Examensarbete

Evaluation of on-line cell viability and L-lactate measurements in soft sensor for mammalian cell cultures

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Abstract
Increasing demand on more effective cell culture reactors has driven optimization works to increase output of products. This has led to development of soft sensors that uses mathematical formulas to increase the available information for the parameters during runs. In the project two parameters was evaluated for use in such a soft sensor, viability by measuring on-line capacitance with Aber probe and L-lactate production using BioSenz apparatus. To determine how well these could be used both were used on batch reactors measuring on a mouse-mouse B cell hybridoma culture which produced IgG₁. On-line measurements were performed by probes which measured directly on the cell suspension or withdrew sterile sample from the reactor. Measuring viability gave results with low error, which can be concluded to the variation in reference cell count, but it could not be determined if measuring L-lactate production with BioSenz works in reactors of this size. More work needs to be done on other types of reactors, like fed-batch or perfusion, or lower working volumes.
Abbreviations
Radio-frequency impedance (RFI)
Immunoglobulin G (IgG)
Monoclonal antibodies (mAbs)
Dissolved oxygen (DO)
High-performance liquid chromatography (HPLC)
Fetal Bovine Serum (FBS)
Penicillin Streptomycin (Pen strep)
Enzyme-linked immunoSorbent assay (ELISA)
Enzyme thermistor (ET)
Dulbecco's modified Eagle Medium (DMEM)
Non-essential amino acids (NEAA)
Master cell bank (MCB)
Volumes of air per volume of liquid per minute (VVM)
1. Introduction

1.1 Purpose of the project
The main purpose of the project is to develop a new soft sensor by combining the BioSenz™ Analyzer for lactate analysis (Senzime) and the Futura Capacitance Probe (Aber) for viability monitoring of Immunoglobulin G (IgG) producing hybridoma cells continuously/on-line during the process. The purpose of this master thesis is to test if a soft sensor can be based upon these sensors and be used to estimate parameters such as specific viable growth, specific lactate production etc. which is used to regulate and control large scale production as well as small scale.

1.2 Boundary conditions
Some of the boundaries that are put on this project are in the form of the sensors that we are going to use which is the Applikon BioSenz and the Futura capacitance Aber probe. The BioSenz apparatus will do on-line measurements of lactate and the Aber probe will do on-line viability monitoring. When it comes to the cell line that is going to be used, certain criteria has been put on it. The cell line needs to be a suspension IgG producing hybridoma cell. Here the IgG does not need to be specific to any specific antigen since we are only interested in the production of the antibody itself. The type of hybridoma cell used is not specified for this project. Another limitation is that only one bioreactor is available for the project, which limits the amount of runs that can be performed during the 20 weeks the thesis has. The time limitation also limits what kind of tests that can be performed, but of course also what equipment is available in the laboratory.

The parameters that will be in focus are lactate production/concentration, which is an overflow metabolite, and the cell viability which is determined by measuring the capacitance over the suspension in the reactor. From this, other parameters can be estimated and thereby increase efficiency and decrease resource allocation of industrial scale production as well as laboratory scale, by managing the resources better since better control over the system gives rise to better resource control by optimization.

1.3 Expected impact of study
Beyond the project the developed soft sensor will help to increase productivity for processes using animal cells for production of monoclonal antibodies (mAb). Optimization of the initial growth step of the animal cells is an important step which sets the limitations for the rest of the process by the time it will take to reach inoculum concentration and production of targeted product. In the end this will save both time and resources for the producers and is also in compliance with PAT (Process Analytical Technology)(FDA, 2004). Being able to fully regulate the process will also enable the producers to increase the production of the targeted product.

The project also has a beneficial effect on society since a decrease in usage of resources is both good in an cost efficient way, lowering consumer prices and thereby increasing availability for more people to purchase, but also an environmental impact because of reduction of raw materials during the process thereby increasing the input/output of the process.
1.4 Objectives
The main objective of the project is to see if a soft sensor of this sort can be made practically and not just theoretically. In the end the soft sensor should work well and give signals that can be used to make estimations of parameters that later on are able to control a bioprocess of this type. In future prospect these parameters are needed to control and regulate the cell growth process and to minimize waste and increase the productivity of the mammalian cells by increasing the yield of product. Some of the sub-objectives in the thesis work include:
- Start-up of the reactor (making it ready before active use)
- Completing and conducting reference test to confirm sensor readings/estimations.
2. Background

2.1 Software sensor

The term soft sensor or software sensor is a technique that’s been getting a lot of traction in industrial processes as well as laboratory scale processes used for process control and regulatory control (Gustavsson and Mandenius, 2012). Soft sensors are used in processes to enable certain processes to do on-line measurements that normally cannot be done due to how the process works or fail to pass operation and process qualifications (OQ/PQ) and GMP validation (Benedikt Warth, 2010). The principal of soft sensors is to use real hardware sensors that can perform on-line measurements and from these measurements derive other information by using mathematical models (Luttmann et al., 2012).

Usual estimations with soft sensors are biomass, substrate and product using kinetic equations. The sensor structure is divided into Black-box and Hybrid sensors. Black-Box sensors requires no prior knowledge of the fermenter system, but rather is an input-output relationship where biomass concentration is the output and is used together with gas analysis to regulate regulatory parameters such as feed rates etc. (James et al., 2000, Benedikt Warth, 2010). Methods used for correlation include multivariate analysis (MVA), regression modeling, artificial neural network (ANN) and chemometric approaches. Hybrid sensors on the other hand are based on differential equations for the fed-batch system.

Less complex soft sensors can be created by combining two sensors with a task to compensate for reactor disturbances. An example of this is where a pH electrode can be compensated for long-term temperature variations in the reactor by a thermistor and an algorithm that adjusts the pH signal (Benedikt Warth, 2010).

Figure 1. Principal of soft sensor

Hardware on-line sensors are used on the bioprocess and parameter signals are acquired. The signal is inputted into a computer program where these signals are incorporated into mathematical models where other parameters is estimated that describes the process such as growth rate, byproducts, products etc. (Gustavsson and Mandenius, 2012).

The down part of using soft sensors is that it lacks a predictive elements and is therefore not adequate to predict upcoming problems during the process and can only react to events that is happening real-time (Gustavsson and Mandenius, 2012).
2.2 Lactate Analysis with Flow Calorimetry enzyme thermistor

BioSenz™ optimizes the bioprocess by continuous measurements of the glucose and lactate level in the bioreactor, where the measured value will be evaluated if it can be used for regulating the process system. BioSenz uses a lactate or glucose biosensor chips with high specificity that utilizes flow through calorimetry (Figure 2) with immobilized enzymes(Senzime, 2014b) with a useful range of 0.2-25mM.

![Flow though system of the BioSenz. Lower part of the figure shows the side and front of the BioSenz apparatus where W is waste, B is buffer and numbered bottles are the calibrator fluids.](image)

On-line readings can be done for up to four reactors at the same time and is using a unique patented heat-flux measuring technology(Senzime, 2014a, Applikon Biotechnology). BioSenz uses calorimetry for determination of amount of product in the sample.

The BioSenz uses a combination of enzyme thermistor (ET), calorimeter and flow system and is ideal for continuous measurement(Ramanathan et al., 1999). ET is based on an immobilized enzyme column that in some cases is called immobilized enzyme reactor. Conventional ET cylinder consists of insulators and uses Plexiglas tubes for column insertion where the reaction enzymes are either immobilized or free depending on the setup. Figure 3.
ET works on the basis that the total heat evolution is proportional to the moles of product molecules that is created in the reaction between the immobilized enzymes and the corresponding substrate (Yakovleva et al., 2013). In the BioSenz the columns function is replaced with a biosensor chip using co-immobilization of Lactate oxidase and catalase. This minimizes the size of the apparatus and increases the sensitivity. This is done since catalase consumes the hydrogen peroxide which is a by-product from the oxidase reaction, reaction (1) and (2), and also increases the heat evolution which doubles the sensitivity of the sensor (Yakovleva et al., 2013, Ramanathan and Danielsson, 2001). Here the biosensor chip is where the enzyme and substrate reaction is performed and uses flow calorimetry.

\[
\begin{align*}
L-\text{lactate} + O_2 & \xrightarrow{\text{Lactate oxidase}} \text{Pyruvate} + H_2O_2 + \text{Heat} \\
2H_2O_2 + O_2 & \xrightarrow{\text{Catalase}} 2H_2O + O_2 + \text{Heat}
\end{align*}
\]
A calorimeter is a device whose purpose is to measure heat flows, or energy that is liberated during physical or chemical changes (Wojcik, 2011). Figure 4 describes the general setup for the heat exchanger in the BioSenz equipment. The assembly’s main parts are the peltier elements, heat sinks and the enzymatic sensor which is surrounded by housing.

![Figure 4. Flow Calorimeter. Substrate is allowed to flow through channels where enzymes are immobilized and the reaction generates energy in form of excess heat. The generated heat is then led through the Peltier elements and generates a current that represents the amount of substrate involved in the reaction. Heat sink absorbs the excess heat to ensure that the heat generated comes from the reaction.

The housing comprises two thermally insulating blocks providing a generally consistent air temperature around the heat sinks, the reflectors and the Peltier elements during operation. (Carlsson, 2009)

When the material passes through the enzymes the reaction generates heat which can be detected (Figure 4). The detection is done by Peltier elements which generates a current that can be measured and used to calculate the amount of material (Guilbault, 1984, Ramanathan and Danielsson, 2001). See equation 3.

\[ Q = -n_p(\Delta H) \quad (3) \]

In eq. (3) Q is the total heat that is generated, \( n_p \) is moles of product and \( \Delta H \) is the molar enthalpy change.

To maximize the peltier elements they are arranged in a way that the contact with the thin foil on the sensor has maximum heat conduction. The heat sink is used to disperse heat that is generated.

\[ I = 0 \rightarrow I = Q \]
Figure 5 shows the setup of the biosensor apparatus that is used. Where (16,16’) is the peltier elements and (11,12) are heat sinks, which dissipates the heat from the peltier elements to the surrounding structure that is made up by a non-conductive material.

![Figure 5. BioSenz enzyme thermistor. Biosensor apparatus for detection of thermal flow. (Carlsson, 2009)](image)

### 2.3 Radio-frequency impedance

Futura capacitance Aber probe uses radio-frequency impedance (RFI) and is regarded as a robust and reliable method for observing the viability of a biomass in a cell suspension. This method is routinely used to monitor mammalian cells as well as high density yeast and bacterial fermentation. The RFI method has a clear advantage for manufacturing because it is an unambiguous reflection of the viable cells in suspension rather than a total number of cells, it can be done with a high degree of accuracy and has a high sensitivity when it comes to detecting changes in physical properties of the materials (Carvell and Dowd, 2006, Srikanth et al., 1999).

When the RFI’s alternating current is applied on a cell suspension an electrical polarization affects the isolating cell by charge separation across the cell membrane which turns the cell into a capacitor (Figure 6). This is due to that ions in the solution are forced to move in both the solution and the cells. The ions inside the cells can only move until they reach the cell membrane. This creates the charge separation because the cell membrane is essentially non-conductive (Figure 7a). By measuring the capacitance of the suspension at different frequencies the biomass can be estimated by applying a cell concentration to capacitance factor called CPM which is specific for the cell type. See equation 4.

\[
\frac{\text{Cell concentration Cells/mL}}{\text{Capacitance pF/cm}} = CPM
\] (4)

The rate which the frequency is changed can be controlled and is used to measure the background capacitance that must be neglected for the measurements. This is done by increasing the frequency. At very high frequencies, around 10 MHz and above, ions in the cells does not have time to move far enough and therefore cannot polarize the cell membrane. This gives rise to the background capacitance that comes from dipoles of the
water in the medium. (Figure 7b). For most cells the running frequency is centered between 0.5 and 3 MHz (Carvell and Dowd, 2006).

This effect only works on whole and living cells since only intact cell membranes can be affected by the charge separation and become capacitors whereas dead or damaged cell membranes cannot separate the charge and therefore does not affect the collective capacitive effect on the suspension, which is measured in pF/cm (Carvell and Taylor, 2013, Carvell et al., 2014). The amount of living cells in the suspension is then in direct correlation to the capacitance over the suspension. The capacitance on the suspension is also dependent on the cell type and to the membrane bound volume (Instruments, 2013).

![Figure 6: Futura capacitance turning cells into tiny capacitors (Instruments, 2013)](image)

One of the positive aspects of this method is that micro-carries and other materials that might be associated with cell growth etc. will not affect the capacitance since they don’t have membranes and does not contribute to the overall suspension capacitance (Carvell and Taylor, 2013).
2.4 Hybridoma cells and Immunoglobulin G
Monoclonal antibodies (mAb) are increasingly being used in diagnostic and therapeutical purposes in medicine for more specific treatments. Over the last couple of decades US Food and Drug Administration (FDA) have approved of more than a dozen mAbs for treatment of just certain cancers (Rosen and Rosen, 2014). These antibodies are designed to specifically target a certain antigen. Today monoclonal antibodies have a wide-spread use in treating disease, including some types of cancers. IgG is the most abundant isotype found in human circulation and is synthesized and secreted by plasma B cells. Its main function is infection response and activation of secondary immune response (Strachan et al., 2011).

If an animal is injected with an antigen, the B lymphocytes that make antibodies recognizing that antigen are stimulated to grow and secrete the antibodies. Each antigen-activated B lymphocyte forms a clone producing the identical antibody. This is a monoclonal antibody. B-

Figure. 7 a) b) illustration of how changing of frequency increases the rate at which ions changes direction and moves towards the membrane. (Carvell and Dowd, 2006)
lymphocyte clones that recognize different epitopes on the same antigen is said to be polyclonal. The first step in producing a monoclonal antibody is to generate immortal, antibody-producing cells. Because B cells have a limited lifespan in culture, it is preferable to establish an immortal cell line (Strachan et al., 2011).

This is done by fusing normal B lymphocytes from an immunized animal with transformed, immortal lymphocytes called myeloma cells. Treatment with certain viral glycoproteins or the chemical polyethylene glycol promotes the plasma membranes of two cells to fuse. Some of the fused cells undergo divisions, and their nuclei eventually coalesce, producing viable hybrid cells with a single nucleus that contains chromosomes from both cells.

Like myeloma cells, hybridoma cells can grow rapidly and are immortal. Each hybridoma produces the monoclonal antibody encoded by its B-lymphocyte parents. The second step in this procedure for producing monoclonal antibody is to separate, or select, the hybridoma cells from the unfused parental cells and the self-fused cells generated by the fusion reaction. This selection is usually performed by incubating the mixture of cells in a special culture medium called selection medium that permits the growth of only the hybridoma cells because of their novel characteristics (Figure 8).

Figure 8. Selective medium for reducing unwanted cells (Lodish, 2013)
The myeloma cells used for the fusion carry a mutation that blocks a metabolic pathway, so a selection medium can be used that is lethal to them and not their lymphocyte fusion partners that do not have the mutation. This allows only the selected type of hybridoma to grow in the selected cell medium and proliferates (Figure 9) (Lodish, 2013).

![Diagram of hybridoma cell fusion process]

*Figure 9. Selective medium for growth of desired hybridoma cells and proliferation (Alberts, 2008)*

### 2.5 Overflow metabolism

Overflow metabolism is a metabolic phenomenon that is induced when the rate of glycolysis and glutaminolysis exceeds the critical value of respiratory capacity in the mammalian cells which results in an increase of aerobic lactate formation and ammonia formation (Amribt et al., 2013, Amribt et al., 2014). The formation of these metabolites can also be attributed to limited oxygen capacity. In mammalian cells overflow metabolism denotes the incomplete oxidation of abundantly supplied glucose, which results in excretion of organic end products. In animal cell cultures it is widely recognized that cells convert a high amount of glucose and glutamine to lactate and ammonia which results in a lower amount of glucose entering the triacarboxylic acid cycle (TCA cycle) when these are in high concentration (Figure 10).
Overflow metabolism of glutamine ends in excess production of ammonium and the amino acids alanine, proline, ornithine, asparagine, glutamate, serine and glycine (Ljunggren and Haggstrom, 1992). Glutamine is utilized for energy production through glutaminolysis, where glutamine is converted to pyruvate and NADH at the same time. Pyruvate is used for ATP generation via the TCA cycle or can be converted into lactate which will be excreted from the cell into the cell suspension. During startup of a new cell culture there is always an overproduction of these overflow metabolites since all cultures start with overflow metabolism. This is hard to avoid in batch reactors since all nutrients and glucose is in the reactor from the beginning.

Both lactate and ammonia are inhibitory for cell production and may even, in high concentration, decrease or halt biomass productivity (Amribt et al., 2014). There is no specific concentration where inhibition is concrete for all hybridoma, but has been observed at concentration between 28-40mM for lactate and as low as 4mM of ammonia (Ozturk et al., 1992, Sen and Roychoudhury, 2013a). During normal batch runs concentrations of 2-5mM ammonia and 25mM lactate is to be expected when using hybridoma (Hassell et al., 1991).
2.6 ELISA

Enzyme-linked immunoSorbent Assay (ELISA) is a quantification method used for concentration calculations for antigen or antibodies produced in cell cultures. Here a modified version of ELISA “sandwich”, (Figure 11), will be used to capture IgG1 instead of antigen. The first step is to fixate capture antibodies onto a surface of a 96 polystyrene well plate. By using capture antibodies that binds the Fc-region of the targeted antibodies we get a proportional relationship between bound antibodies.

Figure 11. ELISA sandwich. Capture antibodies are coated to a plate. Sample is added and targeted antigen binds to capture antibody. Detection antibody is added and binds to bound antigen. An enzyme-linked secondary antibody is added which binds to the detecting antibody. Substrate is added which is converted by the enzyme to detectable form (Motif).

After washing to remove unbound material a secondary enzyme-linked/conjugated antibody is added and bound to the analyte. After another wash substrate is added that is converted by the enzyme and converts a colorless substrate into a colored substrate which can be measured with absorption photo spectroscopy. The intensity of the color is proportional to amount of bound targeted antibodies.
3. Materials and Methods

3.1 Materials

3.1.1 ELISA
Mouse-IgG ELISA kit was used to quantify the amount of IgG that was produced during the large scale cell batch cultures. IgG ELISA uses sheep anti-mouse antibody (Fc specific, Roche) and a peroxidase-conjugated anti-mouse κ chain antibody from sheep (Fab-fragments, Roche) as coating and secondary antibody (Sen and Roychoudhury, 2013b). ABTS will be used as the coloring substrate.

3.1.2 Bioreactor
The reactor that was used were a Belach Bioteknik model LMS 2002 AB BR Pilot bioreactor with a volume of 10L using an gas mix station that regulates air, O\textsubscript{2}, CO\textsubscript{2} and N\textsubscript{2} which is computer regulated with computer software BioPhantom.

3.1.3 Cell line and medium
The cell line that was used was 520C9 [520C9.C3B10T] (ATCC \textsuperscript{®} HB-8696™) mouse-mouse suspension B cell hybridoma. This was delivered in a 1mL ampule with a cell concentration of 4.6 * 10\textsuperscript{6} cells/mL with an expected viability of 50%. For reactor runs HyClone DMEM/High Glucose SH30243 was used as medium with 5% FBS, 1% 15140 GIBCO pen strep 10.000 units/ml and 1% HyClone MEM Non- Essential amino acids solution 100x (NEAA). The inoculum was grown in Dulbecco's modified Eagle Medium (DMEM) with addition of 20% FBS, 1% 100 UI Pen. Strep. and 1% Non-essential amino acids (NEAA). Medium was changed every 2-3 days to maintain an sufficient amount of nutrients.(Zhen et al., 2009)

3.1.4 Sensors
Certain sensors were used as a standard for normal runs, such as pH, Temperature and DO but specific here will be the BioSenz analyzer with lactate chip sensor to measure produced lactate during batch runs and Futura capacitance Aber probe for viable cells growth measurement.

3.2 Methods

3.2.1 Cell bank
A master cell bank was created from the acquired cell line from ATCC biological resource center (Atcc.org). This was done for the first generation cell line, denoted as HB8692#2, which was one of the initial cultures from the cryopreserved cells from ATCC. In this case different protocols were used to accommodate these specific types of cells since none of the protocols was adapted to the cell line. The cell bank was grown in a T-75 T-flask using DMEM complemented with 20% FBS. Trypan blue and Bürker plate was used to calculate the cell viability. For cryopreservation of this cell a concentration of 1 * 10\textsuperscript{7} cells/mL is needed(Fuller et al., 2001) which was achieved by centrifugation at 300 x g (1400 rpm) for 15 min in accordance with ATCC specification for these cells. The pellet was then re-suspended in cryopreservation medium to achieve the right cell concentration. Cryopreservation medium consisted of HyClone DMEM/High glucose with 10% DMSO as cryopreservative (Aldrich, 2010). The cryopreserved cells were then transferred to 2mL
ampules and placed in a Nalgene™ Cryo 1°C Freezing Container which then was placed in a -80°C for 4 hours before being placed in liquid nitrogen storage tank.

3.2.2 Reference analysis
Since validation is needed to ensure that the parameters for a software sensor are good enough to estimate other controlling variables, reference measurements is needed. This is needed because some of the methods used are new and is based on factors that are specific for the cells used and therefore needs validation from more robust and tested methods.

To confirm cell viability during runs, coloring with trypan blue dye was used combined with Bürker haemocytometer. The dye will only color the dead cells which can determine a viable cell concentration and dead cell concentration which is used to determine the CPM factor. The factor is later used to convert the measured capacitance which is measured by the Aber probe. In a sense this is not a reference measurement since the on-line viability measurements is derived from Bürker cell count.

For BioSenz lactate analysis HPLC (Shimadzu, Tokyo, Japan) was used with an ion-exclusion resin (Aminex® HPX-87H, 300 9 7.8 mm; BioRad) placed in a column oven (60 °C; CTO-20A) which analyze organic acids, alcohols and carbohydrates in the samples using a refractive index detector (RID-10A). This has been used in the past for this purpose since HPLC is a robust method.

Samples were taken every day through a needle mounted in the reactor. 2,5ml sized samples was taken to ensure that there would be enough sample for all tests. These tests included ELISA, HPLC and cell count. Since all ELISA test would be done at the same time the samples where frozen to ensure that the IgG did not denature over time.

3.2.3 Inoculum culture
The cell line that was used was ATCC HB8696 mouse-mouse hybridoma and was grown in two separate T-75-flasks. This was done to ensure that the cell line will survive and give an extra amount of cells for inoculating the reactor which required 480ml of inoculum to reach a start concentration of 100 000 cells/ml. The cells were suspended in a 25 mL growth medium HyClone DMEM/High Glucose containing 20% FBS, 1% pen Strep and 1% NEAA. During initial steps after thawing addition of medium was done at a slow rate so not to shock the cells in the new environment and thus which increases cell survivability. First 4,5 mL growth medium was added to the cells and was allowed to sit for 5 min before adding the remaining 20 mL growth medium. Bürker chamber confirmed that both T-flasks were seeded and had a sufficient amount of cells since a low concentration will increase the lag phase. Following ATCC guidelines cell concentration was kept between $1 \times 10^5$ — $1 \times 10^6$ during the cultivation by centrifugation and addition of new medium. This is to ensure that the cells stay in log/exponential phase under the duration of the cell concentration scaling.

The cells were transferred from T-flask to spinner flasks when the concentration of the cells was over $1 \times 10^6$ cells/mL in the T-75 flasks. When transferred to spinner flask the volume was increased to 125 mL and step by step increased to 250 mL to keep cell concentration in specified range. From each spinner flask 240 ml was transferred to reactor and the remaining 10 mL was used to keep a continuous inoculum culture active during runs.
When the cells entered log/exponential phase cell growth increased to an unexpected fivefold per day. To try and limit cell division during this phase medium was changed to contain 10% FBS from 20%.

3.2.4 Reactor runs

Working volume of 4L was used during first run and 4,5L during the second run. This was done because the DO sensor was replaced after the first run because of an inability to calibrate the sensor. Growth medium used was DMEM complemented with 1% NEAA, 1% Pen. Strep. and 5% FBS. Aeration was done with both sparger and through the top of the reactor to ensure a DO value of 50%. Since gas diffusion is hard in the great amount of working volume and low stirrer speed top aeration has set to maximum (2 l/min) and sparger was set at 0,1 l/min. Sparger was increased on demand to try and maintain a DO value around 50% as long as possible during the run, a problem that was not anticipated during the first run. Sparger rate of 0,1 VVM (volumes of air per volume of liquid per minute) was chosen based on previous work and literature but was exceeded since air alone was not able to keep the DO over recommended value and pure oxygen was not available during runs (Enfors and Häggström, 1998). Before autoclaving the reactor pH meter was calibrated and Aber probe and BioSenz filter was inserted. To be able to ensure sterile transference of both medium and inoculum two filter flasks was prepared and autoclaved. This made it possible to pump medium and inoculum into the reactor without it increasing the risk for contamination. First the medium was transferred into the reactor to be able to stabilize the environment with the right temperature at 37°C, pH at 7,2 and dissolved oxygen between 40-60%. This to minimize the stress placed on the cells when transferred into a new environment. Once the parameters stabilized the Aber probe was zeroed so that background noise from the medium could be subtracted from the overall data.

Inoculation was done when stability could be ensured in the reactor and was pumped in at a slower speed to decrease the cells stress from the pump itself. Sample was taken after inoculation to record start concentrations of lactate, glucose and IgG1 produced during spinner flask stage. Sampling and monitoring was done every day. Increasing of sparger was done every other day to try and keep the DO at sufficient level.

3.2.5 ELISA

Quantification of produced IgG1 was done with an acquired ELISA kit Mouse-IgG from Roche lifescience with a specificity to all IgG-subclasses (IgG1, IgG2a, IgG2b and IgG3) independently of the type of light chains (κ or λ). This kit utilizes absorbance to quantify amount of bound product and was done at a wavelength of 405 nm. A Nunc-immuno F96 MAXISORP plate was used for analysis. Samples taken from the reactor has to be prepared before they are used in the assay. Cells and cell debris is removed by centrifugation and the supernatant is used as analyte. A standard curve is needed in these cases since the intensity from the color is measured and needs to be put in an absorbance to amount relationship. 7 samples were prepared at fixed concentrations at 1000, 200, 100, 50, 25, 12.5 and 6.25ng/ml and then plotted against measured intensity in a logarithmic curve. Sample fitting must be done with the samples to ensure that the standard curve gives a reliable value. This was done by diluting the supernatant of the samples until it has a concentration between 25-80ng/ml. Since the concentration is not known in forehand a dilution series is done. 1:100 and 1:1000 dilutions were used based on Roche recommendations and literature values. Both samples and standards were done in double estimation to ensure accuracy of the test results. Since
the normal growth medium already contains IgG from the FBS blanks with diluted medium is done together with the standard curve. Figure 12. All samples were done on one plate, Figure 13, and had a duplicate below itself and goes from high to low concentration. P1 is 1:100 dilution and P2 is 1:1000 dilution and then next sample starts.

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*Figure 12. Layout ELISA 96 well plate for measurement of standard and background measurement in the form of medium.*

<table>
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<tr>
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<td>P3</td>
<td>P4</td>
<td>P5</td>
<td>P6</td>
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<tr>
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<td>P3</td>
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<td>P15</td>
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<td>P23</td>
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<td></td>
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<td>H</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

*Figure 13. Layout ELSA 96 well plate. Samples from both reactor runs.*

A shaker table was not available at the time and the well plate had to be incubated in room temperature during preparation. The well that is used is prepared the same way and is first incubated with capture antibodies. Incubation for 1h. Washing solution is used to remove remaining capture antibodies and then blocking factor is added and incubated for 15 min. Washing is done once again and antibody solution (standard/samples) is added to the wells according to figure 12 and figure 13 and incubated for 1h. Another washing is done to remove extra material and conjugate solution is added and incubated for 1h. Washing. After this the substrate solution is added to the wells and is allowed to incubate for about an hour. Absorbance is then measured using a FLUOstar Galaxy Labvision spectrophotometer.
3.2.6 Aber Probe RFI
Data is acquired from continuous measurements of the cell suspension every 30 seconds and is recorded in the computer program. Biomass, conductivity and capacity were measured during the run. Since the CPM factor is calculated from the acquired data after the runs the factor was set as 1 in the beginning. Since the apparatus runs constantly when turned on commands are set to mark events during runs, such as start, inoculation and other parameter changes that might affect the measured value. Zeroing is the only thing that changes the output data since it removes the background data so that the only measured output comes from the living cells and nothing else. Once the runs were completed the data was exported and used to calculate the CPM value by putting it against measured cell counts.
4. Results & Discussion

4.1 Inoculum growth

Growth period of the different cell cultures varied from the original 520C9 [520C9.C3B10T] (ATCC\textsuperscript{®} HB-8696\textsuperscript{™}) cells since different cell medium and composition was used and might have given different results. This cannot be confirmed since problem with the other inoculum cultures did not yield any cell growth during a 10-20 day period and then went into natural cell death. The reason for this was deduced to a faulty buffering and a pH beyond ideal environment for the cells to proliferate.

A certain amount of lag time before the cells to start to proliferate is not unexpected and is usual after thawing cells and could be between 3-6 days for most cell lines (Atcc, 2014). Data from all failed inoculum cultivations was collected, though the growth is non-existent starting viability was used to confirm viability for the master cell bank (MCB) and was derived from freezing concentration and cell count after thawing. Table 1. This show that average cell viability after thawing is over 52\% which is close to standard viability for this specific cell line according to Atcc documentation for the cells.

<table>
<thead>
<tr>
<th>Nr</th>
<th>Cells</th>
<th>%of ampull conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>#2</td>
<td>6519000</td>
<td>65.19%</td>
</tr>
<tr>
<td>#3</td>
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<td>61.48%</td>
</tr>
<tr>
<td>#4</td>
<td>5760000</td>
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<td>54.24%</td>
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<tr>
<td>#7</td>
<td>5035000</td>
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</tr>
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<td>43.46%</td>
</tr>
<tr>
<td>Avg</td>
<td></td>
<td>52.19%</td>
</tr>
</tbody>
</table>

Figure 14 shows the growth for the MCB and first cell inoculum culture, here denoted as HB-8696\#1 and HB-8696\#2-cryopres, and the later successful inoculum growth that was used during larger scale batch runs, denoted as HB-8696\#10\#1/#2. The extra sub number on the culture name refers to sub-cultivation of \#10 cultivation. Sub-cultivation was done to reduce the amount of MCB ampules that was used. This is why the graphs overlap during the first days of growth since sub-cultivation was done after 220 hours into growth. The reason we do not get the same increase in cell growth is because a homogeneous cell solution was not transferred to the second spinner flask and different cell amounts resulted. To explain in more depth is that an uncertain amount of cells that is not show during cell count (form of lumps and thin film of cells at the bottom and around flask) results in a faster growth for the original spinner flask which was culture \#10\#1.
As mentioned before, different composition of the medium was used for MCB and inoculum culture. This was done by mistake and was discovered later when pH was measured on dead cell cultures and it was well above pH 8. The first medium had an extra addition of 1.5g/L NaHCO$_3$ which is used as a buffer in the medium and buffered at 5% CO$_2$. As it turned out there already was an amount of buffer already in the medium which also buffers at 10% (Freshney, 2005). This was only noticed during larger scale growth since other experiments had not been affected by this. For inoculum growth this was then changed so it no longer contained any extra NaHCO$_3$ and the incubator was changed so it would have a 10% CO$_2$ atmosphere.

Since all other inoculum comes from the MCB it is important to take extra care of them since they grew in different medium. Figure 15 illustrates the handling of the inoculum growth. Seeding was done in accordance to previous stated handling but differs in mid runs. Addition of extra medium was done during the first day of growth and showed positive effect in cell growth. After noticing this maintenance handling was changed and centrifugation and cells where transferred into fresh medium after 140h since seeding. This increased the cell growth and after 212h transference could be done to spinner flasks. The cell culture was split into two spinner flasks. Since the cells needed to stay in log phase removal and dilution was done from time to time to sync the inoculum growth with reactor runs so that they could be transferred to the reactor in good health. During cultivation change in medium was done from 20% FBS to 10% FBS and by looking at the graphs angle it did seem to slow down the growth rate to some extent.

Figure 14. Inoculum growth. Growth of MCB and inoculum for reactor runs.
4.2 Cell growth
During the project two runs with the reactor was performed. The reason that only two runs was performed was because of change in the reactor from what originally was going to be used. The change in reactor size from about 1L working volume to 4-4.5 resulted in an increased amount of medium was needed and since time was a factor more could not be ordered in time.

Figure 16 shows both dead/dying and living cells during both batches. What can generally be said about these runs is that batch 2 managed a higher cell concentration then the first batch. The reason for this can be explained to amount of air in the reactor. The reactor system used lacked pure oxygen regulation and only air inlet was available. This gave rise to a aeration problem that was not foreseen beforehand and was handled better during the second batch. By increasing sparger inlet more air was able to enter the reactor and managed a higher cell concentration. Increasing sparger gave rise to another side effect of batch 2 instead. An increasing cell death. If compared to batch 1 cell death, batch 2 has a higher elevation in the slope. Even though cell death increased because of the increased air flow the batch was able to run longer before all cells were dead which in both batches happened over night.
Even though cell concentration differs between the batches, consumption and production of glucose and lactate is almost the same except after about 72h where lactate from batch 2 seems to increase. The reason for this can be that cell concentration in batch 2 managed to reach a higher concentration and that consumption of glucose reached similar levels in both batches even though batch 1 and 2 had different starting concentration of glucose (Figure 17).
An unexpected challenge during the project has been the growth of the inoculum and many batches died early in the log phase and even at the beginning of the lag phase. During inoculum growth in T-flasks there was a peak at around $3 \times 10^5$ and then started to slow down and even decreasing in viable cell concentration. This was found out to be a buffer problem where the medium buffer was optimized for a 10% CO$_2$ and not 5% which was used. After that problem was solved specific handling of the cells still needed to be done, since the first cell batch that was used as MCB the first days they were adapted to this environment and needed coaxing to start to grow. Slow addition of medium increased the available sustenance for the cells which increased cell proliferation until log phase began.

To maintain exponential growth in a batch culture, it is necessary to stimulate cell proliferation with repeated additions of serum or pure growth factor. Wasteful overproduction of amino acids in hybridoma cells is a result of excess glutamine, and can be avoided by glutamine limitation but was not deemed as a problem for this project. Substrate limitation (glucose and glutamine) decreases by-product formation and increases metabolic efficiency in all these cell lines but can greatly reduce product production and is mostly used in fed-batch and perfusion reactors (Doverskog et al., 1997). This can only be done in early stages when spinner flasks are used since they practically are smaller fed-batch reactors.
4.3 Inhibitory effects
Another reason for lower cell concentration could be the accumulation of metabolites such as lactate and ammonia which do have a synergetic effect and has been shown to accumulate early in the batch phase since overflow metabolism is hard to avoid in the early phase (Sen and Roychoudhury, 2013a, Amribt et al., 2013). Measurement with HPLC have indicated that lactate accumulation does not reach inhibition level but since we cannot detect ammonia we cannot rule out the synergetic effect that these to have together (Hassell et al., 1991).
Since overflow metabolism of L-glutamine, which is in the used DMEM, ends in excess ammonium (Ljunggren and Haggstrom, 1992) a limited L-glutamine medium could be used to try and reduce eventual ammonium production. But using a medium containing no L-glutamine is not recommended since this leads to slow cell proliferation and apoptosis at certain degrees (Harnett et al., 2013). Proliferation in batch cultures has the tendency to decrease at low cell densities even though the medium appears nutritionally sufficient and metabolic by-products have not accumulated to inhibitory levels. It has been shown that increasing the inoculum density shortens the lag phase and increases the growth rate (Doverskog et al., 1997).

4.4 BioSenz and Lactate production
The BioSenz reactor measured on-line measurements from the bioreactor during the batch runs and measured lactate production. Samples were taken every two hours from reactor using filter adapter that was connected to the reactor and allowed the BioSenz to withdraw samples directly. Figure 18 and Figure 19 shows measured lactate from reactor against reference measurement which was made with HPLC from samples withdrawn directly from the reactor.
The results show that there is a difference between the measured amounts of lactate between the BioSenz and HPLC. This difference also increases over time. There can be many explanations for this. When checking the patterns of the graphs there are similarities between them. Since they seem to follow similar production rates one reason can be mixing in the reactor.
Figure 18. Comparison between measured on-line values from BioSenz apparatus and samples from Batch 1 and run through HPLC.

The measurements from the BioSenz ran into some problems during both batches since concentrations of lactate became high at the end of both runs, so that the software could not give absolute values from the measurements. This affected the last two measurements for batch 1 but affected every measurement after 84h for batch 2. This is due to limitations of the apparatus itself since at this high of concentration the apparatus cannot guarantee the measured values.

Figure 19. Comparison between measured on-line values from BioSenz apparatus and samples from Batch 2 and run through HPLC.
Figure 20. End sample from Batch 2 homogenous mixture. Double measurements with HPLC were used and gave a little to none difference and is only displayed as a single line in the figure.

As a validation for the sampling done by both the BioSenz and manual an end sample was taken at the end of the batch run. The sample went through mixing to ensure that a homogeneous solution was sampled. Figure 20 indicates that well-mixed samples yields better result than when withdrawn directly from the reactor. But even here result might not be the best since even here the apparatus warned for unreliable measured results. This was expected since batch runs yields lactate concentrations that can be well above the recommended 25mM (2.2 g/L) which results in less accurate measurement for the BioSenz apparatus.

As could be seen in measurement graphs there was not a good fit between lactate production from the BioSenz apparatus and the reference method using HPLC. By studying the graphs a similar form of both graphs can be seen as they increase and slope at similar times. Here the specificity of the BioSenz can be the problem since it is specific to only measure L-lactate, whereas HPLC cannot differentiate between D-lactate and L-lactate. D-lactate is produced in mitochondria through the methylglyoxal pathway in mammalian cells (Ewaschuk et al., 2005, de Bari et al., 2013). D-lactate is unlikely to be the only reason for this high difference since production lies in a scale of nano-micro molar concentration. This would mean that for this to be a reason for the increase would be a higher than normal production of D-lactate and then consummation of the lactate. The consumption of L-lactate would be faster since metabolism of D-lactate is only one-fifth of that of L-lactate(Ewaschuk et al., 2005). But for this to be true would be seen in a higher mismatch between the forms of the curves which is not big enough to be the only reason.
It would be unlikely for the difference to be measurement error since accuracy is both specified and confirmed in Figure 27 where measurement on a lactate standard where general error did not exceed 2% during a 22h period.

![Graph showing measured lactate over time](image)

**Figure 27. Measured lactate by BioSenz on a lactate standard**

Since there is a distance for the sample to travel from the reactor and the sensor itself, referred as dead volume, there could be an error from just old medium being measured more than once. Before runs this volume was calculated to be around 15min which gives a good safety margin and should not be a problem. If this was the problem there would not be a concentration error but a time lag since the BioSenz measurements would only come later. Since we see a plateau on the curve this is impossible since this should be on the same concentration level if it was a time lag.

HPLC also gives rise to a certain problem since it works on the basis that a certain substance takes a certain amount of time to travel through the system and is then the factor that distinguishes the substance. The problem is that some substances takes the same amount of time to travel and can give a false positive. For instance ethanol was a substance that was detected from times to times when measuring the cell suspension, which mammalian cells cannot produce. This could mean that there could be a substance that shares the retention time of lactate and adds to the real amount of lactate. This is realistic since it would explain that the HPLC measured a higher amount then the specific BioSenz apparatus.
4.5 Aber probe RFI

After batch runs was completed and stopped the data was exported and then put against the cell concentration obtained from manual cell counting. Here matching with that time point capacitance with cell concentration gives rise to that specific points CPM value which is the factor used to calculate the conversion of capacitance to cell concentration. By using this for all counted point plotting of CPM value is done to try and find the best values to use. Figure 21. Theoretically all points should give the same value, but is practically not possible in this case. This is because cell counting using Bürker is an imprecise method which only gives an estimate cell concentration. But another problem is that the cell solution inside the reactor is not completely homogeneous and lumping and thin films may exist, which would make sampling imprecise and not totally representative for the total amount of cells in the reactor.

![CPM values from both batches](image)

*Figure 21. Calculated CPM values from both batches. Selection of clustered points and then plotting against Aber data to find best fit.*

In both batches the first values spike a lot from the other values from later and are removed from the list. Table 2 shows the chosen values for average calculation of CPM and was chosen on basis on clustering and level. Using average on these values gives the new CPM value of 317130,9 which is multiplied with all measured capacitance values. This plot is then fitted with cell counted concentration to see how well they fit together. The results of this can be seen in Figure 22 and Figure 23 which shows fitting for Batch 1 and Batch 2.
Table 2. Selected CPM values based on clustering and best fit against cell count.

<table>
<thead>
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<th>Batch</th>
<th>Nr</th>
<th>CPM</th>
<th>Time point</th>
</tr>
</thead>
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<td>2</td>
<td>263591,4333</td>
<td>19:51</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>337730,8707</td>
<td>43:20</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>343582,8877</td>
<td>68:27</td>
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<tr>
<td>2</td>
<td>3</td>
<td>331060,1364</td>
<td>41:53</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>271059,216</td>
<td>65:40</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>355761,1438</td>
<td>93:08</td>
</tr>
</tbody>
</table>

Figure 22. Measured capacitance with multiplied CPM value against viable cells/mL from cell count from batch 1.
When plotted together there is a good fitting for batch 1 which would lead us to think that the chosen CPM value is a good match for this cell type. Batch 2 on the other hand does not have a perfect match at the end of the run where counted cell concentration seems to dip at a faster rate than measured with Aber probe. An explanation for this can be the high cell concentration and previous stated problems with a none-homogeneous cell solution but also an interpretation of dying cells which is subtracted from the viable cell count. But this is that coloration of cells can be vague in some states and instead of counting them as living they are counted as dead. There is also the fact that when concentration of living cells diminish and the number of cells in the chamber becomes fewer the method becomes less reliable.

Mixture becomes a problem here as well since this effects the distribution of living cells compared to manual sampling. This was talked about briefly in the results and is the reason for the big difference in calculated CPM factors. It is recommended to measure on a living culture before a run where a better control of cell concentration can be done. In this case this would not have been reasonable since it would require measurements with the reactor that was going to use this medium to get a base line and the addition of living cells. This would have been too time consuming and expensive because of the volume. But good fitting on first batch shows that it is accurate to a good degree and the bad fitting on the end of batch 2 is probably only error in cell counting.

Figure 23. Measured capacitance with multiplied CPM value against viable cells/mL from cell count from batch 2.
4.6 IgG$_1$ ELISA

Production of IgG$_1$ is measured spectrophotometric and gives rise to an absorbance value that needs to be translated into a concentration. By using a standard curve from the ELISA kit a value can be derived by putting the measured absorbance on the standard curve and following the intersection and see what concentration it would represent. Figure 24. The standard curve is cut of at 10ng and 80ng and the line function is derived from this. The resulting function obtained was (5) and is used to calculate X (6) which is concentration for measured y.

\[
y = 841,24 \times \ln(x) - 526,43 \quad (5)
\]

\[
X = e^{\frac{y + 526,43}{841,24}} \quad (6)
\]

Figure 24. Standard curve for IgG measured from ELISA at fixed concentrations. Line function is calculated between 10ng to 80ng on the curve and is used to calculate measured samples. Here we use line function instead of doing it manually, which is illustrated.
Figure 25. ELISA absorbance test with duplicates test wells below and lower concentration on the right side of the sample. The higher concentration of IgG₁ the well contains the more color is shown and measured.

Since there are both 1:100 and 1:1000 dilution only those samples that are above 10ng/mL which is the minimum recommended value for the ELISA kit. This became a problem for B1P1 and B1P2 and might not be accurate. Table 3. Figure 26 illustrates the amount of IgG₁ in the samples. Production of new IgG₁ seems to start after 24h since samples from starting value and after 24h is almost the same. Since there is a certain amount of IgG₁ from the start of the reactor, which is produced in the spinner flasks during inoculum growth, an estimate 5,69 µg/mL in batch 1 and 12,26 µg/mL in batch 2 is produced in the spinner flasks before inoculation. This is not unexpected since a higher concentration in lower working volume has been shown in previous work.

Table 3. Calculated concentration from batch samples. Dark grey samples falls outside of recommended minimum concentration for the ELISA kit.

<table>
<thead>
<tr>
<th>Sample</th>
<th>µg/mL</th>
<th>Sample</th>
<th>µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1P1</td>
<td>0.654493</td>
<td>B2P1</td>
<td>1.362808</td>
</tr>
<tr>
<td>B1P2</td>
<td>0.57598</td>
<td>B2P2</td>
<td>1.301843</td>
</tr>
<tr>
<td>B1P3</td>
<td>1.181637</td>
<td>B2P3</td>
<td>2.774311</td>
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<td>B1P4</td>
<td>2.667588</td>
<td>B2P4</td>
<td>2.849515</td>
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<tr>
<td>B1P5</td>
<td>2.892172</td>
<td>B2P5</td>
<td>4.731008</td>
</tr>
<tr>
<td>B1P6</td>
<td>4.546311</td>
<td>B2P6</td>
<td>4.35586</td>
</tr>
<tr>
<td>B2P7</td>
<td>5.340866</td>
<td></td>
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</tr>
</tbody>
</table>
To reduce the amount of dilution series for each sample 1:100 and 1:1000 dilutions was performed to ensure that the sample concentration was kept within the range of the ELISA kit. Estimation of the production of mAb for the hybridoma cells was based on both batch and perfusion reactors. This lead to adapt the experiments for a concentration to levels as high as 50 µg/l. This was based on a working volume of 25ml. From what we know is that production decreases at higher working volume since maintenance becomes harder and environment becomes increasingly harder to keep at optimal levels (Ozturk et al., 1992, Sen and Roychoudhury, 2013b).

The results showed that production of mAb is much lower than for batch reactors at lower working volumes which almost have a ten-fold higher concentration. This means that even the lowest dilution of 1:100 was almost too high and a new dilution series would be better for future runs, since it would give extra results to validate the measured concentration since in most cases both dilutions would fall in range of the test.

4.7 Reactor type
The bigger the reactor that is used the more problems arise to keep an effective production. Stirring becomes a problem at higher working volumes since faster stirring is needed which harms the cells. This results in both increased cells death from the propeller itself and from the turbulent flow which leads to an increased shear effect on the cells (Sinnadurai, 2007, Enfors and Häggström, 1998). But since stirring is needed to ensure sufficient nutrition mixture and to avoid cell lumping it might be beneficial to use a multiple level stirrer (Cimander, 2002) that can give a better mixture at a lower stirrer rate.
Aeration is still the biggest problem to maintain a reactor with high working volume. A aeration rate of 0.1 VVM is a standard for this type of reactor and cells (Enfors and Häggström, 1998) but is not sufficient for the reactor type using a sparger in this case. Sparger releases bubbles around 0.5 cm in diameter and is at the chosen aeration rate good for the cells since they has a minimum shear stress on the cells since they rise to the surface faster and the bubbles pop fast, which reduces the cell loss (Chisti, 2000). Here the problem is both diffusion and the lack of O₂. Not enough O₂ can dissolve in the solution from the top of the reactor which only leaves the sparger to maintain the O₂ level. This results in higher rates of air flow which leads to increased cell death and stress on the cells.

Reactor setup may also influence the results of the runs. Since on-line measurements are used on the reactor sampling might affect the results. The increased working volume has seemed to affect the suspension and it cannot maintain homogenous mixture. Since in this reactor setup sampling is done by the BioSenz and manual sampling is done on opposite sides of the reactor. This can result in different values. This was also discussed in the BioSenz part of the discussion. In the BioSenz results it was also mentioned that a better mixture of the cell suspension gave a closer value when comparing BioSenz and HPLC (Figure 20).

4.8 Soft sensor
Basing the soft sensor on lactate and capacitance seems to come with some problems. Capacitance seems to be suitable since it can handle non-homogenous suspensions without losing precision. This would also apply to other types of reactor setups in the form of fed-batch and perfusion reactor. Using BioSenz comes with a bigger challenge since it comes with lower and upper limitations for concentration measurements where the ladder is often surpassed during normal batch reactor runs which make it imprecise during later phases of a run. For it to be a good parameter for use in a soft sensor error needs to be reduced since it would be used as a control parameter which requires good precision.

5. Conclusions and Future work
From what have been observed it is possible to make a soft sensor with BioSenz and RFI with Aber probe for this kind of culture since they have shown to have low error on measured cultures. This is concluded by comparison between reference tests and the resulting BioSenz data and RFI values. But with higher working volume it becomes harder to use it for the same reasons culture are harder to maintain for physical and practical reasons. Batch however seems to be an unideal principal to use with this type of soft sensor since overflow metabolism is unavoidable in this setup, but would be more practical in fed-batch where feed rates could be controlled or perfusion reactors where change of medium would make accumulation inhibitory metabolites a lesser problem as well as a smaller working volume system, which would increase controllability over the process.

For future work extra efforts needs to be done for an implementation of BioSenz in a soft sensor by validating that the measurement error lies in fault with reactor setup and sampling. Evaluation of what the soft sensor should control or what calculations can be derived from them needs to be done by checking its model to real life measurements.
6. Acknowledgment
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7. Reference
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