic acid was performed.

3.8. Fed-batch shift experiments

3.8.1. Temperature shift

Three different experiments concerning temperature have been performed: constant 33 °C, shift to 33 °C at 30 hours and 76 hours after inoculation. Shift experiments were done by simply changing the setpoint of the controller to 33 °C. The time the temperature control needed to cool the reactor system from 37 °C to 33 °C was negligible and considered to have

no effect. In the case of the constant 33 °C experiment, cells were inoculated into a reactor already controlled at 33 °C.

3.8.2. Osmolarity shift

The shift of osmolarity was performed at the end of the exponential and beginning of the stationary phase after 76 hours by the addition of a defined volume of 6 M NaCL solution. Before and after the shift, the osmolarity of the culture was measured with an OsmoLab One osmometer (LLA Instruments GmbH, Germany).

3.9. Cell cycle analysis

3.9.1. Cell fixation

A cell culture sample containing 2 x 10^6 cells was centrifuged at 300g for 3 minutes at room temperature. While vortexing 0.5ml ice cold PBS 1x buffer was added, then drop by drop 1.5 ml ice cold 100% analytical ethanol was added. Fixed cells were stored at -20 °C.

3.9.2. Cell staining

Before staining, fixed cells were washed twice with phosphate-buffered saline 1x (PBS) containing 1% fetal bovine serum (FBS) (PAN Biotech GmbH) at 300 g for 3min. The resulting cell pellet was resuspended in 200 µL PBS 1x with 1% FBS giving a concentration of approximately 10^7 cells/ml. 100 µL of that cell suspension was transferred into a fresh tube and 5 µL FITC conjugated Mouse Anti-Human Ki-67 antibody (BD Pharmingen) was added. Samples containing the antibody were incubated at room temperature in the dark for 20-30 minutes. Following another wash with PBS 1x plus 1% FBS the cells were stained by the addition of 0.5 ml PBS 1x staining solution containing 1µg/ml propidium iodide (Sigma-Aldrich) and 100 µg/ml RNase (Thermo Scientific). Again incubation for 20-30 minutes was

performed to get efficient staining. Using 100 µL of the fixed and washed cell samples, PI and FITC single color control samples were provided to optimize cell cycle analysis during the acquisition. All samples were transferred into 5 ml BD round-bottom tubes and analyzed by flow cytometry.

3.9.3. Flow cytometry (FACS)

Samples were analyzed on a FACS Calibur flow cytometer (Becton-Dickinson) equipped with two lasers, a blue laser at 488 nm and a red laser at 635 nm wavelength. The blue fluorescence given by the PI and FITC fluorochromes were collected after passing through a 530/30 nm (FITC) and a 585/42 nm (PI) band pass (BD) filter. To reduce the spillover of FITC into the PI channel, compensation was done using a FITC single color control sample. When analyzing samples over a full course of a fed batch process, variation in the staining is common. Therefore the G0/1 peak of every sample was placed manually at a value of 200 to guarantee consistent data acquisition. Furthermore a collection gate was used to ensure the acquisition of 20000 PI stained single cells. The analysis software CellQuest Pro was provided by the manufacturer (BD).

3.9.4. FACS data analysis with Flow Jo

Within this analysis we simultaneously detected the cell cycle distribution (G1, S, G2/M) and quiescent cells (G0) using a two color fluorescence plot. The first step of the analysis was the determination of the morphological viable cell population by excluding outliers on a forward scatter (FSC x-axis)/side scatter (SSC y-axis) plot (Figure 3A). Most of the excluded particles/cells were characterized by high granularity or low granularity and size, and were most likely dead and aggregated cells or cell debris. Due to the fact that high levels of debris and aggregates negatively affect data analysis, a second segmentation of the morphological viable population was performed. Therefore the width and the area of the PI signal were

plotted against each other to resolve aggregates, diploid continuum (cells that have doubled amount of DNA but do not divide) and apoptotic cells from viable single cells. Aggregates are characterized by a high width value whereas diploid continuum cells show high values for the area (Figure 3B). Taking into consideration that early apoptotic cells characteristically have fragmented DNA (low values for area and width) accurate determination of the early apoptotic cell fraction was feasible via a single gate in the PI histogram (Figure 3C). The viable single cell population is composed of overlapping G0/1, S and G2/M cell cycle fractions. Because of this, the viable single cell population was displayed in a histogram to de-convolute the populations and assign percentage values to each using a modeling program included in the software (Dean-Jett-Fox fit without use of constraints) (Figure 3E). At that time the fraction of cells in G0 and G1 is not yet separated. The FITC labeled Ki-67 monoclonal antibody was used to stain selectively proliferating cells (G1,S, G2/M), giving a low or no signal for non-proliferating cells (G0). Therefore distinction between G0 and G1 can be achieved when the PI signal (area) is plotted against the FITC signal (height) and a gate is drawn to give a percentage value to G0 (Figure 3D). When setting the gate for G0 some important points have to be elucidated. Due to the fact that the G0 fraction is simply subtracted from the G0/1 fraction, the width of the gate (Figure 3D) should not exceed the width of the PI signal given by the G0/1 peak (Figure 3E). Furthermore it was observed that the FITC signal of the negative control (PI single color) increases with the duration of the fed-batch culture thus, making G0 gate adjustment along the y-axis necessary. The increase for the unspecific FITC signal most likely can be explained by decreasing cell quality (constantly increasing amount of cell debris) which results in higher auto-fluorescence. Final outcome of the analysis is an allocation of the morphological viable cell population into cells in G0, G1, S and G2/M and early apoptotic cells.

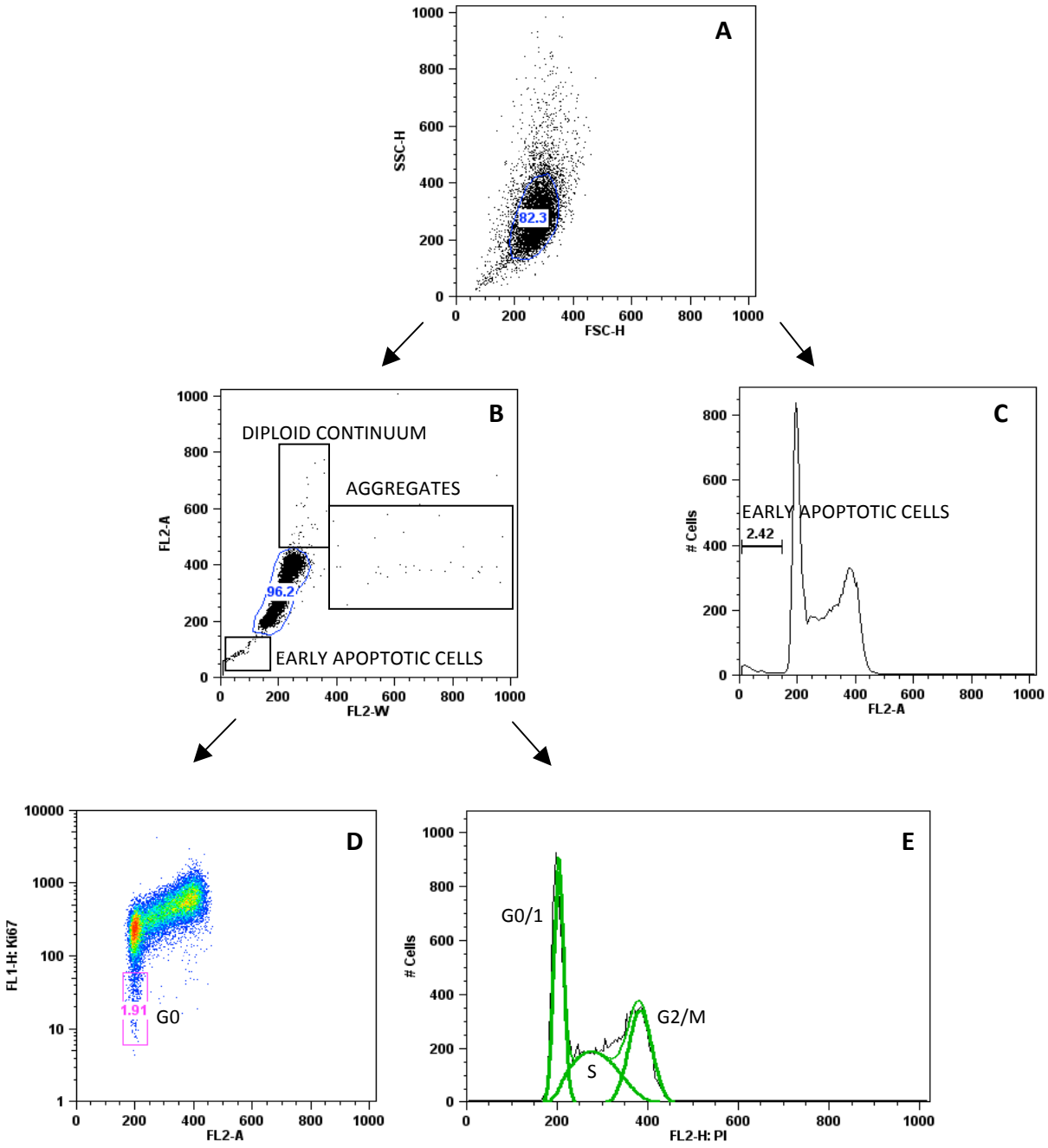

Figure 3. Flow chart of the cell cycle analysis using Flo Jo. Cell cycle phases were determined using propidium iodide (PI) and the FITC conjugated monoclonal antibody Ki-67.FL1 refers to FITC signal FL2 to PI signal. H= height of the signal, A= area of the signal, W= width of the signal. Only cells within the gate undergo further analysis, outlying cells are excluded. (A) Morphological viable population was gated (blue line) according the parameter FSC and SSC. (B) Diploid continuum cells, aggregates, early apoptotic cells and viable single cells can be easily resolved (blue line represents the gate for the viable single cells). (C) Early apoptotic cells show morphologically unremarkable characteristics, though the PI signal reveals DNA fragmentation. (D) Quiescent cells (G0) do not express the Ki-67 proliferation marker thus having a reduced FITC signal (pink line represents G0 gate). (E) Overlapping cell cycle phase populations are de-convoluted via PI histogram and modeling (Dean-Jett-Fox fit, green lines).

4. RESULTS

4.1. Standard fed-batch culture

CRL-1606 cells were cultivated at standard conditions with temperature at 37^{o}C, pH control at 7.2 and dissolved oxygen equal to 50% air saturation. Figure 4 shows the standard profiles of viable cell concentration, glucose and lactate concentration and the monoclonal antibody concentration often referred to as titer. In addition, the viability of the cell culture and the reactor volume is reported. Cell growth profile indicates a lag phase after cell inoculation and after approximately 3 days the culture growth was reduced and stationary phase initiated. Interestingly, the peak viable cell density was not reached right after the exponential phase though the concentration of 6.34 x 10^{6} cells/ml was achieved at day 6 (Figure 4A). Viability was above 95% until day 3 and declined throughout the stationary phase (Figure 4B).

The process was started with an initial glucose concentration of approximately 20 mM hence allowing the glucose to drop to the desired set point of 10 mM. Once the set point was reached, glucose concentration was kept constant with oscillation of ±5 mM (Figure 4C).

Looking at the lactate profile 3 phases can be assigned: production phase I (day 0 to 2), consumption phase (day 2 to 5) and production phase II (day 5 to end of process). The lactate concentration reached 19.5 mM at day 2, followed by a consumption state where lactate declined to 13.7 mM. Afterwards cells returned to a lactate production state, accumulating lactate up to 23.6 mM (Figure 4D). When comparing the growth profile with the lactate profile a clear correlation between cellular growth and lactate production can be observed. Either lactate accumulation affects cell growth through inhibition or lactate production is growth or nutrient regulated as reported earlier for hybridoma cells (Ozturk and Hu, 2005; Zhang et al., 2004). Due to the fact that growth inhibition by lactate for CRL-1606 hybridoma

cells is reached at 40 mM (Ozturk *et al.*, 1992), we conclude that in our experiment growth inhibition due to high lactate concentration can be neglected. Therefore lactate metabolism of CRL 1606 cells is regulated by the growth rate.

A final titer of 0.31 g/L was achieved using fed batch operation mode (Figure 4E). Even though nutrient feeding goes hand in hand with rising reactor volume, the initial decrease of the volume in the bioreactor was attained because of frequent sampling (Figure 4F). Based on the fact that the calculation of the feeding flow rate includes the actual reactor volume (Vr) we consider Vr to have no effect on culture performance.

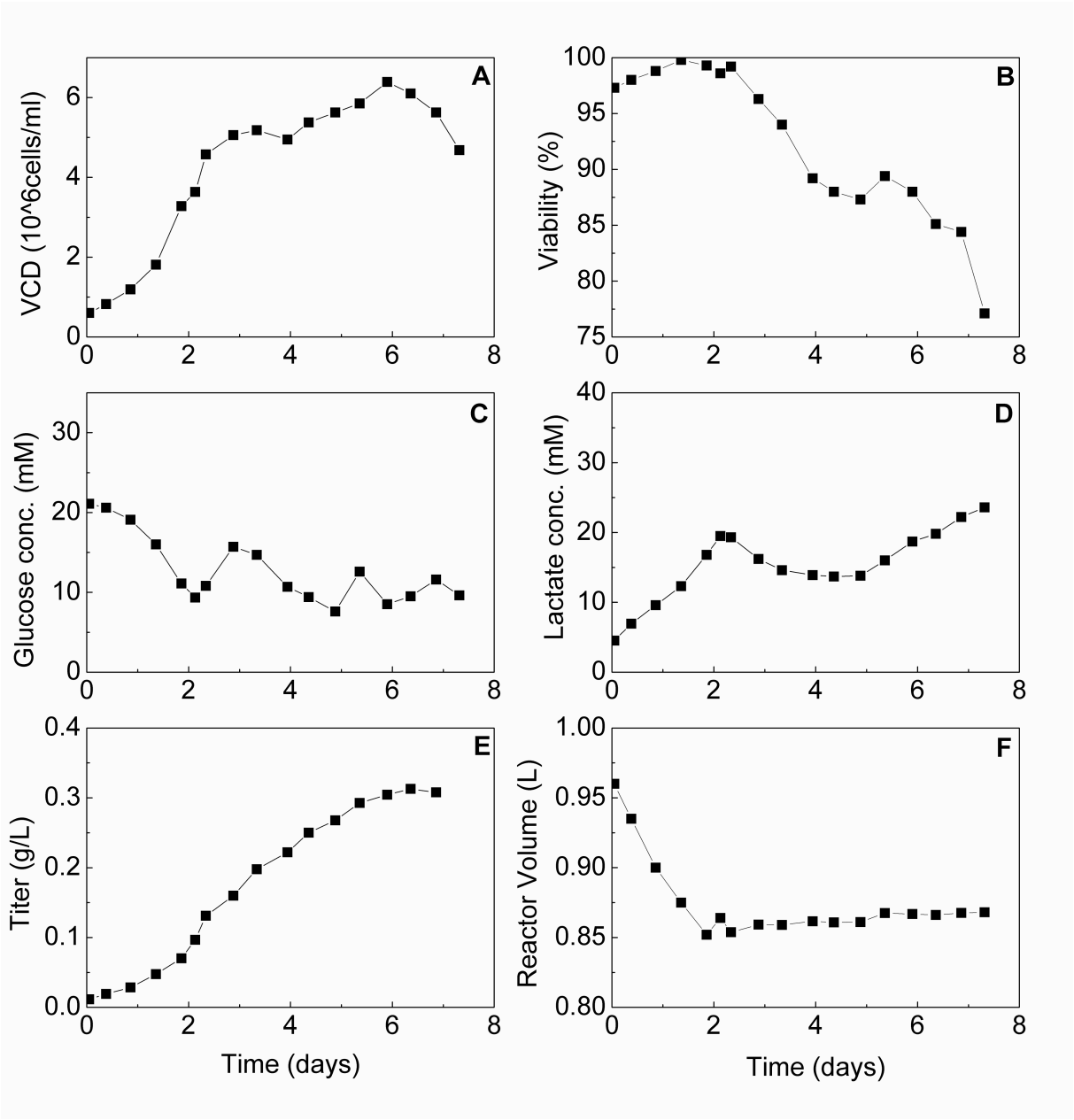

Figure 4. Offline data of a standard fed-batch cultivation at a temperature of 37°C, DO of 50% and pH of 7.2. Hybridoma cells CRL-1606 were grown in suspension in a controlled bioreactor system. Glucose feeding was performed according to the controlled glucose feeding strategy. Feeding of amino acids, vitamins and trace elements (feed 2) was started 30 hours after inoculation. Shown are the viable cell concentration (A), viability (B), glucose concentration (C), lactate concentration (D), monoclonal antibody concentration (E) and reactor volume (F).

Besides the measured parameters reported in Figure 4, online parameters such as pH, DO, O_2 and CO_2 were recorded and are shown in Figure 5. pH and DO were controlled within the desired range (Figure 5A and B). Taking into consideration that lactate has a pKs of 3.9 (25 °C) increasing lactate concentration acidifies the culture broth, thus the pH controller acts with a decrease in the CO_2 inlet gas fraction to keep the pH constant. The same explanation is valid for the increasing CO_2 fraction after day 2 (Figure 5D). O_2 fraction is connected to growth therefore constantly increasing until day 3. After a slight decrease of the fraction the curve remains constant till the viable cell density starts to decrease at the end of the culture (Figure 5C).

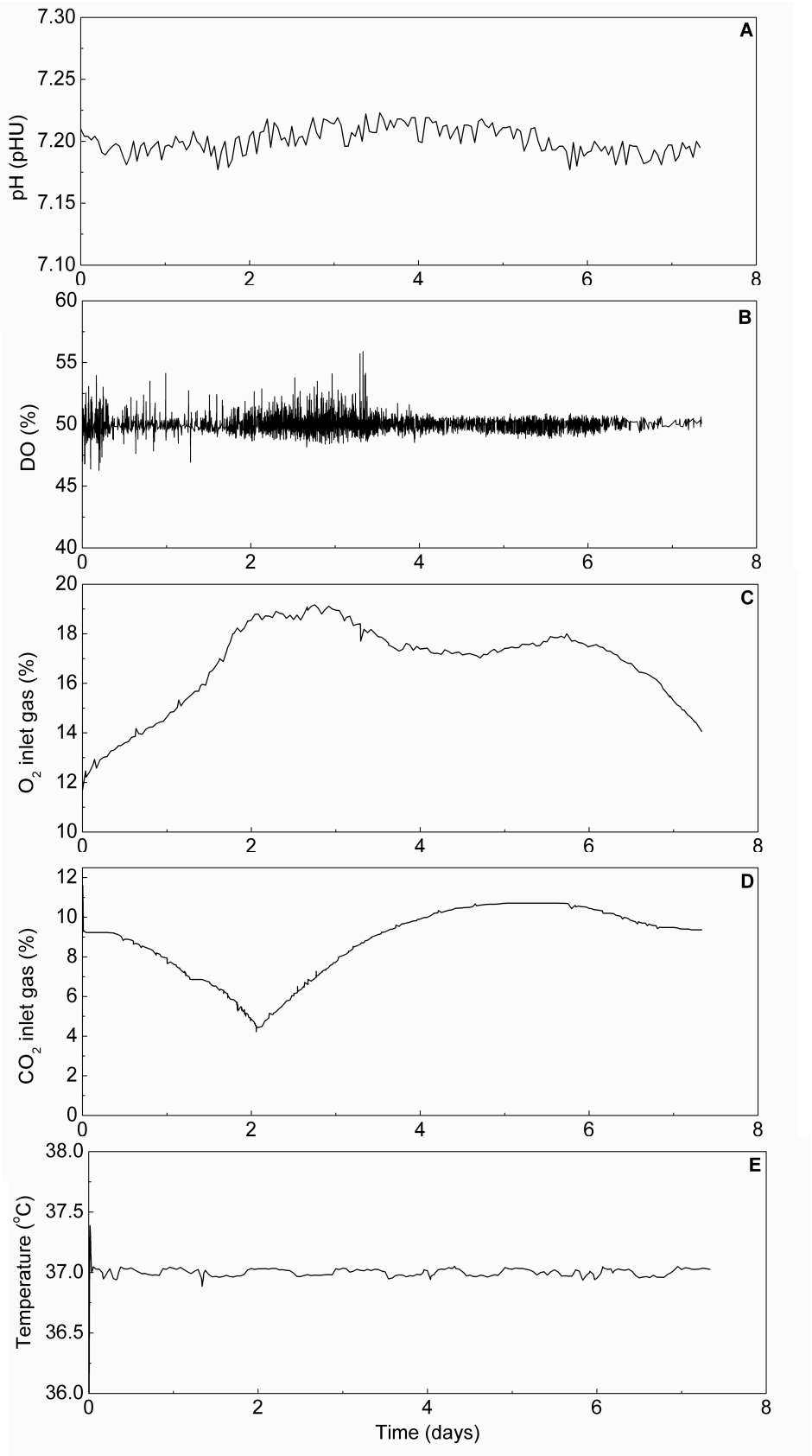

Figure 5. Online data of a standard fed-batch cultivation at a temperature of 37°C, DO of 50% and pH of 7.2. Hybridoma cells CRL 1606 were grown in a bioreactor system with online probes for dissolved oxygen, pH and temperature. Shown are pH (A), dissolved oxygen in % to air saturation (B), O_2 and CO_2 inlet gas fraction in % (C and D).

Furthermore, the amino acid profiles alanine, leucine, lysine, methionine, histidine, glutamine, glutamate, cysteine, isoleucine, aspartic acid, asparagine, arginine, phenylalanine, proline, serine, threonine, tyrosine and valine have been kindly provided by Heeju Noh and are shown in Figure 6. The profiles are only illustrated for completeness of the standard fed-batch process and therefore not discussed further within this work.

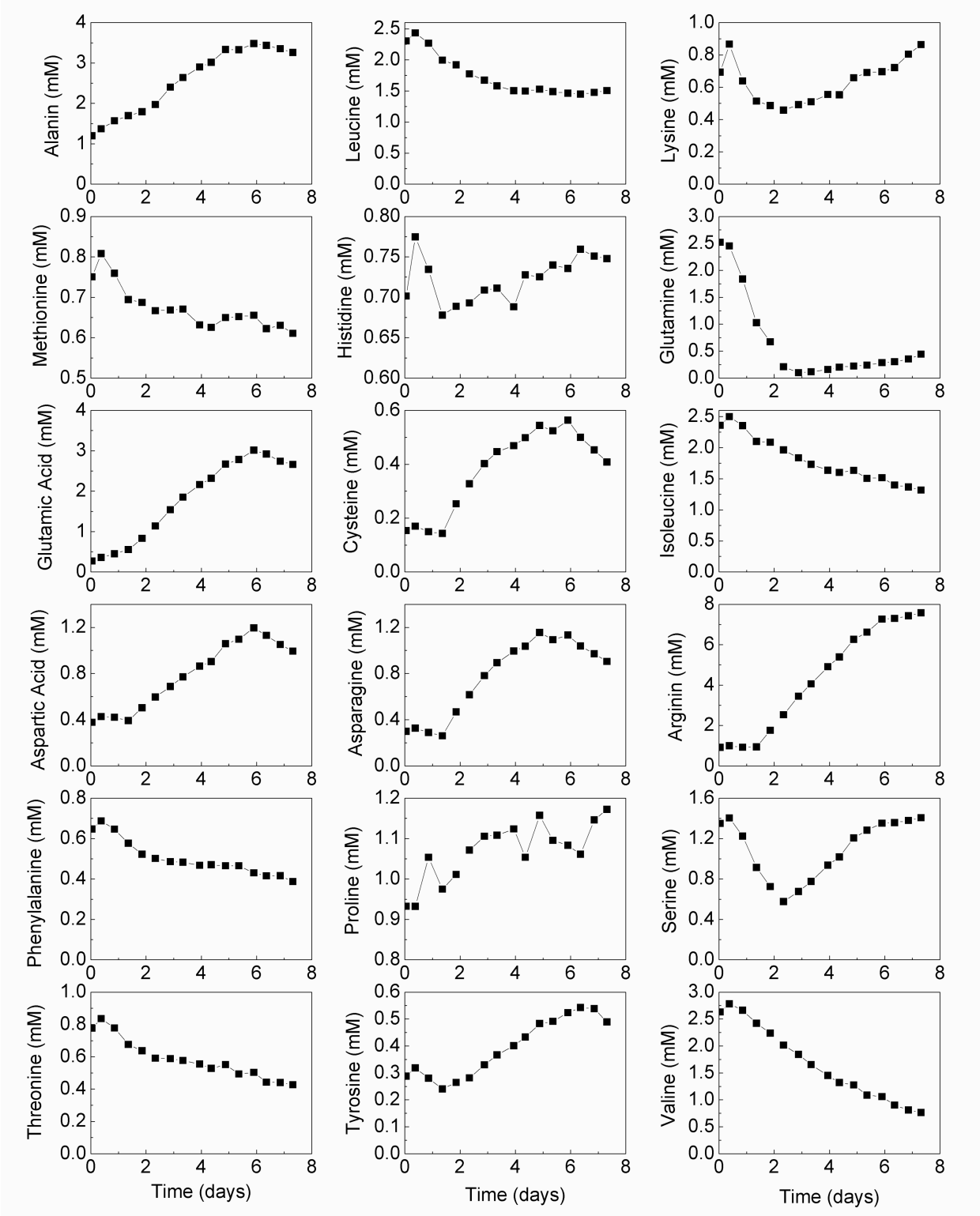

Figure 6. Concentration profiles of the amino acids over time in a standard fed-batch cultivation. Hybridoma cell CRL-1606 cell culture samples were taken approximately every 12 hours. Cells were removed and the culture supernatant was measured using HPLC. Concentration profiles of the amino acids over time are shown (Noh, 2013).

4.2. *Effect of different glucose concentrations on metabolism*

4.2.1. Initial glucose feeding versus initial glucose consumption

Several different glucose feeding strategies have been investigated to study their effect on the metabolism. Current industry practices for cell culture typically employ a bolus feeding strategy thereby allowing the glucose concentration to be reduced dependent on the cellular glucose consumption rate. Once the glucose concentration is below a certain value (setpoint), a defined amount of glucose is added in order to obtain the initial glucose concentration. To overcome high glucose oscillation and reduce the risk of depletion we implemented a controlled glucose feeding strategy. Based on this strategy we investigated a series of glucose feeding regimes in which we started at high glucose concentration of 21±0.5 mM and at intermediate concentration of 10±0.2 mM. Hence we kept the concentrations constant at 20 mM and compared it to an initial consumption state to 10 mM and 2 mM glucose. Likewise we maintained constant glucose concentrations of 10 mM over the fed-batch culture and compare it to the initial consumption to 2 mM (Figure 7C and c). Once the desired lower glucose concentration was reached, the setpoint was kept constant from then on (Figure 7C and c 20-10 mM GLC, 20-2 mM GLC and 10-2 mM GLC).

Looking at the viable cell density and titer concentration, we consider all glucose scenarios to have no effect on growth and productivity (Figure 7A, a and B, b). It is worth noting that in 1988 Miller, Blanch, and Wilke found the apparent Km of glucose for a hybridoma cell to be 0.5 mM. This leads to the conclusion that all experiments were performed in a range where glucose depletion was avoided. Beside growth and productivity there was a clear effect of glucose concentration on lactate metabolism (Figure 7D and d). It has been broadly shown in the past that the formation of lactate is affected by the concentration of glucose (Wong *et al.*, 2005; Hu *et al.*, 1987; Kromenaker and Srienc, 1994; Ozturk *et al.*, 1992).

For the set of experiments starting with high glucose concentration it was observed that holding up a high glucose concentration at 20 mM results in increased lactate concentration, thus ending up with 38.6 mM compared to 23.6 and 17.2 mM (Figure 7C). The lactate consumption phase was less developed in both time and intensity when compared to the same. Thus, the rate and time in which the cells consumed lactate were reduced. Experiments with the setpoints 10 and 2 mM revealed comparable lactate concentrations around day 2 (19.5 and 18.6 mM). Final concentrations were again in accordance with glucose concentration, although the difference was relatively small. Interestingly, the lactate profile between high and low glucose starting experiments were highly comparable when looking at the cultivations where the glucose concentration was kept constant from the beginning (Figure 7C and c 20 mM GLC and 10 mM GLC). Thus, for hybridoma cells CRL-1606 a constant glucose environment within the range of 20 and 10 mM had no varying effect on growth, antibody production and lactate metabolism.

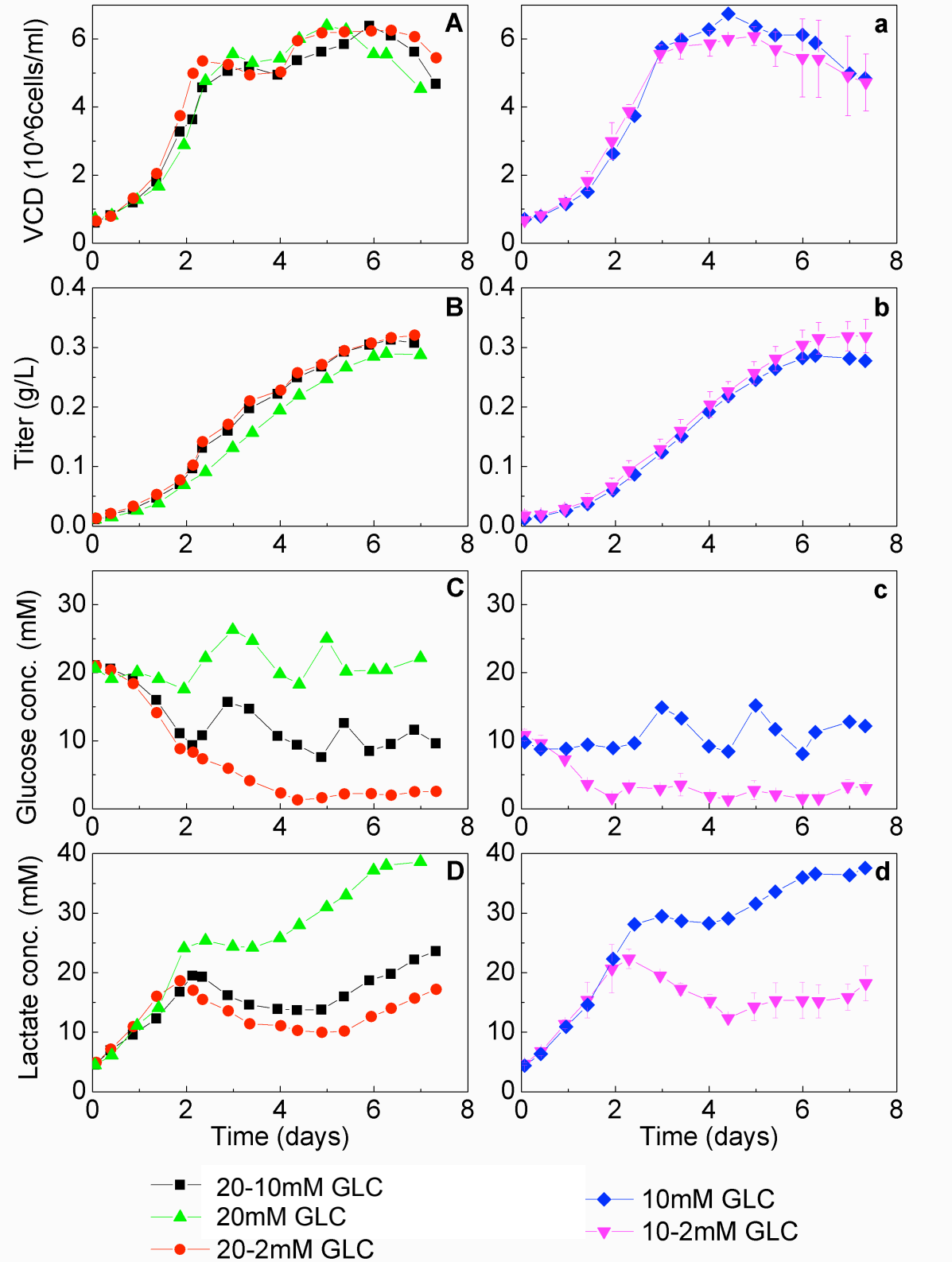

Figure 7. Fed-batch cultivation with controlled glucose feeding strategy starting with high (20 mM) and low (10 mM) glucose concentration. The effect of different initial glucose concentrations and setpoints (C and c) on the viable cell concentration (A, a), antibody concentration (B, b) and lactate metabolism (D, d) are shown over time. The glucose experiment 10-2 mM was repeated once with cells originating from the same working cell bank. Error bars display the calculated standard deviation from the experimental data.

Similarly, looking at experiments with initial glucose consumption (Figure 7C and c 20-10 mM GLC, 20-2 mM GLC and 10-2 mM GLC), comparable lactate profiles independent from the setpoint were attained. Those experiments were characterized by decreased production phase I and stronger developed consumption phase. This resulted in significantly lower lactate concentrations from day 2 onwards, when compared to the experiments where initial glucose was equal to the setpoint concentration. Also for these conditions we can conclude that glucose addition within the initial growth phase, from the point of inoculation to day 2, has a greater impact on the lactate metabolism than the glucose concentration/setpoint itself. Consequently, we consider two glucose feeding scenarios to influence lactate metabolism: (1) Keeping a constant concentration from the beginning; (2) starting with a higher concentration thereby allowing the glucose to drop to the setpoint.

4.2.2. Extended glucose range

Based on the previous experiments our goal was to investigate if substantially increased glucose concentration has a direct influence on the lactate metabolism. Therefore we performed standard fed batch cultivation with high initial glucose (20 mM) and a glucose setpoint of 10 mM. Once the glucose concentration hit the 10 mM threshold we increased the glucose flow in order to reach a concentration above 30 mM within 24 hours. Then again we allowed the glucose concentration to drop to the previous setpoint of 10 mM (Figure 8C).

It could be argued that overshooting glucose revealed a slightly decreased growth performance around day 3 when compared to the cultures with a glucose concentration of 20mM and 10-2mM (Figure 8A), however the titer profiles are comparable for all conditions (Figure 8B). No difference in the secretion of the antibody can be exhibited. However, focusing on the lactate metabolism it was found that exposure of hybridoma cells to constantly increasing glucose concentration starting with 10 mM at day 2, did not necessarily

result in high lactate concentration. Even if the glucose reaches a final level of 31.4 mM at day 3, cells exhibit a developed lactate consumption phase marked by the reduction of the lactate concentration (Figure 8D). Once the peak concentration of 31.4 mM was reached, glucose was consumed to the initial level of 10 mM without any further increase of the lactate concentration.

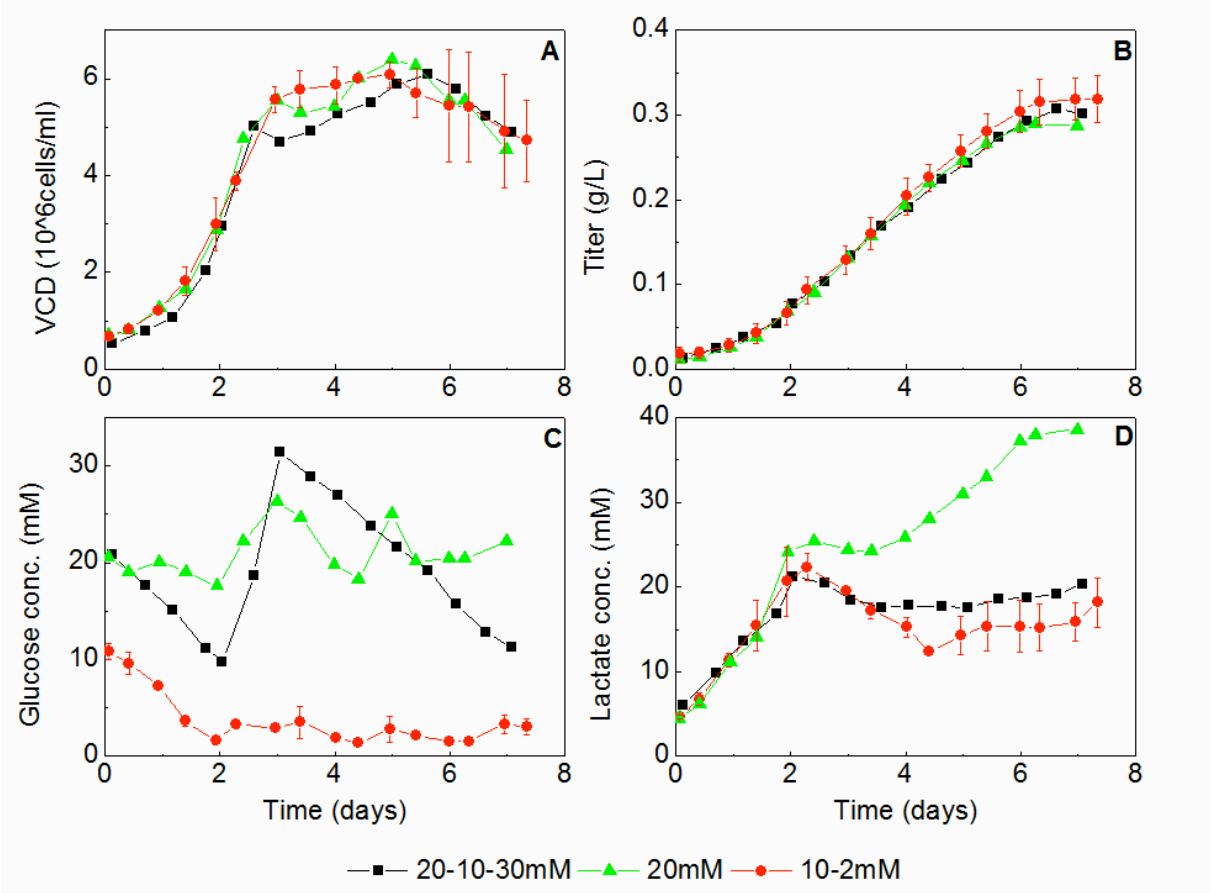

Figure 8. Fed-batch cultivation with extended glucose range above 30 mM. After an initial decline phase from 20 to 10 mM, glucose concentration was constantly increased above 30 mM in a hybridoma CRL-1606 fed-batch culture. The glucose experiment 10-2 mM was repeated once with cells originating from the same working cell bank. Error bars display the calculated standard deviation from the experimental data. Viable cell concentration (A), antibody concentration (B), glucose and lactate concentrations (C and D) were measured offline and plotted over time.

Comparing the glucose pulse with the 10-2 mM glucose experiment, the difference in glucose of approximately 30 mM at day 3 had no effect on the lactate metabolism and profiles were highly comparable (Figure 8D). In contrast, when initial glucose feeding was kept constant at 20 mM, enhanced lactate concentrations from day 2 onwards were attained. With this data we were able to sustain the above mentioned argument that for hybridoma cells CRL-1606 glucose feeding within 48 hours after inoculation is influencing lactate metabolism. Substantial increase of the glucose concentration after 48 hours has insignificant effect.

4.2.3. Limiting glucose condition

For completeness a fed-batch cultivation with limiting initial glucose (2 mM) and setpoint (2 mM) was performed. Limiting glucose concentration showed substantial effect on growth, productivity and lactate metabolism (Figure 9). The growth curve exhibited a prolonged lag phase and a shortened stationary phase when compared to the experiments with glucose at 20 mM and 10-2 mM (Figure 9A). A peak cell density of 4.77×10^6 cells/ml was reached and lactate concentration constantly increased to a concentration of 29 mM. It should be mentioned that no consumption of lactate could be observed (Figure 9D). Starting the cultivation with 10 mM, lactate is decreased despite the increased glucose concentration within the first 48 hours of the cultivation. Further, glucose starting concentration of 2 mM is not sufficient to achieve optimal growth. Again, the remarkable reduced glucose concentration does not affect lactate metabolism by reducing the concentration significantly.

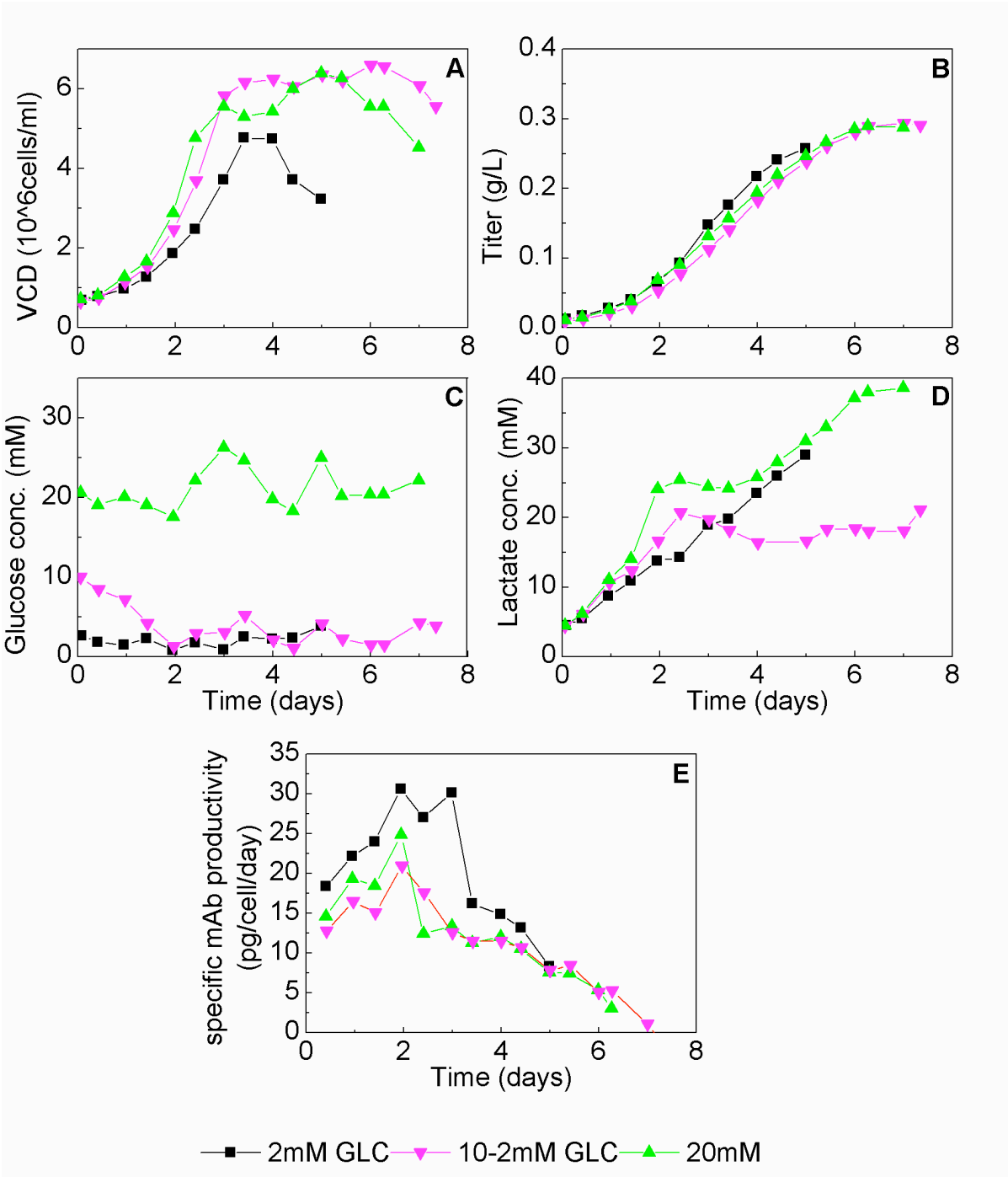

Figure 9. Fed-batch cultivation with limiting glucose concentration at 2 mM. Cells were inoculated into a medium containing 2 mM glucose. Using the controlled glucose feeding strategy the setpoint of 2 mM was kept constant throughout the culture. Viable cell concentration (A), antibody concentration (B), glucose and lactate concentrations (C and D) were measured offline and plotted over time. Based on the viable cell and antibody concentration, the specific productivity (E) of the monoclonal antibody was calculated by $\Delta p / \Delta t * 2 / (Xv_t + Xv_{t-1})$ where p is the antibody concentration, t the time and Xv the viable cell concentration.

In spite of reduced growth and culture lifetime, the antibody concentration for limiting glucose was increased between day 3 and 5 when compared to 20 mM setpoint (Figure 9B). Temporary the specific productivity was increased by 122% (Figure 9E). Due to shortened cultivation time of 2 days the final antibody concentration was decreased by 10%, though. In the past it was reported that media osmolarity has an effect on growth, metabolism and antibody productivity (Ozturk and Palsson, 1991; Wu *et al.*, 2004). Taking into account that the amount of glucose added into the reactor is affecting the osmolarity, we measured the values over the entire course of both cultivations. Values are constantly increasing over time due to the addition of feed 1 and 2 starting at 319 and going up to 352 mOsm/kg (20 mM setpoint). The difference in glucose concentration of approximately 18 mM resulted in a 6.3% decreased osmolarity at the beginning of the cultivation. Calculating the averaged difference gives a value of 6%. We do not consider the variation in media osmolarity of 6% to have the described effect on the cell culture.

4.3. Determination of the cell cycle fractions

Cell cycle phases G1/0, S, G2/M were determined based on the relative DNA content per cell using a flow cytometer. Towards the end of the G1 phase cells reach a so called restriction point. At this point either they stop dividing and enter into the quiescent phase (G0) or they continue with growth thus passing through another cell cycle. High fraction of cells in the G0 cell cycle phase is an indication for unfavorable growth conditions. It has been found that exactly in the G0 phase, where cells are metabolically active but they do not cycle, the immunoglobulin production rate for myeloma cells is increased (Byars and Kidson, 1970). Based on this fact our aim was to distinguish between G1 and G0 using a FITC conjugated Ki-67 monoclonal antibody in order to find out if the productivity is increased within a certain cell cycle phase.

For the determination of the population dynamics we performed a fed-batch culture with a constant glucose setpoint at 20 mM. In Figure 10 profiles of viable cell concentration, titer concentration, and glucose and lactate are illustrated. According to those parameters no distinctive features could be observed and the culture performance is comparable to a standard fed-batch culture as described earlier.

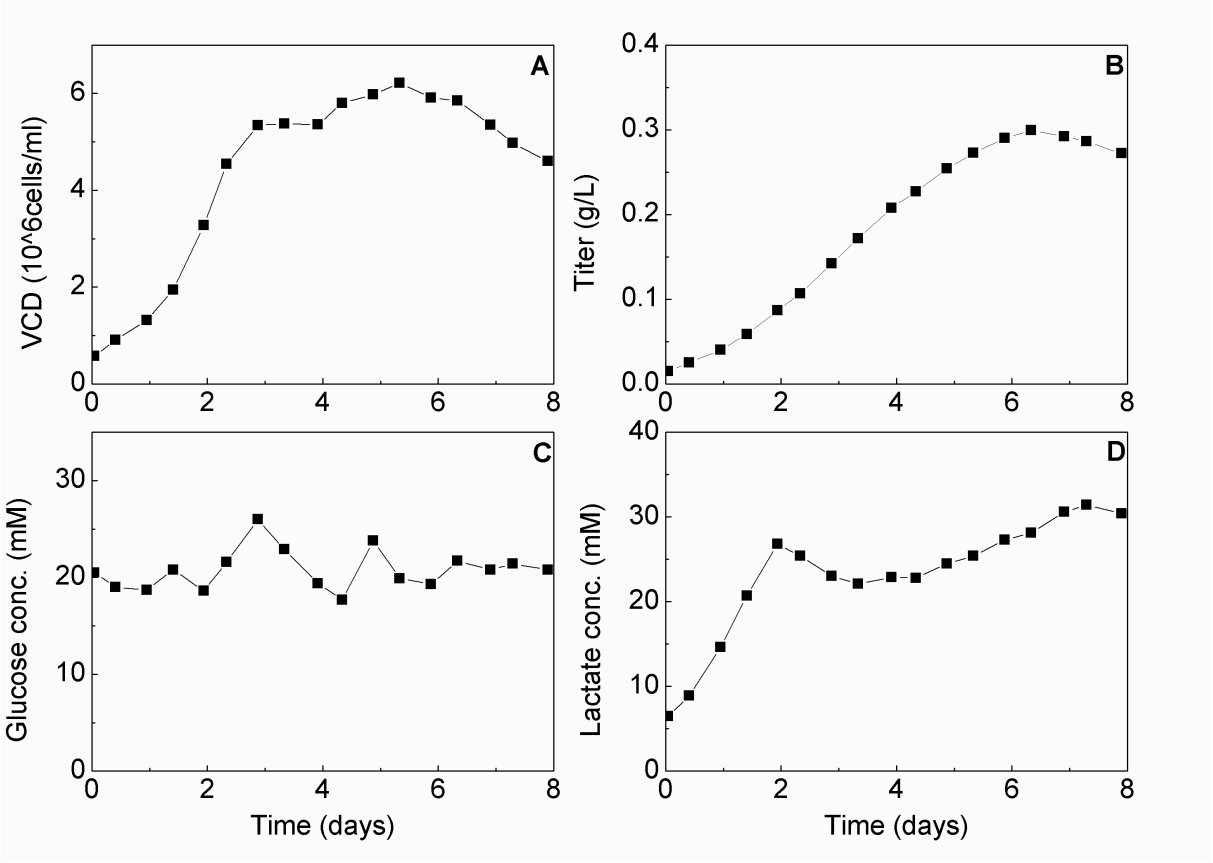

Figure 10. Fed-batch cultivation at standard conditions for the determination of the cell cycle phase distribution. Hybridoma cells CRL-1606 were grown as a 1 L dispersed suspension culture in a controlled bioreactor. Glucose was controlled at 20 mM using the glucose feeding strategy. Viable cell concentration (A), antibody concentration (B), glucose and lactate concentrations (C and D) were measured offline and plotted over time.

The fraction of the morphologically viable population was separated into two groups: the viable and apoptotic fraction (Figure 11). The determination of the apoptotic fraction was performed without additional staining. This was achieved by excluding cells with morphologically unviable characteristics within the first plot of the cell cycle analysis. Early

apoptotic cells reveal fragmented DNA and therefore they were easily detectable within the propidium iodide plot as described earlier.

In the beginning of the culture the fraction of apoptotic cells is below 5% and probably reflects a response to the inoculation procedure (Figure 11). Inoculated cells are descended from an environment where cell viability is starting to decrease due to nutrient limitation. In this stage it is not excluded that early apoptosis is initiated. The apoptotic fraction decreased to 0% at day 2, followed by a constant increment until the end of the culture to 75%. In contrast, we conclude that the highest fraction of viable cells is reached over the span of the exponential growth phase. Once the early apoptosis is induced a series of events leads to the programmed cell death (Krampe and Al-Rubeai, 2010). Inevitable, rising fractions of early apoptotic cells clearly indicated the stationary phase of the fed-batch cultivation followed by the declining phase.

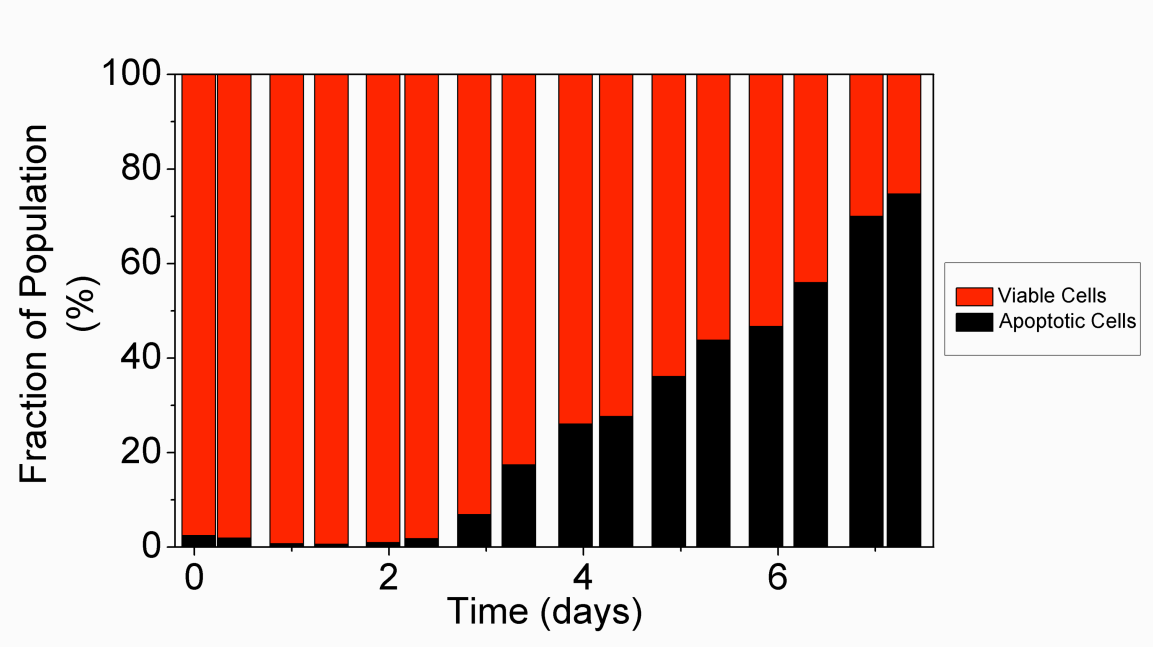

Figure 11. Morphologically viable population dynamics of a hybridoma CRL-1606 fed-batch culture. Approximately every 12 hours cell culture samples were taken and cells were fixed in 75% ethanol. Fixed cells were stained and analyzed using a FACS Calibur (BD) flow cytometer and Flo Jo. Viable (red bar) and apoptotic cell fraction (black bar) dynamics are shown over the entire cultivation.

In fed-batch cultivation, the accumulation of ammonia and lactate, or the depletion of essential nutrients, usually limits the process performance. Since the feeding strategy was designed to overcome amino acid depletion, and vitamins and trace elements in the feed were adjusted according to the amino acid feed without further measurements, waste metabolite accumulation can be further considered. Lactate accumulated to a concentration outlying inhibition whereas ammonia did not. It was reported by Glacken in 1987 that a concentration of 5 mM of ammonia reduces cell growth in hybridoma CRL-1606 by 50%. As can be seen from Figure 12, the viability of the culture is constantly decreasing once toxic ammonia concentrations are reached. Taking into consideration that the 5 mM threshold was already exceeded at day 3 and kept rising to 13.5 mM at the end of the culture, it is suggested that ammonia is responsible for the constantly increasing amount of cells within the apoptotic subpopulation.

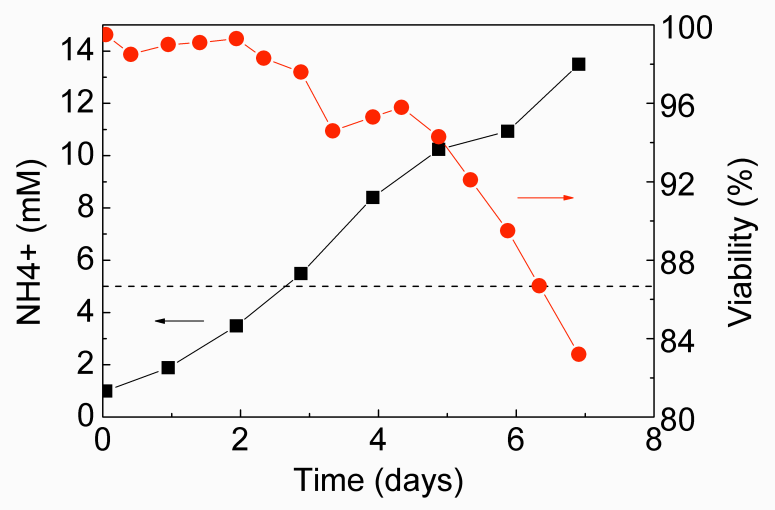

Figure 12. Influence of the ammonia concentration on the viability in a hybridoma fed-batch culture. Viability (red) and ammonia concentration (black) over culture time.

Subpopulations of the viable fraction of the fed-batch culture at 37°C are shown in Figure 13. The cycling fractions S and G2/M remained above 60% until day 2 and approve conclusion to an exponentially growing cell culture. Interestingly, after day 2 the viable population is characterized by a sudden increase of the G1 fraction. Following the transition from exponential to stationary phase, some of the G1 cells re-enter the cycling fraction, but the majority enter the G0 phase. The transition from G1 to G0 was most likely due to unfavorable growth conditions and can be attributed to ammonia accumulation (Figure 12). Therefore the increasing G1 peak can be interpreted as a precursor for limiting nutrients or accumulation of waste products such as ammonia. The partial reentering of cells from the G1 cell cycle phase to the cycling fraction in the early stationary phase can be explained by the adaptation of hybridoma cells to inhibiting ammonia concentrations as reported in literature (Matsumura et al., 1991).

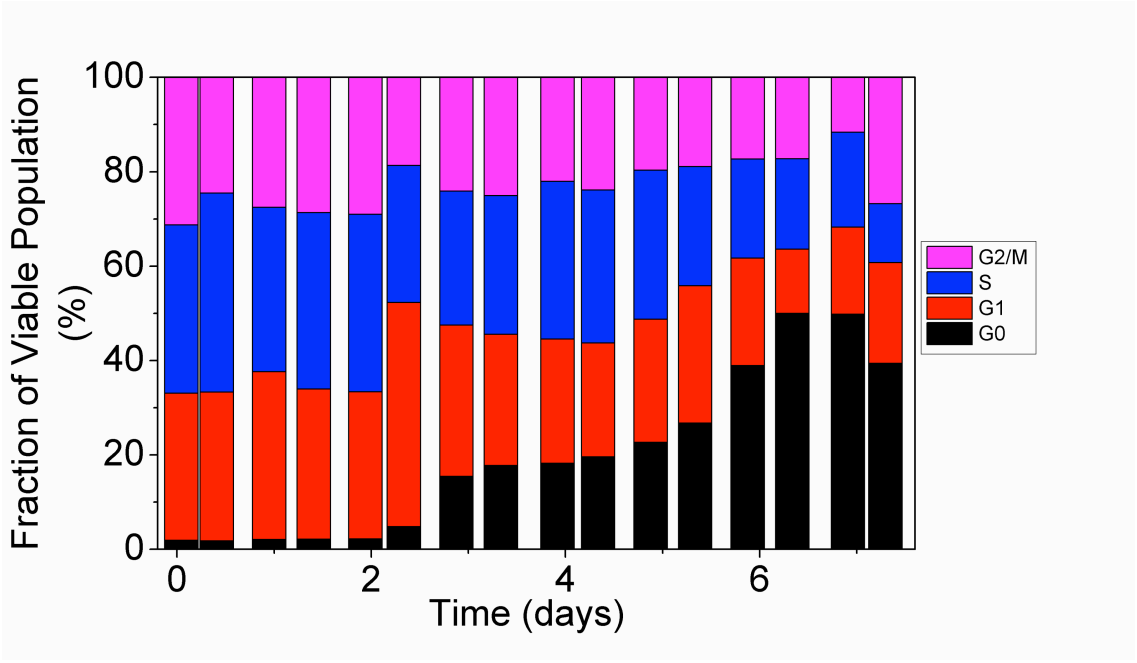

Figure 13. Cell cycle phase distribution of the viable population of a hybridoma CRL-1606 fed-batch culture. The viable population derived from the morphologically viable population and is divided into four subpopulations namely G0 (black bar), G1 (red bar), S (blue bar) and G2/M (magenta bar).

When comparing the morphologically viable population (viable and apoptotic cells) (Figure 11) with the viable population (G1, G0, S and G2/M phase) (Figure 13) we can clearly see that the rise of the G0 fraction occurs just prior to the increase in the early apoptotic fraction. It was suggested by Dutton, Scharer, and Moo-Young in 2006 that cells enter apoptosis from the G1 phase. Even though, their experimental set up did not include the analysis of G0. According to our data it is suggested that cells enter apoptosis from the G0 phase. Nevertheless, initiation of apoptosis is independent from the cell cycle phase, what underlines the hypothetical character of the above mentioned argument.

Increasing the fraction of cells re-entering the cycling instead of initiating apoptosis can be achieved as soon as the culture exhibits better growth conditions. Under conditions of this experiment it is inevitable that over the course of the cultivation cell debris and waste products accumulate. Consequently G0 and early apoptotic cells are constantly augmented. Finally, apoptotic cells reach a level above 70% of the morphologically viable population (Figure 11). When integrating the G0 fraction into the remaining percentage of the viable population, the fraction is below 10% while the amount of apoptotic cells is substantial (above 70%). The increasing apoptotic fraction and the decreasing viable fraction represent a clear evidence for the cultures declining phase.

Based on the description of Leelavatcharamas, Emery, and Al-Rubeai that asynchronous growth is characterized by relatively constant G2/M at 8–10% until the late exponential growth phase, and S phase fractions between 25% and 45% during the exponential phase of a batch culture, we suggest that cycling behavior in our cultivation with G2/M fractions around 30% are not typically asynchronous (Figure 13).

4.4. Effect of temperature and osmolarity on the cell cycle distribution and productivity

4.4.1. Effect of temperature

Temperature has a substantial influence on growth, productivity and metabolism of hybridoma cell culture (Barnabé and Butler, 1994). In our study we focused on the effect of temperature on the cell cycle and aimed to explain in more detail the connection between productivity with individual cell cycle phases.

At constant temperature of 33 °C the culture showed a significant reduction in growth compared to the control culture at 37 °C (Figure 15A). In both cases the peak viable cell concentration was reached around day 5 with concentrations of 2.37 and 6.22 x 10^6 cells/ml, respectively. Similar to growth, the final antibody concentration at 33 °C was reduced by 40% (Figure 15B). When the temperature was shifted to 33 °C after 76 hours the peak viable cell concentration was already reached and there was no further effect on cellular growth. In the case of the earlier shift after 30 hours, growth was not directly affected. Instead, the cells initiated exponential growth and reached a peak viable cell concentration of 5.22 x 10^6 cells/ml which is slightly reduced compared to the 37 °C culture (Figure 14A). However, the culture longevity was positively affected leading to the situation where both viable cell density and mAb concentration were enhanced compared to the 76 hours shift. Nevertheless, the final antibody concentration was in both shift experiments reduced when compared to the control culture. To summarize, we conclude that a shift in temperature to a lower value (33 °C) at different time points showed no prolongation of the stationary growth phase or increase of the antibody concentration. Nevertheless, depending on the time of shift different growth performances were achieved.

Regarding the cell cycle distribution subpopulations of the control and early shift experiment (30h) appeared to be highly comparable (Figure 14C, D, E, F). Cells accumulate in G1 at late exponential phase followed by a substantial amount of cells exiting the cycling fraction hence increasing the G0 fraction. This is most likely due to inhibiting ammonia concentration. From then on G1 is constantly decreased contrary to G0 which is increased. Interestingly, the shift in temperature after 30 hours did not provoke the G1 arrest as it was described in the past (Barnabé and Butler, 1994; Moore *et al.*, 1997).

In contrast to those findings for the 76 hour shift experiment it was observed that the change in temperature was followed by a drop of the G1 fraction and a boost for the S fraction (Figure 14C, D, E, F). Based on this result the temperature shift in the late exponential phase stimulated the cells to cycle. The peak in the S phase right after the temperature shift preceded the peak in the G2/M phase, suggesting that the viable population traverses the cell cycle in partial synchrony. Synchronized growth is favored by environmental step changes as it was the case with the drop in temperature. The G0 fraction in the 30 and 76 hours shift was neither positively nor negatively affected and was comparable with the control culture.

At this point it is worth noting that with culture age the error for the cell fraction within the individual phases is increased (Figure 14). In the late phase of the culture, early apoptotic cells represented the major part of the population with values above 50%. Therefore the quality of the PI signal was decreased whereby fitting the PI signal in order to de-convolute the overlapping populations revealed to be more error-prone.

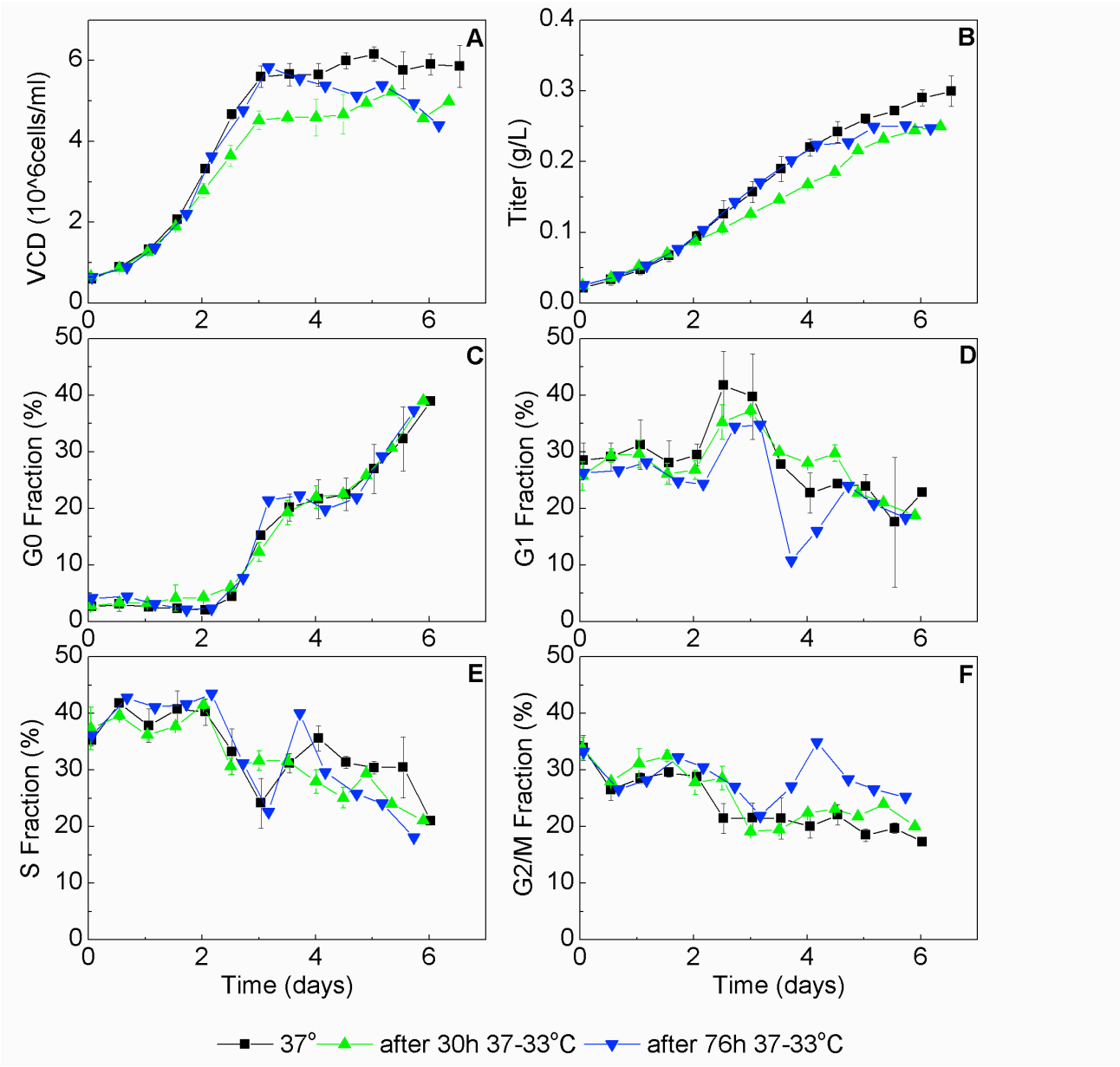

Figure 14. Cell cycle distribution for a hybridoma fed-batch culture with temperature shift 30 and 76 hours after inoculation. Temperature was shifted from 37°C to 33°C 30 and 76 hours after the inoculation of hybridoma cells CRL-1606 into a controlled bioreactor. The 37 °C experiment was repeated once with cells originating from the same working cell bank. Error bars display the calculated standard deviation from the experimental data. Viable cell (A) and antibody concentration (B), G0 (C), G1 (D), S (E) and G2/M (F) cell cycle phases over time.

Looking at the 33 °C profile the G1 peak is enhanced right after inoculation (Figure 15D). Equivalent for all experiments cells were expanded in the incubator at a constant temperature of 37 °C. The sudden change in temperature when the cells were inoculated into the 33 °C environment of the bioreactor caused the distribution of cells in the G1 phase to increase. Cells exited the G1 and entered into G2/M in partial synchrony as it can be seen from the G1 followed by G2/M peak (Figure 15D and F). The accumulation of cells in the G1 phase and the reduction of the cycling fraction, especially the S phase, observed within the first days is in accordance with the findings of Moore *et al.* (1997). The increase of G1 at the end of the cultivation is most likely a result of both reduced temperature and worsening culture conditions. Ultimately the reduction of the temperature to 33 °C could not prolong the culture longevity and viability was reduced equally to the control (Figure 16C) due to the ammonia environment.

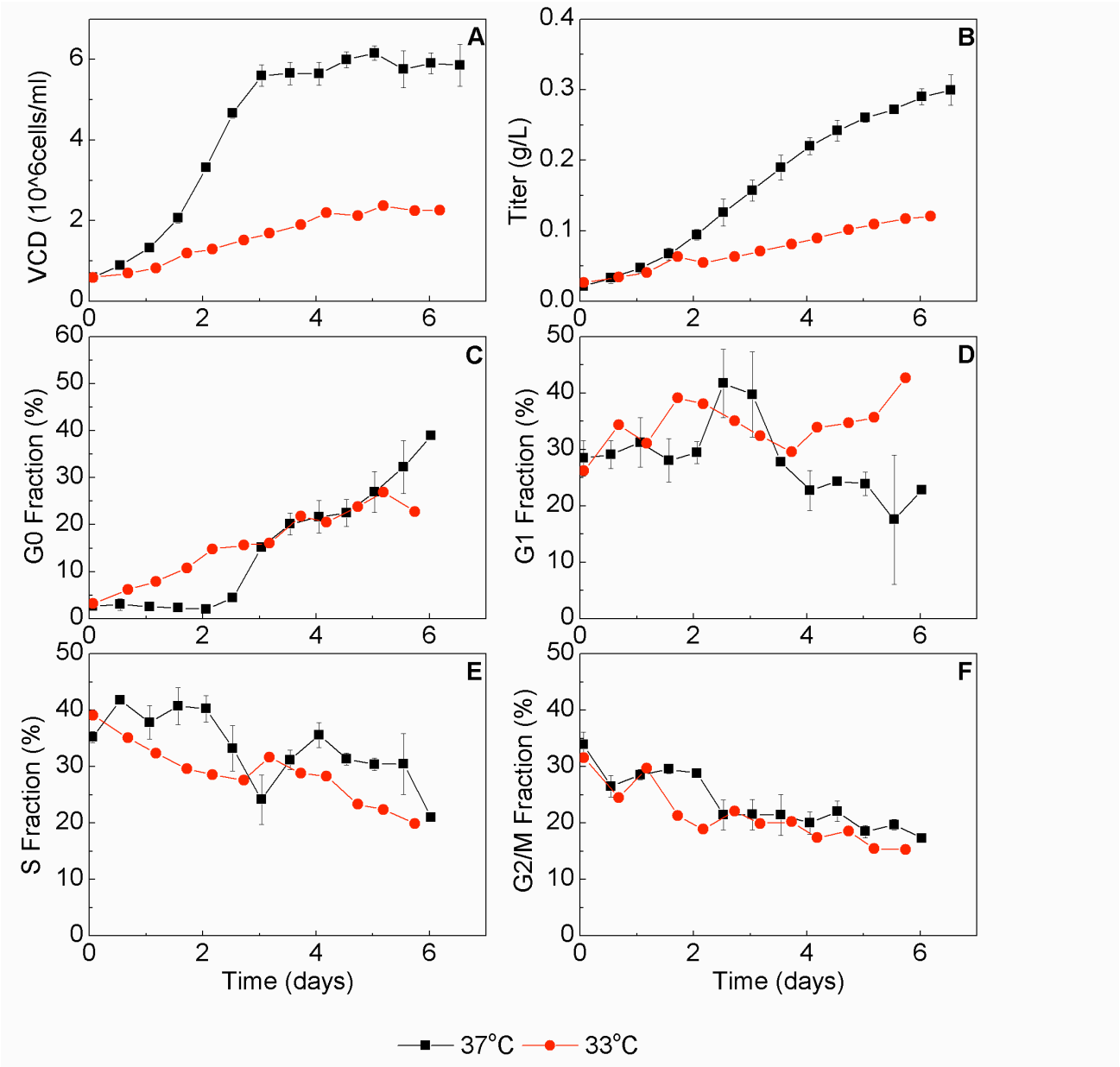

Figure 15. Cell cycle distribution for a hybridoma fed-batch culture at 33°C. CRL-1606 cells were inoculated into a reactor with controlled temperature at 33°C. The 37 °C experiment was repeated once with cells originating from the same working cell bank. Error bars display the calculated standard deviation from the experimental data. Viable cell (A) and antibody concentration (B), G0 (C), G1 (D), S (E) and G2/M (F) cell cycle phases over time.

Noticeable, the G0 fraction was augmented from the beginning, pointing to growth conditions diverged from the optimum. Furthermore the fraction of apoptotic cells is slightly increased. Despite the increase, the threshold of 20% was exceeded approximately 1 1/2 days later when compared to the control at 37°C (Figure 16B). In the course of the continuing cultivation the trend of decreased apoptosis with lower temperature was not confirmed. Interestingly the ammonia concentration was comparable to the control, indicating that the ammonia formation is not directly correlated to the cell concentration but rather to the glutamine concentration that is provided through feeding (Figure 16A).

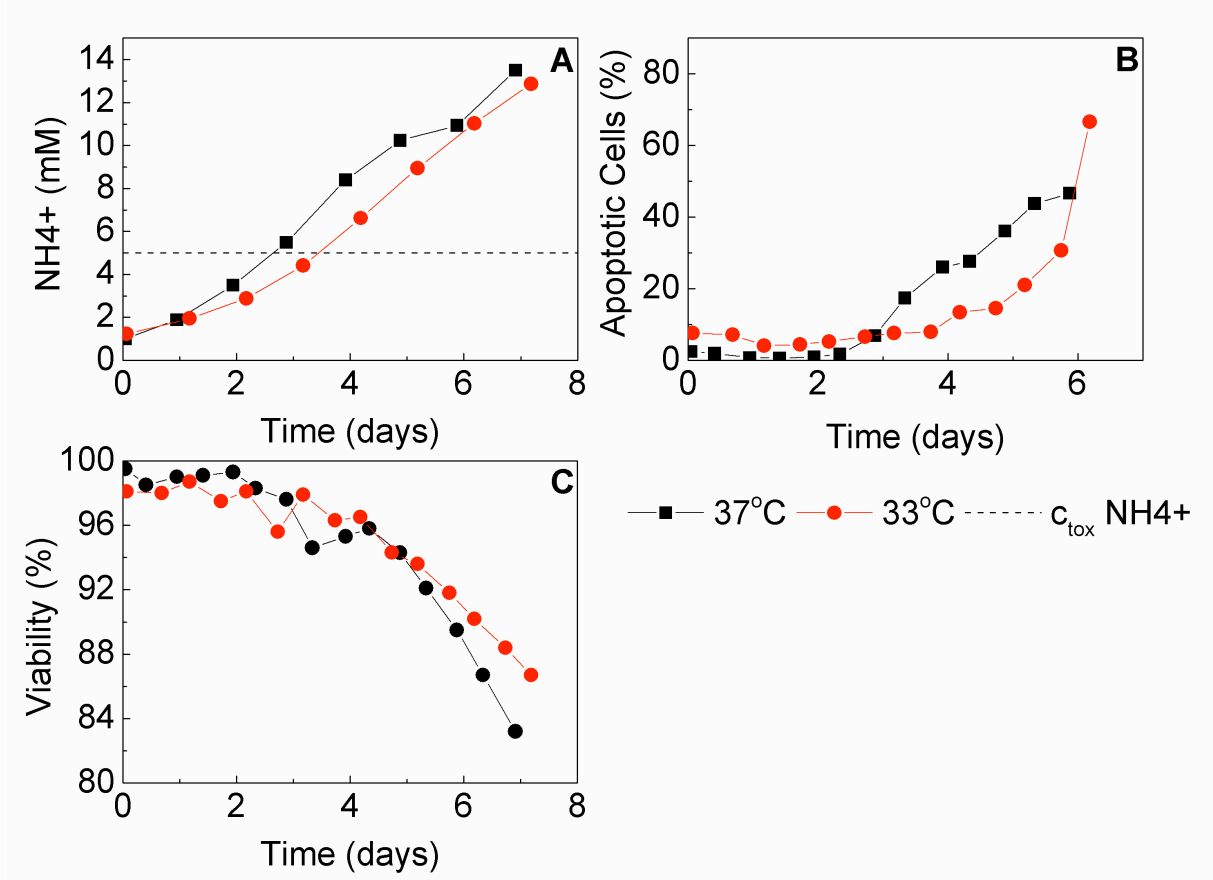

Figure 16. Influence of ammonia concentration on apoptosis and viability of hybridoma CRL-1606 fed-batch culture at 33 and 37°C. (A) ammonia concentration with dashed line that indicates c_{tox}, (B) apoptotic cell fraction derived from the morphologicaly viable population, (C) cell viability.

4.4.2. Effect of osmolarity

76 hours after inoculation osmolarity of 337 mOsm/kg was measured. After the addition of a defined volume NaCl solution in order to reach 450 mOsm/kg the osmolarity was verified (giving 455 mOsm/kg). The impulsive boost in osmolarity has a tremendous effect on the cell culture. Cell growth was directly affected and the viable cell concentration dropped rapidly in the following days (Figure 17A). It is known that hyperosmotic pressure has negative influence on hybridoma cell growth. It was shown for batch cultivation that increasing osmolarity from 290 to 435 mOsm/kg decreased the specific growth although increased the specific productivity (Ozturk and Palsson, 1991). Nevertheless the impact on growth in our experiment was far more tremendous. It is hypothesized that cells receive an osmotic shock due to the step change in osmolarity whereat adaptation is not possible. As a consequence to the stalling cultivation the antibody concentration was remarkable decreased, when compared to the control (Figure 17B).

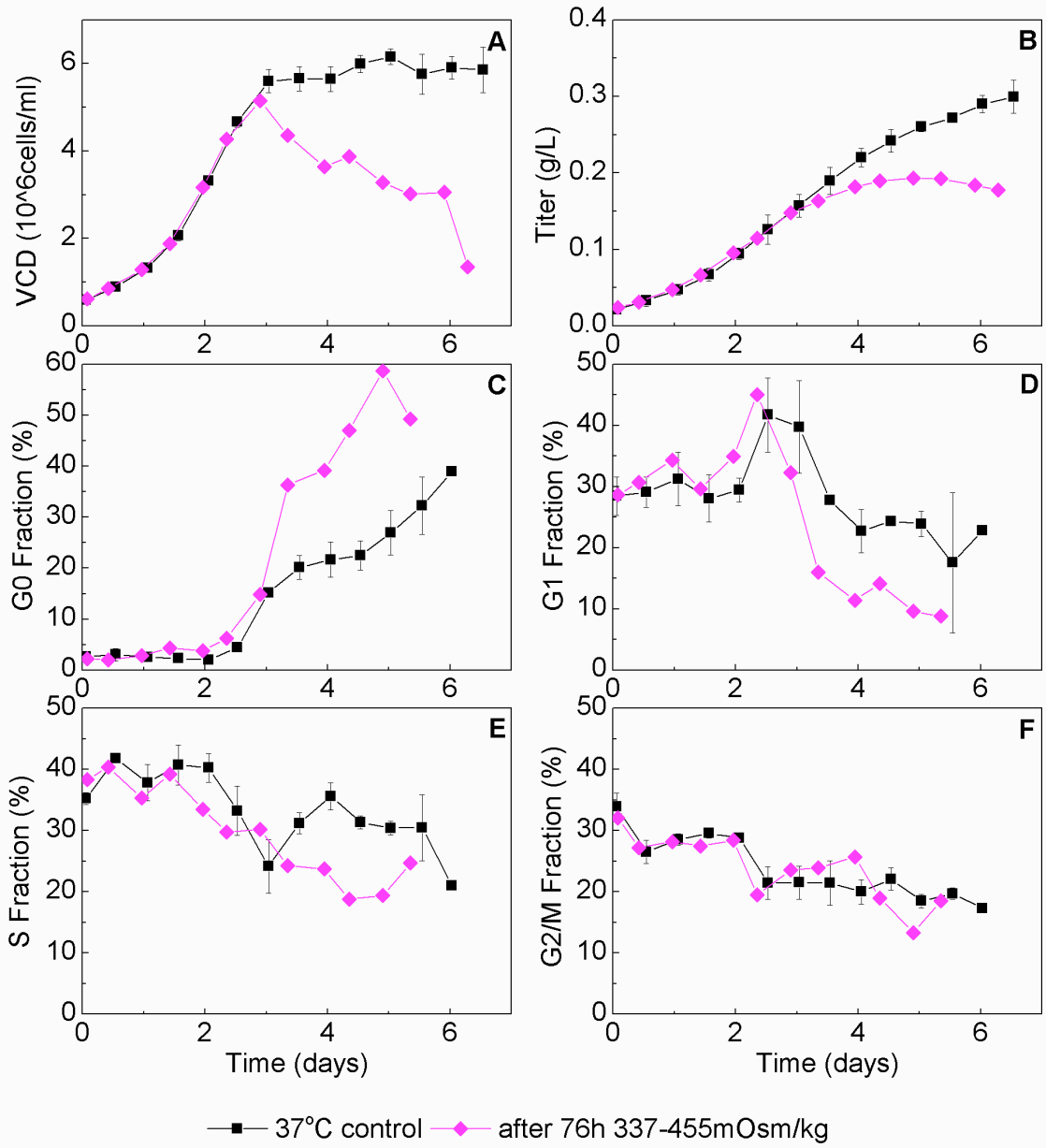

Figure 17. Cell cycle distribution for a hybridoma fed-batch culture with a shift in osmolarity 76 hours after inoculation. Osmolarity was increased from 337 to 455 mOsm/kg 76 hours after the inoculation of hybridoma cells CRL-1606 into a controlled bioreactor. Viable cell (A) and antibody concentration (B), G0 (C), G1 (D), S (E) and G2/M (F) cell cycle phases over time.

Cell cycle fraction profiles were highly comparable within the first 76 hours (Figure 17C, D, E, F). After the shift a substantial amount of cells entered into the G0 compartment accompanied by the reduction of the G1 and S phase whereas G2/M phase was not directly affected. In the following days G0 reached a peak of 58.7%. The apoptotic fraction increased from 8.4% to 47.5% (Figure 18A). Furthermore, the viability was reduced after the osmolarity shift at day 3 (Figure 18B). However, the immediate negative effect of increased osmolarity was not reflected in the viability profile since they were lysed and thus not detectable as dead cells. Summarized, the cells were neither pushed into the cycling fractions S and G2/M nor accumulated in the G1 but rather G0 and apoptosis were induced.

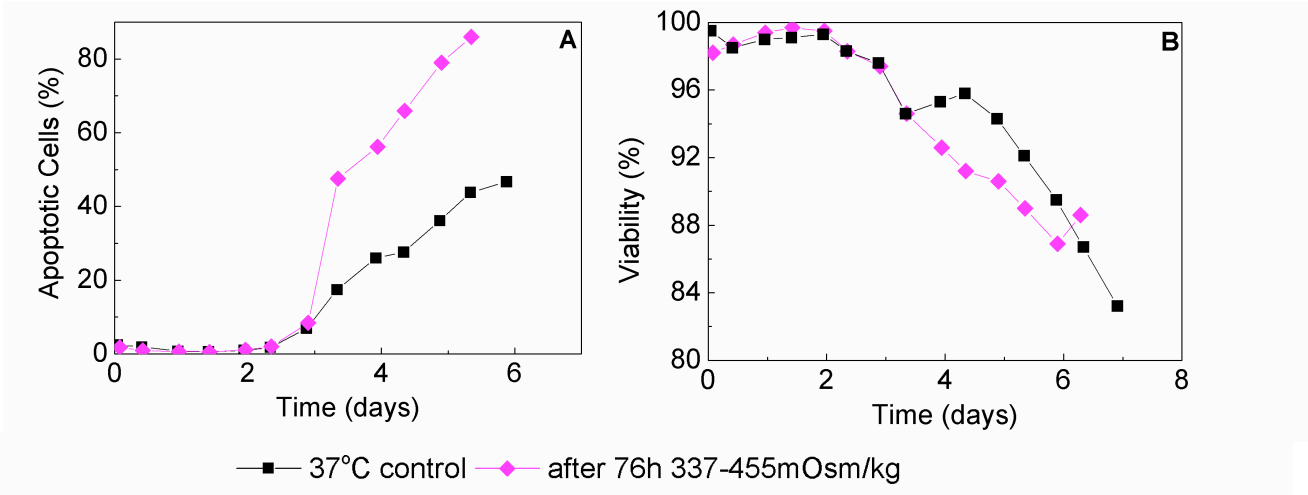

Figure 18. Apoptotic cell fraction and viability of hybridoma CRL-1606 fed-batch culture with osmolarity shift. Osmolarity was increased from 337 to 455 mOsm/kg 76 hours after the inoculation. Apoptotic cell fraction derived from the morphologically viable population (A) and cell viability (B) over culture time.

4.4.3. Effect of the cell cycle distribution on productivity

4.4.3.1. Growth dependent productivity

Over the entire course of a fed-batch culture, cells run through different physiological states. Every single cell cycle phase can be considered as an individual physiological state and considering that every state has special characteristics, it is of high interest to elucidate how and when cells change their physiological state and these characteristics are defined. In terms of productivity this would mean that knowing the specific productivities within the cell cycle phases and understanding which factors affect the cell cycle distribution a specific trigger to enhance antibody production could be defined. In the last years, much attention is focused on the connection between productivity and cell cycle phases (Balcarcel and Stephanopoulos, 2001; Dutton *et al.*, 2006; Hendrick *et al.*, 2001; Kromenaker and Srienc, 1994; Ramirez and Mutharasan, 1990; Suzuki and Ollis, 1989). Basic profiles, such as antibody concentration and growth profiles are not sufficient to describe the connection. Our approach was based on a method published earlier which applies a simple numerical integration technique (cell-hours) to determine the relationship between cell productivity and cell population dynamics (Dutton *et al.*, 2006).

Our first aim was to assign productivity patterns based on correlations to specific growth rates. For this analysis the accurate evaluation of the parameters q_{mAb} and μ is of importance. Therefore, the experimental data (Xv and antibody concentration profiles) were fitted using a polynomial fit 4^{th} order. From the arising functions the derivatives were calculated in order to get q_{mAb} and μ (Adams *et al.*, 2007) over culture time. In Figure 19 the linear relationship between specific growth rate and specific antibody production rate is shown for temperature and osmolarity shifted conditions. Hybridoma cells CRL-1606 clearly represent growth-associated mAb production. Independent from the changed or shifted parameters there is a

positive correlation between growth and production, suggesting that the antibody is preferentially or exclusively produced during G1,S, G2 or M phase. These findings forebode that increased specific productivity in G0 is unlikely. At this point we would like to indicate that in the literature the G1 cell cycle phase is often excluded from the cycling fraction. This is most likely the case because no distinction is made between G0 and G1. In our analysis we were able to separate cell distributions in the G1 and G0 phase and refer to them as two separate physiological phases.

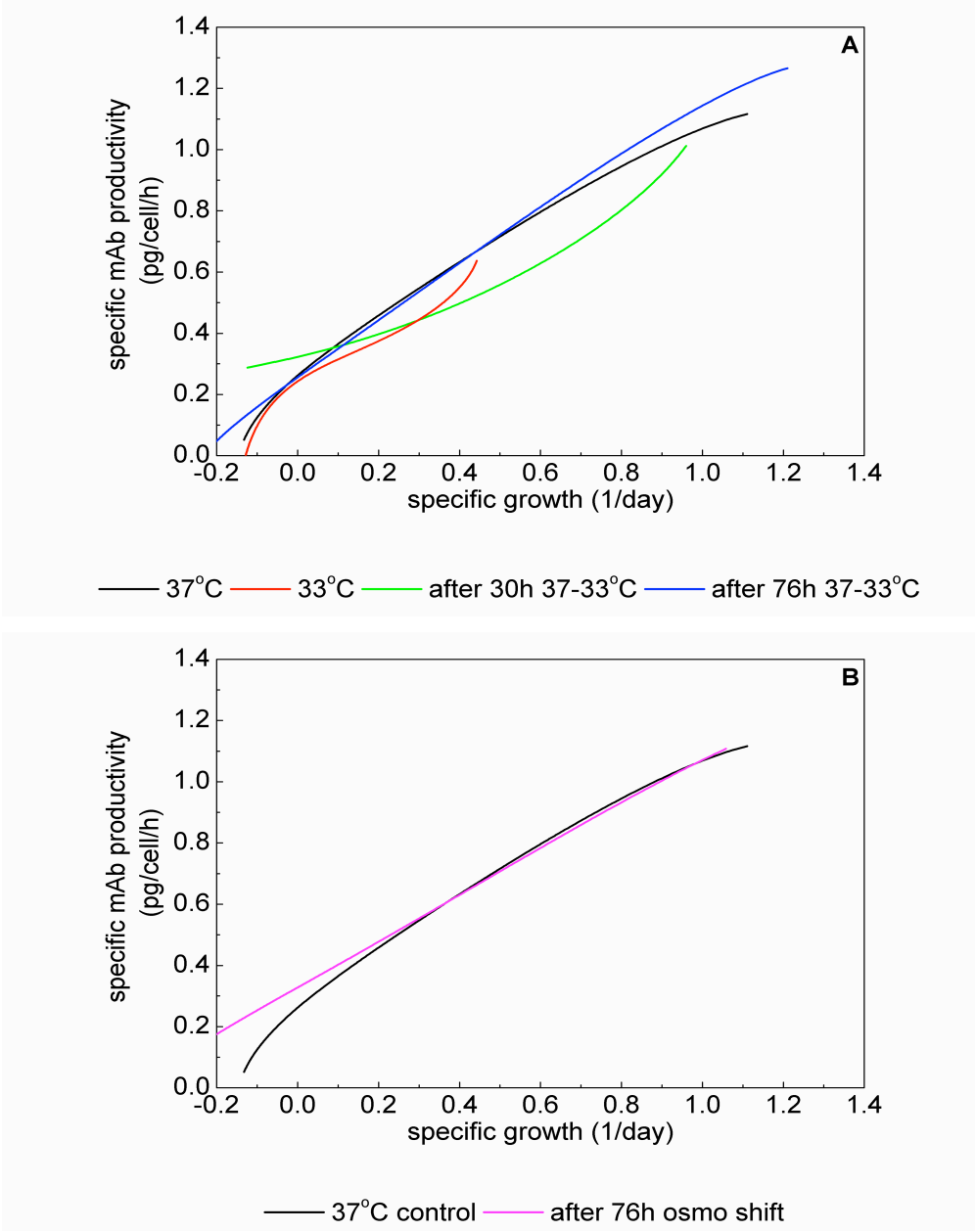

Figure 19. Specific productivity as a function of specific growth for shifted temperature and osmolarity conditions in hybridoma fed-batch cultivation. Viable cell and antibody production profiles were fitted using a polynomial fit 4th order. Arising functions were then used to get μ (specific growth) and q_{mAb} (specific mAb productivity). (A) Shifted temperature conditions, (B) shifted osmolarity

If productivity would be strictly connected to growth, the specific productivity should approximate zero when μ=0. Though, this is not the case when looking at the corresponding profiles in Figure 19. The negative value for μ is a result of the analysis, given due to the negative slope of the fitted Xv curve at the end of the culture. Decreasing values for the viable cell concentration does not necessarily mean that the fraction of cells undergoing the cell cycle is 0. This argument is strengthened when looking at the cell cycle distribution. Even though the fractions of G1, S and G2/M are remarkably reduced within the morphologically viable population at the end of the culture, this could be a plausible explanation for the antibody production with negative values for μ.

4.4.3.2. Cell cycle phase dependent productivity

Theoretically, cell cycle phase dependent productivity can be assigned from a non-segregated population. For example, if the production of the antibody is increased as the specific growth rate decreases, most probably the productivity is restricted to G0 or apoptotic cells. Nevertheless for growth-associated production patterns it is not possible to allocate productivity to the cycling phases.

The method described by Roshni L Dutton, Scharer, and Moo-Young (2006) seems to be plausible to determine whether antibody production is restricted to individual cell cycle phases. Therefore the cumulative cell hours (CHvol) were calculated for every cell cycle phase over the entire cultivation defining the cumulative number of hours spent in the medium by viable cells. If the mAb production correlates linearly with CHvol of the individual cell cycle phase, it is supposed that the antibody is produced at constant rate within this cell cycle phase. The method was applied for temperature and osmolarity cultivations and results are depicted in Figure 20. It was found that the antibody production was directly proportional to G1, S and G2/M, whereas G0 revealed no linear correlation. As it was already

suggested in the section above, hybridoma CRL-1606 is denoted by growth dependent production within G1, S and G2/M. These findings were independent from the shifted parameters (data not shown), and therefore temperature and osmolarity did not affect productivity in any cell cycle phase differentially.

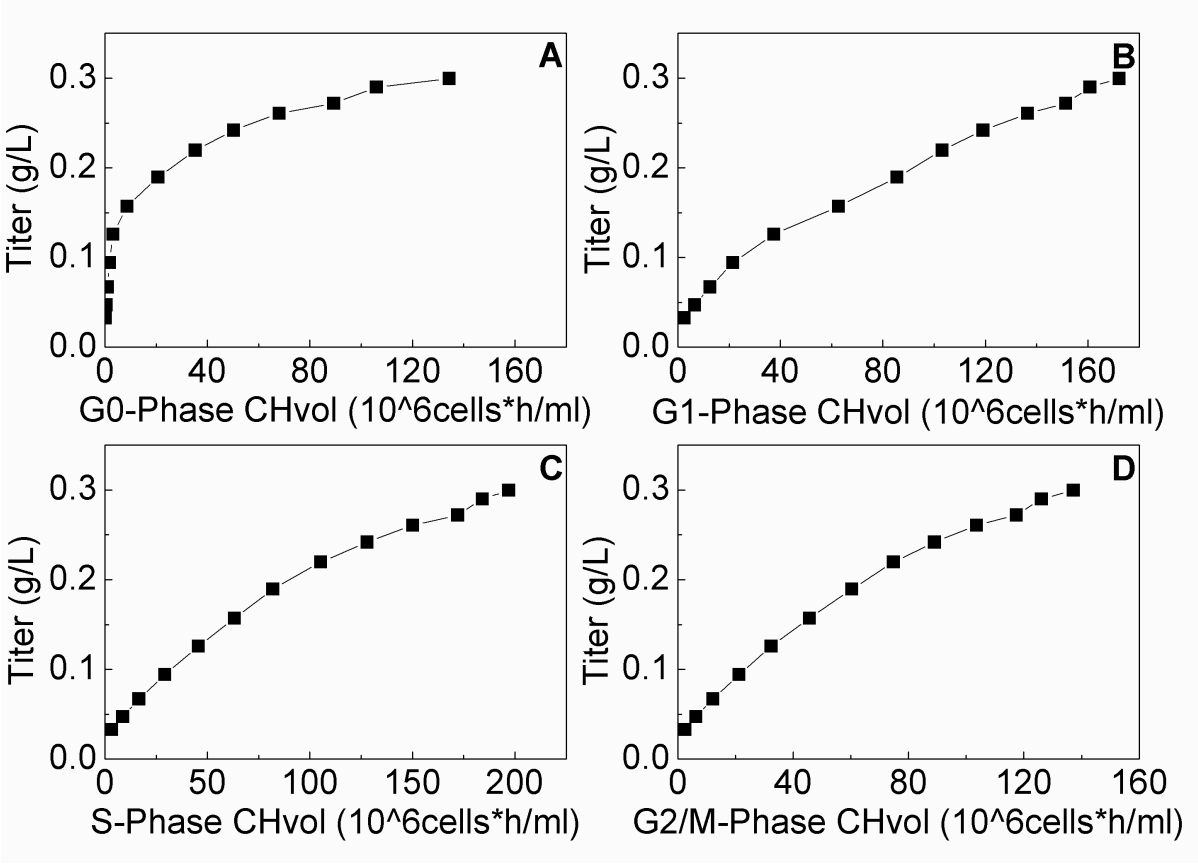

Figure 20. Cumulative antibody concentration as a function of cumulative volumetric cell hours of the individual cell cycle phases. (A) antibody concentration as a function of G0 CHvol, (B) antibody concentration as a function of G1 CHvol, (C) antibody concentration as a function of S CHvol, (D) antibody concentration as a function of G2/M CHvol.

5. DISCUSSION

5.1. Switch in lactate metabolism

It has been previously reported in our group that lactate consumption is induced at pH 6.8, whereas a highly increased production of lactate is observed at pH 7.8. pH shift to 6.8 showed the highest mAb concentration, even though growth was reduced compared to standard conditions. The lowest growth and antibody production was observed at pH 7.8. Thus implementing that pH control is crucial for lactate production and consumption. In our case pH as trigger for the change from lactate production to consumption and vice versa can be eliminated due to constant pH control at 7.20 ±0.02.

Further, lactate metabolism is connected to cytosolic NAD+ and NADH. High glucose consumption rates are accompanied by a substantial NADH production in glycolysis. In order to keep the glycolytic processes running as well to maintain a NADH balance, NAD+ has to be recycled. On one hand extinguishing NAD+ levels can be replenished by lactate metabolism or by the malate aspartate shuttle. When lactate is metabolized into pyruvate NAD+ is regenerated out of NADH. Due to the fact that the metabolic switch from lactate production to consumption occurs invariably around day two, it is hypothesized that altering culture conditions are influencing the cytosolic NAD+/NADH balance. Although ammonia accumulation or nutrient deprivation is decisive, it is not evident. Further it is not excluded that instead of NADH, proton gradients across the cellular membrane trigger the switch in lactate metabolism. Nevertheless, the factors that cause the metabolic switch seem to play a global role in the metabolism of hybridoma cells, as evident from decreased growth and initiation of the stationary phase.

5.2. Effect of initial glucose feeding

When the initial glucose concentration was equal to the desired setpoint, feeding from the very beginning was necessary to get a constant concentration profile. This was the case for the cultivation starting with high glucose and the setpoint of 20 mM, and for low initial glucose with the setpoint of 10 mM. Both experiments showed comparable results independent of the varying glucose concentration. Nevertheless, first addition of glucose at or after day 2 resulted in reduced lactate concentrations. According to these findings it is hypothesized that glucose addition within the first 2 days of cultivation is affecting the lactate metabolism independent of the concentration. In addition it is not excluded that CRL-1606 cells may have sensory characteristics to recognize the trend of the glucose concentration in the culture environment. This feature could permit the cells to adapt their metabolism according to the nutritional resources.

However, in the 20 mM and 10 mM glucose experiments increased specific glucose consumption could be observed when compared to the experiments where glucose declined within the first 2 days. Increased consumption may have caused a less developed lactate consumption phase and thus directed to lactate augmentation. As lactate is released into the culture, the pH is reduced. To be specific, rising lactate is accompanied by a decreasing fraction of CO_2 inlet gas in order to maintain a constant pH at 7.2. Once the CO_2 fraction approximates 0 and lactate is still accumulating, pH control with NaOH is needed. It is hypothesized that the addition of NaOH has an influence on the lactate metabolism. However, we currently have no mechanistic understanding of the influence of NaOH on CRL-1606 cultures.

5.3. Limiting glucose condition

In contrast to the findings of Miller *et al.* in 1988, who found the apparent Km of glucose for a hybridoma cell to be 0.5 mM, a glucose concentration of 2 mM is not sufficient to obtain optimal growth conditions for hybridoma cells CRL-1606. Apart from the growth, lactate was not decreased. It is hypothesized that an initial glucose concentration of 2 mM is experienced by the cells as nutrient limiting condition. This may cause cell stress and be responsible for increased lactate.

To be more specific, when averaging the specific glucose consumption rate within the first 36 hours, the specific consumption is 46% decreased when compared to the 20 mM glucose cultivation, giving rates of -3.1 and -5.8 pg/cell/day, respectively. When comparing the same experiments within the same time interval the lactate concentration was decreased by only 18%, though. This suggests that the glucose utilization and oxidative metabolism were not enhanced while glucose was controlled at a low level. Finally the relative amount of pyruvate metabolized through the TCA was decreased. This gives a possible explanation for the extended lag phase and reduced growth at the beginning of the cultivation. Nevertheless, there is no mechanistic understanding for the increase of the specific productivity with limiting glucose concentration at 2 mM.

5.4. Apoptosis and cell cycle phases

Apoptosis is initiated in response to environmental stress, which may lead to programmed cell death. The effect of ammonia on apoptosis has been investigated in the past and revealed a positive correlation (Singh *et al.*, 1994). As ammonia accumulates throughout the process and feeding was performed in order to overcome nutrient depletion, it is most likely that ammonia proved to be the major stress factor. However, it is not excluded that depletion of essential

media components, which have not been recorded, exerts additional stress on the cells. Despite this fact, the increase of the apoptotic cell fraction in the 33°C experiment was not directly correlated with the ammonia concentration. It is hypothesized that reduced growth and metabolism could minimize the consequences of toxic ammonia concentrations. To resume the above mentioned argument, it could be argued further that because of substantial reduced growth depletion of essential nutrients or accumulation of toxic metabolites is shifted backwards. However, once apoptosis was initiated the fraction reached values comparable to the control at 37°C. This observation is in accordance with temperature shift experiments in CHO batch cultures (Moore *et al.*, 1997).

Taking into consideration that ammonia is the only measured media component that creates unfavorable growth conditions, the increase of the G0 fraction in experiments without shifted parameters (control fed-batch at 37 °C) can be attributed as well to toxic ammonia concentrations. Nevertheless, ammonia is most likely not exclusively responsible for the transition to the stationary growth phase.

Whether cells enter into G0 prior to the initiation of apoptosis, cannot be proved. However, rising G0 fraction occurred just prior to the increase in the early apoptotic fraction when looking at the standard fed-batch experiment. If this would be the case though, it is hypothesized that transition rate from G0 into apoptosis occurs considerably fast. Nevertheless, a cell entering into the apoptotic fraction via another cell cycle phase is not expulsed. This is because initiation of apoptosis is independent of the cell cycle phases and can occur at all times.

5.5. Cycling behavior of hybridoma cells in fed-batch

Typically, a large proportion of the culture resides in the G1 phase after inoculation. This effect is a response to the step change in environment and is consistent with the lag phase (Dutton *et al.*, 2006). In our experiments the increase of the G1 fraction at day 1 indicates these phenomena, although it is weakly defined. This observation is in accordance with the growth profile, exhibiting a weakly developed lag phase.

Generally cells can traverse the cell cycle synchronous or asynchronous. Synchronized cycling is characterized by peaks in one of the cell cycle phases that are preceded by peaks of the previous cell cycle phase. To give an example, a high fraction of cells in the G1 phase followed by a high fraction of cells in the S or G2/M phase indicates synchrony of the population. Even though synchronized cycling could be observed in the 33^{o}C and 76 hours shift 37-33^{o}C experiments, it is deduced to the step change in environment but not considered to be the general cycling behavior of hybridoma cells CRL-1606.

Asynchronous cycling behavior was described in the literature (Leelavatcharamas *et al.*, 1999) and was neither in accordance with our findings. Though, it is worth mentioning that those results were attained with CHO cells in a perfusion culture and that cycling behavior is considered to be cell line dependent. Finally it is suggested that CRL-1606 cells go through the cycle randomly without following the described asynchronous characteristics.

5.6. Growth and cell cycle distribution with shifted parameters

The effect of shifted temperature on growth and cell cycle distribution was shown to be highly dependent on the cultures growth phase. Independent from the cell cycle distribution, it was reported in the literature that a temperature shift to a lower value results in a prolonged active life time during which a higher amount of product was produced (Hendrick *et al.*, 2001). In

contrast to this, culture longevity could be prolonged neither in the 33°C cultivation nor in the shift experiments after 30 and 76 hours. Accumulating waste products could give a possible explanation for this observation. In both 33°C and 37°C experiment ammonia accumulated to toxic levels. Concentration profiles were highly comparable and thus independent from temperature and viable cell concentration. Consequently, we consider ammonia limiting the culture longevity and disregard the temperature effect.

In the case of the 33°C cultivation, the effect of low temperature on the cell cycle could be shown. However, the change in the cell cycle distribution was not connected with increased productivity. Thus, in hybridoma cells CRL-1606 increased fractions of G1 and G0 are not connected with increased productivity. This is because the relationship between growth and productivity or cell cycle distribution and productivity is cell line specific.

Shifting the osmolarity from 337 to 455 mOsm/kg at the end of the exponential phase 76 hours after inoculation resulted in abrupt cell death. In 1991 Ozturk and Palsson showed that increasing osmolarity from 290 to 435 mOsm/kg decreased the specific growth by a factor of about 2. Despite reduced growth, the cells proliferated and did not die abruptly. Finally it was shown that increased osmolarity had a positive effect on productivity. The fact that the osmolarity shift 76 hours after inoculation caused cell death makes the interpretation of the effect of increased osmolarity on the cell cycle distribution and productivity difficult. Nevertheless, it could be shown that prior to cell death the G0 and apoptotic fraction are increased substantially.

We conclude that with increased culture age cells react more sensitive to changing environmental conditions. Further, culture age is accompanied with worsening culture conditions such as nutrient deprivation and waste product accumulation. The combination of

both worsening conditions and changing environmental parameters results in additional stress and may lead to stalling conditions.

5.7. Cell cycle phase dependent productivity

The method described by Dutton *et al.* in 2006 is based on the representation of the cumulative antibody concentration as a function of the cell hours that the viable population spends in each individual cell cycle phase. It was claimed that a linear correlation between those parameters indicates a constant antibody production rate within this particular phase. Further, the specific production rate could be easily calculated according to the slope of the straight line.

In the case of negatively-growth associated antibody production and assuming that production is restricted to G0 (scenario in literature), the determination of the specific production rate is restricted to one single phase and is therefore plausible. But if the protein production is growth dependent and occurs in G1, S and G2/M as in our results, it is not reasonable to calculate specific production rates. This is because the calculation of the production rate of an individual cell cycle phase is based on the cumulative mAb concentration. Even if a linear correlation between cumulative mAb and CHvol of every single cell cycle phase is given, the resulting mAb concentration is most probably a product of the CHvol of G1, S and G2/M. This argument is strengthened by the fact that the mAb concentration is increasing constantly, although the ratio between G1, S and G2/M fractions are changing. Using the described approach, linear correlation between cumulative mAb concentration and CHvol of an individual phase is possible even if this particular phase would be designated by no antibody production.

The antibody production analysis as a function of the segregated cell population confirmed that mAb secretion occurs primarily in the G1, S and G2/M phase. However, more detailed insights into the production kinetics could not be achieved with this approach.

6. CONCLUSION

6.1. Glucose study

Using the controlled glucose feeding strategy, real time glucose demand was met and environmental oscillation was reduced. Apart from this, the implemented feeding strategy was highly reproducible and was characterized by its general applicability in mammalian cell culture processes. By the use of the strategy a broad range of glucose concentration profiles was investigated. The resulting lactate profiles were characterized by a lactate production phase, lactate consumption phase and additional lactate production phase. Independent of the glucose profile and concentration, the switch between production and consumption revealed to be growth dependent and occurred at day 2. The underlying mechanism is not understood, though.

It has been shown further that glucose feeding over the initial growth phase is affecting lactate metabolism and is independent from the setpoint. This indicates that the trend whether glucose is declining or constant is more decisive than the concentration itself. However, it is hypothesized that pH control using NaOH has an additional effect on the lactate metabolism.

Generally the relationship between glucose and lactate was weak. On the one hand, glucose concentration above 30 mM did not increase the lactate concentration; on the other hand, 2 mM did not reduce lactate accumulation. Apart from that, in none of the experiments the lactate concentration exceeded the threshold of 40 mM where specific growth is reduced by 50% of maximum. Thus, using the controlled glucose feeding strategy no optimized growth performance was observed, though better understanding between glucose feeding and lactate metabolism could be achieved. In summary, lactate metabolism is not influenced solely by

glucose, rather it is connected to the complex network of internal metabolic reactions of hybridoma cells CRL-1606.

Finally, to study the effect of glucose on lactate in more detail, enzymatic activities and regulation factors could be investigated further. Based on such measurements the metabolic network could be characterized using flux balance algorithms.

6.2. Cell cycle analysis

Population dynamics are influenced by the environmental conditions. To be specific, temperature and osmolarity have an effect on hybridoma CRL-1606 cell cycle distribution. Nevertheless, the impact of parameter alteration is highly dependent on the growth state of the culture. As can be seen from the temperature experiments, shifting at the beginning of the exponential phase (30h), early stationary phase (76h) or even inoculating the cells into a controlled 33 °C environment diversifies the growth performance substantially. Apart from growth, only for the 33 ^{o}C cultivation the cell cycle distribution was affected. No noteworthy effect on the cell cycle could be observed for the shifted conditions when compared to the control at 37 ^{o}C.

This also applies to the osmolarity where the shift after 76 hours initiated instantly the declining phase characterized by impulsive increase of the G0 and apoptotic fraction. Performing the parameter shift at an earlier stage of the fed-batch cultivation might however alter the outcome.

To put it concisely: In order to achieve an effect of the culture parameter on the cell cycle distribution, the point of the shift is essential. A parameter shift at the end of the culture could be an additional stress for the cells besides the accumulation of waste products and worsening culture condition, as it was the case for the osmolarity and the shift from 37-33 ^{o}C after 76

hours. Parameter change at the beginning could lead to substantially reduced growth, as it was observed for the 33 ^{o}C cultivation. Inevitably, parameter alteration is connected to a detachment from the optimal culture conditions, and thereby growth performance is decreased. The aspired goal is to find a compromise between effective growth and parameter shifted conditions. Though, performing such a shift is just reasonable if the altered conditions are accompanied by increased specific productivity. Hence, protein production kinetics of the individual cell line is crucial.

In hybridoma cells CRL-1606 production of the monoclonal antibody was found to be growth dependent. The correlation of the specific productivity to the specific growth rate revealed to be a fast and simple method to analyze protein production kinetics as a function of the non-segregated cell population. Measured concentrations of antibodies and viable cells form the basis. Specific productivity and growth can be then calculated. Using this approach, detailed cell cycle studies are not necessary and process optimization is still feasible.

The correlation between viable population cell –hours and antibody concentration was able to prove that productivity is growth dependent and thus restricted to G1, S and G2/M. Taking into consideration that the cell cycle analysis was time and labor intense, this approach was not considered to describe the monoclonal antibody production kinetics more in detail. Nevertheless, the acquired cell cycle data can be used in a cell cycle model to describe better the impact of temperature on the cellular growth rate.

Connection between growth and antibody production indicates process designs that prolong the exponential growth phase. This means that arresting cells CRL-1606 in the G1 or G0 phase by changing the culture parameters is contra-productive, rather continuous cultivation is a more appropriate operation mode for hybridoma cells CRL-1606.

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