Letter of Transmittal

Dear Dr. Kastner and Kevin Wu,

Our team is excited to present our capstone design report on optimizing L-asparaginase production, a critical enzyme used in the food industry to mitigate the formation of acrylamide. Through genetic engineering techniques, we introduced four different plasmids containing genetic material encoding for L-asparaginase. From this study, we identified one specific gene that can synthesize L-asparaginase in sufficient quantities for investigation into purification and quantification purposes.

To optimize the production process, we conducted a literature review and identified a submerged, fed-batch reactor as the optimal method for L-asparaginase manufacturing. Using Python programming, mass and energy balances were conducted to identify key kinetic and cell growth parameters. An economic analysis was performed to evaluate production costs, and an environmental analysis assessed potential environmental impacts.

Our research has significant implications for the food and pharmaceutical industries by enhancing the production of a vital food additive drug. As L-asparaginase gains more widespread adoption within the food industry, our research can continue to make significant contributions to promoting sustainable and responsible practices.

Thank you for the opportunity to undertake this capstone project. We hope that this report meets your expectations and provides valuable insights into optimizing L-asparaginase production. Please feel free to contact us if you have any questions or concerns.

Sincerely,

Alma

Alma: Biological Product Production Optimization

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May 2nd, 2023

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Key:

W: Primary writer (wrote a significant portion of the section).
P: Proofreader (primarily fixed grammatical errors and added small changes).

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Executive Summary

This capstone project aimed to optimize the production of L-asparaginase, a vital enzyme used in the food industry to mitigate the formation of acrylamide, a carcinogenic compound found in baked and fried food products. To achieve this goal, genetic engineering techniques were used to enhance L-asparaginase production in Escherichia coli. Four different plasmids encoding L-asparaginase were introduced, derived from an investigation into homologous genes to ans B, which encodes for L-asparaginase in *E. coli*. While only one of the four genes successfully synthesized L-asparaginase in quantities that were insufficient for purification and quantification purposes, the promising results encourage further exploration of additional genes to improve production levels.

A literature review was conducted to optimize the production process and identified a fed-batch reactor as the optimal candidate for L-asparaginase manufacturing. Using Python programming, mass and energy balances were established to identify key kinetic and cell growth parameters, facilitating the development of an optimized production process with increased yields. An economic analysis was performed to evaluate production costs, and an environmental analysis assessed potential environmental impacts of the optimized production process, ensuring its sustainability and identifying areas for improvement.

The results of this investigation found that using 0.1 g/L *E. coli* cells that are recombinant with the *Campylobacter corcagiensis* gene and about 39.4 g/L of glucose would help us reach a competitive production rate of enzyme at 6,960 kg/day to allow us to produce a protein product for $40.

Future steps include protein engineering techniques to optimize the structure of L-asparaginase for enhanced stability, activity, and specificity, ultimately resulting in a more effective and economically viable product. This research has significant implications for the food and pharmaceutical industries by enhancing the production of a vital food additive drug, ultimately reducing associated costs and risks and promoting a more responsible and sustainable industry. As L-asparaginase gains more widespread adoption within the food industry, consumers can benefit from increased confidence in the safety of their food due to effective acrylamide mitigation.

Background

L-asparaginase Applications

L-Asparagine is a non-essential amino acid that is present in all living cells. The main function of asparaginase is that it catalyzes the reaction of L-asparagine (ASN) into aspartic acid (ASP, also known as L-aspartate) and ammonia (NH_3) (Figure 1). Breaking down L-asparagine into these two chemicals can mitigate the presence of carcinogens in cells and other industrial manufacturing processes.

Figure 1. Reaction mechanism of L-asparaginase (Nguyen et. al., 2016)

Production of L-asparaginase as a food additive has been widely successful. Maillard reactions are commonly used in the cooking of food to brown the ingredients and provide flavor. This reaction occurs between asparagine and reducing sugars (Figure 2), but this change produces acrylamide, a known carcinogen, as a byproduct.

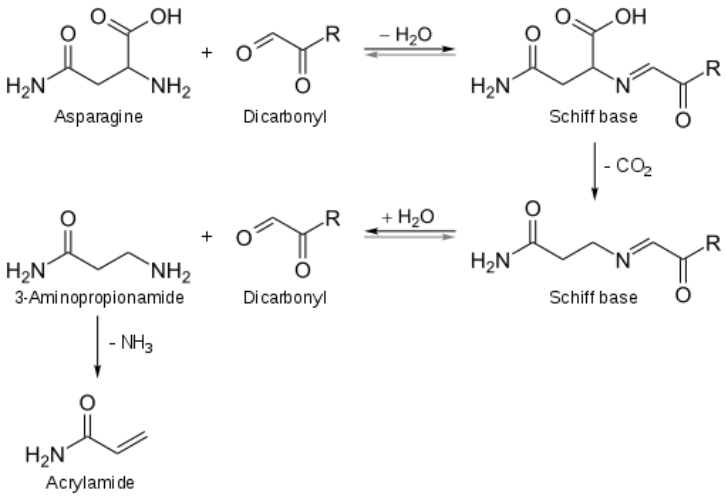


Figure 2. Maillard Reaction Mechanism ("Maillard Reaction," 2022)

As mitigation, asparaginase can be employed to deplete the amounts of asparagine present and thereby reduce the amount of acrylamide produced, which is the application that Alma strives to improve. Issues have arisen in the production of asparaginase as a food additive since the most common form in this application is sourced from fungi. While fungi are known for their high protein production capabilities, fungi are not as easily used in industry because they are significantly more difficult to genetically manipulate. Unlike bacteria, fungi are multicellular, and, because of this, they have more intricate genetic encoding. This makes them difficult to engineer since it is less likely that a gene will turn on since a plasmid with a promoter cannot just be inserted into the system. The only widely used way to manipulate fungi is through manipulation of their fermentation systems; a process susceptible to problems and variations when produced on a larger scale. Thus, asparaginase production using this methodology can be faulty and expensive.

L-asparaginase is used as a chemotherapy agent as well, providing a potential future market for Alma to get involved in. Normal healthy cells are able to synthesize their own asparagine, whereas carcinogenic cells cannot produce asparagine de novo and depend on asparagine circulating within a system to survive (Cleveland Clinic). The most predominant types of asparaginase for cancer use are derived from *Escherichia coli* and *Erwinia chrysanthemi* since they proved to have the most effective anti-cancer ability and are easily producible on a large scale. Asparaginases from *E. coli* and *Erwinia chrysanthemi* differ in their pharmacokinetic and immunogenic profiles, making the *Erwinia*-derived form a sufficient alternative to those who react negatively to the *E. coli*-derived form.

Alma's objective within this project is to genetically and metabolically manipulate *E. coli* cells to produce L-asparaginase for use in the food industry by experimenting with four selected genes. These genes were selected to be similar to the gene *ansB*, which is derived from *E. coli* and codes for L-asparaginase. It is hypothesized that at least one of these genes will produce a higher yield and a more active enzyme when compared to the current competitors.

What is a plasmid?

The injection of the four genes into *E. coli* requires the modification and insertion of a bacterial *plasmid*. Put simply, a *plasmid* is a circular piece of double-stranded DNA that codes for protein production. In nature, plasmids are used to give their host a useful characteristic to help them survive in their environment. A common example is antibiotic resistance (Brown, 2020). However, for industrial applications, they are used to artificially input genes into bacteria, forcing them to produce a desired protein, which in our case is L-asparaginase.

Compared to other genetic input methods, plasmids are inherently easy to use. For one, they contain *multiple cloning sites (MCS)*. In essence, these are short segments of DNA that contain several restriction sites- allowing for the easy insertion of the desired genetic code. Plasmids also contain an *origin of replication (ORI)*. This genetic code is a DNA sequence that initiates the replication of the plasmid. Essentially, it allows the plasmid to self-duplicate and without the ORI, there won't be enough plasmids for further generations of bacteria (Monroe, 2020).

The functionality of plasmids is tightly controlled by a *promoter region* that "turns on" the transcription of genes. This transcription is the process of generating the protein product from the genetic code. Without the promoter region, the cells would be unable to use the plasmid at

all. This region can be designed to only “turn on” in certain circumstances- such as an absence of glucose. By using this functionality, plasmid performance can be precisely designed.

While the main objective of plasmids is to input a desired gene into bacteria, a gene for antibiotic resistance is commonly inserted in addition. This incorporation is important because the cells will be grown on specific lab-based antibiotic plates, killing any cells that did not take in the plasmid. This process is done to verify that only the plasmid-containing cells grow. If this gene was not added, the industrial process would be contaminated with unwanted cells. These unwanted cells would make the process less efficient as a lower percent of the cells would be producing the desired protein. It is important to note that this resistance is to lab-based antibiotics not often used in medicine, such as carbenicillin.

Last, a key characteristic of plasmids that encourages their use in industry are *primer-binding sites*. These are coded regions where primers, short DNA sequences, can easily bind and identify a specific portion of the plasmid. Having these primer sites is advantageous because primers are easily synthesized in labs and can be used in PCR amplification, a process that copies and clones a desired DNA fragment to produce high quantities of fragment copies. This means a small amount of DNA can be purchased or excised from a genome and amplified a thousandfold, so more desired DNA is readily available for a lower cost. Thus, Alma strives to produce a meaningful plasmid containing the selected genes in addition to the corresponding primer sequences. This will ultimately produce recombinant *E. coli* that can increase the production of L-asparaginase, and they can be fermented later for industrial application.

L-asparaginase Metabolics

To further understand how to engineer cells to produce more L-asparaginase, the metabolic pathway involving the enzyme should be considered. The *metabolic pathway* is essentially a path of chemical reactions that converts one molecule into another. These molecular conversions are done for a wide variety of reasons, such as energy storage, or cellular regulation. Creating one simple molecule, such as glucose, requires many different reactions all involving many different proteins. To grasp the most important reactions and molecules involved, the metabolic pathway is determined.

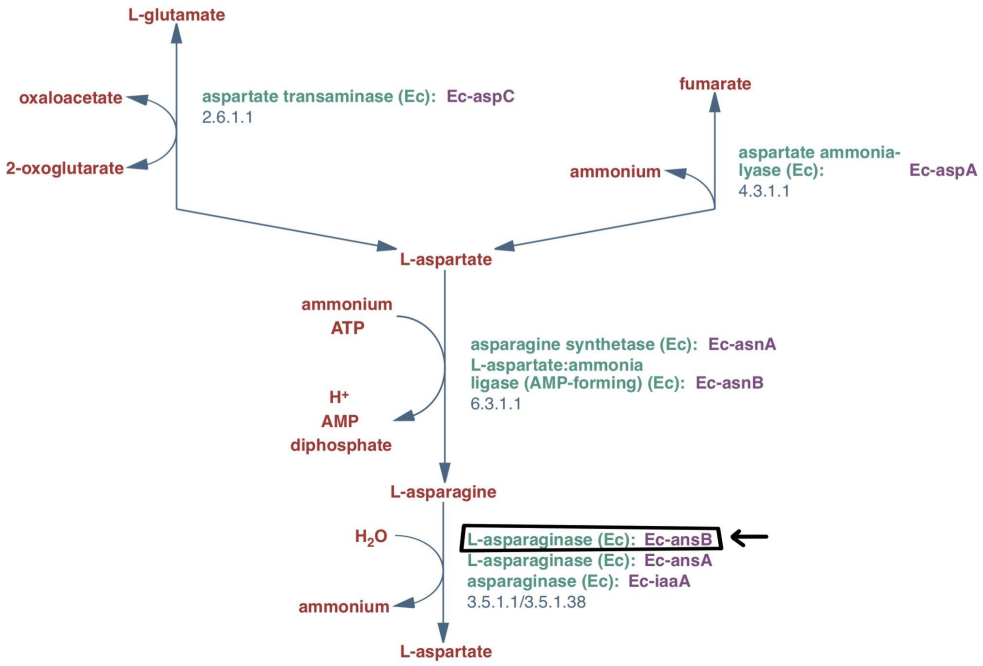


Figure 3**.** Superpathway of aspartate and asparagine biosynthesis; interconversion of aspartate and asparagine. L-asparaginase is boxed and pointed to by an arrow. (Caspi, R. et al, 2018)

L-asparaginase is primarily involved in the *superpathway of aspartate and asparagine biosynthesis* (Figure 3). Note how the reaction described in Figure 1 is involved. Shown in this pathway are the many molecules involved to produce the L-asparagine that is converted into

L-aspartate through L-asparaginase. By changing the concentrations of these molecules in the cell, perhaps more L-asparagine could be produced, requiring the cell to make more L-asparaginase to compensate. Another possibility would be to decrease the amount of ammonium produced, as too much ammonium can negatively affect cell growth. Both of these ways could be inflicted through further genetic engineering or through changes in the fermentation environment of the cells.

Design Objective

Alma's objective is to examine new approaches and characteristics for the manufacturing process of L-asparaginase to be applied in the food processing industry. This will be done by using plasmids to insert four different genes into samples of recombinant *E. coli* cells. Each *E. coli* sample will receive a different gene to be experimented with in the lab. This experimentation will produce data on each gene regarding how much of a desired enzyme is produced and at what activities.

All of the genes selected for experimentation are similar to a gene currently used to produce L-asparaginase in *E. coli,* however, they have not been used for the production of asparaginase previously. To provide a potential novel aspect to the project, one of the selected genes codes for an uncharacterized protein, meaning its function has not yet been determined experimentally. By examining this protein, data on its activity can be obtained. If this protein is more effective, it is possible for Alma to publish these findings and share the results within the scientific community.

Following the selection of these variables, fermentation conditions will be experimented with to ensure the process is optimized to the fullest potential. Alma expects to assess these enzymes, document the process of genetically and metabolically engineering them, and compare them to the existing literature to determine if the resulting process is marketable.

Alma hopes to market its process as a more efficient means of producing asparaginase. A quicker and more efficient production process can allow for a cheaper food additive product. This can entice more food companies to use asparaginase in their products, further limiting the number of carcinogens exposed to the general public.

Stakeholders and Values

In order to understand the best methods to produce this enzyme, the team has reached out to a multitude of stakeholders who could benefit from this novel process (Figure 1A). The following stakeholders provided needs that could optimize the production of this vital enzyme and create a more efficient process for these industries.

Novex Innovations LLC, a full-service contract development and manufacturing organization, is an external stakeholder for Alma. Alma conducted a phone interview with Doug Drabble, the COO of Novex, and inquired about the difficulties with upscaling a process, as well as the most expensive parts of drug manufacturing (Table 3A). Drabble mentioned that companies like Novex strive to decrease the cost of labor, improve the design of these processes, and maintain the integrity of biological organisms over time.

Takeda Pharmaceuticals, a research and development-driven pharmaceutical company, is another external stakeholder for Alma. Alma conducted a phone interview with Justin Rey, the former Global Operational Excellence Lead at Takeda (Table 4A). Mr. Rey provided valuable insight into the most important variables in process engineering for biological products in particular. Mr. Rey emphasized the importance of abiding by regulatory standards, and that every manipulated variable in a manufacturing process requires approval from regulatory heads.

Dr. Kastner, a biochemical engineering professor at the University of Georgia, is an internal stakeholder and mentor for Alma. The team met with Dr. Kastner via Zoom biweekly, where he provided valuable insight on how to approach the project. He suggested developing a

process flow diagram for the production of L-asparaginase, along with an organized timeline of the tasks for the project.

Dr. Yan, a biochemical engineering professor at the University of Georgia, is an internal stakeholder for Alma. The team held in-person meetings with Dr. Yan to inquire about the use of his lab equipment for the in-lab process. He granted the team permission to use his equipment given that the lab protocol is fully developed.

Our last significant internal stakeholder is Kevin Wu, a design engineer with the UGA startup program. Alma meets with Kevin weekly, and in these meetings, he has given financial advice along with strategies on how to approach external stakeholders. He wants Alma to fully understand the value and opportunities of producing L-asparaginase, as well as make clear what the FDA regulations are for this project.

Specifically, Alma strives to synthesize a novel microorganism that produces L-asparaginase. We believe there is potential for at least one of our four genes to produce asparaginase in a more efficient and cost-effective way compared to current existing processes. The asparaginase market has been rapidly growing in recent years, in both the food and pharmaceutical sectors. In 2021, the global asparaginase market was valued at $650 million and is projected to rise to $1.57 billion by 2028. Asparaginase is a highly sought-out enzyme due to its chemotherapy and degradation properties, particularly towards the carcinogen, acrylamide.

With the current health craze in modern media, many individuals are increasingly concerned with what is going into their bodies. The mitigation of carcinogens through an

asparaginase food additive can give food companies a leg-up over others who do not invest as much in cancer prevention. Additionally, since many baked snacks such as crackers and cookies have a higher concentration of acrylamide, parents can worry less about the impact of their children's snacks on their future health.

Engineering Specifications

The primary engineering specifications for the project were determined by cross-examining stakeholder needs with the decision matrix to provide insight into which design concept would prove fruitful. Based on these factors, it was decided that a Michaelis-Menten constant, K_M, of less than 15 μM was the target for the enzyme along with activity over 280 IU/mg. The Michaelis-Menten constant indicates how much substrate is needed to catalyze the reaction, so a lower value means less substrate is required to completely catalyze the reaction, indicating a more effective enzyme. A high activity signifies this as well. These targets are based on literature data found for L-asparaginase derived from wild-type *E. coli*, so, if one of the four genes codes for an enzyme that meets these criteria, it will be utilized in further lab experimentation.

Additionally, a goal of fewer than 24 hours for incubation time was desired based on literature data for the fermentation of wild-type *E. coli*. Thus, to optimize the process, Alma seeks to decrease this incubation time to produce more enzymes at a quicker rate compared to that of the existing process. Coinciding with this, Alma is seeking to model a process that produces over 58,000 kg of enzyme per day based on literature and competitor data. Producing a higher quantity would signify a successfully optimized process, which is Alma's primary goal.

Another target of interest is the dissolved oxygen levels within the fermenter. Dissolved oxygen is significant to the process because it dictates the maximum amount of oxygen that the bacterial cells can uptake. Decreasing these levels from the usual 60-80% could asphyxiate the cells and cause them to produce less product, or change their metabolic pathways to those seen under anaerobic conditions, which is undesirable for Alma's objective. Increasing this level,

however, could lead to a possible increase in production, so another engineering specification is a dissolved oxygen level of greater than 80% to push the cells toward maximum production.

Lastly, other specifications to note are the physiological conditions the fermenters will operate under; a pH of 7 and a temperature of 37 ℃ is common for microbial procedures, and this tends to be ideal for enzymes. These parameters, unlike dissolved oxygen or media, cannot be altered as much. At variations of these parameters, cellular features such as key proteins, will denature- a process where a protein's three-dimensional shape deteriorates and loses its integrity. This denaturing renders the cells nonfunctional, so these specifications are likely to be the optimal conditions and should remain unchanged.

Benchmarking

L-asparaginase has been on the market for years, and there are multiple established competitors. Novozymes, a leading biotechnology company, has created a line of Acrylaway products, which have asparaginase in them for the removal of acrylamide; they have a series of both liquid and granular products with some variations. These products are commended for their use in infant crackers and cookies, and they cite up to a 95% decrease in acrylamide levels in baked goods. The source of this asparaginase is derived from *Aspergillus oryzae*, a common type of fungus.

DSM, a nutritional company, has developed PreventAse. PreventAse was created to reduce acrylamide levels in baking, and this product's launch coincided with the launch of Novozymes' Acrylaway. PreventAse also boasts a 95% decrease in acrylamide levels generated when cooking foods. DSM's asparaginase source varies from Novozymes and is derived from *Aspergillus niger*.

Lastly, Kerry is a food and nutrition group that developed a product called Acryleast for the removal of acrylamide using asparaginase in yeast. This product however, can only be used in foods that exclusively use yeast and are baked. Unlike the other two products, Acryleast cannot be used in fried foods. Acryleast only provides an 80% cut in acrylamide levels compared to the other products.

All three competitors use fungi to produce their enzymes which is common in the industry due to their high protein production capabilities. However, given the multicellular nature

of *Aspergillus* and the eukaryotic nature of yeast, they are significantly more difficult to genetically manipulate than other unicellular organisms, such as bacteria. Despite this, all three alternative processes use the native genes of the fungi to produce their enzyme. Additionally, the competitors use solid-state fermentation, a process that uses solid materials such as agricultural waste, as feed into the fermenter. Along with this, the fungi are grown on solid scaffolds to improve the transfer of oxygen and nutrients into the cell due to the increase in exposed surface area.

While our process provides an alternative to the pre-existing methodologies of producing L-asparaginase, there are possible limitations to the process. Bacteria can be difficult to maintain in industrial conditions since they have the ability to become recombinant. This means they can mutate, which can result in decreased efficiency. Thus, this can harm the rate at which Alma's enzyme is produced, which is why a portion of the process is dedicated to optimization. To mitigate this, Alma proposes the use of fed-batch fermentors to ensure a decrease in genetic drift compared to that of a continuous process.

Additionally, Alma proposes the use of a 60,000 L, fed-batch, submerged fermentation system, a process involving the agitation of microbes submerged in a nutrient-dense medium, as a means for producing the enzyme. This route is a more suitable industrial process for microbes because of its high moisture content and its use of defined media compared to solid-state materials.

Design Concepts

The design concepts for this process were proposed on the basis of a few select criteria; first, the genetically modified *E. coli* will use the gene that produces the highest quantity of the most active enzyme. This can be determined from lab scale experiments using four different genes which were selected based on their similarity to the native ansB gene in wild-type *E. coli,* and these values ranged from 43.7% to 73.2% similar. The gene codes come from different types of bacteria- specifically from *Salmonella arizonae*, *Campylobacter corcagiensis*, *Actinobacillus succinogenes*, and *Aeromonas hydrophila subsp. hydrophila*- and they encode for L-asparaginase. These codes were then modified and edited to reduce any sources of error that may occur during cloning, specifically hairpin formations- a type of RNA fold that prevents the transcription of DNA- and internal cut sites- regions on the gene that can be physically cut by restriction enzymes. This modification was necessary to ensure the genes could successfully be used in the lab experiments.

The next criterion for design is derived from stakeholder needs, and it is the use of the most active enzyme with the highest yield. This is based on the results and data from the lab experiments, which will be performed next semester, and whichever gene fits this description will be used in the theoretical scale-up model with its corresponding data.

Additionally, both a continuous and batch system were proposed for the fermentation process; continuous fermentation involves the continuous adding and removing of media within the fermentation where steady-state conditions are eventually reached. This quasi-steady state has its advantages: it is simpler to model and design within the system, and, in industry, it allows for the possibility of recycling streams which can be cost-effective. However, continuous

fermentation uses the same microorganisms in the process, so if the process is run for long periods of time, the microbes are more prone to mutate, leading to an increase in genetic variation.

For a batch system, specifically a fed-batch system, the media flows into the reactor, but is not removed at a continuous rate; thus, the working volume of the reactor changes with respect to time, and the product stream is removed intermittently. While this process is more labor-intensive to model and requires strict oversight and operation, it reduces the amount of variation among the microbes within the system.

Last, proposed fermentation designs included solid-state and submerged fermentation. Solid-state utilizes solid substrates as feed into the fermenter; materials such as tea waste, saw dust, soybean waste, or apple pomace may be used. This style of fermentation is especially conducive to fungi because the reactor possesses a lower moisture content than that of a submerged fermenter, reducing the chances of contamination. Additionally, various scaffolding materials may be used to immobilize the cells and allow for successful growth, and the selection of the scaffold provides an opportunity for improved control over the various parameters as the material can have a large impact on key features, such as fixation, oxygen uptake, cell seeding, etc.

The other method proposed was submerged fermentation, a process using liquid media flowing into a fermenter that contains various nutrients and high amounts of water. Thus, this method has a much higher moisture content compared to the solid-state fermentation model. Given the liquid nature of the process, this fermentation uses continuous agitation to ensure

proper stirring and distribution of dissolved oxygen and nutrients, and it prevents the build-up of toxic waste products. No scaffolding or cell fixation is used in the system.

The team developed a quality functions diagram to identify key variables in our process and to determine the most important aspects that should be focused on (Figure 10A). From research and input from our stakeholders, several customer requirements were identified. Improving enzyme production and generating highly active enzymes were of the highest importance, followed by making the overall process cheaper and faster, and optimizing fermentation growth parameters. Various functional requirements were quantified based on their importance and correlation with customer needs. It was found that the selection of our gene and the efficiency of our scale-up simulation were the most integral parts of the project.

Design for Scale-Up

Alma proposes using a 60,000 L fed-batch fermentation system because of its ability to produce the necessary quantity of enzyme to compete with Novozymes and our other competitors. This large volume allows for high yield, but the system is not too large where dead zones, areas where there is limited agitation, will occur. Thus, glucose at high flow rates can enter the system, and agitation is feasible without excessive amounts of heat or energy. The fermenter will be inoculated with recombinant *E. coli* containing our plasmid after being cultured in shake flasks.

Following inoculation, the fermenter will be run for about 24 hours, allowing for the bacteria to grow to an optimal density, and they will synthesize the L-asparaginase. After, the cells will be lysed via homogenization. Then, the protein will be harvested using centrifugation, and this will help to separate the solid and liquid components so any debris can later be separated from the protein. The supernatant will be removed as waste, and the impure solids will move on to the downstream purification stages. The enzyme will be freeze dried into a powder form and stored for commercial use.

Multiple factors had to be considered for this design. First, it was necessary to determine which plasmid should be used in the large scale system. To discover this, bench-scale experiments were conducted with the aforementioned genes and plasmids to determine which could successfully produce the enzyme. This was verified using SDS-PAGE gels and DNA gel electrophoresis. This was considered the prototype for the project, and thus it was tested and evaluated in the lab. Based on the bench-scale results, our gene of interest, from *Campylobacter*, served as the prototype for the scale-up model.

Then, the large scale process was determined based on competitors' information. A yield of 6,960 kg/day of L-asparaginase was calculated to remain competitive, and this value, along with mass and energy balances, determined how much equipment was needed. An economic analysis was performed to calculate the cost of the process using this information that ultimately determined how much the product would need to be sold for, and this value was approximately $40. In addition, based on the large scale mass and energy balances, an environmental analysis was performed to determine the footprint of this industrial production.

The remainder of this report will detail how and why prototypes were tested and evaluated, and it will provide the mass and energy balances in addition to the economic and environmental analyses. Finally, regulatory information based on the final product will be outlined and described based on the findings of the previously mentioned analyses and tests.

Prototype Evaluation

To evaluate the utility of each gene, they were inserted into individual plasmids using basic PCR and gene cloning protocols, and the XL-1 Blue *E. coli* were transformed to take in the DNA. Once the bacteria had the respective plasmids, they were grown on ampicillin plates to ensure minimal contamination occurred. Following this growth, plasmid purification was performed to ensure the proper plasmids were being cloned within the cells. These results can be seen below (Figure 4).

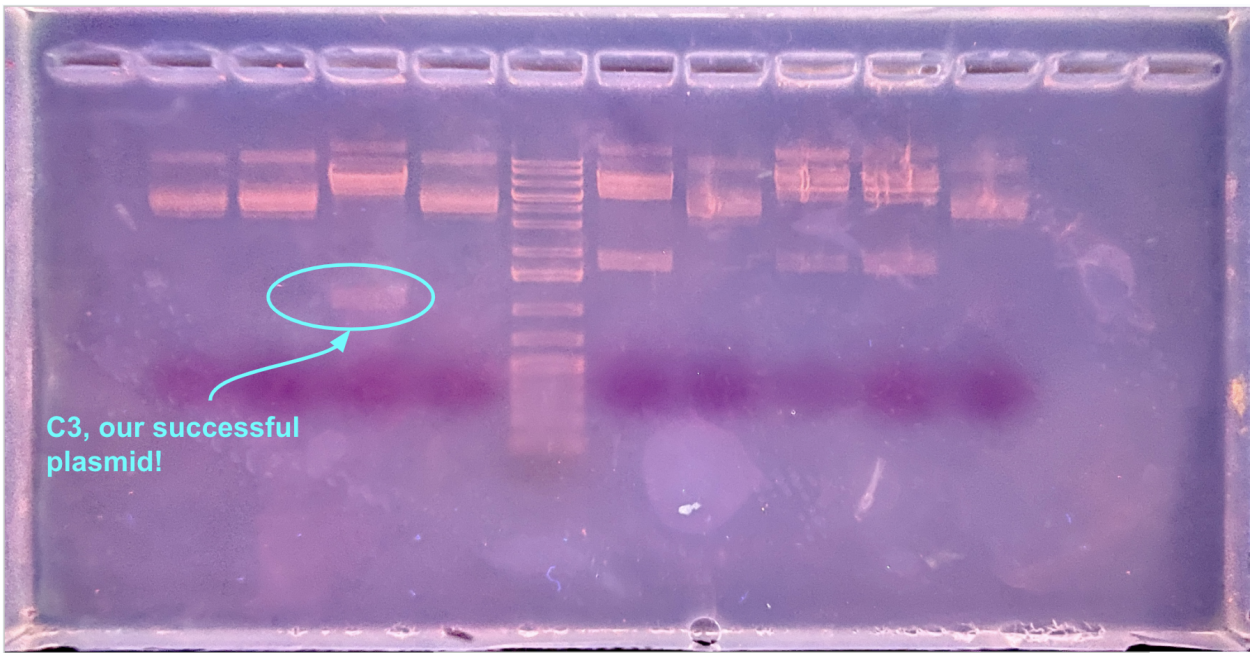


Figure 4**.** Gel electrophoresis verification of plasmid C3.

To determine if the plasmids had been cloned correctly, their sizes were examined by comparing the positions of the plasmid to a standardized DNA ladder. As shown by the blue circle in Figure 4, plasmid C3, indicating the plasmid containing the gene from *Campylobacter*, was determined to be the proper size. This gel revealed that this plasmid could be used to synthesize protein and should be inoculated into BL21 *E. coli*.

The plasmids were transformed into this strain of *E. coli*, and they were grown in culture flasks for approximately 24 hours, but they were spiked with IPTG about 2 hours into this time period to promote protein production. After this time, the protein was purified from the overnight culture by adding a His-tag to the protein. The purified protein was then verified for correctness using an SDS-PAGE gel, which is also based on size. The results can be seen below in Figure 5.

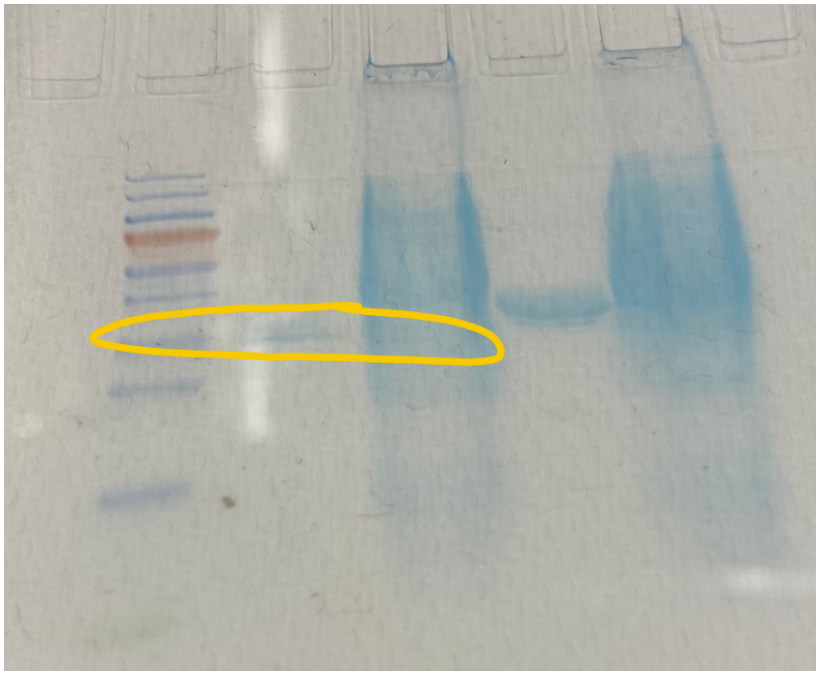


Figure 5. SDS- PAGE verification of L-asparaginase.

As demonstrated by the yellow circle in Figure 5, a slight band can be seen at the expected size for L-asparaginase, indicating that the protein was successfully created. Thus, plasmid C3, derived from *Campylobacter*, was named the most promising prototype, and it would be used for the recombinant *E. coli* going into the scale-up process.

Creativity and Innovation

In our capstone project, we aimed to address the challenge of producing L-asparaginase more efficiently and sustainably. To achieve this, we employed creative and innovative strategies, setting our work apart from previous studies and potentially revolutionizing the production of this enzyme.

One of our key innovations was the use of different genes and host organisms for L-asparaginase production. We opted for *E. coli*, a well-studied and versatile bacteria, rather than the more commonly used fungi. This decision allowed us to explore the potential for further optimization and efficiency improvements in the production process. Additionally, we experimented with novel genes not previously used for L-asparaginase production, which could lead to a more cost-effective and sustainable method for large-scale manufacturing.

Our team also designed a unique process for creating and extracting L-asparaginase. We identified optimal conditions for the process and sought to improve them by using a specialized bioreactor, ion exchange purification, and chromatography techniques. This novel process has the potential to be patented, showcasing our commitment to innovation.

To ensure the practicality and efficiency of our process, we developed a custom Python algorithm that accounted for various operational variables. This code allowed us to identify the best settings for our equipment, maximizing production efficiency. Our hands-on approach to experimentation and our ability to translate theoretical models into tangible outcomes further demonstrate our dedication to innovation through the use of this novel process.

Moreover, we proposed future experiments and research directions to advance L-asparaginase production. For instance, we recommended exploring protein engineering

techniques, codon optimization, and metabolic pathway engineering to enhance enzyme performance and yield.

In conclusion, our capstone project exemplifies creativity and innovation in the field of L-asparaginase production. By using different genes and host organisms, designing a novel process, and proposing cutting-edge research directions, we have taken a significant step towards a more efficient, sustainable, and cost-effective production method. Our work has the potential to transform the food processing and medical industries, making L-asparaginase more accessible and affordable for various applications.

Process Flow Diagram and Material Balances

Process Flow Diagram

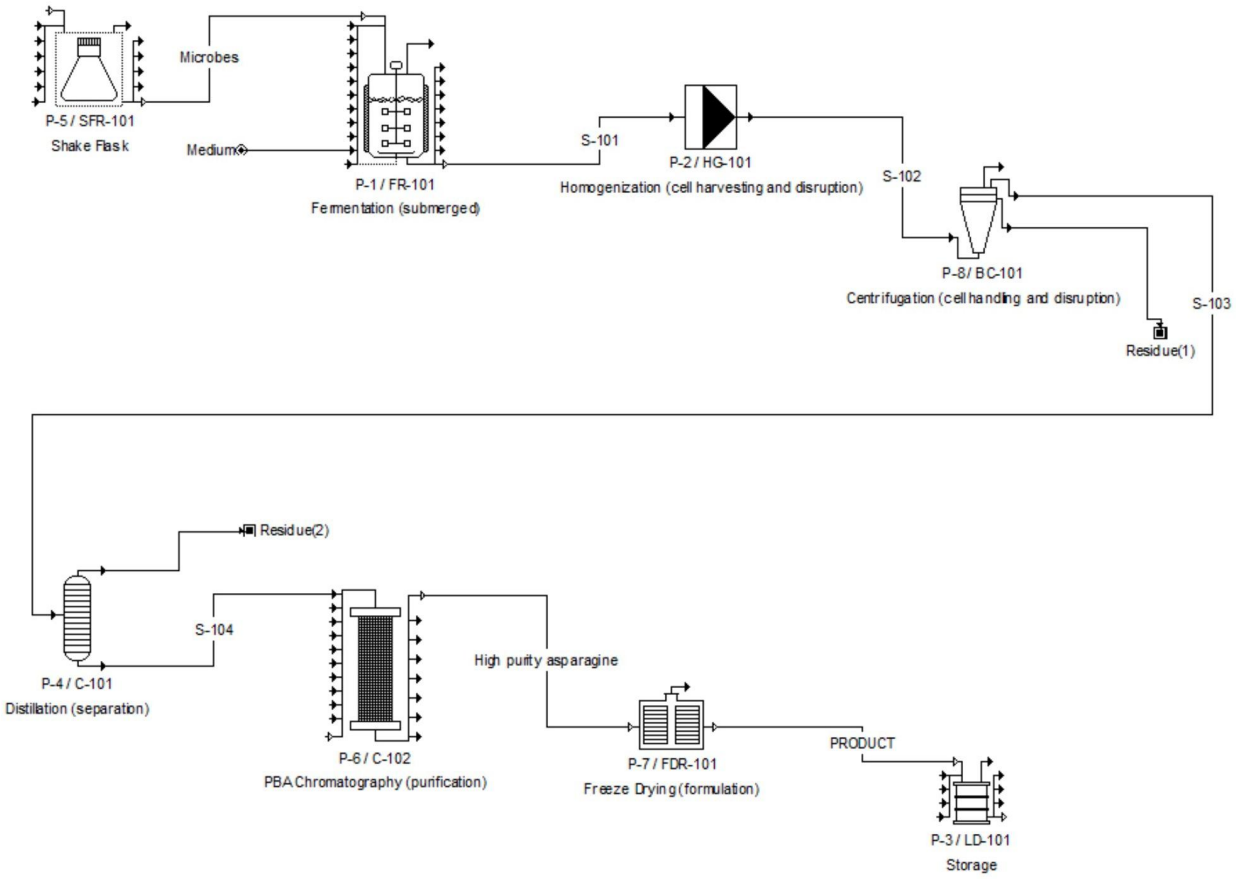


Figure 6: Process flow diagram for scaled-up process.

The production of L-asparaginase from recombinant *E. coli* involves multiple stages to ensure optimal growth, expression, and subsequent purification of the protein. In this section, we describe the process flow diagram (PFD) and the significance of each stage.

- Shake Flasks: Shake flasks are employed during the initial stage of the process for the cultivation of recombinant *E. coli.* This stage is crucial as it facilitates the growth of *E. coli* cells in a controlled environment while ensuring adequate aeration and mixing. The

flasks are filled with a suitable growth medium containing the appropriate nutrients, and the cells are cultured until they reach the desired cell density.

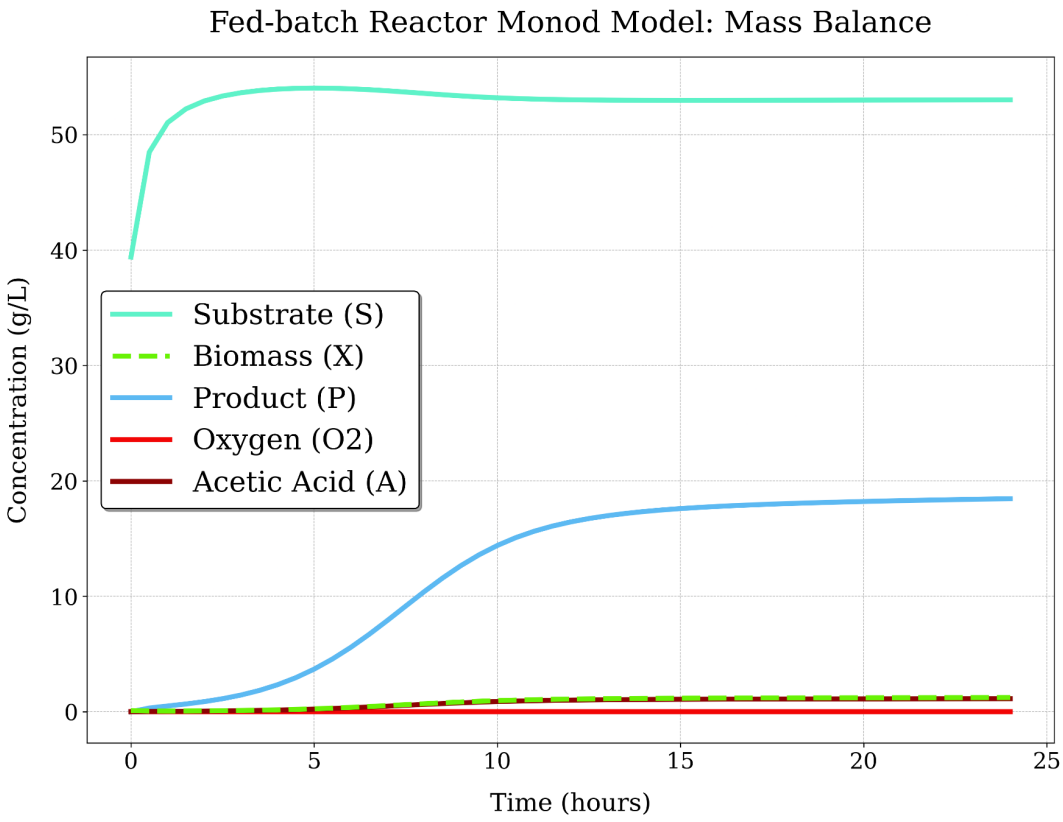
- *Fermentation:* Once the cells have grown to the required density, they are transferred to a fermenter for large-scale production. The fermenter provides a controlled environment for optimal growth, expression of L-asparaginase, and aeration. The fermentation process is carefully monitored and maintained at ideal conditions such as temperature, pH, and dissolved oxygen levels to maximize L-asparaginase production.
- *Homogenization (Cell Harvesting and Disruption):* Following the fermentation process, the cells are harvested through a centrifugation step. The cell pellet is then subjected to homogenization to disrupt the cells and release the intracellular L-asparaginase. This step is essential for obtaining the desired protein, as L-asparaginase is expressed within the cytoplasm of the recombinant *E. coli* cells.
- *Centrifugation (Cell Handling and Disruption):* After homogenization, the cell debris and unwanted components are separated from the soluble protein fraction through centrifugation. This step ensures the removal of insoluble cellular components, leaving behind a clear supernatant containing the target protein, L-asparaginase.
- *Distillation (Separation):* Distillation is employed to separate the target protein from other soluble components in the supernatant. During this process, the volatile components are evaporated and condensed, while the non-volatile target protein remains in the liquid phase. This step aids in the initial separation and concentration of L-asparaginase from other proteins and contaminants.
- *Chromatography (Purification):* The protein sample is further purified using chromatographic techniques such as ion-exchange, size exclusion, or affinity

chromatography. These methods selectively bind and elute the target protein, effectively separating it from other proteins and impurities. Chromatography is critical for achieving the required purity and quality of L-asparaginase for its intended applications.

- *Freeze Drying:* The purified L-asparaginase is then subjected to freeze-drying (lyophilization) to remove any remaining water content. This process involves freezing the protein solution, followed by the application of a vacuum to sublimate the ice directly into vapor. Freeze-drying helps to stabilize the protein and enhance its shelf-life, making it suitable for long-term storage and transportation.
- *Storage:* Finally, the freeze-dried L-asparaginase is stored at appropriate conditions to preserve its stability, activity, and quality. Proper storage conditions are crucial for maintaining the functionality of the protein for its intended applications.

PFD Conclusion

The PFD for recombinant *E. coli* producing L-asparaginase highlights the critical stages involved in the production, purification, and storage of this valuable therapeutic protein. Each stage plays a significant role in ensuring the final product is of the highest quality and suitable for its intended use.



Mass Balance of the Fed-Batch Reactor

Figure 7: Mass balance concentrations vs. time in fermenter model.

In this section, we discuss the mass balance of the fed-batch reactor, focusing on the substrate, biomass, product, oxygen, and acetic acid concentrations. The mass balance equations are important for understanding the dynamics of the reactor and are used to model and optimize the production of L-asparaginase. The mass balance equations are derived based on the Monod model, which accounts for substrate uptake, biomass growth, and product formation, along with inhibition terms for acetic acid, glucose, and oxygen. The python code can be found in Appendix B.

Mass Balance Equations

The mass balance equations are given by the following set of differential equations:

$$\frac{dS}{dt} = -\frac{\mu X}{Y_{\mathrm{xs}}} + \frac{F(S_{\mathrm{f}} - S)}{V}$$
$$\frac{dX}{dt} = \mu X - \frac{FX}{V}$$
$$\frac{dP}{dt} = \mu X Y_{\mathrm{px}} - \frac{FP}{V}$$
$$\frac{dV}{dt} = F\left(1 - \frac{V}{V_{\max}}\right)$$
$$\frac{d\mathrm{O}_2}{dt} = -q_{\mathrm{O}_2} X + kL_{\mathrm{a}}(\mathrm{O}_{2,\mathrm{sat}} - \mathrm{O}_2)$$
$$\frac{dA}{dt} = \mu X Y_{\mathrm{ax}} - \frac{FA}{V}$$
$$\mu = \frac{\mu_{max} \cdot S}{K_s + S} \cdot \left(1 - \frac{A}{K_{i,acetic_a cid}}\right) \cdot \left(1 - \frac{S}{K_{i,glucose}}\right) \cdot \frac{O_2}{K_{O_2} + O_2}$$

Where:

- S, X, P, V, O_2, and A are the substrate, biomass, product, reactor volume, oxygen, and acetic acid concentrations, respectively.
- μ is the specific growth rate given by the Monod equation with inhibition terms.
- Y_{xs}, Y_{px}, Y_{xo2}, and Y_{ax} are the yield coefficients for biomass, product, oxygen consumption, and acetic acid production, respectively.
- F is the feed rate.
- S_f is the substrate concentration in the feed.
- V_{max} is the maximum reactor volume.
- q_{o2} is the oxygen consumption rate.

- kL_a is the mass transfer coefficient.
- $O_{2,sat}$ is the saturation concentration of oxygen.

Code Implementation and Optimization

The mass balance equations are implemented in the Python code using the SciPy library's odeint function, which solves the system of ordinary differential equations (ODEs). The Monod model function monod_model takes the initial conditions, feed rate, and substrate concentration in the feed as input arguments and returns the time derivatives of the concentrations.

To optimize the mass balance for profit, the DEAP library is used to create an optimization problem with the objective function objective_function, which calculates the profit based on the final product mass, total glucose mass fed, and initial biomass concentration. The optimization problem is set up using the Differential Evolution (DE) algorithm, with custom mutation and crossover functions. The optimal values for the initial substrate, biomass, and reactor volume, as well as the substrate concentration in the feed, mass transfer coefficient, and feed rate, are determined to maximize profit.

Results and Discussion

Upon running the code, the optimized mass balance equations provide the final product, biomass, substrate, oxygen, and acetic acid concentrations, as well as the reactor volume. The final values are given as follows:

- Final product concentration: 18.46 g/L
- Final biomass concentration: 1.23 g/L
- Final substrate concentration: 53.02 g/L

- Final oxygen concentration: 0.00727 g/L
- Final acetic acid concentration: 1.13 g/L g/L
- Final reactor volume: 4715.78 L

These final values and the supplementary graphs for the mass balance offer valuable insights into the performance of the fed-batch reactor for L-asparaginase production. By optimizing the mass balance, the reactor conditions can be tuned to maximize L-asparaginase production while minimizing undesired byproducts such as acetic acid. Moreover, optimizing the feed rate, mass transfer coefficient, and substrate concentration in the feed ensures a cost-effective and economically viable process.

The optimized reactor volume allows for efficient production while keeping equipment and operational costs within reasonable limits. The final product concentration of 18.46 g/L indicates that the optimization process successfully improved L-asparaginase yield.

The graphical representations of the mass balance show the progression of the different concentrations throughout the process. The biomass concentration initially increases due to microbial growth but later plateaus as the microorganisms reach the stationary phase. The product concentration continuously increases throughout the process, signifying effective conversion of the substrate into the desired product.

In conclusion, implementing and optimizing the mass balance equations using Python and the SciPy and DEAP libraries have demonstrated the potential for improving L-asparaginase production in a fed-batch reactor. The optimized parameters can serve as a basis for designing and operating the reactor at a larger scale, ultimately leading to a more efficient and profitable bioprocess. Future studies may explore additional optimization techniques, such as multi-objective optimization, to further enhance the reactor's performance while considering

other factors, such as environmental impact or resource constraints. Furthermore, integrating process control strategies and real-time monitoring systems could help maintain optimal conditions during the production process, ensuring consistent product quality and high yields.

Energy Balance and Utility Requirements

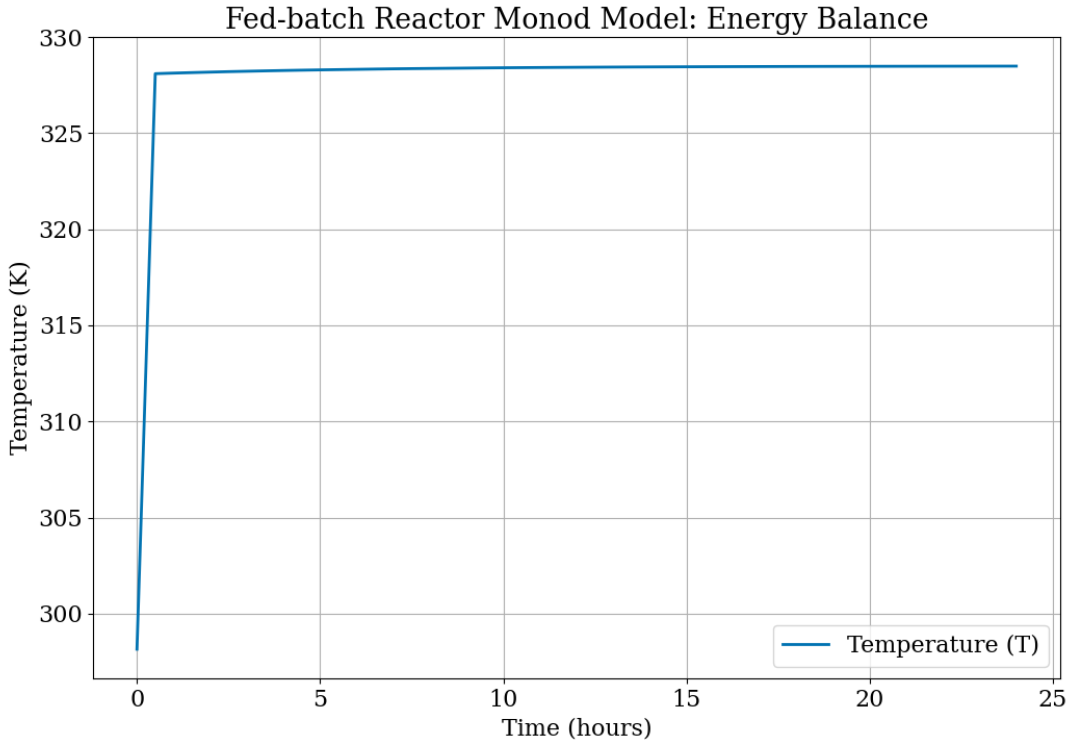


Figure 8: Energy Balance Temperature vs. Time of the Fed Batch Reactor

The energy balance of the fed-batch reactor plays a significant role in determining the utility requirements, including energy consumption and resource utilization, to maintain the reactor's optimal conditions for *E. coli* growth and l-asparaginase production. By optimizing the energy balance, we can minimize the utility requirements, resulting in reduced operating costs and a lower environmental footprint. Some of the key utility requirements associated with the energy balance are:

- *Electricity consumption:* The agitation system and the heat exchanger system require electrical energy to operate. By optimizing the energy balance, we can minimize the

electricity consumption, leading to reduced operating costs and a lower environmental footprint.

- *Cooling and heating utilities:* The heat exchanger may require external cooling or heating utilities to maintain the reactor temperature within the desired range. Optimizing the energy balance allows for more efficient use of these utilities, reducing the overall consumption and costs associated with heating and cooling.
- *Feedstock utilization:* The energy balance helps determine the optimal feed rate, ensuring efficient utilization of the feedstock, minimizing waste, and lowering costs associated with raw materials.
- *Water consumption:* The heat exchanger system may use water as a coolant, depending on its design. By optimizing the energy balance and reactor temperature, we can minimize the water consumption, contributing to more sustainable operations.

In conclusion, optimizing the energy balance in the fed-batch reactor is essential not only for achieving the optimal conditions for *E. coli* growth and L-asparaginase production but also for minimizing the utility requirements. This approach helps reduce energy and resource consumption, decrease operating costs, and lower the environmental impact of the process.

Enhanced Energy Balance of the Fed-Batch Reactor and Its Importance

In this section, we delve into the energy balance of the fed-batch reactor, a critical aspect for maintaining optimal conditions for *E. coli* growth and efficient l-asparaginase production. The energy balance ensures that the reactor temperature stays within the ideal range, which is essential because *E. coli* can produce a significant amount of l-asparaginase at a specific

temperature. It also helps to optimize the utility requirements, such as energy consumption, and minimize any negative environmental impact.

Energy Balance Equations

The energy balance of the fed-batch reactor encompasses the following components:

- Heat generated due to the fermentation of *E. coli*, modeled using the heat of fermentation constant. This value is vital as the metabolic activity of *E. coli* during fermentation directly influences the amount of heat generated, which in turn affects the overall energy balance of the reactor.
- Heat transfer between the reactor and the environment, calculated using the heat transfer coefficient and the surface area of the heat exchanger. Efficient heat transfer is crucial for maintaining the desired reactor temperature, ensuring optimal conditions for *E. coli* growth and l-asparaginase production.
- Heat introduced to the system due to the feed, which depends on the feed rate, specific heat capacity, and temperature difference between the feed and the reactor. Controlling this heat input is essential for achieving a stable energy balance and maintaining the reactor temperature within the desired range.
- Heat generated by the agitation system, which depends on the agitation power per volume and reactor volume. Agitation is vital for maintaining adequate mixing within the reactor, ensuring uniform temperature and nutrient distribution, and promoting *E. coli* growth and product formation.

The energy balance code can be found in Appendix B and the equation is given by:

- dU/dt = -X * mu * heat_of_fermentation_ecoli - heat_transfer_coefficient * current_reactor_area * (T - ambient_temperature) + F * rho * specific_heat_capacity * (T_feed - T) + agitation_power_per_volume * V

To calculate the temperature change in the reactor (dT/dt), we divide dU/dt by the product of reactor volume (V), specific heat capacity (Cp), and density (rho):

- dT/dt = dU/dt / (V * specific_heat_capacity * rho)

Heat Exchanger and PID Controller

The heat exchanger is a critical component in maintaining the reactor temperature within the desired range. It serves to dissipate excess heat generated during the fermentation process or supply additional heat if required. In our model, we implemented a PID controller to optimize the heat exchanger area based on the reactor volume and temperature, ensuring efficient heat transfer and contributing to the overall energy balance.

The PID controller takes proportional (Kp), integral (Ki), and derivative (Kd) gains as inputs and calculates the heat exchanger area correction. It uses the setpoint_temperature, the current reactor temperature (T), integral error, and derivative error to achieve the desired control:

- heat_exchanger_area_correction = pid_controller(Kp, Ki, Kd, setpoint_temperature, T, integral_error, derivative_error, timestep)

The current reactor area is updated based on the heat exchanger area correction:

- current_reactor_area = heat_exchanger_area(V) + heat_exchanger_area_correction

Optimization of PID Parameters

To find the optimal PID parameters (Kp, Ki, and Kd), we used the Particle Swarm Optimization (PSO) algorithm, implemented with the pyswarm library. The objective_function takes PID parameters as inputs, sets up and solves the ODE with the given PID parameters, and returns a performance metric based on the control performance (e.g., the sum of the squared temperature errors).

The PSO algorithm searches for the best set of PID parameters that minimize the objective function, resulting in better control of the reactor temperature.

Final Values and Their Importance

Upon solving the ODEs and simulating the fed-batch reactor, we obtained the following final values:

- Final internal energy: 111313.47 kJ
- Final reactor temperature: 328.49 K

These values are crucial as they offer insights into the reactor's performance, enabling us to determine the optimal conditions for l-asparaginase production. A stable reactor temperature ensures that *E. coli* can thrive and produce significant amounts of l-asparaginase, contributing to the process's overall efficiency. Furthermore, the energy balance helps optimize utility requirements, reducing energy consumption and minimizing any potential negative environmental impact.

Equipment List and Unit Description

Our team conducted research on the various pieces of equipment required for the scale-up design process of L-Asparaginase, as depicted in the process flow diagram Figure 6. We compiled a list of equipment along with their respective capacities and brief unit descriptions, as outlined below in Table 1.

Table 1. Equipment list and Unit Description

Equipment	Capacity	Unit Description
Shake Flask	500 mL	A cylindrical container used for culturing and growing microorganisms or cells in liquid media. The flask can be agitated to enhance oxygen and nutrient supply to the cells.
Fermenter (Submerged)	60,000 L	A large vessel used for growing microorganisms or cells on a large scale in submerged culture. The culture is agitated to provide oxygen and nutrient supply to the cells.
Homogenizer	120 L	A device used for breaking down and emulsifying substances into smaller particles or droplets.
Centrifuge	5 L	A device used for separating particles in a liquid or mixture by spinning it at high speeds, causing the particles to settle according to their density.
Distillation Device	3.5 L	A device used for separating and purifying different components of a mixture based on their boiling points.
Purification Device	2000 L	A device used for purifying substances, such as proteins or chemicals, from a mixture through a series of steps such as chromatography, filtration, or crystallization.

Freeze Dryer/Storage	50 L	A device used for removing water from a substance by freezing it and then applying a vacuum, resulting in the sublimation of water. It is also used for storing samples in a low humidity environment to prevent degradation.

In summary, the equipment research we conducted for the scale-up design process of L-Asparaginase has identified the key pieces required and their capacities, as outlined in Table 1. These findings show the significance of selecting appropriate equipment for efficient and cost-effective large-scale production of L-Asparaginase, while also considering specific requirements and limitations. With this in mind, we can now move forward with the next steps of the design process, which is discussing the cost summary of this equipment.

Equipment Cost Summary

To prepare a cost summary for our company's large-scale production, we needed to determine the cost per unit, quantity required, and total cost of each piece of equipment. Since the production goal for L-Asparaginase is around 7,000 kg/day, the required quantities varied based on the capacity size of each unit. After thorough research and calculations, we created Table 2, that outlines the total cost of each equipment.

Table 2. Equipment Cost Summary for the large-scale production of L-Asparaginase

Equipment	Cost Per Unit ($)	Quantity Required	Total Cost ($)
Shake Flask (500 mL)	$7	2,000	$14,000
Fermenter (Submerged) (60,000 L)	$1,500,000	9	$13,500,000
Homogenizer (120L)	$37,113	8	$296,904
Centrifuge (5L)	$19,397	192	$3,724,224
Distillation Device (3.5L)	$154,990	274	$42,467,260
Purification Device (2000L)	$170,000	1	$170,000
Freeze Dryer/Storage (50L)	$100,000	1	$100,000

According to Table 2, the 500 mL shake flask, 5 L centrifuge device, and 3.5 L distillation device were found to require the largest quantities to achieve the desired production rate of L-Asparaginase. Among the equipment used in this process, the 60,000 L fermenter stands out as the most expensive, costing approximately $1.5 million, and serving as the core of the production line for our product. The final two steps of the production, the 2000 L purification device, and the 50 L freeze dryer storage unit, require only one each. It is important to note that the equipment mentioned above are one-time purchases that necessitate maintenance throughout the years of production. As such, they can be regarded as fixed capital investments, which will be discussed further in the next section.

Fixed Capital Investment Summary

As previously noted in the equipment cost summary, the equipment represents a significant portion of our company's fixed capital investment. Fixed capital investments refer to permanent purchases made by investors. In Table 3, we have compiled a list of these necessary purchases required to initiate the production of L-Asparaginase.

Table 3. The Fixed Capital Investment List

Fixed Capital Investment	Cost Per Unit ($)	Quantity Required	Total Cost ($)
Shake Flask (500 mL)	$7	2,000	$14,000
Fermenter (Submerged) (60,000 L)	$1,500,000	9	$13,500,000
Homogenizer (120L)	$37,113	8	$296,904
Centrifuge (5L)	$19,397	192	$3,724,224
Distillation Device (3.5L)	$154,990	274	$42,467,260
Purification Device (2000L)	$170,000	1	$170,000
Freeze Dryer/Storage (50L)	$100,000	1	$100,000
Building	$1,225,000	2	$2,450,000
		Total Fixed Capital Investment	$62,722,388

Table 3 provides a comprehensive breakdown of each capital investment, including its corresponding price and quantity. In addition to the equipment list, we have also included the building, which is crucial for housing this manufacturing plant. Our company intends to have two warehouses, each spanning approximately 25,000 sq ft, that will accommodate all the machinery, except for the fermenters. These will be located outside of the warehouses but will be connected to the building to ensure the sterility of the process. The total fixed capital investment was then calculated for all of the machinery which came out to be $62,722,388.

Biosafety, Health, and Environmental Consideration

L-asparaginase is widely used in the food industry to reduce carcinogens and improve human health. However, its production process can generate several waste streams, including biomass, effluent, solid wastes, and harmful chemicals that can potentially contaminate the environment. The biomass generated during the cell homogenization step can be mutagenic and, if released into the environment, can harm other organisms. To address this, it is necessary to remove the biomass and properly dispose of the effluent. Ultrafiltration and recycling of wastewater can reduce the overall amount of wastewater generated, thereby reducing energy consumption and environmental impacts.

Acetic acid, another byproduct of the fermentation process, is corrosive and can cause significant damage to skin and tissue. Dilution with high amounts of water is the conventional mitigation strategy. However, acetic acid is also used in petroleum production and as a food additive. Collecting the crude material and selling it to companies for these applications is a viable alternative that not only limits environmental damage but also generates income for the process. As of current, only about 1.13 g/L of acetic acid are generated throughout the process, which has both positive and negative aspects. Less acetic acid produced means less can be sold for other industrial uses, and this results in less side profit. However, since such a small quantity is actually inside the fermentation system, pH is unlikely to drop significantly. This means less control is necessary, and it reduces the likelihood that the acid will need to be neutralized with a base, so the recycle stream is a dependable, albeit small, source of additional income for the company.

Ammonia, a byproduct of the fermentation process, is a severe environmental concern. Excess ammonia can cause acidification of the atmosphere, fine particulate matter, and health problems such as COPD and asthma in humans. Filtering all gasses exiting the facility and reducing ammonia production through optimization of the fermentation process can help prevent ammonia from escaping into the environment.

Reducing the production process's overall footprint is the easiest way to minimize negative impacts on the environment. Alma has optimized the entire process by using larger fermenters and running multiple cycles simultaneously to reduce waste and energy input. The economics of this environmental plan seem promising as it can reduce unnecessary waste and minimize costs while keeping prices low and remaining competitive in the industry. Additionally, the production of L-asparaginase is relatively green as it requires few toxic chemicals and incorporates biosafety measures, making it economically feasible to mitigate any potential damage.

Operation Costs

In the next phase of the scale-up design process, our team considered the operational costs associated with the production of L-Asparaginase. We identified three cost categories for this portion of the design: raw materials, equipment and facilities, and labor costs. Raw materials consist of biomass and substrates used as inputs in the fermentation process. The equipment and facilities category encompasses the cost of maintenance and utilities required to keep the process running. Lastly, labor cost involves three different types of workers: process monitors, equipment operators, and quality control testers. We determined the annual costs for each category and summarized them in Table 4 below.

Table 4. Annual Operation Cost

Cost Category	**Annual Costs ($)**
Raw Materials	$4,623,913
Equipment and Facilities	$14,698,535
Labor Cost	$8,488,440
Total Operation Costs	**$27,810,889**

Table 4 summarizes the annual costs for each category, which allowed us to determine the total annual operating cost of $27,810,889 for the production process of L-Asparaginase. The largest cost contributor was the equipment and facilities category, while raw materials had the lowest cost. It's important to note that these operating costs are subject to fluctuations over time due to inflation rates for workers and our company's ability to develop strategies on saving on the utilities in the plant.

Economic Analysis

Upon finalizing the equipment costs, fixed capital investment, and annual operation costs, our team conducted a comprehensive economic analysis to assess the feasibility of our L-Asparaginase scale-up design process. Our analysis revealed a robust market demand for L-Asparaginase, which was estimated to be worth $365 million according to "Market Watch" in 2023. Next, we evaluated the projected timeline for setting up our production process, which was estimated to take approximately three years, and included the fixed capital investments outlined in Table 3.

Upon completion of the initial investments, we began our production process and started accruing annual profits. A profit analysis chart was created in Figure 6 to estimate the annual and cumulative profits for our L-Asparaginase production over a period of 10 years. This analysis provides key insights into the project's profitability over time, including any fluctuations in profitability from year to year.

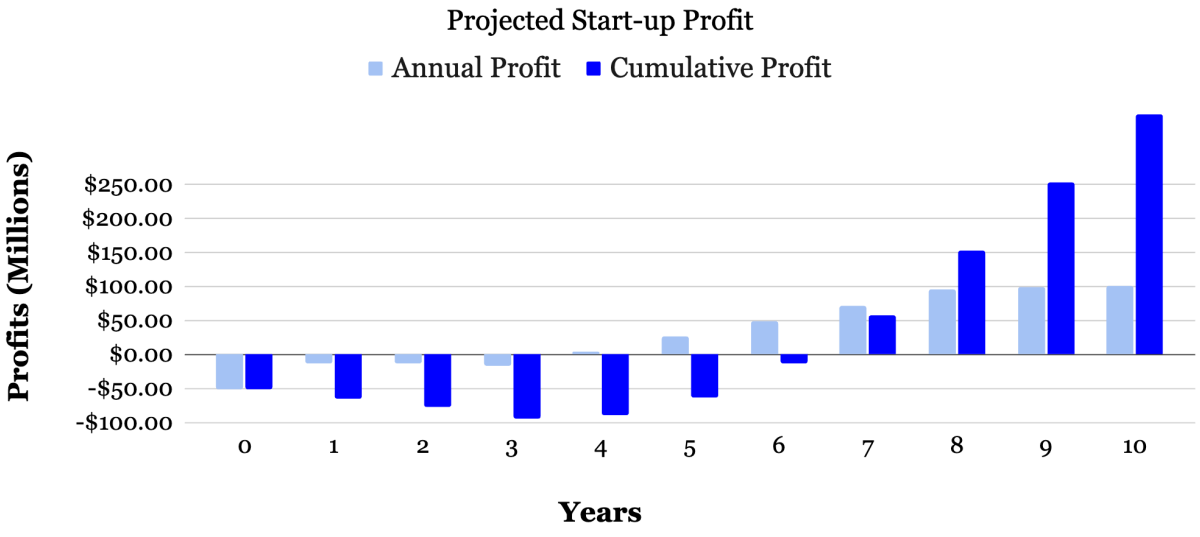


Figure 6. Projected Start-up profit analysis for first 10 years

According to the profit analysis chart in Figure 6, the first three years of the 10-year period were dedicated to capital investments, which were spread out over the period. During year 3, we initiated our production process, which incurred the annual operation costs listed in Table 4. In year 4, we started generating annual profits, based on our projected market share, which was set to increase over time.

As demonstrated in the chart, the cumulative profits accrued by the production process are projected to exceed the initial capital investments by the end of year 7. This indicates the viability of the project in the long run. Our profitability is expected to increase with a greater share of the L-Asparaginase market, demonstrating the potential for sustained growth over time.

Budget

In order for Alma to develop an optimal production process of L-asparaginase, an accurate budget is needed to help in gathering the materials for this project. The main components contributing to this budget include the technology and materials being used for the lab scale production of the enzyme, the software used to design the finalized plasmids for this process, and the purchased plasmids designed from this software. As mentioned before, one of our key stakeholders, Dr. Yan is providing our team access to his metabolic engineering lab, which greatly reduces our budget. This lab includes all the necessary resources for the lab production process mentioned in the lab protocol, like a PCR machine and a centrifuge (Table 1A).

As for the software being used for the designing portion of the plasmids, we chose to use SnapGene. An annual subscription for this software is estimated to be $150 for students. The base pair (BP) sequences specified for each modified plasmid in SnapGene were then transferred over to Twist BioScience. This is the company we are purchasing our four chosen genes from, which has a price breakdown of $0.07 per BP. The estimated cost for each gene is approximately $75.00, giving a total purchase cost of $300 from Twist Bioscience. Therefore, the total budget is currently estimated to be around $500 including taxes and fees from these online orders.

Our team at Alma gathered this information pretty early during the development process of our plasmids and lab protocol. So, we have successfully been granted $550 from the UGA start-up program to cover the expenses for the software and genes in this project. The additional $50 granted by this program will assist with miscellaneous costs not yet determined in the

upcoming months. Next semester, participation in the University of Georgia's Kickstarter Funds will also potentially add funding for additional lab materials and reagents.

Using financial data gathered throughout the entire process, an economic analysis will be fully completed. Completion of this analysis will likely be the final step of the project, as it requires data gathered from the scale-up bioprocess simulation. Ultimately, this analysis will give insight into the economic feasibility of the proposed process (Figure 11A), evaluating key parameters such as raw materials cost, the market price of L-asparaginase, and net present annual worth.

Regulatory Information

Since this project involves the addition of genetically modified microorganisms and enzymes to food, it is crucial to abide by the many codes and regulations regarding a product. The team is actively reading into the related regulatory requirements and will continue to review the production process accordingly. As of current, the team will study the safety data sheets regarding each reagent and organism involved in the process, including that of the cells used in the production of vectors containing the genes, the cells used to express L-asparaginase, and the isolated L-asparaginase itself. The team will reference the FDA Guidance for Industry Acrylamide in Foods (2016) as well as the Codex Alimentarius Code of Practice for the Reduction of Acrylamide in Foods (2009).

Thus far, the only regulatory code deemed necessary by the team is the U.S. Code of Federal Regulations Title 21 Food and Drug, Part 170 Food Additives, Section 30 Eligibility for classification as generally recognized as safe (GRAS) (21 C.F.R § 170.30). This necessity was determined by looking into five total GRAS Notifications (No. 604, 476, 428, 214, and 201) relating to L-asparaginase presented on the FDA's inventory of GRAS notices, which have provided sufficient amounts of information regarding the enzyme's production and regulation.

The team will also abide by Current Good Manufacturing Practices (cGMPs) for Food and Dietary Supplements and the Occupational Safety and Health Administration (OSHA) guidelines. Following these codes, jurisdictions, and regulations will allow for a healthy work environment and ultimately a product that will decrease carcinogens in processed food

Future Experiments

Based on the findings of this project, there are several promising avenues for future research in this area. One potential area for exploration is protein structure optimization. This method involves altering the three-dimensional structure of L-asparaginase to improve its performance and efficiency, by modifying specific amino acids within the protein. Optimizing the structure of L-asparaginase can improve the protein's function, stability, and expression levels, which in turn can lead to higher production yields.

Another area of interest is codon optimization, which involves modifying the nucleotide sequence of the genes to optimize the codons that encode each amino acid within the protein. This process is used to improve gene expression and improve the translational efficiency of the chosen gene, by taking the preferred codons of the host organism into account. Codon optimization can improve the efficiency and accuracy of protein synthesis, and can additionally lead to higher yields of the protein. Metabolic pathway optimization is also a potential method that could increase the overall efficiency of the production process. This can be done by modulating the expression levels of genes involved in the biosynthesis of asparagine or altering the metabolic flux through the pathway.

In addition to these optimization strategies, there are also several experimental approaches that can enhance protein production. Further investigation can be done into the shake flask conditions of our laboratory experiments, and the potential addition of different salts to improve bacterial growth and protein expression levels. Additionally, the exploration of additional genes beyond the four that we examined can allow for the identification of a more effective alternative for l-asparaginase production. It would also be worthwhile to retest the four original genes in our study to ensure the validity of the results and confirm our previous findings.

Another potential area of exploration is the use of fungi as a potential expression system. Fungi can offer several advantages over bacterial expression and generally have a higher capacity for protein production. Certain fungi such as *Aspergillus* or *Penicillium* have been shown to produce L-asparaginase with high yields and activity. Experimentation with different fungal strains and growth conditions can expand the scope beyond bacterial expression systems, and can potentially allow for a more efficient method of producing L-asparaginase.

Conclusion and Recommendations

Overall, based on our preliminary investigations using gene cloning and our scaled-up model, Alma proposes engineering recombinant *E. coli* with the gene from *Campylobacter corcagiensis* to help produce L-asparaginase. This enzyme is commonly used in the food industry to mitigate carcinogen production during baking processes that utilize the Maillard reaction. Thus, Alma focused primarily on the production of L-asparaginase for this application. Following the construction and cloning of plasmids containing our gene, Alma suggests culturing BL21 *E. coli* in shake flasks to an appropriate density as the first step in production and then inoculating a 60,000 L fed-batch submerged fermenter with approximately 0.1 g/L of *E. coli* cells. After a 24 hour residence period, the fermentation product should move through homogenization and centrifugation steps to eventually obtain a concentrated protein solution. This will be evaporated and freeze-dried for storage, ultimately resulting in a finalized protein product, with the company making 6,960 kg/day to remain competitive in the market. The unit price for this product was estimated to be $40 once completed. Within our facilities, we will treat waste streams for ammonia, acetic acid, and excess biomass while maintaining green chemistry, and we will make attempts to minimize our environmental footprint at all steps of the process. Additionally, we will abide by FDA guidelines for good manufacturing processes for the production of a food-additive. Alma wishes to do further research by modifying our gene, examining other genes, and making the fermentation process more efficient by metabolically engineering the fermentation reaction.

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Appendix A

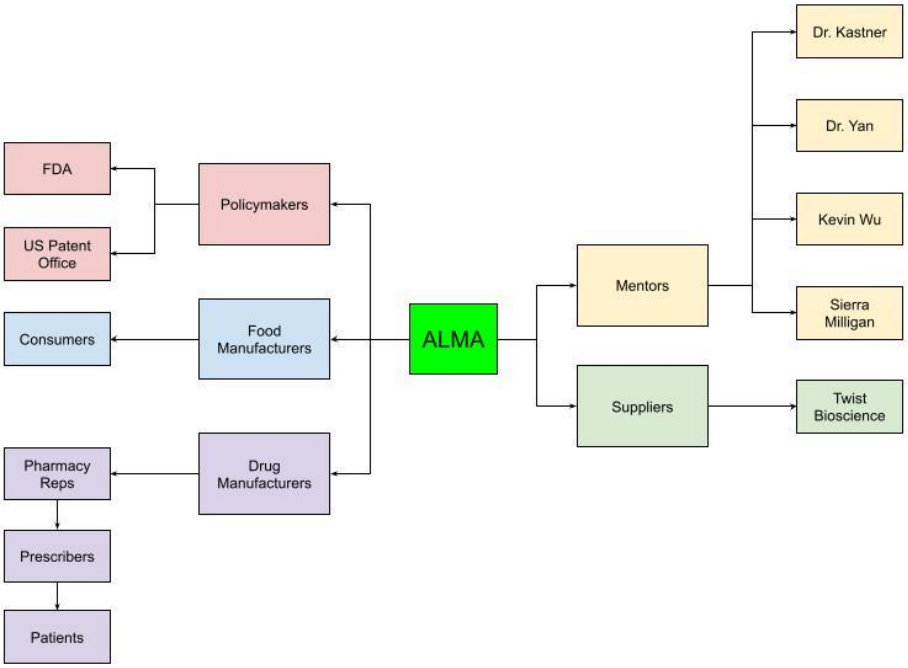

Figure 1A. Stakeholder Map

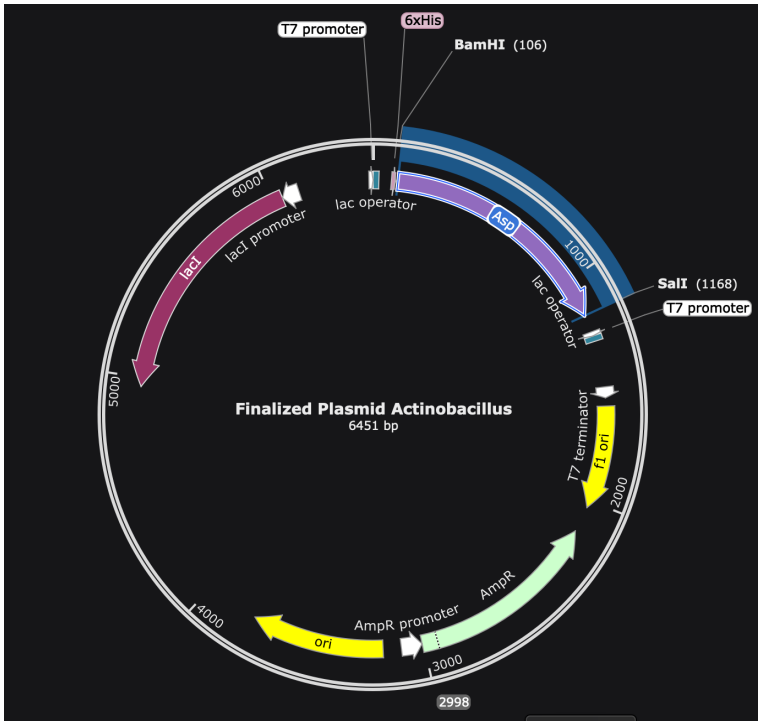

Figure 2A. *Actinobacillus*-derived Plasmid Design

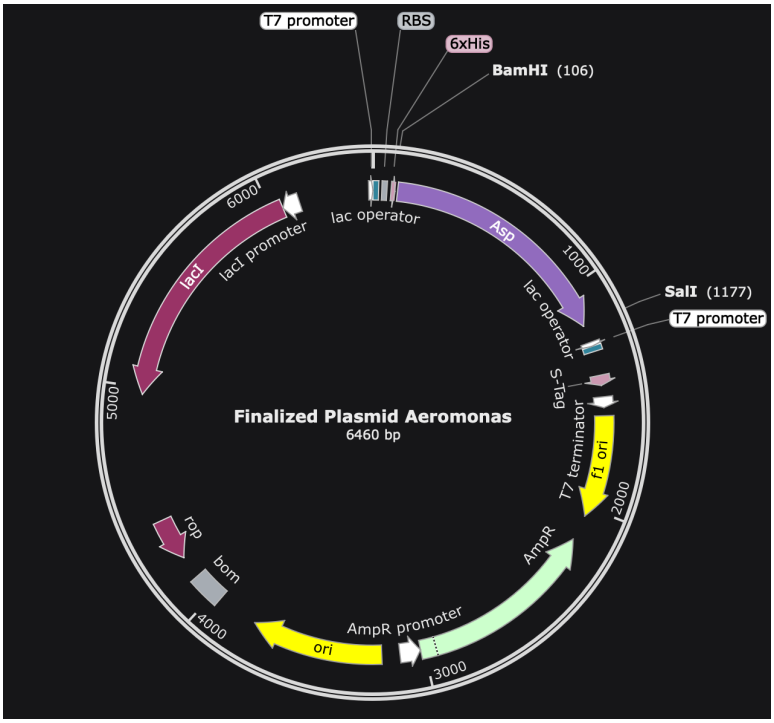

Figure 3A. *Aeromonas*-derived Plasmid Design

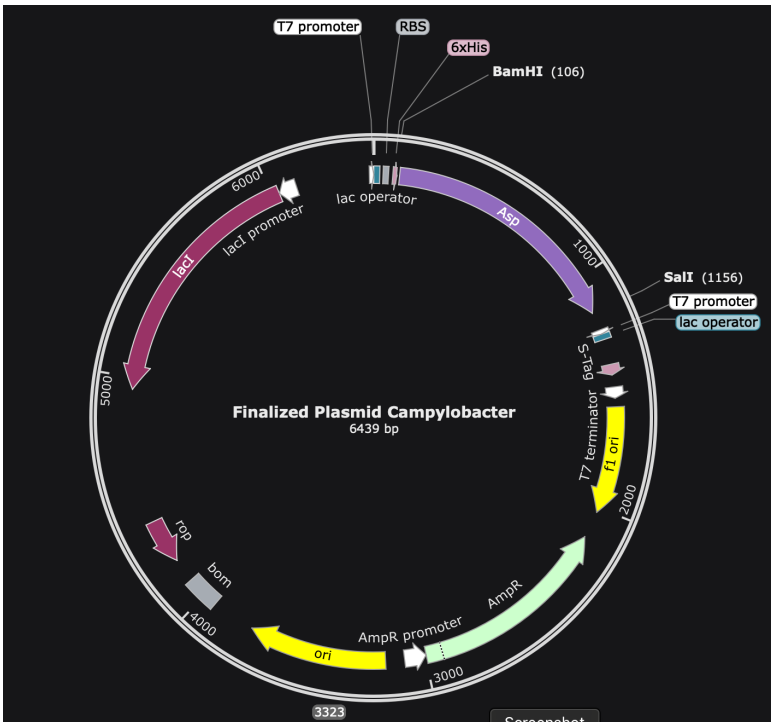

Figure 4A. *Campylobacter*-derived Plasmid Design

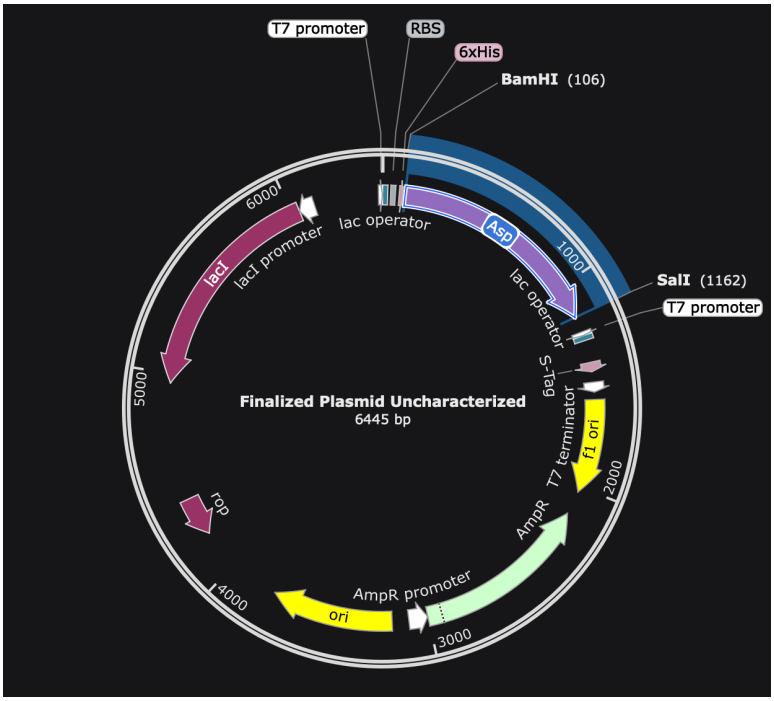

Figure 5A. *Salmonella*-derived Plasmid Design

Table 1A. BOM Lab Scale Production

Bill of Material for Lab Scale Production of L-Asparaginase		
Type of Product	**Name of Inventory**	**Qty**
Gene Software	Annual SnapGene Subscription	1
Gene	Uncharacterized	1
Gene	Actinobacillus	1
Gene	Campylobacter	1
Gene	Aeromonas	1
Equipment	PCR	1
Equipment	Centrifuge	1
Equipment	Plates	48
Equipment	Shake Flask	48
Equipment	Incubator	2
Materials	Media	2
Materials	Antibiotics	2

Materials	Cells culture	2

Table 2A. BOM Large Scale Production

Bill of Material for Large Scale Production of L-Asparaginase		
Type of Product	**Name of Inventory**	**Qty**
Machinery	Shake Flask	2000
Machinery	Fermenter (Submerged)	9
Machinery	Homogenizer	8
Machinery	Centrifuge	192
Machinery	Distillation Device	274
Machinery	Purification Device	1
Machinery	Freeze Dryer/Storage	1

Table 3A. Interview Q/A with Novex Innovations

Questions	**Answers**
What is the biggest difficulty faced when scaling a process up?	Reproducibility at a larger scale. Specifically with organisms, a 1ml vessel and a 10L vessel will not necessarily work in the same way. Testing critical parameters and the effects of scale should be evaluated early on to have a good understanding of scale effects.
From design to regulations to manufacturing to distribution, what part of the process is the slowest or most inefficient?	Of what you have listed, the initial two, design and regulation would be the more lengthy and most inefficient activities.
What is typically the biggest regulatory hurdle faced when manufacturing a drug product?	With respect to a generic type product, that may have a different route of manufacturing or increased throughput, which may carry a different impurity profile.The testing will be a large component of time and money in making sure the impurity profile is comparable (easiest) or new impurities would not

	create any problems.
Generally speaking, what part of a drug manufacturing process is the most costly?	In most cases the most expensive component of manufacturing is related to labor costs. Therefore the key in the process development stage, a lot of effort should be applied to an efficient process.
What does a typical day look like for you? What are some challenges you face most often in your position?	A cross section of an average day would be evaluating ongoing activities, maintaining customer relationships/requirements for future work, work on new projects that are being onboarded, evaluating new technologies and regulatory environments, document maintenance/creation and employee interactions to ensure proper positioning of experiences. The biggest challenge is balancing financial aspects related to properly costing projects and ensuring an appropriate return to support the business and support a successful path into the future.
What are some characteristics of a patented process that would entice you to use it?	Patents are interesting animals. The first I would look at is what type of holes are in the patent that could be taken advantage of, by not needing to use it. If the patent were to be licensed I would make sure it was solid so that no one could take advantage of process variables that don't fall within a viable option that would allow a competitor to circumvent something that you would be paying for. It is very critical to negotiate the best possible licensing fees to ensure that it doesn't impact your performance as a company in the future.
Do you often speak with the actual consumers of your drug products? If so, what features of your product are they satisfied with? What features are they dissatisfied with?	All the time. This is the most important aspect of successful customer relationships. Providing insight into their program along the way. Personal connection with them to be a part or component of their

	success and setbacks. Sometimes price and some wish they had/controlled 100% of our time.
I understand you do contract work with other companies; what encourages them to contract their work instead of manufacturing or developing the process themselves?	With a new company, they don't have the resources, infrastructure and intellectual know how to operate a business. They need to focus what little resource they have at driving the product to market as the source of income from a marketed product is the key component to the survival of the company.
When working with biologics or living organisms, what is one issue your company faces specific to them?	Understanding the proper support and maintenance of cell lines to ensure pure colonies now and in the future. Also ensuring the ability to segregate and ability to ensure a complete kill during the cleaning process.

Table 4A. Interview Q/A with Takeda

Questions	**Answers**
What does a typical day look like for you? What are some challenges you face most often in your position?	I am part of a team in charge of a process line for an albumin product. There are about 16 people in our department and I work with a smaller team of 3 or 4. I was the person to say if a product was good or bad, and dealt with variables like pH and temperature. I worked on pilot scale equipment as well and was responsible with creating the mathematical models and conversion rates for the reactions.
What is typically the biggest regulatory hurdle faced when manufacturing a drug product?	Every single person that works at Takeda has to keep regulatory standards in mind. If I wanted to change any parameter to the process I was working on like flowrate for example, I had to go to the quality department to get approved. The quality department acts as the lawmakers of the site.

When working with biologics or living organisms, what is one issue your company faces specific to them?	The Takeda plant in Covington mainly works on plasma-based biologics. Purifying plasma involves processes like fractionation, centrifugation, precipitation, and filtration. One thing to keep in mind with this product in particular is that different proteins purify at different points, such as different temperatures or pHs. It was important to identify the characteristics of your desired product.
How has your lab experience during university aided you in your career at Takeda?	Working with Dr. Yan for my Master's degree taught me how to problem solve, specifically how to translate scientific language into something meaningful and conveyable.

Project title: ALMA: L-Asparaginase Production
Project Team: Liam, Jordan, Bella, Taylor
Date: 29-Nov-22

	1: low, 5: high	Desired direction of improvement (↑,0,↓)	0	↓	0	↑	0	
	Customer importance rating	Functional Requirements (How's) → / Customer Requirements - (What's) ↓	Gene Selection	Production Costs	Fermentation Conditions	Enzyme Purification	Scale-up Simulation	Weighted Score
1	4	Improved Enzyme Production	9	3	9		9	120
2	4	Highly Active Enzyme	9			9		72
3	3	Cheaper Process	3	9				36
4	2	Faster Production Process	1	3	1		9	28
5	1	Optimal Growth Parameters		1	9		3	13
6								0
7								0
8								0
9								0
		Technical importance score	83	46	47	36	57	269
		Importance %	31%	17%	17%	13%	21%	100%
		Priorities rank	**1**	**4**	**3**	**5**	**2**	

Figure 10A. Quality Functions Diagram

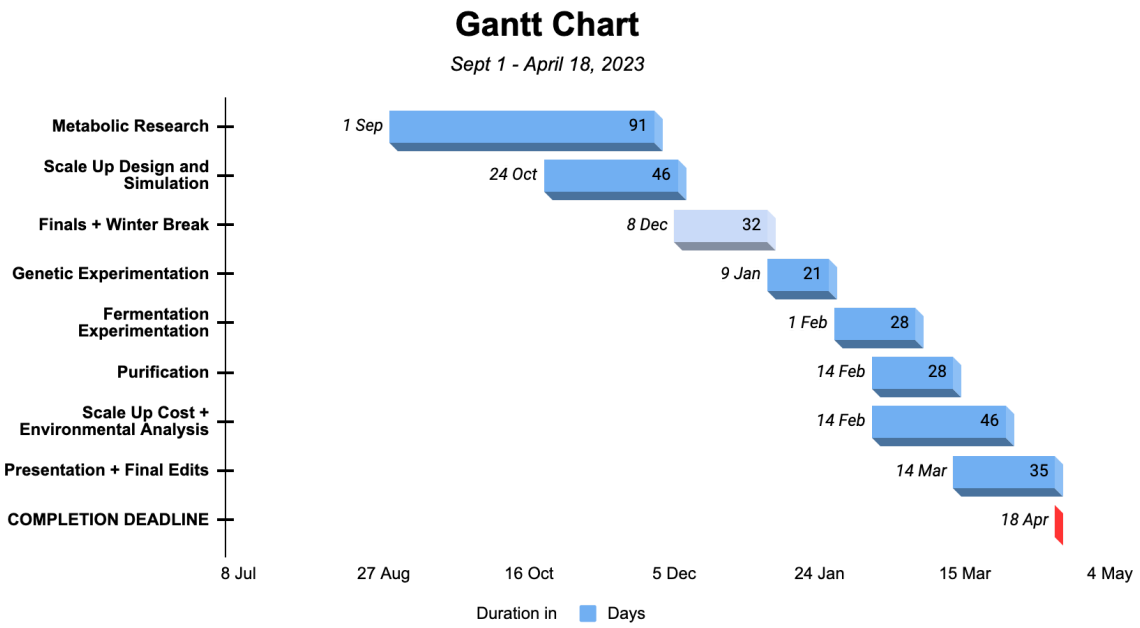

Figure 11A. Gantt Chart

Appendix B

Mass and Energy Balance Python Code:

```
import numpy as np
from scipy.integrate import odeint
import matplotlib.pyplot as plt
# Import rcParams from matplotlib
from matplotlib import rcParams
import seaborn as sns
import math

# Set the seaborn color palette
sns.set_palette("colorblind")

# Set the global font size and family
rcParams.update({"font.size": 16, "font.family": "serif", "font.serif": "DejaVu Serif"})

# Constants
S0 = 39.40  # g/L
X0 = 0.10  # g/L
P0 = 0.0  # g/L
mu_max = 1.2  # 1/hr
Ks = 1  # g/L
Yxs = 0.5
Ypx = 15.05  # find the correct value later
Yxo2 = 1.06  # g O2/g X, assumed yield coefficient for oxygen consumption
Yax = 0.92  # g acetic acid/g X, assumed value, find the correct value later
O2_0 = .008
A0 = 0.0  # g/L
V0 = 79.21  # L
F = 201.34  # L/hr, assumed constant feed rate
T_feed = 298.15  # Feed temperature (K), assuming room temperature (25°C)
Sf = 55.73  # g/L, assumed constant substrate concentration in feed
kLa = 258.13  # h^-1, assumed mass transfer coefficient
O2_sat = 0.0075  # g/L, assumed saturation concentration of oxygen
Vmax = 60000  # L, maximum reactor volume
aspect_ratio = 3
endtime = 24  # hours
timestep = 0.5  # hours

# Inhibition constants
Ki_acetic_acid = 1.2  # g/L, assumed acetic acid inhibition constant
Ki_glucose = 200  # g/L, assumed glucose inhibition constant
K_o2 = .001 #g/L, assumed half saturation constant of oxygen
```

```
# Energy balance constants
heat_of_fermentation_ecoli = -20000  # Heat of fermentation for E. coli (J/g)
heat_transfer_coefficient = 500  # Heat transfer coefficient (W/m^2K)
#reactor_area = 400  # Reactor surface area touching heat exchanger (m^2)
ambient_temperature = 400  # Ambient temperature (K)
specific_heat_capacity = 4.186e3  # J/kg·K
rho = 1 #kg/L
T0 = 298.15  # Initial temperature value (K), assuming room temperature (25°C)
initial_internal_energy = V0 * rho * specific_heat_capacity * T0  # J
agitation_power_per_volume = 10  # W/L

# Heat exchanger area
volume_m3 = Vmax / 1000
D = (6 * volume_m3 / (math.pi * aspect_ratio))**(1/3)
initial_H = V0 / (math.pi * (D/2)**2)  # Initial height of the liquid in the bioreactor (m)
H_max = aspect_ratio * D  # Maximum height of the bioreactor (m)
H_exchanger_max = H_max  # Maximum allowed height for the heat exchanger (m)

def heat_exchanger_area(V):
    V_m3 = V / 1000  # Convert the volume from liters to cubic meters
    H = V_m3 / (math.pi * (D/2)**2)  # Calculate the height of the liquid in the bioreactor based
on its volume
    return 2 * math.pi * (D/2) * H  # Lateral surface area of the cylindrical bioreactor

def pid_controller(Kp, Ki, Kd, setpoint, current_value, integral, derivative, dt):
    error = setpoint - current_value
    P_term = Kp * error
    I_term = Ki * integral * dt
    D_term = Kd * derivative / dt
    return P_term + I_term + D_term

temperature_errors = []

def monod_model(t, y, F, Sf):
    S, X, P, V, O2, A, U, T, int_error = y

mu = mu_max * S / (Ks + S) * (1 - A / Ki_acetic_acid) * (1 - S / Ki_glucose) * O2/(K_o2+O2) #
Monod equation with inhibition terms ADD OXYGEN
q_o2 = Yxo2 * mu  # Oxygen consumption rate

 # Mass balance equations
    dSdt = -mu * X / Yxs + F * (Sf - S) / V
    dXdt = mu * X - F * X / V
    dPdt = mu * X * Ypx - F * P / V
    dVdt = F * (1 - V / Vmax)
```

```
    dO2dt = -q_o2 * X + kLa * (O2_sat - O2)
    dAdt = mu * X * Yax - F * A / V

    # PID controller for the heat exchanger area
    setpoint_temperature = 328.5
    Kp = 852.3879772964561  # Proportional gain
    Ki = 472.39665277036545 # Integral gain
    Kd = 141.95811974998108  # Derivative gain

temperature_errors.append(setpoint_temperature - T)
integral_error = int_error + (setpoint_temperature - T) * timestep

derivative_error = (setpoint_temperature - T) / timestep
heat_exchanger_area_correction = pid_controller(Kp, Ki, Kd, setpoint_temperature, T,
integral_error, derivative_error, timestep
current_reactor_area = heat_exchanger_area(V) + heat_exchanger_area_correction



# Energy balance equation
    dUdt = -X * mu * heat_of_fermentation_ecoli \
      - heat_transfer_coefficient * current_reactor_area * (T - ambient_temperature) \
      + F * rho * specific_heat_capacity * (T_feed - T) \
      + agitation_power_per_volume * V

# Temperature balance equation
    dTdt = dUdt / (V * specific_heat_capacity * rho)
return np.array([dSdt, dXdt, dPdt, dVdt, dO2dt, dAdt, dUdt, dTdt, setpoint_temperature - T])

# Initial conditions
y0 = [S0, X0, P0, V0, O2_0, A0, initial_internal_energy, T0, 0]  # Add initial_internal_energy
and initial_temperature_error here

# Time points
t = np.arange(0, endtime + timestep, timestep)
t_array = t  # Store the time points array for use inside the monod_model function

# Solve the ODE
from scipy.integrate import solve_ivp
result = solve_ivp(lambda t, y: monod_model(t, y, F, Sf), (0, endtime), y0, t_eval=t,
method='BDF')
```

```
# Extract the results
S_result = np.interp(t, result.t, result.y[0, :])
X_result = np.interp(t, result.t, result.y[1, :])
P_result = np.interp(t, result.t, result.y[2, :])
V_result = np.interp(t, result.t, result.y[3, :])
O2_result = np.interp(t, result.t, result.y[4, :])
A_result = np.interp(t, result.t, result.y[5, :])
U_result = np.interp(t, result.t, result.y[6, :])
T_result = np.interp(t, result.t, result.y[7, :])

heat_exchanger_area_result = np.array([heat_exchanger_area(V) for V in V_result])

logoblue = "#5CB9F2"
logolightblue = "#5EF2C8"
logogreen = "#68F205"
logored = "#F20505"
logodarkred = "#8C0303"

# Plot the mass balance results
fig1, ax1 = plt.subplots(figsize=(14, 10))
ax1.plot(t, S_result, label="Substrate (S)", linewidth=4, color=logolightblue)
ax1.plot(t, X_result, label="Biomass (X)", linewidth=4,  linestyle='--', zorder=10,
color=logogreen)
ax1.plot(t, P_result, label="Product (P)", linewidth=4, color=logoblue)
ax1.plot(t, O2_result, label="Oxygen (O2)", linewidth=4, color=logored)
ax1.plot(t, A_result, label="Acetic Acid (A)", linewidth=4, color=logodarkred)

ax1.set_xlabel("Time (hours)", fontsize=20, labelpad=15)
ax1.set_ylabel("Concentration (g/L)", fontsize=20, labelpad=15)
ax1.set_title("Fed-batch Reactor Monod Model: Mass Balance", fontsize=24, pad=20)

ax1.legend(loc='best', fontsize='x-large', shadow=True, framealpha=1, edgecolor='black')
ax1.grid(True, linestyle='--', linewidth=0.5)
ax1.tick_params(axis='both', which='major', labelsize=18)

fig1.savefig("fed_batch_reactor_monod_mass_balance.png", dpi=300)

# Plot the energy balance results
fig2, ax2 = plt.subplots(figsize=(12, 8))
ax2.plot(t, T_result, label="Temperature (T)", linewidth=2)

ax2.set_xlabel("Time (hours)")
ax2.set_ylabel("Temperature (K)")
```

```
ax2.set_title("Fed-batch Reactor Monod Model: Energy Balance")
ax2.legend()
ax2.grid()
min_temp = np.min(T_result)
max_temp = np.max(T_result)

#ax2.set_ylim(min_temp * 0.9999, max_temp * 1.0001)

fig2.savefig("fed_batch_reactor_monod_energy_balance_heat_exchanger_area.png")



# Display final values
final_values = result.y[:, -1]  # Get the final values from the last column of the result.y array

print("Final values:")
print(f"Substrate (S): {final_values[0]:.2f} g/L")
print(f"Biomass (X): {final_values[1]:.2f} g/L")
print(f"Product (P): {final_values[2]:.2f} g/L")
print(f"Volume (V): {final_values[3]:.2f} L")
print(f"Oxygen (O2): {final_values[4]:.5f} g/L")
print(f"Acetic Acid (A): {final_values[5]:.2f} g/L")
print(f"Internal Energy (U): {final_values[6]:.2f} J")
print(f"Temperature (T): {final_values[7]:.2f} K")
```

Profit Optimization Python Code:

```
import numpy as np
from scipy.integrate import odeint
import matplotlib.pyplot as plt
from deap import base, creator, tools, algorithms
import random

# Existing Monod model code (monod_model function and constants) should be placed here

# Constants
S0 = 27.5  # g/L
X0 = 1.8  # g/L
P0 = 0.0  # g/L
mu_max = 1.2  # 1/hr
Ks = 1  # g/L
Yxs = 0.5
Ypx = 15.05  # find the correct value later
Yxo2 = 1.06  # g O2/g X, assumed yield coefficient for oxygen consumption
Yax = 0.92  # g acetic acid/g X, assumed value, find the correct value later
O2_0 = .008
A0 = 0.0  # g/L
```

```
V0 = 3  # L
F = 170  # L/hr, assumed constant feed rate
Sf = 100  # g/L, assumed constant substrate concentration in feed
kLa = 250  # h^-1, assumed mass transfer coefficient
O2_sat = 0.0075  # g/L, assumed saturation concentration of oxygen
Vmax = 60000  # L, maximum reactor volume
endtime = 24  # hours
timestep = 0.5  # hours

# Inhibition constants
Ki_acetic_acid = 1.2  # g/L, assumed acetic acid inhibition constant
Ki_glucose = 200  # g/L, assumed glucose inhibition constant
K_o2 = .001 #g/L, assumed half saturation constant of oxygen

def monod_model(y, t, F, Sf):
    S, X, P, V, O2, A = y
mu = mu_max * S / (Ks + S) * (1 - A / Ki_acetic_acid) * (1 - S / Ki_glucose) * O2/(K_o2+O2)

 # Monod equation with inhibition terms ADD OXYGEN
    q_o2 = Yxo2 * mu  # Oxygen consumption rate

# Mass balance equations
    dSdt = -mu * X / Yxs + F * (Sf - S) / V
    dXdt = mu * X - F * X / V
    dPdt = mu * X * Ypx - F * P / V
    dVdt = F * (1 - V / Vmax)
    dO2dt = -q_o2 * X + kLa * (O2_sat - O2)
    dAdt = mu * X * Yax - F * A / V

return [dSdt, dXdt, dPdt, dVdt, dO2dt, dAdt]

# Initial conditions
y0 = [S0, X0, P0, V0, O2_0, A0]

# Time points
t = np.arange(0, endtime + timestep, timestep)

# Solve the ODE
result = odeint(monod_model, y0, t, args=(F, Sf), rtol=1e-6, atol=1e-6)

# Objective function to optimize
def objective_function(individual):
    S0_opt, X0_opt, V0_opt, Sf_opt, kLa_opt, F_opt = individual
    y0 = [S0_opt, X0_opt, P0, V0_opt, O2_0, A0]
    result = odeint(monod_model, y0, t, args=(F_opt, Sf_opt))
    P_result = result[:, 2]
```

```
    # Calculate profit
    final_product_mass = P_result[-1] * V0_opt
    total_glucose_mass_fed = F_opt * Sf_opt * endtime
    profit_product = final_product_mass * 127  # $/kg
    cost_glucose = (S0_opt * V0_opt + total_glucose_mass_fed) * 0.56  # $/kg
    cost_biomass = X0_opt * V0_opt * 78400  # $/kg

    profit = profit_product - cost_glucose - cost_biomass
    return profit,

# Set up DEAP components
creator.create("FitnessMax", base.Fitness, weights=(1.0,))
creator.create("Individual", list, fitness=creator.FitnessMax)

toolbox = base.Toolbox()
toolbox.register("S0", random.uniform, 10, 50)
toolbox.register("X0", random.uniform, 0.1, 10)
toolbox.register("V0", random.uniform, 0, 15000)
toolbox.register("Sf", random.uniform, 50, 150)
toolbox.register("kLa", random.uniform, 100, 400)
toolbox.register("F", random.uniform, 50, 500)
toolbox.register("individual", tools.initCycle, creator.Individual, (toolbox.S0, toolbox.X0,
toolbox.V0, toolbox.Sf, toolbox.kLa, toolbox.F), n=1)

toolbox.register("population", tools.initRepeat, list, toolbox.individual)

toolbox.register("mate", tools.cxTwoPoint)
toolbox.register("mutate", tools.mutGaussian, mu=0, sigma=10, indpb=0.1)
toolbox.register("select", tools.selBest)
toolbox.register("evaluate", objective_function)

def custom_mutate(individual, mu, sigma, indpb):
    for i in range(len(individual)):
        if random.random() < indpb:
            individual[i] += random.gauss(mu, sigma)
            if i == 0:  # S0
                individual[i] = max(min(individual[i], 50), 10)
            elif i == 1:  # X0
                individual[i] = max(min(individual[i], 10), 0.1)
            elif i == 2:  # V0
                individual[i] = max(min(individual[i], 15000), 0)
            elif i == 3:  # Sf
                individual[i] = max(min(individual[i], 150), 50)
            elif i == 4:  # kLa
                individual[i] = max(min(individual[i], 400), 100)
```

```
return individual,

toolbox.register("mutate", custom_mutate, mu=0, sigma=5, indpb=0.1)

def optimize_model(ngen, population_size, cxpb, mutpb):
    pop = toolbox.population(n=population_size)
    hof = tools.HallOfFame(1)
    stats = tools.Statistics(lambda ind: ind.fitness.values)
    stats.register("avg", np.mean)
    stats.register("min", np.min)
    stats.register("max", np.max)

    pop, logbook = algorithms.eaSimple(pop, toolbox, cxpb=cxpb, mutpb=mutpb, ngen=ngen,
stats=stats, halloffame=hof, verbose=True)

return hof[0]

optimized_params = optimize_model(ngen=50, population_size=100, cxpb=0.8, mutpb=0.2)
S0_opt, X0_opt, V0_opt, Sf_opt, kLa_opt, F_opt = optimized_params
total_glucose_mass_fed = F_opt * Sf_opt * endtime
print("Optimized parameters:")
print(f"Initial glucose concentration (S0): {S0_opt:.2f} g/L")
print(f"Initial biomass concentration (X0): {X0_opt:.2f} g/L")  # Print optimized biomass
concentration
print(f"Initial volume (V0): {V0_opt:.2f} L")
print(f"Glucose concentration in the feed (Sf): {Sf_opt:.2f} g/L")
print(f"Mass transfer coefficient (kLa): {kLa_opt:.2f} h^-1")
print(f"Optimized feed rate (F): {F_opt:.2f} L/hr")
```

PID Constant Optimization Code:

```
import numpy as np
from pyswarm import pso
from scipy.integrate import odeint
import matplotlib.pyplot as plt
# Import rcParams from matplotlib
from matplotlib import rcParams
import seaborn as sns
import math

# Constants
S0 = 39.40  # g/L
X0 = 0.10  # g/L
P0 = 0.0  # g/L
mu_max = 1.2  # 1/hr
Ks = 1  # g/L
```

```
Yxs = 0.5
Ypx = 15.05  # find the correct value later
Yxo2 = 1.06  # g O2/g X, assumed yield coefficient for oxygen consumption
Yax = 0.92  # g acetic acid/g X, assumed value, find the correct value later
O2_0 = .008
A0 = 0.0  # g/L
V0 = 79.21  # L
F = 201.34  # L/hr, assumed constant feed rate
T_feed = 298.15  # Feed temperature (K), assuming room temperature (25°C)
Sf = 55.73  # g/L, assumed constant substrate concentration in feed
kLa = 258.13  # h^-1, assumed mass transfer coefficient
O2_sat = 0.0075  # g/L, assumed saturation concentration of oxygen
Vmax = 60000  # L, maximum reactor volume
aspect_ratio = 3
endtime = 24  # hours
timestep = 0.5  # hours

# Inhibition constants
Ki_acetic_acid = 1.2  # g/L, assumed acetic acid inhibition constant
Ki_glucose = 200  # g/L, assumed glucose inhibition constant
K_o2 = .001 #g/L, assumed half saturation constant of oxygen

# Energy balance constants
heat_of_fermentation_ecoli = -20000  # Heat of fermentation for E. coli (J/g)
heat_transfer_coefficient = 500  # Heat transfer coefficient (W/m^2K)
reactor_area = 400  # Reactor surface area touching heat exchanger (m^2)
ambient_temperature = 300  # Ambient temperature (K)
specific_heat_capacity = 4.186e3  # J/kg·K
rho = 1 #kg/L
T0 = 298.15  # Initial temperature value (K), assuming room temperature (25°C)
initial_internal_energy = V0 * rho * specific_heat_capacity * T0  # J
agitation_power_per_volume = 10  # W/L



# Heat exchanger area
volume_m3 = Vmax / 1000
D = (6 * volume_m3 / (math.pi * aspect_ratio))**(1/3)
initial_H = V0 / (math.pi * (D/2)**2)  # Initial height of the liquid in the bioreactor (m)
H_max = aspect_ratio * D  # Maximum height of the bioreactor (m)
H_exchanger_max = H_max  # Maximum allowed height for the heat exchanger (m)



# Define an objective function that takes the PID parameters as input
def objective_function(pid_params):
```

```
    Kp, Ki, Kd = pid_params

    # Set up and solve the ODE with the given PID parameters
    # (insert your existing code to simulate the bioreactor with the given PID parameters)

    def heat_exchanger_area(V):
        V_m3 = V / 1000  # Convert the volume from liters to cubic meters
        H = V_m3 / (math.pi * (D/2)**2)  # Calculate the height of the liquid in the bioreactor
based on its volume
        return 2 * math.pi * (D/2) * H  # Lateral surface area of the cylindrical bioreactor

    def pid_controller(Kp, Ki, Kd, setpoint, current_value, integral, derivative, dt):
        error = setpoint - current_value
        P_term = Kp * error
        I_term = Ki * integral * dt
        D_term = Kd * derivative / dt
        return P_term + I_term + D_term

    temperature_errors = []


    def monod_model(t, y, F, Sf):
        S, X, P, V, O2, A, U, T, int_error = y


        mu = mu_max * S / (Ks + S) * (1 - A / Ki_acetic_acid) * (1 - S / Ki_glucose) *
O2/(K_o2+O2) # Monod equation with inhibition terms ADD OXYGEN
        q_o2 = Yxo2 * mu  # Oxygen consumption rate

        # Mass balance equations
        dSdt = -mu * X / Yxs + F * (Sf - S) / V
        dXdt = mu * X - F * X / V
        dPdt = mu * X * Ypx - F * P / V
        dVdt = F * (1 - V / Vmax)
        dO2dt = -q_o2 * X + kLa * (O2_sat - O2)
        dAdt = mu * X * Yax - F * A / V

        # PID controller for the heat exchanger area
        setpoint_temperature = 328.5
        Kp = 10000000  # Proportional gain
        Ki = 0 # Integral gain
        Kd = 0  # Derivative gain

        temperature_errors.append(setpoint_temperature - T)
        integral_error = int_error + (setpoint_temperature - T) * timestep
```

```
        derivative_error = (setpoint_temperature - T) / timestep
        heat_exchanger_area_correction = pid_controller(Kp, Ki, Kd, setpoint_temperature, T,
integral_error, derivative_error, timestep)
        current_reactor_area = heat_exchanger_area(V) + heat_exchanger_area_correction



        # Energy balance equation
        dUdt = -X * mu * heat_of_fermentation_ecoli \
          - heat_transfer_coefficient * current_reactor_area * (T - ambient_temperature) \
          + F * rho * specific_heat_capacity * (T_feed - T) \
          + agitation_power_per_volume * V

        # Temperature balance equation
        dTdt = dUdt / (V * specific_heat_capacity * rho)

       return np.array([dSdt, dXdt, dPdt, dVdt, dO2dt, dAdt, dUdt, dTdt, setpoint_temperature - T])



    # Initial conditions
    y0 = [S0, X0, P0, V0, O2_0, A0, initial_internal_energy, T0, 0]  # Add initial_internal_energy
and initial_temperature_error here


    # Time points
    t = np.arange(0, endtime + timestep, timestep)
    t_array = t  # Store the time points array for use inside the monod_model function



    # Solve the ODE
    from scipy.integrate import solve_ivp
    result = solve_ivp(lambda t, y: monod_model(t, y, F, Sf), (0, endtime), y0, t_eval=t,
method='BDF')

    # We'll minimize the mean squared error between the desired setpoint and the actual
temperature
    setpoint_temperature = 328.5
    T_result = result.y[7]  # Extract temperature values (index 7 corresponds to dTdt)
    mse = np.mean((T_result - setpoint_temperature)**2)


return mse

# Set bounds for the PID parameters
```

```
lb = [0, 0, 0]  # Lower bounds for Kp, Ki, and Kd
ub = [1000, 1000, 1000]  # Upper bounds for Kp, Ki, and Kd

# Optimize the PID parameters using the particle swarm optimization algorithm
best_pid_params, _ = pso(objective_function, lb, ub)

# Extract the optimized PID parameters
Kp_optimized, Ki_optimized, Kd_optimized = best_pid_params

print("Optimized Kp:", Kp_optimized)
print("Optimized Ki:", Ki_optimized)
print("Optimized Kd:", Kd_optimized)
```