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Standard Operating Procedures for a Single-Use Fermenter

B.Sc. Project Thesis

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Preface

This thesis is made upon the project that makes the completion of my Bachelor of Science education in Biotechnology at the Department of Systems Biology at the Technical University of Denmark. This project is formed in coorperation with the Department of Systems Biology, DTU, and CerCell ApS. It is aimed at giving a better understanding of how to use a single-use fermenter from CerCell ApS. The duration of this project was from February to June 2016. The experiments were carried out at the Fermentation platform in building 223 at the Department of Systems Biology. Most of the kit and tools used for the experiments were provided from DTU. Some kit was provided with the single-use fermenter from CerCell ApS. The Escherichia *coli* wild type strain used for the experiments was originally delivered from SSI.

I would like to thank my former supervisor Mhairi Workman for her help and guidance throughout this project from February until June 1st 2016. I would like to thank my supervisor Uffe Hasbro Mortensen as well for his helpful guidance throughout June 2016.

I want to thank Tina Johansen from the fermentation platform at DTU Systems Biology for helping me with the experimental difficulties in the laboratory. I wish to thank Alexander Sonne Munch for helping me with information of some of the inital steps of the experimental setup.

I would also like to thank Per Stobbe and all of CerCell ApS for their help and support of this project, and for the preparation and lend of the single-use fermenter used for the experiments carried out for this project.

I would finally also thank my family for being supportive during my time working on this project.

Kristian Krakau

Abstract

Fermentation processes play a very important role in the bio-process industry. Many products are synthesized with fermentation, and the demands of the fermentation processes increase. Industry is beginning to look for methods that are sustainable and have potential.

Single-use fermenters (abbreviated SUFs) are alternatives to the convential stainless steel fermenters when a fermentation process needs to be carried out. The essential expectation of single-use fermenters is to have an alternative that can save time for the production and eventually costs and also reduce the risks of contamination rapidly.

This project is based upon the composition of standard operating procedures for a 3 Liter single-use BactoVessel fermenter from CerCell ApS. The 5 fermentations carried out in this project with the SUF were compared with growth data of Optical density (OD), dry weight, and CO_2 of a wild-type strain of E. *coli*.

Operating procedures are quite important both in research and also in the industry as it makes up the fundamental step-by-step procedures in order to run a successful fermentation.

The SUF was able to grow up E. *coli* confidentially like any other convential fermenter. The E. *coli* fermentations varied from each other, but natural inevitable sources of error were most likely involved, and sensible conclusions were made compared to the errors in the standard operating procedures.

Areas within the operating procedures that seemed to be of high importance was sterility, as the risks of contamination can be high regarding the use of a SUF, and control conditions, which had an impact on the fermentations.

Contents

Introduction

Fermentation

Fermentation in general

Fermentation comes from the Latin word "fermentare", meaning “to leaven.” The definition of fermentation is known as the process in which an agent causes an organic substance to break down into simpler substances. This is especially seen with the anaerobic breakdown of sugar into alcohol. Thus, fermentation as a term in microbiology covers the principles of the metabolic processes in which microorganisms such as bacteria or yeast break down sugar or starch into products such as acids, gases, or alcohol.[43]

Fermentation is a natural process. People applied fermentation to make products such as wine, mead, cheese, and beer long before the biochemical process was understood. In the 1850s and 1860s Louis Pasteur became the first person to study fermentation when he demonstrated fermentation was caused by living cells. The science of fermentation is known as zymology, and a person studying fermentation is a zymurgist.[79]

In industry, as well as other areas, the uses of fermentation progressed quickly after Pasteur's discoveries. Between 1900 and 1930, ethyl alcohol and butyl alcohol were the most important industrial fermentations in the world. Everso, by the 1960s, chemical synthesis of alcohols and other solvents were less expensive and interest in fermentations decreased. Nonetheless, Interest in microbial fermentations experienced a renaissance in 1995. Now Plant starch, cellulose from agricultural waste, and whey from cheese manufacture are abundant and renewable sources of fermentable carbohydrates. Alternatively, these materials, not utilized, represent solid waste that must be buried in dumps or treated with waste water.[22]

Microbial fermentations have other advantages. They do not use toxic reagents nor require the addition of intermediate reagents. Microbiologists are now looking into finding naturally occurring microbes that produce chemicals of demand. In addition, they are now capable of engineering microbes to enhance production of these chemicals. In recent years, microbial fermentations have been revolutionized by the application of genetically-engineered organisms, so-called microbial cell factories. Many fermentations use bacteria, but more fermentations are starting to use mammalian

cells.[22] Nevertheless, Mammalian cells are eukaryotes and much more difficult to work with than bacteria as their requirements for nutrients and environment are more demanding.

Bacteria perform fermentation in which they convert carbohydrates into lactic acid. This is done via the pyruvate molecules from glucose metabolism (glycolysis) which is fermented into lactic acid. It also occurs in animal muscle tissue when the required energy rate is faster than the oxygen that is able to be supplied. The chemical equation for lactic acid production from glucose is[79]:

$C_6H_{12}O_6$ (glucose) $\rightarrow$2 $CH_3CHOHCOOH$ (lactic acid)

The production of lactic acid from lactose and water may be summarized as:

$C_{12}H_{22}O_{11}$ (lactose) + H_2O (water) $\rightarrow$4 $CH_3CHOHCOOH$ (lactic acid)

Lactic acid bacteria have been used to ferment or culture foods for at least 4000 years. They are used in particular in fermented milk products from all over the world, including yoghurt, cheese, butter, buttermilk, kefir, and koumiss.[53]

In this project a wild type strain of E. coli from SSI was used as a microrganism for project fermentations carried out.

Basics of cell- and bacterial growth

Cell growth is a vital part of all life. In eukaryote cells the growth occurs as either mitosis or meiosis. Mitosis results in two identical daughter cells, and meiosis results in four sex cells.

However, the essential principle in cell growth is cell division. In both cases the inner cell compartments are replicated before the cell divides into new cells. Cell growth in general can then mathematically be described as an exponential development. Nevertheless, the number can only assumed as a maximum as not every cell will survive division.[23]

The same applies to bacteria. They even have a simpler approach as they only have to copy their DNA before they can divide. This cell division is known as binary fission.

There are 4 phases of bacterial growth in which the growth in a fermenter is supposed to follow [50], [47], [51] :

- Lag phase: In this phase there is increase in cell size but not multiplication. Time is required for adaptation (synthesis of new enzymes) to new environment. During this phase, vigorous metabolic activity takes place, but cells do not divide. Enzymes and intermediates are formed and accumulated until they are present in concentration that permits growth to start. Antibiotics have little effect on cells at this stage.
- The exponential phase: This phase (sometimes called the log phase or the logarithmic phase) is a period characterized by cell doubling. The number of new bacteria appearing over time is proportional to the present population. If growth is not limited, doubling will continue at a constant rate so both the number of cells and the rate of population increase doubles with each consecutive time period. For this type of exponential growth, plotting the natural logarithm of cell number against time produces a straight line.

- Deceleration growth phase: This is a very short phase where deceleration growth occurs due to depletion of essential nutrients, accumulation of toxic products, and unbalanced growth due to cells undergoing internal restructing in order to survive.
- Stationary phase: This phase is when population growth levels off as the rate of cell death begins to equal the rate of cell division. After several hours of rapid cell division, two things are bound to occur: nutrients will be consumed, and toxic byproducts will be released. In either case, cell division cannot continue at the same rate because it becomes too hard to find nutrients or the bacteria cannot survive the toxins.
- Death phase: If incubation continues after the population reaches stationary phase, a death phase follows, in which the viable cell population declines. If counting by spectrometric (OD) measurements or microscopic counts, the death phase cannot be observed. During the death phase, the number of viable cells decreases exponentially, essentially the reverse of growth during the log phase.

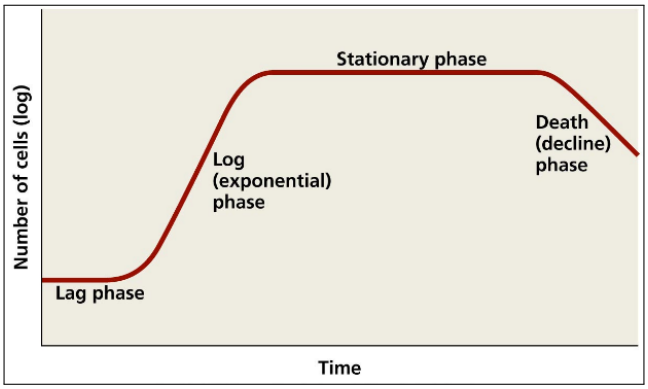

Figure 1: *curve illustrating bacterial growth with its phases.[61]*

Cells are only able to grow under proper conditions. For E. *coli* around 37 °Celcius and pH 7 are the best conditions. A sufficient supply of oxygen is also good. Escherichia *coli* can double every 17-20 minutes under the proper circumstances.[67],[73]

The balance of a batch cell growth is:

$$\frac{dx}{dt} = \mu \cdot x$$

Where x is biomass, t is time, and μ is the specific growth rate.

When $t = 0$ then $x = x_0$ where x_0 is the starting biomass concentration. When $t = t$ then is $x = x$

Then integration is made on the formula in order to know growth over time:

$$\int_{x_0}^{x} \frac{dx}{x} = \int_{0}^{t} \mu dt <=> ln(\frac{x}{x0}) = \mu \cdot t <=> x(t) = x_0 \cdot e^{\mu \cdot t}$$

The formula on the far right is then the formula for biomass over time. By knowing this formula, it is possible to calculate the maximum growth rate from a batch fermentation (see appendix A11).

Another important aspect is the generation time or doubling time. It is the time it takes for the cells to have doubled in number. This means mathematically that:

$x_0 \rightarrow 2 \cdot x_0$ when $t_0 \rightarrow t_d$ where t_d is the doubling time.

This means that:

$$2 \cdot x_0 = x_0 \cdot e^{\mu \cdot t_d} <=> ln(\frac{2 \cdot x_0}{x_0}) = \mu \cdot t_d <=> Ln(2) = \mu \cdot t_d <=> t_d = \frac{ln(2)}{\mu}$$

There by only knowing μ, it is possible to calculate the doubling time for that μ and by knowing μ_{Max} the fastest doubling time can be calculated.[51]

Not much technology for determining the exact cell count of an experiment has made a breakthrough yet so a share of other methods are mostly consulted in microbial experiments. In this project, OD and dry weight measurements were used. Other methods include wet weight, total cell count (microscopy), and viable count (Colony forming units (CFU) on agar plates).[33]

Principles of fermentation technology processes

A fermentation process in a fermenter can be carried out by the three main methods: as a batch fermentation, a fed-batch fermentation, or as a continous fermentation.

- Batch: This fermentation has a starting concentration of substrate (nourishment), biomass (cells), and a starting volume. It can be run aerobically (with oxygen) or anaerobically (without oxygen). In most cases a batch is run aerobically. When the fermentation is run, the substrate and O_2 will be used to create biomass and CO_2 and waste products. The volume will stay constant.

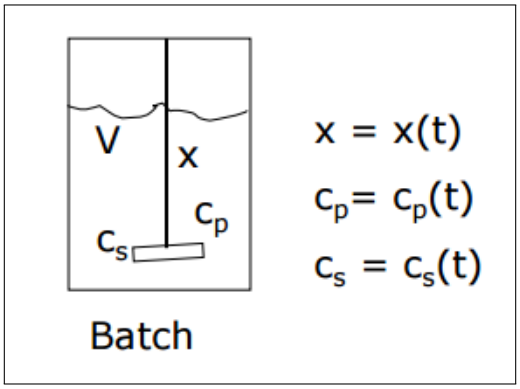

Figure 2: *Batch fermentation. V represents volume, x is biomass, c_p is product concentration and c_s is substrate concentration. Biomass-, product-, and substrate concentration are all develop over time as shown.[80]*

The advantage for a batch fermentation is first of all that it is versatile because it can be used for various different processes. Moreover, there is a limited risk of contamination, and a complete conversion of substrate is possible. Disadvantages are that it has a high cost of labour, and much idle time due to cleaning and sterilization after fermentation. Batch processes are currently used in the chemical and food process industries. Their automation and optimization pose difficult issues mainly because it is necessary to operate concurrently with countinous (algebric or differential equations) and discrete (state machines) models.[60]

- Continuous: This fermentation is also known as a chemostat. It has a flow through the reactor. The volume is kept constant and so will the concentrations of biomass substrate and products. It will let the fermentation run to the stationary phase and then keeping the stationary phase constant as long as the fermentation runs.

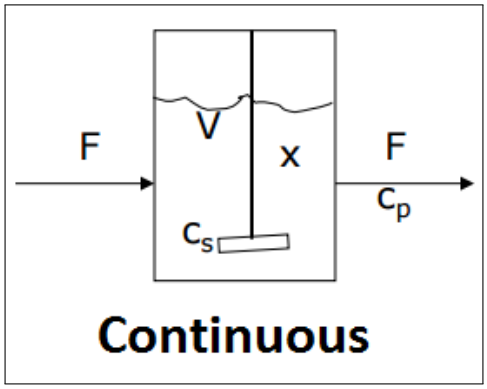

Figure 3: *Continuous fermentation. Every parameter is theoretically supposed to be constant. That is flow, volume, product-, biomass-, and substrate concentration.[80]*

The advantage for this type of fermentation is constant product quality, simple automation, long periods of high productivity, and high efficience of the reactor capacity. Disadvantages are infection problems, low-producing mutants occurence for long operations, inflexibility due to the incapability of running different processes, and adjustment of downstream processing.

- Fed batch: This fermentation has the same starting criteria as a batch fermentation, but addition of substrate is playing a part. This changes the growth rate for the bacteria, and the volume will eventually increase. However, the substrate will still decrease, and the biomass and products will increase.

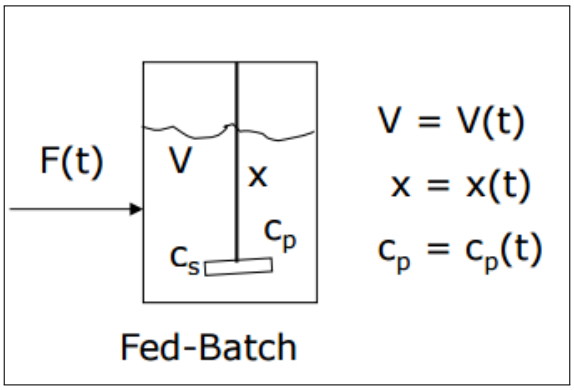

Figure 4: *fed-batch fermentation. It can be seen from this model that most parameters develop over time. That is flow, working volume, biomass, and product. Substrate is theoretically supposed to be constant[80].*

Advantages are allowance of operation under well controlled conditions, and allowance of very high cell density, which results in high final titers. Disadvantages are some of the same as for the other fermentation types, but the disadvatages are a little more subtle with this operation mode.[25]

For this project only batch fermentations were carried out.

The general microbial growth process kinetics for a batch fermentation are the following[38]:

$$\frac{dx}{dt} = \mu \cdot x$$
$$x(t=0) = x_0$$

and for substrate:

$$\frac{dc_s}{dt} = -r_s \cdot x$$
$$c_s(t=0) = c_{s,0}$$

Where r_s is the rate of substance broken down over time and c_s is the concentration of substrate. By making a differation of the substrate kinetic formula, it is possible to find the equation for substrate over time

$$c_s(t)$$

It has already been calculated previously that the equation for biomass over time is:

$$x(t) = x_0 \cdot e^{\mu \cdot t}$$

So for substrate that is:

$$\int_{c_{s,0}}^{c_s} \frac{dc_s}{-r_s} = \frac{c_{s,0} - c_s}{r_s}$$

$$\frac{c_{s,0} - c_s}{r_s} = \int_0^t x dt$$

$$\frac{c_{s,0} - c_s}{r_s} = x(t) \cdot t$$

$$c_s(t) = -r_s \cdot t \cdot x_0 \cdot e^{\mu \cdot t} + c_{s,0}$$

What can be concluded now is that the subtrate concentration over time is a declining exponential function, and biomass over time is exponentially increasing.

This is illustrated with this graph:

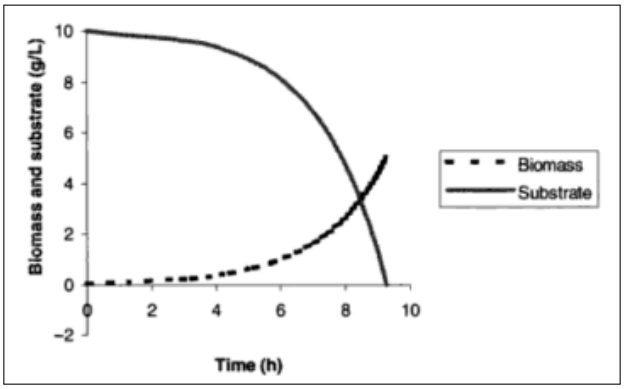

Figure 5: *Graph showing biomass and substrate concentrations over time in a batch fermenter.[38]*

General composition and configurations of a standard fermenter

A fermenter is also known as a bioreactor. It is a fermentation vat for the production of living organisms, mostly bacteria or yeast, used in industrial processes such as waste recycling or in the manufacture of drugs or other products. [20]

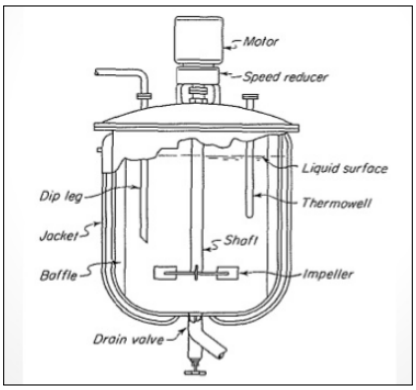

Figure 6: *Typical composition of a batch fermenter.[81]*

In this project, a benchtop fermenter was used with a 3-Liter volume. A benchtop fermenter is of smaller scale typically 1 to 12 Liters, sometimes even more. These types of smaller fermenters are used primarily for research purposes. A fermenter is typically connected to a control station - a computer with software able, through the connected sensors and equipment, to analyze and calibrate temperature, agitation, airflow, disolved oxygen, pH etc. A fermenter with a thorough setup typically has applied or is built with different configurations such as:

- Temperature sensor: The most commonly used type of all the sensors is the sensor which detect Temperature or heat. These types of temperature sensor for fermenters are typically made as a simple ON/OFF thermostatic device which control a domestic hot water heating system. It is placed in the thermowell and connected to the control station.[71]
- Thermowell: This feature is a holder that is integrated into the top of the fermenter and reaches down into the fermenter media. As the fermenter is filled with media so will the thermowell, and by applying a temperature sensor in the well it is possible to measure the temperature inside the fermenter.
- pH sensor: pH is measured by a combined, sterilizable pH electrode sensor. Due to the multiple Variopin plug it can be sterilized without any protection. It is screwed into a sensor well after the media has been added to the fermenter, and it is then connected to the control station.[64]
- DO (disolved oxygen) sensor: A DO sensor measures the partial pressure of both dissolved oxygen and gaseous oxygen. The oxygen sensor coating is integrated into high grade stainless

steel fittings. A filter measures the oxygen-dependent red light generated through luminescence (fluorescence) caused after exitation by the blue light generated by the sensor. It is screwed into a sensor well and connected to the control station.[63],[49]

- Sensor well: These are normally closed with screwable caps but by removing them and screwing on sensors instead it is possible to measure the different conditions such as pH and DO in the fermenter.
- Motor: This is a servomotor customized for the fermenter that it is meant for. The motor used in these project fermentations was made by the company Kollmorgen.
- Impeller: The impeller for fermenters is a rotating device with a plurality of radially extending blades. It serves multiple purposes in a fermenter. It has to dispense air and oxygen, transferring heat, maintain a uniform environment throughout the the vessel contents, and enhance mass transfer between dispersed phases.[6] Impellers can be designed in different models such as Smith, Rushton, Hjort or Bakker. It is driven by the motor placed ontop of the fermenter.[75]
- Acid- and base flasks: These are connected with tubes via the control station to the fermenter and provide the culture with acid and base calibration. For the experiments in this project, sulfuric acid and sodium hydroxide were used.
- Gas exhaust tower: A long pibe on top of the fermenter supposed to lead all the exhaust gas out. A filter is placed ontop of the pibe so the gas is pure before coming out. The pibe is additionally built with a thermo cooling jacket.
- Cooling jacket: A jacket on the exhaust tower with the help of cold water running through is able to cool down the exhaust gasses so they can run through the gas analyzer.
- gas analyzer: Device that via the exhaust gas flow analyzses the gas compartments. It is connected to a computer where the data is saved.
- Gas inflow tube: This tube is connected to the control station through a filter and leads the airflow into and down to the bottom of the fermenter and out of the sparger.
- Sparger: This process equipment introduces the smallest bubbles into a fermenter. As the size of the fermentation vessel increases, the gas requirements increase. The sparger can be made bigger for the purpose but the purpose is to keep bubbles as small as possible. Therefore, for larger aeration needs, a ring-sparger is incorporated. The ring-sparger has many holes, and the sum of which equals the maximum anticipated air introduced into the fermenter. Modern practice is to aim for a diameter that will deliver the air at about the speed of sound because that will tend to insure small bubbles. The Sparge line is located directly under the disk of the lower impeller so that the air stream is directed directly through the impellers.[42]
- Baffles: Baffles are obstructing vertical arranged vanes or elongated plates inside the vessel that stops the radial swirl inside the fermenter and convert the rotational flow to axial mixing. Without baffles, the tangential velocities coming from any turbine causes the entire fluid mass to spin, creating a central vortex. Baffles increase the friction to the vessel inner wall surface. [15]

- Heating jacket: During a microbial process heat will be generated and this needs to be kept under control. The heating jacket is wrapped around the fermenter working volume and is able with help of the control station to calibrate temperature with water flowing through the heat jacket that creates heat transfer.
- Harvesting outlet/sample tube: A tube going out of the the fermenter where a syringe can be applied and can tap media for samples. This tube is ought to be closed with a clamp as media will leak from this tube during fermentation.

In the following sections some theory about Escherichia *coli* will be clarified as it was the organism used in this project.

Escherichia *coli*

Escherichia *coli* as an organism

Escherichia *coli* or simply E. *coli* is one of the most common infecting organisms around. E. *coli* was initially discovered in 1885 by the German bacteriologist Theodore Escherich. Escherich showed that specific strains of the bacterium were responsible for infant diarrhea and gastroenteritis. This was an important public health discovery. Although E. *coli* bacteria were originally called Bacterium *coli*, the name was later changed to Escherichia *coli* to honor its discoverer. E. *coli* is sometimes referred to as the most studied free living organism. The E. *coli* types that are responsible for the several reports of contaminated foods and beverages are those that produce Shiga toxin. This toxin is virtually identical to that produced by Shigella dysenteria type 1. Shiga toxin–producing E. *coli* (STEC) cause approximately 100,000 illnesses, 3,000 hospitalizations, and 90 deaths annually in the United States.[34]

Escherichia *coli* is a rod-shaped bacterium. Each E. *coli* bacterium measures approximately 0.5 μm in width by 2μm in length. E. *coli* is a Gram-negative bacterium. E. *coli* cells stain Gram negative because they have a thin cell wall with only 1 to 2 layers of peptidoglycan. E. *coli* is a facultative anaerobe, which means it does not require oxygen, but grows better in the presence of oxygen.[40]

E. *coli* has only one circular chromosome, some along with a circular plasmid. Its chromosomal DNA has been completely sequenced by lab researchers. E. *coli* has a single chromosome. 70% of the chromosome is composed of single genes (monocistronic). About 30% of the sequenced open reading frames have unknown functions.[41]

E. *coli* is found in various places like foods, in the environment, and inside the intestines of mammals. E. *coli* inhabit intestinal tracts of warm blooded animals such as humans, mammals and birds. The majority of E. *coli* strains are harmless, and some even have importance for the health of human intestinal tract. However, some can be hazardous to the health. These hazards can include diarrhea, urinary tract infections, respiratory illnesses, and pneumonia.[39]

The diarrhea causing E. *coli* strain is transmittable through contaminated water, foods, or from other humans or animals infected. Pathogenic E. *coli* can be categorized into six pathotypes. These pathotypes are mainly involved in foodborne outbreaks[46]. These are:

- Shiga toxin producing E. *coli* (STEC)
- Enteroagregative E. *coli* (EAEC)
- Enteroinvasive E. *coli* (EIEC)
- Diffusely adherent E. *coli* (DAEC)
- Enteropathogenic E. *coli* (EPEC)
- Enterotoxigenic E. *coli* (ETEC)

Newborns have a steril alimentary tract that is quickly colonized after two days by E. *coli*. 700 serotypes have been identified. The shiga toxin producing E. *coli* serotypes are the ones responsible for the several cases of contamination in foods. Serotypes are cells classified with their antigens. Mostly Shiga toxin is one of the most hazardous toxins known to humans. It is even listed as a bioterrorist agent by CDC (Center for Disease Control and Prevention). It is believed that Shiga toxin DNA from shigella bacteria was transferred to harmless E. *coli* via a bacteriophage and giving them the genetic opportunity to produce shiga toxin.[56]

Humans get an E. *coli* infection mostly by coming into contact with the feces of humans or animals. This can happen when drinking water or eating food that has been contaminated by feces. Human or animal feces contains E. *coli* and this can sometimes get into lakes, pools, and water supplies. Humans get infected when a contaminated city or town water supply has not been properly cleaned or when humans accidentally swallow contaminated water while swimming in a lake, pool, or irrigation canal. Therefore, the concentration of E. *coli* in water can be an indicator of faecal pollution. The bacteria can also spread from one person to another. This happens usually when an infected person does not wash his or her hands well after a bowel movement. E. *coli* can spread from an infected person's hands to other people or to objects.[35]

Escherichia *coli* in biotechnology

E. *coli* is an ideal model for understanding fundamental aspects of biology. It has been studied widely because of fast growth and easy propagation. It can easily be isolated, it grows on simple media and mutant selection is easy. The genome is only 4 million basepairs and hence many gene functions have all ready been resolved.[66]

E. *coli* plays an important role in current biological engineering because of its manipulation and long laboratory history. It has been widely used to synthesize DNA and proteins. Most results from E. *coli* research can be applied to animals and humans. The most useful contribution of recombinant DNA from E. *coli* is to use the manipulation of E. *coli* to produce human insulin for diabetes patients. Since E. *coli* can produce human enzymes through recombinant DNA techniques, it is widely used as a very good tool to produce useful compounds or enzymes for medication. In a study back in 2007, scientists explored a new method to cure Alzheimer's disease (AD), which is a neurodegenerative disorder characterized by a progressive loss of cognitive function due to extra deposition of the longer form of the amyloid peptide Abeta. In the study, scientists use E. *coli* to enable rapid production of the antigen and its purification. By other means, E. *coli* plays an important role to enable the rapid, continuous production and purification in large amount by its unique expression system. [41]

E. *coli* lives well in warm conditions but not too warm. This is due to E. *coli* being a gut bacterium. It grows best at body temperature (37.4 °Celcius). This temperature is easy to comply for scientists in the laboratory. E. *coli* can obtain energy from a wide variety of sources. In the gut, E. *coli* grows anaerobically. However, unlike some anaerobic bacteria, E. *coli* also grows well in aerobic environments, such as a culture flask in a laboratory. Under ideal conditions, individual E. *coli* cells can double every 20 minutes. At that rate, it would be possible to produce a million E. *coli* cells from one parent cell within about 7 hours. This allows for preparation of log-phase (mid-way to maximum density) cultures overnight and genetic experimental results in few hours instead of

several days. Faster growth also means better production rates when cultures are used in scaled up fermentation processes. All together experiments involving E. *coli* can be done quickly, conveniently and cheaply. [37],[74]

E. *coli* is a preferred host for gene cloning due to the high efficiency of introduction of DNA molecules into cells. It is a preferred host for protein production due to its rapid growth and the ability to express proteins at high levels. Bacterial conjugation can be used to transfer large DNA fragments from one bacterium to another. E. *coli* is a preferred host for the study of phage biology due to the detailed knowledge of its nucleic acid and protein biosynthetic pathways. The ability of E. *coli* to grow on chemically defined media coupled with its extensive genetic tools makes it a key system in study of bacterial metabolic pathways.[29]

The microorganism Escherichia *coli* has a long history of use in the biotechnology industry and is still the microorganism of choice for most gene cloning experiments. Although E. *coli* is known to the general population for the infectious nature of one particular strain (0157:H7) few people are aware of how versatile and useful E. *coli* is to genetic research. There are several reasons E. *coli* became so widely used and is still a common host for recombinant DNA. E. *coli* cells only have about 4,400 genes whereas the human genome project has determined that humans contain approximately 30,000 genes. Also, bacteria, including E. *coli*, live their entire lifetime in a haploid state, with no second allele to conceal the effects of mutations during protein engineering experiments. The E. *coli* genome was the first to be completely sequenced. The knowledge of the E. *coli* genetics were made possible by the discovery of conjugation. E. *coli* is the most studied microorganism and an advanced knowledge of its protein expression mechanisms makes it simpler to use for experiments where expression of foreign proteins and selection of recombinants is essential. Most gene cloning techniques were developed using this bacterium and are still more successful or effective in E. *coli* than in other microorganisms. E. *coli* is readily transformed with plasmids and other vectors, easily undergoes transduction, and preparation of competent cells (cells that will take up foreign DNA) is not complicated. Transformations with other microorganisms are often less successful.[74]

In fermentation technology E. *coli* is also well studied. Studies have shown that under anaerobic conditions Escherichia *coli* converts sugars to a mixture of products by fermentation. The major soluble products are acetate, ethanol, and formate but also some succinate. Also the gaseous products hydrogen and carbon dioxide are produced in substantial amounts.[32]

The process of engineering Escherichia *coli* strains for applied production of ethanol, lactate, succinate, or acetate was initiated several decades ago and is still ongoing. The pathway generating fermentation products is branched and the flow down each branch is varied in response both to the pH of the culture medium and the nature of the fermentation substrate (See appendix A13 for the fermentative pathway of E. *coli*).[32],[2]

Escherichia *coli* in the project fermentations

In this project Escherichia *coli* was used as the experimental microrganism in order to be grown up confidentially in the fermenter.

The Escherichia *coli* strain used for the fermentations was a wildtype strain originally delivered to the fermentation platform of DTU systems biology from SSI (Statens Serum Institut) to another CerCell project.

Statens Serum Institut has a strain collection containing more than 64,000 E. coli strains, of which most are clinical isolates. The collection contains both wild types and reference strains.

The strains are primarily sold as freeze-dried ampoules, whilst most wild type strains are sold as agar slant in transport medium. Freeze-dried strains can be stored for many years in unopened ampoules at room temperature. Once the ampoule is opened, the strain must be used immediately. Strains sent in agar slant must be plated out within a week from receipt and resown, as strains cannot survive in transport agar for long. All strains are typed for the antigen they are sold under just before sale.[36]

Single-Use technology

History of Single-Use Technology

Single-use technology started with the use of membrane filters, which have been in use in biopharmaceutical processing since the very beginning of the fermentation industry. Their use can be traced to the mid-1970s, when the United States Food and Drug Administration required non-fiber releasing filters to be used in the production of injectables. However, the innovation for other unit operations started with the development and introduction of single-use bags for the storage and transport of buffer and media by Hyclone in the 1990's. The popularity of single-use membrane filters and single-use bags set the stage for innovation for other unit operations that started with single-use bioreactors.[21]

The first introduction to single-use systems came in the late 1990s with a single-use, wave-rocking bioreactor as an easy-to-use, flexible, cost-efficient alternative to stainless steel bioreactors, which require continuous time-consuming cleaning and validation between batches.[77]

Single-use manufacturing was further facilitated in the early 2000s by the introduction of large-scale tube welders and sterile connectors that enabled the connection of two sterilized fluid pathways/systems while maintaining the sterility of both.

Several single-use options have been introduced for 21st-century bioproduction, including disposable flasks and spinner bottles, hollowfiber bioreactors, and specialty devices including bag-based systems such as the Wave technology[68]

Today, the term "single-use technology" encompasses a broad range of primarily plastic disposable technologies that are suitable for a wide variety of scales and applications. This goes from upscale bioprocessing to final formulation and filling. They can be found in manufacturing processes for licensed drug and vaccine products around the world.[57]

Disposable tissue culture flasks and roller bottles have been used in the laboratory for many years. Some early biopharmaceutical products were even manufactured using roller bottle cell cultures. Everso, single-use technologies in large-scale biopharmaceutical production facilities have traditionally been confined to the early inoculum stages of the cell expansion process, which involved the use of shake flasks or T-flasks. The first single-use systems for large-scale production came about with the introduction of the WAVE bioreactor in 1996. The first single-use stirred-tank bioreactor was introduced by Hyclone in 2004 and had a working volume of 250 liters. This system used a self-contained plastic bag that was placed in a stainless steel shell. Larger single-use stirred-tank bioreactor systems have been developed more recently such as 1000 liters in 2006 and 2000 liters in 2009 along with various fermenters where mixing is achieved by rocking or orbital shaking has also come.[1]

Single-Use fermenters from CerCell ApS

CerCell ApS was founded in 2009. It is a small company with approximately 10 employees located in Holte, Denmark. The products of CerCell ApS are aimed to replace every corresponding product in glass and steel with single-use products such as fermenters, fed-batch bioreactors, and batch bioreactors. Additionally the company also expertises in customizeable reactors that can be configurated as the customer wants. [24] The mission of CerCell ApS is to be a high-tech international supplier of innovative and unique process equipment to the biotech industry. Their vision is to improve the quality of life for mankind by introducing cost efficient and flexible single-use equipment to the biotech industry.[28]

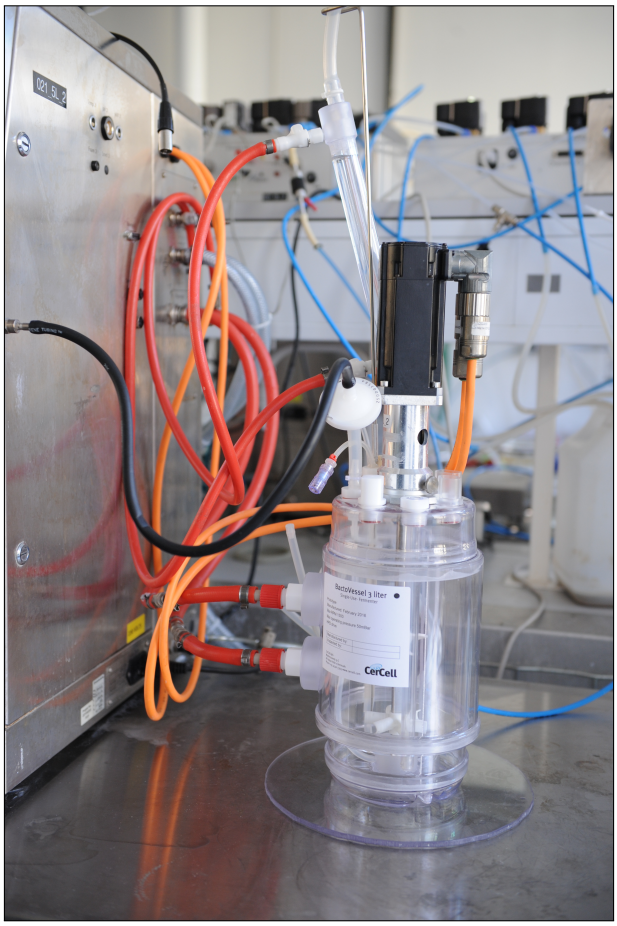

Figure 7: *The fermentations carried out for the experiments upon this project were done with this 3 liter single-use fermenter prototype from CerCell ApS.*

The single-use fermenters from CerCell are manufactured under the trademark BactoVessel. They

are unique and fully configurable. From their website it is possible to create ones very own SUF with help of the "Configurator tool". [44]

In 2013, CerCell realized that the pharmaceutical industry was looking for a single-use fermenter. The fully customisable and scalable single-use fermenter is of world first high-performance. The product BactoVessel changed from the original size of 5 liter SUF now to range from one litre to 30 litre Working Volume. All materials are from rigid plastics. The products are avoiding all technology associated with bags and focus only on rigid plastic fermenters.[14]

BactoVessel's configurable single-use-Fermenters are selectable in diameter, height and volume. The ratio between diameter, height, baffle width, baffle orientation, turbine diameter, and turbine orientation are all done according to standards of the industry. [13]

Throughout this thesis the single-use fermenter will mostly be referred to by the abbreviation "SUF".

Advantages and disadvantages of Single-Use technology

The function of the fermenter or bioreactor is to provide a suitable environment in which an organism can efficiently produce a target product. This target product might be cell biomass, metabolite, or a bioconverted product. The fermenter must be so designed that it is able to provide the optimal environments or conditions that will allow supporting the growth of the microorganisms.[6] One can therefore argue if single-use technlogy has the upper advantages in the future of fermentation.

The benefits of single-use technologies in both upstream and downstream operations are now widely acknowledged by the biopharmaceutical industry, and have led to changes in the design and operation of several bioprocesses. Those changes typically provide more resilient processes and have increased the production flexibility.[4]

Fermenters have been used for many years to produce a range of biomolecules. This is possible by giving the operators the opportunity to monitor and control environmental conditions continuously throughout the culture period with the benefit of maintaining an entirely closed system. Introduction of single-use technology including disposable bioreactors to manufacturing processes has created a cost-effective platform.[8]

Direct market opportunities for parallel disposable bioreactors range from lowprofile fermentations to high-profile pharmaceutical-compliant cell cultures. The high containment level of disposable bag systems is not only very useful for production of biopharmaceuticals, but also for the production of starter cultures in the food, nonfood, and feed industries. The linear scalable and variable culture volume properties of such bioreactor systems can provide flexible cultures in large-volume production runs. The risk of contaminated seeds is minimized when using disposable bags together with decreasing the number of handling steps.[5]

Conventional stainless steel installations presents technical, cost, and reliability difficulties. Biological processes require hygienic environments to grow cells and microorganisms as process environments are exposed to contamination from different agents. When looking this problem, the single-use concept was developed. Rather than validating processes for cleaning equipment with

a large number of variables and risks involved, manufacturers instead implement sustainably produced equipment by replacing stainless steel with plastics. Applications included pumps, singleuse bioreactor vessels, tubing, and respective connectors.[11]

Recently available Disposable Bioreactors having good productivity compare to classical multiple use bioreactors. Having advantages such as[6]:

- Minimizes investments and maximizes returns.
- Simplifies validation process.
- A single-use alternative to conventional stirred tank fermenters
- Scalable technology to support increasing volume demand.

Today, most biological products are synthezised by using fed-batch mode. Perfusion is an alternative culturing mode based on continuous medium feed. On paper, the productivity gains of perfusion operation over fed-batch can be very appealing and can be approximated by a factor of 10 over fed-batch productivity levels and, on the surface, make smaller single-use systems appear even more desirable.[21]

Establishing a new production process is expensive and can take years to set up. At each step, from biosynthesis to formulation, materials must be validated to eliminate contamination risks. Disposable, single-use technologies eliminate cleaning validation steps, which provides significant time and cost savings. testing, validating, and setting up a new process in a fixed platform may take between two to four years. With single-use technology, this time span can be reduced by two thirds. Single-use fluid management and bioreactor bags cut the time of cleaning- and sterilization from days to minutes. Single-use technologies also have a smaller footprint and reduce consumption of resources such as labour, high purity water, and cleaning agents.[69]

Key challenges facing the developers and users of single-use bioprocessing technologies include their limited scale, the restricted diversity of options, and the lack of standardization. Most disposable technologies have been developed for a maximum upstream production scale of 2000 liters cell culture. It seems clear that the scales are limited compared to conventional technologies but it should be emphasized that these restrictions are not technological but are based on demand, thus larger systems will probably be introduced as the demand for them grows. The diversity of options is a current limitation of disposable technologies. This is increasingly frequent when moving towards process scale manufacturing, and it correlates with the number of vendors offering particular categories of equipment. For instance, many different vendors offer disposable filters and tubing, a handful offer disposable bioreactors, and two supply disposable centrifuges. Competition between vendors often drives innovation so the diversity of disposable devices is bound to increase as more vendors enter the market. Another challenge that influence single-use technologies is the absence of standardization and regulation of the quality of materials used. One of the key reasons cited by manufacturers for not taking up disposable technologies is the lack of a validation process to determine the nature, quantity and risk associated with the disposable plastics, which could potentially contaminate product intermediates.[1]

For mammalian cell cultures, cleanable multiuse glass or stainless steel stirred-tank reactors (STRs) have been used successfully for growth of suspension-adapted cell lines in various scale systems.

However, achieving the same or better performance from a single-use bioreactor presents a significant challenge to product designers and developers.[4]

some other disadvantages of single-use technology worth of mentioning are[45]:

- Disposable sensors are limited
- Non-standard, multiple connection options
- Recirculating processes that require high pressure and high flow rates
- Lab to commercial scalability within the same reactor design/configuration
- Mixing/Buffer/Media preparation gives a lower rate of liquid transfers

In the following chapter of materials and methods, the standard operating procedures will be described in step-by-step on how to handle the SUF, and how the fermentations were carried out.

Materials and Methods

Preperation of Media and E. coli

Overview of preperation

In order to be able to carry out a fermentation experiments, it is necessary to prepare different things. First of all, the growth media has to be prepared. In order to grow Escherichia coli in a fermenter and in precultures, LB media must be prepared.

Furthermore, it is also necessary to incubate the cells that are needed for the fermentation in order to make precultures, and then eventually inoculate the fermenter. It is also a good idea to prepare the dry weight filters the day before (see appendix A3).

In the following, these procedures will be described.

Basics of Inoculation

Inoculation originally comes from the method of introducing material of smallpox into the skin of infected patients as a way of treatment against smallpox. In modern meanings, it is generally related to vaccination in which vaccine is inoculated into a person. In microbiology it simply means introducing or inserting one substance into another. This could be either safely introducing a chemical compound into a bacteria culture, or introducing microorganisms into a culture for instance an agar plate or a luquid media. The material that is inoculated is referred to as the inoculum.[78], [52]

For a fermentation process, there are usually three steps of inoculation:

- First step. Inoculate microorganism to an agar plate. This is to make sure the cells are active and are in a proper environment and in good condition. It is also done in order to investigate if the plate is pure or has not been contaminated with other microorganisms.
- Second step. Inoculate single colonies into preculture flasks. This is done in order to transfer the the cells into a liquid environment.

- Third step. Inoculate liquid preculture inoculum into fermenter. This is obvious since a fermentation needs a starting amount of cells to grow up.

These steps will be more thoroughly explained. In order to carry out the fermentation, it is necessary to prepare LB media. The needed volume for the SUF is 2 liters for working volume, but it is also necessary to have LB media for the precultures.

LB Media

LB stands for Lysogeny Broth, originally, but has also been interpreted as Luria Broth or Lennox Broth. The name comes from an old study of Lysogeny for phages infecting Escherichia coli cells where this broth was used to grow the Escherichia coli cells on.[17]

LB media is a highly referenced microbial growth medium used for the purpose of cultivation of Escherichia coli. It is a nutrient rich broth for microorganisms full of peptides, amino-acids water soluble vitamins, and low salt formulation carbohydrates. The benefits of using LB media for growing Escherichia *coli* is because the tryptone and yeast supply essential growth factors that the E. *coli* would otherwise have to synthesize. LB contains essential electrolytes as well for transport and osmotic balance due to the NaCl component.[54]

The recipe of LB media is fairly simple and can be found in Appendix A1. LB media can be made with differently depending on what NaCl concentration is wanted. The variances are as following[26]:

LB media type	**NaCl component**
LB Luria	0.5g/L NaCl
LB Lennox	5g/L NaCl
LB Miller	10g/L NaCl

LB is also used for agar to agar plates on which Escherichia *coli* is grown upon. Nonetheless, also 15 g/L of agar is needed before autoclavation. The substance is after autoclavation cooled to 55 °Celcius before being added to petri dishes and cooled down in a refridgerator.[55]

For the fermentation experiments in this project, only LB Miller media was used. in LB media is an inevitable component that contains all the nutrients, which the cells must live off in order to grow. If one wants to measure a proper cell growth when running a fermentation with Escherichia *coli* then LB media is an inevitable component.

Inoculating E. *coli*

Inoculation with E. coli is not particulary challenging because E. coli is a very dominating bacteria and has a rapid growth rate. A primary requirement for Escherichia *coli* is a nutritious broth to grow on and a temperature of 37°Celcius.

Escherichia *coli* is an organism that is very hardy and can survive under harsh environment. However, to have a confident growth with Escherichia *coli*, it is paramount to choose a broth on which Escherichia *coli* gets a supply of the essential nutrients. LB broth is optimally chosen for Escherichia *coli*. Making an agar plate with LB broth is initially required. Afterwards one must have an active cell colony that can be inoculated onto the agar plate. The cell colony can come from an another agar plate or from a liquid culture. Then prepare an agar plate with E. coli cells (see appendix A2), which further on can be used to prepare precultures.

Preculture

A preculture is is a liquid culture with grown up cells meant for a preperation in advance for an experiment. It is essential for all fermentations because fermentations are carried out with liquid media. [65]

According to theory the growth of bacteria in precultures should be 4-48 hours for a frozen culture and 4-24 hours for a refrigerated culture. [66]

It is necessary to prepare precultures in order to get a cell growth in a new environment, which the cells eventually need to live in when inoculated into a fermenter. A preculture is inoculated with only one single colony from an agar plate. This is because there is no wish for too much genotypic variation between the cells. Cells from a single colony are originally grown from one cell, which means that the genotypic variations between the cells are low. The precultures also needs to be prepared well in advance, at least 18 hours before the initiation of the fermentation. This is because of the incubation being in a shakeflask at only 150 RPM. This gives a slower cell growth.

Prepare at least 2 shakeflasks of precultures the day before the fermentation is supposed to start (see appendix A3).

Conditions of fermenter and start up

Conditions for fermentation

The conditions for the single-use batch fermentations of this project with Escherichia coli are the following:

Conditions	**Value**
Total volume (L)	3
Working volume (L)	2
Agitation (RPM)	750
Airflow (L/min)	1
VVM	0.5
Temperature (°Celcius)	37
pH	7

The conditions are able to being adjusted at the control station, which the fermenter compartments are connected to.

Fermentation start up

Just before the fermentation is able to be initiated, the SUF is placed in a LAF (laminar flow cabinet) bench in order to avoid contamination. The SUF is prepared with LB media and afterwards sensors are sterilized and applied (see appendix A5).

The SUF is then connected to the control station. All the sensors are connected to their correspondant wires, and the water tubes are connected to the control station (the cooling water system for the gas outflow, and the heating water system for the temperature calibration of the fermentation). The gas exhaust pipe must be standing, and that is done with help from a steel holder. The motor is applied to the SUF. 2M sulfuric Acid and 2M Sodium Hydroxide flasks are connected with tubes to the control station and then to the SUF. The tube from the gas exhaust pipe is connected to a gas analyzer. All the conditions are set and turned on.

Before the inoculum is added to the SUF, it is important first to take a reference sample in order to measure the media OD and the starting parameters of the fermentation (see appendix A7 and A8).

Then the inoculum is added.

Fermenter inoculation

When inoculating a fermenter, the inoculum is of liquid preculture. This is due to the cells grown up used to being in a liquid enviroment, which is vital when growing up cells in a fermenter. They also need to be in a certain quantity or else it would simply take too long for the fermenter to grow up the cells and reach an exponential phase.

Take a sample from a chosen preculture shakeflask and measure the OD (see appendix A8). Then use the inoculum volume formula (see appendix A6) in order to calculate the volume of the inoculum that needs to be added to the fermenter. By deciding the starting OD of the fermenter, theory supports the prospect of having a starting OD in a fermenter between 0.1 and 3.0 for bacteria.[66] The inoculum is added with a syringe. The well for the media addition can be used by temporarily removing the cap and apply the cap again right after inoculation. Alternately in order to avoid unnecessary contamination, a sample tube can be used if sterile water is applied to the tube afterwards in order the flush out any inoculum that is trapped inside the tube. The time of inoculum addition is noted and referred to as 0 hours. Another sample is taken for OD measurements for measuring the beginning OD for t=0.

Sampling and methods for cell growth determination

Sampling

During the fermentation, samples are to be taken once in a while. For an Escherichia *coli* fermentation the time interval between the samples are not very long because it grows relatively fast. Therefore, samples are to be taken every 45 minutes the first 3-4 hours, and then every 30 minutes when the exponential growth phase is ongoing. According to the experiment results in this project, Escherichia *coli* should reach its maximum growth point within 10 hours depending on stirring veolicity, gas flow, and initial OD.

OD sample measurements must be taken at every sample but dry weight samples are only necessary to take once the exponential phase has been reached and only once every hour.

OD measurements

OD stands for Optical Density and is one of the most common methods to measure cell growth. OD is more officially known as Absorbance.

The formula of absorbance is [72], [7]:

$$A = log_{10}(\frac{I_0}{I})$$

A is absorbance, I_0 is the itensity of the reference, and I is the itensity of the sample. The higher the absorbance is, the lower the intensity of the sample is. This is because if more light is not passing through, a lower itensity is measured and thus a higher absorbance.

It is possible to measure cell concentration with OD according to Lambert-Beer's law.

Lambert-Beer's law is known as this equation:

$$A = \epsilon \cdot b \cdot c$$

where A is the absorbance, ϵ is the wavelength depending molar absortivity coefficient, b is the pathlength, and c is the concentration.[16]

As it can be seen, the equation shows a linearity between absorbance and concentration in mass per volume or moles per volume. Therefore, if the cell concentration increases then the Absorbance or OD increases as well.

The OD for Escherichia *coli* is measured via a spectrometer with a light at 600 nm. The reason that 600nm is chosen is because the absorbance of light by other molecules in the cell is minimal at this wavelength.[48]

However, the major importance about OD measurements is that they only measure biomass and not the number of actual cells. A quantitiy of biomass can also be dead cells or just fewer but bigger cells. Therefore it is only a superficial method to determine a cell concentration but it is still the most effective,fast, and easiest method there is.

The OD measurement must be measured with a spectrometer in between 0.1 and 0.4 because research has shown that the linearity between OD and concentration becomes very unstable after 0.4.[3]

It has been estimated that a OD value of 1 is approximately equal to 1,000,000,000 Escherichia *coli* cells in 1 ml.[26]

Sample liquid is transferred to a cuvette in order to measure the OD (see appendix A8). It is important to take the OD measurements as fast as possible since biomass will sink to the bottom right after transfer.

Dry weight measurements

Dry weight measurements are also used to measure the cell growth in a fermenter. According to theory, the OD of a sample measured in a spectrophotometer is correlated to either the dry weight, which is in fact a measure of the number of cells per volume. More precisely, according to Lambert-Beer's law, the correlation between dry weight and OD should be linear.

The cells in a sample can be separated from the broth media and are possible to be weighed while wet. Nevertheless, The dry weight measurement usually gives a much more consistent result than the wet weight.[59]

Dry weight measurements are not meant to be taken as often as OD measurements, partly because there is a larger uncertainty by taking dry weight measurements, but also because the quantity of biomass must be efficient to be noticeable. It is usually only necessary to take dry weight samples when involved in large scale production.[12]

Hence, the first dry weight measurement is not needed to taken before the exponential growth phase occurs.

Before taking dry weight measurements, dry weight filters must be prepared (see appendix A3). The filters have milipores of 0.2 μm milipores. The filters are able to hold E. coli cells as they have an areal size of approximately 1 μm^2 [40]

The measurement unit is g/L (grams of biomass), but normally not more than 5 ml are used. Therefore, a multiplying factor is used to get a unit of g/L.

Start taking dry weight samples every half or every hour from 3 to 4 hours for an E. coli fermentation (see appendix A9).

Ending of fermentation and precautions

Ending fermentation

Once the last sample is taken, let the fermenter run until the next day in order to get useful CO_2 measurements. All the connecting wires and tubes from the control station are detached. The motor is removed from the fermenter. The acid and base flasks are replaced with water flasks, and the control station is set to run with both base and acid calibration. This makes sure that the tubes are cleansed from any acid or base leftovers. Finally, the control station is shut down, and the tubes from the water flasks are taken off. Afterwards, the fermenter can be cleaned if one wants to reuse it (see appendix A10). However, for a single-use manner this is normally not necessary. It is only necessary to pour the biowaste from the fermenter into a biowaste container.

Setup and handeling

In order to get good results from a fermentation, it is crucial to correctly prepare a setup of the Fermenter. These following sections will address some of the important precautions that needs to be taken care of before and during fermentation.

The fermenter must stand fastened at any point during the fermentation so that it is not shaking, and it is important not to run the motor at a high speed. It is not particulary necessary to run the fermentation with an agitation over 1000 RPM. For the fermentations in this project the motor ran with a speed of 750 RPM, which was sufficient for cell growth to occur still. Place the sensors and the motor so that they are not in the way for each other, and so it is still easy to consult the media well. The fermenter must also be placed conveniently so that the sample tube is easy accessible. It is a good idea to place a cloth under the cooling tubes in case of leak, which is more or less inevitable.

The acid and base flasks must be placed in a steady position because it is hazardous liquids so the flasks should of course be avoided getting broken. Also make sure the Acid and Base flasks are placed away from the fermenter so that one avoids direct consult with them during fermentation. The tubes, however, must be laid properly so that tangling is avoided. The pH tubes must be put correctly into the automatic squeezers on the control station.

The propes must be fastened at the fitting ports and the motor, and cooling tubes must be fastened tightly.

When handeling a SUF, one must be aware of the fragility of the fermenter as it is made of plastic material and not glass and steel. First of all, it is off limits to autoclave the fermenter at any point because it is not able to withstand the heat from the autoclave. Additionally, it is necessary to treat it in a gentle manner because the material is not as robust as a conventional glass and steel fermenter. Never use a tool in order to tighten items onto the SUF. It is made of plastic and not meant for powerful tools.

Moreover, is it also essential to write everything down that takes place during the fermentation.

This is necessary in order to discover fatal errors and in order to improve the results for the current fermentations and the fermentations to come. Most importantly is writing down the conditions of the fermentation for each sample such as temperature, pH and dissolved oxygen. If they are not within an acceptable range, they must be dealt with. Additionally, it is also crucial to write down the different aspects that might seem to be the reason for the fermentation to go not as expected in order to prevent them further on.

Contamination and sterility

Contamination means making something impure or insuitable by contact with something unclean or bad. In microbiology, contamination is often associated with unwanted cell growth in a culture only meant for one particular organism. Sterility is the prevention of contamination. It originally means keeping something clean an unaffected. In microbiology, sterility refers to a process in which all life is eliminated.[27], [70]

Therefore, in order to keep a SUF steril, it must be put into a LAF bench when anything such as media and sensors are to be added. Make sure also that the LB media flasks are always opened in a LAF bench. Do not autoclave more LB media than you need. It is very likely to be contaminated if not treated under sterile conditions.

One major important aspect is wearing a Labcoat. This is due to avoiding the risk of one not to contaminate the fermentation, but also for own safety regarding not being infected with microorganisms. The labcoat must be put on prior to consulting the SUF and taken off once leaving the working area or the laboratory. Another aspect is wearing gloves. This is not an essential requirement, but it is a good idea in order to lower the risks of contamination. A lot of germs are on the hands, and they must be kept away from the SUF. Alternatively, the hands must be cleaned with a hand sanitizer or ethanol each time entering the working area and every time leaving.

Ethanol is an effective compound for the elimination of cells. 70% ethanol is an essential liquid to have in the working area of the fermentation. After fermentation, all the sensors must be cleaned with ethanol and afterwards washed in destilled water. The sensors are not of single-use technology and must be properly cleaned for reuse. Also use ethanol for sterilizing and cleaning other equipment prior to applying them to the SUF. However, it is essential to wash it in destilled water afterwards as it is not wanted to eliminate the cells meant to be grown up inside the SUF. This is of course also done in LAF bench. If fermentation culture is accidentally leaking and gets on the skin or table then use ethanol to sterilize.

Results

General results

The results in this project are based upon fermentations with E. *coli* grown in LB media with a 3 liter SUF by CerCell. The general results are the following:

Fermentation	**1**	**2**	**3**	**4**	**5**
Time run (hours)	8.25	9	9.5	7.5	9.5
OD$_{Max}$	25.869	23.752	32.945	44.07	34.44
μ_{Max} **(h**$^{-1}$**)**	0.4557	0.6128	1.3499	1.3851	0.5783
Doubling time of μ_{Max} **(h)**	1.52	1.13	0.51	0.5	1.12
DW$_{Max}$ **(g/L)**	3.7	1.8667	0.8	3.1	x
Accumulated CO$_{2,Max}$ **(g)**	12.8196	8.0561	9.9318	12.216	2.6777
Accumulated CO$_2$**,**μ_{Max} **(g** $\cdot h^{-1}$**)**	1.0414	0.9164	0.9787	1.2942	0.81

In the following the respective results will be presented. Starting with the OD results, followed by the dry weight results, and finally the CO_2 results.

OD results

The first results to be presented is figure 8, which is the OD measurements over time for the 5 fermentations:

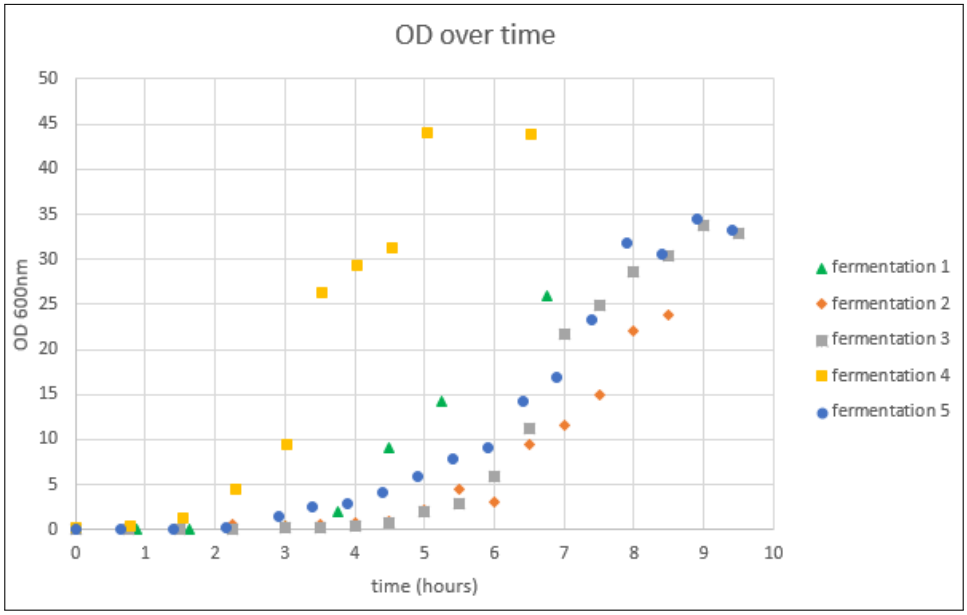

Figure 8: *This shows the OD measurements for all the fermentations completed. The OD measurements were diluted in order to have an OD value between 0.1 and 0.4 and afterwards multiplied with the dilution factor. The lag phase for the majority is approximately between 0 to 3 hours, then the exponential phase between 3-8 hours, and then a stationary phase after 8 hours (only measured with fermentation 3 and 5). However, for fermentation 4 there were observed some quite different OD measurements.*

Here is figure 9 ; the values when ln(OD) is plotted over time:

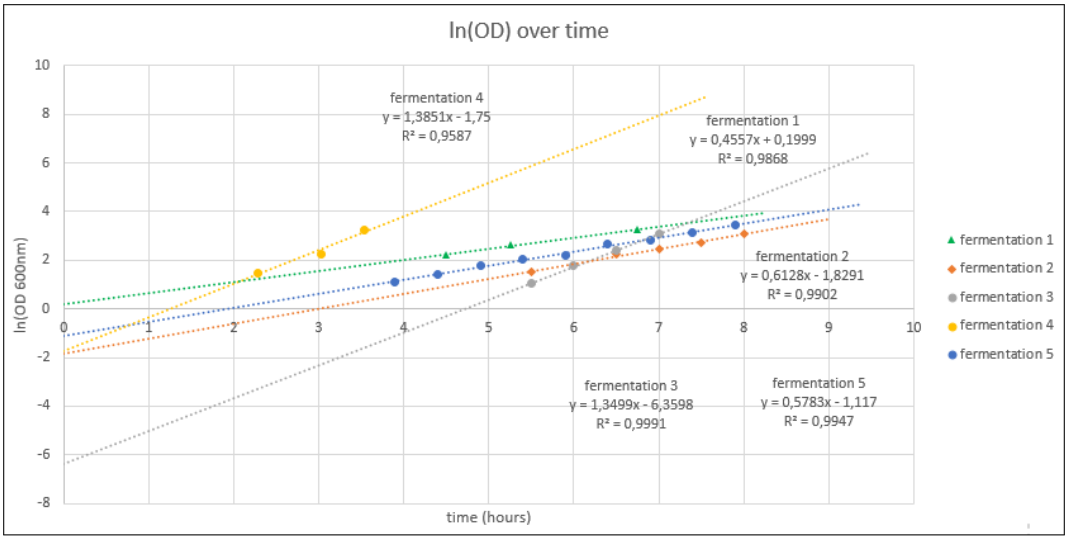

Figure 9: *This figure shows the natural logarithm for the OD measurements for all the fermentations. As it can be seen a linear regression has been made for all of the series. The regression is made on the exponential parts of the fermentations only. The R values clearly shows that the linearity is acceptable. The slope of the regression is* μ_{Max}*. (See appendix A11 for* μ_{Max} *calculation).*

Dry weight results

Represented here is figure 10, which is the dry weight measurements over time for 4 of the fermentations:

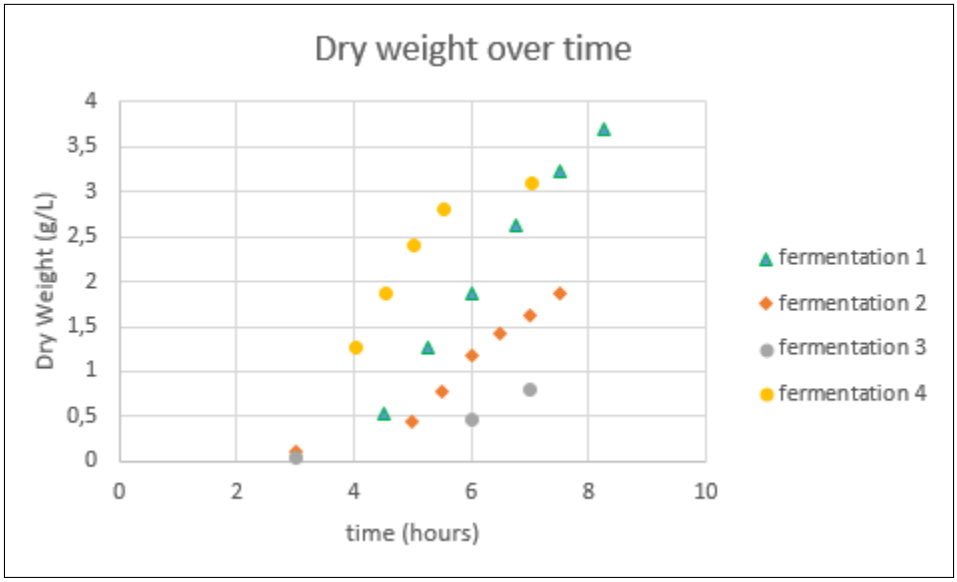

Figure 10: *This shows the correlation between the measured dry weight over time for 4 of the fermentations where dry weight measurements were made. According to the theory, they should follow a similar development as the OD measurements, which is difficult to observe in this graph.*

Here is figure 11 represented with the correlations between the OD measurements and the dry weight measurements.

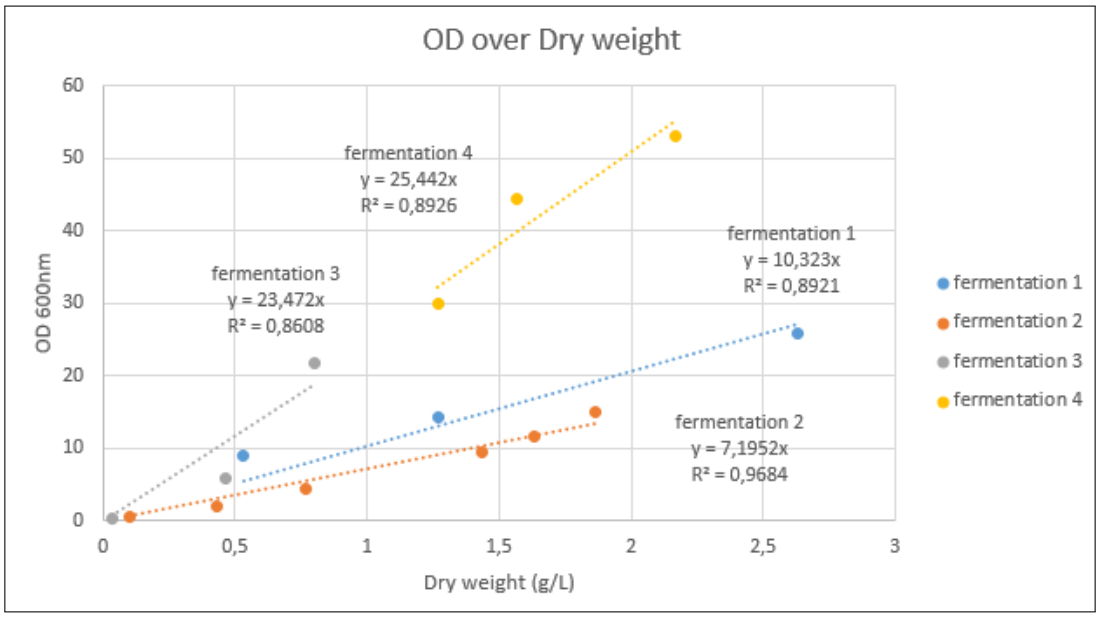

Figure 11: *This shows a correlations between measured OD and the corresponding dry weight. Dry weight is shown on the x scale and OD on the y scale. The slopes are approximate OD for 1 gram of dry weight. The R values are somewhat not very good, indicating that the linearities might not be accurate. Due to the rather significant uncertainties with dry weight measurements, the correlations are very different compared to the fact that the fermentations are supposed to be alike.*

CO_2 results

Represented now is figure 12, which is the CO_2 percent of over time:

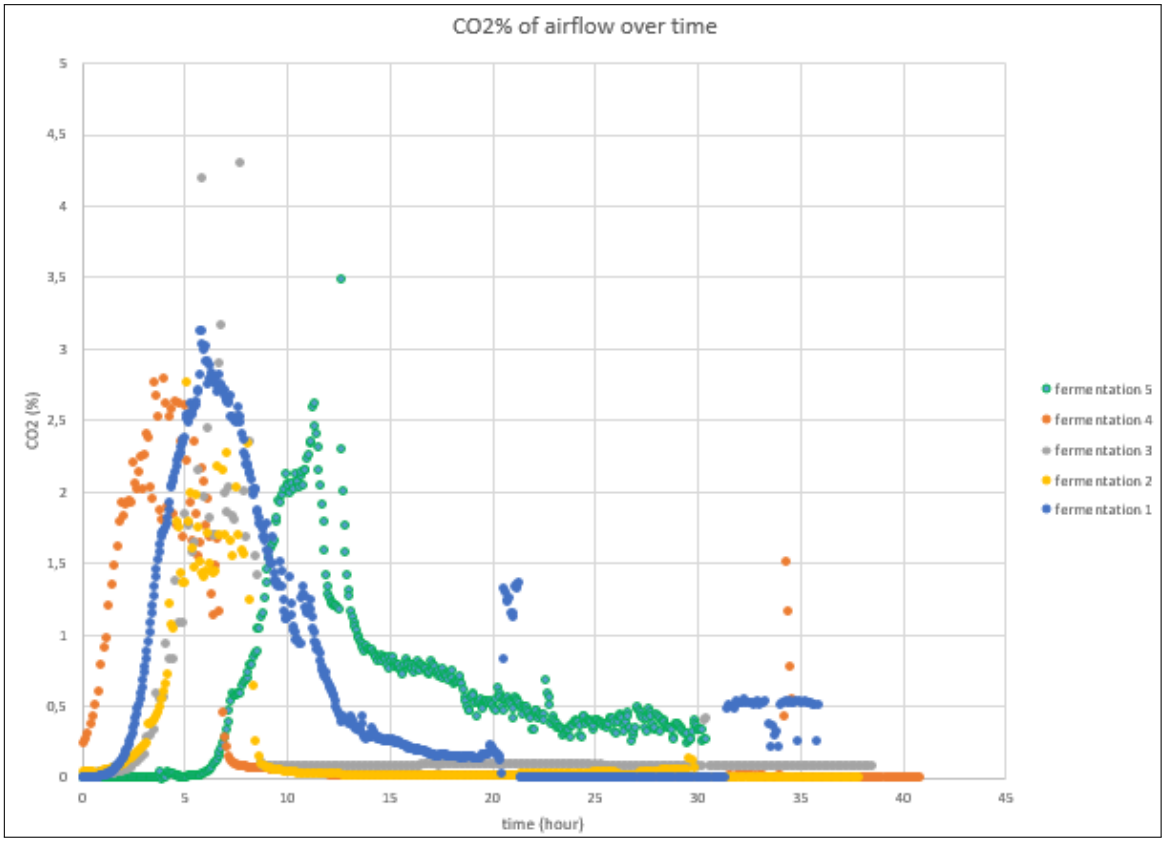

Figure 12: *The percent of CO_2 over time. This graph is made in order to get the idea of the period of maximum growth. It can be seen that fermentation 1,2, and 3 have their peak at around the same time (7 hours). Fermentation 4 is a little faster with a peak around 4 hours and fermentation 5 is a little behind with a peak at around 12 hours. This holds with the data of OD and dry weight.*

Here is figure 13 represented

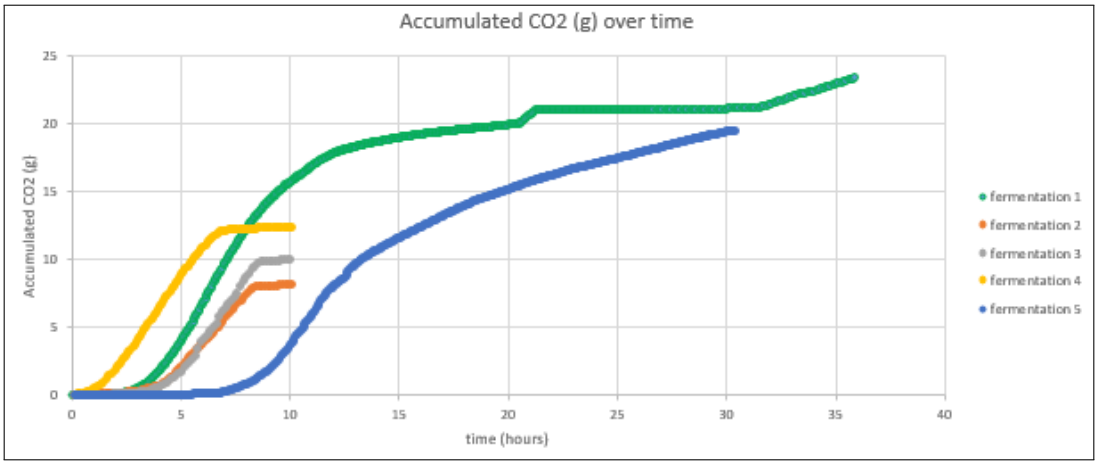

Figure 13: *The accumulated CO_2 in grams over time. It can be seen that fermentation 1 and 5 have values given until after 30 hours. This is due to them being the only fermentations that were run over night. The others had technical difficulties in order to be run over night. Of 1 and 5 it is seen that 1 reaches the steady state faster than 5. Steady state for accumulated CO_2 is theoretically supposed to be the time where all cells are dead. The growth rate in the exponential phase, however, is measured for all 5. the fastest growth is seen for fermentation 4, and the slowest for 5. 1,2, and 3 follow quite the same growth rate.*

Here is figure 14 represented. Natural logarithm of accumulated CO_2.

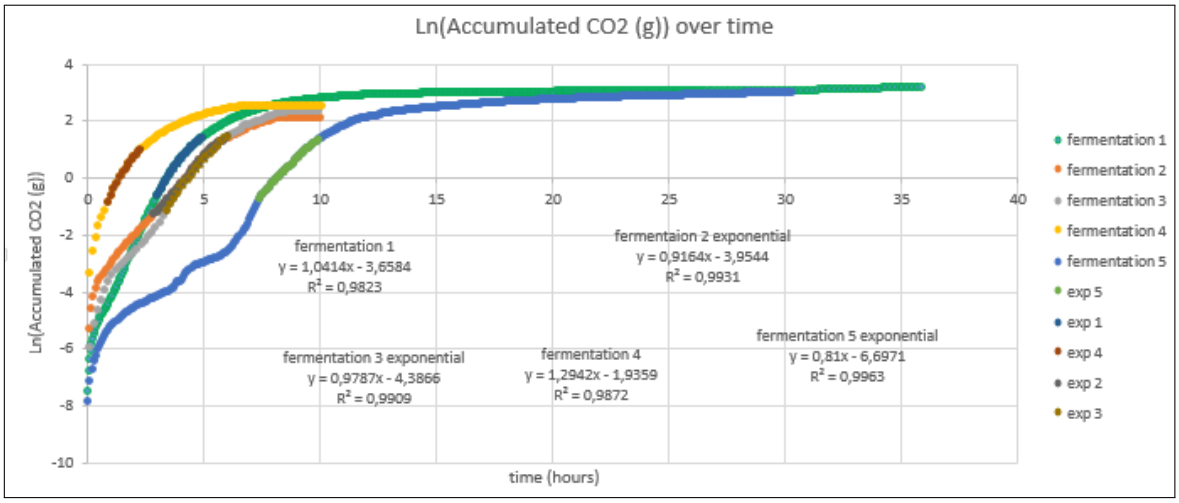

Figure 14: *These graphs are made in order to calculate the μ_{Max} of the CO_2 for the different fermentations. They are the slopes of the different equations. Fermentation 5 is again the slowest and fermentation 4 is the fastest. The method for CO_2 μ_{Max} calculation is the same as for OD (see appendix A11).*

Discussion

When running scientific experiments, a lot of sources of error and uncertainties are always present and inevitable. In this chapter, it will be discussed how the fermentations went, and what the causes were for the fermentations that did not run as expected. In addition, the concept of single-use technology will also be discussed.

The results show that OD and dry weight are increasing over time for all the fermentations. The results show with the OD how a lagphase and an exponential phase is visible. The OD and dry weight values vary to a certain extent from each other. This is not supposed to be expected because they are theoretically supposed to be identical only with a factorial difference in values. This is due to the same wildtype E. *coli* strain, and the same fermentation conditions being used for all the fermentations. Additionally, it can also be seen that a lot less values are represented for the dry weight measurements than the OD. This is because the dry weight is more time demanding and more likely to go wrong and represent an odd value.

The results also show the correlation between OD and dry weight. It shows a linear relationship which indicates that the SUF does not have an impeding influence on the grown E. *coli*. The results are logical in the manner that the E. *coli* strain is the same used for all fermentations. However, the measurements of dry weight are deviating to a certain extent because there are many sources of error that can cause deviations from linearity. The R values also indicate that the linearity is not quite within the acceptable. Nonetheless, for the measurements they are still linear proportional with the OD, which means that OD and dry weight increases proportionally.

Moreover, the results shows the values of the specific growth rates μ_{Max}. It can seen for the majority of the fermentations that exponential growth phase occurs after 3 hours and ends around 9 hours. Fermentation 1, 2, and 5 have a maximum growth rate of around 0.5, but fermentation 3 has a max growth rate of 1.34 which is much higher. Fermentation 4 has the highest of 1.38. The doubling time for both of them were around half and hour. When knowing that E. *coli* can dobule every 20 minutes these fast fermentations were still slower. It is also not as expected for them to vary as they are theoretically supposed to be around the same, being that the microorganism and the conditions are the same all over. Fermentation 4 had multiple problems, but it is still strange for fermentation 3, which was run quite as the expected. Looking at fermentation 4, it is interesting that it is so oddly out of range. It reaches it log phase much faster than the others. additionally, it also reaches its maximum OD value at 45 while the others with the most successfully run (fermentation 3 and fermentation 5) have a max OD at around 35.

The cause most likely lies in the fact that some of the LB media used seemed to be contaminated with other sources of microrganisms before it was autoclaved. The dead cells became biomass remains that went to the bottom and became a part of the medium used for fermentation 4. This is a serious topic and case to avoid because it is very likely to ruin a whole fermentation which also most likely was the case with fermentation 4. E. *coli* growth occured however. Looking on the other hand the dead cell biomass created some nutritious feed for the E. *coli* cells so that an explosive growth occured.

Everso, sterilization of the media is very important and it must be done rather quickly after the preparation. LB media is very nutritious not only to E. *coli* but other bacteria as well. Bacterial growth will occur in a couple of hours if not autoclaved.

It is seen that the majority of the growth curves reaches the stationary phase after 9 hours. This is relatively late as E. *coli* is a fast growing microorganism. This can be due to the agitation only being at 750 RPM, and the flowrate at 0.5 vvm and not 1 vvm. A technical mismatch made the fermentation run at 0.5 vvm (1 liter of air per minute) instead of 1 vvm (2 liters of air per minute). If more air had been supplied to the cells and if the air was distributed faster with a faster agitation then a sooner growth maximum might have taken place. Nonetheless, an agitation much faster than 750 RPM tends to be of surplus energy waste as the primary purpose of the agitation is to keep a uniform environment in the fermenter.

Another aspect worth mentioning is the fact that autoclaved LB media might have an affect on the growth rate. A study has shown that prolonged autoclaved LB media lower the inital growth rate of E. *coli*. [9]

The specific growth rates for accumulated CO_2 are naturally not supposed to be of the same values as growth rate of OD since they are two completely different factors. But one can still analyze the growth rates in order to get an idea if they are the same and if not how they are varying from each other. Again fermentation 4 is the fastest, and fermentation 5 is the slowest.

The idea of having CO_2 measurements, and not just OD, is that with CO_2 measurements, it is possible to estimate when the living cells begin to decrease and eventually the time when all the cells are dead. If the growth curve of accumulated CO_2 over time is reaching a stationary phase then no more CO_2 is produced and therefore no more cells are alive. Only CO_2 data for fermentation 1 and 5 was able for over 30 hours. There was some problems with the pH calibration during fermentation 2, 3, and 4 and if pH calibration was not taking place then the cells would eventually die earlier by their own products such as ethanol, and that would results in faulty results.

It can be seen that for fermentation 1, the cells stop accumulate CO_2 at around 20 hours while fermentation 5 did not reach a full stationary phase even after 30 hours.

One major source of error is the inoculum and the media OD. Even though the formula for calculating inoculum size (see appendix A6) is theoretically true, it is very hard practically to experience that the OD in the fermenter becomes exactly the wanted starting OD (media OD plus starting OD). This is possibly due to the small variations in the media OD. The media is still just a mixture of different components, which means that these components could eventually sink down through the water if not stirred. This will make the OD of the upper media lower and the OD of the bottom higher. These OD variations of the media are also taken into account for the media inoculated in the reactor. So when taking an OD measurement of the media, it will still give a certain source

of error. This being of faulty assuming that the measured OD is the overall OD of the fermenter, which will affect the inoculum volume size.

Another important source of error is the OD measurements themselves. As soon as the diluted media has been mixed in the cuvette, the biomass will rapidly sink to the botton in the cuvette. Unless an OD measurement is not taken immediately, it can give a measurement that is relatively off the actual.

Another topic is the pH calibration. It was noticed throughout the fermentations that the acid was used much more than the base in order to calibrate pH at 7. This must be because of the products yielded by E. *coli*. As mentioned earlier the major products that E. *coli* produces are among others acetate and formate. These compounds are negatively charged and will take the H^+ ions from water molecules and leave OH^- ions. Formate and acetate have thus basic abilities in a aquatic solution, which holds up with the observed empiri.

The actual aim for a SUF is to be able to function just as well as a conventional fermenter. A fermenter is ought to fulfill many things. The SUF is able to grow E. *coli* up but perhaps not every organism. What is certain is that the SUF can grow cells up that are able to feed on a simple media. The SUF can provide all the necessary fundamental conditions there are needed in order to make a simple cell growth take place.

Allready back in 1980, the proper fermenter design was still uncertain and there were a lot of ideas to how a fermenter should be designed. But one thing was common for all proposed design suggestions. The oxygen supply had to be sufficient and the distribution of heat and oxygen had to be eminent.[76]

The essential requirements for a fermenter is having a vessel that makes it possible to control the environmental conditions such as temperature, oxygen and carbon dioxide concentrations, pH and nutrient levels, so that microorganisms can grow and respire without being limited and can work as efficiently as possible.[18]

The SUF can unddoubtly live up to the task of these fundamental requirements. But it might not be able to handle all sorts of requirements when looking on an industrial scale.

When looking at the aspect of having single-use systems replacing the conventional fermentation technology completely different concerns come in focus. First of all a single-use systems must be sustainable. Many single-use bioprocess systems are made of multiple materials or layers including polyethylene (PE), polypropylene (PP), ethylene vinyl alcohol, or nylon. This means that recycling for the most part is not an option for such products because they require thorough efforts to separate the components into homogeneous components. As a result, most single-use systems and components are not amenable to recycling efforts.[19]

According to CerCell the chemical compounds of the material that makes up the SUF are among others Thermoplastic co-polyester, Polyethylene Terepthalate Glycol, polycarbonate, acrylic polymer, polyethylene, polymethylpentene, Polyamide, Silicone, and parylene.[58] Most of these compunds are not dangerous for the environment if going through a complete incineration besides yielding CO_2. Only acrylic polymer might create some toxic compounds such as NO_x gasses when incinerated. Being incinerated at a proper facility will take care of these gasses, but it could be a problem if it is incinerated outside of these facilities. This is of course only depending on the fact that they

will even be incinerated which is not the intention of single-use Technology. According to CerCell, The BactoVessel SUF's are made of rigid plastics. Rigid plastics have a lot of potential for being reused as it is high density plastic mostly used for packaging [62] Another aspect is the use of Polyethylene and Polycarbonate. Plastic bottles made of these plastics have shown to contain hormone disrupting chemicals, which could be a problem when growing microorganisms. Nonetheless, the research was only done with plastic bottles, which also contains antioxidants that might be the true cause and hence nothing to do with the materials used for the SUF.[31]

One can just not be certain that when growing a hazardous possibly gene modified organism (GMO) in a SUF that it is safe to reuse the materials if they are not cleaned thoroughly afterwards. If the SUF is send back uncleaned and with possible GMO inside to the manufacturer, it must also be very important to have it safely sealed when being shipped. There are no certain guaranties when shipping, so this is also another serious aspect. If not, then it is crucial to pay attention to what organism there has to be grown.

Although not tested in this project, the SUF might be able to run at very high or low pH. It has not been possible to find any information on which pH values the SUF is able to withstand. However, According to CerCell the SUFs are not able to withstand temperature of over 50 °Celcius and ethanol with a concentration higher than 60%. [44]

One very great aspect of the SUF is the fact that it can be used for research at institutions. Research fermentations are often of benchtop scale reaching from 1L to perhaps 20 L, which a 3L SUF nicely fits with. When doing research at facilities cleaning is a time demanding factor of the process and desirable to avoid. This problem is what the SUF perfectly fulfills the requirement for. Everso, single-use fermenters might to a certain extend of size be able to be used in the industry.

Single-use stirred-tank bioreactors for mammalian and insect cell cultures have recently been successfully used in scales up to 2,000 L working volume and are installed in different sorts of drug manufacturing facilities. A problem that lies in the single-use technology is the lack of high oxygen transfer rates but these are ought to be achieved in disposable fermenters as well by applying general bioengineering principles and designs.[30]

The most critical reasons to embrace single-use technologies are to reduce capital investment in facility and equipment and to increase speed to clinical trials. The impact of single-use systems in the future must be considered when time to market is a significant factor. New stainless-steel–based facilities can take up to four years to build, validate, and become fully functional, which requires significant capital investment. Many emerging companies are looking for faster and less capital-intensive routes to drug production. They also may need to process multiple products in the same facility, which requires more efficiency and flexibility.[10]

All together, As it looks at the moment there are not any clear signs that single-use technology will completely replace all the conventional stainless steel alternatives there are available even though the potential for having single-use systems is promising, and the demand for single-use systems is increasing. The conventional stainless steel fermenters have been around for many years so it is very unlikely that they will be completely expendable. Currently, the conventional bio-process technology still have its important industrial advantages which makes it irreplaceable with single-use technology.

Conclusion

The aim for this project was to be able to convey the operating procedures that are necessary in order to be able to handle a SUF from CerCell ApS. A lot of prospects have been shed light upon in order to get a deeper understanding of the single-use technology and the potential of using a SUF.

The cell growth was able to run as theoretically expected for the most of the fermentations having a lag phase and afterwards an exponential phase and eventually a stationary phase. This means that the SUF is able to grow up E. coli as well as a conventional fermenter.

The fermentation results varied from each other but no scientific experiment can be run perfectly. The CO_2 data indicated that the E. coli cells have a living span in this sort of fermentations for approximately 20 to 30 hours depending on the exact conditions of the fermentation. OD and dry weight results varied from the expected but were able to be analyzed. Sources of error are inexorable, which is most likely the causes for varity of the results. The stationary phases were also obtained relatively late but parameters such as agitation and media sterility might be major influences. Due to limitations in time and boundaries of the project, it was not possible to go into further detail with this, but if time was available it might have been possible to examine these problems even further. There are several steps in order to handle a SUF, but the possibly most important one is the procedures for sterility. Due to autoclavation being off limits, one must pay supplementary attention to contamination risks when preparing the SUF.

It can be concluded that a fermentation process for a SUF requires ardous preperation, but in the long run the advantages of the technology makes up for it. The expenses lie in the manufacturing of the SUF as the purpose is to have no costs of cleaning.

For further investigation it would be interesting to find out what the potential futural opportunities for a SUF from CerCell ApS are. This will give an illumination of the frame for what is possible with these types of fermenters.

The single-use technology has a lot of advantages, but aspects such as environmental impact and industrial production potential are still topics to be discussed. These are topics that are still challenges of single-use technology so it could be interesting to investigate the futural perspectives and find out whether single-use technology can have a greater impact on the bio-process industry or not.

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Appendix

A1 LB media

The following substances with concentrations are needed to make LB media (LB Miller):

- Bacto Tryptone (10g/L)
- Yeast Extract (5g/L)
- NaCl (10g/L)

For a fermentation media the following needs to be added:

- Antifoam (50 μL/L)

Procedures for a 10 Liter volume of LB media:

1. 100g Bacto Tryptone, 50g Yeast Extract and 100g NaCl are mixed together in a large plastic jug.
2. Add 9 Liters of destilled water and stir well so that all of the substance is dilluted.
3. Add 500 μL antifoam
4. Fill up the rest of the destilled water that is needed to make a total of 10 Liters.
5. The media is added to a flask.
6. In order to have a steril media, the media must be autoclavated at 121°Celcius before use.

A2 Incubation of E. coli

In order to Incubate active Escherichia colonies one must follow these procedures:

1. Pick a single colony from a agarplate containing E. coli colonies. Alternatively one can dip an inoculation needle into a liquid culture containing active E. coli cells.
2. Use streak plate methods in order to grow up single colonies on the another agar plate. Make sure the agar is LB or any other broth that is neat for E. coli. Also make sure that the inoculation takes place in a LAF bench to avoid contamination.
3. the agar plate containing the inoculated cells is put into an incubation chamber for approximately 3-4 days or as soon as growth is visible.
4. The agar plate is able to be incubated in a incubation chamber for about three weeks before the single colonies become too big for usage.

A3 Preperation of dry weight filters

The preperation of dry weight filters is done through the following:

1. The number of filters needed are chosen and are carefully handeled with forceps.
2. The filters are numerated and for a convenient manner tagged with the name of the user.
3. The filters are weighed on a fine weigh scale and the masses are noted.
4. The filters are carefully wrapped into paper towels. Avoid any direct contact with the filters after weigh measurement

A4 Preperation of precultures

The Preculture inoculum is prepared through the following steps:

1. Two or more shakeflasks are each prepared with 100 ml of LB media. Make sure the shakeflasks have been autoclaved before use. It is important to have at least two shakeflasks in case of one fails to grow up cells.
2. One single colony from an incubated agar plate is picked with an inoculation needle. This is done in a LAF bench in order to avoid contamination.
3. The colony is dilluted into a shakeflask
4. Step 2 and 3 is repeated for the other shakeflasks
5. The shakeflasks are applied with a cotton cap in order to give the cells a sufficient oxygen suply.
6. The shakeflasks are put into an incubation shake chamber at 150 RPM and 37 °Celcius degrees overnight (18-20 hours).
7. After the incubation, the density of the shakeflasks media should be quite thick and the precultures are hence ready.

A5 Fermenter preperation

The preperation of the Single-Use Fermenter before fermentation is done through the following steps:

1. Clamps are applied on every tube on the fermenter. This includes the gas in fliter, the gas out filter under and above the filter, the pH calibration tubes, and the sample tubes.
2. The fermenter is put in a LAF bench
3. The fermenter is filled with destilled water
4. The water is poured into the biowaste bucket
5. 2 liters of LB media is poured into the fermenter
6. Sensors for temperature, pH, and DO_2 are sterilized by gently pouring 70 percent ethanol 2 times onto the sensors and afterwards pour with destilled water.
7. The propes are applied to the fermenter
8. the fermenter is put back to its work place and the clamps are taken off.

A6 Inoculum volume calculation

The formula that is used for inoculum volume is:

$$c_1 \cdot v_1 = c_2 \cdot v_2$$

Where the following parameters are:

c_1: The concentration of the inoculum (in this case the concentration is measured by OD).

v_1: The inoculum volume that needs to be added

c_2: The concentration or OD that is wanted in the fermenter. Usually 0.1 is mostly used because it is easy to use in calculations.

v_2: The working volume of the fermenter. The working volume for a 3 Liter Single-Use fermenter is 2 Liters.

By knowing that it is the inocolum volume v_1 that needs to be found the formula can be expressed as:

$$c_1 \cdot v_1 = c_2 \cdot v_2 <=> v_1 = \frac{c_2 \cdot v_2}{c_1}$$

A7 General sample procedure

The standard operating procedure for taking a sample consists of the following:

1. 20 seconds before the sample needs to be taken, about 6 ml are taken with the sample syringe by applying the syringe to a sample tube. The liquid is poured into a biowaste bucket. This is to avoid interferences from a prior sample because remains of prior samples are still in the tube.
2. take a 3-6 ml into the syringe.
3. note the different parameters for your sample (pH, temperature, percent of DO2). The parameters are not necessarily crucial for calculation afterwards but it is important to notice if the parameters are out of range as it can have an effect on the cells.
4. approximately 1 ml of the sample is needed for OD measurement.
5. 3-5 ml are used for dry weight measurement if this is necessary.
6. after the sample has been used for dry weight, the syringe is put back onto the sample tube and a clamp is applied to the tube.

A8 OD measurement procedure

The standard procedures for taking an OD measurements are the following:

1. Pour approximately 1 ml of the sample liquid into a cuvette.
2. Make sure that the surface onto which the light goes through is untouched and completely covered with sample liquid on the inside.
3. OD measurements for E. coli are taken at 600nm and the OD interval must be in between 0.1 and 0.4 because the sources of error become too big with a OD higher or lower than this.
4. Use a reference cuvette with destilled water to autozero the OD measurer.
5. If the OD is not in the wanted interval it is necessary to make dilutions of the sample with destilled water.
6. Note the dilution you have made for your measurement and multiply it with the measured OD in order to calculate the real OD.

A9 Dry weight measurement procedure

The procedures for taking a dry weight sample is the following:

1. A chosen prepared filter is placed onto a flask with a filter platform connected to the vacuum pressure machine which creates a pressure in the flasks that leads to draining of the filter.
2. pour 3-5 ml of sample onto the filter. Use the same volume every time.
3. Turn on the machine.
4. Pour sterile water onto the filter. Be thorough in order to wet the whole area of the filter.
5. Repeat step 4 two times.
6. Take off the filter and let it dry it on paper towel for a bit.
7. Place it in a microwave oven at 160 W for 20 minutes
8. Put the filter in a dry box and let it dry until the next day in an anti-humidity box.
9. repeat step 8 for other filters.
10. The following day filters, now with biomass, are weighed on a fine weigh scale and the original filtermass is subtracted to calculate the dry weight.

A10 Single-Use Fermenter wash after fermentation

The wash of the Single-Use Fermenter (SUF) is only necessary if one wants to reuse a specific SUF which is in fact rarely relevant and not meant to be done in most cases. Nonetheless, after a fermentation is completed then complete the following steps:

1. Clamps are applied on every tube on the fermenter. This includes the gas in fliter, the gas out filter under and above the filter, the pH calibration tubes, and the sample tubes.
2. The fermenter is placed in a LAF bench
3. The biowaste in the fermenter is poured into a biowaste bucket
4. The fermenter is filled with destilled water
5. The water is poured into the biowaste bucket
6. Step 4 and 5 are repeated.
7. The fermenter is filled with 70 percent ethanol and the ethanol is left in the fermenter for 15 to 20 minutes.
8. The ethanol is poured into a flask and can be reused another time.
9. Step 4 and 5 are repeated.
10. use a syringe on the sample tube in order to get the remaining water out of the fermenter.
11. The fermenter is put back to its work place and the clamps are taken off.

A11 How to calculate μMax

ln(OD) is plottet over time. Then a linear regression is made for the values.

When the cells are in the exponential phase the growth in OD can be expressed mathematically as:

$$OD = a_0 \cdot e^{(\mu \cdot t)}$$

where t indicates time, a_0 is the starting OD, and μ is the maximal growth rate also called μ_{Max}.

When taking ln(OD) we get the following equation:

$$ln(OD) = ln(a_0 \cdot e^{(\mu \cdot t)})$$

which ends up with the following:

$$ln(OD) = ln(a_0) + ln(e^{(\mu \cdot t)})$$
$$ln(OD) = ln(a_0) + ln(e) \cdot \mu \cdot t$$
$$ln(OD) = ln(a_0) + \mu \cdot t$$

This means by making a linear regression for values of ln(OD) over time, the μ_{Max} will be the slope in the regression.

A12 How to calculate accumulated CO_2

Source: "CO2 calculations from gas - PDF by Milica Randelovic - November 2015"

1. Copy the CO2 data from the excel file into a real excel file.
2. Change the formatting of the cells to show time.
3. Make a new column called hours.
4. choose a corresponding time and multiply it with 24. Change the formatting of the cell to number. Use the first value as the starting point. This is done by substrating the value of the cell by its value. For the following values remember to substract the value of 24
5. insert next to the corresponding hours the CO_2 % values
6. Subtract the background CO_2 percent in a new coloumn. It is set to 0.0407.
7. Divide with 100 in order to go from percent to decimal.
8. multiply the decimal of CO_2 with 60 in order to go from per minute to per hour and then multiply with the airflow volume per minute.
9. Divide the value with v_m. The value v_m is the volume of 1 mole of a gas given a constant temperature and pressure. It can mathematically be expressed as: $v_m = \frac{R \cdot T}{P}$ where P is pressure in bar, T is temperature in Kelvin and R is the gas constant set to 0.08314472 $L \cdot bar \cdot K^{-1} \cdot mol^{-1}$
10. Make a partial intergration value in order to calculate the number of produced moles in between two values of mol per hour at a given time. This formula is used:

$$\int_{t_1}^{t_2} \frac{mol}{hour}(t)dt = (t_2 - t_1) \cdot (\frac{\frac{mol}{hour}(t_1) + \frac{mol}{hour}(t_2)}{2})$$

    The value of $\frac{mol}{hour}(t_0)$ is set to 0. Calculate for the remaining values.
11. Finally calculate the accumulated CO_2 in moles. The produced CO_2 moles at t_0 is set to zero. All the following values are calculated by adding the last accumulated value to the next individual value.
12. Calculate to accumulated CO_2 in grams by multiplying with 44.0095 $\frac{g}{mol}$

A13 Fermentative pathways of E. *coli*

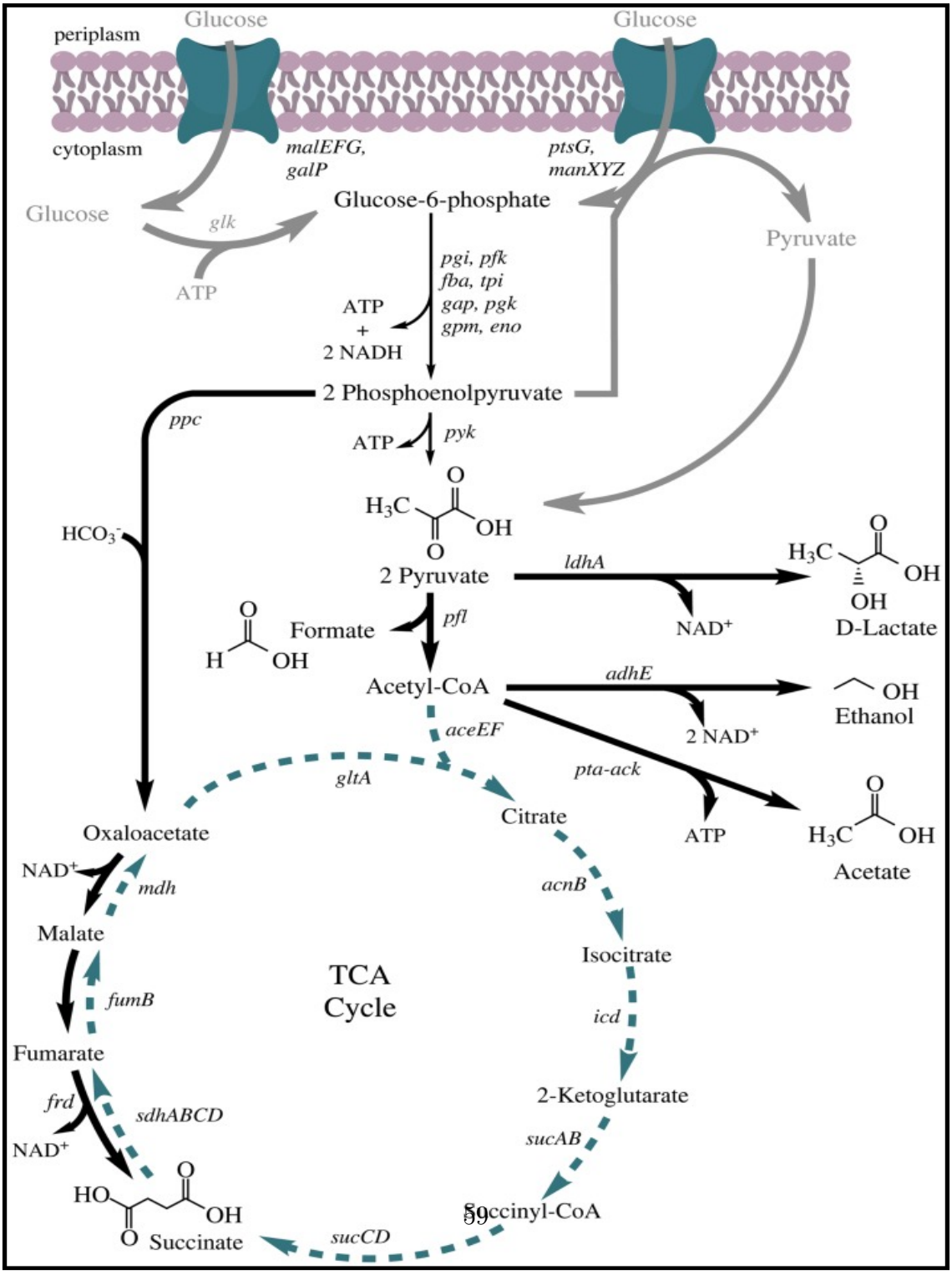


Figure 15: *The fermentative pathways of Escherichia coli.[2]*

A14 Application Note: Standard Operating Procedures for a Single-Use Fermenter

The Application Note begins on the following page. This page is left intentionally plank.

STANDARD OPERATING PROCEDURES FOR A SINGLE-USE FERMENTER

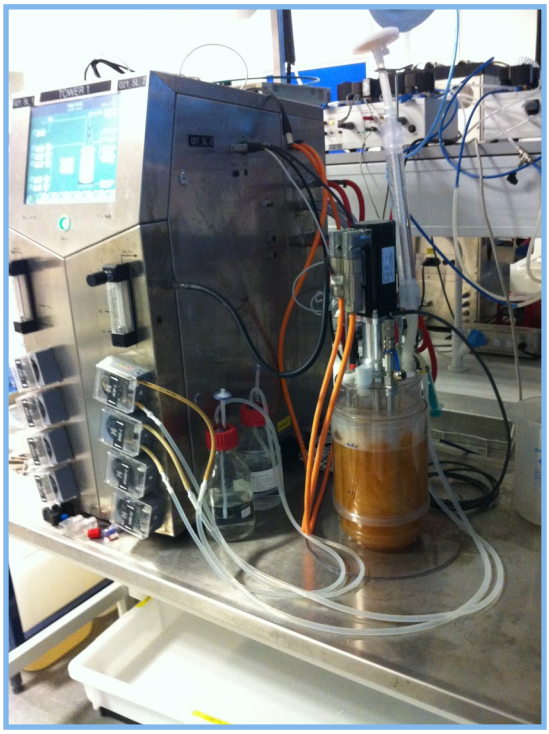
By Kristian Krakau

June 2016

Introduction

Operating Procedures are fundamental in every fermentation experiment. Single-Use fermenters are getting of higher demand and knowing the operating procedure differences between a Single-Use fermenter and a conventional fermenter is necessary in order to experience a succesful fermentation and hence be able to use a Single-Use fermenter from CerCell ApS.

This Application Note describes a step-by-step procedure for growing E. *coli* in a 3 Liter Single-Use benchtop fermenter from CerCell ApS. The Single-Use fermenter are of the BactoVessel™ series meant for microbial applications. They are unique and fully configurable fermenters in a scalable platform. The BactoVessel family is in size of 2.1 Liters and up to 30 Liters.



3 Liter Single-Use fermenter (SUF) running a fermentation with E. coli.

Materials and Methods

The fermenter

A 3 liter BactoVessel™ Single-Use fermenter (SUF) was prepared in a LAF bench. Clamps were applied to every tube on the fermenter and 2 liter LB media was added to the fermenter. Temperature-, pH-, and DO sensors were sterilized and applied to the fermenter. The Fermenter was then put on top of the working bench next to its control station. Wires and tubes were fixed, acid– and base flasks were connected and a Kollmorgen motor was applied. The exhaust gas was connected to the gas analyzer. Clamps were removedand the control conditions were set.

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Materials and Methods (continued)

Control Conditions

The conditions were set at the touch screen on the control station before the inoculation

- Temperature (°Celcius)……...37
- Agitation (RPM)……..……..…..750-1000
- pH……..…7
- Airflow (vvm)…..……0.5-1

After the control conditons were the fermenter was running. The day before the fermentation was started a preculture with 100 ml LB media and a single colony of E. *coli* was mixed in a shakeflask at 150 RPM overnight (18-20 hours).

LB Media Composition (LB Miller)

- 10 g/L NaCl
- 10 g/L Tryptone
- 5 g/L Yeast Extract
- 5 µL/L antifoam

The media was autoclaved at 121 °Celcius before use.

Inoculum size

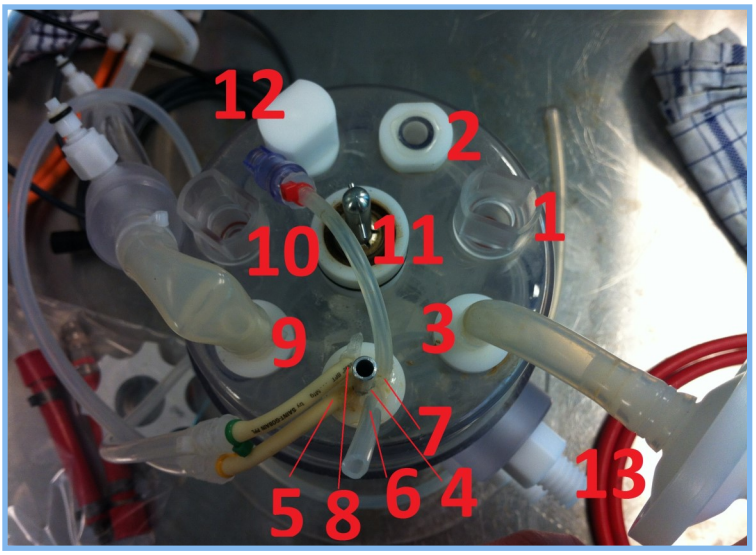
The inoculum size for the fermenter inoculation was calculated with this formula:

$$V_2 = \frac{V_1 \cdot C_1}{C_2}$$

Where V_2 is the inocululum size in ml, C_1 is the wanted starting OD of the fermenter (0.1-3) C_2 is the observed OD of the inoculum and V_1 is the working volume of the fermenter (2000 ml)

Sampling

After the inoculum size was calculated and added to the fermenter the fermentation started. Samples were taken every 45 minutes the first 3 hours and then every 30 minutes for the duration of the fermentation. OD measurements were taken for every sample while dry weight measurements were taken every hour after the first 3 hours.



The different compartments of the SUF. 1) sensor port 2) thermowell 3) ingoing airflow tube 4) gas exhaust holder port 5) acid/base tube 6) harvest tube 7) harvest tube 8) acid/base tube 9) gas exhaust tube 10) sensor port 11) motor holder 12) media port 13) water jacket inlets.

Ending fermentation

After the last sample has been taken the fermenter is run ideally for a total of 36-48 hours in order to let the cells use up all the substrate and to get useful graphs for CO_2.

all the tubes and wires are detached expect for the acid– and base flasks which are replaced with water flasks and run in order the cleanse the tubes of acid and base. The control station is shut off, The biowaste in the fermenter is poured into a biowaste container.

Setup and handeling of SUF

Here is a list of setup procedures necessary to pay attention to:

- The fermenter has to be fastened in a steady position so nothing is shaking during fermentation.
- Place the fermenter so the media port and harvesting tube are easy accessable.
- Put aside the acid and base flasks so that they are not interveining your working area.
- Place a cloth on the working bench but under the water cooling tubes against leak.

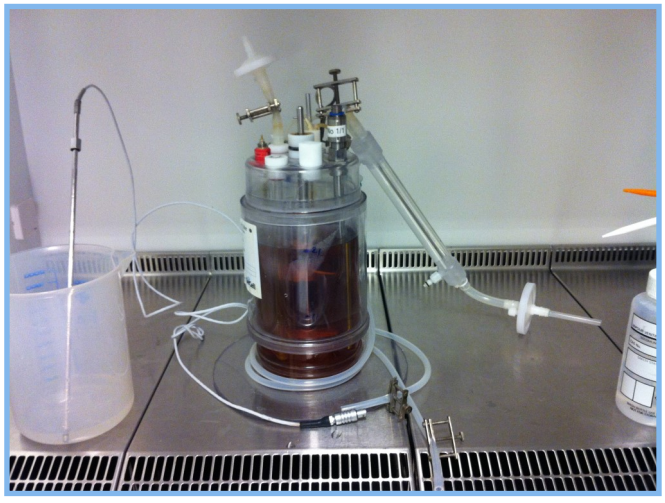
Materials and Methods (continued)

- Fit every sensor tightly with your own hands and not any tool. The material cannot hold against the forces of powerful tools.
- Never autoclave the SUF before use. It is made of rigid plastics and cannot withstand heat over 50° Celcius
- Make sure tubes are laid out properly in order to avoid tangeling
- Write everything down during fermentation for controlling conditions and afterwards for examining things that went wrong and things to pay attention to.

Sterility

In order to keep the fermentation steril it is necessary to:

- Wear a Labcoat in and only in the working area of the SUF. This is to avoid being exposed to cells and also not carrying cells out of the area.
- Always work in a LAF bench when preparing the SUF
- Wear gloves whenever in contact with SUF culture
- Use 70% ethanol for elmination of cells and for cleaning when exposed to cell culture, but never use it on compartments involved directly with the current fermentation.
- Only autoclave the media you need. Contamination risks of media are very high



Preparation of SUF in LAF bench.

Results

Five E. *coli* fermentations were carried out each between 7 and 10 hours. The controlling conditions were the same for all five of them.

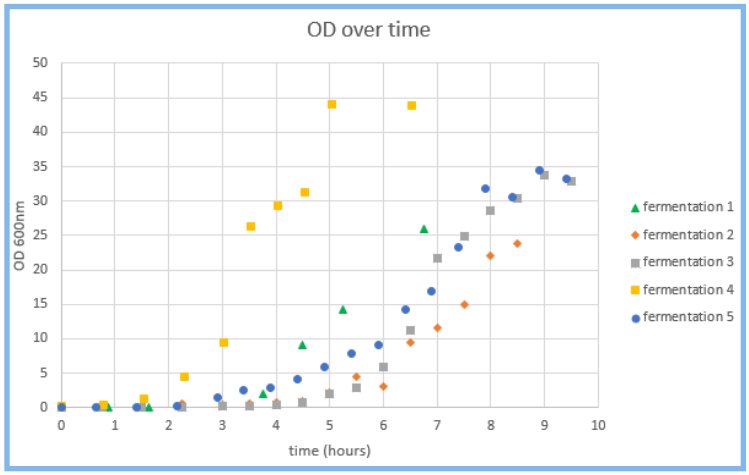
The first results display the OD measurements over time. It can be seen that fermentation 4 is a bit out of range from the others. This is due to the fermentation media being precontaminated before autoclavation. The media was filled with dead biomass material.

However the max OD for the other fermentations varied but were around 24 for number 1 and 2 and around 34 for fermentation 3 and 5.

It can be seen that with the controlling conditions chosen the exponential phase starts approximately after 3 hours for everyone except number 4 which starts after 1½ hours.

The stationary phase can be seen reached for fermentation 3 and 5 at around 9½ hours.

When making a plot like this it is possible to calculate the maximum growth rate of the fermentation called µMax. When taking a linear regression of the logarithm of the exponential phase the slobe will be the value of the µMax.



Results of OD measurements over time for 5 E. coli fermentations

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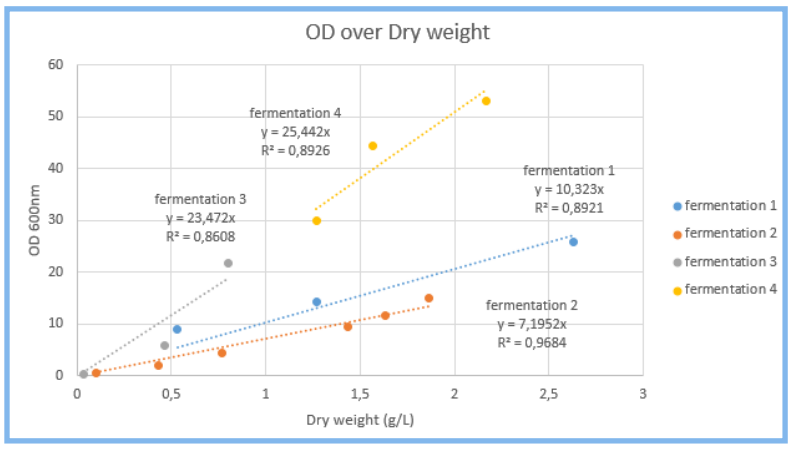
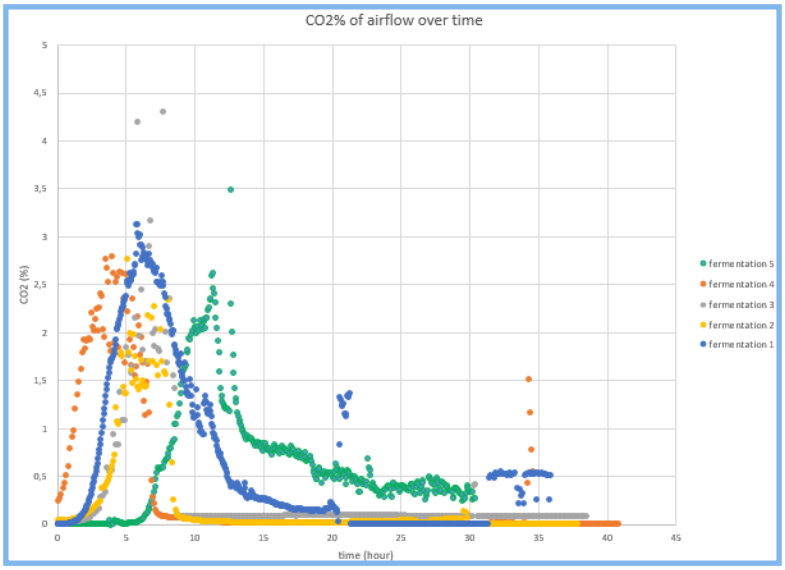
Results (continued)



Results of the linearity between OD and dry weight for 5 E. coli fermentations.



Results of the CO_2 % profiles of the exhaust airflow over time for 5 E. coli fermentations

Results next to be displayed is the correlation between dry weight measurements and the OD measurements for fermentation 1-4. According to the theory of Lambert-Beer's law the concentration (dry weight) and the absorbance (OD) are in a linear relationship. This is shown to a certain extent with the results but dry weight measurements are quite difficult to handle without multiple sources of error so usually many samples will be invalid. Compared to the fact that the fermentations were run with the same conditions it is odd to see that the correlations are varying so much from each other.

Last to be displayed is the results for the % CO_2 profiles for the 5 fermentations. It can be seen how most of the fermentations peak at around 5 to 10 hours only with fermentation 4 being a little earlier (as expected) and fermentation 5 to be a little later. These results can be intepreted as when the cells are most active and when they begin to die. When no more CO_2 is produced then no more cells are expected to be alive.

For further information read the B.Sc. Project Thesis "Standard Operating Procedures for a Single-Use Fermenter" by Kristian Krakau or visit cercell.com

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