Part 1: Model objective

Introduction

1.1 Introduction

Our co-culture system is achieved by using sucrose as the sole carbon source. The sucrose is produced and secreted by an engineered *S elongatus* equipped with a CscB gene. Using sucrose as an energy source, *E. coli* produces two enzymes: PETase and MHETase, which breaks down polyethylene terephthalate (PET) into two monomers: ethylene glycol (EG) and terephthalic acid (TPA), with trace amounts of BHET.

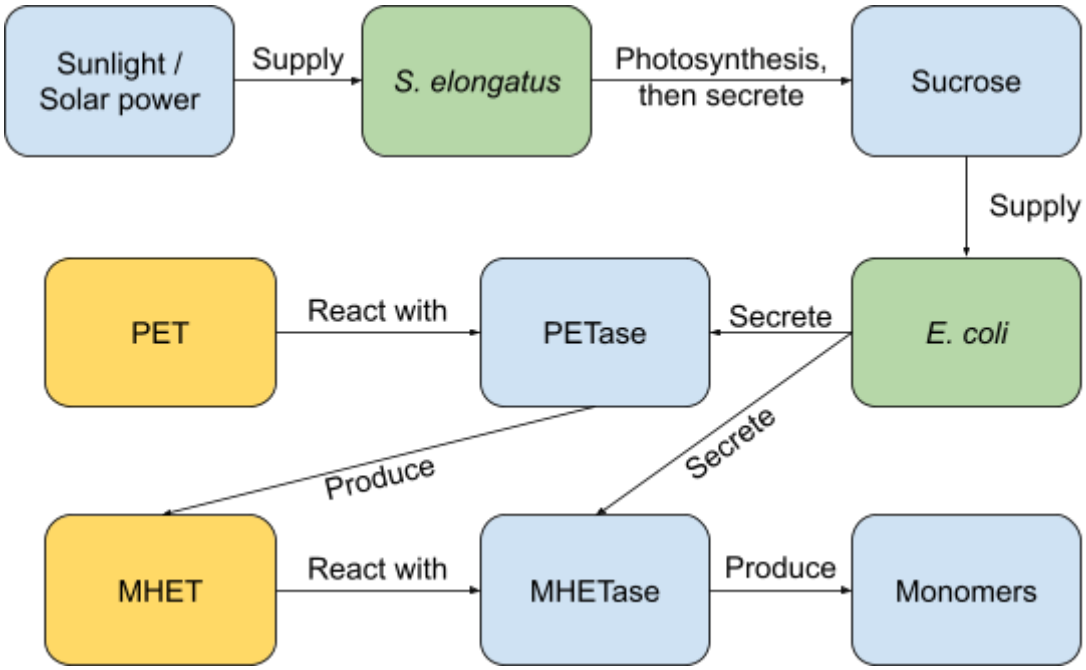


Figure 1: A summary of our system

1.2 Exploration Outline and Aim

The co-culture model consists of the growth functions of the two populations, cyanobacteria and *E.coli.* The specific growth rate equations take into account the effects of substrate availability, namely sucrose and oxygen for *E. coli*, and carbon dioxide for *S. elongatus*.

With the equations written out, a sensitivity analysis was conducted to determine the robustness of results and to understand how each parameter impacts the outcome of the production of enzymes and the growth of the populations. By interpreting the results and finding the production-determined factor, the system can be understood and controlled to optimise production. Overall, we hope the model can improve our understanding of the co-culture

system, help us determine the ratios of *E. coli* and *S. elongatus* and the optimal production rates of MHETase and PETase to digest PET.

We attempt a novel and different approach to optimizing enzyme production using methods from control theory. A thorough literature review showed rates of cell growth are highly dependent on the external conditions. However, media used to co-culture the two bacteria differ in papers, as do the specific medium recipes, as well as the suggested co-culture ratios of the two bacteria. This makes it hard to find a paper detailing cell growth that also matches our exact conditions. Hence, we take a more empirical approach and propose a more dynamic method of controlling the yield.

2. General assumptions and parameters

E. coli is dependent on *S. elongatus* for sucrose. This means the concentration of *S. elongatus* will be much higher than *E. coli* concentration, ensuring low levels of carbon dioxide and sucrose saturation. It follows that the pH will remain reasonably constant, and the low carbon dioxide concentration will yield relatively low amounts of oxygen gained in the co-culture through the photosynthetic process. This creates a stable low-growth situation which we can manipulate through the addition of dissolved oxygen. The limiting factor for the entire process then becomes the concentration of oxygen. Increased oxygen level will allow *E. coli* to grow and multiply, thus consuming more oxygen, with carbon dioxide as a byproduct. This then feeds the cyanobacteria, which generates more oxygen and sucrose, until the population approaches its carrying capacity, which ensures equilibrium.

3. Modelling and Control

With the assumption and parameters outlined above, we propose a simple control structure for the production of MHETase and PETase.

The biggest challenge in the creation of the co-culture is ensuring that:
(i) bacteria do not interact with each other in a negative way i.e. too much carbon dioxide,
(ii) bacteria do not oversaturate, and *E. coli* is
(iii) capable of steady state production

We first model the growth rate of *S. elongatus* and *E.coli* using the Monod model based on premise that oxygen availability is the growth limiting factor in aerobic bacteria, as oxygen has a low solubility in BG-11 medium[1]. To do so, we start by modelling the amount of substrate required for additional biomass yield. This is analogous to the relationship between cell growth and substrate available. This is important as protein products, including PETase and MHETase, are a function of metabolism and cell growth. This is represented by the following equation:

[1] Garcia-Ochoa, F., & Gomez, E. (2009). Bioreactor scale-up and oxygen transfer rate in microbial processes: an overview. *Biotechnology advances*, *27*(2), 153-176.

$$(1)\quad Y = \frac{dX}{dS} = \frac{mass\ of\ new\ cells}{mass\ of\ substrate\ consumed}$$

Where S represents a substrate required for growth, X as the cell mass, and Y as the yield coefficient. Substrates for *E. coli* include oxygen and sucrose, and substrates for *S. elongatus* include carbon dioxide.

We then investigate the relationship between substrate concentration and growth of bacteria: the specific growth rate. This is represented by the following two equations:

$$(2)\quad \mu = \frac{dX}{X_o} = \frac{1}{X_o}\frac{d}{d}$$

$$(3)\quad \mu = \mu_{max}\left(\frac{S}{S+K_s}\right)$$

where
$S = substrate\ concentration$
$K_s = substrate\ concentration\ at\ \frac{1}{2}\mu_{max}$
$\mu_{max} = max\ growth\ rate$

The second equation describes bacterial growth rates, while the third equation is a Monod equation that examines the relationship between substrate concentration and growth. This has been determined empirically through K_s, the substrate concentration of the max growth rate, μ_{max}. The equation shows that the bacterial growth continues until μ_{max}, at which population growth plateaus.

From the previous equations on bacterial growth rates and the relationship between substrate concentration and bacterial growth, we can also derive the following, which gives us the population at any given time:

$$(4)\quad \frac{dX}{dt} = \mu X_o = \mu_{max} X_o \left(\frac{S}{X+K_s}\right)$$

Using equations (1) and (3), we can also obtain an expression for the substrate utilisation rate at any given point in time:

$$(5)\quad \frac{dS}{dt} = \frac{\mu x}{Y} = \frac{\mu_{,max} X_o}{Y}\left(\frac{S}{S+K_s}\right)$$

As the substrate concentration needs to be large enough to support the bacterial population and its continual growth, we take the substrate concentration to be much larger than K_s (i.e. $S >> K_s$). Hence, we reach the following:

$$\frac{dS}{dt} = \frac{\mu_{,max} X_o}{Y} = constant$$

We see that zero-order kinetics describes *E. coli*, whereas *S. elongatus* is best described by first-order kinetics in our system. *S. elongatus* is substrate starved, while *E. coli* is substrate abundant, meaning that any change in substrate concentration will make little difference.

With all the above in mind, we propose to control the amount of light and oxygen in the culture with an automatic control system to ensure that the concentration of bacteria (measured via oxygen, and opacity feedback) stays constant. Through the use of such a system and data logging procedures, we will be able to find the optimal amount through the use of a linear regressor.

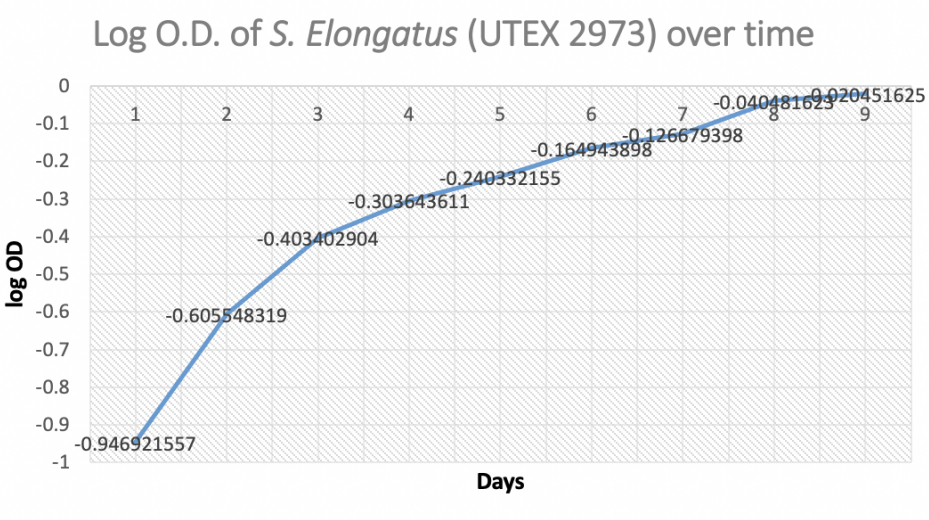
Concentration (g/L)	Representation / Symbol
E. coli	*E*
S. elongatus	*C*
PETase	*P*
MHETHase	*M*

Testing Assumptions

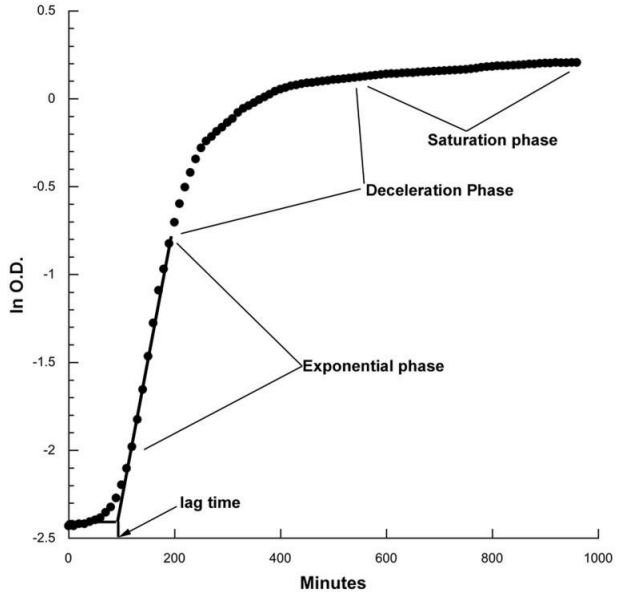
We have sought empirical data to test and apply our model assumptions. Data was obtained from literature and from our own laboratory data. We will also describe methods for improvement and data we would gather in the future where we would prefer to have more data. In order to obtain data, we have simplified our system and divided it into three main parts: cyanobacteria growth; interaction between *S. elongatus* and *E. coli*; and production and functioning of PETase and MHETase.

Cyanobacteria Growth

The following graph depicts data we collected on the growth of *S. elongatus* (UTEX 2973) over a period of a week. The optical density of the culture was taken every 24 hours.



This curve differs from the cyanobacteria growth curve that would be expected, shown below.



Hall, Barry & Acar Kirit, Hande & Nandipati, Anna & Barlow, Miriam. (2013). Growth Rates Made Easy. Molecular biology and evolution. 31. 10.1093/molbev/mst187.

In the future, we could record several more trials of bacterial growth using various *S. elongatus* strains, and compare them to available literature of strain growth rates. Should the curve continue to differ from Hall et al.'s (2013) curve, it may be that the strains have a different curve from a generalised cyanobacteria curve. We would also ideally have the same bacterial growth data for the strain we have chosen, PCC 7942. Regardless of the strain, having data on how amount of BG-11 medium (amount of substrate) impacts bacterial growth would be able to demonstrate the accuracy of our model in predicting the relationship between biomass yield and substrate availability.

Interaction between *S. elongatus* and *E. coli*

We are mostly interested in the optimal co-culture ratio between *S. elongatus* and *E. coli* for this section. As *E. coli* depends on sucrose from *S. elongatus* for survival, it is important to maintain a certain ratio between these two types of bacteria to secure food supply. Ideally, there should be no shortage or excess of sucrose in the system, but an excess in sucrose may be permitted if the growth rates of both bacteria are different, especially since they are being incubated in a similar environment (e.g. same temperature) inside a self-made bioreactor.

Liu et. al. (2021)[2] claims the co-culture ratio between *E. coli* and *S. elongatus* should be 1:1 or 1:4 (volume/volume). We would like to use this ratio to predict the sucrose balance over time, and to see if the system can be maintained.

It is assumed that the uptake to secretion ratio is maintained at a steady range as both bacteria grows. In general, that means a bacterial concentration change should not disrupt the equilibrium. This assumption is validated by Hays et. al. (2017)[3]'s results, where they even after they diluted the *E. coli* and *S. elongatus* consortia, the two species were able to grow again at a constant rate.

Secretion of nutrients by cyanobacteria

Lin et al. (2020)[4] showed that the CscB gene offered a cell the ability to utilise a hydrogen ion gradient across the plasma membrane to take up sucrose. CscB gene is thus a key sucrose/H+ symporter. This paper also mentioned that the highest rate of sucrose export from PCC 7942 - the cyanobacteria strain we are using in our co-culture system - can be up to 0.9g/L/day, with maximum amount up to 2.6g/L.

Uptake of nutrients of *E. coli*

Mohamed et. al. (2019)[5] offers data on different sucrose uptake rates of different *E. coli* strains. The number can be compared with the secretion rate by PCC 7942 from Lin et al. (2020), which would allow us to see what ratio of two bacteria would be ideal.

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[2] Liu, H., Cao, Y., Guo, J., Xu, X., Long, Q., Song, L., & Xian, M. (2021). Study on the isoprene-producing co-culture system of Synechococcus elongates–Escherichia coli through omics analysis. *Microbial Cell Factories*, *20*(1), 1-18.

[3] Hays, S.G., Yan, L.L.W., Silver, P.A. et al. Synthetic photosynthetic consortia define interactions leading to robustness and photoproduction. J Biol Eng 11, 4 (2017). https://doi.org/10.1186/s13036-017-0048-5

[4] Lin, PC., Zhang, F. & Pakrasi, H.B. Enhanced production of sucrose in the fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973. *Sci Rep* 10, 390 (2020). https://doi.org/10.1038/s41598-019-57319-5

[5] Mohamed, E.T., Mundhada, H., Landberg, J. *et al.* Generation of an *E. coli* platform strain for improved sucrose utilization using adaptive laboratory evolution. *Microb Cell Fact* 18, 116 (2019). https://doi.org/10.1186/s12934-019-1165-2

Co-culture Ratio

A comparison of sucrose secretion and uptake rates of *E. coli* and *S. elongatus* was conducted to determine the ideal co-culture ratio.

Table 1: Sucrose uptake rates of various E. coli strains

	Sucrose uptake rate (mmol/(gCDW*h))	**Final density (gCDW/L)**	**Sucrose uptake rate (mmol/(L*h))**	**Sucrose uptake rate (mmol/(L*day))**	**Sucrose uptake rate (g/(L*day))**
K-12 MGcscBKA starting strain	7.56	1.47	11.1132	266.7168	91.29622713
K-12 MGcscBKA 1	8.71	1.91	16.6361	399.2664	136.6674913
K-12 MGcscBKA 2	6.54	1.52	9.9408	238.5792	81.66482513
K-12 MGcscBKA 3	7.84	1.68	13.1712	316.1088	108.2029359
K-12 MGcscBKAp starting strain	7.01	1.48	10.3748	248.9952	85.23018548
K-12 MGcscBKAp 1	9.89	1.38	13.6482	327.5568	112.1215462
K-12 MGcscBKAp 2	9.50	1.44	13.6800	328.32	112.3827869
K-12 MGcscBKAp 3	8.98	1.51	13.5598	325.4352	111.3953299

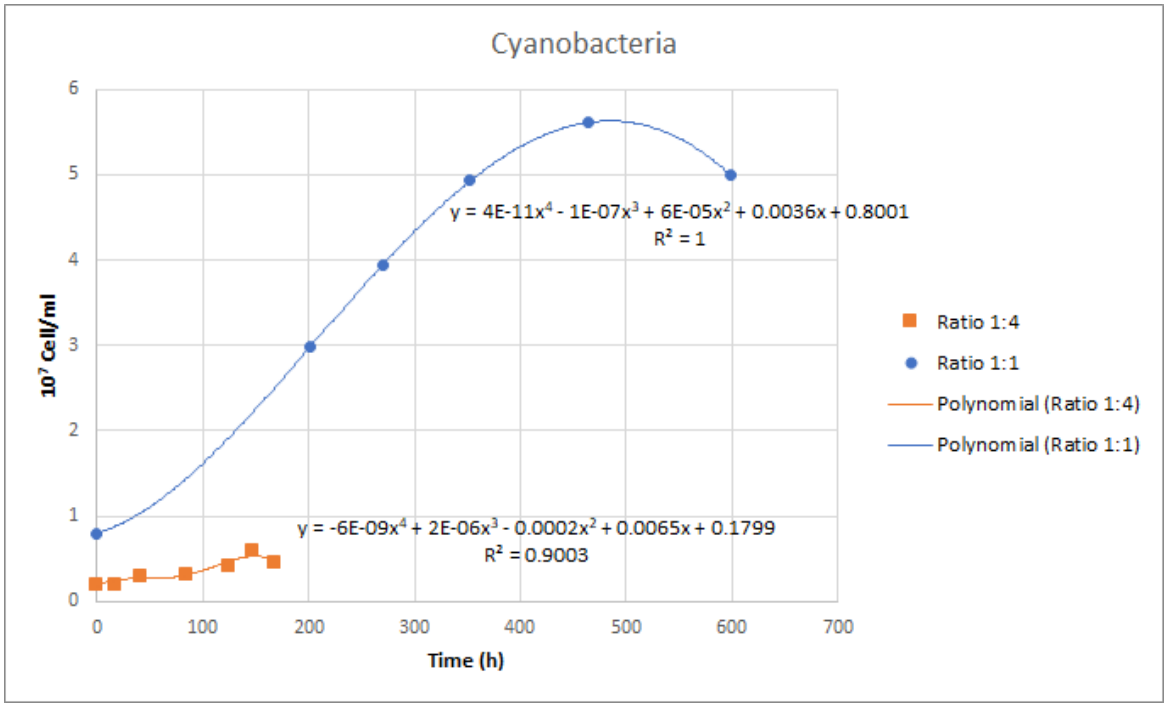
Table 2: Comparison of sucrose uptake and secretion rates of E. coli and S. elongatus

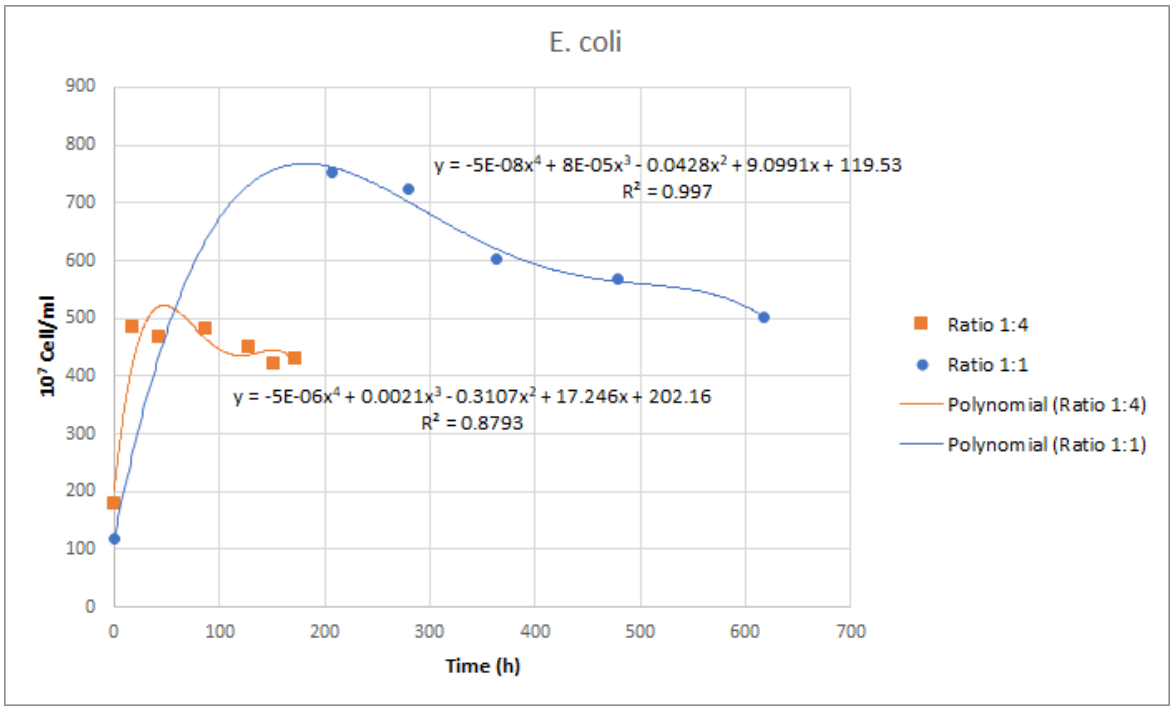
	Sucrose uptake rate by *E. coli* (g/(L*day))	**Sucrose secretion rate by *S. elongatus* (g/(L*day))**	**Uptake rate : secretion rate** **i.e. *S. elongatus* : *E. coli* (cells/cells)**
K-12 MGcscBKA starting strain	91.29622713	0.9	101.440252
K-12 MGcscBKA 1	136.6674913	0.9	151.852768
K-12 MGcscBKA 2	81.66482513	0.9	**90.7386946 (MIN)**
K-12 MGcscBKA 3	108.2029359	0.9	120.225484
K-12 MGcscBKAp starting strain	85.23018548	0.9	94.7002061
K-12 MGcscBKAp 1	112.1215462	0.9	124.579496
K-12 MGcscBKAp 2	112.3827869	0.9	**124.869763 (MAX)**
K-12 MGcscBKAp 3	111.3953299	0.9	123.772589

The comparison shows that the range of the *cell* ratio of *S. elongatus* and *E. coli* should be between 90.74 and 124.87.

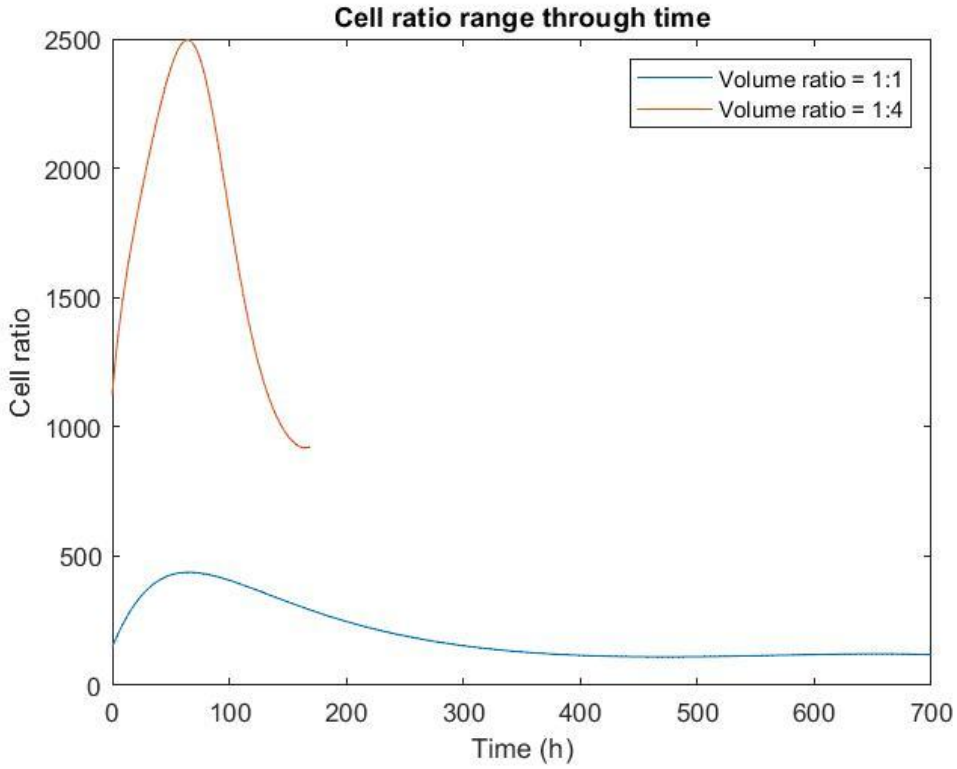
We can derive an equation from Liu et. al. (2021) to check if our cell ratio range is valid. If the range is valid, we should take care to control our ratio of bacteria within this range to make sure

our sucrose levels are balanced within our system (For more information about control, see: Limitations of the Monod Model).





Equations in the above figures show the growth curve of both bacteria under different volume ratios over time. With that, we can derive the cell ratio range. By further dividing the equations with each other for each volume ratio, we obtain cell ratio changes through time.



We can see that for volume ratio 1:4, the cell ratio is generally above 1000, i.e. *S. elongatus* : *E. coli* is over 1000:1, which may not be ideal for us, as we would prefer more *E. coli* to produce PETase secretion. This preference may be different to others who create co-culture systems with similar organisms (eg. Liu et al. (2021)), who may be engineering *S. elongatus* to produce the proteins of interest (eg. isoprene)..

For volume ratio 1:1, the cell ratio falls into our ideal range of 90.74-124.87 when time reaches around 370h. In conclusion, we predict that a 1:1 volume ratio would generally yield better results for our project that seeks for higher secretion of PETase and MHETase from *E. coli*.

Reaction Rates of PETase and MHETase

Enzymes are affected by different environmental conditions including temperature, pH, and enzyme concentration. Assuming that PETase and MHETase will denature at high temperatures and see low activity at lower temperatures, we modeled PETase reaction rates at 30°C, 48°C, and 58°C. We found that PETase has highest reaction rate at 58°C. We then looked into the changes in reaction rates as PETase concentration varied. Equations below were derived using data from Zhong-Johnson et al. (2021)[6].

[6]Zhong-Johnson, E. Z. L., Voigt, C. A., & Sinskey, A. J. (2021). An absorbance method for analysis of enzymatic degradation kinetics of poly (ethylene terephthalate) films. *Scientific reports*, *11*(1), 1-9.

The equation below describes the relationship between PETase concentration (nM) and reaction rates (mA260/min):

$$y = 3E\text{-}07x^3 - 0{,}0002x^2 + 0{,}0458x + 0{,}6593$$

We then modeled the reaction rates of PETase as pH varied. Reaction rate of PETase is measured by measuring the amount of products (mM) produced after 6 hours and 18 hours of PETase activity. The following equations describe the effect of pH on the reaction rate of PETase at 6 hours and 18 hours respectively:

$$y = 0{,}077x^4 - 2{,}6917x^3 + 34{,}952x^2 - 199{,}71x + 423{,}66$$
$$y = -0{,}1253x^4 + 3{,}6898x^3 - 40{,}055x^2 + 190{,}58x - 336{,}12$$

From the above, it is seen that PETase has the highest reaction rate at pH 9 at both time points (6h and 18h after enzymatic activity).

Limitations of the Monod Model

The Monod equation of growth is only applicable for balanced growth. That is, when a pseudo-steady state inside the co-culture has been established. Therefore, it is not applicable in the lag phase. Given that we are currently unable to completely verify the vigour of our model through empirical data, we have instead opted to incorporate both available empirical information and insights offered by our model in future work, using sensors and artificial intelligence to further understand our system and maintain optimal conditions, which is detailed in this section. The lack of a pseudo-steady state during early stages of the co-culture can be dealt with through this, measuring the delay and accounting for it when coding our system.

Algorithm

Given sufficient time, the co-culture will reach equilibrium. Upon reaching that, the sensors will measure the concentration of colonies. We start from a lower bound of *E. coli* and increase the oxygen by a small amount. The yield is approximated to be proportional to the stable *E. coli* colony.

Implementation of Oxygen Control and Sensing Method

For the control method to work, we need to measure the biomass. For *E. coli*, we opted to measure the concentration of *E. coli* based on optical density. The optical density of the colony is measured by passing light through the co-culture. The intensity of the light is measured with a photoresistor, connected to a microcontroller to process data. The photoresistor is capable of measuring the RGB value of the light. With this, we are able to measure the intensity of green light, which will reduce with a lower *S. elongatus* population, as *S. elongatus* is green and

reflects green light. Likewise, *E. coli* absorbs wavelengths of 600nm[7]. Using this, we are capable of measuring the concentration of bacteria in our culture system.

Oxygen levels are measured using an oxygen probe. CO_2 concentration on the other hand is assumed to be small relative to the amount of oxygen as we oversaturate the cyanobacteria concentration, hence, most carbon dioxide will be converted to oxygen by the colony.

Figure 2: An oxygen probe

[7] BioTek. (2008, October). *Monitoring the growth of E. coli with light scattering using the Synergy™ 4 multi-mode Microplate Reader with hybrid technology™: January 15, 2008*. BioTek. Retrieved from https://www.biotek.com/resources/application-notes/monitoring-the-growth-of-e-coli-with-light-scattering-using-the-synergy-4-multi-mode-microplate-reader-with-hybrid-technology/.