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Expression Optimization of the GST-GFP Fusion Protein through the Alteration of Induction Conditions

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EXPRESSION OPTIMIZATION OF THE GST-GFP FUSION PROTEIN THROUGH THE ALTERATION OF INDUCTION CONDITIONS

by

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ABSTRACT

This research sought to determine which induction condition resulted in the greatest GST-GFP fusion protein expression. It will hopefully serve as a guide for future researchers trying to produce their own recombinant protein containing GST and GFP-tags. The CDNB Enzyme Assay was used to determine the quantity of GST-GFP fusion protein present and tested three variables: IPTG concentration, duration, and temperature of induction. The findings showed that IPTG concentration, temperature, and induction duration all had a significant impact on protein expression. Induction temperatures of 20 °C and 25 °C showed better protein expression at IPTG concentrations of 1.0 mM IPTG over 0.1 mM IPTG. Induction durations of 20 hours and 5 hours were better than 3 hours. The 25 °C condition had the greatest protein expression, followed by 20 °C condition. In a follow-up experiment, using 1.0 mM IPTG and 20-hour induction duration, the 20 °C condition showed higher GST activity. Analysis of the 20 °C Western blots revealed the presence of possibly truncated/degraded fusion protein (not seen in the 25 °C Western blots). Future experimenters should use C-terminal GST-tags, as opposed to N-terminal GST-tags, to prevent accidental purification of truncated proteins in addition to the target protein. If this is not possible, then the recommendation is to use the 25 °C temperature for induction, as this temperature had similar GST-GFP fusion protein expression and resulted in much cleaner Western blots.

ACKNOWLEDGMENTS

I want to give my greatest thanks to committee member Prof. Nicole Verity, who continuously provided her support and assistance in the lab and greatly assisted in the research process. Without her, this research would have had no direction nor foundation. Also, I would like to express my deepest gratitude to my thesis chair Dr. Robert Borgon for his incredible help. Without his trust, time, and resources, this research would never have been possible. I am also extremely grateful to committee member Dr. Sean Moore, who continuously gave incredible insight using his vast knowledge and expertise of the field. Without him, the experimentation and analysis would not nearly be as thorough.

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I also want to thank my parents for their continuous support and encouragement. They are my role models for their ceaseless hard work. I also want to thank my sister Lydia for being my best friend.

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LIST OF ABBREVIATIONS

°C: Degrees Celsius

cAMP: Cyclic adenosine monophosphate

cDNA: Complementary deoxyribonucleic acid

CDNB: 1-chloro-2,4-dinitrobenzene

CRP: cAMP receptor proteins

C-termini: Carboxyl-terminal

DNA: Deoxyribonucleic acid

E. coli: *Escherichia coli*

g: grams

GFP: Green fluorescent protein

GSH: Glutathione

GST: Glutathione-*S*-transferase

IPTG: Isopropyl β-D-thiogalactoside

kDa: Kilodaltons

L: Liters

LB: Lysogeny broth

M: Molar

N-termini: Amino-termini

OD_{600}: Optical density at 600 nm

QBM: Quantitative Biological Methods

RNA: Ribonucleic acid

Rpm: Rotations per minute

SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SOC: Super Optimal broth with Catabolite repression

US FDA: United States Food and Drug Administration

CHAPTER 1: INTRODUCTION

BACKGROUND

Recombinant protein technology has already saved millions of lives as a form of medical treatment. In 1982, insulin was the first recombinant protein used as treatment. Since then, recombinant protein technology has become a billion-dollar enterprise, with over 130 recombinant proteins approved for clinical use by the US FDA, that can be used to treat a wide range of diseases (Phuc & Pham, 2018). This technology can be used to produce recombinant antibodies, vaccines, enzymes, and growth factors that effectively treat diseases ranging from dwarfism to congestive heart failure (Koths, 1995). More recently, recombinant protein technology has been used as a teaching tool. The GST-GFP fusion protein can be used to demonstrate a multitude of biological laboratory techniques and is currently an educational component in college classrooms (Verity et al., 2021).

Glutathione-*S*-transferase (GST) catalyzes the addition of electrophilic substrates to glutathione (GSH) to detoxify xenobiotics (Josephy, 2010, Hayes et al., 2005). The attraction between these two molecules makes GST useful as an affinity tag for protein purification (Frangioni, & Neel, 1993). GST as an affinity tag also does not affect the NMR data of the fused partner (Kachel, 2016). The widespread availability of GSH columns exemplifies the popularity and effectiveness of GST as an affinity tag.

Green fluorescent protein (GFP) is commonly used as a biological marker (Zimmer, 2002), due to its bright green glow under fluorescent light. It has been widely studied and its entire molecular structure is well documented (Yang et al., 1996). GFP can be used as a tag for both N- and C- termini, without its loss of function (Topell et al., 1999), exemplifying the versatility of GFP as a protein tag.

GST and GFP are both commonly used as tags in recombinant protein expression, however, in depth-optimization of their expression has not yet been fully accomplished. This research aims to find the optimum induction conditions to maximize GST-GFP expression and will act as a starting point to those trying to optimize their own recombinant proteins containing GST and GSH.

STAGES OF RECOMBINANT PROTEIN EXPRESSION

TRANSFORMATION

Transformation is the uptake of extracellular DNA into an organism's genome. It is also the first step of recombinant protein expression, because it can be utilized to take advantage of an organism's routine metabolism. To do this, a plasmid that contains the selectable marker, origin of replication, and gene of interest (made by the researcher) is taken up by the organism.

Before the plasmid has been made, however, a suitable host that has the machinery necessary to produce the protein of interest must first be identified.

Escherichia coli (E. coli) is an ideal organism for most protein expression and is the most widely used bacteria for recombinant protein expression (Rosano & Ceccarelli, 2014). There are several reasons for this. (i) *E. coli* has an incredibly fast double time at 20 minutes in Lysogeny broth (Sezonov et al., 2007). (ii) *E. coli* can be grown at high concentrations upwards of over 100 grams of dry *E. coli* cells per liter of culture broth (Lee, 1996). (iii) The media required for *E. coli* growth is readily available and cheap. (iv) *E. coli* transformation can be done in as little as 5 minutes (Pope & Kent, 1996).

Specifically, pET vectors are commonly used as the expression system and utilize the T7 *lac* promoter system. In native E. coli, the *lac* operon's purpose is to switch to lactose as the primary metabolite, when glucose is not present. This operon has a positive and a negative regulatory site. The *lac* repressor LacI binds to the operator LacO, the negative regulatory site, and is disabled by the inducer allolactose, an isomer of lactose. CAP binds to the CAP binding site, the positive regulatory site, and is only activated when bound to cAMP, which is produced by the cell when glucose levels are low (Reece et al., 2011). Isopropyl β-D-thiogalactopyranoside (IPTG) is an analogue of allolactose and can be used to induce gene expression (Beel & Lewis, 2000).

When using the *lac* operon system, choosing a medium that contains no glucose is crucial for protein expression. Lysogeny broth (LB), also known as Luria broth, Lennox broth, and Luria-Bertani medium was first developed in 1951 by Giuseppe Bertani (Bertani, 1951). It is

a rich media commonly used for *E. coli* growth and recombinant protein expression, due to the absence of glucose. Glucose inhibits the expression of cAMP, which is a transcriptional activator of the *lac* operon. It binds to cAMP receptor proteins (CRP) and forms the CAP complex, which increases the binding affinity of the promoter (Santillan & Mackey, 2004). Since glucose prevents activation of the lac operon, glucose needs to be fully metabolized from the growth media for recombinant protein expression (Rosano & Ceccarelli, 2014).

INDUCTION

Induction is the stage of recombinant protein expression when the environment of the organism is altered, so that the protein of interest is created. Bacterial cultures are usually induced after a specific optical density at 600 nm (OD_{600}) is reached. Which is why, the OD_{600} is a regularly used method of estimating bacterial growth and is used to determine when a batch of *E. coli* is ready to be induced. OD_{600} readings of 0.4, 0.8, 3.0, and 6.5 correlate to specific stages of *E. coli* growth, such as the starts of: early logarithmic phase, late logarithmic phase, early stationary phase, and late stationary phase, respectively (Kobayashi et al., 2006). It is recommended to begin induction during the early phase of the logarithmic growth curve, specifically anOD_{600} of 0.5 to 0.7 (Harper & Speicher, 2011).

Induction conditions have an important impact on protein expression. Many researchers have identified correlations between temperature, inducer concentration, and time on protein

expression. This research seeks to determine the optimal induction conditions for the GST-GFP fusion protein.

While protein is translated at higher rates at increasing temperatures, that does not necessarily correlate with better protein expression. As induction temperature is increased, ribosomal peptide chain elongation rate also increases, up to 37 °C (Farewell & Neidhardt, 1998). Excess protein expression can lead to the formation of inclusion bodies (Williams et al., 1982). An inverse correlation between temperature and protein solubility exists for recombinant protein rbSRY, which found that the optimum IPTG concentration and temperatures for wtbSRY and cobSRY induction were 0.3 mM at 27 and 32 °C, respectively (Soleymani & Mostafaie, 2019). At high expression levels, protein quality control in the cell can be overwhelmed, and lead to the formation of misshapen proteins that can lead to inclusion body formation (Carrio & Villaverde, 2005). Proteins that become insoluble in inclusion bodies are significantly more difficult to purify than soluble proteins (Marston, 1986, Rudolph & Lilie, 1996, Singh et al., 2015). One study found that decreasing the induction temperature to 4°C for the expression of soluble recombinant P5βR2 led to the greatest soluble protein yield when induced at an OD_{600} of 0.1 (San-Miguel et al., 2013).

Optimal IPTG concentrations change based on induction temperature and time of induction. For the recombinant protein production of EcFbFP in an E. coli Tuner (DE3), the optimal IPTG induction ranged from 0.05 and 0.1 mM IPTG with the lower concentration being optimal for higher temperatures (Muhlmann et al., 2017). This study also found that higher temperatures (37 °C) had optimal protein expression at shorter induction durations than lower

temperatures (28 °C). Optimal IPTG concentrations also vary significantly depending on the target protein being expressed. It should be noted that different IPTG concentrations only affect different levels of population expressing the protein in an “all or nothing phenomenon” (Novick & Weiner, 1957). This means increasing IPTG concentration does not affect cells already induced but increases the likelihood that an uninduced cell will be induced. IPTG has an inhibitory effect on *E. coli* growth (Patil et al., 2013), therefore it is important to take into consideration the pre-induction OD_{600}, before choosing an IPTG concentration.

CHAPTER 2: LITERATURE REVIEW

GST-TAGGED FUSION PROTEINS

There is no general consensus among researchers about the optimal induction conditions for expressing GST-bound proteins. A study, currently cited 69 times, detailing a procedure for GST-fusion protein expression, recommended induction at 37 °C for 3 hours at 250-300 rpm and 1.0 mM IPTG (Harper & Speicher, 2011). In their notes, they add "a general guideline for incubation time is as follows: 3 h at 37 °C, 5 h at 30 °C, and overnight for 25 °C or lower". A different GST-fusion protein (GST-Src) showed better protein yield at 22 °C than 30 °C, with the best IPTG concentration being 0.1 mM, as opposed to 1.0mM and 10 mM (Gong et al., 2006). This same study also found increasing the duration of induction past 5 hours led to minimal increases in protein expression. A concentration of 1.0 mM IPTG was found to be the best concentration for induction of chloramphenicol acetyl-transferase (Bentley et al., 1991), and is the recommended starting concentration for most recombinant protein expression (Donovan et al., 1996).

GST-GFP FUSION PROTEINS

At induction temperatures at and above 30 °C, significant His-GST-GFP fusion protein aggregation was seen in cultures induced at IPTG concentrations of 0.1 mM (Schrodel & Marco,

2005). These induction conditions were in effect for 20 hours, however, which is significantly longer than the recommended 5-hour incubation time by Harper and Speicher. A research team expressing the GST-GFP fusion protein found success when inducing their cells with 0.25 mM IPTG at 30 °C for 4-5 hours (Tessema et al., 2006). While another experiment expressed the GST-GFP fusion protein at 1 mM IPTG with an induction temperature of 25 °C overnight (Buhrman et al., 2012). A study optimizing the expression of GST-hIL-18wild-GFP, found that the optimum temperature for induction was 26 °C compared to a spectrum of temperatures (between 15 and 37) using 5 hours of induction and 1.0 mM IPTG. The optimum IPTG concentration at this temperature was 1.0 and 2.0 mM IPTG (compared to 0.5 and 0.1 mM IPTG) and maximum expression occurred at 15 hours of induction (Kentaro et al., 2004). Currently the GST-GFP lysate preparation for Quantitative Biological Methods (QBM) Lab uses an induction temperature of 20 °C at 1.0 mM IPTG for an overnight induction (Borgon & Verity, 2019).

As shown above, there is great variability in the induction conditions used for GST-GFP fusion protein expression, even more so for differing GST-fusion proteins. This research seeks to find the optimal temperature, IPTG concentration, and duration of induction to maximize the expression of soluble GST-GFP and standardize an induction protocol for GST-GFP fusion protein expression.

CHAPTER 3: METHODS AND MATERIALS

PART I

TRANSFORMATION

The E. coli used came from a glycerol stock of E. coli cells (BL21) transformed with pET expression vectors from VectorBuilder.

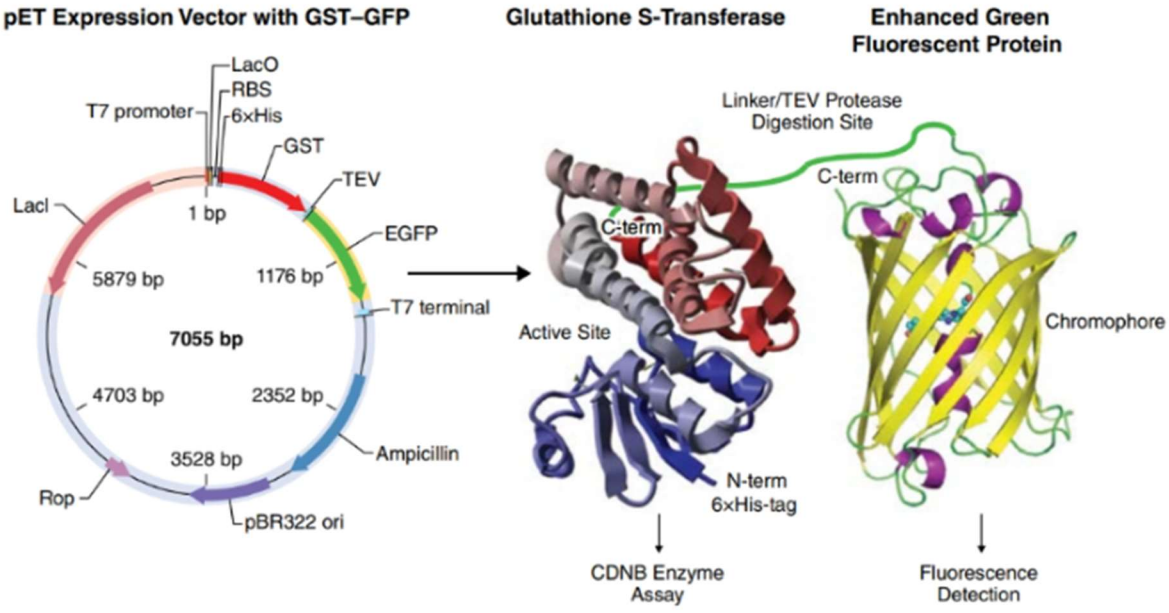


Figure 1: pET Expression Vector used in the experiment
Source: *Quantitative Biological Methods* by Robert Borgon Ph.D. and Nicole Verity, M.S.

Transformation was done using Transformation Storage Solution (TSS) and a heat shock procedure of 10 minutes on ice, 10 minutes off ice, and 10 minutes on ice. Super Optimal broth with Catabolite repression (SOC) media was added to the cells, and incubation took place for 20 minutes at 37 °C. A blue/white screening was then performed to ensure the transformation was successful.

GROWTH

A pipette swab of the frozen glycerol stock was added to 25 ml of pH 7 LB (25g/L) and 100 mg/ml ampicillin and grown overnight at 37 °C at 180 rpm. 10 ml of this inoculated broth was transferred to 190 ml of pH 7 LB (100 mg/ml Ampicillin) and grown at 37 °C and 180 rpm until an OD_{600} of about 0.6 was reached. 8 ml of broth was then transferred to 12 different falcon tubes, each with a different label for their corresponding condition: trial 1 (20 °C, 1.0 mM IPTG), trial 2 (20 °C, 1.0 mM IPTG), etc. The first set of conditions tested was three trials of 20 °C and 1.0 mM IPTG, 20 °C and 0.1 mM IPTG, 37 °C and 1.0 mM IPTG, and 37 °C and 0.1 mM IPTG.

INDUCTION

Induction took place at 20 °C 150 rpm for 20 hours. At hours 3, 5, and 20 of induction, 1.5 ml of broth was taken from the falcon tubes. 0.5 ml of it was used for an OD_{600} reading and the rest was spun down and resuspended in 100 µl 50mM Tris 150mM NaCl pH 8 with HCl. 1 µl

of 100X EDTA and 1 µl of protease inhibitor was added to each 100 µl sample. Samples were frozen at 20 °C for 5 hours.

LYSIS

Cells were lysed using 10 µl of 10 mg/ml Lysozyme and 1 µl of 2500 U/ml DNase for 1 hour. The resulting lysate was centrifuged at 13.3k rpm for 2 minutes and the supernatant was collected.

ANALYSIS

The Bio-Rad ChemiDoc XRS+ and ChemiDoc MP Imaging System were used to take pictures and detect fluorescence within the samples. The cells were put in a freezer until a CDNB enzyme assay was performed. Using a 64-well plate, 9 wells were filled at a time with 269 µl of CDNB Master Mix [7040 µl dH2O, 800 µl 10X Reaction Buffer (685 ml of 1 M KH2PO4, 315 ml of 1 M K2HPO4), 80 µl 100mM CDNB], 8.16 µl lysate, and 20.72 µl 100 mM GSH. Solutions were immediately mixed by pipetting up and down, and then read in the plate reader at 340 nm at 30 second intervals. This was done for each condition. The GST activity was calculated using the equation in Figure 2.

$$\frac{\text{Reaction Velocity/GST Activity}}{(\text{units/ml, or } \mu\text{mol/ml/min})} = \frac{(\Delta A_{340})}{(\varepsilon_{GS-DNB\ conjugate})(\Delta t)(\text{volume})}$$

ΔA_{340} = change in absorbance, or A_{340} (final read) − A_{340} (initial read). Linear below 0.8.

ε_{CDNB} = the extinction coefficient of CDNB conjugate at 340 nm, which is 9.6 mM^{-1} cm^{-1}

Δt = change in time in minutes, or time (final read) − time (initial read)

volume = volume of sample in milliliters, or 0.001 for 1 µl, 0.010 for 10 µl, etc.

Figure 2: Equation to calculate GST activity
Source: Quantitative Biological Methods by Robert Borgon Ph.D. and Nicole Verity, M.S.

PART II

The reagents and techniques followed in this experiment mirror that done in the QBM Lab Lysate preparation, documented in *Quantitative Biological Methods* by Robert Borgon Ph.D. and Nicole Verity, M.S.

TRANSFORMATION AND GROWTH

The glycerol stock from part I of the experiment was further analyzed. The transformed E. coli was added from the glycerol stock to 100 ml pH 7 LB (25g/L) and ampicillin (100 mg/ml) and grown overnight at 37 °C and 180 rpm. 28 ml of this culture was transferred to 532 ml LB and 532 µl of 100 mM ampicillin. The 28 ml transfer was done 3 times (one for each trial). The 3

560 ml cultures grew to an OD_{600} of about 0.6, and then transferred to split into 6 250 ml cultures. The preinduction OD_{600} values were recorded.

Table 1:
Pre-induction OD_{600} Values

	Trials		
Temperature (°C)	1	2	3
20	0.755	0.695	0.771
25	0.767	0.693	0.783

OD_{600} values were taken using a Bio-Rad SmartSpec Plus Spectrophotometer

INDUCTION AND LYSIS

Six 250 ml cultures were induced with 250 µl 1.0 M IPTG (1 mM). And grown for 20 hours at 180 rpm. Half of the cultures were grown at 20 °C and the other half were grown at 25 °C. Unfortunately, the 25 °C incubator did not have a cooling system, so after the ice inside the incubator melted, the temperature increased to 27 °C. I do not know when the incubator's temperature started to increase nor how fast. The samples were then spun down at 20 minutes at 3000g, and resuspended in 10 ml of 50 mM Tris, pH 8, 150 mM NaCl + 100 µl EDTA + 100 µl Protease Inhibitor.

The cultures were lysed using a French press 2 times and spun down at 14000 rpm for 1 minute.

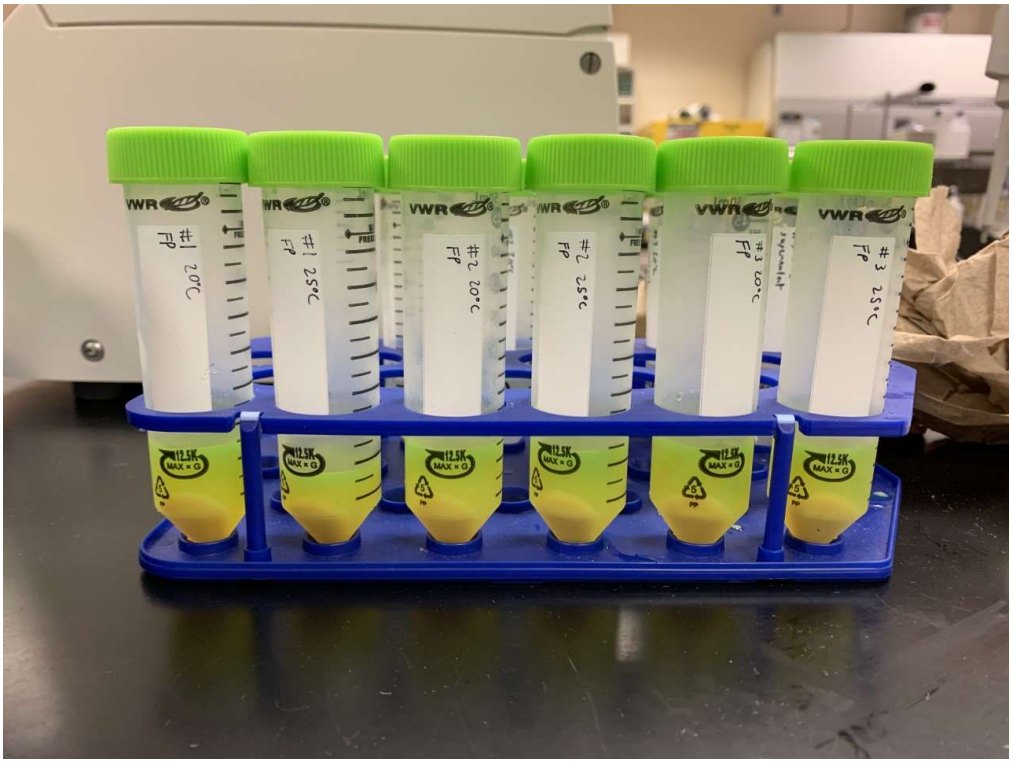


Figure 3: Pellet and supernatant of all samples post-French press and spin down.

The supernatant was collected and a CDNB enzyme assay and Bradford assay were performed on the samples. The samples were pooled and another CDNB enzyme assay and Bradford assay were performed. 1 ml of the supernatants were diluted by a factor of 5 (added to 4 ml of 50 mM Tris, pH 8, 150 mM NaCl). The 5 ml samples were then syringe-filtered using 3 ml syringes with Luer-Lok Tips.

PURIFICATION

The materials used for the affinity chromatography were as follows: wash buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM Calcium disodium EDTA), elution buffer (50 mM Tris pH

8.0, 150 mM NaCl, 10 mM GSH, 1 mM Calcium disodium EDTA), Bio-Scale mini Profinity GST Cartridge, and Bio-Rad Biologic LP. Wash buffer was run over the GSH column at 1 ml/min. Followed by a 50 to a 100% gradient of elution buffer at 1 ml/min, finished with a run of the wash buffer at 2.5 ml/min.

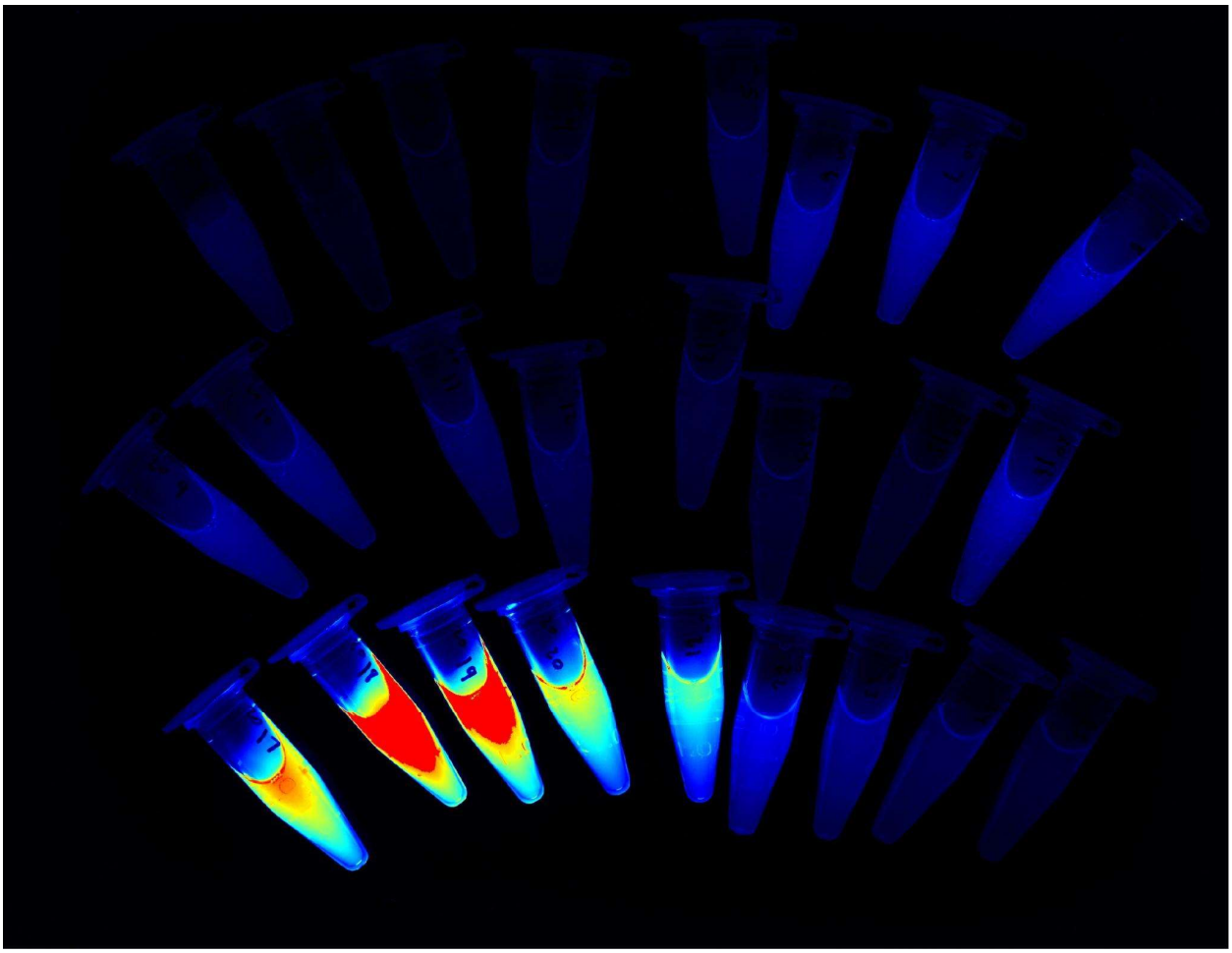


Figure 4: 20 °C condition affinity chromatography microcentrifuge tubes.

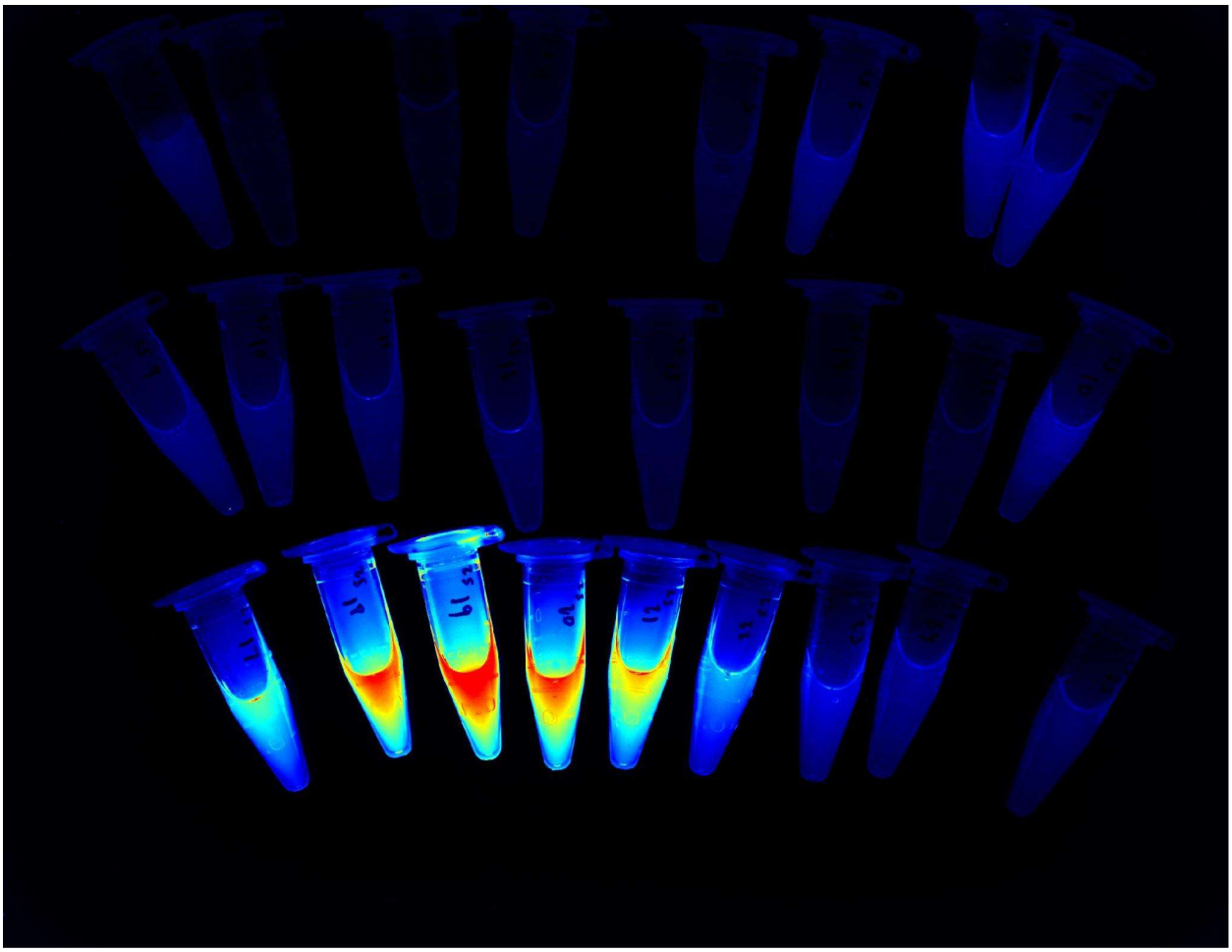


Figure 5: 25 °C condition affinity chromatography microcentrifuge tubes.

Tubes 17, 18, and 19 of the 20 °C condition were kept and pooled. Tubes 18, 19, and 20 were kept of the 25 °C condition.

IEX chromatography was then run on the lysates. The column used was the Bio-Scale Mini UNOsphere Q cartridge (5 ml). The wash buffer was the same as the one used in the affinity chromatography, while the elution buffer is 50 mM Tris pH 8.0, 1 M NaCl. The flow rate

was 2 ml/min, and began with 2 ml of wash buffer, then a 0 to 100% gradient of 6 ml, followed by 3 ml of elution buffer, and finished with 18 ml of wash buffer.

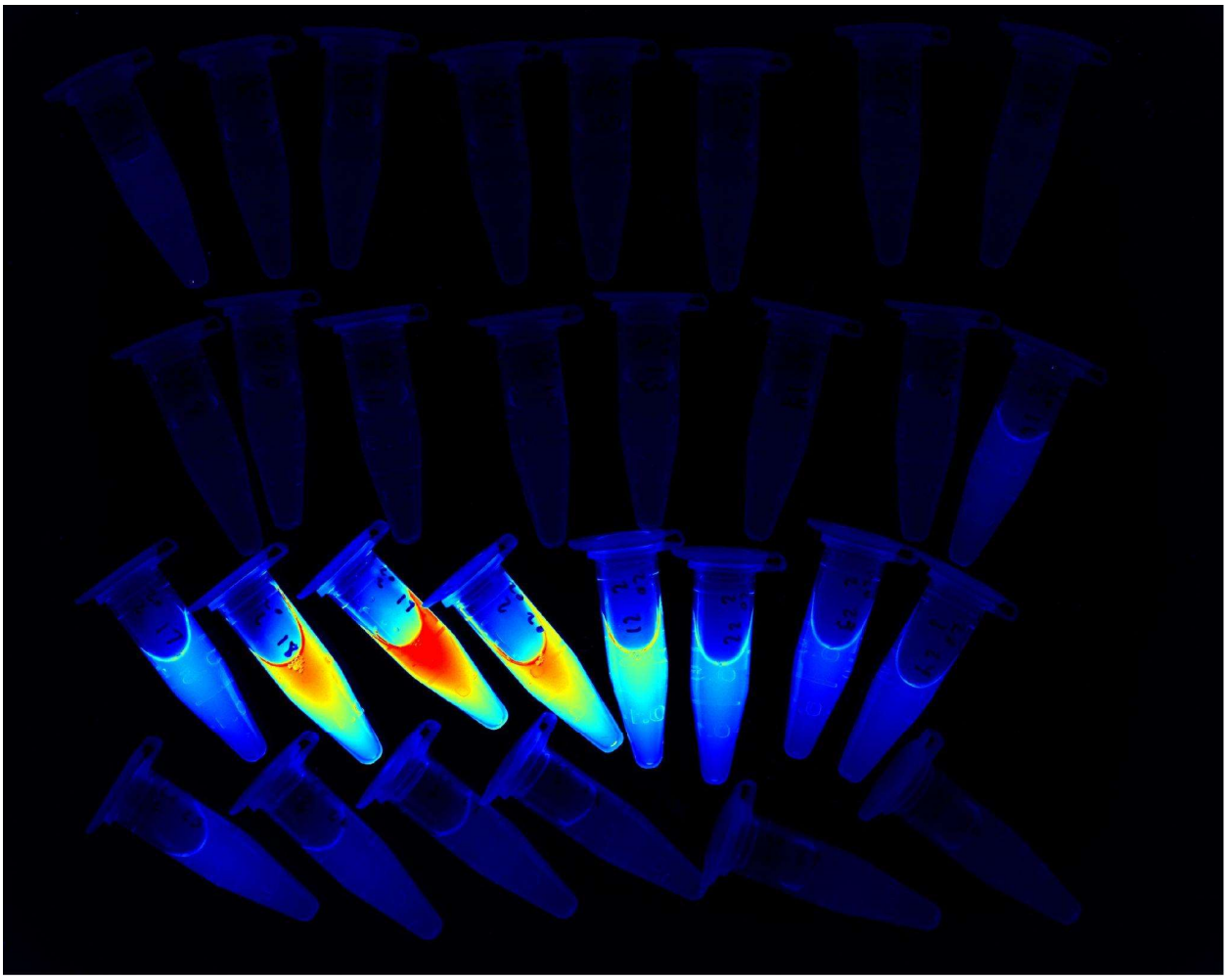


Figure 6: 20 °C condition IEX chromatography microcentrifuge tubes.

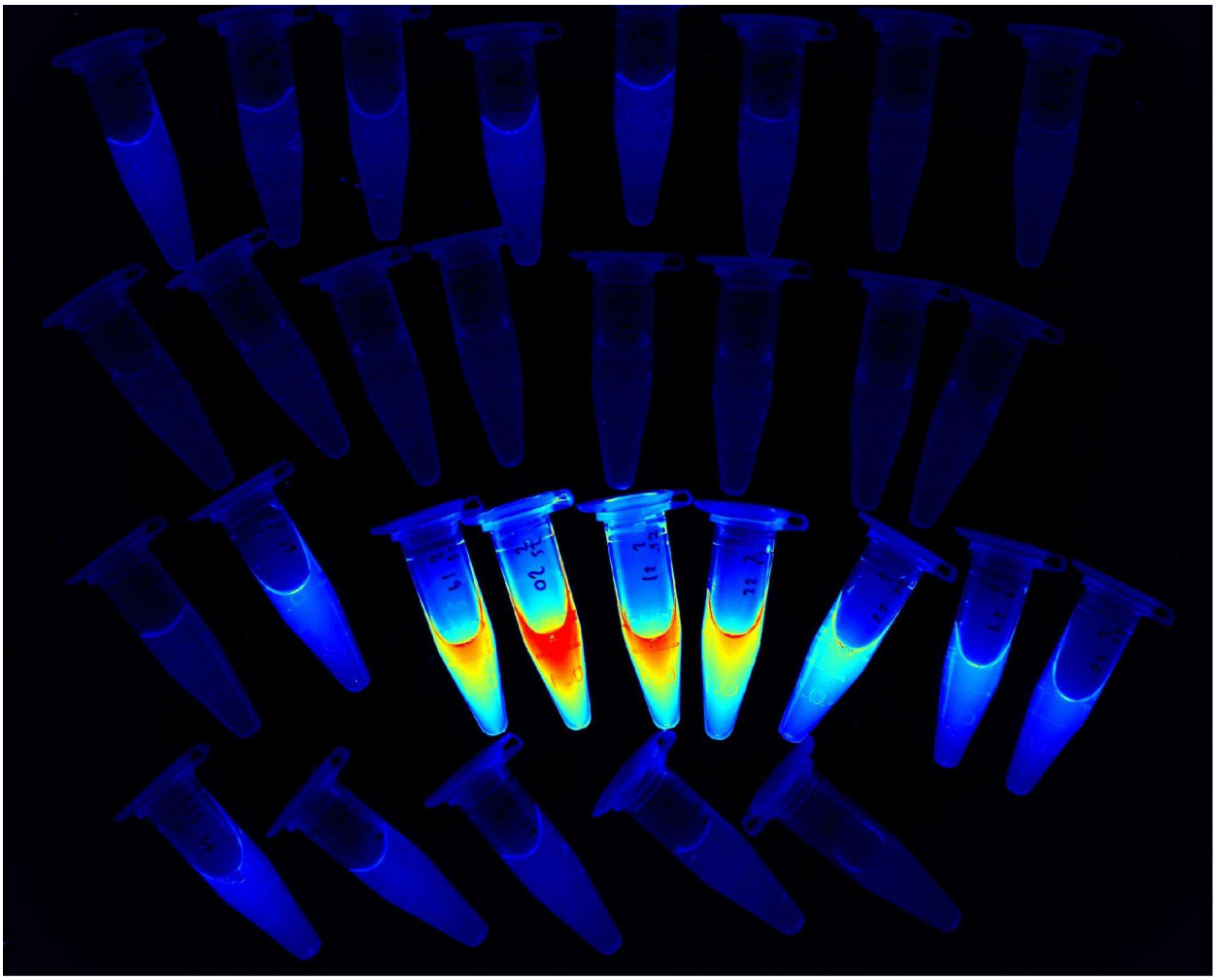


Figure 7: 25 °C condition IEX chromatography microcentrifuge tubes.

Tubes 18, 19, and 20 were kept from the 20 °C IEX chromatography and tubes 19, 20, and 21 were kept from the 25 °C IEX chromatography.

ANALYSIS

A CDNB enzyme assay was performed at each step of the purification: after lysis, after affinity chromatography, and after IEX chromatography.

One SDS-PAGE gel was made using a Bio-Rad Mini-PROTEAN Tetra electrophoresis. The resolving gel will be composed of 1.62ml dH20, 1.25ml 1.5 M Tris pH8.8, 2 ml 30% Acrylamide Mix, 25 µl 99% TCE, 50 µl 10% SDS, 50 µl 10% APS, 5 µl TEMED. The stacking gel will be composed of 892 µl dH2O, 375 µl 0.5 M Tris pH 6.8, 200 µl 30% Acrylamide Mix, 15 µl 10% SDS, 15 µl 10% APS, and 3 µl TEMED. The other SDS-PAGE gel was a premade Mini-PROTEAN® TGX™ Precast Gels from Bio-Rad

The lanes contained the molecular weight marker, a GST-control, a GFP-Control, the original lysate, affinity flowthrough, affinity elution, IEX flowthrough, and the IEX elution. 5.5 µl of 10 X SDS Sample Loading Buffer (containing Tris, SDS, glycerol, β-mercaptoethanol, EDTA, and bromophenol blue), was added to the samples, excluding GST-Control, GFP-Control and the molecular weight marker.

Microplate protein quantification using a DC Protein Assay was done to normalize the samples for the SDS-PAGE.

The SDS-PAGE was run using the Bio-Rad Mini-PROTEAN Tetra electrophoresis equipment, and the 1X SDS-PAGE Running Buffer. It ran for 35-45 minutes at 200 volts. The 20 °C gel was made in the lab, while the 25 °C gel was bought from Bio-Rad.

After visualizing the gels on the gel doc, a western transfer was performed for 7 minutes at 25 volts using the Bio-Rad Trans-Blot Turbo Transfer System.

A Western blot was performed using 1X TBS (Tris-buffered saline), BLOTTO (5% non-fat milk in TBS-T), Rabbit anti-GST IgG (polyclonal antibody), StarBright Blue 700 Goat Anti-Rabbit IgG, mouse anti-GFP IgG (monoclonal), and StarBright Blue 520 Goat anti-Mouse IgG. The results were visualized on the gel doc.

A normalized CDNB enzyme assay (lysate added was proportional to total protein) was done at the very end (Table 7), to give numerical values to the Western Blot results and to be used in Table 6. The CDNB enzyme assay results following each step (Table 8) were not used in Table 6, due to differing room and material temperatures, which I found to influence reaction velocities.

CHAPTER 4: RESULTS

PART I

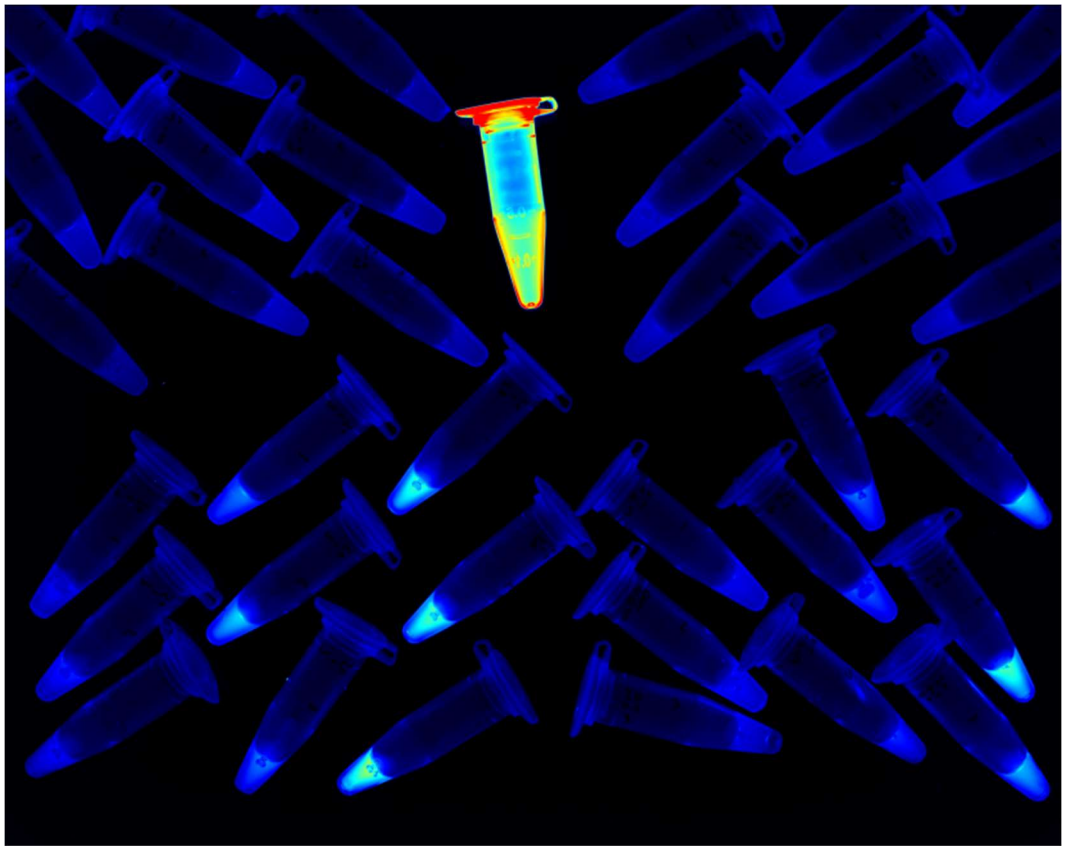


Figure 8: 20 °C and 37 °C sample fluorescence with positive control.
Fluorescent picture of the 20 °C and 37 °C lysate samples taken after cell lysis and filtration, but before the CDNB enzyme assay. From left to right the columns are 3 hours (1.0 mM IPTG), 5 hours (1.0 mM IPTG), 20 hours (1.0 mM IPTG), 3 hours (0.1 mM IPTG), 3 hours (0.1 mM IPTG), and 20 hours (0.1 mM IPTG). From top to bottom the rows are Trial 1 (20 °C), Trial 2 (20 °C), Trial 3 (20 °C), Trial 1 (37 °C), Trial 2 (37 °C), and Trial 3 (37 °C). A fluorescent microcentrifuge tube has been added as a positive control that shows the gel doc's maximum fluorescence, so that Figure 8 can be compared to Figure 9.

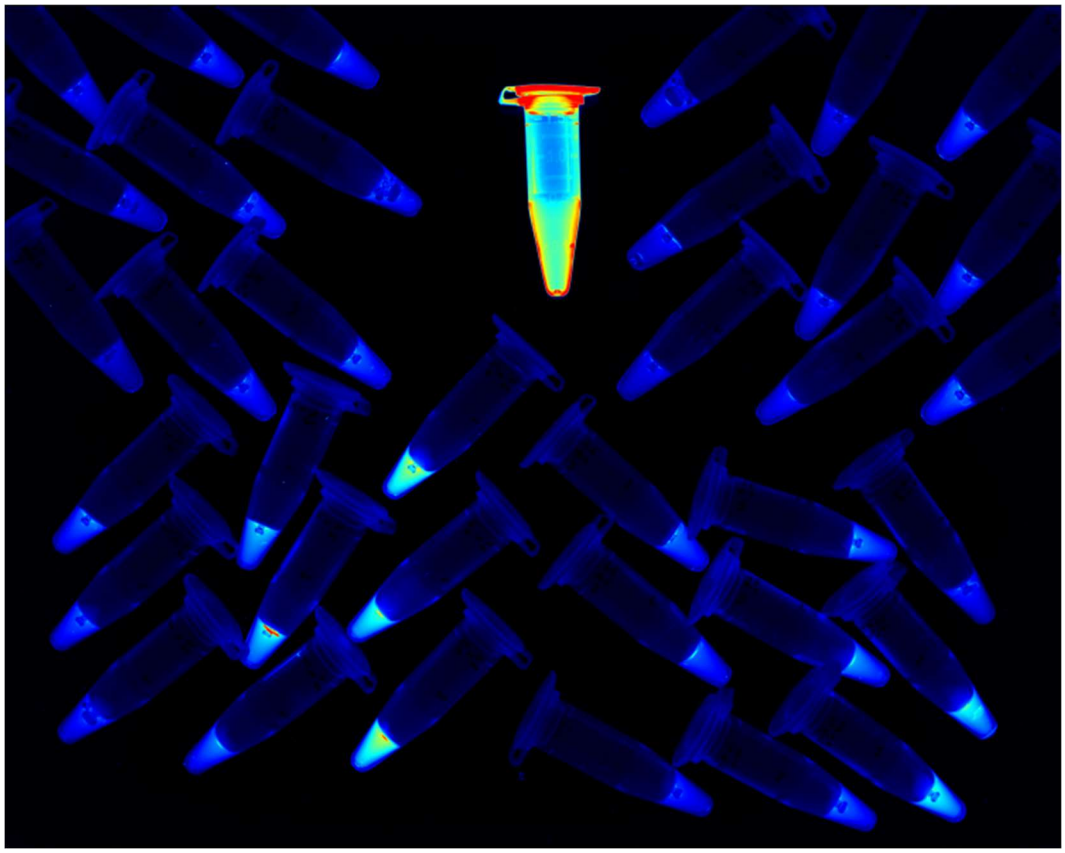


Figure 9: 25 °C and 30 °C sample fluorescence with positive control.
Fluorescent picture of the 25 °C and 30 °C lysate samples taken after cell lysis and filtration, but before the CDNB enzyme assay. From left to right the columns are 3 hours (1.0 mM IPTG), 5 hours (1.0 mM IPTG), 20 hours (1.0 mM IPTG), 3 hours (0.1 mM IPTG), 3 hours (0.1 mM IPTG), and 20 hours (0.1 mM IPTG). From top to bottom the rows are Trial 1 (30 °C), Trial 2 (30 °C), Trial 3 (30 °C), Trial 1 (25 °C), Trial 2 (25 °C), and Trial 3 (25 °C).

The gel doc always shows the most fluorescent object in the image as the deepest red. The fluorescent microcentrifuge tube acts as the gel doc's "maximum fluorescence", so that Figures 8 and 9 can be compared.

The trials of the 20 °C, 1.0 mM IPTG, 20-hour condition and the 25 °C, 1.0 mM IPTG, 20-hour condition showed the greatest fluorescence, followed by the trials of the 20 °C, 0.1 mM IPTG, and 20-hour condition, 25 °C, 0.1 mM IPTG, and 20-hour condition, and 20 °C, 1.0 mM

IPTG, 5-hour condition, and the 25 °C, 1.0 mM IPTG, 5-hour condition. The 30 °C samples showed very little fluorescence, while the 37 °C samples show almost no fluorescence at all. I would expect the largest GST activities of the samples to correspond to the samples with the most fluorescence, since the E. coli should be making the GST-GFP fusion protein. Therefore, Table 1 and Figure 3, should give values that mirror the results of the gel doc.

Table 2
GST Activity (units/ml) of the Various Temperatures, IPTG Concentrations and Durations of the Differing Induction Conditions

		Duration of Induction (Hours)		
Conditions	Trials	3	5	20
20°C, 0.1mM IPTG	1	0.033	0.077	0.084
	2	0.046	0.106	0.181
	3	0.044	0.082	0.130
	Average	0.041	0.088	0.132
20°C, 1.0mM IPTG	1	0.080	0.169	0.156
	2	0.093	0.167	0.124
	3	0.080	0.143	0.184
	Average	0.084	0.155	0.156
25°C, 0.1mM IPTG	1	0.037	0.089	0.104
	2	0.067	0.203	0.195
	3	0.146	0.154	0.214
	Average	0.083	0.149	0.169
25°C, 1.0mM IPTG	1	0.082	0.197	0.216
	2	0.201	0.118	0.221
	3	0.187	0.193	0.354
	Average	0.159	0.170	0.291
30°C, 0.1mM IPTG	1	0.052	0.037	0.086

	2	0.040	0.039	0.064
	3	0.028	0.009	0.057
	Average	0.040	0.028	0.069
30°C, 1.0mM IPTG	1	0.046	0.058	0.048
	2	0.033	0.072	0.072
	3	0.007	0.042	0.058
	Average	0.050	0.057	0.059
37°C, 0.1mM IPTG	1	0.031	0.027	0.029
	2	0.025	0.048	0.023
	3	0.030	0.022	0.019
	Average	0.029	0.032	0.023
37°C, 0.1mM IPTG	1	0.022	0.017	0.024
	2	0.008	0.022	0.040
	3	0.029	0.024	0.042
	Average	0.002	0.021	0.035

GST activities were rounded to the nearest thousandths place.

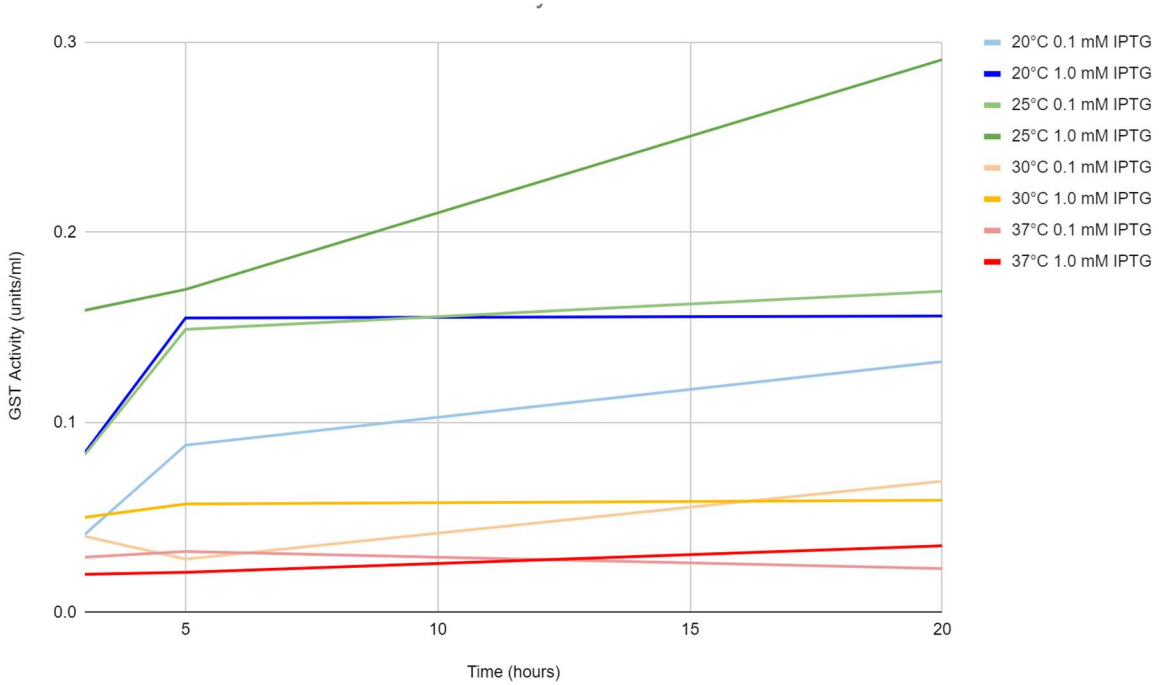


Figure 10: The effect of every induction condition on GST activity.

The CDNB enzyme assay positively correlated with the various samples' fluorescence, except with respect to the trials of the 20 °C, 1.0 mM IPTG, and 20-hour induction condition. Based on the fluorescence, I expected to see these trials have a higher GST activity average, closer to the results of the 25 °C trials at the 1.0 mM IPTG and 20-hour duration condition.

INDUCTION DURATION

At lower temperatures (20 °C and 25 °C), the GST Activity increased with induction time. At induction conditions 20 °C and 0.1 mM IPTG, each trial doubled in GST Activity between 3 and 5 hours as it changed, on average, from 0.041 Units/ml to 0.088 Units/ml. Between 5 hours

and 20 hours, the GST Activity increased further, to 0.132 Units/ml. At the same temperature but 10X the IPTG concentration, between 3 and 5 hours an almost doubling of concentration occurred, from 0.084 Units/ml to 0.155 Units/ml, however, the GST Activity change from 5 hours to 20 hours was not significant.

At 25 °C and 0.1 mM IPTG conditions, all three trials showed increases in GST activity between 3 hours and 5 hours, with the average increasing from 0.083 Units/ml to 0.149 Units/ml. Between 5 hours and 20 hours two of the three trials saw increasing GST activity, with the average increasing from 0.149 Units/ml to 0.169 Units/ml. At the same temperature but with 10X the IPTG, the results greatly varied between the 3 to 5-hour times, with each trial showing either decreasing, increasing, or no change in GST Activity. On average, the GST Activity increased from 0.159 Units/ml to 0.170 Units/ml between 3 hours and 5 hours of induction time. All three trials, however, showed large increases overall from 5 hours to 20 hours, with the average increasing to 0.291.

At higher temperatures (30 °C and 37 °C), almost across the board, there were increases in GST activity as time increased, however these changes were significantly smaller than their lower temperature counterparts. The largest change occurred for the 30 °C 0.1 mM IPTG condition, as it increased from 0.040 to 0.069 units/ml at 3 and 20 hours, respectively. The highest percentage change occurred at the 37 °C 1.0 mM IPTG condition with a 75% increase between hours 3 and 20, with GST activities of 0.02 units/ml and 0.035 units/ml, respectively. Other conditions, however, did not show such a large increase, such as the conditions 30 °C and 1.0 mM IPTG, where there was a 0.009 units/ml change between the 3 hours and 20 hours

sample, as it increased from 0.05 to 0.059 units/ml. Only at conditions 37 °C 0.1 mM IPTG, did a decrease occur with the GST Activity decreasing to 0.023 units/ml from 0.029 units/ml at 20 and 3 hours, respectively.

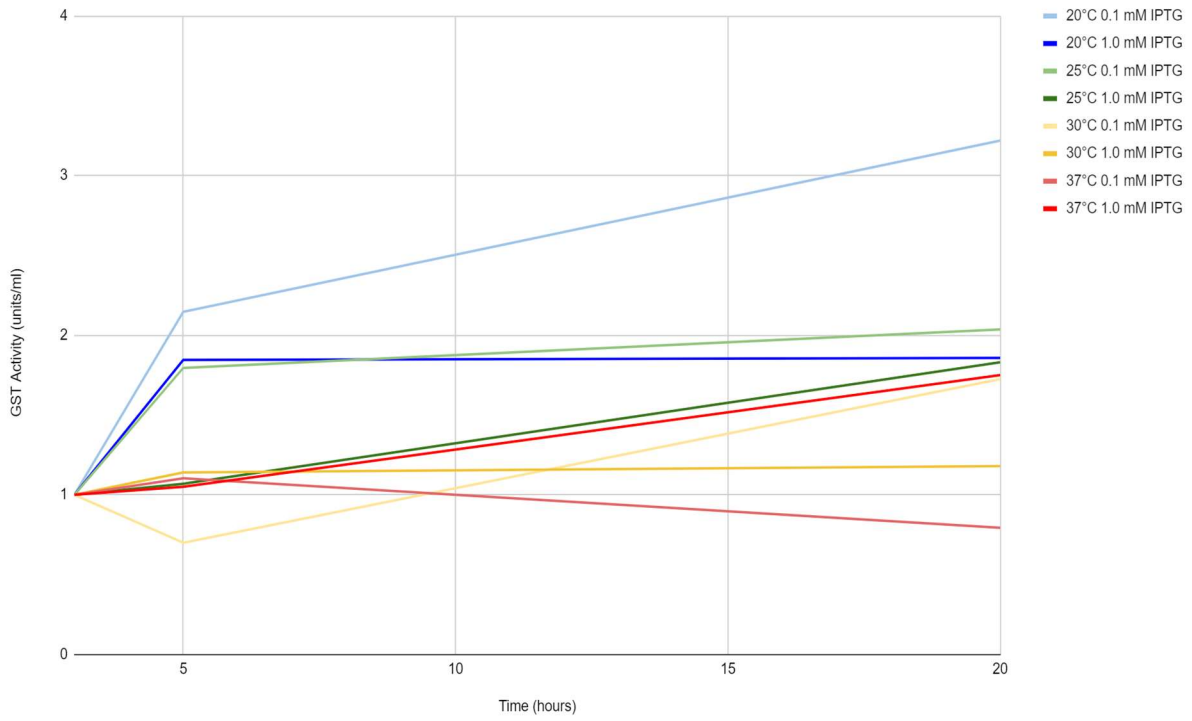


Figure 11: The effect of induction time on the factor increases of the 3-hour GST activity.

Looking at the effect of Induction time on factor change based on the 3-hour GST activity, a majority of differing temperatures and IPTG concentrations at hour 20 converge to a 1.8X factor increase of the corresponding 3-hour GST Activity. Another similar convergence point is at 5 hours, with a 1Xfactor increase. A heteroscedastic, one-sample, one-sided *t-test* was performed using the 5-hour average factor changes and compared to the null mean of 1. With 95% confidence ($p < 0.0265$), I can say that increasing the induction time from 3 hours to 5

hours increased the GST Activity. That same *t-test* was also performed using the 20-hour average factor changes compared to the 3-hour null mean of 1. With 99% confidence ($p < 0.0033$), I can say that increasing the induction time from 3 hours to 20 hours will increase GST Activity. However, when performing a heteroscedastic, two sample *t-test* comparing the 5-hour and 20-hour average factor changes, I can only say with 90% confidence ($p < 0.0614$) that increasing the duration from 5 to 20 hours increased the GST activity.

IPTG CONCENTRATION

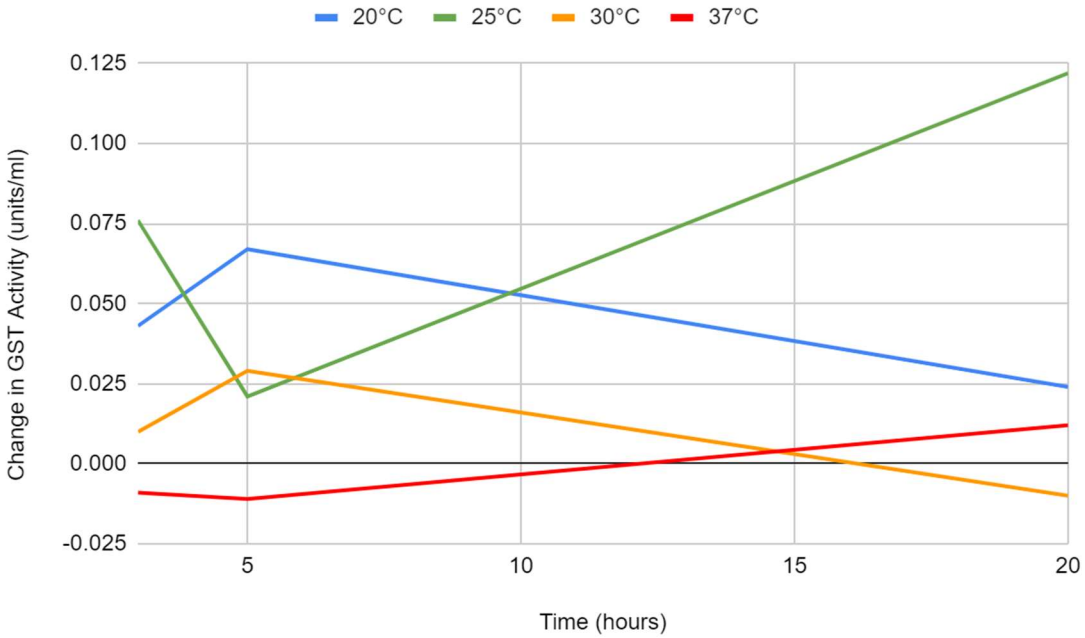


Figure 12: The change in GST activity between the induction conditions.
Change in GST activity = GST activity (1.0 mM IPTG) – GST activity (0.1 mM IPTG)

Just like with induction duration, a heteroscedastic, one-sided, single-sample *t-test*s (but this time the null mean was 0) was performed to determine statistical significance within the

data. The GST activities of the 1.0 mM IPTG trials were subtracted by the 0.1 mM IPTG corresponding hour and trial number at the respective temperature. These numbers were then used in the *t*-tests and compared to 0. The null being that the 1.0 mM IPTG did not result in a better GST Activity than the 0.1 mM IPTG conditions. Using a one-sided, heteroscedastic *t*-test, with 99% confidence ($p < 0.00425$), inducing at 1.0 mM IPTG led to a greater GST Activity than inducing at 0.1 mM IPTG at 20 °C regardless of time. At 25 °C, I can say with 95% confidence ($p<0.0125$), that inducing at 1.0 mM IPTG led to a greater GST Activity than inducing at 0.1 mM IPTG regardless of time.

Due to the abundance of both positive and negative numbers of the resulting difference at the higher temperatures, I used a double-sided, heteroscedastic *t*-test. The null in this case was that there was no difference between the difference of the differing IPTG conditions and 0. At 30 °C and 37 °C the P values were 0.287 and 0.608, respectively. At both of the higher temperatures, the P values were not significant enough to reject the null hypothesis.

I was unable to find any correlation with decreased IPTG concentrations improving protein expression at higher temperatures, despite some articles suggesting otherwise. At lower temperatures, specifically 20 °C and 25 °C, it was evident that the 1.0 mM IPTG induction

condition led to better GST Activity and therefore better GST-GFP fusion protein expression than the 0.1mM IPTG condition.

TEMPERATURE

The most obvious impact on protein expression came from induction temperature. At the 20-hour mark of both IPTG concentrations, 25 °C had the greatest GST Activity followed by 20 °C, 30 °C, and 37 °C, respectively.

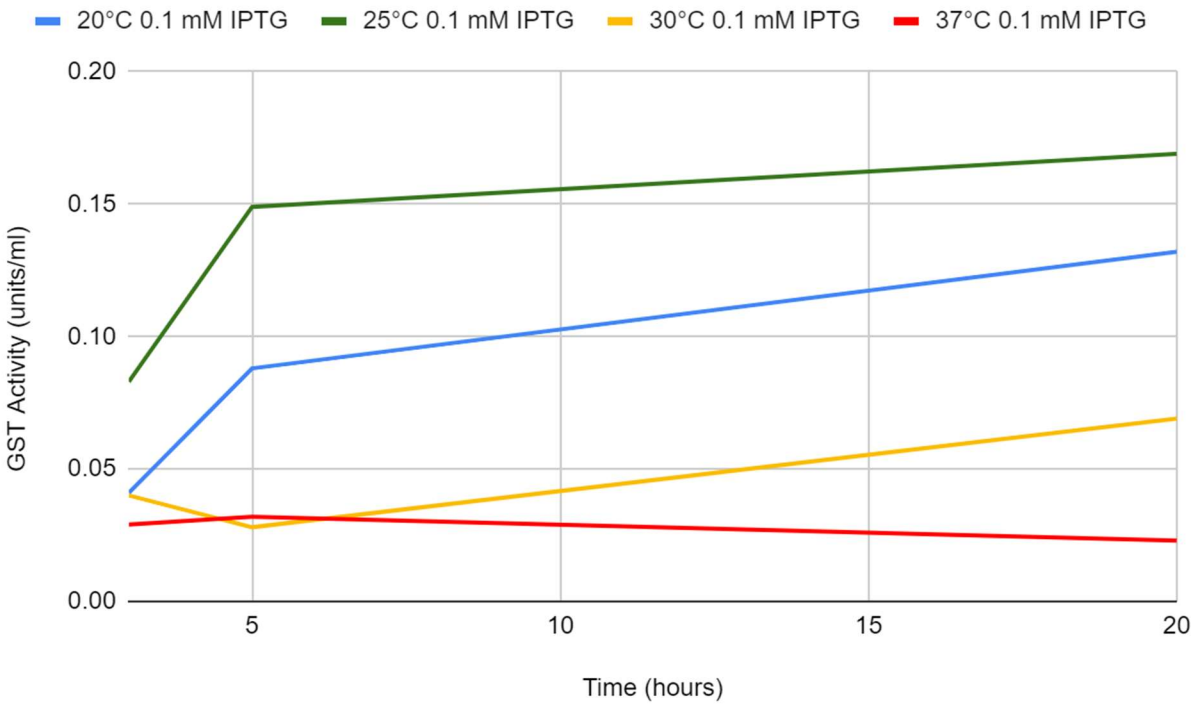


Figure 13: The effect of temperature on the GST Activity of the 0.1 mM IPTG samples.

Table 3:
P values of *t*-tests at 0.1 mM IPTG Condition

	Temperature (°C)		
Temperature (°C)	25	30	37
20	0.023	0.178	0.168
25		0.063	0.059
30			0.348

Single-Sampled, Two-sided, Heteroscedastic *t*-tests were done. P values were calculated by taking the difference of each trial at each hour of the two temperatures being tested, averaging the values for hours 3, 5, and 20, and the resulting three numbers were used in the *t*-tests with n=3, and m=0.

At 0.1 mM IPTG, the only statistically significant (alpha level of 0.05) difference in temperature at all three hours occurred at 20 °C and 25 °C. The difference for 25 °C between 30 °C and 37 °C was significant at alpha = 0.1. I believe these P values are higher than expected, because the difference at hour 3 for many of the temperatures was much smaller than the difference in temperatures for hours 5 and 20.

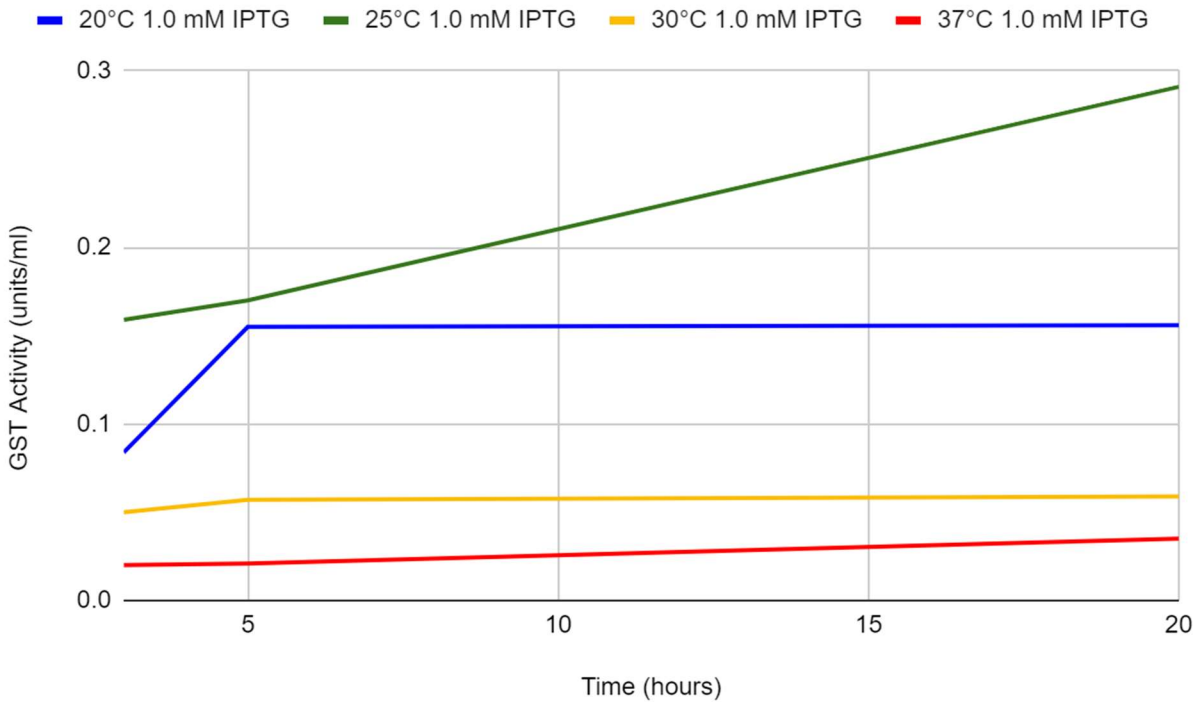


Figure 14: The effect of temperature on the GST Activity of the 1.0 mM IPTG samples.

Table 4:
P values of *t*-tests at 1.0 mM IPTG Condition

	Temperature (°C)		
Temperature (°C)	25	30	37
20	0.163	0.067	0.037
25		0.064	0.040
30			0.013

Single-Sampled, Two-sided, Heteroscedastic *t*-tests were done. P values were calculated by taking the difference of each trial at each hour (of the two temperatures being tested), averaging the values for hours 3, 5, and 20, The resulting three numbers were used in the *t*-tests with n=3, and m=0.

At 1.0 mM IPTG there was much a greater difference at the 3-hour mark for all four temperatures, and that is why I think the P values are on average lower at this IPTG concentration. The 20 °C, 25 °C, and 30 °C conditions all showed statistically significant (alpha of 0.05) differences from the 37 °C condition. The 20 °C and 25 °C conditions showed statistically significant differences from the 30 °C condition at alpha equals 0.1. When doing a one-sided *t*-test, the 20 °C and 25 °C conditions resulted in statistically significant greater GST activities than the 30 °C and 37 °C conditions. However, there is not a statistically significant difference in GST activities between the 20 °C and 25 °C conditions.

PART II

During induction of the samples used for part II, the 25 °C condition eventually increased to 27 °C overnight. There is no way to know when the increase started or how fast it progressed. The 25 °C condition will continue to be referred to as the "25 °C condition" for the rest of the thesis, but it is important to note that the induction temperature did increase to 27 °C.

Table 5:
Original Lysate's OD_{600} and GST Activity

Temperature (°C)	Trial	OD_{600} Absorbance	GST Activity (Units/ml)	Average Protein concentration (mg/ml)
20	1	0.755	4.375	2.596
	2	0.695	4.917	2.816
	3	0.771	5.771	2.908
	Pooled Trials	-	6.458	2.699
25	1	0.767	4.083	2.412
	2	0.693	3.883	2.416
	3	0.783	3.438	2.672
	Pooled Trials	-	5.396	2.449

Values for GST Activity were calculated using the CDNB enzyme assay
Values for Average Protein Concentration was calculated using a Bradford Assay
Pooled trials are composed of Trials 1, 2, and 3 of the respective temperature.

COMPARING OPTICAL DENSITY

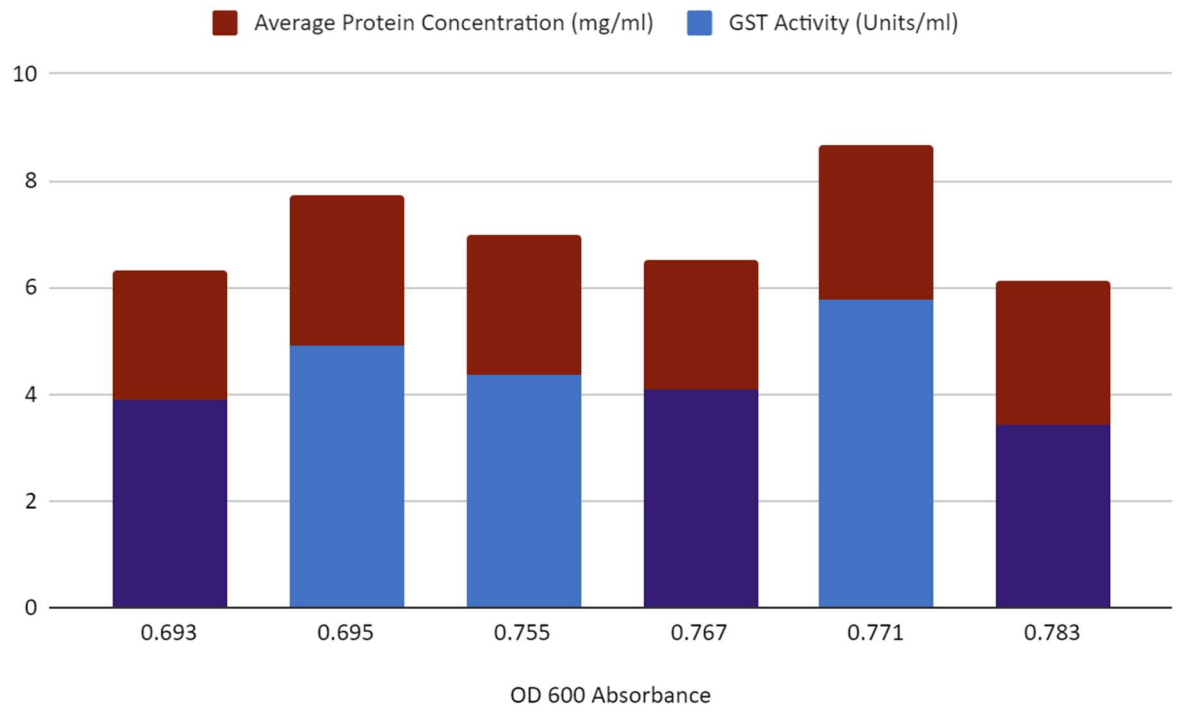
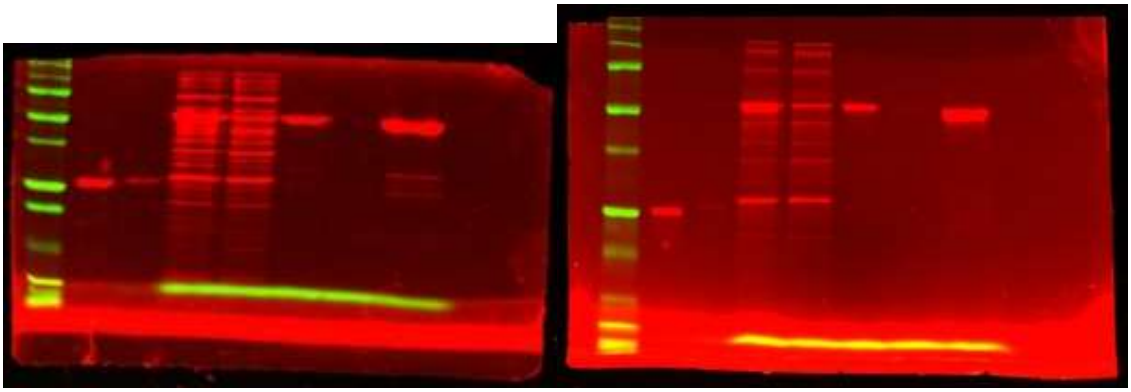


Figure 15: Average protein concentration and GST activity of the various OD_{600} absorbances. Darker blue denotes 25 °C condition, while light blue is 20 °C condition.

The OD_{600} that the cultures were induced at seemed to have no noticeable effect on protein production between the OD_{600}s of 0.693 and 0.783. The major differences between the OD_{600} and the average protein concentration and GST activity came from whether the trial was induced at 20 °C or 25 °C.

There exists a trend that the cultures with the higher protein concentration also had a higher GST activity, however, the results from this experiment did not show a strong *linear* correlation between average protein concentration and GST activity (R^2 = 0.532).

SDS-PAGE



(a) 20 °C (b) 25 °C

Figure 16: SDS-PAGE gels of the 20 °C and 25 °C conditions.
The columns from left to right are the: molecular weight marker, GST control, GFP control, original lysate (OL), affinity flowthrough (AF), affinity elution (AE), IEX flowthrough (IF), and IEX elution (IE). The samples in the columns were normalized to 12.95 µl of total protein. The images were cropped to the size of the gels, and the sizes of the images were adjusted so that the bottoms of the gels were of equal width, since the stacking gel was chopped off at unequal heights.

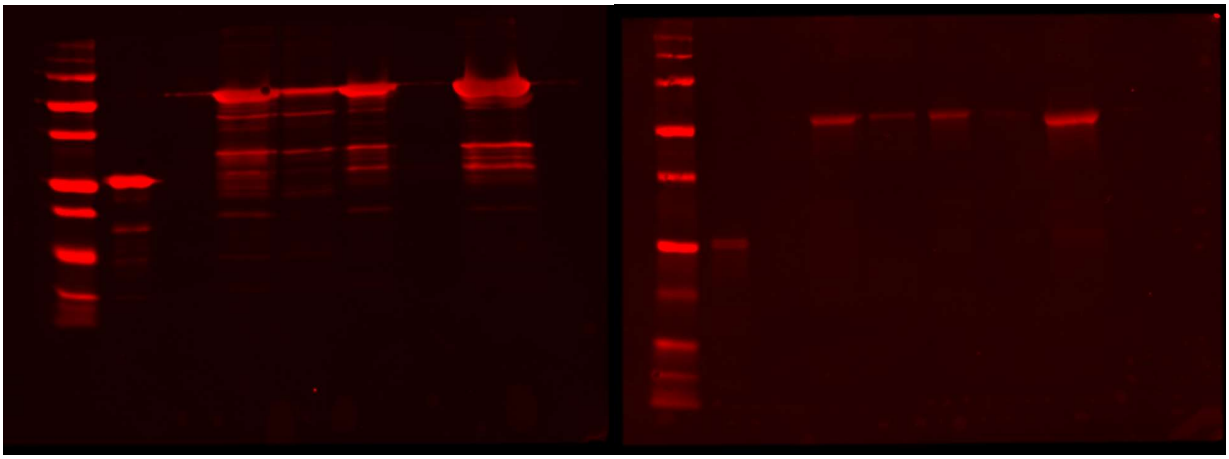
Sample normalization was done to determine how much lysate would be added to each column. This was calculated using a DC Protein Assay, which had a BSA standard equation of y= 0.1607x + 0.0823 with an R^2 of 0.9928. Since the two gels come from different sources, the sizes of the bands are less important than the presence of the bands in the various columns. A normalized CDNB enzyme assay was done later in the experiment to quantify the sizes of the bands.

Looking at the gels, in the OL columns of both temperatures, there appears to be a significant band at ~54 kDa. Presumably this band is the GST-GFP fusion protein (which was confirmed by the Western blot). A significant band also appears at ~27 kDa, however, a band of equal size is also present in the AF column and not visible in the columns of future purifications.

Initial thoughts were that this band might indicate a significant amount of free GFP, but a band of similar size was not present in the GFP Western blots.

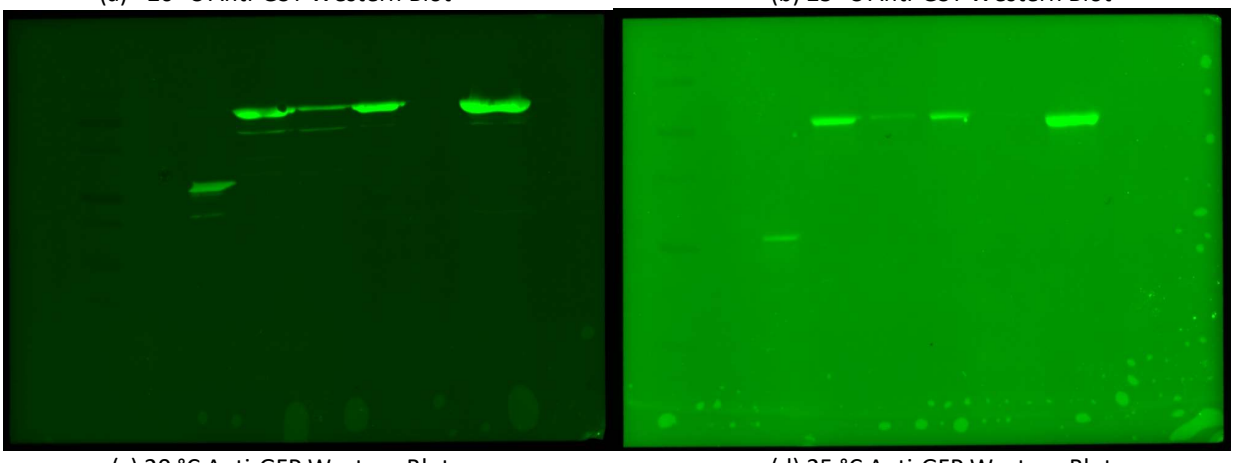
The 20 °C, IE column has a couple of bands at about 25-30 kDa, which also appear on the GST Western, indicating possible free GST or truncated GST-GFP fusion protein.

WESTERN BLOT



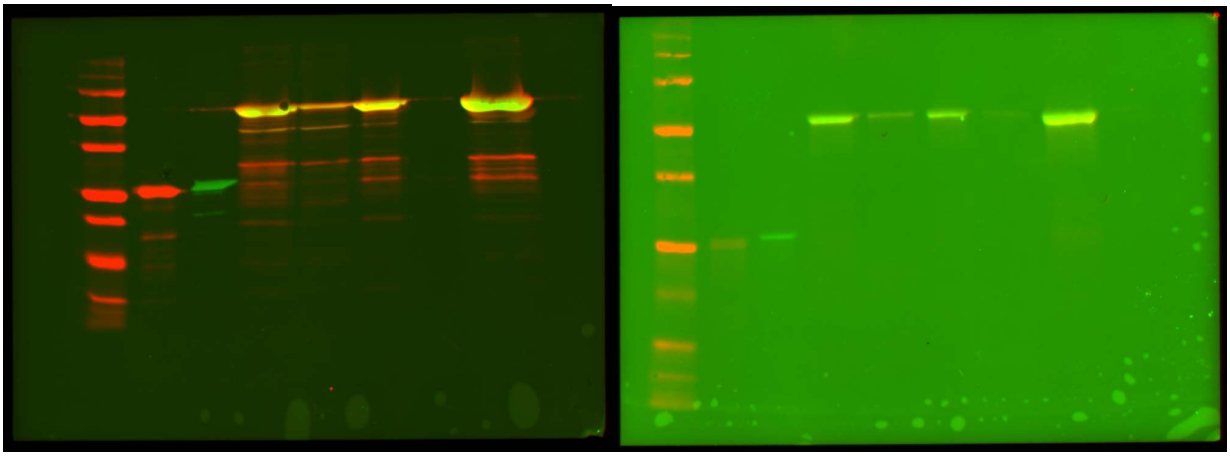
(a) 20 °C Anti-GST Western Blot

(b) 25 °C Anti-GST Western Blot



(c) 20 °C Anti-GFP Western Blot

(d) 25 °C Anti-GFP Western Blot



(e) 20 °C Combined Western Blot (f) 25 °C Combined Western Blot

Figure 17: Anti-GST, anti-GFP, and combined western blots.
The Western blots are from the SDS-PAGES of Figure 10. The columns from left to right are the: molecular weight marker, GST control, GFP control, original lysate (OL), affinity flowthrough (AF), affinity elution (AE), IEX flowthrough (IF), and IEX elution (IE).

These Western blots confirm that the large bands at ~54 kDa are in fact the GST-GFP fusion protein, as these bands are present on the anti-GST and anti-GFP gels. Figure 17a has a significant amount of unexpected bands, across most of the columns. These bands do not appear in the GFP Western blots, indicating they are likely truncated/degraded GST-GFP fusion proteins. It is important to note that these extra bands are not present in the 25 °C Western blots.

The size of the bands across the columns are indicative of the fold purification of the GST-GFP fusion protein. Noticeable bands are present in the AF of each Western, indicating GST-GFP fusion protein was lost at this step across the board. The bands at 54 kDa in the AE column of the 25 °C Western blots are noticeably fainter than the bands in the same gel's OL bands, indicating a possible fold purification of less than 1. Those same bands in the 20 °C Western blots are of comparable size. The largest band of each Western occurs in the IE

column, indicating the purification steps eventually lead to a greater fold purification, which is seen in table 6.

PURIFICATION TABLE

Table 6:
Purification Table

Temperature (°C)		20			25	
Condition	Original Lysate	Affinity Elution	IEX Elution	Original Lysate	Affinity Elution	IEX Elution
Protein Conc. (mg/ml)	2.60	1.71	0.32	2.34	1.99	0.26
Total Volume (ml)	3	3	1	3	3	1
Total Protein (mg)	7.79	5.14	0.32	7.03	5.97	0.26
Reaction Velocity (units/ml)	3.24	1.72	0.90	3.16	1.48	0.65
Total Activity (units)	9.73	5.16	0.90	9.48	4.45	0.65
Specific Activity (units/mg)	1.25	1.00	2.83	1.35	0.75	2.49
Percent Yield (total)	100	65.94	6.21	100	84.94	4.34
Percent Yield (GST-GFP)	100	53.02	17.52	100	46.96	14.50
Fold Purification (GST-GFP)	1	0.80	2.26	1	0.56	1.84

Values were rounded to the nearest hundredths place
Reaction Velocity calculations come from Table 7

The purification table shows how the different temperatures compare at each of the purification steps. Ideally, the total protein should decrease faster than the total activity across the purification steps, which would indicate that the samples were keeping the GST-GFP fusion protein and losing the other proteins. The most important row to look at to determine the successfulness of the purification steps is the fold purification, which compares the concentration of target protein at that step to the starting concentration.

Looking at the last row of Table 6, it is obvious that a lot of GST-GFP protein was lost during the affinity chromatography compared to other proteins, especially for the 25 °C lysate. The 25 °C affinity elution had only a 46.96% percent yield for GST-GFP, compared to an 84.94% yield for total proteins. This is a major blunder in the purification step. The 20 °C also lost a lot of GST-GFP relative to total protein, with a GST-GFP percent yield of 53.02% and a total protein percent yield of 65.94%. This aligns with the less-than-ideal fold purifications of the 20 °C and 25 °C affinity elutions at 0.80 and 0.56, respectively.

The IEX chromatography resulted in much better results, as the fold purifications of the 20 °C and 25 °C condition's lysates finished with a fold purification of 2.26 and 1.84, respectively. In both cases, the percent yield of the GST-GFP fusion protein decreased less than the percent yield for the total protein. At 20 °C the total protein percent yield was 6.21%, while the GST-GFP percent yield was 17.52%. At 25 °C the total protein percent yield was 4.34% and the GST-GFP percent yield was 14.50%. These results indicate that the IEX column used was much more effective at purifying the GST-GFP fusion protein than the GSH affinity column. I suspect this may be due to the speed at which the protein was loaded onto the column and the

size of the column. Some studies have suggested that GST and GSH have a slow binding kinetic (Harper & Speicher, 2011) but a separate experiment should be conducted to test if this is the case with the GST-GFP fusion protein.

The reaction velocities used in Table 6 came from normalized CDNB enzyme assays shown in Table 7. These assays give numerical values to the results of the GST Western blot.

Table 7:
Normalized CDNB enzyme assays

Temperature (°C)		20			25	
Condition	Original Lysate	Affinity Elution	IEX Elution	Original Lysate	Affinity Elution	IEX Elution
Normalized Reaction Velocity (units/ml)	0.323	0.260	0.734	0.349	0.193	0.646
Western Blot Reaction Velocity (units/ml)	1.615	1.300	3.670	1.745	0.965	3.230
Volume of Lysate Added (µl)	0.996	1.512	8.120	1.104	1.300	10.00
Reaction Velocity (units/ml)	3.24	1.72	0.90	3.16	1.48	0.64

All samples received 10 µl of solution [10 µl solution= X µl Lysate + (10-X) µl dH2O], the solutions were normalized to 2.59 µg of total protein.
(Western Blot Reaction Velocity) = 5 x (Normalized Reaction Velocity)
(Reaction Velocity) = (Normalized Reaction Velocity) x (10 µl) / (Volume of Lysate Added)

The normalized reaction velocities are important as they give numerical values to compare the 20 °C and 25 °C Western blots (whose gels are from different sources). It is

important to note that the CDNB enzyme assay used 2.59 µg of total protein, while the SDS-PAGE and Western blot used 12.95 µg of total protein. That is why the normalized reaction velocities were multiplied by 5 to get the Western blot reaction velocities.

Using the normalized reaction velocities as size indicators for the SDS-PAGEs and Western Blots, it becomes immediately obvious that the bands of the premade gels from Bio-Rad are more tightly compact than the bands from the gels made in the lab. For example, the 25 °C original lysate column should technically show a bigger band than the 20 °C original lysate column in their respective Anti-GST Western blots, however this is not the case. When it comes to purification, the 20 °C condition ended up having a larger fold purification, which is evident from the increased normalized reaction velocities. The 0.734:0.323 ratio is greater than the 0.646:0.349 ratio found in the 25 °C normalized reaction velocities over the course of purification. By this metric, the 20 °C would be the better temperature for GST-GFP protein expression due to its greater fold purification and higher concentration of target protein post-purification.

By looking at the reaction velocities, however, we can see how much total GST-GFP fusion protein was produced per 10 µl of lysate. While the 20 °C temperature showed higher values across the board, the difference was minimal for the original lysates, with only a 2.5% increase reaction velocity increase over the 25 °C temperature. After the purification steps, the difference increased to 40%.

COMPARING REACTION VELOCITIES

Table 8:
CDNB Enzyme Assays (Not Normalized)

Temperature (°C)		20			25	
Condition	Original Lysate	Affinity Elution	IEX Elution	Original Lysate	Affinity Elution	IEX Elution
Reaction Velocity (units/ml)	6.458	2.448	0.969	5.396	2.266	0.693

Reaction velocities were taken immediately after purification steps

While the normalized CDNB enzyme assay reduced confounding variables to determine fold purification more accurately and to numerically describe the Western blots, these CDNB enzyme assays taken immediately after the purification steps accurately compare the conditions at each individual step. These CDNB enzyme assays were not used for Table 6, because they were taken on separate days in differing room and material temperatures with differing stocks of GSH and CDNB between purification steps. While the normalized assay was done at a consistent temperature and the same reagents. The reaction velocities of the 20 °C conditions are 19.7%, 8.03%, and 39.8% greater than the reaction velocities of the 25 °C condition for their original lysates, affinity elutions, and IEX elutions, respectively.

Table 9:
GST Activity Ratio of the 20 °C Condition to the 25 °C Condition

CDNB Enzyme Assay	Original Lysate	Affinity Elution	IEX Elution
Not-Normalized, Immediately Taken	1.197	1.080	1.398
Normalized at the End	0.926	1.347	1.136

Ratio compares the 20 °C condition to the 25 °C condition: i.e. X : 1, with 1 being the 25
Calculation: Factor increase = (20 °C GST activity) / (25 °C GST activity)

Hypothetically, the GST activity ratios for each purification step between the CDNB enzyme assays should be the same. This gives comparative insight to how the GST proteins were affected between the purification steps and the SDS-PAGE. After lysis, it appears more GST-GFP fusion protein was lost in the 20 °C condition than the 25 °C condition. The opposite occurred for the affinity elutions and IEX elutions, where more GST-GFP fusion protein was lost in the 25 °C condition than the 20 °C condition.

CHAPTER 5: DISCUSSION

PART I

The first experiment showed the effects of temperature, IPTG concentration, and duration of induction on GST-GFP fusion protein expression.

As the induction duration increased so did the reaction velocity of the GST, implying that more GST-GFP fusion protein was expressed at these higher induction durations. The 5 and 20-hour conditions were statistically significantly better than the 3-hour condition, for GST-GFP fusion protein expression. With 90% confidence, induction for 20 hours resulted in greater GST-GFP fusion protein expression than 5 hours at all the temperatures IPTG concentrations tested in this experiment.

At the lower temperatures of 20 °C and 25 °C, the 1.0 mM IPTG condition resulted in statistically significant increases in GST reaction velocities than the 0.1 mM IPTG condition, indicating the greater IPTG concentration condition resulted in greater GST-GFP fusion protein expression. At the higher temperatures of 30 °C and 37 °C, there was no statistically significant difference in GST-GFP fusion protein expression between the 0.1 mM IPTG and 1.0 mM IPTG conditions.

The 25 °C condition resulted in statistically significant increases in GST-GFP protein expression compared to the 30 °C, and 37 °C conditions at all IPTG concentrations and

durations. The 20 °C showed statistically significant increases in GST-GFP protein expression compared to the 30 °C and 37 °C at the 0.1 mM IPTG concentrations and all durations.

Based on the three variables tested, it is evident that temperature had the greatest effect on GST-GFP fusion protein production. The GST activity differences between different temperatures was statistically significant across the board for some of the temperatures. IPTG concentrations only seemed to affect the lower temperatures, with the E. coli expressing more fusion protein at the higher IPTG concentration. Contrary to previous studies, decreasing the IPTG concentration for higher temperatures had no beneficial effect on protein expression for the GST-GFP fusion protein. The experiment also showed that increasing the induction time from 3 hours to 5 hours or 20 hours increased the average GST activity. However, the time increase from 5 hours to 20 hours was only statistically significant at alpha equals 0.10. Overall, the 20-hour condition at a temperature of 25 °C and 1.0 mM IPTG had the greatest GST activity, and therefore produced the most GST-GFP fusion protein.

Despite these conclusions, there was one unexpected result, which was a non-significant difference in GST activity between hours 5 and 20 of the 20 °C, 1.0 mM IPTG condition. When looking at the fluorescence of the 20 °C, 1.0 mM IPTG, 20-hour condition samples in Figure 8, one would expect to see an increase in GST activity compared to the hour 5 condition. To test if these suspicions were true, the experiment would be run again, however some variables would be changed. Part II of the experiment aimed to only compare the conditions that had the greatest fluorescence in the gel doc: the 1.0 mM IPTG, 20-hour duration, 20 °C and 25 °C conditions.

PART II

There is an important variable to keep in mind when analyzing the results of this experiment. Unfortunately, the incubator was unable to hold the temperature at 25 °C, and after 20 hours of induction, the temperature inside the incubator was 27 °C. It is impossible to determine when or how fast the temperature of the incubator increased from 25 °C to 27 °C.

Despite this, there are a few important takeaways from this set of experiments. Firstly, there were no obvious trends in the reaction velocities of the lysates inducted at different OD_{600}s. The 20 °C condition, however, seemed to have a higher reaction velocity on average, as shown in Table 5. The 20 °C condition also resulted in greater fold purification (Table 6) and normalized reaction velocities (Table 7). This result was unexpected, because the results of part I suggested that the 25 °C condition would have the higher GST activities.

Despite having a greater GST activity, there is a major point to be made regarding the Western blots of the 20 °C lysates. A significant amount of GST-containing protein can be seen on the Anti-GST Western blots at molecular sizes below 56 kDa. These bands were not seen in the Anti-GFP Western blots, implying that these were either degraded or truncated GST-GFP fusion proteins.

If proteases were the cause of the bands, then proteolysis would have had to occur during induction, as protease inhibitors were added before cell lysis via the French press. The results from Table 9, also indicate that more GST-GFP fusion protein was lost in the 25 °C elutions than the 20 °C elutions between the purification steps and the SDS-PAGE. If proteolysis

had occurred after lysis, then bands similar to those seen in the 20 °C Anti-GST Western blot would also be present in the 25 °C Anti-GST Western blot. This implies it is unlikely that the GST-GFP fusion proteins degraded after the first CDNB Assay was run.

Despite not being fully formed GST-GFP fusion proteins, the bands seen on the 20 °C Anti-GST Western blot, indicate that the GST of these proteins was fully formed, indicating that these proteins contributed to the increased reaction velocities of the 20 °C condition. With the technology at hand, it is impossible to determine how much of an impact these shortened proteins had on the GST activity of the 20 °C condition. Therefore, it is difficult to conclude that the 20 °C condition resulted in greater GST-GFP fusion protein production than the 25 °C condition, despite the higher GST activities.

Future experiments should use a C-terminal GST-tag, so that only fully completed GST-tagged fusion proteins will bind to the GSH column and positively charged beads. If results similar to the 20 °C Anti-GST Western blot are observed, then it would imply that proteases are present and causing the degradation of the proteins. If a researcher is required to use N-terminal GST-tagged fusion proteins, the 25 °C temperature might be the better option, as the Western blots for this induction condition were significantly cleaner.

CONCLUSION

Future experiments attempting to express the GST-GFP fusion protein should use an IPTG concentration of 1.0 mM over 0.1 mM, induce for at least 20 hours, and try to keep the

induction temperature below 25 °C. Furthermore, future studies comparing the 20 °C and 25 °C conditions should rearrange the recombinant protein used, so that the GST-tag is on the C-terminal, to prevent purification steps from including truncated proteins.

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