THE 3RD ANNUAL CSTEP SUMMER RESEARCH PROGRAM SYMPOSIUM

June 28, 2024 from 12 pm to 3 pm

Exploring the Impact of Novel H-bonds on Enzymatic Activity: A Computational and Experimental Study on Amino Acid Mutagenesis

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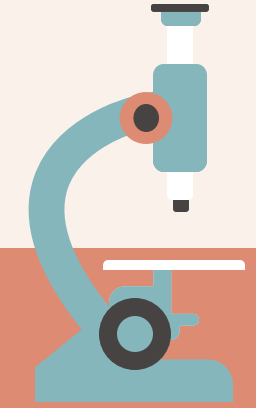
CSTEP Summer Research Program 2024
Mercy University



Overview

- This study explores the impact of newly discovered hydrogen bonds on enzymatic activity.
- Utilizing computational analysis and experimental procedures to find binding affinity and thermostability improvements.
- Investigating the effects of amino acid mutagenesis on enzyme function and benefits, with a main focus on the significance of novel hydrogen bonds.

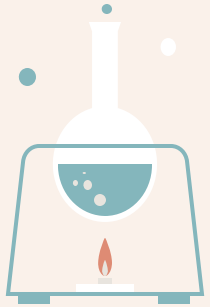
Introduction

Clean Energy:

- Clean energy refers to energy sources that have minimal environmental impact and reduce greenhouse gas emissions. These sources are sustainable, renewable, and contribute to a cleaner planet. (Awogbemi, et.al)

Ethanol:

- Ethanol is a biofuel produced by the natural fermentation of sugars, like glucose. With the use of yeast, sugars are metabolizing into ethanol anaerobically.



Free Energy

- **Free energy:**

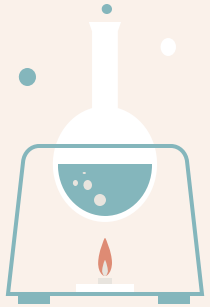
An energy-like property or state function of a system in thermodynamic equilibrium. Its value depends on the system's state and not its history. Free energy helps determine how systems change and how much work they can produce.

Lower Gibbs Free Energy:

- More stable and higher thermostability.

Higher Gibbs Free Energy:

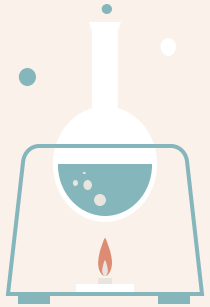
- Less stable and lower thermostability.



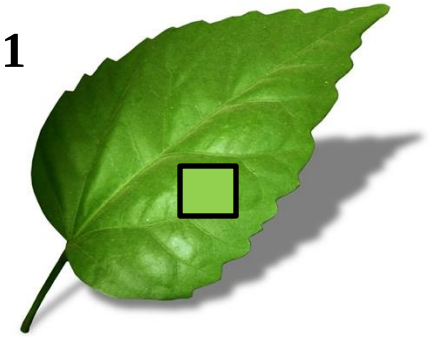
Production of Ethanol

The Role of BglB in the Production of Ethanol

- BglB is a beta-glucosidase enzyme crucial for breaking down sugars, like oligosaccharides, into beta-glucose monomers.
- Key role in ethanol production process by converting biomass into fermentable sugars.

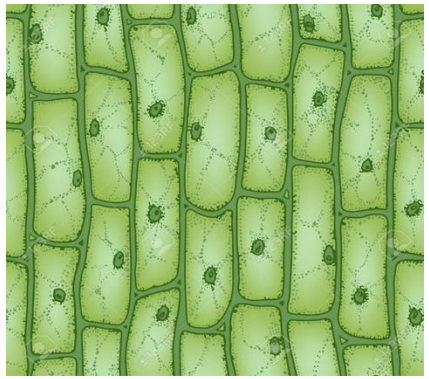


1



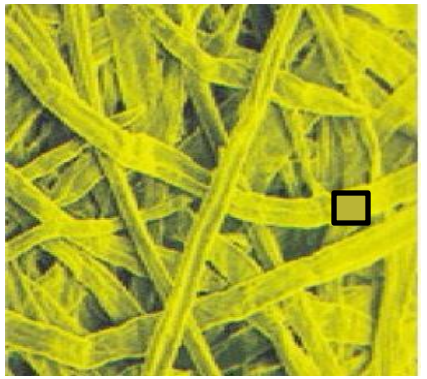
Leaf

2



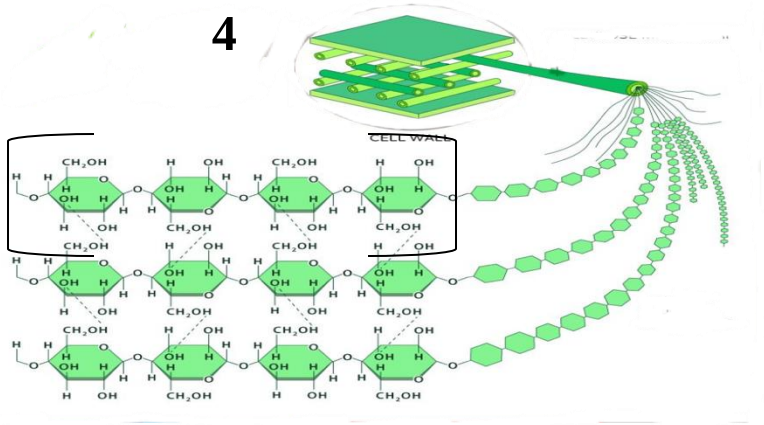
Plant Cells

3



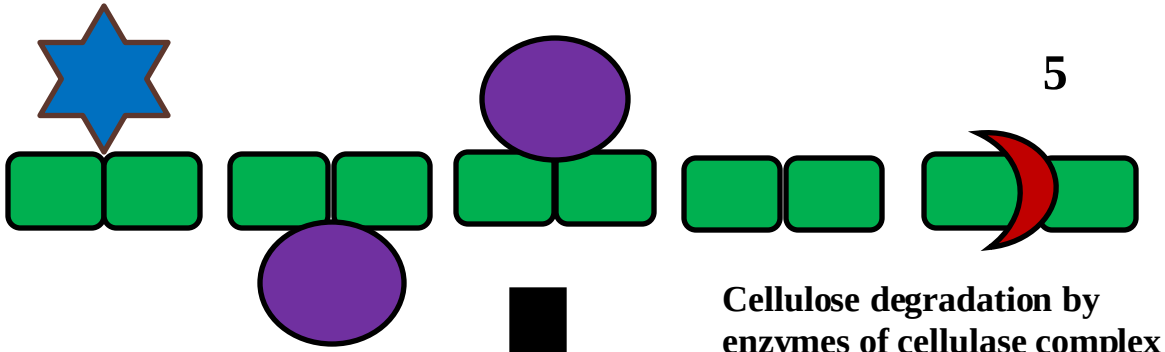
Cellulose microfibrils in cell wall

4



Cellulose glucose chains

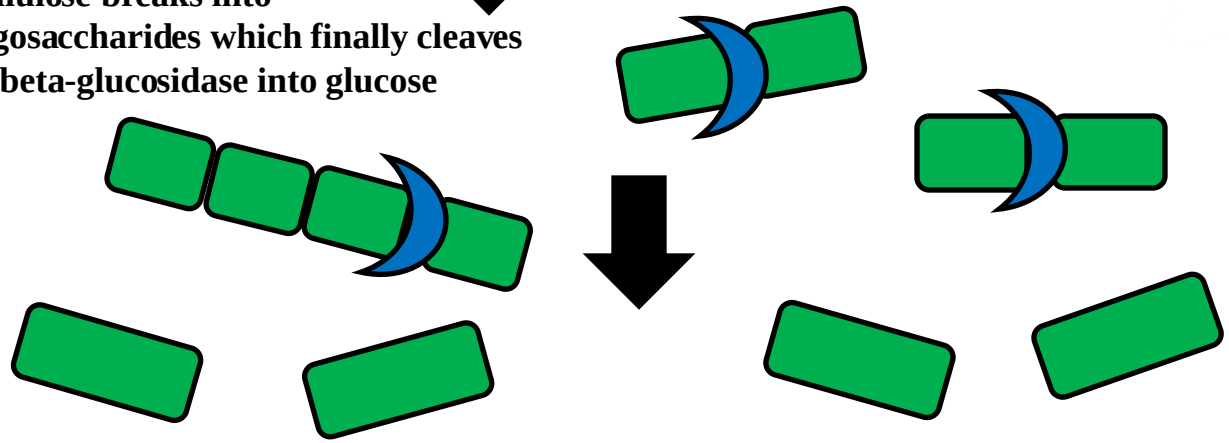
5



Cellulose degradation by
enzymes of cellulase complex

Cellulose breaks into
oligosaccharides which finally cleaves
by beta-glucosidase into glucose

6



Free Beta-
glucose (monomers of cellulose)

Foldit Standalone

1. Tool for manipulating amino acids and forming hydrogen bonds.
2. Enhance understanding of protein folding
3. Contribute to developing new, stable protein designs
4. Provide 3D models for interactive study.

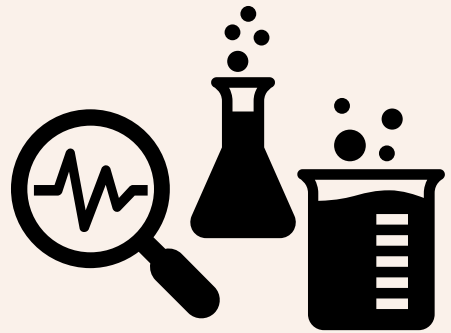
Research Objectives

Hypothesis:

- New H-bonds resulting from amino acids mutations enhance BglB enzyme stability.

Goals:

- Computational biology, using the Foldit Standalone software, aimed to identify new beneficial mutations.
- Mutations identified either lowered the energy score or facilitated the creation of novel H-bonds.
- These mutations led to increased thermostability, as evidenced by reduced energy scores, and improved binding affinity through stronger intermolecular interactions.



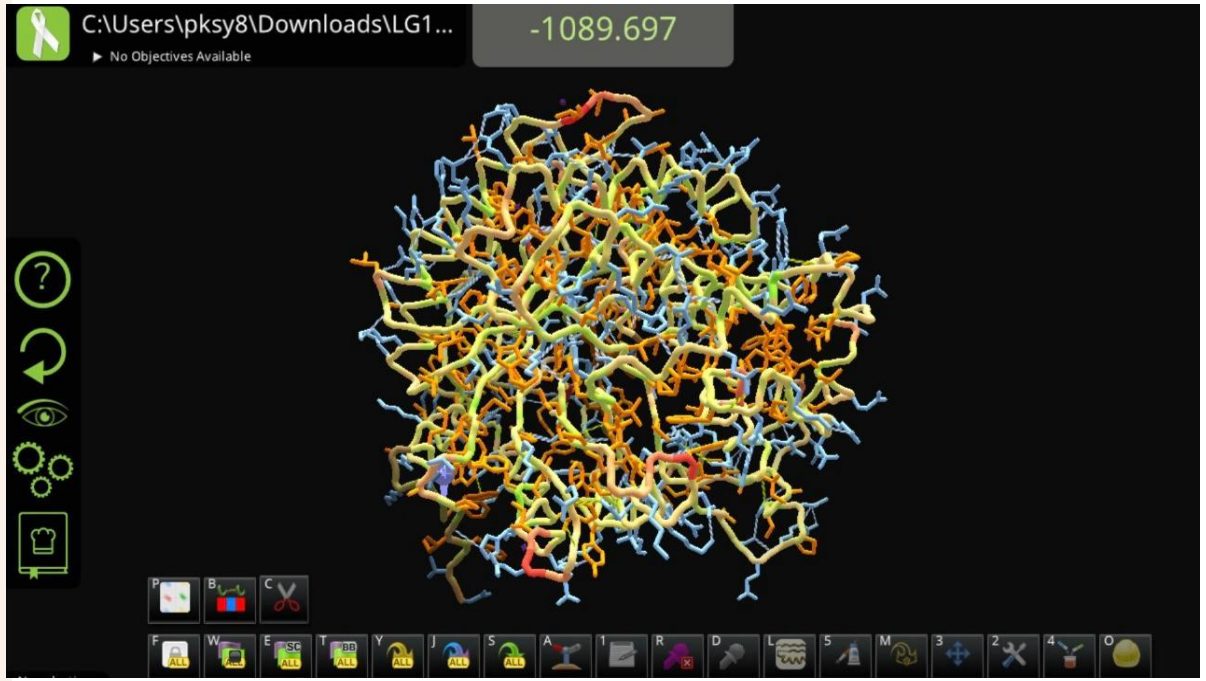
Methods and Materials

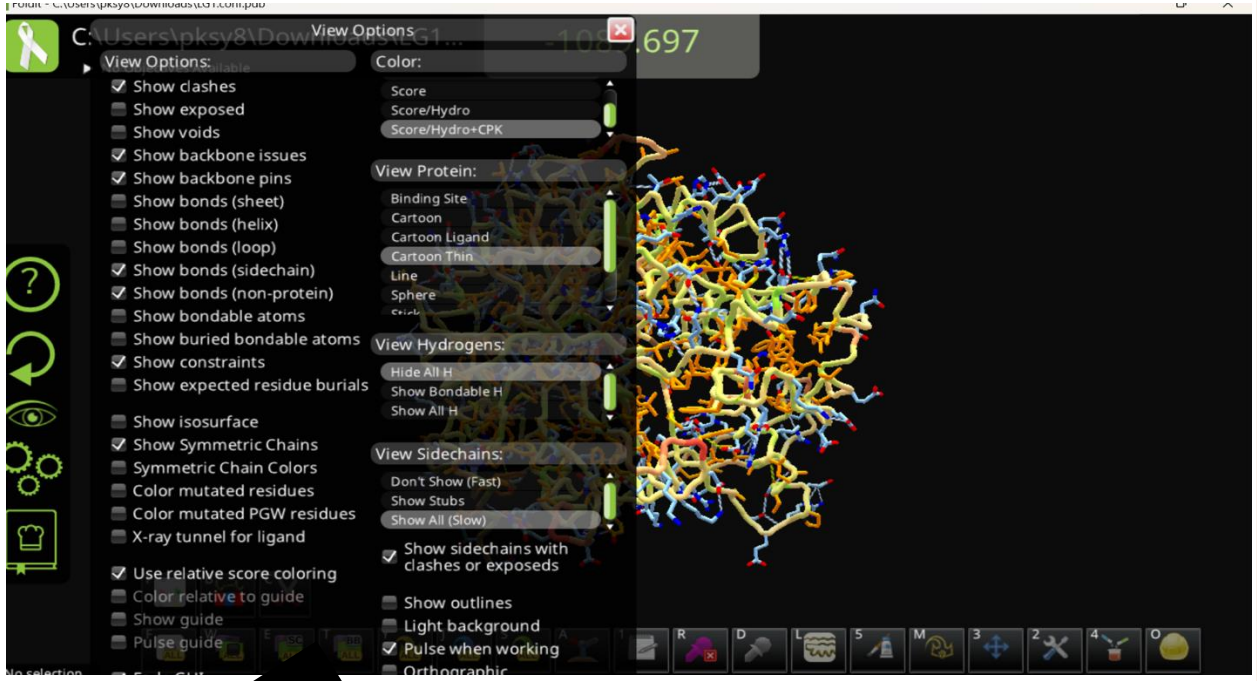
Computational Biology Methods: Foldit Standalone

- Protein modeling and structure predictions for the 3D structure of proteins with introduced mutations.
- Researchers manipulate protein structures to achieve the lowest energy state.

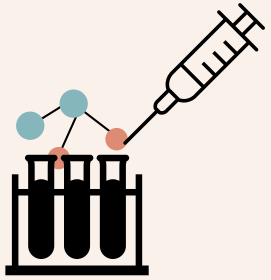
Foldit Standalone

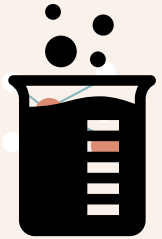
Energy Score





These were the settings utilized to improve enzyme design visualization and identify a.a. locations.

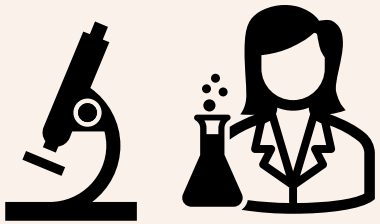




Results

On enzyme BglB, 15 mutational trails were conducted to test for new beneficial mutations. Out of those 15 mutations, 3 novel mutations were identified, V320T, T212S, and T296R. However, mutation T296R was selected to proceed with to conduct experiments as it is located in the active site and novel H-bonds were formed.

On enzyme BglB, 40 mutational trails were conducted to test for new beneficial mutations. Out of those 40 mutations, 6 novel mutations were identified, N293A, H373L, N293A, E136R, F77H and E136K. However, mutation F77H was selected to proceed with to conduct experiments as it is located in the active site and novel H-bonds were formed.



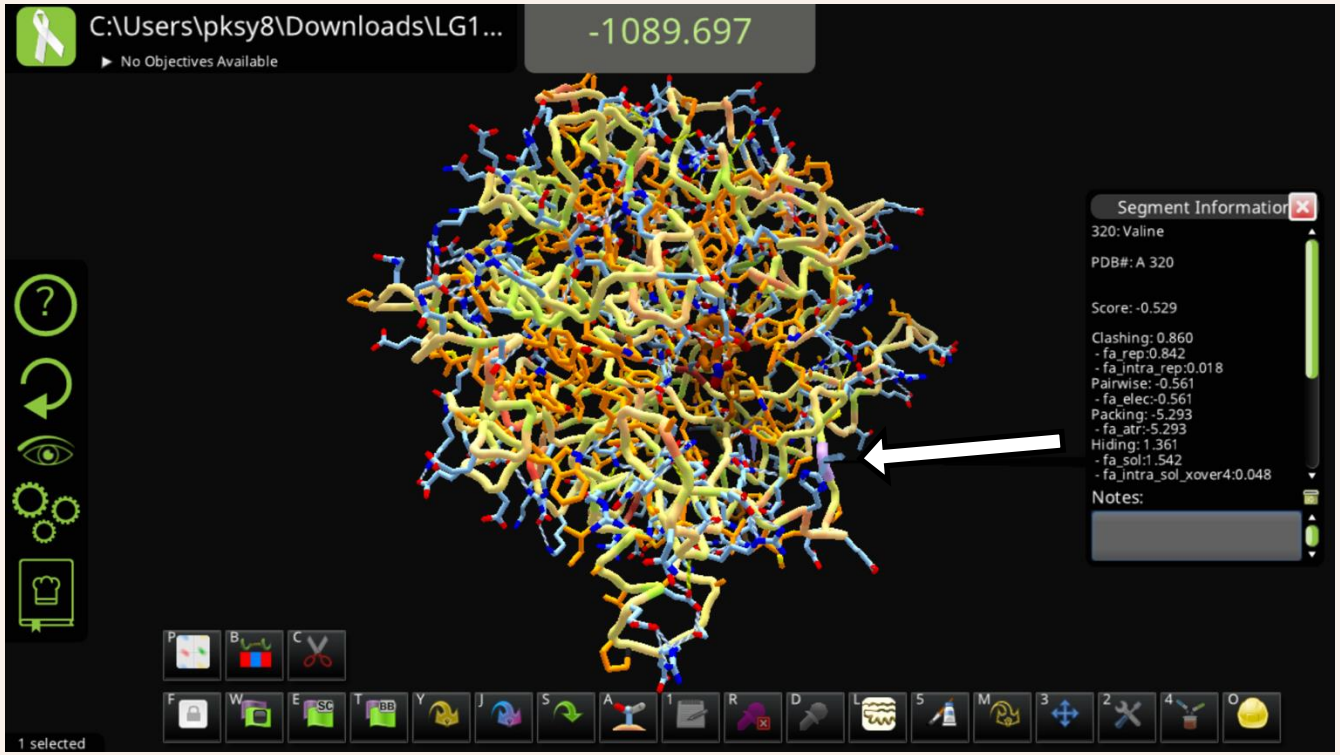
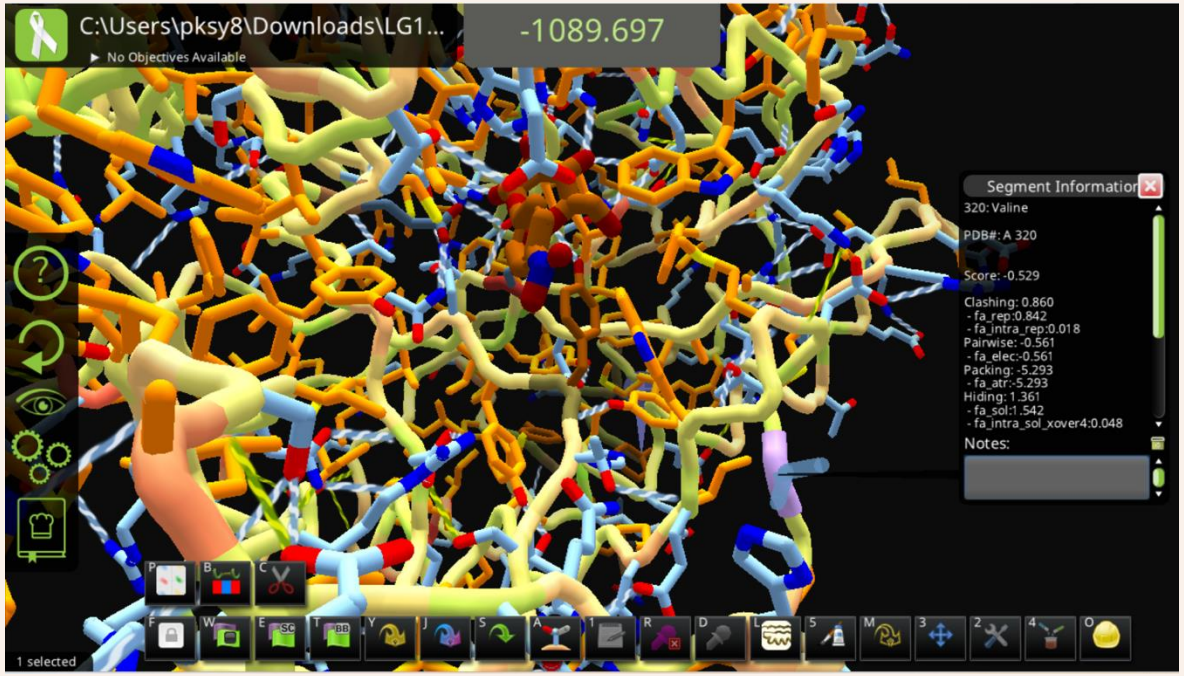


Figure 1: 3D structure of WT and mutant V320. Mutant is shown by the arrow and highlighted in purple. WT energy score shown as –1089.697.



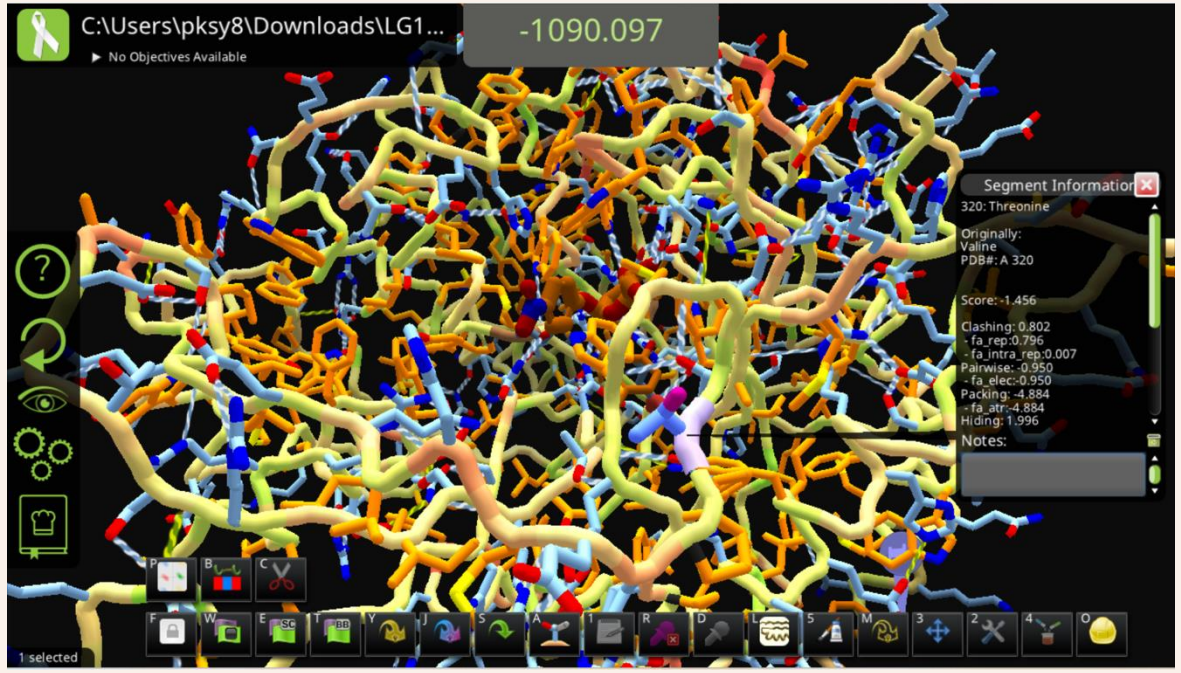


Figure 2: Zoomed in mutant V320 with WT energy score (right) and mutation V320T with precise energy score -1090.097 (left). Precise energy score found using Minimize and Shake to model chemical interactions. These test find optimal interactions between residues after mutating the A.A.

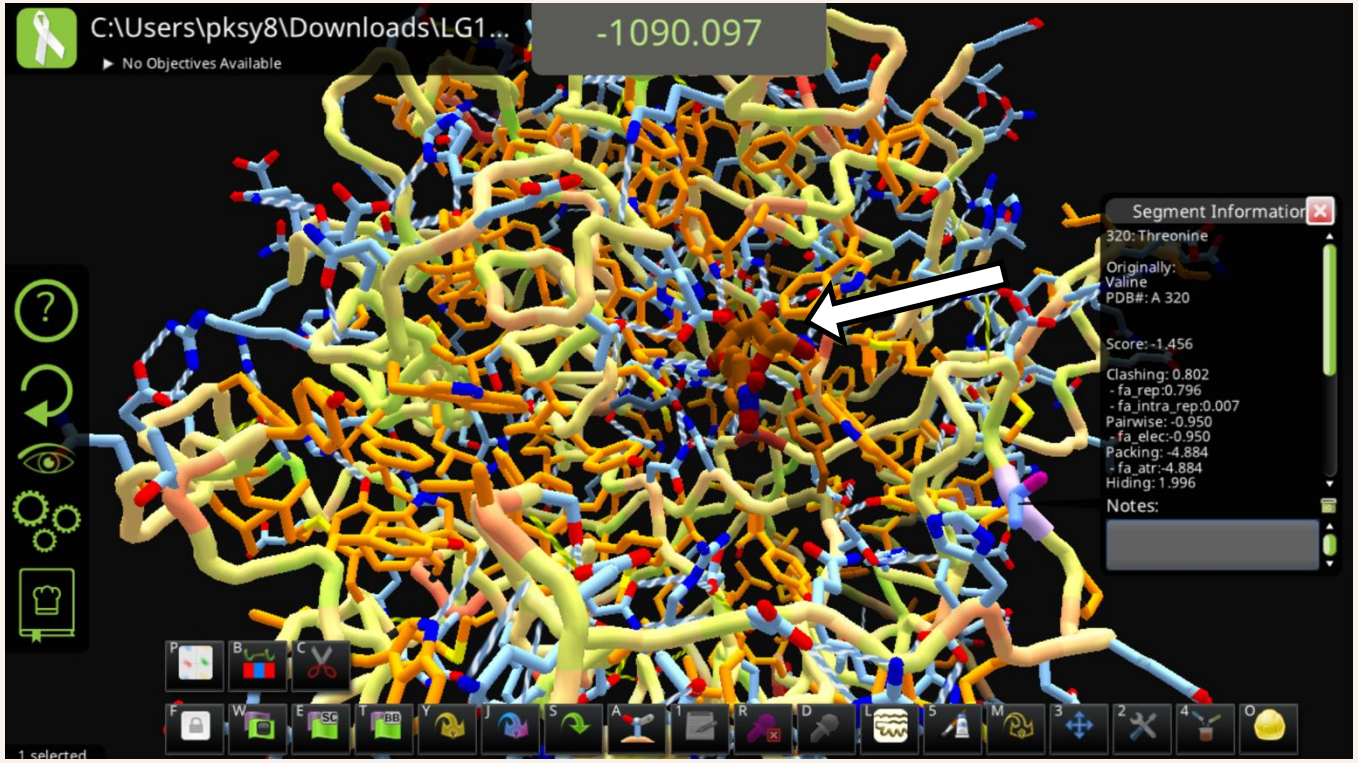


Figure 3: 3D structure of mutation V320T in relation to substrate. The mutated a.a. (highlighted in purple) is located on the peripheral edge of the enzyme in regard to the brown substrate in the center (shown by arrow).

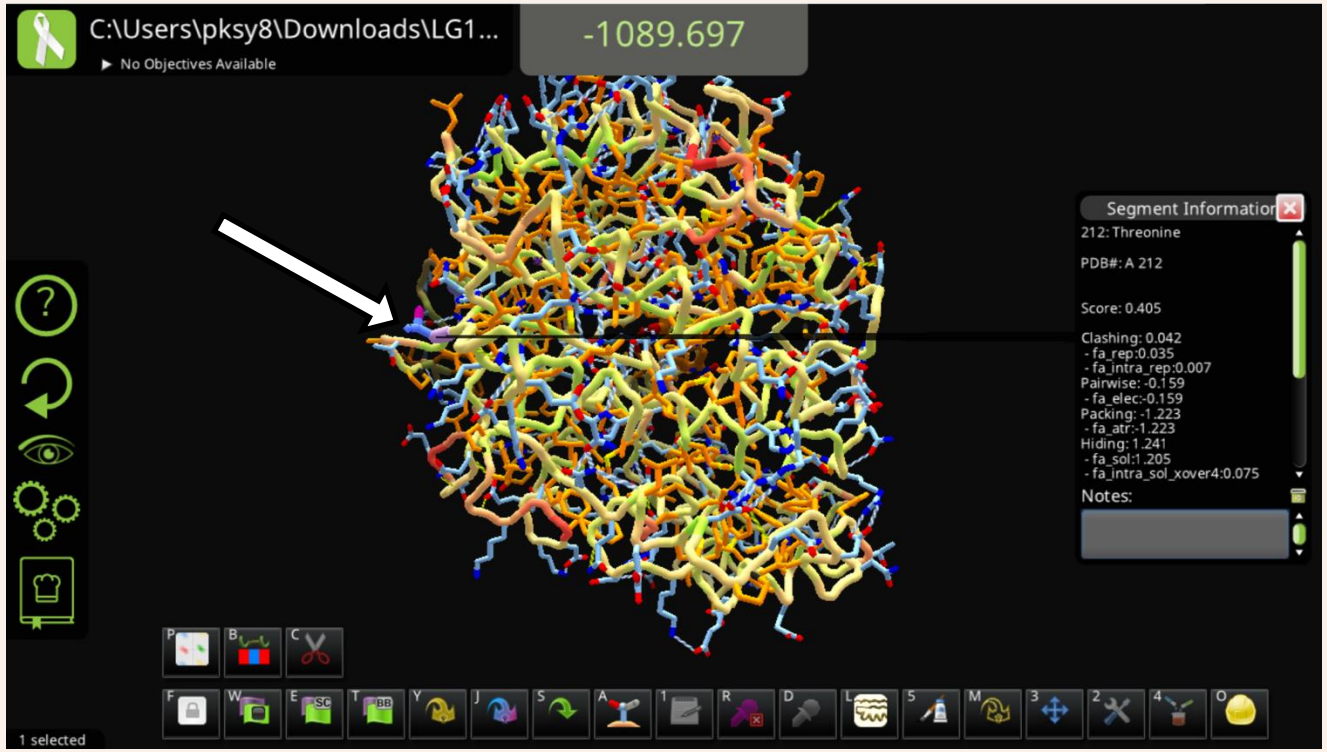


Figure 4: 3D structure of WT and mutant T212. Mutant is shown by the arrow and highlighted in purple. WT energy score shown as –1089.697.

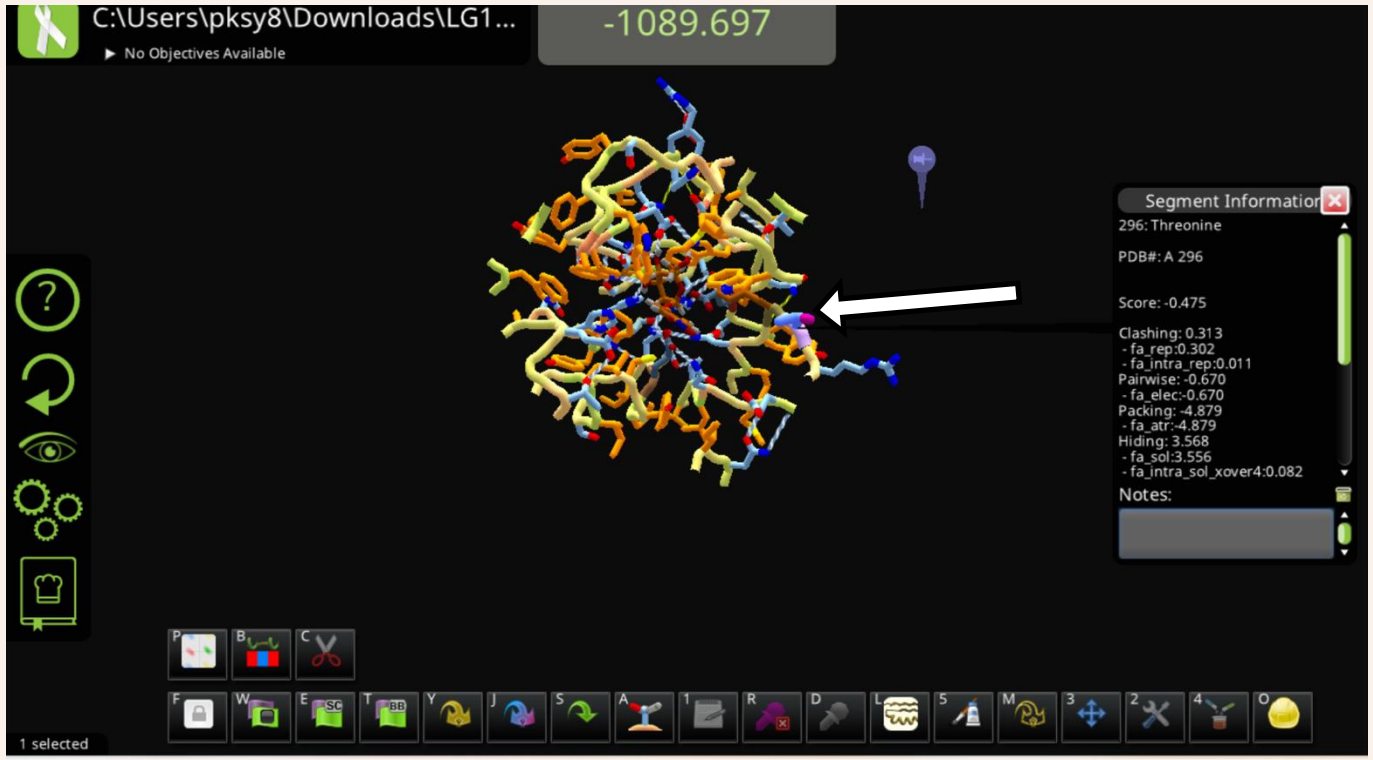
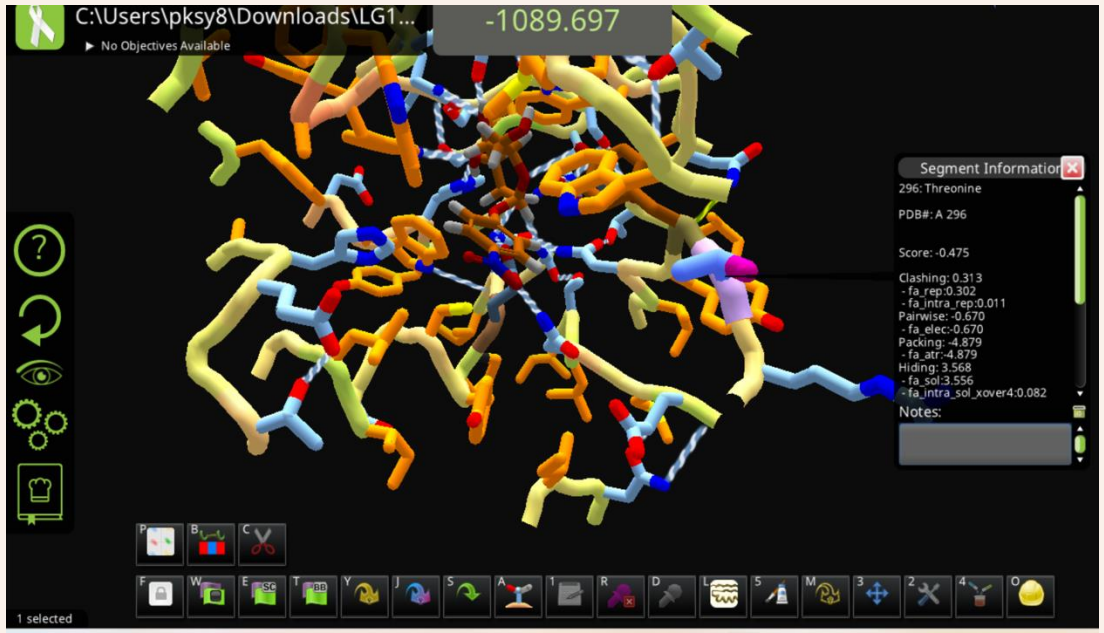


Figure 7: 3D structure of WT and mutant T296R. Mutant is shown by the arrow and highlighted in purple. WT energy score shown as –1089.697.



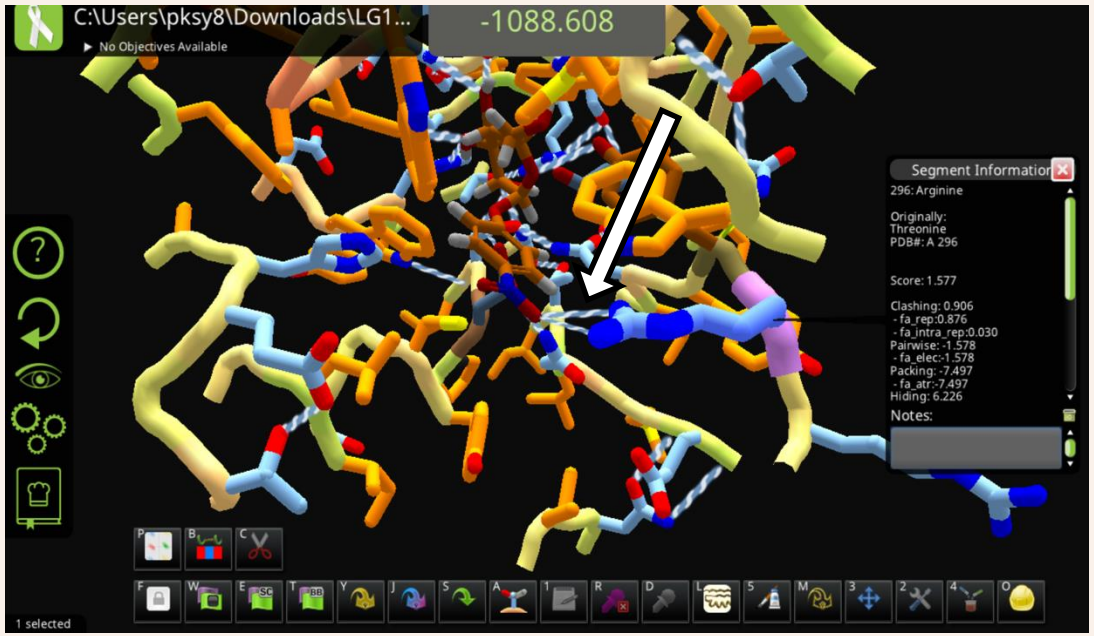


Figure 8: Zoomed in mutant T296 with WT energy score (right) and mutation T296R with a precise energy score -1088.608 (left). Precise energy score found using Minimize and Shake to model chemical interactions. These test find optimal interactions between residues after mutating the A.A. Arrow shows formation of a new hydrogen bond.

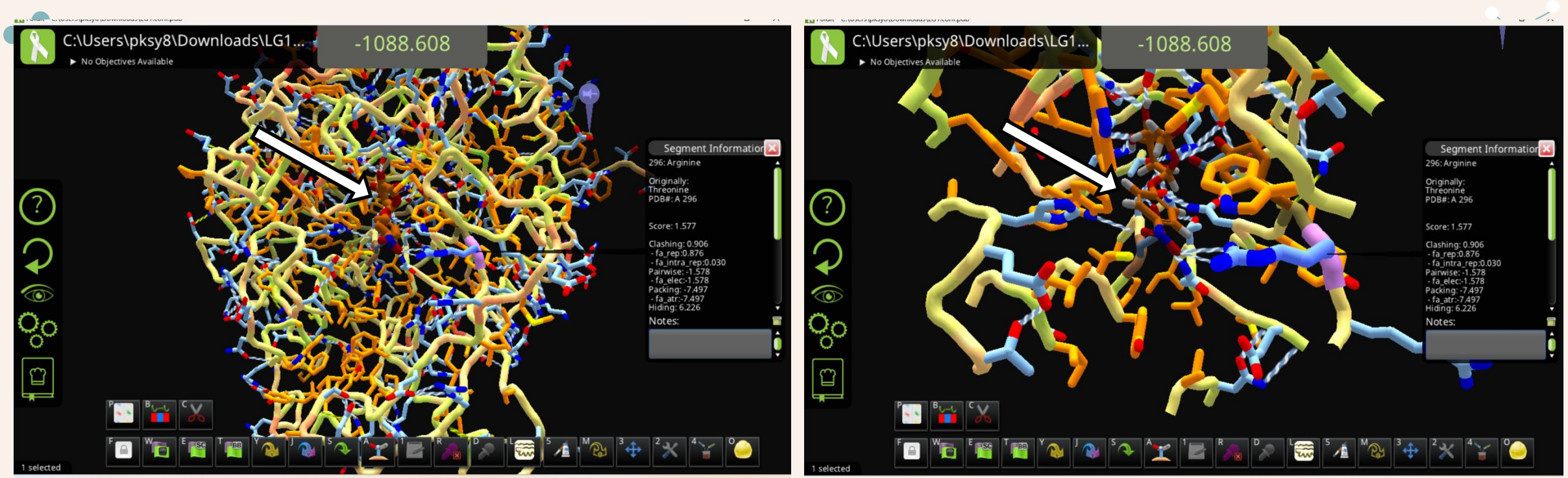


Figure 9: 3D structure of mutation T296R in relation to substrate. The mutated a.a. (hightlighted in purple) is located at the active side of the enzyme in regard to the brown substrate in the center (shown by the arrow).

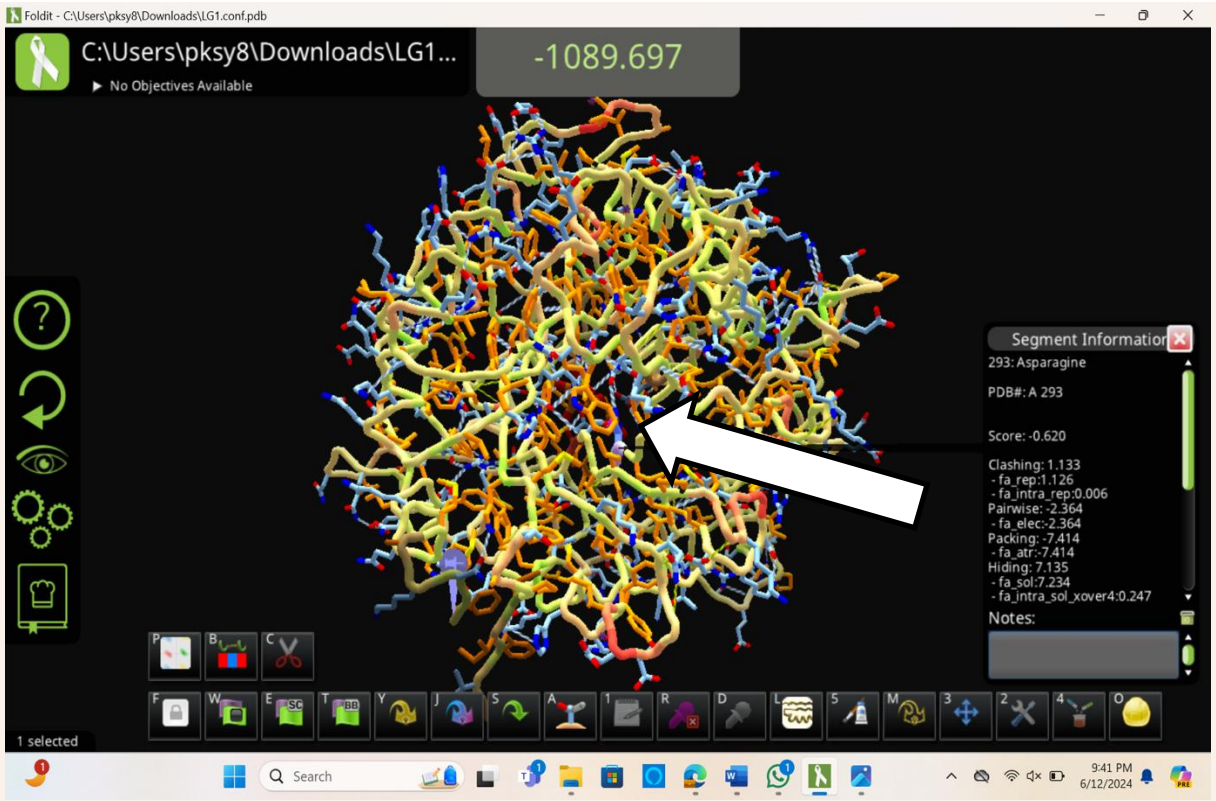


Figure 12: 3D structure of WT and mutant N293. Mutant is shown by the arrow and highlighted in purple. WT energy score shown as –1089.697.

Figure 13: Zoomed in mutant N293 with WT energy score. Precise energy score found using Minimize and Shake to model chemical interactions. These test find optimal interactions between residues after mutating the A.A.

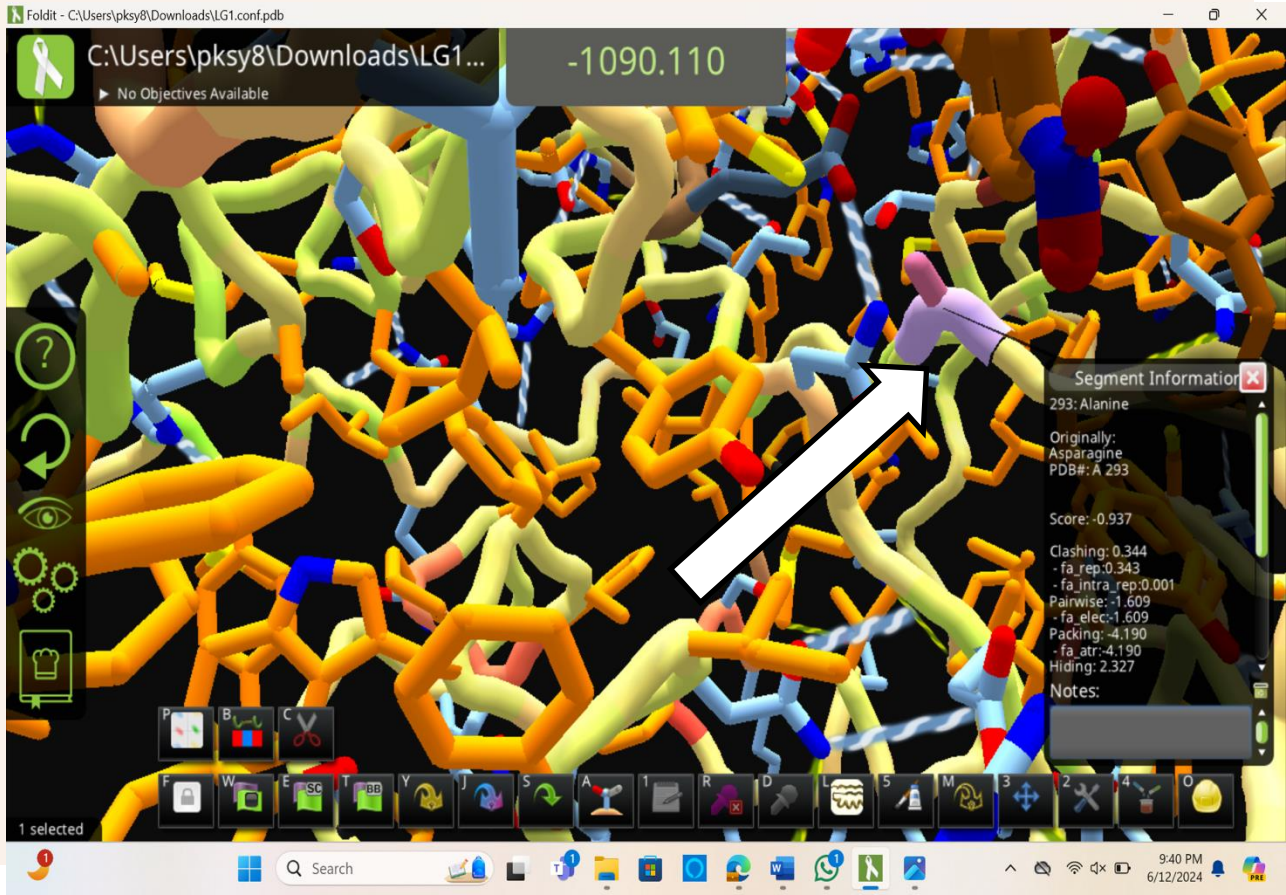
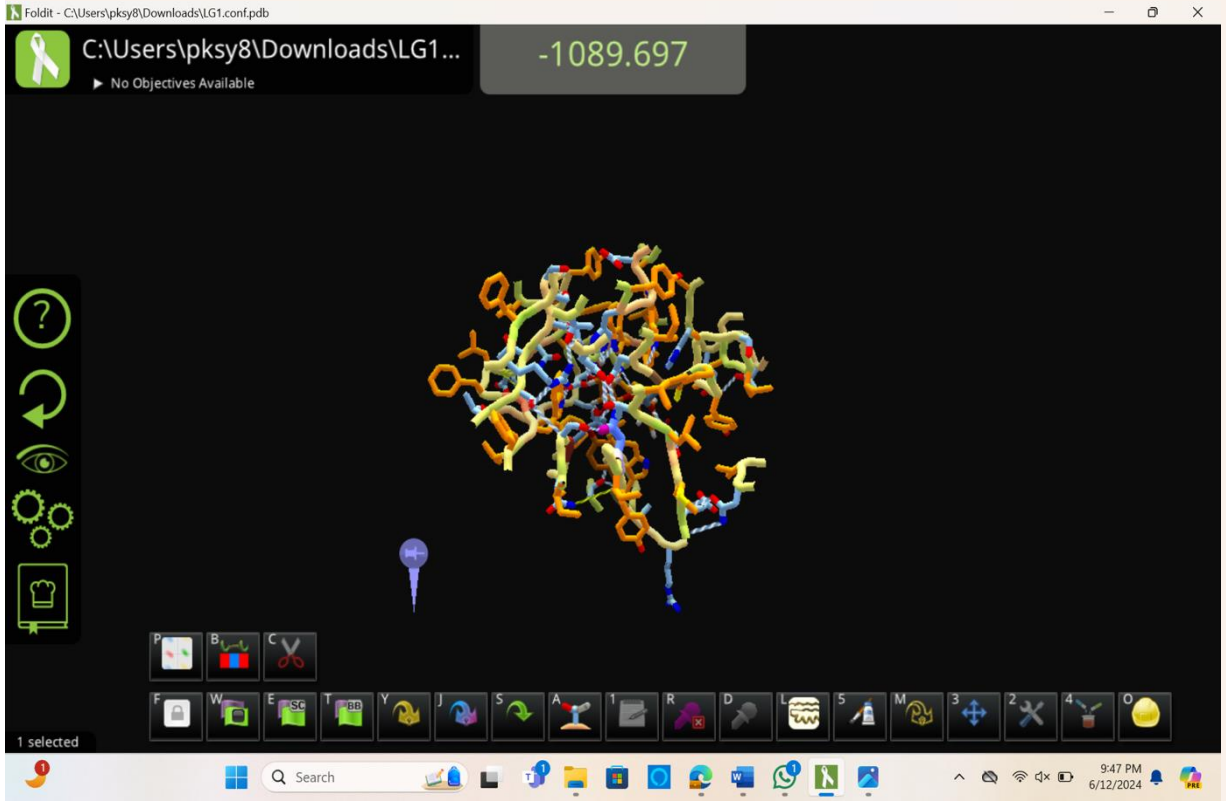


Figure 14: 3D structure of WT and mutant F77. Mutant is shown by the arrow and highlighted in purple. WT energy score shown as –1089.697.



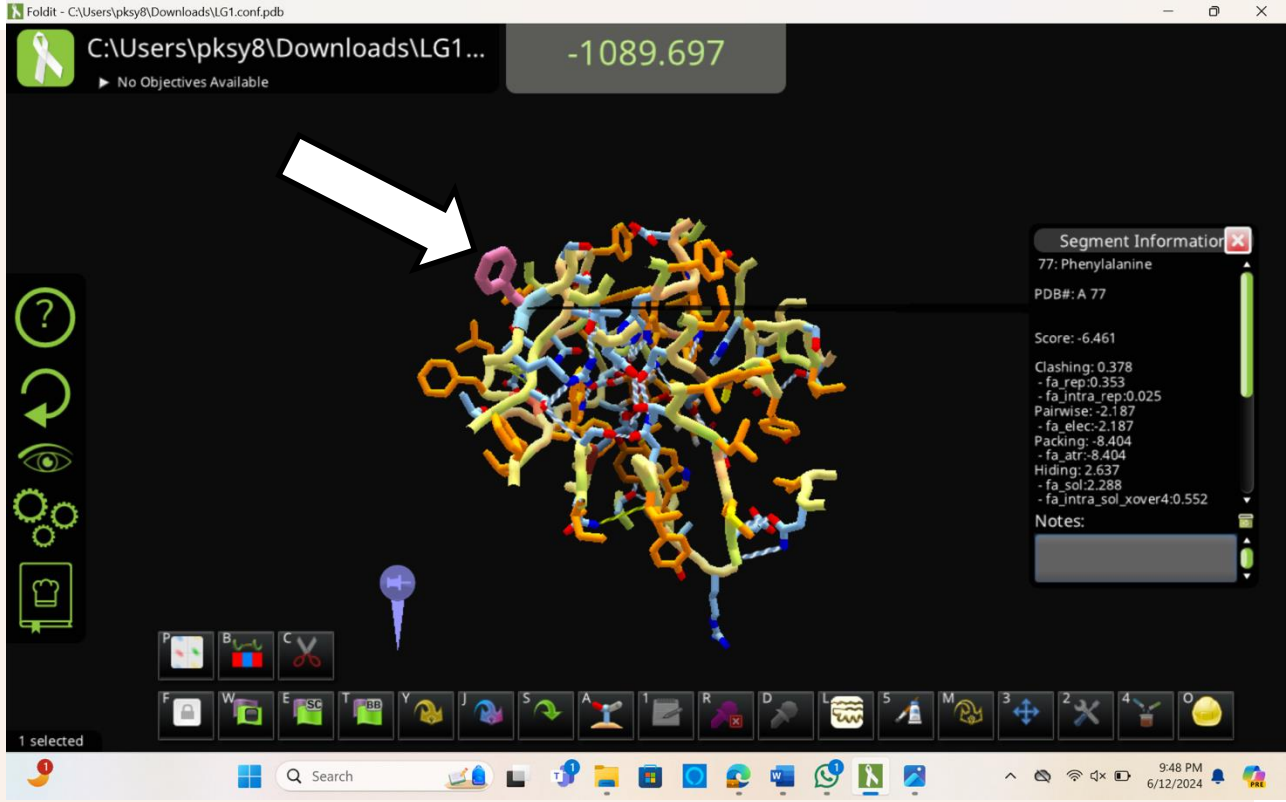


Figure 15: Zoomed in mutant F77.

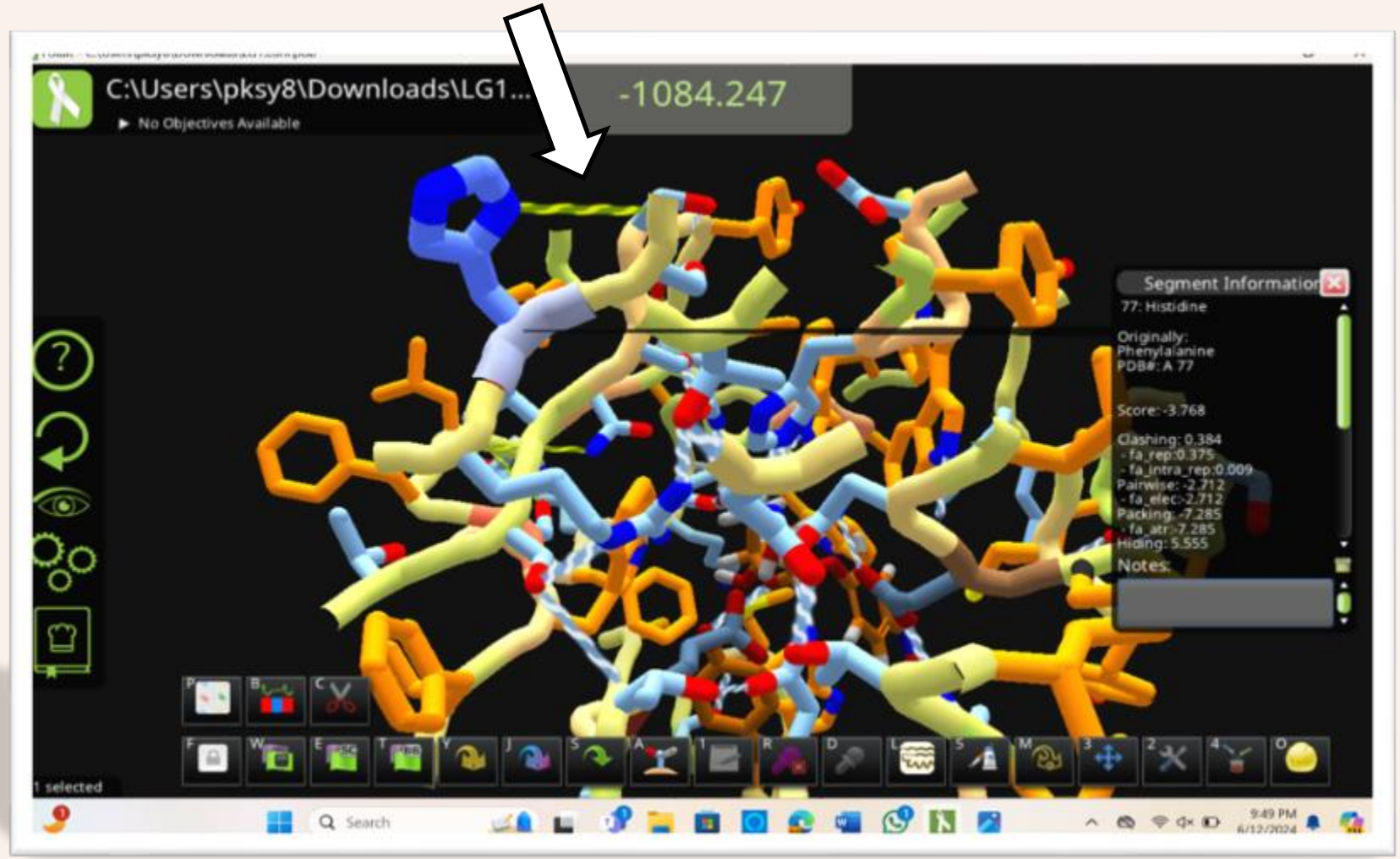
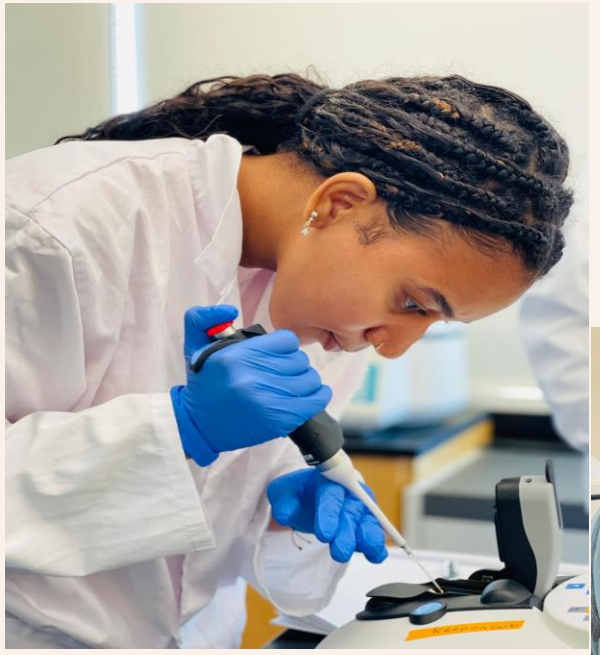


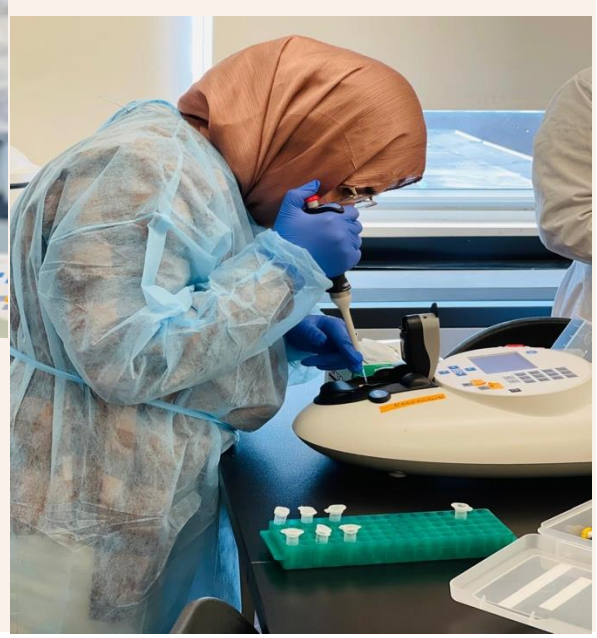
Figure 16: Zoomed in mutation F77H with a precise energy score. Precise energy score found using Minimize and Shake to model chemical interactions. These test find optimal interactions between residues after mutating the A.A. Arrow shows formation of a new hydrogen bond.

Methods and Materials

Experimental Methods: Hands-on

- Phosphorylation of primers
- PCR on site-directed mutagenesis
- Ligation reaction
- Transformation reaction
- Plasmid DNA Purification
- DNA sequencing preparation



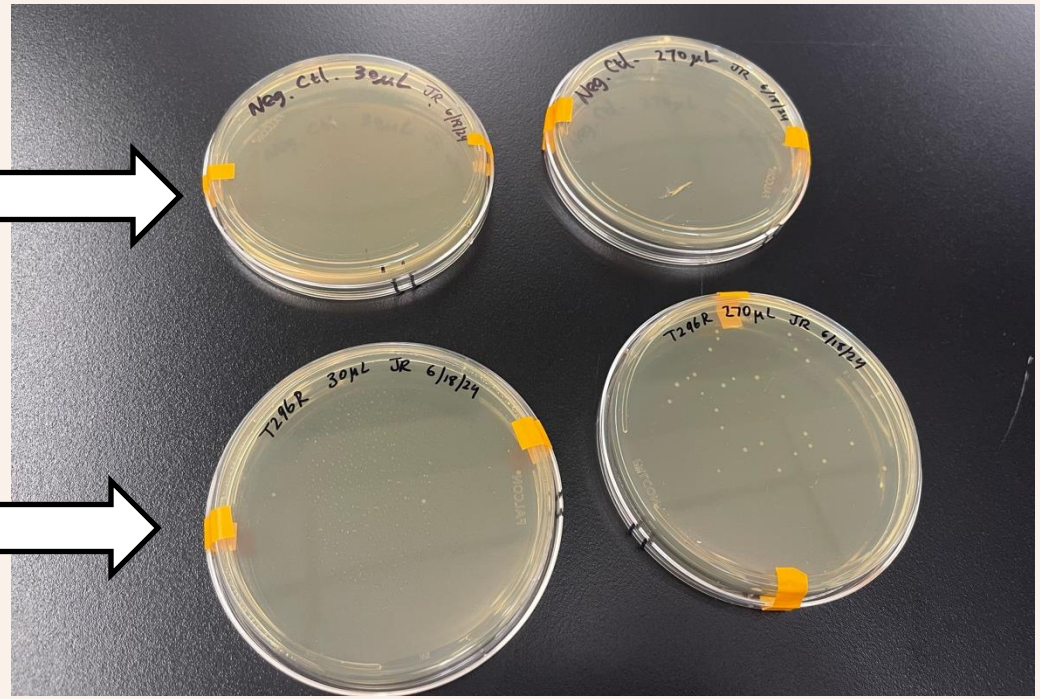


Experimental Results

30 µL 270 µL

Negative Control Colonies

Mutation T296R Colonies

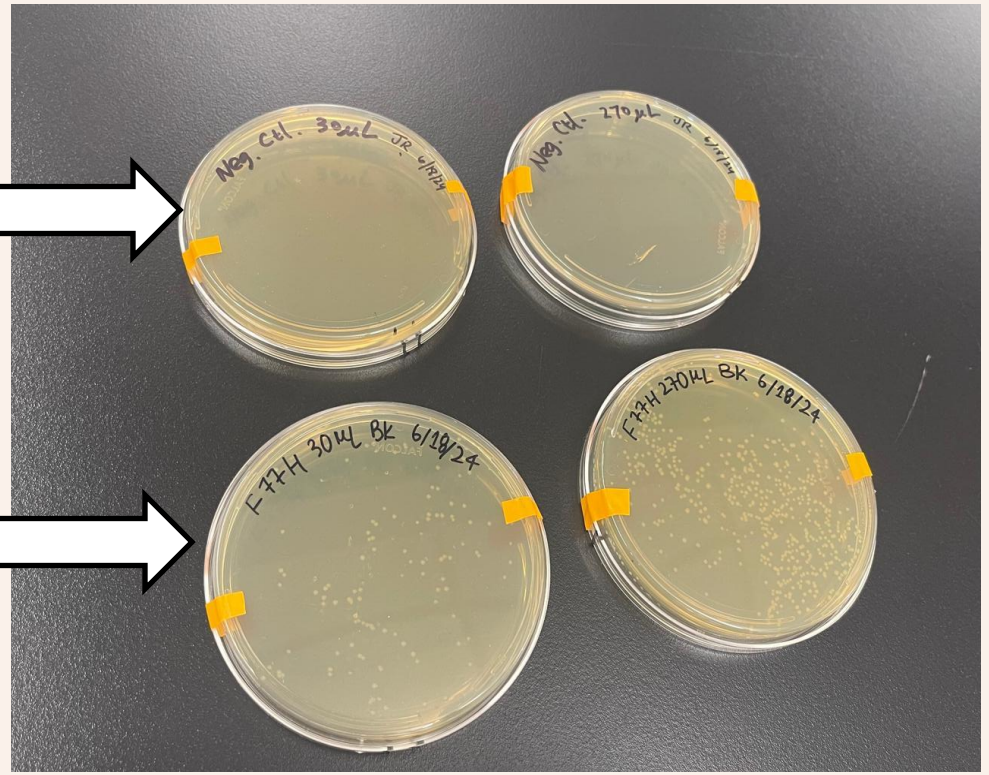


Experimental Results

30 µL 270 µL

Negative Control Colonies

Mutation F77H Colonies



Experimental Results

Figure 17: DNA sequencing demonstrating that T was mutated to R.

Experimental Results

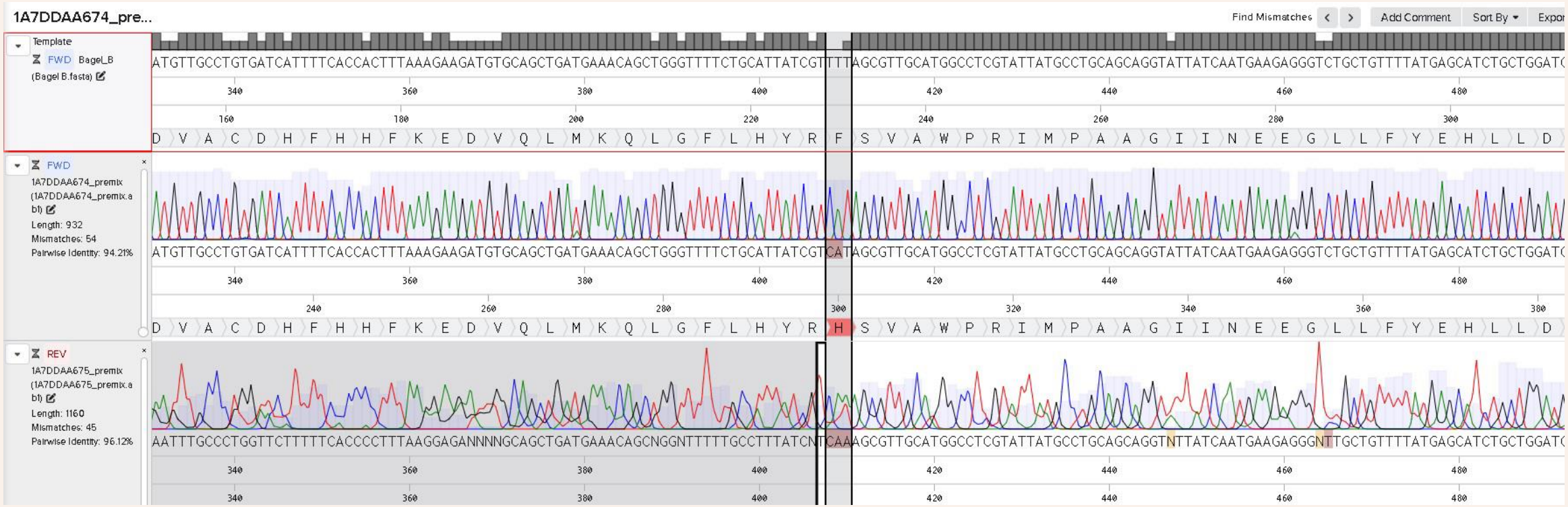


Figure 18: DNA sequencing demonstrating that F was mutated to H.

Discussion

- Key findings indicate that the introduction of novel hydrogen bonds significantly enhanced enzymatic activity.
- The presence of specific hydrogen bonds in amino acid mutations correlated with increased substrate binding and catalytic efficiency.
- Future studies include purification of mutant proteins and characterizing their enzymatic activity and thermostability.

References

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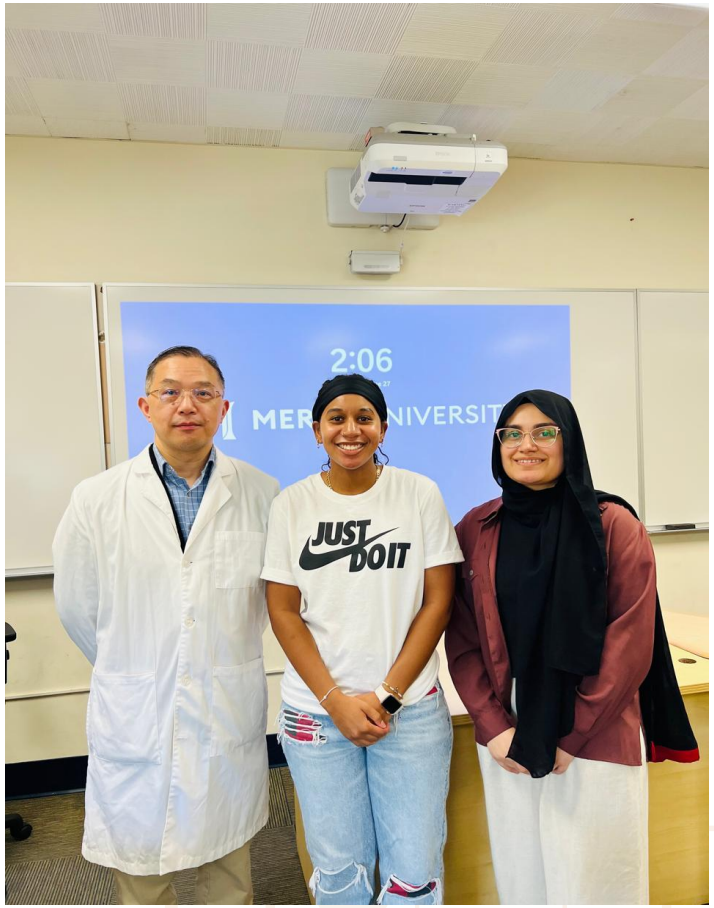
Singh, G., Verma, A. K., & Kumar, V. (2015). Catalytic properties, functional attributes and industrial applications of β-glucosidases. *3 Biotech*, *6*(1). https://doi.org/10.1007/s13205-015-0328-z

Acknowledgements

We would like to thank Dr. Zhou for the valuable lessons taught during the Spring workshops, which helped facilitate deep learning this summer. This summer program has shaped us into promoting ourselves into leaders, teammates, and ultimately achievers.

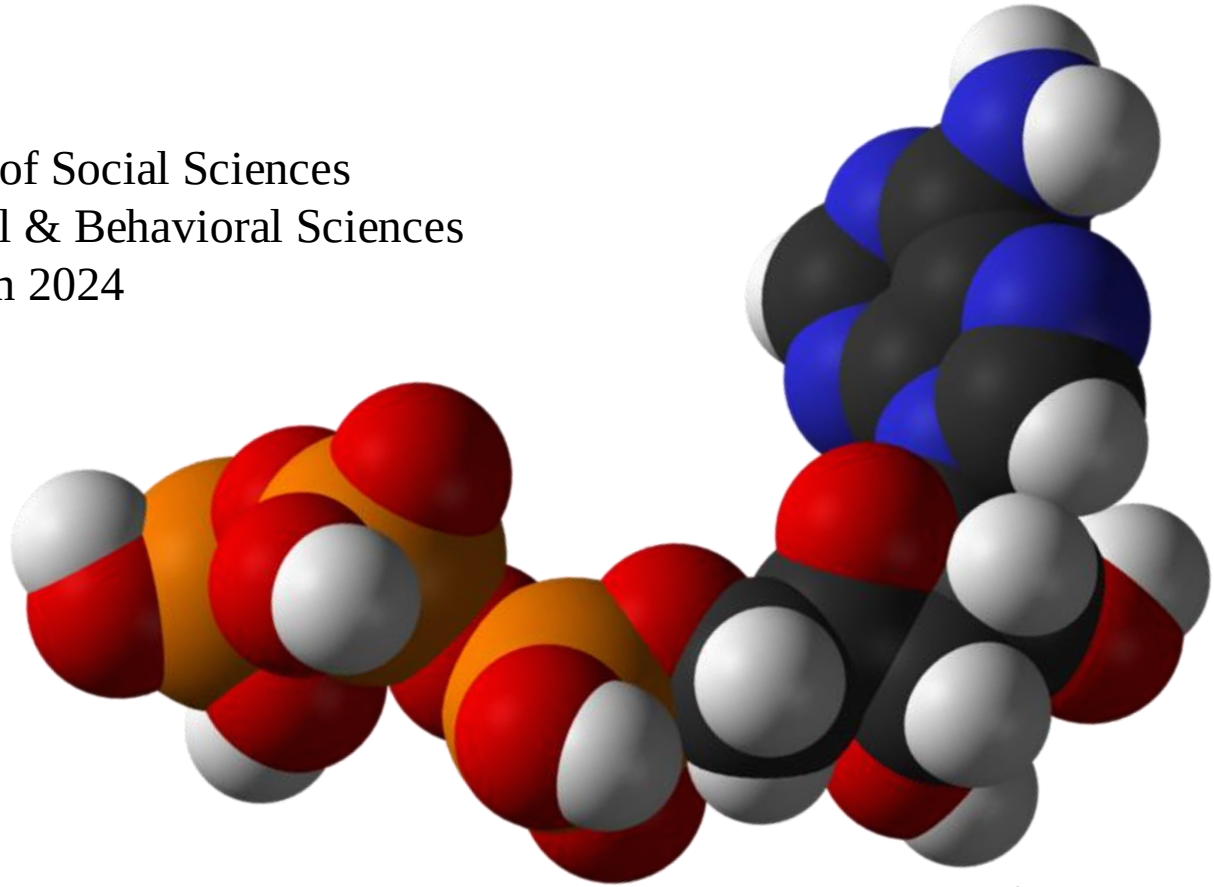
We would also like to thank CSTEP and Edith for this opportunity in enhancing and strengthening not only our research skills, but improving our collaboration and cooperation skills.



Improving the Enzyme Beta-Glucosidase B through Mutagenesis

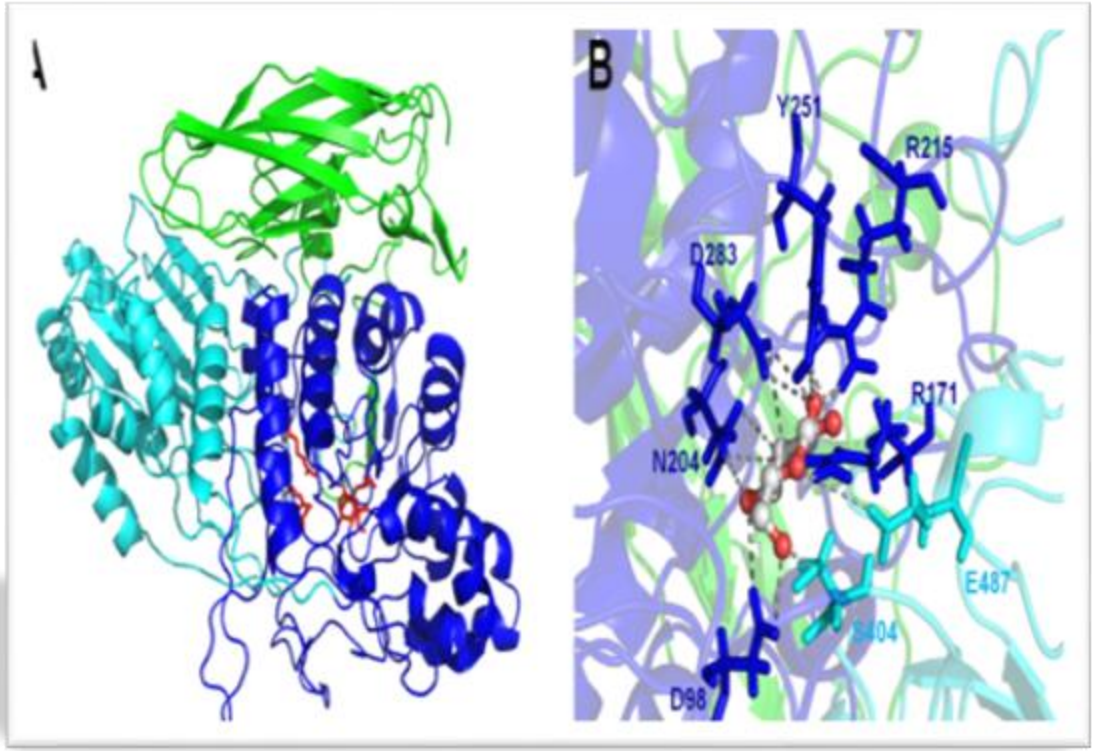
Cheyenne Wright, Abiah Castro

Department of Natural Sciences & Department of Social Sciences
School of Health & Natural sciences, School of Social & Behavioral Sciences
CSTEP Summer Research Program 2024
Mercy University

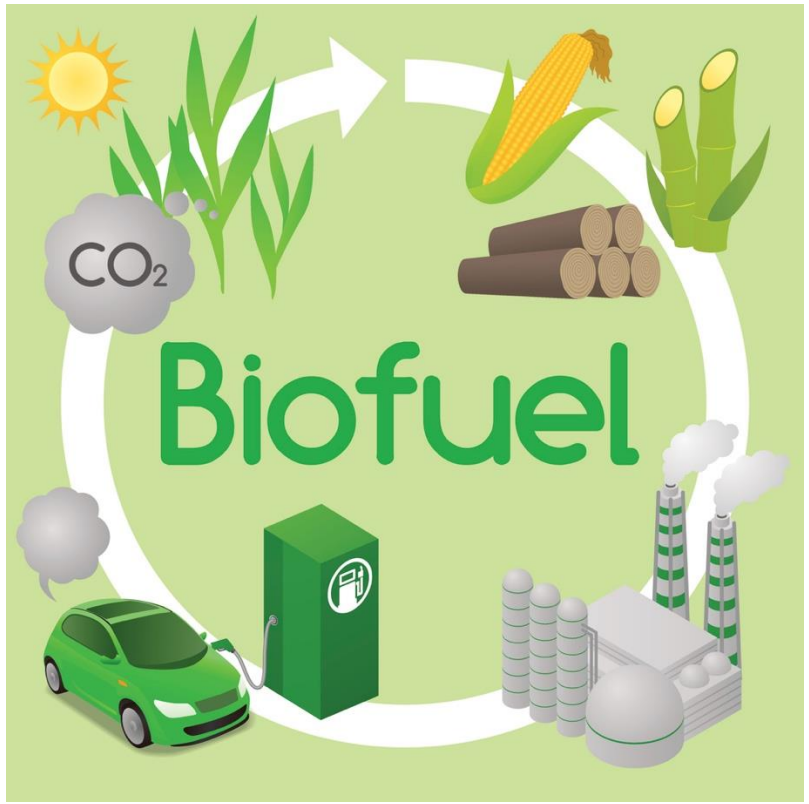


Introduction

- Bacterial β-glucosidase B (BglB) is an enzyme found in bacteria that aids in digesting complex carbohydrates.
- BglB can cleave glucose from polysaccharide such as cellulose of plant biomass, together with other enzymes
- Improving BglB activity and stability is crucial for industrial use.



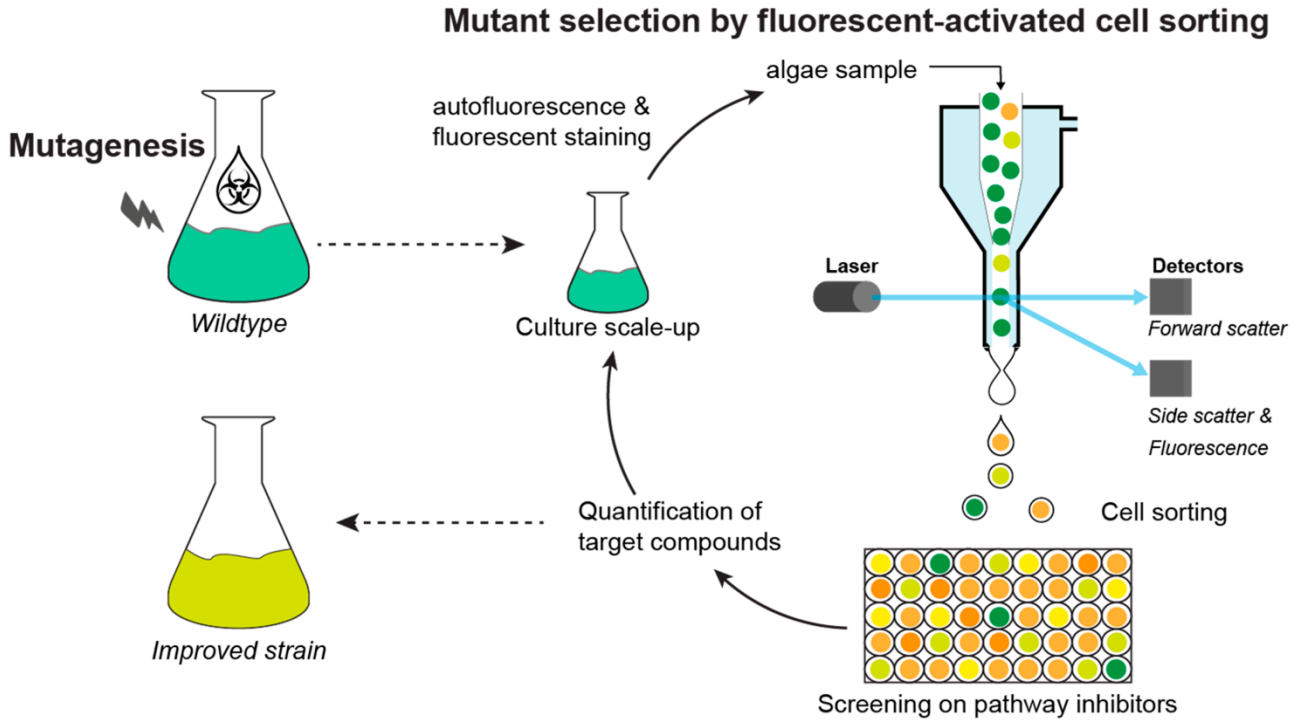
Clean energy & Ethanol



- **Clean energy** is used to describe energy sources that release little or no greenhouse gases such as carbon or nitrogen that have little to no negative environmental impact. Clean energy aims to lessen pollution and fight climate change by producing sustainable and renewable energy for the future.
- **Ethanol** is regarded as a renewable energy source that plants can use to be refined again. Typically, biomass such as corn, sugarcane, or other plant materials is used to make it.

Mutagenesis generating novel enzymes in industry and medicine

- Mutagenesis is the intentional or natural alteration of an organism's DNA sequence, which can be used in biotechnology to create unique proteins and enzymes with desired characteristics for industry and medicine.
- This process can modify proteins like hormones, enzymes, and antibodies for extended half-life and improved treatment effectiveness.



Research Questions

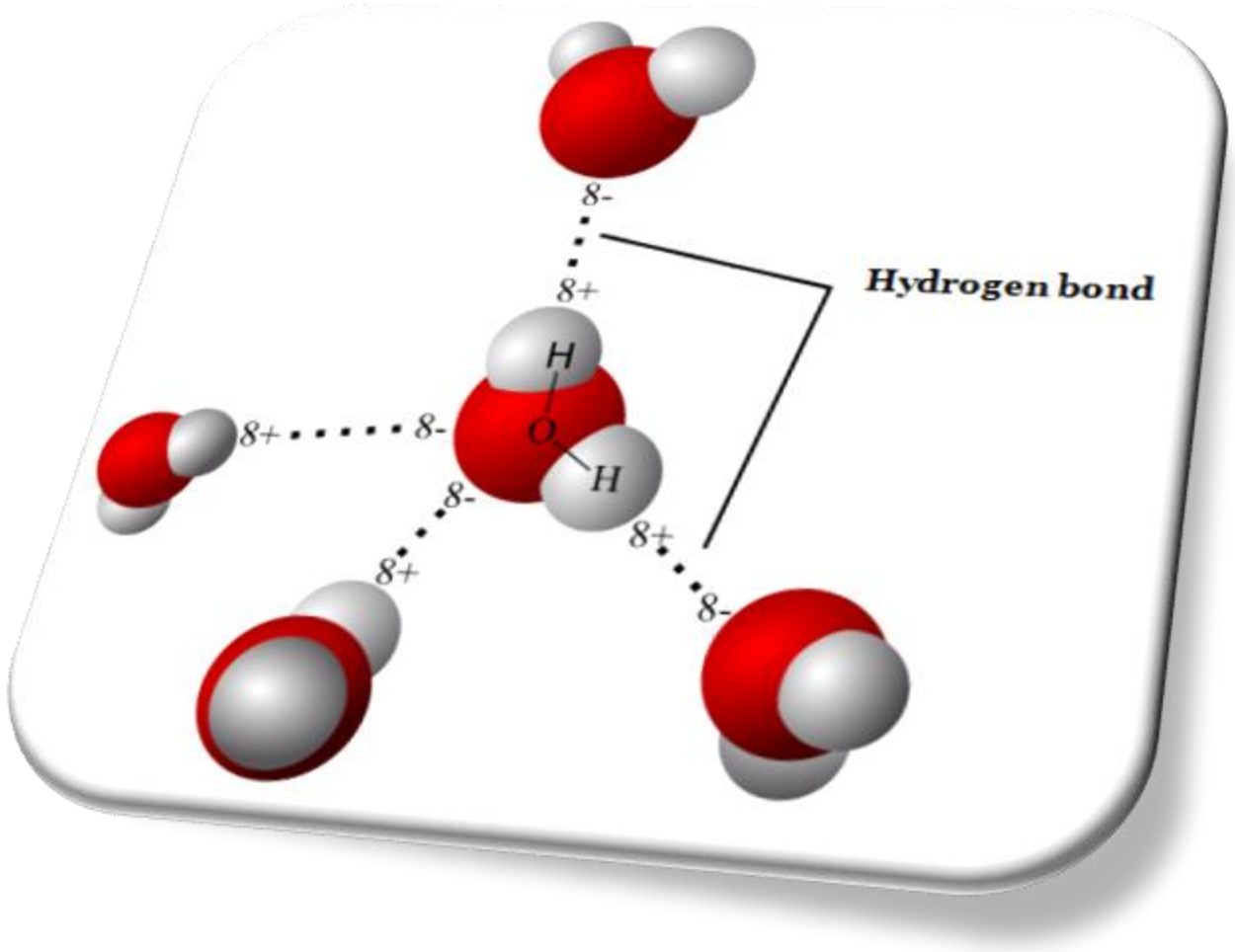
- Do mutations that create hydrogen bonds make proteins more stable than the score it's presenting?
- How do the wild-type and mutant proteins' stability scores compare?

Research Goals

- Determine and classify mutations that generate hydrogen bonds.
- Generate the design of a mutation
- Create models for predicting for stability brought on by mutations.
- Apply research to different kinds of proteins.

Our Hypothesis

Generating additional hydrogen bonds can stabilize protein structures



Foldit Standalone Computer Software

- Foldit Standalone allows users to manipulate proteins in a user-centered manner to solve challenges relating to 3D structure in biochemistry, including protein structure prediction and design.
- Foldit Standalone is an add-on for Rosetta that offers a comprehensive user interface for both novice and professional biochemistry structure research.

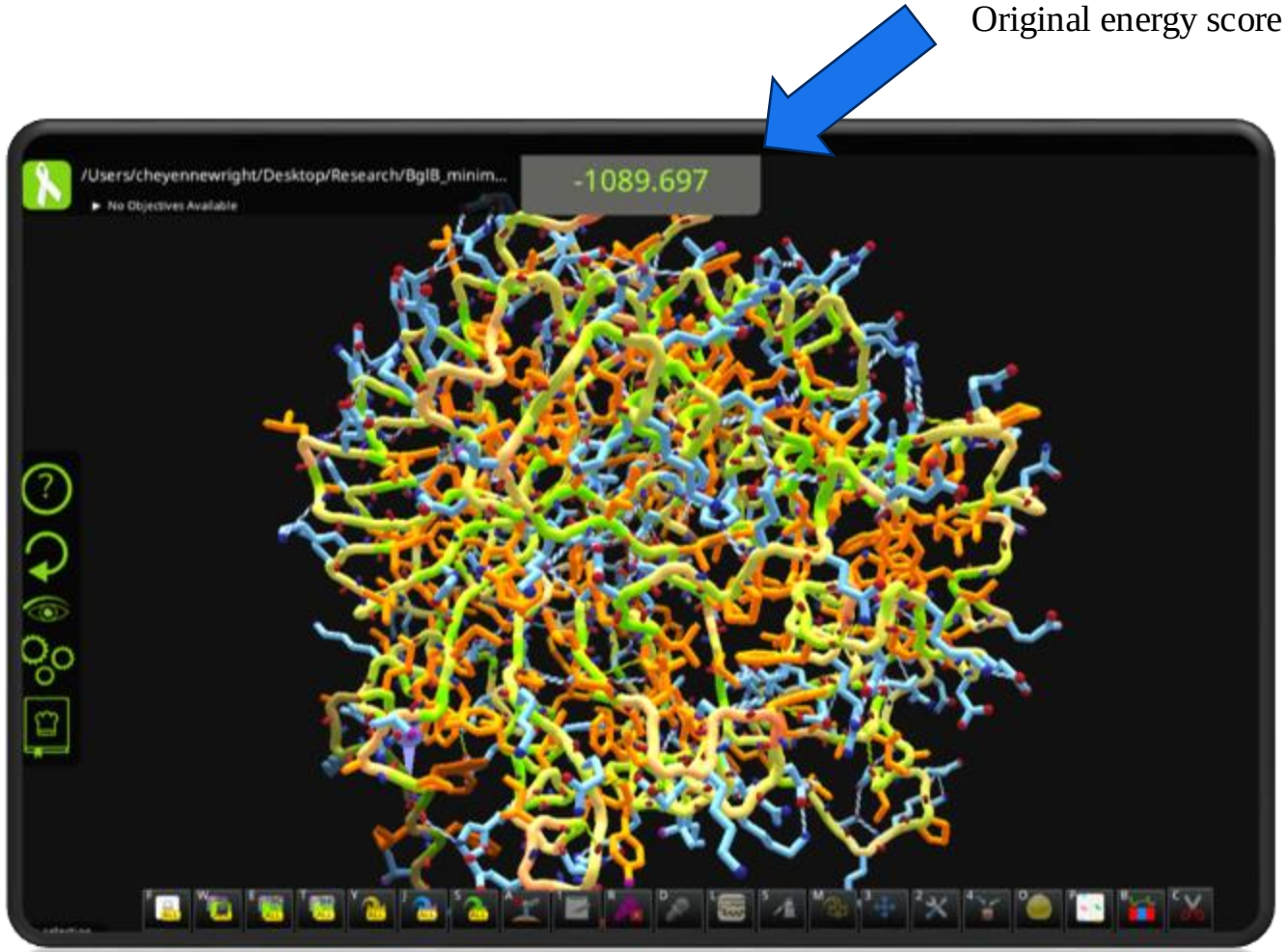


Figure 1: In figure 1 it shows the original form of the enzyme before any mutation occurred with it's original energy score.

Materials & Methods

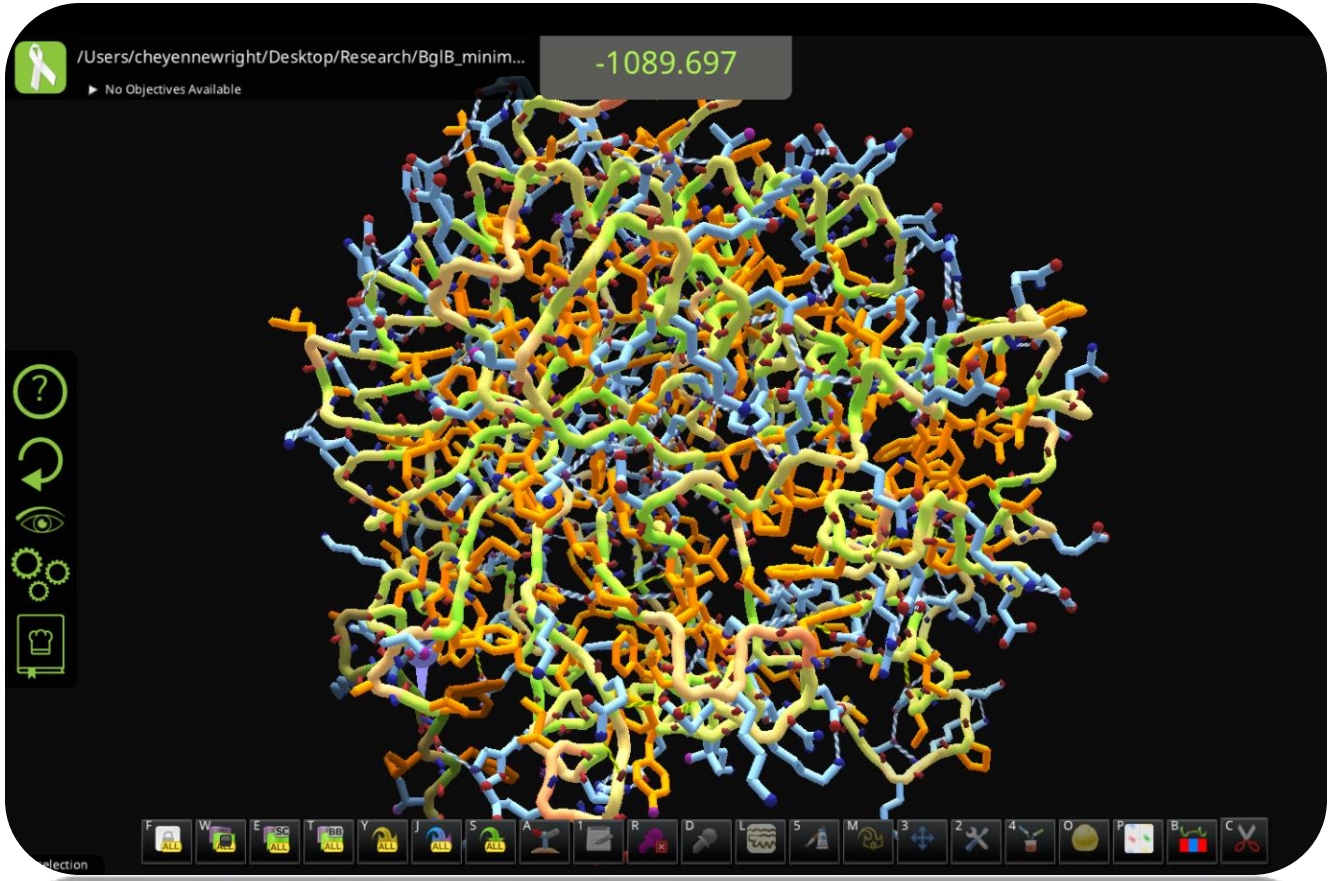


Figure 1: In figure 1 it shows the original form of the enzyme before any mutation occurred with it's original energy score.

- Using the computer software Folditstandalone, we stabilized the enzyme Bacterial bifunctional β-glucosidase (BglB) by clicking on over 20 randomized amino acids
- We mutated the enzyme with different amino acids to generate new hydrogen bonds while keeping the energy score similar to the energy score of the wild-type

After performing many mutations, we decided on the mutations 294 tyrosine to asparagine with a score of -1084.127 (Figure 2) and 398 Valine to glutamine with a score of -1087.572 (Figure 2A) which were found in the active site of the enzyme.

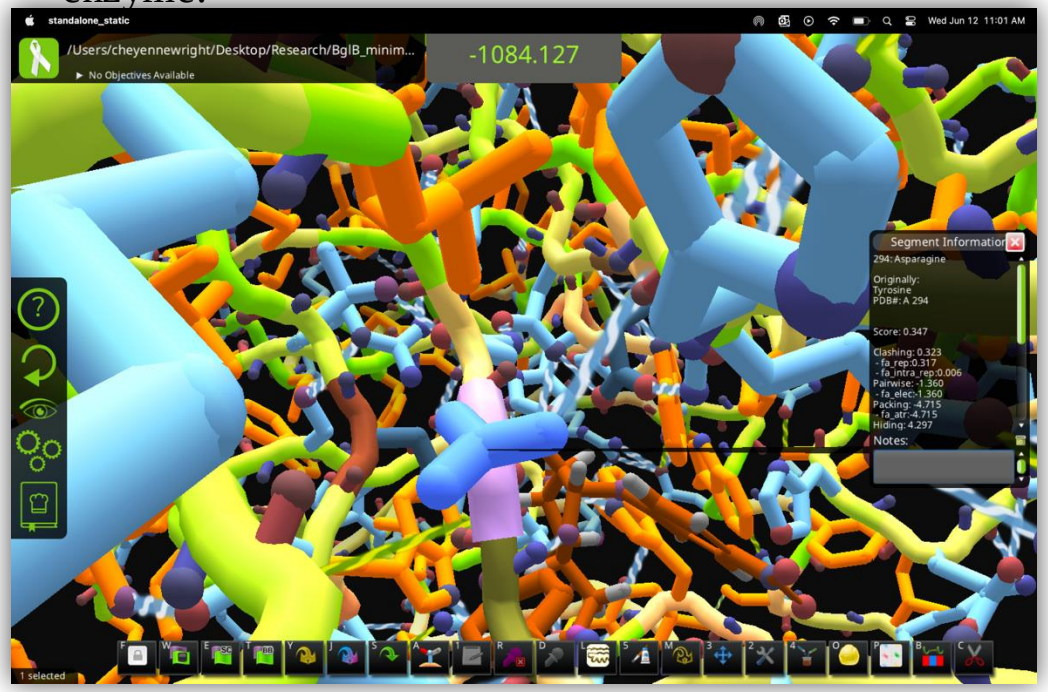


Figure 1: In figure 2 we see the wild-type that was 294 tyrosine with a hydrogen bond. The energy score is -1089.697 which was the original score

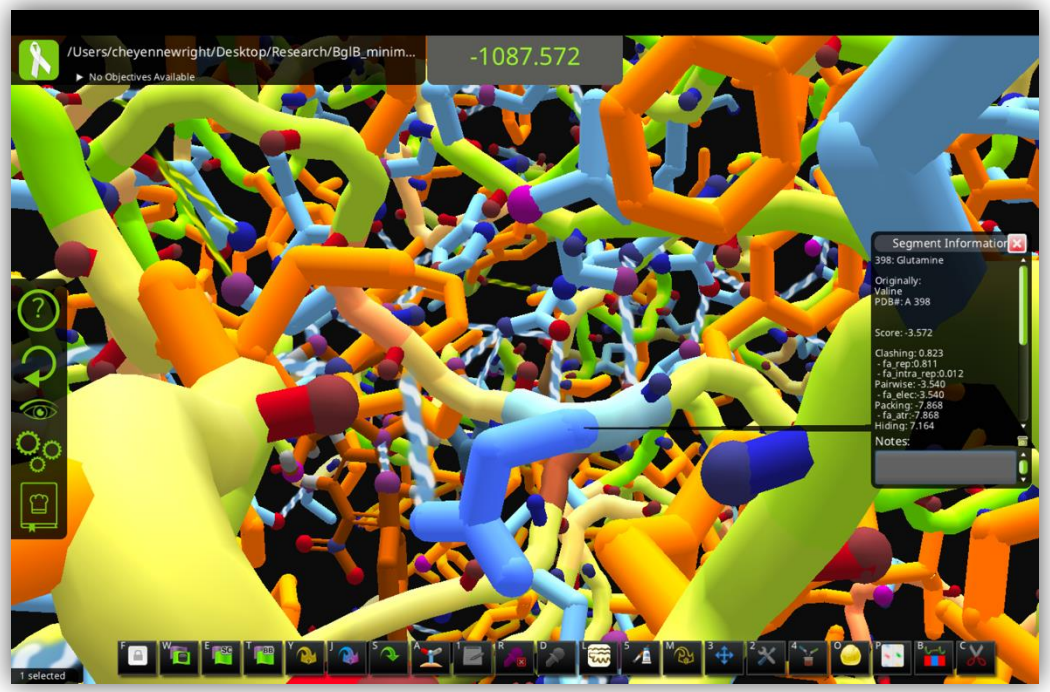


Figure 2A: In figure 3 we see the wild-type 398 Valine mutated to glutamine creating a hydrogen bond. We also see the energy score becoming higher than the original score.

Experimental Methods

- Computational biology study to identify normal mutation with additional hydrogen bonds.

- Generation of the newly identify mutations.
  - Phosphorylation of Oligonucleotides
  - PCR
  - Ligation
  - Transformation
  - Purify plasmid DNA
  - DNA sequencing preparation

Day 1: June 14th, 2024 - Phosphorylation of Oligonucleotides

Materials:

- 1000, 200, 20, 10 micropipette
- Microcentrifuge tube 1.5ml
- Oligonucleotide primer (100 μM) - oligo
- 10x T4 ligase buffer w/ 10 mM ATP - 10x butter
- t4 Polynucleotide Kinase (10 U/μL) - T4 kinase
- H_2O

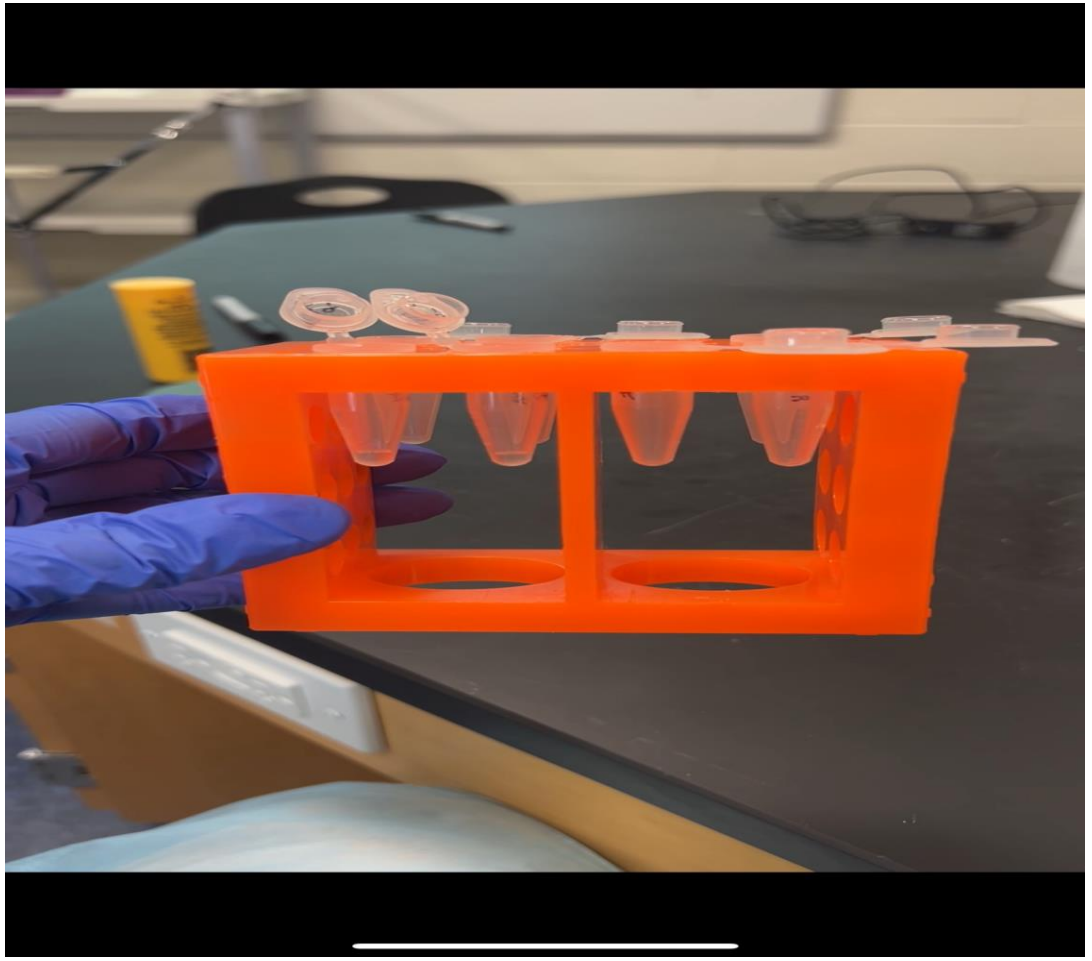


Figure 4: Visual after adding the Oligonucleotide primer

Day 2 - June 17th, 2024: PCR

Total volume in microliters = 50 µl

H2O	**28.36 µl**	**170**
5x HF butter	**10 µl**	**60**
50x 10mm dNTPs	**1 µl**	**6**
200x PF (5 µl)	**5 µl**	
200x PR (5 µl)	**5 µl**	
10 ng wt plasmid	**.14 µl (73.5 ng/ml)**	**0.84 = 1 µl**
Pol	**.5 µl**	**3**

Step	**Temp**	**Time**	**# of cycles**
Initial denaturation	**98 °C**	**60s**	**1**
Denaturation **Annealing** **Extension**	**98 °C** **72 °C** **72 °C**	**10s** **30s** **210s**	**25**
Final extinction	**72 °C** **4 °C**	**10 min** **Hold**	**1**

- Master mix 6x of everything except the primer
- Aliquot the master mix into 40 µl for each pcr tube. Each tube, add 5 µl of the F & R primers
- Used the thermal cycler using standard conditions
- PCR product stored at -20 c

Day 3: June 18th, 2024 - Ligation/Transformation

Steps

1. Transfer product into 1.5 microliter tubes
2. Add 1 µL of FastDigest DpnI enzyme directly to the mutagenesis PCR reaction
3. Centrifuged the mix
4. incubate at 37°C for 15 minutes
5. Prepare 10 µl ligation mix

Component	10 µl reaction	Master mix x7
PCR-Dpnl reaction	5 µl	-
5x Rapid ligation buffer	2 µl	14 µl
H2O	2.5 µl	17.5 µl
T4 DNA Ligase	0.5 µl	3.5 µl

Figure 5: visual of the microliter tubes shaking vigorously (250 rpm) in INCU-Shaker

Day 3: June 18th, 2024 - Ligation/Transformation

6. Add 5 µL Master Mix into each 1.5-mL tube
   - Add 5 µL of the PCR-DpnI reaction into the 1.5-mL tube to make a total of 10 µL ligation reaction.
   - Mix thoroughly
   - Centrifuge briefly
7. incubate at room temperature (25°C) for 10 minutes.
8. Chill on ice, then transform or store at −20°C

Day 4 - June 20th, 2024: Purify plasmid DNA, Prepare sequence reaction

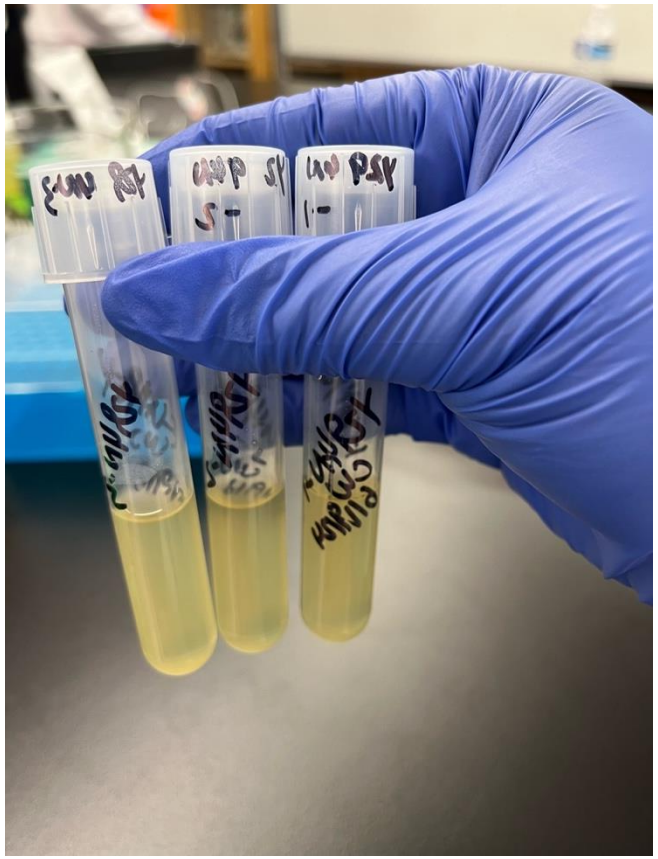


Figure 6: Added 250µl of each of buffer P1, Buffer P2, and Buffer N3 into a microcentrifuge tube of mutation Y294N

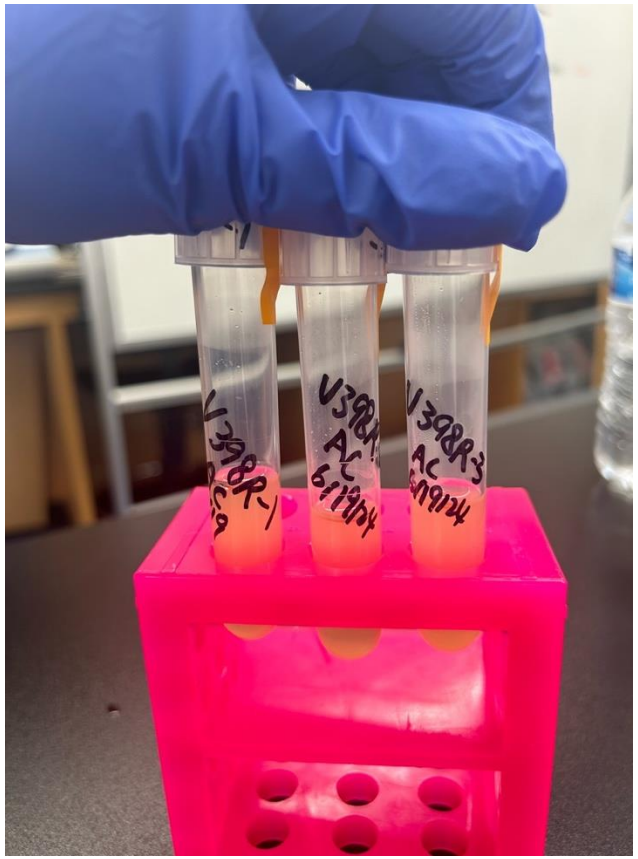


Figure 6A: Added 250µl of each of buffer P1, Buffer P2, and Buffer N3 into a microcentrifuge tube of mutation V398Q

Day 4 - June 20th, 2024: Purify plasmid DNA, Prepare sequence reaction

Measurement of DNA concentration

Y294N

A260/A280	Concentration ng/µl
1.868	71.0
1.869	57.0
1.837	78.0

V398Q

A260/A280	Concentration ng/µl
1.919	35.5
1.939	47.5
1.865	84.5

Results

294 Tyrosine to Asparagine with a score of -1084.127

Figure 2A: In figure 2A we see the wild-type that was 294 tyrosine with a hydrogen bond. The energy score is -1089.697 which was the starting score

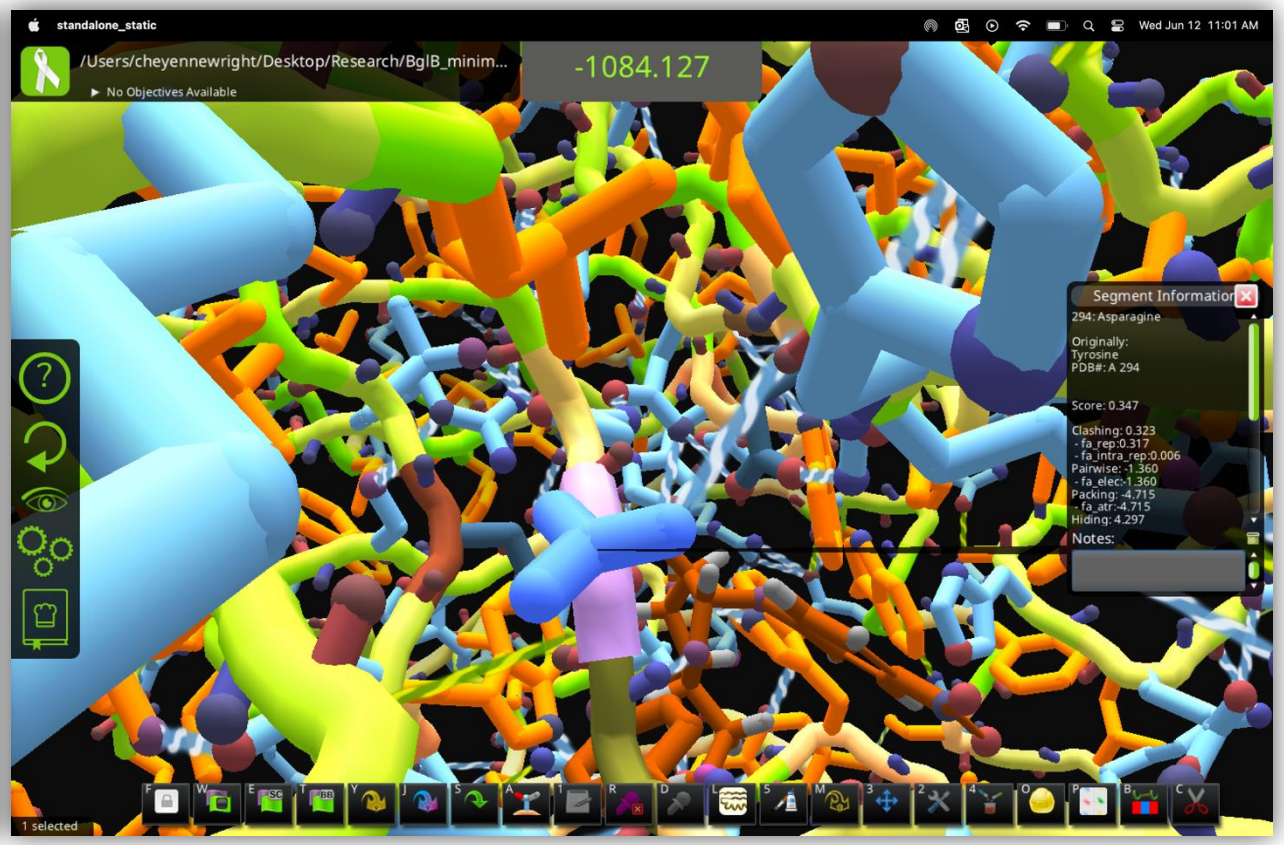


Figure 2: In figure 2 we see the wild-type 294 tyrosine mutated to 294 asparagine maintaining its hydrogen bond after mutation and showing a higher score than the original.

Results

398 Valine to Glutamine with a score of -1087.572

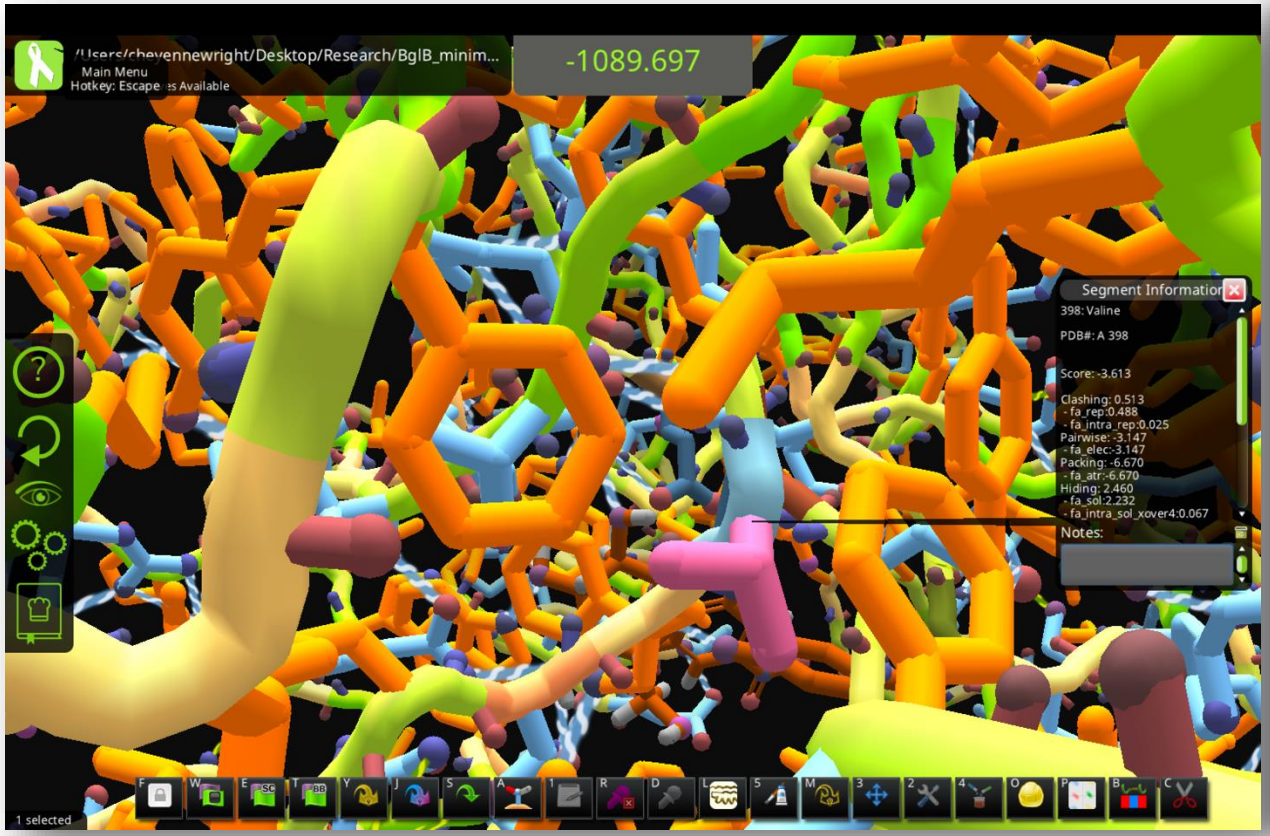


Figure 3A: In figure 3A we see the wild-type that was 398 Valine. The energy score is -1089.697 which was the starting energy score

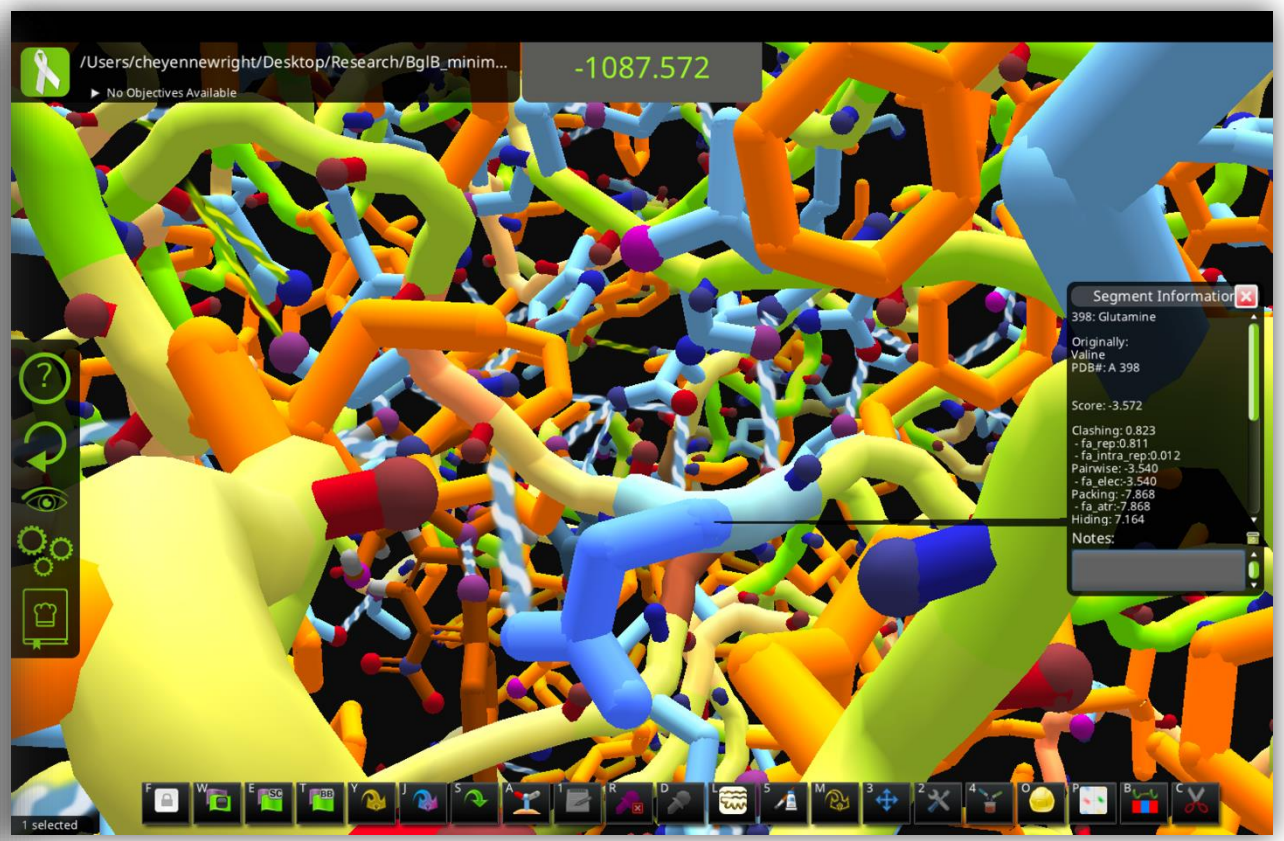
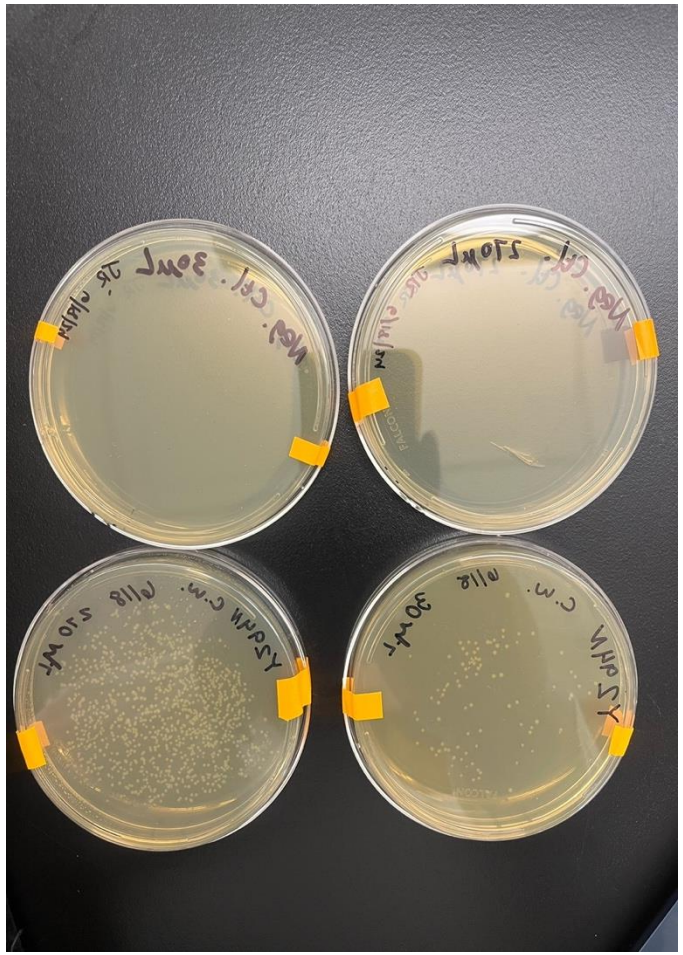


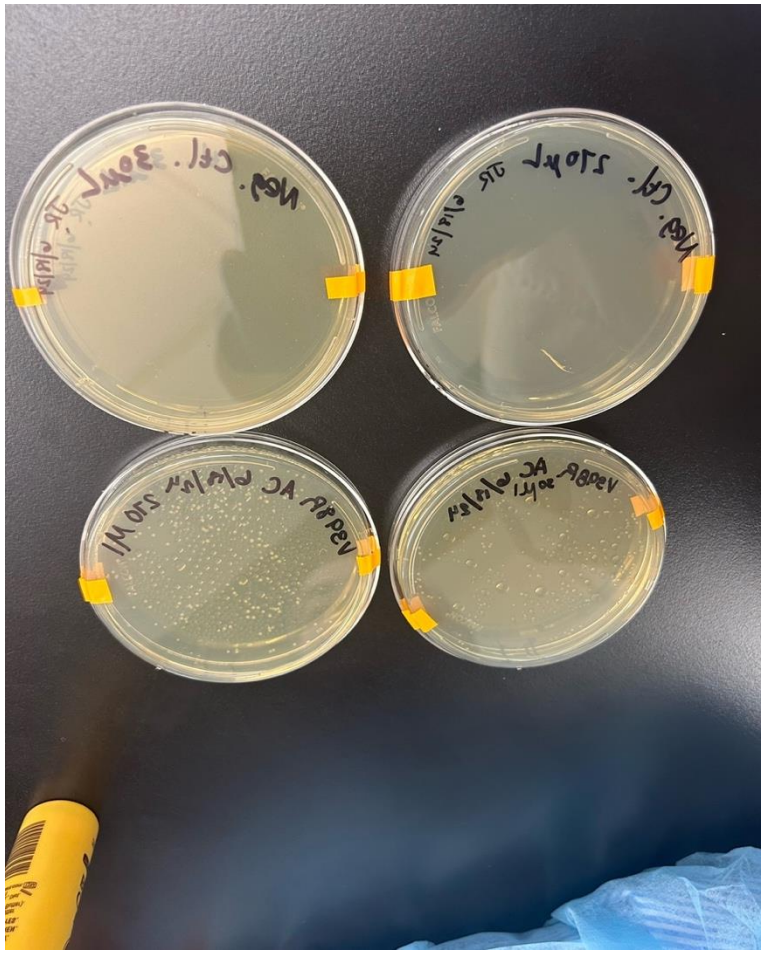
Figure 3: In figure 3 we see the wild-type 398 Valine mutated to glutamine creating a hydrogen bond. We also see the energy score becoming higher than the original energy score.

Experimental Results



Negative control

Mutation Y294N



Negative control

Mutation V398Q

Experimental Results

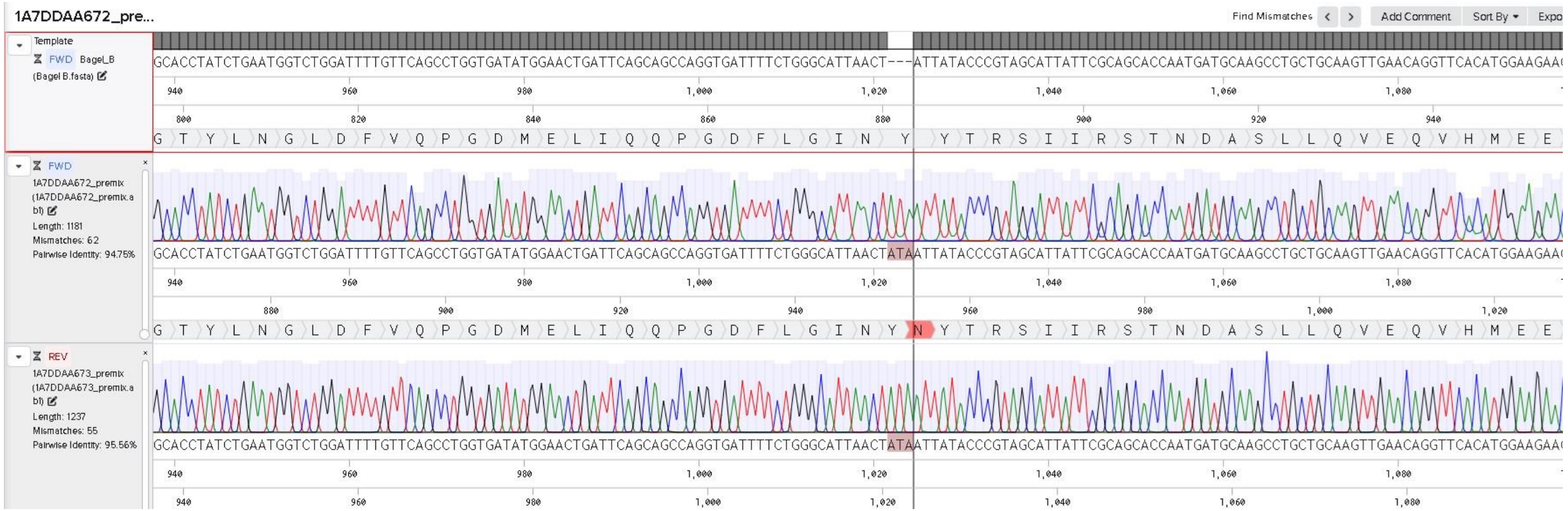


Figure 8: the results after DNA sequence. Tyrosine was mutated to Asparagine successfully.

Experimental Results

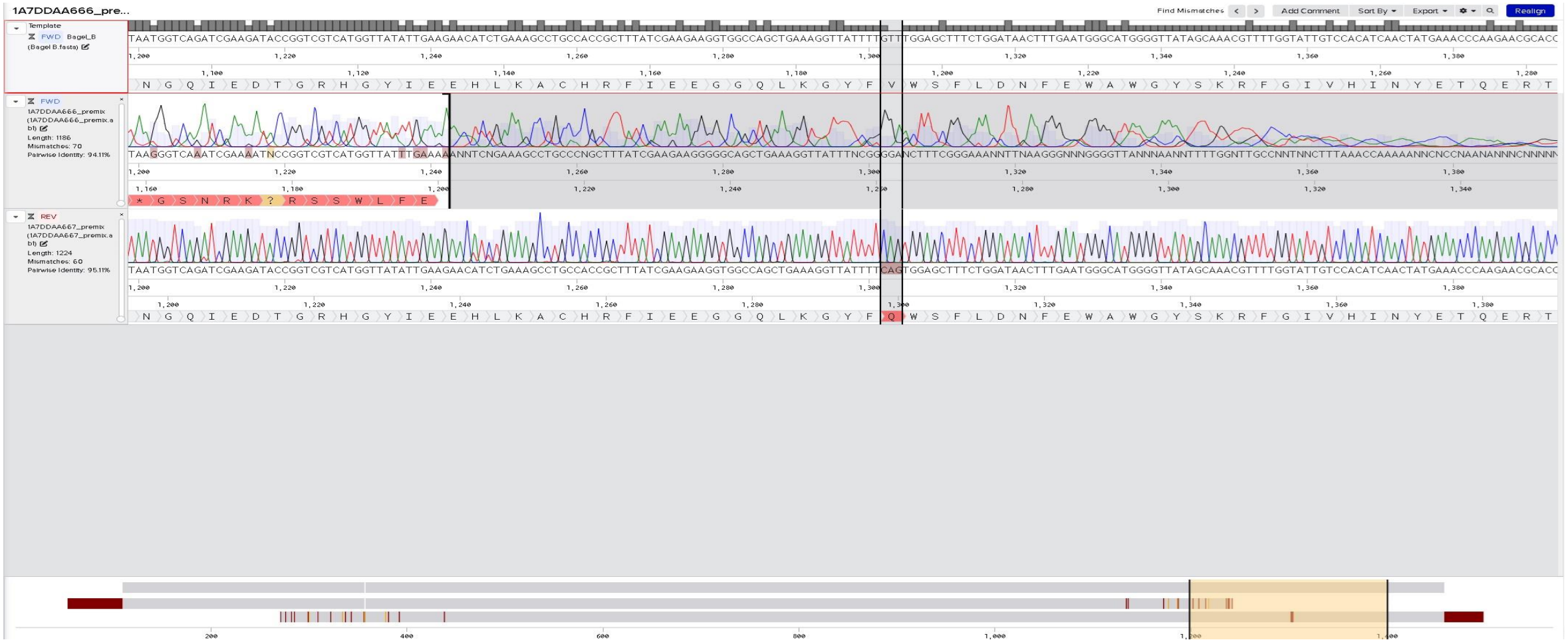


Figure 8A: the results after DNA sequence. Valine was mutated to Glutamine successfully.

Discussion

- The structure and functionality of BglB depend heavily on hydrogen bonding, especially when it comes to preserving the stability and configuration of its active site.
- In summary, the newly identify two mutations have the potential to improve enzymatic activity of BglB by providing additional hydrogen bond.
- the current analysis expands on the knowledge that already exists. It is possible to address important scientific issues and promote innovation in a variety of fields by expanding our knowledge of the chemical mechanisms behind hydrogen bond stabilization.
- Future studies are to purify the mutant proteins and characterize their enzymatic activities and thermostability

Acknowledgement and References

We would like to give a special thanks to Dr. Zhou for the valuable teaching he provided during this program. We are also grateful for the chance to investigate this subject, which was provided by the Department of Natural & Social Sciences, School of Health and Natural Sciences, School of Social & Behavioral Sciences, Mercy College, and CSTEP.

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IDENTIFICATION OF NOVEL MUTATIONS

BACTERIAL BETA-GLUCOSIDASE

BY: CLARISSA PEREZ HERNANDEZ, LITZY AMBRIZ, MARLY GUZMAN

SCHOOLS OF HEALTH , NATURAL AND BEHAVIORAL SCIENCES & NURSING

CSTEP SUMMER RESEARCH PROGRAM 2024

MERCY UNIVERSITY

Research questions, Research goals, Hypothesis, and Aim

01 RESEARCH QUESTIONS

What conditions can be modified to reach optimal conditions that will satisfy stabilization of an enzyme for continued use?

02 RESEARCH GOALS

Improve function and stability of an enzyme through mutagenesis.

03 HYPOTHESIS

To be able to analyze and determine ideal conditions for any hydrogen bond that will stabilize the enzyme

04 AIM

To demonstrate how we can contribute to sustainable practices and innovative solutions across multiple domains to successfully use clean energy

Clean Energy

- Clean energy is produced by systems that do not emit pollution, especially greenhouse gases like CO2, which contribute to climate change.
- As clean energy continues to develop, it supports environmental conservation
- Helps mitigate the crisis caused by reliance on non-renewable fuels like gas and oil.
- There are many different types of clean energy such as hydroelectric energy, geothermal energy, tidal energy, and wind energy.
- Most clean energy is reusable

Ethanol

- Ethanol can be blended with gasoline to reduce greenhouse gas emissions and reliance on fossil fuels.
- Ethanol is also a key component in the production of hand sanitizers, pharmaceuticals, and personal care products.

Exploring environmental friendly approaches of converting fossil fuels into energy

- One approach is Carbon Capture and Storage (CCS), which captures CO2 emissions from power generation and industrial processes for underground storage, thereby reducing atmospheric CO2. Combined Heat and Power (CHP) systems increase efficiency by generating electricity and useful heat simultaneously.
- Transitioning to electric and hybrid systems, integrating renewable energy sources, and using fuel cells further reduce fossil fuel dependence.

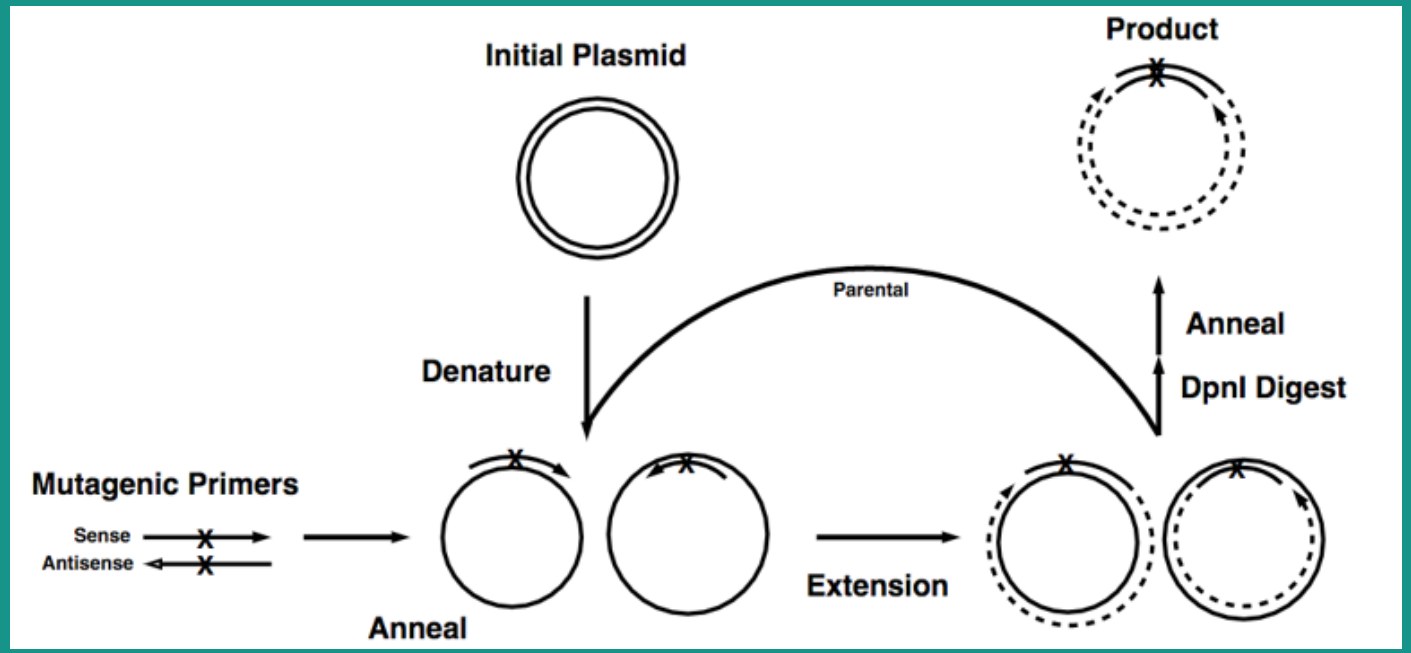
B-GLUCOSIDASES AND ITS ROLE IN PRODUCTION OF ETHANOL

- β-glucosidases B (BglB) is an enzyme that catalyzes the hydrolysis of glucose monosaccharides from larger molecules at a β-glycosidic linkage.
- β-Glucosidases are common among plants, fungi and bacteria
- β-Glucosidases are widely used in the production of biofuel and ethanol from cellulosic agricultural wastes and synthesis of useful β-glucosides

MUTAGENESIS AND ITS APPLICATION IN GENERATING NOVEL ENZYMES OR PROTEINS IN INDUSTRY AND MEDICINE

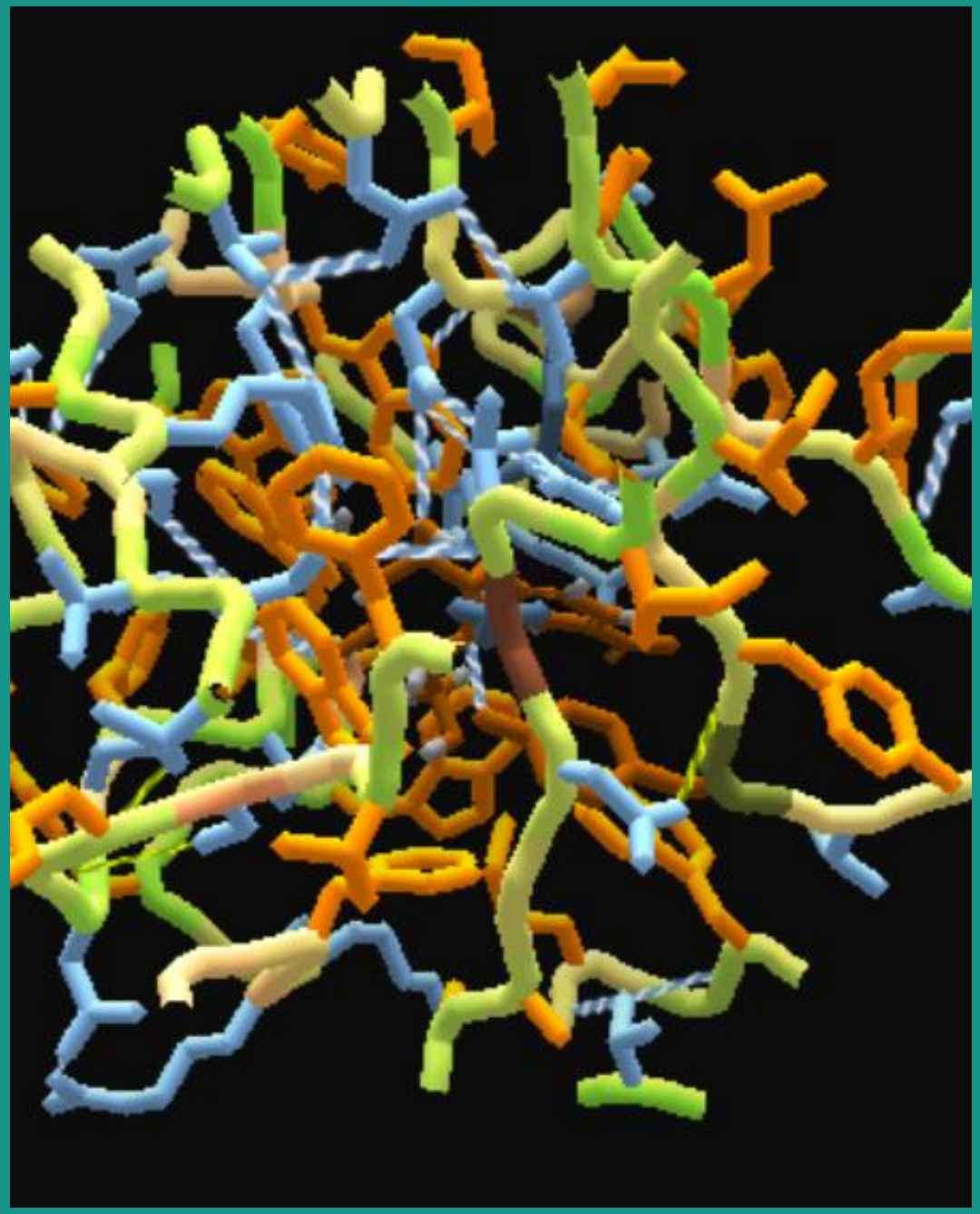
- Mutagenesis is the process by which the genetic information of an organism is changed, resulting in a mutation.
- This can occur naturally through errors in DNA replication or exposure to environmental factors, such as radiation or chemicals.

- Mutagenesis is a powerful tool in genetic research and biotechnology with broad applications across medicine, agriculture, industry, and environmental science.



FOLDIT STANDALONE, FREE ENERGY, H-BOND

- Foldit Standalone, an interface of the Rosetta Molecular Modeling software package.
- This software evaluates protein models for structural thermodynamic stability
- 3D model representation of the structure that can be manipulated and modeled, engaging human spatial intuition.



MATERIALS

Pipette tips>

<Centrifuge machine

Bacterial Shaker ^

Pipettes >

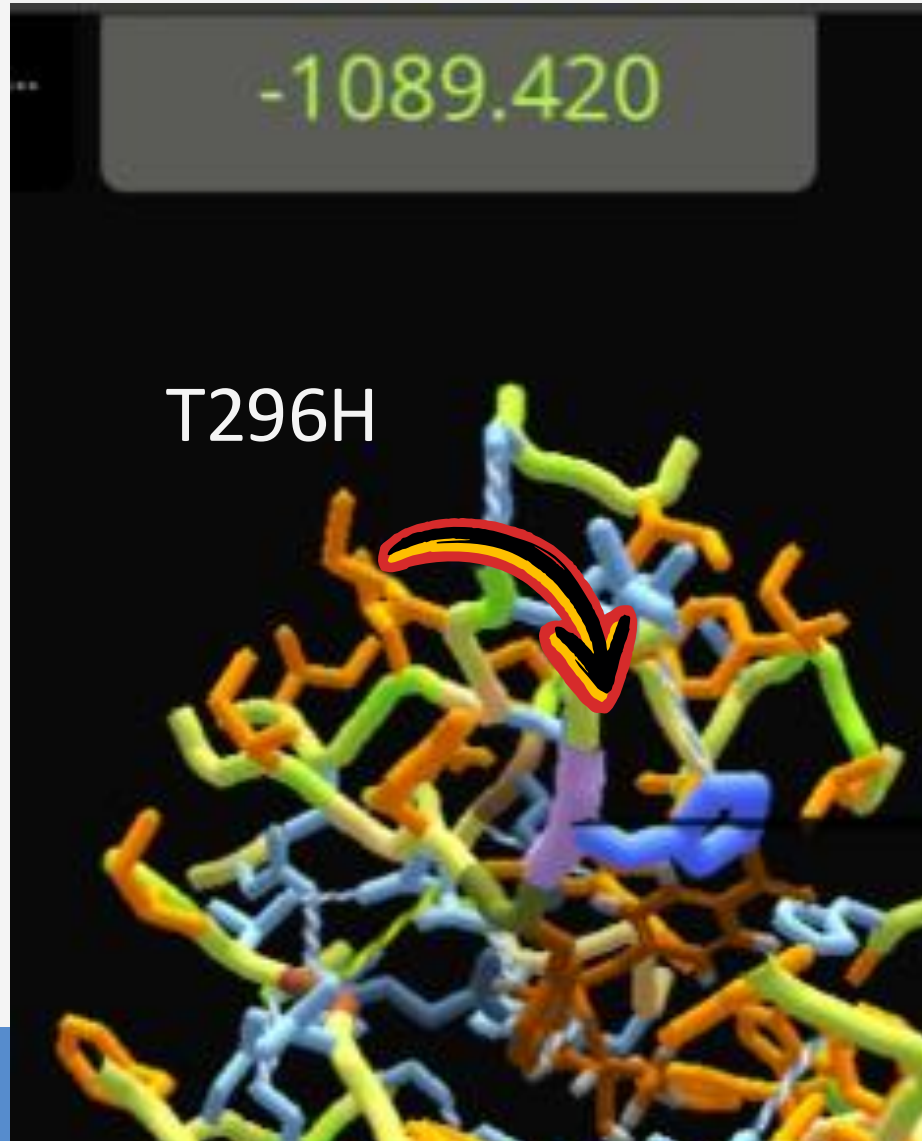
Pipette tips>

METHODS

- Learning how to analyze protein models
- Differentiating protein strands
- Matching proteins and amino acids
- Creating higher energy scores based off the low energy score of normal enzymes
- learning how to pipette

AMINO ACID-THREONINE MUTATED INTO HISTIDINE

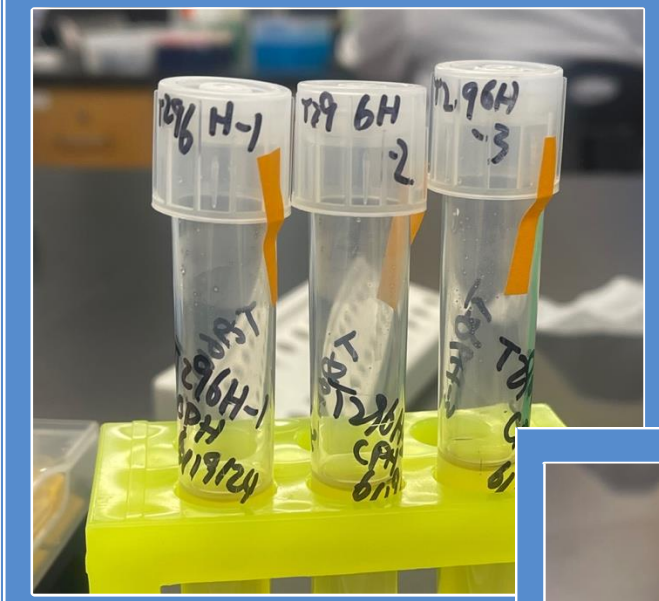
- Threonine is encoded by the codons ACU, ACC, ACA, and ACG.
- Histidine is encoded by the codons CAU and CAC.
- Threonine is critical for the protein's function (e.g., active site of an enzyme or a binding site), substituting it with histidine could alter the protein's ability to perform its normal biological function.
- Threonine (Thr) is a polar amino acid with a hydroxyl group.Histidine is also a polar amino acid but has a different structure and chemical properties, including an imidazole ring in its side chain

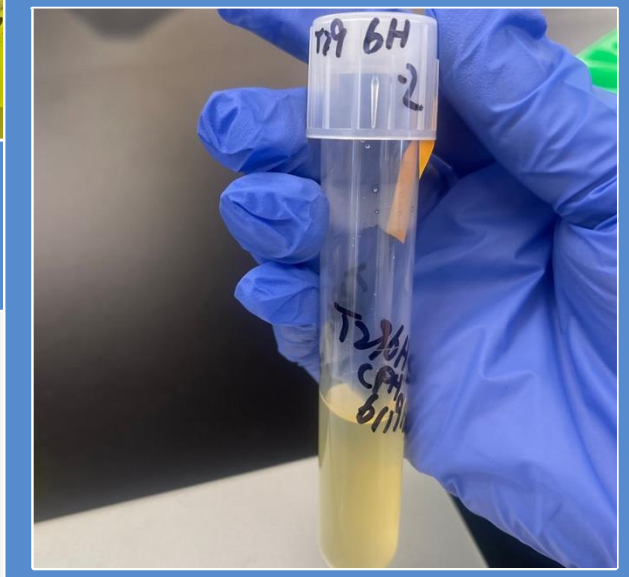


MUTATION T296H

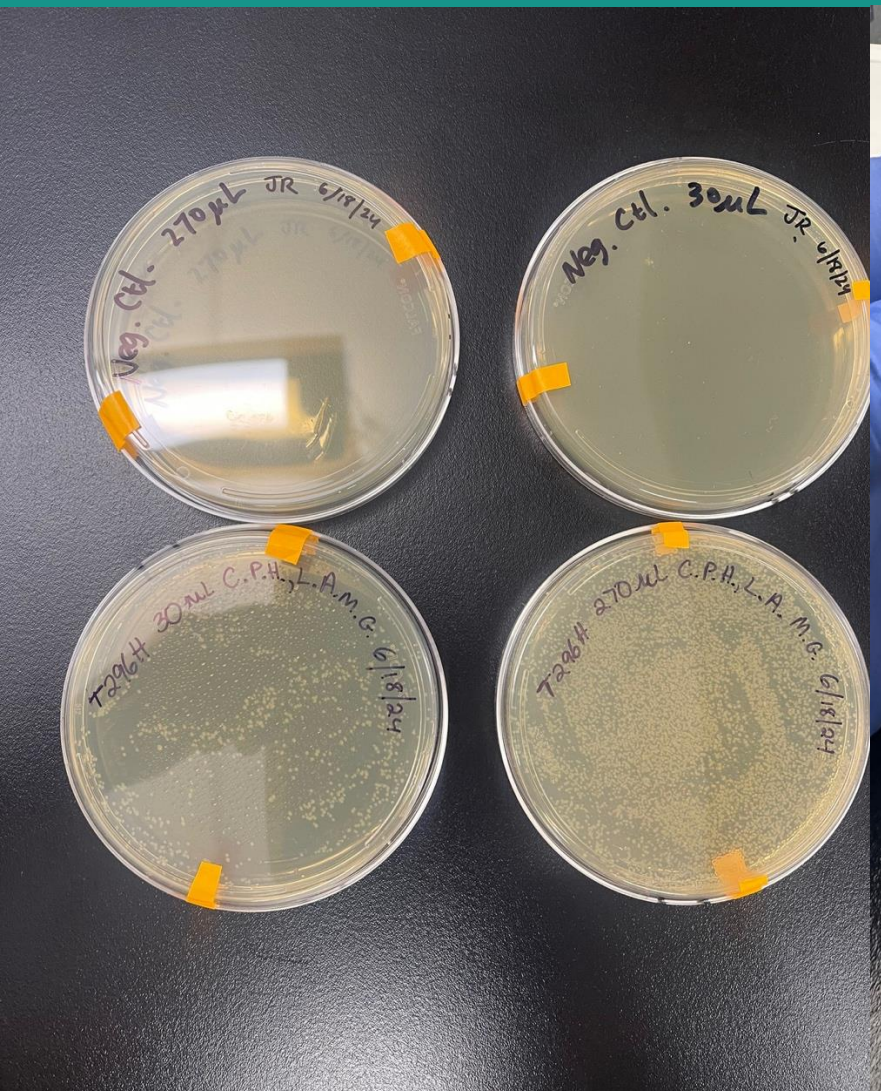
Inside the tubes :

- **Oligonucleotide primer** (100 µM) 2.5 µL (250 pmol)
- **10x T4 ligase buffer** w/ 10 mM ATP 5 µL
- **T4 Polynucleotide Kinase** (10 U/µL) 1 µL
- **H2O** 41.5 µL





TRANSFORMED BACTERIA COLONIES



RESULTS

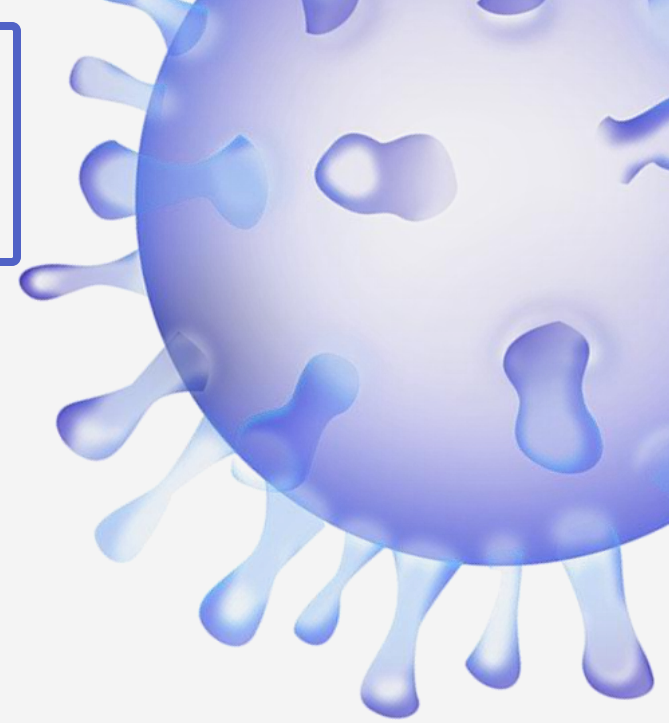
Precision and meticulous technique are crucial for obtaining reliable results. During the process of plasmid purification, our experiment unfortunately resulted in a low amount of plasmid residue. While encountering this setback was disappointing, due to limited research time, we were unable to continue our experiment. However, this technical error presents an opportunity for future learning and improvement.

Future Studies

Although our outcome was incomplete, the mutation T296H holds promise for future research to potentially achieve positive results. The pursuit of such research aims not only to validate existing hypothesis but to also have impacting discoveries that could serve as a breakthrough in medicine and molecular biology.

ACKNOWLEDGEMENTS

We extend our heartfelt gratitude to Dr. Zhou, Edith, and the entire CSTEP team for their unwavering support and guidance throughout this project. Your dedication and expertise have been invaluable, and this journey would not have been possible without your encouragement and assistance. Thank you for fostering an environment of learning and discovery.



THANK YOU !

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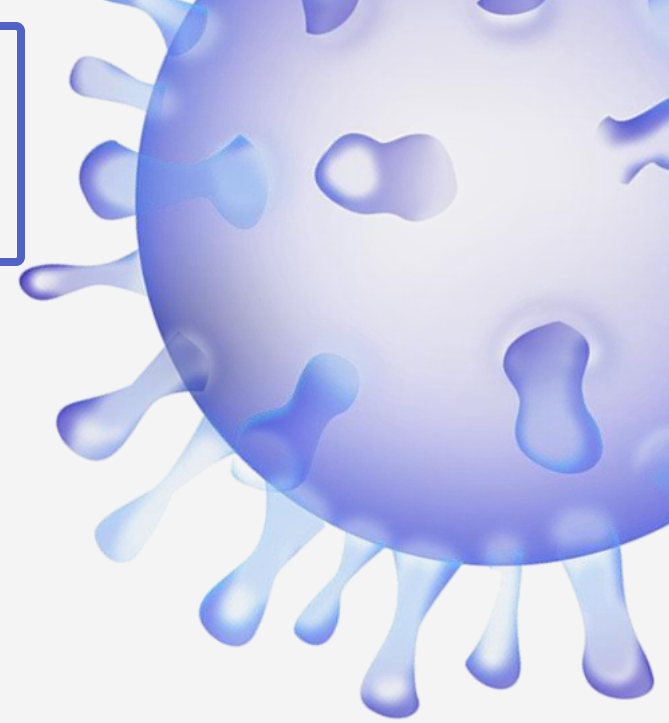


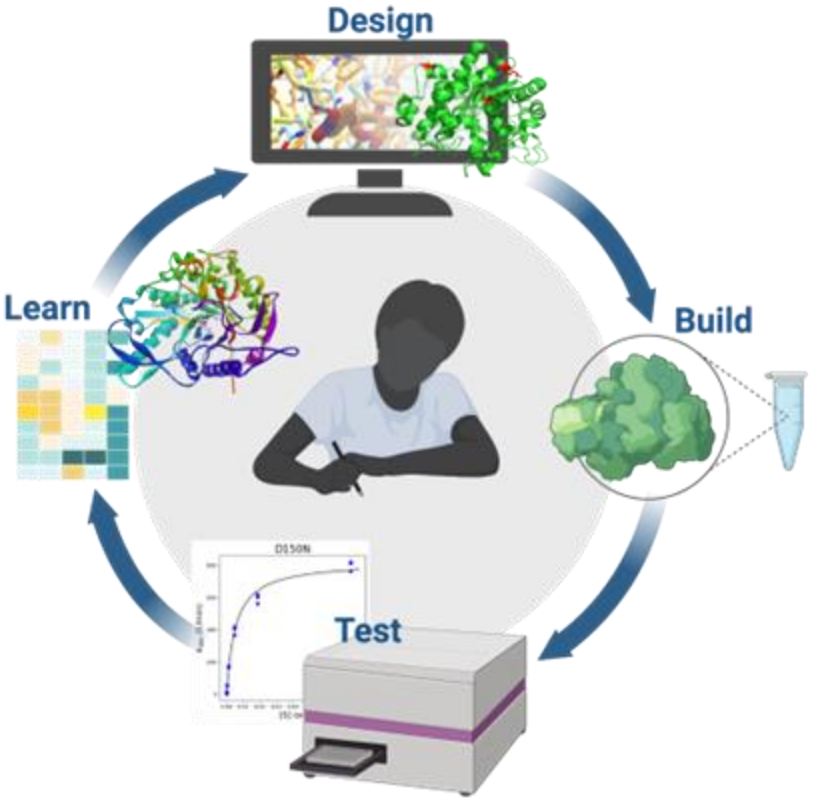

30 MINUTE BREAK

Production and purification of mutant BglB enzymes

By Laetitia Saint Hilaire
CSTEP summer research Program
Department of Natural Sciences
School of Health and Natural Sciences
Mentor: Professor Chun Zhou

BglB enzyme and D2D project

- The D2D project contributes to the understanding of computer assisted mutagenesis and its impact on the thermostability of proteins.
  - -The Folditstandalone is a software used to design mutations that can be beneficial for the function and stability of proteins.
  - - Our research utilizes Folditstandalone to design different mutations of BglB.



- BglB or beta-glucosidase B is an enzyme that generates glucose from polysaccharides.

Labonte, Jason William. “D2D Cure: About.” *D2D CURE* |

Research Question:

How can we improve the efficacy of BglB in generating ethanol from the breakdown of cellulose?

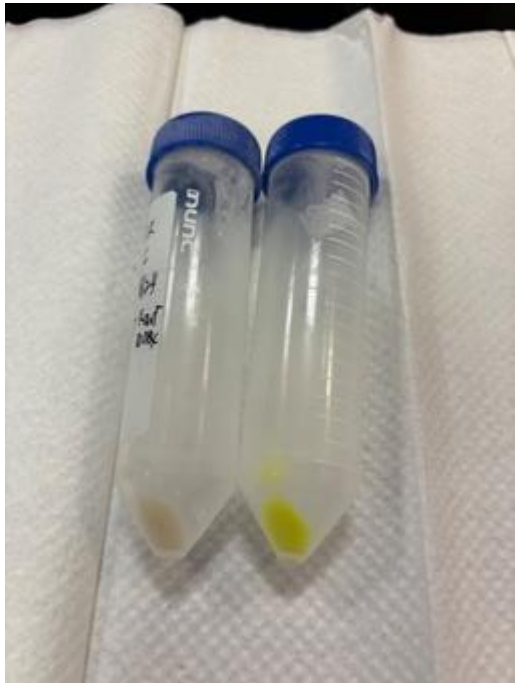
Research Hypothesis:

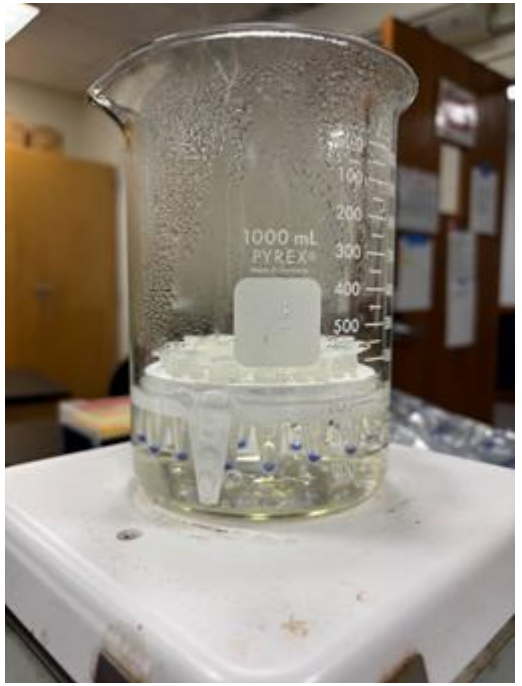
Mutations of amino acid residues will improve the thermostability of the enzyme.

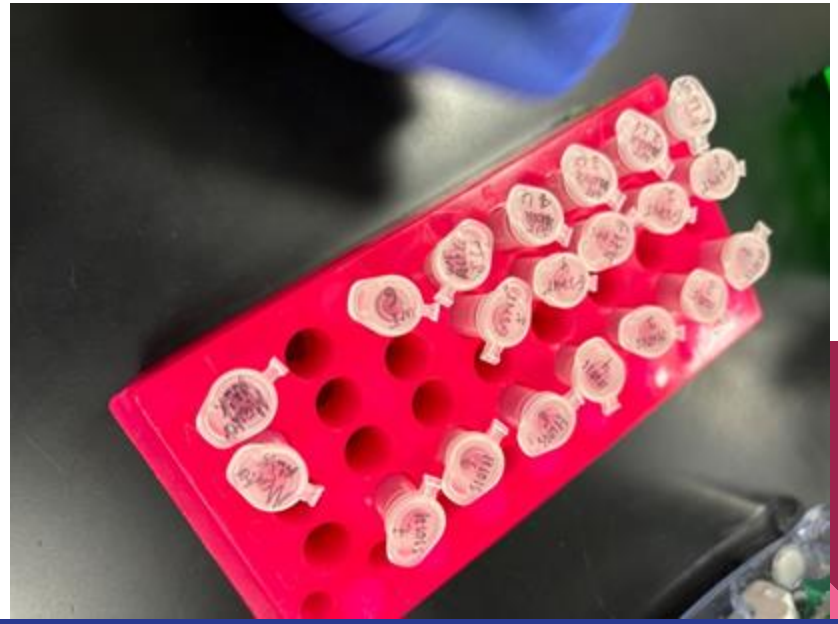
Research Approach: The designing of the new mutations using computational biology and the expression of proteins through the lac operon mechanism and purifying the proteins using the affinity binding chromatography technique.

Mutations and controls used in this experiment

- Computational Biology
- H101S
- G374T
- GFP control
- Wild type BglB
- Score energy level







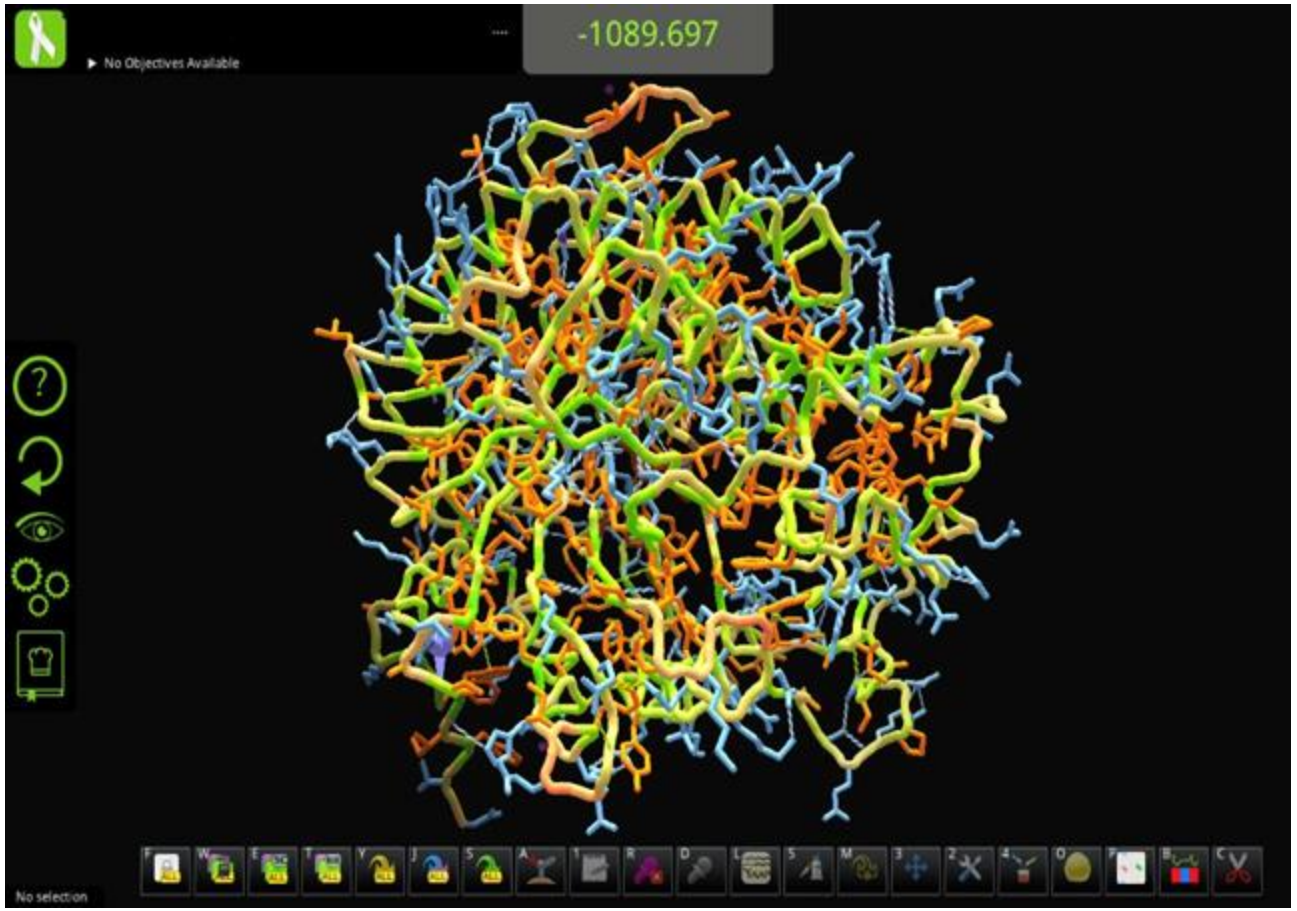


Figure 1a- Wild type BglB enzyme

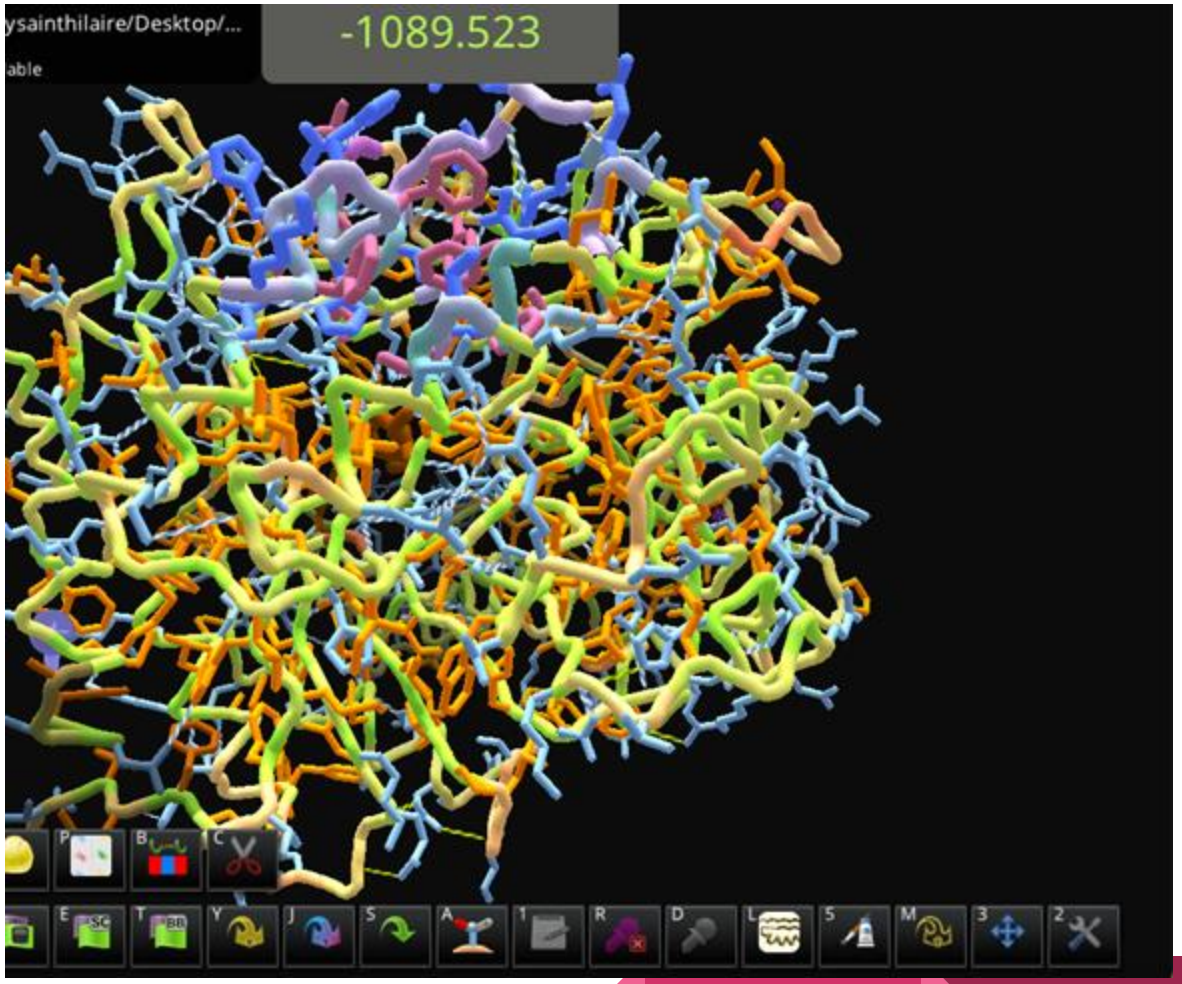
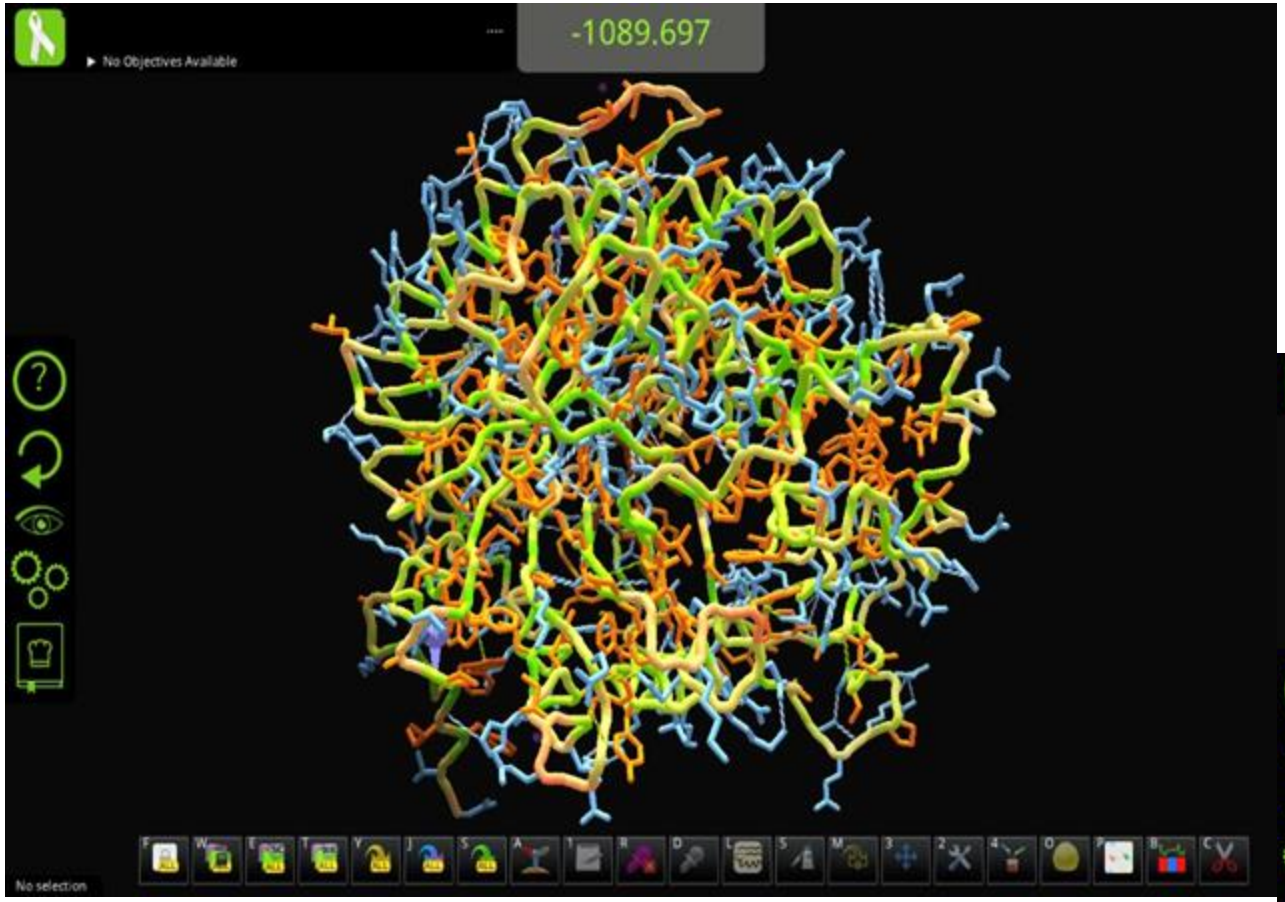
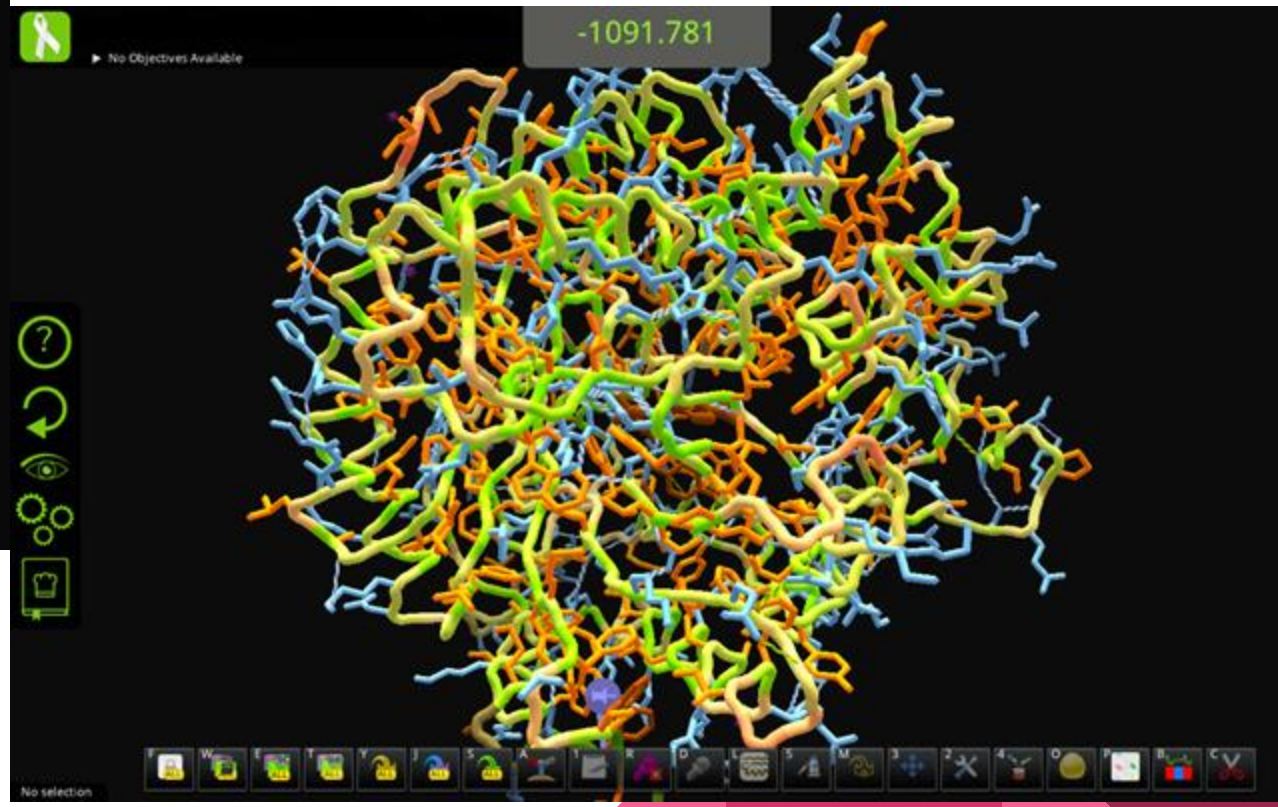


Figure 1b. First mutated gene (H101S)



G374T mutation



Materials and methods

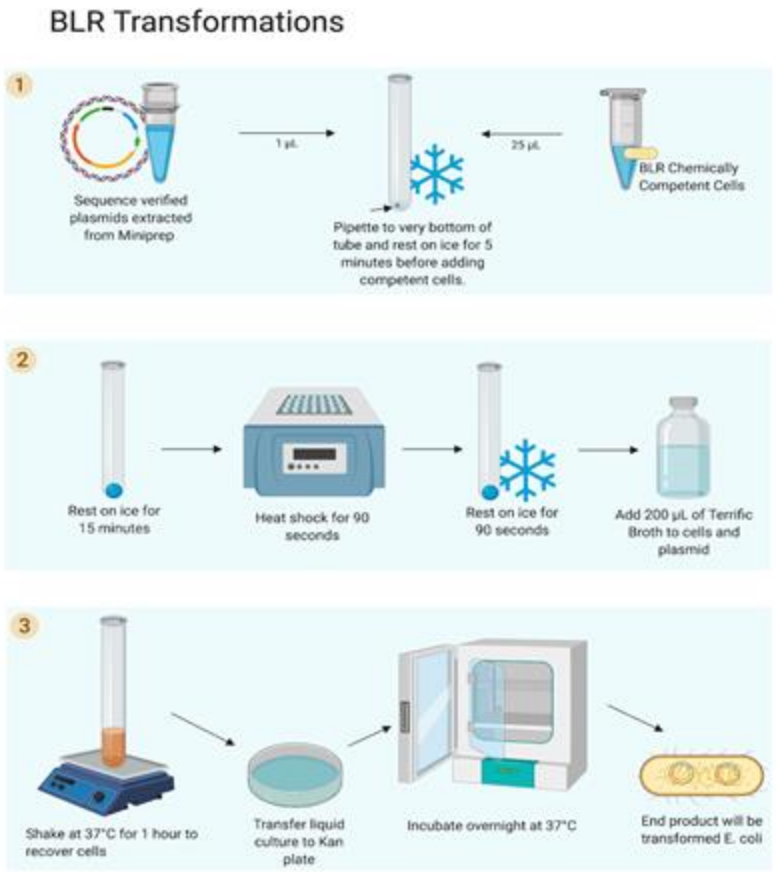
1) Design mutations of BglB using folditstandalone.
2) Generation of mutant BglB enzyme
3) Production of mutant BglB proteins

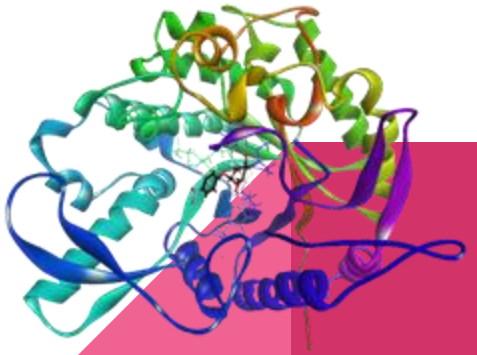
-Transformation of the bacterial cells by introducing the plasmid with the mutation (H101S, G374T) and the wild type enzyme to the BLR (DE3) competent cells.

- a series of bacterial culture for protein production, induced by IPTG.

4) Protein Purification using Affinity chromatography.

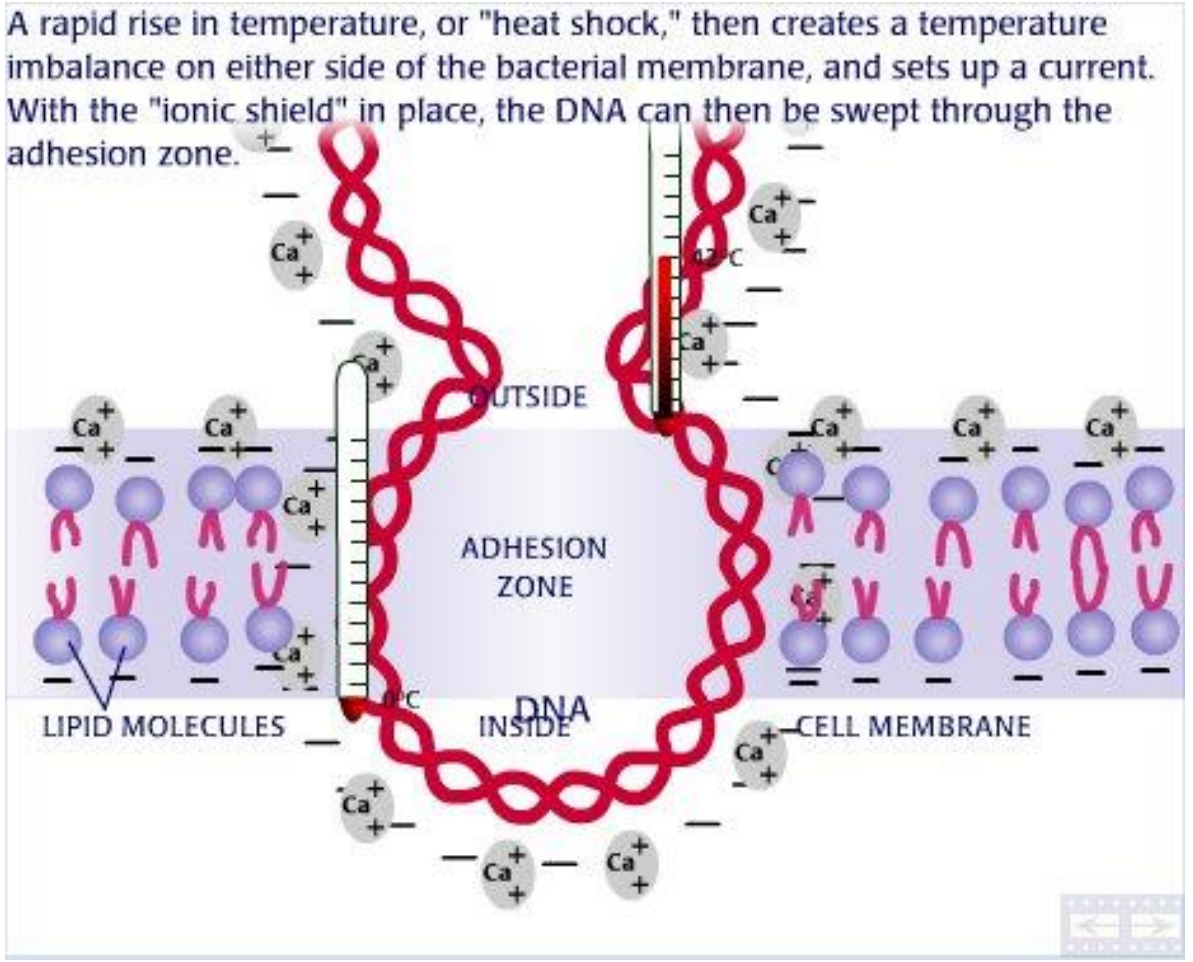
5) Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS- PAGE) to confirm purified protein.





Transformation of the bacterial cells

- The transformation process is an important process in incorporating the gene sequence of the mutation, introducing the plasmid to the bacterial cells.
- During the heat shock, the calcium ions surrounding the cell membrane are attracted to the negative charge of the plasmid.
- Attachment
- Repair damage (when culturing the bacterial cells).



https://pharma e.info/wp-content/up 07/transformatio

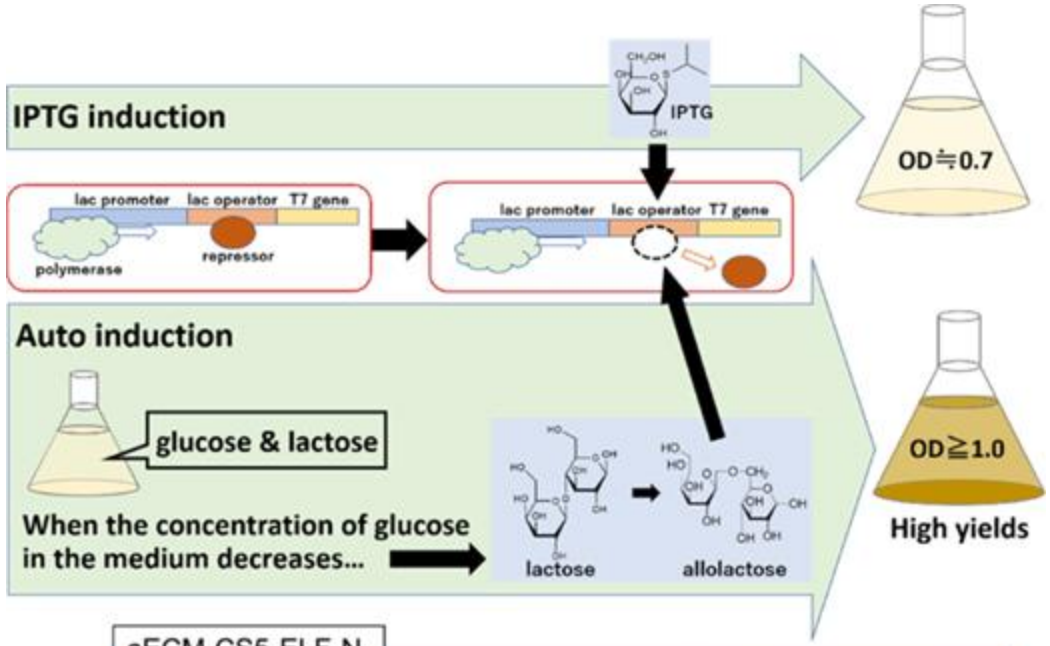
The Lac operon ?

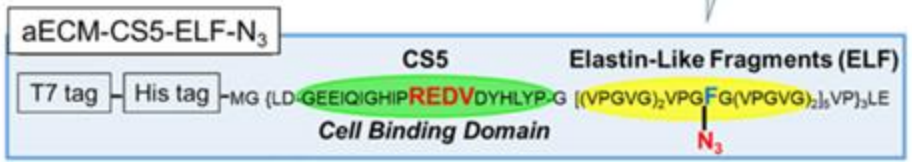
- The Lac operon in bacteria facilitates the breakdown of the sugar lactose.

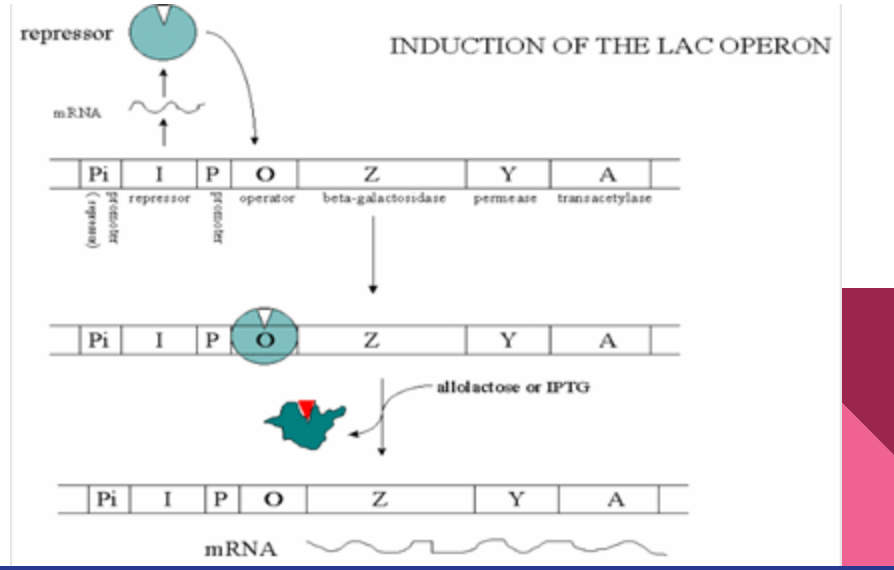
- In the bacterial gene, there are lac A, Y and Z. Each participate in the uptake of the lactose specially in the cell membrane, and in the process of the lactose converted in less complex sugars.

- The operator is the site where the repressor binds to induce the expression and transcription of lactose.

- The allolactose induces the transcription and expression of the lactose when binded to the repressor.





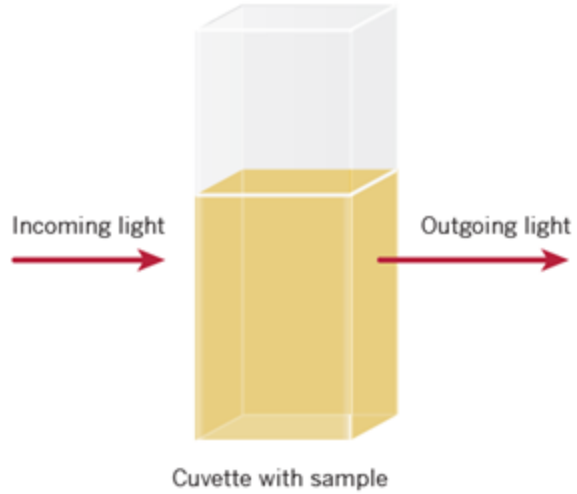


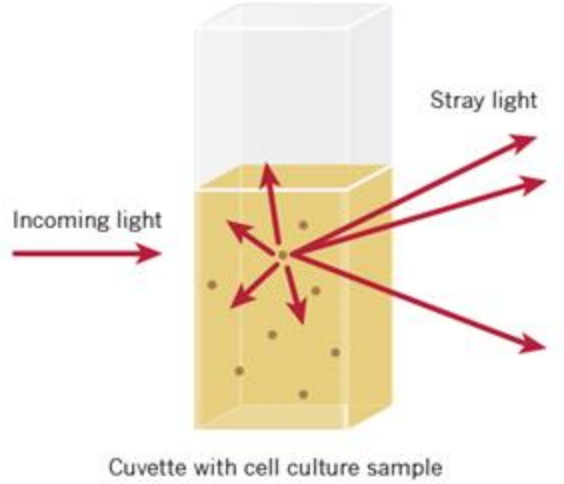
The OD_{600} ?

- After the transformation of the bacterial cells and using a single colony to proceed with the overnight culture to measure how fast the bacteria are growing per generation, the optical density 600 is monitored and recorded gradually.

- For the optical density (OD_{600}) to double per generation, the tubes were put in the shaker at beneficial conditions overnight for the rate of growth anticipated.

- When in the spectrophotometer, the OD_{600} value is determined based on the light reflected when blocked by bacteria in the mixed solution.





"The OD600 Basics: Best OD600 Tool to Generate Microbial Growth Curves." *Implen*, 17 Feb. 2024.

Results

Transformation of the H101S mutation

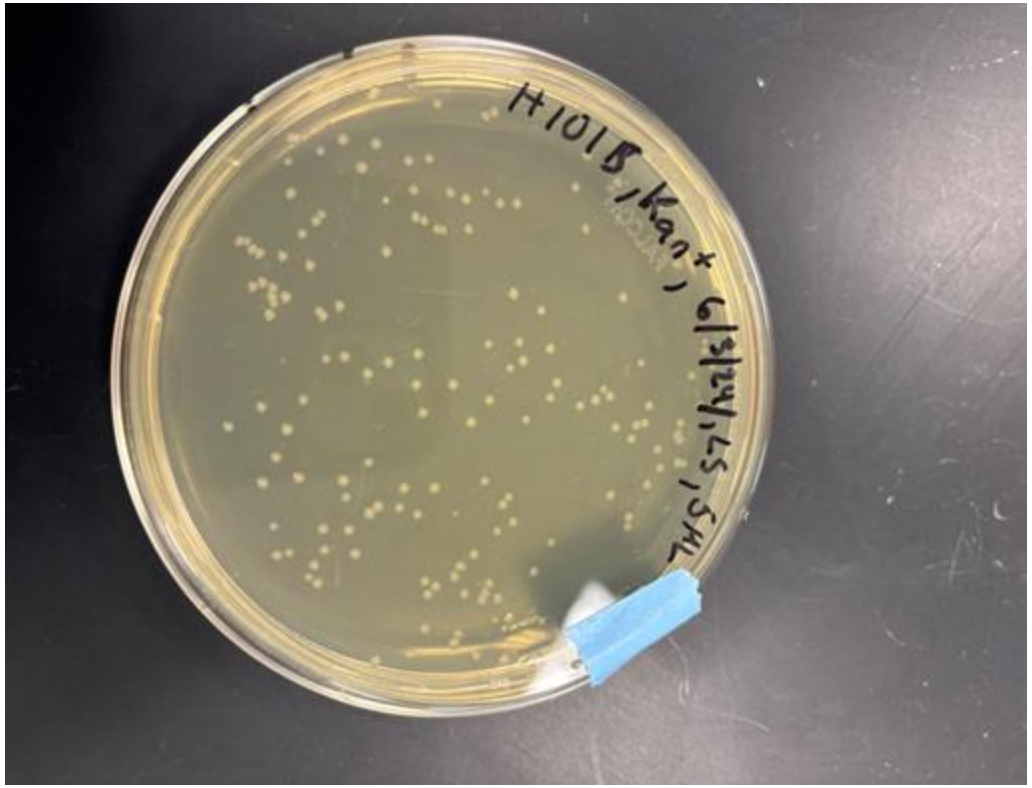


Figure 2a- 5 microliters of bacterial cells + plasmid with 100 microliters of LB solution.

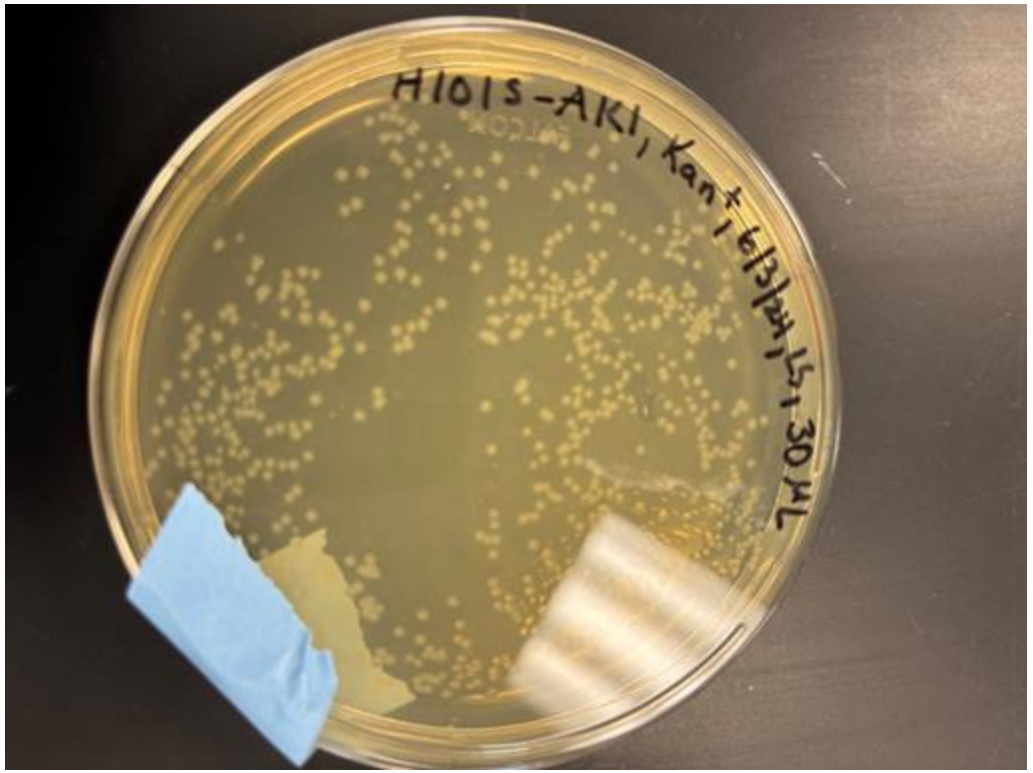
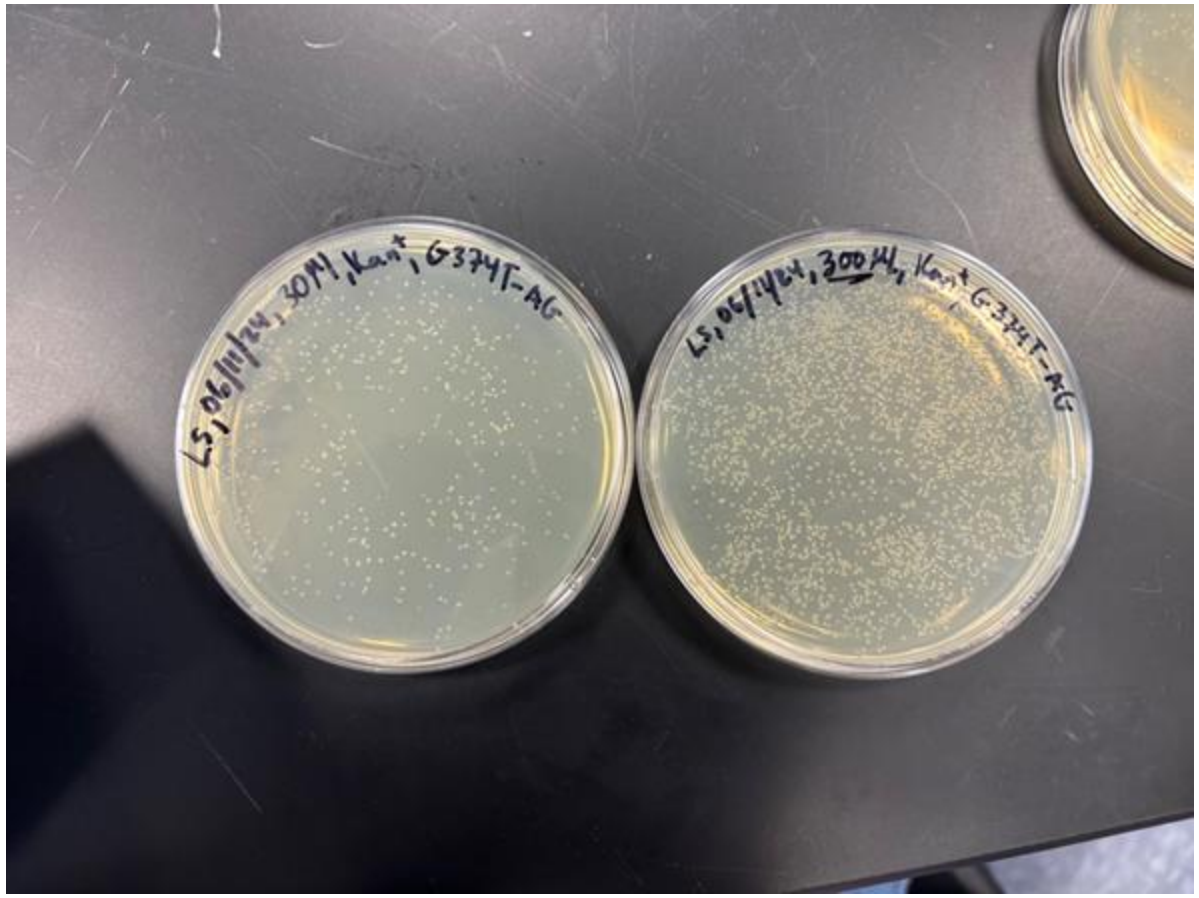


Figure 2b- 30 microliters of bacterial cells + plasmid with 100 microliters of LB solution.

Results



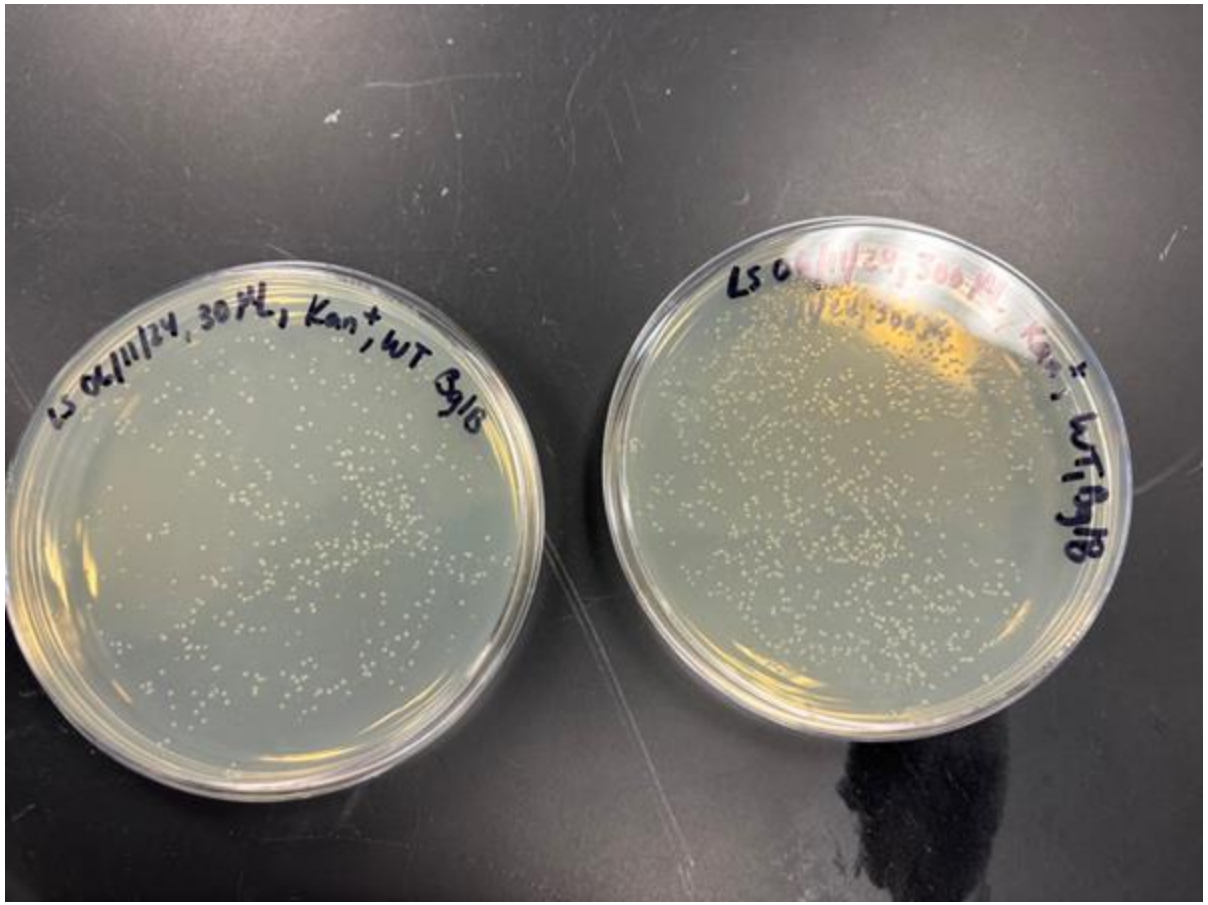


Figure 3a- Transformation of the G374T mutation using 30 and 300 microliters of the bacterial cells with plasmid and the LB solution.

Figure 3b- Transformation of the wild type Bglb using 30 and 300 microliters of bacterial cells with plasmid and LB solution.

The OD measurements- Results

When?	OD 600 value	Rounded to
H101S (first time) 06/5/24	OD= 0.939	1
After 16 hours in shaker 06/6/24	OD= 1.767	2

OD measurements taken after overnight culture growth

Mutation?	OD 600 value?
Wt BglB	0.313
H101S	0.485
G374T	0.316

OD 600 measurements taken after taking the solutions out of the shaker.

Mutation ?	OD 600 Value?
Wt BglB	0.489
H101S	0.685
G374T	0.496

Results

After inoculation, and using the shaker for the overnight culture

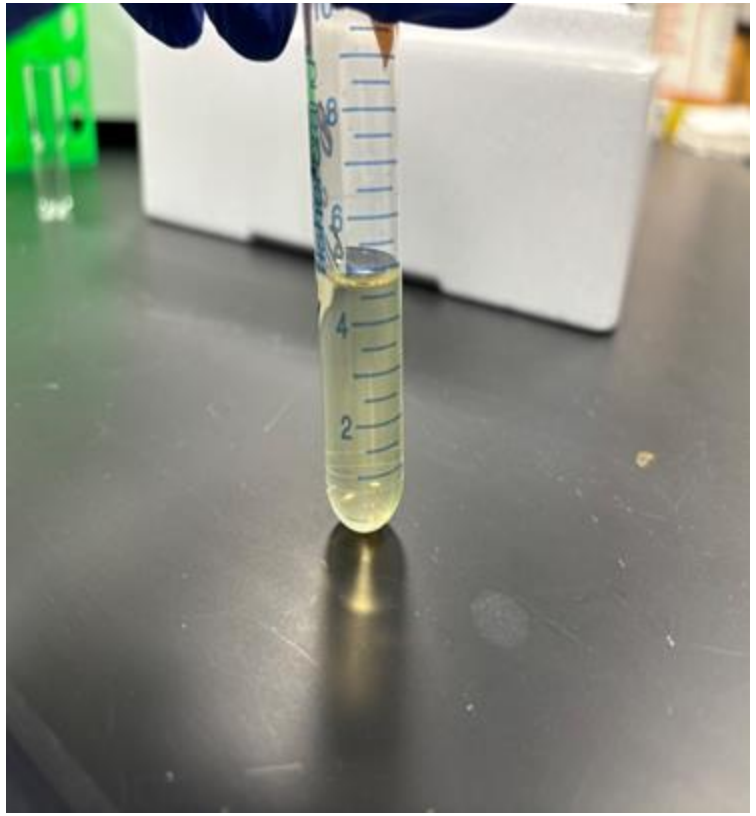


Figure 4a- A negative Wild type BglB growth culture.

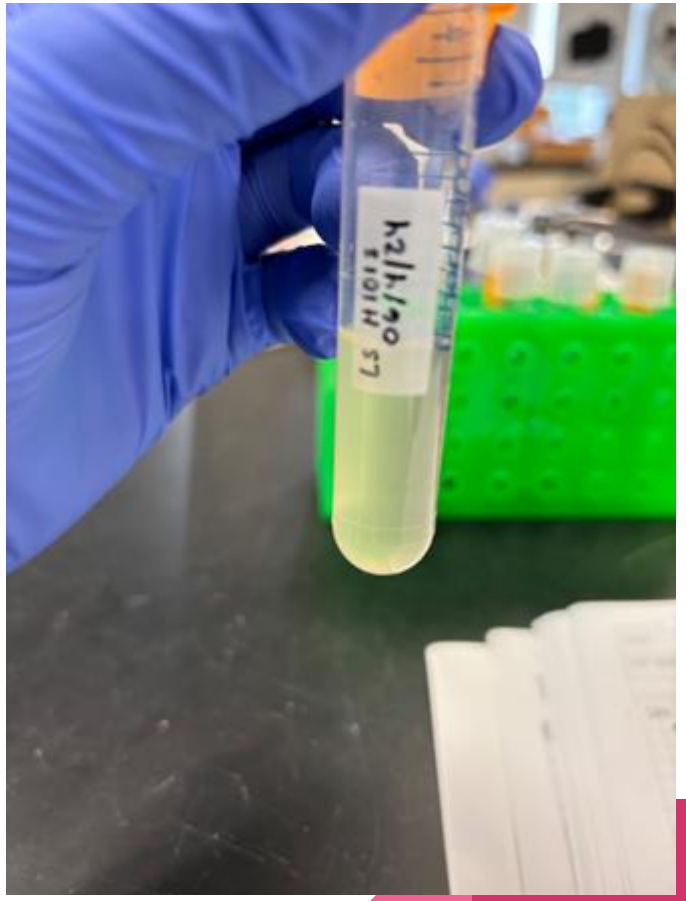
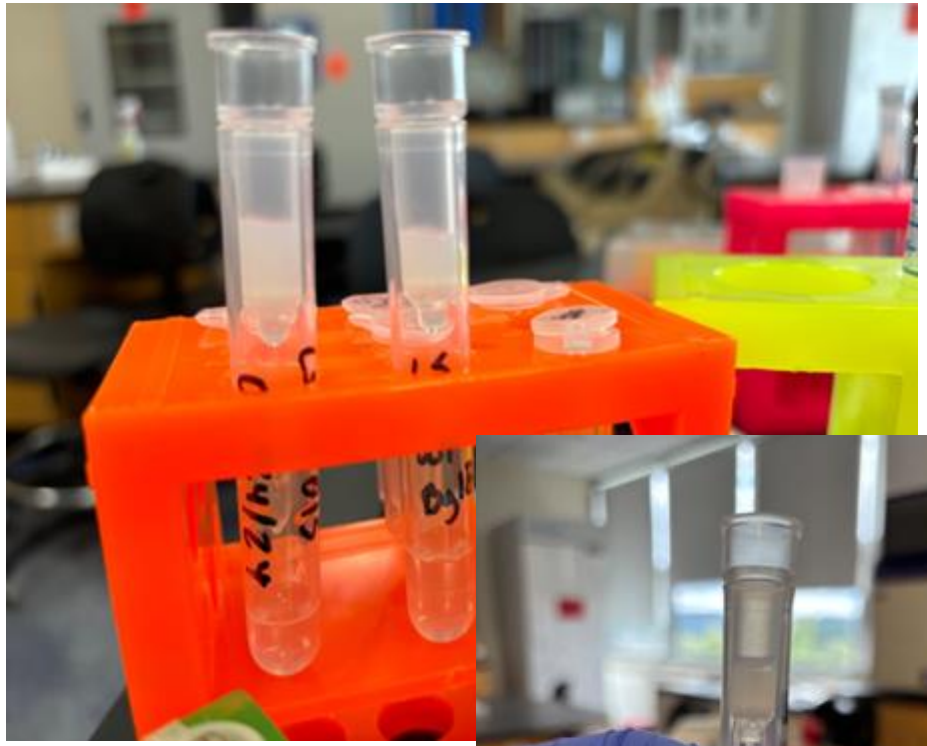
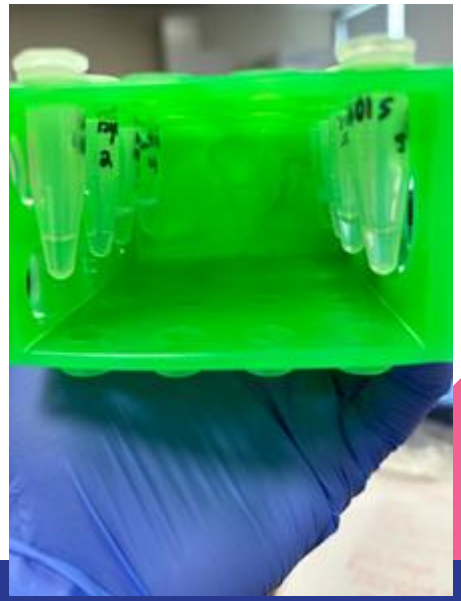


Figure 4b- The mutation H101S's growth culture.

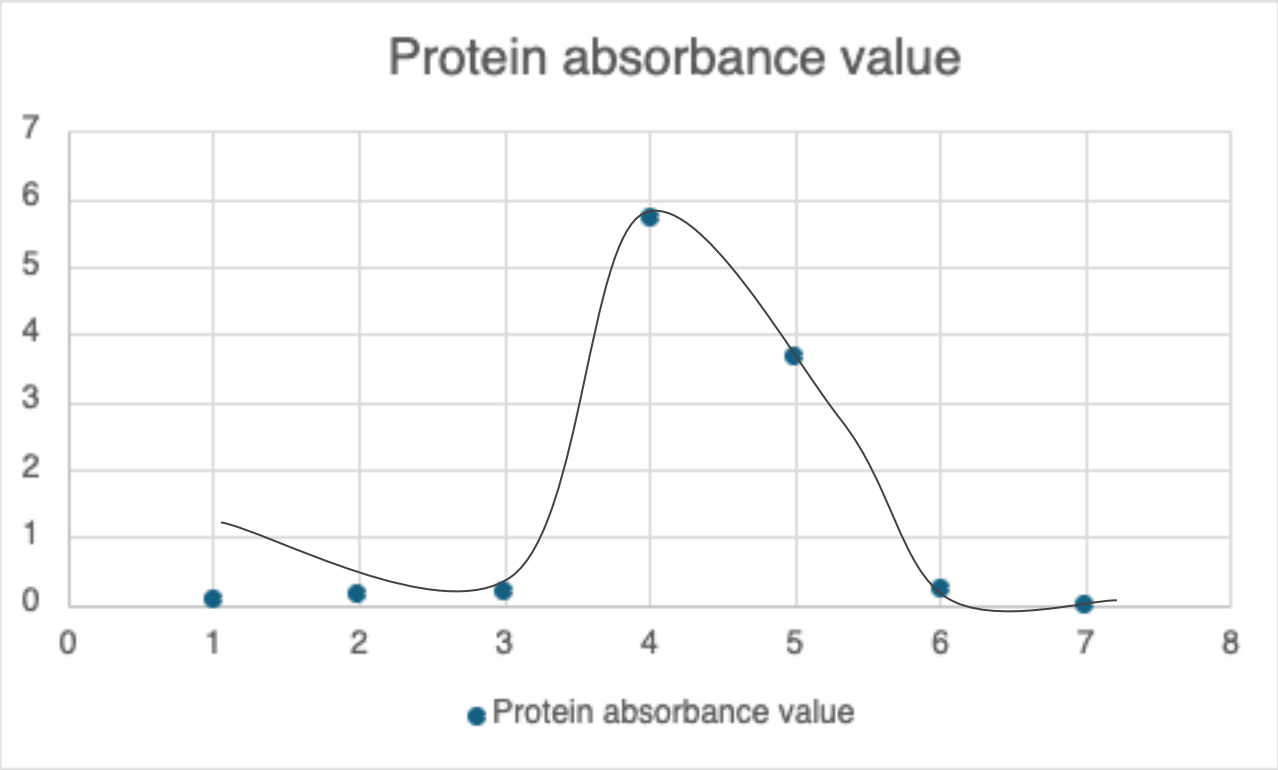
Protein Purification

- The mechanism behind the purification is the affinity binding chromatography.
- The nickel beads are positively charged and the elution buffer is negatively charged.
- The elution buffer was used to collect the protein binded in the column in clean tubes.
- 5 different tubes were used to collect the protein purification samples.
- Absorbance of each protein sample was measured using the spectrophotometer.



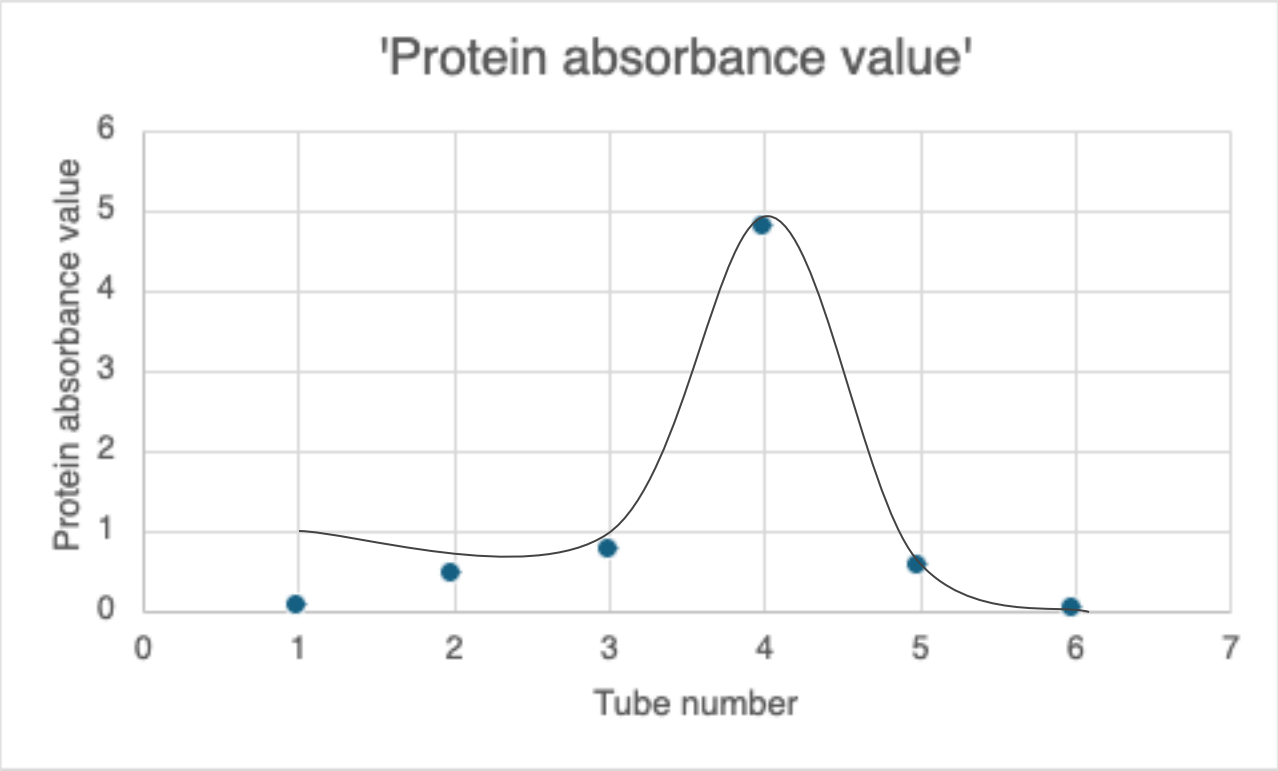


Protein purification graph and data for mutation H101S- Results



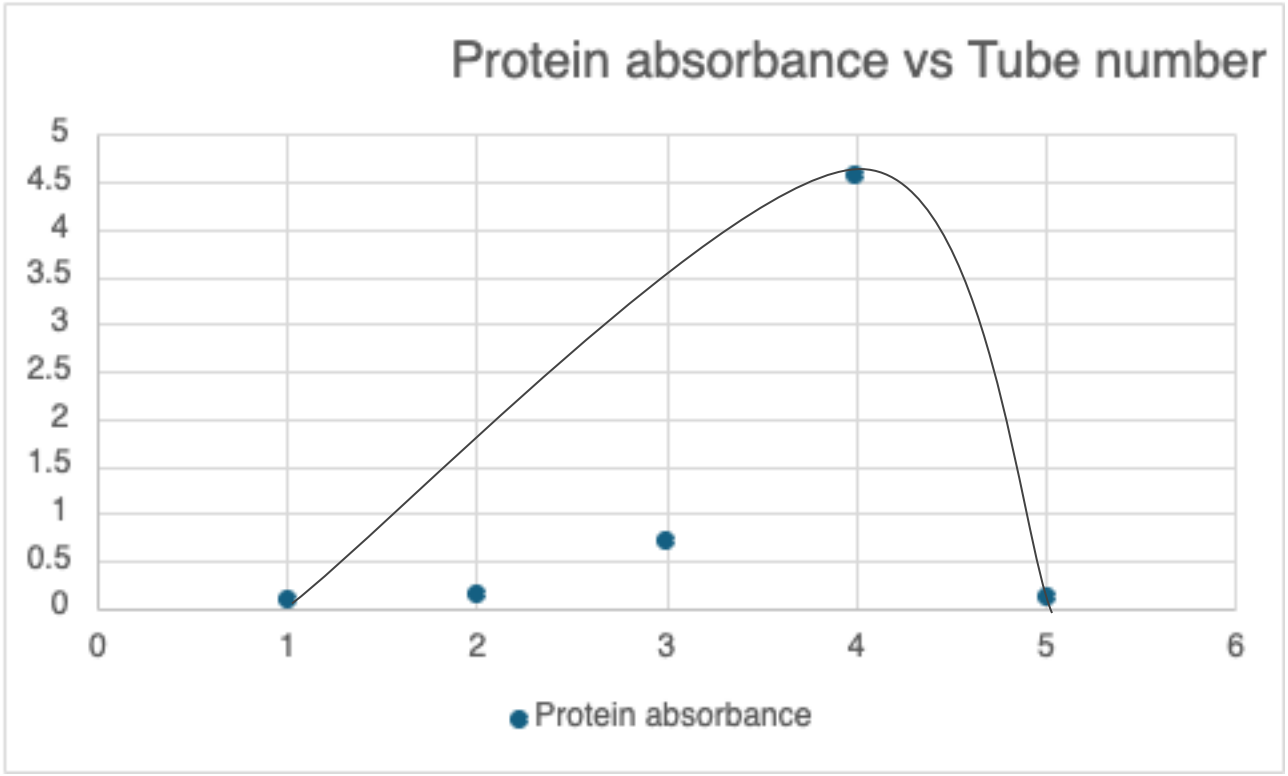
Tube number	Protein Absorbance value
1st tube	0.094 mg/mL
2nd tube	0.148 mg/mL
3rd tube	0.196 mg/mL
4th tube	5.731 mg/mL
5th tube	3.685 mg/mL
6th tube	0.260 mg/mL
7th tube	0.002 mg/mL

Protein purification graph and data for mutation WT-BglB -Results



Tube number	Protein absorbance value
1	0.059 mg/mL
2	0.448 mg/mL
3	0.735 mg/mL
4	4.790 mg/mL
5	0.534 mg/mL
6	0.030 mg/mL

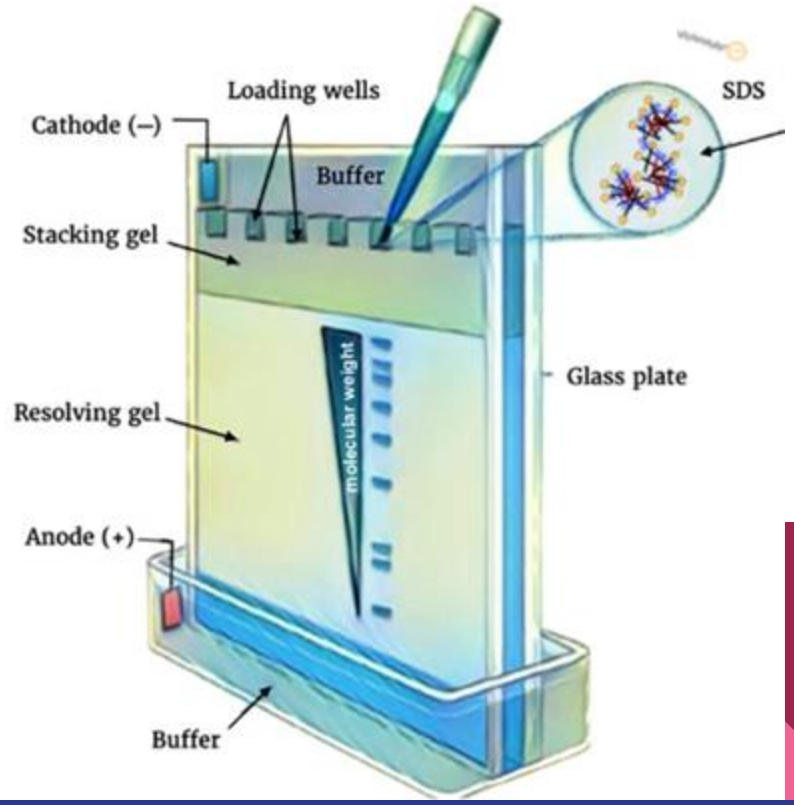
Protein purification graph and data for mutation G374T-Results



Tube number	Protein absorbance value
1	0.082 mg/mL
2	0.136 mg/mL
3	0.721mg/mL
4	4.566 mg/mL
5	0.124 mg/mL

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis or SDS-PAGE

- It is a laboratory technique utilized to isolate proteins based on their molecular weight.
- Another important component of the SDS-PAGE allowing the migration of protein fragments is the gel matrix which works with the intervention of an electrical field.
- The goal is to destroy the dimensional structure of the protein into linear molecules to facilitate the separation of protein fragments.



SDS PAGE- gel result (1)-Wild type BglB

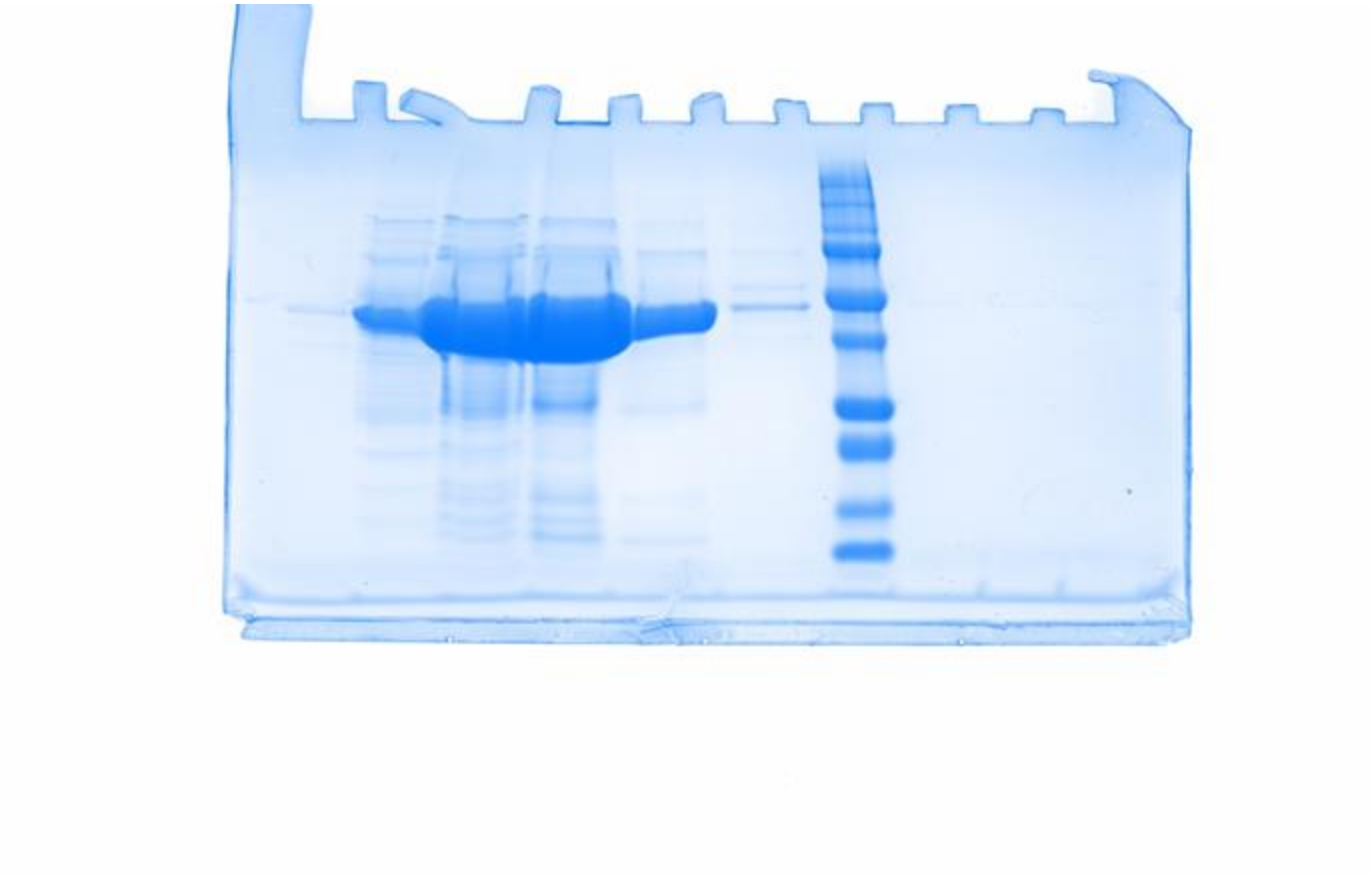


Figure 5-This is the gel picture of the Wild type BglB enzyme protein expression.

SDS PAGE- gel result (2)- G374T mutation

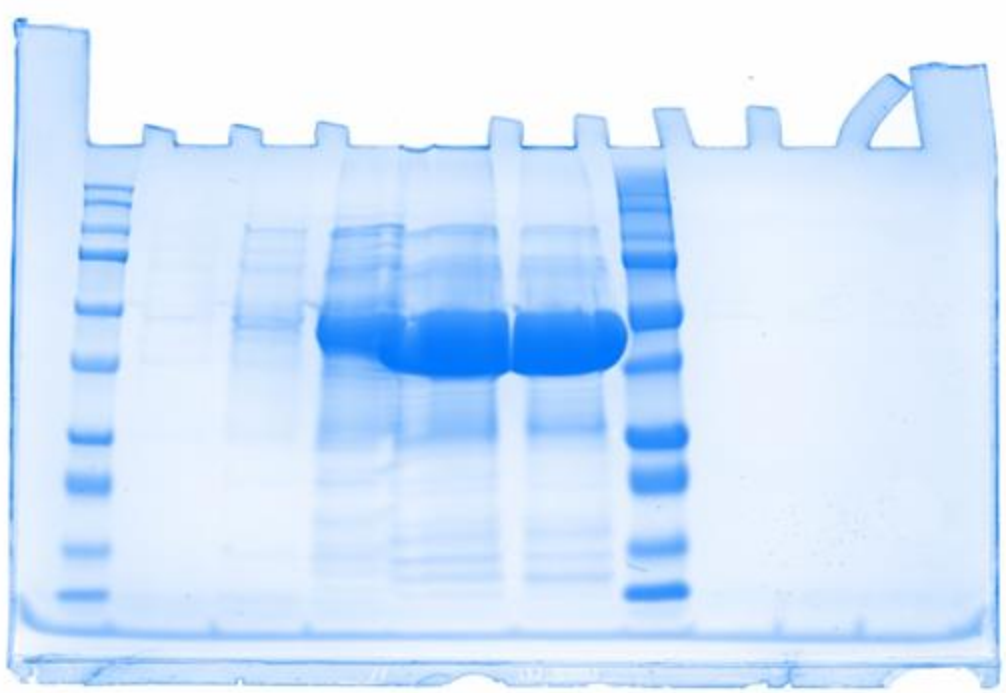


Figure 6- This picture displays the gel results obtained for the protein expression of the G374T mutation.

SDS PAGE- gel result (3)- H101S mutation

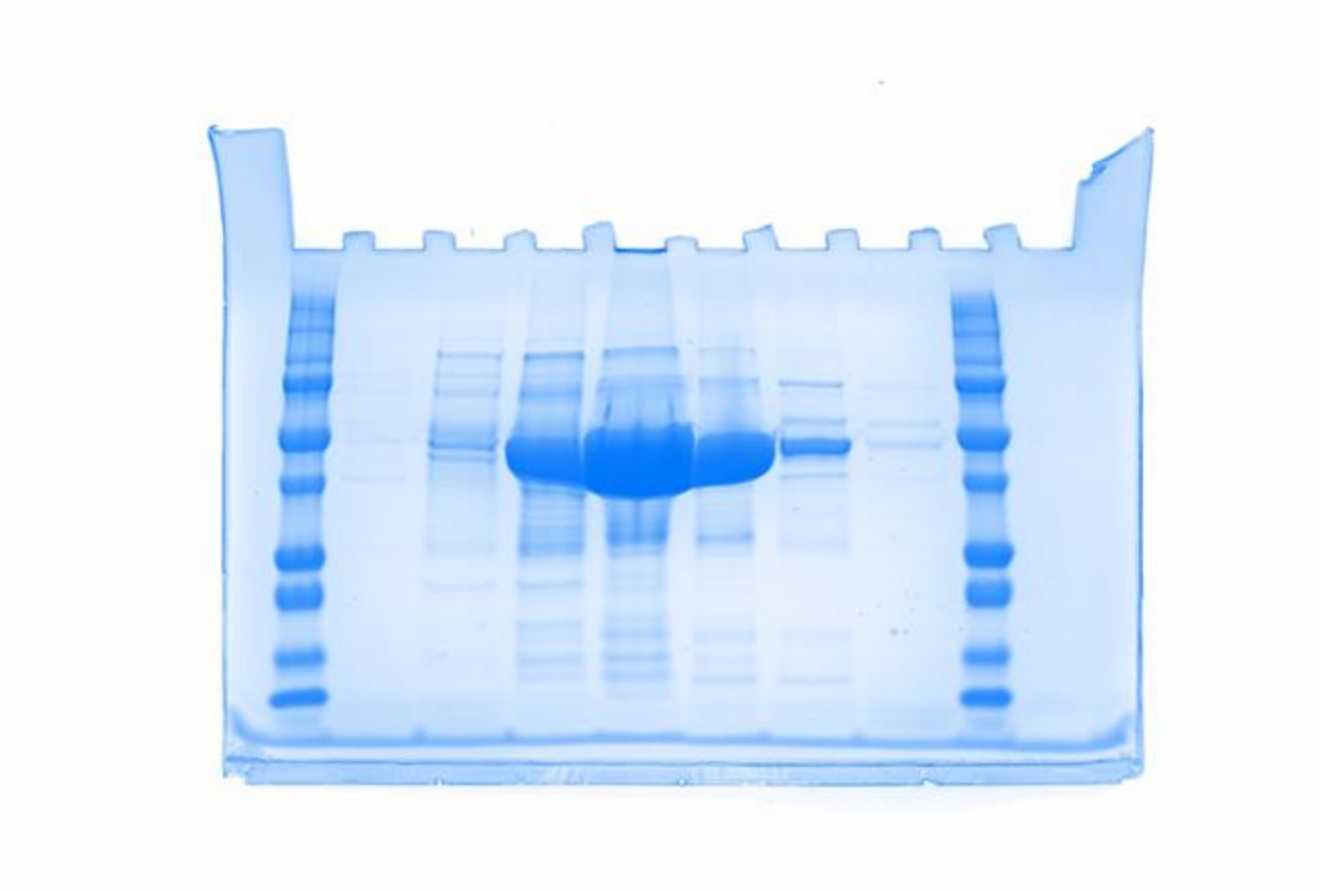


Figure 7- This is the gel representing the concentration of protein expression confirming the presence of the H101S mutation protein.

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Acknowledgements and resources

My acknowledgements to the mentor of the Summer Research Program 2024, Dr. Zhou, who worked tirelessly to make this project successful and possible, to all the CSTEP students who participated in this summer research program, and to CSTEP for sponsoring this hands-on Summer Research Program.

Vater, Ashley W. “D2D Lab Manual.” *Design2Data*, 20 July 2022, d2d.ucdavis.edu/d2d-lab-manual .

Expression & Purification of Mutated Beta-Glucosidase B (BglB) Enzymes in BLR21 (DE3) E. coli Cells

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Introduction

- Beta-glucosidase (BgIB) enzymes are a class of enzymes found in all living domains but most commonly found in fungi, plants, & bacteria.

- Beta-glucosidase enzymes serve many different functions, however, their most notable contribution in bioengineering applications is their ability to produce biofuel from biomass through cellulose hydrolysis.

- Through cellulose hydrolysis, beta-glucosidase can produce glucose, which yeasts will subsequently use in fermentation to produce ethanol.

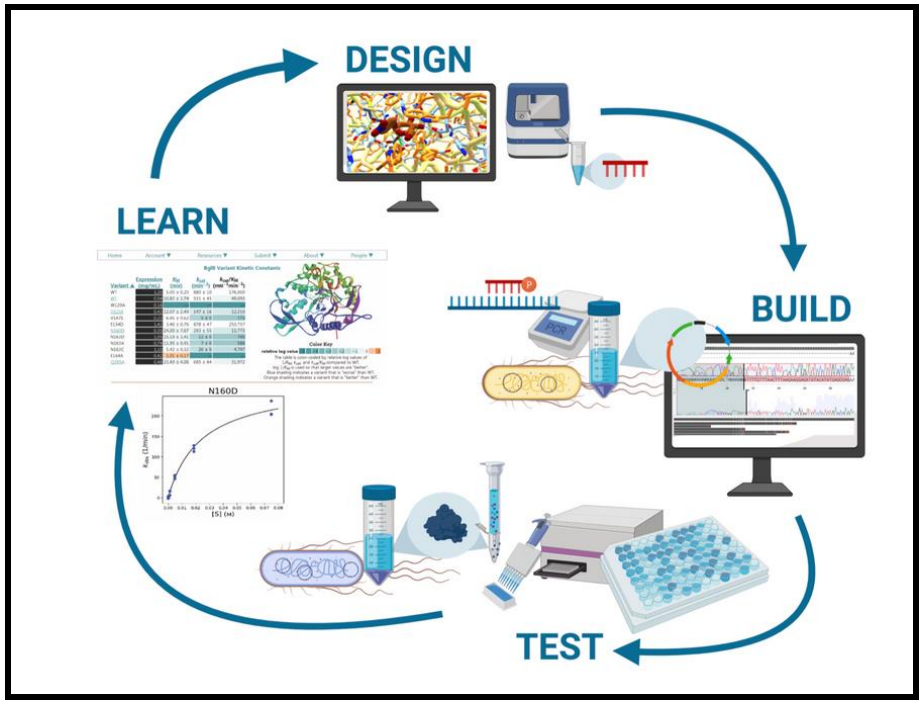
- In recent years, there has been an increased biofuel demand and depletion of fossil resources, thus, creating a crucial need for biofuel, such as ethanol.

- Current methods for biofuel production include using starch-rich sources such as corn, wheat, or sugar feedstock, however, these sources raise environmental concerns.

- Researchers have turned to the possibility of utilizing beta-glucosidase (BgIB) enzymes for biofuel production, however, data suggests that as more glucose is produced through fermentation, thus releasing heat, the more the enzymatic activity is compromised.

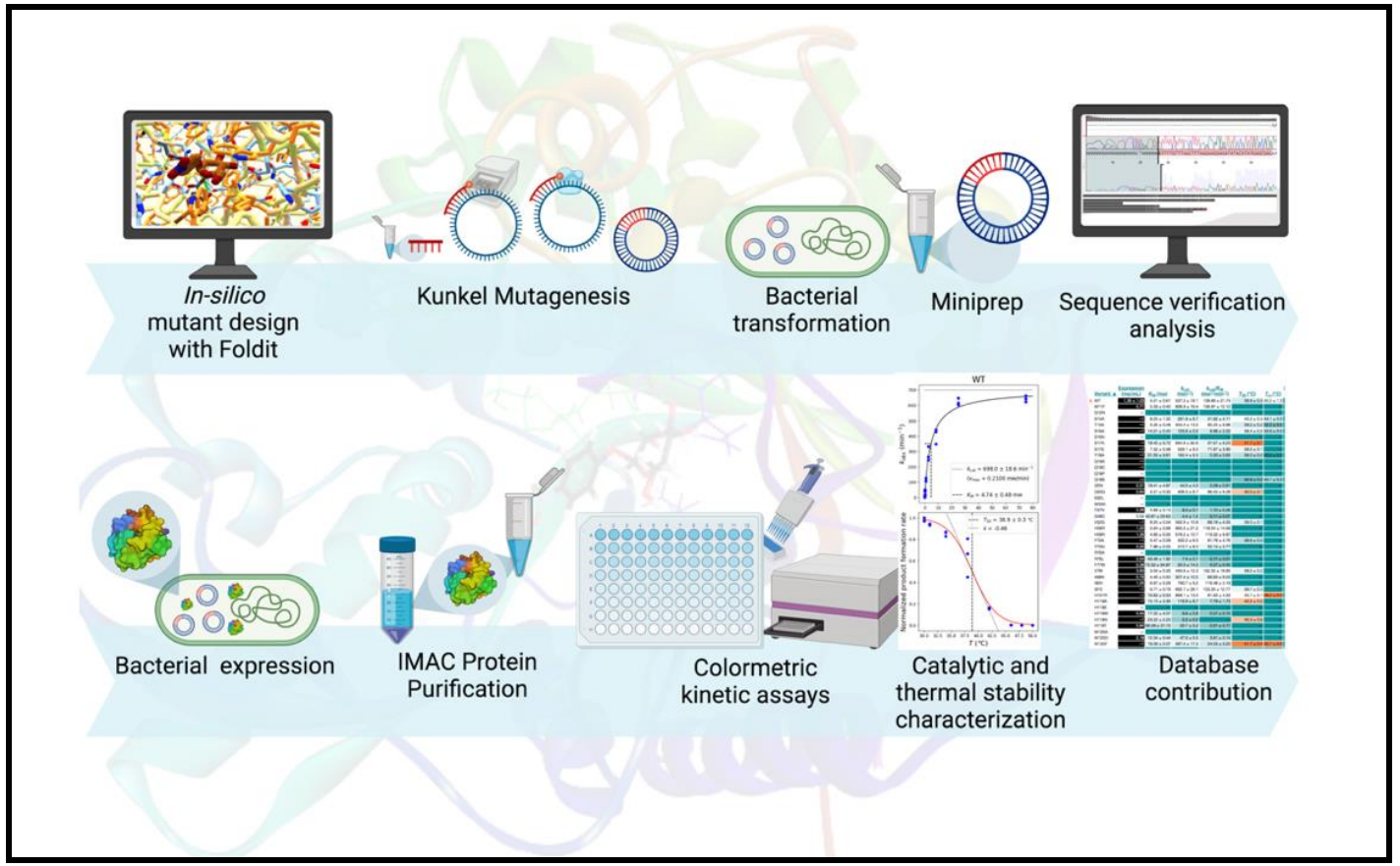
- Utilizing site-specific mutagenesis of beta-glucosidase to create a mutant type can offer a promising alternative for ethanol production while reducing production costs.

Design2Data (D2D) Software



Source: https://siegel.ucdavis.edu/design-2-data

- Design-to-Data workflow was developed at the University of California at Davis (UC Davis) and was created to facilitate enzyme design-build-test workflow for undergraduate students.
- Design-to-Data was also developed to improve the current predictive limitations of protein modeling software by functionally characterizing single amino acid mutants in a robust model system.
- Design-to-Data was utilized in our research for the design and visualization of our in-silico mutant design with Foldit Standalone software.

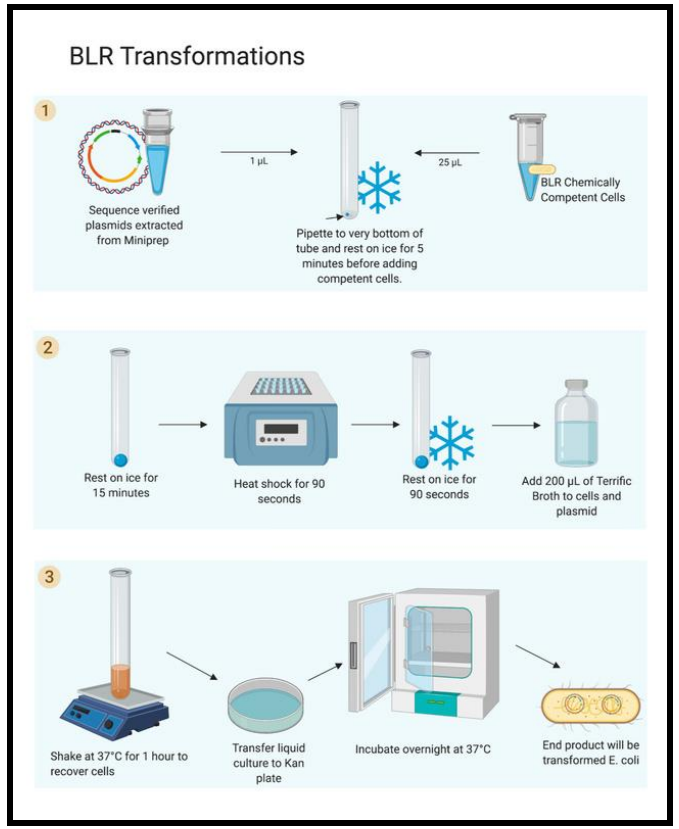


Source: https://d2d.ucdavis.edu/d2d-lab-manual

Materials and Methods

- Chemically Competent BLR Transformation
- Protein Production: Growth and Expression Overnight Cultures
- Protein Purification
- SDS-PAGE

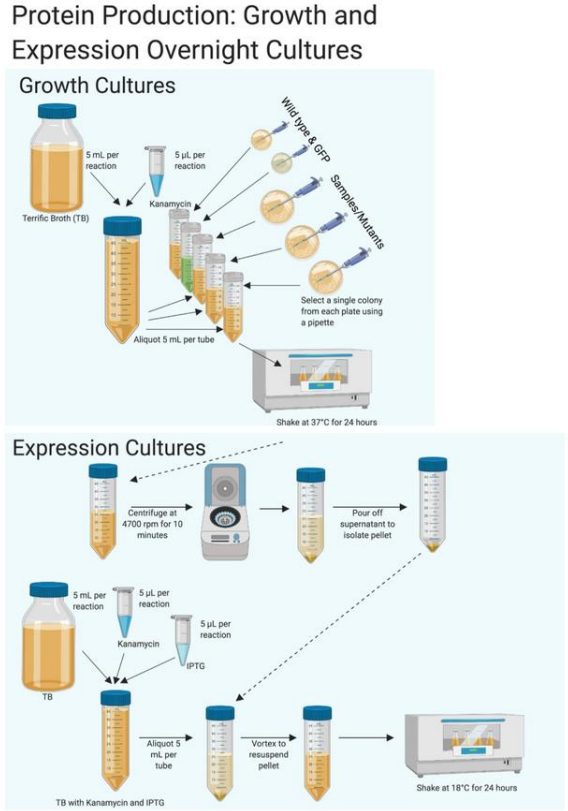
Chemically Competent BLR Transformation



Source: https://d2d.ucdavis.edu/d2d-lab-manual

- Transformation allows chemically competent cells to uptake the DNA (plasmid) from the environment, which will later incorporate it into its chromosome.
- Positive cultures would result in multiple E. coli colonies with the anticipated mutated plasmid.
- LB Agar Kanamycin plates were used to promote the sole growth of our Kanamycin resistant BLR-competent cells while ensuring other bacteria will not grow or contaminate our culture.

Protein Production: Growth and Expression Overnight Cultures



Source: https://d2d.ucdavis.edu/d2d-lab-manual

- For the induction of protein expression of our BgIB mutant enzymes, we needed to grow and multiply our transformed BLR cells.
- This step consisted of a three-day liquid inoculation process to allow sufficient time for our transformed BLR cells to multiply/expand.
- On Day 3, we induced protein expression by adding IPTG, an inducer that has been applied in the lac-operon mechanism.

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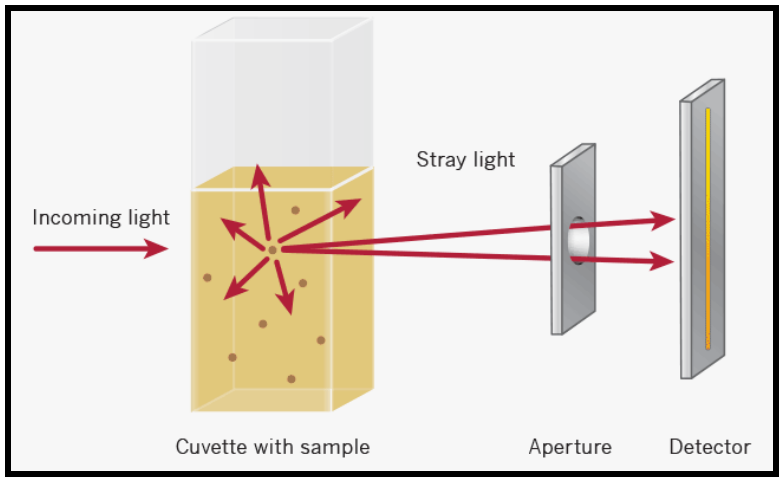
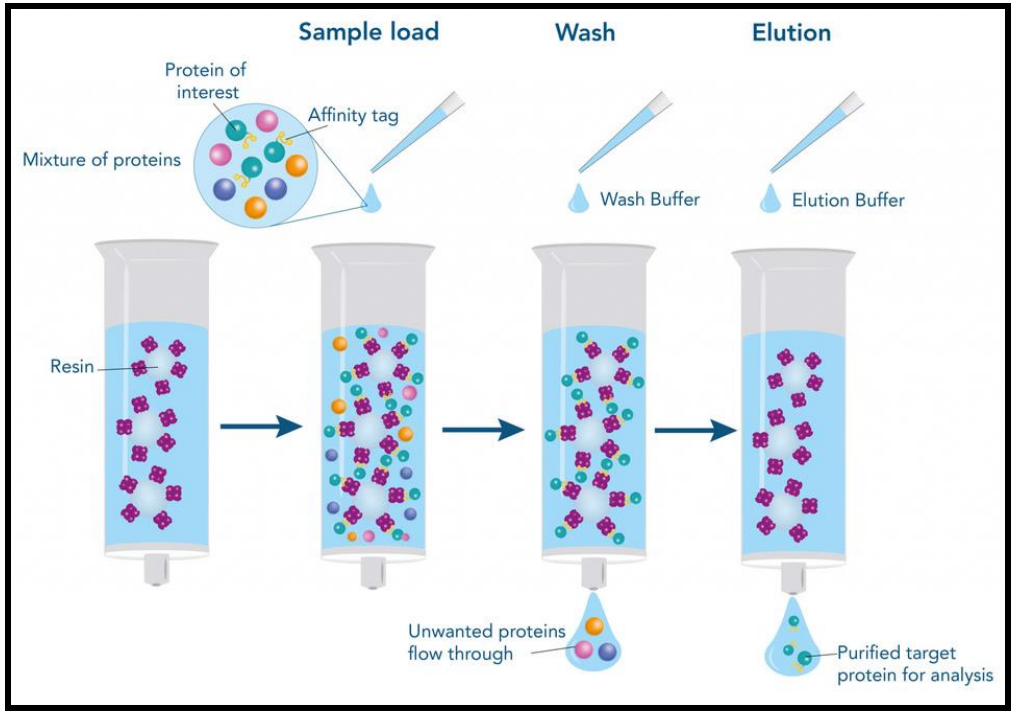
Optical Density (OD600) Significance



Source: https://www.implen.de/od600-diluphotometer/od600/

- Optical density, or absorbance, is a resourceful tool for measuring light transmission through a given sample.
- Based on the linear relationship between light absorbance and particle concentration.
- Utilizing a UV-Vis spectrophotometer, we were able to assess the concentration of the proteins in our cell cultures at 600 nm.
- Optical density (OD600) values helped us determine whether our cell cultures were multiplying appropriately.

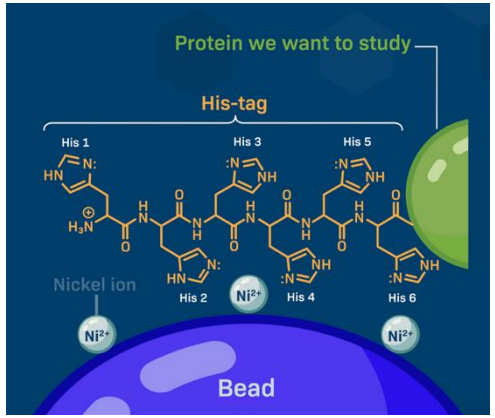
Protein Purification

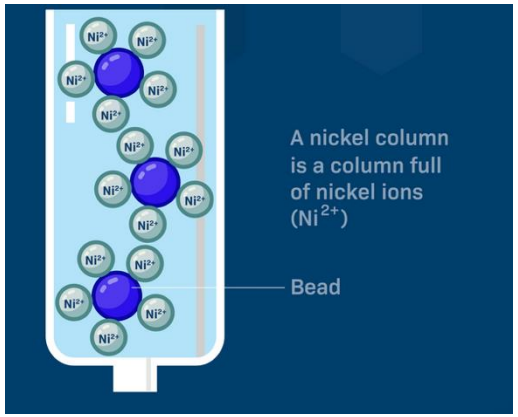


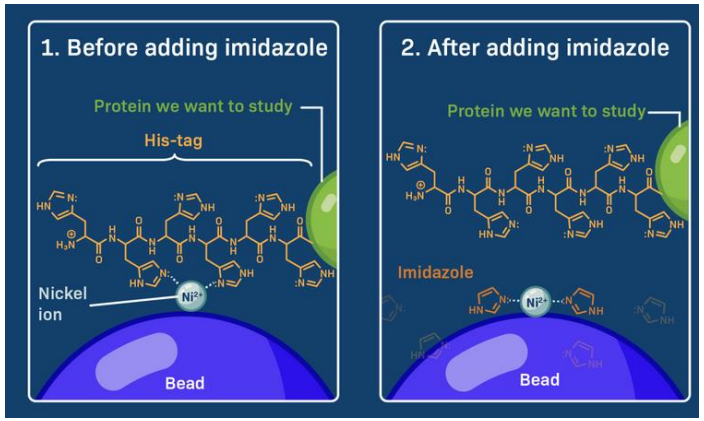
Source: https://www.neuromics.com/protein-affinity-chromatography

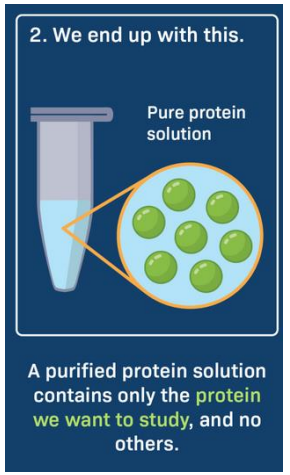
- The goal of this experiment was to isolate the BgIB enzyme (produced in the culturing steps) from the other cell debris.
- For protein purification, we applied a technique called immobilized metal affinity chromatography to separate our protein of interest from other proteins.
- Prior to the protein purification, we lysed the cells to release the proteins from the cells into the supernatant which we then utilized to purify our proteins using microcolumns.

Histidine-Nickel Binding Affinity



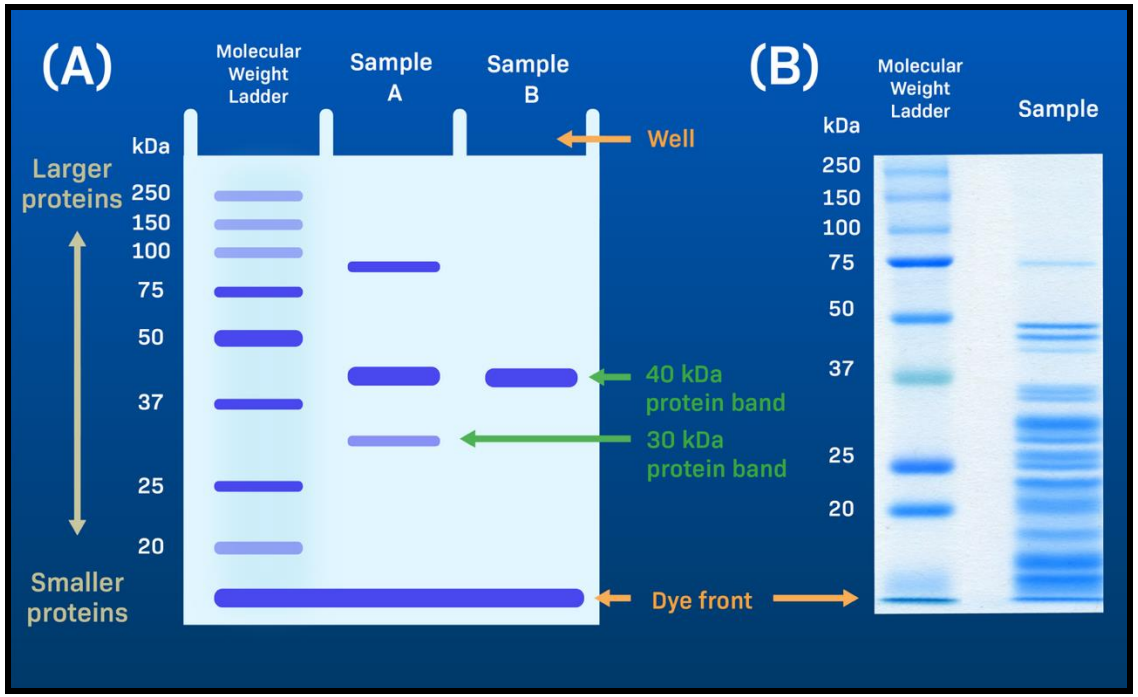






Source: https://labxchange.org.

SDS PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)



Source: https://labxchange.org; Lee N, unpublished data, personal communication.

- The goal of this experiment was to determine protein yield and protein purity.
- SDS, known as sodium dodecyl sulfate, is a detergent used in this experiment to give all proteins a uniformly negative charge to eliminate the effect of charge.
- Since charge does not play a factor, the rate of movement will be dependent solely on the molecular weight/size of the protein where smaller proteins will move the fastest.

Results

Figure 1

A

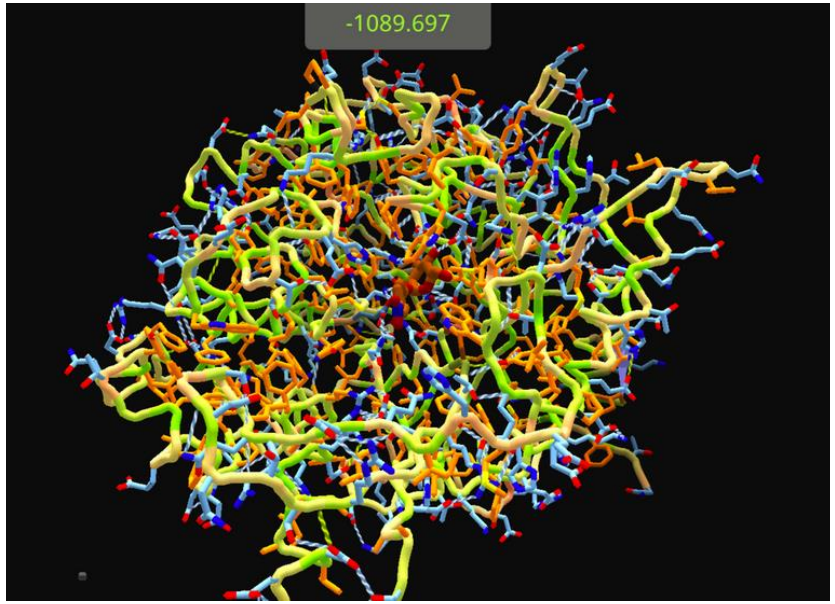


Figure 1A. BgIB wild-type enzyme structure. The energy/starting score as shown in the figure above is -1089.697.

B

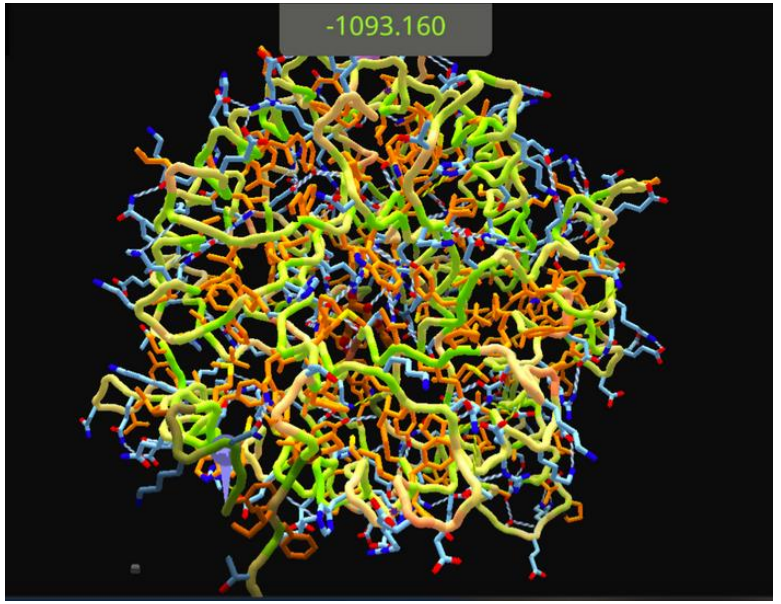


Figure 1B. BgIB mutant-type enzyme structure. The energy score as shown in the figure is -1093.160 following the mutation of isoleucine to serine at the 91st position.

Figure 2

A

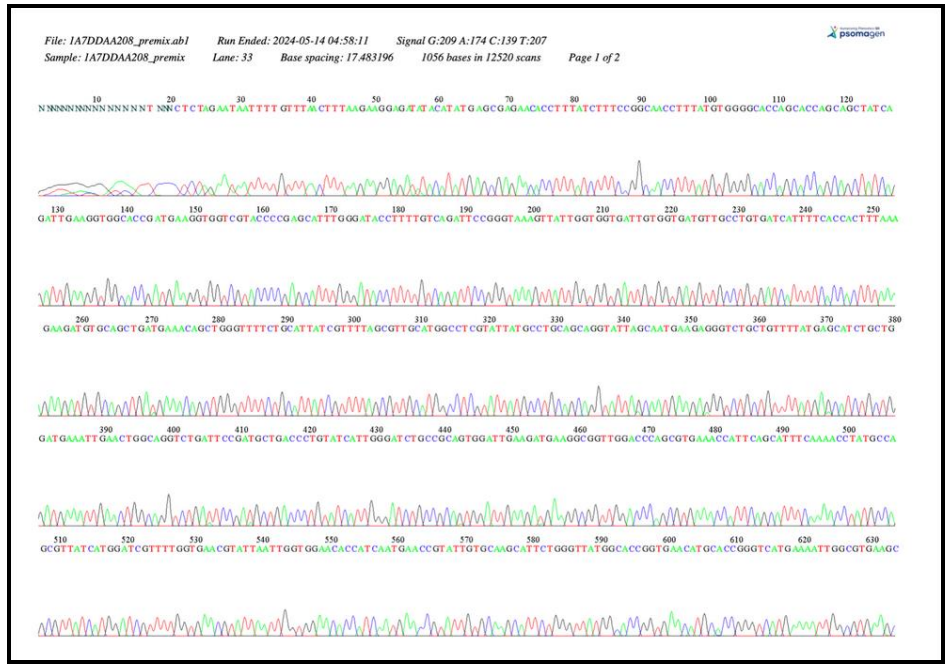


Figure 2A. Picture depicting the DNA sequence analysis of our mutated plasmid.

B

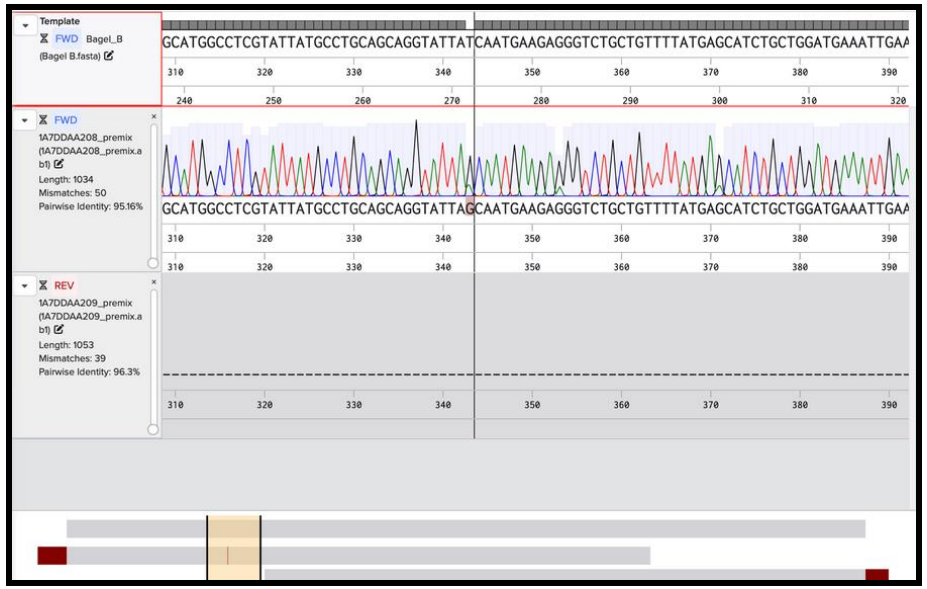


Figure 2B. Picture depicting the alignment orientation of our DNA sequence with the mutant amino acid residue from isoleucine (I) to serine (S) at the 91st position shown at the margin of the nucleotide sequence.

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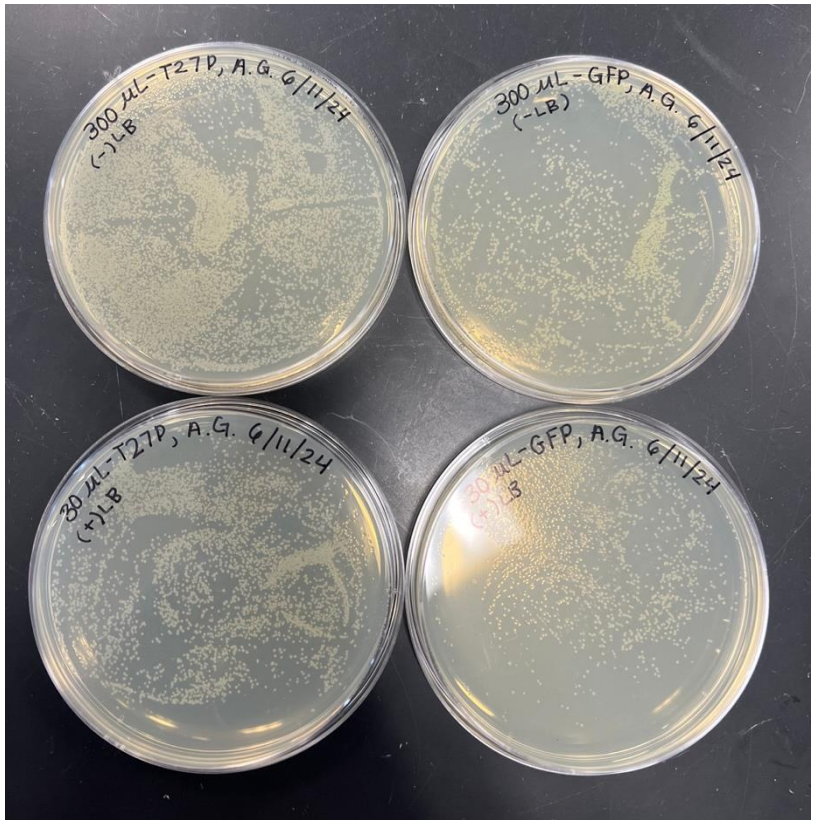
Figure 3



Picture depicting the results of the chemically competent BLR transformation. Positive results would illustrate multiple E. coli colonies with the anticipated mutant plasmid.

Figure 4

GFP

A

Elution Fraction	Protein Absorbance Value (mg/mL)
1	0.120
2	0.242
3	0.247
4	0.374
5	0.215

Figure 4A.Table depicting the elution fraction values obtained during protein purification and their respective protein absorbance values obtained from a UV-Vis spectrophotometer.

B

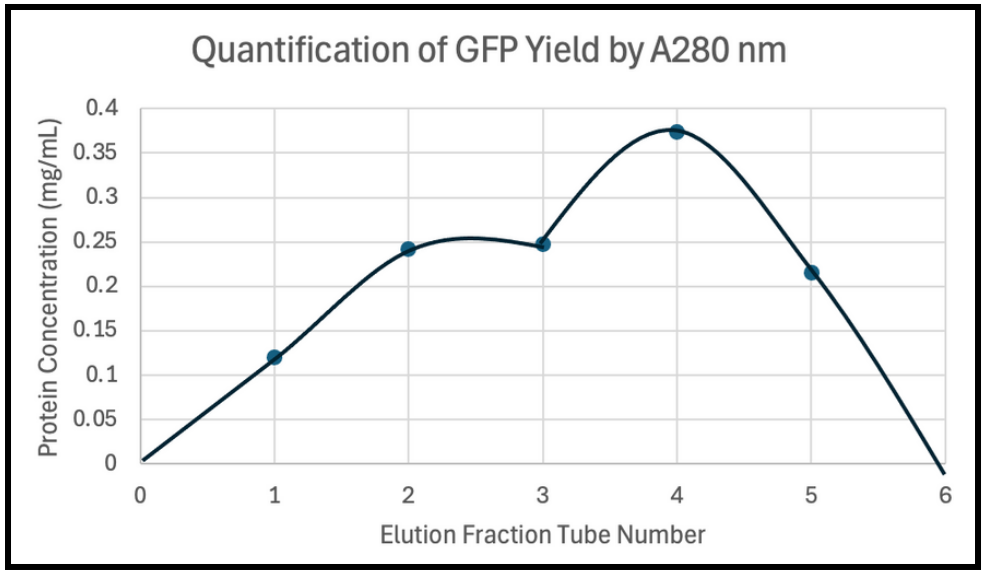


Figure 4B.Scatterplot graph depicting the elution fractions of the GFP and their respective protein absorbance values. The higher the number of the protein absorbance value, the more concentrated the protein sample, thus indicating whether or not the protein was present in the sample/elution fraction.

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T267D

C

Elution Fraction	Protein Absorbance Value (mg/mL)
1	0.148
2	1.877
3	3.365
4	1.178
5	0.130

Figure 4C.Table depicting the elution fraction values obtained during protein purification and their respective protein absorbance values obtained from a UV-Vis spectrophotometer.

D

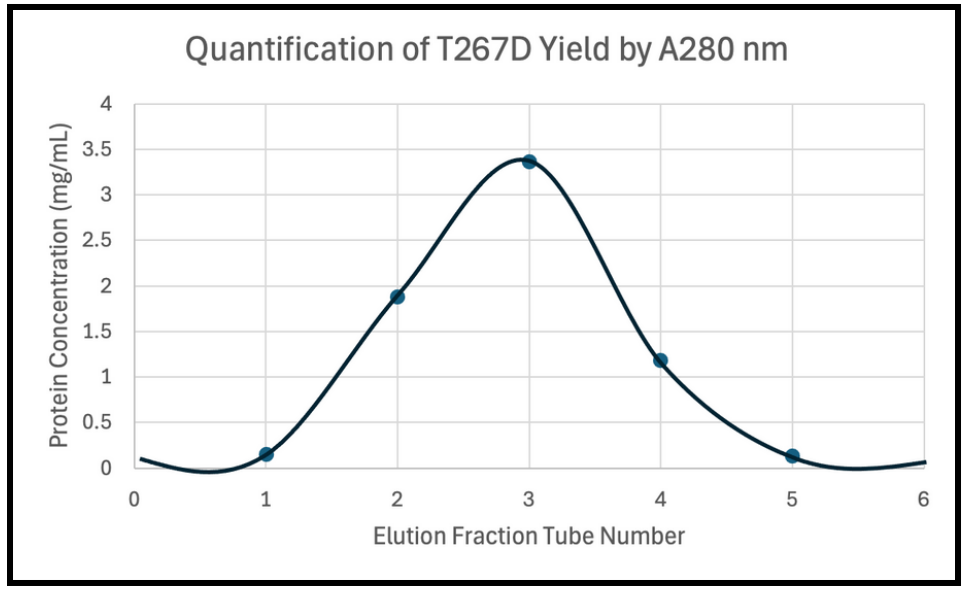

Figure 4D.Scatterplot graph depicting the elution fractions of the T267D mutant type and their respective protein absorbance values. The higher the number of the protein absorbance value, the more concentrated the protein sample, thus indicating whether or not the protein was present in the sample/elution fraction.

I91S

E

Elution Fraction	Protein Absorbance Value (mg/mL)
1	0.066
2	0.121
3	0.108
4	1.438
5	0.160

Figure 4E.Table depicting the elution fraction values obtained during protein purification and their respective protein absorbance values obtained from a UV-Vis spectrophotometer.

F

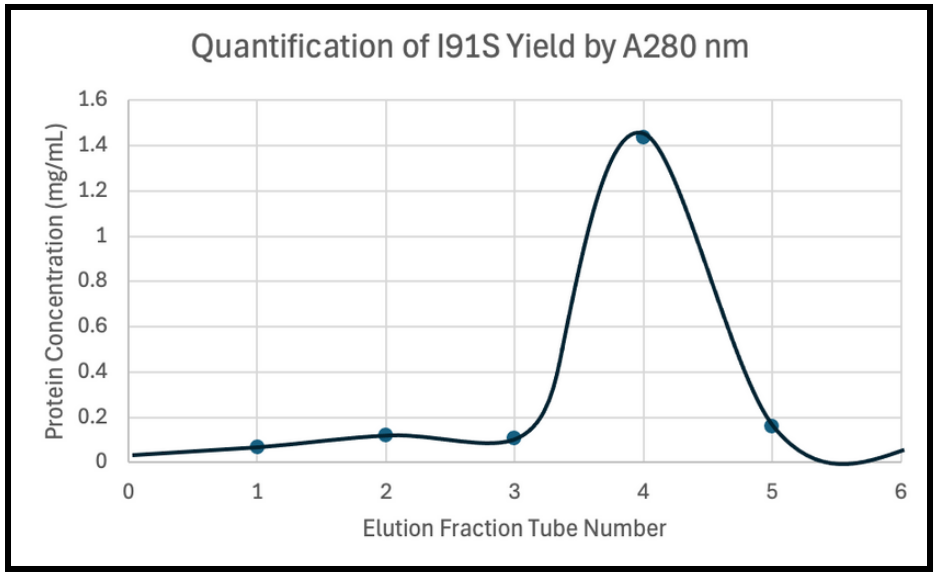

Figure 4F.Scatterplot graph depicting the elution fractions of the I91S mutant type and their respective protein absorbance values. The higher the number of the protein absorbance value, the more concentrated the protein sample, thus indicating whether or not the protein was present in the sample/elution fraction.

Figure 5

I91S

A

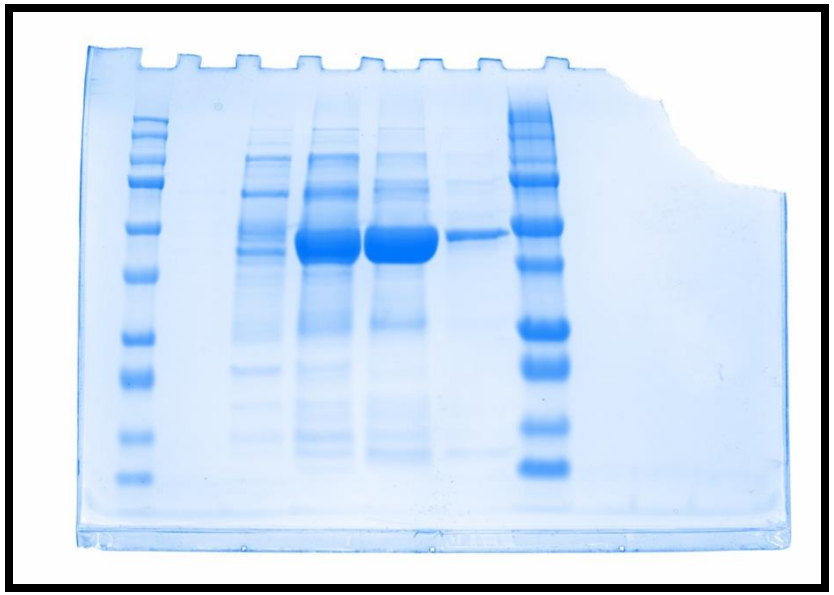

Figure 5A. Picture depicting the results of our SDS PAGE experiment with the I91S mutant and the confirmation of the purification of our target protein (BgIB) illustrated as the thicker bands shown in the 3rd and 4th columns.

T267D

B

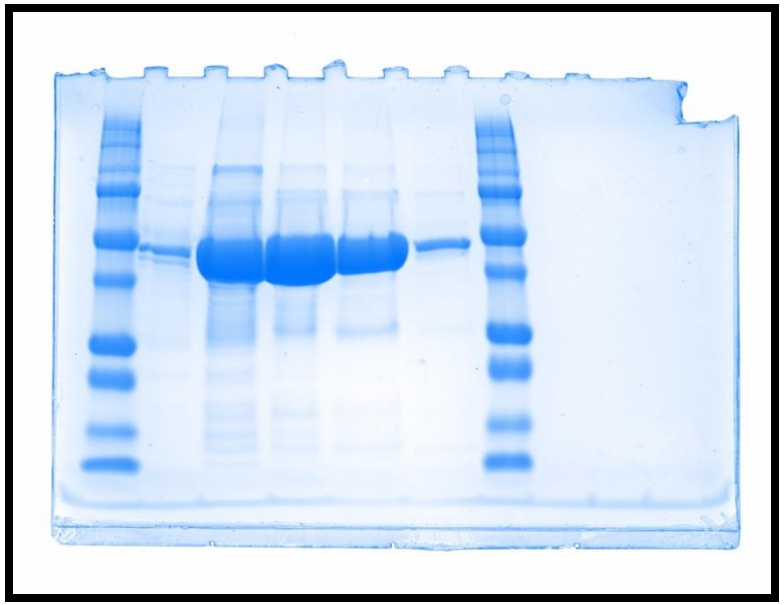

Figure 5B. Picture depicting the results of our SDS PAGE experiment with the T267D mutant and the confirmation of the purification of our target protein (BgIB) illustrated as the thicker bands shown in the 2nd, 3rd, and 4th columns.

Discussion/Conclusion

- Further assays, such as kinetic and thermal stability heat assays, must be used to determine the catalytic efficiency and thermal stability of our mutant enzymes.
- With acceptance of our null hypothesis, these mutant enzymes can be implemented to efficiently produce ethanol while lowering the production costs.
- With substantial and cost-effective production of ethanol, ethanol can be used in various beneficial applications such as antiseptics, fuel, medicine, or beverages.

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Edith Perez

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Mercy University Faculty & Staff

“Navigating the Landscape of DNA Mutations Through the D2D project”

Audrey Kawala Charles

Department of Health and Natural Sciences
Mercy University School of Health and Natural Sciences

C-STEP Summer Research Program
Mentor: Dr. Chun Zhou

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The D2D Project

- Originally designed by UC Davis faculty networks whose main objective is to speed up the process of answering protein design challenges that would often take decades to resolve in isolated labs by facilitating academic collaboration.

- This computational biology software enables us to forecast the impact of a single-point mutation on the stability of proteins.

- The experiment, in the previous semester involved phosphorylation, PCR, and ligation reactions using T4 DNA Ligase and FastDigest DpnI enzyme

Introduction

- The experiment aims to create a new mutation in the BglB protein and observe its impact on stability, substrate binding, and catalysis.
- The learning objectives include selecting amino acid substitutions, developing hypotheses, and demonstrating comprehension of the universal genetic code.
- Benchling DNA analysis software is used to visualize the BglB DNA and protein sequence, and codon optimization and benchling technologies are used to create a functional mutagenic oligonucleotide.
- Beta-glucosidase is a rate-limiting enzyme essential for the final step of hydrolysis of lignocellulose, converting cellobiose and short cellodextrins into glucose.
- It is found in bacteria, fungi, plants, and animals and has industrial applications in cosmetics, textiles, detergents, and food industries.

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Procedures and Techniques

Designing β-Glucosidase Mutants: Foldit standalone

- Download the latest Foldit Standalone version, use the 20230408 version with "shift+Q" feature, open the setup zip drive on your Mac, and launch it from within your applications.

Navigating Foldit standalone

- Launch Foldit, select "Import PDB/Fasta" or "Load Session" on a splash page, and select the extracted data files from the Google Folder.

Modeling your mutation in Foldit standalone

- Foldit allows users to model mutations by locating active sites, avoiding protein conformation, and returning to original score using "Control" or "Command + Z".

PCR

- Two tubes of reactions were prepared using the L108S mutation for oligonucleotide phosphorylation. Phosphate was added to primers and mutagenesis was performed. The reactions were incubated at 37°C, 75°C, and stored at -20°C for further experimentation.

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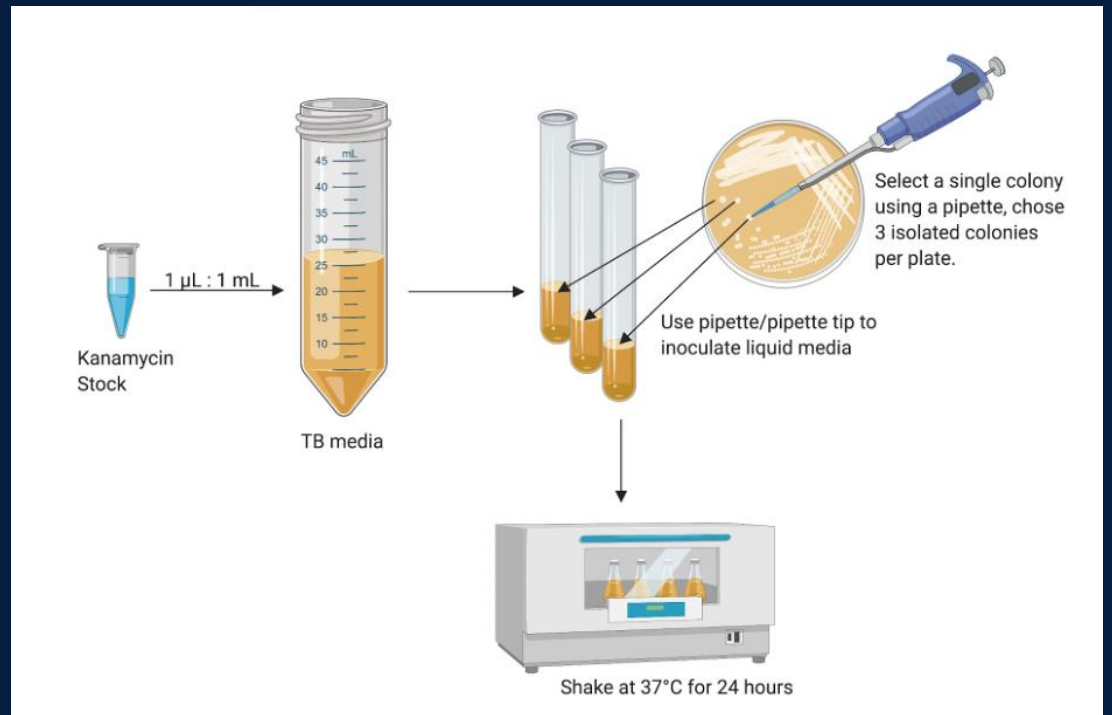
Transformation- Day 1

- To combine ingredients for a cell culture, set up a water bath at 42ºC or 37C. Thaw DH5α chemically competent cells on ice, label sterile tubes, and pipette Kunkel Product to them.
- Incubate the cells and Kunkel Product on ice for 5-15 minutes. Heat shock the cells 30 seconds in a 42°C water bath, then place them on ice for at least two minutes.
- Add room temperature TB medium without antibiotics. Shake at 225 rpm for 1 hour to recover transformed cells. Label LB kanamycin plates for each mutant and label blank/control plates.
- (Cells Plating)Pour 200 µL of culture on individual plates, shake to distribute cell suspension evenly, and incubate at 37°C overnight.

Overnight Cultures - Day 2



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Cell Lysis

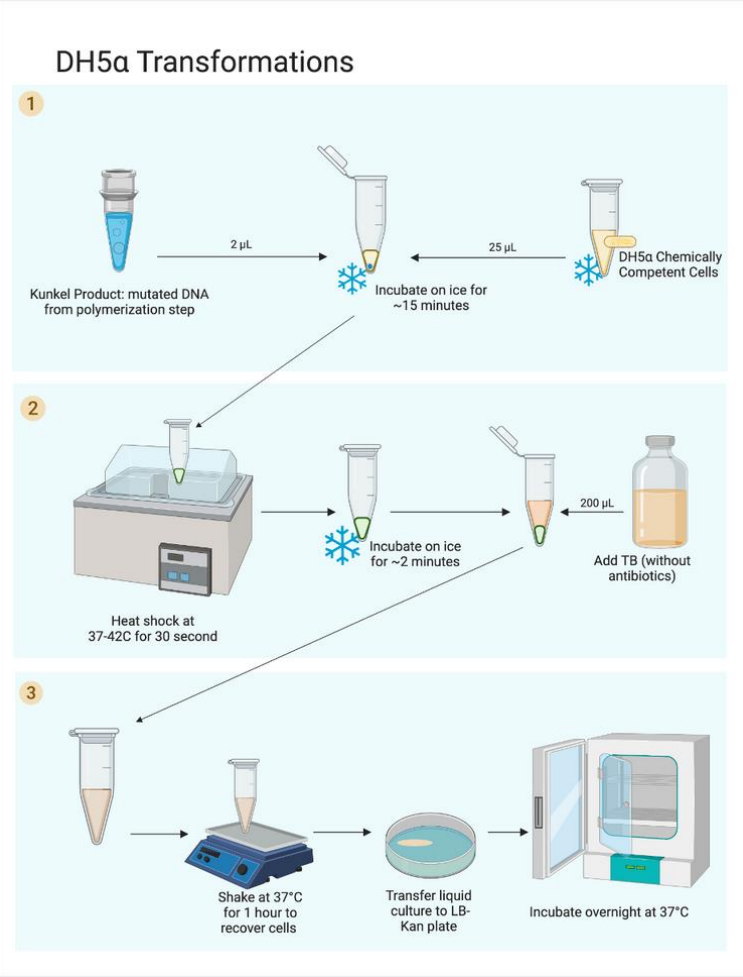
- Transfer overnight cultures to microcentrifuge tubes, pellet 2 mL of bacterial culture, and resuspend in chilled Buffer P1.
- Mix Buffer P2 with resuspended mixture, invert six times, and mix with buffer N3.
- Centrifuge for ten minutes, then pour the lysate onto the spin column membrane and discard the pellet. Discard the solution washed through at the bottom.

Elution

- Transfer the GeneJet spin column into a microcentrifuge tube, add Elution Buffer, incubate, centrifuge, discard the spin column,
- Keep solution, and store eluted DNA in an ice bath
- And store purified plasmid DNA at -20ºC.

Sequencing

- To find a mutation, multiply the amino acid number by 3 to find the nucleotide sequence number, then use the sequence axis to identify the mutation's spot.



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Protein Purification

- This step involves purifying mutant enzymes using immobilized metal affinity chromatography to isolate the BglB enzyme from cell debris.
- The enzyme has a poly-histidine tail and interacts with nickel beads.
- The protein is eluted with an EDTA solution, outcompeted by the beads. The assays should be run within 48 hours after purification.

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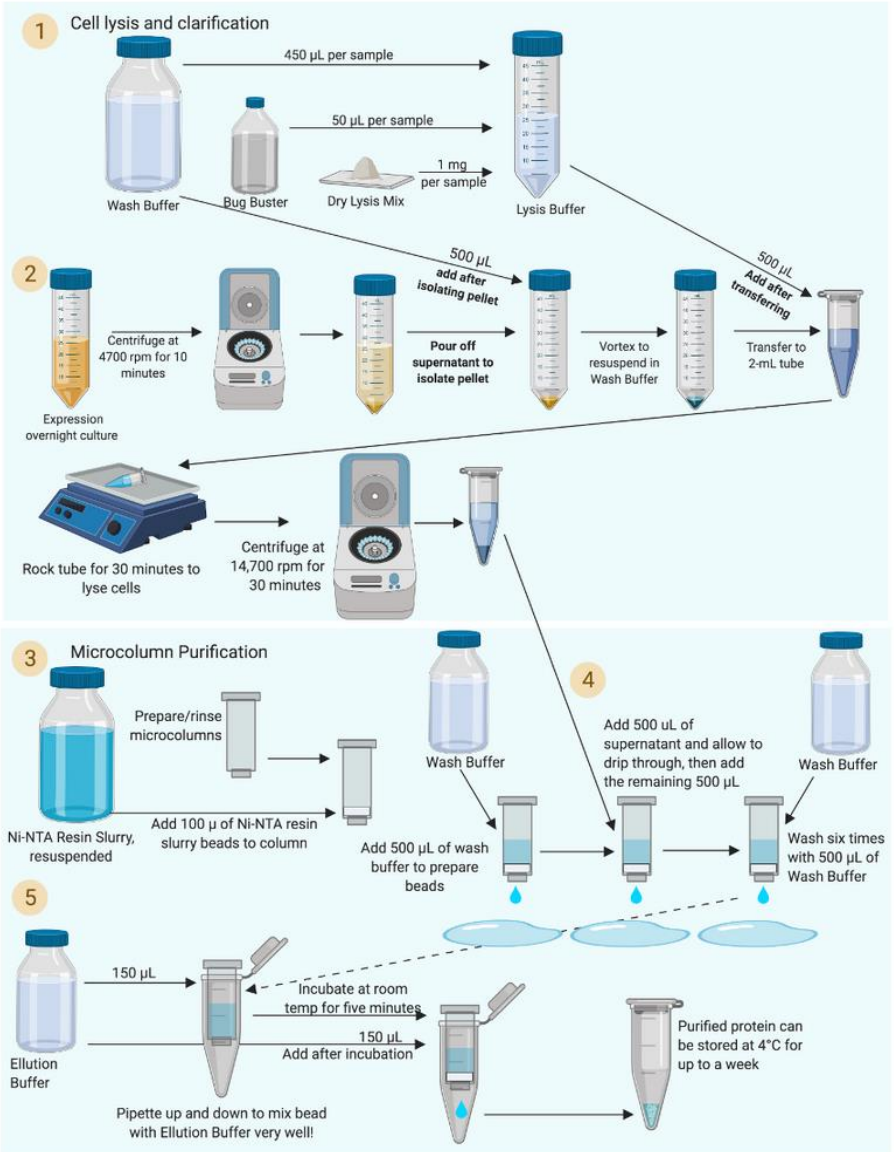
Protein Purification



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Gel Electrophoresis

- To prepare samples for SDS-PAGE, mix 4x concentrate loading dye and 12 µL of protein in labeled PCR tubes. Heat to 95°C for ten minutes.
- Prepare the protein gel and electrophoresis apparatus, create 1x MES Buffer Solution, and load the protein standard and samples.
- Run the gel for 30 minutes, check for bubbles, and remove the gel.
- Rinse with DI water and pour in Coomassie Blue gel stain. Two techniques for staining and destaining the gel are the overnight method and the quick microwave method.

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Results

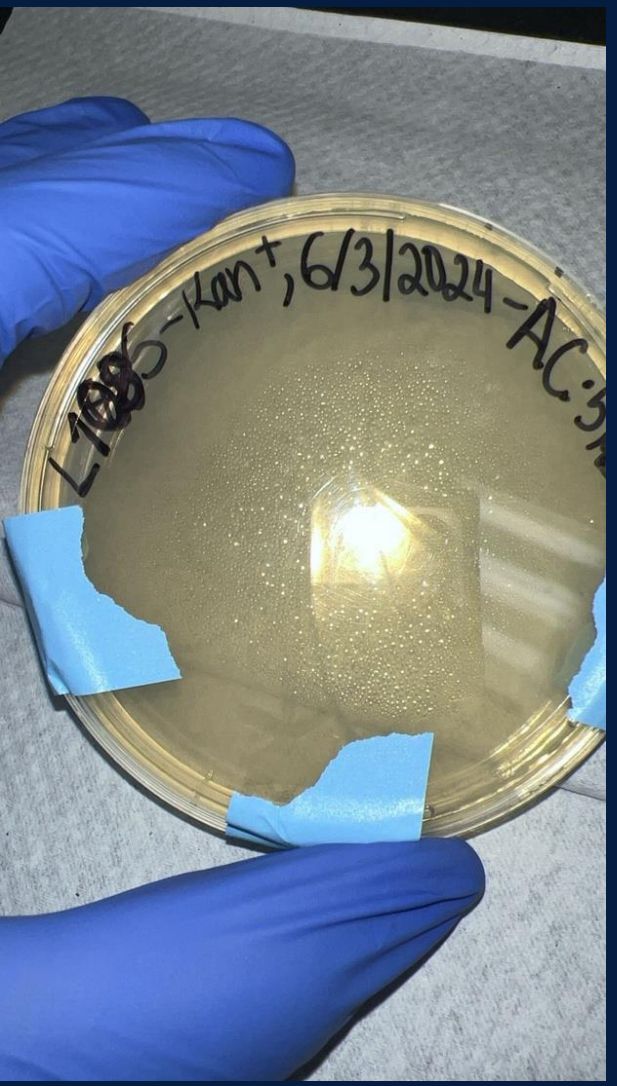

Figure 1 A-Single Colonies from plating 5 uL of transformed bacteria

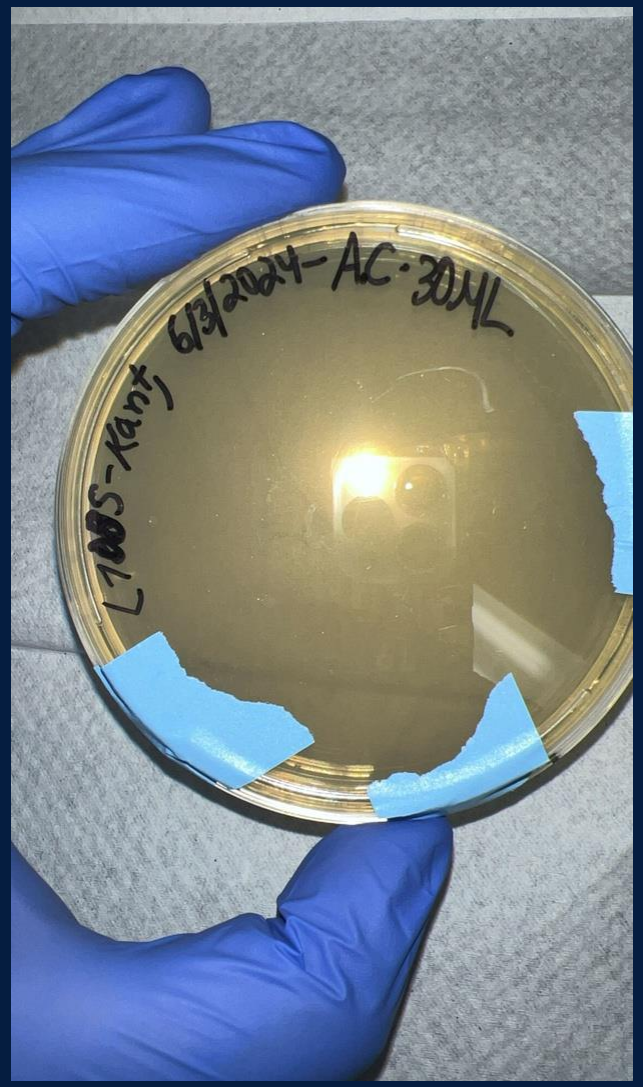

Figure 1 B-Single colonies from plating 30 uL of transformed bacteria

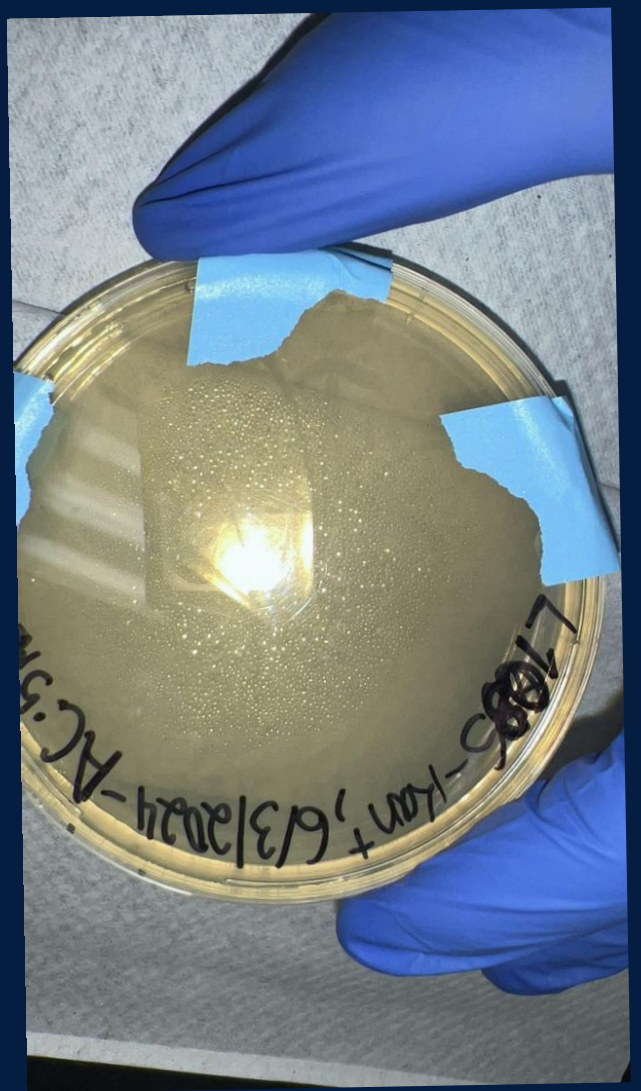

Figure 1 C-Single Colonies from plating 5 uL of transformed bacteria

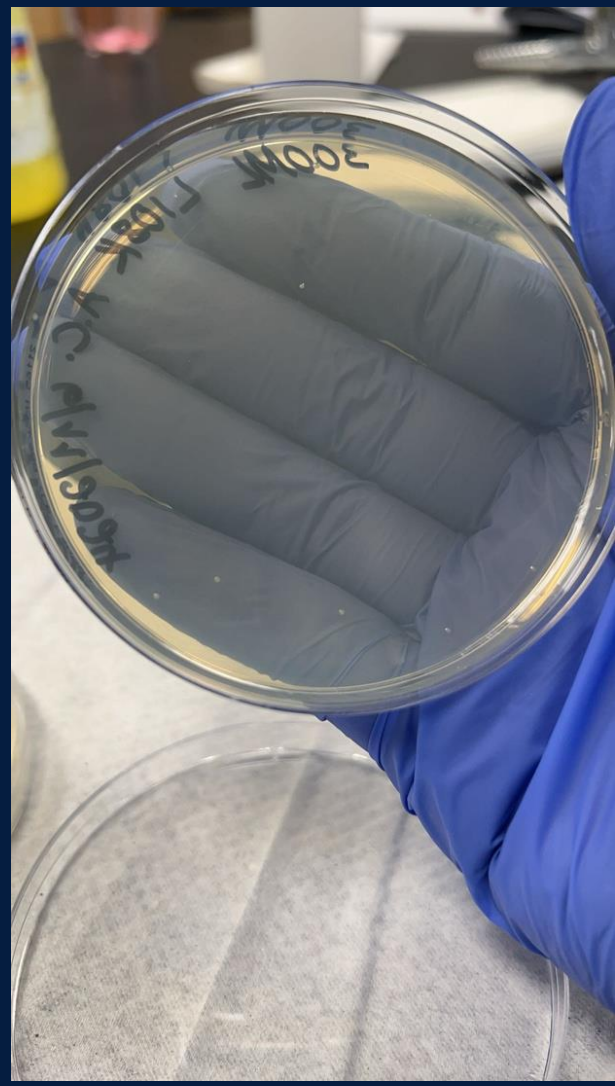

Figure 1 D-Single colonies from plating i 300 uL of transformed bacteria

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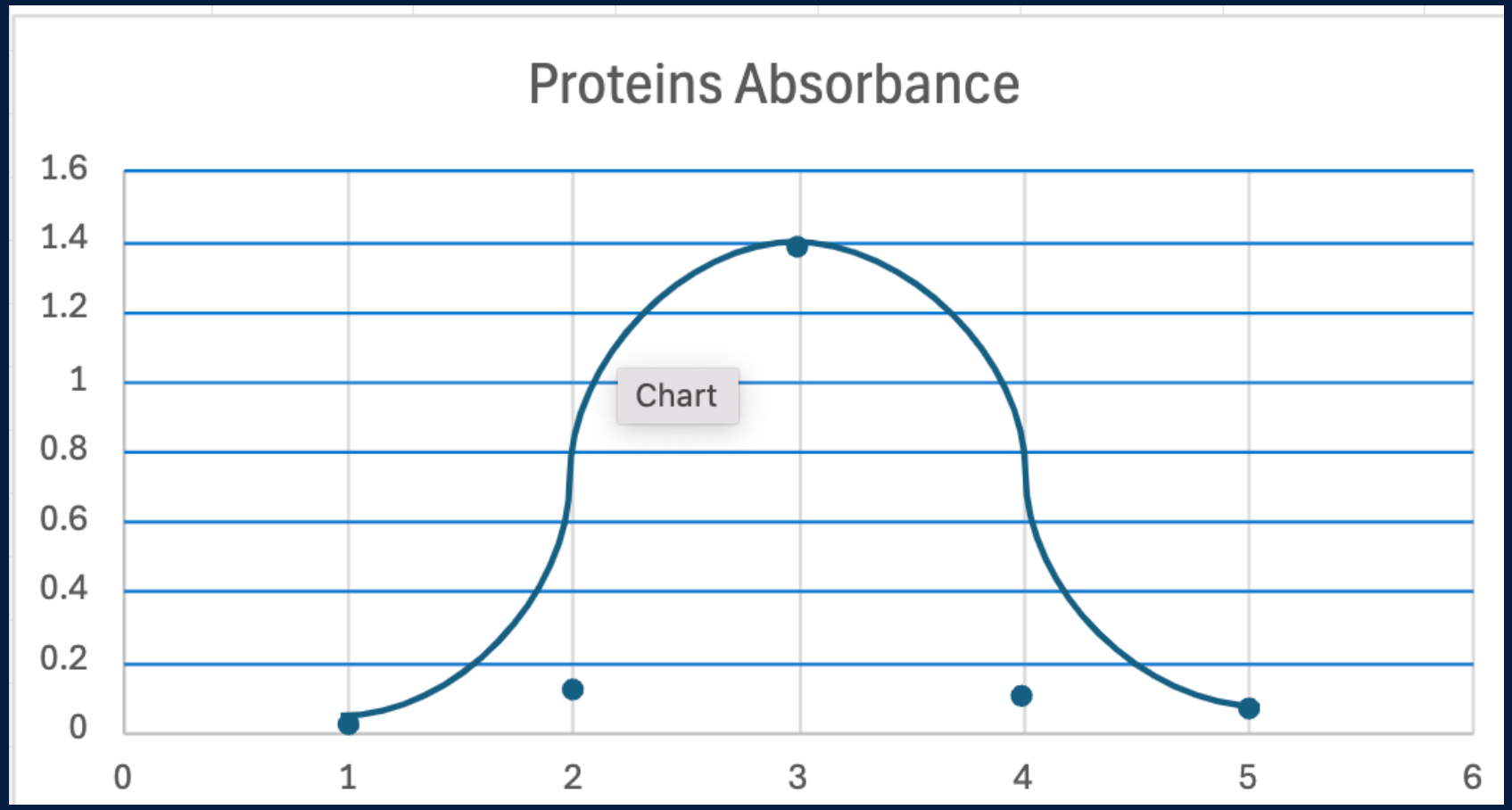

Figure 3 - OD concentration results (see curve)

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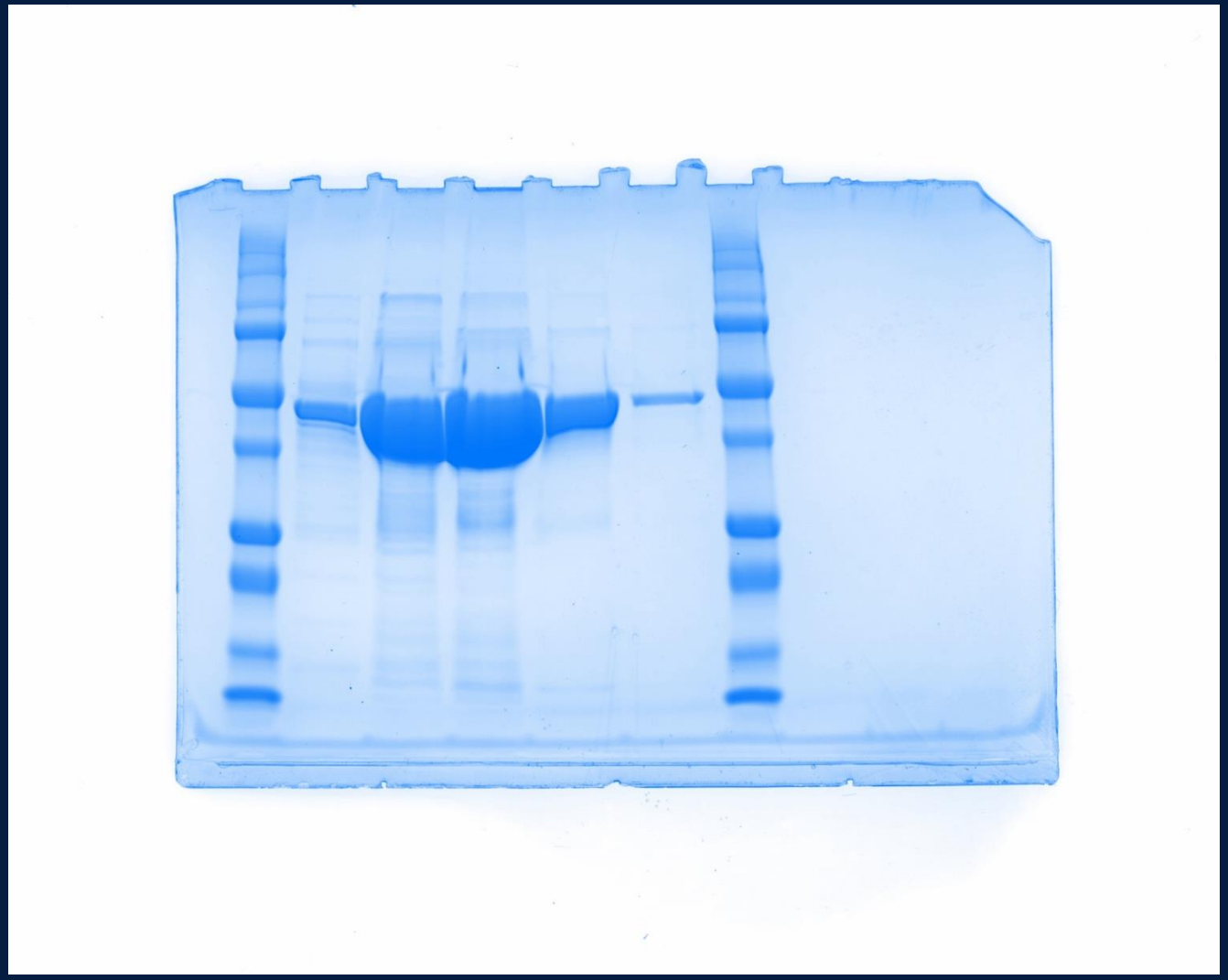

Figure 2 A-Gel electropheris showing single band from mutation L108S

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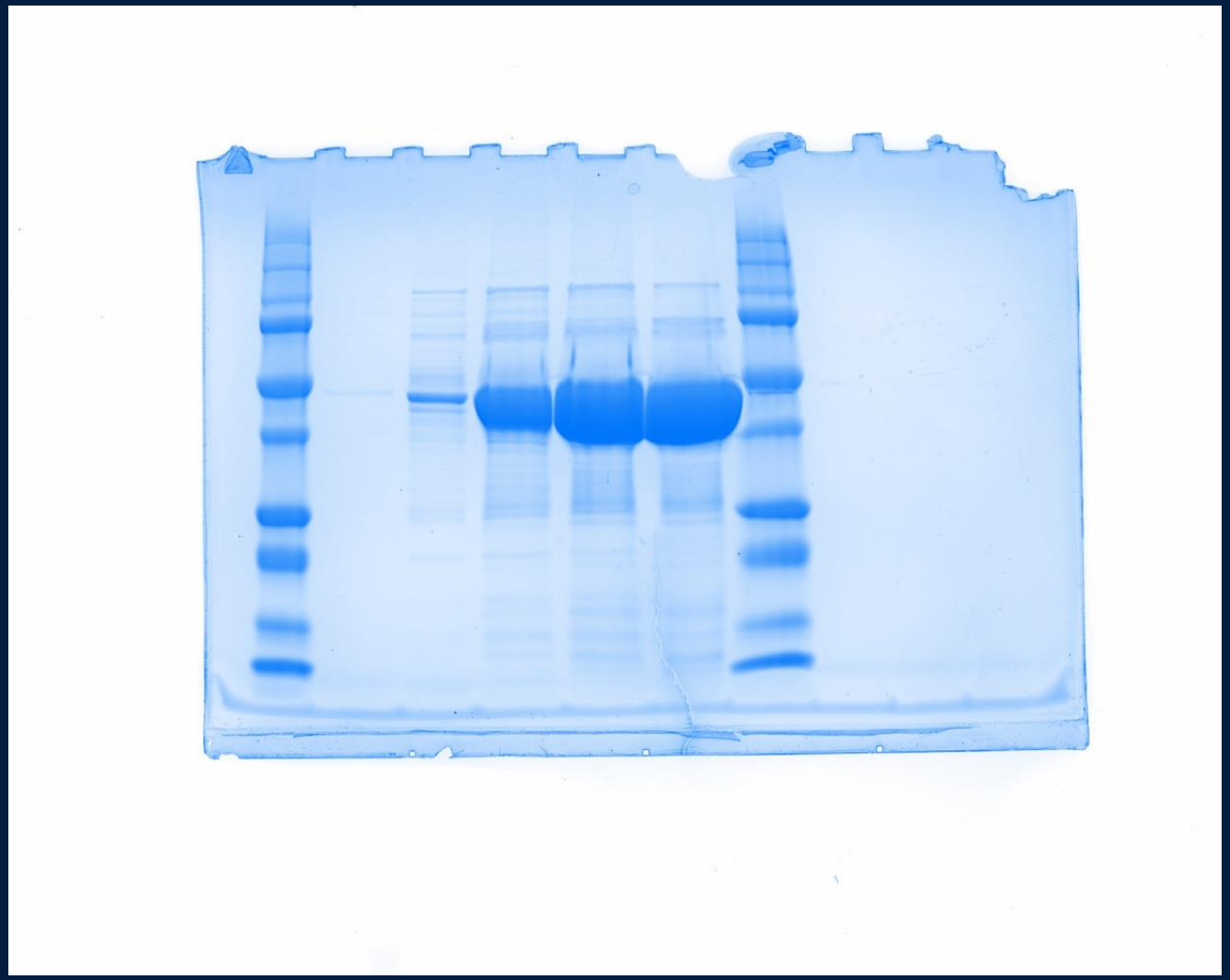

Figure 2 B-Gel electropheris showing single band from mutation L108K

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Discussion Topics

- Conclusion on research project and techniques
- Colonies on Day 2
- OD concentration
- Curve on graph based on absordance
- Experimental error and future research
- Future development of our research project
    - In addition to modifying BgIB function and stability, we are in the process of understanding medically important proteins' structure and functions in order to create more effective therapeutics.

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THANK YOU FOR JOINING US TODAY!