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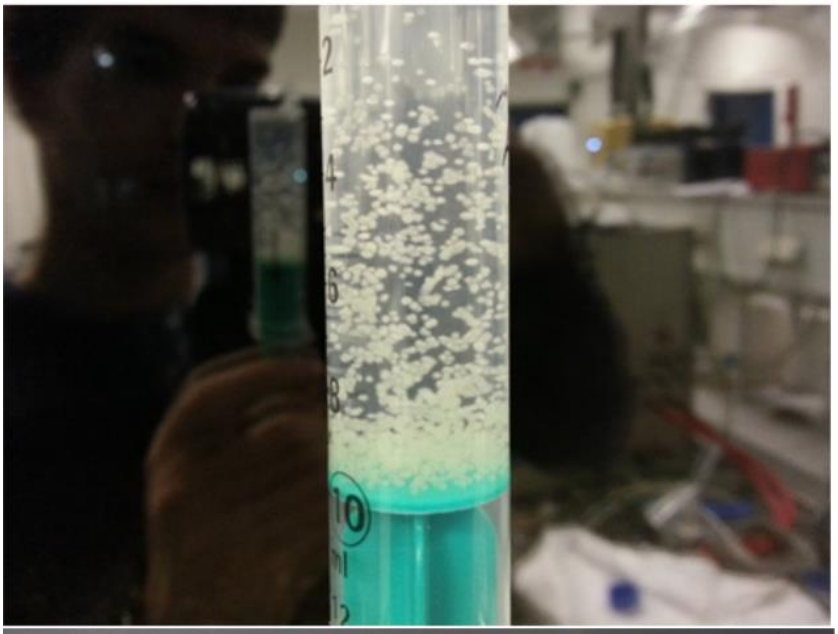

Comparison of cell density methods for conventional fermenters and Single-Use-Bioreactor

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- Alexander

Abstract

Within fermentation and cultivation technologies, it is an important parameter to know the cell concentration/cell density. If one wants to know how quickly the cells grow in an experiment, it is necessary to have some sort of cell counting method. Or it can be necessary to know the cell density in order to adjust the optimal process parameters.

It can also be desired to know the amount of viable cells in your experiment. This can, for example, be used to check if your culture can survive under the process parameters you have chosen. Or identify harvest points, infection points and similar.

This project shows that capacitance measurements, under the right conditions, can be used to accurately measure the viable cell density in your experiment. And that capacitance measurements correlate nicely with well-established cell density measurements as OD, DW and online accumulated CO_2 off-gas.

Experimental results shows that there is a linear correlation between the amounts of cells and the capacitance measurements, with a correlation of determination of $R^2 = 0{,}9998$. Experiment done for SiC.

The experiments show that in order to have accurate capacitance measurements, it is either required to produce a high cell density in your fermentation or have a low stirrer speed. A high stirrer speed makes the capacitance measurements significantly less accurate.

This project shows that state-of-the-art Single-Use capacitance equipment targeted Single-Use-Bioreactors gives results that are comparable to conventional multi-use capacitance equipment.

Dansk resumé

Inden for fermenteringsteknologi er det vigtigt viden at kende cellekoncentrationen. Hvis det ønskes at vide hvor hurtigt ens celler formere sig, er det nødvendigt at have en form for metode til at tælle ens celler. Eller alternativ kan man kende cellekoncentrationen for korrekt at kunne justere procesparametre i ens fermentering.

Det kan også være nyttigt at vide hvor mange aktive/levende celler i ens eksperiment. Det kan bl.a. bruges til at tjekke om ens kultur kan overleve under de valgte procesparametre, og det kan bruges til at identificere tidspunkt for hvornår cellerne ens kultur bør høstes, eller tidspunkt hvis cellerne bliver inficeret.

Dette projekt viser at kapacitansmålinger, under de korrekte forhold, kan bruges til præcist at måle den aktive/levende cellekoncentration. Kapacitansmålingerne korrelerer fint mod veletablereret cellekoncentrationsmålinger som OD, DW og online akkumuleret CO_2 off-gas.

Experiment lavet i dette projekt viser at der er en lineær korrelation mellem mængde af celler og kapacitansmålingen. Der haves en determinantkoefficient på $R^2 = 0{,}9998$. Eksperiment udført for SiC.

Eksperimenter udført viser at hvis det ønskes at have præcise kapacitansmålinger et det enten nødvendigt at producere en høj cellekoncentration i ens fermenter (High cell density ferementation), eller have en fermentering med lav omrøring. En høj omrøring medfører at nøjagtigheden af kapacitansmålingerne bliver markant mindre.

Projektet viser at 'state-of-the-art' engangs kapacitans udstyr for Single-Use-Bioreactors giver resultater der er sammenlignelige med konventionelle kapacitans målinger.

Table of content

Introduction - Purpose of the project

This project has two purposes. The first one is to obtain competences within fermentation technology and learn state-of-the-art technology for Single-Use-Bioreactors. The second purpose is working with the development of capacitance measurement system for Single-Use-Bioreactors. This is done in collaboration with the Company CerCell.

Getting competences within fermentation technology

Prior to this project, I had zero lab experience with fermentations. I've only done cultivation in shake flasks of *E. coli* prior to this project, but was intrigued to learn more about fermentation technology. Therefore, the first objective of the project was to get experience with fermentation experiments.

During the experiments, there have been two focuses. The first focus in the experiments has been to correlate different biomass/cell density and investigate if the methods accurately can measure the maximum exponential growth rate μmax. The different cell density methods used are DW, OD, CO_2 off-gas and capacitance.

The second focus of the experiments have been on the cell density method, capacitance. In the experiments, the capacitance measurements for *Saccharomyces cerevisiae* and *E. coli* were checked and compared with other cell density methods. This was done with actual cell cultures of *Saccharomyces cerevisiae* and *E. coli* to check if capacitance measurements truly can be used as a viable cell density method.

Development of Chloris; the capacitance system for Single-Use-Bioreactor

The second purpose of the project was to work with the development of a capacitance system, specially designed for Single-Use-Bioreactor. This part was done in collaboration with CerCell, who are the only manufacturer who has developed a Single-Use capacitance probe for Single-Use-Bioreactor. The capacitance system for Single-Use-Bioreactor is called Chloris. Currently, there are two main manufacturers that also produce capacitance probes, and they are both designated for conventional fermenters. The two companies are Hamilton and Aber. In this thesis, capacitance probes from Hamilton have been used as reference for comparing the results gotten from Chloris.

The main goal here has been to compare how well the Single-Use capacitance probe compares against the conventional, multi-use capacitance probe from Hamilton. This has been done by making experiments where both Hamilton and Chloris probes are used in the same fermenter. No one has ever done that, before now. These experiments show that the capacitance system, Chloris, works similar to Hamilton capacitance measurements. The Chloris system has the same advantages and cons as the Hamilton system. The experiments show that a high cell density is still required and high stirrer speeds reduces the accuracy of the measurements greatly.

The development of Chloris is not done yet. Chloris is still in the alpha phase, and while it has its quirks regarding software and user interface, it is intended that a commercial version will be finished in a few years. This project confirms that such ambitions may very well be possible to fulfill.

Theory

Cell counting

According to different studies, online detection of biomass is one of the most sought after parameters in industrial cell cultivation. [1]

There are different methods to do cell counting. The different methods are usually classified as direct cell counting or indirect cell counting. For direct cell counting, you actually count the amount of cells for a give volume, for example in a Bürker-Türk counting chamber. Indirect cell counting, you measure a parameter correlated to the amount of cells, for example, optical density, dry weight or even capacitance measurements. [2]

Offline measurements

The following methods feature techniques where samples are taken during fermentation and measured via laboratory equipment.

Cell count

Determination of the cell count concentration requires one to count the number of cells, e.g. via a microscope. This requires all the cells to be separated from each other, in order to count the number of single cells. If the cells are aggregated, it may be required to either make a dilution or disintegrate them in order to use this method.

Cellular substances

When using a medium which contains particulate matter, it is not an option to use methods such as dry weight, since part of the medium would then be included in the weighing. Searching for specific cellular substances in the medium such as ATP, DNA, protein, ergosterol is an option in order to specify the biomass concentration, e.g. when using a complex medium. The methods are very time-consuming, laborious and hard to validate, though. [3]

Dry weight

During the fermentation, samples are taken from the fermenter broth. Using a vacuum, the sample is poured onto a filter in order to separate the liquid and cells. By weighing the filter with and without cells, the mass of the cells can be weighed. The amount of sample used is also noted, in order to get a cell concentration (g biomass/L fermenter medium).

One of the disadvantages with using dry weight is that it requires a high cell concentration and/or a large sample volume in order to actually be able to weigh the dry cells. Because the mass of the cells is relatively small compared to the filter's mass and relative small compared to the analytical balance's precision, the variance of the measurements are high.

Dry weight measurements does not differentiate between viable and dead cells, and will give incorrect results when used to measure cell concentration of viable cells when the fermenter process enters the death phase. This is because dead cells will also be weighed.

Dry weight measurements cannot be used for complex mediums, since insoluble particles and debris will also be weighed. [4]

Optical density (OD)

Optical density is a method to measure how much light is absorbed when a source of light is shone through the sample. If there is a lot of biomass in the sample, most of the light will be absorbed, and by comparing the absorbance with a sample with pure water, the optical density is found.

Other OD-sensors can also be based on the reflection or scattering of light.

There are some disadvantages with using optical density. Dead cells and cell debris are measured. Small air bubbles are measured and counted as living cells. If the sample is colored, it will also distort the estimation of the optical density.

It should be noted that it's also possible to make OD measurements online with modern equipment. [5]

Online measurements

The following methods can be used to measure the biomass online, to give continuous information back to the user about how the fermentation is going. This helps to give deeper process knowledge and allows the user to quickly adjust parameters in the fermentation when needed.

Density (ARD)

Acoustic Resonance Densitometry is a method where the difference in the specific gravity of the medium is used to determine the biomass. The formula for determining the biomass is given as:

$$RD_{biomass} = RD_{composite} - RD_{medium}$$

Where RD is Relative Density. $RD_{composite}$ is the relative density of the fermenter broth (containing biomass and convertible substrates in aqueous media). RD_{medium} is the relative density of the medium that does not contain any cells/biomass.

One of the difficulties with this method is that you need a blank control sample of the medium without any cells. You need to measure the RD_{medium} at the same time you measure the density of the fermenter broth $RD_{composite}$. This is because the density of the medium varies a great deal, perhaps even more than the difference between the fermenter broth density and medium density [5].

Possible future leading biomass measurement instruments

Ovizio - Differential Digital Holographic Microscopy (DDHM)

Differential Digital Holographic Microscopy is a new digital image cell counting program, developed by Ovizio. It was first showcased in Barcelona on May 31, 2015. [6]

When a light source hits an object (in this case cell biomass) the light will be scattered. The scattered light will be compared with a reference beam light, which makes it possible to recreate a 3D hologram of the cells in the fermenter broth. By analyzing these images with their software, they are able to determine the amount of the cells in the fermenter, and also present the user with real-time images of the cells. [6]

Figure 1: *Screenshot of real-time monitoring of the cells. According to Ovizio the software is capable of distinguishing viable and dead cells based on light refraction. Viable cells will create a light cone, which will give a very high intensity light (lens effect), while dead cells will create a more scattered light. In this image viable cells are marked by green, and dead cells are marked by red. Whether or not a cell is classified as viable or dead is based on light scattering and cell diameter [6].*

Avenisense - Density and viscosity measurements for liquids

Avenisense offers real-time measurements of density and viscosity that can be used as an alternative to measure cell growth. According to Avenisense, a noticeable density increase can be observed for concentrations with at least $5 \cdot 10^9\ cells/ml$. By measuring the culture's density you can monitor how fast your cells grow. [7]

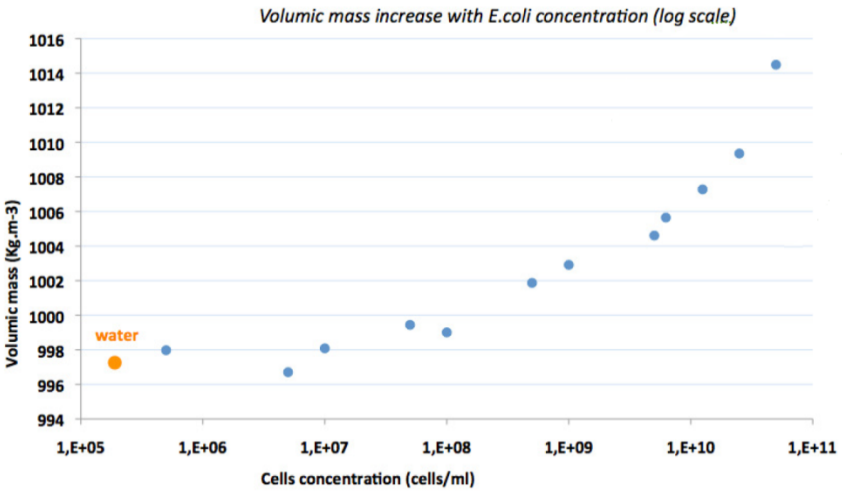


Figure 2: *Data from Avenisense. For the bacteria Escherichia coli, there is only a noticeable change in the broth density after the cell concentration is larger than* $5 \cdot 10^9\ cells/ml$*..*

Capacitance measurements

Capacitance measurement is an online indirect cell counting method than can measure the viable cell density in your broth. There is a linear relationship between the capacitance and the viable cell density.

Dielectric spectroscopy has been used for over 2 decades for measuring the live cell concentrations of animal cells. [8] Online measurement of viable cell density makes it easier to detect event and respond in real time to the fermentation, without having to do fermentation. It helps define points such as feeding, harvest or infections points. And it helps early detection of deviation in the process. [9]

Offline methods as visual cell counting or similar methods are still a lot more widespread for biomass measurements. The main advantage of capacitance measurements is that it monitors the process continuously, and it's possible to get a lot of data points (measurements can be taken every 6 seconds if desired). [1]

How does capacitance work?

The technology works by applying an alternating electrical field. When an electrical field is applied to a solution, the ions in the solution are forced to move. The positive ions will go in the direction of the electrical field, while the negative ions will go the opposite way. The ions in the cell will move to the side of the cell membrane, where the cell membrane act as an insulating physical barrier, preventing further movement, as shown in *Figure 3.* [10]

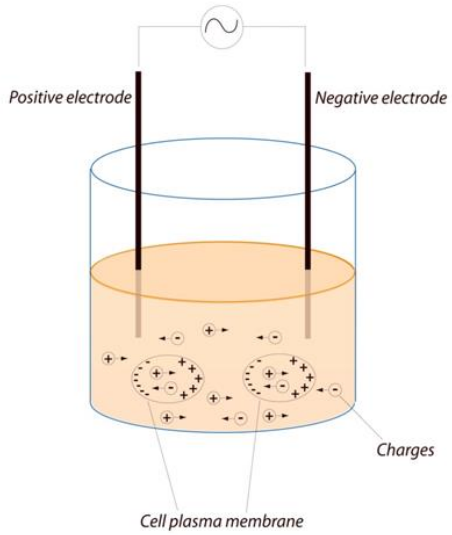


Figure 3: *Cells are being polarized as they are being influenced by an electric field. The positive ions are pushed toward the negative electrode and vice versa. [11]*

This separation of the positive and negative charges results in a polarization of the cells. The magnitude of this polarization is measured by its capacitance, in pico-farad (pF). The more cells that are polarized, the higher the capacitance is. [10]

Unlike live cells, dead ones cannot store charges inside them and are therefore invisible to the technology. Live cells are able to store ions within their cell membrane. Dead cells, dead cell debris, air bubbles and solid media particles, on the other hand, cannot contain the ions and they are therefore invisible to the technology. An illustration is shown in *Figure 4*. Dead cells have a raptured membrane that can no longer hold the ions. [1] Because of this, the capacitance measured is proportional to the live bio-volume of the culture. [8]

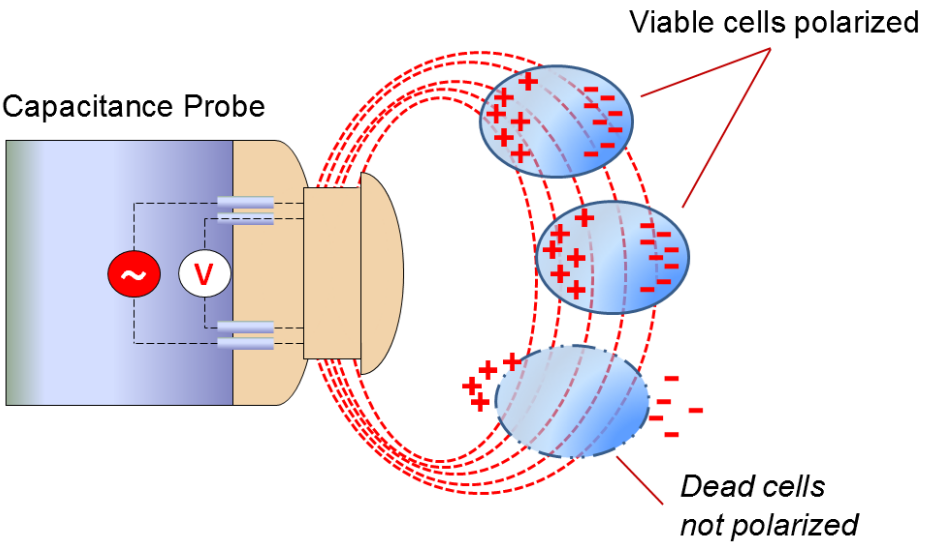


Figure 4: *Because the dead cells do not have an intact plasma cell, they are unable to store charge and will not be polarized* [8]

Experiments done by Aber biotech shows that there is a linear relationship between the capacitance measured and the amount of cells in a fed batch culture. Using a capacitance probe is a reliable alternative to traditional offline measurements as shown in *Figure 5*. Experiment is done by a biopharmaceutical company producing a therapeutic protein by genetically engineered SP2/0 cells using a high cell density perfusion technology. [12]

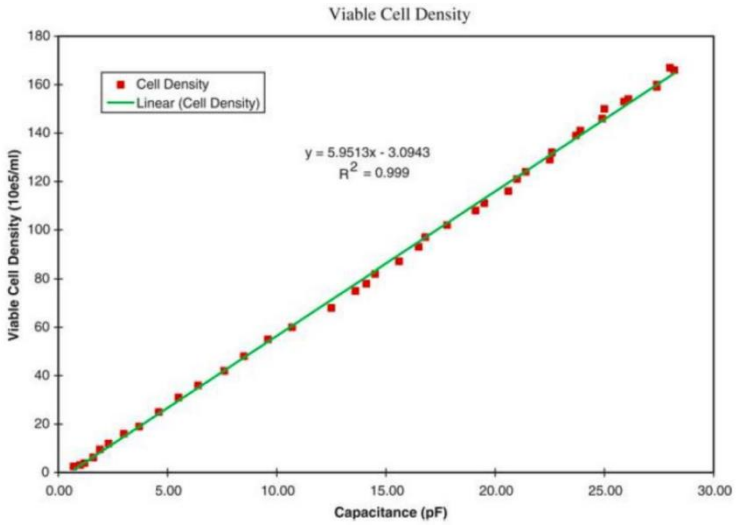


Figure 5: *Data points show the linear relationship between the capacitance and offline viable cell count.* [8]

Capacitance for Conventional fermenters and Single-Use-Bioreactor

In this project, two different capacitance systems are investigated: one capacitance system for conventional fermenters or bioreactors, and a capacitance probe for Single-Use-Bioreactor. The conventional capacitance system is called Hamilton. The Single-Use capacitance system is called Chloris.

Hamilton probe

The conventional capacitance probe used in this project was originally made by Fogale and is now owned by Hamilton and will be used as reference for comparing measurements. The capacity probe by Hamilton measures the permittivity in the medium. The unit is pico-farad per cm (pF/cm).

Chloris probe

The Single-Use capacitance probe is developed by CerCell in collaboration with the Germany University IBA. This capacitance system is still under development, and a large part of the project was to help them with the development of this probe. The capacitance probe by CerCell measures the capacitance in the medium. The unit is just pico-farad (pF).

For reference, permittivity is the most widespread unit for measurements (pF/cm). Hamilton, Aber and Sartorius use the unit permittivity (pF/cm) for capacitance measurements.

The capacitance measurement from the Chloris probe (pF) is still very comparable, though. Basically, the capacitance measurements just need to be multiplied by a factor to convert from capacitance (pF) to permittivity (pF/cm). [13]

Single-Use-Bioreactor

As the name suggests, a Single-Use-Bioreactor is a reactor that is only being used once before it is disposed. Instead of a conventional steel vessel, the reactor can be a disposable bag, or as in this thesis, a disposable plastic vessel. (The reactor is made of hard plastic.)

Using Single-Use-Bioreactors eliminates the need for autoclavation and cleaning. The bioreactor already comes pre-sterilized, and after the experiment is done, the bioreactor is simply discarded. This reduces the risk of cross-contamination and saves time. In many cases, using Single-Use-Bioreactor reduces the cost of experiments, compared to using conventional steel fermenters. While a conventional steel fermenter can be used multiple times, the initial cost is a lot higher. This requires the company to make a bigger investment and plan the experiments ahead to make sure the fermenter actually fulfills all the process parameters for the fermentations. It usually takes a long time to produce a steel fermenter, and the initial high cost of a fermenter requires the company to have a higher liquidity. [14]

Single-Use-Bioreactors, on the other hand, are more flexible, have a lower cost, and make it easier to make new experiments quickly and cheap. Overall, the costs of using Single-Use-Bioreactors are in many cases cheaper than buying a steel fermenter. [15][16]

Single-Use-Bioreactors are best suited for small-scale cultivation, usually implemented in the screening, development and pilot phase. For large-scale production in vessels of approximately 1000 L or over, conventional steel fermenters are still more beneficial. [17]

Single-Use-Bioreactors are a relatively new technology compared to conventional steel reactors, but there's a growing trend of more companies implementing Single-Use-Bioreactors. [18] This creates a demand for development of Single-Use-Components, since it's preferred to have all component pre-installed and pre-sterilized before delivery, so the user only has to add media and inoculate.

Single-Use-Components include equipment such as single use temperature probe, pH, air inlet, impellers, etc. Alternatively, non-invasive equipment can be used, which still eliminates the risk of contamination.

If the components do not come pre-installed, the user will need to autoclave the equipment separately before adding them to the Single-Use-Bioreactor under sterile conditions (e.g. in a LAF bench). This is a more time consuming procedure and also adds to the risk of contamination.

This is one of the incitements for creating the Single-Use-Capacitance system Chloris. The capacitance probe is single use and comes pre-installed in the Bioreactor. The user only has to connect the probe with a pre-amplifier and connect it to the computer via a HDMI-cable.

Currently, Chloris is the only capacitance system for Single-Use-Bioreactor available on the market. No one has ever developed a Capacitance probe for Single-Use-Bioreactor before now. The development of components and measuring methods for Single-Use-Bioreactor is definitely on its way, though. The company Sartorius has, for example, developed a viable biomass sensor for their Single-Use Flexsafe RM bag. [1]

Stoichiometry

When writing a chemical equation, it is important that the equation is balanced, e.g. the number of carbon moles on the reactant side must be the same as the number of carbon moles on the product side. This principle is built on the law of mass conversation. [19]

Saccharomyces cerevisiae

Saccharomyces cerevisiae is known for its application in food production. Its ability to convert sugar into ethanol makes it widely used in fermentation processes for brewing beer, wine and liquor. It is also used as a leavening agent for bakeries, where *Saccharomyces cerevisiae* is known by its more common name *Baker's Yeast*. [20]

There exist three different stoichiometric equations for the growth of *Saccharomyces cerevisiae.* [21]

$$C_6H_{12}O_6 + a\ O_2 + b\ NH_3\ [NH_3] \rightarrow b\ C_1H_xO_yN_z + c\ CO_2 + d\ H_2O \quad (1)$$

$$C_6H_{12}O_6 + g\ NH_3\ [NH_3] \rightarrow g\ C_1H_xO_yN_z + h\ CO_2 + i\ H_2O + j\ C_2H_6O \quad (2)$$

$$C_2H_6O + k\ O_2 + l\ NH_3\ [NH_3] \rightarrow l\ C_1H_xO_yN_z + m\ CO_2 + n\ H_2O \quad (3)$$

The produced biomass, X, has the chemical formula $C_1H_xO_yN_z$ where x, y and z, are variables that can be calculated by elemental analysis. From the above equations, it can be seen that *Saccharomyces cerevisiae* is capable of making both oxidative reaction where O_2 is consumed (reaction 1 and 3 are oxidative) and one anaerobic reaction where no oxygen is consumed (reaction 2 is reductive). *Saccharomyces cerevisiae* is therefore capable of growing under aerobic and anaerobic conditions, which is called a facultative anaerobe. [22] Reaction 2 (anaerobic reaction) is also known as the crab tree effect, where glucose is turned into ethanol. The production of ethanol is not favored, since the reaction has a lower ATP yield compared to the respiratory mechanism (2 ATP vs. approximately 18 ATP). [23] Reaction 2 happens when the glucose concentration is high, because a high uptake of glucose limits the respiratory pathway.

Fermentation terminology

μmax

The maximal specific growth rate, μmax, is a measurement for how fast the cells maximally grow under their optimal conditions. If a batch fermentation, for example, has a maximum growth rate of $\mu_{max} = 0{,}30\ \mathrm{h}^{-1}$, the amount of time it will take before the cells have doubled is given as: [24]

$$Doubling\ time = \frac{\ln(2)}{0{,}30\ h^{-1}}$$

$$Doubling\ time = 2{,}31\ h$$

With a maximum growth rate of $0{,}30\ \mathrm{h}^{-1}$, it will take 2,31 hours (2 hours and 19 minutes) before the cells have doubled.

Error bars

For most of the offline measurements taken in this project, there were usually several measurements taken at the same time in order to have accurate measurements. This is the case for OD and DW, and Single-Use capacitance measurements.

Data points that include several measurements are plotted on the graphs with error bars. For this project, error bars are equal to $\pm\ 1$ standard deviation. This is equivalent to the error bars indicating a 68% confidence level. It is 68% certain that the true mean of the measurements are within the error bars. [25]

List of experiments

A total of 4 experiments, where 3 of them were actual fermentations, were conducted. The specifications of the experiments are shown in *Table 1*.

Table 1: *Timeline for the project.*

	Experiment 1	Experiment 2	Experiment 3	Chloris
Date	14/9 - 17/9-2015	2/11 - 5/11/2015	7/12 - 8/12-2015	21/10 - 24/10-2015
Cell type	*S. cerevisia*	*S. cerevisia*	*E. coli*	*SiC*
Fermentation type	Batch	Batch	Batch	Cont.
Fermenter Material	Steel	Steel	Hard Plastic	Hard Plastic
Working Volume	2 L	2 L	3 L	1L
Actual Volume	1,8 L	1,8 L	1,8 L	600 ml
Number of towers	2	2	1	1
Starting conc. (OD)	0,001	0,001	0,1	- *NA* -
pH	5	5	7	7
^{o}C	35	35	37	22
Air inlet (L/min)	1,8	1,8	1,8	Open
Stirrer Speed (RPM)	800	800	800	350
OD	✓	✓	✓	%
DW	✓	%	%	%
Accumulated CO_2	✓	✓	✓	%
HPLC	✓	✓	✓	%
Capacitance (Hamilton)	%	✓	✓	✓
Capacitance (Chloris)	%	%	✓	✓

Experiment 1: *Saccharomyces cerevisiae*

Objective of the experiment

The first experiment was conducted to verify that biomass production of *Saccharomyces Cerevisiae* has an exponential growth. This was done by comparing the maximum specific growth rate µmax for OD, DW, and accumulated CO_2 off-gas from the MS.

Conclusion

The experiment gave nice exponential growth for the first phase. The maximum specific growth rate µmax is comparable to other experiments and the 3 different methods have values that are close to each other (OD, DW and accumulated CO_2 off-gas)

Table 2: *Growth properties of Saccharomyces cerevisiae in aerobic fermentation.*

Method	µmax h^{-1} (glucose)	µmax h^{-1} (ethanol)
OD	0,40	0,10
DW	0,37	0,086
Accumulated CO_2	0,39	0,044

Other researchers have achieved a maximum specific growth rate of $\mu max = 0{,}44\ h^{-1}$ in a continuous culture where the C-source was likewise glucose. [26]

In an anaerobic cultivation with glucose as C-source, $\mu max = 0{,}41\ h^{-1}$ has been obtained. [27]

Setup of the experiment

For full setup on how the fermenter was setup, media composition, etc. please see Appendix 3

The setup of the experiments is shown in the following Table 3**.**

Table 3: *Specification of experiment 1.*

	Experiment 1
Date	14/9 - 17/9-2015
Cell type	*S. cerevisia*
Fermentation type	Batch
Fermenter Material	Steel
Working Volume	2 L
Actual Volume	1,8 L
Number of towers	2
Starting conc. (OD)	0,001
pH	5
^{o}C	35
Air inlet (L/min)	1,8
Stirrer Speed (RPM)	800
OD	✓
DW	✓
Accumulated CO_2	✓
HPLC	✓
Capacitance (Hamilton)	%
Capacitance (Chloris)	%

Data acquisition for the fermenters began right after the yeast *Saccharomyces Cerevisiae* was inoculated in the fermenter. The amount of inoculum injected was based on the fact that the desired starting concentration in the fermenter should be 0,001 OD.

The next offline sample was taken after 16,9 hours of cultivation. From then on, samples were taken with an interval of 1,5 hours until the first exponential growth stopped. The first exponential growth stopped at 24 hours.

Samples were then taken at interval of 3 hours until the second exponential growth stopped. The second exponential phase stopped at 39,5 hours.

OD, DW and HPLC samples were taken for a total of 27 hours.

Results

The experiment went as expected. *Figure 6* shows results from the off-gas. (The result does not take the reference air into account.)

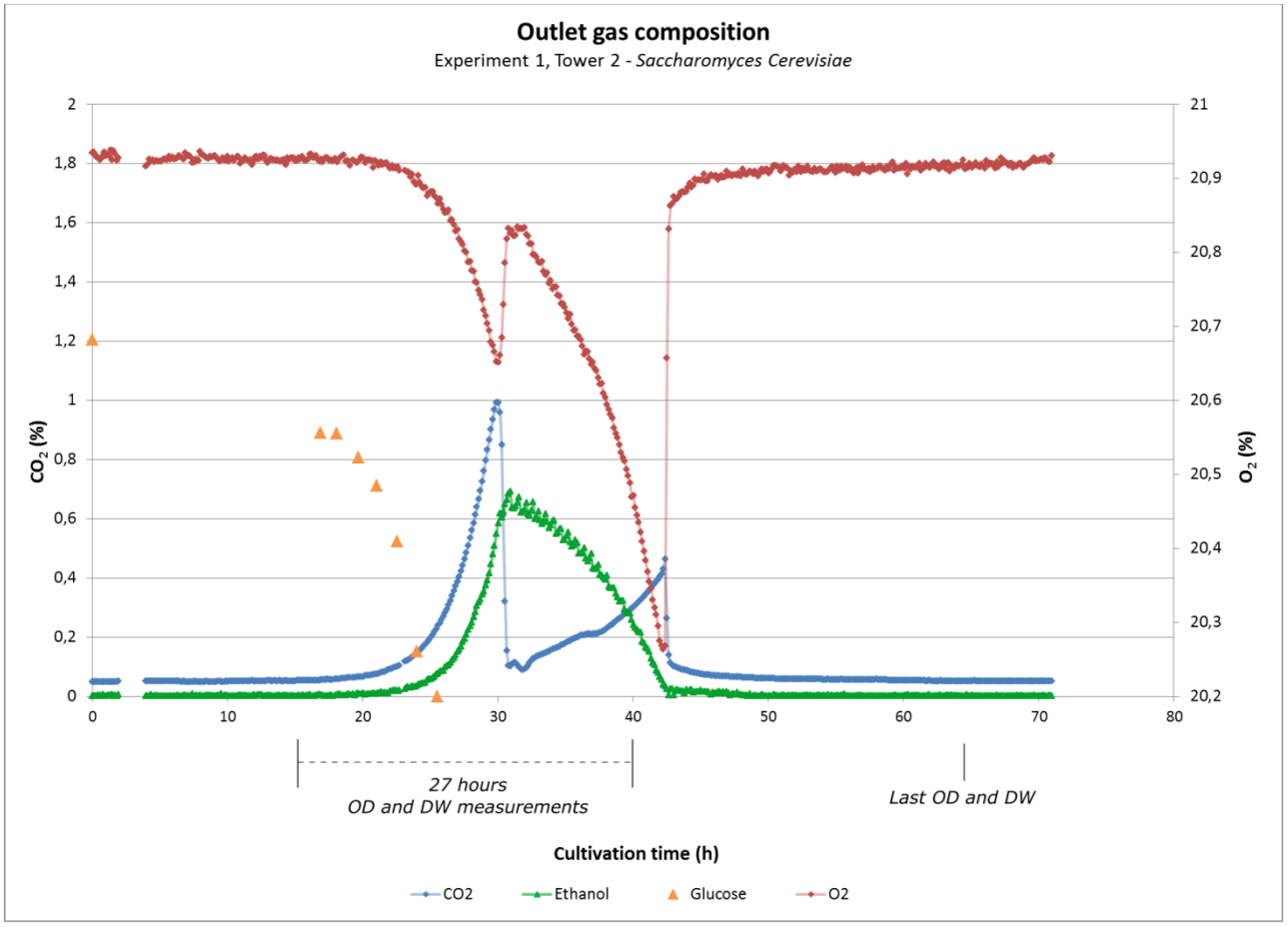


Figure 6: *Outlet gas composition for experiment 1. After 30 hours of cultivation, the first exponential growth phase is over. After 43 hours, the second growth phase is over.*
Please note that there is no corresponding y-axis for ethanol or glucose. The maximum amount of ethanol in the outlet gas was measured to have a composition of 0,048 %. The start concentration of glucose was measure with HPLC to 10,8 g/L glucose.

In the beginning of the fermentation, the fermentation is in its lag phase and the amount of CO_2 is near constant. At some point, the acceleration phase begins and the growth rate increases. Around 20 hours, the growth rate is near its highest, and the exponential yeast growth is at its maximum specific growth rate µmax. For determination of µmax, the first data point used was at 21,90 hours.

As the growth of *Saccharomyces Cerevisiae* increases, the production of CO_2 likewise increases nearly proportionally. The fact that the respiratory mechanism consumes oxygen and produces CO_2 can be seen from the graph. The relationship between CO_2 and O_2 is inversely proportional. As the production of CO_2 increases, the amount of O_2 in the composition decreases. This is given by reaction:

$$C_6H_{12}O_6 + a\ O_2 + b\ NH_3\ [NH_3] \rightarrow b\ C_1H_xO_yN_z + c\ CO_2 + d\ H_2O \qquad (1)$$

After 30 hours of cultivation, the amount of CO_2 suddenly drops. This is because the substrate has already been consumed (verified by HPLC). After the cells are adapted to the new conditions, they begin to consume the ethanol they just produced a few hours ago. The reaction for consuming of ethanol is given as:

$$C_2H_6O + k\ O_2 + l\ NH_3\ [NH_3] \quad \rightarrow\ l\ C_1H_xO_yN_z + m\ CO_2 + n\ H_2O \qquad (3)$$

Because the reaction is oxidative, it can likewise be seen that the amount of oxygen in the composition decreases as the amount of CO_2 increases. At 43 hours, the ethanol has been consumed and there is also a sudden drop in CO_2 as there is almost no substrate left for the yeast to use. The growth of *Saccharomyces Cerevisiae* is finished at 43 hours. OD and DW samples were taken from the 17 hour to the 40 hour marks, as shown in the bottom of the figure. The sample taking lasted 27 hours. At 64 hours, one last sample was taken to make sure that the growth had actually ended. (This was the case.)

μ_{max} for *S. Cerevisiae*

For the first experiment, offline OD, DW and online CO_2 off-gas measurements were conducted, which can be used to determine the maximum specific growth rate μ_{max}. [28]

μ_{max} based on OD.

Only 1 OD measurement was taken at each data point. (And therefore, no error bars.)

In order to determine which data points you should use to calculate μ_{max}, it is important to identify at which interval the process is undergoing an exponential phase with maximum growth rate. In order to do identify it easier, the OD values are plotted on a semi-logarithmic scale. If the OD values have an exponential growth, it will be seen as a straight line, which visually can be easier to detect, rather than identifying an exponential curve.

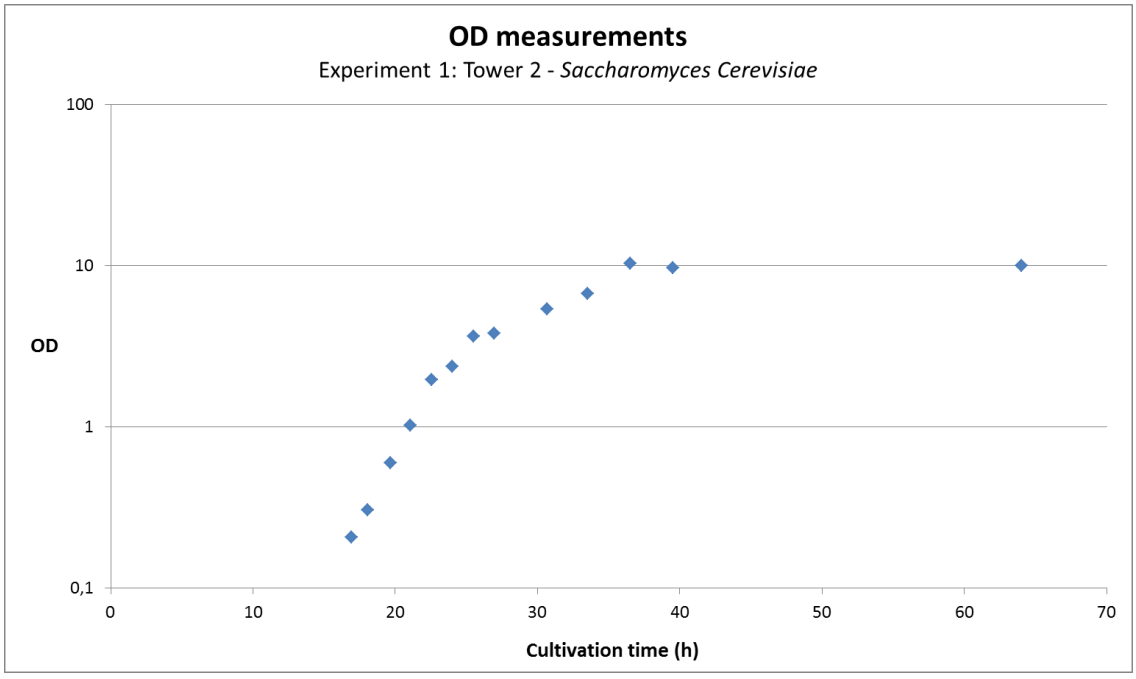


Figure 7: *OD measurements with semi-logarithmic scale. The first 5 points are on a linear line, which indicates exponential growth.*

Based on these data, it is chosen to determine the maximum growth rate based on the first 5 data points. By plotting the value $LN(1/OD)$ the maximum growth rate μ_{max} can be determined as the negative value for the slope of the trend line. From *Figure 8*, it can be seen that the slope of the trend line is -0,3988. The specific maximum growth rate can therefore be determined to be $\mu_{max} = 0{,}3988\ (h^{-1})$.

The coefficient of determination $R^2 = 0{,}9984$ validates that the growth of *Saccharomyces Cerevisiae* is indeed exponential.

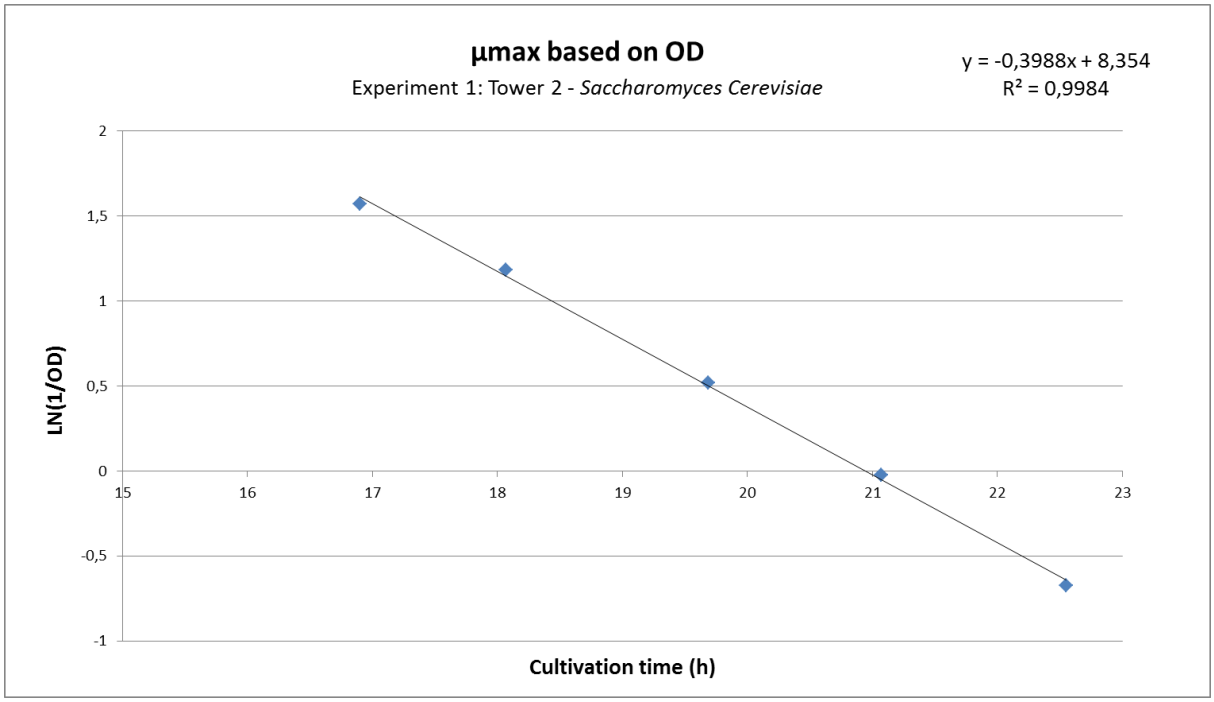


Figure 8: *Determination of the maximum growth rate μ_{max} based on OD measurements. OD measurements were only taken once which is the reason for no error bars*

μ_{max} based on Dry Weight

2 dry weight samples were taken at each sample point.

The semi-logarithmic plot of DW values is shown in *Figure 9*. The correlation of a linear line is a bit more unclear. From the error bars, it can be seen that the DW measurements are quite inaccurate when the mass of dry weight is less than 1 g/L. For values above 1 g/L, the dry weight measurements are much more accurate.

For determining the first maximum exponential growth rate, it looks like 6 points should be included, instead of 5 for the OD measurements. In order to keep it consistent, though, and in order to compare the measurement of OD and DW, the first 5 data points were chosen in the same time range.

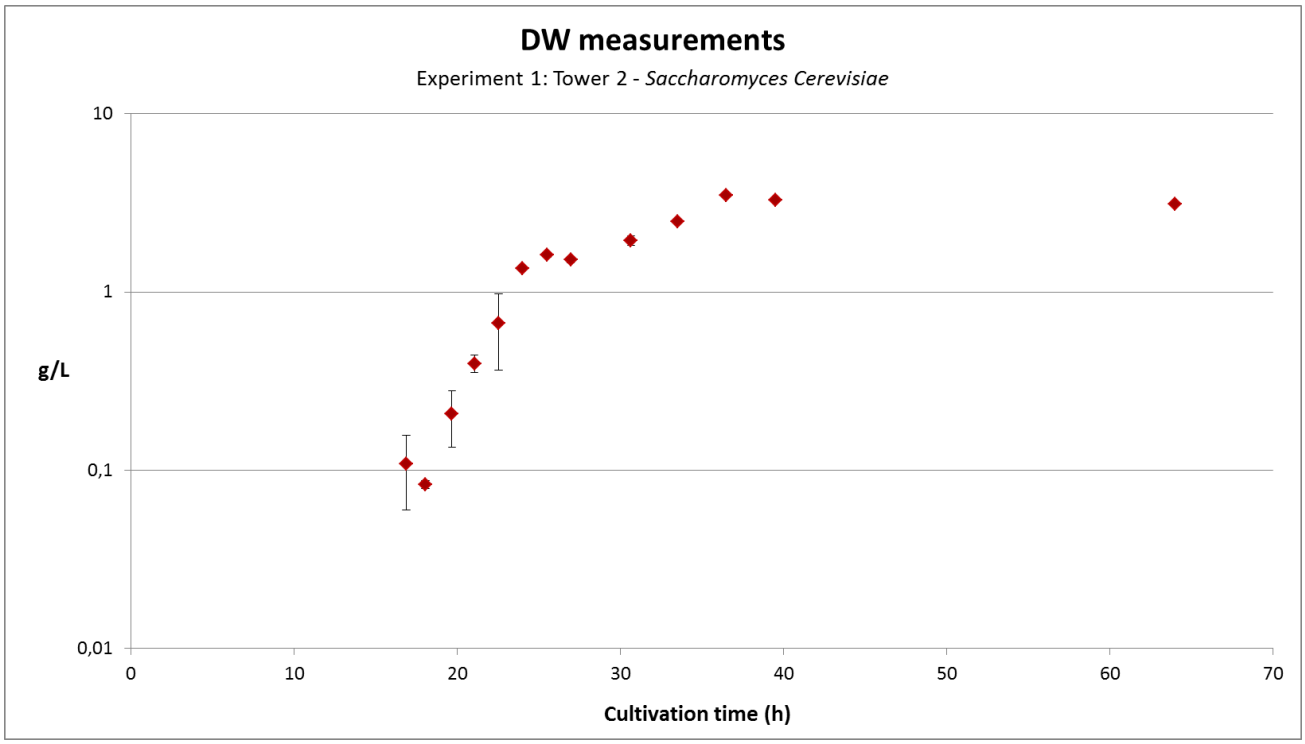


Figure 9: *Determination of data points to include for the maximum growth rate. The error bars equal one standard deviation. For samples above 1 g/L, the error bars are so small that they cannot be seen.*

Based on the same 5 data points as for OD, the µmax based on the DW measurements are shown in Figure 10.

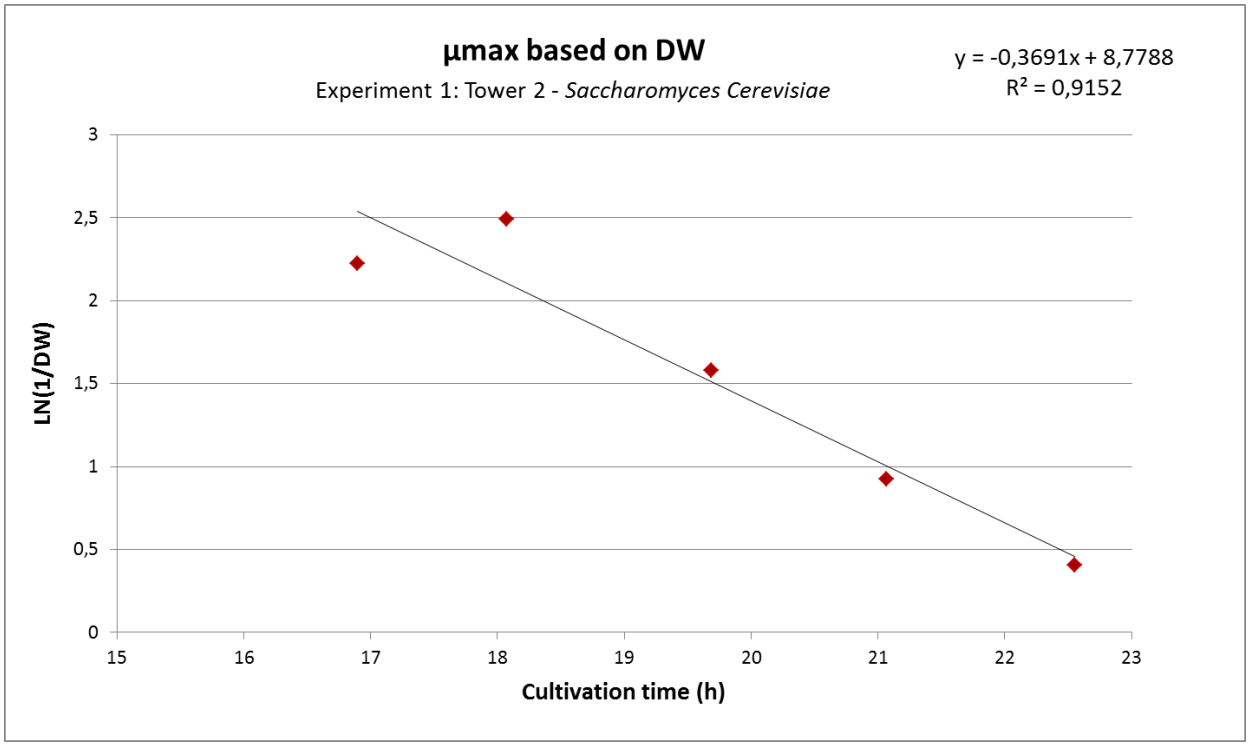


Figure 10: *Determination of the maximum growth rate μ_{max} based on DW measurements.*

μ_{max} determinations for OD and DW measurements are summarized in *Figure 11*. The figure shows µmax determination for the two growth phases. The first with growth with glucose as C-course, and the second growth phase with ethanol as C-source.

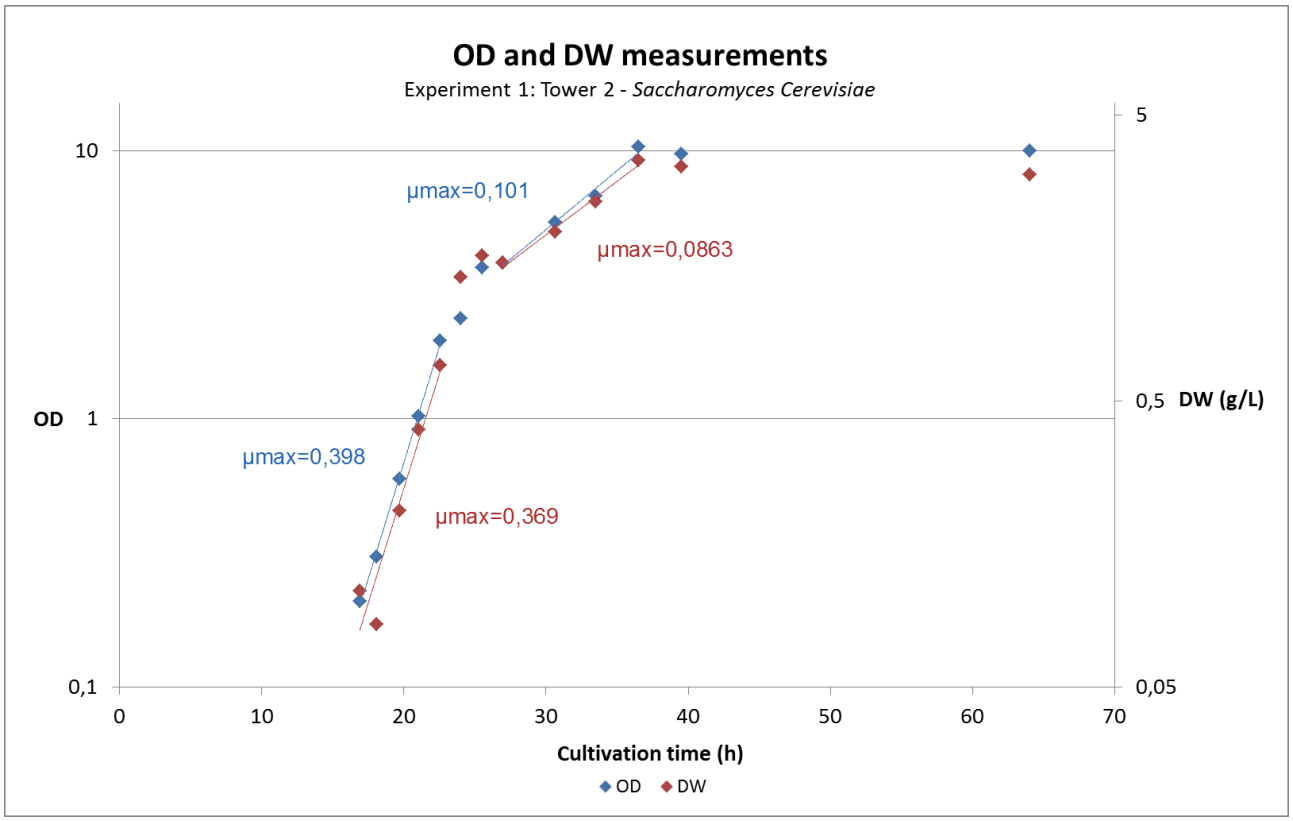


Figure 11: *μ_{max} based on OD and DW for the two exponential phases.*

The OD and DW measurements seem to correlate nicely.

μ_{max} based on accumulated CO_2 off-gas

The maximum exponential growth rate can also be based on the amount of accumulated CO_2 in the off-gas. The graphical presentation is shown in *Figure 12*. With this method, the maximum growth rate μ_{max} for the first exponential phase is $\mu_{max} = 0{,}3895\ \mathrm{h}^{-1}$. The coefficient of determination is solid at $R^2 = 0{,}9998$.

The accumulated CO_2 was calculated by converting the MS signal from CO_2-44 (%) to accumulated CO_2 (g/L). This was done by using the air inlet, working volume, and CO_2 content in the air reference.

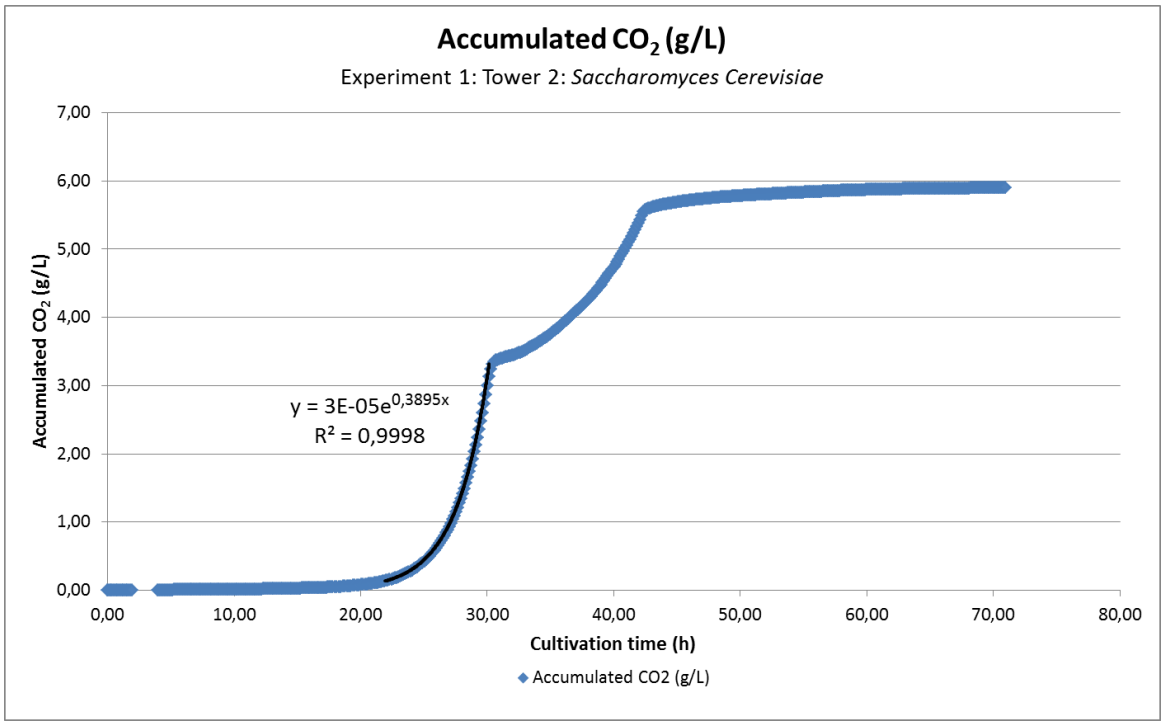


Figure 12: *μ_{max} based on accumulated CO_2 in the off-gas. μ_{max} = 0,3895 h^{-1}*

The two different growth phases are clearly separated from each other and easily identified. After the glucose is consumed at 30 hours, it looks like Saccharomyces cerevisiae adapts relatively easily and begins the second growth phase.

Based on the accumulated CO_2, it looks like it is only the first phase that grows exponentially. Or at least the second growth phase is significantly slower than the first. This can be seen more easily from a semi-logarithmic plot as shown in *Figure 13*. The second growth rate gives a µmax of just $0{,}0438\ h^{-1}$.

For the second growth phase based on ethanol, it seems more like the cell growth like a second order polynomial.

While the coefficient of determination R^2 for an exponential growth rate gives $R^2 = 0{,}9856$, a second order polynomial fit gives a coefficient of determination of $R^2 = 0{,}9994$.

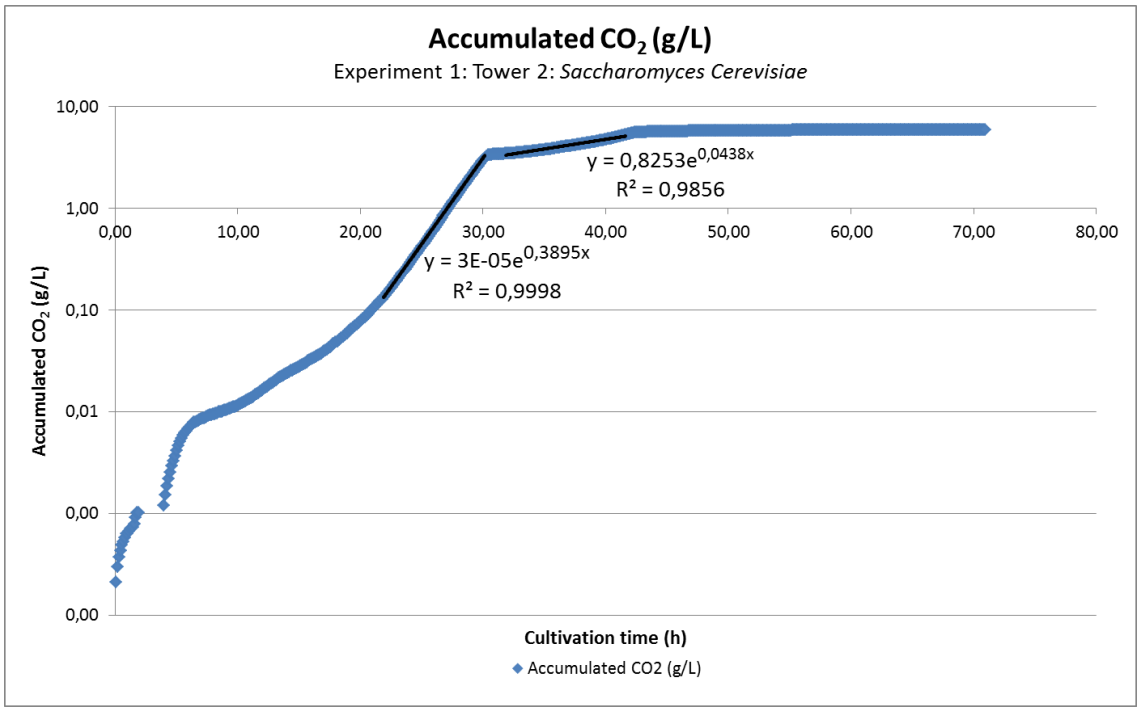


Figure 13: *Accumulated CO2 shown in a semi-logarithmic plot. Linear trend lines equals to an exponential growth*

$\mu max1 = 0{,}3895\ h^{-1}$. $\mu max2 = 0{,}0438\ h^{-1}$

Cell Yield

The cell yield of *Saccharomyces Cerevisiae* Y_{SX} is the ratio between how many cells are formed against how much substrate has been used. It tells how efficiently the carbon source is converted into cells. The formula is given as:

$$Y_{SX} = \frac{Biomass\ formed\ (g/L)}{Substrated\ consumed\ (g/L)}$$

'Biomass formed' is based off the last dry weight determination, after both glucose and ethanol is consumed. This is done since the initial concentration of ethanol was zero, and the ethanol was produced from consuming some of the glucose.

'Substrate consumed' is the initial glucose given from HPLC-0 minus the remaining glucose at the time the last DW measurement were taken. (In this experiment, no glucose was left, so it is just the initial concentration of glucose).

This gives the following yield:

$$Y_{SX} = \frac{DW\ (g/L)}{C_6H_{12}O_6\ (g/L)}$$

$$Y_{SX} = \frac{3{,}48\ g/L}{10{,}8\ (g/L)}$$

$$Y_{SX} = 0{,}32$$

Other data has given yields of $Y_{SX} = 0{,}51$. [29]

Experiment 2: *Saccharomyces cerevisiae*

Objective of experiment

The objective of this experiment is to compare the Hamilton capacitance measurements with the conventional cell density methods OD and online CO_2 off-gas.

This experiment was very similar to experiment 1. The difference from experiment 1 was that a capacitance probe from Hamilton/Fogale has been added. This probe replaced the DO sensor (Disolved Oxygen) since there were no other available places to insert the capacitance probe.

Conclusion

The capacitance measurement compares nicely with the OD measurements. The capacitance measurements were very noisy though. If one wants to use capacitance measurements for detecting the growth phases of *Saccharomyces cerevisiae,* it is recommended to make a fermentation that yields a high cell density. Or alternatively, to use a strain that does not require high aeration. The µmax values are shown in Table 4.

Table 4: *µmax values for the two growth phases. Please notice that µmax values for capacitance are estimated*
Experiment 2: tower 2 - *Saccharomyces cerevisiae*

Method	µmax h^{-1} (glucose)	µmax h^{-1} (ethanol)
OD	0,27	0,075
Accumulated CO_2	0,27	0,041
Capacitance (estimated)	0,26	0,07

Pictures of *Saccharomyces cerevisiae*

Taking pictures of the colony can be a good way to check that there is no contamination in your fermenter. It can also give a better understanding of the morphology of your strain.

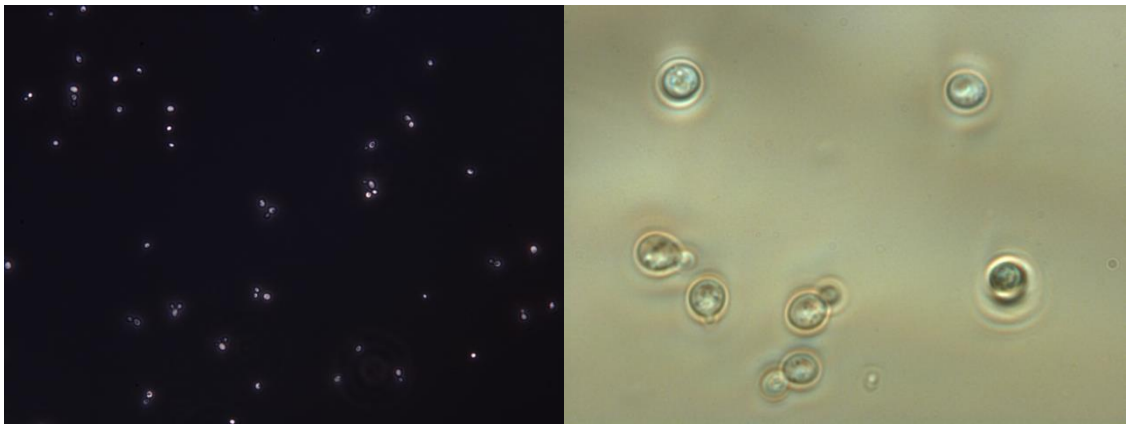


Figure 14: *20x microscope of Saccharomyce cerevisiae after the fermentation had reached stationary phase. The image shows that there has there has been no contamination.*

Figure 15: *100x microscope of Saccharomyces cerevisiae cells after the fermentation has reached stationary phase.The picture shows the cells reproducing by budding.*

Setup of the experiment

The experiment was setup very similar to experiment 1. Media composition, strain, fermenter parameters (temperature, air inlet flow, pH, etc.) were all the same. There were only 3 differences:

- The DO probe was replaced by a capacitance probe from Hamilton.
- Experiment 1 used tower 1 and 2. This experiment used tower 2 and 3. Inside the control box assigned for Tower 1, there was a leakage in the air inlet hose. Because of this, no oxygen could be delivered to the fermenter.
- The only offline samples taking were OD and HPLC. It was decided to discard taking DW measurements because the measurements were relatively inaccurate and took a long time. By discarding DW measurements, it was possible to take 2 OD and 1 HPLC measurements every 1 hour, instead of 1 OD, 1 DW and 1 HPLC every 1,5 hour.

None of the above changes in this experiment should have any impact on the actual fermentation, and experiments 1 and 2 should be comparable.

For the first growth phase, 2 OD samples were taken every hour. After the first growth phase ended after 30 hours of cultivations, 2 OD samples were taken every three hours. Sample taking lasted a total of 29 hours.

Results

The fermentation went as expected. The two growth phases are easy distinguishable. There can easily be seen an exponential growth for the first growth phase based on glucose.

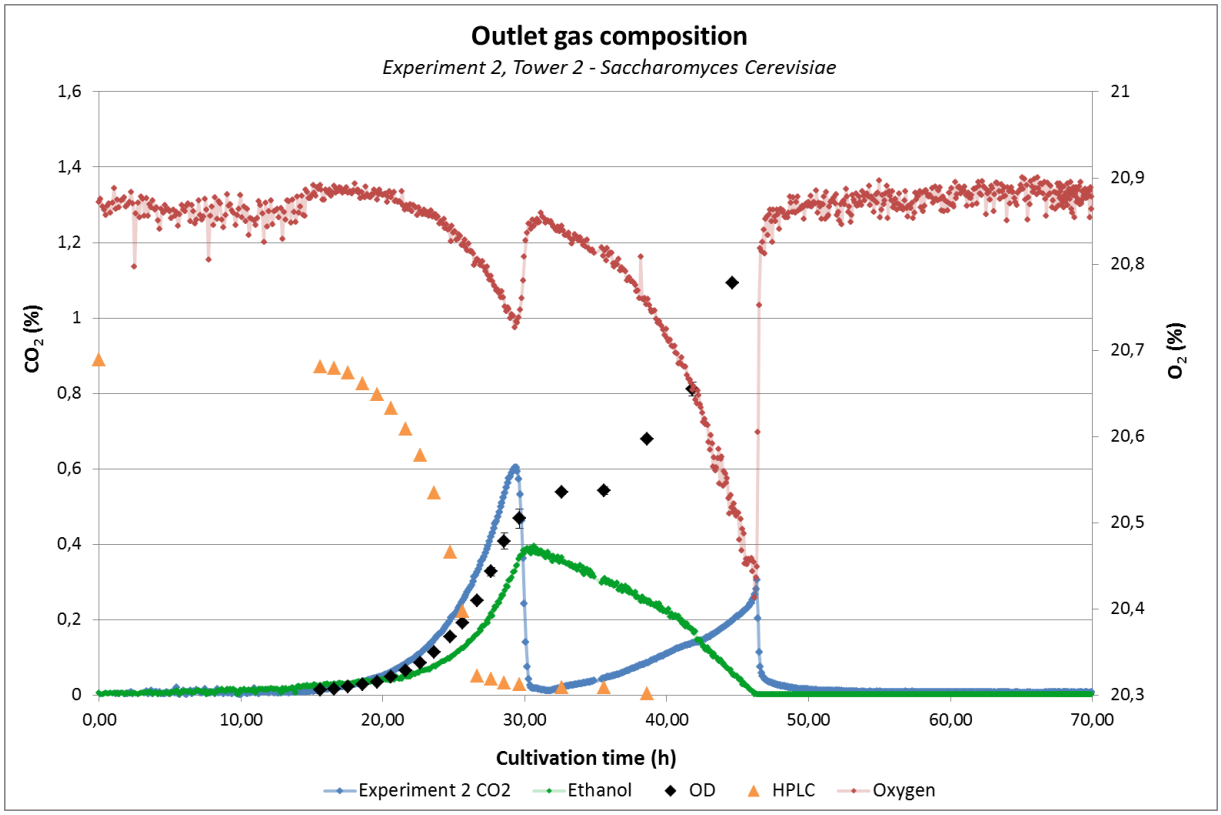


Figure 16: *Outlet gas composition + OD measurements. Please note that there is no corresponding y-axis for ethanol, HPLC and OD measurements. The highest ethanol content is 0,05%. The highest OD measurement is 7,29. Start concentration of HPLC = 9,87 g/L.*

The trend of the graphs looks very similar to experiment 1. One difference, though, is that in this experiment, the maximum obtained content of CO_2 is 0,6% around 30 hours. For experiment 1 the maximum CO2 content was slightly over 0,9%. This could be because of a slight difference in the amount of cells inoculated (higher start OD). It could also be the phase the cells are in (from the pre-culture/shakeflask).

µmax determined by OD measurement

OD measurements for tower 2 are displayed below:

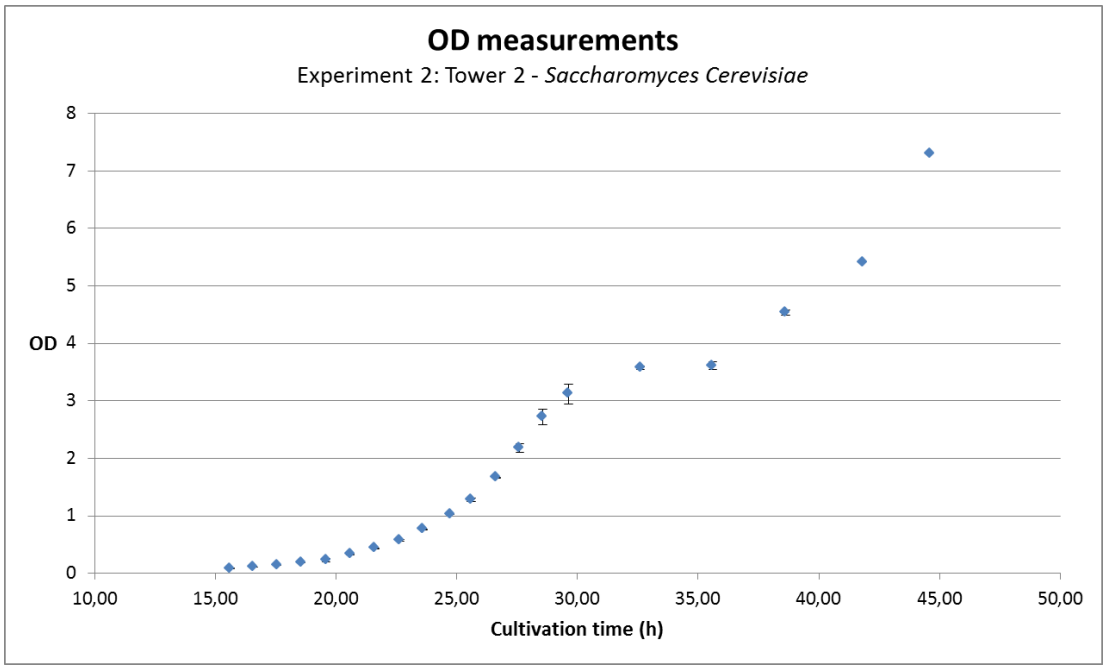


Figure 17: *OD measurements from Tower 2. The first growth phase lasts until 30 hours.*

It is a good idea to plot the data on a semi-logarithmic plots. This makes it easier to see where the data truly are exponential. The semi-logarithmic plot of the OD measurements is shown in *Figure 18*.

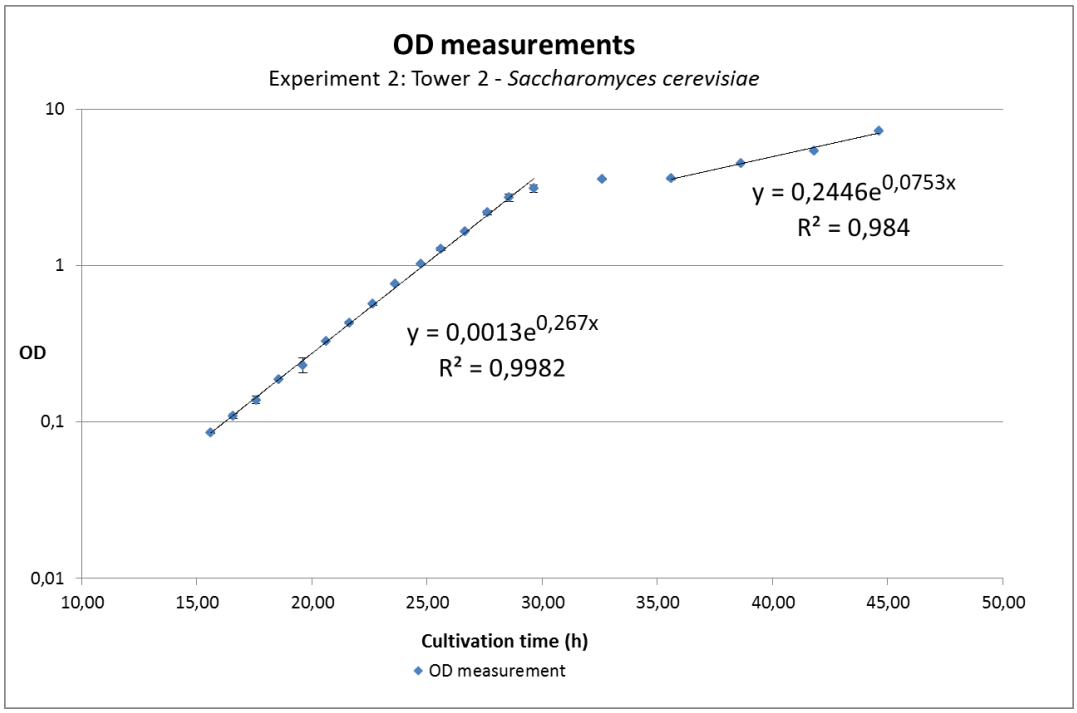


Figure 18: S*emi-logarithmic plot of the OD measurements. The added trendline is exponential, because a linear line on a semilogarithmic gives an exponential function.* $\mu max = 0{,}267 h^{-1}$; $\mu max2 = 0{,}0753 h^{-1}$

The OD measurements fit an exponential trend line nicely at $R^2 = 0{,}9982$. The maximum specific growth rate is $\mu max = 0{,}267\ h^{-1}$.

Tower 3 gives a maximum specific growth rate of $\mu max = 0{,}3575\ h^{-1}$. The two towers from experiment 2 are compared in *Figure 19*. The cells in tower 2 grow a bit slower. For the first phase, it looks like almost the same amount of cells have formed, but it takes a bit longer for tower 2.

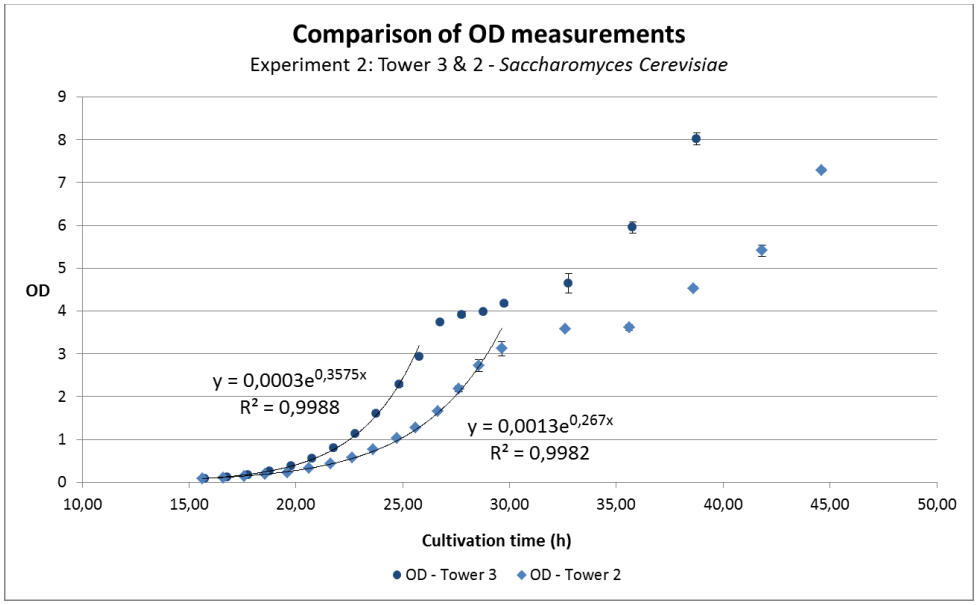


Figure 19: *OD-Measurements from both towers. They both have exponential growth in first phase, but the maximum exponential growth rates differ a bit.* $\mu max\ (3) = 0{,}3575\ h^{-1}$; $\mu max\ (2) = 0{,}267\ h^{-1}$

There is no apparent explanation of why the two towers give different specific maximum growth rates, or why µmax is different from experiment 1 and 2. Both experiments and both towers have run under the same conditions. The media for both towers was made at the same time and were inoculated with inoculum from the same shake flask.

The cells are grown in a minimal media. The difference in µmax in the two towers is also almost identical to the difference in experiment 1, though. Here, the maximum specific growth rates were: $\mu max = 0{,}29\ h^{-1}$ and $\mu max = 0{,}40\ h^{-1}$.

All OD measurements for both experiment 1 and 2 are plotted in *Figure 20.* They are plotted on a semi-logarithmic plot for easier identifying exponential growth. It can be seen that all 4 fermentations have an exponential growth for both phases. Some of the fermentations have slightly higher µmax values when you compare them, but the overall trends correlate very well. They all have a clear exponential growth rate for the first phase, and a second growth phase on ethanol with significantly lower µmax values.

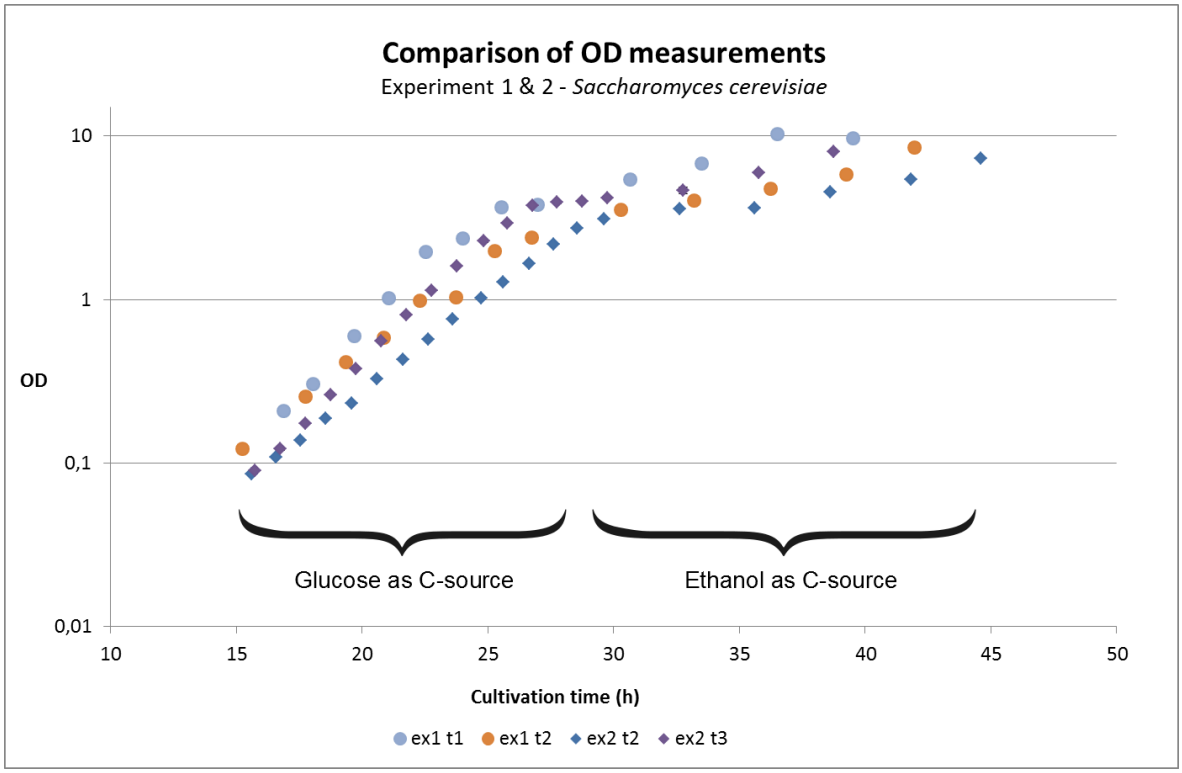


Figure 20: *OD plotted on semi-logarithmic plot. The growth is exponential for all experiments.*

Comparison of experiment 1 and 2 – CO_2

If we compare experiment 1 and 2, the trends of the fermentation looks similar. For experiment 2 though, the fermentation is a bit lower and is lagging behind. Please notice that the air reference has not been taken into account for the CO_2 values.

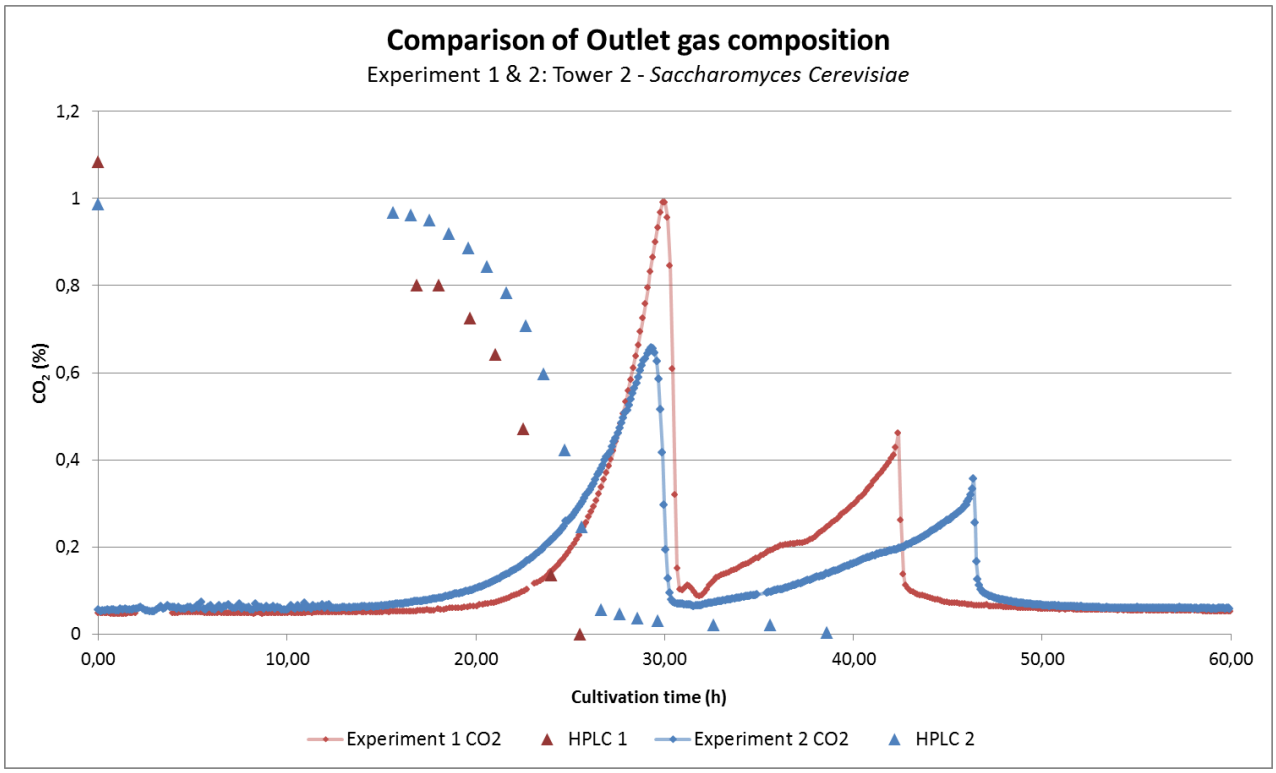


Figure 21: *Comparison of experiment 1 and 2. There is no corresponding y-axis for the HPLC values. The start-concentration of glucose for HPLC 1 is 10,8 g/L. The start-concentration of glucose for HPLC 2 is 9,87 g/L*

Both experiment show two grow phases, and they both consumer the glucose in the first phase. Experiment 2 was conducted 49 days after experiment 1. The medium for experiment 1 and 2 was made at the same time, meaning that the medium for experiment 2 had been stored at 3 °C. for approximately 49 days before use. (Neither glucose or vitamins were added during storage.)

Otherwise both experiments have been conducted the same way.

µmax based on accumulated CO_2 off-gas

Accumulated CO2 (g/L) is calculated the same way as in experiment 1. The trends are very comparable to experiment 1. The first growth phase based on glucose has a perfect exponential growth based on CO_2, with a perfect coefficient of determination at $R^2 = 0{,}9999$.

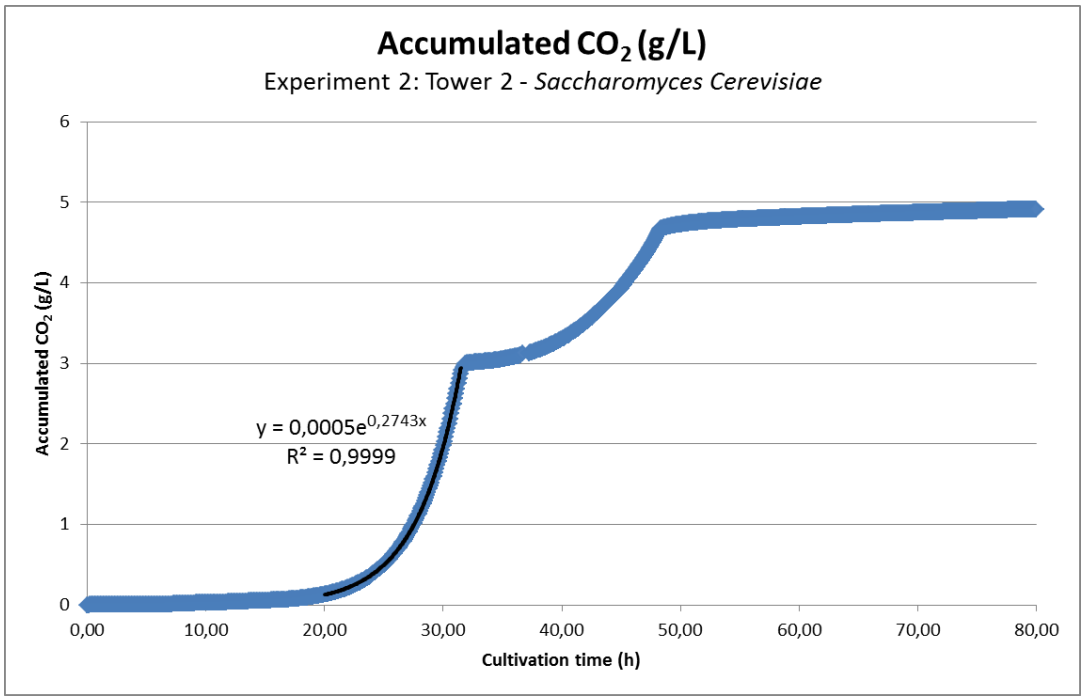


Figure 22: *Accumulated CO_2. The trendline for the first growth phase perfectly fits an exponential trend line.*

$$\mu max = 0{,}2743\ h^{-1}$$

Just as in experiment 1, the accumulated CO2 off-gas gives a lower µmax for the second growth phase based on ethanol. Likewise the second growth phase fits a second order polynomial much better.

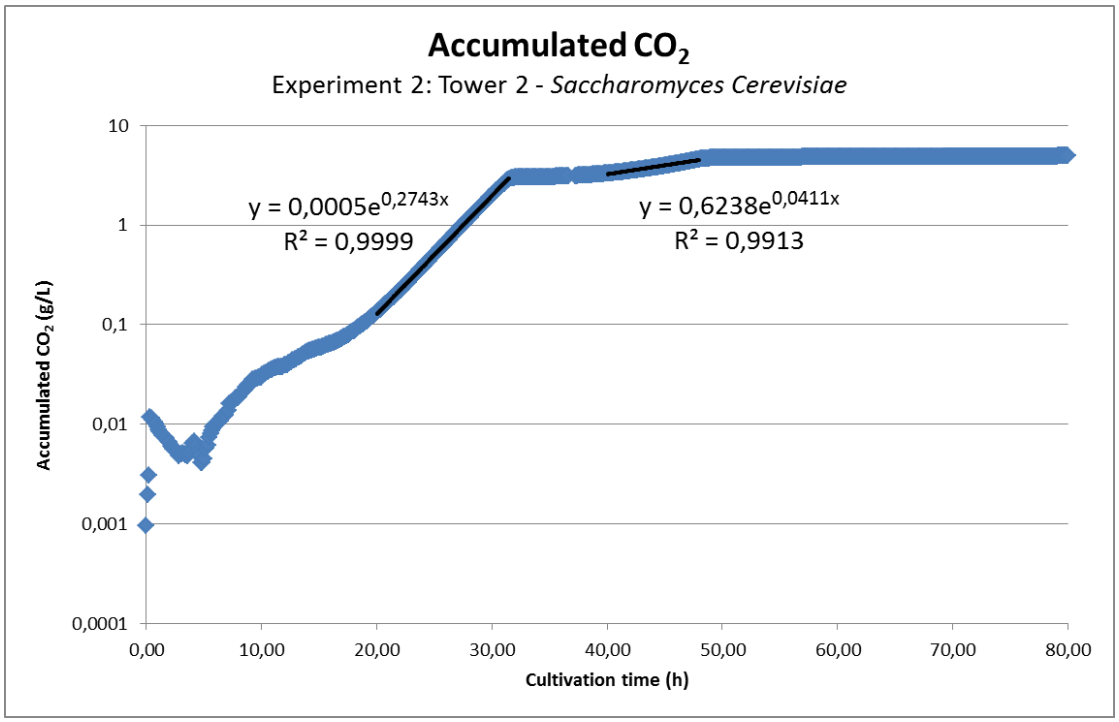


Figure 23: *Accumulated CO2 shown in a semi-logarithmic plot. Linear trend lines equals to an exponential growth*

$\mu max1 = 0{,}2743\ h^{-1}$. $\mu max2 = 0{,}0411\ h^{-1}$

Capacitance measurements

In this experiment, a capacitance probe was added to the fermenter, in order to measure the viable cell density. All the different cell density methods are plotted together in *Figure 24*. All three methods seem to have decent correlation which each. All three methods manage to show a diauxic growth curve, but the variance in the capacitance measurements is very high. (There is a lot of noise in the capacitance measurements).

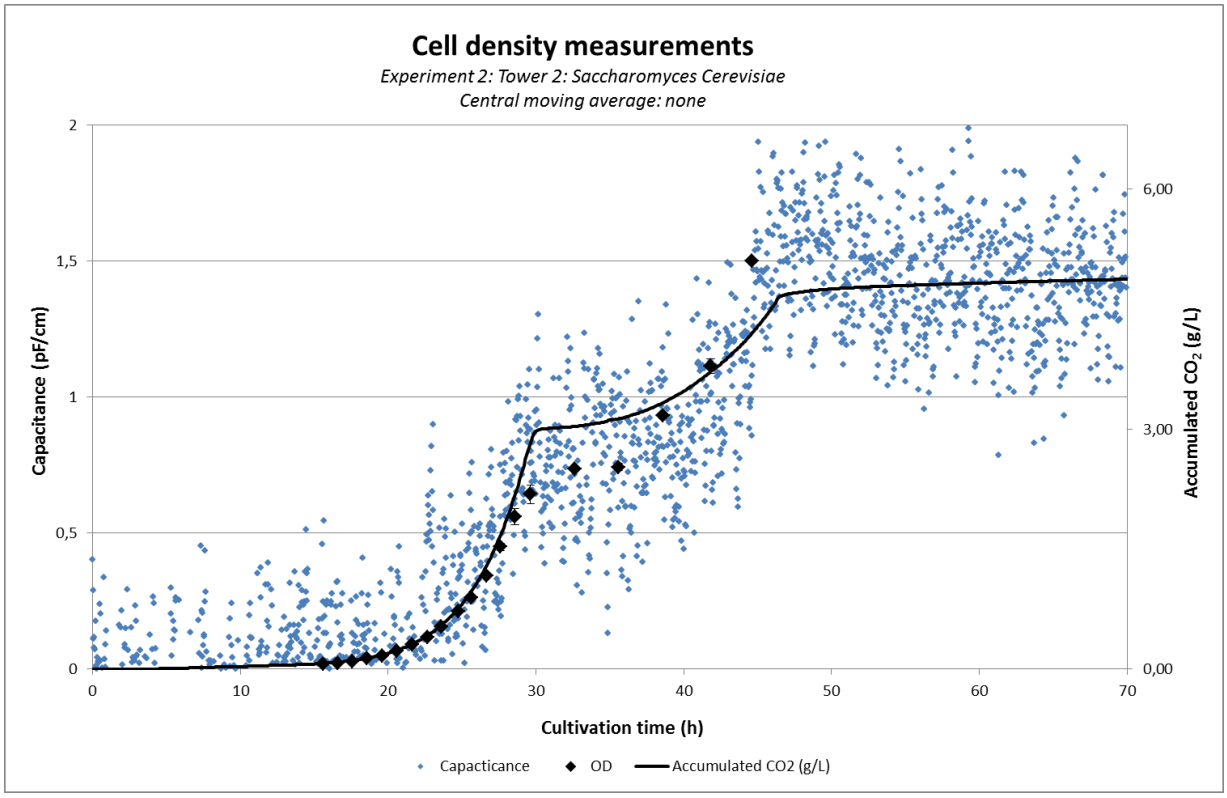


Figure 24: *All cell density methods plotted in the same graph. Please notice that there is no corresponding y-axis for the OD measurements. The highest OD value was OD = 7,29.*

The reason for the high variance in the capacitance is because of air bubbles. The high agitation at 800 RPM produces a lot of air bubbles, which interferes the capacitance measurement. If the agitation had been run at lower RPM, the accuracy of the capacitance measurement would have been a lot better (shown later).

While the variance in the capacitance measurements is extremely high, the Hamilton software has actually already tried to reduce the amount of noise, by a function called integration. Basically, the software takes a moving average of the measurements, and thus reduces the noise. [30] In all experiments, the integration function has been set to "Integration: High." Despite the high integration, the noise is still very noticeable. To minimize the noise even further, one can try to reduce it by adding a moving average. In this case, a simple central moving average has been applied to the data set. A simple central moving average uses data before and after the point to calculate the mean. A simple average is unweighted, meaning that each data point contributes equally to calculate the mean.

Listed below are the capacitance measurements with different degrees of moving averages applied. The more data points are applied to calculate the average, the smoother the curve gets. At some point, too many data points are used, though, as you will lose more and more information as you smooth out the curve.

Capacitance measurement with different moving averages applied

Experiment 2: Tower 2 - *Saccharomyces cerevisiae*

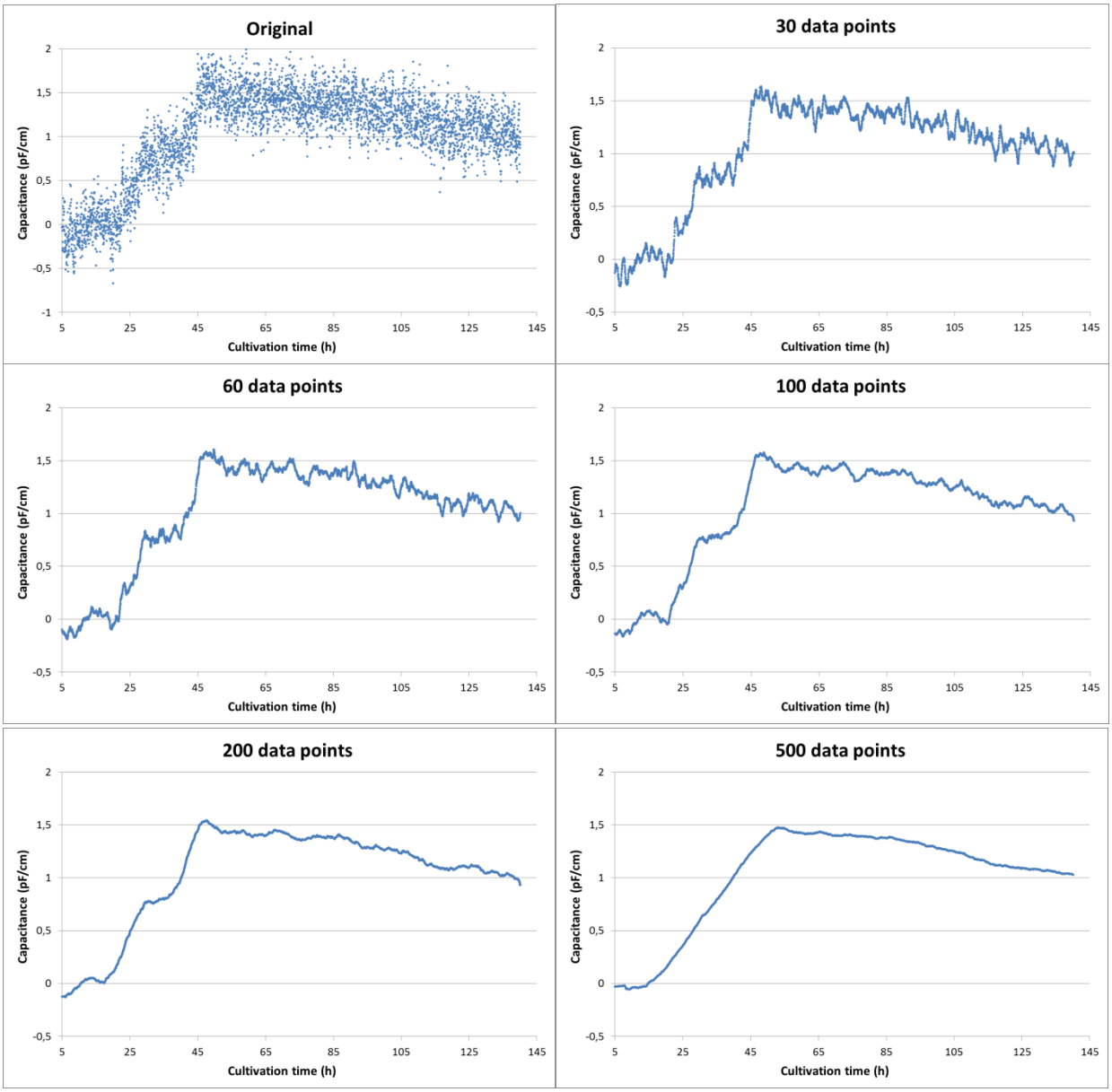


Figure 25: *Capacitance measurements with an added moving average. A moving average will reduce the noise, but at the cost of losing information.*

How much you want to smooth out the curve depends on the situation and how many details you need. But choosing an integration of 500 points, as shown in the last graph, is clearly too much. In the last graph, it looks like there is only one linear growth phase, which is wrong. There are two exponential growth rates, which are verified by OD and CO_2. Therefore, care must be taking when manipulating the data.

µmax based on capacitance measurements

Calculating the maximum specific growth rate base on capacitance measurements was slightly more challenging. The problem is that it is not possible to use negative data points when calculating an exponential trend line. That is because the logarithm of any negative value is not defined. This means that in order to calculate an exponential trend line, it's necessary that all data points are positive.

A lot of the capacitance measurements had negative values in the beginning of the fermentation. There was found no sensible way to transform the data set to only include positive values. Taking a rolling average of the capacitance measurements still resulted in having negative values. It was also tried to add a positive constant to all the capacitance values, but doing so impact the µmax value significantly.

Because of this it was instead chosen to estimate the trend lines shown in *Figure 26*. The trend lines are not directly based on the capacitance measurements.

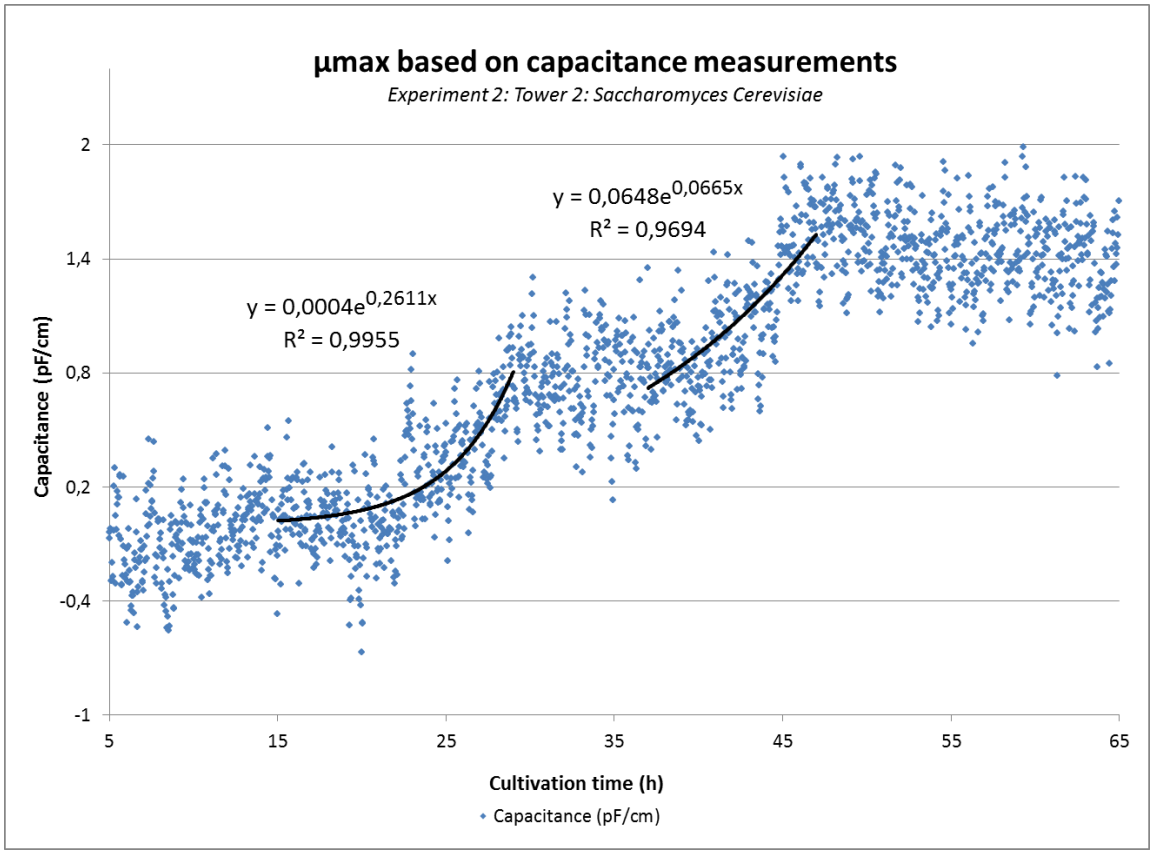


Figure 26: *µmax based on capacitance measurements. The exponential trend lines are estimated.*

$$\mu max1 = 0{,}2611\ h^{-1} \quad ; \quad \mu max2 = 0{,}0665\ h^{-1}$$

The estimated µmax values correlate nicely with the µmax values determined by OD and accumulated CO_2 off-gas. Though, it should be taken into account that the estimation of the µmax value based on capacitance may be biased.

Capacitance noise

Because experiment 2 gave very inaccurate capacitance measurements, it was investigated what caused the noise in the measurements. For this experiment, it turned out that the agitation in the fermenter was almost the sole reason for inaccurate measurements. After experiment 2 was concluded, the fermentation was run again with the now inactive yeast (*Saccharomyces cerevisiae*). Under the exact same conditions, the capacitance was measured with varying agitation (rotation of the impellers). The capacitance was measured at 100, 200, 300, … ,800, 900 RPM.

The graphs in *Figure 27* are results from 4 different rotations and show a clear correlation between the rotation of the impeller and the noise in the capacitance measurement. At 100-300 RPM, the measurements are very accurate for the 2 L fermenter with *Saccharomyces cerevisiae*. Beyond 300 RPM, the amount of noise increases exponentially.

Capacitance measurement of *Saccharomyces cerevisiae*

Experiment 2 – conducted after the death phase was concluded

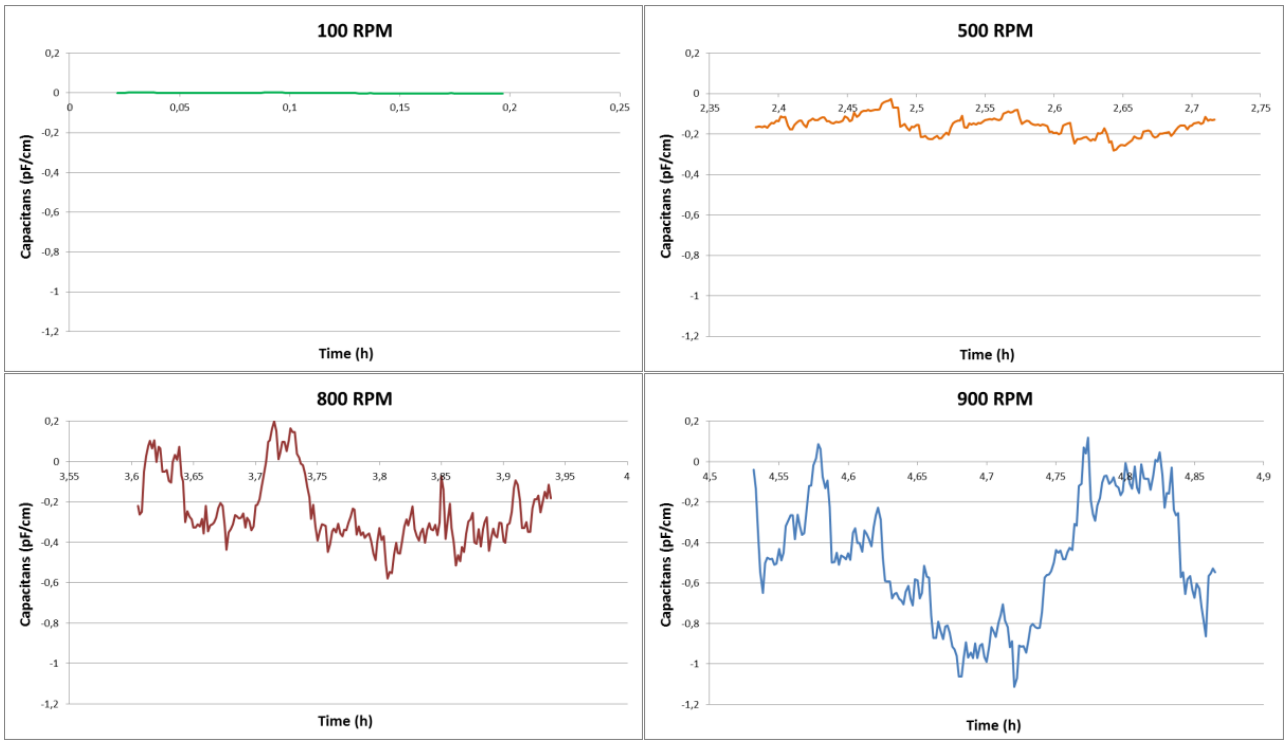


Figure 27: *Capacitance measurement at different levels of agitation. High agitation results in noisy measurements.*

This experiment shows the importance of having a low stirrer speed, if it is desired to have accurate measurements. This is very problematic, and it is probably the biggest drawback of using capacitance measurements. A lot of cells require oxygen to grow, and dissolved oxygen is often a limiting factor in fermentations. Therefore, they have to a run the fermentation with a high stirrer speed to increase the amount of dissolved oxygen. In practice, this means that a great deal of fermentations will not be able to run capacitance measurements with a decent accuracy, unless they produce a high cell density.

Experiment 3: *Escherichia Coli*

Objective of experiment

The objective of this experiment was to compare CerCell's single use Chloris capacitance probe to Hamilton's capacitance probe. By placing two Hamilton and two Chloris probes in the same fermenter, the two systems can be directly compared.

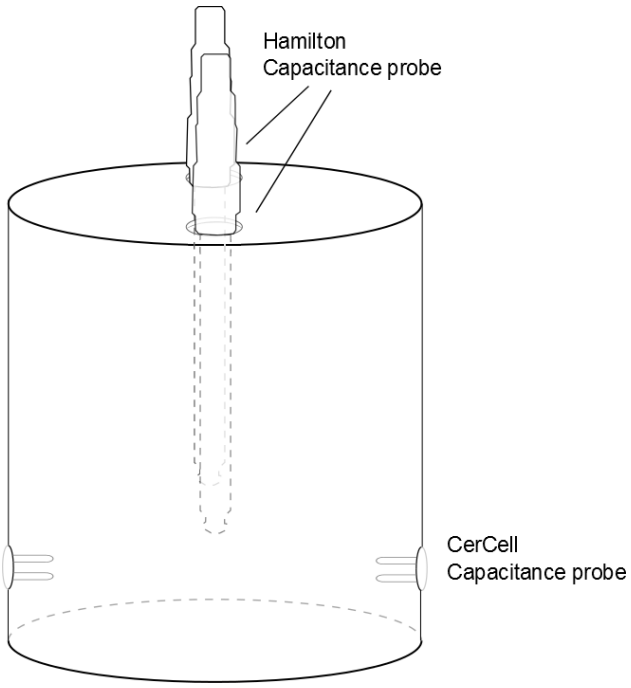


Figure 28: *Sketch of capacitance probe placement. Probes were planned to be placed as far as way as possible from each other, in order to avoid interference/interaction between the capacitance probes. (E.g. the electricity travels from one probe to the other.)*

Conclusion

This experiment, unfortunately, gave disappointing results. Several mistakes were made for the actual preparation of the fermentation, but even if the experiment had been run flawlessly, it would still have given unsatisfactory results regarding the capacitance measurements.

For *E. coli,* the conclusion is that a very high cell concentration is necessary if you want to get accurate capacitance measurements. Since *E. coli* grows via an oxidative reaction and is very dependent on a large oxygen supply, it is necessary to have a high agitation and therefore a large amount of bubbles in the fermentation. Because there are a lot of air bubbles, capacitance measurements are inaccurate, and a high cell density is necessary to get somewhat accurate capacitance measurements.

In this experiment, the cell density was far below the necessary amount to get accurate capacitance measurements. The variance in the capacitance measurements were far too big, so detecting cell growth was not possible.

This is both the case for the Hamilton capacitance probe and CerCell's single use capacitance system, Chloris.

Set up of experiment

Since the CerCell capacitance probes are plugged in from the side, it was necessary to make a custom fermenter/Single-Use-Fermenter, because none of DTU's regular steel fermenters include this feature. Since the fermenter includes a cooling/heating jacket, it was necessary to custom design it, to allow space for the CerCell capacitance probes.

A 3D model of the Single-Use-Fermenter can be seen in Figure 29:. [31]
The 3D models include all of the single use components needed for the fermentation.

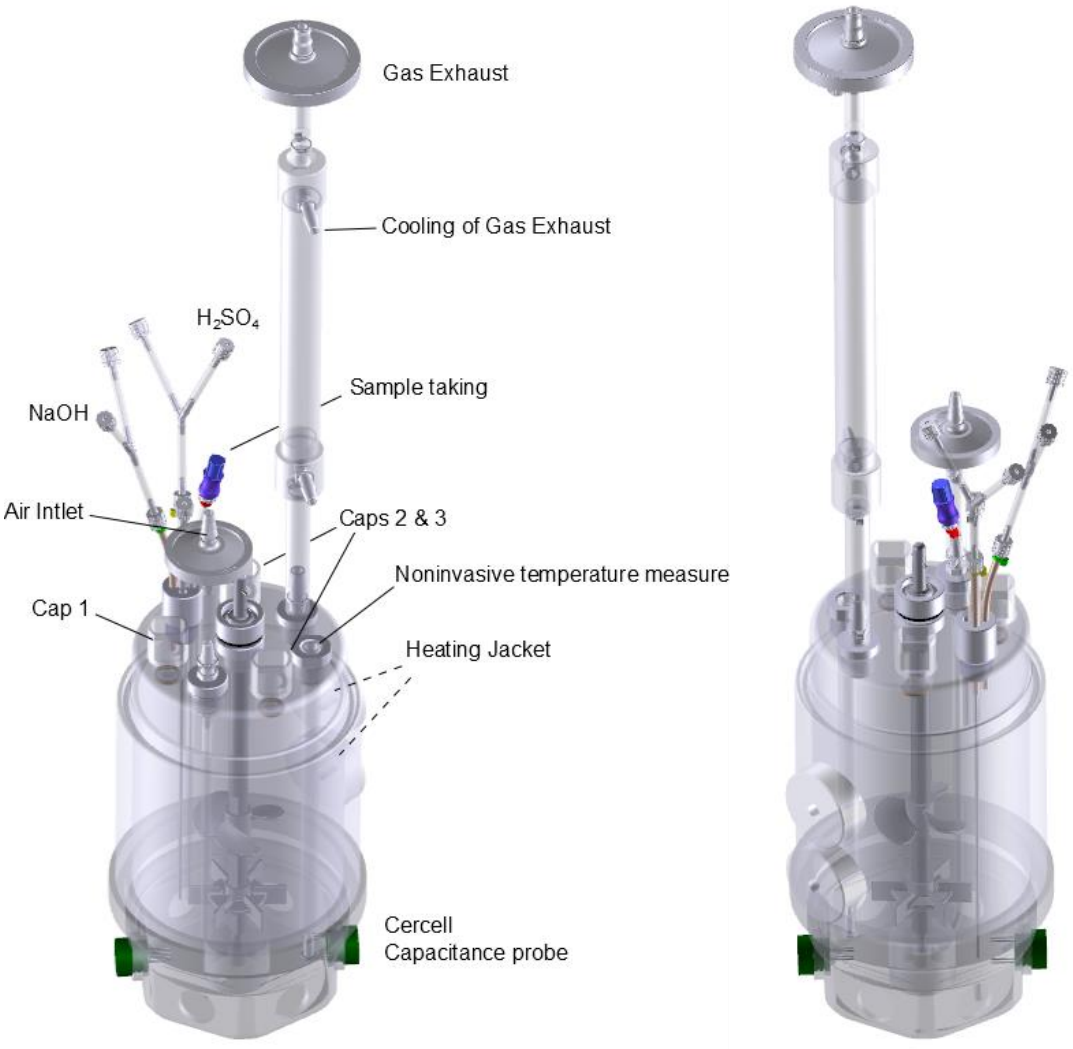


Figure 29:
a: *Sketch of Single-Use-Fermenter custom designed for this experiment. Cap 1 is assigned for pH probe.* Caps 2 and 3 are assigned for the two Hamilton capacitance probes.
b: *View of the fermenter from the back.*

Beside the Single Use Fermenter, the following components were afterwards added:

- 2 Hamilton/Fogale capacitance probes
- 2 Preamplifiers for the CerCell Capacitance probe (Called Karpos)
- 1 bio stat HPD adapter (with 7 cogs)
- Nylon Cogwheel (Adapter for the motor)

And the following Standard equipment is used:

- pH probe
- Temperature probe
- Hoses for gas exhaust and heating jacket
- Hoses for acid and bases (H_2SO_4 and NaOH, respectively)

The finished product of the Single-Use-Fermenter deviated slightly from the proposed 3D model. The Fermenter also included baffles, to improve aeration, but this resulted in a rearrangement of the caps in order to avoid collision with the baffles. This resulted in the Hamilton capacitance probes being placed closer to the CerCell capacitance probes than originally intended. Else, the finished fermenter was identical to the 3D model shown in Figure 29.

Sterilization of the fermenter/reactor

The advantages of using Single-Use-Bioreactors is that it is easy and fast to do experiments, since no cleaning or autoclavation is needed. When companies order Single-Use-Bioreactors, they are normally delivered autoclaved/sterilized, so the customer only has to add their media and inoculum. And when the fermentation is done, the fermenter is discarded, to save time from cleaning.

A Single-Use-Bioreactor cannot be autoclaved at the usual 121 °C, because the hard plastic begins melting at around 60-80 $°C$. [32] Therefore, the Bioreactor is pre-sterilized at a special place, where the sterilization takes place at a lower temperature. Because the Single-Use-Bioreactor is delivered pre-sterilized, it is usually desired to have single-use-components that are already integrated in the Single-Use-Bioreactor prior to sterilization. That way, it is faster to perform experiment, and the risk of contamination is reduced.

If the user wants to add conventional equipment, they can be autoclaved normally and be added to the Single-Use-bioreactor under sterile conditions, e.g. in a LAF bench.

In this experiment, most equipment was single-use-components already integrated in the Single-Use-Fermenter prior to sterilization. E.g. the impellers, air inlet, gas exhaustion, sample ports, and last but not

least, the Chloris capacitance probe are all single-use-components and were integrated in the Fermenter before it was sterilized.

The pH probe and the 2 Hamilton capacitance probes are conventional equipment and had to be added afterwards under sterile conditions in a LAF bench. When the fermenter was delivered, caps were added to the fermenter to avoid contamination. In a LAF bench, cap 1 would be replaced by the pH probe and caps 2 and 3 would be replaced by the Hamilton probes. (See Figure 29).

Failures of the experiment

Result-wise the experiment was a disaster. The following things went wrong.

- The gas exhaust and air inlet filter was stopped. This resulted in the air not being able to flow, and give the prober aeration to the *E. coli* cells. Since *E. coli* grow via an oxidative reaction and are often limited by the O2 supply, this was a fundamental mistake that likely limited the growth of the cells. This problem was first found in the middle of the fermentation.
- The MS was not connected probably and gave false measurements. This problem was first found 3 hours after inoculation. While this problem does not affect the fermentation, it means that there are no MS measurements for the first 5 hours of the fermentation. (It took approximately 2 hour to fix the problem.)

As described earlier, even if these problems had not occurred, the goal to compare capacitance measurements of the Hamilton probe and the Chloris system would not had been very successful, since both measurements have too high a variance.

Results

The capacitance measurements from the two Chloris probes are plotted together with offline OD measurements in *Figure 30*. While there seems to be a decent correlation between the capacitance measurements and OD measurements, the variance in the capacitance measurements is too high to conclude anything. (The error bars are too big)

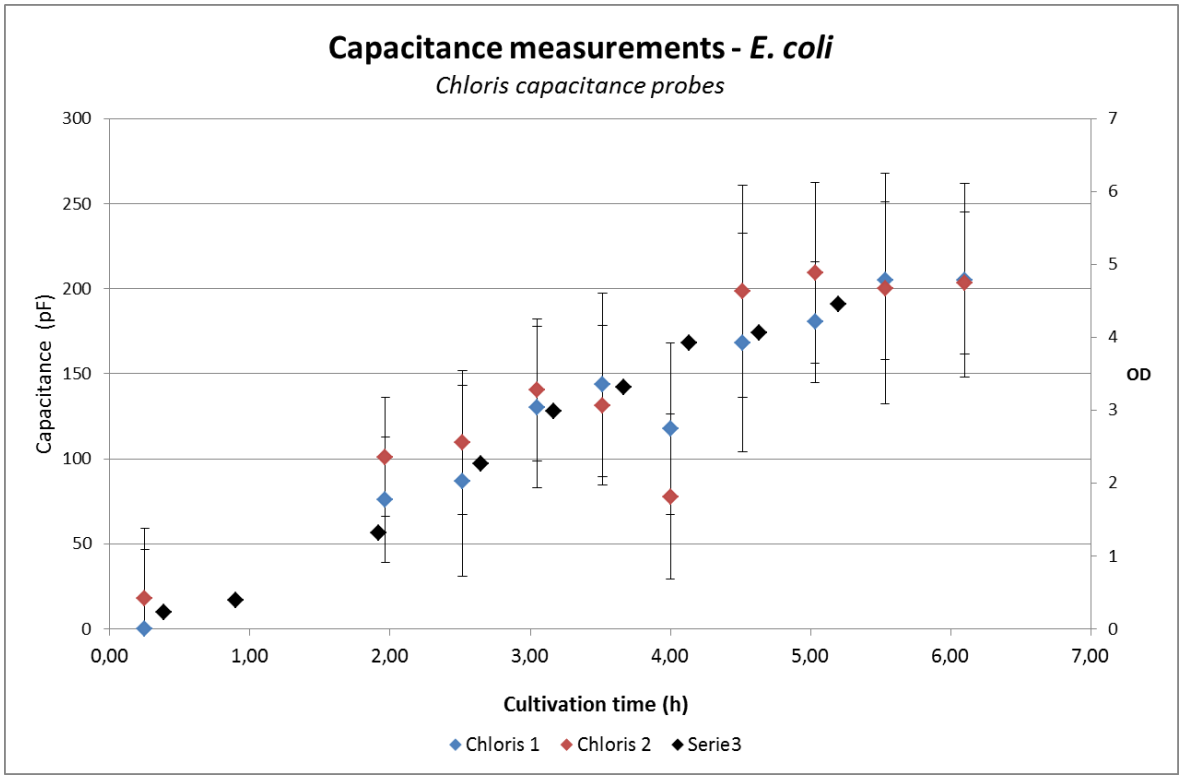


Figure 30: *Capacitance measurements from Chloris plotted together with OD measurements. Each data points for the Chloris measurements consist of 20 measurements.*

Suggestions for future experiments

To give more accurate capacitance measurements, there are two ways the experiment could be improved. Either you could have a higher cell density and/or you could use another cell that requires less agitation and thus create fewer bubbles.

1. Make a fed-batch/high cell density instead

E. coli is not the best cell for measuring the capacitance accurately, since it requires a high amount of aeration. But if the user/customer insists that they want to test it for *E. coli*, it is crucial that the cell density is high. *Figure 31* shows the results from a high cell density fed-batch fermentation of *E. coli*. [33] The graph shows that it is necessary to produce a high cell density in order for the capacitance measurements to be accurate. If they had only run a batch fermentation, it would have been very difficult to follow the cell growth accurately, since the noise is too high.

The capacitance measurements form experiment 4 have also been photoshopped in the graph to display that the experiment would not have given any useful capacitance measurements, even if the experiment had gone as planned. A higher cell density is needed when you are making fermentations with high stirrer speed.

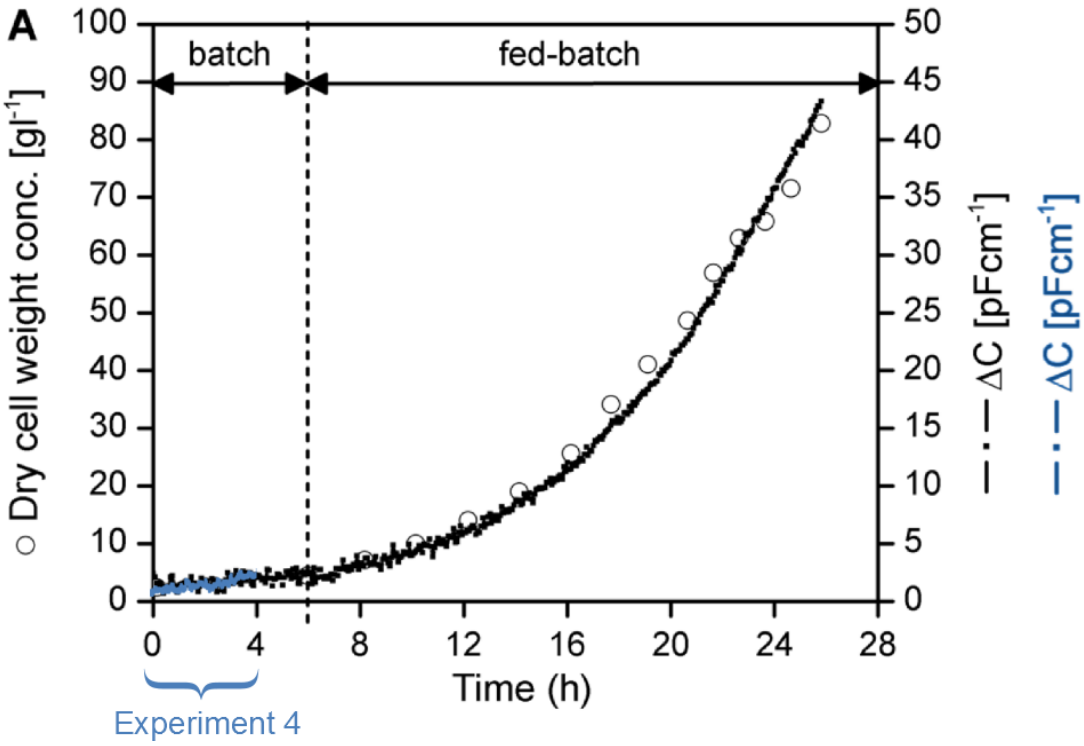
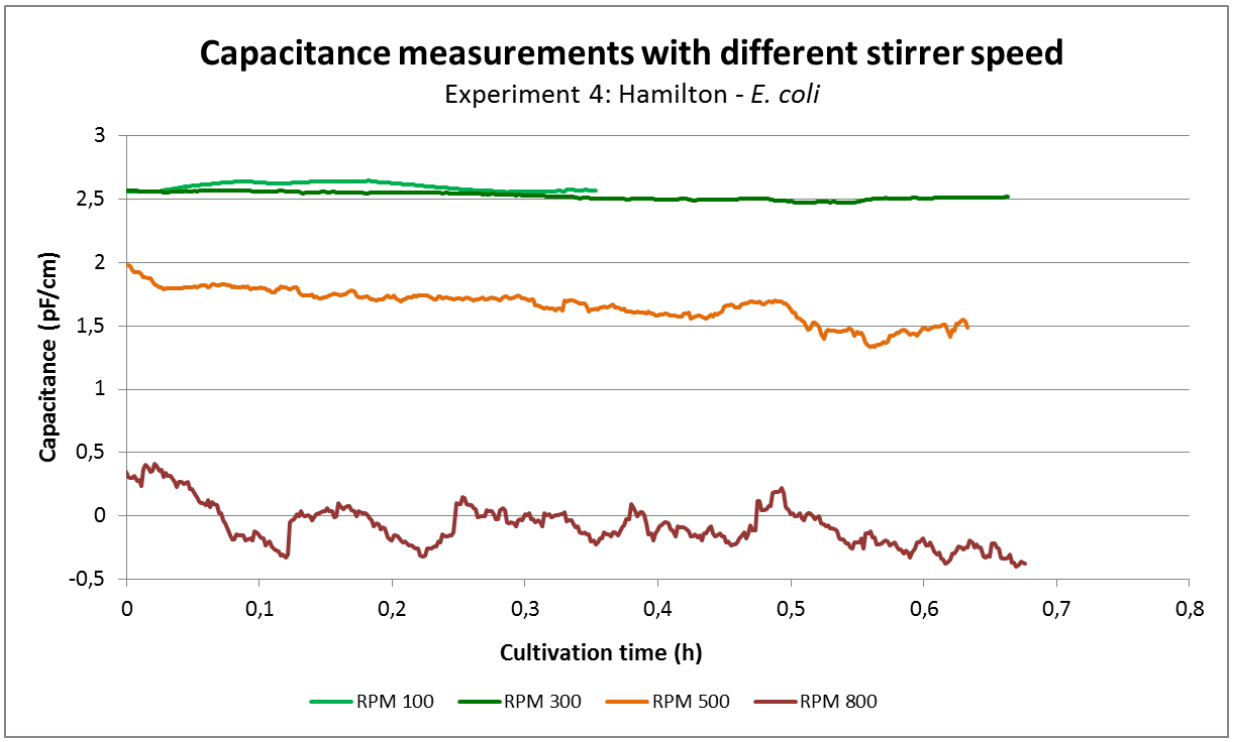


Figure 31: *Original graph is a high cell-density cultivation of E. coli VH33 in minimal medium with glucose as the C-source. Experiment 4 was a batch cultivation of E. coli in complex medium. The capacitance measurements from experiment 4 have been photoshopped into the graphs. The maximum value from the experiment was 1,5 pF/cm.*

Stirrer speed – Hamilton and Chloris

After 6 hours of cultivation, it was decided to stop the experiment and measure the Hamilton capacitance at different stirrer speed (RPM).

The capacitance was measured at 4 different stirrer speeds: 100, 300, 500 and 800 RPM. The results for the Hamilton system and Chloris system are shown in the figures below:



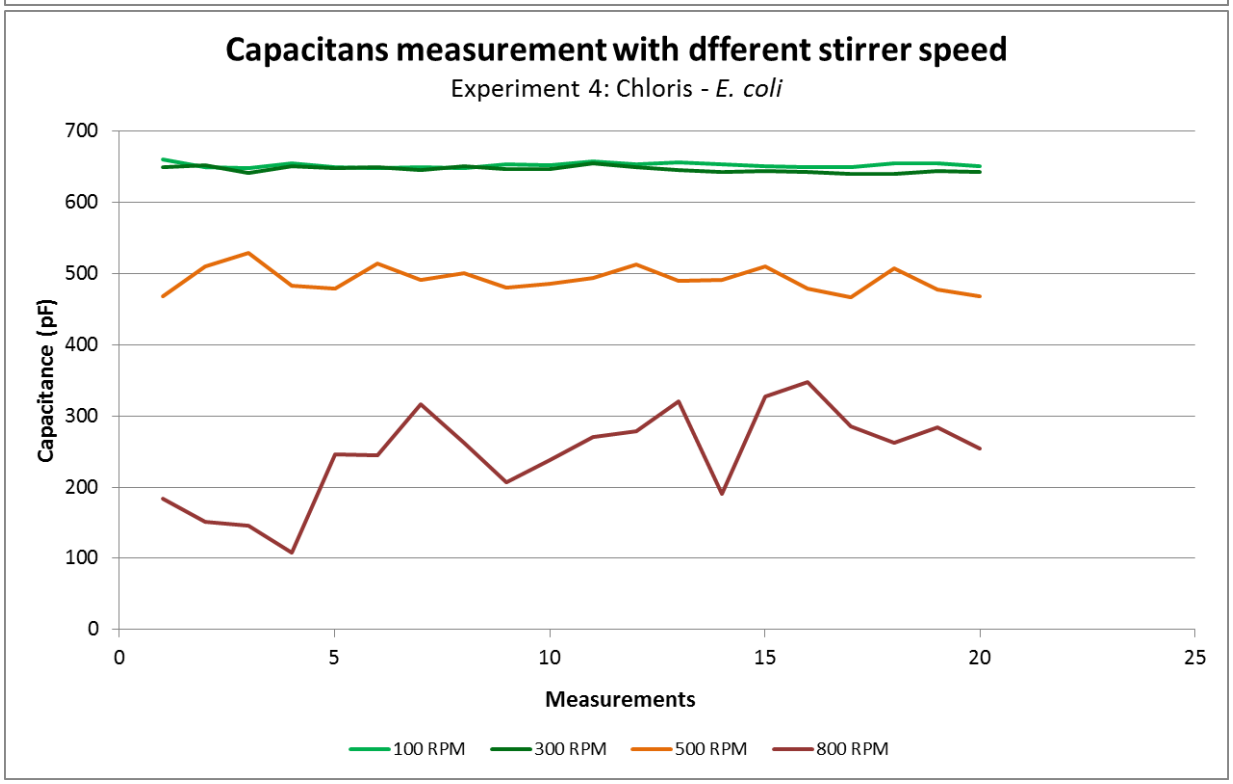


Figure 32: *Hamilton system: Higher agitation/stirrer speed gives more noisy results and a lower capacitance.*
Figure 33: *Chloris system: Higher agitation/stirrer speed also gives more noisy results and a lower capacitance.*

These measurements are in agreement with the previous experiment. Up to 300 RPM gives accurate measurements, while higher stirrer speeds make the measurements more inaccurate.

The two systems, Hamilton and Chloris, seem to suffer from the same problem. A high stirrer speed also gives inaccurate measurements for the Chloris system and gives significantly lower capacitance values.

The fact that a higher stirrer speed gives a lower capacitance measurement is already a known problem. [34] A high stirrer speed results in the medium containing more air bubbles, which causes it to have a higher volume. Since the amount of cells in the medium is the same, whether the medium contains air bubbles or not, a high volume results in a lower concentration of cells. The capacitance probe measures the cell density in the fermenter and not the total amount of cells. You'll manually have to take into account the change in volume to get a correct measure of the viable biomass. [1]

Capacitance measurements for Single-Use-Bioreactors

In collaboration with CerCell Aps, a big part of the project has been to develop their system for measurement of biomass/cell density for Single-Use-Bioreactors. The product is officially launched under their website: http://cronus-pcs.com/products/niobe-sensors/chloris/

The product is part of an easy configurable Process-Control-System. The concept behind the product is that all components for The Process-Control-System are easily interchangeable and work as stand alones. It is also the world's first open software platform for Process-Control-Systems, giving the user more flexibility and choices. [35]

Currently, there are only two main distributors of capacitance measurements, Aber and Hamilton. Both of these systems are only implemented for traditional steel bioreactors.

An objective for this part of the project is to investigate the accuracy of the capacitance measurement system called Chloris. This is done by placing the Hamilton and Chloris probe in the same fermenter and comparing the results. At the start of the project, the capacitance probes and an alpha version of the software had already been developed, but no extensive testing or validation of the system had been done.

Figure 34 shows the Chloris system. The metal box contains all the hardware and electrical components. Via a HDMI cable, the white pre-amplifiers are connected. The pre-amplifiers are inserted in the side of the bioreactor to measure the capacitance. Data is transferred via a USB cable to a computer/laptop with the Chloris software installed.

Figure 34: *Picture of Chloris, currently the only system to measure capacitance for Single-Use-Bioreactors.*[36]

Correlation between Chloris and Hamilton

In order to test how accurate the Chloris system is able to detect biomass/cell density, an experiment was conducted where both Chloris and Hamilton probes were used simultaneously in the same bioreactor. This was done to compare if the two systems would give the same capacitance measurement. In this experiment, 1 Hamilton probe and 2 Chloris probes were used.

Setup of experiment

Step 1

In an open non-sterile Single-Use-Bioreactor, 500 ml demineralized water was added together with 0,8% KCl (4 grams KCl). The Bioreactor was stirred at 280 RPM at room temperature (ca. 22 ^{o}C). One Hamilton probe and two Chloris probes were added to measure the capacitance.

Step 2

After the Hamilton Evo 200 and Chloris system had been turned on for more than 30 minutes, the first capacitance measurement was noted. (It takes both systems approximately 30 minutes to warm up, in order to give accurate measurements.)

Step 3

After the capacitance had been measured, 2 grams of fine SiC powder were added to the water and KCl solution. After 10 minutes, the SiC powder had been dissolved and dispersed in the solution. The new, higher capacitance was noted.

Step 3 was repeated 16 more times, and the capacitance was plotted as a function of the amount of added SiC powder, shown in *Figure 35*. The Hamilton system measures the permittivity in unit pF/cm, while the Chloris system measures the capacitance in pF.

The correlation between concentration of powder and permittivity for the Hamilton system is excellent. The coefficient of determination for a linear trendline for the Hamilton system is $R^2 = 0{,}9998$

This shows that capacitance measurements can be a very accurate method to determine the cell density (at least for simple particles such as SiC).

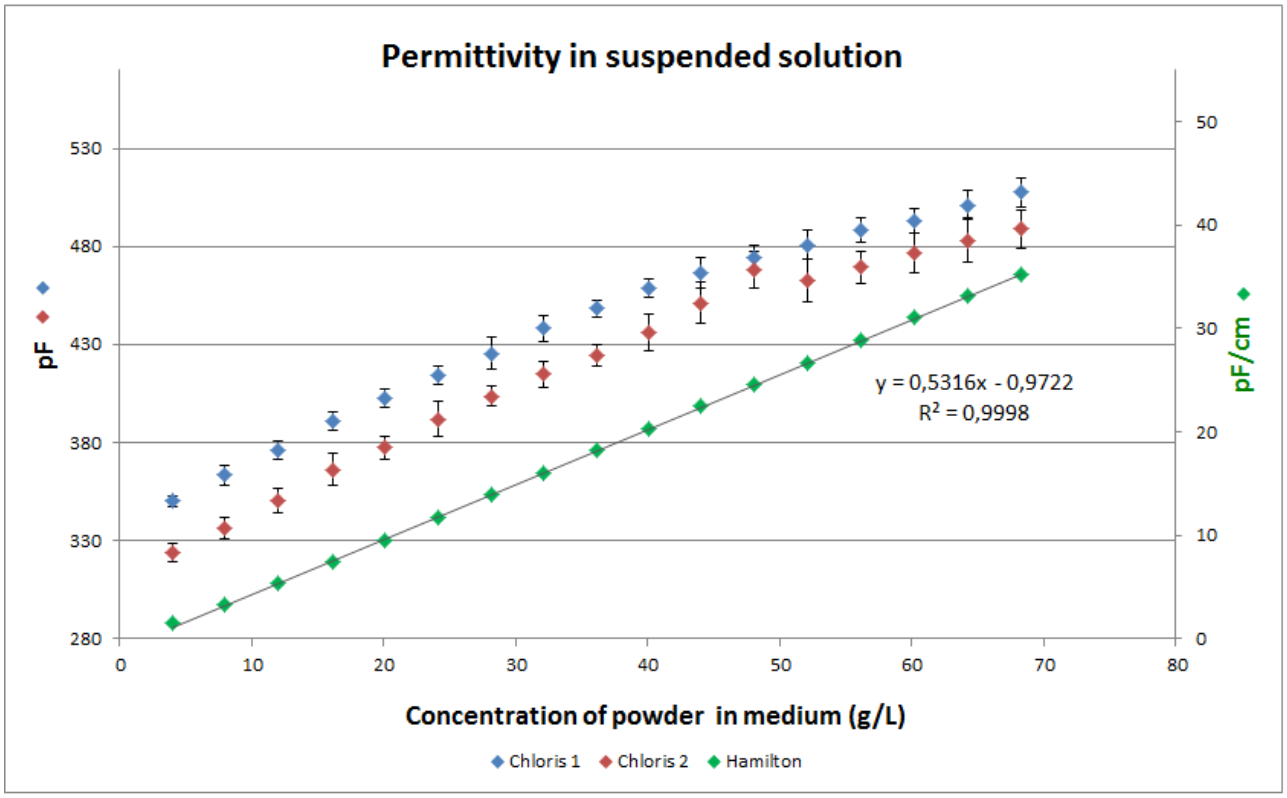


Figure 35: *Capacitance/permittivity as function of amount of SiC powder added. Error bars for Chloris equals to* $\pm 1\ SD$*, and are based off 50 measurements. There are no error bars for the Hamilton measurements.*

The Hamilton probe clearly has accurate measurements. But the Chloris system also seems to have an okay correlation between the concentration of powder and the capacitance pF.

To get a more precise comparison, the two systems Hamilton and Chloris 1 are plotted against each other in *Figure 36*. Initially, it was tried to fit a linear trend line.

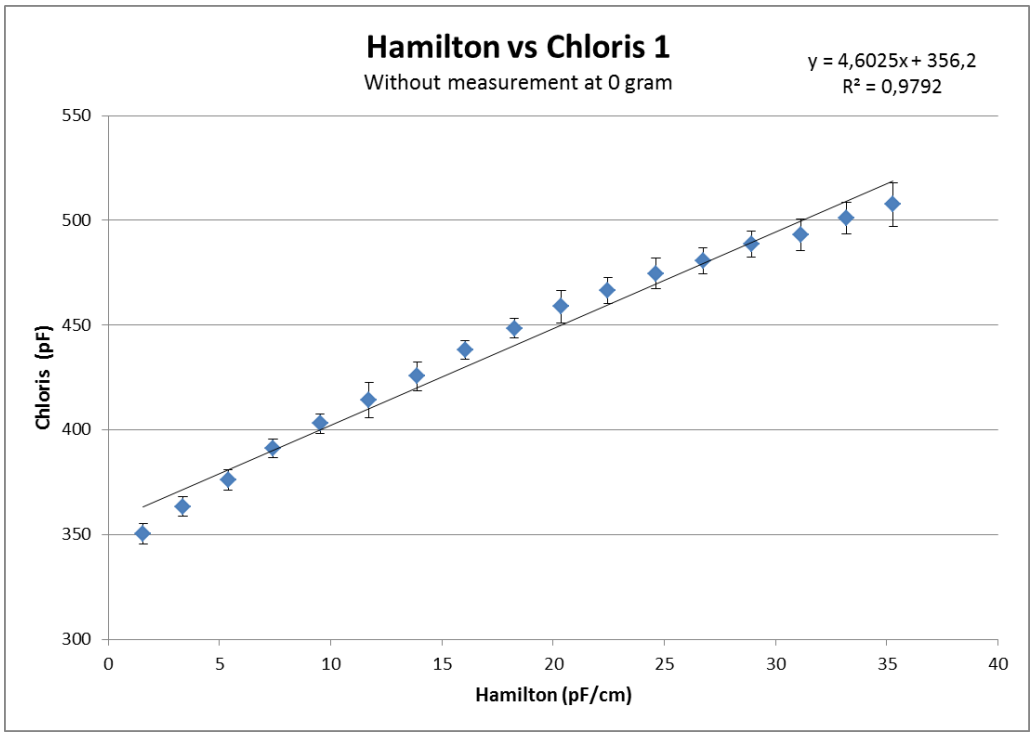


Figure 36: *Comparison of the 2 capacitance measurement systems. The error bars equals to one standard deviation.*

As seen on *Figure 36*, the data points do not fit a linear trend line perfectly, with a coefficient of determination of only $R^2 = 0{,}979$. Instead, it turns out that a polynomial regression fits a lot better, as shown in *Figure 37*.

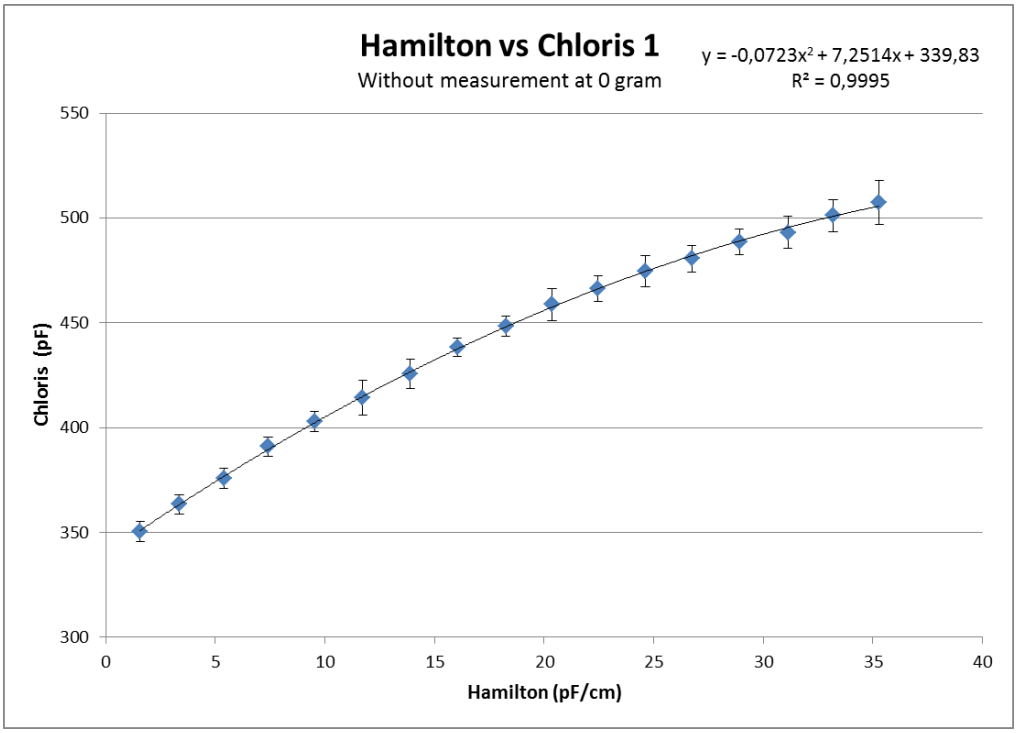


Figure 37: *Polynomial trend line of the capacitance measurements turn out to fit a lot better. The data points are exactly the same as in Figure 36.*

From *Figure 37*, it can be seen that when the concentrations of powder increases, the measured capacitance becomes relatively lower than expected. It would have been expected that the capacitance measurement would increase linearly, but this is not the case since electrical interaction between the particles increases with higher concentrations.

So if the capacitance measurements follows a polynomial, how come the Hamilton measurements follows a linear trend line perfectly? The answer is that Hamilton has already taken into account that the electrical interaction between particles increase with higher cell density. The actual capacitance measurements are calibrated by an algorithm/formula before they are displayed to the user. [30]

It is a problem that the Chloris measurements are not linear proportional to the cell density. It makes it difficult for the user to interpret the results and determine the actual viable cell density. One way to solve the problem, though, could be to linearize the function, using a Taylor polynomial.

Definition of Taylor polynomial:

Let $f(t)$ be an *n* times differential function. In this case *f(t)* is our polynomial trendline for Chloris 1.

$$f(t) = -0{,}0723 \cdot t^2 + 7{,}25 \cdot t + 339{,}8$$

This function can be differentiated 2 times, without returning zero, so $n = 2$.

The n^{th} *order Taylor polynomial* $\tilde{f}_n(t)$ *for f(t)* can be approximated near *a,* by the following formula.

$$\tilde{f}_n(t) = f(a) + f'(a)(t-a) + \frac{f''(a)}{2}(t-a)^2 + \frac{f'''(a)}{6}(t-a)^2 + \ldots + \frac{f^{(n)}(a)}{n!}(t-a)^n$$

Where $n!$ is faculty and defined as:

$$n! = 1 \cdot 2 \cdot 3 \cdot \ldots \cdot (n-2) \cdot (n-1) \cdot n$$

For example:

$$4! = 1 \cdot 2 \cdot 3 \cdot 4$$

$$4! = 24.$$

The point *a* indicates where the approximation will be most correct. The nearer our values are to the point *a,* the more precise the approximation is from the real value, as shown in *Figure 38*. *f(x)* is the original function and $f(a) + f'(a)(x-a)$ is the first order Taylor Polynomial approximation.

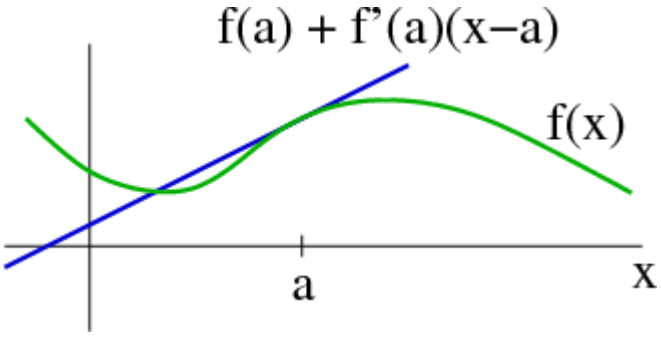


Figure 38: *First order Taylor polynomial approximation of the function f(x).* [37]

In order to get a simple function, we will approximate our function by a first order Taylor Polynomial as this correspond to a linear approximation.

For a first order, the Taylor Polynomial is given as:

$$\tilde{f_n}(t) = f(a) + f'(a)(t - a)$$

$f(a)$ is the function $f(t)$ where all variables *t* are replaced with *a*.

$$f(t) = -0{,}0723 \cdot t^2 + 7{,}25 \cdot t + 339{,}8$$

$$f(a) = -0{,}0723 \cdot a^2 + 7{,}25 \cdot a + 339{,}8$$

$f'(a)$ is found by differentiating the function $f(a)$.

$$f'(a) = \frac{d}{da}(-0{,}0723 \cdot a^2 + 7{,}25 \cdot a + 339{,}8)$$

$$f'(a) = -0{,}1446 \cdot a + 7{,}25$$

The values for $f(a)$ and $f'(a)$ are inserted in the first order Taylor Polynomial.

$$\tilde{f_n}(t) = f(a) + f'(a)(t - a)$$

$$\tilde{f_n}(t) = -0{,}0723 \cdot a^2 + 7{,}25 \cdot a + 339{,}8 + (-0{,}1446 \cdot a + 7{,}25) \cdot (t - a)$$

In this experiment *a* is chosen to be 15 pf/cm, as is lies somewhat near the middle of the graph. Replacing *a* with 15 gives:

$$\tilde{f_n}(t) = -0{,}0723 \cdot 15^2 + 7{,}25 \cdot 15 + 339{,}8 + (-0{,}1446 \cdot 15 + 7{,}25) \cdot (t - 15)$$

$$\tilde{f_n}(t) = 5{,}0824 \cdot t + 356{,}066$$

This is the function for the first order Taylor polynomial.

The two functions $f(t)$ and $\tilde{f_n}(t)$ are plotted together in *Figure 39.*

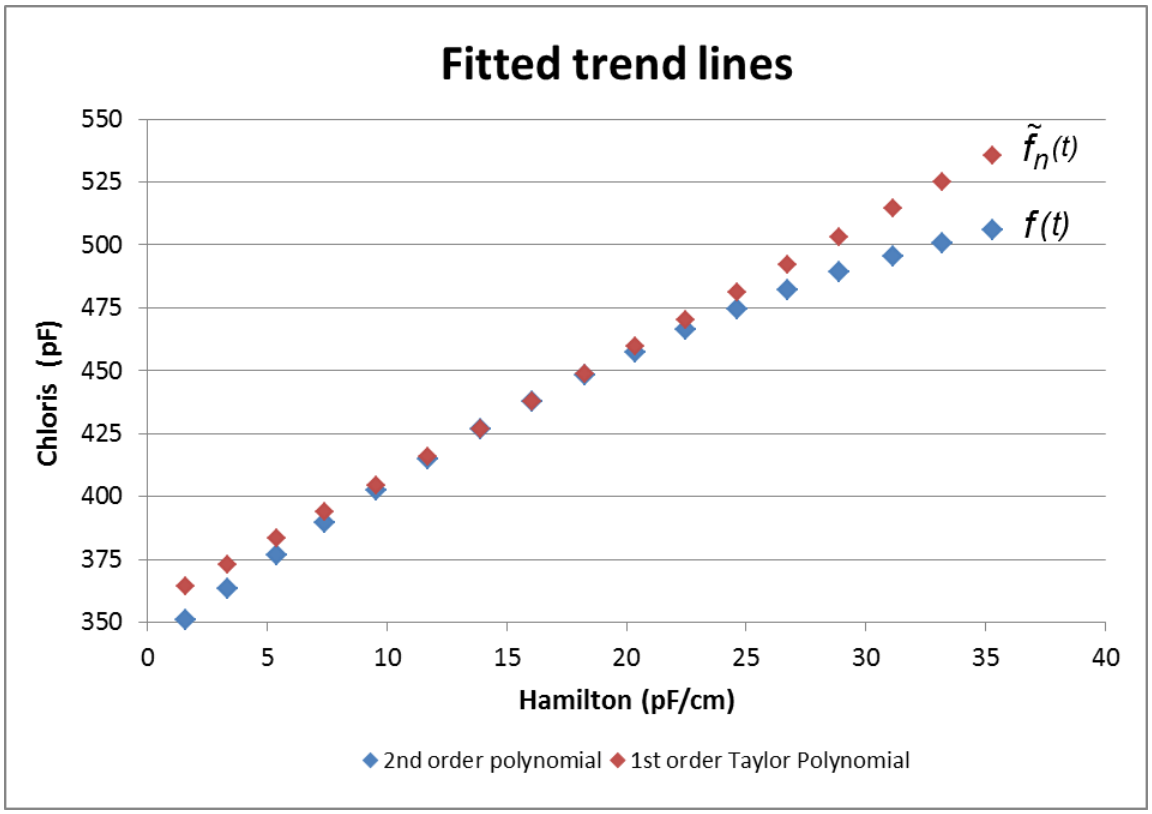


Figure 39: *Graph of the two calculated trend lines.*

The difference between $\tilde{f}_n(t)$ and $f(t)$, are added to the original capacitance measurements from Chloris 1. This is done for all data points. The linearization of the capacitance measurements are now complete and can be seen in *Figure 40*.

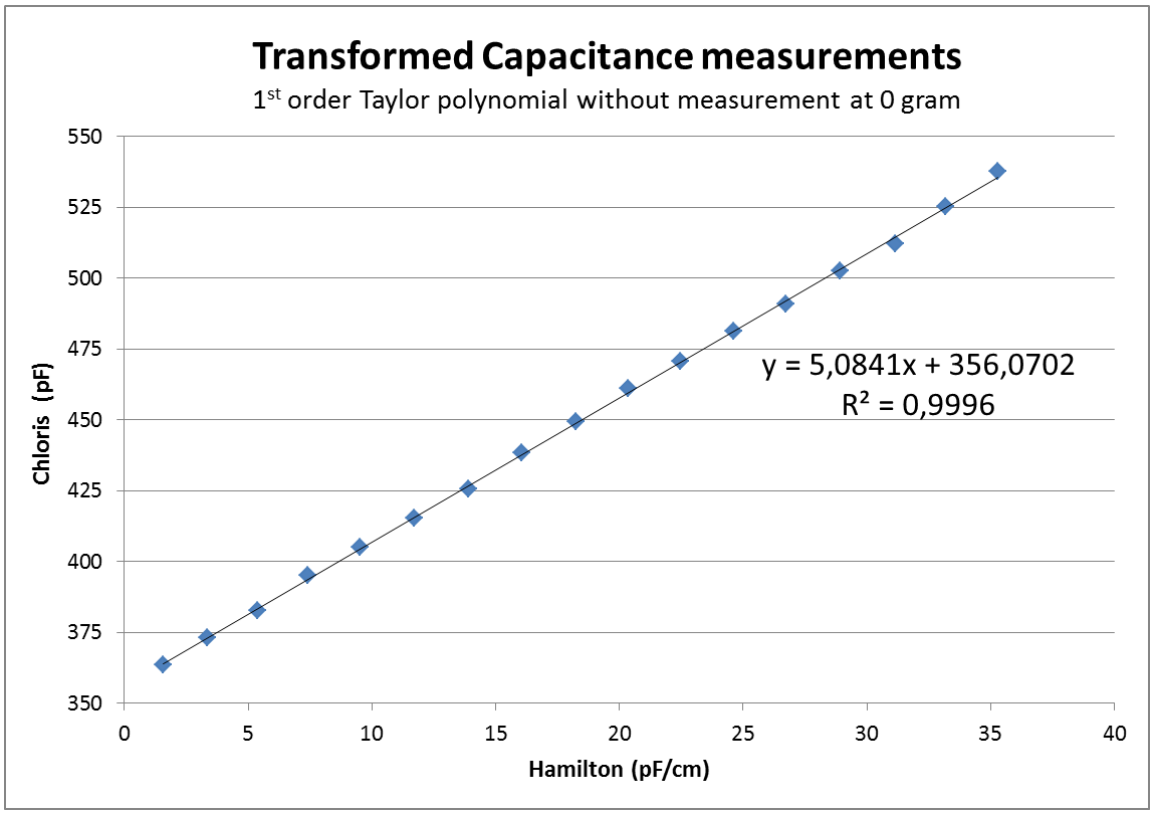


Figure 40: *Transformed data of the capacitance measurement from Chloris 1.*

The reason why *Figure 40* has such a great coefficient of determination ($R^2 = 0{,}9996$) is because the trend line of the second order polynomial in *Figure 37* had a great coefficient of $R^2 = 0{,}9995$. If the Chloris measurement did not fit a second order polynomial, the transformation would not have been nearly as successful.

This result shows that, at least for SiC powder, it is possible to calibrate/adjust the capacitance measurements to get a linear correlation between the capacitance measurements and the actual cell density in the bioreactor.

For Chloris 2, the procedure is the same for calibrating the measurements. The trend line for the second order polynomial had a coefficient of determination of $R^2 = 0{,}9959$.

Problems with the Chloris system

Since the Chloris system is still in its development phase, it is to be expected that software errors or similar problems can occur. This section is dedicated to show the flaws that have been found for the Chloris system.

Outliers

Sometimes, the Chloris system produces measurements that are completely incorrect from the other measurements. Figure 41 is an example of a randomly produced outlier in the measurements. No explanation for this problem has been found. In this example, the outlier happened for Chloris 2, but similar outliers have also been produced from Chloris 1, as shown in Figure 41.

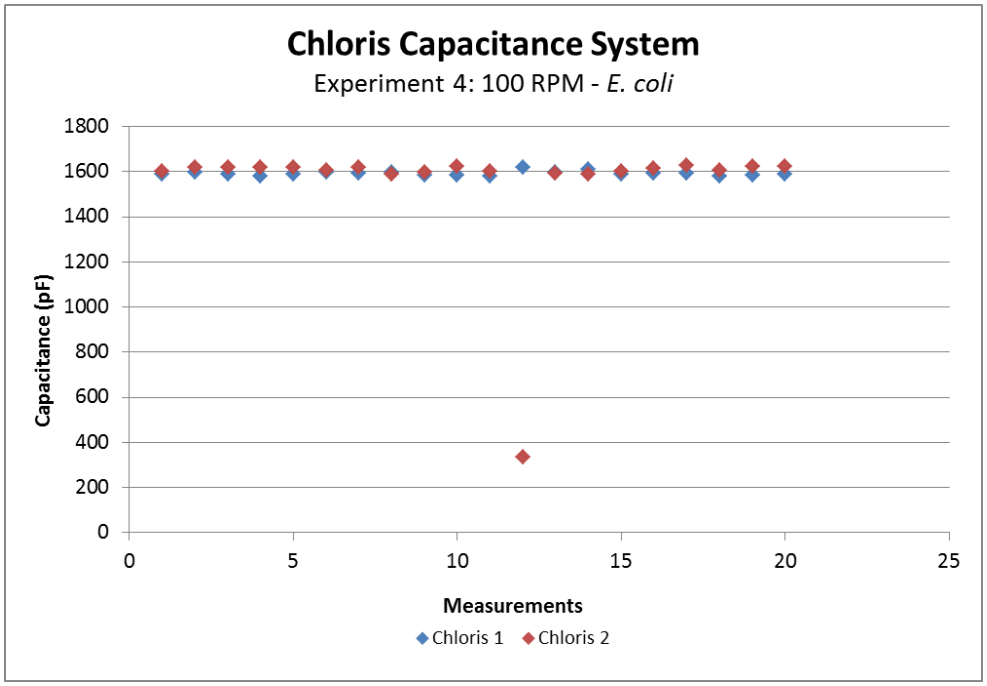


Figure 41: One of the measurements from Chloris 2 produced an outlier.

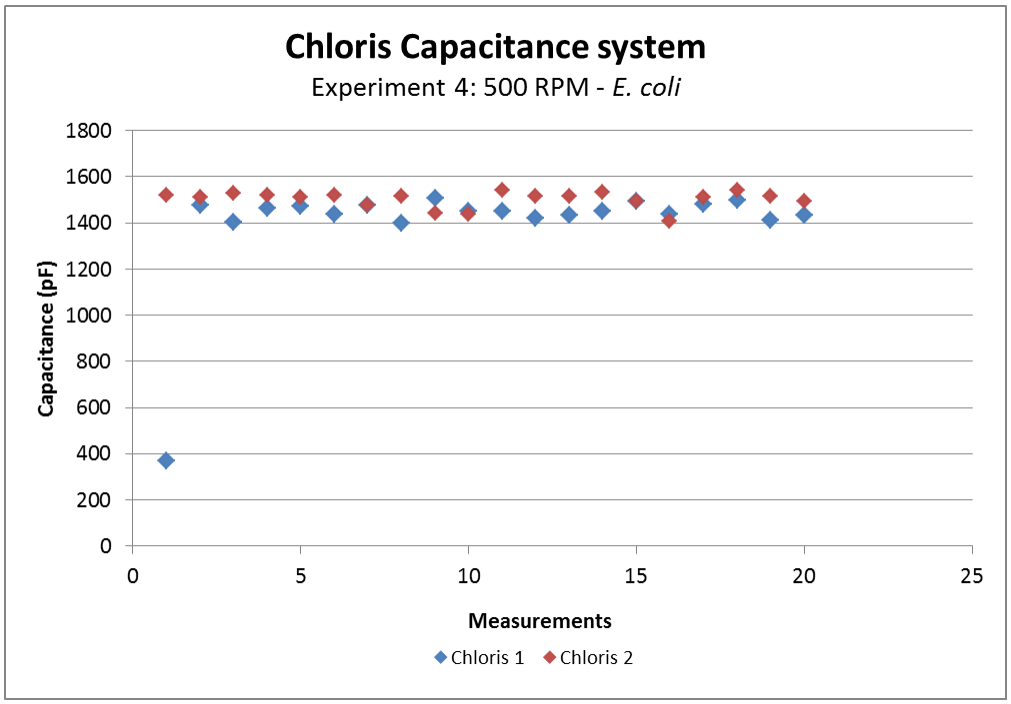


Figure 42: One of the measurements from Chloris 1 also produced an outlier.

Software problems

Most of the work needed for the Chloris system to become a commercially viable product is actually improving the user interface and debugging. This has not even been mentioned in this thesis, before now, but it is mostly fixes that will make the program more accessible. The current version is not very user friendly, and you cannot directly see the growth/capacitance trend. You will need to export the data into an Excel file first and then analyze the data to get the desired graphs. There are other general software problems such as:

- Program can **only** run on Windows 7.
- Pressing the "Stop" button, to stop collecting data will crash the program 100% of the time. (And you will not be able to retrieve your data.) This means that you have to time when you want to make measurements very precisely. Let us say you want to take capacitance measurements for 1 hour. In that case, you will have to wait 1 hour before you can do anything else. You cannot stop the program and you cannot prolong the time you want to take samples, either.
- It is not possible to open multiple measurements in the program, after you have saved them. It is only possible to open one measurement session at a time.
- If you set your computer into hibernate mode, the program crashes.
- If your computer goes into snooze mode, the program will stop collecting data. It will most of the time continue when you open up the computer again.
- The program is not always able to export all data into Excel. Sometimes you will only get some of the measurements exported into Excel.
- Changing scales on the graphs result in the scales being permanently stuck at the same settings, even when new measurements are conducted.

Conclusion

Fermentations of *Saccharomyces cerevisiae* were successfully conducted. It was possible to calculate the same μmax values with different cell density methods. OD, DW and accumulated CO_2 off-gas correlated nicely with each other, and are all feasible methods for determine the cell density and maximum specific growth rate μmax. For detecting the total cell density, all these methods give accurate measurements. Especially the OD and accumulated CO_2 off-gas method gave precise measurements and nice coefficients of determination.

The capacitance measurement method has a couple of requirements that must be met in order to measure accurately. In fermentations that require high stirrer speed, a high cell density is necessary to get accurate measurements. If the cell density is low, it's required to run at low stirrer speed to get accurate measurements.

Chances in stirrer speed during the fermentation affects the capacitance measurements. A higher stirrer speed results in lower capacitance measurements. If possible, it should be avoided to change the stirrer sped during the fermentation.

If these conditions are met, though, capacitance measurements correlate well with other cell density methods, and the correlation between cell density and capacitance measurements are linear for the Hamilton probe. For the fermentation of *Saccharomyces cerevisiae,* the estimated μmax value was $0{,}26\ h^{-1}$. This correlated nicely with OD and accumulated CO_2 off-gas, that both gave $0{,}27\ h^{-1}$.

The Hamilton probe also gave excellent for the experiment with SiC. The correlation between the amount of SiC and capacitance had a coefficient of determination of $R^2 = 0{,}9998$.

The Chloris probe, also gave a nice correlation with a coefficient of determination of $R^2 = 0{,}9996$, after a Taylor polynomial has been applied. The Single-Use capacitance system targeted for Single-Use-Bioreactors seems to work very similar to Hamilton's capacitance system. It has the same advantages as well as the same problems regarding stirrer speed. All experiments in this thesis supports that the Chloris system can indeed be used for measuring capacitance. The system requires further development, though, before a commercial version may be launched. Mainly improving user interface and fixing software bugs.

Future Work

Fermentations with other cultures

This thesis only examined capacitance measurements for the two cells, *Saccharomyces cerevisiae*, and *E. coli*. If one wants to use capacitance measurements with other cell types, it may be a good idea to make a test run first, to check if the capacitance probe is able to detect the cell density accurate enough under the desired stirrer speed.

In this project, the capacitance measurements have only been compared to **total** cell density methods. It could be interesting to compare the capacitance to other **viable** cell density methods. That way, you could quantitatively determine if the capacitance system is able to detect the death of cells. Experiment with cultivation of Chinese Hamster Ovary cells (CHO), indicates that capacitance measurements may be a bit better to detect the growth of cells than the death of cells. [38]

Chloris system

The timeframe for the Chloris system is set for a few years. As mentioned, most of the work needed is making the system more user friendly, i.e. coding and interface.

The Chloris system has been developed by researchers at IBA University who also handle development and coding of the software used to run the Chloris. The biggest user interface that would improve the system is to allow the user to view how the capacitance changes during the cultivation/fermentation (just like Hamilton does),

Further tests with the Chloris system not having linear correlation between the amount of cells and capacitance measurements should also be done. This could be done by making a fermentation with both Hamilton and Chloris probe and checking if the applied Taylor polynomial calculated in the experiment with SiC can still be used.

Appendix 1: Manipulation of data

When making experiments, you will sometimes get data points which clearly make no sense. Whether the hardware has technical difficulties, equipment makes incorrect measurements, etc.

In order to make sure this report is reliable and trustworthy, this section is dedicated to show the unedited results from the experiments. To make sure the author has not unrightfully removed data in order to make the results look nicer than they really are.

All forms of data removal or similar are shown here, that has been done in order to present the results.

Experiment 1: *Saccharomyces cerevisiae*

During the fermentation of experiment 1, the MS had a few data points that were clearly incorrect. These data points were deleted when analyzing the results. For the lag phase, the data points could just be deleted, since it did not have any impact on the results since the fermentation was still in the early lag phase. There was no significant contribution to the accumulated CO_2.

At around 23 hours into the fermentation, there were 2 data points which had an unrealistic high amount of CO_2, at 1,5 %. These data points were a bit more critical, because they were during the exponential growth rate. Therefore, the two missing data points were interpolated, which was deemed fair, since there was a clear trend line of the CO_2 content. Using the raw data would have given a gap in the exponential growth rate, which would have looked like the growth of the cells was stopped for a short while.

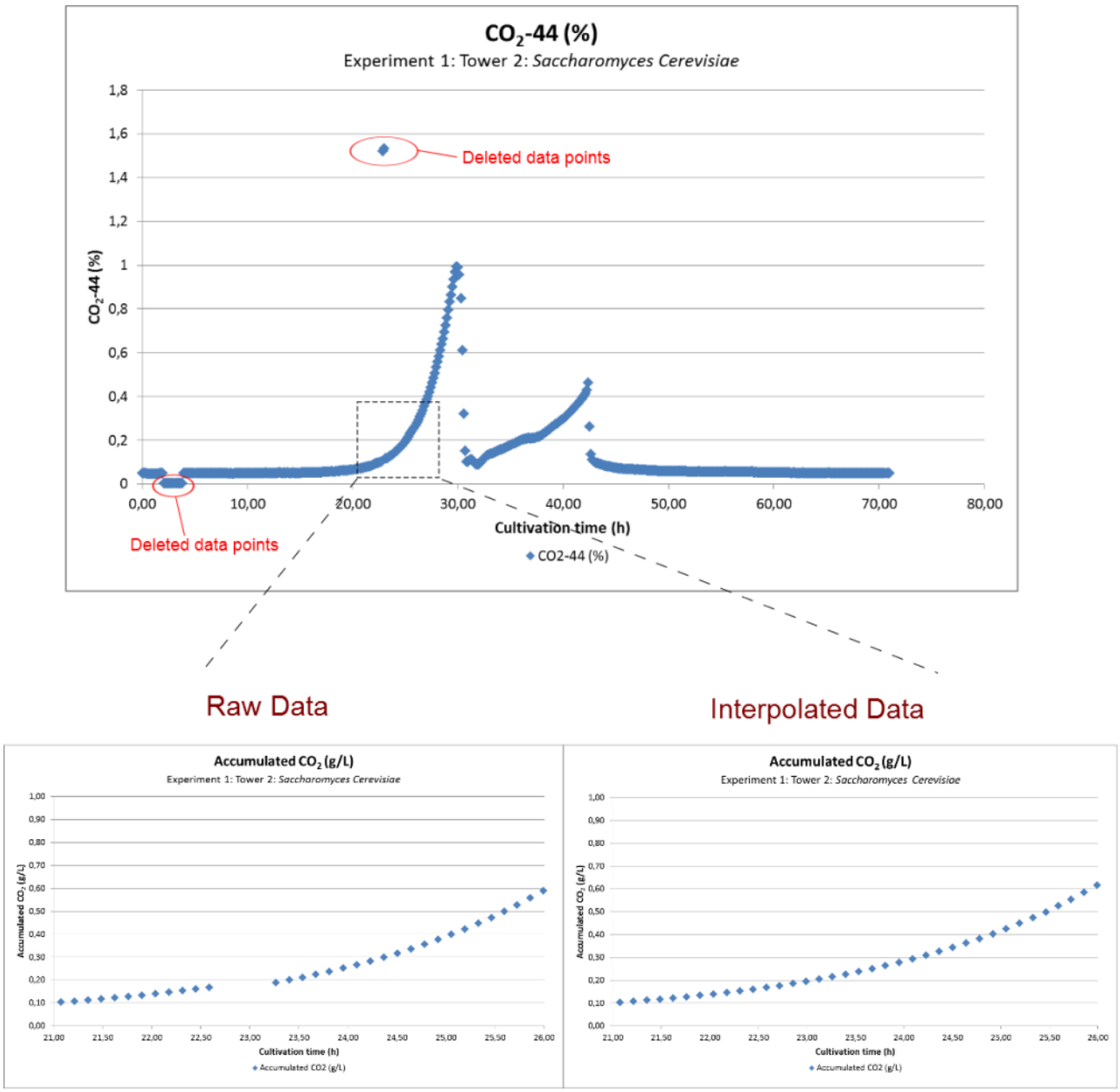


Figure 43: *Deletion and interpolation of data points.*

Experiment 3: Single-Use-Bioreactor – *E. coli*

At the end of the experiment where the capacitance was measured at different stirrer speeds, one of the Hamilton probes gave very counter-intuitive results that were the complete opposite of the other probe and the 2 Chloris probes. It is expected that a high stirrer sped gives a lower capacitance measurement, because air bubbles results in a lower cell concentration. This was not the case for the Hamilton probe 1, in fact, the capacitance measurement increased at higher stirrer speed.

It would be expected that two (supposedly) identical Hamilton probes should give the same capacitance measurements. Especially when they are both placed in the same Bioreactor and they measure simultaneously.

It seems peculiar that two Hamilton probes give results that are so different from each other. An explanation for the difference between the two probes has not been found. One guess could be that the Hamilton and Chloris measurements interferes each other. The Chloris and Hamilton probes were place quite close to each other. (Originally, it was intended to place them as far from each other as possible, but baffles made this impossible.) But it cannot explain why Probe 1 gets higher capacitance measurements when the stirrer speed is increased.

It was decided not to include results from probe 1. More experiments should be run to test if this is a repeating problem, or if it was only one instance. It's strange that this inconsistency happened for the Hamilton probe. Both Chloris probes worked fine.

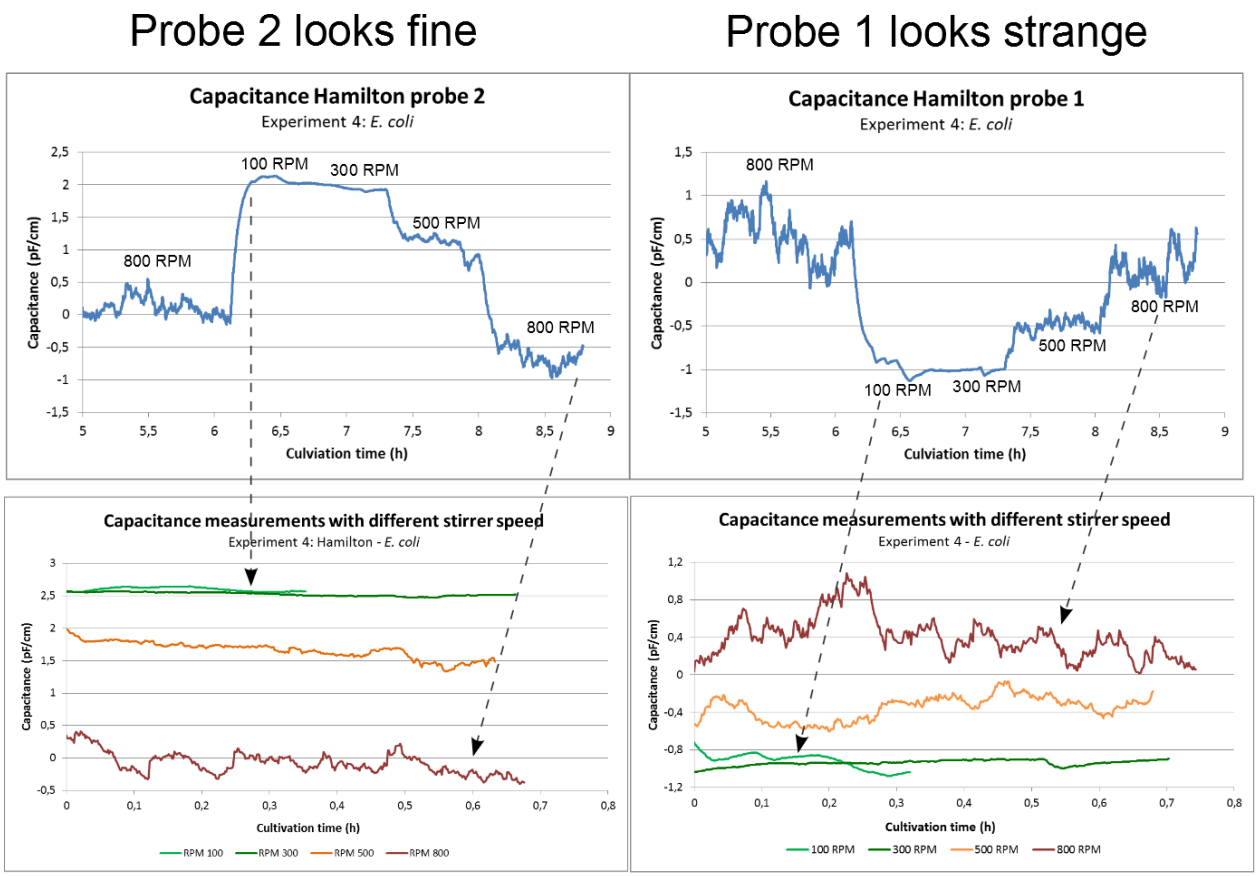


Figure 44: *Hamilton capacitance measurements at different stirrer speeds. Both probes were in the same bioreactor, and measurements were done simultaneously.*
The top pictures show the raw data measurements from Hamilton. Bottom pictures show the graphs used in the rapport.

Appendix 2: Media Composition

Minimal CBS medium:

The minimal CBS medium was used for the two first experiments for fermentation of *Saccharomyces cerevisiae*. This minimal CBS medium was used for both the shake flasks and fermenters.

Compounds

- 5 g/L $(NH_4)_2SO_4$
- 3 g/L KH_2PO_4
- 0,5 g/L $MgSO_4 \cdot 7H_2O$
- 1 ml/L Trace metals stock solution
- 50 µL/L Sigma 204 antifoam

Autoclaved separately

- 11 g/L $C_6H_{12}O_6 \cdot H_2O$

Added after Autoclavation

- 1 ml/L vitamin (d-biotin 50 mg/L)

The vitamins are added through a sterile filter, just before inoculation.

LB-media for *E. coli*

The LB-media was used for the experiment with *E. coli.*

The following medium was used for both the shake flasks as well as fermenters

- Bacto Tryptone: 10 g/L
- Bacto Yeast extract: 5 g/L
- NaCl: 10 g/L
- Sigma 204 antifoam 50µL/L

Everything is mixed together and autoclavated together.

Appendix 3: Fermentation of *Saccharomyces Cerevisiae*

This following guide is done in chronological order, thus make it practically easier to follow, should one wish to reproduce the results. This guide is based on the very first fermentation in this project that had the following specifications:

Table 5: *Specification of experiment 1.*

Specifications: Experiment 1 - *Saccharomyces cerevisiae*	
Date	14/9 - 17/9-2015
Cell type	*Saccharomyces cerevisiae*
Fermentation type	Batch Fermentation
Fermenter Material	Steel
Working Volume	2 L
Actual Volume	1,8 L
Number of towers	2
Starting concentrations (OD)	0,001
pH	5
^{o}C	35
Air inlet (L/min)	1,8
Stirrer Speed (RPM)	800
OD	✓
DW	✓
Capacitance	%

Time schedule for the fermentation can be seen in *table* Days highlighted with light blue indicate days where the actual fermentation took place. Data acquisition from the fermenters began, right after the yeast *Saccharomyces Cerevisiae* was inoculated in the fermenter.

The next offline sample was taken the next morning. From then on, samples were taken with an interval of 1,5 hour suntil the first exponential growth stopped.

Samples were then taken at interval of 3 hours until the second exponential growth stopped.

Pre-Preparation

- Set a couple of shake flasks and pipettes up for autoclavation.
- Filters for dry weight measurements are dried in the microwave, cooled in desiccator and their weight is noted. Remember to write a number on each filter, and place lint free paper in the microwave to avoid the filters from sticking to the surface.

Plate streaking

In a LAF bench, a colony of *Saccharomyces Cerevisiae* was spread over a section of the LB plate as shown in *Figure 45*. Only one colony was placed at the beginning of streak 1.

After the plate streaking, the LB plates where placed in a heating cabinet at 30 ℃.

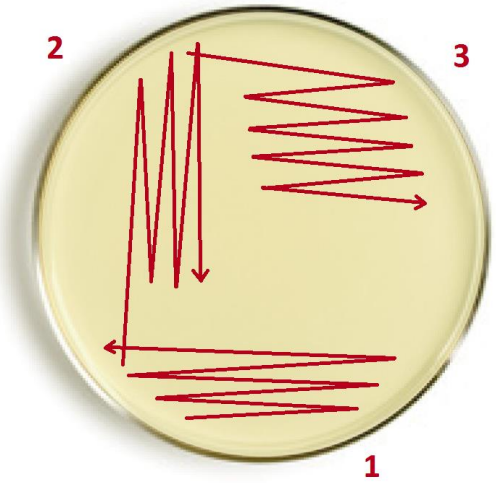


Figure 45: *Plate streaking on a LB plate.*[39]

Setup of reactors

The reactors were assembled using SOP 1.1 from experimental fermentation technology.

The top part for the fermenter is show in *Figure 46*. The “screw for autoclavation“ is put loosely on top during the autoclavation to prevent overpressure. When the fermenter is taken out from the autoclave, the screw is immediately put back on to prevent contamination.

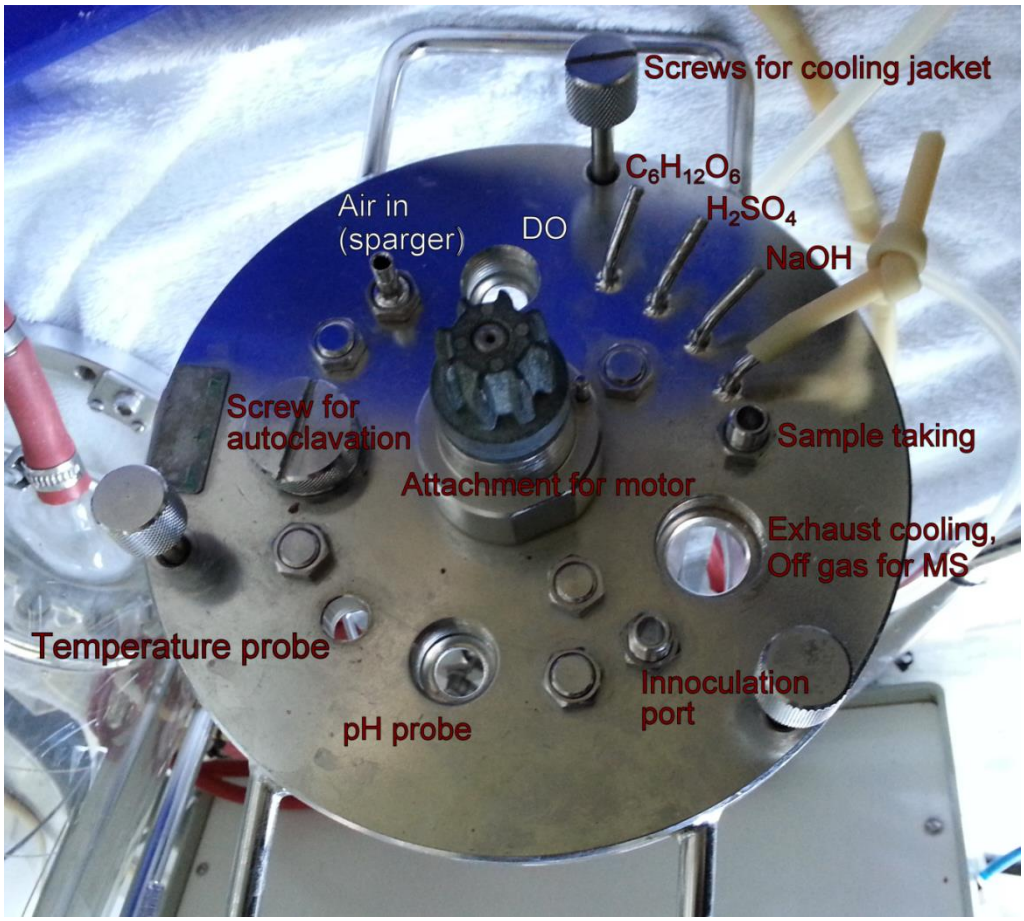


Figure 46: *Top part of the fermenter for the first experiment with Saccharomyces cerevisiae.*

Preparation of medium

The medium was made using SOP 1.4 for a minimal CBS medium. Glucose was in separate blue cap bottles with amounts so the final total concentration in the fermenter amounted to 10 g/l. (The actual glucose concentration from HPLC was measured to be 10,8 g/L.)

Shake flasks, and autoclavation of fermenter

The fermentation medium was added to the fermenter and pressure checked. After validating that there were no leaks, the fermenters were autoclaved. After the autoclavation had ended, the “screw for autoclavation” was put back on immediately and vitamins were added to the fermenter via the inoculation port.

Two shake flasks were added for cultivation medium + glucose solution + vitamins. One cell colony was taken from the LB plate and transferred to each shake flask. The shake flasks were set over for cultivation.

Inoculation of fermenter

Around 6 PM, inoculation of the fermenters began. First, a small sample from the shake flask (around 1 ml) was taking out for OD measurements. This was done to calculate the volume that needed to be added to the fermenter in order to give a starting concentration of OD = 0,001. The reason for using such a small starting concentration was to have a long lag phase, since sample taking first started the following morning.

The calculation for the volume of inoculum that needed to be added to the fermenter was based on the formula:

$$c_1 V_1 = c_2 V_2$$

c_1 and V_1 is the concentration and volume of the shake flask, and c_2 and V_2 is the concentration and volume of the fermenter respectively.

The working volume for the fermenter is $V_2 = 1{,}8\ L$

The desired initial concentration in the fermenter is $c_2 = 0{,}001\ (\mathrm{OD})$

The OD value for the first shake flask was: $c_1 = 2{,}13\ (\mathrm{OD})$

The volume added to the fermenter is calculated to:

$$V_1 = \frac{c_2 V_2}{c_1}$$

$$V_1 = \frac{0{,}001 \cdot 1800\ ml}{2.13}$$

$$V_1 = 0{,}845\ ml$$

Therefore, 0,845 ml of the *Saccharomyces cerevisiae* solution from the shake flask was added to the fermenter via the inoculation port. This was done using a syringe.

All sample taken from the shake flask were done under sterile conditions in a LAF bench.

Sample taking

The following morning at 10:59 AM, the first sample was taken. The time of the first sample was based off the amount of CO_2 in the off-gas, calculated by MS. As a rule of thumb, the exponential phase roughly begins when the amount of CO_2 is around 0,9 %

There were samples being taken for the following measurements:

- 1 High-performance liquid chromatography (HPLC)
- 2 Dry weight (DW)
- 1 Optical density (OD)

For the first exponential phase, samples were taken in intervals of 1,5 hours.
When the second exponential phase began, samples were taken in intervals of 3 hours.
The sample taking for OD, DW and HPLC lasted 27 hours, as shown in ***Figure 6***. The sample taking ended slightly early, compared to when the second exponential phase ended.

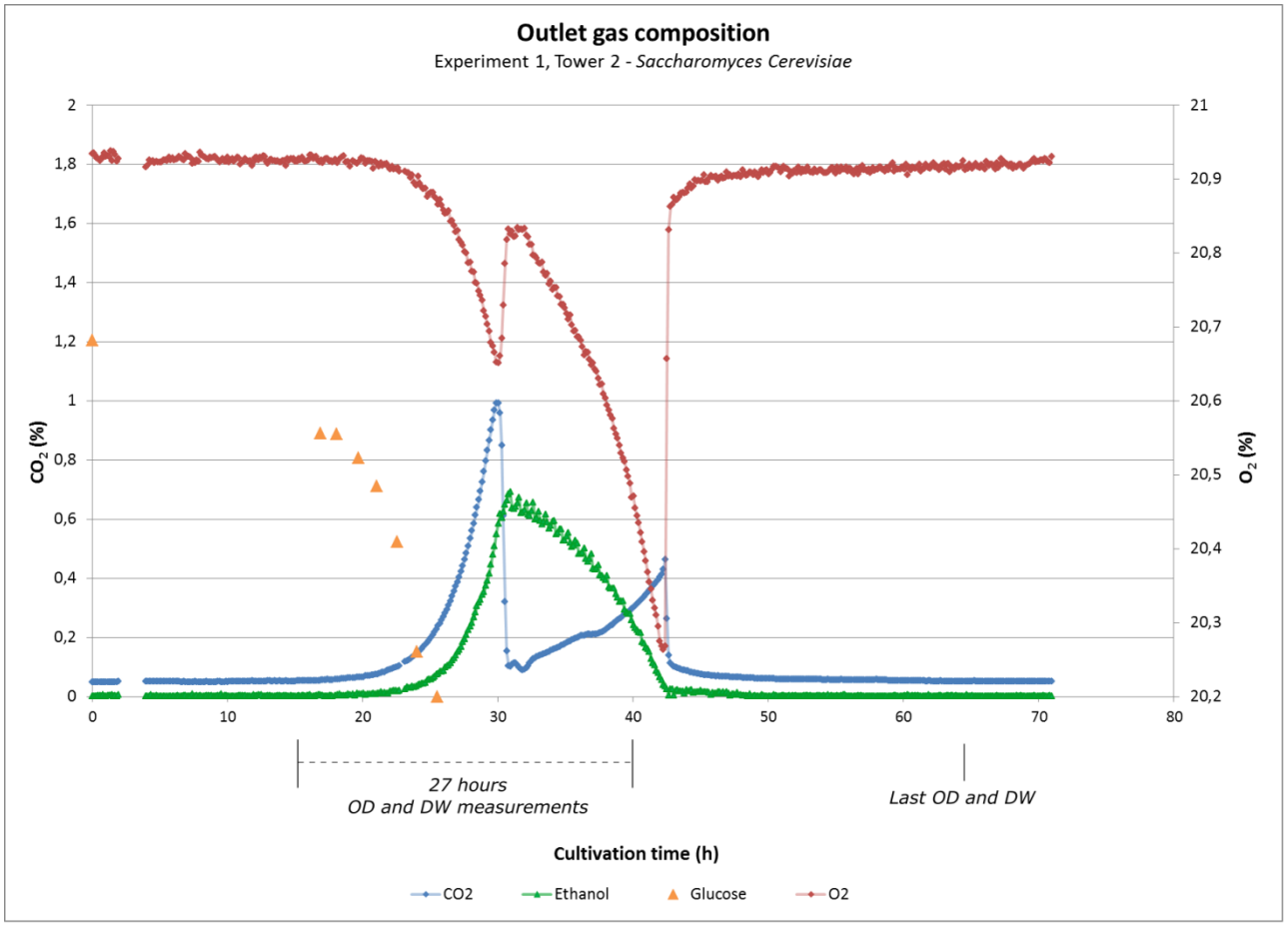


Figure 6: *Outlet gas composition for experiment 1. After 30 hours of cultivation, the first exponential growth phase is over. After 43 hours, the second growth phase is over.*

Please note that there is no corresponding y-axis for ethanol or glucose. The maximum amount of ethanol in the outlet gas was measured to have a composition of 0,048 %. The start concentration of glucose was measure with HPLC to 10,8 g/L glucose.

Dry weight measurements

Preparation

First a small filter is heated in the microwave for 20 minutes, at a low frequency. After the heating is done it is immediately put into a desiccator to cool. After 30 minutes, the filter is weighed.

Measuring your sample

First around 1-2 ml. of sample is taken out and put into a waste bottle. This is because the liquid stuck in the tube is not a representative sample of the fermenter broth.

After that, an appropriate amount of sample is taken out of the fermenter. The amount needed depends on your cell concentration. If the cell concentration is low, which is the case in the start of the fermentation, a large volume of sample is needed in order to get accurate measurements. Note the amount of sample taken so the dry weight can be expressed in g/l.

Under a vacuum, the sample is filtered through your filter. The filter is rinsed with demineralized water, to make sure only cells are on the filter. Afterwards the filter is again heated in the microwave for 20 minutes at low frequency and immediately afterwards put into a desiccator to cool. After 30 minutes the filter is weighed.

The mass of the cell is calculated as the difference of the filter's mass after and before. To get the dry weight expressed in g/l, the volume of the sample is divided.

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