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Scale Up of a Novel Method to Maximize Malonyl-CoA in Escherichia Coli

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SCALE UP OF A NOVEL METHOD TO MAXIMIZE MALONYL-COA IN *ESCHERICHIA COLI*

A Thesis

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Abstract

The instability of oil's price along with its limited availability and impact on the environment motivate the search for an alternative feedstock that can sustain the profitability of chemical companies. Industrial biotechnology can promote renewable sources of energy and products by using microorganisms to produce a wide range of chemical compounds.

The three-carbon metabolite, malonyl-CoA, can serve as a precursor to a variety of industrial chemicals. The major hurdle with using malonyl-CoA in industry is that its intracellular concentration in *Escherichia Coli* is very low. Previous attempts to increase the intracellular level of malonyl-CoA have ranged from genetic engineering of proteins involved in fatty acid biosynthesis to adding inhibitors of enzymes.

Tatiana Mello, a graduate researcher at Louisiana State University, recently developed a clean, low-cost method that increases the level of malonyl-CoA in *E. Coli* [1]. At 5 mL, the method produced flaviolin, and indicator of malonyl-CoA at 68 mM/gWW with a titer of 1.053 g/L. The first goal of this project was to scale up the method by three orders of magnitude from a shake flask (milliliters) to a benchtop bioreactor (liters). At the 5L scale, *E. Coli* produced 74.1 mM/gDCW of flaviolin with a titer of 0.04 g/L. Compared to the production and titer at the 5 mL scale, the 5 L experiments showed similar target chemical production per unit biomass, but significantly lower target chemical concentration per batch. This suggests that the individual cells are producing similar amounts of the target chemical, but that the cells are not reproducing to similar concentrations.

Another interesting finding at the 5 mL scale was the effect of CO_2 concentration on malonyl-CoA production. Unpublished findings by Mello, at the 5mL scale, showed that an

optimum concentration of approximately 1.6 mM CO_2 exists where flaviolin production is significantly increased. This trend was investigated at the 5 L scale, but was not observed.

The production of industrial chemicals is the end goal of this work, so experiments were also done to produce 3-hydroxypropionic acid (3-HP), a precursor to acrylics, from *E. Coli.* Qualitative results from thin layer chromatography were promising for this application of the method.

1. Introduction

1.1. Motivation

The world relies heavily on petroleum based products. It is widely recognized that a dependence exists on a limited supply of petroleum. The energy and products sourced from petroleum suffer from price instability and contribute to atmospheric pollution. These factors create an increasing need for alternative methods to create the products traditionally derived from petroleum. Many methods for making products from petroleum alternatives exists, but are not yet economically competitive with traditional petroleum based methods. This challenges researchers to not only investigate new methods of producing the chemicals and energy the world needs, but also to optimize these methods such that they are economically competitive.

One such alternative method for sourcing the products traditionally obtained from petroleum is the use of naturally occurring biological pathways. Microorganisms, such as bacteria, are capable of producing industrially useful chemicals. The process of producing chemicals from microorganisms provides a major advantage over petroleum processes in that it relies on a renewable feedstock, sugar. Alternatively, petroleum based products rely on a limited, and unequally distributed, supply of fossil fuels. Carbon Dioxide (CO_2) is released as a by-product of petroleum based products, increasing the concentration of greenhouse gasses in the atmosphere. Contrarily, the production of bio based industrial chemicals has the potential to consume atmospheric carbon. Work published by Mello shows that bacterial production of malonyl-CoA may serve as a potential solution to many of the problems created by the limited supply of petroleum [1]. This research attempts to further these findings.

1.2. Background

Malonyl-CoA is a naturally occurring metabolite of *E. Coli*. Malonyl-CoA is useful for its ability to be converted into a variety of manufactured products. Acetyl-CoA carboxylase is a biotin-dependent carboxylase that catalyzes the first committed and regulated step in fatty acid biosynthesis in *E. Coli*. The reaction catalyzed by ACC involves two half-reactions and is shown in Figure 1. Bacterial ACC is composed of three proteins: biotin carboxylase (BC), carboxyl transferase (CT), and biotin carboxyl carrier protein (BCCP). In the first half-reaction, BC catalyzes an ATP-dependent carboxylation of biotin, which is covalently attached to BCCP. In the second half-reaction, CT transfers the carboxyl group from biotin to acetyl-CoA to produce malonyl-CoA. Enzymatic activity requires all three of these proteins to form a macromolecular complex [2].

$Reaction\ 1$:

$$BCCP - biotin + MgATP + HCO_3^- \underset{Mg^{+2}}{\overset{BC}{\rightleftharpoons}} BCCP - biotin - CO_2^- + MgADP + P_i$$

$Reaction\ 2$:

$$BCCP - biotin - CO_2^- + Acetyl\text{-}CoA \underset{a}{\overset{CT}{\rightleftharpoons}} BCCP - biotin + Malonyl\text{-}CoA$$

Sum:

$$MgATP + HCO_3^- + Acetyl\text{-}CoA \underset{a}{\overset{BC-BCCP-CT}{\rightarrow}} MgADP + P_i + Malonyl\text{-}CoA$$

Figure 1. The reactions of biotin carboxylase (BC) and carboxyl transferase (CT) along with the sum of the entire acetyl-CoA carboxylase reaction.

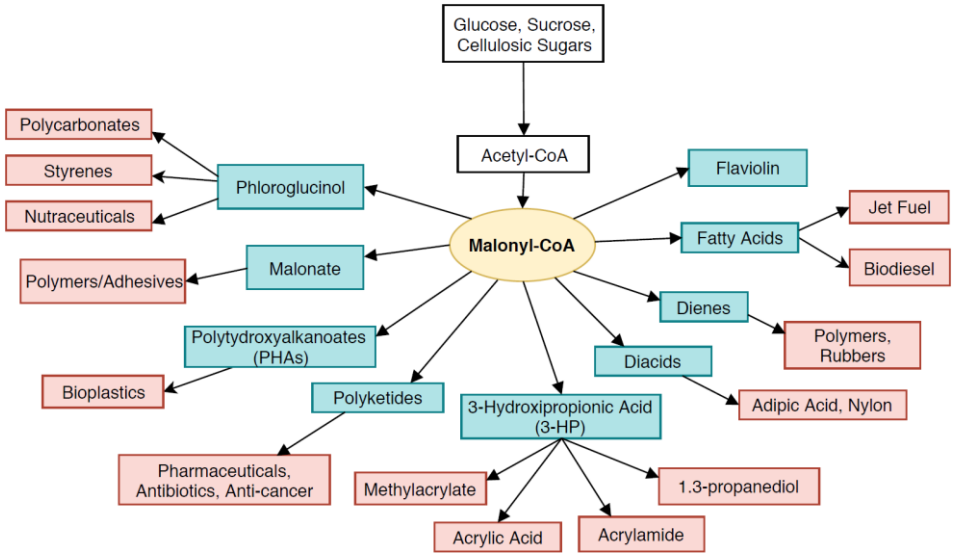

Figure 2. Malonyl-CoA and its derived products [1].

The many end products that can be produced by malonyl-CoA are illustrated in Figure 2. A simple and inexpensive method to assay Malonyl-CoA *in vivo* has been described by Yang [3]. This method takes advantage of the colorimetric malonyl-CoA biosensor, flaviolin. Specifically, 1,3,6,8-tetrahydroxynaphthalene synthase (THNS) converts malonyl-CoA to 1,3,6,8-tetrahydroxynaphthalene (THN), which is secreted out of the cell and then auto-oxidized to flaviolin. Flaviolin serves as an easily measured indicator for production of malonyl-CoA because it is light absorbent at 340 nm. Determining the flaviolin concentration of a sample allows for calculation of the malonyl-CoA produced to create such a concentration of flaviolin. This calculation is based on the reaction shown in Figure 3. Here, the enzyme (THNS) catalyzes condensation of 5 molecules of malonyl-CoA to form (THN) which is converted into 2,5,7-trihydroxy-1,4-naphthoquinone (flaviolin) by autoxidation and secreted out of the cell [4]

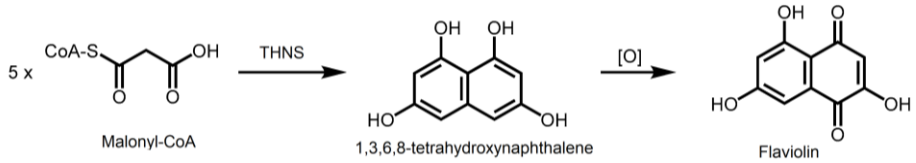

Figure 3. Flaviolin Production [5]

Understanding the mechanisms by which Malonyl-CoA is produced is useful for maximizing its production. The barrier to industrial production is that Malonyl-CoA naturally exists at relatively low concentrations (35 µM) in *E. Coli* [4]. However, manipulation of the environment in which the bacteria is grown can lead to higher production levels.

Increasing the initial concentration of any reaction's reagents will ultimately increase the concentration of the reactions' final products, as the reaction approaches equilibrium. However, the final concentration of the products is ultimately limited by the reagent whose supply is first exhausted, or the limiting reagent. Mello theorized that HCO_3^- was the limiting reagent of the reaction shown in Figure 1. Therefore, the simplest way to increase production of Malonyl-CoA would be to provide excess HCO_3^- to cultures of *E. Coli* by incubating them in a CO_2 rich environment.

Similar to flaviolin, 3-hydroxypropionic acid (3-HP) is a product derived from malonyl-CoA as shown in Figure 2. 3-HP is an industrially useful for its ability to be converted to acrylic acid. Acrylic acid is commonly used to make acrylics, surface coatings and the super absorbent polymers found in diapers. While acrylic acid is traditionally a derivative of petrochemicals, it can be renewably produced from 3-HP producing *E. Coli.* A strain of *E. Coli* transfected with a vector containing the DNA that codes for the enzyme malonyl-CoA reductase (MCR) can be used to produce 3-HP. This reaction is depicted in Figure 4.

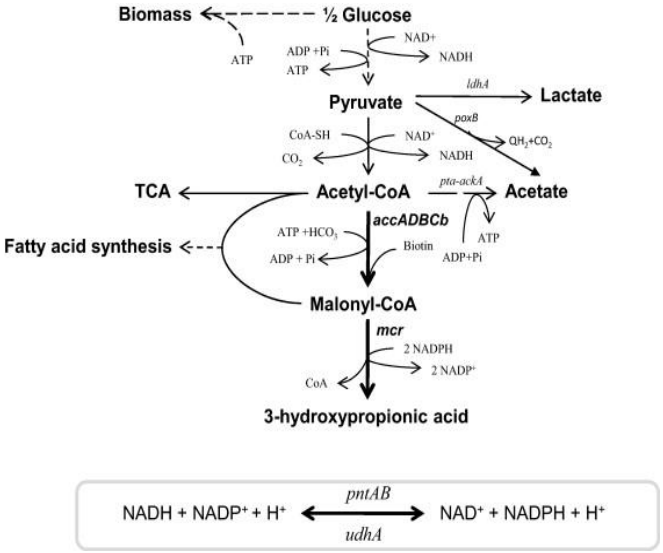

Figure 4. The 3-hydroxypropionic acid pathway [6].

Being that malonyl-CoA is the substrate for 3-HP production, the ability of this method to maximize 3-HP production is another industrially significant consideration. To investigate this potential, the method which was used to maximize malonyl-CoA production with the THNS (flaviolin) strain was again studied using the MCR (3-HP) strain.

1.3. Literature Review

Other researchers have attempted a variety of strategies to maximize malonyl-CoA production in *E. Coli.* A review conducted by Mello found that the highest published yield was obtained by Lynch et al by knocking out genes of the fatty acid synthesis pathway [7]. More recent investigation suggests this is still the next highest published yield. Other attempts involved the use of other genetic modifications and expensive antibiotics such as, Cerulenin ($20/mg). The results of these attempts can be found in Table 1.

Table 1. Competing method's malonyl-CoA titer.

Product	**Fermentation**	**Strategy**	**Inducer**	**M-CoA Titer**
3HP: 2.0 mM [8]	Shake flask	Genetic modification	IPTG	1.7 [g/L]
3HP: 49.0 g/L [7]	Fed-batch 1L bioreactor	Genetic modification, Cerulenin	IPTG	464.7 [g/L]
Flaviolin: 2.6 mM [1]	Shake flask	Medium optimization	Lactose	10.9 [g/L]

Commented [HL1]: 49 g/L (3HP)->464.7 g/L Mcoa

Unpublished experiments carried out by Mello displayed a positive relation between environmental concentration of CO_2 and production of Malonyl-CoA. Using a strain of *E. Coli* that produced flaviolin as a indicator of Malonyl-CoA production, Mello tested the effect of varying CO_2 concentration on 5 mL shake flask cultures in an incubator.

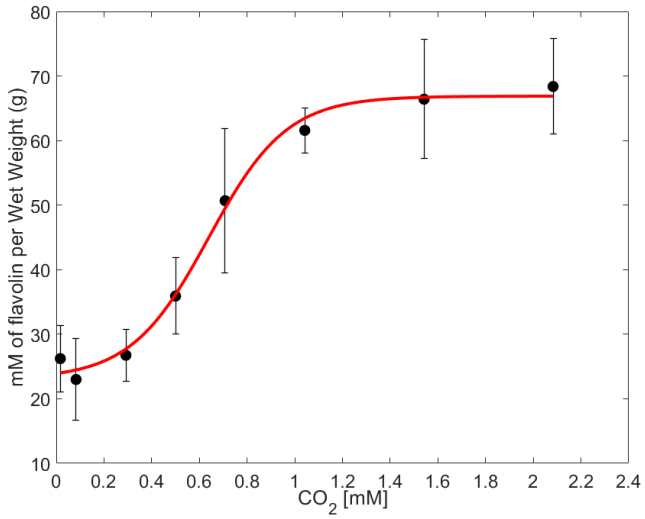

Figure 5. *E coli* cultures were incubated at several CO_2 concentrations. Cultures were grown in atmospheric air (0.04% CO_2 or 0.017 mM) and external CO_2 was adjusted in an incubator varying from 0.2% (0.08mM) to 5% (2.08 mM) (black points). Red curve represents the sigmoidal curve that best fits the data. The error bars represent the standard deviation of three replicates [9].

It is important to note that Mello frequently reported malonyl-CoA production as "the concentration of flaviolin (M) per gram wet-weight of bacterial cells in a 1 mL sample" [1]. For the sake of comparison to Mello's results, production levels in this thesis are reported in the same manner. As seen in Figure 5, Mello observed an increase in flaviolin production with increased CO_2, up to a plateau in flaviolin production near a concentration of 1.6 mM CO_2, or roughly 3.6%. The method produced by Mello utilized a 5 mL culture in a 125 mL shake flask. For this phenomenon to be industrially meaningful, it must be reproducible and scalable.

1.4. Research Goal

It was determined that the next logical step for validating Mello's method would be to scale up of the method's volumetric production in order to demonstrate its commercialization potential. After consulting industry experts (potential industrial partners), it was determined that a 1,000 fold increase in production volume from Mello's work would be industrially meaningful if successful. Therefore, the specific goal of this project was to scale up the method by three orders of magnitude from a shake flask (milliliters) to a benchtop bioreactor (liters).

Initially, a batch type 1 L bioreactor with working volume of 500 mL, seen in Figure 6, was borrowed from the Louisiana State University Department of Chemical Engineering for experimentation.

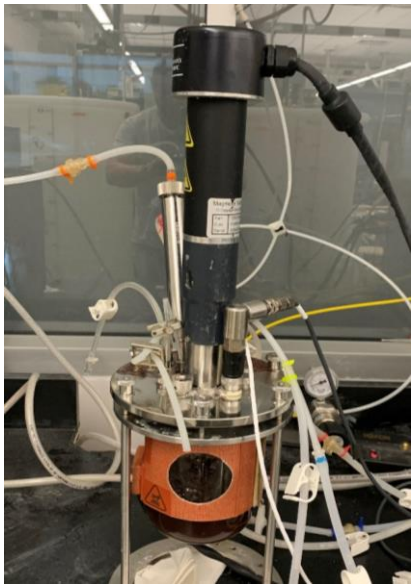

Figure 6. LSU Department of Chemical Engineering 1 L Eppendorf bioreactor.

The method produced by Mello was scaled up from 5 mL to 500 mL by replicating culture conditions. Optimal temperature, run time, and reagent concentration identified by Mello were utilized at this larger scale. Additionally, agitation speed of 300 RPM, and gas sparging rate of 5 SLPM were used according to [10]. Experimentation at this scale gave insight to the proper practices and procedures for bioreactor operation. At 500 mL, a spectrophotometric analysis showed flaviolin production in the same order of magnitude as Mello. This confirmation of scalability led to the sourcing of a 7.5 L bioreactor for continued experimentation, shown in Figure 7.

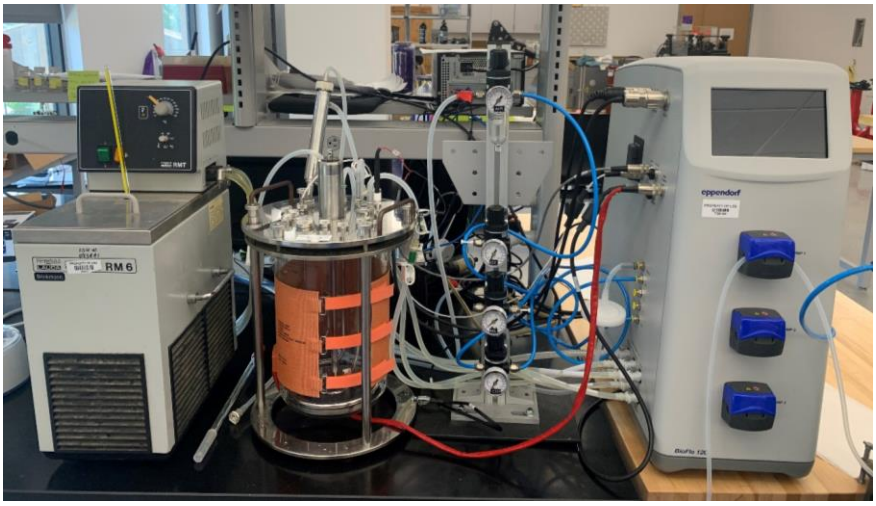

Figure 7. Bioreactor vessel by New Brunswick Scientific with Eppendorf BioFlo 120 controller.

Another interesting finding at the 5 mL scale was the effect of CO_2 concentration on malonyl-CoA production. Therefore, another objective of this research was to study the effects of CO_2 concentration of malonyl-CoA production at the larger scale. The bioreactor seen in Figure 7 is equipped with four gas regulators and a mass flow sensor that allow for precise mixing of the sparge gas supplied to the vessel.

The end use of this method will be the production of industrially useful chemicals. Therefore, the final objective of this study was to produce 3-HP from *E. Coli.*

2. Experimental Methods

2.1. Batch Culture Preparation

The *E. Coli* strain BL21 (DE3) used to produce flaviolin was acquired from Novagen. This strain contained the gene for THNS, which was a gift from Dr. Hans Liao, formerly of OPX Biotechnologies in Boulder, CO. Genetic modification of the *E. Coli* strain BL21 (DE3) to

contain the gene for THNS was conducted by Mello in 2018 [1]. The strain permanent was stored at -80 °C prior to and during this study.

Petri dishes were prepared by combining components listed in Table 2. Streptomycin (50 mg/mL) was added after autoclaving the liquid media.

Table 2. Agar plate components.

Agar Plates	
Deionized Water	1 L
Luria Bertani	20 g
Bacteriological Agar	12 g
Streptomycin (50 mg/mL)	1 mL

An incubator, set to 37°C, was used to culture agar plates streaked with *E Coli*. Plates were incubated for 24 hours before an isolated colony was taken from the plate and introduced to an Erlenmeyer flask containing 100 ml of LB and 350 µL of Streptomycin (50 mg/mL). A water bath shaker (Figure 8), set to 37°C and 200 RPM was used to culture the Erlenmeyer flasks, supplemented with 0.18 g of Dextrose, for 24 hours.

Figure 8. Water bath shaker used to culture 100 mL inoculum.

The 7.5 L glass bioreactor vessel produced by New Brunswick Scientific was used in conjunction with an Eppendorf BioFlo 120 controller to carry out the method at increased scale. 5 L of Luria Bertani (LB) were prepared for the bioreactor using the components of Table 3.

Table 3. Luria Bertani components.

Luria Bertani	
Deionized Water	5 L
Tryptone	50 g
Yeast Extract	25 g
NaCl (Sodium Chloride)	25 g

Tryptone and yeast extract, used to make LB, were obtained from Life Technologies Corporation. In an effort to reduce production costs, Sodium Chloride was obtained from Walmart under the generic “Great Value” brand. The media was autoclaved on a liquid cycle

before being added to the bioreactor's glass vessel. Prior to inoculation, trace metals, lactose, and Antifoam B Emulsion (Figure 9) were added to the bioreactor.

Figure 9: Antifoam B Emulsion.

Table 4 provides the quantities in which trace metals, lactose, and antifoam were added to a 5 L batch.

Table 4. Trace metals and inducer used in culture broth.

Other Additions	
1 M $MgSO_4$	41 µL
1 M CaCl	197 µL
0.1 M MnCl	11 µL
Lactose (Inducer)	25 g
Antifoam B Emulsion	0.75 mL

Each 5 L batch was inoculated at a 1 % (v/v) ratio by adding 50 mL of inoculum to the batch. Inoculation was performed by injecting the saturated culture into the assembled bioreactor through a port in the head plate (Figure 10).

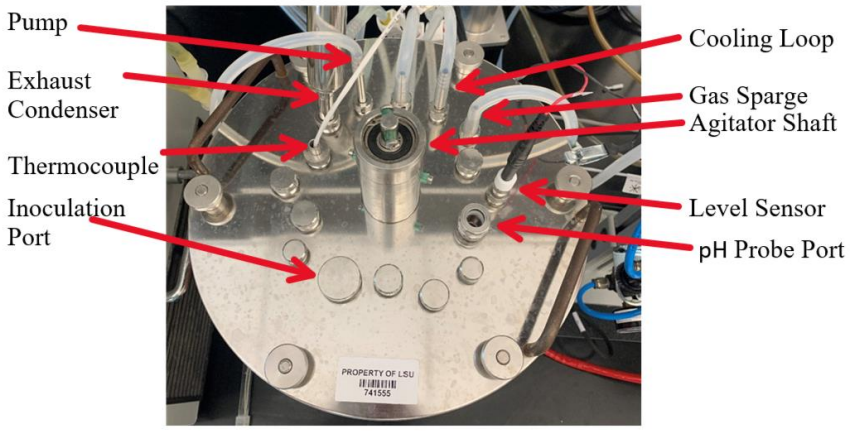

Figure 10: Bioreactor vessel headplate with labeled ports.

The BioFlo 120 is capable of monitoring and controlling temperature, gas flow, gas mixture, agitation speed, and pH. Culture conditions were controlled and monitored using Eppendorf's Track and Trend desktop software. Set points for the various processes values were as follows according to [10]:

Table 5. Bioreactor control set points.

Control Set Points	
Temperature	37 °C
Agitation	300 RPM
Gas Flow Rate	5 SLPM
Air	100%
CO_2	0%

2.2. Sampling

Three batch samples, 1 mL each in volume, were taken at inoculation (time 0) and then every hour from 19 to 24 hours after inoculation. A benchtop microfuge from VWR (Figure 11) was used to spin down batch samples at 7,500 RPM for 5 minutes. A scrolling pipette was used to separate supernatant from pellets. Supernatant samples were stored in a Forma Scientific BioFreezer at -50°C prior to spectrophotometry.

Figure 11. VWR microfuge used to pellet culture samples.

3. Analytical Methods

3.1. Absorbance and Concentration

To test samples for flaviolin concentration, a Cary 60 UV-Vis spectrophotometer from Agilent Technologies was blanked using the supernatant of the time 0 sample at a wavelength of 340 nm. Absorbance (A) was recorded for each sample in a cuvette of path length (L) 1 cm. Beer's law allows for the calculation of flaviolin concentration (C) from absorbance and path length, knowing that the extinction coefficient of flaviolin (ϵ) is 3,068 $M^{-1}cm^{-1}$ [11]

$$A = \varepsilon * L * C$$

3.2. Dry Cell Weight

Dry cell weight was obtained by subtracting the weight of the empty microfuge tube from the weight of the tube containing the dried pellet. Each individual microfuge tube was weighed prior to sampling. Microfuge tubes containing pellets were dried at 37 °C for 1 week before being weighed.

4. Experimental Results and Discussion

4.1. Scale Up Results

The primary goal of this research was to reproduce the levels of malonyl-CoA produced by Mello [1] in a volume 1,000 fold larger. At the 5 mL scale, Mello produced an average of 68 mM/GWW of flaviolin. While weighing cells wet is not traditional, it provides an overly conservative measurement of flaviolin yield. This 68 mM/gWW of flaviolin corresponds to 340 mM/gWW of malonyl-CoA, given the conversion ratio of 5 molecules of malonyl-CoA to 1 molecule of flaviolin previously discussed. Quantifying the concentration of a product per unit cell weight (mM/gDCW or mM/gWW) provides insight as to how productive the bacterial cells are at making the target chemical.

In a particular study, Mello produced the relationship shown in Figure 12 between flaviolin production and time.

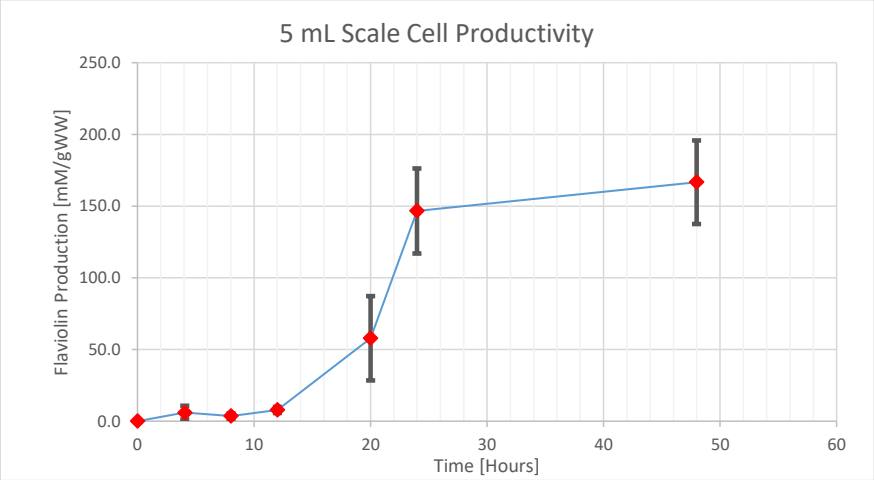

Figure 12. Flaviolin production vs. time at 5 mL [1].

While this single study outperformed the reported average yield of 68 mM/gWW flaviolin at the 5 mL scale, it still shares some insight as to the relationship between production level and culture time. It can be seen in Figure 5 that from approximately 0 to 12 hours of culture time, flaviolin production is relatively low. However, from approximately 12 to 24 hours, the rate at which flaviolin is produced reaches its maximum.

Carrying out the method at the 5 L scale produced a similar relationship between flaviolin production and time, as seen in Figure 13. In order to make comparisons to Figure 5, units of flaviolin concentration per unit biomass (mM/gDCW) are again used. In this study, cell weight was takin after drying the cells, as is more commonly found in literature. Taking the mass of dried cells reduces the ambiguity created by water content in wet cells.

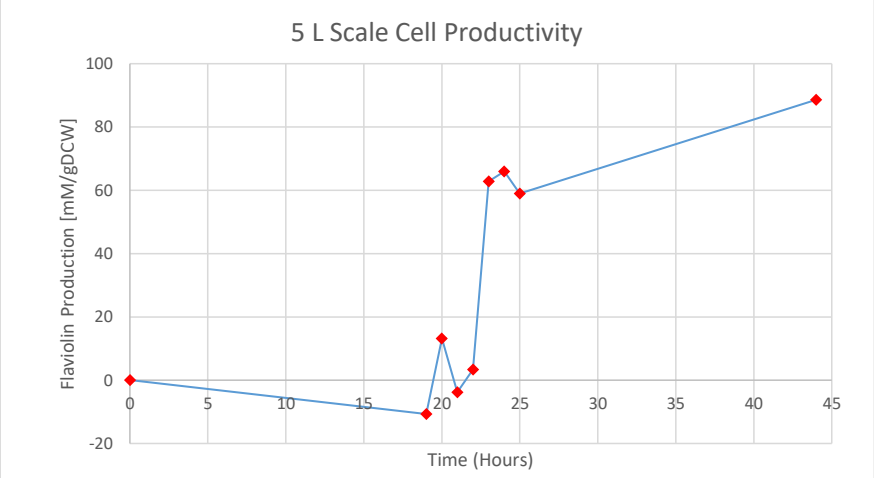

Figure 13. Flaviolin production vs. time at 5 L. Error bars are too small to be observed on plot.

Similar to Mello's results at the 5 mL scale, results at the 5 L scale exhibit a lag phase from approximately 0 to 19 hours. In this time frame, the production of flaviolin is presented as negative. This can be explained by the bacterial consumption of Yeast extract in the culture

media, prior to significant flaviolin production. Yeast extract, present in the LB growth media, is also light absorbent at 340 nm. Therefore, the bacteria's consumption of the growth media from 0-19 hours is likely more significant than its production of flaviolin. Theoretically, the light absorbance at time 19 hours could be considered the "zero flaviolin production" benchmark. However, this has been avoided in order to be more conservative.

From an industrial perspective, it is important to notice that the highest production rate of flaviolin per hour is seen between 20 and 23 hours, for both the small and large scale. This suggest the ideal product harvest time of a batch type bioreactor remains in this time range, regardless of culture volume.

As this study focuses on the commercial viability of the method, it is also important to consider how much product is created per unit batch volume. To do this, concentration of the product, flaviolin, in the batch media is plotted as a function of time in Figure 14.

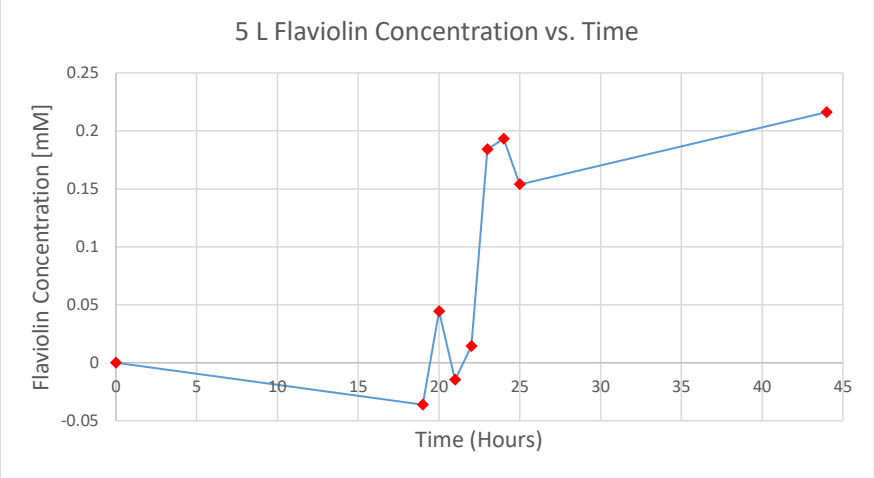

Figure 14. Flaviolin concentration vs. time at 5 L. Error bars are too small to be observed on plot.

As with cell productivity plotted in Figure 13, flaviolin concentration also reaches a peak at 24 hours after induction of 0.19 mM. Compared to results at 5 mL, the bacterial cells show similar productivity at 5 L (mM/gDCW). However, the ultimate concentration of flaviolin in the batch is much lower at 5 L (0.19 mM) when compared to flaviolin concentration at 5 mL (2.55 mM) [1]. This means that the ratio of product to biomass scales up well, but the ratio of product to batch volume does not. Obtaining a higher concentration of viable cells in the batch would likely increase the product to volume ratio at the large scale.

4.2. CO_2 Results

Mello observed an interesting relationship between the concentration of environmental carbon dioxide (CO_2) and *E. Coli's* production of flaviolin at the 5 mL scale. As previously mentioned, she hypothesized that bicarbonate (HCO_3^-) may be the limiting reagent in the production of malonyl-CoA. In order to provide ample HCO_3^- to a culture, the amount of CO_2 supplied to the growth media can be increased externally. CO_2 will dissolve in water to form carbonic acid (H_2CO_3). This carbonic acid then disassociates to from one molecule of bicarbonate (HCO_3^-) and one proton (H^+). This is illustrated in Figure 15:

$Reaction$ 1:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$

Figure 15. Formation of bicarbonate from dissolved carbon dioxide.

To test this hypothesis, *E. coli* strains were incubated with varying amounts of atmospheric CO_2. The amount of CO_2 available to the cultures was regulated via incubator in which the cultures were grown. Mello's unpublished results at the 5 mL scale are shown in Figure 16.

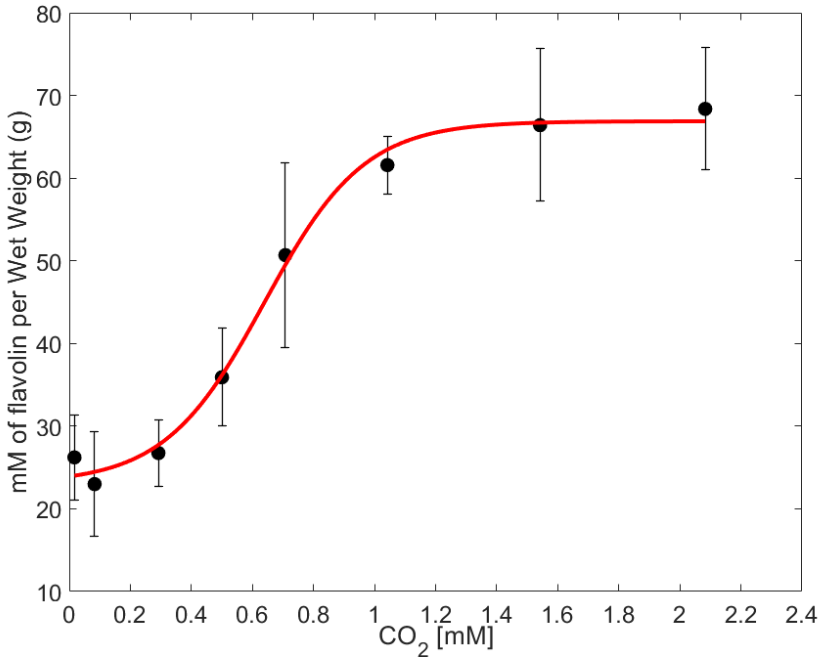

Figure 16. Effect of environmental CO2 concentration on production of flaviolin in E. Coli. [9]

Four experiments were carried out in attempt to reproduce the positive effect of CO_2 concentration on flaviolin production at the 5 L scale. The method described in the original scale up was repeated, and all experimental parameters remained constant between experiments with the exception of the rate at which CO_2 was sparged through the batch. It is important to note that the aeration method by which CO_2 was provided to the 5 L cultures differed significantly from Mello's method at 5 mL. At the 5 L scale, aeration is accomplished by sparging from the bottom of the bioreactor as illustrated in Figure 17. Contrarily, Mello aerated cultures by placing flasks in an incubated water bather shaker inside capable of controlling the internal atmospheric composition.

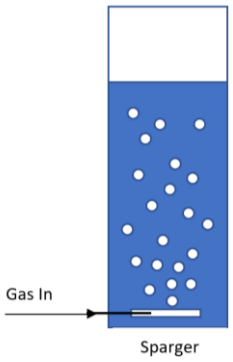

Figure 17. Bioreactor gas sparging.

In order to observe the effects of increasing the concentration of CO_2 available to 5L *E. Coli* cultures, different batches were sparged with the gas compositions shown in Table 6.

Table 6. Sparge Gas Compositions.

% CO_2 [Concentration]	Flow Rate CO_2 [SLPM]	Flow Rate Air [SLPM]
0% [0 mM]	0	5.00
1% [0.45 mM]	0.05	4.95
2.5% [1.12 mM]	0.125	4.875
5% [2.23 mM]	0.25	4.75

As previously shown, the batch sparged with 0% additional CO_2 produced flaviolin similar to Mello's findings, however experiments involving additional CO_2 were not so conclusive. The 1, 2.5, and 5% sparged CO_2 batches did not exhibit the expected behavior for flaviolin production as a function of time. Additionally, the maximum production of flaviolin for each experiment

occurred at a variety of time points during the 24 hour run times. The maximum flaviolin yield as a function of CO_2 concentration is shown in Figure 18.

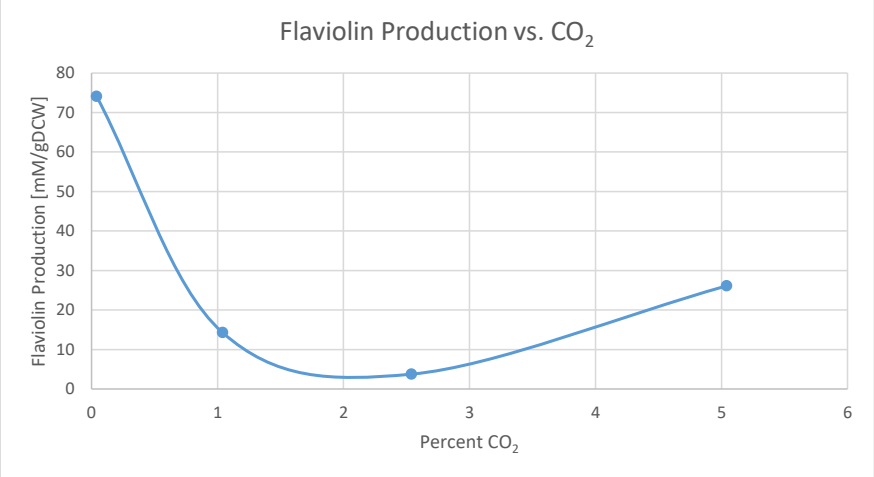

Figure 18. Effects of CO2 concentration on flaviolin production in *E. Coli*.

No distinct relationship was observed between increased concentration of CO_2 and the production of flaviolin in *E. Coli* at the 5 L scale. In fact, cultures exposed to increased CO_2 concentrations consistently produced less flaviolin than cultures exposed to air. The different methods of aeration (sparging versus shaking) may give rise to the inability to scale up the positive effect observed by Mello. It was observed that cultures exposed to increased CO_2 concentrations produced higher levels of cell growth, as shown in Figure 19.

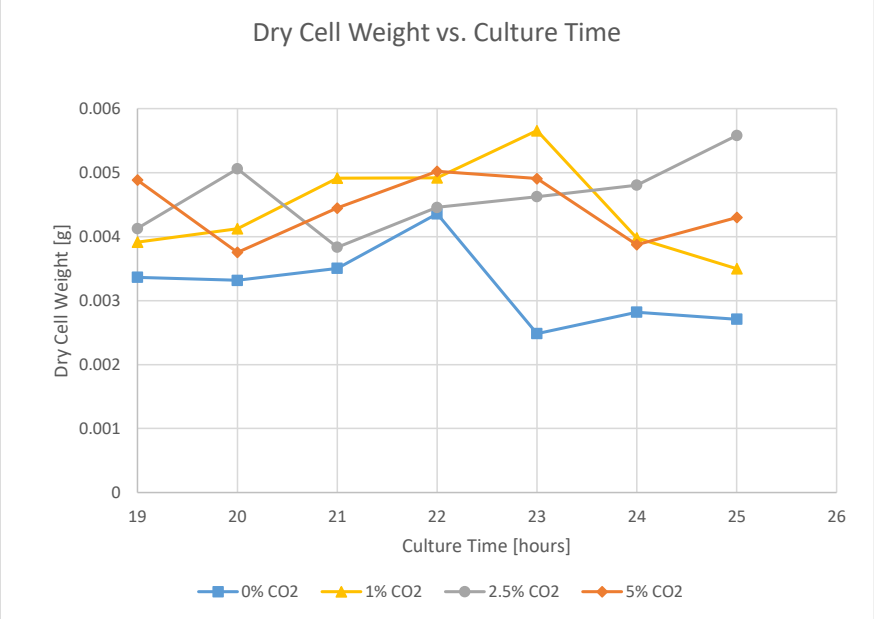

Figure 19: Dry cell weight of 1 mL samples versus culture time for varied CO2 concentration.

This suggests that when exposed to increased concentrations of CO_2, *E. Coli* directs more malonyl-CoA towards the fatty acid synthesis pathway (needed for cell division) and away from the pathway that produces flaviolin. A potential solution to this problem would be the use of an antibiotic. After the cells have grown to saturate the medium, treating them with an antibiotic that inhibits fatty acid synthesis would reduce competition against the target chemical's pathway.

While it is still possible that an optimal range of CO_2 concentration exists for increased production of malonyl-CoA at the 5 L scale, it was not observed in this study. Therefore the study of the effects of CO_2 on *E. Coli's* production of flaviolin, and in turn malonyl-CoA, at the 5 L scale was inconclusive and merits further investigation.

4.3. 3-HP Results

Two indicators of 3-HP were apparent. Thin liquid chromatography (TLC) is an inexpensive, but straightforward test of the presence of a target chemical in a sample. A sample of purified 3-HP was purchased for use as a control in the TLC analysis. Samples from both the experiment in which the bacteria were sparged with air and CO_2 enriched air were spotted next to the control. As seen in Figure 20 , a noticeable “band” formed in the control region due to the presence of purified 3-HP. The sample cultured in the presence of 5% CO_2 also produced a noticeable band in the same region as the control, suggesting the presence of 3-HP in the sample.

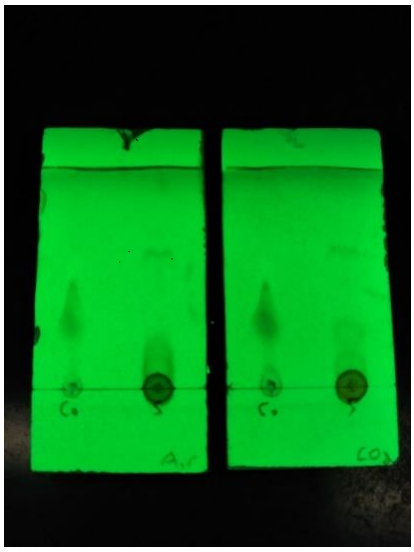

Figure 20: TLC conducted on *E. Coli* samples containing the MCR gene for 3-HP production.

The second indicator provided further assurance to the bacterial production of 3-HP. A noticeable drop in pH was recorded over time as the batch was cultured. A drop in culture pH could likely be explained by the production of an acid by the bacteria, specifically 3-HP. This pH

drop, plotted in Figure 21, is only seen in the strain of *E. Coli* with the MCR gene that produces 3-HP and not the strain of *E. Coli* containing the THNS gene that produces flaviolin.

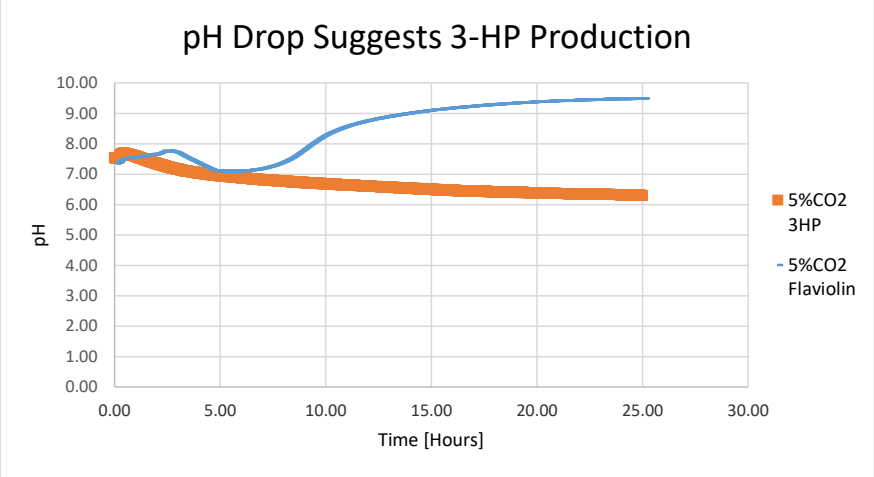

Figure 21: Noticeable pH drop is seen in culture with genetics for producing 3-HP when compared to culture with genetics for flaviolin.

Although means were not available to quantify the amount of 3-HP the method produced, these results strongly suggest the method's ability to produce an industrially useful chemical. Both the noticeable change in pH as well as the TLC are encouraging factors towards the method's further development for commercialization.

5. Process Economics

5.1. Cost of Materials

The most significant cost associated with the process followed in this work is that of the materials required. Table 7 details the required materials for a 5 L batch along with their unit costs.

Table 7: Cost of Materials

Consumable	Unit Cost	Quantity in 5 L Batch	Item Cost per 5L Batch
Streptomycin	0.91 $/g	0.0275	$0.03
Yeast Extract	0.19 $/g	25 g	$ 4.70
Tryptone	0.21 $/g	50 g	$ 10.70
Sodium Chloride	6E-3 $/g	25 g	$ 0.02
Agar	0.24 $/g	0.5 g	$ 0.12
Dextrose	0.21 $/g	0.18 g	$ 0.04
Calcium Chloride	0.06 $/g	0.7055 g	$ 0.04
Magnesium Sulfate	0.18 $/g	0.1185 g	$0.02
Manganese Chloride	0.10 $/g	0.0025 g	$ 3E-4
Lactose	0.18 $/g	90 g	$16.47
Antifoam B	0.20 $/mL	0.75 mL	$ 0.15
Premixed LB	0.14 $/g	0.86 g	$ 0.03
		TOTAL	**$32.31**

As detailed in Table 7, the cost of materials for a 5 L batch is $32.31. The measured production of flaviolin and calculated production of malonyl-CoA for a 5 L batch is detailed in Table 8. These results give a production cost of 7.84 $/g for malonyl-CoA.

Table 8: Production Yields

	Flaviolin	Malonyl-CoA
Max Experimental Concentration	0.000193 M	0.000966 M
Max Experimental Titer	0.03953 g/L	0.824623 g/L
5 L Batch Yield	0.199 g	4.123 g

6. Conclusions

6.1. Method's Scalability

The goal of this study was to explore the scalability of a novel method that maximizes production of malonyl-CoA in *E. Coli.* Using a 5 L bioreactor batch, it was successfully demonstrated that specific production of flaviolin, an indicator of malonyl-CoA, at the large scale very similarly follows trends and specific production levels previously demonstrated at the 5 mL scale. Using spectrophotometry, a maximum specific production of 65.9 mM/gDCW of flaviolin was measured with a titer of 0.04 g/L. This measurement implies a specific production of 329.5 mM/gDCW of malonyl-CoA and a titer of 0.82 g/L. Similar to trends at the 5 mL scale, it was observed that malonyl-CoA concentration is highest 24 hours after induction.

The ratio of product to biomass (expressed in mM/gDCW) at the 5 L scale was similar to that of the 5 mL scale. However, the titer (expressed in g/L) of the product was much lower at the 5 L scale. This suggests that the *E.Coli* cells in a 5 L culture are not reproducing to the extent as seen at 5 mL. However those cells that are present at 5 L are just as productive as those at 5 mL.

The positive effect of CO_2 on malonyl-CoA production previously observed at the 5 mL scale was not observed when the method was carried out at 5 L. This is likely due to a greater uptake of malonyl-CoA for fatty acid synthesis when compared to the uptake for target chemical (flaviolin) production.

The method's potential for production of an industrially useful chemical, 3-HP, was explored. While these results were not yet quantifiable, TLC results and pH trends were encouraging for the method's industrial potential.

6.2. Future Directions

The next immediate step in the method's development should study cell growth in the 5 L batch. It was observed that cells remain productive at increased scale, but in lower growth concentrations. Therefore, work should be done to maximize the viable cell population in a 5 L batch. Measuring batch turbidity over time and varied culture conditions would provide valuable insight towards this effort.

The application of antibiotics that inhibit fatty acid synthesis should be studied to further investigate the positive effect of CO_2 on malonyl-CoA production previously observed at the 5 mL scale. Although it was not directly observed, it is still possible that CO_2 had a positive effect on production of malonyl-CoA at the 5 L scale. It is likely that more malonyl-CoA was directed towards fatty acid synthesis than towards production of measurable flaviolin. Therefore the use of an antibiotic that inhibits fatty acid synthesis could provide valuable insight towards the effect of CO_2.

Further development of this method to maximize malonyl-CoA production in *E. Coli* should also focus on the production of industrial chemicals, such as 3-HP. While flaviolin is a useful indicator of malonyl-CoA production, the method should be perfected for the biomechanisms that produce industrially useful chemicals. The next logical step towards this goal will be to develop an effective assay for 3-HP in bacteriological media. Being able to quantify the method's ability to produce industrially useful chemicals will greatly determine its viability for further development. Should the method continue to prove for viable industrial production, obtaining a commercial partnership with an established chemical manufacture would be a significant milestone.

Appendix A. Process Logistics and Detailed Procedure

Below, the step-by-step procedure for carrying out the method is described.

Day 1

a. Streak plate from the strain permanent with a sterilized wire hoop. If the desired strain has been cultured on a plate in a previous experiment, an isolated colony from the previous plate may be used instead of the permanent to streak a new plate.
b. Incubate the freshly streaked plate at 37 °C for 24 hours.

Day 2

a. To make the bioreactor media, prepare 5 L of media in Erlenmeyer flasks filled no more than half capacity (e.g. five 2 L flasks filled with 1 L of media each). Cover flasks with aluminum foil and autoclave on a Liquid Cycle (slow exhaust) for a minimum of 60 minutes.

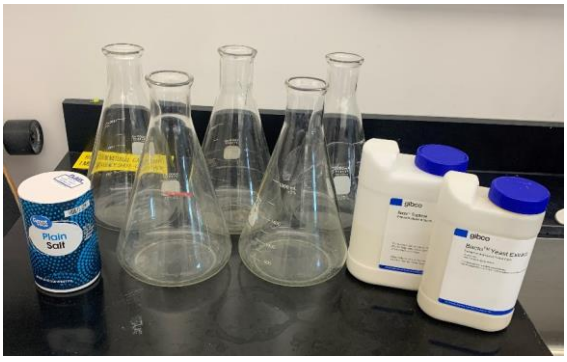

Figure A1. Preparation of media.

b. To prepare the inoculum, make 100 mL of media in a 250 mL Erlenmeyer flask. Supplement media with 350 µL of 50 mg/mL antibiotic, or equivalent dosage.

Supplement media with 180 mg of dextrose. Remove the plate prepared on the previous day from incubator. Swab a single isolated colony from the plate into the 100 mL of media. Cover the 250 mL flask with aluminum foil. Use a needle to poke small vent holes in the foil covering. Secure the 250 mL flask in a water bath shaker set at 37 °C and 200 RPM for 24 hours.

Day 3

a. To turn on the BioFlo 120 control unit, flip the black power switch located on the back of the BioFlo 120 unit.

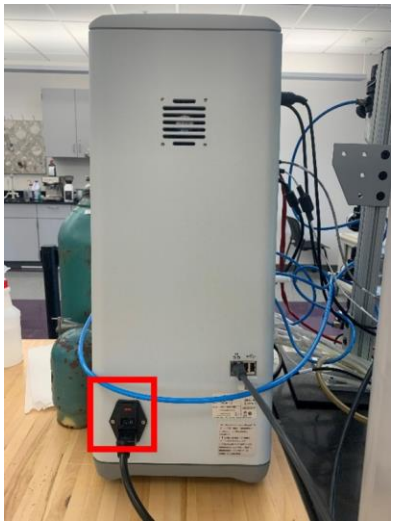

Figure A2. BioFlo 120 power switch.

b. Open the valves to supply air and additional gases (CO_2) to the regulator tower. Pressure at the regulator tower should read between 4-6 psi.

Figure A3. Air supply valve.

c. Proceed by filling the reactor vessel with media, antifoam, inducer, and any other required additions.

d. Insert thermocouple, pH probe, and level sensor through headplate of bioreactor vessel. Wrap the orange heat jacket around the base of the glass vessel. Use the hook and loop straps to secure the heat jacket.

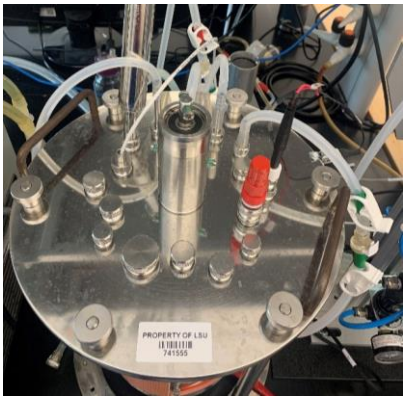

Figure A4. Bioreactor vessel headplate assembled with probes.

e. Reassemble the bioreactor head plate to the vessel. Slide the agitation motor over the stirrer and turn on the desired control loops on the touch screen of BioFlo 120.

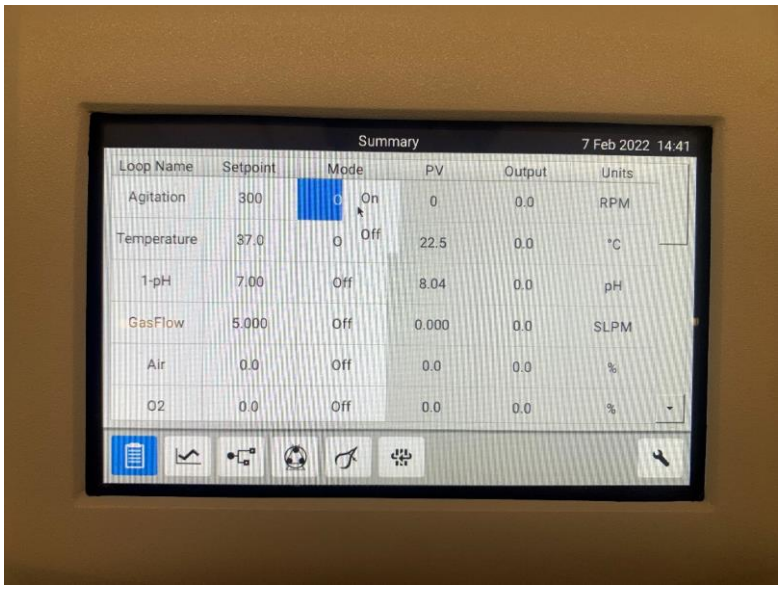

Figure A5. BioFlo 120 touch screen.

f. Remove threaded plug in headplate of bioreactor to inoculate. Replace the threaded plug.

g. Launch the BioCommand Track and Trend software on the desktop computer. Select "New Batch" on the home screen and follow the BioCommand prompts.

Day 4

a. To end the experiment, first close the air and gas supply valves and power off the water chiller.

b. To disassemble and clean the bioreactor, first clamp and disconnect all connected hoses.

Figure A6. Hose clamps and connections.

c. Remove and sterilize the connected probes. Remove the heat jacket from vessel and lay it flat for storage. Remove the five knurled nuts from head plate. Sterilize and wash the head plate assembly using bleach and deionized filtered water. Properly dispose of the remaining media. Wash the glass vessel using first bleach then deionized water. The

vessel and headplate may also be autoclaved prior to the next run, but all plastic fittings and O-rings will need to be removed and replaced. Export Track and Trend data to the desktop computer before powering off the control unit.

Appendix B. Cost of Materials

Table A1. Materials pricing and package sizing sourced from Thermofisher Scientific, Sigma Aldrich, and Walmart

Item	Cost [$]	Package [g]	$/g	Per Batch 5L [g]	Itemized Run Cost [$]	Itemized Cost Per L [$]
Streptomycin	91.25	100	0.913	0.0275	0.02509375	0.005019
Yeast Extract	94.00	500	0.188	25	4.7	0.94
Tryptone	107.00	500	0.214	50	10.7	2.14
Sodium Chloride	0.48.00	737	6.5E-4	25	0.016282225	0.003256
Agar	119.00	500	0.238	0.05	0.119	0.0238
Dextrose	107.00	500	0.214	0.18	0.03852	0.007704
Calcium Chloride	57.60	1000	0.058	0.7055	0.0406368	0.008127
Magnesium sulfate	88.80	500	0.178	0.1185	0.0210456	0.004209
Manganese chloride	25.40	250	0.102	0.0025	0.000254	5.08E-05
Lactose	183.00	1000	0.183	90	16.47	3.294
Antifoam B	98.80	500	0.198	0.75	0.1482	0.02964
Premixed LB	135.00	1000	0.135	0.867	0.027	0.0054

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Vita

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