COVID-19 Virus Extraction (Snow Lab)

A senior design report submitted to

the Faculty of

The Department of Chemical and Biological Engineering

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by

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Executive Summary

This project seeks to create a novel material for packing in waste water filtration devices for collection of SARS CoV-2 viral particles. Whereas previous products in this industry have used polymers to collect viral genomic material, this report explores the use of porous protein crystals with spike protein binding domains. These binding domains are concentrated inside of porous protein crystals, which are able to store viral material. The use of E. Coli to produce protein for porous protein crystals and binding domains leads to a highly economical process with a high return for investors. Additionally, the use of entirely organic materials means that there is very little risk to the environment stemming from use of the proposed devices.

Chapter 1. Project scope and timeline

The objective of this project is to design a filter for extracting SARS CoV-2 viral particles from wastewater using a porous protein crystal containing a binding protein specific to SARS CoV-2. In order to accomplish this, there are several components which must be engineered. There is an existing protocol for creating porous protein crystals from the bacterial protein Campylobacter jejuni(CJ) using a sitting drop technique. CJ proteins have been demonstrated to form hexagonal crystals with regularly spaced 13 nm pores throughout the surface. These pores have been noted to collect substrates from solution beyond the concentration that would be expected through diffusion through non-covalent interactions. However, in order to optimize a filtration device, it is necessary to design a method for covalent installation of a binding domain. A CJ variant, CJ^{OPT}, has been previously designed with an exposed cysteine residue within each crystalline unit cell. (Huber et al., 2018) These thiol binding sites allow for the installation of a guest molecule under oxidizing conditions. While this reaction is reversible, an irreversible variant may be engineered using an additional linker molecule, such as dibromobimane. Installing a viral binding domain into these crystals using one of these methods will create a dense packing material with the ability to extract and concentrate viral particles for later sequencing and testing.

Origin and motivation

This work is motivated by the growing need for wastewater testing during the Covid-19 pandemic. Wastewater testing is an efficient method of tracking outbreaks without the need for individual testing. Collection of viral particles also allows for variant detection and tracking on a large scale. However, existing materials are expensive, and are quite labor intensive. Loading a viral binding domain in a porous protein crystal is a relatively inexpensive, scalable method for wastewater testing.

Research on this topic is ongoing across many regions and countries, including at Colorado State University. Dr. Susan De Long of the Civil Engineering Department has spearheaded wastewater testing on and around campus using a variety of testing sites. These tests can give advance notice of outbreaks, as individuals do not need to present themselves at a given location for testing. Furthermore, wastewater testing includes samples from those infected individuals who are asymptomatic, and therefore would not know they require a test. (DeLoss, 2020) Cities around the world would benefit from this easy and reliable means of monitoring outbreaks before they can spread further. Additionally, the design of the porous protein crystals, which are able to covalently bind to any guest molecule containing a thiol group, makes this technique easily and quickly applicable to any future viruses of interest.

The viral binding domain selected for this project is the P8 binding domain: an engineered Ace-2 receptor mimic designed to bind to the spike protein on SARS CoV2 with high affinity. (Karoyan et al., 2021) This binding domain will be installed throughout the porous protein crystals, which in future may be packed into a filtration device and submerged in wastewater to collect a representative sample.

Product or service delivered (Problem definition)

The product to be manufactured in this project is a porous protein crystal containing a viral binding domain that will, in the future, be packed inside of filters and used in wastewater testing. This product will be significant as it is a mass producible means of filtering and collecting viral particles from solution. Given the ongoing pandemic, this is invaluable, as it allows cities to track outbreaks and variants on a large scale. The design of this device will be highly selective for Covid-19, and able to be quickly altered to target a different virus or variant.

Project goals

- Install dibromobimane in CJ^{OPT} Greg crystals
- Verify installation of spike binding domains using confocal microscopy
- Verify loading of P8 peptide using fluorescently labeled peptides
- Design and analyze a bioprocess model for scale-up

Project timeline

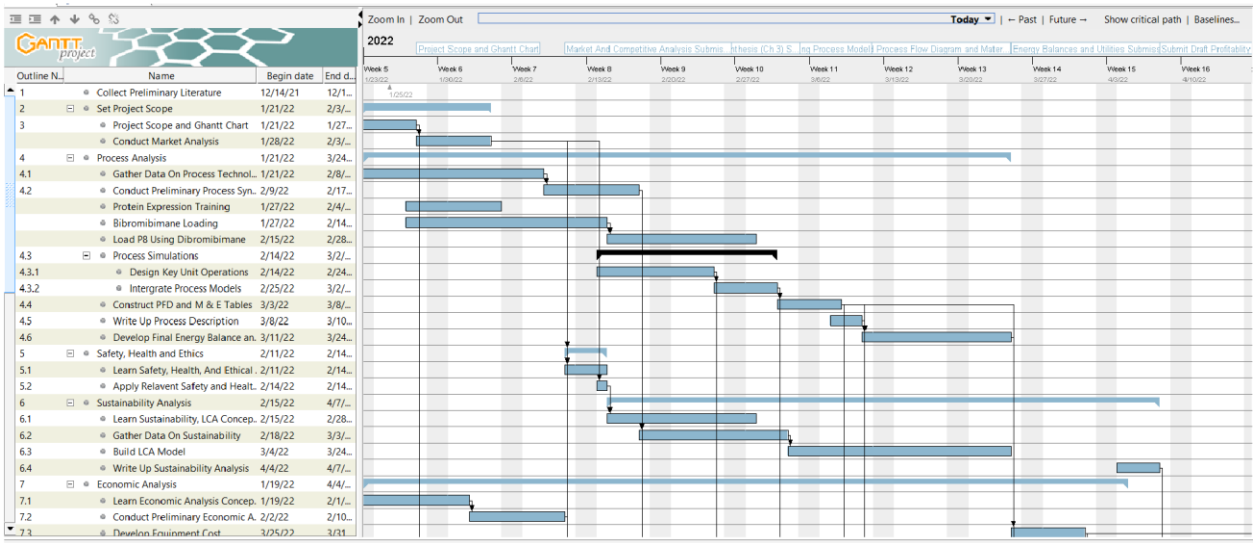

Figure 1. Project goals, objectives and timeline. Tasks will be assigned to team members on an ongoing basis according to research progress.

The Gantt chart above outlines the key milestones and deliverables that need to be met throughout the semester. The main activity taking precedence is laboratory experiments which will help determine a narrower approach to both the process and economic analyses. As a relatively small team, we plan to take a combined approach at the beginning of the semester and as research progresses we will evaluate the possibility of assigning individual tasks.

Design constraints

There are several design constraints to be considered over the course of this project. Firstly, many tasks undertaken in a laboratory setting are highly labor intensive and require specialized training to complete. These requirements often render scale up and mass production difficulties. To overcome these difficulties, we will consider alternative methods of scale up, such as using bioreactors in place of shake flask expressions. It is also important to consider health and safety concerns with respect to the Escherichia coli(E.coli) used for expression. While a modified, less dangerous version is used for all expressions, there is still a possibility of infection and mutation. For these reasons, several safety measures are in place. All individuals present in the lab must wear safety glasses, closed-toed shoes, long pants, and lab coats. Furthermore, all bacterial cultures, and containers that have contained live bacteria will be sterilized with bleach and either washed, or disposed of in hazardous waste as is appropriate. Any other hazardous waste, such as the nickel used for metal affinity columns, will be disposed of using proper hazardous waste protocols so as to avoid any environmental impact.

Methods and tools

Laboratory experiments

To complete this project, several laboratory experiments were needed. Firstly the CJ^{OPT} variant was crystallized. This is a variant of the wild type CJ protein that includes a cysteine residue, which is capable of forming disulfide bonds. All protein used was expressed in E.coliusing a five liter scale shake flask expression of BL21 gold cells. Various conditions, including salt concentration, protein concentration, and pH must be optimized to crystallize this protein. Figure two shows the molecular structure of CJ^{OPT}, with cysteine residues, and therefore possible binding sites highlighted in teal.

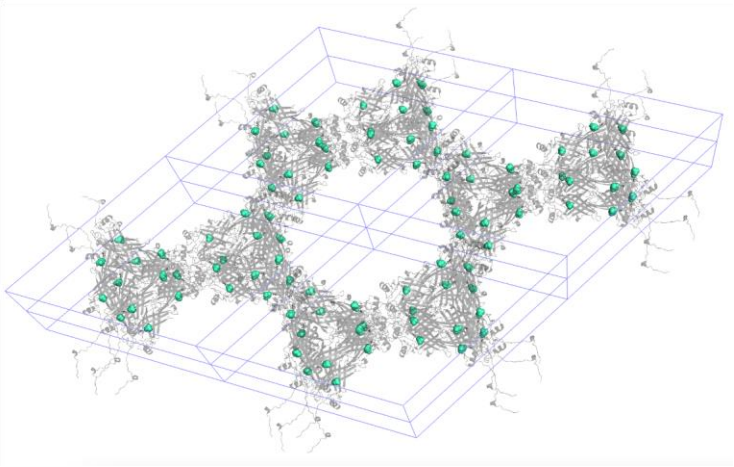

Figure 2. Crystalline structure of porous protein crystals. Unit cells highlighted in blue, and binding sites highlighted in cyan.

The first experimental milestone was to validate the availability of the cysteine binding sites within the protein crystals. Due to the ability of cysteine residues to form disulfide bonds, there is a possibility that in the process of crystallization, dimers could be formed which render the crystals incapable of participating in additional reactions. This validation was completed using dibromobimane, a molecule which contains two bromines which may act as leaving groups in a substitution reaction. The removal of a single bromine in the process of binding to a cysteine residue within the porous protein crystals generates an increase in fluorescence, and therefore validates the availability of the binding sites. The addition of a second nucleophilic group to displace the second bromine causes a further increase in fluorescence, and may be used as an irreversible installation method. As this is a novel technique, several methods were explored, including installing the dibromobimane before and after crystallization. Installation will be verified using the inherent fluorescence of this molecule after a bromine has been removed. Following this validation, two methods were explored to install the viral binding domain. The P8 peptide may be irreversibly installed using a second SN2 reaction with dibromobimane as a linker molecule. Alternatively, the P8 peptide may be modified to include a thiol group which is able to bind directly to the cysteine residues. After consideration, the latter method was selected as it opens the possibility of reusable porous crystals through exploiting the reversible nature of a disulfide bond.

Process analysis

Process analysis was conducted both in SuperPro, for the purposes of selecting and optimizing equipment, and in Excel for the purposes of economic analyses. SuperPro allows for facile simulation of a variety of biological processes, and for an exploration of various bioreactor sizes and temperatures. SuperPro also serves as a source of pricing information, especially in relation to process units. Some limitations for this method of modeling include the fact that SuperPro requires many parameters as an input, such as centrifugation efficiency, and crystallization yield. While the values input were based on experimental findings from the Snow Lab, they were collected during processes on a much smaller scale than is proposed in the SuperPro model. The scaled up processes could lead to differing efficiencies and yields that are difficult to produce at a smaller scale.

Economic analysis

To conduct a rigorous economic analysis, current materials prices were collected from common scientific supply sources, such as Thermo Fisher. Required quantities were modeled using data taken from both

SuperPro analysis, and from data collected using 2 L bioreactors in the Snow Lab. Sales data was modeled through using similar products, such as the Ceres particle, and cost data collected from the current wastewater testing team here at CSU.

Life cycle analysis

For this product, a rigorous life cycle analysis will be difficult due to the novelty of this technique, and the demand for viral waste water testing.

This product will likely be fairly robust, and there are strategies that could be implemented to improve the lifespan of a single device. One such method would be to use a reversible binding method for attaching the covid binding domains to the porous protein crystals. While irreversible installation methods have been the focus of this project, it would be relatively simple to use a disulfide installation method, which is reversible under reducing conditions. Porous protein crystals, once chemically crosslinked, have been demonstrated to remain stable for a long period of time, and in a variety of different conditions. This stability could be utilized to design partially reusable devices. Should a used device be returned to a manufacturing facility, the crystals could be exposed to a reducing agent that would cause the covid binding domains, and any bound viral particles, to leave the crystals. The crystals could then be incubated in a fresh store of the viral binding domains, and the devices would be ready for redistribution. This would greatly improve the life cycle and sustainability of the devices.

The equipment purchased for use in production would also likely have a long life cycle. The production process for porous protein crystals takes place at entirely moderate temperatures, and at atmospheric pressure. Furthermore, there are no extremely corrosive materials used. This would mean that should proper preventative maintenance be conducted regularly, the equipment would not experience a great deal of stress, and would not have to be replaced often.

The combination of the possibly reusable devices and the low strain level on equipment means that this process and these products could have a long life cycle, making it a viable investment both economically and environmentally.

Chapter 2. Market and competitive analysis

The current market for viral wastewater testing has become quite expansive with the recent successes in Covid-19 wastewater surveillance. Monitoring viral particles in wastewater allows communities to get a general sense of infection as a whole and doesn't rely on individuals getting tested. Furthermore, it allows community leaders to make informed decisions based on the trends they see in the wastewater tests. Implementing a surveillance wastewater system is quite attractive to businesses such as universities, correctional facilities, nursing homes, and cities with dense populations. The monetary impact an outbreak could have on these facilities is potentially detrimental which has been seen throughout the past few years. Covid-19 outbreaks have the potential to stop everyday life and change the way society lives. It causes jobs to be lost, restaurants to lose business, and the economy to drop. Having the ability to track an outbreak and stop it from affecting an entire city, university, or facility prevents the severity of pandemics which is why viral wastewater testing is important going forward. Given that it is a brand new market, it is likely that companies and research entities will be introducing projects to explore and build upon methods of viral wastewater testing in the near future. Not only is it urgent to develop an efficient and effective method of measuring Covid-19 in wastewater, but developing a method that can be adapted in the future to other viral particles is important to keep in mind for future market uses.

While this market is quite new, there is one notable competitor in thIS space: the Ceres virus capture kits.
Ceres particles are able to capture viral particles out of solution, and have been used in combination with RT-PCR to track viral outbreaks.These capture kits aim to concentrate and extract the nucleic acid from the Covid-19 particles (Virus Capture Kits | CeresNanosciences, Inc. | United States, n.d.) The Ceres virus capture kits still require a large sample of wastewater to be obtained. This requires a more labor-intensive and unsafe process which this project's technology aims to reduce. Furthermore, these particles have not worked well for Colorado State University's

wastewater testing efforts, and are quite expensive. Because this project's method will be composed mainly of protein, the cost per unit once scale-up has been completed will likely be relatively low. However, Ceres has received several grants in recent months to scale up and improve their product, so they present significant competition. It will be important in the coming months to monitor this market, as it is quickly evolving.

More broadly, this project's product will also enter into the protein capture market. For this, there are several established techniques. One common technique for removing proteins specifically from solution is a packed resin column. In this technique, a metal, commonly nickel, is loaded to binding sites on microscopic resin beads and packed in a flow-through column. One major producer, Sigma Aldrich, sells a resin for His-Tag mediated purification for $367 for 25 mL, or $2,480 for 200 mL (Complete™ His-Tag Purification Resin Suspension, Suitable for Protein Purification, Matrix Sepharose-CL 6B | Sigma-Aldrich, n.d.). Aside from the high price tag, there are limitations to this technology. His-Tags, or histidine tags, are a segment of repeating histidine residues. They are commonly used because it is easy to add this feature to an existing protein, and they do not tend to interfere with protein structure or function. Furthermore, they have a relatively high affinity for metals such as nickel, which allows for easy purification. However, these repeating segments are not typically found in wild type proteins. This project's product will likely be less expensive upon scale up, and can be specified to many different viruses in their natural states.

Another common existing technique for viral particle detection is the ELISA assay. In this technique, immobilized enzymes are used to extract viral particles from solution. While this technique is very reliable, the reagents are very expensive and they can be difficult to set up for each virus. Moreover, centrifugal ultrafilsV-2 particles from wastewater (Anderson-Coughlin et al., 2021). This technique is utilized in combination with RT-qPCR, to perform particle-size dependent membrane filtration and virus quantification. While this technique has been historically quite successful at isolating other types of viral particles from wastewater, it is not an effective means of extracting SARS-CoV-2 particles. Centrifugal ultrafiltration utilizes ddPCR, which is a technology that is widely unavailable and requires additional development.

The technology this project aims to achieve will use materials and technologies that are more easily accessible. Additionally, when the engineered crystals are packed into a filter, this will allow the testing method to be less labor-intensive as well as safer for the personnel conducting the collection.
Furthermore, the technology created in this project for the wastewater testing devices has the ability to be adapted for future viruses. This would allow society as a whole to be more prepared for the next pandemic and would reduce the severity of the effects it has on society's everyday lives. For these reasons, we believe that this project's product will be competitive in today's market.

Chapter 3. Preliminary process synthesis

Summary of data-gathering for process synthesis

Review of relevant published literature, patents and reports

There is a great deal of literature that is relevant to this project. Primarily, in the last year, there has been rapid progress regarding various means of detecting the spread of SARS-CoV-2. One promising emerging strategy is waste water testing. The Centers for Disease Control and Prevention (CDC) have accelerated research into this strategy. This technique captures viral material shed from infected individuals and concentrates it. Regular sampling of wastewater allows researchers to track outbreaks without requiring individuals to be tested. Furthermore, wastewater testing detects outbreaks from asymptomatic individuals who may not realize they require a test. Wastewater testing is also a promising long term measure to prevent ongoing outbreaks that will not interfere in the general populaces' lives. (CDC, 2022) Currently, Ceres Nano manufactures particles used in these filtration devices. According to a patent filed in 2019, the Ceres product is a small particle composed of specialized polymers .(US20090087346A1 - Method for Harvesting Nanoparticles and Sequestering Biomarkers - Google Patents, 2013)

With respect to our product, there are several relevant published articles. One critical publication is that describing the design of the P8 peptide, which was developed in 2020. (Karoyan et al., 2020) The P8 peptide is a partial mimic of the hACE2 receptor, which is known to bind to the SARS-CoV-2 spike protein. Through computational prediction and experimental validation, the P8 peptide was designed to bind the spike protein with extremely high affinity. (Karoyan et al., 2020) Another relevant category of literature is that describing the chemistry behind dibromobimane, which we are using to install the P8 peptide in porous protein crystals. Dibromobimane is a chemical crosslinker that uses an SN2 reaction to bind specifically to thiol groups. This binding causes an increase in fluorescence, making it easily detectable. (Kim & Raines, 1995)

In sum, relevant literature shows that there is a space in the market for a novel technique, and that the dibromobimane installation of a P8 peptide is a viable method. Therefore, it is reasonable to proceed with experimental testing to validate this strategy.

Summary of design options considered

To produce a complete project, there are two main process flows: experimental and computational. The experimental process flow entails experimentally validating the installation of the P8 peptide within porous proteins crystals, and the computation process flow entails designing a mass-production model for these crystals. To optimize each of these components, many different designs were considered.
Figure 3 shows the basic process components required for the synthesis of protein crystals..

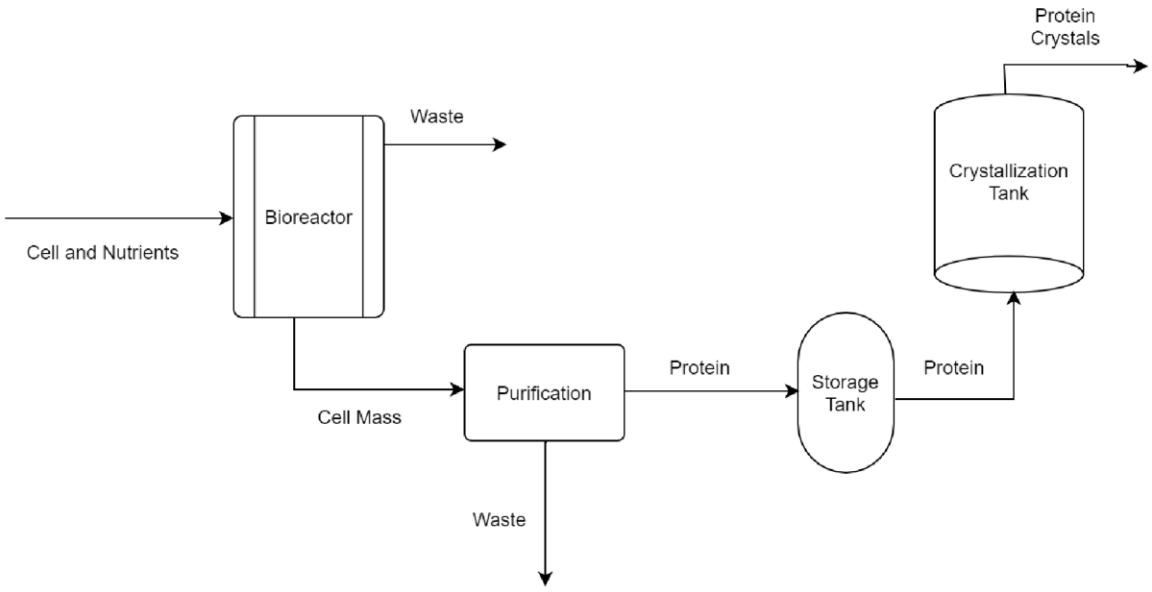

Figure 3. Block flow diagram showing basic components of producing protein crystals.

The primary required component is a bioreactor, in which E. coli cells containing a plasmid encoding the genetic information for CJ^{OPT} will be cultured. E coli was selected due to the wide range of literature available in relation to its growth. This will allow for easy condition selections and troubleshooting should any issues arise. This reactor could be either a batch reactor, or a continuously stirred tank reactor (CSTR). As summarized in table 1, there are appealing facets to both options.

Table 1: Batch Reactor versus CSTR.

Batch Reactor	Continuously Stirred Reactor (CSTR)
Larger volumes	Smaller Volumes
Easy Operation	Better Temperature Control
Variability in Product	Continuous Process
Reach High Conversion	High Mixing Rates

After consideration, for the sake of this project a batch reactor was selected. This choice was made due to the reproducibility of batch conditions, and the high degree of control regarding occupancy time.

Several options were also considered to optimize the purification process. Design choices were made using the current protein purification protocol in use in the Snow Lab. Changes were made to this existing protocol, such as omitting a dialysis step, due to the difficulties of scaling up these processes. The units selected for purification were selected in an attempt to balance the purity requirements for effective protein crystallization with minimizing the initial cost of equipment.

Process flowsheet options

To optimize viral binding domain-containing crystals, there are many processes in the design of the final product that should be considered. The three main processes include peptide installation, and dibromobimane installation. P8 peptide installation can be achieved either covalently or noncovalently. These choices are summarized in figure 4 below.

Figure 4. Methods of peptide installation decision tree.

A non-covalent installation could be achieved using the noted propensity of porous protein crystals to collect small molecules within the crystal pores. While this option would be the fastest and the most economical, the small molecule retention under varying conditions and over a long period of time could be unpredictable. A second option is to use an irreversible covalent installation. This could be achieved using dibromobimane as a linker molecule for a series of two SN2 reactions. This option is appealing as the dibromobimane facilitates a very strong bond that would make the crystals extremely durable. Furthermore, dibromobimane shows an increase in fluorescence following the removal of each bromine. This would make installation very easy to validate. Even if not used as a linker, dibromobimane serves as a useful tool for validating the availability of binding sites within the crystals. The final option is to use a reversible covalent installation through a disulfide bond between a thiol residue on the P8 peptide and a cysteine residue in the crystal pores. This option is appealing as the viral binding domains could be installed and removed many times to allow for reusable devices. The chemistry involved in these covalent installations is demonstrated below.

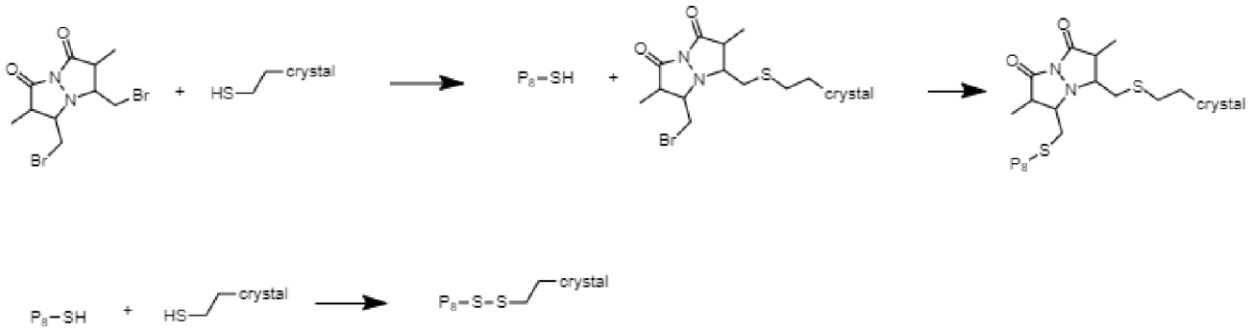

Figure 5. Chemical routes for covalent peptide installation. Dibromobimane facilitated irreversible covalent installation is shown above the method of disulfide-mediated reversible covalent installation

In considering these processes, it is important to note intermediates and byproducts. These are important factors, as they can be potentially dangerous. Process intermediates and byproducts are shown below.

Table 2: Raw Materials, Intermediates, and Byproducts

Raw Materials	Intermediates	Byproducts*
Dibromobimane	P8-SH	Excess ammonium sulfate
Ammonium Sulfate	SH-crystal	Excess protein
Bis Tris Buffer		

As this table demonstrates, these processes do not involve any dangerous chemicals, and therefore all choices are favorable for worker and environmental safety.

Should dibromobimane be used, there are several options for its installation. Primarily, it can be installed before or after crystallization. Installing the dibromobimane prior to crystallization is an appealing option as should it bind to peptides in solution prior to crystallization, it could prevent the formation of disulfide bonds between CJ monomers that would hinder the crystallization process. This facet raises further issues regarding the concentration of dibromobimane relative to protein concentration. A reaction at low protein concentrations and high dibromobimane concentrations increases the likelihood that a CJ monomer would encounter and bind with a dibromobimane molecule prior to forming a dimer through a disulfide reaction. However, performing the reactions at these concentrations requires an extra step of concentrating the protein. This could be accomplished using an amicon filter, or with dialysis driven by a high concentration of polyethylene glycol outside a membrane. Installation following crystallization is also a valid option as it allows for imaging of the loading. Recording an increase in fluorescence is an easy way to validate the availability of binding sites within the crystals. In this case, the dibromobimane concentration must also be carefully chosen. Excess dibromobimane would lead to more favorable conditions for each crystalline cysteine to be reacted with dibromobimane, but could also lead to excess fluorescence which would make measurements difficult. For this reason, a 1:1 molar ratio may be more reasonable. These choices are summarized below.

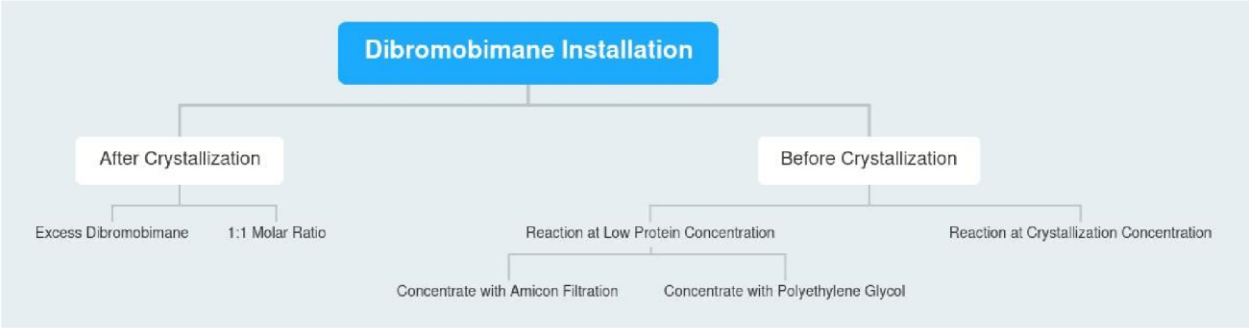

Figure 6. Synthesis tree representing options for dibromobimane installation either before or after crystallization.

Through consideration and trials, the procedure selected was to use a disulfide-mediated reversible covalent installation of the peptide. However, dibromobimane was retained as a method of validating the availability of binding sites within the protein crystals. These options were selected as it allows for the possibility of reusable devices, which increases the sustainability of the product. However, using dibromobimane as a validation method allows for a more robust data set and understanding of the chemistry at play within the crystal pores.

Summary of laboratory data collected as part of the project

This project requires a great deal of experimental data. The first data collected was the conjugation of dibromobimane to thiol sites in CJ^{OPT} porous protein crystals. As the dibromobimane installation causes an increase in fluorescence, this installation may be validated using confocal microscopy. CJ^{WT}, which does not contain a thiol, was used as a control to validate specific binding. Dibromobimane installation validates the availability of thiol binding sites within the CJ^{OPT} crystals for binding to viral binding domains. The chemistry involved in this reaction is shown below in Figure 7. To test this installation, one CJ^{WT} crystal and one CJ^{OPT} crystal were incubated in a solution containing dibromobimane and imaged using a spinning disk confocal microscope using laser excitation. The results of this trial are shown below.

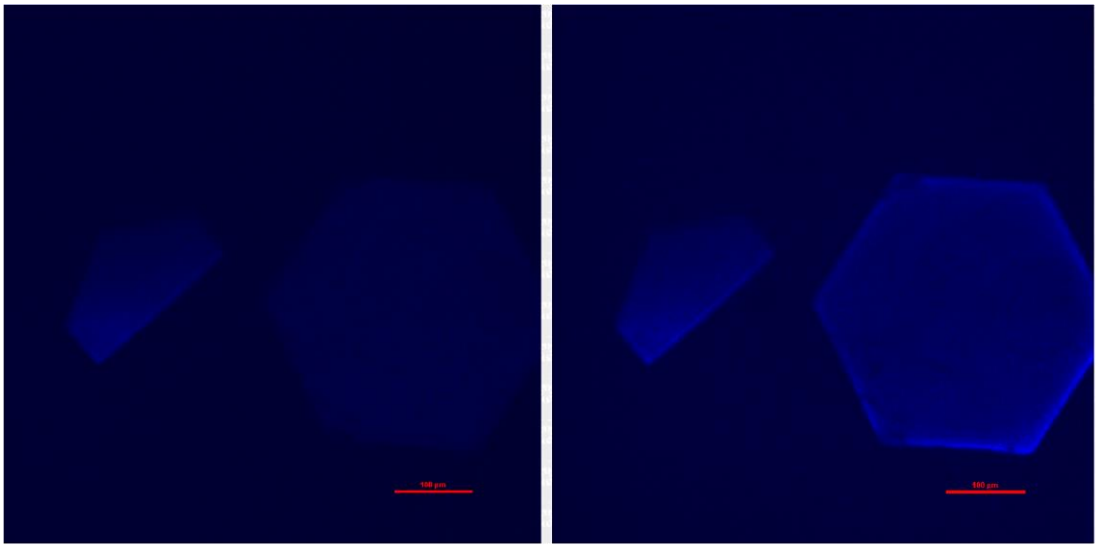

Figure 7. Control crystal (left) and crystal with binding domains (right) incubated with binding-dependent fluorescent molecule (30 mins).

As shown in figure 7, the fluorescence of the control crystal remains static throughout the 30 minute time series while the crystal containing cysteine residues shows a dramatic increase in fluorescence. This increase confirms that increased fluorescence is dependent upon bromine displacement, and demonstrates the availability of the thiol groups within the CJ^{OPT} crystals for participating in reactions.

The next experimental data collected was to validate the binding of P8 peptides within CJ^{OPT} crystals. For this trial, a version of the P8 peptide which was labeled with a red-fluorescent fluorophore, and had an additional thiol group was obtained. A CJ^{OPT}crystal was incubated in this solution for 15 minutes, and imaged throughout using spinning disk microscopy and laser excitation. The results of this experiment are shown below.

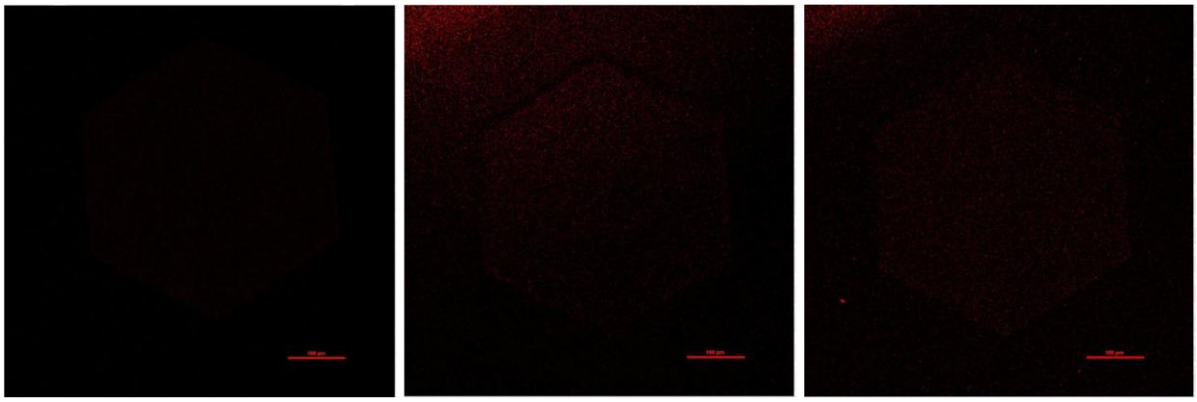

Figure 8. Loading of fluorescently-labeled viral binding domain. Preloading (left), upon introduction of binding domain (middle), and following 15 min incubation (right).

The image on the far left in figure 8 is an image taken of the crystal prior to loading the peptide into solution. This image serves as a baseline, as it shows that there is no autofluorescence. The middle image shows the time at which the fluorescent peptide was introduced into the solution. The peptide is visible as a cluster of small red particles in the upper left corner of the image. As shown in the third image, over the span of a 15 minute time series, the fluorescently labeled peptides congregated within the crystal, demonstrating the ability of these crystals to bind the viral binding domains. The viral binding domains were able to bind within the crystal through a disulfide bond, formed between the peptides and a cysteine residue in the crystal.

The combination of these two experimental results demonstrates the high potential of this design to produce usable viral-collection devices that could be used around the world in wastewater testing infrastructure.

Effect of design constraints on process synthesis choices

Environmental considerations

Wastewater is typically treated in wastewater treatment plants for use as freshwater. However, the presence of SARS-CoV-2 particles in wastewater not only increases the likelihood of wastewater acting as a transmission pathway for COVID-19 infections. It also decreases the availability of freshwater because of the difficulty associated with using current technologies to completely remove the virus from wastewater. This protein engineering project will help in designing a virus trap that will, ideally, capture all viral particles in wastewater and help in the long-term mitigation of viral wastewater load. Due to its focus on removing SARS-CoV-2 particles, this project does not harm the environment, but rather takes into account environmental factors as project objectives. Furthermore, all materials used in this filter are protein, and would therefore not be harmful to the environment even should there be an issue that causes materials to be released into the environment.

The primary environmental consideration for this project is to ensure that the engineered crystals are capable of capturing high amounts of viral particles. To do this, different wastewater samples or different viruses have to be tested in the presence of the crystals and analyzed using confocal microscopy (as mentioned earlier). Another environmental factor being considered is the time it would take for a wastewater sample to be completely clear of viral particles, to determine the impact on freshwater recycling processes.
Moreover, a preliminary life cycle assessment (LCA) is critical for the design of this project to ensure that the scale-up of this project will allow for successful removal of viral particles. Additionally, the LCA will allow for the determination of the cost-effectiveness of this technology, especially when considering the cost of implementing and maintaining other technologies, like the Ceres capture kits, over long periods of time. The highest potential for

environmental waste in this product lies in the filter disposal. Therefore, it is critical to ensure that the designs are as high quality as possible so as to avoid degradation. It is also important to explore options which make the filters reusable, such as the reversible method of viral binding domain installation.

Safety and health considerations

There are multiple potential hazards and risks associated with this project. Specifically speaking, the scale-up of this technology requires bioreactors, which must be operated and maintained at a steady temperature and pressure (once the optimal values have been determined). To ensure that the bioreactors are operated properly, all team members must have a thorough understanding of how bioreactors work, and how to identify potential faults in the bioreactor itself. Additionally, all team members must be aware of the potential safety hazards associated with the use of the chemicals in this project, and must always wear PPE (gloves, safety glasses, long pants, lab coat) when working with these chemicals. A thorough review of the MSDS's of the primary chemicals that will be used in this project yields the following safety information:

Ammonium sulfate (3-4 M): skin, eye, and respiratory irritant
Bis-Tris buffer (0.1 M, pH 5.5-6.5): very mild irritant
Dibromobimane: skin irritant

Prior to working on the design of this project, it is highly important to conduct a HAZOP analysis. This analysis will not only be critical in determining other potential hazards that have not yet been identified, but will also help in understanding how to mitigate any safety issues that arise throughout the course of this project. Additionally, a HAZOP analysis will allow for the determination of safer techniques that could be substituted in place of the ones currently being implemented for the process design. In any production facility, it is important to put into place clear standard operating procedures to mitigate any possible spills or other hazards.

Broader societal considerations

In general, this project has a myriad of societal goals. The primary goal of this project is to reduce the impacts of the COVID-19 pandemic, and introduce a new, cost-effective technology that can be sustainable in the long-term. Other goals include being able to eventually utilize this technology for other viruses, and implementing this technology worldwide especially in communities that lack the resources necessary to mitigate the effects of the pandemic.

Chemical data

Table 3: Principal Thermophysical and Transport Property Data for Principal Chemicals

Chemical	Property*							
	Molar Mass [g/mol]	pH	Solubility [g/mL]	Melting Point [ºC]	Boiling Point [ºC]	Heat Capacity (specific or molar)	Density [g/cm^3]	Index of Refraction
Ammonium Sulfate	132.13	5.0 - 6.0	0.77	280	330	144.7 [J/mol*K]	1.13	1.396
Bis Tris Buffer*	209.24	5.8 - 7.2	0.50	102	-	-	-	-

Dibromobimane	271.11	-	-	170	-	-	1.98	1.503
Polyethylene Glycol	6000	4.5-7.5	0.50	60	>250	-	1.2	1.47

*Some information not available. All data obtained from sources listed in the Works Citedsection.

Chapter 4. Process Description

Process overview

The simple block flow diagram below outlines the key process steps involved in making CJ crystals.
These key processes include a batch bioreactor step, a purification step, and a crystallization step. Temperatures are maintained at, or slightly above, room temperature, while pressures are all maintained around 1 atm.

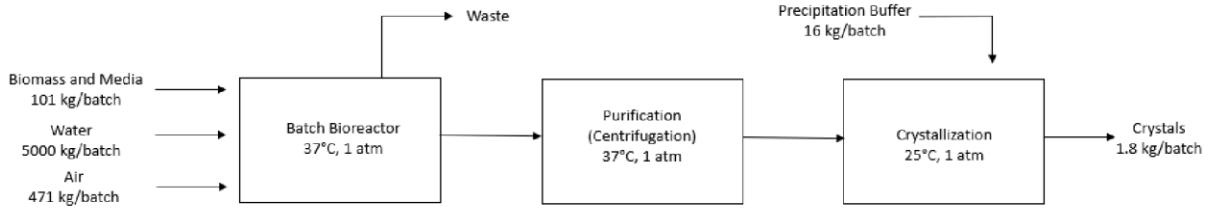

Figure 9. Simple block flow diagram demonstrating the key process steps of making CJ crystals.

Detailed process description

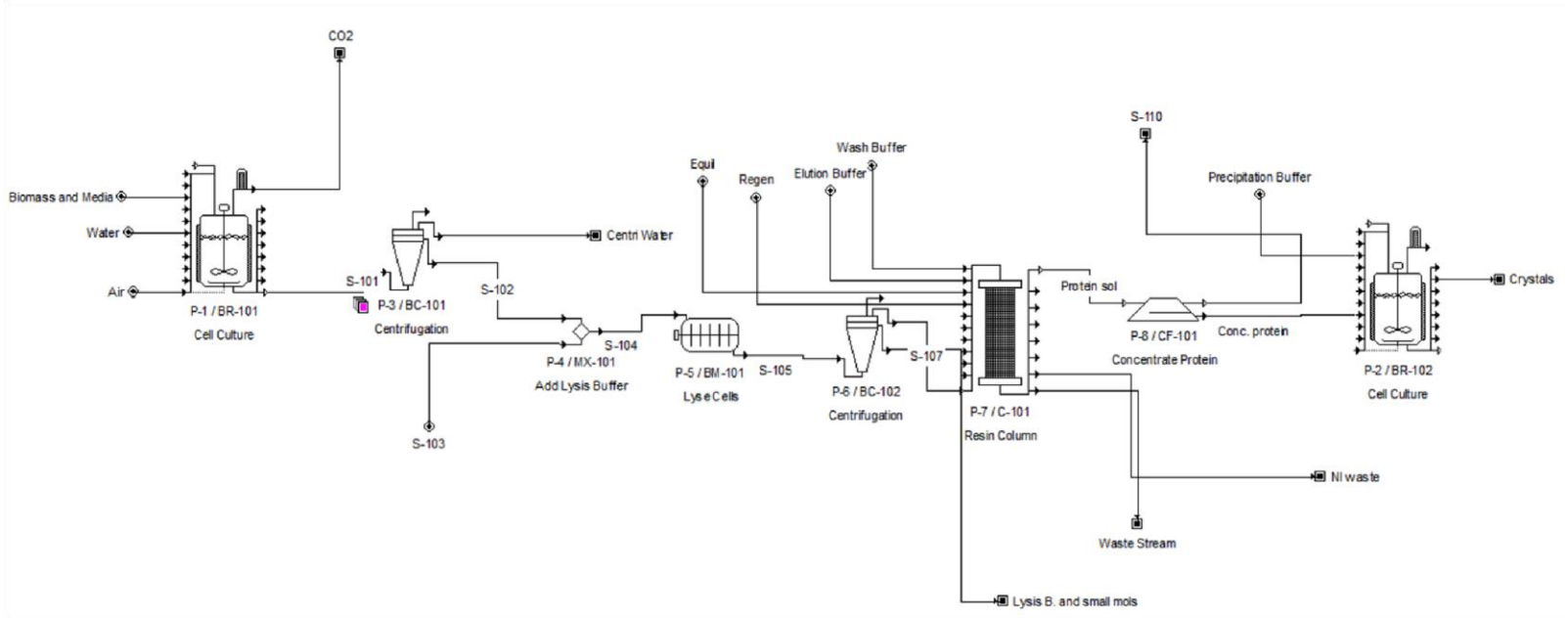

Figure 10. Detailed process flow diagram for production of CJ crystals, designed in SuperPro Designer.

The detailed process flow diagram (shown in Figure 10) demonstrates a bioreactor with three input streams. The bioreactor has two output streams, one of which enters a bowl centrifuge. The output stream from the centrifuge is mixed with a lysis buffer and then fed into the bead mill. The product of the bead mill, lysed cells, then enter into a centrifuge, after which there is a purification process done using the resin column. Finally, the leaving product is fed into another centrifuge and then passed through a bioreactor for crystallization. Pertinent

information concerning the construction and operating parameters of each of the units utilized in the process is given in Table 4. Additionally, the process is described in more thorough detail in the next section of this report.

Table 4: Process Development - Key Equipment Information (sizing, construction and operating conditions).

Equipment	Size (Capacity)	Materials of Construction	Operating Conditions
Bioreactor 1 BR-101	6438.44 L	Stainless steel	• 24 hours operation • 37ºC • aerobic • batch process
Centrifuge 1 BC-101	640.83 L/h	Metal alloys (mainly aluminum and titanium)	• 240 mins operation • 37ºC • 10,000 RCF
Mixer MX-101	24.29 kg/h	Stainless steel	• Rated throughput = 24.28 kg/h
Bead Mill BM-101	0.87 L	Stainless steel	• 85.0% packing density
Centrifuge 2 BC-102	5.84 L/h	Metal alloys (mainly aluminum and titanium)	• 240 mins operation
PBA Column C-101	81.0 L	Stainless steel	• Bed height: 0.250 m • 2.52 hr cycle duration
Centritech Centrifuge CF-101	40.81 L/h	Metal alloys (mainly aluminum and titanium)	• 24.7 hour cycle duration • membrane cutoff of 10 kDa
Bioreactor 2	27.87 L	Stainless steel	• batch process • 25ºC • 24 hours operation*

Detailed functional description of major equipment

The major pieces of equipment required for this process are continuously-stirred bioreactors, centrifuges, a mixer, a bead mill, and a resin column. The first bioreactor (shown in Figure 10as BR-101) serves as a medium for cell growth. The input streams for this bioreactor are biomass and media (i.e., nutrients for cell growth), water, and air. The output stream of this bioreactor has cells (after cell growth) containing the protein of interest, CJ, in their cytoplasm. The continuously stirred bioreactor is ideal for this process because it allows all the cells to have access to their growth nutrients, and as a result, allows for maximization of cell growth. Additionally, the operating conditions of the bioreactor (24-hour operation time at 37ºC) help ensure that the cells are grown in conditions

suitable for maximal proliferation. These operating conditions, which can be input into the SuperPro Designer model, are summarized in Table 4. Table 5: Temperature and Pressure Set for Batch Bioreactor, BR-101.

Unit Operation	BR-101*
Temperature ( )	37
Pressure (bar) ℃	1.52

*Note: This is the only unit operation for which SuperPro Designer allows users to pick temperature and pressure conditions.

The outlet stream from the first bioreactor enters the bowl centrifuge (BC-101 shown in Figure 10). The purpose of this equipment is to ensure that all cell mass is pelletted. A bowl centrifuge is selected for this process because this type of centrifuge separates solutions at high centrifugal forces (10,000 RCF is required in this case). The high centrifugal force allows for the effective pelleting of cell mass, and is comparable to laboratory procedures. The temperature of this unit is maintained at 37ºC so that the cells are not exposed to multiple varying factors at once, and can continue to remain in their ideal growth conditions.

After centrifugation, the supernatant is discarded and the pelleted cell mass is resuspended in a lysis buffer, represented by the mixer, MX-101, in Figure 10. The lysis buffer contains 50 mM HEPES, 500 mM NaCl, 10% glycerol, and 20 mM imidazole at pH 7.4. The purpose of this mixer is to ensure that the cell mass heavily interacts with the lysis buffer, and its rated throughput of 24.28 kg/h helps ensure efficient mixing. The cell mass resuspended in the lysis buffer is then moved into a bead mill (BM-101 in the figure above), which lyses the cells (i.e. disrupts the cell membrane), releasing and solubilizing the protein initially expressed in the cytoplasm of the cells.

The second centrifuge (BC-102 in Figure 10) plays a crucial role in pelleting the solution that comes out of the output stream of the bead mill. This centrifugation step ensures that the protein remains in the supernatant, so that the pellet can be discarded. Similar to before, this centrifuge is run at a high rate of 10,000 RCF to ensure that high amounts of protein are suspended in the supernatant. The supernatant is then utilized in C-101, which is a resin column with a height of 0.250 m. This column's features allow it to concentrate the protein through osmosis, using a size exclusion membrane and a 4M ammonium sulfate solution. The protein solution then goes through the centritech centrifuge, CF-101 in Figure 10, for additional protein concentration. This centrifuge concentrates and purifies the protein using a size exclusion membrane within it, and utilizes a membrane cutoff of 10 kDa.

Finally, the concentrated and purified protein is sent to the second bioreactor, BR-102. This bioreactor is selected so that protein is continuously mixed with ammonium sulfate, and maximal crystallization is achieved. The 24-hour operation time of this bioreactor allows for sufficient crystal formation time, and aids in ensuring that the crystals are uniform. Also, this reactor operates at 25ºC, as per experimental conditions that indicate that 25ºC is the optimal temperature for crystallizing the CJ protein. The bioreactor's operating condition helps ensure that the output quantity ensures approximately 90% yield, which aligns with experimental findings.

Additional information about the process design in SuperPro Designer is provided in the tables below.

Table 6: Material and Energy Balance Table Summarizing Flow Rates for Each Species.

Stream Name	Biomass and Media	Water	Air	CO2
Source	INPUT	INPUT	INPUT	P-1
Destination	P-1	P-1	P-1	OUTPUT
Stream Properties				
Activity(U/ml)	0.00	0.00	0.00	0.00
Temperature (°C)	25.00	25.00	25.00	37.00
Pressure (bar)	1.01	1.01	1.01	1.01
Density (g/L)	995.22	994.70	1.18	1.18
Total Enthalpy (kW-h)	2.95	145.91	3.31	4.91
Specific Enthalpy(kcal/kg)	25.11	25.11	6.05	8.85
Heat Capacity (kcal/kg-°C)	1.00	1.00	0.24	0.24
Component Flowrates (kg/batch)				
Biomass	1.00	0.00	0.00	0.00
Carb. Dioxide	0.00	0.00	0.00	69.78
Media	100.00	0.00	0.00	0.00
Nitrogen	0.00	0.00	361.39	366.05
Oxygen	0.00	0.00	109.71	41.35
Water	0.00	5,000.00	0.00	0.00
TOTAL (kg/batch)	101.00	5,000.00	471.10	477.18
TOTAL (L/batch)	101.48	5,026.62	399,502.87	405,796.30

Stream Name	S-101	Centri Water	S-102	S-103
Source	P-1	P-3	P-3	INPUT
Destination	P-3	OUTPUT	P-4	P-4
Stream Properties				
Activity(U/ml)	0.00	0.00	0.00	0.00
Temperature (°C)	25.00	40.29	40.29	25.00
Pressure (bar)	0.96	0.96	0.96	1.01
Density (g/L)	995.00	989.46	989.41	1,091.44
Total Enthalpy (kW-h)	148.85	238.80	0.62	0.27
Specific Enthalpy(kcal/kg)	25.11	40.39	40.39	20.99
Heat Capacity (kcal/kg-°C)	1.00	1.00	1.00	0.84
Component Flowrates (kg/batch)				
Biomass	29.00	28.94	0.06	0.00
CJ Protein	2.00	0.00	2.00	0.00
Glycerol	0.00	0.00	0.00	3.90
HEPES	0.00	0.00	0.00	0.06
imidazole	0.00	0.00	0.00	0.00
Impurities	10.00	9.98	0.02	0.00
Sodium Chloride	0.00	0.00	0.00	0.30
Water	5,060.00	5,048.82	11.18	6.76
TOTAL (kg/batch)	5,101.00	5,087.73	13.27	11.02
TOTAL (L/batch)	5,126.62	5,141.95	13.41	10.10

Stream Name	S-104	S-105	S-107	Lysis B. and small mols
Source	P-4	P-5	P-6	P-6
Destination	P-5	P-6	P-7	OUTPUT
Stream Properties				
Activity(U/ml)	0.00	0.00	0.00	0.00
Temperature (°C)	34.02	18.00	100.00	100.00
Pressure (bar)	0.96	0.96	0.96	0.96
Density (g/L)	1,032.93	1,039.33	1,006.59	3.26
Total Enthalpy (kW-h)	0.89	0.47	2.41	0.41
Specific Enthalpy(kcal/kg)	31.58	16.75	92.71	185.58
Heat Capacity (kcal/kg-°C)	0.93	0.93	0.93	0.84
Component Flowrates (kg/batch)				
Biomass	0.06	0.06	0.00	0.06
CJ Protein	2.00	2.00	1.80	0.20
Glycerol	3.90	3.90	3.61	0.29
HEPES	0.06	0.06	0.06	0.00
imidazole	0.00	0.00	0.00	0.00
Impurities	0.02	0.02	0.01	0.01
Sodium Chloride	0.30	0.30	0.27	0.02
Water	17.94	17.94	16.63	1.31
TOTAL (kg/batch)	24.28	24.28	22.39	1.90
TOTAL (L/batch)	23.51	23.37	22.24	582.70

Stream Name	Wash Buffer	Elution Buffer	Equil	Regen
Source	INPUT	INPUT	INPUT	INPUT
Destination	P-7	P-7	P-7	P-7
Stream Properties				
Activity(U/ml)	0.00	0.00	0.00	0.00
Temperature (°C)	25.00	25.00	25.00	25.00
Pressure (bar)	1.01	1.01	1.01	1.01
Density (g/L)	1,032.19	1,031.91	999.92	1,012.76
Total Enthalpy (kW-h)	6.91	6.91	4.71	4.67
Specific Enthalpy(kcal/kg)	23.69	23.71	25.01	24.48
Heat Capacity (kcal/kg-°C)	0.94	0.94	1.00	0.98
Component Flowrates (kg/batch)				
EDTA Disodium	0.00	0.00	0.00	2.72
Glycerol	25.08	24.75	0.00	0.00
HEPES	1.43	1.43	0.00	0.00
imidazole	0.09	2.19	0.00	0.00
Nickel	0.00	0.00	0.95	0.00
Sodium Chloride	7.10	7.10	0.00	0.00
Sodium Hydroxid	0.00	0.00	0.00	3.24
Water	217.12	215.27	161.04	158.10
TOTAL (kg/batch)	250.82	250.75	161.99	164.07
TOTAL (L/batch)	243.00	243.00	162.00	162.00

Stream Name	Protein sol	Ni waste	Waste Stream	S-110
Source	P-7	P-7	P-7	P-8
Destination	P-8	OUTPUT	OUTPUT	OUTPUT
Stream Properties				
Activity(U/ml)	0.00	0.00	0.00	0.00
Temperature (°C)	25.57	25.00	29.37	25.57
Pressure (bar)	1.01	1.01	0.96	1.01
Density (g/L)	1,031.41	1,006.34	1,030.83	994.96
Total Enthalpy (kW-h)	4.75	9.37	11.48	3.86
Specific Enthalpy(kcal/kg)	24.26	24.74	27.78	25.66
Heat Capacity (kcal/kg-°C)	0.95	0.99	0.94	1.00
Component Flowrates (kg/batch)				
CJ Protein	1.22	0.00	0.59	0.12
EDTA Disodium	0.00	2.72	0.00	0.00
Glycerol	16.50	0.00	36.95	0.17
HEPES	0.96	0.00	1.97	0.01
imidazole	1.48	0.00	0.82	0.01
Impurities	0.00	0.00	0.01	0.00
Nickel	0.00	0.95	0.00	0.00
Sodium Chloride	4.73	0.00	9.74	0.05
Sodium Hydroxid	0.00	3.24	0.00	0.00
Water	143.52	319.14	305.50	129.16
TOTAL (kg/batch)	168.38	326.05	355.58	129.52
TOTAL (L/batch)	163.26	324.00	344.94	130.18

Stream Name	Conc. protein	Precipitation Buffer	Crystals
Source	P-8	INPUT	P-2
Destination	P-2	P-2	OUTPUT
Stream Properties			
Activity (U/ml)	0.00	0.00	0.00
Temperature (°C)	25.57	25.00	25.00
Pressure (bar)	1.01	1.01	10.15
Density (g/L)	1,174.86	1,369.29	1,225.84
Total Enthalpy (kW-h)	0.88	0.24	1.11
Specific Enthalpy (kcal/kg)	19.57	13.16	17.39
Heat Capacity (kcal/kg-°C)	0.76	0.53	0.69
Component Flowrates (kg/batch)			
Amm. Sulfate	0.00	10.00	10.00
BisTris	0.00	5.00	5.00
CJ Protein	1.09	0.00	0.11
Crystal	0.00	0.00	0.98
Glycerol	16.34	0.00	16.34
HEPES	0.95	0.00	0.95
imidazole	1.44	0.00	1.44
Polyethylene Gl	0.00	1.00	1.00
Sodium Chloride	4.69	0.00	4.69
Water	14.35	0.00	14.35
TOTAL (kg/batch)	38.86	16.00	54.86
TOTAL (L/batch)	33.08	11.68	44.75

Table 7: Additional Equipment Utilization Details obtained from SuperPro Designer

BM-101 (Bead Mill)		
Equipment size was calculated		
Number of Units	1.00	
Number of Standby Units	0.00	
Number of Staggered Units	0.00	
Installation Factor	0.50	
Maintenance Factor	0.10	
Cost Allocation Factor	1.00	
Usage Rate	100.00	$/equipment-h
Availability Rate	100.00	$/h
Material of Construction		SS316
Purchase Cost (system model for Bead Mill)	47,000.00	$/unit
Grinding Volume	0.87	L
Packing Density	85.00	%

BR-102 (Bioreactor)		
Equipment size was calculated		
Number of Units	1.00	
Number of Standby Units	0.00	
Number of Staggered Units	0.00	
Installation Factor	0.30	
Maintenance Factor	0.10	
Cost Allocation Factor	1.00	
Usage Rate	100.00	$/equipment-h
Availability Rate	100.00	$/h
Material of Construction		SS316
Purchase Cost (system model for Bioreactor)	245,000.00	$/unit
Max Volume	40,000.00	L
Min Working/Vessel Volume	0.00	%
Max Working/Vessel Volume	90.00	%
Volume	49.74	L
Height	0.63	m
Design Pressure	1.52	bar
Vessel is constructed according to ASME standards		
Diameter	0.32	m

BR-101 (Bioreactor)		
Equipment size was calculated		
Number of Units	1.00	
Number of Standby Units	0.00	
Number of Staggered Units	0.00	
Installation Factor	0.30	
Maintenance Factor	0.10	
Cost Allocation Factor	1.00	
Usage Rate	100.00	$/equipment-h
Availability Rate	100.00	$/h

Material of Construction		SS316
Purchase Cost (system model for Bioreactor)	1,583,000.00	$/unit
Max Volume	40,000.00	L
Min Working/Vessel Volume	0.00	%
Max Working/Vessel Volume	90.00	%
Volume	6,438.44	L
Height	3.20	m
Design Pressure	1.52	bar
Vessel is constructed according to ASME standards		
Diameter	1.60	m

BC-102 (Bowl Centrifuge)

Equipment size was calculated		
Number of Units	1.00	
Number of Standby Units	0.00	
Number of Staggered Units	0.00	
Installation Factor	0.50	
Maintenance Factor	0.10	
Cost Allocation Factor	1.00	
Usage Rate	100.00	$/equipment-h
Availability Rate	100.00	$/h
Material of Construction		SS316
Purchase Cost (system model for Bowl Centrifuge)	123,000.00	$/unit
Sigma Factor	496.22	m2
The unit is aseptic		

BC-101 (Bowl Centrifuge)

Equipment size was calculated		
Number of Units	2.00	
Number of Standby Units	0.00	
Number of Staggered Units	0.00	
Installation Factor	0.50	
Maintenance Factor	0.10	
Cost Allocation Factor	1.00	
Usage Rate	100.00	$/equipment-h
Availability Rate	100.00	$/h
Material of Construction		SS316
Purchase Cost (system model for Bowl Centrifuge)	292,000.00	$/unit
Sigma Factor	54,436.60	m2
The unit is aseptic		

CF-101 (Centritech Centrifuge)		
Equipment size was calculated		
Number of Units	1.00	
Number of StandbyUnits	0.00	
Number of Staggered Units	0.00	
Installation Factor	0.50	
Maintenance Factor	0.10	
Cost Allocation Factor	1.00	
Usage Rate	100.00	$/equipment-h
Availability Rate	100.00	$/h
Material of Construction		SS316
Purchase Cost (system model for Centritech Centrifuge)	362,000.00	$/unit
Unit Cost of Consumable: Dft Centritech	800.00	$/item
Disposal Cost of Consumable: Dft Centritech	0.00	$/item
Rated Throughput	40.81	L/h

MX-101 (Mixer)		
Equipment size was calculated		
Number of Units	1.00	
Number of StandbyUnits	0.00	
Number of Staggered Units	0.00	
Installation Factor	0.50	
Maintenance Factor	0.10	
Cost Allocation Factor	1.00	
Usage Rate	100.00	$/equipment-h
Availability Rate	100.00	$/h
Material of Construction		CS
Purchase Cost (system model for Mixer)	0.00	$/unit
Rated Throughput	24.28	kg/h

C-101 (PBA Column)		
Equipment size was calculated		
Number of Units	1.00	
Number of Standby Units	0.00	
Number of Staggered Units	0.00	
Installation Factor	0.05	
Maintenance Factor	0.10	
Cost Allocation Factor	1.00	
Usage Rate	100.00	$/equipment-h
Availability Rate	100.00	$/h
Material of Construction		SS316
Purchase Cost (system model for PBA Column)	416,000.00	$/unit
Unit Cost of Consumable: Dft PBA Chrom Resin	1,500.00	$/L
Disposal Cost of Consumable: Dft PBA Chrom Resin	0.00	$/L
Column Diameter	0.64	m
Bed Height	0.25	m
Column Height	0.25	m
Bed Volume	81.00	L
Column Volume	81.00	L

Summary of utility requirements

Table 8: Utility Demands for the Production of CJ Crystals

Power Type	Start (h)	End (h)	Cons. Time per Batch (h)	Power (kW)	Demand kW-h/batch	Usage (%)
Std Power	0.00	33.28	33.28	(14.40)	479.37	
Main Section	0.00	33.28	33.28	(14.40)	479.37	100.0
P-1	9.29	33.28	23.99	(2.58)	61.79	12.9
FERMENT-1	9.29	33.28	24.00	2.57	61.79	12.9
P-3	0.00	4.00	4.00	(45.28)	181.14	37.8
CENTRIFUGE-1	0.00	4.00	4.00	45.28	181.14	37.8
P-5	0.00	6.00	6.00	(33.66)	201.96	42.1
HOMOGENIZE-1	0.00	6.00	6.00	33.66	201.96	42.1
P-6	0.00	4.00	4.00	(2.62)	10.48	2.2
CENTRIFUGE-1	0.00	4.00	4.00	2.62	10.48	2.2
P-8	0.00	4.00	4.00	(6.00)	24.00	5.0
CENTRIFUGE-1	0.00	4.00	4.00	6.00	24.00	5.0

Heat Transfer Agent	Start (h)	End (h)	Cons. Time per Batch (h)	Rate (kg/h)	Amount (kg/batch)	Usage (%)
Chilled Water	0.00	33.57	33.57	(1426.80)	47897.68	
Main Section	0.00	33.57	33.57	(1426.80)	47897.68	100.0
P-1	9.29	33.57	24.28	(972.07)	23601.83	49.3
FERMENT-1	9.29	33.28	24.00	419.58	10069.85	21.0
COOL-1	33.37	33.57	0.20	67659.91	13531.98	28.3
P-5	0.00	6.00	6.00	(4048.74)	24292.46	50.7
HOMOGENIZE-1	0.00	6.00	6.00	4048.74	24292.46	50.7
P-2	0.36	24.36	24.00	(0.14)	3.39	0.0
CRYSTALLIZE-1	0.36	24.36	24.00	0.14	3.39	0.0
Steam	9.09	9.29	0.20	(674.80)	134.96	
Main Section	9.09	9.29	0.20	(674.80)	134.96	100.0
P-1	9.09	9.29	0.20	(674.80)	134.96	100.0
HEAT-1	9.09	9.29	0.20	674.82	134.96	100.0

Table 8 shows that the primary utility demands that must be considered for the process design are heating, cooling, homogenizing, and power consumption. To meet the demands for cooling, a pump connected to the bioreactor setup will be utilized to circulate cooling water throughout the process. To meet the demand for heating, the temperature control knob of the bioreactor will be used. The temperature will be monitored to ensure that minimal heat is lost to the surrounding contents of the bioreactor. If there is a high amount of heat being lost, additional mechanisms for insulation surrounding the steam pipe will be designed and installed. The steps taken to minimize the heating and cooling demands of this process will inherently help minimize the utility demands of the homogenizing steps. Preliminary experiments will be conducted to understand how to account for the power demand of both the bioreactors and the centrifuges. In general, optimizing the size of the units and the bioreactor impeller speed will help curtail some of the power consumption needs of the process.

Important design assumptions and calculations

To design a computational model for scaled-up production of CJ protein crystals, several design considerations were taken into account. Firstly, to calculate cell growth, Monod kinetics were used. Monod kinetics describe bacterial growth as a fraction of a maximum growth rate, dependent on the presence of substrate, and species-specific affinity constant K_m. (Elsevier Science, 2016) Using this equation, the rate of growth, μ, may be calculated for substrate concentration [S] as:

$$\mu = \mu_{max} \frac{[S]}{K_s + [S]}$$

For the purposes of this model, S is assumed to be the growth media. Experimentally, this is an enriched form of LB media. In the model, this is represented as “media”. While this model provides a useful overview of cell growth, there are some notable limitations. Firstly, this model assumes the system is at a steady state, in which all intermediates have a functional concentration of 0. (Elsevier Science, 2016) Secondly, this model is most accurate in the exponential growth phase, which, while a significant portion of growth, does not describe the entire life cycle of the cells. However, for the purposes of this design project, the approximation is suitable. In this model, μ_{max} was set to 0.2 1/h, and K_S was set to 35 mg/L. These values are commonly used to model E. coligrowth. To further refine the model, these values could be experimentally verified using the BL21 gold E.colistrain, which was used in this project to express CJ protein.

A production process was modeled using SuperPro Designer. For the process, several units were chosen to model the process. Firstly, a batch model was selected. This is because it lends itself well to bacterial growth, such that all

grown cells and their produced protein may be processed as a unit. For the bioreactor, a continuously stirred model was selected (BR-101 in Figure 10). The continuous stirring will ensure a uniform distribution of nutrients, and thus uniform growth. The temperature of the reactor was set to 37 ºC- a temperature which has been experimentally demonstrated to facilitate bacterial growth. Cells, media, and water are combined in this reactor. The initial cell to media ratio is 1:100. This is intended to provide the cells with enough media to spend the majority of the subsequent 24-hour growth period in the exponential phase, which will produce the most cells. Additionally, air is sparged into the tank to provide oxygen to the cells.

Following growth, the cells are sent to a bowl centrifuge unit (BC-101 in Figure 10). This unit is utilized as it provides the most similar configuration to the centrifuge used to express cells experimentally. Process time on this unit is set to 240 minutes- the total experimental process time.

The final step of the modeled process is to incubate the produced protein in a high salt concentration in a second batch bioreactor (BR-102 in Figure 10). This unit is temperature controlled at 25 ºC, and is continuously stirred. The protein is mixed inside with a 4 M ammonium sulfate solution to facilitate crystallization. The bioreactor unit allows for uniform conditions throughout the tank.

Chapter 5: Techno-economic analysis

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Fixed capital investment summary

There are many financial considerations for this project that merit careful analysis. To assemble a process capable of mass-production of porous protein crystals, substantial fixed capital investments are needed. For the purposes of this analysis, all facilities are assumed to be indoors in Colorado. This assumption was made as Colorado was the site of development for many of these processes, and therefore the cost and environmental factors used in this report are the most relevant for this state. Furthermore, all units are designed for a process at atmospheric pressure, and moderate temperatures ranging from 25-37 °C. The equipment recommended for purchase below are not rated for higher temperatures and pressures to minimize the initial required investment.

The largest initial investment would be devoted to equipment purchase and assembly. The required units for purchase are summarized in Table 9. All pricing data is taken from SuperPro Designer, which uses price indices taken from the Chemical Engineering Plant Cost Index to extrapolate to current unit costs (Intelligen). For the purposes of this analysis, the costs of pipes and valves were neglected, as those are highly variable, and would be relatively small in comparison to the overall cost. Furthermore, all calculations were performed for a final protein crystal output of roughly 1 kg/batch.

Table 9: Summary of units and cost generated using SuperPro Designer.

Main Equipment				
Quantity/ Standby/ Staggered	**Name**	**Description**	**Unit Cost ($)**	**Cost ($)**
1 / 0 / 0	BR-101	Bioreactor Vessel Volume = 6438.44 L	1,583,000	1,583,000
1 / 0 / 0	C-101	PBA Column Column Volume = 81.00 L	416,000	416,000
1 / 0 / 0	CF-101	Centritech Centrifuge Rated Throughput = 40.81 L/h	362,000	362,000
2 / 0 / 0	BC-101	Bowl Centrifuge Throughput = 640.83 L/h	292,000	584,000
1 / 0 / 0	BR-102	Bioreactor Vessel Volume = 49.74 L	245,000	245,000
1 / 0 / 0	BC-102	Bowl Centrifuge Throughput = 5.84 L/h	123,000	123,000
1 / 0 / 0	BM-101	Bead Mill Bead Volume = 0.87 L	47,000	47,000
		Unlisted Equipment		840,000
			TOTAL	4,200,000

As shown in Table 9, the most significant equipment investment is for the bioreactor. This unit is critical for porous protein crystal production, because in this vessel bacteria are cultured to produce protein. The high price is likely due to the large volume, which allows for a high throughput of protein, and a return on investment in the long term. The prices estimated for all vessels assume stainless steel construction. This material was selected as it will have minimal corrosion through large volumes of production. This will also allow for easy cleaning when units require sanitation, which should be performed carefully due to the involvement of bacteria in production. These assumptions and materials lead to a total bare module cost (TBM) of $4,200,000. This cost could be reduced by selecting different materials of construction, or by adjusting unit volume. However, based on the calculations described previously in this report, the chosen vessel volumes provide optimal production. For further calculations, an order of magnitude method was selected. This was deemed the appropriate method due to the early stage of this project. Therefore, all cost estimates mentioned in the following paragraphs are assumed to be within 50% of

the correct final value. This deviation may result from shifting cost or unconsidered factors. The first financial metric calculated with this methodology was the total direct permanent investment (DPI), which accounts for the sum of the TBM, site preparation, service facilities, and utilities. (Seider, 2019) This calculation assumed an indoor facility and a grass-roots plant. Based on these calculations, the DPI calculated was $10,920,000. Continuing with the order of magnitude methodology, the total depreciable capital (TDC) was calculated as 140% of the DPI. This additional 40% cost increase accounts for contingencies and contractor fees. Using the order of magnitude estimate, the TDC was calculated to be $15,300,000. Similarly, an additional 10% was added to calculate the total permanent investment, TPI. This 10% accounts for the costs related to royalties, land, and start-ups, and brings the total to $16,400,000. (Seider, 2019) Finally, the total capital investment, TCI, was calculated as 115% of TPI. This calculation brings the TCI to $18,800,000. These values are summarized in Table 10.

Table 10: Summary of cost metrics.

Metric	Cost (USD)
TBM	4,200,000
DPI	10,900,000
TDC	15,300,000
TPI	16,400,000
TCI	18,800,000

In summary, as detailed in the table above, there are significant costs associated with building this facility. While the cost of equipment is relatively low, the cost of building and beginning production in a new facility adds significant costs to the project. These considerations are opportunities for optimization as this project is refined.

Operating cost summary

In addition to the initial fixed capital investment, there are costs associated with day-to-day operation. One component of these costs is the cost associated with materials supplied to the process. Table 11, generated using SuperPro, summarizes the materials consumed both in a single batch, and annually. The annual calculations assume 227 batches run annually, which amounts to 5 batches run each week, for a typical working week of Monday through Friday, 45 weeks each year. The assumed downtime of approximately 65 days per year provides a conservative cost estimate and allows for repairs and maintenance.

Table 11: Material use per batch and per year.

Material	kg/yr	kg/batch
Air	106,940	471.10
Amm. Sulfate	2,270	10.00
Biomass	227	1.00
BisTris	1,135	5.00
EDTA Disodium	618	2.72
Glycerol	12,198	53.74
HEPES	665	2.93
imidazole	518	2.28
Media	22,700	100.00
Nickel	216	0.95
Polyethylene Gl	227	1.00
Sodium Chloride	3,291	14.50
Sodium Hydroxid	735	3.24
Water	1,307,132	5,758.29
TOTAL	1,458,873	6,426.75

Each input material has an associated purchase cost. Current price data was gathered from Sigma Aldrich, Thermo Fisher, and Colorado utilities databases to estimate total cost. The costs estimated below likely provide a high-end estimate, as they do not account for the typical decrease in prices associated with purchasing materials in bulk. The calculated costs are summarized in Table 12.

Table 12: Itemized cost of materials.

Material	Cost/Batch (USD)	Cost/Year (USD)
E coli	$0.26	$60
TB broth	$4,400	$998,800
Ammonium Sulfate	$800	$181,600
Ampicillin	$2,000	$454,000
Glycerol	$7,700	$1,747,900
HEPES	$1,900	$431,300
Imidazole	$228,000	$51,756,000
Sodium Chloride	$1,000	$227,000
Water	$7	$1,600
Nickel	$2,500	$567,500

EDTA 0.5 M	$800	$181,600
Sodium Hydroxide 0.5M	$150	$34,100
BisTris	$2,400	$544,800
Polyethylene Glycol 1500	$120	$27,200
Total	$251,800	$57,158,600

As shown in Table 12, the total estimated annual cost of materials is roughly $57,200,000. Within this, the most significant material costs are TB broth, glycerol, and imidazole. While these are critical materials, and could not be entirely omitted from the process to reduce the cost, trials could be performed to decrease the concentrations used to ultimately lower the overall cost. The price of TB broth in particular could also be reduced by using a similar broth, such as LB broth. While this could cause a decline in cell mass yield, it could be worth the price reduction.

Additional operational costs stem from personnel. To ensure smooth operation, two operators per shift were accounted for, with 5 shifts per day. Furthermore, one of those operators is responsible for technical assistance with manufacturing, and for the control laboratory. (Seider, 2019) Overall, 2 operators are needed at each of the five shifts per day. This totals to 10 operators per day. The results of these calculations are summarized below in Table 13.

Table 13: Itemized personnel costs.

Category	Cost (USD)
Direct Wages and Benefits	$624,000
Direct Salaries and Benefits	$93,600
Operating Supplies and Services	$37,440
Technical Assistance to Manufacturing	$52,000
Control Laboratory	$57,000
Total	$864,040

The final accounted cost is utility cost. The amounts shown below in Table 14 reflect the energy consumption for the production process, and the associated costs.

Table 14: Production goals and estimated sales summary.

Utility	Unit Cost ($)	Annual Amount	Ref. Units	Annual Cost ($)
Std Power	0.10	136,022	kW-h	13,602
Steam	12.00	31	MT	368
Chilled Water	0.40	10,873	MT	4,349
TOTAL				18,319

Combining materials, personnel, and utility costs yields a total operating cost of approximately $59,000,000 annually. This cost is for batch production. While other methods, such as continuous and semi batch were considered, they are not practical for protein crystallization due to changing conditions throughout the process, and were therefore discarded.

From this materials investment, there is a significant product yield. If the process assumes 227 annual batches, the protein crystal yield is roughly 225 kg of crystals. As each crystal is capable of concentrating a significant amount of viral material, each wastewater filtration device would need only a small amount of crystals.

In sum, there would be significant operating costs associated with operating this facility as shown below in Table 15. However, these high costs could realistically be offset by selling the product, due to the high demand for viral detection globally. Furthermore, this price would likely decrease, as bulk purchasing costs tend to be lower than those advertised for smaller amounts. For these reasons, this could be a viable process.

Table 15: Summary of overall costs.

Category	Cost (USD)
Materials	58,000,000
Personnel	900,000
Utility	18,000
Total	$59,000,000

Profitability analysis

In order to further analyze the feasibility of this venture, a profitability analysis was conducted. For this analysis, pricing was set at $16 per device, as per information provided by Dr. Susan De Long, who oversees the wastewater testing efforts at Colorado State University. This device estimate takes into account current wastewater testing methods used by Dr. Susan De Long's team. As the current production model only accounts for the production of porous protein crystals, and not of the filtration devices themselves, a value of $8 for the crystals per device was used for analysis. Furthermore, each device was assumed to require 1 mg of crystals. This value was selected given the low detection threshold of modern diagnostic techniques, and the ability of porous protein crystals to collect a relatively large amount of viral material per crystal. Furthermore, the plant was assumed to be built over a period of 3 years to account for current supply chain shortages, and the low availability of labor. For the first year following construction, the plant was assumed to be operating at 50% capacity, and in the second year at 75% capacity. The assumed operating capacities were chosen to allow the facility to ease into production of the protein crystals. This allows the facility to smooth out the operating process and make any necessary adjustments before full capacity is reached. Additionally, a 10 year class life was used for depreciation analyses using the Modified Accelerated Cost Recovery System (MACRS) method. A middle ground class life of 10 years was used for this analysis given the longevity of the equipment chosen for the process. For the period between 2024 and 2040,

which includes both plant construction and plant operation, cash flow analyses were conducted as a measure of fiscal success. As shown in Figure 11, there is a significant increase in cash flow over the period analyzed.

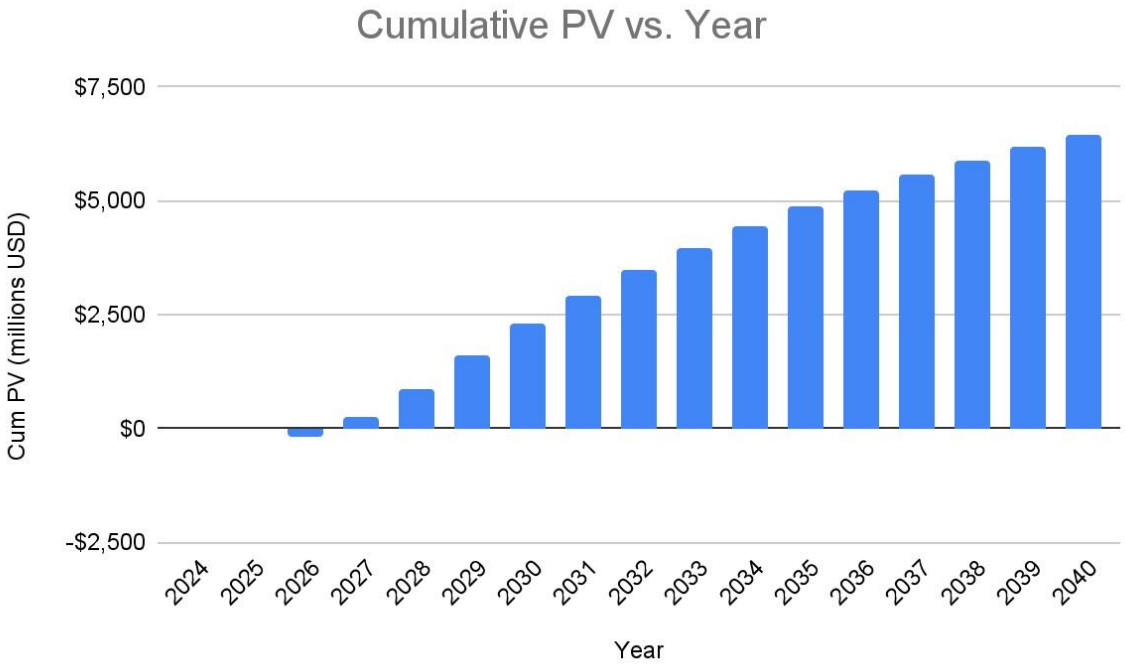

Figure 11. Cumulative PV vs. year beginning at initial investment.

The increasing cash flow trend is likely due to the high market cost of specialized purified protein products, and the relatively low cost required for production in bioreactors. As all pressures are atmospheric, and all temperatures have a range of 25-37 ºC, there is not a high unit cost. This leads to a large return for initial investors, with an return on investment of 4.98 units, a payback period of 0.0121 years, and an investor's rate of return of 2.46 units. These values yield a venture profit of approximately $1,200,000,000. This indicates that it has the potential to be an extremely profitable investment. These values are summarized in Table 16.

Table 16: Key profitability values.

ROI	4.98
PBP	0.0121 years
VP	$1,200,00,000
IRR	2.46
NPV	$6,700,000,000

The profitability calculations yield interesting results. For starters, the return on investment (ROI) value appears to be quite high. Although a value of about 20% is typically expected, the calculated ROI value is close to 500%. A possible reason for this value being high may be that the total capital investment (TCI) estimation is too low. This may be due to the initial cost estimations being inaccurate. However, considering that industry standards were utilized for these calculations, the aforementioned scenario is unlikely. To combat the issue of the TCI potentially being too low, higher cost estimations may need to be made to account for the manual labor required to install

the equipment, as well as the cost of site preparation. Moreover, the high ROI value causes a decrease in the payback period (PBP) value, which is why the PBP is lower than expected. Once again, this could potentially be attributed to the initial cost estimations, but does meet the standards previously utilized in this report. Furthermore, while the PBP and the ROI are not as expected, the net present value or worth (NPV) and investor's rate of return (IRR) values of $6.7 billion and 2.46, respectively, take into consideration the full scope of the facility and show that it will be monetarily advantageous.

In summary, the profitability measures analyzed above demonstrate the high profitability potential of this production process. Therefore, it would be financially beneficial to proceed.

Chapter 6. Sustainability and other considerations

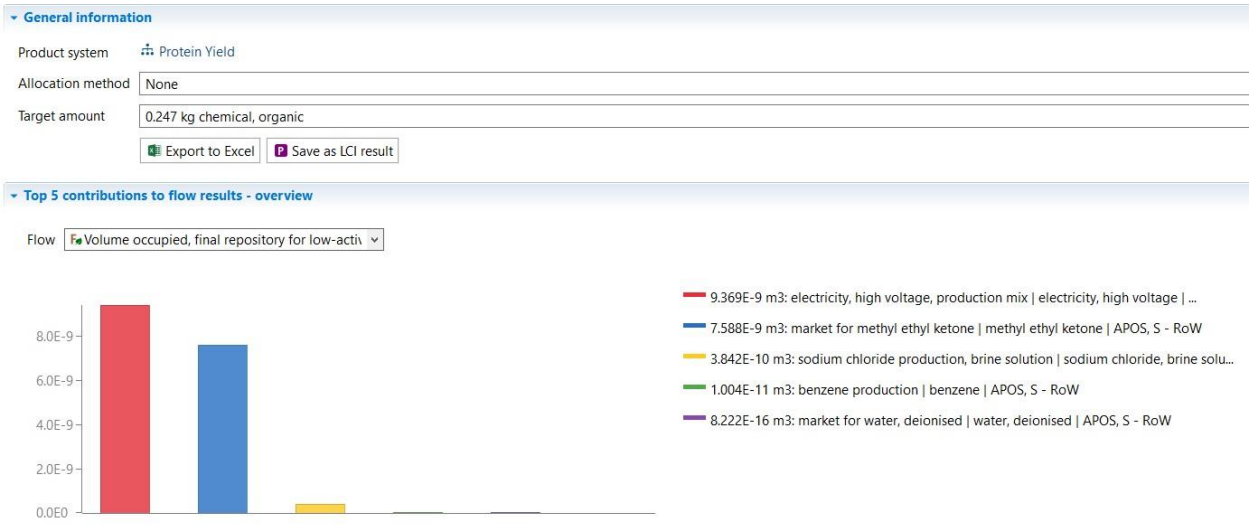

Figure 12. Results from openLCA analysis

Environmental impacts

The main goal for this project with respect to environmental impact is to minimize carbon emissions and other negative environmental impacts. The key indicators that we will be using to measure success are emissions and efficiency in order to minimize energy loss throughout the process. To perform the LCA analysis, several assumptions were made. Firstly, as the components for media and cell mass are not available in the LCA platform, individual chemical components of media were used as inputs. The electricity consumption input was taken from the SuperPro model described in above chapters. These assumptions likely resulted in some inaccuracies with respect to the final analysis. As shown in the above figure, there is not a significant energy demand for the production system, which is a strength of the process. This number will likely increase as production continues due to inefficiencies resulting from wear on production units. This can be minimized by performing regular maintenance on process units. Furthermore, the fact that all processes are conducted at mild temperatures and pressures leads to an overall low energy demand. Decreasing environmental impact is a common goal across the bioproduction industry. According to the Ceres Environment web page, they are actively “seeking out opportunities to manage their environmental impact” (Ceres, 2020) Because of this, the viral extraction device described in this report must rigorously maintain high environmental standards to compete.

Societal impacts

The societal goal for this project is to minimize the impact of possible future Covid-19 outbreaks on the general population. Waste water testing will reduce the need for individuals to seek out testing when they become symptomatic, and will reduce outbreak severity by detecting outbreaks before symptoms spread through the population. Upon deployment of the product, success will be measured through the accuracy of results obtained, and the speed with which outputs are detected. This should result in decreased outbreak severity.

Additionally, this technology could be applicable to other viruses as well, whether they be future pandemics or more common like the Avian flu virus. By being able to monitor and filter out other viral particles, this technology could help minimize the spread of future outbreaks, help with resource distribution for communities more severely affected by the virus, and help increase public awareness on the issue as well.

Workplace and community health and safety impacts

There are several goals for workplace health and safety. The primary goal is to maintain a safe working environment through properly maintaining equipment and communicating safety standards. A SOP will need to be designed for all facility workers to understand how to handle the equipment so as to not damage it. Workers would also be trained on understanding how to mitigate equipment mishaps or maneuver safety incidents that occur.

Producing a functional waste water testing device will also improve the overall health and safety of the community through decreasing the severity of outbreaks and slowing the spread of Covid-19. Communities would be better able to understand when they should get tested and isolated, in a more time efficient manner. This would, ideally, help the community feel safer, while also improving the quality of water the community has access to because of the wastewater filtering.

Sustainability Summary

In sum, the goal of this product is to safeguard the health and safety of the community through monitoring the spread of Covid-19 with up to date and accurate data. In doing this, it is critical to maximize efficiency in order to decrease environmental impact. This will both increase the longevity of the product and ensure the product remains competitive in an industry determined to reduce its carbon footprint.

Chapter 7. Conclusions and recommendations

Based on the results and analyses described in the preceding sections, there are several necessary next steps. Primarily, based on the promising initial experimental results such as the dibromobimane and peptide loading, as well as the positive economic analyses, using porous protein crystals as a material for viral collection in wastewater testing is a viable project and should be continued. The economic benefits of this endeavor are demonstrated by a short payback period and a high return on investment. Due to the premium market price for both purified protein and research materials, these devices will be able to be sold for much more than the cost of production. Additionally, the adaptability of the technology allows the protein crystals to be utilized for various other viral particles showing its future potential. The technology explored in this project combined with packing the crystals into filters makes the process less labor-intensive and safer in practice. Additionally, the demand for this product helps offset the costs of production. The profitability values obtained indicate that this technology is an extremely appealing prospect for investors. To continue progression towards a final product, there are two lines of optimization to pursue: experimental optimization and device production optimization.

Experimentally, there are two further results to be gained using small amounts of porous protein crystals. Primarily, fluorescently-labeled viral particles will be used to validate the ability of the peptide binding sites to attach to the viral particles and collect them at various concentrations. This is a key finding to demonstrate the viability of these crystals as a material for wastewater testing. Secondly, the crystals must be demonstrated to retain the availability of thiol binding sites following a chemical crosslink to stabilize the crystal outside of a high salt solution. For the purposes of this report, all experimental tests with porous protein crystals were conducted in a high salt solution to stabilize the crystals, as in their natural state they dissolve outside of high salt concentrations (3-4 M). However, should the crystals be used in wastewater testing, these conditions will not be maintained. Therefore, the crystals will be stabilized with a chemical crosslinker, such as glyoxal. The crosslinking process forms covalent bonds between adjacent residues, allowing the crystals to move to solutions with no salt, a variety of temperatures, and a variety of pHs while maintaining its crystalline structure. However, this crosslinking process may also interfere with the exposed crystalline thiols which allow for covalent peptide loading. Therefore, a method of protecting these groups during a chemical crosslink must be established. There are several proposed methods for this, including loading a protective group prior to crosslinking, but a definitive protocol has not been established. Once these additional conditions are met, the porous protein crystals will be ready for use in wastewater testing devices.

In terms of device production, there are several goals which are yet to be met. The foremost challenge is to design a device capable of stability containing porous protein crystals while allowing for adequate liquid flow through the packed crystals to collect a representative sample. One possible scheme is to use tangential flow, which would allow for flow through the packed crystals, with a lower risk of obstructions from wastewater blocking flow entirely. This proposed design is shown below in figure 13.

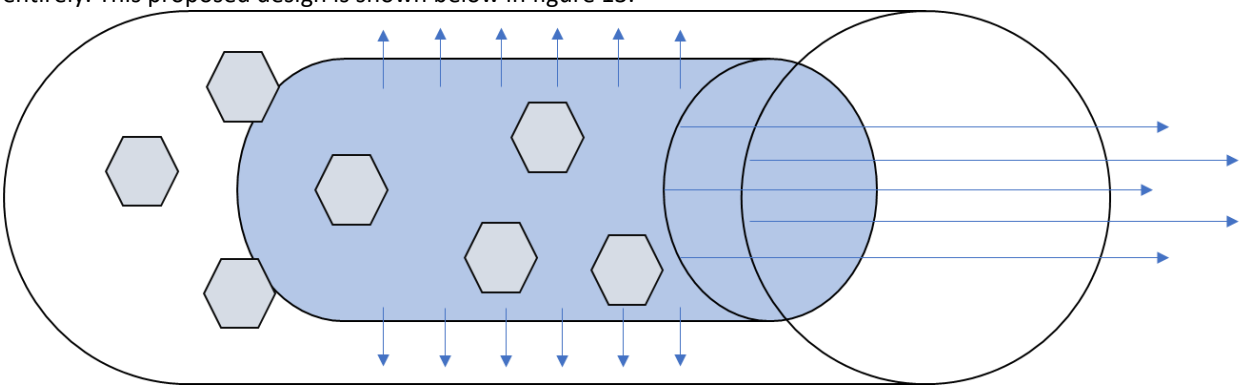

Figure 13. Possible wastewater testing device demonstrating tangential flow.

The optimization of this device will require investigation of various semi-permeable membranes, as well as elucidation of the fluid mechanics involved in such a device. Tangential flow typically requires a pressure differential to remain functional; this difference will need to be finely tuned. These designs will also have to consider the varying temperatures experienced within a sewer where wastewater testing takes place.
In sum, while the evidence presented in this report conveys the high possibility of this project, there are several further avenues to be explored before it is fully viable. Should the crystals be validated to bind viral particles, and retain binding sites following a chemical crosslink, and should these crystals then be loaded into a properly tuned flow device, these devices will be ready for use by cities around the world to monitor future viral outbreaks.

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Appendices

SuperPro Model