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Diploma Thesis

In-situ microscopy for online monitoring of yeast propagation in culture broths

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1 Abstract

Contemporary times request optimization of every possible aspect of the product value chain. This challenge includes, also, the very process of producing products inside large-scale reactors. These reactors, though, because of their great dimensions, work in conditions far from ideal. The concept of continuous stirring, which promises homogeneous conditions across the volume of the reactor is restricted to just a small volume around the stirrer, whereas across the rest of the reactor gradients of many kinds occur (substrate, DO, pH, dissolved carbon dioxide etc.). This phenomenon becomes worse, the more viscous the reactor's solution is. This, in turn, poses a much more serious problem: inhomogeneous conditions are very difficult to monitor and subsequently to control. In order to get a realistic picture of what's happening inside the reactor, multiple sampling from different regions should be taken. The analysis in the vast majority of situations include at-line and off-line analytic tools. This procedure, though, is in one hand rigorous and requires time and work, and on the other hand, since it is time consuming, it doesn't offer the possibility to quickly react and take the appropriate measures, if certain conditions in the reactor change. A novel solution for this problem is online analytic tools, and specifically in-situ light microscopy, which was used in this study.

In-situ light microscopy, consists of a microscope that uses light in the visible range to magnify and take pictures of the suspended cells, while mounted inside the reactor. These pictures are then processed by image-analysis software and provides information about the cell size distribution, cell count, variance of size etc. Commercially there have been microscopes that work, though, at-line and off-line. In this study, an in-situ light microscopy sensors, SOPAT (**S**mart **O**nline **P**article **A**nalysis **T**echnology), was used to measure the cell size distribution of yeast cells S.Cerevisiae cultivated in a CSTR reactor and in scale-down shake flasks under optimum and non-optimum conditions. The results were compared with results from at-line microscopy Ovizio and off-line analytics, which included HPLC, GC-FID, glucose, pH, OD etc.

The results are very promising, providing good correlation with at-line analytics at a much faster and less rigorous way. The detection, though, still needs to be improved, since it loses accuracy in high cell concentrations.

2 Περίληψη

Η ανταγωνιστικότητα της σύγχρονης εποχής καθιστά επιτακτική την βελτιστοποίηση κάθε σταδίου της παραγωγικής διαδικασίας ενός προϊόντος. Η πρόκληση αυτή περιλαμβάνει φυσικά και την αυτή καθ'αυτή παραγωγή του προϊόντος σε αντιδραστήρες βιομηχανικής κλίμακας. Οι αντιδραστήρες αυτοί, όμως, εξαιτίας των τεραστίων διαστάσεών τους, λειτουργούν σε συνθήκες μακριά από ιδανικές. Η παραδοχή της συνεχούς ανάδευσης, η οποία μεταφράζεται σε ομοιογενείς συνθήκες σε όλη την έκταση του αντιδραστήρα περιορίζεται σε μικρές περιοχές γύρω από τον αναδευτήρα, ενώ στην υπόλοιπη έκταση του αντιδραστήρα δημιουργούνται βαθμίδες διαφόρων ειδών (υπόστρωματος, διαλυμένου οξυγόνου, διαλυμένου διοξειδίου του άνθρακα κλπ). Το φαινόμενο αυτό γίνεται πιο αισθητό όσο μεγαλύτερο ιξώδες έχει το περιεχόμενο του ανιδραστήρα. Αυτό με την σειρά του προκαλεί περαιτέρω προβλήματα: ανομοιγενή συστήματα είναι πολύ δύσκολο να παρακολουθηθούν και κατά συνέπεια να ελεγχούν. Για να παρθεί μία ρεαλιστική εικόνα του τι γίγνεθαι στο εσωτερικό του αντιδραστήρα, χρειάζονται πολλαπλές δειγματοληψίες από διαφορετικά τμήματα του αντιδραστήρα. Η ανάλυσή τους περιλαμβάνει στην συντριπτική πλειοψηφία των περιπτώσεων χρήση μεθόδων και οργάνων at-line και off-line. Η διαδικασία αυτή απετεί αφενός εργατοώρες και χρόνο και αφετέρου, επειδή ακριβώς χρειάζεται χρόνο, δεν δίνει την δυνατότητα για γρήγορη αντίδραση και την λήψη κατάλληλων μέτρων, όταν συγκεκριμένες συνθήκες στο αντιδραστήρα αλλάζουν. Μία ρηξικέλευθη μέθοδος είναι η χρήση online αναλυτικών οργάνων και συγκεκριμένα η in-situ μικροσποπία φωτός, η οποία χρησιμοποείται σε αυτήν την έρευνα.

Η in-situ μικροσκοπία φωτός αποτελείται από ένα μικροσκόπιο, το οποίο χρησιμοποιεί φως στο ορατό φάσμα και μεγεθύνει και τραβά φωτογραφίες των κυττάρων, ενώ είναι τοποθετημένος μέσα στον αντιδραστήρα. Οι εικόνες αυτές, επεξεργάζονται από λογισμικό ανάλυσης εικόνας και παρέχει πληροφορίες για την κατανομή μεγέθους κυττάρων, αριθμό κυττάρων, διακύμανση μεγέθους κλπ. Εμπορικά υπάρχουν διαθέσιμα μικροσκόπια, τα οποία λειτουργούν at-line και off-line. Σε αυτήν την έρευνα, ένα μικροσκόπιο φωτός in-situ της εταιρείας SOPAT (**S**mart **O**nline **P**article **A**nalysis **T**echnology) χρησιμοποιήθηκε για να μετρήσει την κατανομή μεγέθους κυττάρων ζύμης S.Cerevisiae καλλιεργημένα σε αντιδραστήρα συνεχούς ανάδευσης και scale-down ανακινούμενων δοχείων σε ευνοϊκές και μη ευνοϊκές συνθήκες. Τα αποτελέσματα έγιναν σε αντιπαραβολή με αποτελέσματα από ένα μικροσκόπιο at-line της εταιρείας Ovizio και άλλων off-line μεθόδων, όπως HPLC, GC-FID, pH, OD κλπ.

Έγιναν 4 σειρές πειραμάτων με ζύμη S.Cerevisiae, τα οποία πραγματοποιήθηκαν στο εργαστήριο του Chair of Bioprocess Engineering του TU Berlin στο Βερολίνο.
Η πρώτη σειρά πραγματοποιήθηκε σε αντιδραστήρα συνεχούς ανάδευσης 10 L. Η προετοιμασία και η ανάπτυξη της καλλιέργειας έγινε σε ανακινούμενα δοχεία (shake flasks) σε 3 φάσεις με την τελική να φτάνει σε οπτική πυκνότητα (OD) 10. Έπειτα, ξεκίνησε η πρώτη φάση της καλλιέργειας, η οποία ήταν σε συνθήκες αντιδραστήρα διαλείποντος έργου (batch), ο οποίος είχε αποστηρωθεί προηγουμένως. Συνολικά 9.5 L μίγματος μικροοργανισμών και μέσου ανάπτυξης παρέμειναν στον αντιδραστήρα για περίπου 20 ώρες, έως ότου η οπτική πυκνότητα (OD) έφτασε 24. Έπειτα για τις

επόμενες 13 ώρες, ο αντιδραστήρας μετατράπηκε σε ημιδιαλείποντος έργου, με την τροφοδοσία να υπολογίζεται μέσω της $F=k*v_L*e^{\mu t}$, όπου $\mu=0.12h^{-1}$ και $k=0,00318h^{-1}$. Οι συνθήκες ήταν σταθερές σε όλη τη διάρκεια του πειράματος με pH=5.5 και θερμοκρασία $T=27^oC$. Κάθε μία ώρα υπήρχε δειγματοληψία και ο αισθητήρας SOPAT, ο οποίος ήταν προσαρτημένος στον αντιδραστήρα, έπαιρνε μετρήσεις κάθε 20 λεπτά και στις 2 φάσεις του πειράματος.

Τα επόμενα 2 πειράματα έγιναν σε ανακινούμενα δοχεία shake flasks, με σκοπό την χρήση του αισθητήρα SOPAT για να παρακολουθηθεί ο κύκλος ανάπτυξης της ζύμης S.Cerevisiae. Για να γίνει ευκολότερη η παρακολούθηση, η καλλιέργεια εκτέθηκε στην ουσία Nocodazole, η οποία συγχρονίζει τα κύτταρα του μικροοργανισμού, ωτσε να βρίσκονται στο ίδιο σημείο του αναπαραγωγικού κύκλου (cell cycle) στην φάση G2/M. Τα δύο πειράματα εκτυλίχθηκαν στις ίδιες ακριβώς συνθήκες, με μόνη διαφορά ότι το μέσο ανάπτυξης στο δεύτερο πείραμα είχε μεγαλύτερη περιεκτικότητα σε πηγή άνθρακα – δεξτρόζη (απο 1.65 σε 22 gr/L), ώστε να αποφευχθούν συνθήκες περιορισμού υποστρώματος άνθρακα και επίσης ο αισθητήρας SOPAT προσαρμόστηκε σε εστιάση στα 0.2mm συγκριτικά με το πρώτο πείραμα, όπου η εστίαση ήταν στα 2mm, ώστε να προκύψουν εικόνες μεγαλύτερης ευκρίνειας. Η προετοιμασία της καλλιέργειας έγινε πάλι σε 3 φάσεις με την τελική να φτάνει οπτική πυκνότητα (OD) 0.6 σε 8 διαφορετικά ανακινούμενα δοχεία όγκου 35 ml έκαστος, όπου και μετά αναμείχθηκαν μεταξύ τους και χρησιμοποιήθηκαν για το πείραμα. Το πρώτο πείραμα διήρκεσε 4.5 ώρες και το δεύτερο 3 ώρες. Η δειγματοληψία γινόταν κάθε μισή ώρα και ο αισθητήρας SOPAT έπαιρνε μετρήσεις κάθε 10 λεπτά.
Το τελευταίο πείραμα αφορούσε την μελέτη της κατανομής μεγέθους των κυττάρων όταν εκτεθούν σε 2 είδη δυσμενών συνθηκών: σε συνθήκες με υψηλή συγκέντρωση αιθανόλης και συνθήκες με υψηλή συγκέντρωση οξικού οξέος. Η προετοιμασία της καλλιέργειας ήταν κοινή με την προετοιμασία της καλλιέργειας, όπως με το πείραμα στον αντιδραστήρα συνεχούς ανάδευσης. Παρασκευάστηκαν 6 πανομοιότυπα δοχεία με 50 ml διαλύματος έκαστος. Στα 2 προστέθηκαν αιθανόλη με τελική συγκέντρωση στο δοχείο 120mM, στα επόμενα 2 προστεθηκε οξικό οξύ με τελική συγκέντρωση 20mM και στα τελευταια 2 δεν προστέθηκε οτιδήποτε, ώστε στο τέλος να συγκριθούν τα αποτελέσματα. Δειγματοληψία έγινε στο ξεκίνημα του πειράματος, στην μιση ώρα, στην μία ώρα και στις 2 ώρες από την έναρξη του πειράματος. Παράλληλα στα ίδια χρονικά σημεία, χρησιμοποιήθηκε και ο αισθητήρας SOPAT.

Τα αποτελέσματα είναι πολλά υποσχόμενα, αφού προσφέρουν καλή συσχέτιση με δεδομένα από αναλύσεις at-line και off-line, πολύ πιο σύντομα και με λιγότερο κόπο. Συγκεκριμένα επιτεύχθηκε συσχέτιση συγκριτικά με δεδομένα από τρισδιάστατο μικροσκόπιο της τάξης του 92%. Η αναγνώριση των κυττάρων, όμως, έχει μεγάλα περιθώρια βελτίωσης τόσο την αναγνώριση τους ακριβές μεγέθους των κυττάρων, όσο και της ακρίβειας σε μεγάλες συγκεντρώσεις. Για αυτόν τον λόγο, δοκιμάστηκε ένα νέο σετ παραμέτρων στο λογισμικό του αισθητήρα SOPAT, το οποίο μπορούσε σταθερά να εντοπίζει και να μετρά τουλάχιστον 1000 κύτταρα ανά λήψη. Όμως η βελτίωση αυτή έγινε σε βάρος της ακρίβειας της μέτρησης ως προς το μέγεθος των κυττάρων και ταυτόγχρονα κύτταρα λίγο πριν την διαίρεση προσμετρόνταν για 2.

3 Theoretical Background

3.1 Introduction to Process Analytical Technology (PAT)

Process analysis (PA) as a field, is evolving in ever-increasing rates in sectors that was not used before, like the pharmaceutical industry as part of the recently adopted process analytical technology (PAT) [1,10]. PA is defined as the action of deploying in-field instrumentation (real-time analysis) and using chemometrics to monitor a chemical of physical attribute (CQA) or detecting events that would otherwise be impossible with conventional variables (flow, temperature, etc.). PA is usually associated with the use of on-line analytics in large-scale production, though this discipline can extend its uses in many other sectors, such as continuous surveillance of hazardous materials such as explosives of chemical agents, or applications in the environmental sector. PAT is considered a very open-ended field, which relies on sum of principles and tools to improve the production process and its control, which includes the automation of the process, chemometrics and in general the process analysis. Quality by design (QbD) cooperates with PAT principles in order to improve the risk management with is applied in the quality assurance of the products. [1,2,10].

The main difference of on-line process analysis with off-line analysis in laboratory scale, σs that the former is performed in a matter of seconds and minutes continuously and the latter requires hours or even days. Moreover, off-line analysis often proves to be inadequate in finding the cause that is in the root of the problem that leads to oscillations in the product quality. A fundamental use of PA, include the application of real-time monitoring instrumentation (RTM). For example, in many industries in situ ultra violet – visual light (UV-VIS) spectrometers are used, which provide a relative picture of the process initially, but after the use of chemometrics, it can provide a much more detailed analysis of the process [3]. The combination of many sets of such data can lead to the derivation of relationships, that were initially very hard to think of. These relationships coupled with on-line monitoring of chosen chemical attributes enables the creation of models that provide a much more controllable process environment, that Is easy to monitor, control and optimize. The highest level of process analytical tools use is, when these models are used to replace older laboratory methods. With process analysis and the use of its tools, eventually, the product quality is improved (steady quality, less need for rework), improved efficiency of the process, reduced process risk and reduced cost of manufacturing goods [4,5,10].

Figure 3.1 show the hard and the soft principles of process analysis. Firstly, the process instrumentation range from easy-to-use to complex technologies. More detailed discussion about on-line instrumentation will be provided in Section 3.1.1.

The next part of PA involves either (1) an inline interface for example an in situ transmission probe or a noninvasive sensor; (2) an autonomous system for sample-taking without unsettling the conditions of the process. The system apart from sample-taking could perform other analytical tasks such as adding a reagent or making a dilution; (3) an on-line or at-line method for transferring the sample taken automatically or manually from the process to an off-line instrument, which is located

close to the process area and not in remote laboratory for quality assurance. Such examples are high performance liquid chromatographers (HPLC), FTIR or Raman spectrometers and other instruments, which will be analyzed later in section 3.1.1 [6-10].

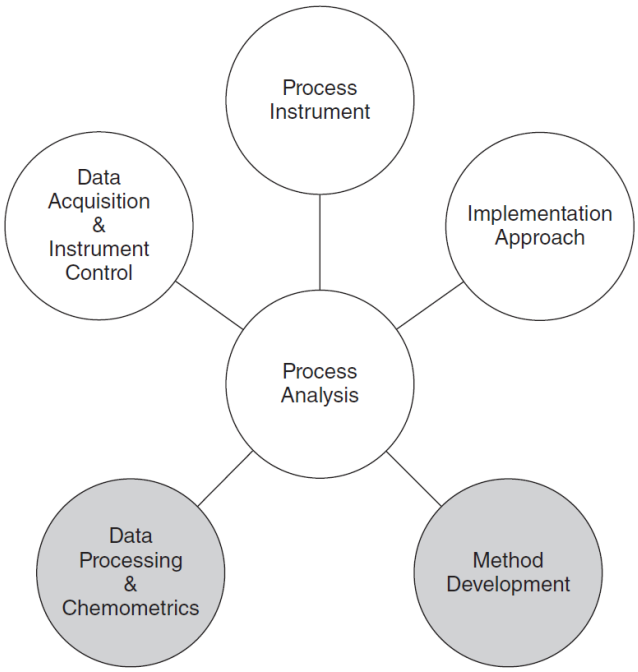


Figure 3-1 Hard and soft (gray) process analysis elements

The data acquisition element is a very important part of PA and consists from an automatic system with the following purpose:

1. Acquiring and archiving data
2. Instrument control from a distance
3. Real time implementation of chemometric derived models
4. Real-time quality assurance

Implementing automation of this level proved to be a very difficult problem to solve from the industrial world. The root of this problem is called 'tower of babble' problem, which simply is the incompatibility of the various process instruments across a production line. Since the results of the instruments are not standardized, each instrument provides results in various different formats, ranging from different wavelengths to different scale of units and even different file formats. Recently automation companies have tried to develop software for automatically converting all results in the same format, though the reporting for these solutions is yet to be determined.

Process analysis applications are found across very diverse industries ranging from petrochemical, pharmaceuticals and electronics to industries in the services sector such as utilities and energy (e.g. sewage, water purification). Usually quality assurance

is the main objective of the process analysis (PA), though there are other applications of it in applications with substantial financial benefits such as safety area monitoring and hazardous materials monitoring are a few examples of applications with much lower financial risk and shorter development life cycle in comparison with quality assurance applications.

3.1.1 Process Instrumentation

Process instrumentation can be grouped in four distinct categories [7,10]. The first category and the most common is physical property analysis instruments, which measure a specific physical attribute such as viscosity or the refractive index. Next category is the electrochemical analysers, which analyse the current or the voltage produced from a standard electrochemical cell coupled with a solute concentration such as pH, conductivity or redox analysers. Combustion analyser measure the different compounds that consist a gas process stream. Last but not least, there are spectroscopic instruments, which monitor attributes vis electromagnetic interactions such as scattering or emission.

3.1.2 Process instrumentation types

In general, instruments used in process analysis can be divided in two main categories: Sensors and analyzers. Analyzers are by definition bulky or large, which consequently need a fix installation, usually side utilities such as electricity and air, enough space inside the process and high cost fiber optics. On the other hand, sensors are more compact, self-contained and lightweight devices with almost every utility it needs onboard. Portable spectrometers and photometers are examples of sensors for process analysis. The difference can be seen, also, from the cost perspective of the analyzers and sensors, where an analyzer cost from 50 to 200 thousand $, while a sensor can cost up to 100 thousand $. Sensors are deemed to be more attractive, since they cost less, are easier to integrate in the process, easier to maintain and replace, mobile, more robust and can be easily deployed across a manufacturing plant. Though, the most current and common commercial process spectroscopic instruments belong to analyzer category.

Figure 3.2 depicts a comparison between common instruments used in process analysis from a ‘business’ and an ‘analytical’ scope. ‘Analytical’ scope focuses in the detection performance of each instrument (e.g. precision, repeatability, sensitivity) and selectivity. The ‘business’ scope focuses on quantifying the various investments from the company: training of the users, capital cost and running costs, ease of replacement etc. While the relative position is widely disputed among experts, the resulted plot highlights the wide range and the differences in complexity among process instruments in these two dimensions.

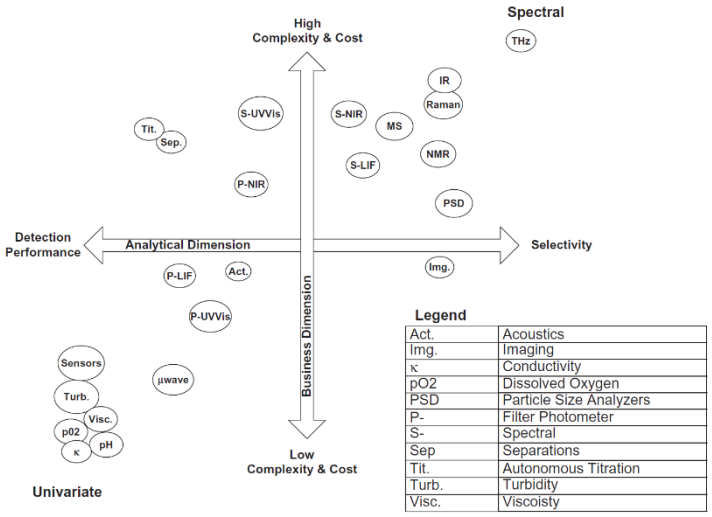


Figure 3-2 Process instrument classification

3.2 In situ monitorization in biotechnological processes

The shift of biotechnology from a traditional discipline to a cutting edge technology was a 30-year results of immense advance and growth in the disciplines proteomics, genomics, molecular biology, microbiology and genetic engineering. Biotechnology is seen nowadays a promising field with countless uses in very diverse sectors, such as environment, new materials and biochemical analysis, energy production, food and pharmaceutical industry etc. That's why, biotechnology and bioprocesses has become a field of immense between international companies.

Control of bioprocesses achieve higher efficiency and productivity, reduced production cost, stable quality and reduces the environmental impact of the production process. The most important compound in all bioprocesses, the biocatalyst, is prone to even the slightest changes of environmental conditions. Every uncontrolled alteration, even in the smallest scale, in pH, feed composition or temperature can lead to alteration in the cell metabolism, which in short time can change the efficiency of the process and in mid-term render the whole process unprofitable. Full process control in bioprocesses aim to achieve and retain the optimal conditions for the cells to grow, proliferate and produce the desired products.

A pre-requisite in process and subsequently bioprocess control is an efficient and robust methodology for monitorization. The monitorization of processes can be divided roughly in two major categories. Off-line monitoring consists of two steps: manual or automatic sample taking from the bioreactor and the analysis of these samples in offline analyser. This methodology, which is the oldest and still the most frequently enables high precision measurements, but sacrifices a lot of time for analysis and delays between measurements. Off-line results, due to their high precision, are usually accumulated and used for developing models for future used in the control of the processes. On the other hand, on-line monitoring provides information during the process. A direct approach of the process provides the chance for detecting production problems early on and taking countermeasures immediately in order to resolve the problem.

The use of on-line monitoring instruments in laboratory or industrial uses is not widespread. This is mainly due to the unique nature of each analyte and the complexity of the medium that these analytes are suspended. One important prerequisite for the success of such monitoring the preservation of the sterility of the reactor. This fact, in combination with the fact that usually, the most important analytes are in very small quantities (e.g. antibiotic, gene expression inductor, hormone) adds a lot of degrees of complexity. Because of these reasons, the concentration, purification and determination of such compounds is very time-consuming and complex, thus the use of off-line analytic tools is suggested.

Each bioprocess is unique, that's why every monitoring technique that will be used must be adapted to the needs of this bioprocess. Some require higher degrees of asepticity, other require high degrees of precision and less sterility. In this study, the focus in on sensors and more specifically in in-situ light microscopy sensors.

3.2.1 In situ sensors

Bioprocesses can last from a couple of hours to a dozen of days, thus the in-situ sensors should be durable enough to endure at least the time needed for the completion of the process. The most common problem is the precipitation of biomaterials contained in the reactor medium, especially proteins, on the surface of the sensors, which to baseline drift and inconsistency in the results given. One important problem that all in-situ sensors have to endure is the extreme conditions of sterilization (high temperature) and the necessity to stay calibrated after the exposure in such conditions. Also, another important prerequisite is the ability of the sensor to be recalibrated, while being mounted in the reactor without compromising the sterility of the reactor. The most commonly used in-situ sensors are reviewed in the following sections.

3.2.1.1 Classical-electrochemical and temperature sensors

Autoclavable , in-situ sensors for pH, dissolved oxygen and temperature have widespread application, both in laboratory and industrial scale. The core of the temperature sensors is resistance temperature detectors (RTD) made out of platinum. The principle of RTD relies on the change of electrical resistance of the wire, which is proportional to the change of temperature. A standard potentiometric glass electrode is used for measuring pH. Dissolved oxygen is measured with Clark amperometric electrodes. Those three variables, in combination with optical density measurements and agitation and aeration rates are the variable that every biotechnologist usually have. The combination of these data can lead to development of models, which can relate these variable with other in-process variables. Though the increased complexity of the medium used and of the processes themselves deem these models inadequate to depict the reality of the process, thus new sensors, which measure directly more important variables are needed. [11,12]

A recent progress has been made with the use of Severinghaus electrodes for the monitorization of dissolved CO_2, which is increasingly important in the cultivation of mammalian cell.

3.2.1.2 Electronic tongues

Electronic tongues (ET) are a very novel idea in the field of chemical analysis. Until now, sensors focus of one specific analyte and try to measure it as accurately as they can. Considering, though, how complex the culture media are and all the possible interferences that can be present, make this task increasingly difficult.

Electronic tongues and subsequently electronic noses are based on the mechanism of the olfactory sense of animals. The sensor, instead of having one specific signal for each analyte, it composes a combination of all the signals. This combination can be used qualitatively very fast, in order to detect for example defective raw material inside the reactor. The quantitative use of such sensors could very well be utilized in on-line process monitoring. Though so far no commercial applications have been made, the use of such sensors is feasible, since the constituents of such sensor are available commercially and are easy to autoclave.

3.2.1.3 Ion selective field effect transistor (ISFET) sensors

Close to the concept of electronic noses and tongues are ISFET sensors. The principle of ISFET sensors are metallic oxides with semiconductor abilities. These oxides absorb into their surfaces gases with different oxidation properties than oxygen, thus changing the semiconductors electric properties. Since these oxides have cross sensitivity, they are used also in the electronic tongues and noses. A great drawback of this sensor is the need to work in high temperatures (400 ^{o}C), thus it restricts the use of such sensors on if off gas analysis, since bioprocesses have a much lower range of working temperatures. [11,15]

3.2.1.4 Optical Density sensors

Optical density is the most widely used measurement in bioprocesses. Usually, it consists from an autoclavable probe with an optical fiber guide. The light emitted is usually in the infrared zone, since it is proven that such wavelengths are more accurate than different wavelengths.

The main problem of OD sensors is their limited range of measurements. Different types of cells in same concentrations provide inconsistent results. Also, an important problem that arises, is the inconsistency of the results in higher concentrations of cells, suspended solids and bubbles. Also the sensor is inadequate in distinguishing living and dead cells. Still though, it is widely used since it offers a fast and easy way of measuring the cell concentration inside the reactor.

3.2.1.5 UV–Vis spectrophotometry

UV-Vis spectrophotometry in-situ is used mainly for measuring online the pH value. This is possible with measuring the color intensity of pH sensitive dyes like phenol red. The sensor monitors the pH by measuring the specific absorption of light with certain wavelength. Modified Severinghaus electrodes have been utilized from Uttamal and Walt to measure pH, while they could also measure at the same time dissolved CO_2. The drawback is that Severinghaus electrodes suffer from long response times. Also they have limited range because of the limitations of each dye used. [11,17]

3.2.1.6 IR spectrophotometry

Near infrared (NIR) and mid infrared (MIR) spectroscopy can be used to measure various process compounds such as glucose, glutamine, proline, fructose and CO_2 [11,21]. The detection limits of near infrared spectroscopy are higher than MIR and biosensors, but is well within the limits of bioprocess monitorization. This method, though, proves to be complex in calibrating and is more expensive than other alternatives. The main drawback is the broad peaks due to overlapping, thus advanced mathematical models such as principal component analysis have to be used. The calibration needed for such instrument should be 6 times the number of the analytes, thus considering that complexity of bioprocesses, the whole monitoring becomes a very time consuming process. The possibility, though, to use the same calibrations for repeated processes, which is the case for industrial applications, makes the use of IR spectroscopy in-situ feasible [11].

3.2.1.7 Raman spectroscopy

Raman spectroscopy is suitable for in-situ monitoring of bioprocesses. It has not gathered so far a lot of scientific attention, since its main drawback is that many molecules in bioprocesses fluoresce in the region of Raman scattering bands. Researchers have reported successful monitorization of glucose, formate, acetate and other intermediate metabolites from fermentation of E. Coli. [11,22]. This method though is appropriate for laboratory scale for the time being, since it take a full 2 minutes to receive a full spectrum. Also it is necessary to investigate more the interactions between Raman spectra and compounds of fermentation broths that fluoresce.

3.2.1.8 Heat exchange monitoring

A novel method for process monitoring is the measurements of heat exchanges of the reactor with its surroundings. Generation of heat is correlated with the metabolic state of the cells inside a bioreactor. Monitoring the heat exchanged from a bioreactor is much less costly than the previous methods described, though it requires precise mathematical models, that include each time many process variables such as the viscosity and the heat capacity of the broth, microorganism morphology, biomass concentration etc. Voisard et al proposed it is easier to monitor heat dissipation in large scale experiments as opposed to laboratory scale experiments, because of the favorable volume to surface ratio. [11,23]

3.2.1.9 Capacitance sensors

Measuring the capacitance of one bioreactor can be a tool for measuring biomass [11,24,25]. Living cells with intact cell membrane can operate like capacitances, therefore it is possible to measure the viable cell concentration. Such sensors are available commercially by Aber Instruments Ltd., Aberystwyth, UK. These sensors, though, are limited due to low sensitivity.

3.2.1.10 NMR

NMR is a technique, which has been established in the research of enzymatic reactions. The greatest advantage of this methodology is that it is completely noninvasive, since the whole bioreactor can be placed in a magnetic chamber, and can also provide a clear insight of the metabolic activity of the cells. The main disadvantage of using NMR real-time is that it requires a lot of time to record a NMR spectrum and also the cell concentration should be high enough in order to have precise results. [11,26].

3.2.1.11 In situ biosensors

In situ biosensors rely on the principle of reaction rate of an analyte with a specific enzyme that the biotechnologist decides to use. So far only glucose has been successfully measured with glucose oxidase mounted in a Clark amperometric electrode. The main drawback is, that the enzymes that are used cannot withstand the high temperatures needed to sterilize the sensor. Also another problem that arises is that since there is an active reaction inside the biosensor, on one hand the analyte is being consumed, unsettling the equilibrium inside the reactor and on the other hand the products of the reaction can be toxic for the cells such as hydrogen peroxide, which is produced by oxidases.

In order to keep the enzymes protected from the thermal shock of the sterilization, the enzyme can be kept in a reservoir together with the electrode. This reservoir is separated for the rest of the reactor with semi permeable membrane. This membrane can be sterilized separately, so it works as a barrier of sterility. The advantages on one hand is that it 1) protects the enzyme 2) permits recharge of the enzyme carrier and the disadvantage son the other hand is that the response time increases to even more than 10 minutes and the semi permeable membrane is prone to fouling after continuous use.

3.2.2 Photo-Optical In-situ light microscopy for cell size detection

In 1990, Kirin brewery, locate in Japan developed the ancestor of modern in-situ microscopes and named it fermend scope [29,30]. This in-situ microscope consisted with the same basic components that today's in-situ microscope is consisted are a CCD camera and a microscope. This microscope offered x10 and x20 magnification and the illumination resulted from an external light source and transferred inside the reactor with a glass fiber. Since it was the first attempt, the images were not analyzed with image analysis software, and offered only an interface to control the image acquisition. All the different constituents were stored in a waterproof boxed, which was immerged inside the reactor. Since this box was not sterilizable, fermend scope could not be used in bioprocesses that required asepticity, and was used only in brewing facilities, which are very robust.

The first to report in situ microscopy in a scientific paper was Suhr et al in 1991 [29,31]. Nowadays many different inline microscopes have been developed, with few of them being in-situ online and most of them being in-situ at-line. The different probes can roughly be separated into 2 categories: transmitted light microscopes using mechanically defined sampling zone and incident light microscopes with an optically defined sampling zones.

3.2.2.1 Transmitted light in situ microscopes with mechanically defined sampling zone

A steam-sterilizable ISM (Fig. 3.3) and implemented IA software was presented for the first time in 1995 [29,31] The ISM consists of an aluminum tube that fits in the bioreactor's closure top. The microscope's sampling zone slide is defined by two sapphire gaps (slide and cover slip) and an oval shaped silicon seal which is located around the slide. The medium liquid flows through the gap freely. Before image capture, a moveable compressor is turned in the direction of the cover slide to shut the gap, thus capturing a predefined volume between slide and cover slip. The moveable compressor is made from a miniaturized lamp and a one-lens condenser incorporated in a cylindrical case. The moveable compressor is pushed by a motor in the control instrument. Particles and cells captured in the chamber stabilize and allow capture of still images. After acquisition, the gap is reopened. An achromatic lens with 20-times magnification is put in the inner tube. Focusing is only possible by the operator and can be done with a micrometer screw. A 45 degrees' mirror reflects the incoming light in the control unit, where a CCD camera resides. The image data are stored on a hard drive and are processed by an image analysis program for calculating variables such as cell count and average cell volume/ cell diameter.

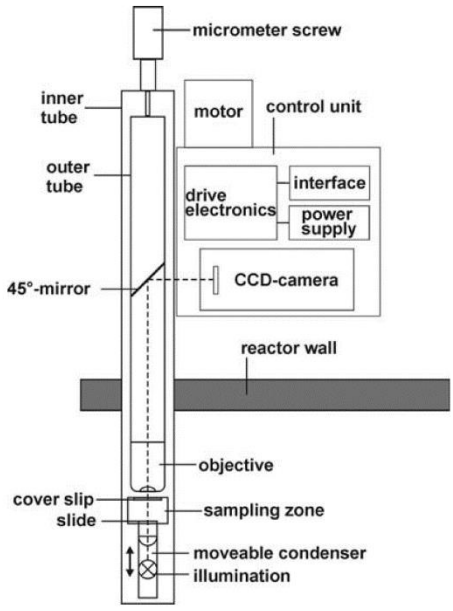


Figure 3-3 Layout of a transmitted light microscope with a mechanically defined sampling zone

3.2.2.2 Transmitted light in situ microscopes with optically defined sampling zone

A transmitted light in situ microscope using a sampling zone optically defined is analyzed by Camisard and Guez et al. The ISM (Fig. 3.4) consists of an outer circular tube with a quartz window and an inner circular tube on which an objective (40-times

magnification) is put. Focusing is also performed by the operator. Attached to the submerged top of the outer tube is an illumination instrument with a pulsed LED (synchronized with the trigger of the CCD-camera) as source of light. The LED emits at a wavelength of around 600 nm is placed on the other side of the quartz window. This microscope does not capture a predefined volume mechanically. In this way, the volume for the calculation of the cell count is also defined optically with the in-depth focus procedure. After the acquisition, an image analysis software processes the image data in regard to cell count and cell size distribution.

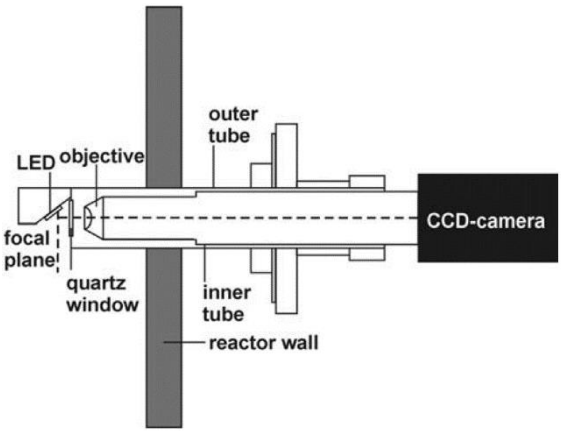


Figure 3-4 Layout of a transmitted light microscope with an optically defined sampling zone

3.2.2.3 Flow Cytometry

Flow Cytometry is a laser-based technique used in biotechnology to assess biological and physiological characteristics of several types of cells. Most applications of flow cytometry are witnessed in human cells, while the analyses of bacterial cells have just recently been under observation. This technology can be employed as an at-line monitoring tool in bioprocess development, for example in microbial biofuels production processes (Silva, Roseiro et al. 2012). It is widely used as a method to simultaneously quantify cellular characteristics and the levels of cellular components. Assays that employ flow cytometric techniques have been established to determine both cell characteristics and the parts of cell components such as protein, DNA, surface receptors, and calcium. A flow cytometer is a combined system of fluidics, optics and electronics. The objectivity, high analytic performance rate, precision and high sensitivity are the reasons for the implementation as a process analytical tool. Basically, a flow cytometer composed of the fluidics system which is responsible for confinement of cells for individual analysis in laser beam. The optics system constitutes the source for the lasers which illuminate the cells in the cell suspension and as well as optical filters which direct the resulting light signals to the appropriate detectors. Finally, the electronics system converts the optical signals into electronic signals, which can be processed by the computer using appropriate software. In contrast to the conventional microbiology methods, it is possible, using several staining dyes, to differentiate cells based on their structure and metabolic activity.

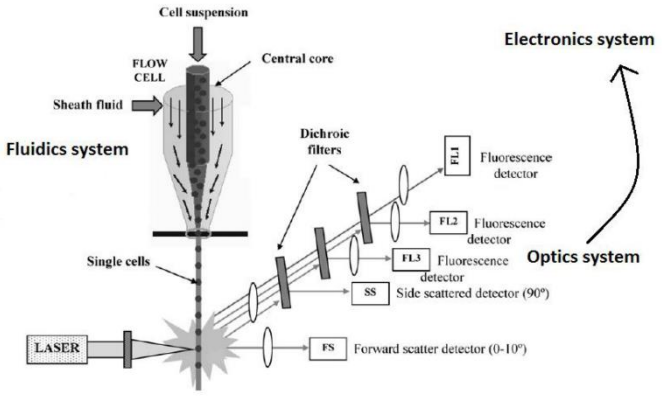


Figure 3-5 The combination of the systems in a flow cytometer

3.2.2.4 Digital Holographic Microscopy

Holography was first introduced and utilized in the late 1940s (Gabor 1948). However, the studies at that time were in sense proof-of-concept demonstrations. Only in contemporary years have computers and digital image sensors become fast enough to reconstruct images with sufficient resolution, speed, and quality. This has recently led to the creation of in-situ instrumentation that uses digital in-line holography to detect particles and organisms, for example, the holosub by Pfitsh et al. (2005) or a 'point-source inline digital holographic microscopes' (Bochdanky et al. 2013). This instrumentation is based on an on-line holographic configuration, where the pattern interference results from the light, that is dispersed by the object with the unscattered beam that encircles the object (i.e., object and reference beams are linear). In-line holography has the advantage that it requires a simple, inexpensive optical setup, but also has some inherent drawbacks (the presence of a ghost image accompanying any refocused object, less accurate optical phase information, a high noise level inherent to the use of a coherent laser beam) [35].

These issues can be resolved by combining an off axis DHM configuration, where object and reference beams are separated and recombined on the camera sensor, with partial coherent illumination (Dubois et al. 1999; Dubois et al. 2004). The principle of digital holographic microscopy (DHM) using an off-axis Mach-Zehnder interferometry configuration is based on a laser beam that is split into two separate components: a so-called reference beam, which travels unhindered from its source to the CCD camera used for the image capture, and a so-called object beam, which travels through the object or sample of interest (Fig 3.11). The object beam interferes with the reference beam to create an interference image or hologram, which is recorded by the CCD camera [35].

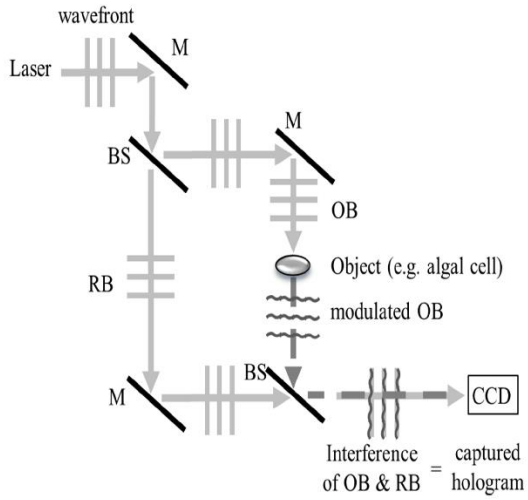


Figure 3-6 Conceptual diagram of the set-up used for off-axis digital holographic microscopy (Mach-Zehnder configuration). A wavefront of light (a laser) is split at a beamsplitter (BS) into an object beam (OB) and a reference beam (RB). The OB passes through the

3.3 The yeast Saccharomyces Cerevisiae

Saccharomyces cerevisiae is a eukaryotic organism. More specifically, it is a globular-shaped, yellow-green yeast, which belongs to the kingdom of fungi. Natural strains of the S.cerevisae reside on surfaces of plants, the gastrointestinal system and body surfaces of warm-blooded animals and insects and soils from all around the world [36]. The most usual place that yeast is found in places where fermentation processes take place such as wine/beer cellars, the surface of rotting food and on equipment used for fermentations [37].

S. cerevisiae is widespread known for its importance in fermentation processes. S. Cerevisiae converts sugar to alcohol on various substrates from wine to beer and every other beverage, that contains alcohol. Also it has a great importance in pastry and bread, since when mixed with dough it produces gases, which create this distinctive texture in bread and cake. Yeast has been used in fermentative process for thousands of years, with indications of fermented drinks back in Ancient Egypt in 7000 BC and ascertained remains of S.Cerevisae cells back in ancient China in 3140 BC.

Isolation of the species, though, occurred in 1938, when Emil Mark isolated it from rotten figs found in Merced, California. Robert Mortimer combined the strain from rotten figs with other strains of contemporary scientists and created strain s288c. Furthermore, this strain was then used to sequence the S. cerevisiae genome [37].

3.3.1 S. Cerevisiae Cell Cycle

S. Cerevisiae has two forms: diploid and haploid. The most common form is the diploid. The diploid form has usually a diameter of 5 to 6 μm with an ellipsoid-like shape, whereas the haploid is more spherical with a diameter ranging to 4 μm. Both forms reproduce with a process called budding. In this process, when the mother cell reaches a critical size, in its surface a daughter cell protrudes. The difference with usual cell division is that instead of one cell getting larger and then dividing in half,

budding yeast mother cell reaches a critical size and stays at this size, while only the daughter cell gets bigger in size.

The process is as follows:

1. As budding begins the DNA duplicates (S phase of interphase).
2. The bud emerges as a protuberance on the surface of the mother cell.
3. As the cell progresses into mitosis (G2/M), chromosomes are divided between the bud and the mother cell.
4. The bud enlarges then separates from the mother cell to form an independent cell.
5. This process is the same regardless of whether the cell undergoing budding is diploid or haploid.
6. Yeast morphology is correlated with progression through the cell cycle. Cells with no buds are in G1, cells with small buds are in S, and cells with large buds have progressed from G2 into mitosis as shown in Figure 3.12. Thus through microscopy, one can determine the cell cycle phase of a particular yeast cell.

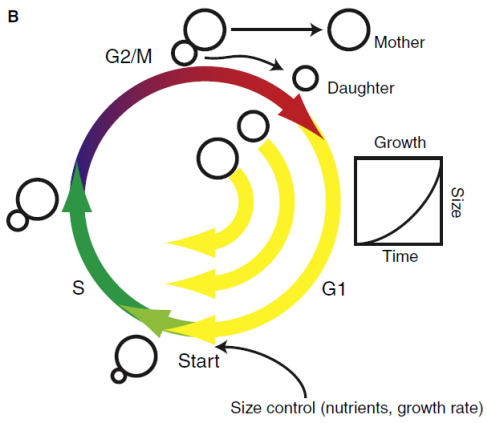


Figure 3-7 Cell cycle of yeast S. Cerevisiae [43]

S cerevisiae can grow in both anaerobic as well as aerobic conditions. In the presence of oxygen, yeast can work with aerobic respiration, where glucose molecules are broken down to carbon dioxide and produces ATP by protons falling down their energy gradient to an ATPase. When there is not enough oxygen, yeast can get their energy only from glycolysis and the sugar is instead transformed into ethanol, a much less energy-efficient process than aerobic respiration. The main source of energy and carbon is glucose, and when glucose concentrations are very high, there is a gene repression of enzymes used in respiration and fermentation enzymes takes over respiration [42]. However, other sugars can also be used as a carbon source. Sucrose can be converted into fructose and glucose by using an enzyme called invertase, and enzyme mannase can break down maltose into two distinct molecules of glucose.

3.3.2 Application in Industry

Ethanol Production

One of the first applications of S. cerevisiae in biotechnology is production of alcohol-rich beverages. In a process named fermentation, yeast grows on sugars from their substrate and transforms it to ethanol, increasing the alcoholic content in these beverages. Depending on the type of beverage, yeast is included into the manufacturing process in many different ways.

Wineries choose their yeast genus based on many factors: local climate, type of grape, the desired taste of the final product and the geographical area [42]. Yeast is then gown in the winery, then poured to the crushed grapes, when fermentation is ready to start. Exception is champagne, where natural-born yeast strains are used, since yeast is added directly into the bottle instead through a huge vat [42]. To create sparkling wine, more gas is pumped into the bottle.

In beer brewing processes, two main different types of yeasts are used in the fermentation, depending on the kind of beer created. Yeasts fermenting on top, known as ale yeasts, create foam on top of the barrel, that contain the sugar that is converted by the yeast to alcohol. The yeast population stays at the surface of the reactor, and the fermentation takes place in warm temperatures. This process is used in the creation of porters, ales, wheat beers and stouts. On the other hand, bottom-fermenting yeasts, the so called lager yeasts, ferment at colder temperatures, and the yeast stays at the bottom of the reactors. [42].

Another yeast process, that is used to produce the beverages known as alcoholic spirits, such as tequila and vodka. Yeast grown in such fermentations of these beverages, are stemmed from sugar cane or beet. Selection reasons for these yeasts are production of ethanol in high concentrations, high tolerance to high ethanol concentration, and able to ferment different substrates depending on the beverage produced [42].

Food Production

S. cerevisiae also works as a leavening agent. During preparation of the mixture, dried yeast is added with the other ingredients. While baking, yeast grows and reacts with its environment and releases carbo dioxide. This gas is trapped inside the dough, creating holes as it bakes. This contributes to the characteristic spongy-like texture of cakes and breads seen after baking. While a leavening agent is included in the dried yeast, in order to add specific flavor to the bread, unleavened yeast is also added. [42].

Yeast used in the brewing process of beer is still useful after the fermentation. After the fermentation process, the yeast that is left ca be dried and sold in various forms. Dried yeast contains B vitamins, proteins and various minerals, and can be taken as a food supplement [42]. Hydrolyzed yeast or else yeast extract still contains many of its nutrients, thus is can be used in a variety of applications, ranging from flavor enhancer to media for growing yeast [42]. Also yeast has probiotic applications, since it can grow

non-pathogenically in the gastrointestinal tract, while killing other pathogens residing there.

Recombinant Protein study and production

Study of secretion of recombinant proteins in S. cerevisiae is generally two-staged: i) it can work as a model organism to study various human diseases connected with ER stress and defective protein folding, such as Parkinson's ,Alzheimer's, atherosclerosis ischemia, diabetes mellitus(Yoshida et al 2007); ii) nowadays more than 20% of protein-based pharmaceuticals commercially are produced through S. cerevisiae (Martínez, Liu, 2012), including hepatitis B surface antigen, insulin, glucagons, urate oxidase, hirudin, and granulocyte macrophage colony stimulating factor (Demain and Vaishnav et al 2009).

S. Cerevisiae is long known for being a model organism for the research of recombinant protein synthesis and production, thus there are many different studies on molecular bioengineering of it, as well as research on production processes. Usually these kinds of studies focus on obtaining very specific results: usually they focus on the production of one specific protein and in some cases a few proteins, and produce in in very small concentrations sometimes even in the milligram scale. This is normal though, since recombinant protein processing is very complex and unique to each protein. In the future, the goal is to derive general models, that work not for one or a few proteins, but a large number of different proteins, that would be produced in concentrations that deem the process of its production economically feasible.

3.4 Scale-down experiments of yeast

In research scale, the metabolic activity of the cells is measured in controlled and homogeneous conditions. On the other hand, in industrial scale bioprocesses with reactor volumes from 10 to over 500 m^3 are inhomogeneous due to inconsistent hydrodynamic conditions, caused by limitations in the mixing. This creates gradients of nutrients, products, cofactors, and cells (especially in processes with many species) within a culture. Cells in an industrial scale reactor move through different concentration zones and experience immediate and harsh changes in their environment. Consequently, the constant change in their environment, adjusts their metabolic activity a lot differently than those grown in homogeneous well-stirred conditions [44].

One of the most important variables in aerobic processes is the dissolved oxygen (DO) concentration (pO_2). [44,47]. In aerobic cultivations, the DO is closely connected to the consumption rate of the sugar/carbon source, often glucose. If the glucose consumption per cell is high, the oxygen drops and becomes insufficient at high cell concentrations. This problem is engorged in large-scale fed-batch processes, where gradients in the substrate are created because the feed has high concentration of the carbon source. In a large-scale glucose-process of Saccharomyces cerevisiae showed enhanced ethanol concentrations and a reduced biomass yield in comparison with smaller scale homogeneous cultures, which were performed under same conditions [44,45].

An underestimated parameter that has great importance in large-scale bioreactors is the pH, since the metabolic activity of the microbes is affecting and affected by the pH of the medium. Also, gradients can be created, due to highly concentrated pH control agents, which can have great impact to local pH [44].

Scale down experiments with yeast have been analyzed by several research papers with several kind of setups [45,46] and all showed that non-steady conditions have an influence on the microbial metabolic activity and therefore on the bioreactor efficiency.

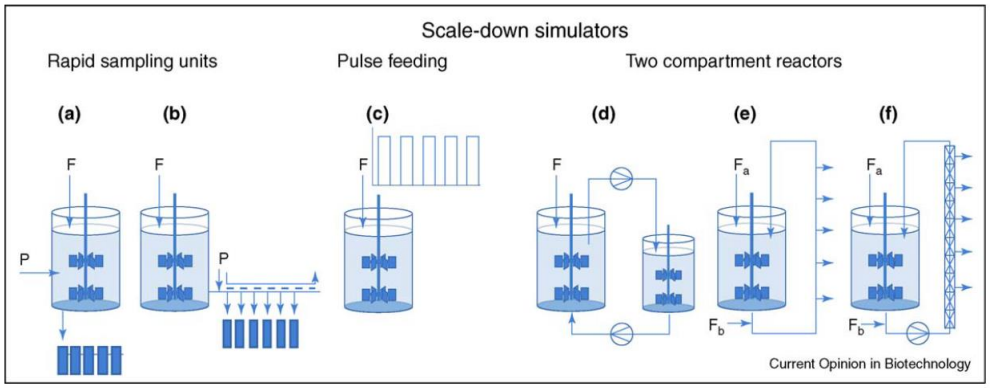


Figure 3-8 Schematic presentation of different scale-down simulators. (a,b) Single pulse simulators. (a) Rapid sampling device after a pulse addition into the bioreactor; (b) stop-flow sampling device (plug-flow reactor) with different sampling positions which rep

4 Aim of the Thesis

The aim of this thesis is to utilize the in-situ SOPAT sensor to monitor online the yeast cell size distribution.

Industrial applications, while they have quite successful methods of measuring physical properties online in reactors, such as temperature, pressure, pH etc., they lack the instrumentation of measuring online important process variables, which have a much more direct impact into the efficiency of the process, and particularly in bioprocesses variables such as the cell size, cell heterogeneity and other. A novel solution for this problem is in-situ light microscopy, which is the core component of this thesis.

So far light microscopy has been used offline and in some applications at-line. This configuration, though, doesn't provide the data needed to take immediate measures to correct the altercation of one condition, that could affect the cell metabolism and render it economically unfeasible, fast enough. The development of a robust and reliable in-situ light microscope will solve this important problem that not only pharmaceutical and biotechnology industries face, but every industry that works with chemical/biochemical reactors.

In this thesis the under-development in situ microscope SOPAT is used to monitor online the yeast cell size distribution under different environmental conditions, try to relate the size distribution with the actual growth state of the cells, coupled with at-line and off-line analyses of them and find the crucial parameters and the weaknesses of the sensors, so that it can become more reliable and ready to be commercially available in the coming years.

5 Materials & Methods

5.1 Strain and cultivation conditions

Media composition and preparation

For the cultivation of yeast S. Cerevisiae YEPD complex medium was prepared. As a carbon source dextrose (Sigma-Aldrich, Steignheim, Germany) was used in various concentrations (Table 2.1) and as nitrogen source a combination of yeast extract (Ohly, Hamburg, Germany) and peptone from casein (Sanofi, Gentilly, France) was used. In the medium also present were NH_4Cl (Roth, Karlsruhe, Germany) and KH_2PO_4 (Roth, Karlruhe, Germany).

Table 5-1 Medium compositions

	Concentration (g/L)	
	Medium 1	Medium 2
Dextrose	22	1.65
Peptone from casein	20	20
Yeast Extract	10	10
NH4Cl	1	1
KH2PO4	14	14

All the aforementioned substances were diluted with deionized water. Important note is that dextrose was autoclaved in separate container from the other substances, since in high temperatures it reacts with phosphate and creates toxic compounds. Autoclavation was done in 120°C and finally 1 liter of each medium was prepared. The pH value of the media was adjusted in 5.5.

Organisms

For both experiments the laboratory strain of *Saccharomyces cerevisiae AH22* (MATa leu2-3 leu2-12 his4-519 can1), stored in -80 °C.

Preculture procedure

For each experiment a different preculture was prepared and each time with 2 repetitions, from which, the one that was better grown was used in the next step. During the cultivation, the optical density of the cultures was monitored in regular time intervals with a spectrophotometer (Amersham BioSciences, Buckinghamshire, UK) at a wavelength of 600 nm. In this way the growth and the cell density was monitored.

Starting with the cell cycle experiments, in the first step, the preculture consisted of 90μl of cryoculture, 5 ml of medium low in dextrose in sterilized 125 ml Ultra Yield Flasks (UYF) and was cultivated in orbital shaker (Infors GmbH, Einsbach, Germany) in 250 rpm and 25°C. Two repetitions were prepared. After 2 days, when the OD reached ~2, the two precultures were reinoculated to a target OD of 0.3 with 10ml of

medium rich in dextrose and 400μl from preculture one IN 2 125 ml UYF. After being cultivated in the same conditions for one day and reaching an OD of ~16, the were reinoculated for a third time with 35 ml of low dextrose medium and 625 ml of preculture 2 to a target OD of 0.3 in 8 250ml UYF. After that, they were cultivated until they reached an OD of 0.6 and were used for the cell cycle experiments.
The whole procedure was repeated, since the whole experiment was repeated, but the difference was that during the third inoculation medium rich in dextrose was used.

For the stress experiments, the preculture consisted of 90μl of cryoculture, 50ml medium rich in dextrose and 500μl sterilized antifoam (Sigma-Aldrich Chemie GmbH, Munich, Germany) in 6 sterilized 250 ml UYF and was cultivated in 250 rpm and 25°C in orbital shaker (Infors GmbH, Einsbach, Germany).

5.2 Analytics

Off line Analytics

Cell Dry Weight analysis

For calculating cell dry mass, a couple of days before the cultivation, 2ml Eppendorf tubes were dried in an oven at 80°C. After they were completely dried, they were weighted in analytic scale (Sartorius,Göttingen, Germany). During the cultivations, samples were taken and stored inside the Eppendorfs, which were later centrifuged (Eppendorf AG, Hamburg, Germany) and their supernatant was subsequently discarded. After that, they were dried at 80°C and weighted again in analytical scale. The difference to their mass before and after was the cell dry weight.

Quantification of sterols with GC-FID

Total Sterol Analysis

For this analysis, the samples were diluted to a volume of 20ml with an OD_{600} of 12, which corresponds to a cell cry weight (CDW) of 3.5grams/liter. Each sample was then centrifuged in 2°C, 6000 rpm for 6 minutes (Eppendorf AG, Hamburg, Germany) twice and resuspended eventually with 2 ml of deionized water. The sample was then mixed with 100μl of Cholesterol (Sigma-Aldrich Chemie GmbH, München, Germany) internal standard. The internal standard had a concentration of 1mg cholesterol per ml, diluted in chloroform (Sigma-Aldrich Chemie GmbH, München, Germany). In the aforementioned mixture, 2.5 ml of HCl (Carl Roth GmbH &Co. KG, Karlsruhe, Germany) of 1N concentration were added and were incubated in water bath at boiling water. After 20 minutes the samples were cooled, the HCl was neutralized with KOH (Carl Roth GmbH &Co. KG, Karlsruhe, Germany) and 12 ml of pyrogallol (Sigma-Aldrich Chemie GmbH, München, Germany) solution in methanol (Carl Roth GmbH &Co. KG, Karlsruhe, Germany) in a concentration of 0.25 g/l were added. The new mixtures were incubated in water bath again at 70°C. After 1 hour and 45 minutes of incubation the samples were cooled in room temperature. The following step was hexane extraction were 15 ml of hexane (VWR International GmbH, Darmstadt, Germany) were added and the samples were shaken in a General Rotator Drive, STR4 (Bibby Scientific Limited, Staffordshire, UK). After 15 minutes the upper phase (hexane phase) was isolated. The whole process was done twice in each sample and the hexane phase was stored in 100ml round bottom flasks. The hexane was later evaporated in a rotary evaporator (Büchi GmbH, Ingolstadt, Germany) at 35°C and 290mbar pressure. The dried samples were then resuspended with 1.5ml of isopropanol (VWR International GmbH, Darmstadt, Germany) and stored in brown vials. The resuspension was done in an ultrasonic bath. The samples were later analysed in a gas chromatographer (Shimadzu Deutschland GmbH, Berlin, Germany)

The temperature program that was used was the following: The initial temperature was 150°C and was constant for 2 minutes, then it started increasing 15°C/min until 250°C, where it was kept constant. After 37 minute the temperature increased again with a rate of 5°C/min until 290°C, where it was kept steady for 7 minutes.

Free Sterol Analysis

For this analysis, the samples were diluted to a volume of 20ml with an OD600 of 12, which corresponds to a cell cry weight (CDW) of 3.5grams/liter. Each sample was then centrifuged in 20C, 6000 rpm for 6 minutes (Eppendorf AG, Hamburg, Germany) twice and resuspended eventually with 2 ml of deionized water. The sample was then mixed with 100µl of Cholesterol (Sigma-Aldrich Chemie GmbH, München, Germany) internal standard. The internal standard had a concentration of 1mg cholesterol per ml, diluted in chloroform (Sigma-Aldrich Chemie GmbH, München, Germany). To obtain the free sterols, the cell membrane had to be ruptured. This was done by mixing the sample with4ml of glass beads (Carl Roth GmbH &Co. KG, Karlsruhe, Germany) in a miller (Retsch Technology GmbH, Haan, Germany). In this mixture, 2 ml of a solution of 4:1 chloroform (Sigma-Aldrich Chemie GmbH, München, Germany)- methanol was added. After 15 minutes, the glass bead was separated using nylon filter (Carl Roth GmbH &Co. KG, Karlsruhe, Germany) and the glass beads stuck on the filter were washed with 2 ml of chloroform. The resulting sample was centrifuged in 9000 rpm at 2°C, so that the two organic phases could separate. After 15 minutes, the lower chloroform phase was transferred to 50 ml around bottom flasks. The chloroform was evaporated in a rotary evaporator (Büchi GmbH, Ingolstadt, Germany) at 35°C and 474 mbar pressure. The dried samples were then resuspended with 1.5ml of isopropanol (VWR International GmbH, Darmstadt, Germany) and stored in brown vials. The resuspension was done in an ultrasonic bath. The samples were later analysed in a gas chromatographer with the same temperature program as in total sterols (Shimadzu Deutschland GmbH, Berlin, Germany).

Quantification of extracellular side products with HPLC-RID

During the experiments, 2 ml sample were taken from each flask during regular time intervals. The samples were centrifuged and the supernatants were stored in -20°C. 200 µl of each supernatant were stored in transparent vials and subsequently analysed in a high performance liquid chromatographer (HPLC-RID) (Agilent Technologies Deutschland GmbH & Co. KG, Waldbronn, Germany). The flow rate was adjusted at 0.5 µl/min in a temperature of 15°C.

Glucose enzymatic test

The glucose analysis was done with a glucose enzymatic testkit (DiaSys Diagnostic Systems GmbH, Holzheim, Germany). Initially a 50 ml stock of 4:1 mixture of solution 1 and 2 was prepared in a falcon tube, which was covered with aluminum foil to protect it from sunlight. Then, 1 ml of this solution was mixed with 10µl of sample, waited 10 minutes and analysed in a UV-Vis spectrometer (Amersham BioSciences, Buckinghamshire, UK) at a 340nm wavelength. The blank was 1ml of enzymatic solution with 10µl of deionized water and the reference was an empty cubette. When

the sample had optical density over 0.8, they were diluted accordingly, since the linear zone of the instrument was between 0.2 to 0.7.

At line Analytics

Optical Density

During the cultivation, a quick measurement that can be indicative of the cell dry weight is the optical density of the sample. One ml of the sample was taken and analyzed in a UV-Vis spectrometer (Amersham BioSciences, Buckinghamshire, UK) at 600nm wavelength. Samples with optical density over 0.8 were diluted, since the linear zone of the instrument is between 0.2 and 0.7.

On line/At line Microscopy

For the single-cell size distribution monitoring (fig. S 1), the 3-dimensional digital holographic microscope oLine-OT40GA (Ovizio, Belgium) and the photo-optical probe SOPAT MM 1 (SOPAT, Germany) were applied. Measurements of the cell size distribution were either performed directly in the culture broth (SOPAT) or on a microscope slide (Ovizio). Since the photo-optical sensor was not mounted directly to the shake flasks or the bag of the single-use bioreactor due to limited space and the lack of sensor ports, samples were taken and directly filled in 50 mL plastic tubes. The SOPAT sensor was dipped into the cell suspension. By moving up and down with the plastic tube, circulation of the fluid through the measurement gap was achieved. In case of the DHM, the cell suspension was diluted to an optical density of OD_{492} = 12, and captured on a microscope slide. Table II provides an overview of main characteristics of the microscopes.

Table 5-2 Overview of the main characteristics of the applied microscopes

Parameter	SOPAT MM 1	oLine-OT40GA
Field Depth	2.32 µm	1.5µm
Camera	2750x2200 CCD with 19fps	2456 x 2058 CCD with 15 fps
Interface	GigE Vision	-
Magnification	10x with an adaptive TV-lensewith a magnification factor of 1.6	x63

Numeric aperture	0.1	0.7
Illumination	Transmission, Xenon flash lamp, 2.6 J, pulse duration 8µs	Transmission, Monochromatic LED at 630 nm
Measuring Gap	200µm	not applicable
Probe length	270mm	not applicable
Probe diameter	24.5mm	not applicable
Software Version	SOPAT v1R.002.0053	OsOne-4.3

Several settings such as size boundaries or the algorithm to be used were adjusted for automated algae cell detection at the DHM and ISM. Parameters are summarized in table 3.3.

Table 5-3 Parameters for the detection of algae cells in the OsOne software version 4.3 (Ovizio) and SOPAT detection software (SOPAT)

Parameter	**OsOne**	**SOPAT**
Background	2.15	-
Median cell size (d_{50}) [µm]	32	18
Background detection algorithm	Phase variance	-
Cell detection algorithm	Local maximum	v1R Algo
Apply refocus	yes	yes
Detect invalid areas	yes	yes
Cell minimum size [µm]	9	8.4
Cell maximum size [µm]	-	23.5
Remove image defects	9	adjusted
Split neighbor cells	6	adjusted
Invalid area- sensitivity	4	-

The Sauter diameter measured with both techniques is equivalent to the diameter of a circle that has the same area as the cell. The circularity feature provided with the DHM is the ratio between the areas of the cell to the square perimeter. The ratio describes the circularity of a cell (1= exactly circular shape,0=no circular shape). The phase homogeneity represents a measure of the spatial closeness of the image of a particle's surface. Values range between 0 and 1, the maximum value is achieved when the co-occurrence matrix is diagonal.

Cell detection was performed by the software and inspected visually for proper detection. Whenever cells overlaid each other, they were restricted from cell detection. In average, 10 captures were taken with the DHM and 100 at the ISM. The slide (cell suspension) was moved between captures of the holographic microscope (photo-optical probe) in order to assure a replacement of cells in the image field.

5.3 Studies in shake flasks and STR with single cell size distribution analysis

Stress experiments

In order to determine the reaction of cells under stress conditions and monitor their growth and the

Yeast cells, when grown in non-optimal conditions, show immediate changes in their cell size distribution. In the first set of experiments, yeast cells where induced in a stressful environment and the change in the cell characteristics were monitored with on-line, at-line and off-line analytic tools. The Design of Experiments (DOE) was the following:

- The preculture that was prepared for these experiments was divided in 6 sterilized 250 ml UYF in volumes of 50 ml. The compounds that would create the stressful conditions were acetic acid (CH_3COOH) and ethanol (EtOH).
- In 2 of the aforementioned UYF nothing was added and they were deemed as the control flasks – their purpose was to compare their results with the results of the other flasks with stressful conditions.
- In the next 2 flasks, some environment rich in ethanol was created. In order the analysis to be more convenient and precise a stock solution of ethanol was prepared. The stock solution of ethanol was prepared for an initial stock of 70% (v/v) ethanol (VWR International GmbH, Darmstadt, Germany) to a final concentration of 2400mM diluted in deionized water. The final volume of the stock was 10ml. The target concentration inside the flasks was 5% v/v or in term of molarity 120mM in ethanol, thus in each flask 0.228 ml of ethanol stock was added. Every thirty minutes the SOPAT sensor was used and samples were taken for off-line analysis.
- In the last 2 flasks, and environment rich in acetic acid was created. Again for the experimenter's convenience and preciseness a stock solution of acetic acid was prepared. The stock solution had a concentration of 400Mm, prepared from initial stock of 100% v/v acetic acid (Carl Roth GmbH &Co. KG, Karlsruhe, Germany), diluted in deionized water. The final volume of the stock was again 10ml. In order to adjust the pH in a value of 6, 1.28 grams of sodium sulfate (CH_3COONa) (Carl Roth GmbH &Co. KG, Karlsruhe, Germany) were added. The target concentration inside the flasks was 5% v/v or in term of molarity 20mM in acetic acid, thus in each flask 0.228ml of acetic acid stock was added. Every thirty minutes the SOPAT sensor was used and samples were taken for off-line analysis.
- The off-line analysis, that the samples were used for, were cell dry weight analysis, intermediate metabolites and glucose enzymatic test.

Cell cycle synchronization methods

A second set experiments was done, this time to monitor the growth of the yeast cells on-line and off-line, while they are synchronized. This was achieved, by cultivating, for a limited time, the yeast cells with specific substances, that block the yeast cell growth in a specific phase. Later, these substances were removed, restarting the cell growth, which was this time synchronized, leading to a greater cell homogeneity and much more concentrated cell size distribution.

The protocol, that was followed, utilized the drug Nocodazole (Sigma-Aldrich Chemie GmbH, München, Germany), which blocks the cell growth in G2/M phase [57,58]. More precisely, a stock solution of 500μg/ml of Nocodazole was prepared dissolved in 10 ml of DMSO (Carl Roth GmbH &Co. KG, Karlsruhe, Germany). In each cell cycle preculture, after if reached an OD of 0.6, 1ml of 100% DMSO (which corresponds to 1%v/v of the sample) was introduced, in order to help the yeast cells to get accustomed to the solvent. After half an hour, 1.1 ml of nocodazole solution was added to a final concentration of 15μg of nocodazole per ml of culture. After 1 and a half hours, the cells were centrifuged (Eppendorf AG, Hamburg, Germany) twice at 5000 rpm for 2 minutes and re-suspended in dextrose medium. Then, the 8 samples were merged and the SOPAT sensor was introduced inside the solution and started measuring. The sensor was to take pictures every 5 minutes and every 30 minutes' samples were taken for at-line and off-line measurements. At-line measurements were pH value, OD, ovizio and optical microscope analysis and off-line measurements were intermediate metabolites analysis with HPLC-RID and glucose enzymatic analysis.

STR Experiment

As part of the thesis, it was deemed important to compare results of SOPAT sensor with results from 3D-Microscopy from samples taken from a cultivation of the same strain in a stirred tank reactor (STR).
The protocol followed was the following:

Preculture medium

A total volume of 1 L YEPD medium was prepared. The composition of the medium is the same as the one used in cell cycle and stress experiments. Important point is the sterilization of glucose separately from the other components. Also 10 ml of antifoam were sterilized and used along the precultures.

Batch-phase medium

The end volume for YEPD medium for the batch phase was 10L. 500 ml of glucose solution was prepared separately.

Fedbatch-phase medium

The end volume was 2 L of YEPD medium, but the concentration of glucose was doubled to a concentration of 40 gr/L. Again glucose was sterilized separately.

Preculture preparation

In each step 2 precultures were made and the one that was better grown was chosen for the next step. Throughout the preculture preparation, the optical density was measured at 600 nm with a spectrometer (Amersham BioSciences, Buckinghamshire, UK) to get an overview of the cell growth. For the first preculture, 50 ml of medium, together with 90μl of cryoculture and 500μl sterilized antifoam were added in 250 ml UYF in an orbital shaker (Infors GmbH, Einsbach, Germany) in 250 rpm and 25°C. After the OD of preculture 1 reached a value of ~28, which happened after almost 3 days, the second preculture was prepared. Again 50 ml of medium were mixed with 500μl of sterilized antifoam and 2ml from preculture one (the one that was better grown) in a 250 UYF in an orbital shaker and cultivated in 250 rpm and 25°C. After almost one day, the third preculture was prepared, in which 10 ml of preculture 2 were mixed with 250 ml of medium and 2.5 ml of antifoam in a 2L UYF and cultivated in 250 rpm and 25°C.

Cultivation in STR

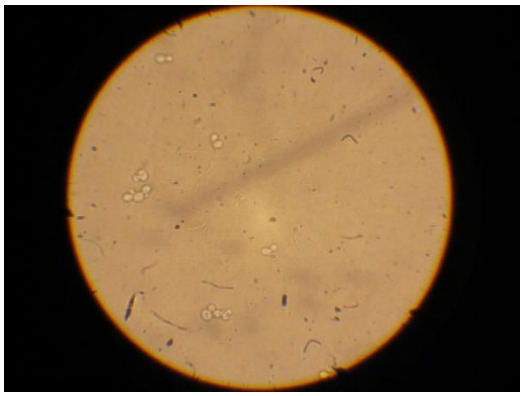
After sterilizing the reactor, the inoculation started. The first phase was a batch-phase, which lasted around 19-20 hours, until the OD reached a value of ~24. Then, the reactor changed to an exponential feed. The profile of the feed was $F=k*v_L*e^{\mu t}$, with $k=0,00318h^{-1}$ and $\mu=0.12h^{-1}$. The initial feed was set at 0.53 ml/min. The fedbatch cultivation lasted another 13 hours. During both phases (batch and fedbatch) the temperature was constant at 27°C. The oxygen flow was set at 0.5 vvm and the stirrer speed at 500 rpm. After 4 hours of cultivation, the flow rate was changed to 0.7 vvm and 650 rpm. The entire process is monitored with the aid of sensors. Throughout the cultivation a DO sensor, a PH sensor (all three Hamilton Germany GmbH, Planegg-Martinsried, Germany) and an OD sensor (Mettler-Toledo GmbH Germany, Giessen, Germany) were used. The pH value was adjusted with the assistance of a connected to the STR caustic soda pump and was maintained between 5.5 and 6. An exhaust gas analysis was also connected and measured the oxygen and carbon dioxide concentration of the exhaust gas. The exact protocol is attached in the Appendix.

6 Results

6.1 Cell cycle 1 synchronization results

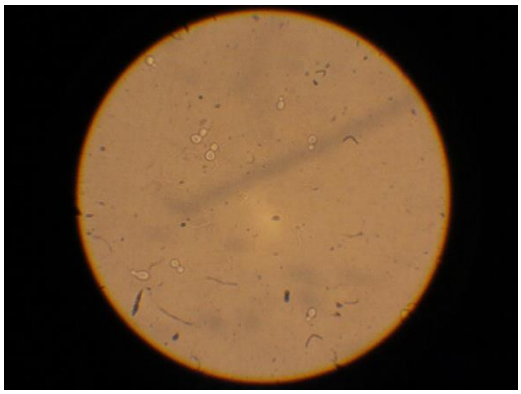
Optical microscope results

In the first cell cycle experiment, before, during and after removing nocodazole, pictures were taken with optical microscope to monitor the influence of the drug to the cells and their growth.



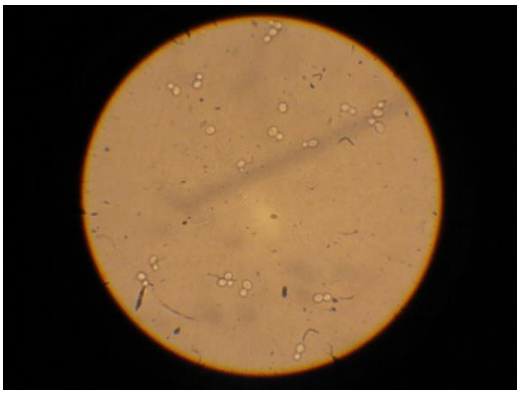
Picture 6-1 Before adding Nocodazole.

Each cell is in different cell cycle



Picture 6-2 35 mins after adding Nocodazole.

Most cells are in G2/M-phase and some still in S-Phase phase

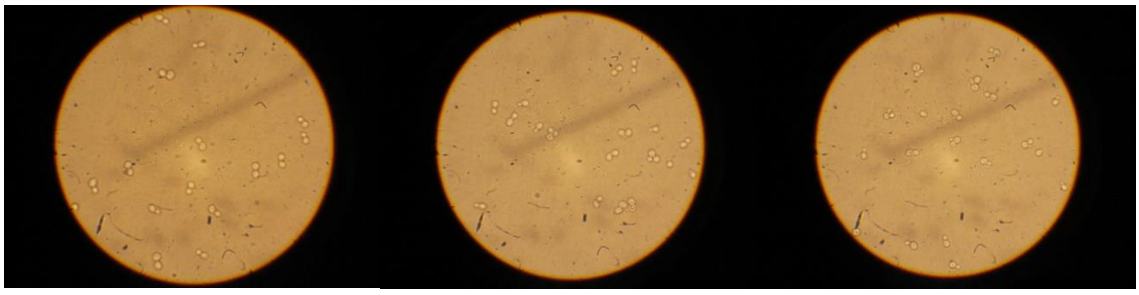


Picture 6-3 90 mins after adding Nocodazole.

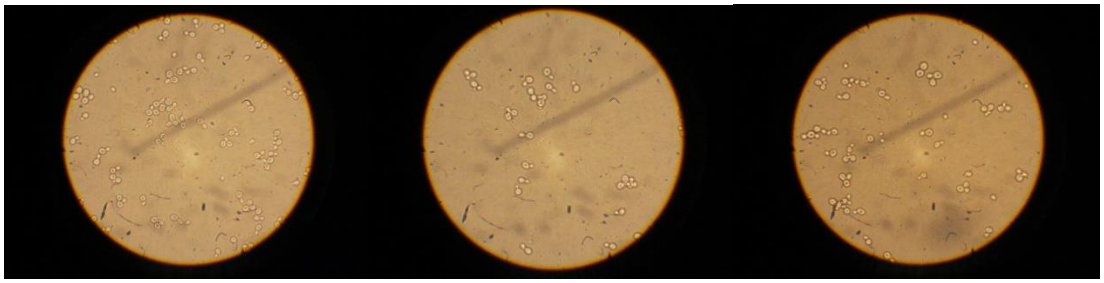
Largest percent of cells are blocked in G2/M-phase

Already after 35 minutes from adding nocodazole, there is a clear tendency of the cells to stop growing in the G2/M phase. In picture 3.3 the largest percent of the cells are in this phase, with very few in G1 phase.

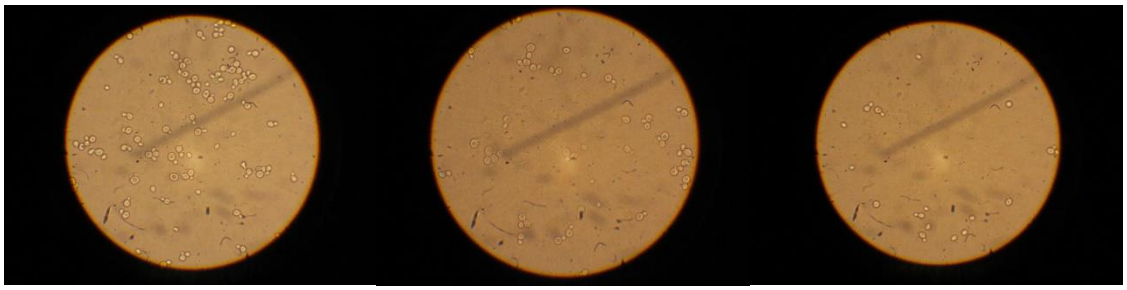
After 1.5 hours, the samples were centrifuged to remove the supernatant, that included nocodazole, and re-suspended with medium low in dextrose. Every half an hour, samples were taken and used for several analyses, including taking photos with the optical microscope.



16:00 – G2/M phase | 16:30 - G1 phase | 17:00 – G1 phase



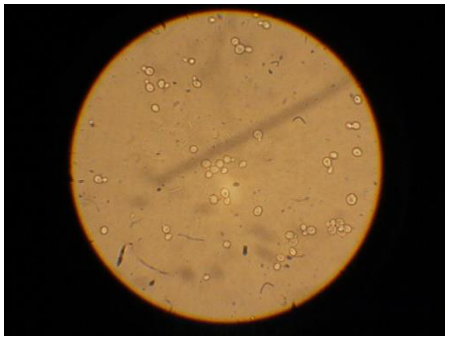
17:30 – S phase | 18:00 – S phase | 18:30 – S/G2 phase

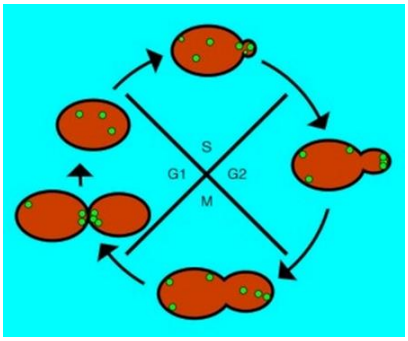


19:00 – G2/M phase

19:30 – G2/M phase

20:00 – G1 phase





20:30 – S phase

Optical Density and pH results

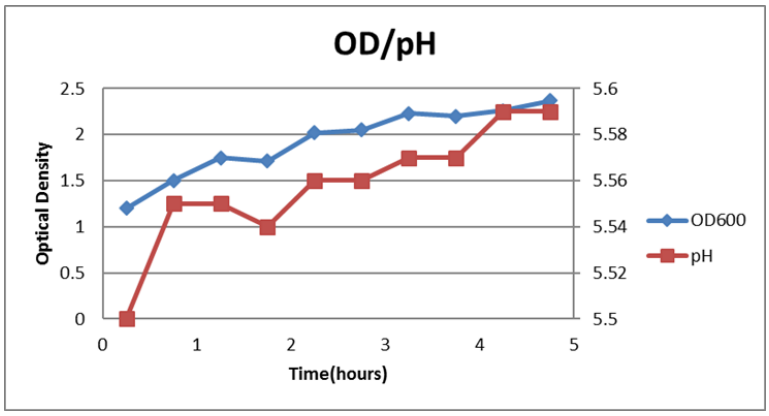


Chart 6-1 Optical Density and pH value across time for the first cell cycle experiment

As it is expected, the OD steadily increases with time. What is important to point out, is that the OD until ~1.5 hours increases with a different gradient than in later times. This will be explained later, mainly due to the fact of the sugar limitation conditions until 1.5 hours and the sugar depletion after 1.5 hours, which naturally resulted in decreased OD increase. The pH is almost constant to a value of 5.5, which is the pH

value of the initial medium. (The pH is slightly increasing, probably because the ethanol is consumed as a carbon source in sugar depletion conditions.)

Glucose enzymatic test results

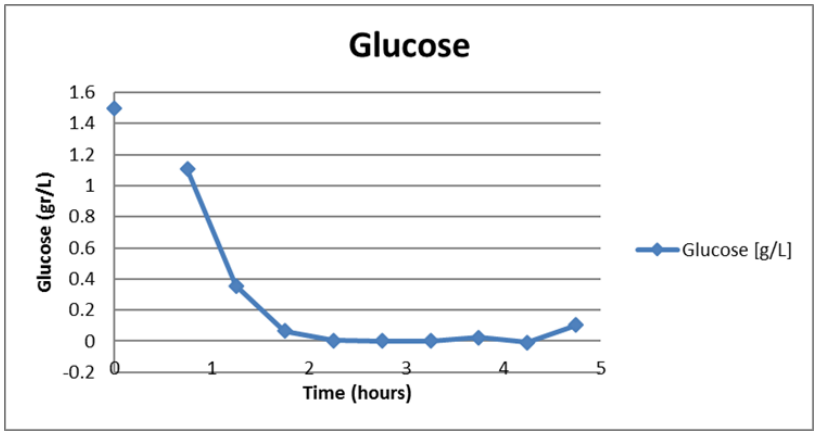


Chart 6-2 Glucose concentration derived from glucose enzymatic kit across time

In the first cell cycle experiment, after nocodazole solution was removed, cells were re-suspended in low-glucose medium with 1.5 grams of glucose per liter of medium. This resulted, as shown in the diagram, quickly to sugar limitation conditions and after ~1.5 hours to sugar depletion conditions.

HPLC-RID results of carbon cycle intermediates

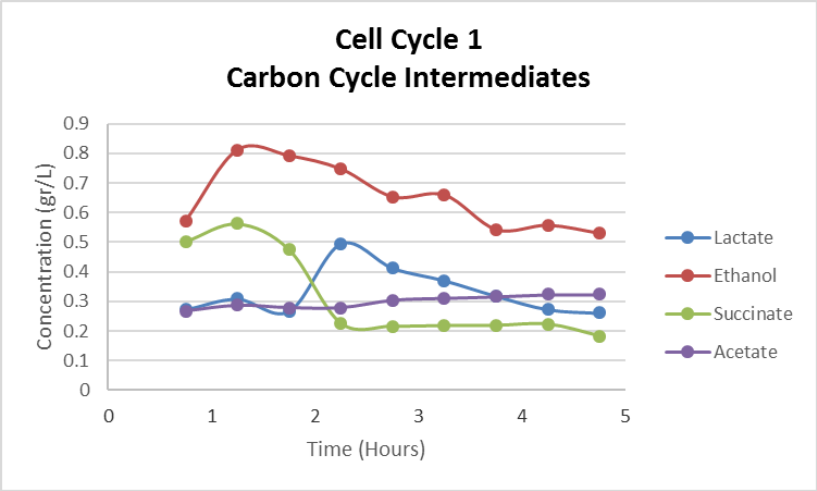


Chart 6-3 Intermediate metabolites concentration 1

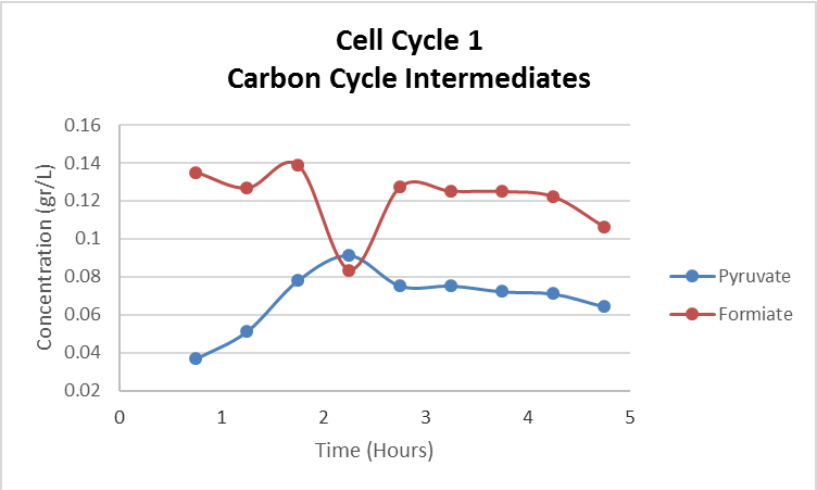


Chart 6-4 Intermediate metabolites concentration 2

Firstly, it has to be pointed out that formiate and pyruvate were secreted in very low concentrations, that's why they were plotted in a different chart.
The charts of the intermediate metabolites can be divided in 2 parts. The first one until ~1.5 hours is the sugar limitation region and after 1.5 is the sugar depletion region. During the first 1.5 hours, all metabolites are increased, especially ethanol, with the exception of formiate. After 1.5 hours, where sugar depletion conditions have been created, all metabolites are being decreased, with the exception of lactate, which slightly increases.

3D Microscopy results

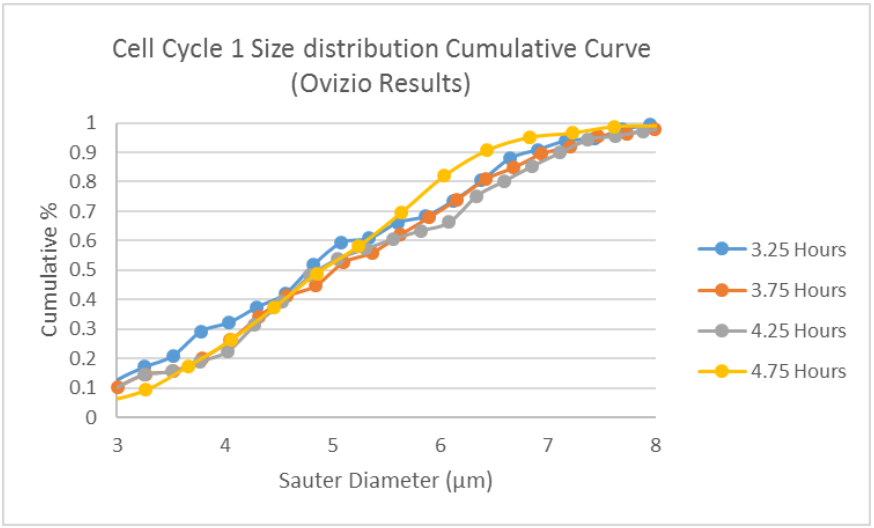


Chart 6-5 Cumulative distribution of yeast Sauter diameter

During the last four measurements, when the cell concentration is above a certain threshold needed for Ovizio analysis, sample were taken for 3D Microscopy.
As referred before, an average cell cycle of S. Cerevisiae lasts ~100 min, which corresponds to the time between the first and the last measurement.
Thus, it is clear that the cell distribution has a tendency to higher diameter across

time, until the cell cycle ends, as can also be seen from the mean Sauter diameter (chart 3.6)

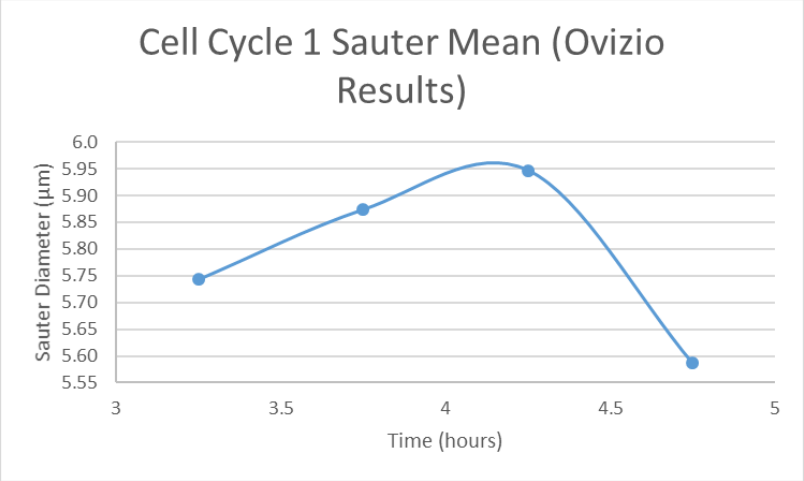
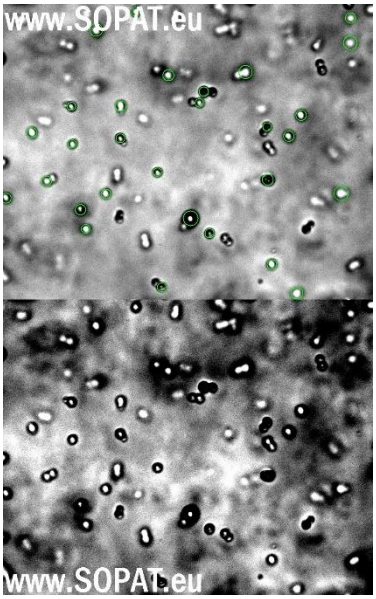


Chart 6-6 Mean Sauter diameter of yeast cells

SOPAT results



Picture 6-4 SOPAT take on bottom picture and the same picture processed from SOPAT software on top

A typical picture taken from SOPAT sensor is shown on picture 6-4. The bottom picture is the raw picture taken from the sensor, whereas the top pictures is the same picture processed from SOPAT software. The software recognizes patterns of cells and encircles them with green circles. Thus it calculates the cell size. In each take, approximately 50 pictures are taken. To be more precise, the instrument takes pictures until 5000 cells are identified or else 50 different pictures are taken.

A convenient measure for calculating an average size for microorganisms is the Sauter Diameter. Sauter diameter is the ratio of the cube of the volume mean diameter to the square of the surface mean diameter; roughly the ratio of the particle volume to its surface area. Since yeast cells are almost spherical, the mean is the same with the maximum and the minimum value of the diameter.

During the first cell cycle experiment, pictures were taken every 10 minutes for 4 and a half hours. Not all the data gathered from this experiment, though, was processed, but only certain time points.
From this data, the average Sauter diameter was plotted, together with the cell count from each processed time point.

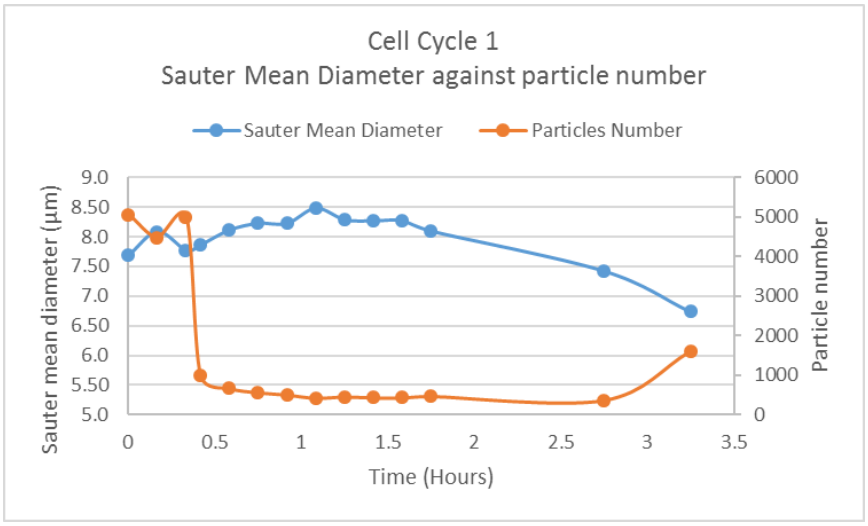


Chart 6-7 Cell Cycle 1 Mean Sauter Diameter against Cell Count

What is important to point out is that the first 3 time points were processed from a different parameter set, thus is lead to totally different cell count.
The software calculates the different sizes of the cells and, then, it can plot the cumulative cell size distribution. Since not many time points were processed and considering that an average yeast cell cycle lasts ~90 minutes, for cumulative size distribution plot points during the same cell cycle where plotted.

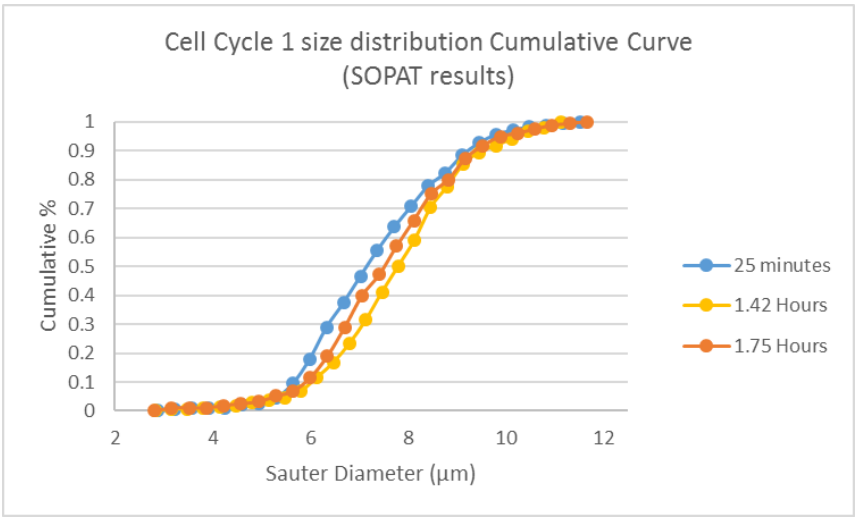


Chart 6-8 Cumulative distribution of yeast Sauter diameter

It's obvious that after 25 minutes, large percentage of the cells have small diameters, meaning they are G1 phase. As time progresses, cells progress to S phase and then to G2/M phase. This results to an increase of the mean Sauter Diameter, which in the cumulative size distribution translates to higher percentages in larger cells. After

almost ~80 minutes the cell cycle comes to an end and returns to the phase that this cycle started. This can be seen from the curve after 1.75 hours, where the distribution resembles more and more with the initial one.

6.2 Cell cycle 2 synchronization results

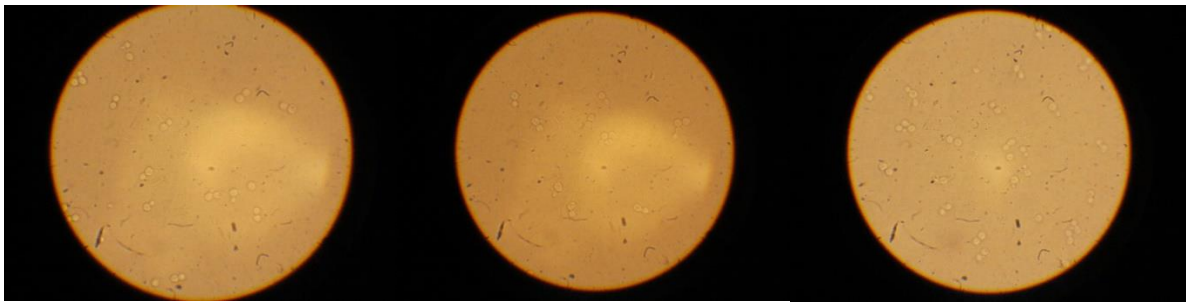
In the second cell cycle synchronization experiment, the conditions under which the experiment was performed were the same except:

- The measuring gap of the SOPAT sensor. The first experiment was performed with a measuring gap of 2mm. This resulted in pictures, taken from the probe, that were not clear enough. Thus, the second experiment was done with 0.2 mm measuring gap, enabling a much clearer and more precise capture of yeast cells.
- During the step of removing the nocodazole and re-suspending the cells in fresh medium, the medium instead of low in glucose, it was rich in glucose (22 grams of dextrose instead of 1.65 grams), in order to avoid sugar limitation conditions.
- The experiment lasted 3 hours, since at the end of the 3rd hour, while adjusting the aeration of the sample, the sample itself was spilled.

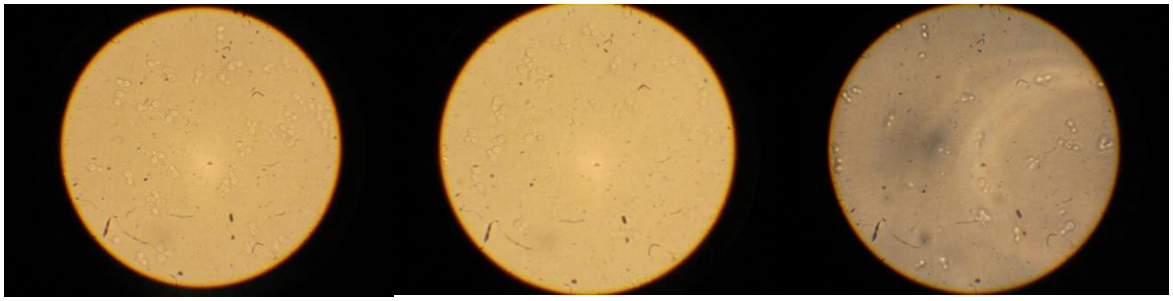
Optical microscope results

Since the experiment was performed under almost the same conditions, pictures before and after the addition of Nocodazole were not taken.

The same protocol was followed throughout the experiment. After the separation of Nocodazole solution and the resuspension of the cells, every half an hour, samples were taken and used for several analyses, including taking photos with the optical microscope.



15:20 15:50 16:20



16:50 17:20 17:50

Optical Density and pH results

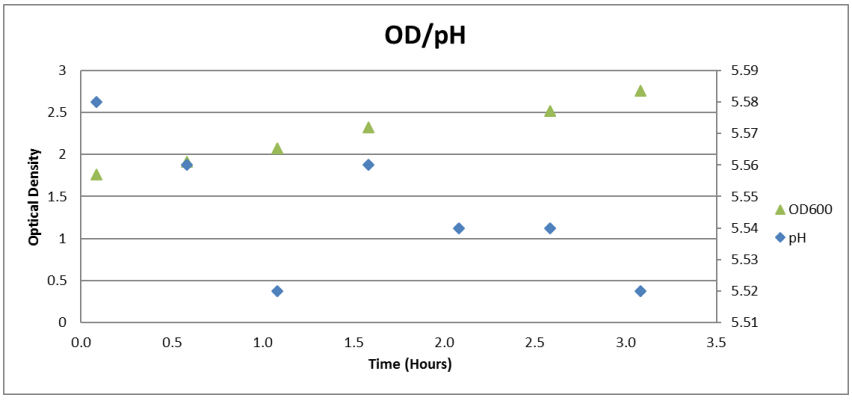


Chart 6-9 Optical Density and pH value across time for the second cell cycle experiment

Just like in the first cell cycle experiment, the OD increases steadily. The pH value is close to the of 5.5, which is again the pH value of the medium, though it now decreases slightly as it is expected, since the cell cycle intermediates and products (especially ethanol) are accumulating.

Glucose enzymatic test results

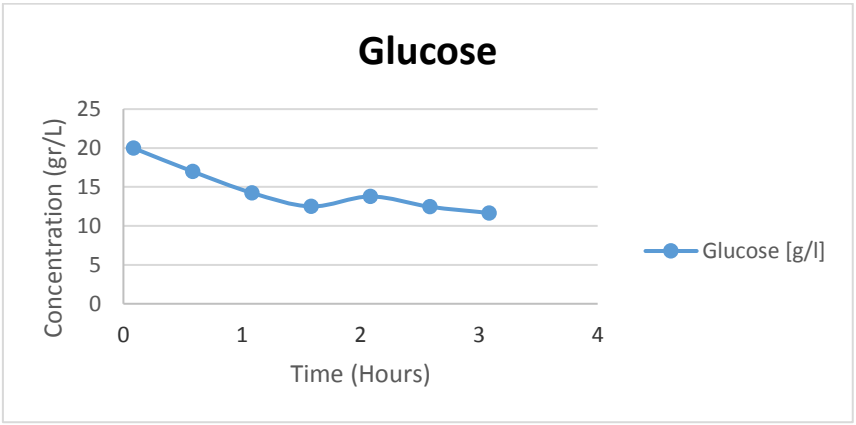


Chart 6-10 Glucose concentration derived from HPLC-RID across time

As mentioned before, the medium that was used in the second cell cycle experiment was rich in dextrose to avoid sugar limitation conditions. This concentration, though, was out of the detection limit of the UV-Vis instrument, that is used to calculate OD and subsequently the concentration, thus the glucose content was calculated using the HPLC-RID instrument, that is used to calculate the intermediate metabolites.

HPLC-RID results of carbon cycle intermediates

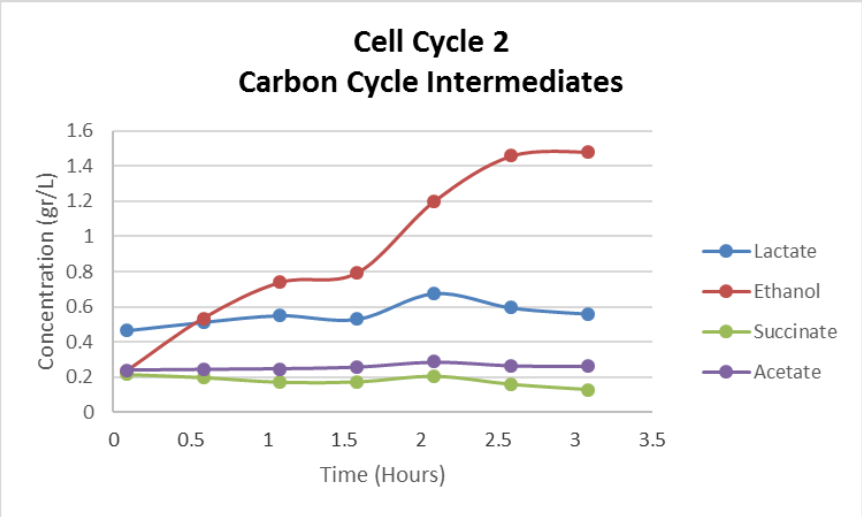


Chart 6-11 Intermediate metabolites concentration for second cell cycle experiment 1

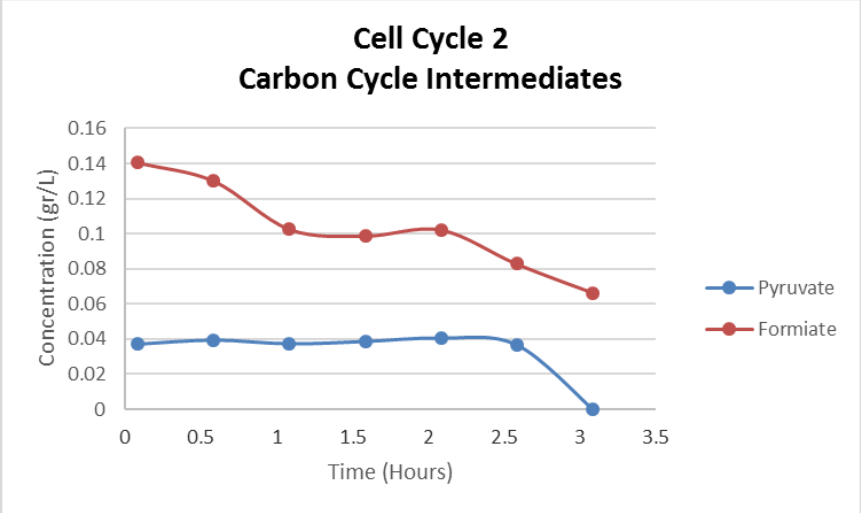
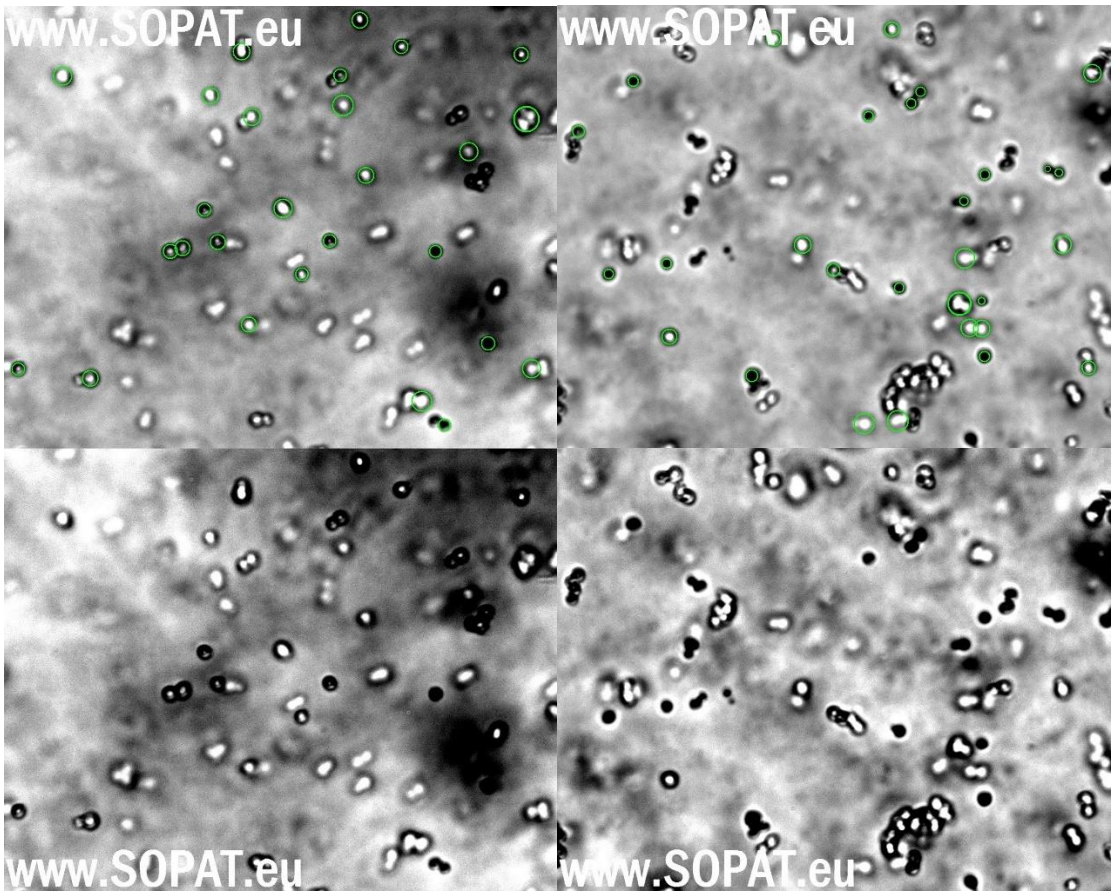


Chart 6-12 Intermediate metabolites concentration for second cell cycle experiment 2

The pyruvate and formiate were plotted (again) on a different chart, since they have lower concentrations than the other metabolites. Also during the last measurement, pyruvate was below detection limits, thus it is pointed out as 0.
It is important to point out the steady increase of ethanol. The main reason for that is the trigger of overflow metabolism, because of the high glucose content of the medium.

SOPAT results

The second cell cycle experiment, as stated before, differs mainly because the SOPAT sensor was adjusted with a 0.2mm measuring gap. This change lead to pictures with better focus.



Picture 6-5 Pictures taken with SOPAT at 2 mm measuring gap (left) and 0.2 mm measuring gap (right)

The SOPAT probe was set to take, in the second cell cycle experiment, pictures every 5 minutes. This time a better parameter set was used giving much more concise and understandable results and shown in the next chart.

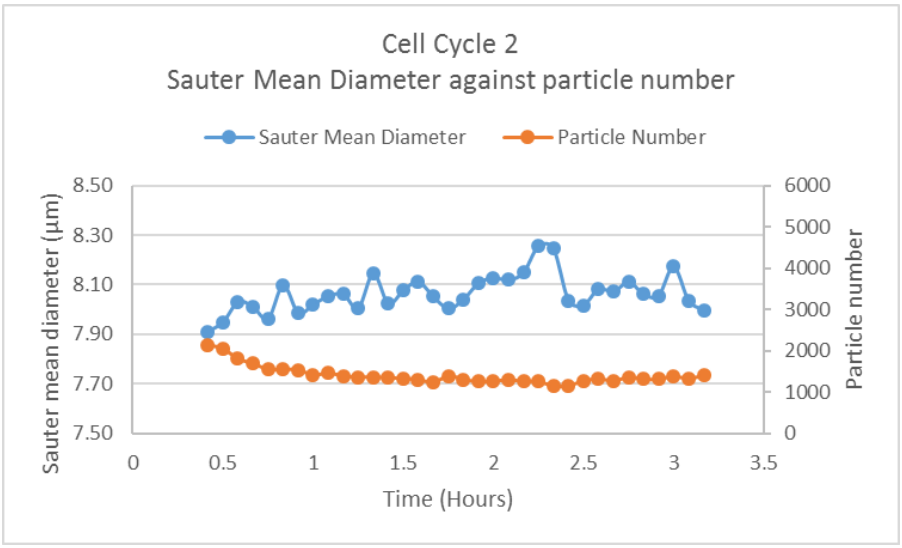


Chart 6-13 Cell Cycle 2 Mean Sauter Diameter against Cell Count

This time, the parameter set helped take pictures with almost the same cell count and constantly above 1000, a fact with great importance as it will be discussed in the results section.

Judging from the chart, we can see a specific pattern, that repeats. After a spike in the diameter of the cell, which corresponds to G1/M phase, there is sharp decline of the diameter, which corresponds to S phase, where the cells divide, then the diameter increases slightly and reaches a plateau, during which the DNA is being multiplied, and when the DNA replication is finished, the maximum diameter is reached and then, the whole process is repeated.
During the 3 hours of the experiment, almost 3 generations of S. Cerevisiae, where cultivated based on the results, which correspond to the average duration of cell cycle for this strain of yeast.

For the cumulative chart time points after 2.5, 2.75 and 3 hours where chosen to show the distribution of cell sizes during one cell cycle.

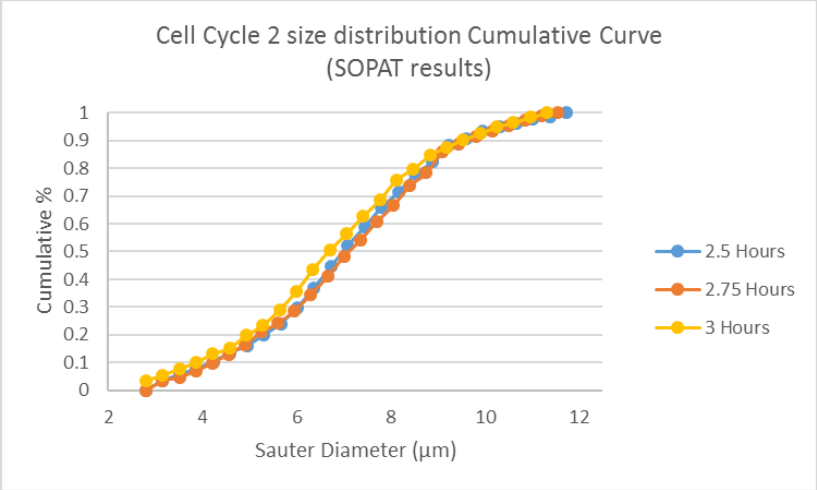


Chart 6-14 Cumulative distribution of yeast Sauter diameter

During this half an hour, the cells increase in size, meaning there is an accumulation of larger cells, as can be seen by comparing the curve after 2.5 hours and the curve after 2.75 hours. Then they reach maximum diameter, after which they divide in many smaller cells, a fact that can be seen in the increased percentages of cells with lower diameters in curve after 3 hours.

6.3 Stress conditions experiments results

Dry Cell Weight

During the stress conditions experiments, samples were taken after half an hour and at after 2 hours of experimentation. These samples were used for offline analyses.
The resulted dry cell weight is the following:

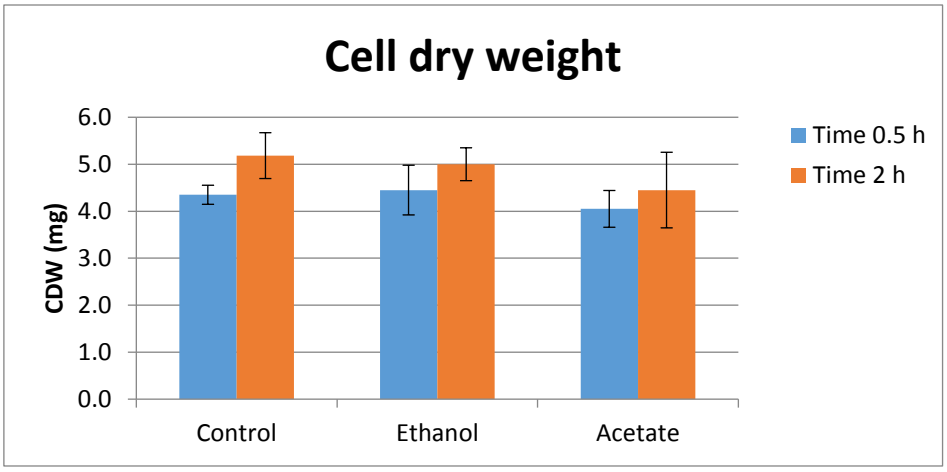


Chart 6-15 Dry cell weight of 2 ml samples from stress experiments samples

As expected, the cell that were grown in optimal conditions resulted in higher DCW, ethanol conditions resulted in a bit lower and acetate conditions resulted in the lowest amount of DCW, almost the same as in the beginning culture.

HPLC-RID results of carbon cycle intermediates

To get a better understanding of the metabolism, a look on the extracellular metabolites should be taken:

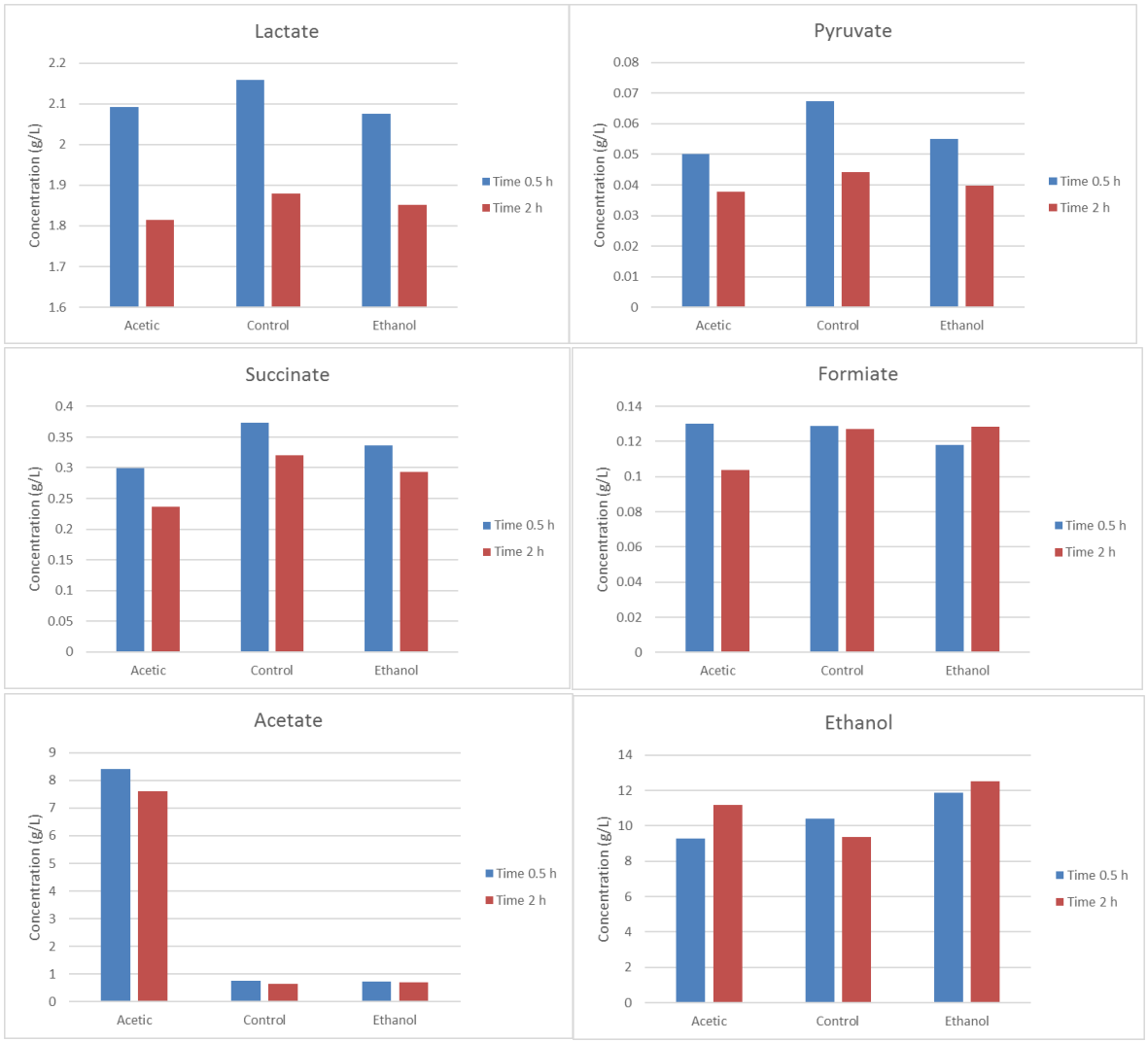


Chart 6-16 Intermediate metabolites concentration after 0.5 and 2.0 hours

The general tendency of the metabolites is to diminish in amount. Since there is no oxygen limitation, we can see that there is not an accumulation of intermediate products. The decrease of the products is associated mainly with the progression of cell cycle. What can also be inferred is that the majority of intermediate products achieve greater values, when they are grown in optimal conditions.
As expected acetate is much greater in cultures grown in acetic environment and ethanol is greater in cultures grown in ethanol-rich environment.

SOPAT Results

After taking samples for offline measurements, the SOPAT sensor was mounted in the shake flask and measured the cell size distribution. In total, measurements were taken during 3 distinct time points with 2 repetitions. The repetitions, where analyzed separately and their results were merged together for the cumulative curves.

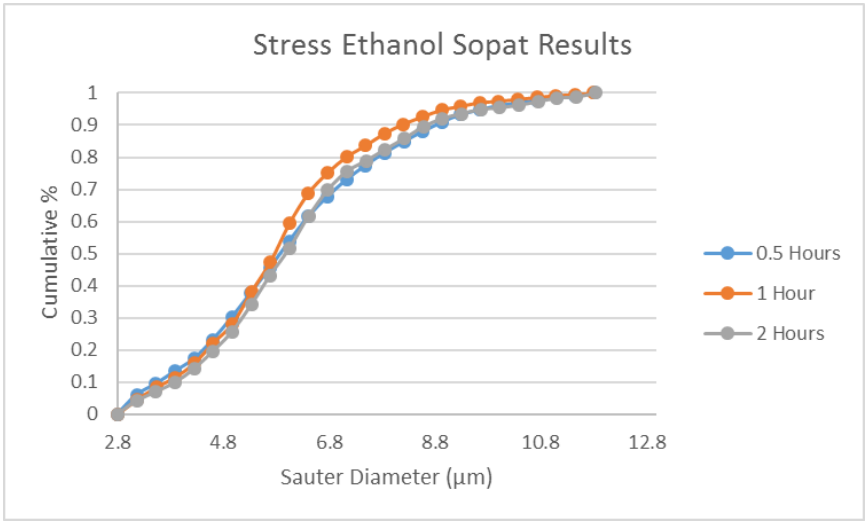


Chart 6-17 Cumulative distribution of yeast Sauter diameter under ethanol stress

In chart 6-17, the cumulative cell size distribution curves can be seen of yeast cultivated in ethanol-rich environment. There are 2 factors that must be taken account for analyzing these results (same factors influence the cumulative curves of yeast grown in acetate-rich environment):

- The natural progression of the cell cycle and the different mean diameters in each phase
- The adaptation of the cells is the non-optimal conditions.

According to literature (Aubergine et al, 1998) yeast cells grown in ethanol rich environment, reach larger critical sizes in comparison with cells grown in glucose. Considering that each yeast cell cycle lasts around ~90 minutes, cells measured after half an hour and 1 and a half hours will be around the same cell cycle phase. Though we can see that in chart 6-17, cells reach greater diameters, a result that corresponds to the aforementioned literature.

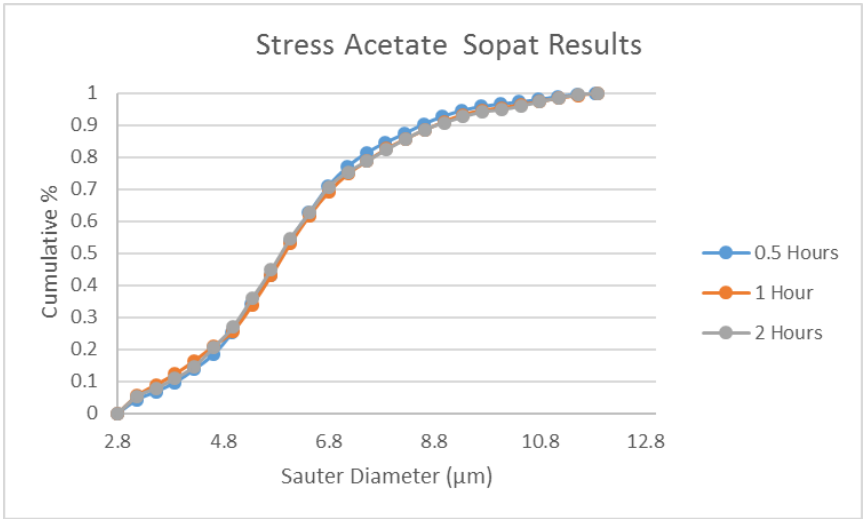


Chart 6-18 Cumulative distribution of yeast Sauter diameter under acetate stress

In contrast, yeast cells under acetate stress as shown in chart 6-18 show much less adaptation as in the previous cell analysis. Since acetate environment is much less prolific for the yeast cells, possibly there is an adaptation to smaller cells, as can be seen from curve after half an hour and after 1 and 2 hours, though it is not that apparent, so this interpretation is not conclusive.

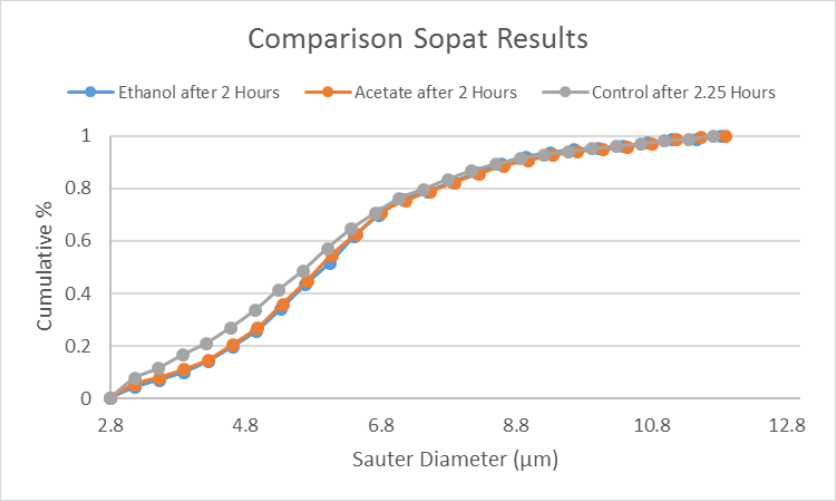


Chart 6-19 Comparison of Control, Ethanol and Acetate sample at the end of cultivation

A more insightful chart is the comparison of ethanol- and acetate-rich samples with a control one, in which no other compounds were added, just the initial culture. Immediately, a first conclusion is that in comparison to control, in which cells are more uniformly distributed, both acetate- and ethanol-grown cells are accumulated in percentiles with larger diameters.

6.4 STR experiment results

Sterol Analysis

The main product of the sterol synthesis pathway is ergosterol, which is shown below:

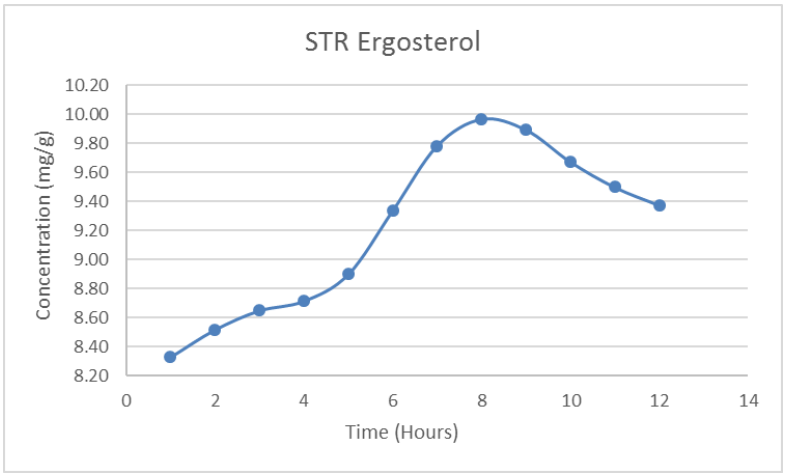


Chart 6-20 Total Ergosterol concentration across time during STR experiment

Ergosterol increases steadily until the fourth hour, in which the stirrer speed is increased. Then the slope becomes steeper, probably because the higher stirrer speed results in better aeration until the 8th hour in which it reaches a peak and then ergosterol starts getting reduced until the end of the experiment.

Squalene, the precursor of the sterol synthesis pathway reaches very low concentrations, since the STR and well-aerated.

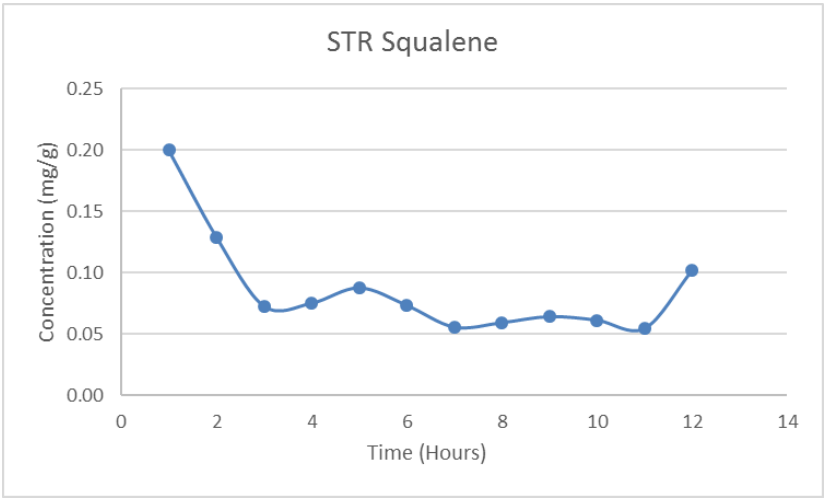


Chart 6-21 Total squalene concentration across time during STR experiment

The intermediate products of sterol synthesis exhibit similar tendencies.

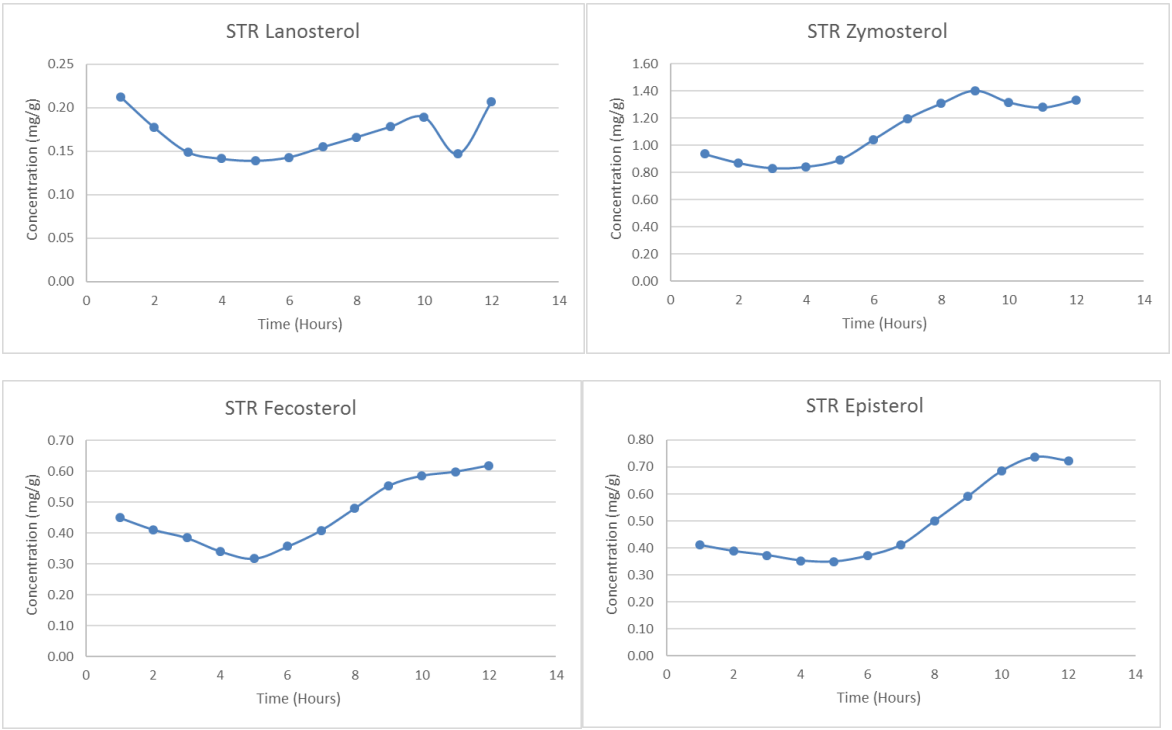


Chart 6-22 Intermediate products of sterol synthesis

An important fact that can be inferred by the charts is that until Zymosterol, previous intermediates(Lanosterol) followed a different tendency that the product, ergosterol. After Zymosterol, though, the next intermediates follow the same pattern, which consistent with the theory that zymosterol to fecosterol is the rate controlling step of the sterol pathway synthesis (Maczek et al,2006).

At line/On line microscopy

STR experiment was monitored both by at line microcopy (Ovizio DHM) and online microscopy (SOPAT).
Every one hour, samples were taken and measured with Ovizio. Using the data generated from the measurements inside the software of SOPAT, cumulative curves of size distribution were generated for each hour. In chart 3.14 for demonstrating purposes, the cumulative curves after 1, 6 and 12 have been graphed (with a zoom between 10 and 90%).

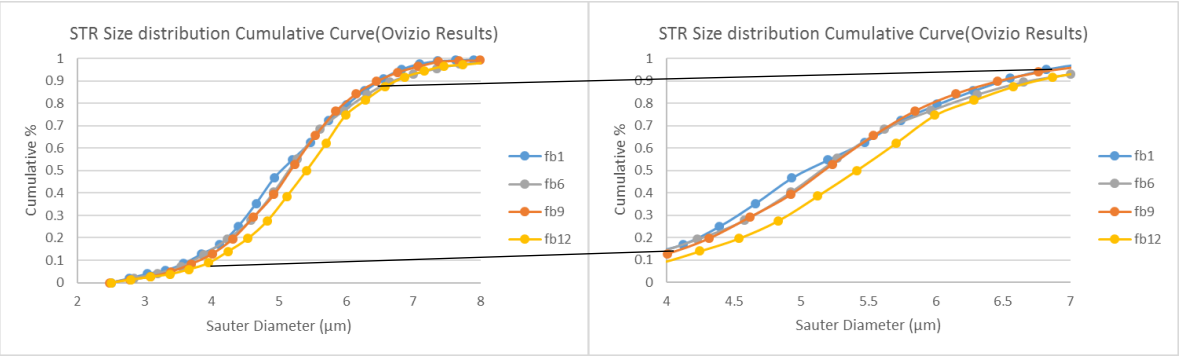


Chart 6-23 Cumulative curves of cell size distribution from Ovizio for STR

In the meantime, the STR reactor was mounted with the SOPAT sensor which took images every 15 minutes. For demonstration purposes again, the cumulative curves of size distribution at the beginning and after 1, 6 and 12 hours were plotted.

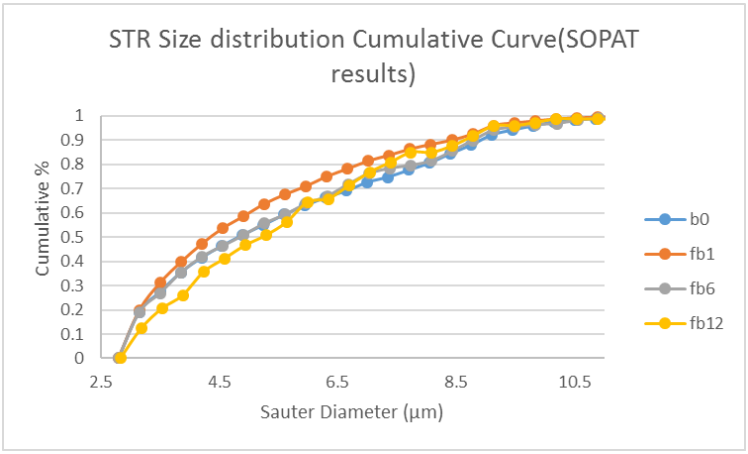


Chart 6-24 Cumulative curves of cell size distribution from SOPAT for STR

What is important, is to examine if there is correlation between the results from those 2 methods. As time points for comparison, after 1 hour and 12 hours were chosen and the results are shown below:

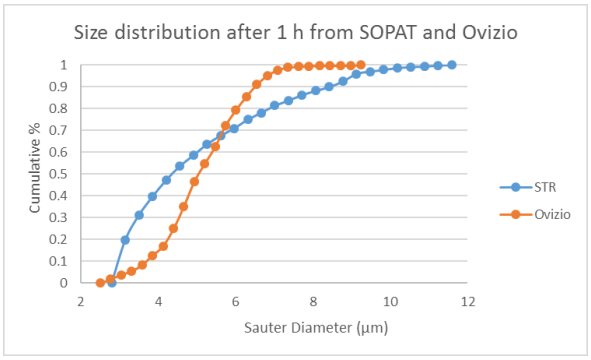


Chart 6-25 Comparison of Cumulative curves of cell size distribution from SOPAT and Ovizio for STR after 1 h

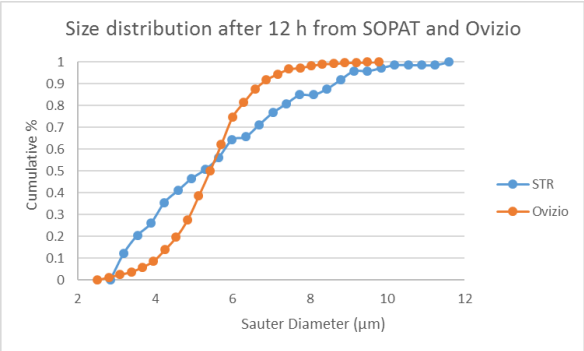


Chart 6-26Comparison of Cumulative curves of cell size distribution from SOPAT and Ovizio for STR after 12 h

After 1 hour, the 2 curves have a correlation of **0.973** and a coefficient of determination of **0.947**, which is quite high considering one was done at-line and the other one on-line. After 12 hours, the correlation and the coefficient of determination drop to **0.963** and **0.929** respectively. The reasons for this change will be analyzed in the Discussion part extensively.

7 Discussion of experimental results

Overall the online SOPAT sensor, as shown with the cell cycle and the stress experiments, provides a very descriptive analysis of the cells inside a working reactor with remarkable correlation with at-line methods (Ovizio) reaching 97% correlation. Though, it stills needs improvement in specific aspects of the information that provides.

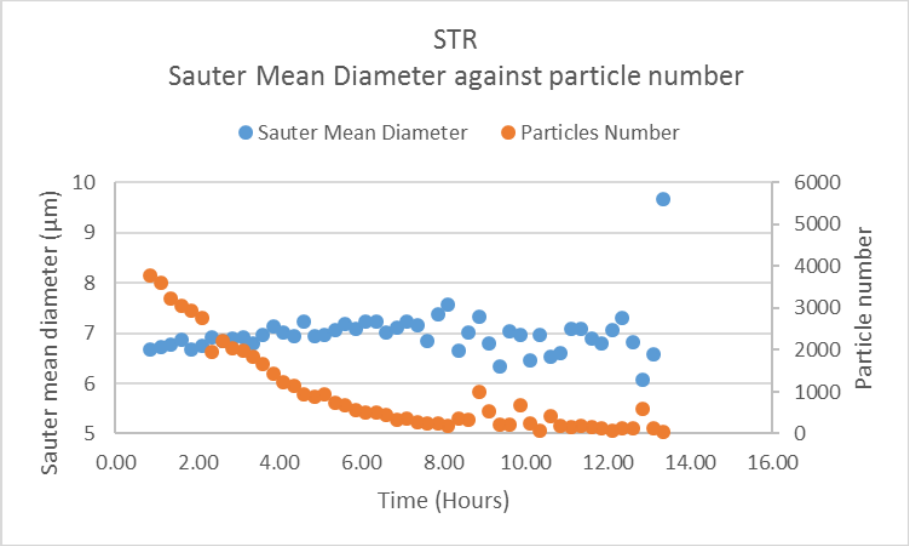
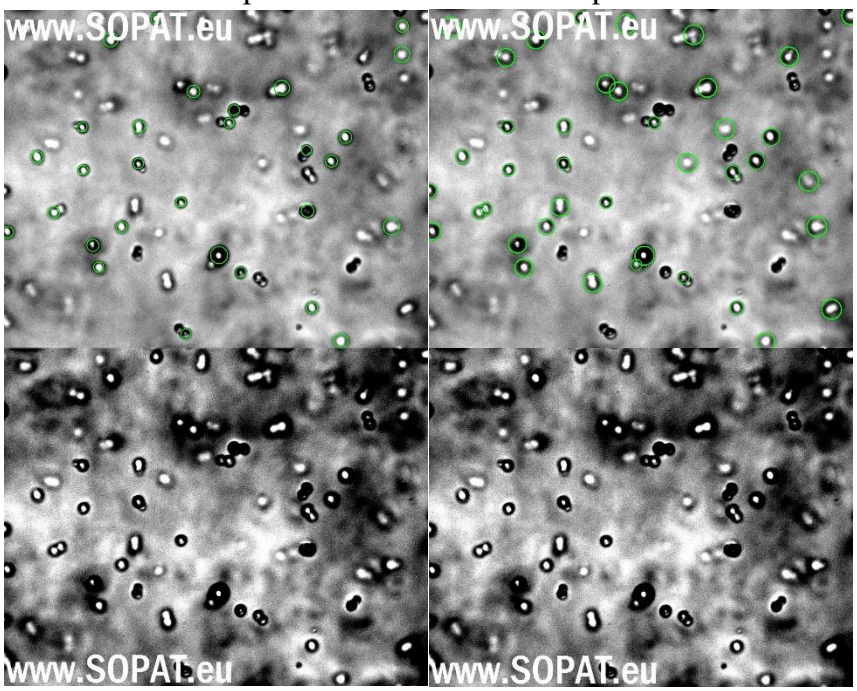


Chart 7-1 STR Mean Sauter Diameter against Cell Count

The parameter set that was used for the CSTR experiment, which preceded the other experiments, provided quite detailed pictures resulting in large number of cells being measured. In higher concentrations though (OD over 30), the overlapping is so high, that the software is incompetent to recognize cell patterns, leading to very few cell measurements (just 50 measurements, whereas at lower concentrations averaged 3000 measurements). This results in non-consistent results with great variances, as can be seen in measurements after 8 hours. The manufacturer company suggests that a time point measurement is reliable, when at least 1000 particles are measured.

Based on these results a new parameter set was developed.



Picture 7-2 SOPAT picture processed with old parameter set (left) and new parameter set (right)

The new parameter set lead to increased number of cells measured. Though this improvement was done in exchange of 2 new problems:

- Cells with large buds, ready to be divided, were counted as 2 cells instead of one cell
- Shaded areas around some cells were counted in the diameter of the cells resulting in mean Sauter diameters 50-70% larger than the real values as shown in chart 7-2

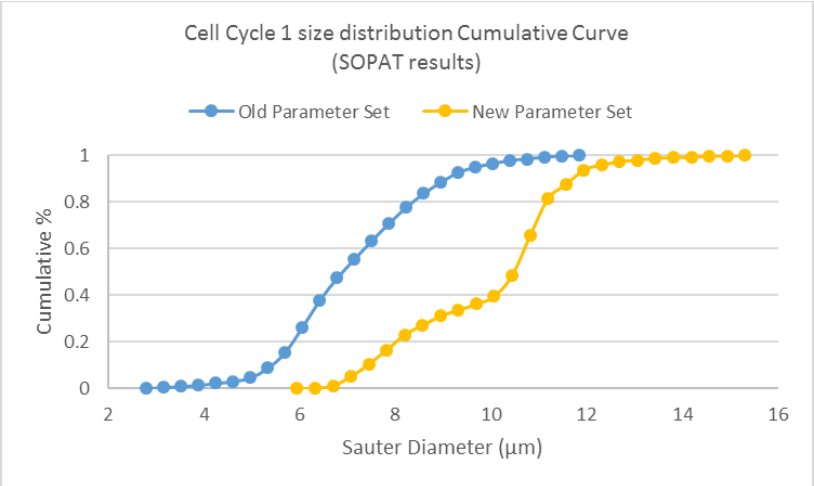


Chart 7-2 Comparison between parameter sets in Cell Cycle 1 size distribution Cumulative curve

It has to be pointed out that with the second parameter set, a sharp increase in the cumulative curve can be seen, which corresponds better to reality, since the growth of the culture is synchronized, meaning the cell sizes should be more concentrated in certain diameters. In this study though, since the diameter presented with the second set were very different from the real ones, the old parameter set was used.

Important point, that has to be discussed concerns the conditions that were created during the first cell cycle experiment. As shown in Chart 6.2, glucose limitation conditions were achieved after 1.5 hours and after 2 hours, glucose depletion conditions took place. This, in turn, had a negative impact in the cell metabolism, as shown in the off-line HPLC carbon cycle intermediate concentrations. That's why, the on-line results after 2 hours doesn't make sense in processing, since now the parameter of cell cycle and cell shrinkage, because of glucose limitation/depletion, are merged, distorting the expected results.

8 Conclusions/Outlook

From the experiments done, the following conclusions were drawn:

- SOPAT sensor measures with high correlation, almost 97%, with 3D Microscope the size of yeast cells in low concentrations, below 30 Optical Density.
- In contrast, in concentrations over 30 Optical Density, the cells overlap that much, that the software is unable to differentiate between the cells, lowering the cell to be detected in less than 100, while the threshold for miniscule statistical error in one thousand particles.
- The importance of off-line measurements, especially HPLC and glucose measurements in order to have an overview of the metabolic activity, as was the case with first cell cycle experiment and the glucose depletion conditions.
- The second parameter set was able to solve the problem with the number of particles in exchange of other problem concerning the detection accuracy and overlapping.
- Cell cycle synchronization of yeast cells was successful, especially in the second cell cycle experiment, though the current parameter sets were not able to detect as it would be supposed to.
- Sterol analysis was not helpful in detecting the metabolic activity in these experiments and providing a better insight in the progression of cell cycle.

The outlook of research with SOPAT sensor could be:

- Scale up experiments of cell cycle synchronization and growth in non-optimal conditions in the 10 L bioreactor.
- Use of flow cytometry, in order to see the correlation with SOPAT microscope results in cell size measurements.
- Analysis of protein content of cells, instead of sterol content to see, if there is better understanding of the cell size results.
- Use of different model organism, for example bacteria, which have different shapes in order to evaluate the accuracy of the detection software.
- Use of compounds that synchronize cells in a different part of the cell cycle (e.g. hydroxyurea)

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10 Appendix A

STR, *S. cerevisiae* AH 22, Cultivation March.2016

1. Time plan

Monday-Wednesday (16/03/16)

- Prepare medium and antifoam for precultures and autoclave it.

Thursday (17/03/16)

- Dry Eppendorf-tubes (2mL) for CDW in the 70°C oven
- Preculture 1

Sunday (20/03/16)

- Preculture 2
- Prepare the syringes (MeOH and $HClO_4$)
- Prepare Phosphate Saline Buffer (1L) and filter at least 500mL, prepare NaCl
- Prepare Eppendorf-tubes for supernatant samples and perchloric acid, Flow Cytometry, Label Falkon tubes for sterol and fatty acids samples
- Prepare glucose solution for batch in Erlenmeyer flask with inoculation needle and autoclave
- Prepare glucose solution for feed in Schott flask
- Prepare 500 mL NaOH 30%. Autoclave an empty Schott flak with inoculation needle
- Autoclave empty Inoculation flask (vol. at least 1L)
- Prepare the stirred tank reactor (STR)
- Prepare feed media in Erlenmeyer flask with inoculation needle and autoclave
- Weight Eppendorfs for CDW

Monday (21/03/16)

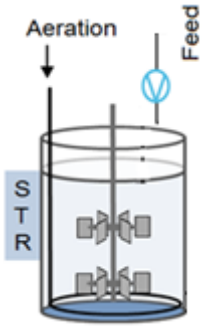
- Preculture 3
- Prepare the batch medium (weight)
- Calibration of the sensors.
- Sterilize the STR including the media (2.5 Hours).
- Inoculation

Tuesday (22/03/16)

- Prepare dye working solutions for flow cytometry
- Connect feed
- Sampling and PAT.

- Finish of the cultivation and cleaning

2. Reactor



3. **Media** (all compounds are dissolved in VE water)

a. *Pre-culture*= 1L End volume

YEPD (complex medium) * (pH=5.5)

Dextrose	22g/l
Peptone aus Casein tryptisch verdaut	20g/l
Hefeextrakt	10g/l
NH4Cl	1g/l
KH2PO4	14g/l

*Autoclave the medium components in the reactor. The glucose must be autoclaved separately!! = e. g. for 1L: 800 mL medium + 200mL glucose solution (containing 20g glc or 22 g dextrose)

During the preparation of highly concentrated dextrose solutions on the heated magnetic stirrer, add the dextrose only step by step to VE water in order to avoid clumping or melting of the sugars!

Sterilize 10 ml antifoam!

b. *Batch-culture*= 10L End volume

YEPD see 1.a.:

- weigh all components for 10 L medium (water is added in reactor)
- separately prepare500mL glucose solution (containing 200g glc, 220g dextrose)

c. *Feed*= 2L End volume

YEPD see 1.a. but with 400g/L Glucose (440g/L Dextrose)!!!

= weigh components for 2L medium but dissolve only in 1 L; separately prepare 1L glucose solution containing 880g dextrose.

4. Preparation of Eppendorf for sampling

1. For Cell Dry Weight(triple estimation)
   a. Dry 2mL Eppendorfs in drying oven
   b. Transfer 2mL Eppendorfs from drying oven to exicator and wait for them to cool down.
   c. Label and weigh the empty Eppendorfs (closed!!!), write the weight on them and make a list.
2. For supernatant
   a. Use 1.5mL Eppendorfs and label them
3. For $HClO_4$ samples
   a. Use 2mL Eppendorfs and label them
4. For MeOH samples
   a. Use 2mL Eppendorfs and label them
5. For sterols and fatty acids
   a. Use 50mL Falcon Tubes (1 for Sterols, 1 for Fatty acids)
6. Label order
   a. Fermentation series (AMM_STR15B=Stirred Tank Reactor 2015 2ond Trial)
   b. Indication of batch / fed-batch (b =batch (before feed start), fb= fed-batch (after feed start))
   c. Kind of sampling: CDW (cell dry weight), Ü (supernatant),$HClO_4$, MeOH (Methanol), St. (Sterols), FA (Fatty acids).

   ➔ e. g. AMM_STR15B_b0_CDW
7. Prepare three boxes for freezing of samples, 1 for supernatant, 1 for $HClO_4$ -quenching, 1 for MeOH

5. Sample preparation (MeOH, $HClO_4$)

Equipment:

- 5mL B.BRAUN syringes
- SARSTEDT adapter
- Methanol (98.9%, HPLC grade)
- $HClO_4$ (70%)
- Beaker

Execution:

$HClO_4$-samples

- Add 5 g/L Butanol to $HClO_4$ $V1*C1=V2*C2$
  - V1= 5g/L*50mL/810g/L

- Pour the solution into a beaker
- Put a Sarstedt adapter on the syringe
- (mark the syringe)
- Fill it with 1mL $HClO_4$ (make sure no air bubbles are present!)
- Store at -80°C

MeOH- samples

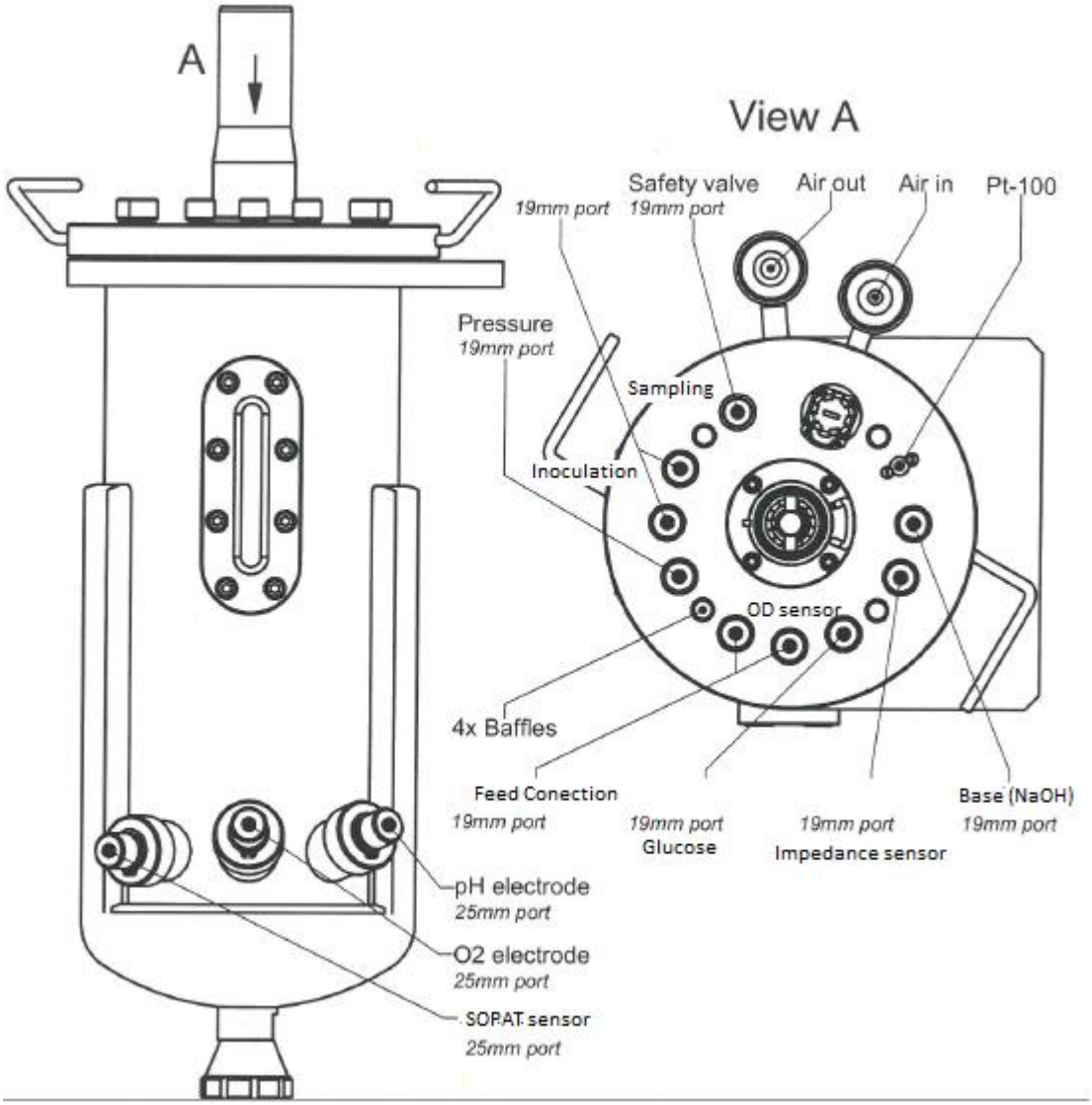
- Pour methanol in a beaker
- Put a Sarstedt adapter on the syringe
- (mark the syringe)
- Fill it with 2mL MeOH (make sure no air bubbles are present!)
- Store at -80°C

6. Pre-Cultures

Pre-culture	Inoculation Date	Inocul. Time (ca.)	Inoculum - volume	Medium-volume	Antifoam 204 Sigma - volume	UYF-Flask-Vol.	Cultivation Conditions	Measured OD before/after inoculation expected
1	17/03/2016	15:30	90µL (from cryo-culture)-80°C First compartment, second box.	2x 50mL	Each 500µl	2 x 250mL	250 rpm, 25°C	----
2	20/03/2016	9:00	2mL (from PC 1) = 4%	2x 50mL	Each 500µL	2 x 250mL	250 rpm, 25°C	A:27.5,B:28.6/ A:1.18,B:1.19
3	21/03/2016	9:00	Each 10mL (from PC 2) = 4%	2 x 250mL	Each 2.5mL	2 x 2L	250 rpm, 25°C	A:34.9,B:33.3 / A: 1.34

7. STR preparation (calibration, construction, sterilization)

- Generally: never tight steel on steel too strong. Afterwards it might be impossible to separate these parts again. The sealing is just provided by the rubber rings, while the screw thread (Gewinde) is just to hold the pressure of autoclavation. They must not be fastened with force!!!
- Open water and compressed air valves.
- Fix the aeration ring and the baffles. The aeration ring is in the rear left.
- Calibration of pH sensor: adjust at “ΔpH” for pH 7 and “mV/pH” for pH 4, recheck after adjustment!
- Fix sensors, plugs, septum in the ports:



- The total VE water volume is 9.5 L. Fill in 7L, start the stirrer with 300rpm, fill in the medium components and fill up to 9.5 L with remaining water --> **add 5 mL Antifoam before sterilization.**
- Start sterilization. The fermenter can be heated up while installing remaining parts
- Put the lid on the fermenter, keep attention of the ring sealing.
- Tight the screws the following way
  - Tight up two opposite screws
  - Turn them not more than 120°
  - Fasten the next two screws manually before using the fork spanner (Maulschlüssel)

- When the temperature is below 40°C **add 500mL glucose solution** (sterile); aeration on when connecting, aeration off for transfer, aeration on for disconnecting.

- Connect the NaOH -flask and place the tube into the base pump, fix it with tape, remove the clamps
- When all medium components are in the fermenter, adjust the flow meter to 5 L/min (0.5 vvm) and the stirrer to 500rpm
- adjust temperature control to 27°C and pH to 5.5
- Set the oxygen sensor to 100% by turning "slope"
- Position needle at sample port also using ethanol
- Take sample and check pH *offline* and OD with suspension
- For inoculation transfer the pre-culture in the inoculation flask (under the clean bench). **Shake frequently all the time**. Add the inoculum to the fermenter while keeping the pre-culture shaking. No oxygen limitation may occur. Turn gasing off for inoculation of the reactor
  Switch on oxygen control to 30% (**O_2 level** should be always **> 20%**!!), adjust stirrer speed to 500rpm, flow meter to 0.5vvm
- Take OD, CDW, pH, supernatant sample after inoculation
- **Adjust flow meter to 0.7vvm and stirred to 650 rpm, 4 h after inoculation.**

8. Reactor Inoculation (Batch)

Inoculation Date	Inoculation - Time (ca.)	Inoculum- volume	Medium- volume	Cultivation Conditions	Measured OD before / after inoculation
21/03/2016	16:45	2125/OD= 133 OD=15.95 Expected: OD=10.6 --> 200mL	10L	STR Conditions	/ 0.2

9. Feed connection

Equipment:

- PFR-pump (hose pump for P-food/pharma 19-GM/FUC, LEWA HOV)

10. Feed start (Fed-Batch)

After ca.20 h of Batch (check OD after 19h)→ when OD ~ 24. Feed rate adjustments are needed, especially after removal of the Sterol samples (large volume). Starting feed rate: 0.53mL/min.

Exponential feed: $F=k*V_L*e^{\mu t}$.

F(t)= F(0)*EXP(μ*t) --> F(t)= 0,0318L/h*Exp(0.12*t)

- **μ=0.12 h-1, F0= 0.53 mL/min**
- **Pump rate= 29, 800 rpm**

11. Sampling and special sensor technologies

- **Intracellular metabolites ($HClO_4$--> short chain fatty acids and alcohols, MeOH-Quenching --> Amino acids)**
  - STR: 1 batch (Tuesday), 3 batch + 13 fed-batch (Wednesday) = 17 syringes for MeOH, 17 syringes for $HClO_4$
  - Frequency: Sampling every hour after feed starts

  Procedure (take the sample directly from the reactor)

  - *MeOH-Quenching:*
    - take a syringe out of -80°C freezer short before taking sample
    - fill the syringe with suspension up to 5mL; mixing
    - transfer to the labelled Eppendorf
    - store immediately in -80°C freezer
  - *$HCLO_4$:*
    - take a syringe out of -80°C freezer short before taking sample
    - fill the syringe with suspension up to 5mL; mixing
    - store immediately on ice on horizontal shaker (7.5min),then shake per hand (shortly), again horizontal shaker (7.5min)
    - transfer complete sample volume to 50mL falcon-tube
    - add step by step 200µL up to 845µL 5 M K_2CO_3 (attention foam building)
    - filter 2mL with a filter with a pore size: 0.8µm and transfer to the labelled Eppendorf
    - store immediately at -20 °C

Take ca. 10mL sample with a 20mL syringe for:Extracellular metabolites, pH *off line* OD, and CDW

- **Extracellular (Supernatant --> Amino acids, short chain fatty acids and alcohols, sugar)**
- Time Points: 1 after inoculation (Tuesday), 3 batch + 13 fed-batch (Wednesday) = 17 syringes
  - Frequency: Sampling every hour after feed starts

  Procedure:

  - filter 1.5mL culture with a 5mL syringe (pore size: 0.8µm) --> store at -20°C

- Glucose enzymatic kit (Enzymatic Kit Glucose Hexokinase)
  - Frequency: Sampling every hour after feed starts

  Procedure:
  - Mix 4 parts of R1+1 part of R2 --> reagent mixture
  - Supernatant --> sample
  - Blank: 10µL distilled water + 1000µLMixture --> Mix
  - Sample: 10µL sample+ 1000µL Mixture --> Mix
  - Prepare each time 1 blank and 2 repetitions for each sample --> Mix, wait 10 minutes and measure at a wavelength of 340 nm.
- **pH *off line***
  - measure before inoculation and directly after inoculation, then every hour
  - measure pH directly from sample in Falkontube with pH meter
- **OD *off line***
  - measure before inoculation and directly after inoculation, then every hour
  - dilute cells until the linear range of the OD meter is reached (0.1-0.8) (1:10 steps)
  - measure blank at 600nm with dilution solution
  - prepare cuvettes with 1 mL volume of diluted cell suspension and measure at 600nm
- **CDW**
  - Time points: 1 before and 1 after inoculation (Tuesday), then every hour

  Procedure
  - centrifuge in previous weighed 2mL Eppendorfs (15000rpm; 10min; 4°C) 1mL ***3 (triple estimation)**
  - wash the pellet with 1mL NaCl 0.9% and centrifuge again
  - store in drying oven

Take together with a 50mL syringe 2x50mLfor Sterols and fatty acids --> store immediately at -80°C