

Optimising IPTG and Lactose Induction of Recombinant Expression with Flow-based Online Analysis

Master Thesis

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1 Abstract

The induction of recombinant expression in bioprocesses is associated with high costs, but still suffers from unoptimised standard procedures with high use of IPTG. A flow-based online method for enzymatic analysis of recombinant expression in *E. coli* could greatly help in mapping induction behaviour, and assembly and programming of a FlowSystem for automatic sampling was done. The process was never able to handle cell samples as they caused counter pressure in the system, and the program and the hardware would have to be further optimised to control dispersion and mixing, but if done properly, this method could be an important tool for deeper induction studies. The enzymatic assay was also used offline and showed that concentrations of IPTG lower than 1 mM could be feasible, at least in some situations, but concentrations as low as 0.1 mM would be too low. Lactose as an inducer was only tested in a smaller study and no clear evidence of whether it could work – either on its own, or in concert with IPTG – was found. In conclusion the study indicates that lower IPTG concentrations could be viable for bioprocesses, but further research is needed to accurately optimise the concentration.

Abbreviations

BSA – Bovine Serum Albumin

CAP – Catabolic Activator Protein

FIA – Flow Injection Analysis

IMAC – Immobilized Metal Affinity Column

IPTG – Isopropyl β -D-1-thiogalactopyranoside

OD₆₀₅ – Optical Density measured at 605 nm

pNPG – p-nitrophenyl- β -D-glucopyranoside

SDS-PAGE – Sodium Dodecyl-sulphate – Polyacrylamide Gel Electrophoresis

TMG – Thiomethyl galactosidase

TnBgl3B – 3 β -glucosidase B from *Thermotoga neapolitana*

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2 Introduction

Throughout history, humans have fed and sheltered living beings to harvest the various substances that the biology of nature spent millions of years developing and refining through evolution. We are highly dependent on these resources as a supply of foods, medicines, building materials and aesthetics, and today our control of biological production is greater than ever thanks in part to the introduction of recombinant protein production. In our modern day society we employ cell cultures as living factories to produce pharmaceuticals to aid against major diseases, biological assays used in medicine, environmental protection or forensics, and a multitude of substances that can aid in other fields such as agriculture or research (Cusabio, 2017).

When producing biological molecules in recombinant microorganisms it is important to be able to control the production. Several factors come into play, but one of the major aspects is *when* the production takes place. In order to maximise the output of a process, a sufficient density of the cell culture must be reached before starting – *inducing* – the expression of the desired molecule, while still not letting the culture grow too dense leading to lowered metabolism and unwanted by-products (Aucoin, et al., 2006). Induction can be done in several ways, but a common one is the activation of the lac operon using IPTG (Isopropyl β -D-1-thiogalactopyranoside) which allows expression to be started at the right moment (Boezi & Cowie, 1961).

Presently when doing expression of proteins in prokaryotic cells it is common to use an IPTG concentration of 1 mM (Chhetri, et al., 2015) (Glifberg & Svensson, 2016). There are several cases where lower concentrations have been argued for (Hausjell, et al., 2018) (Taylor, et al., 2017), but this conduct does not seem to have seeped through to the industry as a whole. It is also not unheard of to try and optimise the conditions for inducing *E. coli* with IPTG, but most of the studies doing so have only done optimisation of the inducer concentration as part of a bigger study, and the main focus has mostly been elsewhere. In Table 1, a selection of studies where optimisation of IPTG concentrations was performed are presented along with the concluded optimal concentrations. The experimental setups were of course very different, and factors such as what protein was expressed, the type of growth medium or incubation parameters played a big role in the results. However, the main idea still shines through, using 1 mM IPTG when inducing is in many circumstances superfluous.

Table 1. Optimised concentrations for induction with IPTG in various studies.

Concentration IPTG	
0.1 mM	(Dagar, et al., 2017)
< 0.2 mM	(Golotin, et al., 2016)
0.25 mM*	(Chang, et al., 2010)
0.4 mM	(Yu & Liu, 2012)
0.4 mM**	(Guan, et al., 2017)

* *The induction was done overnight.*

** *The induction was done in Bacillus subtilis.*

Even though IPTG is an efficient inducer it suffers from some drawbacks. For example the metabolism of the organism is affected, unwanted production of inclusion bodies has been reported (Hausjell, et al., 2018), it is expensive and it is bad for human health (Gombert & Kilikian, 1997). Alternatives have been investigated like lactose

(Gombert & Kilikian, 1997) (Aucoin, et al., 2006), TMG (Thiomethyl galactosidase) (Marbach & Bettenbrock, 2012) (Narang & Oehler, 2017), heat (Aucoin, et al., 2006) or light (Chang, et al., 2017), but none of them are as widely used as IPTG. This is probably because they are not as well documented, and stable methods have yet to be developed.

One of the alternative inducers that stands out is lactose, as it is currently used in a method called autoinduction where lactose is included in the growth medium and induction starts once all the glucose available has been consumed (Fox & Blommel, 2009). There have been attempts to combine lactose and IPTG in *E. coli* BL21(DE3). In a study IPTG (1 mM) was added as a preactivating inducer and then the cells were moved to a fresh medium containing lactose. This method gave a much higher yield than simple IPTG induction. (Kim, et al., 2007) However, if one considers making this method into an industrial process moving the cells to a new medium when inducing might prove too complicated in a normal batch process.

For bioanalysts, precision and repeatability are virtues. Potent enzymes and antibodies require only the tiniest concentrations to give clear responses, and therefore can the smallest of errors easily be turned into monstrous discrepancies if extreme care is not taken. This is where the increased use of Flow Injection Analysis (FIA) is such a promising sight today. FIA is the use of pumps, valves and tubings to automatically control sampling and processing of samples and it gives the ability to handle small amounts of samples with extremely high precision – ideal for bioanalysis. (Ruzicka & Hansen, 1975)

When considering the current use of IPTG induction and the lack of precise monitoring of the process, it would certainly be very interesting and valuable for the industry, academia and anyone who uses heterologous protein expression, to investigate this area. This project will therefore try and bring clarity by putting the following hypothesis to the test:

The 1 mM concentration of IPTG used as industrial standard for induction of protein expression is not optimised, and the efficiency of fermentative production could be increased ubiquitously by doing so.

And

Induction can be made more resource-efficient by combining lactose and IPTG as inducers.

And

Flow-based online analysis can be a useful tool to investigate the induction with real-time monitoring.

3 Background

3.1 The lac operon

The lac operon is a natural unit of genetic material present in the genome of bacteria that regulates the expression of the genes included in the operon. The lac operon works in concert with a protein called a lac repressor that attenuates the transcription of the genes in the operon, as long as a ligand does not bind to and rearrange the structure, see Figure 1. When the gene is not expressed, the protein encoded cannot be produced by the cell. The natural ligand is allolactose, a product of β -galactosidase which is coded for in the lac operon, but several other molecules have the capability of binding the lac repressor and inducing transcription. (Berg, et al., 2012) Molecules that induce expression in the lac operon include IPTG, TMG and lactose – all of them sugars or modified sugars – but their efficiency vary greatly (Marbach & Bettenbrock, 2012).

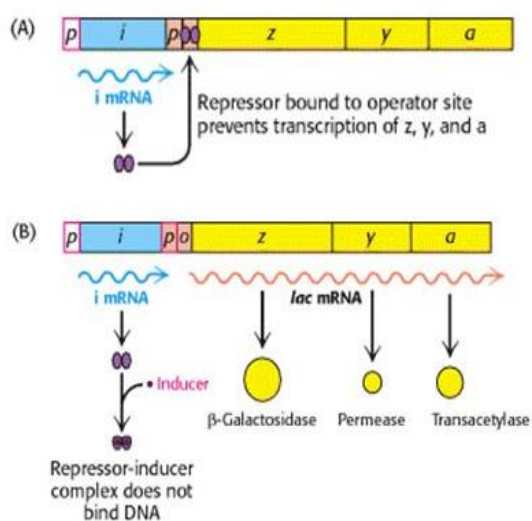


Figure 1. The mechanism of the lac operon. **A:** Normally the repressor binds to the operator and blocks transcription. **B:** When an inducer is added, it binds to the repressor which can no longer bind to the operator and transcription is activated. (Berg, et al., 2012)

In addition to being activated by various substances, expression of the lac operon can also be induced by physical factors such as temperature or attenuated by built-in mechanisms. An important attenuating factor to be considered is glucose which reduces the levels of cAMP which in turn reduces the stimuli of the Catabolite Activator Protein (CAP). The role of CAP is to activate expression in the lac operon and when it is attenuated by glucose, production of the proteins coded for in the operon is slowed down. This is important because when lactose is present, expression is still not activated as long as glucose remains. (Berg, et al., 2012)

3.2 Induction of recombinant expression

The process of inducing expression is a straight-forward one where an organism in its exponential growth phase is stimulated to start transcription of a target gene. For example, when using IPTG as an inducer, at the time of induction IPTG is added to a sufficient concentration, the lac repressor is deactivated and transcription commences. Protein production starts almost immediately, and the precision is high when it comes to timing because the transport of IPTG over the cell membrane of the cells is both spontaneous through diffusion and aided by proteins (Fernández-Castané, et al., 2012). At high concentrations the cells are saturated quickly, and induction is fast. A problem can occur if concentrations are lower than the commonly used standard, as some cells will take up the inducer whereas others will not. This bimodal behaviour is thought to

depend on the presence of lactose permease – a protein aiding in the transport of lactose and some other molecules like IPTG – and the presence of this protein *before* induction (since it is coded for in the operon) is seemingly random. Once concentrations are high enough for spontaneous diffusion to occur all cells will be induced irrespectively of whether they had any lactose permease present beforehand (Marbach & Bettenbrock, 2012). Bistability range is so low that it shouldn't be an issue at operating concentrations as it is mainly observed around 8-40 μM IPTG (Fernández-Castané, et al., 2012). However, this is an explanation to why too low concentrations of inducer wouldn't be effective.

3.3 *Escherichia coli* BL21 (DE3)

One of the most frequently used bacteria in heterogenous protein expression is *Escherichia coli* – a well-documented and easily handled organism – and in particular the BL21 (DE3) strain. *E. coli* is naturally present in animal digestive tracts and the BL21 (DE3) strain has a history tracing back to 1966 when it was first selected by humans. It is often combined with the T7 plasmid expression system which allows foreign genes to be transcribed and the encoded proteins to be produced in a bacterial host cell. The BL21 (DE3) strain is especially useful for expression as it has a T7 RNA polymerase protein that is useful when transcribing from T7-plasmids, and it has had some common proteases deleted to protect the product. (Gileadi, et al., 2017)

Although *E. coli* is easy to handle and grow, it is not optimal for some uses. Especially when expressing eukaryotic proteins, *E. coli* will struggle as different codons are used and many post-translational modifications are lacking. To tackle this problem, it is common to use other cells such as *Pichia pastoris*, which is a yeast that produces stable recombinant proteins in large concentrations. Other vectors used are insect or mammalian cells that offer high quality expression, or even cell free expression systems that give very precise control of the process, but unfortunately these systems are much more expensive. (Broadway, 2012)

3.4 β -glucosidase-B (TnBgl3B)

There is a protein present in many different bacteria that is called β -glucosidase-B, an enzyme with the ability to hydrolyse bonds in carbohydrates. It either produces smaller sugars from oligosaccharides or transfers glucose-groups to other molecules and is part of the metabolism. It also serves in digesting certain toxic compounds in the environment. It is a useful enzyme that is used in various areas such as production of biofuels, food-processing and various biotechnological applications. (Singh, et al., 2016)

Turner et al. have described a variant of β -glucosidase-B called TnBgl3B that is naturally occurring in a thermophilic bacterium called *Thermotoga neapolitana*. It has a molecular weight of 81.1 kDa, and is non-aggregating and thermostable – with an optimum for hydrolysis at 90 °C. One of the useful features of TnBgl3B is its ability to catalyse the hydrolysis of p-nitrophenyl- β -D-glucopyranoside (pNPG) into p-nitrophenol. p-nitrophenol has an absorbance maximum at 405 nm and can therefore be spectrophotometrically detected and quantified. (Turner, et al., 2007)

3.5 Cultivation principles

Heterologous expression in bacteria is most efficiently performed in some type of bioreactor with precise control of preparatory steps and growth parameters. When preparing for fermentation of a cell culture it is of utmost importance to work under *sterile conditions*. This includes sterilisation of all components that are added to the reactor, either through autoclavation or filtration in order to remove contaminants such as foreign cells, spores and biological particles. Special areas such as laminar flow benches are employed while preparing or mixing components in order to maintain the achieved sterility after autoclavation or filtration. When the cultivation has been started, sterile barriers must be maintained to prevent contamination during the process, with particularly crucial steps being addition or removal of components from the reactor. Air supply has to be delivered through a filter and samples are taken as quickly as possible to prevent any contamination from occurring while the system is exposed to the surrounding atmosphere. Factors that have to be monitored and/or manipulated during the cultivation include pH, dissolved oxygen, temperature, stirring, cell density and glucose levels. (Rouf, et al., 2017)

3.6 Lysis using BugBuster or sonication

Proteins expressed in microorganisms are sometimes transported to the outside of the cell to perform a certain task, but the vast majority of proteins remain within the cytoplasm of the cell. In order to retrieve the trapped recombinant proteins that are produced the cell must somehow be opened, a process called lysis. Lysis can be performed using several methods divided into two categories: chemical and mechanical lysis. (Brown & Audet, 2008)

An example of chemical lysis is the use of BugBuster, a readymade mixture containing detergents that disrupts the cell wall, lysozyme that hydrolyses bonds in the cell wall and nucleases to remove nucleic acids from the sample or lower viscosity. Some variants only contain some of the ingredients. (Novagen, 2008) When constructing a flow-based method it is important that all steps can be easily incorporated, and chemical lysis is perfect for automatic treatment of a sample.

Mechanical lysis can for example be done by sonication. Sonication is the use of high frequency soundwaves that disrupt the cell walls of the bacteria, releasing its content into the surrounding buffer. Sonication is often done in pulses while cooling the sample to avoid overheating. A big advantage is that it is simple and easy to use and therefore it is one of the most common methods when disrupting cells. (Feliu, et al., 1998)

3.7 IMAC column purification

When expressing and analysing recombinant proteins it is common to genetically alter the protein to include a tail of six histidine residues that can easily be distinguished in separation methods. By letting the protein solution pass through a column with metal ions immobilised in a matrix, 6Histidine tagged proteins will adhere, like in Figure 2, meaning that unwanted content can be washed from the sample. The target protein is finally released from the column with the use of an elution buffer with a high concentration of imidazole that causes the matrix to release the protein. Before the sample is added, a binding buffer containing a low concentration of imidazole should be used to equilibrate the column and remove unwanted contaminants. (IBA Lifesciences, 2012) (Porath, 1975)

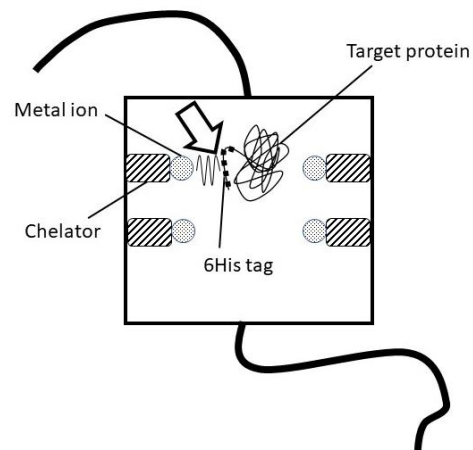


Figure 2. The binding of the 6xHistidine tail to an immobilised metal ion in the IMAC column.

3.8 SDS-PAGE

Proteins can be separated by size in a method called SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). The principle of this method is that voltage is applied over a gel where the proteins are added, and the proteins will move a distance depending on their molecular weight – smaller proteins move further than larger ones. (Laemmli, 1970)

After staining the proteins with a dye, they appear as bands and the position and size of the samples can be identified and compared to a known standard – a ladder – included in the experiment. By scanning the gel and letting a software identify the bands and determine their relative intensity, the relative amount of different proteins in a sample can be found. (Carter, et al., 2013)

3.9 Bradford analysis

When determining the concentrations of a protein sample there is a useful method that uses a compound called the Bradford reagent. It contains Coomassie Brilliant Blue G-250, a dye that when bound to a protein – especially to aromatic sidechains and arginine – takes on a blue colour that has an absorbance maximum at 595 nm. The intensity of the colour is proportional to the amount of protein in the sample and thus a calibration curve can be made using a known protein standard, such as bovine serum albumin (BSA), tracking the absorbance with a spectrophotometer. (Bradford, 1976) (Bio-Rad, n.d.)

3.10 Enzymatic assay

Enzymes are complex catalysts with high specificity and large efficiency. Because of this, it is very easy to detect small amounts of molecules in a sample using enzymatic techniques. A central concept in enzyme kinetics is the turnover number (V_{max}), which describes how many substrate molecules an active site on an enzyme converts to a product in one time unit when the enzyme is fully saturated. Equation 1 describes how the turnover number is dependent of the number of active sites ($[E]_T$) and the rate constant (k_2), which is specific to a given reaction. (Berg, et al., 2012)

$$V_{max} = k_2[E]_T \quad (1)$$

$[E]_T$ is directly proportional to the concentration of enzyme molecules in the solution, and thus V_{max} is directly proportional to the enzyme concentration. Therefore, if one can find out how fast an enzymatic reaction develops, the *reaction rate*, at the point of saturation, the concentration of said enzyme can also be determined.

When treating a thermostable enzyme such as TnBgl3B, a problem is that a higher than ambient temperature has to be used. Heating and reading of product formation might not be the easiest to perform at once, and thus a different approach would have to be used. By utilising a vast excess of substrate, saturation of the enzyme can be achieved. The reaction must then be initiated by heating the sample to the reaction temperature, lower than the optimal 90 °C in order to make sure that no enzyme is denatured by the heat. (Turner, et al., 2007) When the sample is removed from heating the substrate concentration is immediately recorded and this is continuously done over time while the sample is cooled by the room temperature. Eventually the rate will decrease over time, but in a predictable manner. This means that if all the samples are recorded at once, an average reaction rate can be determined for each sample that is based on all the points measured for that sample.

The reaction rate is also the slope of a curve describing the amount of product formed over time. The slope can in turn be seen as a simple estimate of the average of the derivative in all the points on the reaction curve, which is equal to the average of the reaction rate in all the points on the reaction curve. However, care should be taken when deciding on which points should be used for determining the slope. Immediately after removing the sample from heating it will show errors, and too far from the starting time will the cooling of the sample start to affect the results. In Figure 3 tangents to a curve have been constructed using the derivative. A linear fit of the three points yields exactly the same slope as the average of the derivative in the same three points. Of course will this not be the case if the curve is not as regular as the one presented here, but using the slope of the linear regression as an estimate of the reaction rate will still be feasible.

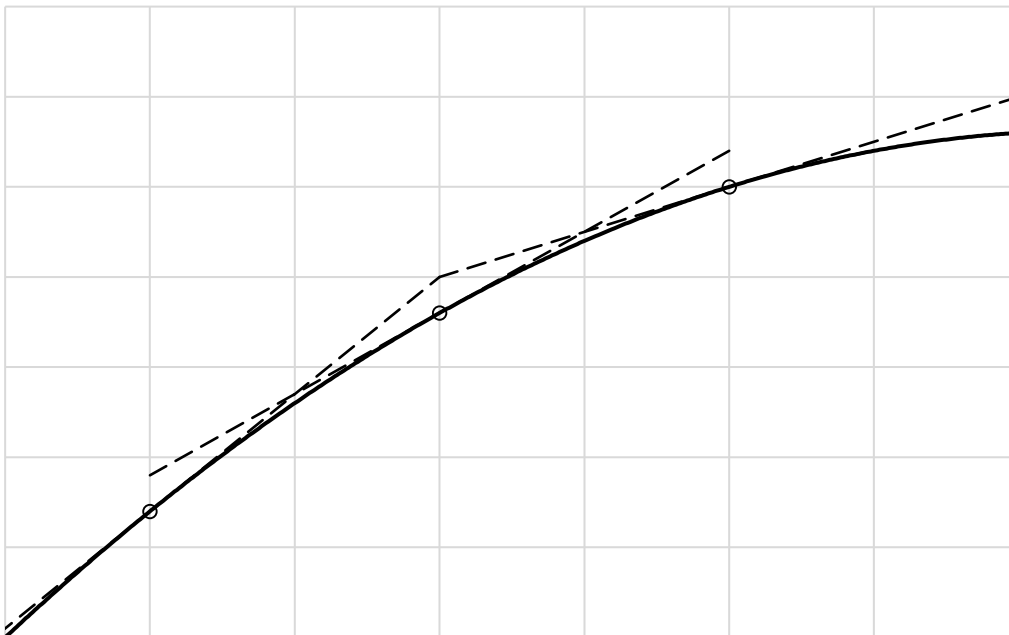


Figure 3. Tangents calculated using the derivative of the curve in different points. A linear fit of the three points yields the same slope as the average of the derivative in the same three points.

3.11 Flow-based systems

Monitoring of a process such as this includes much sampling and analysing that takes a lot of time and work, but also leaves room for a large human factor. To cope with these problems, automated sampling systems are often used to ensure reliable and streamlined processes, a technique called Flow Injection Analysis (FIA). An important aspect of FIA systems is the capability of processing real-time data and doing low-intrusive online analysis. (Ruzicka & Hansen, 1975)

3.11.1 Mixing and dispersion in flow-based systems

When talking about liquid behaviours in tubes, Reynold's number (Re) is a very useful tool. Reynold's number is calculated using equation 2 where U = liquid velocity, D = tube diameter and ν = kinetic viscosity, and if Reynold's number is greater than around 2300 this means that the flow is *turbulent* as opposed to *laminar* at lower Reynold's numbers. Turbulent flow is characterised by a lot of axial movements and mixing, where laminar flow typically has a very consistent flow profile with little mixing. (Avila, et al., 2011)

$$Re = \frac{U * D}{\nu} \quad (2)$$

In flow-based systems, liquids tend to behave in certain ways depending on the nature of the hardware and the methods employed. When placing a sample within a piece of tubing one phenomenon that will affect it is *dispersion* – linear spreading and dilution of the sample into the surrounding carrier liquid – or as defined in a textbook by Karlberg & Pacey: “the amount that the chemical signal is reduced by injecting a

sample plug into an FIA system” (Karlberg & Pacey, 1989). Dispersion is a product of the friction between the liquid and the walls, heterogenous movements in the liquid body and particle diffusion. Oftentimes this phenomenon is unwanted like for example when a sample has to be moved through a system with an analytical instrument at the end and a concentrated sample zone with sharp interfaces is desired. However, if the purpose of the analysis is to let a reaction take place between different liquids in the system a certain amount of dispersion is useful to ensure proper mixing, and if a titration gradient is desired, high levels of dispersion can be crucial. (Ruzicka & Hansen, 1978)

Because of its importance in various applications, control of dispersion is essential to flow-based systems. There is a number of factors that will increase dispersion such as higher flow rates, turbulent flow profiles, longer tubings and lower sample volumes. (Ruzicka & Hansen, 1978) This means that optimal dimensions and length of tubings has to be chosen to fit the considered method, but sometimes this is not enough. Controlling mixing can be just as important and here the use of confluence points (Ruzicka & Hansen, 1978) – which allows for great mixing of two separate streams – stopped-flow injection analysis (Ruzicka & Hansen, 1978) – which can give higher residence times without altering the physical dimensions of the system – and reactor design (Karlberg & Pacey, 1989) – where endless variations are possible – is central.

When optimising the mixing of a reactor, one important parameter is the shape. A straight reactor has poor mixing, whereas a coiled reactor is somewhat better and a knitted reactor, being the best, has both the least dispersion and the most mixing. In addition, the inclusion of glass beads in the reactor can give an increase in turbulence, leading to improved mixing. (Karlberg & Pacey, 1989)

3.12 Software programming

The FlowSystem developed by CapSense Biosystems AB (Lund, Sweden) has an interface that can be programmed to perform several tasks such as moving pumps and valves, and starting or turning off analytical equipment. By carefully considering when and how the pumps and valves are moved, complex online analytical procedures can be performed automatically with high precision and minimal manual work required. (Kumar, et al., 2011)

4 Materials and Methods

4.1 Equipment

For the automatic sampling, CapSense FlowSystem (CapSense Biosystems AB, Lund, Sweden) was used. It is a versatile system using Cavro Centris Pumps and Cavro Smart Valves with 12 connections from Tecan (Männedorf, Switzerland) to transport and analyse various liquids. In this setup it was combined with an integrated spectrophotometer (Runge GmbH, Potsdam, Germany), a self-packed chromatography column (referred to as the His column or the IMAC column), a Jetstream 2 oven (LabVision, Värmdö, Sweden) and an external spectrophotometer – WPA Biowave II (Biochrom Ltd, Holliston, USA). The whole system was connected with 0.8 mm tubes. A

software was used to operate the FlowSystem (CapSenze Biosystems AB, Lund, Sweden).

The reactor cultivations were performed in a 2 L reactor (New Brunswick Scientific Co., Edison, USA) with a pH electrode controlled by a feed-back system (Inventron, Istanbul, Turkey) and an MR Hei-Standard combined heater and magnetic stirrer (Heidolph, Schwabach, Germany).

In addition to the main cultivation and analysis setup, a number of free-standing analysis instruments were used. An ELx808™ Absorbance Microplate Reader (BioTek, Winooski, USA), a WPA Biowave II (Biochrom Ltd, Holliston, USA), an Accu-Chek Aviva blood glucose meter (Accu-Chek, Solna, Sweden), a 744 pH Meter (Metrohm, Herisau, Switzerland) and an SDS-PAGE system consisting of a PowerPac 300 Electrophoresis Power Supply with a Mini-PROTEAN Tetra Cell (Bio-Rad, Hercules, USA). The SDS-PAGE gels were analysed using a MiniBIS Pro Gel Documentation System (DNR Bio-Imaging Systems, Neve Yamin, Israel) with Image Lab Software (Bio-Rad, Hercules, USA).

4.2 Chemicals

BugBuster® Protein Extraction Reagent (EMD Millipore Corp., Burlington, USA)

Ethanol 99.5 % (Solveco, Rosersberg, Sweden)

4-Nitrophenyl β-D-glucopyranoside (Sigma-Aldrich, St. Louis, USA)

Citric acid (Merck, Darmstadt Germany)

Dibasic sodium phosphate (Sigma-Aldrich, St. Louis, USA)

PBS tablets pH 7.2 (Medicago AB, Uppsala, Sweden)

Sodium Hydroxide (Duchefa Biochemie, Haarlem, Netherlands)

Chloric acid (Unknown)

Ammonium hydroxide solution 28.0-30.0 % (Sigma-Aldrich, St. Louis, USA)

Glycerol >= 99.5 % (Sigma-Aldrich, St. Louis, USA)

LB-Agar – Powder according to Lennox (PanReac AppliChem, Darmstadt, Germany)

Albumin Bovine Serum (BSA) (Sigma-Aldrich, St. Louis, USA)

Glass beads 0.1 mm (Supelco, Bellefonte, USA)

Imidazole (ACS grade) (Sharlau, Barcelona, Spain)

D(+)-Glucose 1-hydrate (PanReac AppliChem, Darmstadt, Germany)

Magnesium Sulphate Anhydrous (Sigma-Aldrich, St. Louis, USA)

Ampicillin Sodium (Duchefa Biochemie, Haarlem, Netherlands)

Ammonium sulfate (Merck, Darmstadt Germany)

di-Potassium hydrogen phosphate (Merck, Darmstadt, Germany)

Sodium phosphate monobasic dihydrate (Sigma-Aldrich, St. Louis, USA)

di-Ammoniumhydrogencitrat (Merck, Darmstadt, Germany)

Calcium chloride dihydrate CaCl₂·H₂O (Merck, Darmstadt, Germany)

Iron(III) chloride hexahydrate FeCl₃·6 H₂O (Sigma-Aldrich, St. Louis, USA)

Zinc sulfate heptahydrate ZnSO₄·7 H₂O (Sigma-Aldrich, St. Louis, USA)

Copper(II) sulfate pentahydrate CuSO₄·5 H₂O (Sigma-Aldrich, St. Louis, USA)

Manganese(II) sulfate monohydrate, 99+%, extra pure MnSO₄·H₂O (Fisher Scientific, Pittsburgh, USA)

Cobalt(II) chloride hexahydrate CoCl₂·6 H₂O (Sigma-Aldrich, St. Louis, USA)

Na₂EDTA (Sigma-Aldrich, St. Louis, USA)
IPTG (Applichem, Darmstadt, Germany)
 α -Lactose (Sigma-Aldrich, St. Louis, USA)
Bradford reagent (Bio-Rad Laboratories, Inc., Hercules, USA)
4-Nitrophenyl β -D-glucopyranoside (pNPG) (Sigma-Aldrich, St. Louis, USA)
Ni-coated agarose beads – Workbeads 40 IDA (Bio-works AB, Stockholm, Sweden)
CuSO₄·5 H₂O (ACS grade) (Sigma-Aldrich, St. Louis, USA)
10x TGS Buffer (Bio-Rad Laboratories, Inc., Hercules, USA)
4x Laemmli Sample Buffer (Bio-Rad Laboratories, Inc., Hercules, USA)
2-mercaptoethanol (Merck, Darmstadt, Germany)
Brilliant Blue R (Coomassie Brilliant Blue R-250) (Sigma-Aldrich, St. Louis, USA)
Acetic acid (Sigma-Aldrich, St. Louis, USA)
2-Propanol (Merck, Darmstadt, Germany)
Precast gels 12 wells (Bio-Rad Laboratories, Inc., Hercules, USA)
Livsmedelsfärg röd/blå (food dyes red/blue) (Dr. Oetker, Bielefeld, Germany)
Milli-Q water (Merck Millipore, Burlington, USA)

4.3 Optimising SDS-PAGE for whole cells

In order to describe a method for doing SDS-PAGE on whole cells without a separate lysing protocol 0.27 g of a cell sample – recombinant *E. coli*, BL21 producing a protein of around 40 kDa – was thawed and resuspended in 1 ml MQ water. A dilution series was made to reach dilutions of 1, 10, 100, 1000 and 10 000 times. 30 μ l of each dilution was mixed with 10 μ l loading buffer that had already been made from 900 μ l 4x Laemmli sample buffer with 100 μ l 2-mercaptoethanol, and the samples were subsequently heated at 100 °C for 10 minutes. The samples were loaded in a gel. As the samples formed blobs – probably due to the composition and concentration of the loading buffer – only 10 μ l of each sample was added and only in every second well. The gel was run for 10 minutes at 100 V and 20 min at 200 V before staining with 0.6 mg Brilliant Blue R in 10 ml 10 times diluted acetic acid for 0.5 hours, and destaining with 25 ml isopropanol and 10 ml acetic acid diluted to 100 ml in water overnight.

4.4 Preparing for cultivation

Before cultivation was initiated a number of solutions had to be prepared, but some could be prepared in bigger stock solutions and stored in fridge between runs to save time and facilitate the weighing of small masses.

The cultivation medium was prepared in parts according to (Holme, et al., 1970). A base medium component solution was made with 2 g/L ammonium sulphate, 14.6 g/L dipotassium phosphate, 3.2 g/L monosodium phosphate and 0.5 g/L ammonium citrate in MQ water. A magnesium sulphate stock solution with 120.37 g/L MgSO₄ solution in MQ water; a trace element stock solution with 0.5 g/L CaCl₂·H₂O, 16.7 g/L FeCl₃·6 H₂O, 0.18 g/L ZnSO₄·7 H₂O, 0.16 g/L CuSO₄·5 H₂O, 0.15 g/L MnSO₄·H₂O, 0.18 g/L CoCl₂·6 H₂O and 20.1 g/L Na₂EDTA in MQ water; and a glucose stock solution with 0.75 g/ml D-glucose in MQ water, were prepared and autoclaved separately at 121 °C for 15 minutes.

A 30 g/ml sodium ampicillin stock solution was prepared in MQ water and filtered using a 0.2 µm sterile filter. An IPTG stock solution was prepared with 0.1787 g/ml IPTG in MQ water and filtered using a 0.2 µm sterile filter. 1 M NH₄OH was prepared by diluting 6.9 ml 28 % solution to a volume of 100 ml and autoclaving it at 121 °C for 15 minutes.

4.5 Cultivation in shake flasks

4.5.1 Preparation and inoculation

Base medium component solution was added – 49 ml to each shake flask and 13 ml to the inoculum flask – before being autoclaved at 121 °C for 15 minutes. After autoclavation, 0.1 ml magnesium sulphate stock solution, 0.667 ml glucose stock solution, 0.1 ml trace element stock solution and 0.167 ml ampicillin stock solution was added to each shake flask. 0.026 ml magnesium sulphate stock solution, 0.173 ml glucose stock solution, 0.026 ml trace element stock solution, and 0.043 ml ampicillin stock solution was added to the inoculum flask, and then a colony from plate was added. The inoculum was incubated at 37 °C and 180 rpm in a shaker overnight. Each of the shake flasks was inoculated with 1 ml inoculum and then incubated at 37 °C and 180 rpm in a shaker.

Table 2. Inducer concentrations for the shake flasks.

Sample number	Lactose (mM)	IPTG (mM)
1	1	0.1
2	5	0.1
3	10	0.1
4	20	0.1
5	1	0.5
6	5	0.5
7	10	0.5
8	20	0.5
9	1	1
10	5	1
11	10	1
12	20	1
13	0	0

4.5.2 Induction

OD₆₀₅ was measured from different flasks until the OD₆₀₅ was over 0.6. The flasks were then inoculated with lactose and IPTG according to Table 2. The incubation was then continued for 3 more hours and OD₆₀₅ was measured regularly. When the cultivation was finished, the content of the flasks was centrifuged at 3000 xg for 20 minutes in 50 ml tubes and the pellets were stored in freezer.

4.5.3 SDS-PAGE

An SDS-PAGE with samples from all the shake flasks was performed using the whole cell method tested preciously with sample diluted 10 µl sample, 20 µl MQ water and 10 µl loading buffer, and 15 µl of the mix loaded to the gel.

4.6 Cultivation in reactor

4.6.1 Preparation and inoculation

Base medium component solution was added – 1500 ml to the reactor and 29 ml to the inoculum flask – before being autoclaved at 121 °C for 30 minutes. After autoclavation, 3 ml magnesium sulphate stock solution, 20 ml glucose stock solution, 3 ml trace element stock solution and 5 ml ampicillin stock solution was added to each shake flask. 0.06 ml magnesium sulphate stock, 0.4 ml glucose stock solution, 0.06 ml trace element solution,

and 0.1 ml ampicillin stock solution was added to the inoculum flask, and then a colony from plate was added. The inoculum was incubated at 37 °C and 180 rpm in a shaker overnight. The reactor was inoculated with the content of the two densest inoculum flasks and then incubated at 37 °C and approximately 250 rpm stirring with a combined agitator and sparger.

4.6.2 Induction

During cultivation, OD₆₀₅ was continuously measured around every 40 minutes to keep track of the cell growth by taking out 1 ml sample and measuring it in a spectrophotometer at 605 nm. Glucose concentration was measured in the same sample using the blood glucose meter after diluting the sample 2 times to reach the range of the instrument. When the cells had reached a cell density of 0.6, they were assumed to be in the exponential phase and were subsequently induced with enough IPTG stock solution to reach the desired concentration. Once the system was induced, OD₆₀₅ and glucose was continued to be measured the same way and a 2 ml sample was taken out every time an online measurement was made and frozen for offline analysis.

4.6.3 SDS-PAGE

SDS-PAGE was used to determine the composition of the purified proteins from the first reactor batch. Cell samples from the same batch were also added from samples taken after induction using the whole cell method tested previously with sample diluted 10 µl sample, 20 µl MQ water and 10 µl loading buffer, and 15 µl of the mix loaded to the gel.

4.7 Preparation of a protein standard from a reactor cultivation

The enzyme assay required a reliable protein standard to accurately determine the amount of protein expressed during the process, and such a standard was prepared from the first reactor cultivation. The reactor was induced to an IPTG concentration of 0.7 mM.

4.7.1 Harvesting the cells

All of the cells were harvested by centrifuging the content of the reactor in 250 ml bottle for 15 minutes at 6000 xg. The cell pellets were resuspended in approximately 15 ml binding buffer (20 mM imidazole) and sonicated at amplitude 60, 0.5 s intervals and 20 000 Hz for 20 min. The sample was then split into 2 ml tubes and centrifuged at 13 400 rpm for 20 minutes. The supernatants were pooled in one tube except for 0.5 ml that was stored in freezer.

4.7.2 Packing the column

A column was prepared with Ni-coated agarose beads by pouring them into the column while applying suction from below. The column was washed and packed with water and then the nickel was stripped using 0.1 M Na₂EDTA. The column was then loaded with CuSO₄·5 H₂O, and washed with binding buffer to equilibrate.

4.7.3 Purifying the protein

The sample was sucked into the copper-loaded column followed by a thorough wash using binding buffer. The sample was then eluted using elution buffer (200 mM imidazole) and the sample was collected in 15 aliquots. The column was then washed with water and 20 % ethanol. The samples were spun in 30 000 Mw spin columns at 5000

rpm for 5 min to remove the imidazole. The aliquots were pooled as 1+2, 3-7, 8-12 and 13-15 with 7 ml PBS (10mM) each used to spin them.

4.7.4 Making the Bradford standard curve

A BSA standard was prepared by diluting 0.500 g BSA in 1000 μ l water. A standard curve was made by diluting the standard with water 10 times twice and then repeatedly making 2 times dilutions to reach concentrations of 0.3123, 1.561, 0.7806, 0.3903 and 0.1952 mg/ml. 10 μ l of each concentration was added to a 96-well plate along with a blank of water. 100 μ l Bradford reagent was added to each well and then the plate was incubated at room temperature for 40 minutes. Absorbance was measured at 600 nm.

4.7.5 Measuring the protein samples

The protein samples were diluted 5 times and then 5 μ l of each was added in triplicates to a 96-well plate. 100 μ l Bradford reagent was added to each well and then the plate was incubated at room temperature for 40 minutes. Absorbance was measured at 600 nm.

4.8 Designing, programming and using the flow based automated sampling system

4.8.1 Hardware

The flow-system was assembled as shown in Figure 4 with the objective of being able to form a continuous line, capable of transporting and processing a sample from the fermenter through a series of process steps. At first the sample should be mixed with BugBuster at a confluence point, using two independent pumps, and then allowed to incubate in a reactor with sufficient volume and mixing, which was achieved by inserting a wider tube with glass beads giving a total volume of 1 ml. Next the lysed sample would have to be purified by running it through a metal ion affinity column that binds to the His6 tag of the protein and then eluting it. Yet another confluence point is needed to mix the sample with the substrate solution where after, the mix should be incubated in a 1.5 ml reactor in an oven at 70 °C. Finally, a spectrophotometer should record the reaction product that is pushed from the reactor.

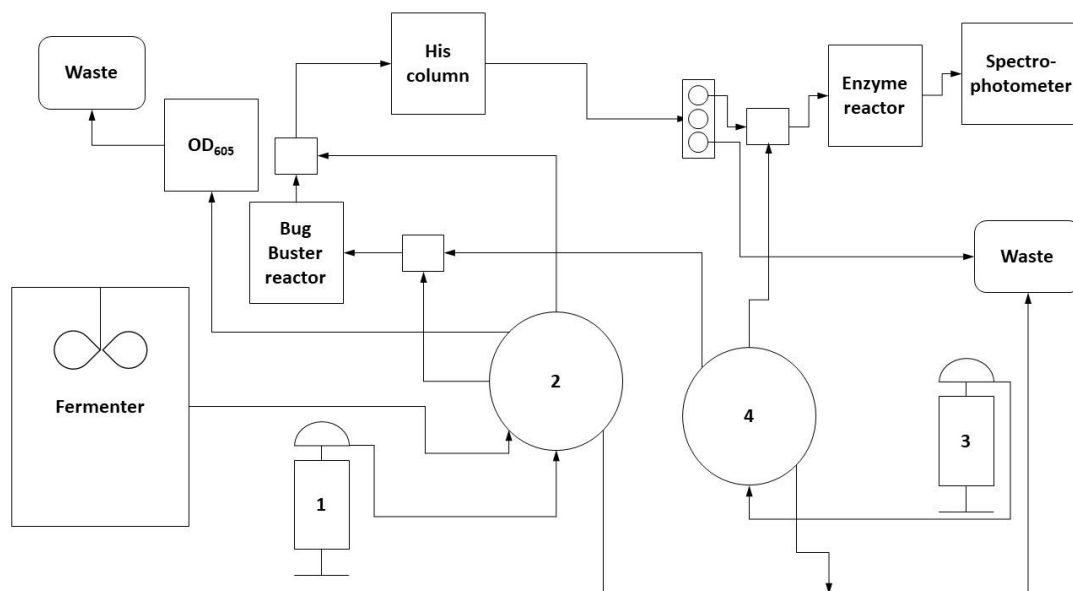


Figure 4. The flow-system setup that was used for the experiments. 1 and 3 are pump, and 2 and 4 are 12-way valves.

4.8.2 Software

When designing the program for sampling and analysis, a block like structure was used. In order to achieve repeatable results, building blocks with very fundamental commands such as pulling or pushing liquids were assembled to form the foundation. The building blocks in turn were assembled into bigger blocks with more specific functionalities that were gathered to form the general program. The overall structure of the main program is shown in Figure 5, but some other programs were also used to perform various tasks such as daily washing routines and troubleshooting. The main idea of the program is that each time it is run, it prepares the system for sampling, takes a sample to two different analyses and finishes with a washing step to keep the system clean between runs.

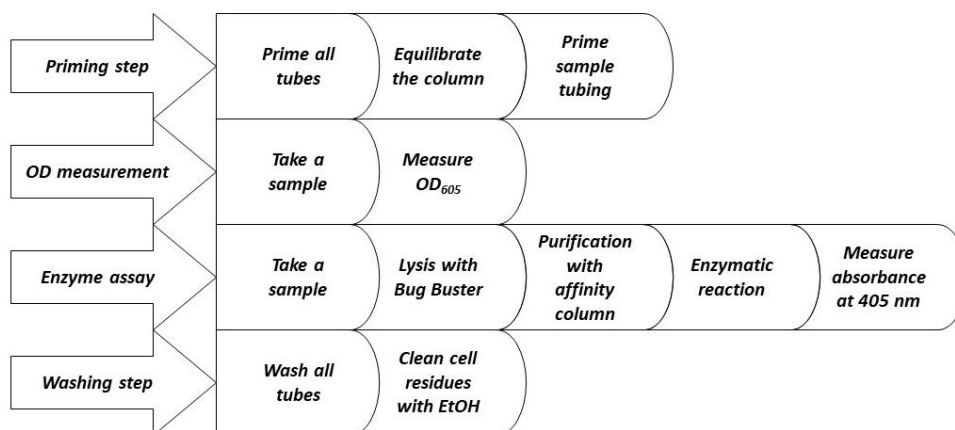


Figure 5. Flow-design for the flow-system program. Each block in the figure is in reality made up of several smaller commands such as pulling or pushing liquids.

4.9 Dispersion study

When designing the flow-system, control over dispersion and mixing was achieved by the choice of reactors. For the enzyme incubation reactor two reactors of equal volumes were tested, one wide straight tube reactor and one knitted coil reactor. One red and one blue food dye was injected simultaneously into the reactor through a confluence point and mixing was visually assessed.

4.10 Induction study

In order to investigate the effects of IPTG induction, different amounts of IPTG were used to induce reactor batches after a cell density of 0.6 had been reached. Volumes added and their corresponding concentrations are shown in Table 3. After induction, samples were taken regularly and frozen for analysis of the protein content.

Table 3. Inducer concentrations for the reactor batches.

Batch No.	Volume IPTG stock solution (ml)	IPTG concentration (mM)
1	2	1
2	2	1
3	0.2	0.1
4	1	0.5
5	2	1

A 5 mM pNPG substrate solution was made by adding 1.50626 mg pNPG to 1 ml citrate buffer. The citrate buffer was made by adding 933.5 mg citric acid and 1.83 g dibasic sodium phosphate to 100 ml water.

4.10.1 Online enzymatic assay

When taking online samples, 100 μ l was taken to either OD₆₀₅ measurements or the enzymatic assay. The main program performed all these tasks and recorded the OD₆₀₅, and with continuous spectrophotometric measurements at 405 nm a curve describing the eluted protein sample was received from the enzymatic assay. The area of the curves was then calculated using a trapezoidal numeric integration method, but the dips from pressure variations occurring when the pump stopped pushing in order to pull new running buffer were removed by taking any point in the middle of the curve that was less than 95 % of the surrounding values and replacing it with the average of the surrounding values. An example of how the areas were calculated is found in Appendix 4.

When taking a sample to the enzymatic assay, 100 μ l sample was taken and mixed with 200 μ l BugBuster and incubated for 2 minutes. The lysed cells were then bound to an IMAC column which was washed with plenty of binding buffer. The sample was then eluted with 200 μ l elution buffer, mixed with 400 μ l pNPG substrate solution and incubated at 70 °C for 5 minutes. The sample was finally pushed to a spectrophotometer which continuously recorded absorbance at 405 nm to yield a curve corresponding to the enzyme concentration of the initial sample.

To make a standard curve for the online assay a sample containing only the purified protein was treated the same way as a sample from the reactor would i.e. the program was run complete with BugBuster mixing, binding to the column, mixing with

substrate, incubation and recording the peak using a spectrophotometer. A slight change was however made to the program so that it would pull various volumes of sample and mix with water up to a volume of 100 μl giving sample concentrations of 100 %, 50 %, 25 % and 0 % of 0.621 mg/ml – the enzyme standard concentration diluted two times.

4.10.2 SDS-PAGE

All the reactor batches in the induction study were examined using SDS-PAGE by taking four of the samples – the one closest to induction time and the three last ones – taken after induction from each reactor and running them together using the whole cell method tested previously. 30 μl of each sample was mixed with 10 μl loading buffer and 15 μl each was loaded to the gel.

4.10.3 Offline enzymatic assay

To determine the production of enzyme in the reactor, 100 μl of each sample taken after induction along with a calibration curve made from a 1.24 mg/ml enzyme standard and diluted 10, 20, 40, 80, 160 and 320 times, was mixed with 400 μl BugBuster in a tube and incubated at room temperature under heavy shaking for 20 minutes. 50 μl of each sample was added to a 96-well plate (see Appendix 1 for layout) and 50 μl pNPG substrate solution was simultaneously added to all the wells. The plate was immediately incubated at 70 °C for 5 minutes and absorbance was then recorded at 405 nm at regular intervals to determine the enzymatic activity in the samples.

The data was used to, for each sample, generate a kinetic curve describing the progression of the enzymatic reaction over time. A representative slope for each sample corresponding to the rate of reaction in that sample and therefore also the concentration of enzyme was generated by making a linear regression. The slopes of the curves were used to make a standard curve with which the slopes of the samples from the reactor batches were converted to the concentrations at different points in the reactors. The inducer efficiency of the different reactors was calculated by dividing the final enzyme concentration with the inducer concentration.

5 Results

5.1 Whole cell study

When looking at the gel in Figure 7 it is clear that the cells have lysed and that the proteins have been separated. The optimal dilution should give visible bands without smearing and in this case the 10 times and 100 times dilutions were the closest. In order to achieve clear results, dilutions of around 30 times should be used which corresponds to concentration of 9 mg/ml wet cells.

5.2 Shake flask cultivation

The final OD_{605} for the shake flasks along with the lactose and IPTG concentrations were used to make a principal component analysis and find potential correlation in the data. A biplot (Figure 6) was made to illustrate the correlations. It is clear that lactose concentration shows no correlation with OD_{605} , but IPTG shows a strong negative correlation with OD_{605} implying that IPTG hinders growth.

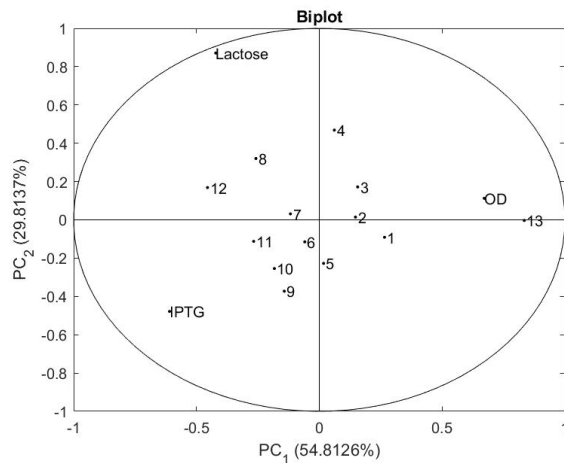


Figure 6. A biplot constructed from the OD₆₀₅ measurements of the shake flasks. There seems to be no correlation between the OD and the lactose concentration which implies that the lactose does not affect growth. There is however a strong negative correlation between OD and IPTG concentration which implies that growth is hindered by IPTG

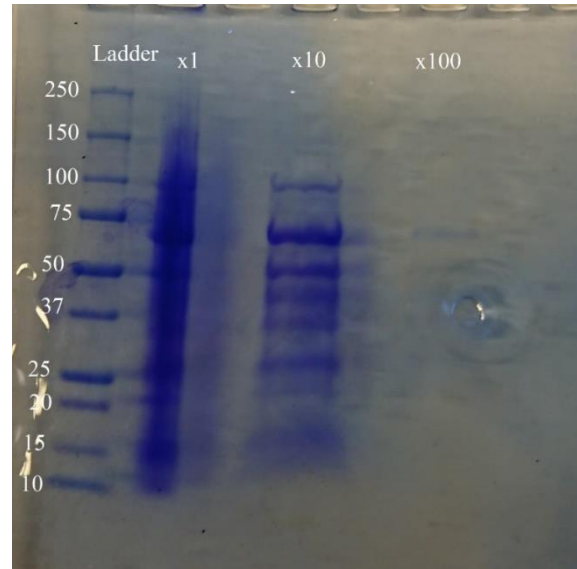


Figure 7. Gel with dilutions of the whole cell sample that was lysed as part of an ordinary SDS-PAGE protocol. The dilutions beyond 100 times were not visible at all and are not included in the picture.

The SDS-PAGE where cells from all of the shake flasks were included generated two gels shown in Figure 8. In all of the shake flasks except for the one without inducer, a distinct band around 80 kDa can be distinguished. However, no clear difference in amount of expressed protein can be distinguished between the different amounts of inducer added.

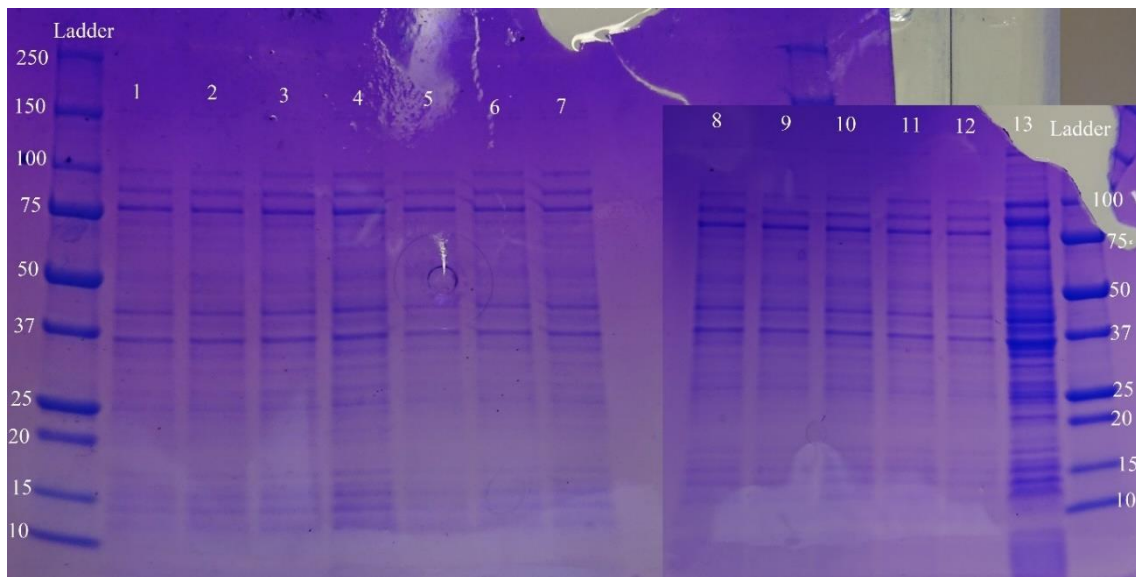


Figure 8. SDS-PAGE gels for shake flasks number 1 through 13 showing all proteins present in the samples. The white arrow indicates a band at a weight of ~80 kDa that is present in all of the induced samples 1-12 but missing in the uninduced sample 13.

5.3 Preparation of a protein standard from a reactor cultivation

The OD₆₀₅ measured during the entire first batch is shown in Figure 9. At the moment of induction, the growth seems to have reached the exponential phase, but after induction the growth rate slowed down compared to the extrapolation made using an exponential curve fitted to the data before induction.

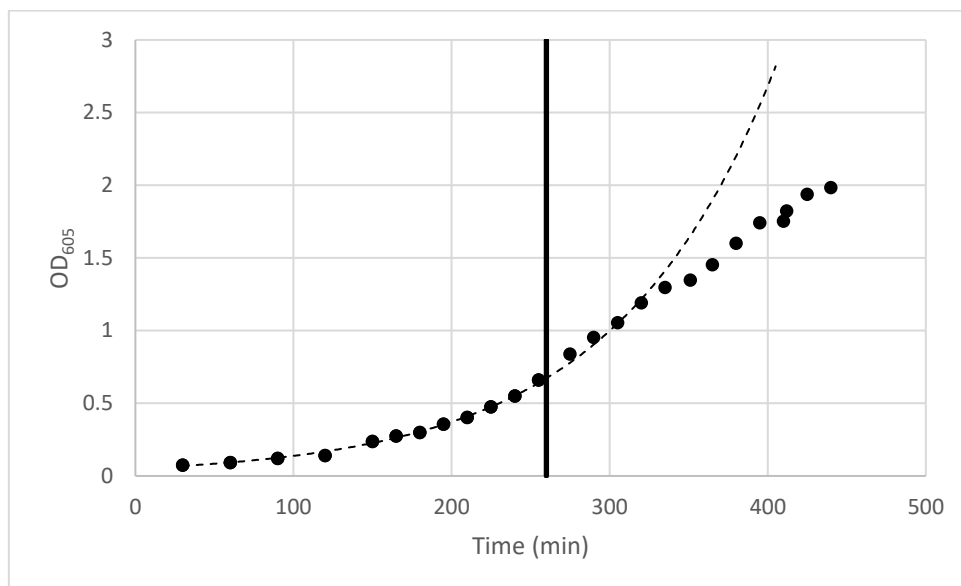


Figure 9. Growth curve for the first batch. The line at 260 minutes indicates the time of induction, after which the exponential phase begins to stagger somewhat. The dashed line is an extrapolation from the growth development before the induction time.

15 fractions were received from the purification using the IMAC column, but for easier handling they were pooled as: 1-2, 3-7, 8-12, 13-15, because those fractions were estimated to have similar concentrations. The pooled fractions were then run in an SDS-PAGE along with cell samples taken after induction and the gel shown in Figure 10 was received. Fractions 3-7 seem to contain the highest amount of enzyme around 80 kDa and 1-2 barely contain any. Many of the samples also have a band around 40 kDa. The same gel was also analysed using the Image Lab software to assess the distribution of different proteins in the samples, and this is presented in Table 4. The 3-7 contained 49.1 % of the target enzyme and the 8-12 contained 64.6 % of the target enzyme.

Lane	Band %	MW
3-7	49.1	76.859
8-12	64.6	78.555
13-15	100	83.465

Table 4. Proportional weight of the biggest bands estimated with the Image Lab software. The fraction expressed protein seems to be higher in the samples containing less total protein, however note that the 100 % of the 13-15 sample probably just means that the software couldn't recognise the weaker bands as other weak bands can be seen in Figure 10.

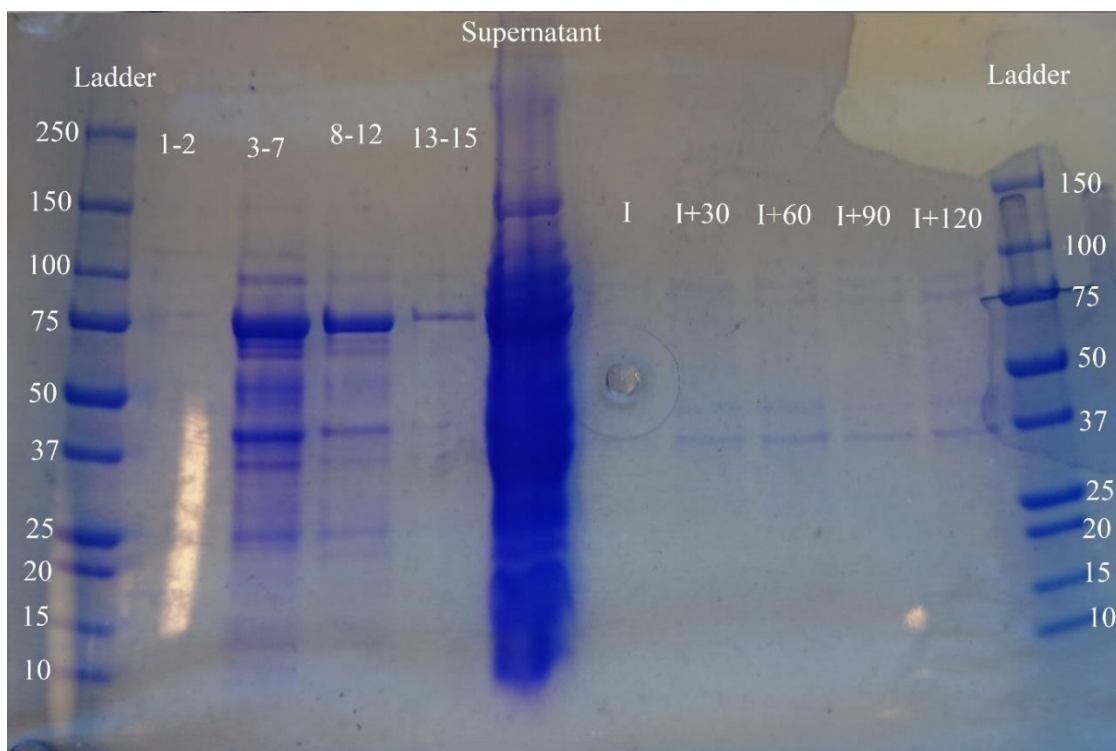


Figure 10. SDS-PAGE gel showing the different fractions 1 through 15 purified from the first reactor cultivation. The supernatant is the unpurified supernatant from the same sample and the I+X are cell samples from the reactor at time X after induction. It is clear that the fractions 3-7 followed by 8-12 contain the most expressed protein as can be seen by the strong band around 80 kDa. Many of the samples also seem to contain a band at around 40 kDa.

5.4 Bradford protein analysis

The Bradford analysis gave the standard curve seen in Figure 11. Using the standard curve and the fractions in Table 4, the protein concentrations were calculated and are presented in Table 5. The concentration of TnBgl3B in sample 3-7 was 1.24 mg/ml.

5.5 Dispersion study

When pushing the two dyes through the confluence point, they tended to form separate streams in a laminar fashion, and this profile was kept throughout the tubings if left untouched. In Figure 12 the flow through the tubes and reactors can be seen. The straight tube reactor had poor mixing and even after passing it, two separate flows could be distinguished in the tubings. The knitted coil reactor on the other hand showed great mixing, and even after just a few turns in the reactor, separate dye streams could not be distinguished anymore.

Table 5. The concentrations of TnBgl3B in the purified protein samples.

Protein sample	[TnBgl3B] (mg/ml)
1-2	0.00144*
3-7	1.24
8-12	0.459
13-15	0.0831

*No fraction from the SDS-PAGE gel was identified and so this is the concentration of all the proteins in the sample.

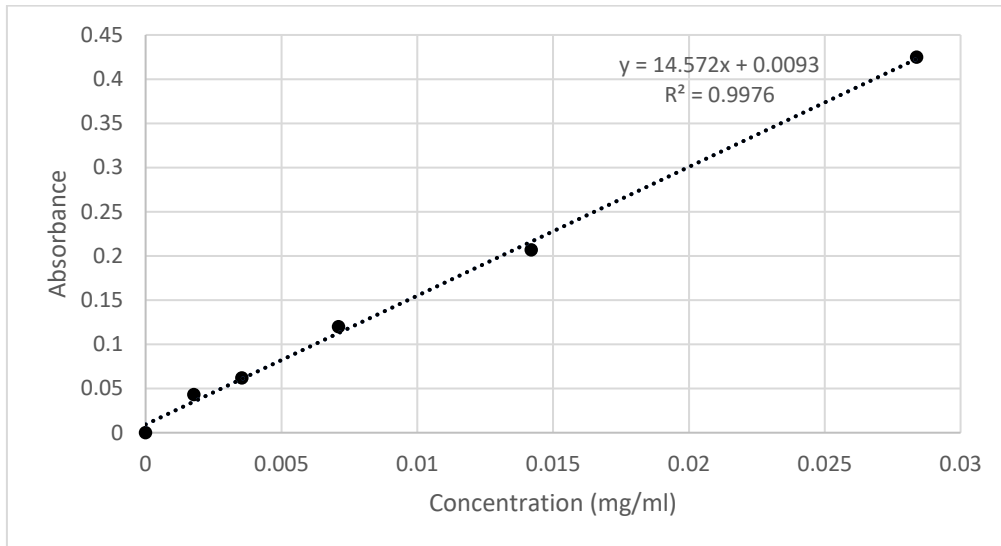


Figure 11. BSA standard curve from the Bradford protein concentration determination.

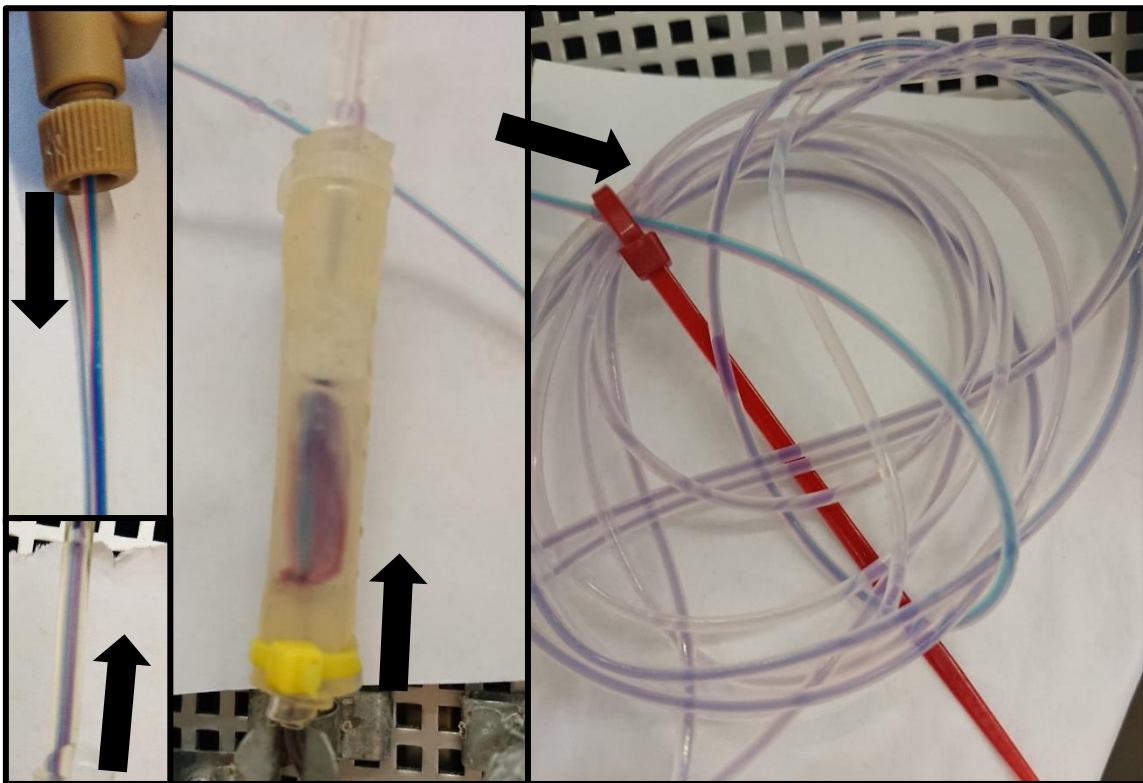


Figure 12. The black arrows indicate flow direction. The two leftmost pictures have had contrast and/or brightness increased. **Top left:** just after the confluence point, the flow was laminar and limited mixing was seen. **Bottom left:** Even after the straight reactor, the dyes were somewhat separated. **Middle:** in the straight tube reactor, mixing was poor and separate streams of red and blue can still be seen. **Right:** At the entry point of the knitted coil reactor, the dyes were separated, but after entering the reactor, the mixing increased and a purple tone can be seen where the dyes have been mixed.

5.6 Induction study

A comparison of results from the internal micron and the external spectrophotometric OD_{605} measurements is presented in Figure 13 using the batch from 2019-05-21. The growth curves follow each other closely over time. In Appendix 5 and Appendix 6 glucose is shown to decrease over time whereas OD_{605} increases. It must be noted that the reactor induced with 0.5 mM IPTG consumed more glucose and reached a higher cell density than any of the other reactors.

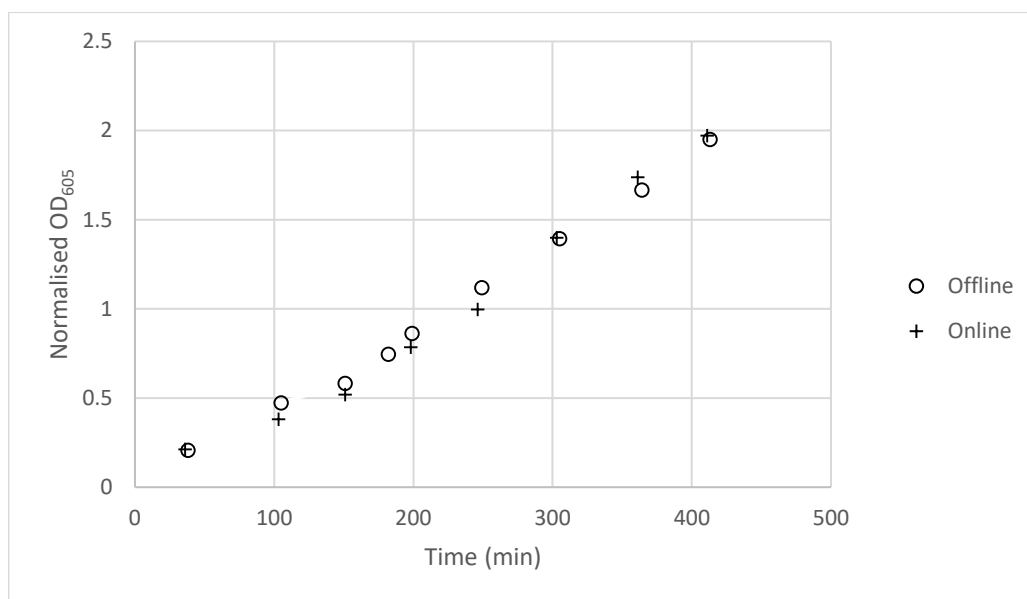


Figure 13. Growth curves for the 2019-05-21 batch. The circles are OD_{605} measurements taken offline, and the crosses are taken online. Overall the results follow each other closely.

5.6.1 Online enzymatic assay

The areas of the curves received from the calibration curve measurements were calculated and plotted versus the enzyme concentrations ($[E]$) in Figure 14. A linear curve was initially fitted to the points but as the residuals seemed to be trending (Appendix 2), a second order curve was instead fitted with equation 3.

$$\text{Area} = -6.7425 [E]^2 + 11.783 [E] + 1.6731 \quad (3)$$

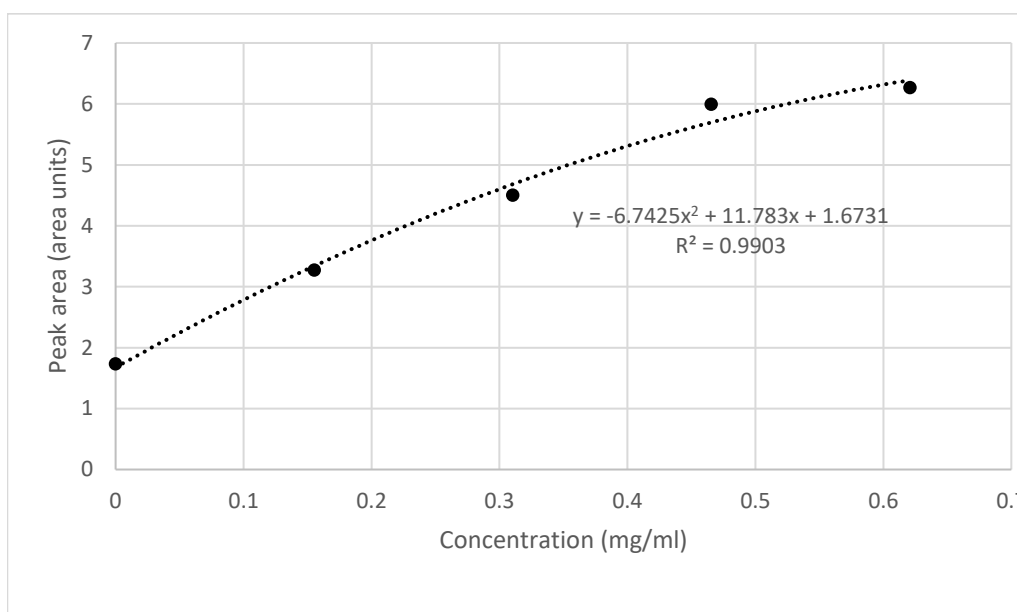


Figure 14. The second order calibration curve for the online enzymatic assay that is fitted to the peak areas at various enzyme concentrations.

5.6.2 Offline enzymatic assay

The SDS-PAGE gels in Figure 15 show that higher amounts of proteins are present in the samples taken later in time. For all the 1 mM samples as well as the 0.1 mM sample, the 80 kDa bands and 40 kDa bands are the strongest. The 0.5 mM sample seems to contain a lot of other bands of various intensity. In general, the lane closest to the induction shows the weakest bands, but there is no big difference between the later samples, except for the 0.1 mM and the 0.5 mM samples. The 0.1 mM is hard to compare since it is spread between two gels, but the intensity of both the 0.1 mM and the 0.5 mM samples seems to increase with longer incubation times.

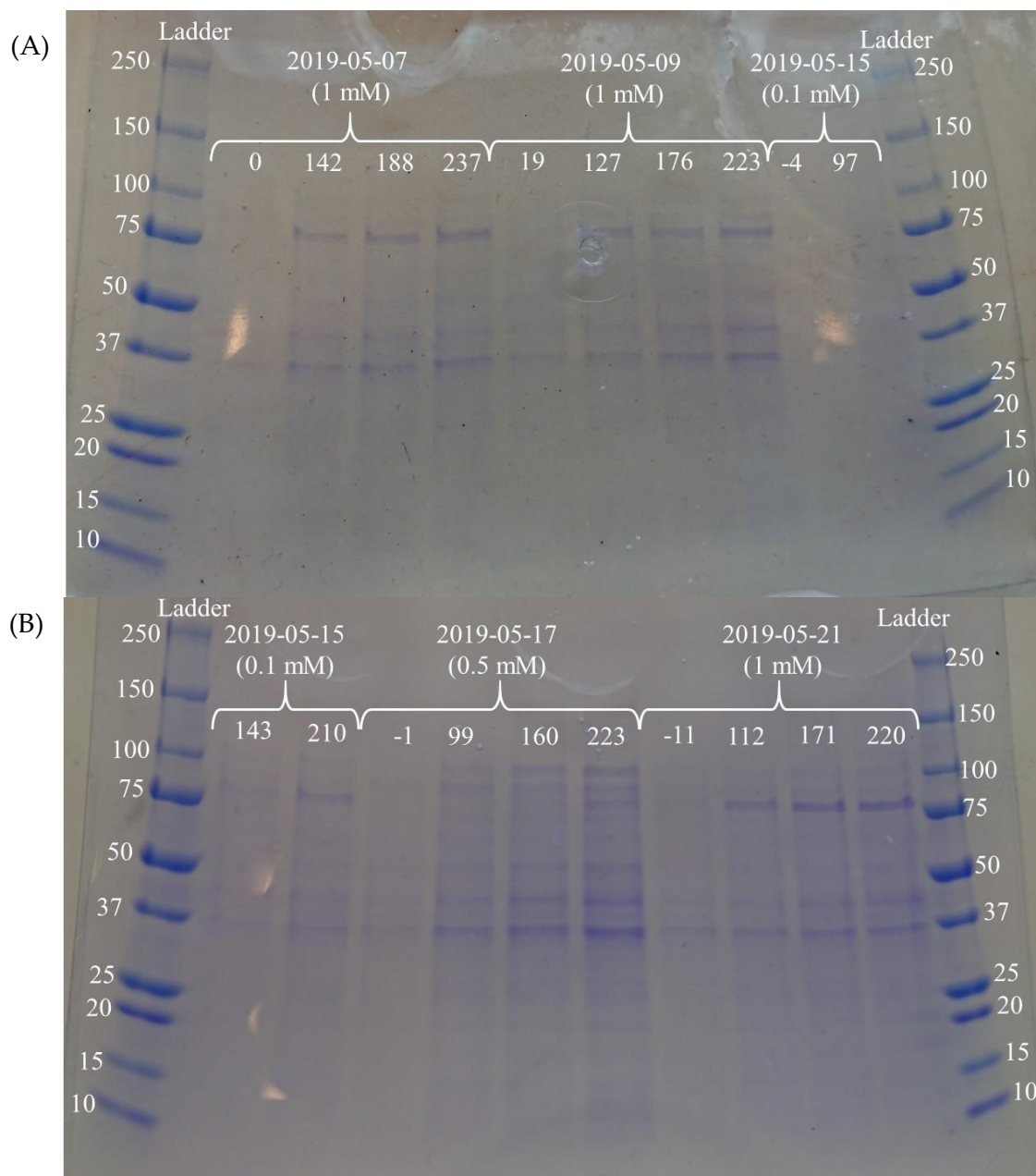


Figure 15. SDS-PAGE gels containing the samples from the five reactor batches.

The results from the offline enzymatic spectrophotometric measurements are found in Appendix 7. The kinetic curves for the calibration curve have been normalised and gathered in Figure 16. It can clearly be seen that a higher concentration of enzyme results in a steeper slope – corresponding to a higher enzymatic reaction rate. Even after 18 hours of reaction time, the absorbance was still increasing meaning that the reaction was still going on and unreacted substrate was still present. The first hour after removing the samples from the oven the reaction was pretty uniform and therefore all of the points were used when describing the reaction rates.

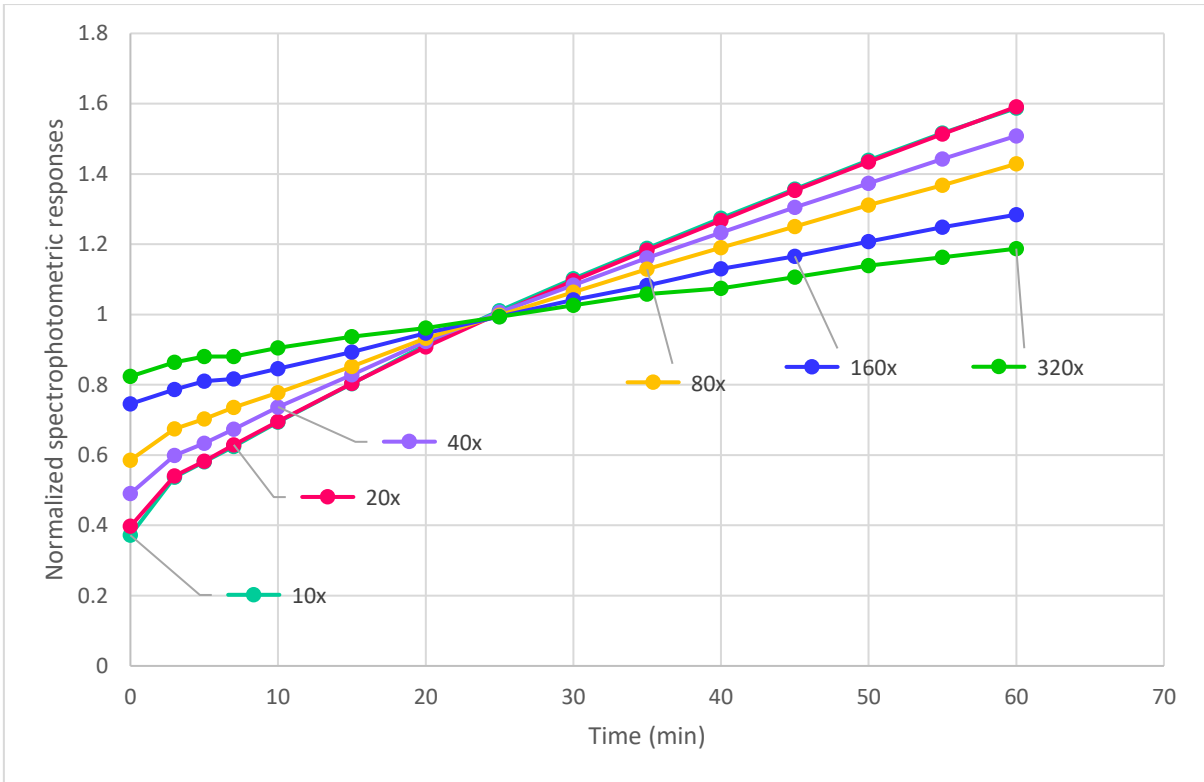


Figure 16. The normalised responses from the spectrophotometric measurements of the calibration curve recorded over time. The lines correspond to the standard solution diluted 10, 20, 40, 80, 160 and 320 times.

The slopes from the kinetic curves were used to generate the calibration curve in Figure 17, and a second order equation is fitted to the curve due to the look of the residuals when plotting a linear curve, see Appendix 3.

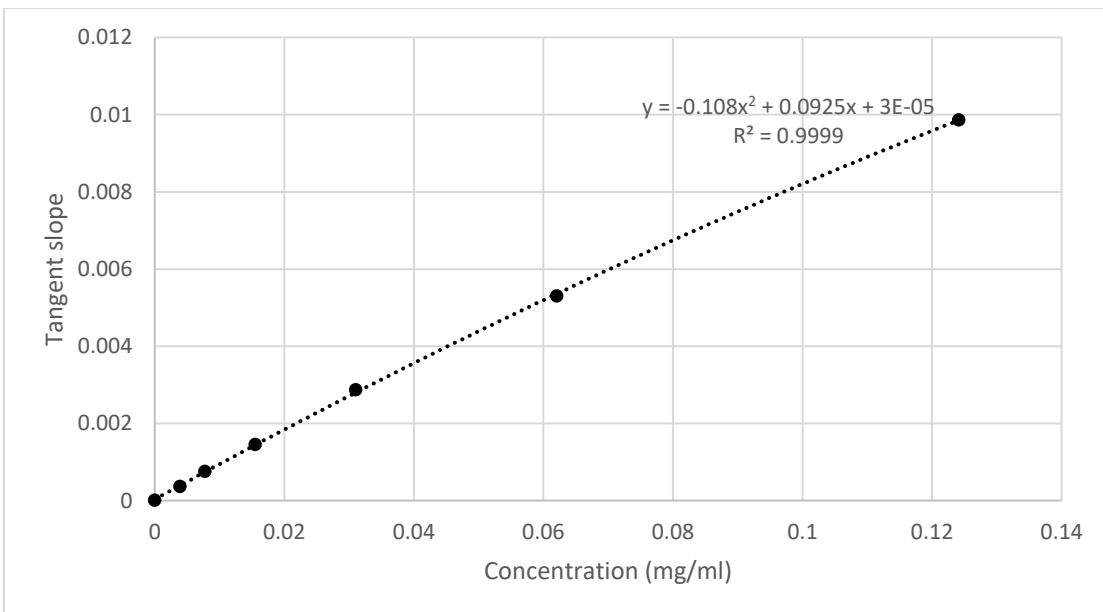


Figure 17. The second order calibration curve for the offline enzymatic assay that is fitted to the slopes of the reactions at various enzyme concentrations.

The calibration curve was used along with slopes from the kinetic curves of the samples to calculate the concentrations at various times in the reactor. The concentrations are presented versus time in Figure 18. The reactors with 1 mM IPTG showed by far the highest concentrations of produced protein compared to lower concentrations of IPTG. There is no clear difference between the 0.1 mM and the 0.5 mM IPTG reactors. The 1 mM reactors show a decrease in production rate over time, and the concentration curve seems to level off after around 4 hours. The final enzyme concentrations of the reactors were 2019-05-07: 3.2 $\mu\text{g/ml}$, 2019-05-09: 4.2 $\mu\text{g/ml}$, 2019-05-15: 0.73 $\mu\text{g/ml}$, 2019-05-17: 0.67 $\mu\text{g/ml}$ and 2019-05-21: 3.2 $\mu\text{g/ml}$ and are shown in Table 6 along with the inducer efficiency.

Table 6. The final enzyme concentrations of the different reactor batches along with the inducer efficiencies.

Batch date	IPTG concentration (mM)	Final enzyme concentration ($\mu\text{g/ml}$)	Inducer efficiency (Final enzyme concentration/Inducer concentration)
2019-05-07	1	3.2	3.2
2019-05-09	1	4.2	4.2
2019-05-15	0.1	0.73	7.3
2019-05-17	0.5	0.67	1.34
2019-05-21	1	3.2	3.2

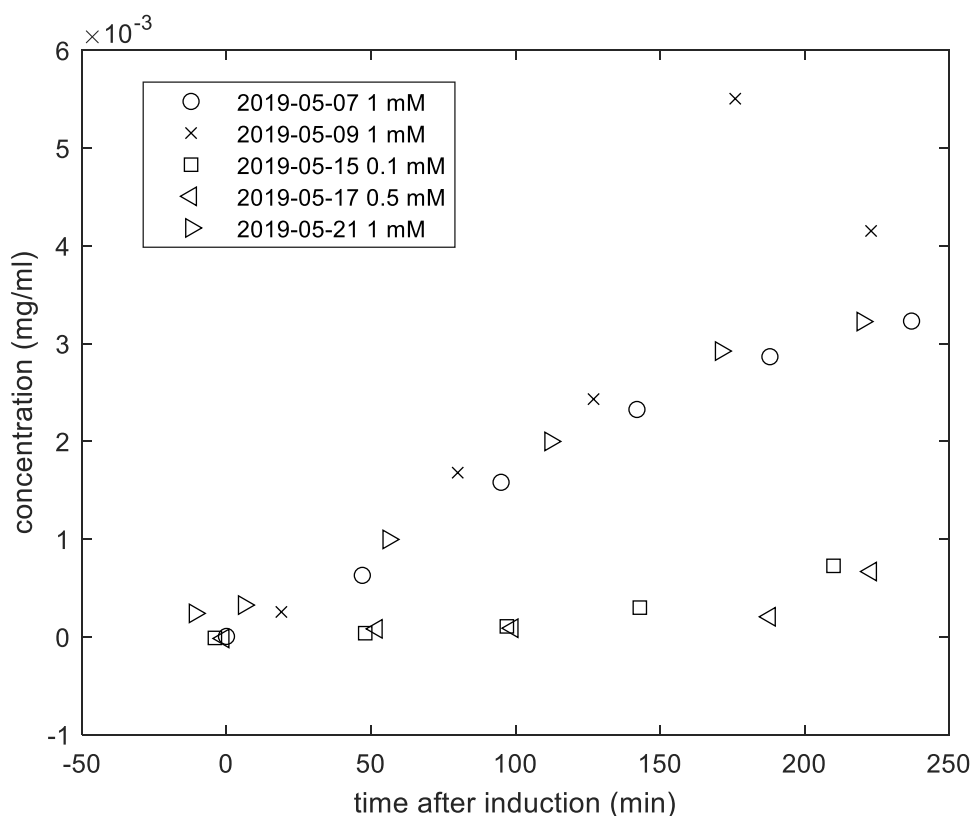


Figure 18. Concentrations of enzyme in the reactors plotted versus the time after induction.

6 Discussion and conclusion

6.1 Initial studies

The initial whole cell study showed that around 9 mg/ml cells could be added to an SDS-PAGE gel to give nice lysis of the cells and separation of the proteins present. This method might not work as well for cells that are not lysed by the boiling, and some proteins might not endure the harsh treatment and denature or break in ways that would affect the assay. Care should therefore be taken when adapting this method for other circumstances, however if *E. coli* is used and relatively heat-stable proteins are expressed, this method should be feasible.

When looking at the shake flask cultivation, it is clear that the addition of IPTG slows down the growth rate, probably because of the switch in metabolism when expression of the recombinant protein starts. The addition of lactose on the other hand implies no such switch in metabolism as growth rate is equal at different lactose concentrations. When looking at the SDS-PAGE gels there is no clear sign that any one of the inducers would start protein expression on its own since all of the samples contain both of the inducers, but the sample without inducer does not show any band at 80 kDa and therefore does not contain any of the recombinant protein. At least one of the inducers is working, and when looking at the metabolic shift when using IPTG, that one

is the more probable one. It would have been interesting to have expanded this initial study by measuring the actual enzyme concentrations of the samples to get a better idea of the induction behaviours of the cultivations. It would also have been good to include samples with only one of the inducers to be able to rule out which inducer works, and which one does not. After this initial study it was however decided that the focus would be on the use of IPTG as the only inducer for the rest of the project because it was believed that an in-depth study of the more interesting inducer would be better than a shallow study encompassing both.

The growth curve that was generated for the first reactor batch (Figure 9) showed the same indication as the shake flask study, namely that the growth rate was lowered after induction. This further supports the idea that IPTG changes the metabolism of the cells when used to induce recombinant protein expression.

The protein standard that was generated from the first batch reactor had only 49.1 % of the recombinant protein, even after purification. This value should be possible to increase, for example by better packing of the IMAC column or more careful washing with binding buffer before elution. Still, some non-specific binding would be expected in the column and thus would 100 % recombinant protein content not be feasible. The reason that the less concentrated fractions actually contains higher shares of recombinant protein is probably because of the limit of sensitivity of the software as it will not recognise the weaker bands once they are thin enough, and thus for the 13-15 fractions only the 80 kDa band was detected.

In most of the SDS-PAGE gels where the recombinant protein was studied, a parallel band at around 40 kDa, half of the target protein's mass, was seen. This could just be that the cells had a protein around this size that was really well expressed, but it could also be a sign that the target protein had dissociated into two dimers. No indication in literature of this being true or false was however found and it must therefore remain a question for future studies.

6.2 The FlowSystem

The online enzymatic assay proved to be a difficult step in the process. The results varied a lot between runs for different reasons such as the substrate self-reacting when not cooled properly, the build-up of counter pressure in the IMAC column and the degrees of mixing in the different reactor designs tested. One problem that arose was that there was too little difference between the blank samples and the ones with enzyme present, probably because of background noise. The samples were effectively diluted and drowned in other substances such as imidazole, cell debris, self-reacted substrate, or other contaminants. This problem was addressed by increasing the mixing in the reactors with the addition of glass beads or a knitted coil reactor, but no stable results were achieved even after these changes. In order to further decrease dilution, shorter tubings, smaller sample volumes and lower flowrates could be used.

It was possible to generate a calibration curve, albeit not a very repeatable one, when using enzyme standards without cells, but as soon as cell samples were used, the process failed. This was in large due to the build-up of counter pressure in the system, and in particular the IMAC column. This could in turn either be because of too small

dimension in the column, but more likely because of the BugBuster lysis not working properly. No proper investigation was done on the lysing protocol of BugBuster, but if comparing the 20 minutes used for the offline lysis with the 2 minutes used for the online lysis it seems reasonable to believe that the incubation time could be increased. An alternative would be to increase the concentration of BugBuster in the reactor, or a redesign of the reactor to improve mixing and allow the sample and BugBuster to interact more.

The online analysis program in itself worked as it should, but improvements could of course be made. The main issue with the program was that a full run complete with priming of the sample tubing and clean-up afterwards took around 45 minutes, which meant that samples could be taken no more frequently than so. A lot of small steps could be altered to reduce the time of the process, and a greater simultaneous use of the two pumps could make certain tasks run in parallel. If the program could be designed so that two samples could be in the system at once, it would be a great improvement. This would however require a new design of the setup, but could actually be feasible if done correctly.

6.3 The induction study

The results of the induction study show that no or little recombinant expression takes place before the point of induction. This can be seen both in the SDS-PAGE where the uninduced samples lack the 80 kDa band, but also in the enzymatic assay where enzyme levels at induction times are several orders lower than the final concentrations, at least in the reactor batches with high levels of inducer. Likewise, expression slows down over time, and 4 hours after induction, the expression is almost finished. It was also shown that the reactors with 1 mM IPTG showed much higher levels of enzyme activity than those with lower concentrations. When comparing with the SDS-PAGE gel however, the 0.5 mM IPTG reactor has very intense bands that would imply higher levels of expression. It must however be noted that the lanes corresponding to that reactor also show intense bands at other molecular weights than 80 kDa, and the band at 80 kDa is fairly weak, which means that other proteins are expressed at high levels. An explanation to this would be that some contamination has occurred in that batch, or that the inducer was somehow not working properly. In support of this is the fact that the glucose consumption and cell density of this batch was much higher than the others. Lack of IPTG would explain both the low enzyme levels and the greater growth rate which in turn explains the presence of other proteins. A higher cell density would of course result in a higher concentration of all the native proteins in the strain. The strange thing is that the 0.1 mM IPTG reactor did not show the same increase in growth rate or native protein concentration, but recombinant expression levels were still very low. A more reasonable explanation here would be that some other factor affected the growth of the 0.5 mM reactor, such as contamination from a very viable bacterial strain that could outpace the recombinant *E. coli* used in this experiment. For example, if the ampicillin added to generate an evolutionary pressure was destroyed by heat or lost due to some other factor, it would be easy for foreign organisms or variants of the same strain without the recombinant plasmid to grow uncontrolled.

According to Figure 18, a concentration of 0.5 mM IPTG is required in order to induce proper expression in this system, but since this reactor batch had questionable characteristics in general, it would not be possible to state this with a high degree of certainty. It is however fairly clear that a concentration of 0.1 mM IPTG yields less enzyme than a concentration of 1 mM IPTG, but when comparing the inducer efficiencies, using 0.1 mM IPTG gives an almost twice as high value compared to using 1 mM IPTG. This result is interesting, because of the high cost of IPTG. If a process that was previously run with 1 mM IPTG were to change that to 0.1 mM IPTG, they would also lower their yields per batch by around a factor 5. Although this sounds like an unreasonably big loss, if the process in itself was very cheap and the majority of the costs associated with one batch came from the inducer it would be feasible to reduce inducer costs 10 times at the expense of the yields decreasing 5 times. This situation is however fairly unlikely since the costs associated with one batch are more than just the medium components used. Staff, facilities, administration and maintenance are all examples of costs that the profits from the produced batch would have to carry, and with this perspective, cutting inducer costs would only be a relatively small gain. Lower batch yields would also face problems such as more complex purification steps and longer production times during which the product might be spoiled while just standing on a shelf in an expensive storage space. In the end the same product quality might not even be achievable anymore!

6.4 Future prospects

The results of this project were of course limited by the resources available. Even though feasible conclusions can be drawn, more and better information could have been wished for. In future studies, the IPTG range would have been the first thing to look further into. The data from the 0.5 mM IPTG reactor batch was not entirely reliable and this would have to be remade. By investigating more different concentrations in the range of 0.1-1 mM IPTG and even beyond, a relationship between cost and yield could be found to determine the optimal concentration of IPTG when inducing.

The use of lactose as an inducer was abandoned early on in the project due to time limits, but if a new study with shake flasks was made, the inclusion of flasks with only one of either inducer present would be the priority. If such a study showed that there indeed was reason to believe that lactose could work as an inducer on its own, it would be of interest to try and combine it with IPTG in a more complex study like the induction study made here.

The FlowSystem never reached its full potential as the transportation of cells in the system proved to be difficult. When redesigning the system, it would be good to consider what steps are necessary. The IMAC column could for example be skipped, letting the substrate mix with the lysed cells. More background noise would of course be present, but shorter tubings and no addition of imidazole would compensate for this. The process would also become much simpler and faster, and sampling could be done more frequently. Other improvements to be made would be any changes that could limit dispersion in the tubings while increasing mixing in the reactors. The knitted coil reactor was probably a good choice, but there might be other alternatives.

6.5 Conclusion

All in all, this project showed good results, even if some of the questions remain unanswered. One of the most important aspects was the development of a flow-based enzymatic online method to study the induction behaviour of a recombinant cell culture. The FlowSystem was assembled and programmed to do automatic sampling, but the process was never able to handle cell samples as they caused too big counter pressure. Further attempts to optimise the program and the hardware would have to be done to control dispersion and mixing, but if done properly, this method should prove an important tool for deeper induction studies. Other parts of the method were the whole cell SDS-PAGE, which proved to be an invaluable tool for the rest of the project, and the preparation of a protein standard, which was a foundation for the subsequent quantifications of the recombinant protein.

The enzymatic assay was used offline as well and showed that concentrations of IPTG lower than 1 mM could be feasible, at least in some situations, but concentrations as low as 0.1 mM are too low in most cases. Lactose was only tested in a smaller study and no clear evidence of whether it could work as an inducer – either on its own, or in concert with IPTG – was not found. The most promising area seems to be the inducer concentration span between 0.1-1 mM IPTG, in which further studies should be able to find the lowest viable concentration for recombinant protein expression processes.

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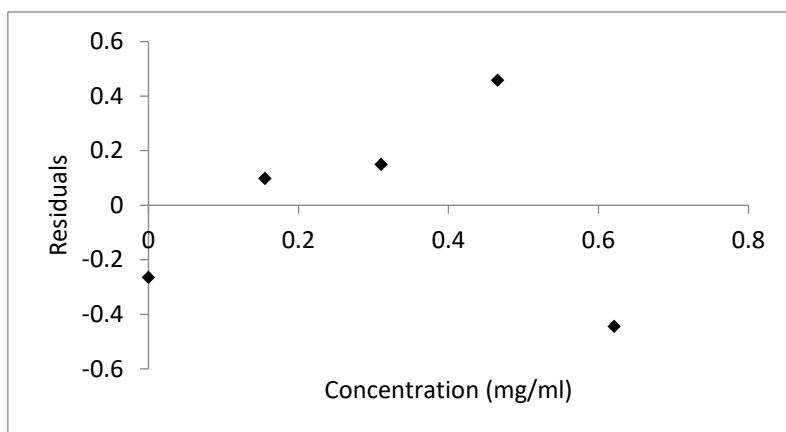
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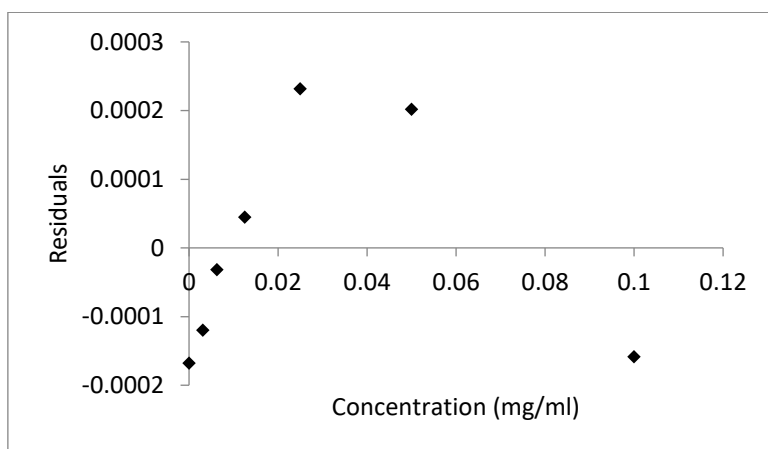
9 Appendix

Appendix 1. The layout of the 96-well plate used for the offline enzymatic assay. The numbers indicated in the columns for the 5 reactor batch dates corresponds to the time after induction that the sample was taken in minutes. The numbers in the calibration curve indicate the dilutions of the 1.24 mg/ml enzyme standard. The blank is made of water treated the same as the other samples.

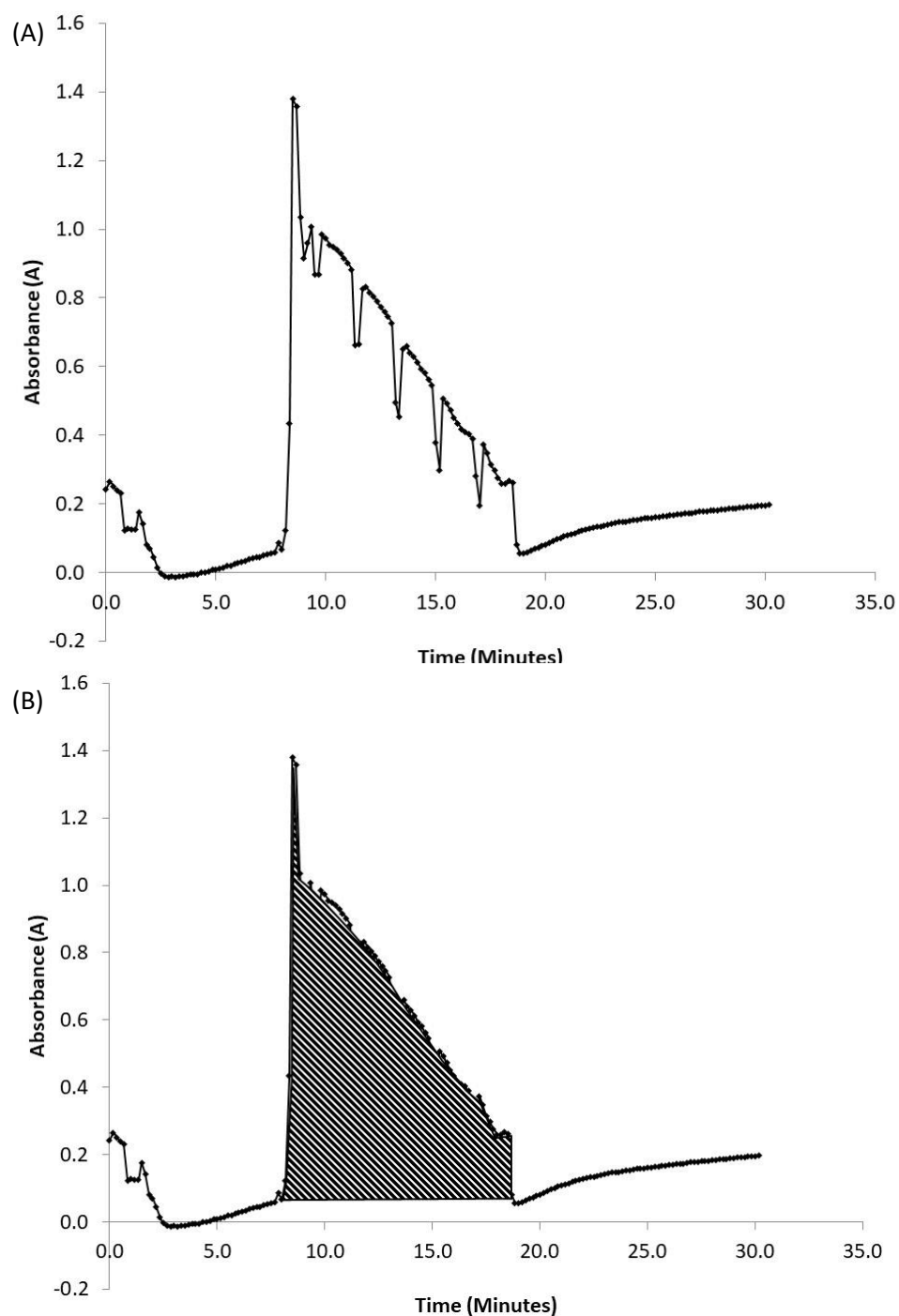
2019-05-07 1 mM		2019-05-09 1 mM		2019-05-15 0.1 mM		2019-05-17 0.5 mM		2019-05-21 1 mM		Calibration curve	
0	0	19	19	-4	-4	-1	-1	-11	-11	10x	10x
47	47	80	80	48	48	52	52	6	6	20x	20x
95	95	127	127	97	97	99	99	56	56	40x	40x
142	142	176	176	143	143	160	160	112	112	80x	80x
188	188	223	223	210	210	223	223	171	171	160x	160x
237	237							220	220	320x	320x
										Blank	Blank



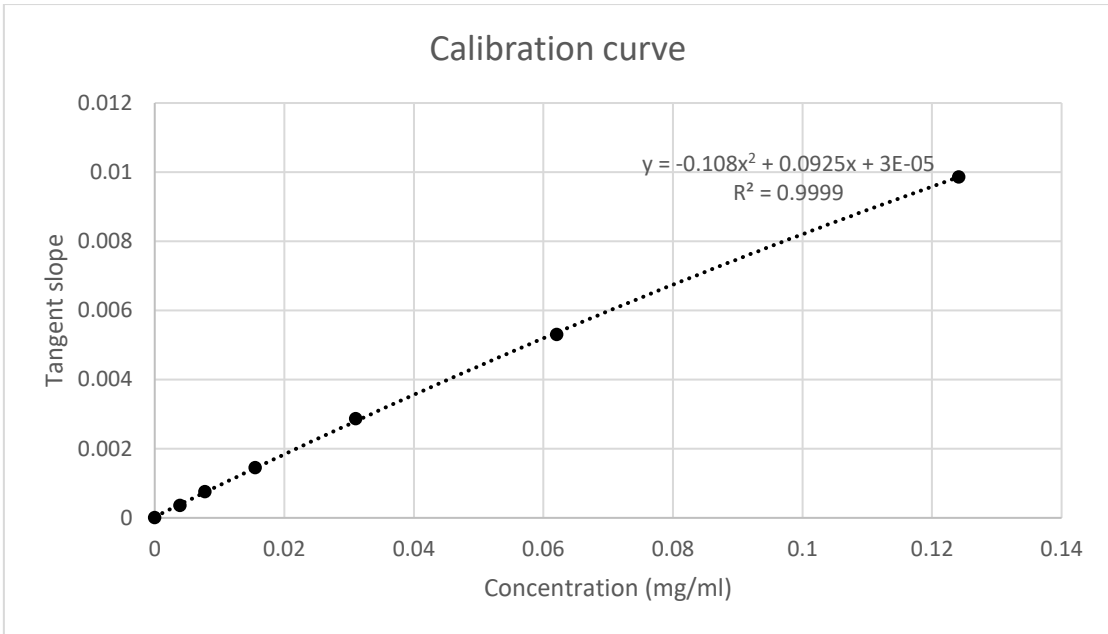
Appendix 2. Residuals for the online enzymatic assay plotted versus enzyme concentration. There is some systematic variation in the residuals, possibly a second order correlation.



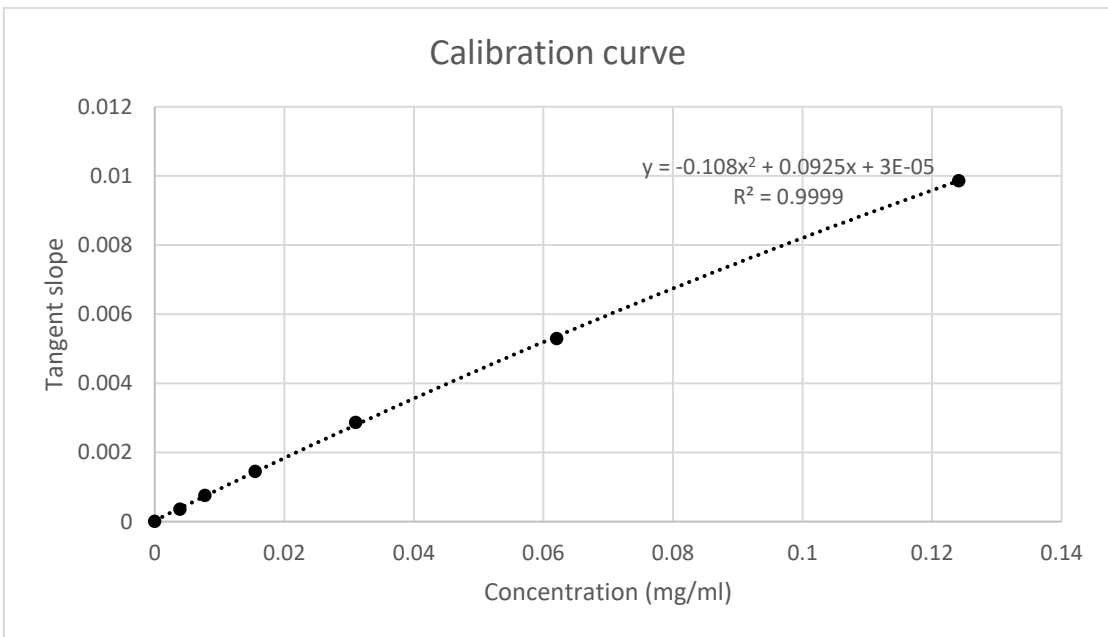
Appendix 3. Residuals for the offline enzymatic assay plotted versus enzyme concentration. There is some systematic variation in the residuals, possibly a second order correlation.



Appendix 4. An example of how the curves received from the online enzymatic assay were treated. **A:** The curve that was received from the calibration curve point with 100 % sample, corresponding to 0.621 mg/ml enzyme. The curve has recurring “pressure dips” at the times when the pump stopped pushing to pull new running buffer. **B:** This illustration is an approximation of how the area used to calculate the enzyme concentration was done. Note that the pressure dips are neglected.



Appendix 5. The glucose concentrations of the batch reactors plotted versus time.



Appendix 6. The optical density at 605 nm for the batch reactors plotted versus time.

Appendix 7. Raw data from the enzymatic assay. Each time represents the measurements of the 96-well plate, and hence, the same position in the matrix contains the same sample at all the different times. The samples are arranged after increasing sampling time, and thus the first sample is found in the top of each column. The calibration curve is instead arranged with falling concentrations meaning that the top values have the highest concentration. Each sample was also added as duplicates, and these wells are always next to each other. **a:** 2019-05-07, 1 mM IPTG, **b:** 2019-05-09, 1 mM IPTG, **c:** 2019-05-15, 0.1 mM IPTG, **d:** 2019-05-17, 0.5 mM IPTG, **e:** 2019-05-21, 1 mM IPTG, **f:** calibration curve, **g:** blank. Values inside the thick line are empty wells.

0.051 ^a	0.051 ^a	0.055 ^b	0.062 ^b	0.049 ^c	0.048 ^c	0.054 ^d	0.069 ^d	0.061 ^e	0.055 ^e	0.175 ^f	0.206 ^f	0 minutes
0.062 ^a	0.064 ^a	0.07 ^b	0.066 ^b	0.053 ^c	0.05 ^c	0.06 ^d	0.056 ^d	0.063 ^e	0.057 ^e	0.108 ^f	0.113 ^f	
0.074 ^a	0.074 ^a	0.075 ^b	0.074 ^b	0.053 ^c	0.05 ^c	0.058 ^d	0.061 ^d	0.075 ^e	0.063 ^e	0.08 ^f	0.091 ^f	
0.087 ^a	0.077 ^a	0.139 ^b	0.124 ^b	0.055 ^c	0.059 ^c	0.069 ^d	0.065 ^d	0.077 ^e	0.086 ^e	0.061 ^f	0.064 ^f	
0.089 ^a	0.083 ^a	0.097 ^b	0.108 ^b	0.063 ^c	0.065 ^c	0.082 ^d	0.077 ^d	0.084 ^e	0.085 ^e	0.068 ^f	0.058 ^f	
0.101 ^a	0.094 ^a	0.039	0.037	0.036	0.037	0.037	0.04	0.103 ^e	0.111 ^e	0.05 ^f	0.052 ^f	
0.044	0.037	0.041	0.04	0.036	0.04	0.041	0.037	0.05	0.04	0.049 ^g	0.047 ^g	
0.044	0.036	0.037	0.039	0.037	0.036	0.036	0.036	0.042	0.04	0.039	0.038	
0.05	0.051	0.058	0.064	0.049	0.048	0.054	0.07	0.063	0.057	0.255	0.295	3 minutes
0.068	0.067	0.084	0.078	0.055	0.05	0.062	0.057	0.066	0.06	0.146	0.155	
0.089	0.087	0.094	0.092	0.054	0.051	0.059	0.063	0.083	0.07	0.097	0.112	
0.111	0.097	0.193	0.174	0.058	0.061	0.071	0.068	0.093	0.109	0.07	0.074	
0.12	0.109	0.132	0.147	0.069	0.072	0.088	0.085	0.106	0.107	0.071	0.062	
0.137	0.13	0.039	0.038	0.036	0.037	0.037	0.04	0.136	0.143	0.053	0.054	
0.044	0.037	0.04	0.04	0.036	0.04	0.042	0.037	0.06	0.04	0.049	0.046	
0.045	0.037	0.037	0.039	0.037	0.036	0.037	0.037	0.042	0.04	0.04	0.038	
0.05	0.051	0.058	0.065	0.049	0.047	0.054	0.07	0.063	0.057	0.279	0.316	5 minutes
0.07	0.069	0.089	0.082	0.055	0.05	0.062	0.057	0.067	0.06	0.158	0.166	
0.093	0.09	0.1	0.098	0.054	0.051	0.059	0.064	0.085	0.073	0.103	0.118	
0.118	0.104	0.209	0.189	0.058	0.061	0.071	0.069	0.098	0.116	0.073	0.077	
0.127	0.117	0.143	0.159	0.071	0.073	0.089	0.086	0.113	0.114	0.073	0.064	
0.146	0.139	0.039	0.038	0.036	0.037	0.037	0.041	0.144	0.152	0.054	0.055	
0.044	0.037	0.04	0.04	0.036	0.04	0.042	0.037	0.06	0.04	0.049	0.046	
0.045	0.037	0.037	0.039	0.037	0.036	0.037	0.037	0.042	0.04	0.04	0.038	
0.05	0.051	0.059	0.064	0.048	0.047	0.054	0.07	0.065	0.057	0.302	0.338	7 minutes
0.072	0.07	0.093	0.086	0.056	0.05	0.061	0.058	0.068	0.061	0.171	0.179	
0.097	0.094	0.107	0.104	0.055	0.051	0.06	0.066	0.087	0.075	0.111	0.124	
0.124	0.11	0.225	0.204	0.059	0.062	0.072	0.07	0.103	0.123	0.076	0.081	
0.134	0.124	0.153	0.17	0.073	0.076	0.091	0.088	0.121	0.121	0.073	0.065	
0.153	0.148	0.039	0.038	0.036	0.037	0.037	0.04	0.152	0.161	0.054	0.055	
0.044	0.037	0.04	0.04	0.036	0.04	0.042	0.037	0.063	0.04	0.049	0.046	
0.045	0.037	0.037	0.039	0.037	0.036	0.037	0.037	0.042	0.04	0.04	0.038	

0.05	0.051	0.06	0.066	0.048	0.047	0.054	0.069	0.065	0.058	0.338	0.373
0.074	0.073	0.099	0.092	0.056	0.05	0.061	0.058	0.069	0.062	0.19	0.197
0.102	0.098	0.116	0.113	0.055	0.052	0.06	0.065	0.09	0.079	0.123	0.134
0.131	0.118	0.248	0.226	0.06	0.063	0.073	0.07	0.109	0.131	0.081	0.085
0.143	0.134	0.168	0.184	0.075	0.078	0.093	0.093	0.132	0.131	0.076	0.067
0.164	0.159	0.039	0.038	0.036	0.037	0.037	0.041	0.162	0.172	0.055	0.057
0.044	0.037	0.04	0.04	0.036	0.04	0.042	0.037	0.063	0.04	0.049	0.045
0.045	0.037	0.037	0.039	0.037	0.036	0.037	0.037	0.042	0.04	0.04	0.038
<hr/>											
0.051	0.051	0.061	0.067	0.048	0.047	0.054	0.069	0.066	0.059	0.395	0.428
0.077	0.077	0.108	0.101	0.056	0.05	0.062	0.059	0.071	0.064	0.221	0.226
0.11	0.106	0.129	0.126	0.056	0.052	0.061	0.067	0.095	0.084	0.14	0.149
0.143	0.13	0.277	0.255	0.062	0.065	0.075	0.072	0.12	0.143	0.089	0.093
0.158	0.15	0.19	0.207	0.079	0.082	0.097	0.098	0.147	0.146	0.08	0.071
0.18	0.176	0.039	0.038	0.036	0.037	0.037	0.041	0.178	0.19	0.057	0.059
0.044	0.037	0.04	0.04	0.036	0.04	0.042	0.037	0.063	0.04	0.049	0.045
0.045	0.037	0.037	0.04	0.038	0.036	0.037	0.037	0.042	0.04	0.04	0.038
<hr/>											
0.051	0.051	0.062	0.068	0.048	0.047	0.054	0.068	0.067	0.061	0.453	0.483
0.081	0.08	0.117	0.109	0.056	0.05	0.064	0.058	0.073	0.066	0.249	0.256
0.118	0.113	0.141	0.139	0.056	0.053	0.062	0.067	0.1	0.089	0.157	0.165
0.155	0.141	0.303	0.281	0.064	0.067	0.076	0.074	0.13	0.152	0.097	0.102
0.174	0.163	0.211	0.229	0.082	0.086	0.101	0.103	0.162	0.161	0.085	0.075
0.199	0.194	0.039	0.038	0.037	0.037	0.037	0.041	0.193	0.206	0.059	0.06
0.044	0.037	0.04	0.04	0.036	0.04	0.042	0.037	0.062	0.04	0.049	0.045
0.045	0.037	0.037	0.04	0.038	0.036	0.037	0.037	0.042	0.04	0.04	0.038
<hr/>											
0.051	0.051	0.064	0.07	0.048	0.047	0.054	0.068	0.069	0.062	0.504	0.531
0.084	0.083	0.125	0.117	0.056	0.05	0.065	0.058	0.074	0.067	0.276	0.282
0.125	0.12	0.153	0.151	0.057	0.053	0.062	0.068	0.105	0.094	0.172	0.179
0.167	0.152	0.328	0.306	0.065	0.068	0.077	0.075	0.138	0.162	0.104	0.109
0.188	0.177	0.23	0.248	0.086	0.09	0.104	0.106	0.177	0.175	0.089	0.079
0.216	0.209	0.039	0.038	0.037	0.037	0.037	0.041	0.209	0.221	0.061	0.062
0.044	0.037	0.04	0.04	0.036	0.04	0.042	0.037	0.062	0.04	0.05	0.045
0.045	0.037	0.037	0.039	0.038	0.036	0.037	0.037	0.042	0.04	0.04	0.038
<hr/>											
0.051	0.051	0.065	0.071	0.048	0.047	0.054	0.068	0.07	0.063	0.551	0.578
0.086	0.086	0.133	0.124	0.057	0.05	0.066	0.058	0.076	0.069	0.301	0.309
0.129	0.127	0.163	0.162	0.057	0.053	0.062	0.068	0.11	0.099	0.185	0.193
0.178	0.163	0.354	0.33	0.066	0.069	0.078	0.075	0.148	0.172	0.111	0.116
0.201	0.19	0.249	0.267	0.089	0.093	0.106	0.109	0.19	0.188	0.093	0.083
0.229	0.224	0.039	0.038	0.037	0.037	0.037	0.041	0.224	0.236	0.063	0.064
0.044	0.037	0.04	0.04	0.036	0.04	0.042	0.037	0.062	0.04	0.05	0.045
0.045	0.037	0.037	0.04	0.037	0.036	0.037	0.037	0.042	0.04	0.04	0.038

0.051	0.051	0.066	0.072	0.048	0.047	0.054	0.068	0.071	0.064	0.595	0.622
0.089	0.089	0.141	0.132	0.057	0.05	0.067	0.058	0.077	0.07	0.325	0.333
0.137	0.135	0.174	0.173	0.058	0.054	0.062	0.067	0.114	0.104	0.199	0.206
0.186	0.174	0.377	0.354	0.068	0.07	0.078	0.076	0.156	0.181	0.118	0.123
0.212	0.202	0.267	0.285	0.092	0.096	0.108	0.111	0.204	0.201	0.097	0.086
0.243	0.235	0.039	0.038	0.037	0.037	0.037	0.041	0.238	0.252	0.065	0.066
0.044	0.037	0.04	0.04	0.036	0.04	0.042	0.037	0.062	0.04	0.05	0.045
0.045	0.037	0.037	0.04	0.037	0.036	0.037	0.037	0.042	0.04	0.04	0.038
35 minutes											
0.051	0.051	0.067	0.073	0.048	0.047	0.054	0.068	0.072	0.065	0.639	0.666
0.092	0.092	0.148	0.14	0.057	0.05	0.067	0.058	0.079	0.071	0.348	0.357
0.145	0.144	0.184	0.184	0.058	0.054	0.063	0.068	0.119	0.108	0.211	0.219
0.199	0.185	0.4	0.378	0.069	0.072	0.079	0.076	0.165	0.19	0.124	0.13
0.226	0.216	0.284	0.305	0.095	0.1	0.11	0.113	0.218	0.214	0.101	0.09
0.258	0.251	0.039	0.038	0.036	0.037	0.037	0.041	0.25	0.268	0.066	0.067
0.044	0.037	0.04	0.04	0.036	0.04	0.042	0.037	0.062	0.04	0.051	0.045
0.045	0.037	0.037	0.04	0.037	0.036	0.037	0.037	0.042	0.04	0.04	0.038
40 minutes											
0.051	0.051	0.068	0.074	0.048	0.047	0.054	0.068	0.073	0.067	0.682	0.708
0.095	0.095	0.156	0.147	0.057	0.05	0.067	0.058	0.08	0.073	0.372	0.381
0.151	0.154	0.195	0.195	0.059	0.055	0.063	0.068	0.124	0.113	0.223	0.232
0.208	0.196	0.427	0.401	0.071	0.073	0.08	0.077	0.173	0.2	0.13	0.137
0.24	0.229	0.304	0.324	0.098	0.103	0.113	0.115	0.232	0.227	0.104	0.093
0.272	0.266	0.039	0.038	0.037	0.037	0.037	0.04	0.264	0.281	0.068	0.069
0.044	0.037	0.04	0.04	0.036	0.04	0.042	0.037	0.062	0.04	0.05	0.045
0.045	0.037	0.037	0.04	0.037	0.036	0.037	0.037	0.042	0.04	0.04	0.038
45 minutes											
0.051	0.051	0.07	0.075	0.048	0.047	0.054	0.068	0.074	0.068	0.725	0.749
0.097	0.097	0.163	0.156	0.058	0.051	0.067	0.058	0.082	0.074	0.394	0.404
0.157	0.166	0.208	0.206	0.06	0.055	0.063	0.068	0.129	0.117	0.234	0.245
0.219	0.206	0.454	0.428	0.072	0.074	0.08	0.078	0.182	0.209	0.137	0.143
0.253	0.242	0.321	0.349	0.102	0.106	0.115	0.117	0.247	0.24	0.108	0.096
0.286	0.28	0.039	0.038	0.037	0.037	0.037	0.041	0.281	0.297	0.07	0.071
0.044	0.037	0.04	0.04	0.036	0.04	0.042	0.037	0.062	0.04	0.051	0.045
0.045	0.037	0.037	0.04	0.038	0.036	0.037	0.037	0.042	0.04	0.04	0.039
50 minutes											
0.051	0.051	0.071	0.077	0.048	0.047	0.054	0.068	0.075	0.069	0.765	0.788
0.1	0.1	0.17	0.165	0.058	0.051	0.068	0.058	0.083	0.076	0.416	0.426
0.164	0.176	0.218	0.218	0.06	0.056	0.064	0.068	0.133	0.122	0.246	0.257
0.229	0.217	0.486	0.453	0.073	0.076	0.081	0.079	0.189	0.217	0.143	0.149
0.265	0.256	0.339	0.366	0.105	0.11	0.12	0.121	0.261	0.252	0.111	0.1
0.3	0.294	0.039	0.038	0.036	0.037	0.037	0.041	0.294	0.314	0.072	0.072
0.044	0.037	0.04	0.04	0.036	0.04	0.042	0.037	0.062	0.04	0.05	0.045
0.045	0.037	0.037	0.039	0.037	0.036	0.037	0.037	0.042	0.04	0.04	0.038
55 minutes											

0.05	0.051	0.072	0.078	0.048	0.047	0.054	0.068	0.076	0.07	0.803	0.823	60 minutes
0.103	0.103	0.179	0.172	0.058	0.051	0.069	0.059	0.085	0.077	0.437	0.448	
0.17	0.182	0.23	0.231	0.061	0.056	0.064	0.068	0.138	0.126	0.258	0.268	
0.239	0.228	0.504	0.48	0.075	0.077	0.082	0.08	0.198	0.226	0.149	0.156	
0.277	0.269	0.359	0.383	0.109	0.114	0.125	0.125	0.275	0.266	0.114	0.103	
0.314	0.308	0.039	0.038	0.037	0.037	0.037	0.041	0.309	0.329	0.073	0.074	
0.044	0.037	0.04	0.04	0.036	0.04	0.042	0.037	0.062	0.04	0.051	0.045	
0.045	0.037	0.037	0.04	0.038	0.036	0.037	0.037	0.042	0.04	0.04	0.038	
0.059	0.068	0.206	0.206	0.065	0.056	0.075	0.079	0.175	0.177	1.774	1.701	18h 31 minutes
0.359	0.394	0.856	0.846	0.076	0.074	0.078	0.076	0.241	0.274	1.663	1.584	
0.713	0.897	1.119	1.137	0.134	0.126	0.113	0.1	0.577	0.562	1.152	1.16	
1.02	1.092	1.817	1.805	0.267	0.262	0.186	0.176	0.945	1.036	0.695	0.71	
1.158	1.255	1.537	1.63	0.479	0.515	0.379	0.391	1.268	1.253	0.422	0.406	
1.252	1.272	0.037	0.038	0.037	0.037	0.037	0.041	1.311	1.399	0.232	0.232	
0.044	0.035	0.037	0.044	0.036	0.04	0.042	0.037	0.058	0.04	0.055	0.046	
0.045	0.038	0.035	0.039	0.038	0.036	0.037	0.037	0.043	0.04	0.04	0.041	