

Department of Physics, Chemistry and Biology

Master's Thesis

**Soft sensor application on lactate controlled fed-batch
cultivation for monoclonal antibody production**

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Thesis work done at IFM

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Abstract <p>Monoclonal antibody producing cells are of great interest and used frequently in the field of biomedical research, diagnostics and therapy with increasing need for better systems to more efficiently produce antibodies at a lower costs. In this project three fed-batch cultivations of hybridoma cells (HB-8696) were cultured in a stirred tank reactor with the use of a soft sensor to monitor the lactate concentration and as well as a dielectric probe for biomass measurements. In addition, a protocol for growing the inoculum was also successfully produced and a previous batch cultivation was also analyzed which gave crucial information about stoichiometrically relation in the feed medium which was used in the fed-batch cultivations. The BioSenz Analyzer was used for on-line lactate concentration monitoring and was later used to control the feed profile to avoid overflow metabolism in two of the three fed-batch cultivations. However, nothing conclusive could be said about the lactate controller as of yet which needs further research.</p>

Nyckelord Soft sensor, fed-batch, hybridoma, lactate, ELISA

Abstract

Monoclonal antibody producing cells are of great interest and used frequently in the field of biomedical research, diagnostics and therapy with increasing need for better systems to more efficiently produce antibodies at a lower costs. In this project three fed-batch cultivations of hybridoma cells (HB-8696) were cultured in a stirred tank reactor with the use of a soft sensor to monitor the lactate concentration and as well as a dielectric probe for biomass measurements. In addition, a protocol for growing the inoculum was successfully produced and a previous batch cultivation was analyzed which gave crucial information about the stoichiometric relation in the feed medium which was used in the fed-batch cultivations. The BioSenz Analyzer was used for on-line lactate concentration monitoring and was later used to control the feed profile to avoid overflow metabolism in two of the three fed-batch cultivations. However, nothing conclusive could be said about the lactate controller as of yet which needs to be investigated further.

Abbreviations

Monoclonal antibody (mAb)

Immunoglobulin G (IgG)

Software sensor (soft sensor)

Process analytical technology (PAT)

Good manufacturing practice (GMP)

Fetal bovine serum (FBS)

Dulbecco's modified eagle medium (DMEM)

Minimum essential medium (MEM)

Nonessential amino acids (NEAA)

Dissolved oxygen (DO)

Enzyme-Linked ImmunoSorbent Assay (ELISA)

High-performance liquid chromatography (HPLC)

Volume per volume (V/V)

Radio-frequency impedance (RFI)

Multivariate data analysis (MVDA)

Artificial neuron network (ANN)

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1 Introduction

1.1 Purpose of project

The purpose of this project was to grow monoclonal antibody (mAb) producing hybridoma cells in a controlled fed-batch cultivation with the help of a soft sensor. The soft sensor was going to be a combination of the Aber probe and the BioSenz Analyzer which measures the viable cell concentration and the lactate concentration respectively. The presence of lactate means that the overflow metabolism is active in the cells due to substrate abundance. Desirably, this is something to avoid because the metabolic products lactate and ammonia from the overflow metabolism have an inhibitory effect on the cell growth.

A software sensor (soft sensor) was constructed to monitor the lactate concentration. A big advantage with soft sensors is that by using mathematical models the soft sensor can be expanded to approximate other parameters such as the production of immunoglobulin G (IgG), consumption of substrates and more by monitoring the biomass and lactate concentrations in the system.

The soft sensor concept can have a great impact in industrial production of therapeutics when cultivating mammalian cells due to reduced resource allocation and cycle times. When controlled, the amount of feed injected in a fed-batch will be just enough for the cells to get the necessary amount of nutrients to ensure optimal growth and thereby avoid production of overflow metabolites and inhibition of the cell growth. This is also in accordance with process analytical technology (PAT) which main purpose is to optimize efficiency of the process and reduce costs such as equipment, raw materials, observation and handling costs due to automated processes.

A more efficient process which can produce more therapeutics at a lower cost will increase the availability for consumers and will also lower the environmental stress due to that less raw materials are used in the process.

This project was a continuation of a previous master thesis project where two batch cultivations were run and that data was used in this thesis for analysis. Both projects were based on the same cell culture (HB8696), equipment and placed in the same timeframe of autumn 2014.

1.2 Boundary conditions

This master thesis was performed at the University of Technology in Linköping over a period of 20 weeks. The mAb producing hybridoma cells (HB-8696, ATCC) was cultured in a 10 L bioreactor with an initial working volume of 4.5 L and a final working volume of 5.5 L. The Biosenz analyzer and the Aber probe was used to monitor the process. The data from the Aber probe was validated with cell counting on a Bürker haemocytometer and the lactate concentrations measured by the BioSenz Analyzer was validated with HPLC. The cells were cultured with a DMEM medium containing fetal bovine serum (FBS) and extra additions of amino acids.

1.3 Objectives of the work

The main objective in this master thesis was to use a soft sensor based on the Aber probe and the Biosenz Analyzer to accurately monitor and control the overflow metabolism and growth of biomass in a fed-batch reactor.

Sub-objectives:

- Establish a protocol for the preparation of the inoculum used in the cultivations
- Establish a protocol for calibration of the Aber probe
- Implement both probe signals on the same computer allowing real-time monitoring of the system
- Implement a soft sensor that uses both probes for monitoring and control of fed-batch cultivations
- Run at least four batch cultivations with frequent data acquisition of cell viability and lactate concentrations
- Run at least two fed-batch cultivations with the soft sensor used to control the overflow metabolism

2 Theory and methodology

2.1 Scientific background

The cultivation of mammalian cells is very different from cultivation from microbial cells and therefore needs other analytical methods to guarantee good growth and control (Mandenius and Gustavsson, 2014). The following section is going to delve deeper into soft sensors for monitoring, control and factors that affect cell growth.

2.1.1 Soft sensors

Process analytical technologies (PAT) and good manufacturing practice (GMP) have gained a lot of momentum in today's industries which helps to reduce cycle time, rejection of batches, lower the use of raw material needed in a process and reduces variations in the products so the quality becomes more consistent (FDA, 2004, Mandenius and Gustavsson, 2014). The soft sensor is a method that efficiently improves processes and has gained much popularity over the years and is used in both laboratories as well in industrial processes for greater process control (Gustavsson and Mandenius, 2013).

The soft sensor uses in-line hardware sensors and with mathematical models it can estimate important information about the system such as biomass, substrate and product concentrations as seen in figure 1. Algorithms can be constructed by using different methods depending on what is measured and what is known of the system. Multivariate data analysis (MVDA) and artificial neuron networks (ANNs) can be used for correlation studies and gives useful information for construction of soft sensors when not much is known about the process. However extensive calibration and fine tuning needs to be done in order to estimate the parameters (Dai et al., 2006, Scarff et al., 2006).

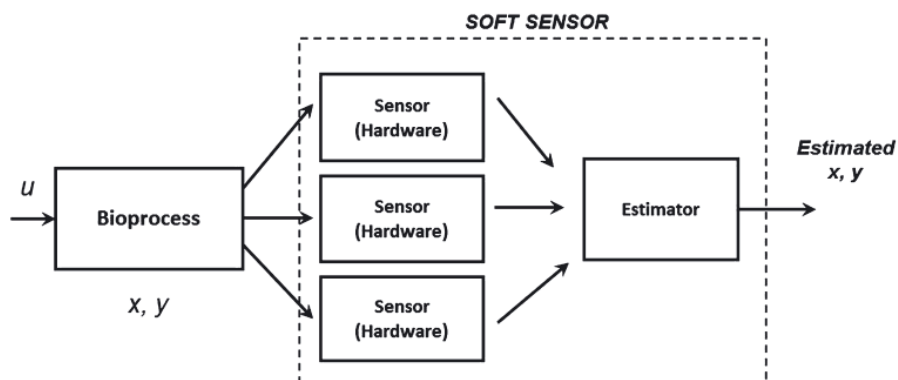


Figure 1. Signals from the bioprocess are picked up by the hardware sensors and is used by a mathematical algorithm to estimate information about the bioprocess (Mandenius and Gustavsson 2014).

Another popular approach is to use dynamic models to describe the system where metabolic pathways can be used in combination with the hardware sensors to predict behavior (Jang and Barford, 2000a). These kinds of models are used frequently in soft sensor applications to approximate for example the consumption rate, growth rate or concentrations of interest in cultivations. The model consists of dynamic mass balances taken from measurements, e.g. substrate concentrations, in combination with kinetics to describe the rates as functions of the mass balances. The dynamic models

can be divided into the two subclasses; structured or unstructured. An unstructured model use constant yield coefficients which can be a drawback when using soft sensors due to their inflexibility to handle upcoming problems caused by environmental changes and is therefore more suited for batch cultivations. A structured model can be used for changing yield coefficients and is therefore more flexible to deal with complex behavior in cultivations and makes it better suited for fed-batch (Luttman et al., 2012).

A drawback with soft sensors however, is that they cannot predict problems that lie outside of the models estimations which can cause instability and in the worst case rejection of batches. Therefore models are often based on vital functions and kinetics coupled to the cell growth and nutrient consumption.

2.1.2 Factors that affect the cultivation of hybridoma cells

For a mammalian cell cultivation to grow efficiently and maximize production and growth many factors must be taken into consideration such as temperature, pH, size of inoculum, media composition, lactate concentration, ammonia concentration, agitation, aeration and cultivation method to name a few. The following sections are going to look deeper into some of these factors to get a better understanding of mammalian cells.

Glucose and glutamine are the main sources for carbon during cell growth and production of mAbs in hybridoma cell cultures. The glucose is broken down to pyruvate and energy through the glycolysis, pyruvate then enters the tricarboxylic acid cycle (TCA cycle) to produce more energy for cell growth in the form of ATP. Glutamine is broken down to ammonia and glutamate which enters the TCA cycle in the form of α -ketoglutarate to then produce energy in the form of ATP. However, when there is an abundance of glucose the respiration capacity of the TCA cycle will not be enough to oxidize all of the substrate, this is denoted as overflow metabolism as shown in figure 2. Through the glycolysis an abundance of pyruvate will be produced where a major part will not be able to enter the TCA cycle and will instead be catalyzed into lactate which inhibits cell growth in high enough concentrations. Similarly, a high concentration of glutamine will produce more ammonia which inhibits cell grow. It will also produce an abundance of malate which is catalyzed to oxaloacetate under normal conditions. In Overflow metabolism however, an abundance of malate will be catalyzed to pyruvate and then to lactate if the pyruvate concentrations are high (Amribt et al., 2013, Bonarius et al., 1996).

It has also been reported that the consumption rate of glucose is three times larger than the consumption rate of glutamine in hybridoma cell cultivations and this ratio should be used when preparing the cell growth media to minimize the production of overflow metabolites such as ammonia and lactate (Jang and Barford, 2000b)

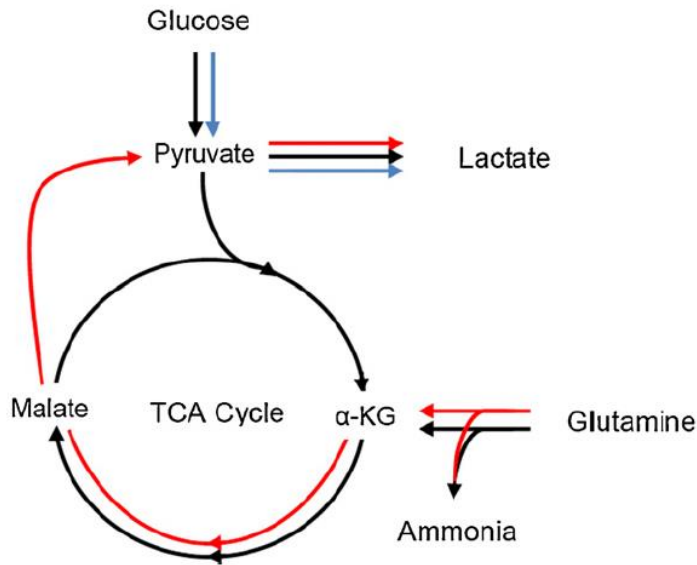


Figure 2. Shows a simplified overflow metabolism in hybridoma cells and the metabolic fluxes of glucose and glutamine (Zakaria Amribt et al 2013)

When cultivating hybridoma cells in large scale the need for agitation is paramount to ensure heat distribution and good mass balance between cells and nutrients. However, the agitation must be gentle to prevent shear stress and damage to the cell membrane which directly affects the viable cell concentration and production of mAbs (Legazpi et al., 2009).

Another important factor to consider when cultivating hybridoma cells is the pH value. These types of cells have an optimal pH value centered around 7.2. Higher or lower pH levels will greatly inhibit cell growth and viability. However, it has been found that by lowering the pH from 7.2 to 7.0 will increase antibody production rate and the average final antibody concentration was increased by 2 folds. The specific growth rate however was lowered by an average of 13%. But by lowering the pH it could be used as a form of inducer to increase antibody production (Sauer et al., 2000).

Mammalian cells require a complex nutrient environment for optimal growth and survival. A typical medium consists of glucose, amino acids, vitamins, inorganic salts, buffers and various other components. It has been found that correlation between the cell growth and the amino acid composition in the media has shown significant differences in cell proliferation. It was concluded that the amino acid composition in the cultivation media could be tailored for specific cells to optimize growth and production which also reduced the production of lactate and ammonia (Selvarasu et al., 2010). Something to be avoided however is depletion of the amino acids, especially threonine and isoleucine which are strongly correlated to decreased cell viability and growth which makes them important for the cell maintenance when cultivating hybridoma cells (Greenfield and Wellner, 1977). Other amino acids that are highly correlated with the cell growth are serine, glycine, tyrosine and asparagine and should never be in shortage. However, it has also been reported that the amino acids aspartate, glutamate and alanine have a negatively correlated effect on the cell growth and should not be in too high of a concentration in the cultivation medium

(Selvarasu et al., 2010). Overall, all the amino acids should be present to ensure growth.

Another factor to consider when deciding upon the growth media is to use animal-derived serum or not. Advantages with using serum is that it is easy to use and contains growth factors, lipids and proteins that greatly boost cell growth, increases cell stability and cell viability. However, there are some disadvantages when using serum which greatly increases the cost of the cultivations and there can be big variations in composition between serums that reduces consistency which affects the reproducibility from batch-to-batch. The high protein content in the serum will make purification of the desired product more complex and there is always a risk of contamination when using serum (Nilsang et al., 2008). Hybridoma cells have been cultivated successfully in a serum-free media and a substitute that consisted of 12 components was used instead of serum. This lead to reduced production of ammonia and lactate which had a positive effect on the cell growth and more viable cells was observed (Shibuya et al., 2008).

2.2 Methodology

2.2.1 Calorimetric measurement of L-lactate concentration

The BioSenz analyzer is an on-line tool for measurement of lactate concentrations. It filters a small volume from the cell cultivation which is then applied on a chip with enzymes that specifically catalyze L-lactate which produces heat as shown by reaction (R1) and (R2). The heat is measured and compared to four lactate standards to determine the L-lactate concentration in the cultivation.



An advantage with the BioSenz analyzer is that it measure either the glucose or lactate concentration depending on the chip used and can also do on-line readings for up to four bioreactors at the same time (Applikon, 2014).

2.2.2 Viable cell concentration determined with capacitance measurements

The Futura Aber capacitance probe uses the radio-frequency impedance (RFI) method to accurately measure the viable cell concentration. Viable cells will act as small capacitors under the influence of an electric field. Charges will build up inside of the cells due to the nonconducting nature of the cell membrane which counteracts the electric field. The resulting capacitance from the cells is directly proportional to the amount of viable cells. An advantage with this method is that only viable cells will act as capacitors due to that nonviable cells have damaged and leaking membranes and thus the charge distribution cannot build up. However, the capacitance from the cells is dependent on cell type due to different sizes and morphology and it is therefore necessary to calibrate the probe with methods such as cell counting (Kaiser et al., 2007).

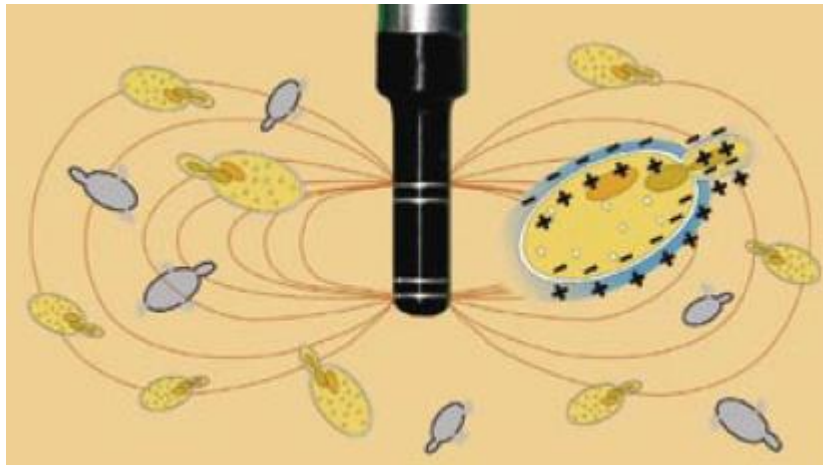


Figure 3. Shows the electric field of the Aber probe which causes the charge distribution in viable cells to change and act like capacitors (Kaiser et al., 2007).

When cultivating mammalian cells one of the most important parameters is the biomass concentration. This method can give good insight in the cell growth and changes in physiology. Another advantage is that the concentration of adherent cells that is grown on microcarriers can be measured without the need removing cells from the carriers and then counting them (Matanguihan et al., 1994).

2.2.3 ELISA

Enzyme-Linked ImmunoSorbent Assay (ELISA) is a method for quantification of antigen in samples, e.g. cell cultivations. As seen in figure 5 the principle of ELISA is to capture the antigen between two antibodies (sandwich) which binds specifically to the antigen.

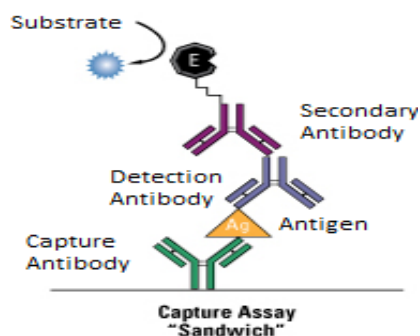


Figure 4. ELISA sandwich - The capture antibody is bound to the surface of the well. A sample with the antigen is then added which will bind to the capture antibody. A detection antibody is then added which will bind to the antigen. A secondary antibody conjugated with an enzyme is then added which will bind to the detection antibody. Substrate is then added which is converted color by the enzyme and the presence of the antigen can be detected.

The ELISA method is divided in several steps where the first is to fixate the capture antibody onto a surface which is done by adding capture antibodies to most commonly a 96 well plate and let it incubate. The surface is then washed to get rid of abundant capture antibodies. In the second step a blocking reagent is then added to ensure that nothing else can bind to the surface of the well, i.e. unspecific binding to the surface is avoided. In step three the antigen of interest is then added which will bind to the Fab region of the capture antibody. If the antigen is an antibody then the

Fab region will bind to the Fc on the tail region of the antibody of interest. The plate is then washed and a detection antibody (primary) is added which will bind to the antigen with the Fab region and thus create a sandwich in the fourth step. The plate is washed again and a secondary antibody conjugated with a reporter enzyme is added in the fifth step which will bind to the Fc region of the detection antibody. In the sixth step the plate is then washed and a colorless substrate solution is added. The enzyme on the secondary antibody will begin to catalyze the substrate in to colored products. Depending on the concentration of antigen in the sample different intensities in color can be observed, i.e. a sample with high concentration antigen will be more colored than a sample with low concentration of antigen. The color intensities can be quantified with an absorption photo spectrometer to derive the antigen concentration in the sample.

3 Materials and methods

3.1 Materials

3.1.1 Cell line and preculture medium

The hybridoma cell line used in this study is HB8696, a mouse-mouse hybridoma producing immunoglobulin (IgG₁) from American type culture collection (ATCC). The culture medium used for the inoculum was Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (25 mM) and glutamine (4 mM). The medium was supplemented with additions of 20 % (v/v) FBS, 1 % (v/v) Penicillin/Streptomycin and 1 % (v/v) MEM NEAA 100X (HyClone).

3.1.2 Sensors

Standard sensors in the bioreactor for measurements of pH, dissolved oxygen (DO), pressure and temperature was used during cultivation. As additions, the BioSenz analyzer (SenZime) and the Aber probe (Futura) were added to monitor the lactate and viable cell concentrations respectively.

3.1.3 Bioreactor set-up and cultivation medium

An autoclavable bioreactor (Belach Bioteknik AB) with a maximum volume of 10 L was used. The reactor was monitored and controlled with BioPhantom version 2000 (Belach Bioteknik AB). The temperature and stirring speed was maintained at 37 °C and 40 rpm respectively. The pH was maintained at 7.2 by flowing CO₂ into the reactor through the headspace. The fed-batch cultivations had an initial volume of 4.5 L and a final volume of 5.5 L after feeding. The initial medium consisted of DMEM medium with low glucose concentration (5.5 mM) and high glutamine (4 mM). The initial medium also contained 5% (v/v) FBS, 1% (v/v) MEM NEAA and 1% (v/v) penicillin/streptomycin. The feed medium for the fed-batch experiments contained DMEM medium with high glucose concentration (25 mM) and high glutamine concentration (4 mM). Extra additions of 5% (v/v) FBS, 3% (v/v) MEM NEAA 100X, 4% (v/v) MEM amino acids solution 50X (Sigma Aldrich) and 1% (v/v) penicillin/streptomycin was also added to get a more concentrated medium.

3.2 Methods

3.2.1 Subculturing and the inoculum culture

The hybridoma cell line was first grown in smaller scale to get a high enough start concentration for the bioreactor. To ensure good growth the cell concentration should be somewhere in the region of $1-3 \times 10^5$ cells/ml after seeding (Sinnadurai, 2007). The inoculum was prepared with two spinner flasks with a working volume of 250 ml to get the inoculation volume of 500 ml.

3.2.2 Off-line analysis

During the cultivation period samples were taken periodically for analysis. A sampling volume of 3 ml was taken from the reactor and divided. 1 ml was used in a high-performance liquid chromatograph (HPLC) (Shimadzu, Tokyo, Japan) using a refractive index detector (RID-10A) to validate the lactic acid concentrations

measured by the BioSenz analyzer. The HPLC was also used to measure the glucose concentration during cultivation. 1 ml was used for cell counting by coloring the cell with trypan blue which allowed viable cells to be counted on a Bürker haemocytometer. Only nonviable cells were colored blue due to that the viable cells actively removed the dye from the cytoplasm. Lastly, 1 ml of the sample was frozen and later used to measure the mAb concentrations during the cultivations with the ELISA kit.

3.2.3 ELISA

The ELISA kit used was Mouse-IgG ELISA (Roche) was used to capture IgG₁ antibodies. When enough samples had been gathered to run a full 96-well plate the samples were thawed. 50 µl capture antibody solution was added into each well and incubated for one hour. The solution was then removed by tapping on a dry cloth and the wells were then washed twice with 200 µl wash solution and removed by tapping. 200 µl blocking reagent was then added and incubated for one hour and the removed by tapping on a dry cloth. Each well was then washed twice with 200 µl wash solution. 50 µl of the antibody sample was then added and incubated for one hour and then washed twice. 50 µl of conjugate solution was then added and incubated for one hour and was then washed twice with 200 µl wash solution. 50 µl substrate solution was then added and incubated for one hour. The color intensities in each well were then quantified with a FLUOstar Galaxy Labvision spectrophotometer.

For greater sensitivity with the ELISA kit each sample from the bioreactor was divided into the categories high concentration and low concentration. The high concentration was diluted 50 times with blocking buffer while the low concentration was diluted 100 times with blocking buffer. A replication was done on each of the dilutions and all measurements were then averaged to get the antibody concentration in the cultivations.

3.2.4 ABER probe calibration

The ABER probe was calibrated off-line by using a 50 ml viable HB8698 cell suspension with known concentrations. The capacitance was first measured on 100 ml stirred DMEM medium with 5% (v/v) FBS, 1% (v/v) MEM NEAA X100 and 1% (v/v) penicillin/streptomycin. A volume of 10 ml was then added and the capacitance measured. This was repeated until all of the cell suspension had been added. The cell concentration was then plotted against capacitance where the slope works as conversion unit between the capacitance and cell concentration (Aber., 2011).

3.2.5 Soft Sensor and controller

The soft sensor was based on the lactate concentration in the cultivation measured by the BioSenz Analyzer to prevent accumulation of lactate and inhibition of cell growth. The lactate concentration was used to generate an error signal ($e(t)$) based on equation (1).

$$e(t) = \frac{S_p - C_L(t)}{300} 100\% \quad (1)$$

The error signal is generated by the difference of the set point (S_p), the allowed concentration of lactate during a cultivation, and the measured lactate concentration at

a given time ($C_L(t)$). The error signal is adjusted by a P-controller in a feedback system shown in figure 5. The P-controller has the advantage that it can compensate for errors in the system and increase stability (Glad and Ljung, 2011). Because it takes time for changes to occur in the cultivation after the feed rate has been changed a time-lag will arise between the changed feed rate and the effects of that change, therefore the P-controller needs to be calibrated to not be overly sensitive to changes in the system to avoid instability and fluctuations.

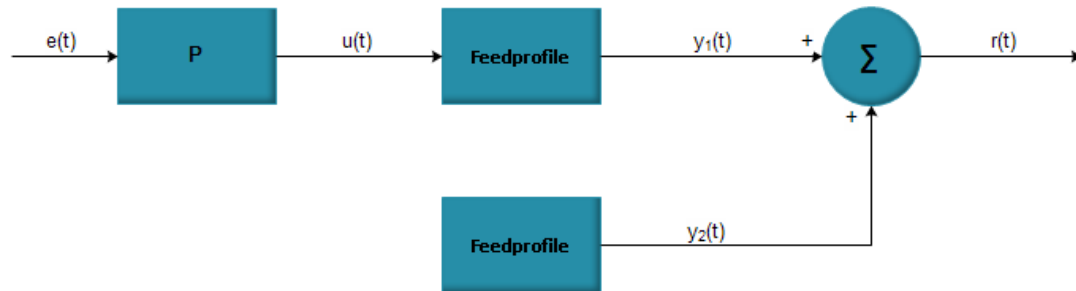


Figure 5. P-controller for the feeding in the fed-batch cultivation where the lactate concentration is used in the $e(t)$ signal to increase or decrease the feed amount depending on the lactate concentration.

The feedback system will use the equations (2)-(5) based on the model seen in figure 6 to adjust the feed rate in a fed-batch cultivation:

$$u(t) = K_P e(t) \quad (2)$$

$$Y_1(s) = F_{Profile}(s)U(s) \quad (3)$$

$$Y_2(s) = F_{Profile}(s) \quad (4)$$

$$R(s) = Y_1(s) + Y_2(s) \quad (5)$$

The error signal has the advantage that it can take on either a positive or a negative value depending on the concentration of lactate with respect to the set point. This will increase or decrease the feed rate in the feedback system. The P-controller will amplify the error signal with the constants K_P to ensure that a high enough compensation in the feed rate occurs. The adjusted signal, $u(t)$, will work as a correction factor with a range of -50% to 50% as lowest and highest value. The adjusted signal is then multiplied with the feed profile, $F_{Profile}$, to give the feed rate correction, $y_1(t)$. The output signal, $r(t)$, which controls the feeding pump is the summation of the feed profile, $y_2(t)$ and the feed rate correction signal.

3.2.6 Lactate measurements intervals

The sampling time for the BioSenz Analyzer was set to take a sample from the bioreactor every two hours for the first fed-batch cultivation with the P-controller turned off. For the two fed-batch cultivations with the P-controller turned on the sampling time was set to 1 hour due to the need for closer updates to get a more precise control of the cultivations. During 24 hours the sampling times of 2 and 1 hours would consume 30.4 ml and 60.8 ml from the bioreactor respectively.

3.2.7 Kinetic analysis

Experimental data from the fed-batch cultivations and data from the batch cultivations taken from the previous work was analyzed by using appropriate kinetic models. The analysis was done on data that accounted for the growth phase of the cultivations. The data for the fed-batch was divided into two phases; the batch phase and feeding phase after the feeding started. The data from the batches and fed-batches were used to obtain the measured specific growth rate (μ_m) and the specific glucose consumption rate (q_{glu}) for the cells with equations (6) and (7) by using the viable cell concentration (X_v), the cultivation volume (V), the feed flow (F), the glucose concentration in the reactor (C_{glu}) and the glucose concentration in the feed ($C_{glu,feed}$).

$$\frac{d(X_v V)}{dt} = \mu_m (X_v V) - F X_v \quad (6)$$

$$\frac{d(C_{glu} V)}{dt} = q_{glu} (X_v V) + F C_{glu,Feed} - F C_{glu} \quad (7)$$

For the fed-batch cultivations the initial feeding flow (F_0) was calculated from the initial batch phase data during the fed-batch cultivations according to equation (8) and (9). Equation (10) was used as the feed profile to keep up with the exponential growth of the cells (Enfors and Häggström, 1998).

$$F_0 = \frac{\mu_m}{Y_{XS} C_{Glu,Feed}} (X_v V)_0 \quad (8)$$

$$Y_{XS} = \frac{\mu_m}{q_{Glu}} \quad (9)$$

$$F = F_0 e^{\mu_m t} \quad (10)$$

To ensure good growth the initial feeding flow and feed profile was calculated with respect to the highest measured specific growth rate.

4 Results and discussion

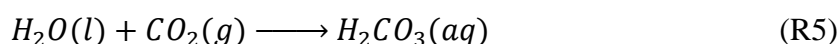
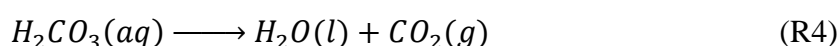
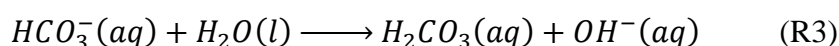
This project is a continuation to that of another master thesis where batch cultivations were performed. Both masters were performed at the Institute of Technology at Linköping University during autumn 2014. The same equipment and cultivation medium was used in both project and therefore data from the batch cultivations was used for analysis to grasp trends and behaviors in the cultivation to better prepare the fed-batch cultivations (Reissig, 2014).

4.1 Inoculum

During the first period of the project there was trouble to grow the inoculum to give a satisfactory cell concentration for use in the bioreactor. Two major problems became apparent.

4.1.1 The pH

The first medium formulation that was used had the additions of 20% (v/v) FBS, 1% (v/v) Penicillin/Streptomycin and 17.8 mM sodium bicarbonate with DMEM medium as base. It was observed that the pH value was around 8 during the cultivations of the cells. 50 ml of only the cultivations medium was incubated in 5% CO₂ for four hours that reached a pH value of 8.02 which is 0.82 above the optimal value for hybridoma cells. Through troubleshooting it was found that the DMEM medium contained 44 mM sodium bicarbonate and thus the extra addition was not necessary. Instead, the incubators CO₂ environment was increased from 5% to 10%. Through this, the pH was successfully lowered to 7.2. The increase in pH was due to that a too high amount of sodium bicarbonate was present in the medium and the 5% CO₂ environment in the incubator was not enough to ensure buffering. When an abundance of sodium bicarbonate is present in a fluid it will dissolve into water and gaseous CO₂ as shown in the reactions (R3)-(R4).



Through this process a hydroxide anion is created when sodium bicarbonate takes a proton from water to produce carbonic acid which then dissolves into water and gaseous CO₂, making the solution more alkaline. This reaction goes on until equilibrium has been reached between solved CO₂ from the environment seen in (R5) and the dissolved CO₂ from carbonic acid (Freshney, 2005).

4.1.2 Nutrients

The cell concentration remained about constant during cultivation in the inoculum with only a slight increase after the problem with the pH had been solved. After looking at the formulation of Hybri-care Medium 46-x from ATCC which was the recommended cultivation medium we found that the nonessential amino acids alanine, asparagine, aspartic acid, glutamic acid and proline along with some vitamins were missing in the DMEM medium. This led us to believe that energy and essential amino acids was rerouted to produce the absent nonessential amino acids and the cell growth

decreased as a result. It has been observed that the nonessential amino acids concentrations would remain constant during cultivation of hybridoma cells whereas the essential amino acids would decrease to keep the steady state (Selvarasu et al., 2009). This implies that a certain amount of nonessential amino acids are necessary for optimal growth and the absence of these in the beginning will divert more energy and resources, thus inhibits the cell growth greatly. Also, leakage could be another reason for production of nonessential amino acids (Freshney, 2005). Due to this we added 1% (v/v) MEM NEAA to reroute energy back to cell growth. Around 80-90% of the medium gets changed every third day to avoid depletion of nutrients and accumulation of lactate and ammonia. Through these steps the cell concentration increased remarkably and more viable cells could be observed.

4.2 Lactate analysis

The lactate concentration was measured with both the BioSenz Analyzer and HPLC during the two batch runs. However, a difference between the two measuring techniques was evident from the start of each batch run as seen in figure 6.

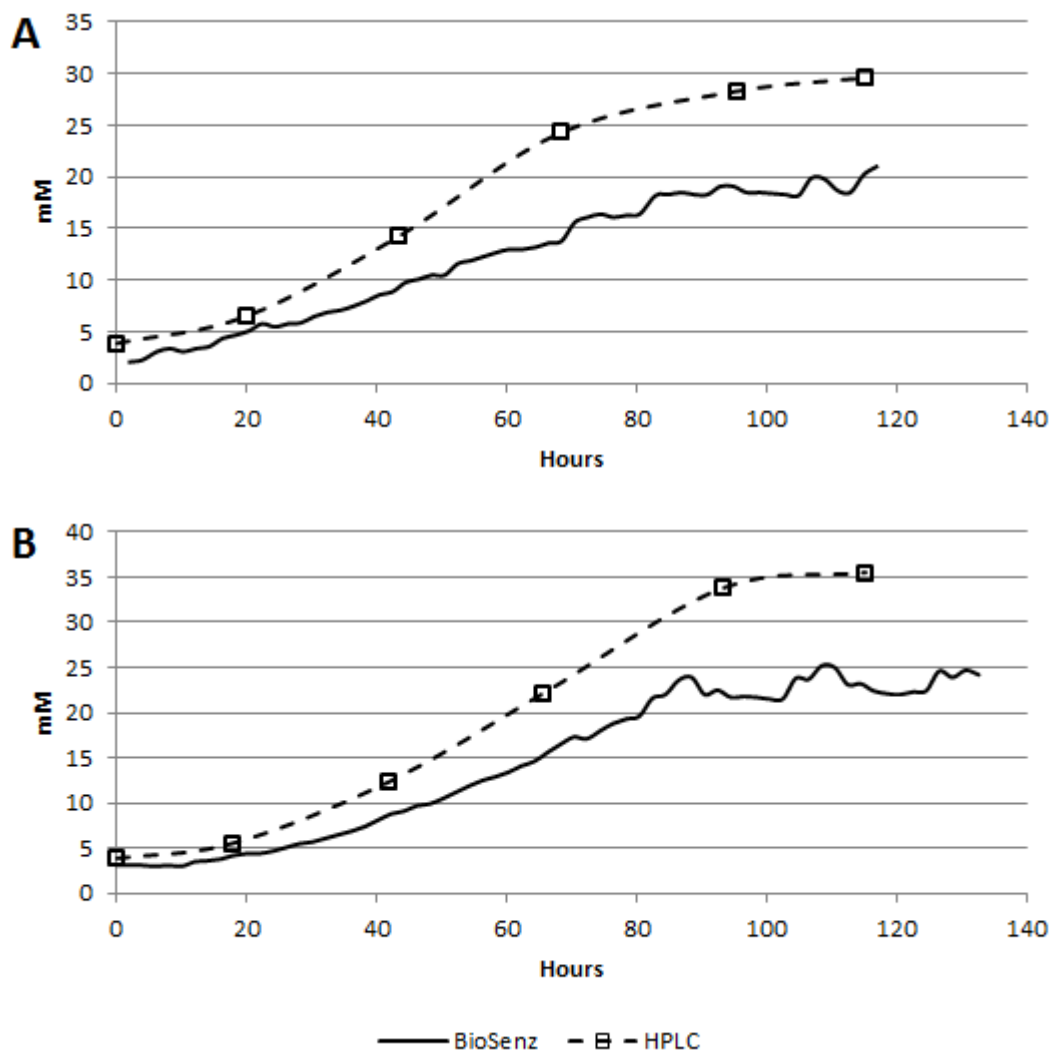


Figure 6. Comparison between lactate concentrations between the BioSenz analyzer and the HPLC from the first batch run (A) and the second batch run (B).

As seen from the HPLC measurements, the lactate concentration during the batch cultivations could be up to 50% higher than the measurements from the BioSenz analyzer. It has been observed that mammalian cells produce D-lactate inside the mitochondria (Flick and Konieczny, 2002). The enzymes in the chip of the BioSenz Analyzer are L-lactate specific and cannot catalyze D-lactate. In the HPLC however, D-lactate and L-lactate will have the same retention time and thus the lactate concentration will consist of both D- and L-lactate which could affect the HPLC measurements and give higher values. However, the production of D-lactate has been observed to be in the concentration of nanomolar (nM) (Ewaschuk et al., 2005) and it is therefore improbable that the difference between the HPLC measurements and the BioSenz Analyzer is dependent on the concentration of D-lactate. Another plausible

explanation would be that another organic component with similar retention time as lactic acid would add to the HPLC measurement.

An experiment in order to test the HPLC calibration curve was done with the use of five lactic acid standards of the known concentrations; 0 mM, 10 mM, 15 mM, 20 mM and 25 mM with the results shown in figure 7.

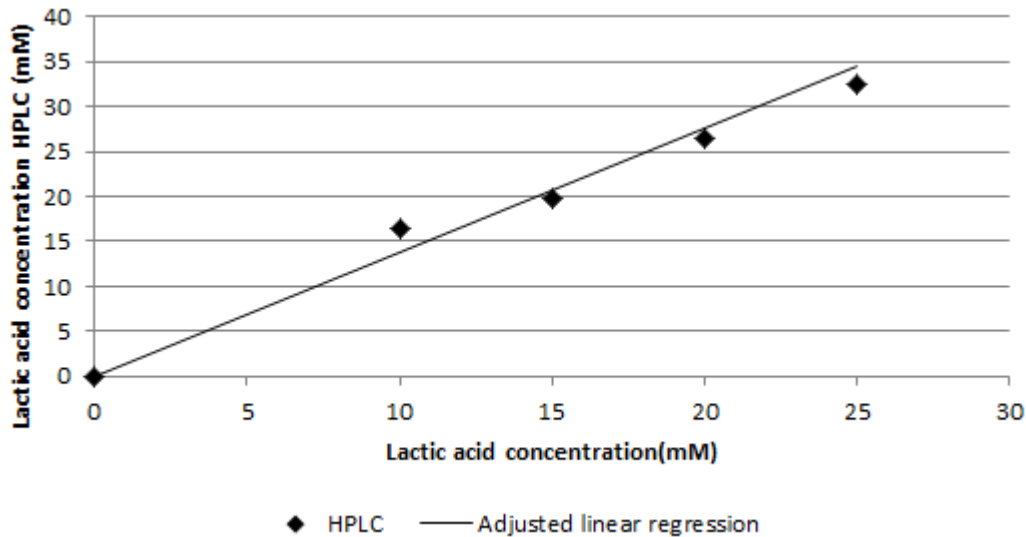


Figure 7. Squares shows the measured concentrations of lactic acid from the HPLC with respect to the true lactic acid concentrations and the adjusted linear regression.

As can be seen from figure 7 the HPLC always gives a larger measured value compared to the known concentrations. A linear regression was done but was adjusted however to make sure the line stayed on origin. This because the origin is a true value where the measured lactic acid concentration was zero in the HPLC and is the only data point that assures no variation. The linear regression was then adjusted to fit the other data points. The slope from the adjusted linear regression was 1.38 which means that the HPLC measured a 38% higher value than in reality. The slope coefficient was used as a correction value to adjust the measurements in batch run 1 and 2 which made the datasets from the HPLC and the BioSenz Analyzer almost overlap. For the fed-batch runs with the P-controller this means that no correction of the BioSenz Analyzer was needed.

The useful range for lactate measurements for the BioSenz analyzer is between 0.2-25 mM. In the batch run 2 the BioSenz Analyzer reached the maximum value for lactate concentration after about 90 hours, which means that given values after this might not be accurate due to less sensitivity. Therefore, the BioSenz Analyzer might not be the best choice for lactate monitoring during a batch cultivation. A fed-batch cultivation however is more appropriate due to controlled feeding to keep the lactate concentrations low.

4.3 Analysis of stirred batch cultivation

From the batch cultivations only the data from batch 2 was used for analysis due to technical problems with the DO and Aber probes during the first batch cultivation (Reissig, 2014). Figure 8 shows the viable cell concentration, glucose concentration

and lactate concentration during the second batch run measured with cell counting and HPLC. The maximal specific growth rate of the cells was calculated to 0.045 h^{-1} during the cultivation. The lactate and glucose concentrations can be seen as mirrors to each other in the figure 8 where the increasing consumption of glucose leads to an increasing production of lactate. This is a sign that an abundance of glucose is present and the overflow metabolism is active.

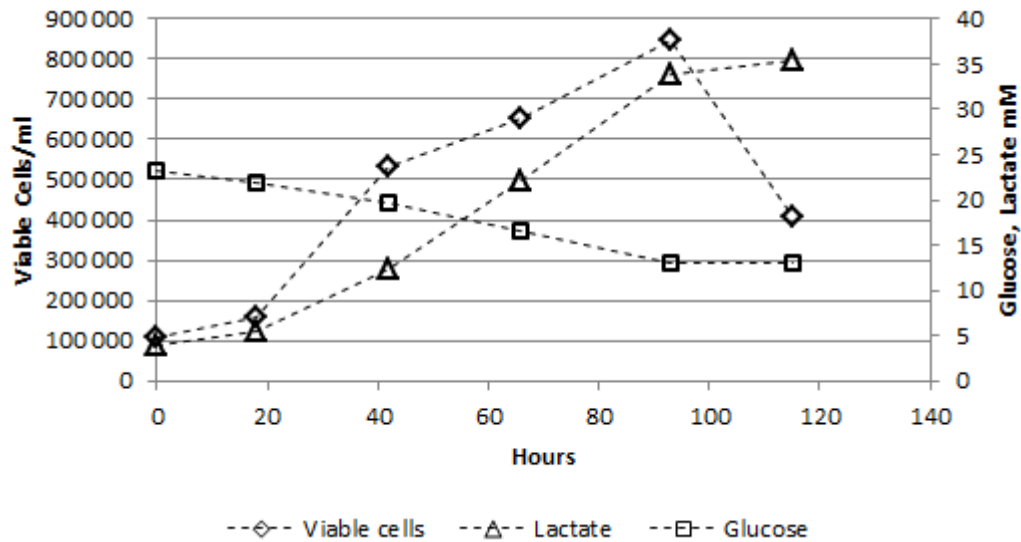


Figure 8. Shows the off-line data of the cell growth, glucose consumption and lactate production during cultivation in batch run 2.

During the cultivation the glucose concentration drops from 23.3 mM and stops at 12.8 mM when the cultivation ended. Coupled with the cell concentration which dropped right before 100 hours, the cells cultivation seems to have depleted sources of vital nutrients and life supporting functions in the cells have come to a halt, i.e. the uptake and glycolysis of glucose. This is supported by figure 9 which shows the on-line monitoring of the cell concentration with the Aber probe and the DO. From the figure it can be observed that the DO drops from the start of the cultivation and follows the growth of the cell concentration. The airflow from the sparger was increased twice during the cultivation to keep the oxygen concentration from getting too low. First increase was at 40 hours from 0.15 L/min to 0.25 L/min and the second at 65 hours and was increased to 0.35 L/min. It can be observed that right before 80 hours the cell concentrations starts to drop and the DO starts to climb, this is an indication that a vital source of nutrient has been depleted and the cells starts to die (Salehmin et al., 2013).

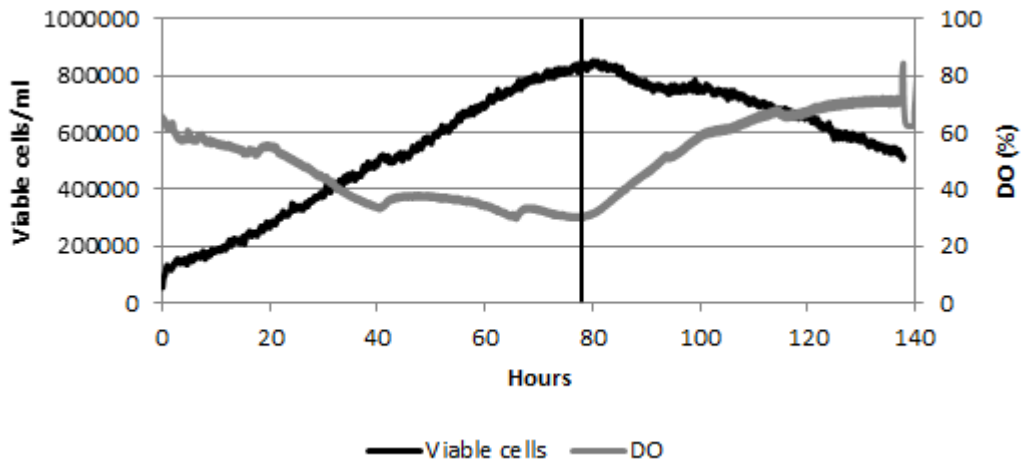


Figure 9. Shows the viable cell concentration from the Aber probe and dissolved oxygen. It also shows the depletion of a vital nutrient after about 80 hours during batch 2 marked by the line.

This means that about 8.3 mM glucose was consumed from the beginning of the cultivation until the DO started to climb. This is important due to that stoichiometrically there were only enough building blocks for 8.3 mM glucose in the cultivation medium. Extra additions of amino acids solutions in the feeding medium for the fed-batch cultivations was therefore done to get a three times more concentrated feeding medium to ensure that all of the 25 mM of the glucose could be metabolized in the fed-batch cultivations.

The mAb production of the second batch run is shown in figure 10 and is growing continuously throughout the cultivation. It can be seen that the mAb concentration still increases somewhat after 80 hours and then remains about constant thereafter meaning that the mAb production was not growth associated. However, the initial mAb concentration in the inoculum was calculated to be 12.2 $\mu\text{g/ml}$, which is more than twice as much compared to the end of the second batch run. One big difference between the inoculum and batch is that no agitation caused by air bubbles was present in the spinner flasks when growing the inoculum. This would make the cells in the inoculum less stressed with a higher growth rate and production rate of mAbs (Legazpi et al., 2009).

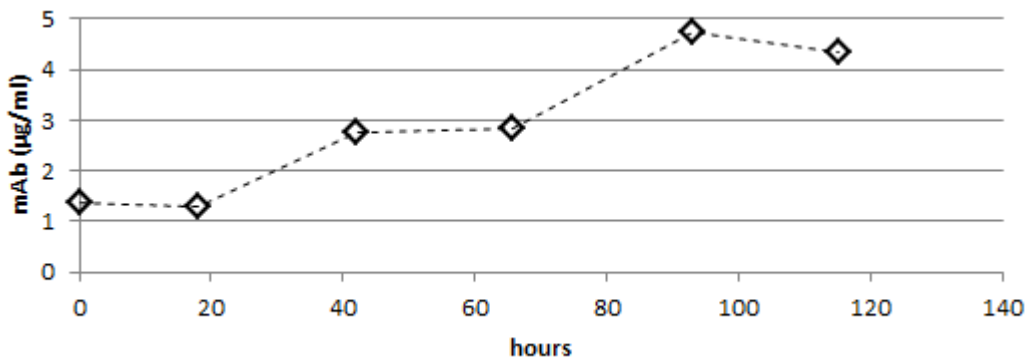


Figure 10. Production of mAbs during the second batch cultivation

4.4 Aber probe calibration and analysis

The in situ Aber probe was calibrated with 50 ml of viable cell suspension with a viable cell concentration of 795 000 cells/ml. Small volumes of 10 ml from the cell suspension was added to 100 ml of cells cultivation medium and then measured. The data gained resulted in a linear regression seen in figure 11 below.

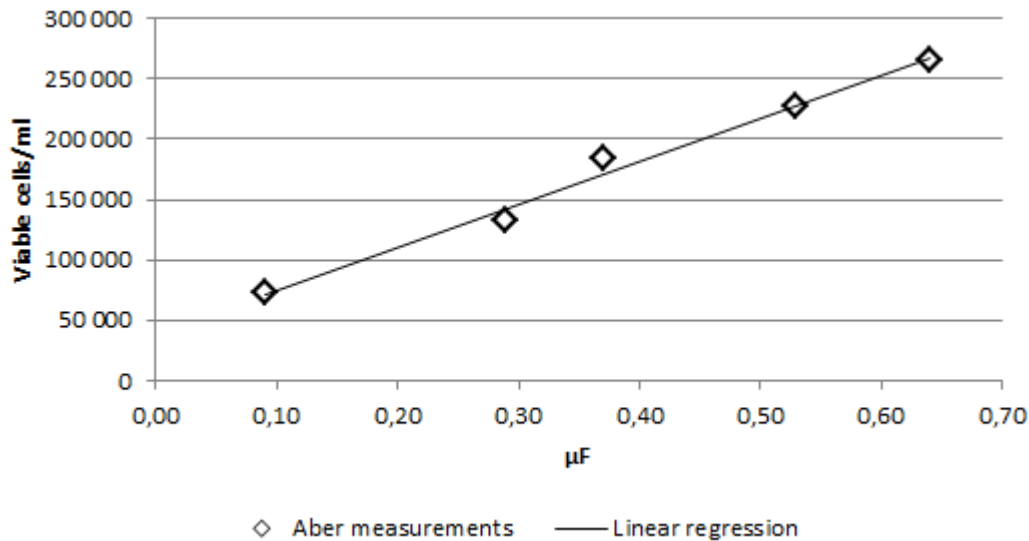


Figure 11. The Aber measurements shows that the capacitance is correlated with the viable cell concentration

The slope of the linear regression had a value of 355 042 cells/µF which was used as the conversion factor from capacitance to viable cells/ml in the three fed-batch runs. One of the biggest advantages with the Aber probe was that it could be calibrated to the medium and then zeroed, thus getting rid of initial conditions from the medium that influenced the probe measurements. The data derived from the Aber probe during the cultivations however always had noise in the data as can be seen in the measurements from the batch cultivation and the three fed-batch cultivations. This is due to that the probe is not only influenced by the growing concentration but also the geometry of the cells and changes in the medium such as consumption, thus creating noise (Kiviharju et al., 2008). As can be seen in figure 12 the Aber probe mostly overlap with the cell counting data points in all of the fed-batch cultivations. Much of the variability comes from the cell counting where different counts can give differences in cell concentrations from only one sample.

4.5 Analysis of stirred fed-batch cultivations

Three fed-batch cultivations were performed where the first was done without the controller to see how the calculated flow and feed profile affected the cell growth and the concentrations of lactate and glucose. The two other fed-batch cultivations was done with the controller activated where the set point and K_p were individually set between the fed-batch cultivations depending on growth during the initial phase.

The growth of the cells in the different fed-batch cultivations are shown in figure 12. As can be seen all cultivations reached a viable cell concentration of 400 000-480 000 cells/ml before the feed was started between 50-60 hours of the elapsed time. Compared to that of the batch cultivation which had a viable cell concentration of 600 000-700 000 cells/ml during the same time frame the cells in the fed-batch

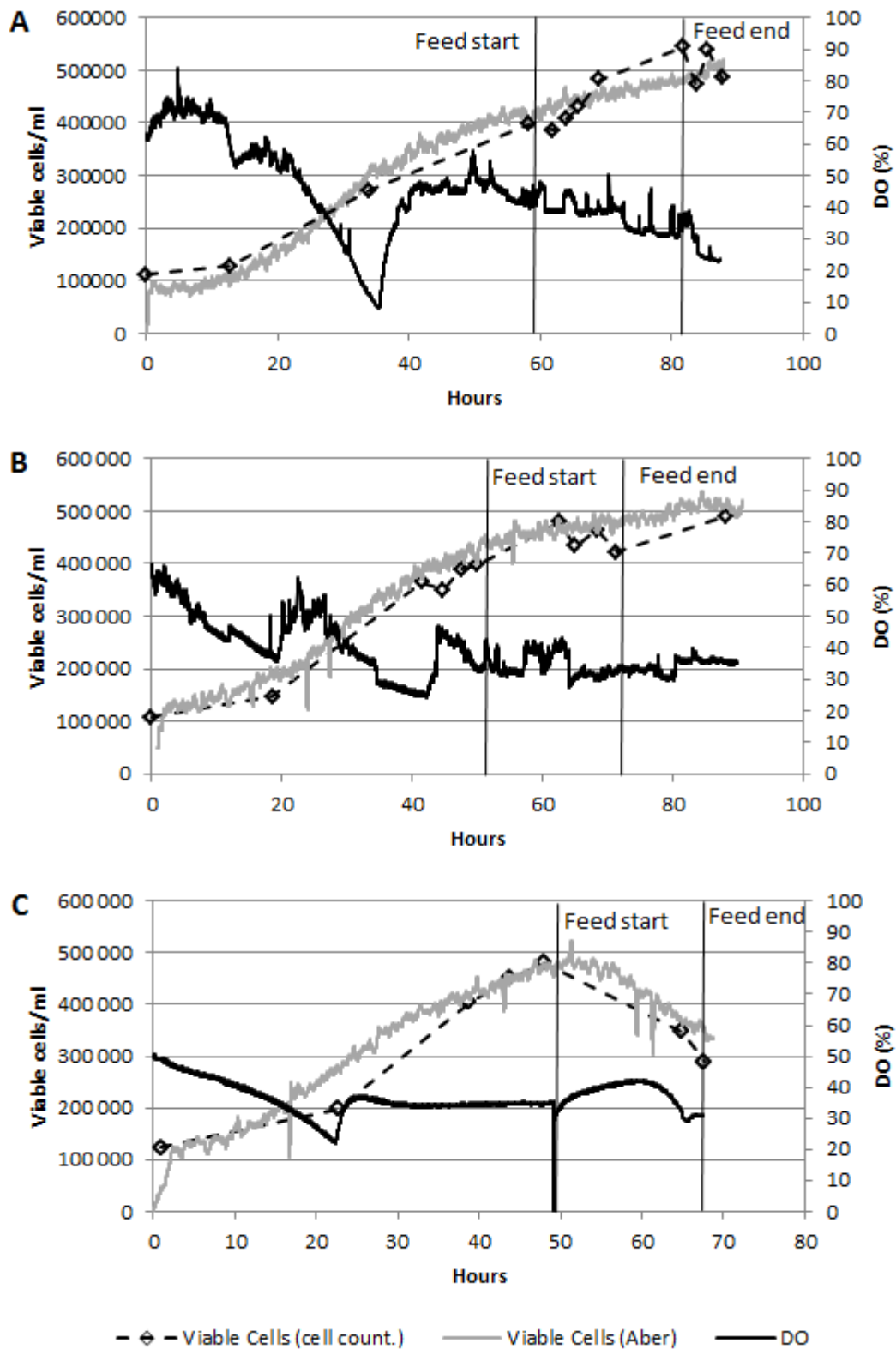


Figure 12. Shows online data from the DO and Aber probe as well as the cell counting samples from (A) the first fed-batch run, (B) the second fed-batch run and (C) the third fed-batch run.

cultivations seemed to grow at a slower rate. This could be explained by the difference in the glucose concentration between the batch with 25 mM and the fed-batch with 5.5 mM glucose. The specific growth rate, μ , of the cells is directly

affected by the concentration of glucose which has a maximal value at high glucose concentration and goes to zero when the glucose starts to run out (Ljunggren and Haggstrom, 1994). This is also supported by the fact that the highest measured growth rate for the fed-batch cultivations was; 0.035 h^{-1} , 0.032 h^{-1} and 0.034 h^{-1} for the first, the second and the third fed-batch cultivation respectively which are all lower than the highest measured growth rate of 0.045 h^{-1} from the second batch cultivation between 0-60 hours. The highest measured specific growth values for the fed-batch cultivations were also used in the feeding profile for their respective cultivation. The specific growth rates for all the fed-batch cultivations before and during the feeding phase are summarized in table 1 at the end of the section.

As in the batch cultivations the dissolved oxygen was controlled manually. This however gives rise to problems of reproducibility between the fed-batch cultivations due to that none of the cultivations are exactly the same in their execution. However, the dissolved oxygen was for most part kept over 20% to prevent the cells from suffocating and below 60% to avoid oxidative stress which could have been the cause for the slow growth in the beginning of the first fed-batch cultivation. The dissolved oxygen was then to be kept around 30-40% to keep down the airflow from the sparger to minimize cell stress from both agitation and oxidative stress (Handa-Corrigan et al., 1989). These precautions and the low stirring speed of 40 rpm helped to keep the cell viability high as can be seen in figure 13.

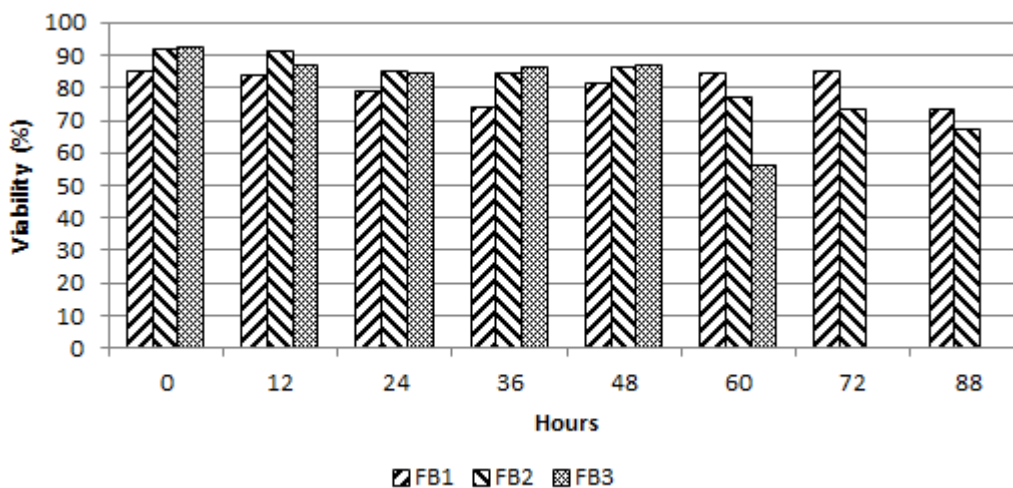


Figure 13. The viability of the hybridoma cells during the fed-batch cultivations.

The feeding phase of the first fed-batch cultivation had an initial flow of 26 ml/h which was calculated from the highest measured specific growth rate during the initial phase. During the feeding the viable cell concentration increased from around 400 000 cells/ml to slightly under 500 000 cells/ml. As can be seen in figure 14 (A) most of the glucose had been consumed when the feed was started. The glucose concentration quickly rises thereafter due to that the cells cannot consume the glucose at the rate at which it is introduced in the reactor. The lactate concentration which stabilized at 14 mM after the initial phase also starts to increase after the feed has been started due to that the increasing glucose concentration triggers the overflow metabolism in the cells.

The second fed-batch cultivation was very similar to that of the first which also had a calculated initial flow of 26 ml/h. The feed was initiated around 2 mM glucose as seen in figure 14 (B) and the set point value was set to 15 mM lactate and K_p set to 0.5. These values were chosen to see how the controller affected the lactate concentration when the set point value was chosen much higher than the measured lactate concentration of 10 mM at the beginning of the feed. The lactate concentration only increased slowly throughout the feeding phase even though the controller increased the feeding flow. It was believed that the lactate concentration would increase faster and then limit the feed. However, the lactate set point was never reached during the feeding phase according to the BioSens Analyzer, which meant that the set point was set too far apart from the measured lactate concentration at the beginning of the feed and the cells were not fast enough to metabolize the added glucose. The set point was also chosen to be as low as possible to avoid cell growth inhibition. This, because it has been observed that at lactate concentration of 55 mM the specific growth rate for hybridoma cells was reduced to only half of its maximum value (Ozturk et al., 1992). As mentioned the effective range of the BioSenz when measuring lactate is 0.2-25 mM which gives a large span to choose the set point of the controller.

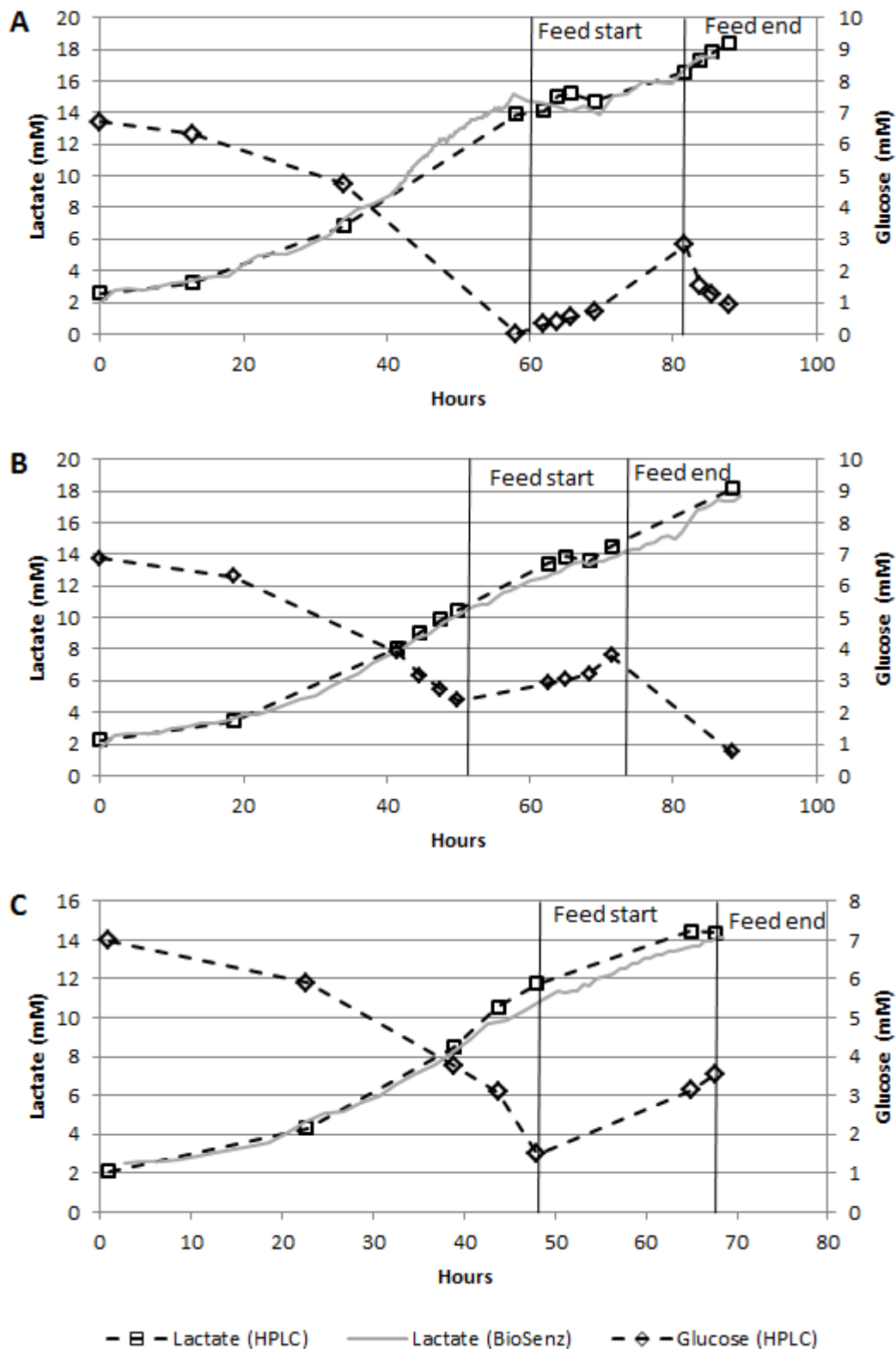


Figure 14. The off-line measurements of glucose and lactate done with HPLC as well as the lactate monitoring by the BioSenz Analyzer from (A) the first fed-batch, (B) the second fed-batch and (C) the third fed-batch.

For the third fed-batch cultivation the initial flow was calculated to 31.3 ml/h and a set point value of 10 mM was chosen to be even closer to the measured lactate concentration of around 11 mM at the end of the initial batch phase as can be seen in

figure 14 (C). The K_p value was also increased to 0.7 to get larger changes in the feeding flow and the sampling time was reduced to 30 min to faster react to changes in the system. However, due to technical problems the controller for the pH stopped working around the time the feed was started which lead to an increased pH and the cell concentration started to drop as can be seen in both figure 12 (C) after the start of the feed and figure 13 where the viability drops from 85 % to 55 % due to growth inhibition and cell death correlated to the increased pH. Unfortunately because of this not much can be said about the feeding phase in the third fed-batch cultivation.

The mAb production from the three fed-batch cultivations can be seen in figure 15.

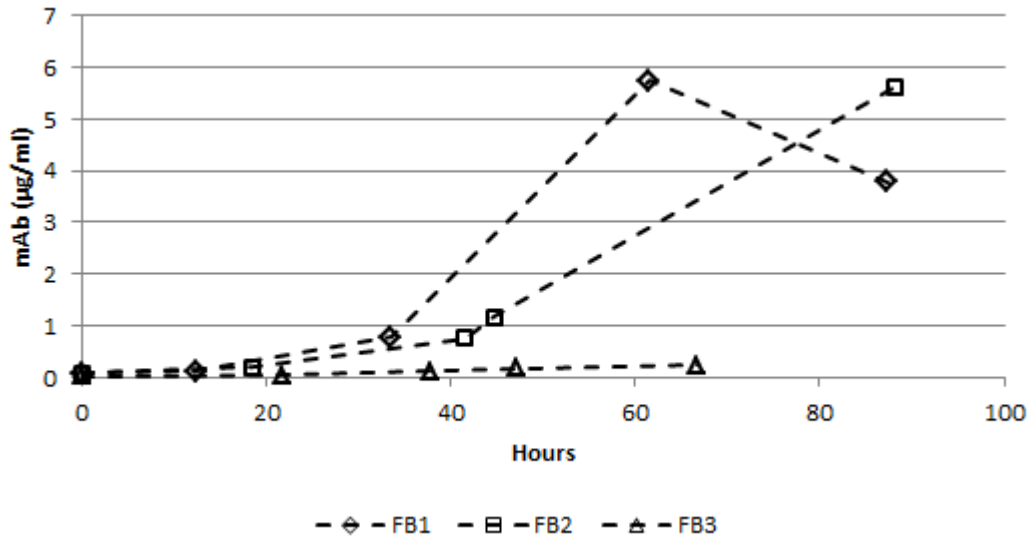


Figure 15. The production of mAbs from the three fed-batch cultivations analyzed by the ELISA sandwich.

Both the first and the second fed-batch reached similarly high mAb concentrations at the end. The third fed-batch cultivation seems to have had a lower production rate of mAbs even though the cells had high viability and were growing well up to the point of the technical problem. It has been found that if the specific growth rate of hybridoma cells is low the production of mAbs could be up to twice as high as when the specific growth rate was closer to its maximum (Jang and Barford, 2000b). However, this does not seem to be the case either due to that the growth rates between the initial phases of the fed-batch cultivations are quite similar to each other.

Table 1. The specific growth rates for the second batch cultivation and the three fed-batch cultivations.

	B2	FB1	FB2	FB3
μ_m (h^{-1}) (Initial batch phase)	0.045	0.035	0.032	0.034
μ_m (h^{-1}) (Feeding phase)		0.028	0.031	-

5 Conclusions and future work

First of all it should be mentioned that more cultivations need to be done for both batch and fed-batch to increase certainty when evaluating the data. The use of only one dataset for the batch analysis can give information of the general trends when cultivating cells but nothing can be said about the deeper details of the cells and the system, e.g. optimal specific growth rate, glucose consumption, stress responses and so on.

Hybridoma cells are very sensitive and need a sterile and good environment to survive. As discovered when making the protocol for growing the inoculum even the smallest changes can make big impacts on the growth of the cells.

The main focus in this project was on the lactate concentration and how to use it to control the overflow metabolism. Ammonia however was not measured in the cultivations and should be a point of interest due to its inhibitory effect on the cells growth. Both the initial medium and feeding medium contained 4 mM of glutamine which is catalyzed into ammonia and it would be interesting to if something could be said from its effect on the system as more ammonia is being produced.

Another experiment to try in the future would be to use glucose instead of lactate as the variable in the controller. The benefits of this would be that the concentration is directly connected to the feed flow. The lactate however is indirectly connected to the glucose feed which must first be broken down into acetate via the glycolysis which is catalyzed into lactate if the TCA cycle is fully loaded which makes the feed-back loop in the controller far more complex. Instead, by controlling with the glucose the overflow metabolism can be avoided and the lactate concentration kept low if the glucose concentration is kept low as well.

The soft sensor based on the BioSenz Analyzer was never expanded and combined with the Aber probe during the project with the biggest reason being due to the noise generated by the Aber probe. Both the BioSenz analyzer and the Aber probe showed variance in the data acquired due to their sensitivity and if combined small separate measuring errors would get larger and probably give rise to more noise and oscillation making it unsuitable for controlling the feed. However, if a soft sensor based on the BioSenz Analyzer and the Aber probe should be used in the future the noise must be reduced from the Aber probe.

The size and type of reactor used in the cultivations is also an issue which should be addressed. Due to the large volume the compartment effect must be taken into account in a fed-batch cultivation meaning that it will take time for newly introduced feed to get properly mixed homogenously because of the low stirring speed. This also creates environments with high and low nutrition value thus leading to overflow metabolism in one place and starvation in another. However, this was not the case in this project due to the glucose concentrations were increasing during the feeding phase in the fed-batch cultivations. But it should be taken in to account when trying to avoid overflow metabolism which means indirectly means to maintain a low glucose concentration. Another concern was to maintain the oxygen levels in the reactor during cultivations. Hybridoma cells are unsuitable to grow in a reactor which makes use of a sparger to increase the dissolved oxygen due to the fact that they lack cell walls which makes them vulnerable to the shear stress from the bubbles. The use of

animal serum increases the resistance to agitation but can contaminate the culture. Another option would be to use perfusion reactors when growing hybridoma cells which would decrease the stress on the cells and inhibitory waste products such as ammonia and lactate can be removed from the cell culture thereby increasing the duration of the cell cultivation.

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