# Estimation of cell viability with high-resolution growth curves

*Master thesis developed under the Erasmus program*

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# Abstract

Fuel grade bioethanol is produced through distillation of a liquid product which comes from fermentation of the sugars constituting biomass (Petrou, 2009). However, fermentation of lignocellulosic materials presents some challenges, one of them being the toxicity of the substrate for the fermenting organism. Toxic effects of lignocellulosic raw material inhibitors and their effect on the metabolism and viability of the fermenting organism is a major research subject. The work presented in this report aimed at developing a superior method for the determination of the fraction of viable cells in a yeast population.

The method is based on growth curve determinations performed with the Bioscreen C MBR instrument. This equipment is a tool for microbiology optimized for growth analyzes (Bioscreen, 2011).

Experiments carried out with Bioscreen allowed developing a method to perform optimal growth curves in this equipment after some experimental parameters were optimized. The best ones are continuous shaking with high intensity and initial optical density = 0.10. The temperature and the volume were set to 30 °C and 145 μl, respectively (Murakami, 2009).

In order to estimate the number of viable cells, several growth curves with different percentages of viable cells were performed in Bioscreen. Doing the comparison between two growth curves with different percentages of viable cells, it is possible to observe a "delay time" to reach a target OD. Based on this delay, it is possible to estimate the number of viable cells presented in the studied population.

Analyzing the data from the experiments with mixed populations with different percentage of viable cells it was possible to develop a method to estimate the number of viable cells in a sample. However, it was possible to successfully apply this method only for populations with percentage of viable cells between 70 and 100 %. Suggestions on future perspectives and strategies to possibly improve the method are presented.

**Keywords:** Bioscreen C MBR; Growth curve; Inhibitors; Lignocellulosic hydrolysates; Viability

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# Notation

| | |
|---|---|
| λ | Wavelength |
| k | Kinetic growth constant |
| r | Bioscreen well radius |
| $r_x$ | Rate of cell growth |
| S | Fraction of viable cells |
| t | Time |
| $t_{lag}$ | Lag phase time |
| V | Volume |
| X | Cell concentration |
| δ | Delay |
| $\mu_{max}$ | Maximum specific growth rate |

# Abbreviations

| | |
|---|---|
| CFU | Colony Forming Unit |
| OD | Optical Density |
| PL | Path Length |
| YPD | Yeast Peptone Dextrose |

# 1 Introduction

Nowadays, the world is heavily dependent on fossil fuels. It's necessary to change this paradigm in order to alleviate problems as greenhouse gases emission and the consequent global warming. A progressive shift of the society towards the use of renewable resources is a prerequisite for a sustainable development. In this context, energy and material produced from biomass are very important (Rittmann, 2008).

Biomass can be converted to different forms of energy: thermal energy, electricity and fuels (such as bioethanol, biodiesel and biogas). First generation bioethanol is produced from starch and sucrose rich raw materials, while the so-called second generation bioethanol is obtained from more complex lignocellulosic material such as wood and agricultural waste (Gressel, 2008).

Conversion of the sugars contained in the raw material to ethanol is obtained by fermentation. Most commonly, the yeast *Saccharomyces cerevisiae* is used, due to its high sugar consumption rate and high ethanol productivity and tolerance. However, commercial production of second generation ethanol is still hindered by low productivity, due to fermentation inhibition by a number of compounds present in the lignocellulosic feedstock. During the pretreatment of the lignocellulosic raw material and its hydrolysis, not only monomeric sugars are released but also inhibitors to the microbial fermentation. Therefore, yeast robustness and viability play a crucial role in the switch from first to second generation bioethanol (Gressel, 2008).

The work in this master thesis is specifically focused on the implementation of a new method for yeast viability determination, since it can be correlated with the potential of the cells to produce ethanol. The method developed should be reliable for measuring the number of viable cells during model lignocellulosic substrate fermentations.

**The Bioethanol Process**

Lignocellulosic ethanol production process can be divided into the following steps (Figure 1): biomass pretreatment, hydrolysis or saccharification, fermentation and distillation.
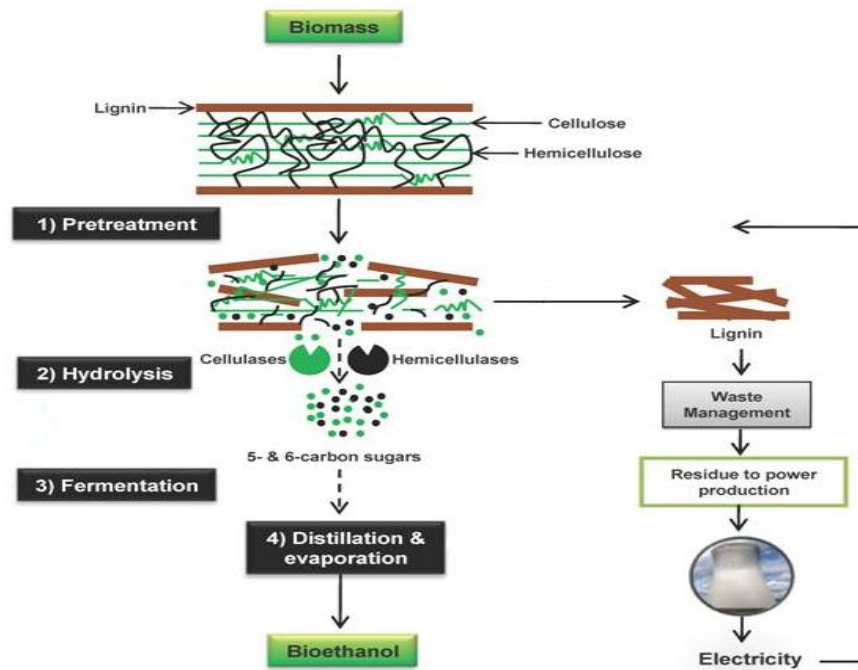
**Figure 1 -** Scheme of ethanol production from lignocelluloses (Adapted from Dashtban et al., 2009).

Prior to fermentation, plant biomass needs to be decomposed in its primary sugar components, which can be used by the fermenting organism for the production of ethanol.

The pretreatment step is not necessary in the starch or sucrose based ethanol production. This step is needed to decrease the recalcitrance of plant material to the following hydrolysis by loosening the connection among the major polymeric components of plant biomass: hemicelluloses, cellulose and lignin (Pandey, 2009). The pretreatment can be physical, chemical, physicochemical and biological and depending on the severity of the conditions used, hemicellulose sugar polymers might be already degraded to monomeric sugars at this stage. In addition, relatively harsh conditions are needed and inhibitory compounds are released or generated from other components, such as sugars degradation. In general, fermentation inhibitors are classified among furaldehydes, weak organic acids and phenolic compounds. Although to different extents, these compounds reduce yeast viability and fermentation performance.

In the next step, called hydrolysis or saccharification, sugar monomers are released from cellulose and the remaining hemicellulose polymers. This step can be done by different ways: acid hydrolysis (diluted acid or concentrated acid) and enzymatic hydrolysis.

During acid hydrolysis, along with the sugars more inhibitors of microbial fermentation are released as well. The type and amount of these inhibitors depends on the type of lignocellulosic raw material (Pandey, 2009). Figure 2 presents a schematic picture for the conversion of lignocellulosic biomass to sugars.
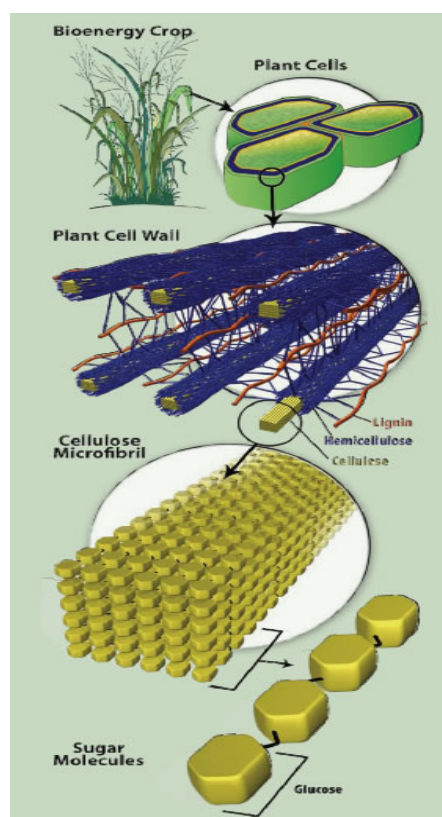
**Figure 2 -** Schematic picture for the conversion of lignocellulosic biomass to sugars (Berkeley Lab, 2011).

The sugars released during the both stages of hydrolysis process can be fermented to ethanol, which is finally separated by distillation. In particular, due to the existence of an azeotropic point between ethanol and water, the highest ethanol concentration is 95 %. However, it is possible increase that value if a drying process is included (Sousa, 2010).

High ethanol titer in the beer to the distillation column is desirable for improving process and water economy (Wringren et al., 2003). However, the inhibitory compounds contained in the hydrolysate can substantially affect and limit the fermentation rate and thus process productivity and final ethanol titer. Although the yeast *S. cerevisiae* has proven to be naturally tolerant to inhibition, more tolerant and robust strains to be employed in large scale processes are needed.

In parallel, the study of the mechanisms underlying strain robustness in order to harness them and create more tolerant strains is nowadays receiving great attention among the research community.

The aim of this thesis is to establish a method useful for understanding how many cells are affected by these inhibitors compounds or different conditions.

It is necessary to know the number of viable cells, cells able to convert the sugars into ethanol, in a certain fermentation set-up, both for fundamental research problems and process optimization. Some methods to count and estimate the number of viable cells are

already available, however, none of them is ideal. It is therefore desirable to develop a high-throughput technique for cell viability determination in lignocellulosic raw materials fermentations, since this parameter can be correlated with the metabolic capacity of the cells and therefore with potential ethanol productivity. This research project has aimed to developing this technique using the Bioscreen C MBR (Growth Curves Ltd, Helsinki, Finland).

## 1.1 Project Outline

The project developed in this master thesis can be divided in 3 different parts: method development for the Bioscreen C MBR, analysis and determination of the lag phase time and, finally, method validation for the estimation of the number of viable cells.

Initially, a method to perform optimal growth curves in the Bioscreen C MBR was developed. In this part, i) shaking parameters, ii) initial optical density and iii) an appropriate correction function to improve the measurement linearity was set.

The second part was focused on the study and the determination of the lag phase. Lag phase was induced purposefully by shifting the cells between media with different carbon sources and lag time was calculated visually and by extrapolation.

In the third and final part, the method for viability determination through curves performed on Bioscreen C MBR was assessed. First, an appropriate way to inactivate cells without destroying them and thus altering their optical properties was found and then different cell populations of increasing fraction of viable cells were created by mixing know proportions of fully viable and inactivated cells. The growth curves were performed with different fraction of viable cells and the data was analyzed in order to verify the method.

# 2 Background

Cell viability is an extremely important parameter for the study of strain robustness and for the characterization of a fermentation process. Cell viability can be measured with several methods. However, none of these methods is completely satisfactory in terms of throughput, reliability or applicability to lignocellulose hydrolysate fermentation. Therefore, the object of the present work is to develop an alternative.

## 2.1 Methods to measure viable cells

Nowadays, there are some methods to measure viable cells, such as plate count or cell staining with metabolically activated dyes like methylene blue or fluorescent dyes (FUN 1 or Funga light). In this chapter these methods will be described.

The traditional way of counting viable cells is using plating methods. The plating methods were developed in the Robert Koch's laboratory and they are very directly and simple (Fankhauser, 2005).

This method is one of the most accurate ways to measure viable cells because it's possible to have a visual contact of each cell and it only counts cells that are able to reproduce. For those reasons, the plating methods are widely used in samples of food, water and soil. In brief, a suitably diluted sample of the microbial culture is spread on plate of rich medium and the resulting colonies, developed after a suitable period of incubation, are counted. The results of this method are expressed in CFU, *Colony Forming Unit* (Willey et al., 2008). The method is inexpensive, although the dilutions needed for having a countable number of colonies and the spreading are highly labor intensive and introduce large variability and a number of replicates are needed for reliable results. In addition, colony development on plates requires several days.

The classical methylene blue staining method allows direct microscope observation of metabolically active cells. This technique is based in the following principle: when the methylene blue enters a viable cell, it is degraded to a colorless product; non viable cells don't synthesize the enzyme needed for methylene blue conversion and will remain blue. A yeast colony stained with methylene blue is shown in Figure 3.
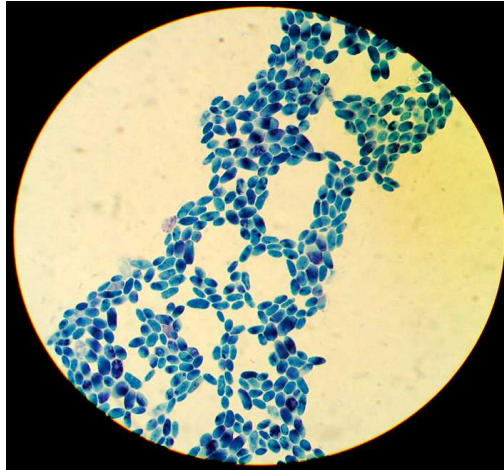
**Figure 3** – Example of a contaminated yeast colony staining with Methylene blue, illustrated at 1000x (Fankhauser, 2005).

The method is a cheap, quick and easy way to determine the viable cells present in a sample, even though it has some disadvantages. First, it is important to know that methylene blue becomes toxic to yeast and the samples should be analyzed within ten minutes. Another problem is that old cells could have residual metabolic activity but not be viable. These ones have the ability to become colorless yet and are counted as viable cells, which is not correct (Glick et al., 1998).

More recently, viable cells staining has been improved by developing commercial fluorescent dyes. When stained with the fluorescent dye FUN 1, viable cells are marked with clearly fluorescent orange-red structures, while dead cells become fluorescent too but with a different green-yellow diffuse fluorescence (Probes for Yeast Viability, 2001). In Figure 4 it is possible observe metabolically active yeast stained with FUN® 1 dye, forming numerous red fluorescent cylindrical structures within their vacuoles.
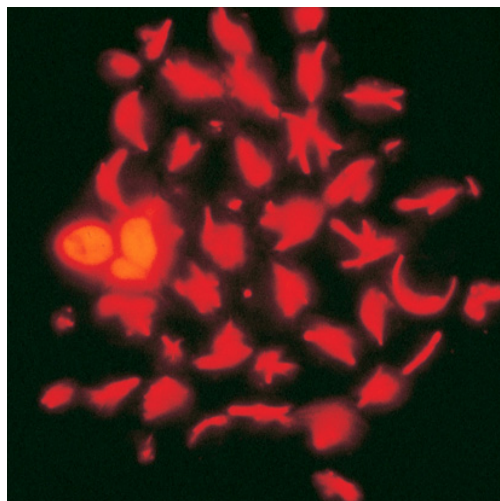


**Figure 4** – Metabolically active yeast stained with the FUN® 1 dye (Invitrogen™, 2011).

There are another fluorescent dyes, such as the commercial fluorescence method "Funga light" which includes 2 compounds, SYTO® 9 and propidium iodide. While SYTO® 9 is a green fluorescent nucleic acid stain, propidium iodide is a red fluorescent nucleic acid stain. The two compounds have different spectral characteristics and ability to penetrate healthy cells. When both dyes are used together, the yeast cells with intact membrane are detected as viable cells and appear fluorescence green. On other hand, yeast with damaged membranes emits red fluorescence (FungaLight[TM], 2005).

## 2.2 Bioscreen: Current Uses

Bioscreen C MBR (Growth Curves Ltd, Helsinki, Finland) is a tool for microbiology optimized for growth experiments. This equipment was developed by the Finnish company Labsystems in the mid 1980s. Initially, Bioscreen was used to mutagenity and carcinogenity testing systems but new applications were implemented very quickly (Bioscreen, 2011). Nowadays, this equipment is developed to analyze microbiology growth curves.

This system allows performing up to 200 growth curves in miniaturized scale. Its temperature gradient over all 200 wells is very constant and it has a sensitive reader measuring turbidity, a technique more sensitive than a spectrophotometer. Bioscreen equipment is shown in Figure 5.



**Figure 5 -** Bioscreen equipment (Bioscreen, 2011).

This system is the only one patented incubator able to maintain temperature with a 0,1°C accuracy. By analyzing high resolution growth curves obtained with Bioscreen it is possible estimate the number of viable cells in a test culture compared to a reference culture.

There are also another landmark study about growth curves in the Bioscreen C MBR, developed by Warringer and Blomberg. For this thesis work, the most important issue in their work is the equation to correct non-linearity observed above OD = 1 (Warringer, 2003). Since

the measurement start to be non linear to higher cells densities, is necessary correct the OD observed, $OD_{obs}$, with the Equation 2.1:

$$OD_{cor} = OD_{obs} + 0.449\,(OD_{obs})^2 + 0.191(OD_{obs})^3 \tag{2.1}$$

This study was also very important because they tested different intensities and ways of shaking. In their work, the best way of shaking the cells an intermittent way with intervals with 60 s shaking and 60 s resting (Warringer, 2003). In order to analyze the importance of shaking, this parameter was also tested in this thesis work with our own conditions.

## 2.3 Microbial growth curves

A microbial growth curve typically presents four phases: lag, exponential, stationary and death phase (Willey et al., 2008). Figure 6 shows a typical microbial growth curve with the different phases identified.
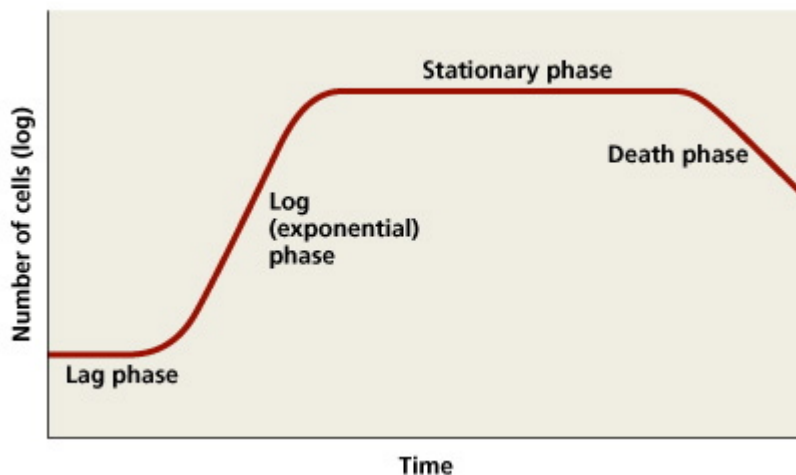


**Figure 6** – Microbial growth curve in a closed system (Bakersfieldcollege, 2011).

The first phase, called lag phase, is a period of time when cells are not dividing and increasing in number yet. This period can change considerably with the conditions of the microorganism or the nature of the medium. The next phase, exponential or log phase, is characterized for the faster cell growth. The rate of growth is constant during this period, which means the cells are dividing and doubling in number at regular intervals (Willey et al., 2008). In this phase, is possible to calculate the maximum specific growth rate which, as it will be shown later, is a very important parameter to estimate the number of viable cells in a sample.

The maximum specific growth rate is calculated assuming that in batch fermentation with ideal conditions the quantity of biomass increases with the time, Equation 2.2 (Adapted from Dunn et al., 2003).

$$r_x = k\,X \qquad\qquad (2.2)$$

Where:

- $r_x$ is the rate of cell growth (g cell/lh);
- $k$ is a kinetic growth constant (l/h);
- $X$ is the cell concentration (g cell /l).

If a batch system is considered, $r_x$ can be substituted for dX/dt, expressing the rate of change in cell concentration over the time, Equation 2.3.

$$\frac{dX}{dt} = k\,X \qquad\qquad (2.3)$$

Finally, if the previous Equation is integrated the Equation 2.4 is obtained, which describes the growth in the exponential phase.

$$\ln X = k\,t + \ln X_0 \qquad\qquad (2.4)$$

In the growth curve, the ln(OD) in the exponential phase follows a linear equation, and the slope is the maximum specific growth rate. Analyzing the Equation 2.4, the k corresponds to the maximum specific growth rate, $\mu_{max}$, while the $X_0$ is the cells concentration for time zero. The variable X, cells concentration, usually appears as OD because is the way used to measure the cells concentration.

After the exponential growth, the stationary phase starts and the growth stops e.g. for nutrient limitation, metabolic waste or oxygen limitation and, finally, in the last phase called death one, the cells are with irreparable harm. In this phase the cells are death and they can also lyse, which leads to a decrease in number. These last two phases are less important to estimate the number of viable cells.

## 2.4 Estimation of cell viability through growth curves

Some studies were already done in order to find a better way to estimate the number of viable cells through the growth curves. The most similar study was done in Department of Pathology, University of Washington by Murakami, C. and Kaeberlein, M.

In the work by Murakami and colleagues, estimation of the fraction of viable cells in a test population was used for the study of yeast chronological life span, defined as the length of time cells can survive in a non-dividing, quiescence-like state (Murakami, 2009). Populations of increasing chronological age were assessed. To develop the method, aging cultures were prepared by cultivation for several days. After that, samples from cultures maintained under constant agitation and temperature long after growth had ceased were taken every 2 or 3 days and inoculated in a rich growth medium in a Bioscreen plate.

The different growth curves obtained with the Bioscreen were plotted and the result is shown in Figure 7.
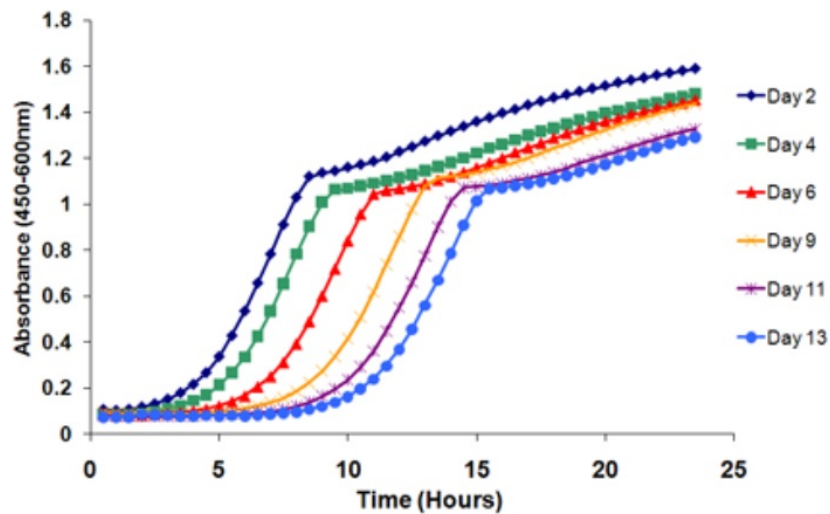


**Figure 7** – Comparison between growth curves obtained from the Bioscreen with different percentages of viable cells (Murakami, 2009).

The number of viable cells in each culture, relative to the reference culture from day 2, was estimated as a function of the "delay time" with which each test culture reached a target OD. The method used in this project is based on this delay time.

Here follows an explanation on how the fraction of viable cells in a population, S, can be determined, as a function of the specific growth rate and the delay displayed by the target population in reaching a certain OD, when compared to a reference culture with 100 % viable cells.

Given:
- $\mu_{max} = \mu_{max}'$ (Specific growth rates of the reference and test culture, $h^{-1}$);
- OD = OD' ("Target" optical density, arbitrarily chosen 0.8, 1.0, 1.2 and 1.4);
- δ (Delay time of the test culture in reaching the target OD).

Figure 8 shows two theoretical growth curves with a different fraction of viable cells. They will be compared to estimate the number of viable cells.
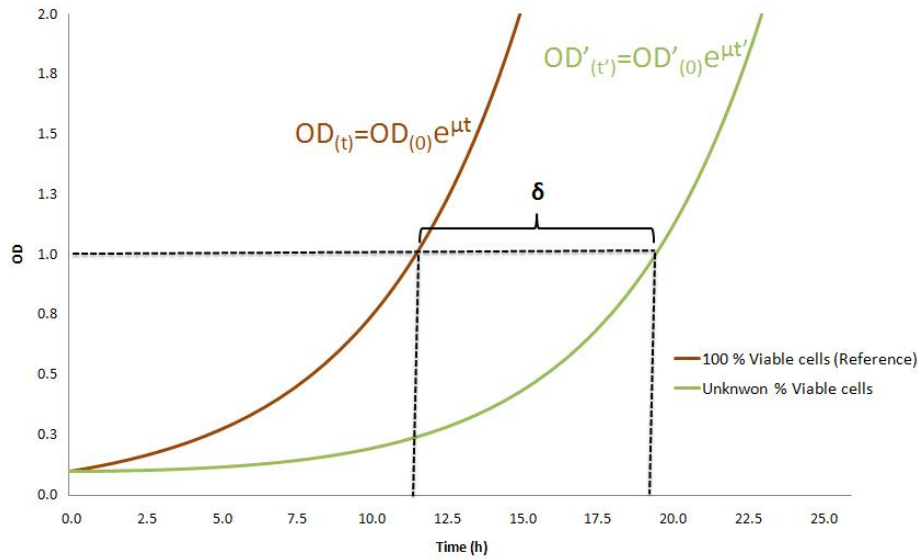
**Figure 8 -** Theoretical growth curves with different fractions of viable cells.

The growth curves can be described by the Equations 2.5 and 2.6:

$$OD_t = OD_0\, e^{\mu_{max}t} \tag{2.5}$$

$$OD_t' = OD_0'\, e^{\mu_{max}'t} \tag{2.6}$$

Since the fraction of viable cells is 1 for the reference culture (brown line in Figure 9) and S in the test culture (green curve in Figure 9), the initial OD of the test culture can be expressed as a function of the fraction of viable cells S, Equation 2.7.

$$S = 1 \ \ and \ \ S' < 1$$

$$OD_0' = OD_0 \times S' \tag{2.7}$$

If all the assumptions referred will be taken, it's possible deduce Equation 2.8 which allows estimate the fraction of viable cells.

$$OD_0\, e^{\mu_{max}t} = OD_0 \times S' \times e^{\mu_{max}(t+\ \delta)}$$

$$S' = \frac{1}{e^{\mu_{max}\ \delta}} \tag{2.8}$$

The parameter δ will be estimated using the Equation 2.5. In this case the $OD_t'$ will be the target OD and the time corresponds to the delay.

# 3 Material and Methods

In this chapter the experimental procedures will be described. In addition, the calculations and the assumptions taken are explained.

All the experiments developed were done with the yeast *Saccharomyces cerevisiae* and the specific strain used was CEN PK 113 7D (Kötter, 2000).

## 3.1 Growth media

Cells were always grown on synthetic mineral medium (Verduyn et al., 1992). Twenty g/l glucose was normally used as carbon source unless otherwise indicated. Medium composition and the experimental procedure are described on the Appendix 1.

## 3.2 Growth in shake flask

Before using the Bioscreen C MBR some experiments were carried out in shake flask to determine a benchmark value for the maximum specific growth rate, $\mu_{max}$. Growth curves were performed by following the OD with a spectrophotometer. The equipment used in this project was always the same (Thermo Scientific – Genesys 20).

### 3.2.1 Preparation of the pre-culture

The day before the experiment, a single colony was taken from a plate and inoculated in approximately 10 ml of medium. With this procedure, it was possible to start the growth curve in the shake flask with the desired initial OD, after appropriate dilution.

### 3.2.2 Cultivation in shake flasks

Cells were inoculated in mineral medium, and incubated at 30 °C with continuous shaking (200 rpm). The cells growth was aerobic with a ratio of 1/10 between the volume of the medium and the volume of the shake flask. A sample was taken each hour and the OD at $\lambda$=600 nm was measured with a spectrophotometer. Since the OD measurement was assumed to be linear only between OD 0.03 and 0.5, when needed samples were diluted in water in order to have a correct measurement.

## 3.3 Growth conditions in Bioscreen C MBR

First, a method to obtain complete growth curves in Bioscreen C MBR was established. In particular, initial cell density (OD) and shaking mode were optimized (see results section). All the experiments were done under the standard conditions defined in this phase. The conditions are continuous shaking (high intensity), initial $OD_{Bioscreen}$ = 0.10, Temperature = 30 °C and Volume = 145 µl.

A multiwell plate with mineral medium and cells from a pre-culture was prepared as it follows. With a multichannel pipette, the wells were filled in with the medium, Figure 9. The cells of the pre-culture were inoculated, one by one, with a single pipette.
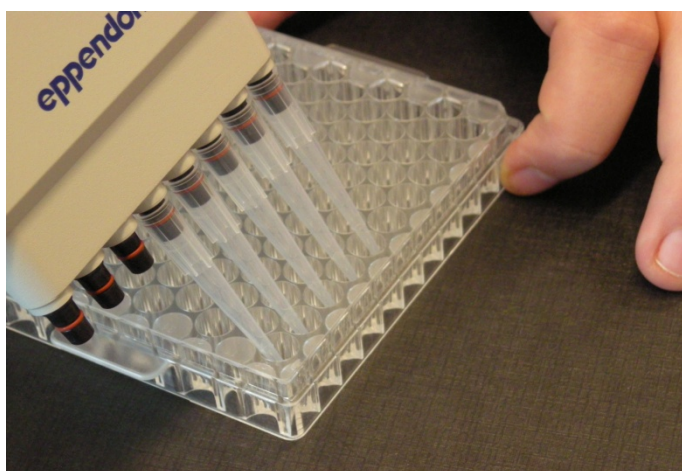


**Figure 9 -** Prepare the multiwell plate.

It's important to note that it is necessary to have some wells only with medium to be used as blank.

## 3.4 Calculation of growth characterization parameters

- *Maximum specific growth rate ($\mu_{max}$):*

OD was plotted over time and the maximum specific growth rate was calculated through an exponential least square fitting of the data points in the exponential growth phase. Equation 2.5 was used.

Growth in the Bioscreen was obtained in 10-plicated, meaning that each time 10 independently inoculated wells were measured. At each time point, the average of the 10 OD values was calculated. Optical path length and the linearity correction were applied to the vector of average values and Equation 3.1 was applied to the exponential part of the average growth curve. Then, the specific growth rate was determined.

- *Lag phase time induction:*

In this project, the lag phase was induced by changing the cells medium: yeast was precultivated in glucose-based medium, while growth curves in shake flask or Bioscreen were performed in galactose based medium (Appendix 1). Between the medium shift, the cells were centrifuged and washed with NaCl 0.9 % (w/V).

- *Calculation of Lag phase time ($t_{lag}$):*

In order to have a reliable method to estimate the number of viable cells, it's central to know how estimate the lag phase length, which has to be subtracted to obtain a net delay time, $\delta$.

The lag time was obtained by two different methods: by extrapolation and visually, Figure 10.
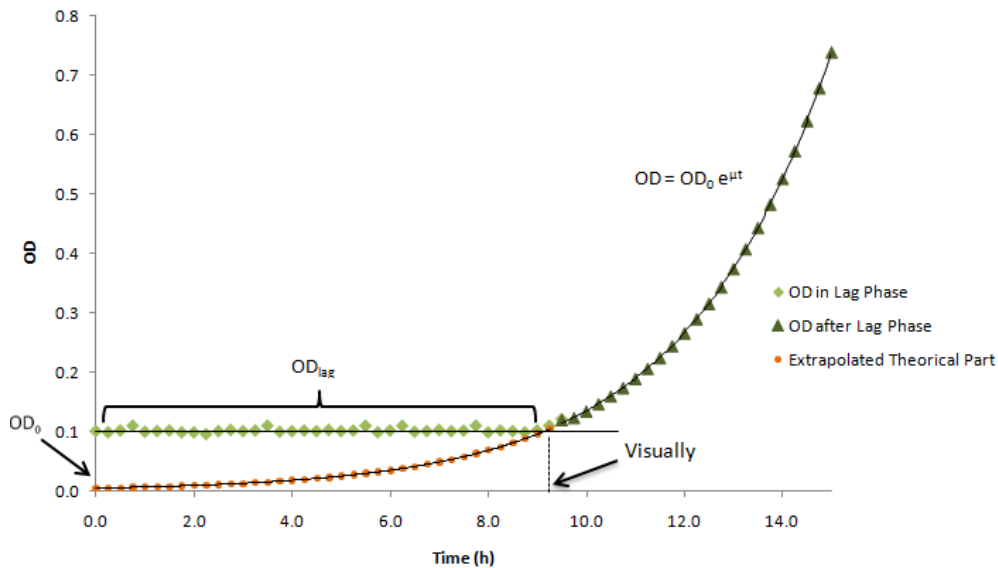


**Figure 10 -** Lag Phase time calculated visually and parameters to calculate it by extrapolation.

The extrapolation method is based in the function that describes exponential growth. To calculate the lag phase time, some parameters in the Equation 2.5 were replaced and it was changed in order to $t_{lag}$. The Equation 3.1 is now used to estimate the lag phase time.

$$t_{lag} = \frac{\ln\left(\frac{OD_{lag}}{OD_0}\right)}{\mu_{max}} \qquad (3.1)$$

To estimate $OD_{lag}$ the average of OD values until it starts increasing was taken (Figure 10). The parameters $OD_0$ and $\mu_{max}$ were determined by the method already explained.

At higher $OD_0$ it's also possible estimate the lag time visually, observing the increase in the OD value.

## 3.5 Preparation of Mixed cell populations

To check the method viability, populations with different percentage of viable cells will be done. In order to create them, viable cells and inactivated cells will be needed.

The viable cells will be prepared by the same way described in section 3.1.1, in a pre culture.

On other hand, it is necessary an efficient method to inactivate the yeast. First, some samples of 1 ml will be taken from the initial pre culture and then they will be subject to high temperatures: 80 °C during 5 minutes. This method will inactivate them and, after check in the microscope, they look similar but they will not grow anymore.

To create the mix populations is just to mix the both viable and inactive cells in the desired proportions (between 0 and 100% with increments of 10 %) and the inoculate them either on shake flask, Bioscreen or plate.

### 3.5.1 Plating Method

The plating method was used as control, to be sure that the populations inoculated on the Bioscreen have the desired percentage of viable cells. This method was also important to check if the method to inactivate yeast works well.

The plates were always done in duplicate and the respective pre-culture was diluted, in fresh medium, 30 000 X. 100 µl of the diluted pre culture were spread on the surface of solidified glucose-based medium (See composition in Appendix 1) and incubated at 30 ℃ during 48 hours. In parallel with the Bioscreen test, plates were always done.

# 4 Results

Method viability: Analyze mixed cells populations

2. A conversion factor to extend the linearity range of OD measurements

Estimate the equation to extend the linearity range of OD measurements

Calibration: Bioscreen C MBR vs Spectrophotometer

2. The parameters to perform optimal growth curves in the Bioscreen were defined. In this part, a comparison between Bioscreen and shake flask growth curves was done as well.

Method to create non viable populations

This is a parameter very important to estimate the corrected delay between two growth curves, to reach a certain OD.

Analysis of the linearity range

3. Find an efficient method to inactivate cells in order to create mix cells populations

Setting the basic parameters on Bioscreen

Following the organization of the experimental work, this section is divided as it follows:

1. Since two different equipments were used to measure OD, a conversion factor allowing to compare OD measurements between the spectrophotometer and the Bioscreen was determined.
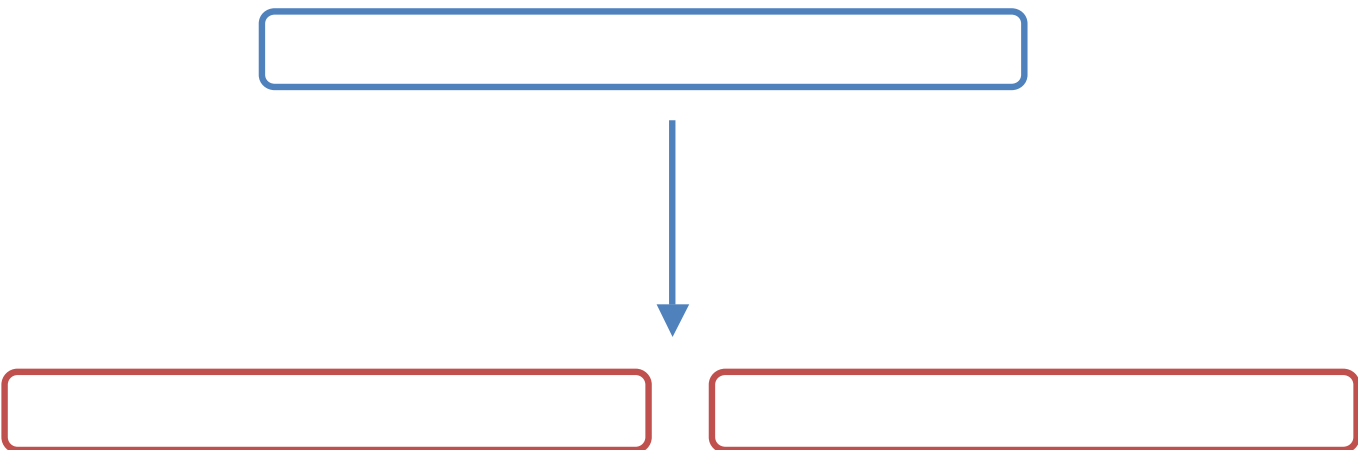
At this stage, the method to perform optimal growth curves in Bioscreen was optimized and it was possible to start with the method to estimate the number of viable cells.
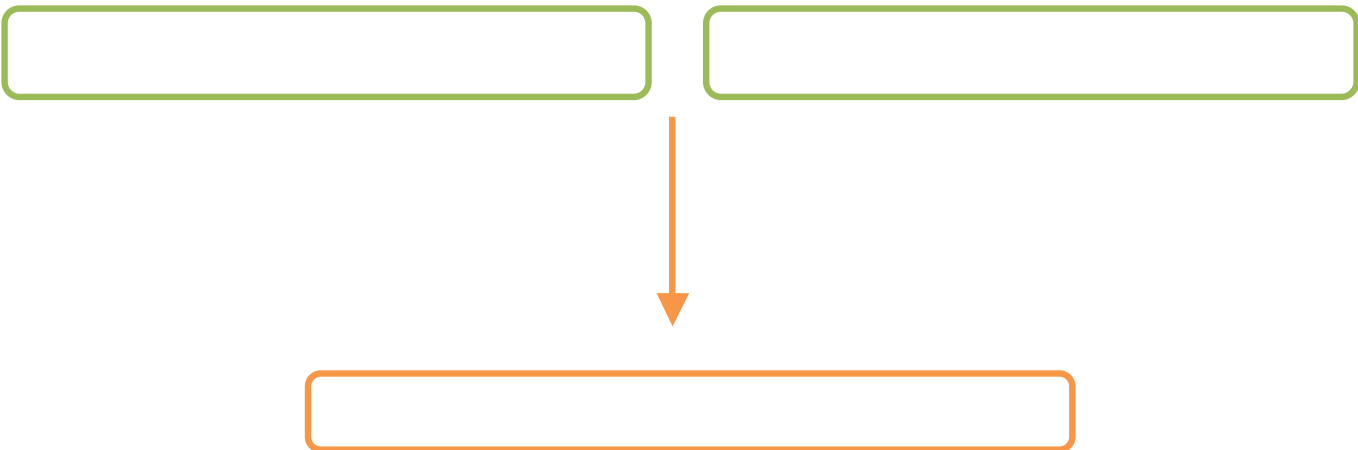
4. Finally, the growth curves obtained with mixed populations were analyzed which allowed to estimate the fraction of viable cells in each sample tested.

## 4.1 Calibration: Bioscreen C MBR *vs* Spectrophotometer

In this project two different instruments were used to measure OD: the Bioscreen C MBR and a regular bench-top spectrophotometer. The optics of the two instruments have slightly different response to OD and, in addition, while OD is measured in the spectrophotomer at λ=600 nm, the Bioscreen uses a wide-band filter, with λ = 420-580 nm. Furthermore, while the light path in the spectrophotometer is constant and equal to 1 cm, in the Bioscreen it is perpendicular to the multiwall plate, meaning that the path length in each well is dependent on the volume it is filled with, Figure 11.
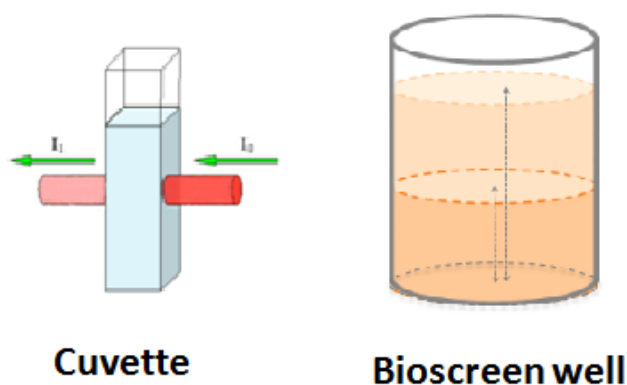


**Cuvette**          **Bioscreen well**

**Figure 11**- Path length's difference between spectrophotometer and Bioscreen.

In order to calculate the path length in the Bioscreen, the Equation 4.1 is used:

$$Path\ length\ [cm] = \frac{Volume\ [mL]}{r^2\ [cm] \times \pi} \qquad (4.1)$$

The radius of the Bioscreen well (r) was measured and it is 0.35 cm.

Due to these factors, for the same nominal cell density different OD readings were obtained. In order to facilitate the inoculation calculations and procedure, preliminary experiments were done in order to establish the relationship between the measurements of the two instruments.

To determine the relation between the measurements obtained with the two instruments, 6 samples were prepared with OD in the spectrophotometer between 0.05 and 0.3 and their OD was also measured in Bioscreen C MBR. The measurements in Bioscreen C MBR were done with three different volumes, and therefore path lengths: 100, 200 and 300 μl, corresponding to 2.6, 5.2 and 7.8 mm, respectively. Table 1 shows the results obtained as well as the respective error.

**Table 1** – OD measurements on Spectrophotometer and on Bioscreen with different path lengths (PL).

| $OD_{Spectro}$ | $OD_{Bioscreen}$ | | |
|---|---|---|---|
| PL = 10 mm | PL = 2.6 mm | PL = 5.2 mm | PL = 7.8 mm |
| 0.053 ±0.007 | 0.108 ± 0.074 | 0.063 ± 0.021 | 0.084 ± 0.056 |
| 0.124 ±0.019 | 0.256 ± 0.165 | 0.126 ± 0.063 | 0.122 ± 0.042 |
| 0.169 ± 0.015 | 0.299 ± 0.176 | 0.196 ± 0.087 | 0.206 ± 0.020 |
| 0.221 ± 0.017 | 0.367 ± 0.174 | 0.279 ± 0.079 | 0.253 ± 0.074 |
| 0.272 ± 0.038 | 0.462 ± 0.308 | 0.356 ± 0.070 | 0.318 ± 0.090 |
| 0.329 ± 0.029 | 0.505 ± 0.290 | 0.435 ± 0.083 | 0.398 ± 0.062 |

The values of $OD_{Bioscreen}$ were already corrected with the respective path length, which means that the observed differences between the two instruments are only due to the different optics response. In order to find an equation that relates the measurements in both equipments, the results shown previously were plotted and fitted with linear regression, Figure 12.
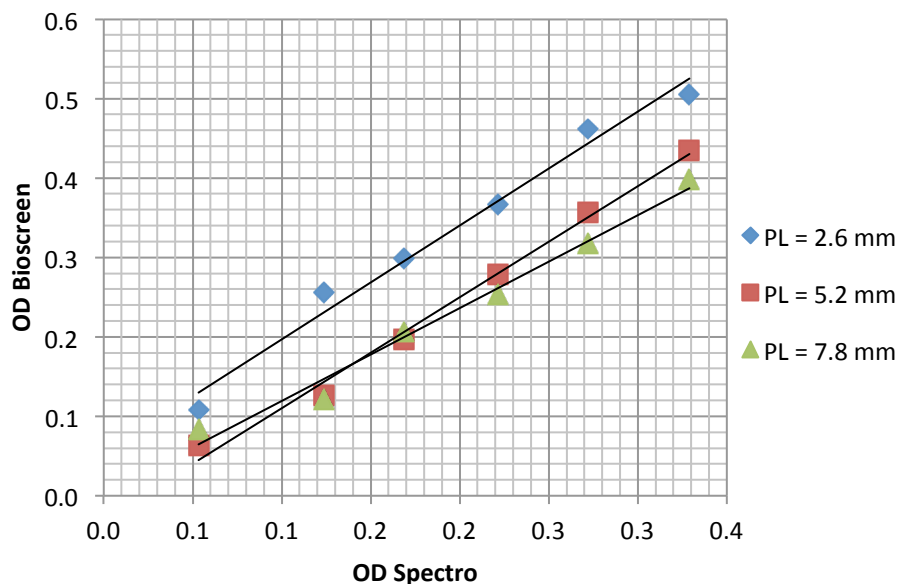


**Figure 12** – Relationship between the OD read in Bioscreen C MBR and Spectrophotometer, for different path lengths: 2.6, 5.2 and 7.8 mm.

The data shows a linear relationship between the equipments. The results were done 4-plicate and Equation 4.1 was determined from the average values obtained:

$$OD_{Spectro} = \frac{OD_{Bioscreen}}{(1.33 \pm 0.14) \times Path\ Length\ [cm]} \qquad (4.1)$$

It is also important to notice that the path length should be in [cm] because the Equation considers 1 cm for the path length in the spectrophotometer.

## 4.2 Analysis of the linearity range

Data obtained from a previous work (Warringer et al., 2003) showed non linearity for OD>1. Figure 13 presents the difference between the OD observed directly on Bioscreen C MBR and the OD corrected with the polynomial function, Equation 2.1 (Warringer et al., 2003). In order to verify the suitability of the equation developed by Warringer and co-workers for the conditions used in the present work, the equation parameters were recomputed by fitting the observed curve to an ideal reference curve (data not shown). The resulting re-computed parameters were essentially unchanged and therefore no correction was applied.
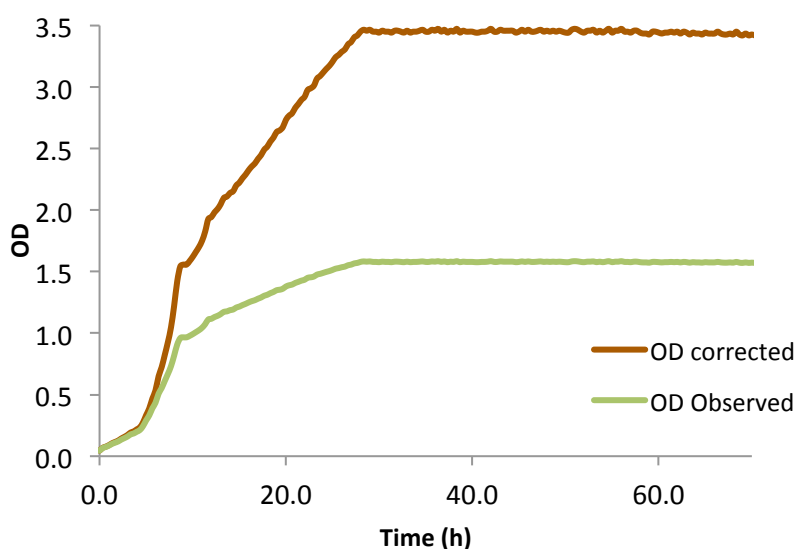


**Figure 13**- Comparison of growth curves before and after apply the linearity equation for OD higher than 1.

From this point on, all the curves shown will refer to OD values already corrected for non-linear response at OD>1.

## 4.3 Setting of the basic parameters on Bioscreen C MBR

In order to obtain representative and unbiased growth curves with the Bioscreen it is necessary to optimize a number of settings, such as shaking intensity and mode and initial inoculation OD. The volume and the temperature were fixed to 145 µl and 30 °C, respectively (Murakami, 2009).

## 4.3.1 Shaking mode

The importance of shaking mode is discussed in the study by Warringer et al., 2003. In particular, shaking intensity can vary and shaking mode can be intermittent or continuous. Two different softwares were use for the control of the instrument: "Norden Lab" and "EZexperiment" which allow different configuration of intermittent and continuous shaking. Figure 14 shows the comparison between three different ways of shaking: high continuous shaking and intermittent shaking with two different softwares. The intermittent mode corresponds to 1 minute long shaking cycles, interspersed with 1 minute long pauses when no shaking is applied.
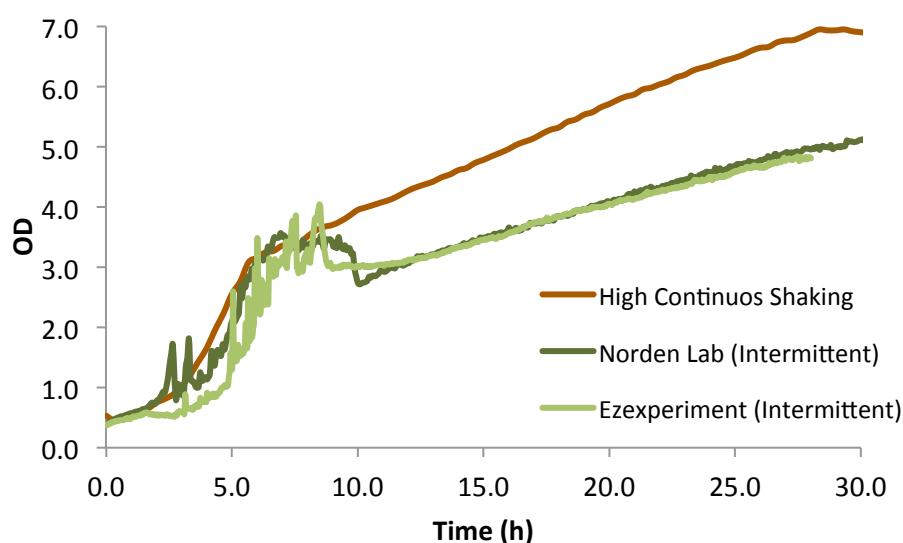


**Figure 14 -** Growth curves obtained with different shaking modes (continuous or intermittent) and with two different instrument controlling softwares ("Norden Lab" and "Ezexperiment").

As clearly visible from Figure 14, high continuous shaking is the best configuration option and it was chosen for all successive experiments.

## 4.3.2  Initial OD

Initial inoculation OD has been to shown to have substantial influence on the quality of growth curves (Warringer et al., 2003).

The amount of cells inoculated in the Bioscreen multi-well plate is calculated through the equation that relates to OD of the pre-culture (as read in spectrophotometer) and the desired OD on the Bioscreen, Equation 4.1. Different initial OD were tested and the results are shown in Figure 15.

**Figure 15 -** Growth curves in Bioscreen with different values to initial OD.

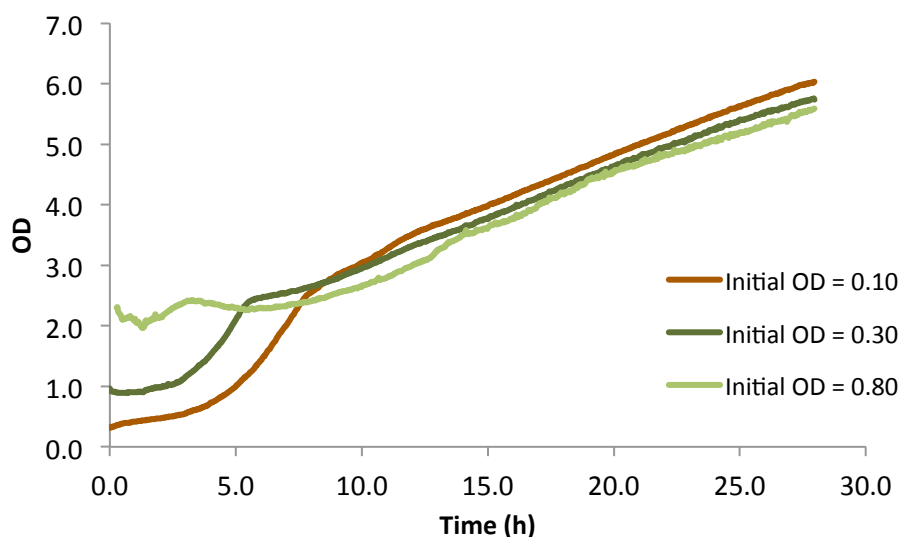When initial OD increases, the growth curve shows some oscillatory behavior and peaks in the first part. Initial OD values <0.3 gave comparable and reproducible results.

### 4.3.3 Comparison of growth curves in Bioscreen C MBR and shake flask

The standard method used for obtaining growth curves was aerobic cultivation of cells in shake flasks. In order to be able to compare the results obtained with the Bioscreen, and analyze the quality of the growth curves, they were compared with shake flasks results obtained with the same strain and the same medium. Table 2 presents the specific growth rate and also the final OD to the both situations. In the Bioscreen, the experiments were done 6-plicate and in the shake flask in duplicate.

**Table 2** – Maximum specific growth rate and final OD for the Bioscreen C MBR and shake flask.

|  | $\mu_{max}$ ($h^{-1}$) | Final OD |
|---|---|---|
| Bioscreen C MBR | $0.43 \pm 0.05$ | $3.1 \pm 0.3$ |
| Shake Flask | $0.35 \pm 0.01$ | $3.5 \pm 0.1$ |

The specific growth rate was calculated through an exponential regression on the data points in the exponential growth phase (see section 3.4 of Materials and Methods). Only absolute OD values below 3.5 were considered for regression. In the case of Bioscreen measurements, the OD values were corrected to the spectrophotometer value with Equation

4.1. Cells did not appear to growth exponentially after reaching OD 3.5. The final value for the specific growth rate was obtained doing the average of all values.

The final OD was taken after 48h incubation either in the Bioscreen or in shake flask. The error presented in this measurement is the standard deviation of all values.

For both equipments, the growth curves were also plotted using ln(OD)according to Equation 4.2.

$$\ln(OD) = \ln(OD_0) + \mu_{max}t \qquad (4.2)$$

Figure 16 shows the comparison between growth curves in log scale performed in the Bioscreen and shake flask.
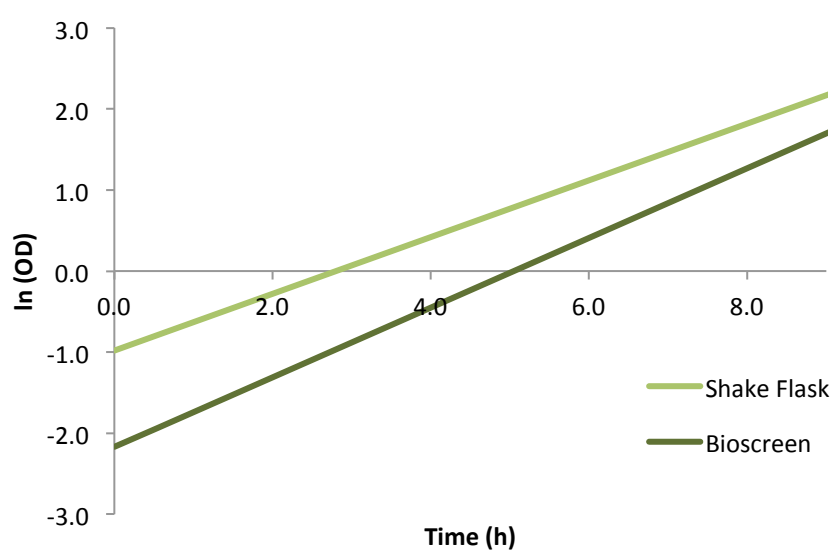


**Figure 16** – Comparison between the growth on Bioscreen and shake flask.

The data points fitted to a linear equation which means the specific growth rate is the slope. With this representation of the growth curves, it is easier to observe that the specific growth rate is similar in both equipments used.

### 4.3.4 Growth curve shape as a function of sugars concentration

As clearly visible from Figure 13, 14 and 15, growth curves obtained with the Bioscreen display at least 3 different phases: exponential growth, non-exponential growth with a reduced rate and, finally, a stationary phase. Sometimes an initial lag phase also appears. The second, non exponential growth phase could be due to nutrient limitation or oxygen limitation during purely respiratory growth after glucose depletion.

In order to verify this hypothesis, an experiment with different sugars concentrations was done. The cells were grown in medium with different glucose concentration: 5, 20 and 50 g/L and the growth curve shapes were compared, Figure 17.



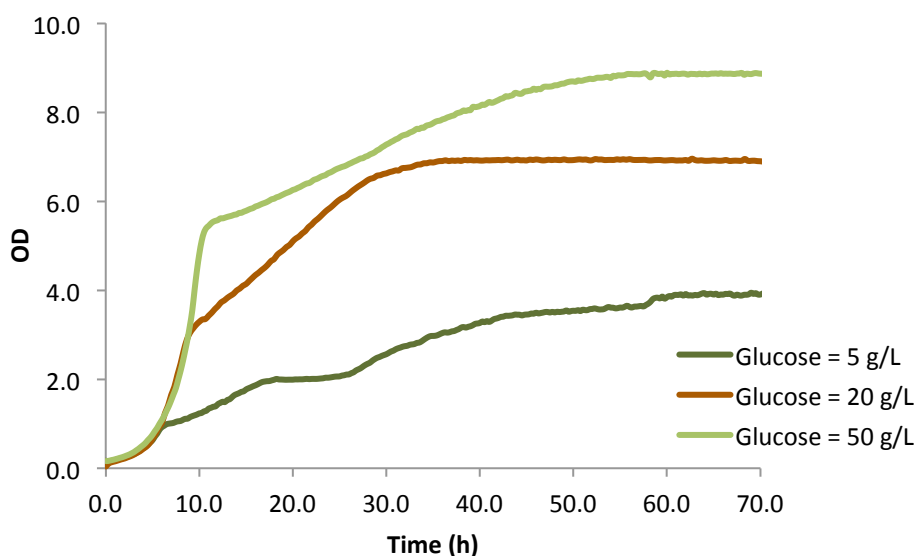**Figure 17** – Analyze cells growing with different glucose concentrations.

As shown in Figure 17, as expected, the sugar concentration in the medium affects the final OD (Table 3) as well as the duration of the exponential phase. As shown by data in Table 3, the specific growth rate in the 3 different conditions tested was not significantly affected.

**Table 3-** Specific growth rate and final OD for growth curves with different glucose concentrations.

|  | $\mu_{max}$ (h$^{-1}$) | Final OD |
|---|---|---|
| Glucose 5 g/L | 0.31 ± 0.03 | 3.7 ± 0.1 |
| Glucose 20 g/L | 0.33 ± 0.01 | 7.9 ± 0.6 |
| Glucose 50 g/L | 0.34 ± 0.01 | 8.9 ± 0.2 |

## 4.4 Estimation of lag phase time

In order to describe a growth curve with sufficient accuracy, it was necessary to estimate the length of the lag phase. In addition, in case a test culture displays a lag phase, its length must be subtracted from the delay in reaching a target OD compared to a reference culture before the determination of the delay δ.

A lag phase was purposefully induced in a cell population by medium shift: cells growing in mineral medium containing glucose as the sole carbon source were shifted to a

fresh medium, containing galactose as sole carbon source. This change will induce a lag phase, since the glucose is the preferred carbon source for *Saccharomyces cerevisiae* (Van Den Brink et al., 2009).

Figure 18 shows two growth curves: a curve resulting from cells pregrown on glucose medium and inoculated in the same medium (brown curve) and a curve resulting from cells pregrown on glucose medium and inoculated in galactose medium (green curve). A lag phase is clearly induced by the medium shift.
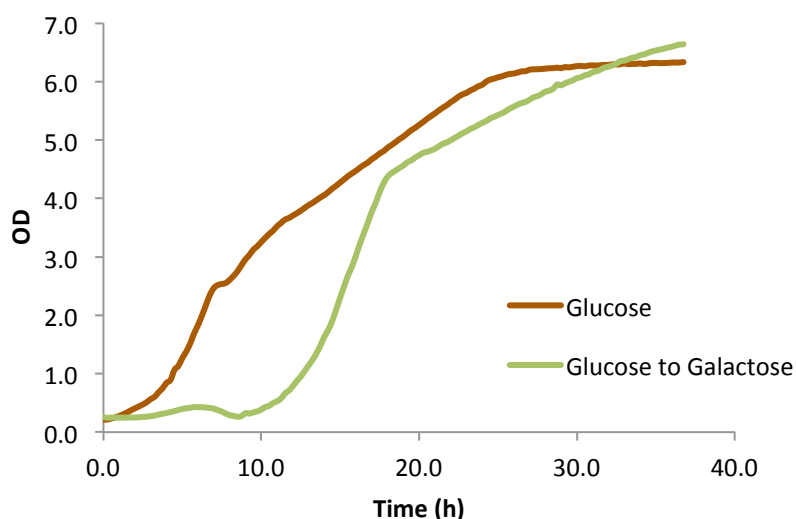


**Figure 18** – Growth curves in Glucose medium and in Galactose medium with an induced lag phase.

The lag phase was estimated with two methods: visually and by extrapolation (see section 3.4 of Materials and methods). The experiment with the induced lag phase was done 4 times and 4 different growth curves were obtained.

In order to calculate the lag time with the extrapolation method, all curves were fitted with an exponential regression and the $OD_0$ and $\mu_{max}$ were calculated as explained before. It was also necessary to choose the values which will be taken to determine the $OD_0$ value (average of the first points, before the OD value starts to increase).

Table 4 shows the results for the lag phase time by both methods for the 4 growth curves obtained, as well as the equation and the parameters used to determine that time.

**Table 4** – Lag phase time determined by extrapolation and with a visually method and parameters used to obtain this time.

| Experiment | Equation | OD | $t_{lag}$ (h) - Extrapolation | $t_{lag}$ (h) - Visually |
|:---:|:---:|:---:|:---:|:---:|
| 1 | $OD = 0.0232\, e^{0.3012\, t_{lag}}$ | 0.324 | 8.8 | 8.5 |
| 2 | $OD = 0.0368\, e^{0.3448\, t_{lag}}$ | 0.784 | 8.9 | 9.0 |
| 3 | $OD = 0.0064\, e^{0.3484\, t_{lag}}$ | 0.173 | 9.5 | 9.0 |
| 4 | $OD = 0.0146\, e^{0.3277\, t_{lag}}$ | 0.274 | 9.0 | 9.3 |

The difference between the OD is due to the different initial OD in the assays.

Doing the average of the previous results, the lag phase time is very similar for both methods, since the results are 9.1 ± 0.3 h and 8.9 ± 0.4 h for the extrapolation and visually method, respectively.

## 4.5 Evaporation

Finally, evaporation from the multiwell plate during incubation was measured. In some experiments, the cells are incubated up to 72h at 30 °C and probably some evaporation occurs. It is very important to assess the extent of evaporation, since it might lead to medium concentration and therefore to a dramatic change in the growth conditions of the cells.

One multiwell plate was prepared with 26 wells, filled with 145 µl each. The plate was incubated for 72h at 30°C and the volume in each well was measured again with a micropipette, Appendix 2. The evaporation was calculated and it is around 5 %, which is considered not influential for the sake of this work.

## 4.6 Mixed cells populations

In order to validate the method developed to estimate the number of viable cells, known percentages of viable and inactivated cells were mixed. Different populations with known percentage of viable cells were inoculated in Bioscreen multi-well plate and growth curves were performed. The same populations were also spread in plates on the surface of solidified glucose-based medium and incubated at 30 ºC during 48 hours, which allows to each cell growth into a visible colony (Fankhauser, 2005).

This section presents the results of both methods and a comparison between them.

### 4.6.1 Plating Method

As explained before, the plating method was used as a control of cell inactivation, where no growth was expected and as a benchmark for the measurement of the percentage of viable cells inoculated in the Bioscreen. Table 5 presents the percentage of viable cells for the plating method. The values shown are the result of 3 replicates.

**Table 5** – Percentage of viable cells obtained with the plating method.

| S $_{Theoretical}$ (%) | Number of colonies | S $_{Experimental}$ (%) |
|---|---|---|
| 100 | 194 | |
| 80 | 172 | 86 ± 10 |
| 60 | 120 | 63 ± 4 |
| 40 | 100 | 45 ± 3 |
| 20 | 54 | 26 ± 5 |

It is important to notice that in each plate 100 µl of the diluted pre-culture were spread. An appropriate dilution was used, so that the number of colonies would be between 30 and 300 (Fankhauser, 2005).

### 4.6.2 Bioscreen C MBR

Several growth curves with percentage of viable cells between 0 and 100 % with 10% increments were performed. Figure 19 presents an example of them, with 100, 70, 50, 20 and 0% of viable cells.
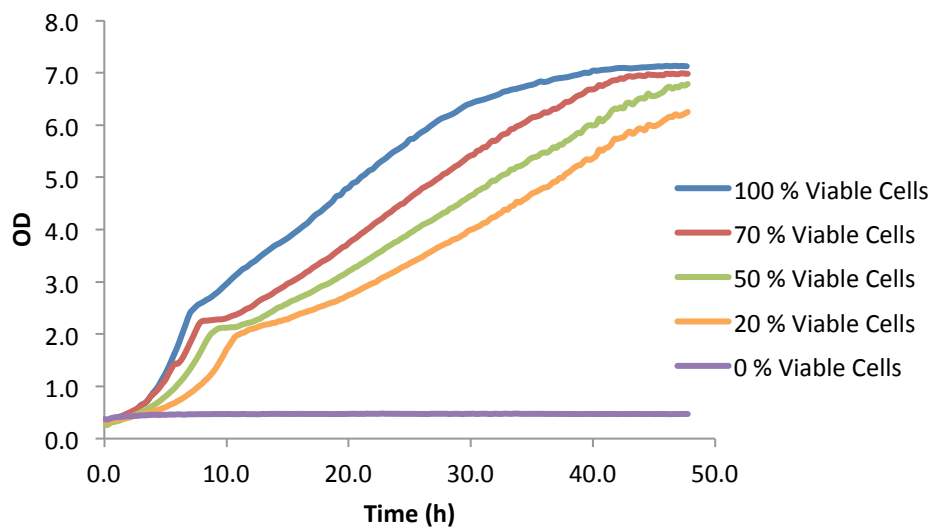


**Figure 19** – Growth curves of mixed populations with different percentage of viable cells: 100, 70, 50, 20 and 0 %.

The experiment was repeated 7 times. Regardless of the different percentages of viable cells used, cultures with 100 and 0 % viable cells were always performed as a reference and control, respectively. In particular, the culture inoculated with inactivated cells only (lilac line in Figure 19), served also as a control for the inactivation procedure. As it is possible to observe in Figure 19, the OD of the control culture is constant throughout the experiment, meaning that cells were not growing neither lysing. The inactivated cells were also observed in the microscope and they were undistinguishable from the viable ones.

The value of the delay "δ" and, consequently, the fraction of viable cells can be calculated from a growth curve and Equation 2.5 and 2.8, respectively (see section 2.4 of Background). Figure 20 shows 10 growth curves with populations between 10 and 100 % viable cells.
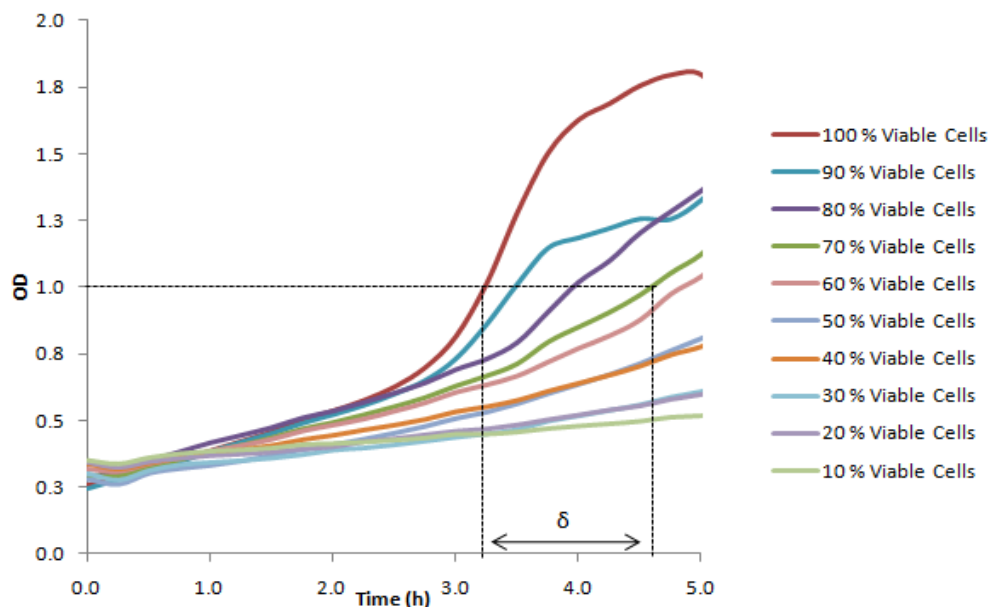


**Figure 20** – Growth curves for mixed populations with percentages of viable cells between 10 and 100 % viable. Dashed lines show the visually determined points used to estimate the delay to reach OD = 1.0 for the curve with S = 70%.

For each of the curves obtained with mixed cell populations, the maximum specific growth rate and the delay "δ" were calculated (see Materials and Methods). The value of δ was calculated for several target OD values (data not shown). However, the best results are obtained with target OD=1 and they are presented in Table 6.

**Table 6** – Specific growth rate for 10 populations and percentage of viable cells for different values of target OD.

| $S_{Theorical}$ (%) | $\mu_{max}$ (h$^{-1}$) | $S_{OD=1.0}$ (%) |
|---|---|---|
| 100 | 0.37 ± 0.08 | |
| 90 | 0.31 ± 0.04 | 87 ± 8 |
| 80 | 0.30 ± 0.05 | 88 ± 3 |
| 70 | 0.29 ± 0.04 | 77 ± 4 |
| 60 | 0.25 ± 0.02 | 73 ± 7 |
| 50 | 0.23 ± 0.02 | 65 ± 12 |
| 40 | 0.21 ± 0.01 | 63 ± 9 |
| 30 | 0.20 ± 0.02 | 54 ± 4 |
| 20 | 0.18 ± 0.01 | 54 ± 10 |
| 10 | 0.17 ± 0.08 | 52 ± 5 |

The tests were done in duplicate (S = 10 and 30 %), triplicate (S = 50, 70 and 90%) and 4-plicate (for the remaining S values). The error associated to each value in the standard deviation between all measurements.

As shown in Table 6, the higher the fraction of viable cells, the higher the reliability of the method. For values of S ≥ 70 %, the method gives results within 10% difference from the expected.

It is important to note that the method assumes that the specific growth rate is the same for all curves compared. However, as clearly visible from Table 6, the estimated value of $\mu$ is also severely affected by S. Therefore, a modification of the method was evaluated as it follows.

Based on the data from the control culture with S = 0 (lilac line in Figure 18), it is possible to consider that the total amount of non-viable cells is constant throughout the experiment, suggesting the possibility of a constant-term correction of the OD values. Since the populations are a mixture of viable and non-viable cells and only the viable cells grow, the inactivated cells represent a constant term in the measured OD values in each growth curve. Therefore, in order to evaluate the influence of the non-growing cells on the maximum specific growth rate calculation, a correction for eliminating the contribution to measured OD given by the inactivated cells was introduced, Equation 4.3, and the maximum specific growth rate was recalculated.

$$OD_{viable\ cells} = OD - OD_0 \times (1 - S) \qquad (4.3)$$

After applying Equation 4.3 to the obtained growth curves, the $OD_{viable\ cells}$ value in fact correspond to the nominal OD value given by growing cells only. The results for the maximum specific growth rate and the percentage of viable cells, after the correction, are presented in Table 7.

**Table 7** – Specific growth rate and percentage of viable cells for 10 populations and for different values of target OD, applying the correction for the inactivated cells.

| $S_{Theoretical}$ (%) | $\mu_{max}$ (h$^{-1}$) | $S_{OD=1.0}$ (%) |
|:---:|:---:|:---:|
| 100 | 0.34 ± 0.04 | |
| 90 | 0.31 ± 0.05 | 85 ± 1 |
| 80 | 0.31 ± 0.05 | 74 ± 4 |
| 70 | 0.29 ± 0.03 | 59 ± 6 |
| 60 | 0.27 ± 0.05 | 51 ± 6 |
| 50 | 0.25 ± 0.07 | 40 ± 2 |
| 40 | 0.26 ± 0.05 | 32 ± 7 |
| 30 | 0.28 ± 0.00 | 26 ± 0 |
| 20 | 0.24 ± 0.09 | 16 ± 6 |
| 10 | 0.28 ± 0.00 | 13 ± 0 |

As it can be seen from the comparison between the values in Table 6 and 7, the spread of the calculated values of maximum specific growth rate throughout the range of theoretical percentages of viable cells is greatly reduced. Consequently, it is possible to observe that when the correction was applied, the reliability of the method improves for the whole range of viability percentages.

Figure 21 presents the results of these two methods to estimate the number of viable cells and also a comparison with an ideal situation, when the $S_{experimental}$ is equal to the $S_{Theoretical}$.
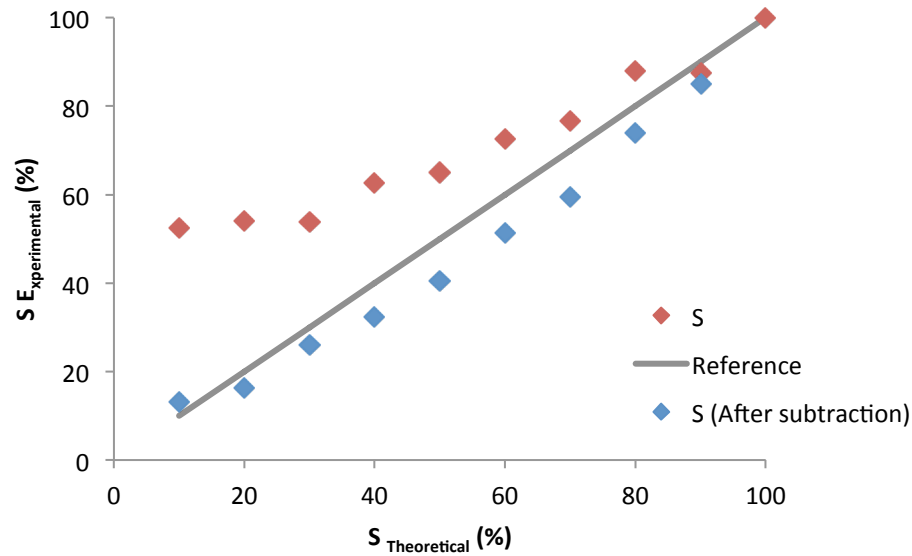
**Figure 21** - Comparison between the theoretical and experimental results for the number of viable cells in a population. Results obtained with the based method and after subtraction of the non viable cells.

It is clearly illustrated that the method improves its reliability when the Equation 4.3 is applied, since all values fitted to the ideal line. However, the correction of measured OD values with equation 4.3 is obviously only possible during the method development, when experimental populations of known S are used.

With real samples from lignocellulose hydrolysate fermentation, the percentage of viable cells is not known which makes impossible to apply that simple correction. In order to solve this barrier, another approach was analyzed (see Limitations and Future work).

# 5 Discussion

The present work aims at finding a reliable method to estimate cell viability through high resolution growth curves. For this purpose, a new equipment was used, Bioscreen C MBR, and at the first stage of this project a method to perform optimal growth curves needed to be developed.

In order to have growth curves of sufficient quality, some parameters were optimized, such as the initial OD and the shaking mode (Warringer, 2003). It was possible to conclude that the continuous shaking with high intensity is the most efficient shaking mode (Figure 14). The intermittent ways of shaking causes peaks in the beginning of the growth which makes the specific growth rate calculation difficult and it does not allow observing all phases of the growth curve. This experiment was done based on published work (Warringer, 2003), which however used intermittent shaking (60 s shaking and 60 s resting). According to Warringer and co-workers, continuous shaking results in accumulation of cells at the periphery of the wells and, because of that, non-reproducible growth curves. However, this parameter is strongly dependent of the yeast strain (Warringer, 2003). The difference in the strains used in this project and in the referred work (CEN.PK 113 7D and FY1679, respectively) could therefore explain the need for different shaking parameters.

To check the method reliability, some growth curves were also done in shake flask and the specific growth rate and the final OD were compared with the values obtained on Bioscreen C MBR. The growth conditions is shake flask were the same as in the Bioscreen, since the cells were incubated at 30 °C during constant shaking. Analyzing Figure 16 it is possible to conclude that the cells are growing at a slightly different rate, because the straight lines which describe the growth curves are not completely parallel. This is also proven by the results showed in Table 2: the specific growth rates are similar but not the same ($0.43 \pm 0.05$ h$^{-1}$ for Bioscreen C MBR and $0.35 \pm 0.01$ h$^{-1}$ for shake flask). On the other hand, final OD is similar, with values of $3.1 \pm 0.3$ for the Bioscreen and $3.5 \pm 0.1$ for shake flask experiments. The variation in the $\mu_{max}$ and final OD can be due to experimental errors in culture's preparation, errors associated to the OD measurement in the spectrophotometer and also because different equipments were used and even correcting the OD based in the Equation 4.1, some error could be associated. Another possible reason for the difference in the values is the method to calculate de $\mu_{max}$: it was calculated through an exponential fitting of the experimental data points in the exponential growth phase. In some cases, mainly in the experiments in shake flask since more experimental error can be associated, the data points didn't fit perfectly with an exponential equation which can also induce some small error to

the value. The difference between final OD is not significant and it can prove that the Equation to correct the non linearity associated to the Bioscreen works well.

As visible in Figures 13, 14 and 15, the growth curves performed in the Bioscreen present different stages. In particular, during the last stage before the stationary phase the cells display slower rate and the growth is not exponential anymore, but almost linear. The experiment done with three different glucose concentrations, Figure 17, indicate that the slow growth rate could be due to glucose exhaustion and consequently growth on ethanol, which would suffer from oxygen limitation in the Bioscreen multiwall plate. Accordingly, the final OD increased with the initial glucose concentration while the variations in the specific growth rates values can be considered as not significant (0.31 ± 0.03, 0.33 ± 0.01 and 0.34 ± 0.01 which correspond to glucose concentrations of 5 g/L, 20 g/L and 50 g/L respectively).

A relevant problem in the study of cell viability is the correct identification of the lag phase time. In the present work, the lag time was defined visually and by extrapolation, providing similar results, 8.9 ± 0.4 h and 9.1 ± 0.3 h, respectively. The average difference between the methods of 0.2 hours is not significant when compared to the absolute length of the lag phase time, in this case ~9h. However, the lag phase time is a determinative parameter to estimate the correct delay between two growth curves to reach a target OD. When the percentage of viable cells increases, the delay time decreases. For that reason, with higher percentage of viable cells the theoretical delay is in the order of minutes and a slightly difference of 0.2 h can be significant. For that reason, the lag phase was determined by the extrapolation method since it is more correct, and the same method was applied to all cases.

The last parameter checked before the method validation was the evaporation in the multiwell plate. It was measured and it is 5 %. This factor was ignored in all calculations because it is very low and doesn't affect the results.

Finally, the experiments to validate the method were carried out and the first task was finding an efficient method to inactivate cells. The method used was the contact with high temperatures (80 °C during 5 minutes). The treatment effectively led to 100% inactivation, as it is possible to observe from Figure 19. The constant OD corresponding to the non viable cells is very important, since it indicates that inactivated cells keep their optical properties constant throughout the whole experiment.

Colony Forming Units counts were always done in parallel to Bioscreen experiments. The results from the plating method were done as a control, to analyze if the populations inoculated in Bioscreen had the desired percentage of viable cells. Results from Table 5 shows that the method to inactivate cells and also the pipetting method to mix and dilute the samples worked well, since the differences between the experimental results and the theoretical ones were always smaller than 6 %.

The plating method was only used in the present study as a control. Although it provided good results, estimating cell viability through this method is time-consuming, labour intensive and it presents high variability compared to the Bioscreen or other methods. In addition, the Bioscreen method presented in this study presents certain advantages when compared to alternative ways of measuring viability such as the fluorescence based method using the viability dependent dye FUN1 (Millard, 1997; Probes for Yeast Viability, 2001). The method based on high resolution growth curves is essentially simpler and less labour intensive. Fluorescent dye-based methods require a specific set of chemicals for the staining procedure and the preparation of the mounted microscope slide. In addition, staining and washing steps are required, the cells have to be adjusted to a specific density and each sample has to be individually analyzed at the fluorescence microscope (Millard, 1997). It is also known that the samples should be analyzed as soon as possible, since the FUN1 is toxic to the cells (Probes for Yeast Viability, 2001). Traditional methylene blue staining (Parkkinen, 1976) is technically simpler, although it might display toxic effects for the cells. In addition, previously published data show that results obtained with this method on experimental population of cells, obtained by mixing living and inactivated cells, do not completely correlate with the expected results (Millard, 1997). All these methods already described (see also Background) allow to obtain acceptable results, however a less labour intensive and simpler method is desirable.

The new method to estimate cell viability was developed with the Bioscreen and analyzing the results, Table 6 allows to conclude that the assumption that the maximum specific growth rate in both curves is the same should not be taken. The values are quite different, meaning that the inactivate cells were influencing this parameter: $\mu_{max}$ decreases when the percentage of inactive cells increases.

The percentages of viable cells were calculated through the delay between the reference curve and the analyzed one, to reach a target OD (in this case, OD = 1.0). The results also from Table 6 show that the method worked better for higher percentage of viable cells (S ≥ 70%). The results when S ≥ 70 % have a difference of 8 % in maximum when compared to the expected value. However, this 8 % is a relative number, since the valued in the plating method also have an associated error of 6%. Once more, the inactivated cells were affecting the results because the delay time is not determined correctly. Since the OD values are counting with the OD corresponding to the non viable cells, the time to reach the target OD will be higher than it really is.

The method to estimate the number of viable cells used in the present project is based on published results from C. Murakami and M. Kaberlein, from University of Washington, USA (Murakami, 2009). Using the raw data from this study, the S values were recalculated and it was possible to conclude that, even presenting high accuracy and

precision, the higher percentage of viable cells tested was 70%, indicating that the results obtained in the present project are consistent with the published ones.

The maximum specific growth rate was also calculated on the published raw data, in order to check if the assumption of constant maximum specific growth rate throughout a range of "S" could be applied to that study. In fact, the published results are in this case not consistent with what found in our study. As shown in Table 6, the maximum specific growth rate decreases with the percentage of viable cells, while it did not when recalculated based on Murakami's data. Nonetheless, this finding is supporting our findings and it is consistent with our hypothesis on the influence of a subpopulation of inactive cells on the calculated maximum specific growth rate.  The results presented in section 4.6.2 and, in particular, Figure 21, demonstrate the feasibility of the method and the assumption of constant growth rate once the contribution of inactivated cells to the measured OD was removed. In fact, in Murakami's populations the presence of such subpopulation is not obvious and it is rather possible that the "non viable" cells in that case had already lysed, due to the prolonged incubation needed in the chronological life span experiments.

In order to increase the method viability and since is known that inactivated cells were affecting the parameters calculated, the Equation 4.3 was used to correct the OD values. Analyzing the results shown in Table 7 is possible observe that the maximum specific growth rate values are quite similar. Using this correction, the assumptions that the maximum specific growth rates in both curves are the same seems to be reasonable. Figure 21 shows that the results for the percentage of viable cells are also better and the method seems to work for all range of viability.

# 6 Conclusion

A method to estimate the number of viable cells in lignocellulose hydrolysate fermentations through growth curves performed in Bioscreen C MBR was developed in this master thesis. However, the method was not valid for all range of viability, since it only gives the reliable results to percentage of viable cells between 70 and 100 %.

To improve the method already developed and to overcome the limitations, a new approach was tested and it was proven that the method works well for the whole range of viability if the OD corresponding to the percentage of inactivated cells were subtracted to all values. In this case, the maximum specific growth rate becomes more similar between the analyzed curve and the reference curve (100 % viable).

Although it works well, it is not possible to apply since the percentage of non viable cells is not known in samples from real lignocellulose hydrolysate fermentations.

# 7 Project Evaluation

## 7.1 Objectives achieved

This thesis was divided in 4 big objectives: i) Method to perform optimal growth curves on Bioscreen C MBR, ii) determination of lag phase time, iii) Analyse the method viability, to estimate the number of viable cells and iv) Test the previous method with real lignocellulose hydrolysate fermentation.

The first two tasks were completed and the results are pretty good. However, in the last two points some future work has to be done.

The method to estimate the number of viable cells was completed but only for a range of percentage of viable cells. When it is between 60 and 100 % it works very well.

The fourth task was not done yet. Only one experiment was carried out, however the results need to be analyzed with more detail bit only after the method will be completed established.

## 7.2 Limitations and Future work

The work developed in this master thesis shown some limitations to develop a reliable method to estimate the number of viable cells in a sample, which bring some more development and future work.

Thinking about the growth curves for populations with an unknown percentage of viable cells, the OD values are the sum of an exponential growth (viable cells) and a constant value (inactivated cells), Equation 7.1.

$$OD' = OD_0 \times S \times e^{\mu'_{max}(t+\delta)} + OD_0(1 - S) \qquad (7.1)$$

Since the assumption of the maximum specific growth rate are the same when curves with 100% and unknown percentage of viable cells doesn't work well, the previous equation displays two unknown variable: $\mu_{max}$' and S. This problem can be solved fitting the raw data to the Equation 7.1, using a simulation program such as Matlab, and the best results for the both unknown variables will be calculated.

The results obtained with mixed populations in order to validate the method (See section 4.6 of Results) are good enough to believe that, if the maximum specific growth rate is not assumed as the same of the reference curve and the OD values are corrected with the percentage of inactivated cells, the method will work pretty well. However, some investigation and development should be done in this direction.

After the method be completed established, the experiments with samples from real lignocellulose hydrolysate fermentations should be carried out in order to assess the method viability.

## 7.3 Final appreciation and opportunities

During these 5 months several good opportunities appeared which improve my knowledge and my general skills as a student in chemical engineering.

Besides my research work, I could present the work developed on my thesis in a Poster Exhibition during the annual departmental meeting of the Chemical and Biological Engineering department and Chemistry department from Chalmers University and University of Gothenburg, respectively. The poster presented had the title: "Yeast cells viability in industrial fermentation media determined by high-resolution growth curves". An updated version of the poster will also be presented to the conference "XIX ISAF-International symposium on alcohol fuels" to be held October 10th-14th, 2011 in Verona, Italy.

During my stay at Chalmers University, I was also given the opportunity to join the students of the local MSc program in Biotechnology for a study trip to Copenhagen (Denmark). During the trip we visited the production plant of Christian Hansen and the Novozymes R&D facilities and Pilot plant.

Since I was responsible of the method development for the Bioscreen C MBR, I was also asked to instruct new users in the use of the new instrument, write a Standard Operating Procedure and a compile a risk assessment declaration for it. In addition to instructing regular staff members of the group, I also had the opportunity to briefly supervise a group of 6 bachelor students, who used the instrument for a subset of experiments included in their BSc thesis final project, entitled "The ability of six yeast strains to produce ethanol from inhibitory lignocellulose hydrolysates".

Chalmers students are required to act as opponent to another student's degree project, meaning that they have to read and be critical about the report and also the presentation. I was approved as opponent for the thesis of Ana Gonçalves, supervised by Paul Gatenholm and entitled: "Preparation and evaluation of material properties of biofilms from spruce xylan".

Finally, after completion of my project I was asked to prepare a Power Point presentation and present my results in English during the public defense of my thesis.

The Erasmus program is the best way of meeting new people, learn new ways of life and find a lot of new opportunities. It was a pleasure to me be part of this family and even better spent my 5 months away in such a prestigious University like Chalmers University of Technology is. It was also great to be part of Industrial Biotechnology group, a group where fellowship is a constant.

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# Appendix 1: Preparation of the medium

## 1.1 Mineral Medium

Almost all experiments were carried out with a mineral medium with glucose as carbon source. There are also some experiments with galactose. In this appendix all the procedure to prepare that medium will be described as well as the composition of all solutions involved.

Table 1 presents the concentration of each solution in the medium.

Table 1 – Volume of each solution present on 1L of mineral medium used.

| Solution | Volume (mL) |
|---|---|
| Base Salts (10 X) | 100 |
| Carbone source (10 X) | 100 |
| Phthalate Buffer (5 X) | 200 |
| Trace Elements (1000 X) | 1 |
| Vitamin Solution (1000 X) | 1 |
| Water | 600 |

All solutions were prepared and autoclaved separately. Here follows an explanation on how the solutions were prepared and also its composition.

- *Base Salts*

The concentration of each component in the base salts solution is presented in Table 2.

Table 2 – Components present in base salts solution and respective concentration.

| Component | Concentration (g/L) |
|---|---|
| $(NH_4)SO_4$ | 5 |
| $MgSO_4 \cdot 7H_2O$ | 0.5 |
| $KH_2PO_4$ | 3 |

These compounds were well dissolved in water and the solution was prepared to be 10 X concentrated.

- *Carbon source solution*

In this project either glucose or galactose were used as carbon source, but almost all the experiments were with glucose. The solutions were prepared in the same way: 200 g of the sugar was dissolved in 800 ml under continuous shaking. The final concentration was 200 g/L since the solutions were 10 X concentrated.

- *Phthalate Buffer*

The buffer consists in a mixture of two compounds: Potassium Hydrogen Phthalate and Potassium hydroxide. The concentration of each compound in the buffer solution in presented on Table 3.

**Table 8** – Molecular weight and concentration for the components of the Phthalate buffer.

| Compound | Molecular weight (g/mol) | Concentration (g/L) |
|---|---|---|
| Potassium Hydrogen Phthalate | 204.22 | 51 |
| Potassium hydroxide | 56.11 | 11 |

The compounds were dissolved in water under constant shaking. The final pH was set to 5.5. The solution is 5 X concentrated.

- *Trace Elements Solution (1000 X)*

The protocol followed to prepare the trace elements solution is done for 300 ml of solution. To prepare this solution is very important to keep the pH at 6.0 since the components are very difficult to dissolve.

The protocol started dissolving 4.5 g of EDTA and 1.35 g of $ZnSO_4 \cdot H_2O$ in 150 ml of water. The pH was set to 6.0 with 1 M NaOH and the components presented in Table 4 were dissolved, one by one.

**Table 4** – Components present in trace elements solution and respective mass.

| Component | Mass (g) |
|---|---|
| $MnCl_2 \cdot H_2O$ | 0.3 |
| $CoCl_2 \cdot H_2O$ | 0.09 |

| | |
|---|---|
| $CuSO_4 \cdot H_2O$ | 0.09 |
| $Na_2MoO_4 \cdot H_2O$ | 0.12 |
| $CaCl_2 \cdot H_2O$ | 1.35 |
| $FeSO_4 \cdot H_2O$ | 0.9 |
| $H_3BO_3$ | 0.3 |
| KI | 0.03 |

When all components were dissolved, the pH was set at 4.0 with 1 M HCl and the volume was adjusted to 300 ml with water.

The solution was sterilized by filtering and stored at 4 °C with aluminum foil around, since the components are light sensitive.

- *Vitamin Solution (1000 X)*

The protocol followed to prepare the vitamins solution was also done for 300 ml. First, 0.015 g ob biotin was dissolved in 3 ml of 0.1 M NaOH solution and then this solution was added to 240 ml of water. The pH of this mixture was set to 6.5 with 1 M HCl or 1 M NaOH and several vitamins were dissolved. The pH was checked each time on vitamin was dissolved and it should be always 6.5. The list of vitamins dissolved and its respective mass is shown in Table 5.

**Table 5** – List of vitamins used to prepare the Vitamin solution and respective mass.

| Vitamin | Mass (g) |
|---|---|
| Ca-pantotenate | 0.3 |
| Nicotinic Acid | 0.3 |
| Myo-inositol | 7.5 |
| Tiamine·HCl | 0.3 |
| Pyridoxine·HCl | 0.3 |
| Para-aminobenzoic acid | 0.06 |

In the end, water was added to complete the volume of 300 ml and the pH was adjusted to 6.5. The solution was sterile filtered and stored at 4 °C. This solution is 1000 X concentrated.

## 1.2 YPD Medium

Besides the liquid medium, also YPD medium with agar was prepared to plates. The components and the respective concentration are presented in Table 6.

**Table 6 -** Components and respective concentration to prepare YPD medium.

| Component | Concentration (g/L) |
|---|---|
| Yeast Extract | 10 |
| Peptone | 20 |
| Agar-Agar | 20 |
| Glucose | 20 |

The yeast extract, peptone and agar-agar were dissolved in water under constant shaking and the solution was autoclaved. The glucose solution was autoclaved separately and mixed with the medium after.

# Appendix 2: Evaporation

Table 1 presents the volume in each well after an experiment of 72 hours and also the respective evaporation. Since the pipette could not be perfectly calibrated, 18 wells were filled with water and the final volume measured was 142.3 μl. This volume was taken as the initial one.

**Table 1** – Volume measured in each well of the Bioscreen and respective evaporation.

| Volume (μl) | Evaporation (%) |
|---|---|
| 125.2 | 12.0 |
| 132.8 | 6.7 |
| 129.6 | 8.9 |
| 130.8 | 8.1 |
| 130.4 | 8.4 |
| 129.2 | 9.2 |
| 135.0 | 5.1 |
| 134.0 | 5.8 |
| 133.4 | 6.3 |
| 135.0 | 5.1 |
| 145.0 | 1.9 |
| 135.0 | 5.1 |
| 144.2 | 1.3 |
| 136.0 | 4.4 |
| 144.4 | 1.5 |
| 135.6 | 4.7 |
| 136.8 | 3.9 |
| 133.0 | 6.5 |
| 138.0 | 3.0 |
| 132.0 | 7.2 |
| 135.4 | 4.9 |
| 143.4 | 0.8 |
| 136.2 | 4.3 |
| 136.4 | 4.2 |
| 136.4 | 4.2 |
| 135.0 | 5.1 |