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RECOVERY OF ENZYMES FROM INDUSTRIAL FERMENTATION AND DETERMINATION OF ACTIVITY ASSAY

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ENTSYYMIEN TALTEENOTTO TEOLLISESSA FERMENTOINNISSA JA AKTIIVISUUSANALYYSIN MÄÄRITYS

Opinnäytetyön aiheena oli luoda MetGen Oy:n tuottamalle entsyymille standardoitu aktiivisuusanalyysi, johon määritettiin käytettävä lämpötila, pH, substraatti sekä puskuriliuos. Entsyymi tuotettiin kahdessa eri tilavuudessa: yhdessä litrassa sekä 250 litrassa. Jokainen fermentointi suoritettiin itsenäisesti alkuvalmisteluista tuotteen talteenottoon saakka. Lopulta analysoitavaksi kerättiin kuusi fermentoinnin lopputuotetta: neljä yhden litran ja kaksi 250 litran fermentoinneista.

Aktiivisuusanalyysi suoritettiin käyttämällä kolmea kaupallista ja kolmea itse valmistettua puskuriliuosta, joiden pH-arvot vaihtelivat välillä 4-6. Analyysin lämpötilana käytettiin kolmea vaihtoehtoa: huoneenlämpötila, 30 °C ja 40 °C. Analyysin substraattina toimi ABTS, jolla aikaansaatiin mitattava värireaktio, jonka absorbanssia mitattiin ajan funktiona. Näiden tulosten perusteella luotiin absorbanssin kasvukaaviot jokaiselle näytteelle sekä laskettiin vertaileva aktiivisuusarvo vertaamalla saatuja tuloksia tunnettuun standardiin.

Aktiivisuusanalyysi antoi selkeitä ja toistettavia tuloksia, joiden perusteella jokaiselle fermentoinnin lopputuotteelle laskettiin standardiin verrattu aktiivisuusarvo. Tuloksissa oli nähtävissä, miten erilaiset olosuhteet vaikuttivat entsyymin aktiivisuuteen. Kaikkien saatujen tulosten perusteella on tehty MetGen Oy:lle ehdotus mitatun entsyymin aktiivisuusanalyysin standardiolosuhteista. Yritykselle on myös tehty ehdotus jatkomittausten suoritusta varten, jotta analyysistä saadaan mahdollisimman tarkkoja vertailuarvoja entsyymin tuleviin käyttöolosuhteisiin verrattuna.

ASIASANAT:

Aktiivisuusmääritys, absorbanssi, entsyymi, entsyymiaktiivisuus, fermentointi

BACHELOR´S THESIS | ABSTRACT

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The aim of the experiment presented in this thesis was to create a standard enzyme activity assay for a produced enzyme. The enzyme was independently produced six times after learning the fermentation procedures and suitable parameters for the assay were defined based on the results of this experiment. The enzyme itself was produced in a mutated *Escherichia coli* in two different volumes: one liter and pilot-scale. The assay was conducted with final end of fermentation samples from all fermentations.

The activity assay was conducted with six different buffer solutions, three commercial and three self-prepared ones. The buffer solutions were adjusted to different pH, ranging from 4 to 6. In addition, three different temperatures were applied: room temperature, 30 °C and 40 °C. The substrate of the assay was ABTS. The assay was carried out in 96 well plates by measuring the absorbance of the samples. The green colored reaction of ABTS dissociation was measured at 405 nm wavelength, and an activity value was calculated by comparing the obtained values to a known standard.

The measurements gave clear and reproducible values, which were used to calculate the activity values. There was variation between different buffers, pH and temperatures, as expected, and based on these results, a suggestion for the oncoming assay parameters was made. It is recommended that the standard be replaced by more suitable standard for this enzyme. Also, a suggestion to conduct extra assays with other possible environmental factors, in which the enzyme can be applied to, has been given to MetGen.

KEYWORDS:

Activity assay, absorbance, enzyme, enzyme activity, fermentation

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GLOSSARY

ABTS	2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
DO	Dissolved oxygen
GSIII	Golden Standard III, a known enzymatic standard for enzyme activity assays
MZ	MetZyme®, general product name for MetGen’s enzymes, refers to enzyme used as a positive control in the thesis
OD	Optical density

1 INTRODUCTION

The research in this thesis aimed to produce an enzyme with MetGen's fermenters and to create a standard protocol on its activity assay. Within the assay, certain factors will be set based on the results

This study was conducted at MetGen, a biotechnology company located in Kaarina. MetGen is a company that produces enzymes for industrial use, e.g. for the wood and paper industry. (MetGen 2016)

Commercial-use enzymes are produced from animal, plant and microbial sources. Microbial enzymes have an advantage over the other two groups in that larger-scale production is easier with current fermentation technology. It is also easier to improve their enzyme production compared to animal or plant based enzymes. Thanks to recombinant technology, it is now possible to produce more difficult animal and plant source enzymes much easier. Recombinant techniques have made it easier to change the producing cell to focus on the production of the desired enzyme. Improving the enzyme producing strain is one of the most important phases in production improvement. (Stanbury *et al*. 1995, 2)

Enzyme activity assays are methods for visualizing the activity of the enzymes. In recent years, a multitude of assays has been developed for research and industrial use. (Reymond *et al*. 2008, 34) The assays always aim to determine the amount of enzyme or identify the enzyme's presence in a sample. This means that they are either quantitative or identification assays. Enzymes are easy to determine by their reactions and the components they produce. This is used in assay planning to develop a suitable assay for a particular enzyme. (Bisswanger 2014, 42)

The aim of this study was to produce an enzyme for industrial use at two different scales: 1 liter and pilot-scale ferment volumes. Samples from the fermentations will be analyzed with enzyme activity assay to determine the enzyme's activity. The focus was on the assay factors producing the most reliable and

reproducible results for both absorbance values and comparable activity values. With the results, standard factors were determined for future assays of this enzyme.

2 FERMENTATION

The term fermentation comes from the Latin word *fervere* which means to boil. Currently in industrial microbiology, fermentation can mean any kind of production by micro-organism mass culture. Fermentation can be divided into five different groups based on what the end product is: production of microbial cells or biomass, production of enzymes, production of microbial metabolites, production of recombinant products and modification of an added product - or transformation process. (Stanbury *et al*. 1995, 1)

Using fermentation to produce a product has certain advantages. Some complex molecules and optically active compounds, such as vitamins, amino acids or enzymes, are impossible to manufacture chemically due to economic aspects. Producing food industry products (e.g. beer) is considered better to use fermentation procedures over chemical production. Fermentations mostly use renewable materials and rarely petrochemicals. This usually makes it more economical choice for production. However, there are also some disadvantages on using fermentation in production. Even if the fermentation waste products are not toxic, they require extensive waste treatment, which requires a lot of resources. In microbial fermentations, contamination by a competitive strain may occur, sterilization is required and the process must be contained to avoid contamination. This is a risk that can in worst case ruin large quantities of product and cause significant economic losses. The recovery of the fermented product is often laborious and expensive compared to chemically produced products. Also, environmental factors (pH, temperature, nutrient levels, etc.) must be strictly controlled when microorganisms are used. Otherwise these factors may slow the process or, in the worst case, kill the organisms. (Reddy *et al*. 2012, 7-8)

Microbial, animal and plant sources have been used for commercial production of enzymes. Of these three groups, microbial enzymes have an advantage as they are easy to produce in large amounts with current fermentation techniques. It is also much easier to enhance the yield of a microbial system than that of an

animal or plant system. With the development of recombinant DNA technology, it has become possible to synthesize animal origin enzymes into the microorganisms. This has considerably facilitated the production of animal based enzymes. The production of the enzymes in the microorganisms is closely controlled and to improve productivity, these controls are altered to find the best combination. Strain improvement is extremely important in the improvement of the production. (Stanbury *et al*. 1995, 2-3)

The fermentation process can be divided into six different parts, regardless of its type. These parts are media formulation, sterilization of the equipment and the medium, pre-culturing a pure, active strain for the actual fermentation, growing the strain to produce the desired product in the actual fermentation, recovery and purification of the product and waste removal. (Stanbury *et al*. 1995, 9-10)

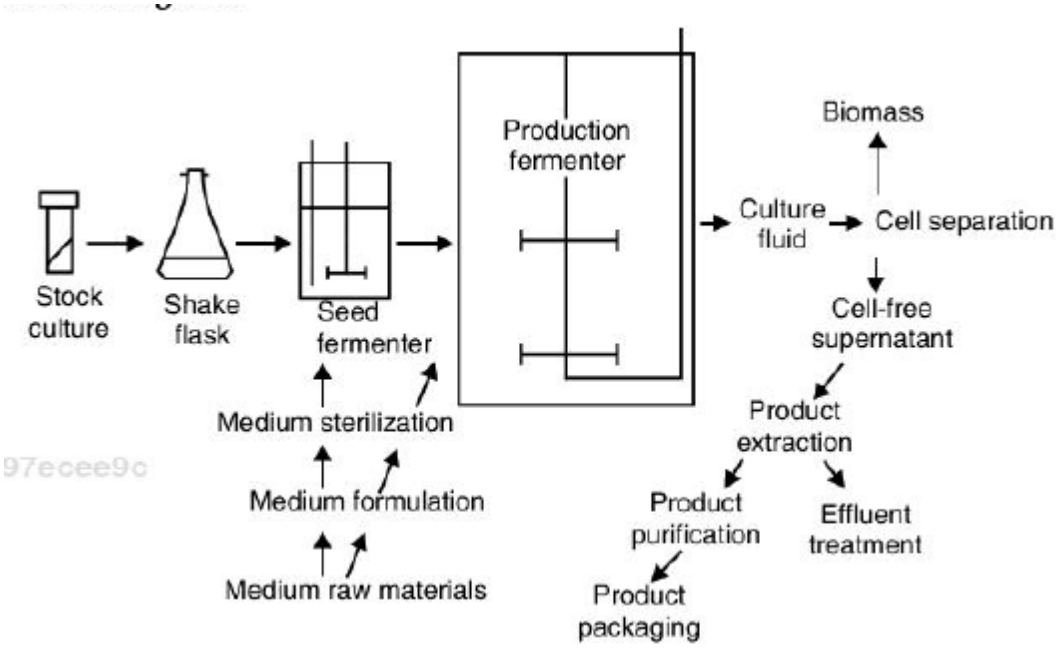

Figure 1. General representation of fermentation process. (Stanbury *et al*. 1995, 10)

In addition to the six steps, there are other important measures required to achieve a proper fermentation. For instance, the process should be analyzed and studied constantly to achieve improvements. Also, before the actual fer-

mentation process starts, the production organism must be identified, isolated and to produce the enzyme in industrial quantities. The process for extracting the final product must be ready before starting the production. The whole process must be thought through and continual improvement should be aimed at throughout the process. (Stanbury *et al*. 1995, 10)

The culture fermenters are classified into three groups based on their operation. These groups are batch, fed-batch and continuous operation. In the batch process, all required nutrients and other necessities for cell growth are present in the medium before inoculation. The only added components are oxygen by aeration and acid and base for pH regulation. The fermentation ends when the limiting nutrient is completely consumed and the growth ceases. Batch processes are mostly used in research and development when the growth and the limiting nutrients are studied but they are also used in production. Fed-batch fermentation resembles batch fermentation but one or more nutrients are added during the fermentation, either continuously or at certain time intervals. Fed-batch processes enable longer fermentations than batch processes, as the added nutrients usually are the limiting ones that run out during batch processes. This means better growth and higher productivity. In a continuous process, the medium is pumped into the fermentation at a constant rate and the cultured medium is pumped out so that the volume remains constant inside the vessel. A continuous process can increase growth and productivity even better than a fed-batch process. Its drawback, however, is the great space it needs due to all the piping. Also, it is inevitable that at some point, the continuous process is contaminated, as more and more material moves through the reactor, multiplying the risk of contamination. This is why fed-batch operation is usually preferred to continuous operation, especially if space is a limiting factor for the user. (Vogel & Todaro 2014, 3-5)

Fermentation performance can be influenced by a number of different factors, e.g., pH, dissolved oxygen and carbon dioxide, temperature, medium composition, choice of operation, shear rates inside the fermenter and mixing. Changes in these factors affect the fermentation rate, yield of product, product properties

(smell, taste, texture, etc.) and toxin generation. This is why formulating the medium is important, as it is vital to have an appropriate medium with functioning instruments to control the various factors. Even with good control of said factors, if the medium is not suitable, the yield will be affected by it. The medium's priority is to provide the growing compound with enough nutrients (e.g. vitamins, nitrogen, and carbon). Some fermentation may need some specific sources for these nutrients and the ratio between nitrogen and carbon must be fixed and controlled. Only by understanding the biochemistry of the fermentation can the optimal medium formulation be achieved. Otherwise, changes in the medium will affect the growth in a negative way. The concentrations of some nutrients may have to vary during the fermentation, whereas avoiding some concentrations is needed. Also economic reasons, such as cost and availability, have an impact on the choice of medium. (Batt & Tortorello 1999, 664)

3 ENZYMES AND ACTIVITY ASSAYS

In 2014, the worldwide market of enzymes was valued at 4.2 billion U.S. dollars (MarketsAndMarkets 2015). Enzymes function as biological catalysts for many important chemical reactions. Their purpose is to catalyze the reaction and to control the rate of the reaction. Mostly this means accelerating a reaction which would otherwise be slow. As enzymes catalyze and thus change the rate of the reaction, the enzymes themselves stay intact. They are proteins that have the ability to change the velocity of a specific chemical reaction. The substance which is affected by the enzyme is called *substrate*. For example, trypsin is a protein that catalyzes peptide bond hydrolysis of proteins and polypeptides. In this example, the polypeptides are the substrates for trypsin. (Mathews *et al*. 2000, 360-361)

3.1 Enzyme assays and parameters

Assays are tools for visualizing the activity of an enzyme. Many different assays have been developed in recent years. Many of these assays are intended for the use of the “white biotechnology”. There, the need for selective enzymes has been great to achieve environmentally and economically friendly processes. (Reymond *et al*. 2008, 34)

The purpose of an enzyme activity assay is normally either to determine the enzyme amount in a sample or to identify a certain enzyme; its absence or presence in the sample. To determine the amount of the enzyme, quantitative methods must produce as accurate data as possible, whereas for identification, a simple positive/negative end result is enough. Enzymes can be easily distinguished from each other and other components by their reactions and the products they produce. Usually the amount of end product exceeds the amount of the enzyme and this can cause difficulties when attempting to reverse the reaction back to the amount of enzyme. (Bisswanger 2012, 42)

There are many different enzyme assay procedures recorded in databases and books, but even with recorded observations of a procedure, the result of an assay is not guaranteed. The activity of a particular enzyme depends on a multitude of factors. Understanding these factors is of fundamental importance for reliable results. In 1913, Leonor Michaelis and Maud Menten showed that the activity of an enzyme is dependent on pH and temperature and the strength of the ions. The only way to compare different assays is to pay careful attention to these factors. However, the vast diversity of enzymes makes this extremely difficult. Enzymes themselves are very sensitive substances. They are naturally not found in high amounts and their activity is often detected only in their most favorable conditions. Most enzymes work best within an average physiological range but there are some which require extreme conditions to work properly, e.g. high temperature or high/low pH. (Bisswanger 2012, 42)

Whereas most of the required conditions can be found in the literature, it is always important to tune these conditions based on the enzyme and the observations of that particular enzyme. This must be taken to consideration especially when a new assay for a new enzyme is developed. Still, what is most important in enzyme activity assays is that they enable simple observation of the reaction. Enzymes turn substrate into a product and usually by monitoring the amount of product formed the enzyme activity against the time consumed can be measured. As with the formation of the product, also its decline can be a measured sign during the reaction. When there are two products formed or more than one substrate involved, only one reaction component, which is most likely to be the easiest to determine, is sufficient for measurement. (Bisswanger 2012, 43)

The detection of the enzyme reaction can, at its simplest, be an observation of color change in the sample. This will give the advantage of getting results without any measuring instruments. However, this way no data is obtained, nor experiment reproducibility. An instrument will enable the data and the reproducibility. The instrument, e.g. a photometer or a colorimeter, will determine the intensity of the color and thus give data about the reaction. Spectrophotometers are commonly used in determining enzymes as they have a broad spectrum of dif-

ferent wavelengths of light, especially in the UV range, and most of them are user-friendly and easy to handle. They are also very resistant to disturbances during measurement. Fluorimetry is another way to measure and it is much more sensitive and accurate, but there are only a few substrates that emit fluorescence. The instruments themselves require expertise from the user as they are more difficult to use compared to a regular spectrophotometer. Other optical possibilities are turbidimetry (e.g. starch particle degradation) and luminometry (ATP reactions). Besides optical methods, electrochemical methods can also be used, especially pH dependent reactions, e.g. the forming of an acid or base. As the enzymes' optimum pH range is narrow, the process of electrochemical determination must be controlled by countering the increase or decrease of the pH by appropriate solution. By following the addition of this neutralizing solution, the progress of the reaction can be monitored. (Bisswanger 2012, 44)

The mentioned methods all allow continuous, time-dependent enzyme reaction monitoring. For that, they are termed *continuous assays*. Continuity is important when determining enzyme activity and the rate of the reaction. It also permits to observe influences and disturbances caused by other molecules and chemical reactions. With it, controlling the course of the reaction (progress curve) is possible. As the substrates deplete during the later stage of the reaction, the reaction itself slows down and, at some point, ends. That is why, when calculating the velocity, only the linear part of the curve should be taken into account. To determine reaction velocity, the reaction itself must be stopped when a certain time point is reached and the amount of formed product analyzed with an appropriate method, e.g. HPLC (*stopped assay*). Compared to the continuous assays, stopped assays give only a single measured point and the curve velocity is calculated from the slope of the curve, connecting to the point of a blank before the start of the reaction. In this method, it is not guaranteed that the measurement is on the linear part of the curve and that is why control values must be measured at different time points during the reaction. This kind of procedure is time consuming, laborious which is why continuous assays are preferred. Stopped assays are as good as continuous assays in qualitative measurement,

but only the presence of enzyme activity should be detected. (Bisswanger 2012, 44-45)

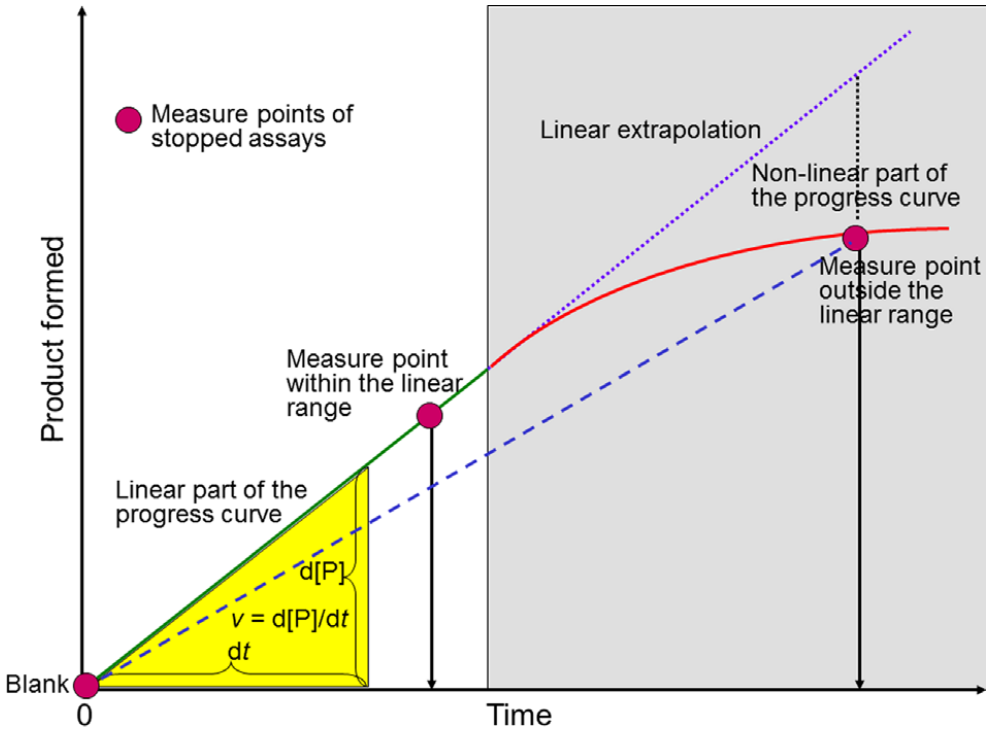

Figure 2. Progress curve of an enzyme reaction. (Bisswanger 2012, 44)

3.1.1 pH

Enzyme activity is highly dependent on the pH. The activity curve usually forms a normal distribution-like curve, as the activity is low in the acid and base region and higher in-between them. (Bisswanger 2012, 45)

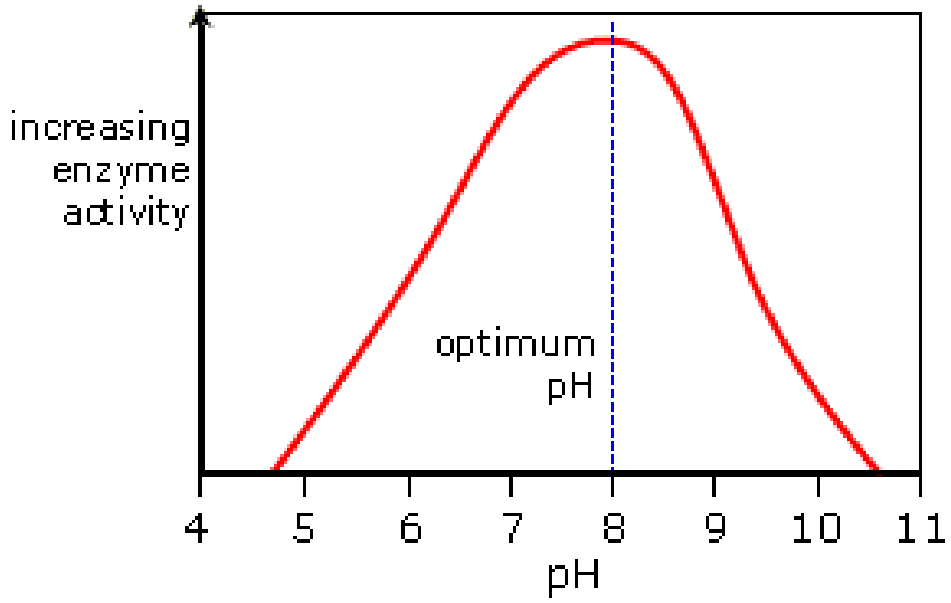

Figure 3. The pH optimum curved based on the pK_a values. (BBC 2011)

This is affected by two different reasons: protonation stage of the cofactors and amino acids in the reaction and native, 3D protein structure of the enzyme. Protonation can be reversed but repairing the damages in the protein structures is not reversible. Protonation of one functional group can be a promoting factor for the activity whereas it can be a decreasing factor with another functional group. For this reason, two titration curves starting from different ends of the pH range are used to form the bell-like curve. Using the pK_a values the optimum pH be determined from the curve. This pH is the point where reaction velocity (V_{max}) is the highest and it should be then used as the assay's standard pH. Many enzymes have their pH optimum in the range of pH 7-8. (Bisswanger 2012, 45)

Not all enzymes have pH optimum in average physiological range. For example, enzyme of the human stomach, pepsin, has its optimum at pH 2, which is considerably more acidic than pH 7-8. Also, there are enzymes that have their optimum in alkaline conditions (alkaline phosphatase, pH 10.5). The activity should be tested at the pH optimum conditions. (Bisswanger 2012, 45)

The enzymes should be stored, as well as tested, at the optimum pH, as they are usually stable in those conditions. The pH must be maintained as the optimum, or close to it, when the enzyme sample is taken from storage to be prepared for the assay. Extreme pH conditions should be avoided, as in such conditions, the enzymes tertiary structure is damaged irreversibly. This is a time-dependent reaction, but when pH reaches values less than three or above 11, the reaction is almost instantaneous. (Bisswanger 2012, 45)

3.1.2 Temperature

The effect of the temperature to the enzymes activity is considerable. When temperatures rise, the activity increases. When the temperature reaches and passes the maximum value, the enzyme activity decreases. This behavior is called *temperature optimum,* but this does not mean that there exists an optimum temperature for enzyme activity. Normally, all chemical reaction velocities tend to increase with higher temperature, two to three times per 10 °C rise can be used as an empirically received rule. This rule applies to the enzyme activities as well. The only limiting factor to this progress is the denaturation temperature. What also needs to be taken into consideration is the 3D structure of the enzymes. The structures themselves are sensitive and are destabilized at high temperatures and, by that, causing denaturation. This factor limits the velocity and is the reason for velocity decrease at high temperatures. The denaturation is dependent on both time and temperature, so no accurate temperature can be determined for enzyme denaturation, but the higher the temperature, the faster the denaturation is. Also, the pretreatment of the enzymes affects the maximum temperature. (Bisswanger 2012, 47)

Testing the enzyme activity's correlation against temperature is needed to establish correct assay temperature. By using the Arrhenius diagram (Figure 4) with the enzyme and temperature, a line should show linearity in lower temperatures, indicating stability, and in denaturation temperatures and close to that, divergence from the line. The assay temperature must be on the line's linear

part, even if the enzyme does not possess its maximum activity in that temperature. (Bisswanger 2012, 47)

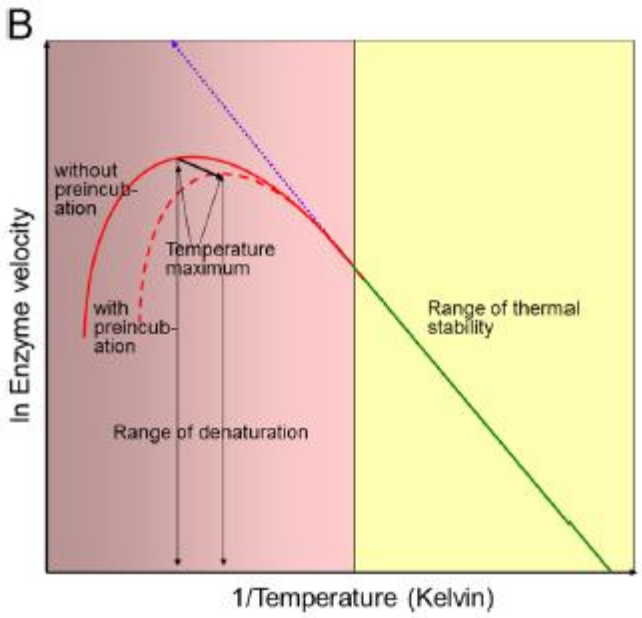

Figure 4. Arrhenius diagram, depicting enzyme activity's dependence over temperature. (Bisswanger 2012, 47)

All in all, there is no universal temperature that works well with all different enzymes. Still, some temperatures have been considered as "general" options for various reasons. For most of the assays, three different temperatures are commonly used. First is the physiological temperature, 37 °C. For many enzymes, it matches their natural temperature. Compared to the two other temperatures, at 37 °C the enzyme usually shows the highest activity which enables using the lowest amounts of enzyme in the assay. It is also closest to the denaturation range, so proper temperature control and regulation is required. As most of the assay samples are stored in low temperatures, they must be let to warm up to the right temperature. Most mistakes in the assays that are performed at 37 °C are somehow connected to improper temperatures of the samples, e.g. the operator has not waited long enough for their warming from storage temperature. This will show a decrease in enzyme activity and incorrect results are gained.

For this reason, a separate system for heating is recommended to heat the samples. (Bisswanger 2012, 47-48)

By using room temperature, the required heating is eliminated from the assay process. However, the room temperature is not a constant value, as it differs between different spaces and also in the same room with the changes of window or door opening/closing, air conditioning and sunlight radiation. Because of these reasons, a slightly higher temperature, 25 °C, is considered a better option. Temperature maintenance is easier and faster, and the changes in temperature do not show high variations from the actual results. Compared to higher temperatures, the activity is clearly lower and the amounts of enzymes required are higher, but its convenience and robustness are the reasons why this 25 °C is recommended for most assays, as long as there are enough enzymes to use. (Bisswanger 2012, 48)

Third frequent temperature is 30 °C. This temperature is somewhat of a compromise between 37 °C and 25 °C. The temperature gives higher activity results than at 25 °C but with less temperature regulation than at 37 °C. And in some cases, none of these three are applicable, as some of the enzymes are only active in very high temperatures. These thermophilic enzymes show low results in the range of aforementioned temperatures, so their testing should be executed in their natural temperatures. Even if these enzymes do work in extreme temperatures, they also have a denaturation point, and that should be taken into consideration during testing the enzyme. Using high temperatures provide a series of obstacles for the assay. Finding thermostable reagents for the assay can be difficult and the oxidation is accelerated in those temperatures. (Bisswanger 2012, 48)

3.1.3 Solvents, substrates and cofactors

For most of the assays, water is appropriate solvent. Some enzymes require organic solvents when their crucial component is insoluble to water. Then a solvent that is miscible with water must be used, e.g. acetone or ethanol. The mix-

ture's aliquot is added to the assay, and is should stay dissolved. To avoid any precipitation, the amount of the non-water-dissolving component should be as low as possible or the amount of the organic solvent should be high enough to keep the substrate soluble. The solubility is connected to the temperature, so by manipulating the temperature, the component can be dissolved. This is a problem if the component is dissolved in the assay temperature, but precipitated in storage temperature. Also, the concentration of the organic solvent will affect the enzyme itself. Either it can denature the enzyme completely or it can affect its activity. If organic solvents are used in the assays, their concentrations must always be kept constant to avoid any deviations in the results because of their concentration variations. (Bisswanger 2012, 46-47)

Substrates, cofactors and co-substrates are, besides the enzyme itself, the most important parts of the activity assay. Their quality and stability are extremely important and these must be demanded by the operator. In many cases, substrates are not defined to specified enzyme. Most enzymes are not specific to one substrate, but accept others as well. The same goes for cofactors. Essential parts of the covalent reactions are divalent cations, and they can be replaced by other divalent cations. Physiological substrates are changed into artificial ones, if the physiological one is not stable enough for the assay. When enzyme accepts multiple substrates, the most optimum one must be chosen. As results with different substrates are not comparable, the K_m value of the substrates must be compared. The lower this value is the better. Sometimes, the optimum substrate is not chosen, as the other substrate can be more stable, soluble, accessible and available for the assay. To get comparable data, the same substrates must be used every time. The only time this may be changed is if the assay is tested with another substrate, e.g. if the new substrate is more stable or economical for the assay. (Bisswanger 2012, 48)

4 METHODS

The process of the work can be divided roughly into two separate sections: fermentation and enzyme activity analysis. Both fermenter sizes used Medium 1 (same consistency in reagents) in the fermentations.

4.1 Fermentation

The target enzyme was produced in two different sized fermenters: Sartorius manufactured 1 liter glass fermenters and pilot-scale fermenter. Both scales of fermentations are fed-batch, meaning that they are batched with initial medium and supplements but also, there are also other supplement flows going into the fermenter while it is running.

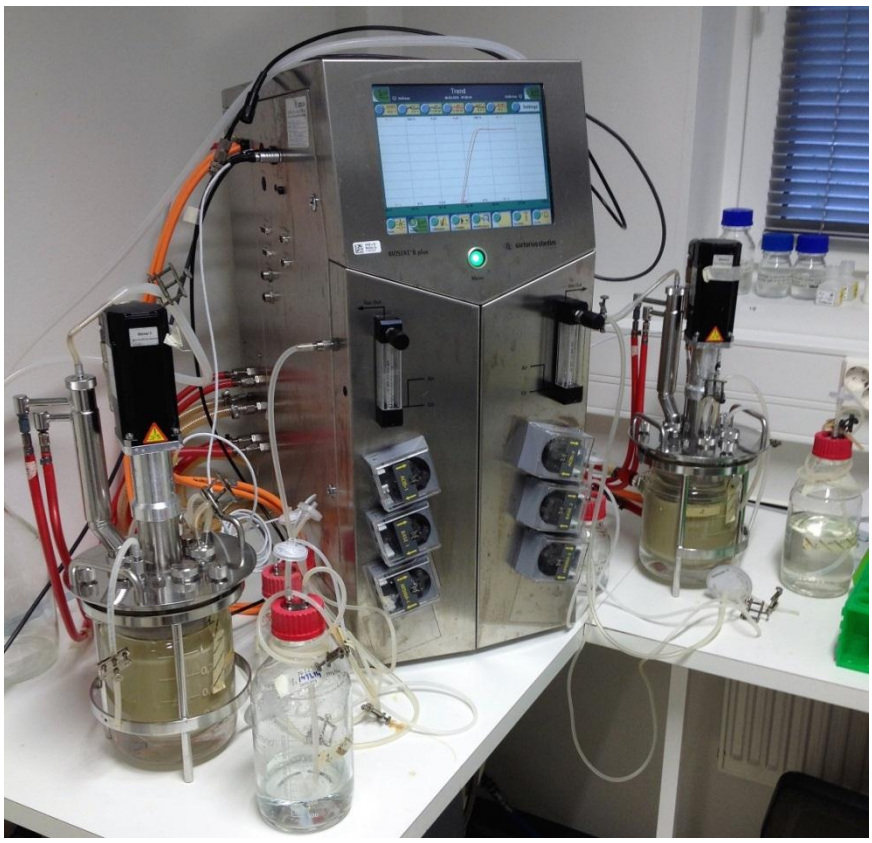

Figure 5. A picture of the two Sartorius 1 liter fermenters and their DCU.

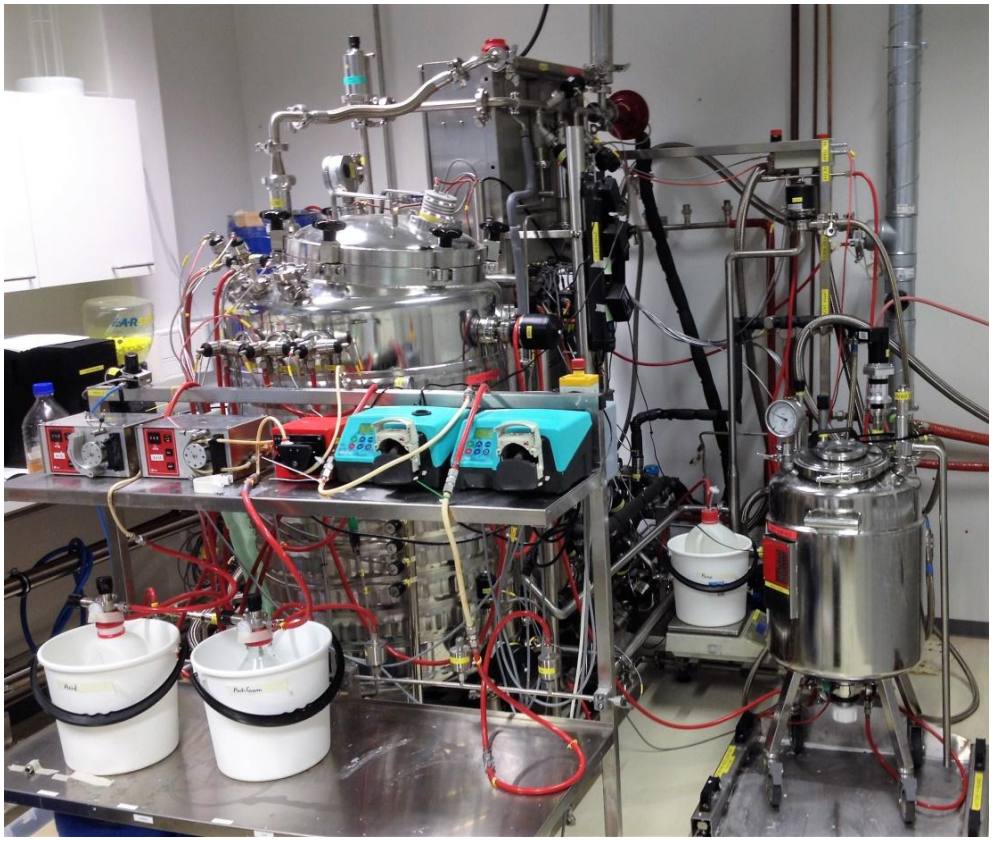

Figure 6. A picture of pilot-scale fermenter (left) and mobile tank unit (right).

The 1 liter fermenters were prepared by first assembling them, adding the Medium 1 in them and then sterilized at 121 °C for 15 min. Temperature, pH and DO sensors were all attached in the fermenters during sterilization. The pH sensors were calibrated before the sterilization. The DO sensors were calibrated after the sterilization, when the fermenters were placed in the fermentation laboratory. Batch glucose was added to the medium via syringe and tube, so that the cells had an energy source. pH was set to the starting point and the cells were inoculated into the fermenters and then the batch phase was started. The medium and the added glucose were optimized so that the cells were left to grow overnight and the glucose feed was started next morning, when there was a clear signal that the batch glucose had run out. The feed glucose was added over time with a programmed feed profile. Hourly samples were taken to monitor the growth inside the fermenter. From these samples, the concentration of glucose and lactate were measured with measurement strips. The tip of the strips were doused into the sample and the devices calculated the concentra-

tions from the liquid. Also, the optical density was measured with spectrophotometer. Small samples were stored in a cold room for further testing. After the fermentation, lysis buffer was added into the fermenters. The buffer was left to affect the medium overnight in a shaker and the next morning it was heat treated, cooled and harvested. Formulation buffer was added to the end products and then they were stored in + 4 °C cold room for analysis.

With pilot-scale fermenter, the preparations were started by sterilizing the mobile feed tank which was batched with the feed glucose. Simultaneously, the fermenter's pH sensors were calibrated and the fermenter was batched with hot water and the Medium 1 reagents. While the medium was mixing, supplements were connected to the supplement lines of the fermenter. When all the supplements were attached, the fermenter was sterilized. After the sterilization, a small amount of glucose was pumped from the mobile tank to the fermenter to act as the batch glucose. The DO sensor was calibrated and the pH adjusted to match the starting pH of the fermentation. Before starting the fermentation, the PID-control values were checked from the control computer.

The inoculation of the cells was performed in the evening to time the start of the feed phase to early next morning. The cells used in the pilot-scale were cultivated in shake flasks. The shake flasks were shaken with 250 rpm at 30 °C. The inoculation was performed with syringe via septum.

The feed phase was started when the glucose in the fermenter had run out, as in the smaller fermenters. The glucose was added according to the programmed feed profile. Hourly samples and measurements were performed as with the 1 liter fermenters. After the fermentation, lysis buffer was added into the fermenter and it was left there overnight. Next morning, the solution was heat treated, cooled and then ultra-filtrated to get the end product into smaller, more concentrated volume. Formulation buffer was added into the concentrated product and then it was stored in + 4 °C cold room for analysis.

4.2 Enzyme activity assay

The assay was performed with two different buffer sets: commercial produced Britton & Robinson buffer and Sodium acetate buffer prepared at MetGen. With Britton & Robinson, three solutions with different pH were used: pH 4, 5 and 6. With Sodium acetate solutions, used pH values were 4, 4.8 and 5. The pH 4 and 5 Sodium acetates were 25 mM solutions and the pH 4.8 a 50 mM solution. The pH range was decided based on initial pH testing done by MetGen's Genetic Department. The substrate of the assay was ABTS. GSIII and MZ were used as a standard and a positive control. The lysis buffer used in the samples was the negative control.

Each sample was centrifuged at 7500 g for 5 min. Then, a 4 ml of the supernatant was pipetted to be used in the experiment. For the pilot-scale samples, a 1:10 dilution was used, as they had been concentrated with ultra-filtration. For the positive control, a 1:50 dilution was used, as its known activity was high. The rest of the samples were used as undiluted. All samples were let to reach the room temperature before pipetting them to the plates. A total of six samples were used, plus a standard, positive and negative control.

When all buffers and samples were pipetted, the plates were sealed with sealing plastic and transferred to incubation at appropriate temperatures. Room temperature samples were left on the laboratory table and the 30 °C and 40 °C samples were put into incubators.

The room temperature samples were transferred to analysis after 30 min of incubation. ABTS was added to the plates with row pipette. Plates were then placed into the spectrophotometer and absorbance was measured every 30 seconds starting from 30 sec after adding the ABTS and ending at 5 min after ABTS addition. The 30 °C and 40 °C samples were let to incubate for 60 min and then they were transferred to the ABTS addition and spectrophotometer. The spectrophotometer was heated up to match the temperatures the plates were incubated in. After all measurements, the results were exported into a flash drive and analyzed.

5 RESULTS AND DISCUSSION

The absorbance values of the triplicate samples were calculated to achieve an average value for absorbance. If large deviation occurred in the triplicates, the deviating one was ruled out. The absorbance values were plotted into a graph for comparison. An example of the graph is presented in Figure 7. All the result graphs are presented in Appendix 1.

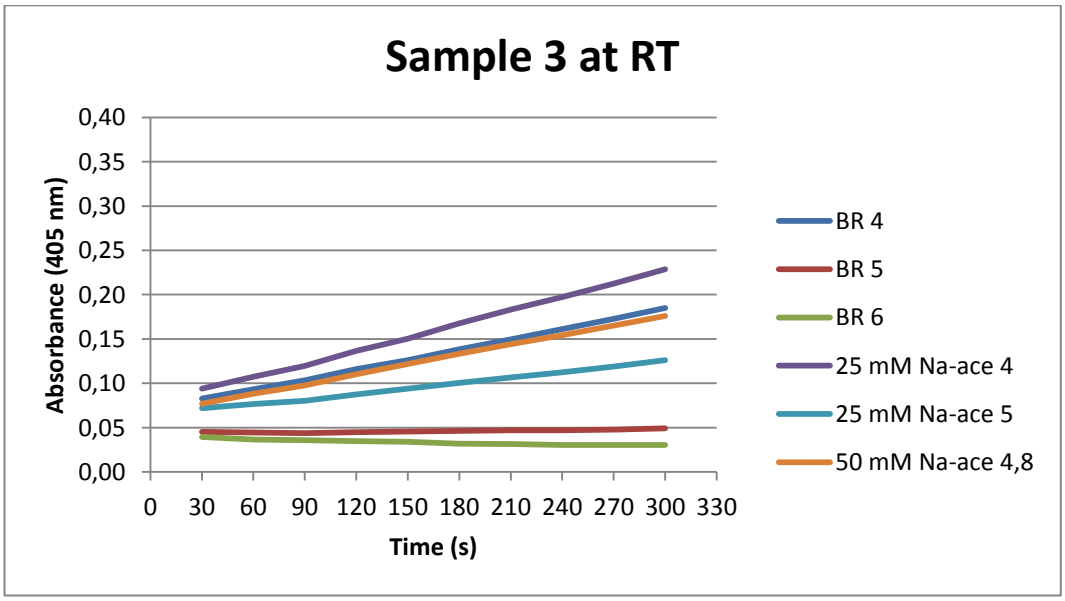

Figure 7. Sample 3 absorbance values at room temperature.

For all the fermenter samples, the highest absorbance values - and their growth - were with 25 mM Sodium acetate at pH 4 as the buffer. Britton & Robinson buffer at pH 4 and 50 mM sodium acetate at pH 4.8 also showed promising results, whereas Britton & Robinson at pH 5 or 6 did not show growth in any of the fermenter samples. 25 mM sodium acetate at pH 5, however, seemed to have slower growth than the other sodium acetate buffers.

These results would suggest that pH 6 is not suitable for the assay in the conditions applied, as no positive results were obtained from the assay. It seems that pH 6 is already so different from the pH optimum for this assay that the enzyme is affected negatively by it. pH 4 seems to be the most suitable pH for this as-

say, as with both sodium acetate and Britton &Robinson buffers, the development of the absorbance during the assay is desirable. In addition, the 50 mM sodium acetate at pH 4.8 showed almost as good results as the pH 4 buffers but the pH 5 Britton & Robinson buffer, on the other hand, was not. It could be that the change of 0.2 units in pH is within the area where the activity starts to decrease rapidly, as depicted in Figure 3. Furthermore, the composition of the two different buffers may have different effects on the activity. That, however, seems unlikely, because absorbance values of the pH 4 Britton & Robinson and 25 mM sodium acetate were still quite close to each other. What is more, the higher concentration of the pH 4.8 sodium acetate compared to the pH 5 sodium acetate may be the factor that explains the better results. This could be verified with further testing.

The absorbance at different temperatures showed slight increase in most samples. Figure 8 shows the differences with Sample 3 in 25 mM sodium acetate at pH 4.

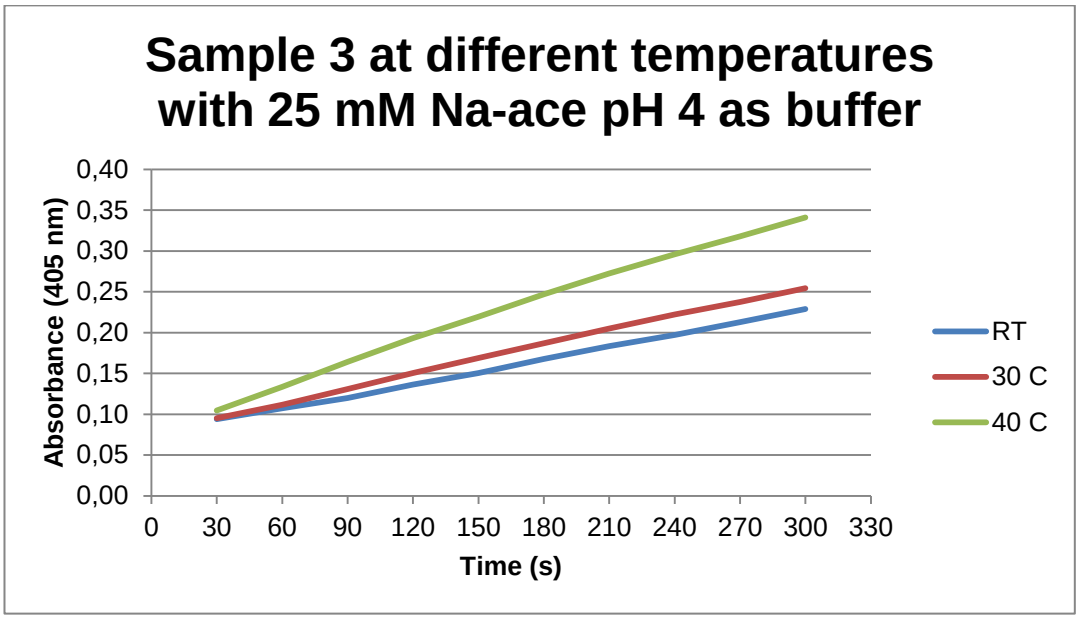

Figure 8. Development of absorbance at different temperatures.

Few of the samples had quite similar results at room temperature and 30 °C. This is because during the assay phase, the room temperatures varied between 23 °C and 25 °C, which is a smaller difference to the 30 °C than the difference between 30 °C and 40 °C. Still, the fluctuation in room temperature during the assay is notable. This can be seen clearly as small differences in the results.

From the average absorbance values, a slope (OD/s) was calculated for the samples. From these slope values, a comparison to the GSIII slope was calculated. With the calculated ratio and the standard's known activity, standard-relative activity values were calculated for each sample. All graphs are presented in Appendix 1.

The results show that the activity is highest with the two sodium acetate buffers: the 25 mM at pH 4 and 50 mM at pH 4.8. The Britton & Robinson at pH 4 shows the next highest values, but it is still much lower. The rest of the buffers exhibited smaller activity results. Of course, these values are compared to the standard, so it cannot be said that there is no activity at all in those conditions. This is proven with the increase of the absorbance values with other buffers except the Britton & Robinson with pH 6.

There are substantial differences between different temperatures, which even more confirms the fact that enzyme activity is dependent on ambient temperature (Bisswanger 2012). The activity in the 50 mM sodium acetate buffer seems to increase linearly, doubling the activity from room temperature to 40 °C. With 25 mM sodium acetate, the difference between room temperature and 30 °C is not so high, with only a 12 % difference, but at 40 °C, the activity had increased substantially. From the calculated results, it can be interpreted that the enzyme's activity is highest at 40 °C. Further experiments should be conducted to see if the activity would rise further at higher temperatures.

6 CONCLUSION

The aim of this experiment was to study the fermentation processes used in MetGen Oy and with that knowledge perform a wide range enzyme activity assay for the produced enzyme. Based on that assay, standard factors were created for prospective assays.

The beginning of this experiment started with studying how to operate the fermenters. As there was no prior experience from using fermenters other than as a part of one laboratory course, the process to get familiarized with the machines and protocols took time. Eventually, enough experience was achieved to operate both fermenter scales and became adept to produce independently any desired enzyme. It was time consuming and an important part of this experiment and unfortunately it is not easily presented on paper.

All six fermentations that were included in the experiment were successful, as the measurements conducted during the fermentation (OD, concentrations of glucose and lactate) suggested that proper cell growth and enzyme production were achieved. By analyzing the fermentation samples, the production of the enzyme was shown to have been on a good level. The analytical part of the study was conducted by using a ready-made protocol basis for the experiment. However, some alterations were made because the experiment was broader than what the protocol provided instructions for. Otherwise, the protocol was followed.

The risk of errors occurring during the experiment is quite limited because the fermenters and the spectrophotometer were automated machines. The greatest error factor may be the operator. The timing of the assay measurements was up to the operator, so the small deviations in the times between each measurement may differ due to the human reaction time factor. Also, during the preparation of the assay samples, errors in the heterogenetic composition of the samples may affect the results. The pipetted volumes were tens of microliters, and thus, even a small change in the sample composition could have had an impact

to the results. Errors might also have occurred in the calculations. There was a great deal of results to analyze and multiple calculations were performed. There could have been a miscalculation during the analysis of the numbers, which could have affected the final results. (Hiltunen *et al*. 2011, 38-42)

These factors were limited to a minimum with precise actions. Each of the assay samples was prepared and pipetted from the supernatant of the fermenter samples. The samples were centrifuged at 7500 g for 10 min before pipetting, which should have cleared the supernatant enough such that no cell debris was present in the assay samples. In pipetting, automatic pipettes and reverse pipetting were used to ensure the right volume of liquid into the assay mix. This should have excluded pipetting error during the preparation of the samples. Also, the pipettes were properly calibrated and maintained. (Hänninen *et al*. 2010, 66-67)

From the tested pH, temperatures and buffers, different results were calculated. Based on the results, the assay was evaluated and standard factors for oncoming assays of this enzyme are proposed:

As the reaction buffer, the 25 mM sodium acetate is recommended. Even if the 50 mM solution gave equally high or higher comparable enzyme activity values, the 25 mM solution showed better results on the absorbance values. It is better to work with clearly increasing absorbance and then convert to activity values. A pH value of 4 is now recommended as the standard pH for the assay. Absorbance in both pH 4 buffers showed clear increase in absorbance and ease converting that into an activity value. The temperature for the assay is to be 30 °C. It requires less energy to maintain during the assay than 40 °C and there will be less fluctuation percentage-wise if there is contact with room temperature. 30 °C also removes the effects of the temperature variations that are present at room temperature. The substrate of the assay will be ABTS as clear and measurable color reaction was obtained with it. It also did not seem to affect the assay negatively in any way so there is currently no need to change it.

The experiment itself was challenging because many new things needed to be learnt from the preparation of the fermentation medium and operating the fermenters to calculating the activity values. Still, the experiment was successful and allowed the determination of standard factors for the activity assay. During the experiment, important guidance was provided by Bas Romein. I would like to thank him for everything he did to enable this experiment, Taina Lahtinen for help with the fermenters and also MetGen Oy in general for the opportunity to work on this Bachelor's Thesis in their facilities.

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Graphs of the assay

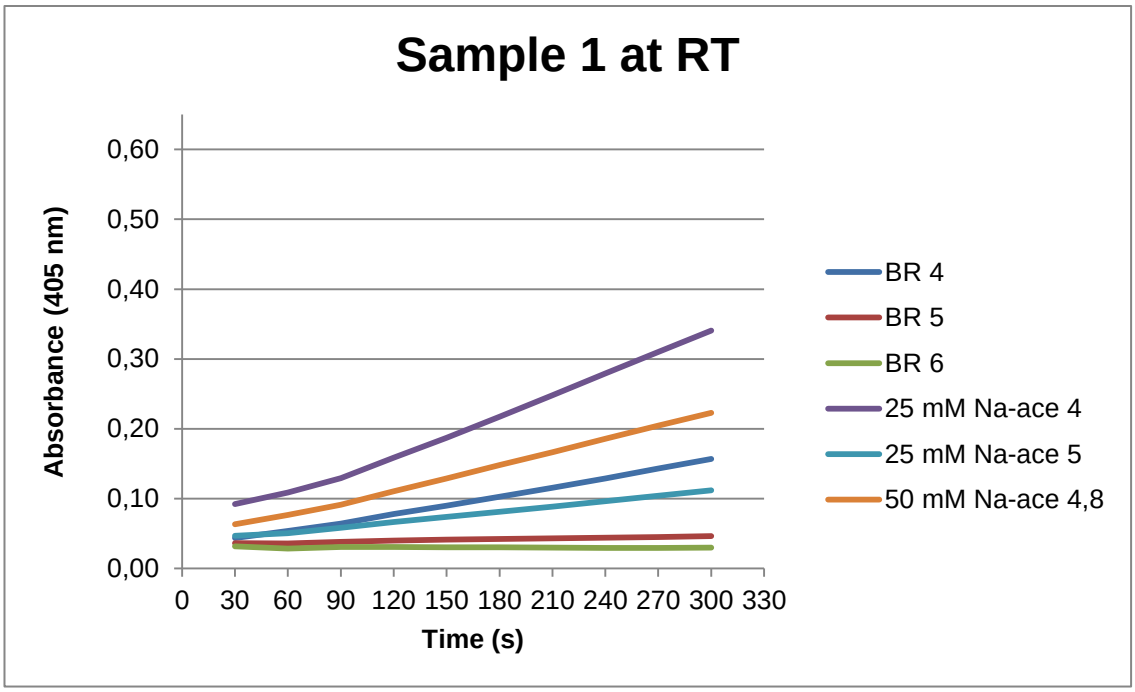

Figure 9. Sample 1 absorbance values at room temperature.

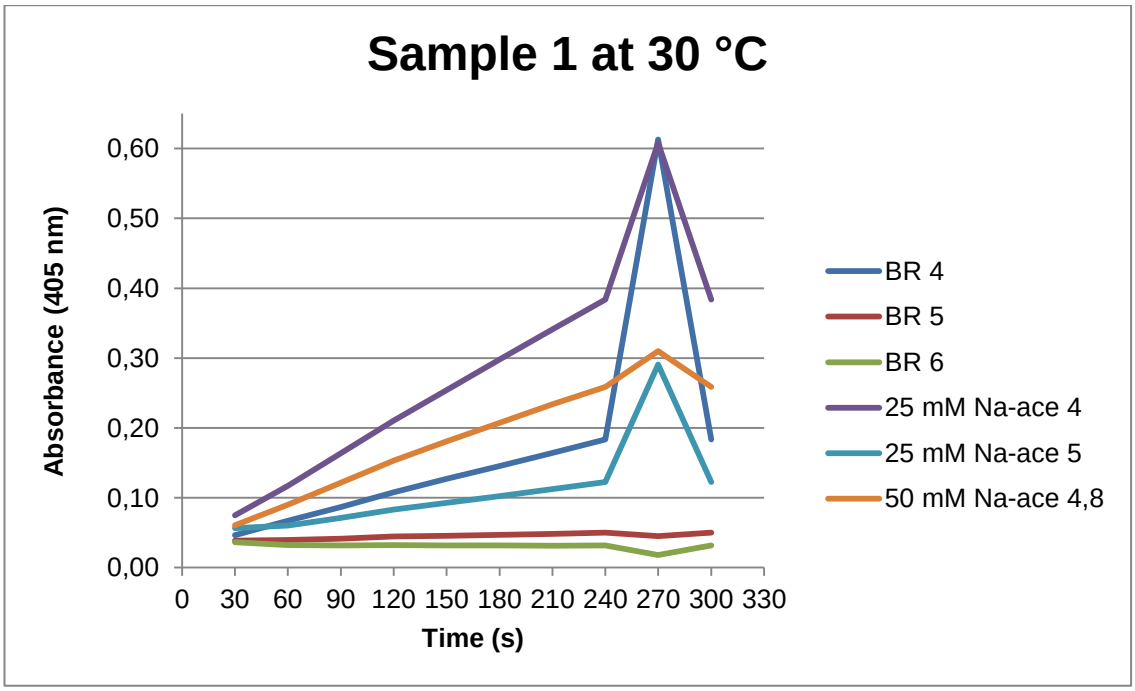

Figure 10. Sample 1 absorbance values at 30 °C.

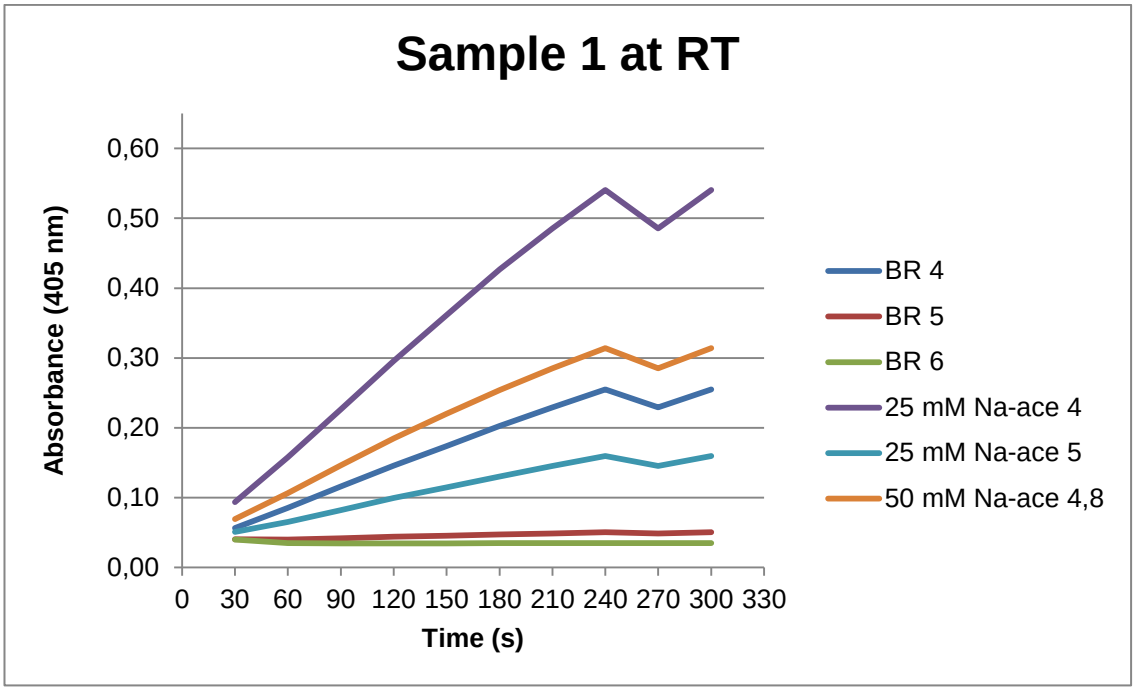

Figure 11. Sample 1 absorbance values at 40 °C.

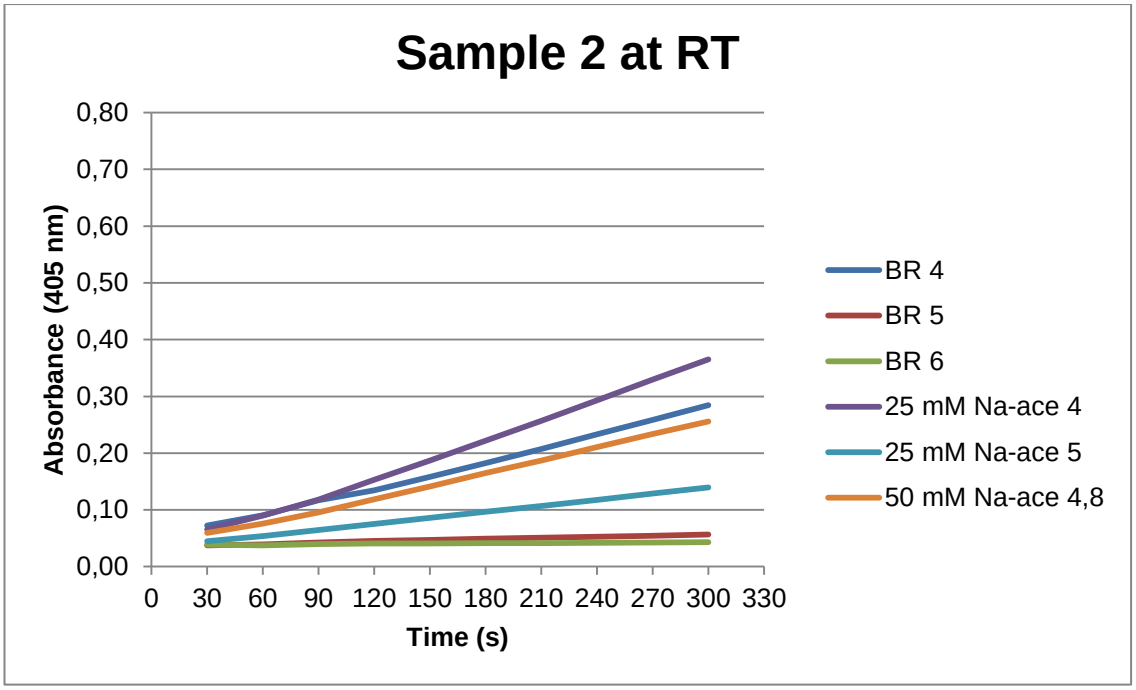

Figure 12. Sample 2 absorbance values at room temperature.

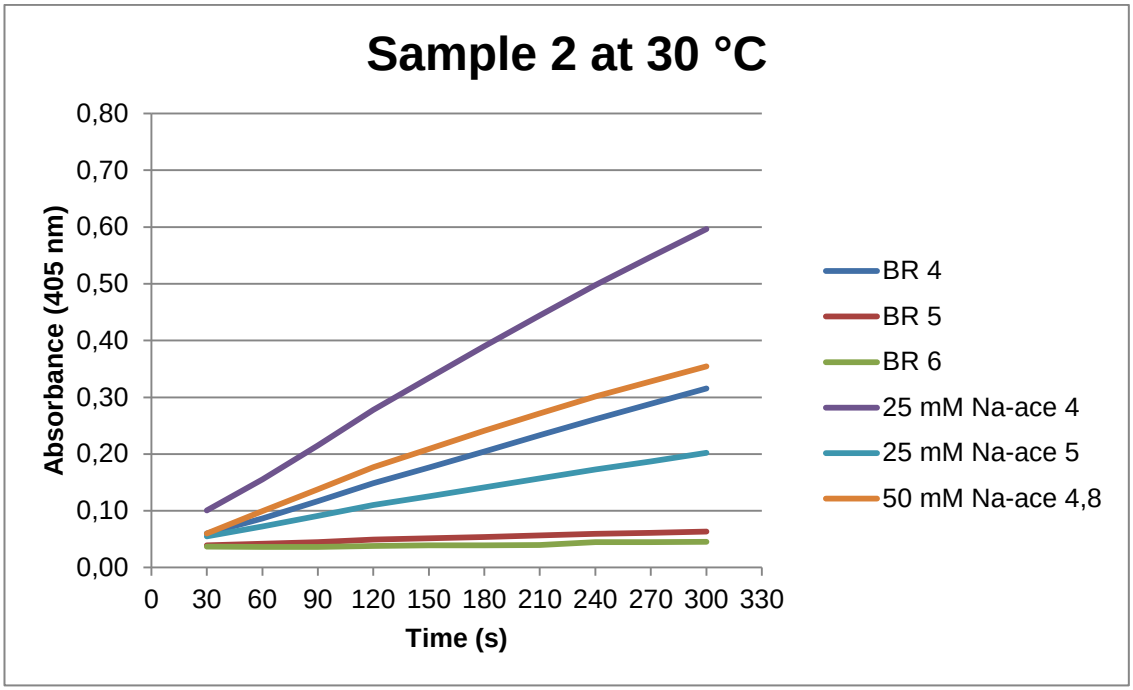

Figure 13. Sample 2 absorbance values at 30 °C.

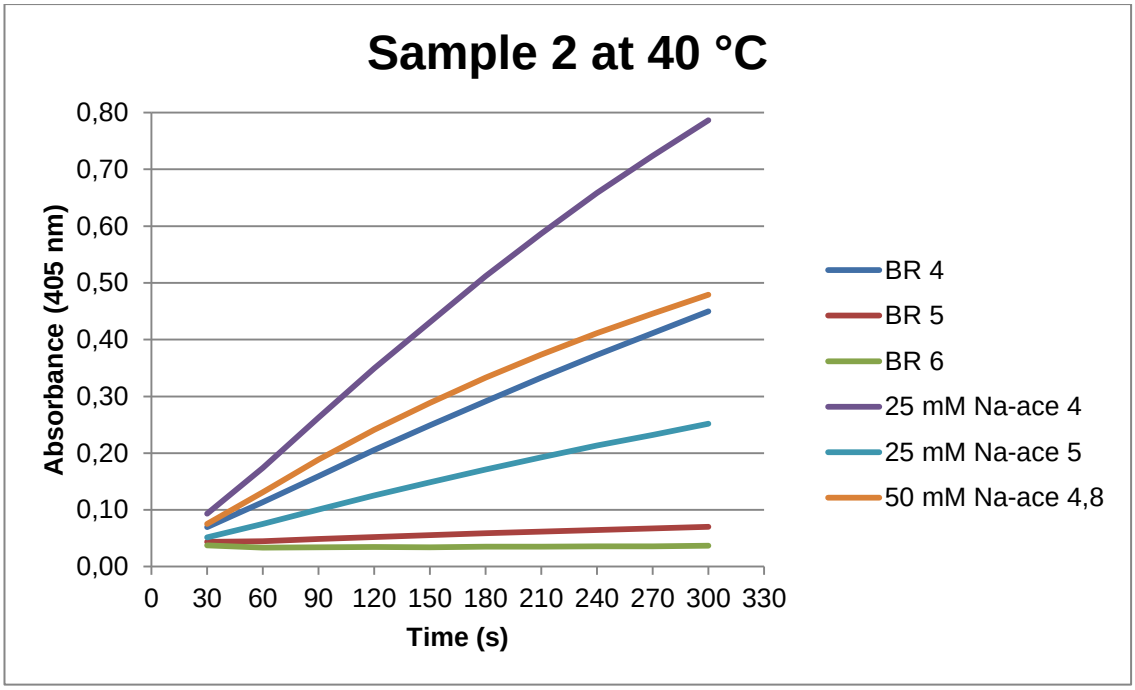

Figure 14. Sample 2 absorbance values at 40 °C.

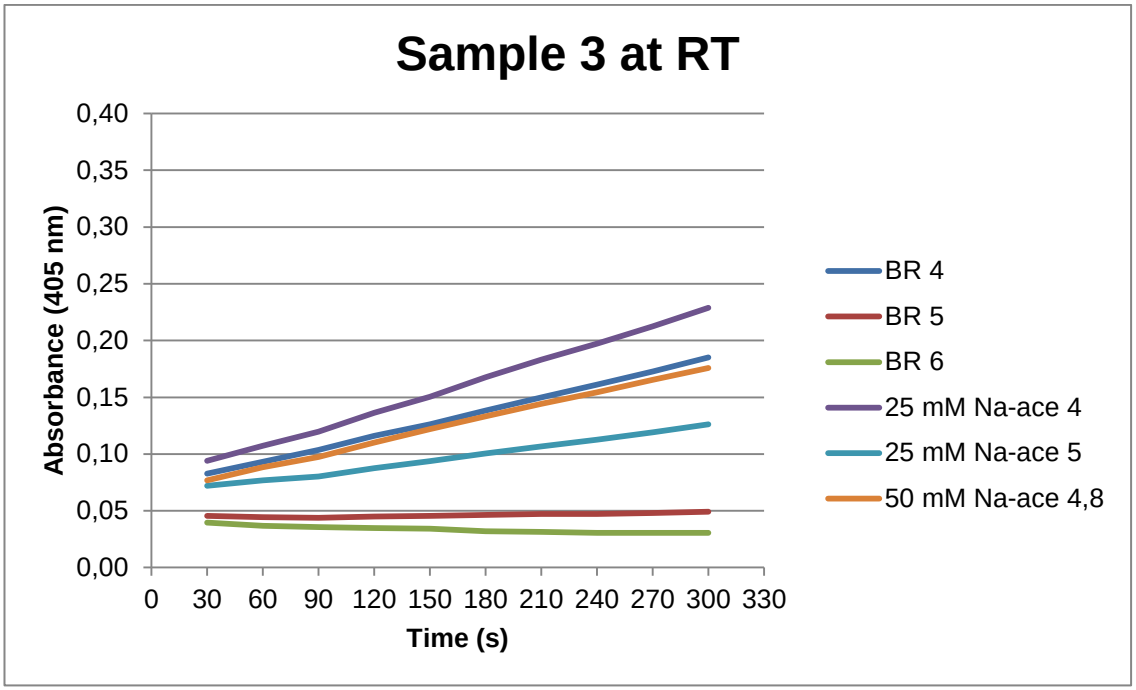

Figure 15. Sample 3 absorbance values at room temperature.

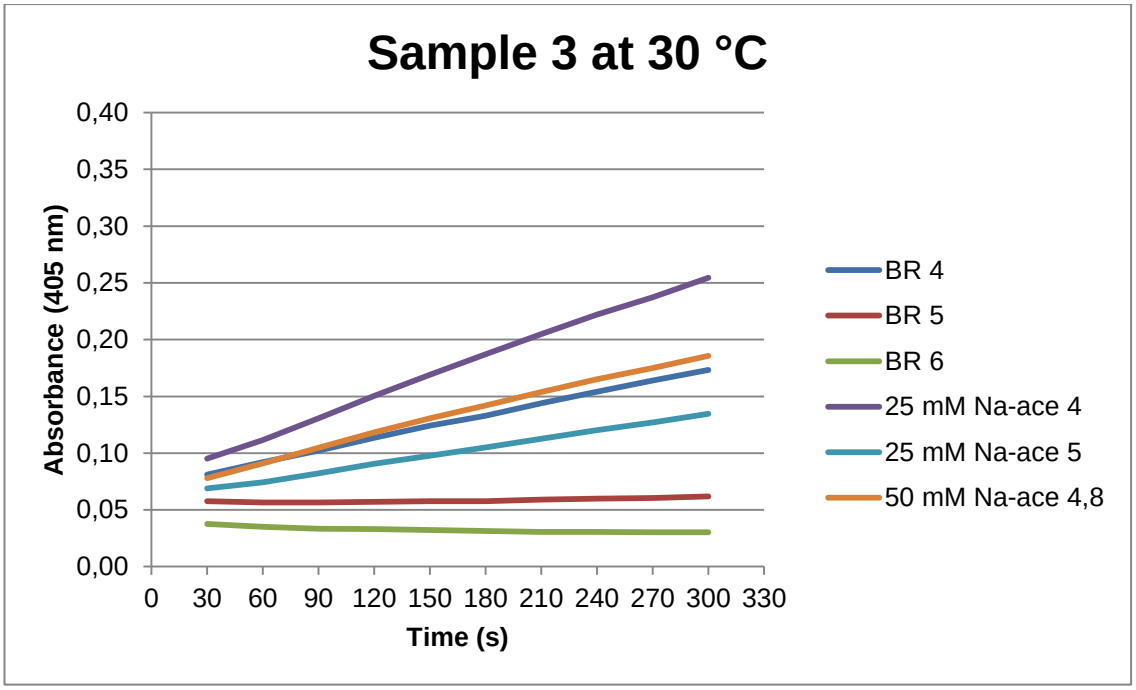

Figure 16. Sample 3 absorbance values at 30 °C.

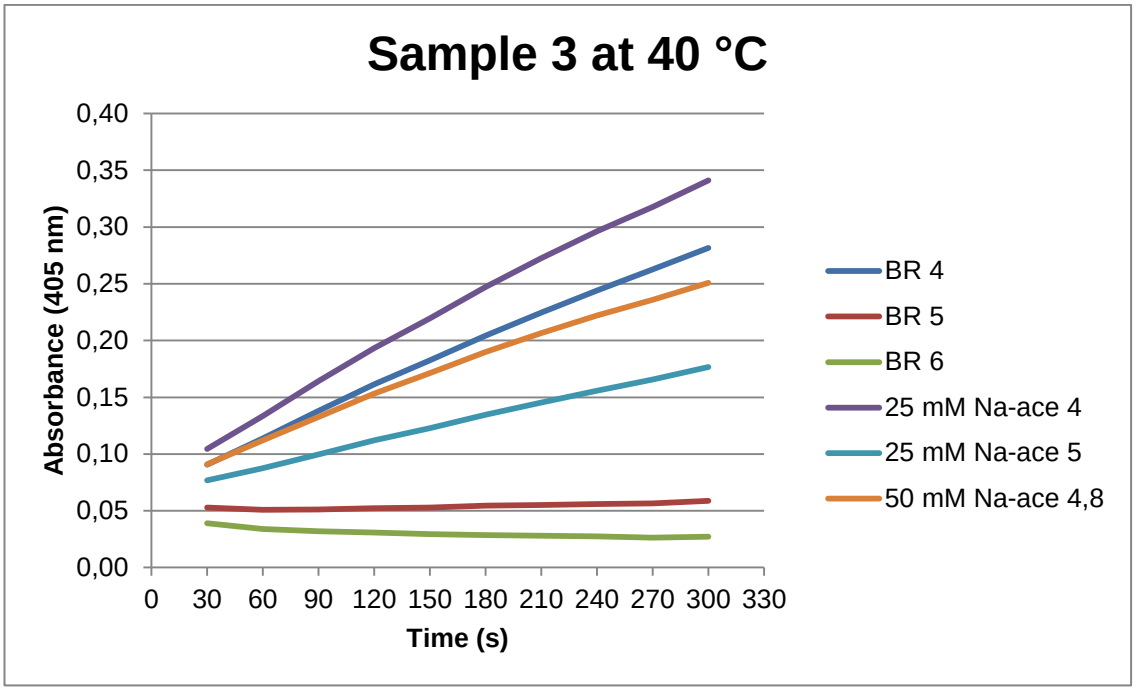

Figure 17. Sample 3 absorbance values at 40 °C.

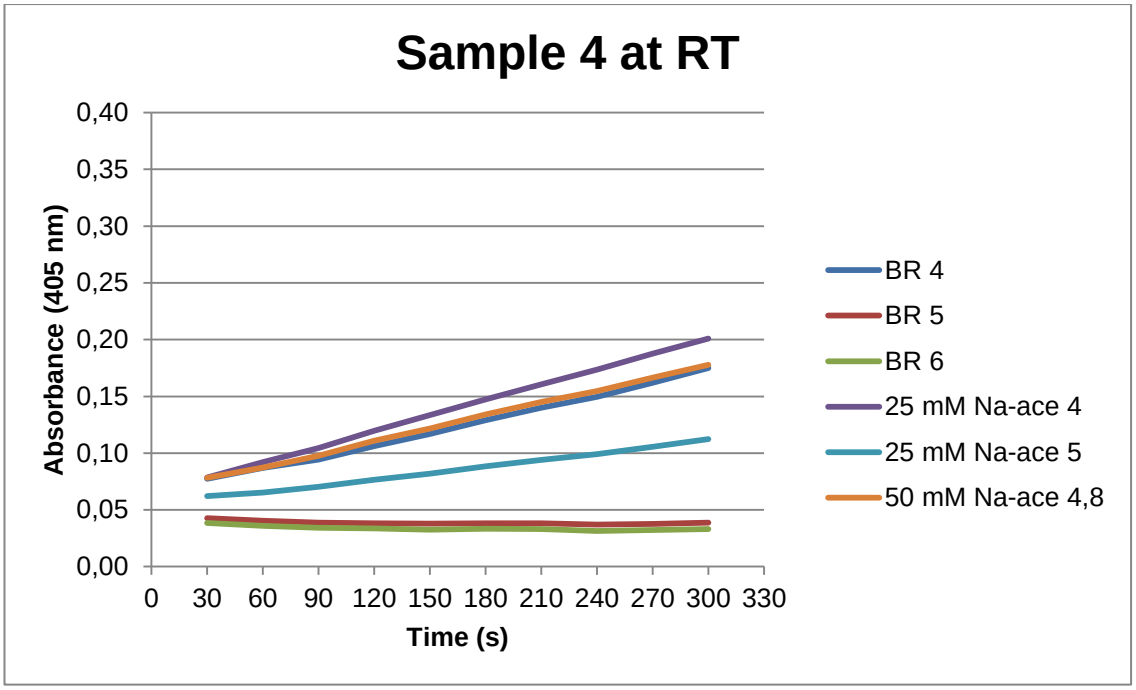

Figure 18. Sample 4 absorbance values at room temperature.

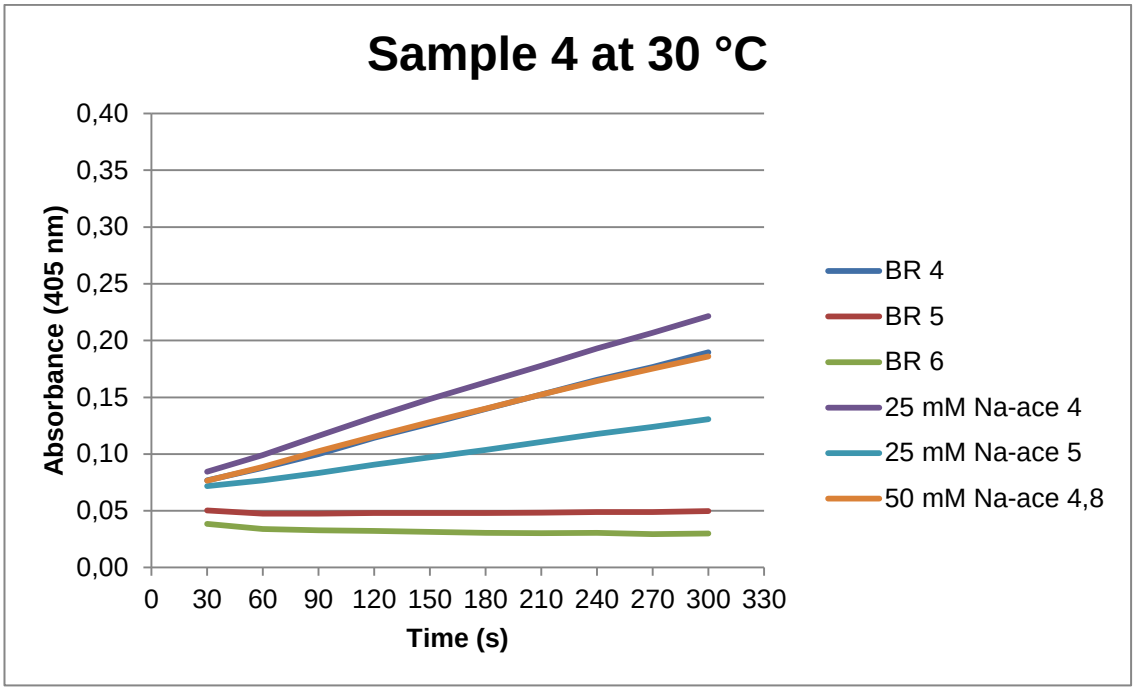

Figure 19. Sample 4 absorbance values at 30 °C.

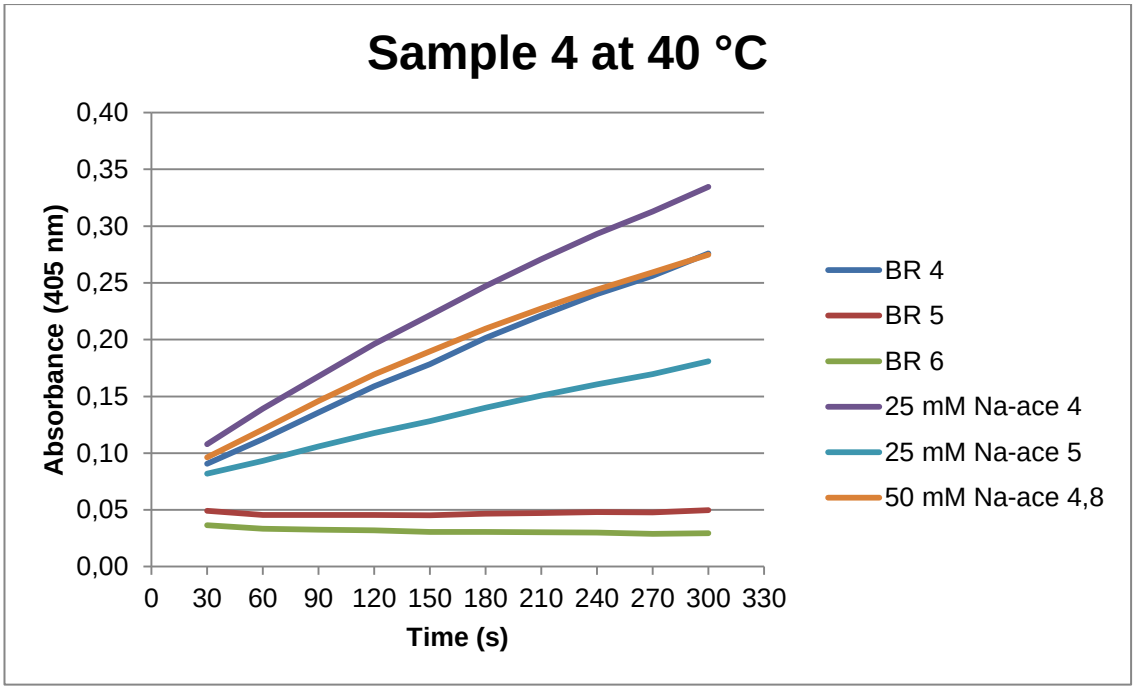

Figure 20. Sample 4 absorbance values at 40 °C.

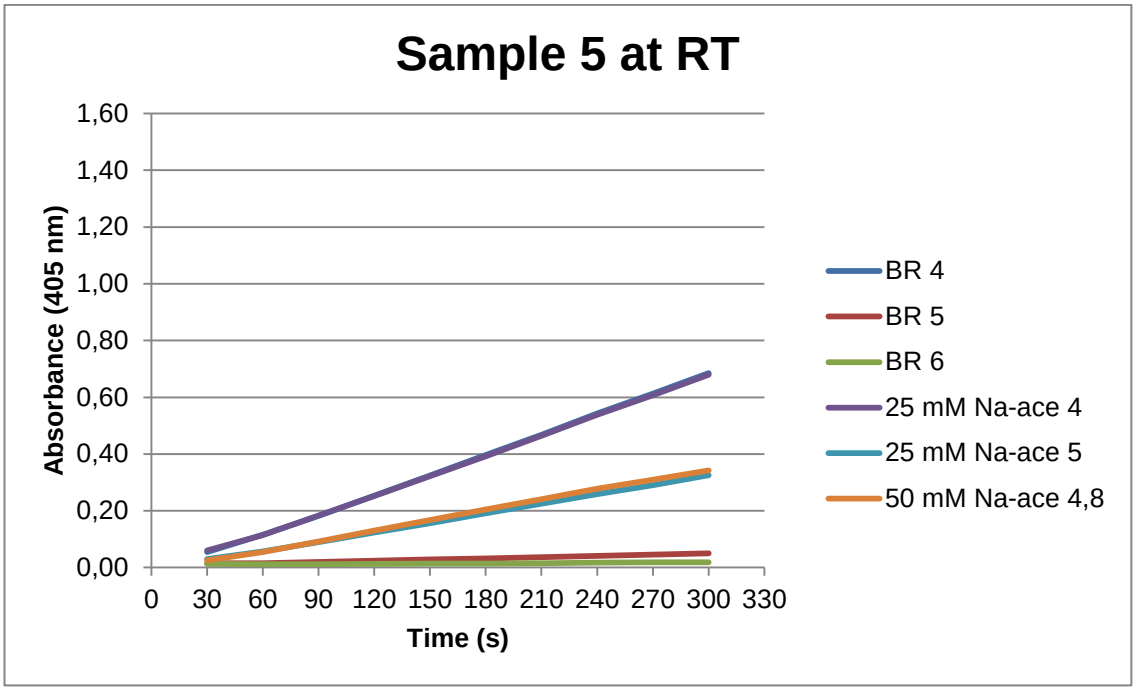

Figure 21. Sample 5 absorbance values at room temperature.

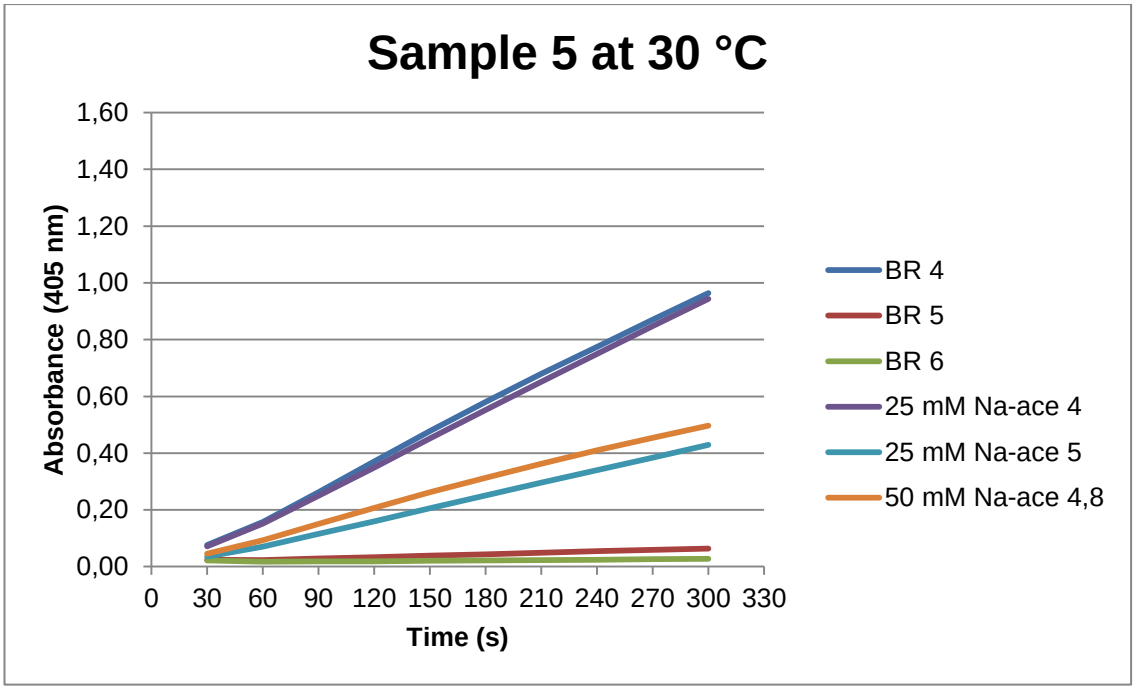

Figure 22. Sample 5 absorbance values at 30 °C.

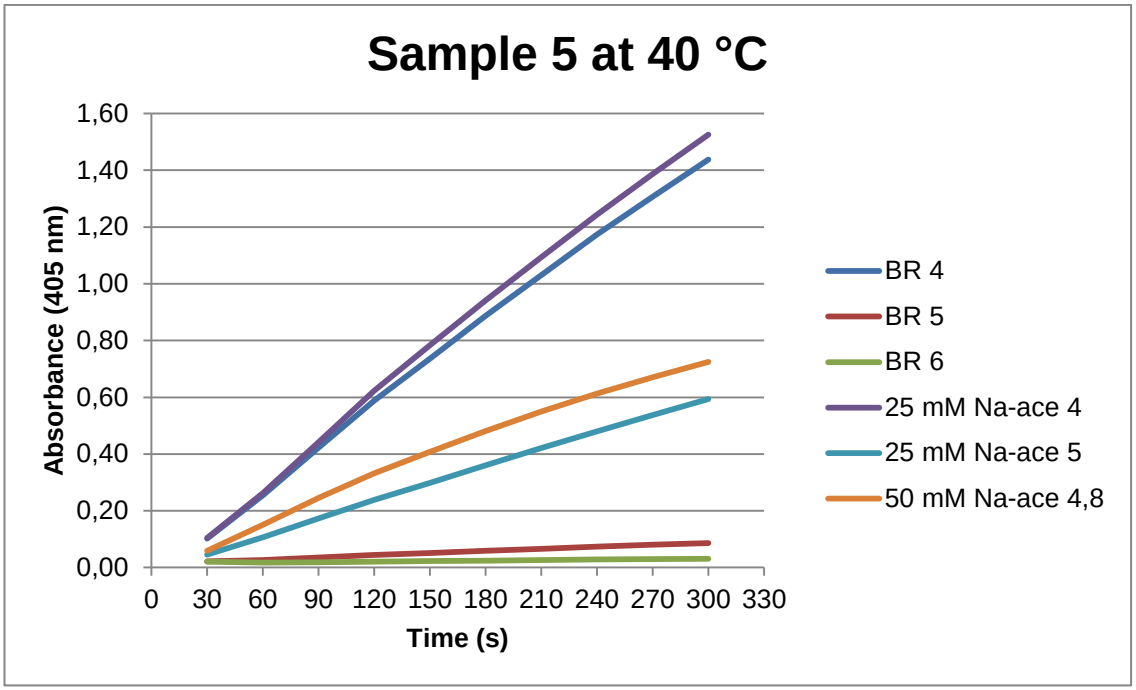

Figure 23. Sample 5 absorbance values at 40 °C.

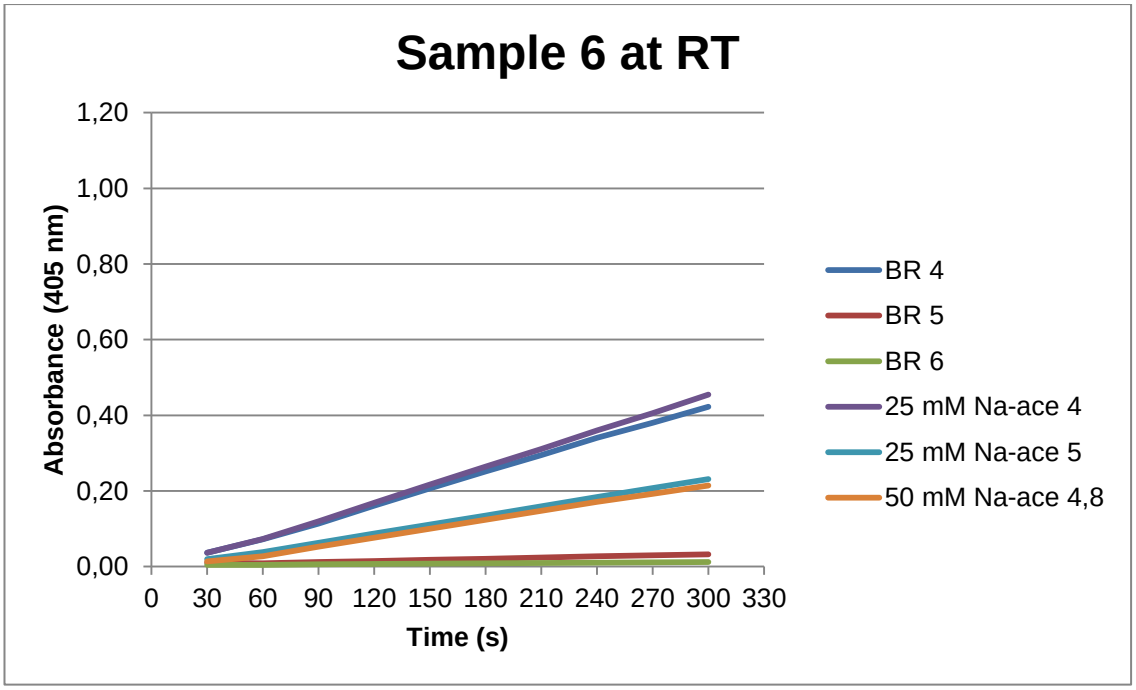

Figure 24. Sample 6 absorbance values at room temperature.

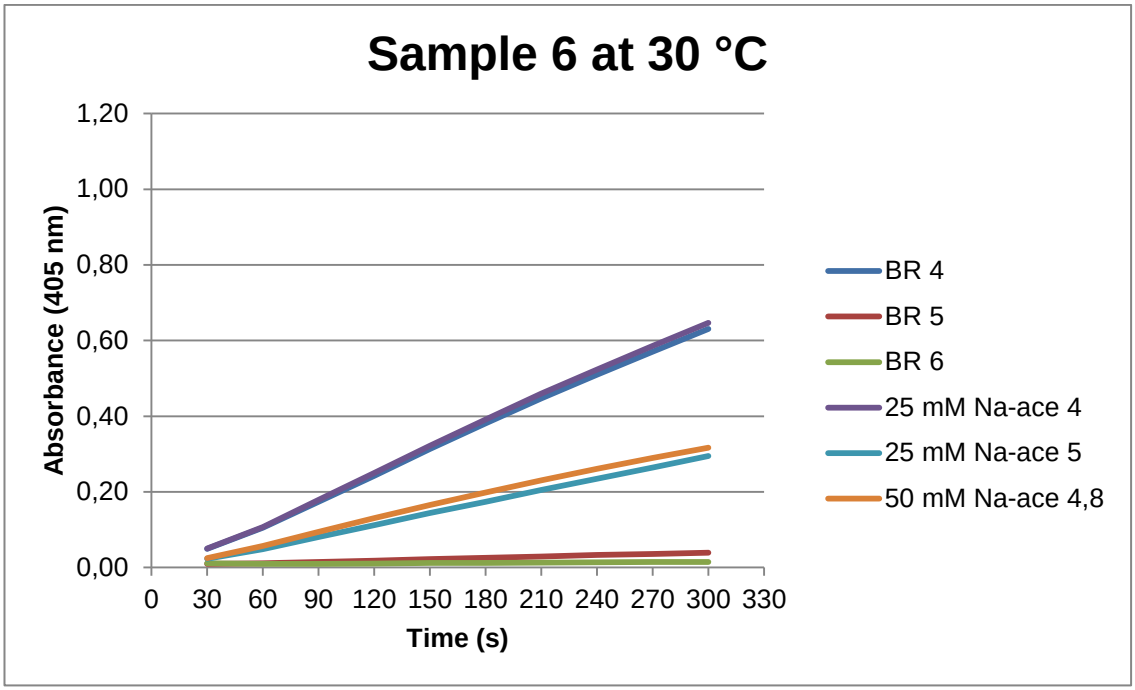

Figure 25. Sample 6 absorbance values at 30 °C.

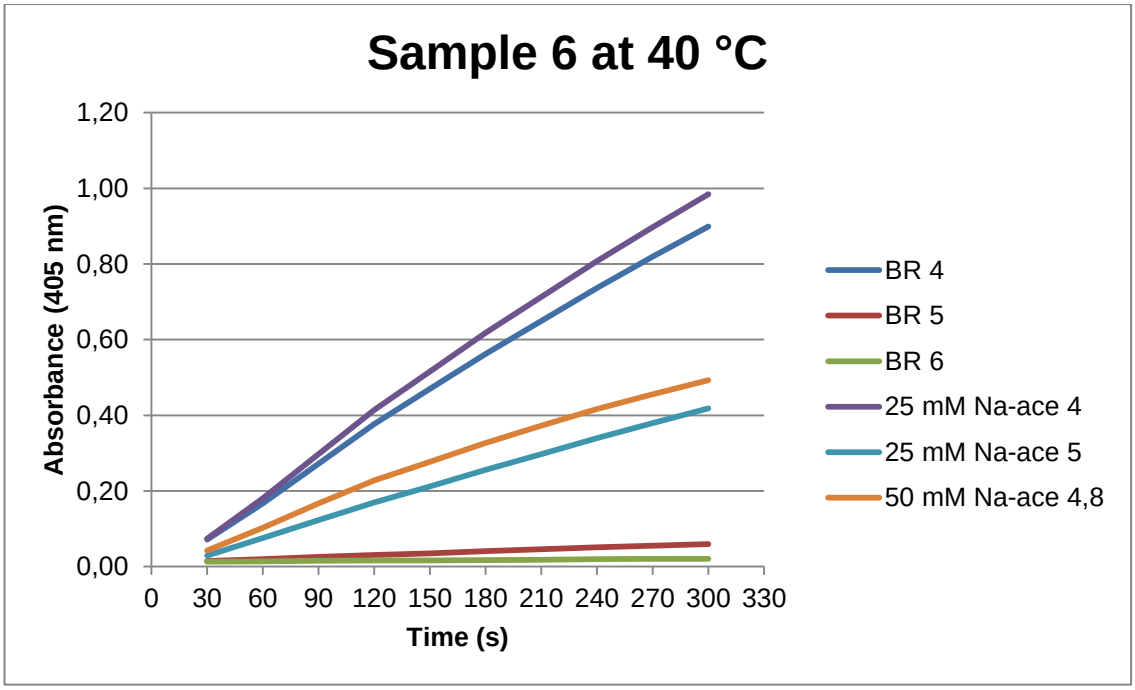

Figure 26. Sample 6 absorbance values at 40 °C.

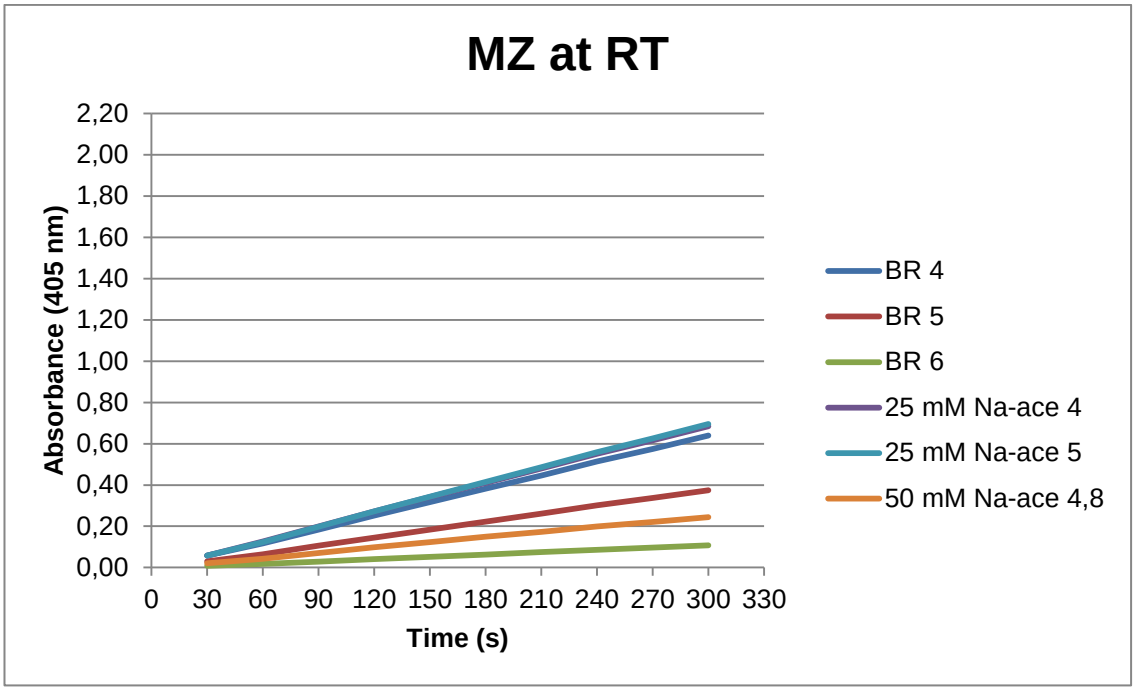

Figure 27. Positive control (MetZyme) absorbance values at room temperature.

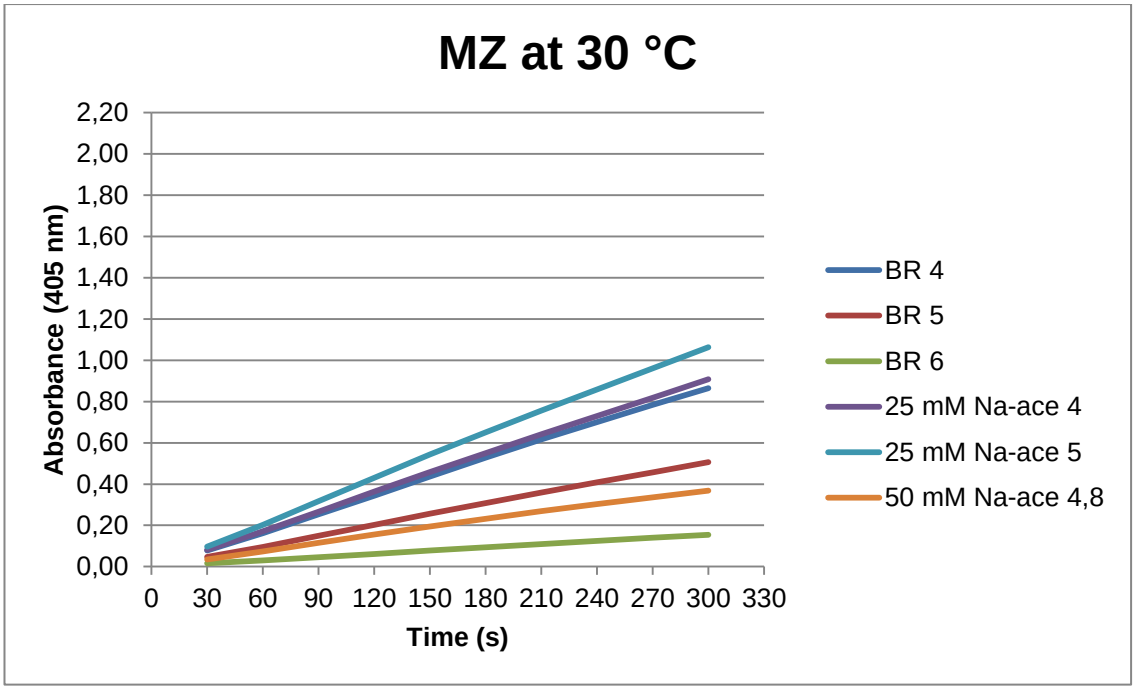

Figure 28. Positive control (MetZyme) absorbance values at 30 °C.

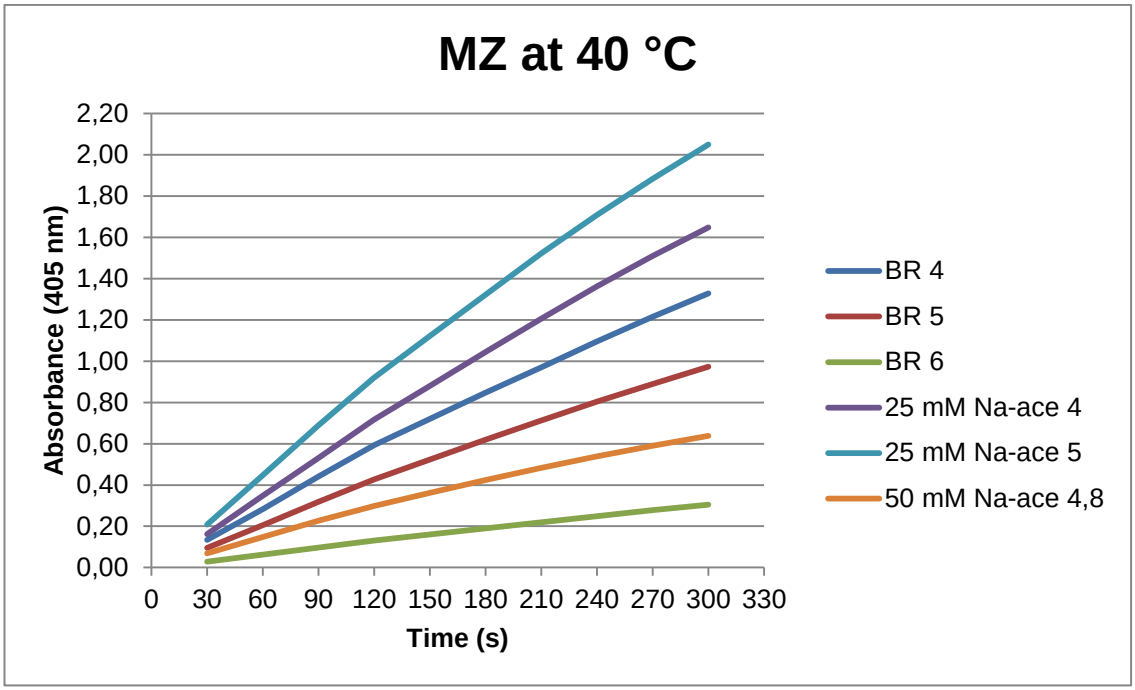

Figure 29. Positive control (MetZyme) absorbance values at 40 °C.

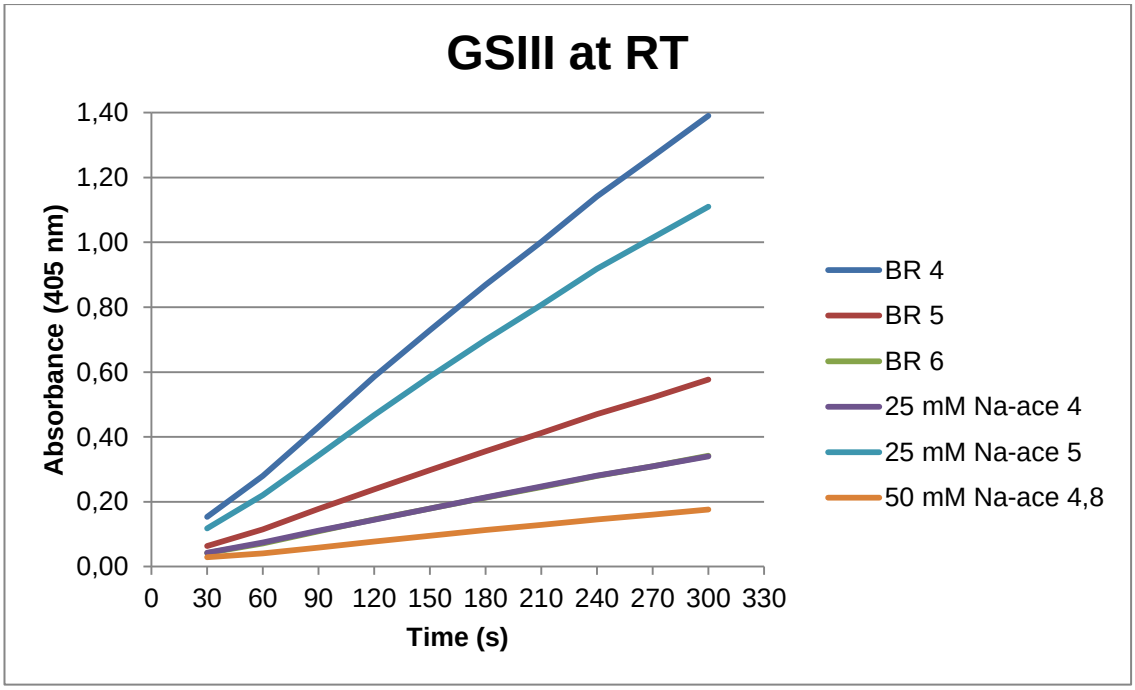

Figure 30. Golden Standard III absorbance values at room temperature.

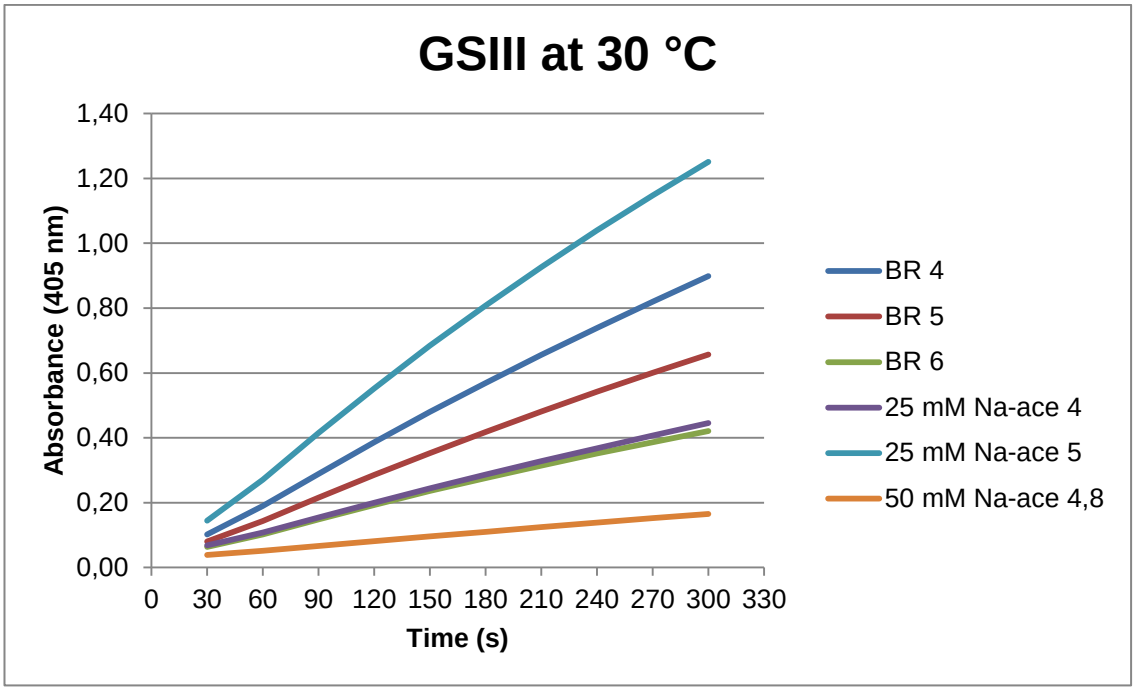

Figure 31. Golden Standard III absorbance values at 30 °C.

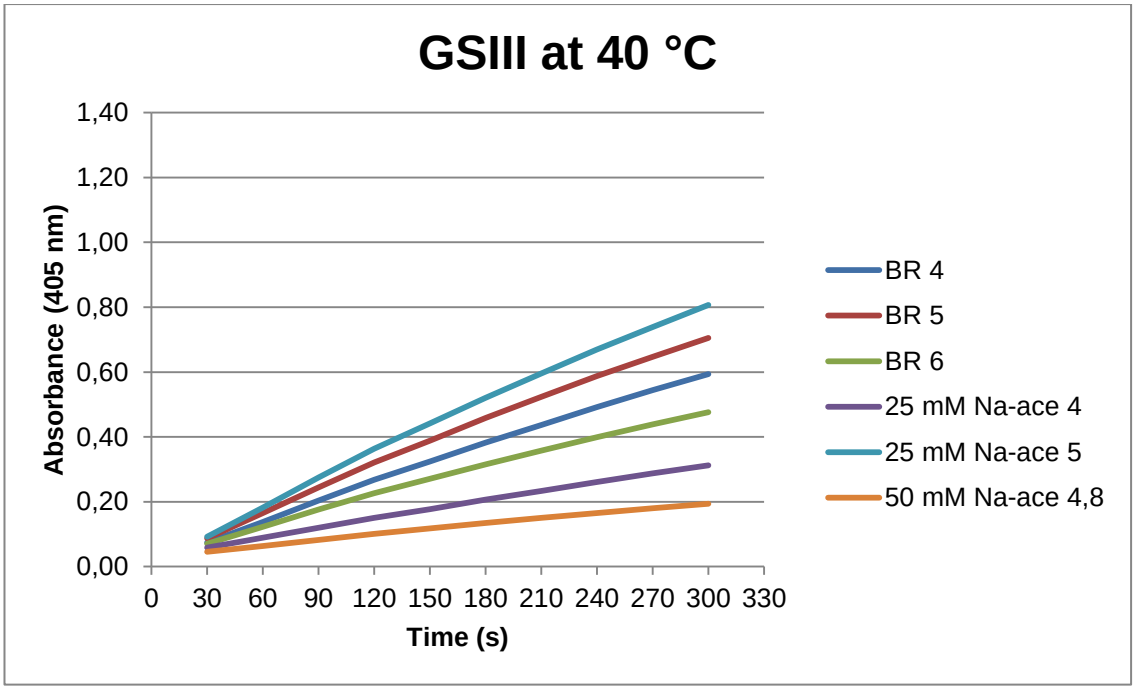

Figure 32. Golden Standard III absorbance values at 40 °C.