Milestone 5 - Using Bromoform to Combat High Methane Emissions from Cattle

IBEHS 2P03 - Health Solutions Design Projects II: Introduction to Genetic Engineering

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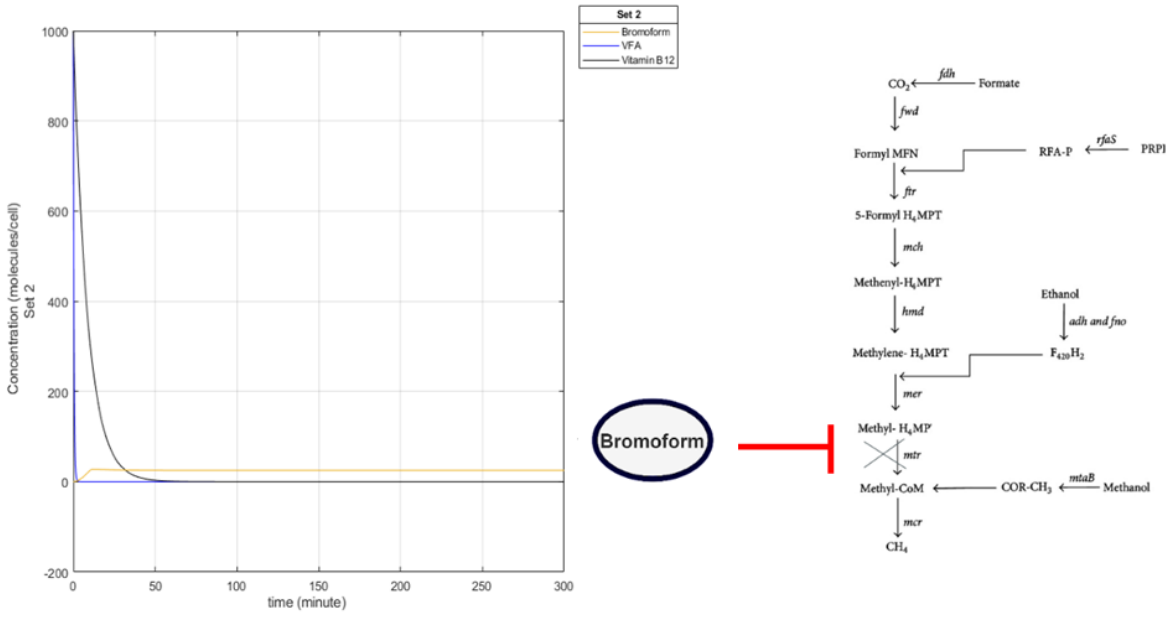
Abstract

Methane is a short-lived byproduct of digestion in ruminant livestock. With an effect 25 times more potent than carbon dioxide, it's an obvious target for the fight against climate change in the agricultural sector [1]. Prior studies have shown that when fed *Asparagopsis Taxiformis*, a particular species of red seaweed, methane production from cows was significantly reduced (68-99% reduction) [2]. The reduction in methane is due to the compound bromoform, which is produced by the species of red seaweed. This research project proposes a recombinant *E. Coli* species that will be ingested by the cow along with the required inputs to activate circuitry to produce bromoform. There are two proposed in vitro testing measures that aid in the investigation of the proposed solution. To complement the in vitro testing, a Simbiology model was constructed to analyze and investigate the circuit created. The results of the simulation support the circuit's logic, indicating that the solution is viable for methane reduction.



Introduction

The problem that this biological solution aims to combat is finding a method to reduce methane gas emissions from the agricultural industry. Worldwide statistics rank emissions from ruminant livestock emissions as 14.5% of all global greenhouse emissions. The leaders in agricultural greenhouse gas production are cattle - a single cow produces roughly 220 pounds of methane in a year. The purpose of utilizing recombinant *E. Coli* bacteria is to reduce the methane emissions released by cattle during digestion, by using it as a vector for a methanogen-reducing agent; bromoform. Global warming is a grave threat that impacts everyone in the world. Its effects can already be felt in every country on every continent, disrupting economies and affecting lives. In the United States alone, more than 15,180 people have been killed by climate change-related disasters, and over $2.195 trillion has already been spent since 1980 as a result [3]. As timely as the issue of methane production is, there are many factors that must be analyzed before a solution can be implemented. In approaching the problem with the production of bromoform intended to stop methanogenesis (as what occurs with red seaweed feed), the issue of bromoform being harmful to the environment if released into the atmosphere is also cause for concern, as it can contribute to ozone layer depletion [4].

Materials and Methods

A full list of materials for the in vitro experiments can be found in *Table 4*. The following proposed efficacy experiments and assembly will be conducted upon verification of the MATLAB SimBiology model that will be analyzed in the **Results** section.

Gibson assembly will be used because it allows the assembly of multiple fragments at once, leaves no scarring (which could alter the properties of the DNA), and eliminates the requirement of restriction sites [5],[6]. While Gibson Assembly is not ideal for parts under 200bp as the endonucleases utilized in this method can damage coding sequences, matching restriction sites were not available for the parts used in the circuit. If it is observed that Gibson Assembly is damaging the smaller parts, 3A Assembly can be used as a last resort.

Experiments needed to demonstrate efficacy of the biological circuit in vitro

In Vitro Experiment 1:

Prior to any in vitro testing, PCR will be used to amplify the biological parts in order to increase the chances of a successful ligation to the plasmid backbone (PCR protocol [7]). To test the results of the ligation process used to create the recombinant plasmids (Ligation protocol [8]), gel electrophoresis trials will be conducted (Gel Electrophoresis protocol [9]). The results of the test will help indicate if the ligation of biological parts and the plasmid backbone was successful. The assumed-to-be ligated PCR product would be run alongside a control test, which would consist of a PCR product created without the forward or reverse primers.

Once the PCR amplification and digestion process have been confirmed as successful by the gel electrophoresis, insertion of the plasmid into the host *E. Coli* will be performed. In order to do so, the heat shock method will be utilized [10]. To verify the recombinant colonies grown, the Sanger sequencing technique (in addition to capillary electrophoresis) will be used to identify if the target DNA has been inserted [11]. After screening the *E. Coli* to ensure the proper recombination, in vitro testing will begin. To simulate the environment in which the *E. Coli* will be introduced, an agar plate mimicking the pH, nutrient-depleted environment of a cow's rumen will be used. Alongside the recombinant *E. Coli*, microbes taken from the tissue of a cow's rumen will also be introduced into the agar plate. Once the target number of colonies have grown, the bacterial plates will be introduced to a gas chamber, where they will be exposed to the necessary inputs to initiate their circuitry (Volatile Fatty Acids (VFAs) and B12). The inputs will stimulate the digestion patterns of a cow, and bacterial samples taken from the cow's tissue will commence the production of methane gas. In theory, if the recombinant circuitry is successful, the readings of methane gas produced in the chamber should be minimal compared to a control test performed solely with bacterial samples taken from the tissue of the rumen.

In Vitro Experiment 2:

To test the efficacy of the bromoperoxidase circuit, the plasmid containing the circuit will be inserted into planktonic microbial communities through heat shock , and tested using Sanger sequencing using the protocol outlined above. Bromoperoxidase catalyzes the de- arylation of aminophenyl fluorescein via H2O2, which can be detected using a fluorometer [10],[11],[12].

Once the plasmid is inserted, the microbial culture will be gravity filtered onto a polycarbonate filter in near-axenic conditions, to limit contamination. *Navicula* species plankton (CCMP 546) will be used as both the experimental and negative control, as this species does not naturally contain bromoperoxidase (where the control has no plasmid insertion) [13]. As a positive control, *Nitzschia* species plankton (CCMP 580), which naturally contains bromoperoxidase, will be used [13].

After filtration, the microbial cells will be lysed through bead beating in a tube, where the cells are broken down to release bromoperoxidase. Zirconia beads are used along with the Retsch Mixer Mill MM 400 for beating in the presence of a non-ionic surfactant [14]. Non-ionic surfactants are neutrally charged and are thereby able to remove organic material [15].

The solution is then to be placed in the MES buffer to undergo further bead beating. At this stage, hydrogen peroxide will be added for the aminophenyl fluorescein to undergo dearylation to fluorescein if bromoperoxidase is present. The Photon Technology International (PTI) QuantaMaster fluorometer will be used to detect the amount of fluorescein present in the solution [12]. It is expected that the negative control solution provides a detection reading of 0 fluorescein, and the experimental solution reading closely resembles that of the positive control. If this is the case, it will show the circuit is working as intended.

Results and Discussion

Synthetic Biology Open Language Model (SBOL)

The circuits depicted are simplistic representations of the overall mechanisms at play in the simulated model. The production of hydrogen peroxide and bromoperoxidase are the key outputs of the circuit, as they are required to reduce/prevent the methane production in the cow's rumen. The VFA-dependent and ROS circuit can be simply expressed as an AND gate, where the inputs required are Volatile Fatty Acids (VFAs) and Vitamin B12. Additionally, the circuit also possesses co-factors; Heme, Flavin, and NAD(P)H that act as secondary inputs in their own AND gate, to form a complex with Mbb2 to produce hydrogen peroxide. Finally, the circuit possesses a third AND gate in order to produce bromoform with the available species created by the preceding circuits' logic gates. The temperature-dependent kill switch is modeled as a fail-safe in order to induce apoptosis and can be simply represented as a NAND gate.

For each circuit, it is assumed that the associations and dissociations for each complex that is formed with multiple cofactors occur in one reaction (one association and dissociation reaction without intermediate steps for binding each cofactor separately). It is assumed that the association, dissociation, and catalytic rate constants are the same for each complex. It is also assumed that the bromide required in the final AND gate production of bromoform is delivered into the rumen along with the *E. Coli* as it is not naturally present in cows. The toxicity of the concentration of bromide will be taken into account, though studies have shown low toxic effect even at large quantities of bromide [16]. Furthermore, it is helpful to assume that there is adequate bromide present in the cell for the reaction, it does not decay or is lost from the cell in any other pathway other than the reaction, and this quantity is only available in finite amounts. The bromide needed for the reaction to take place is assumed to be supplied by the vector used for delivery (1000 molecules per cell). Finally, it is assumed that any substance present in the rumen required as inputs or cofactors is brought into the cell through endocytosis, making them available for reactions.

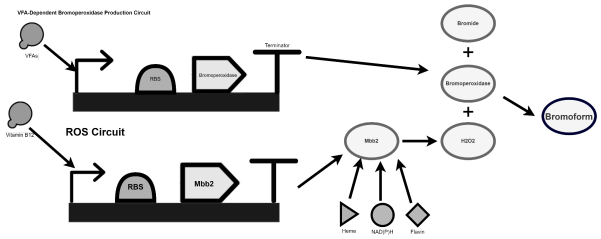


Figure 1. SBOL diagram depicting bromoform production in an E Coli bacterial culture.

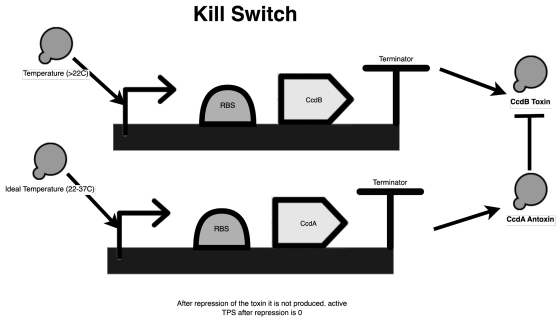


Figure 2. SBOL diagram depicting the temperature-dependent kill switch used as a fail-safe to protect cow by-products from recombinant *E. Coli* cultures.

ROS Production Circuitry - MatLab

The ROS production circuit works to produce Mbb2, an enzyme that forms Hydrogen Peroxide to react with Bromoperoxidase to produce Bromoform, which is dependent on the presence of Vitamin B12. Please note that for the decay rates of compounds and cofactors, the rates were calculated by dividing ln(2) by the researched half-life, in most cases. Additionally, the enzyme produced by this circuit, Mbb2, is dependent on the presence of Heme, Flavin, and NADPH as cofactors. It is assumed that Heme, Vitamin B12, Flavin, and NADPH, are always available in sufficient and large (at least 1000 molecules per cell) amounts as they are common inside animals. To accurately model the appropriate in-vivo conditions of this circuit, Vitamin B12 will be varied to illustrate the dependence of H2O2 production on it. Its half-life of 6 days will be included as well. The model will also include the transcription rate of Mbb2, its translation rate, and Mbb2 mRNA decay rate constant. These conditions should aid in the accurate representation of Mbb2 production.

Once Mbb2 is produced, the model must account for interactions of Mbb2 and its cofactors in the production of H2O2. The half-lives of Heme, Flavin and NADPH (1 hour, 75 minutes, and 2 hours respectively) were included to account for the concentrations of Mbb2's cofactors over time since they will directly impact its function. Additionally, since Mbb2 forms a complex to produce H2O2, the complex formation constant and dissociation constant were also considered to help determine the rate of Hydrogen Peroxide production from the circuit. To model enzyme kinetics, the mass action rate assumption was used, hence the constants for complex association, dissociation, and catalytic rate needed to be found. Michaelis-Menten Kinetics was not used, so as to model the interactions between the intermediates instead of only the final products. The constants used to determine association and dissociation rates (asp and dsp in the Appendix) were taken from the DT3 material as the values for this specific complex were not readily available. The Mbb2 complex was the most complicated reaction in the model as it required the largest number of cofactors. It is expected that with no B12 present there will be little to no production of Hydrogen Peroxide since transcription and translation of the Mbb2 coding sequence would not occur. On the other hand, it is expected that with high initial concentrations of B12

and appropriate levels of all cofactors, there will be increased production of Hydrogen Peroxide since Mbb2 should be produced and has the necessary cofactors to function properly.

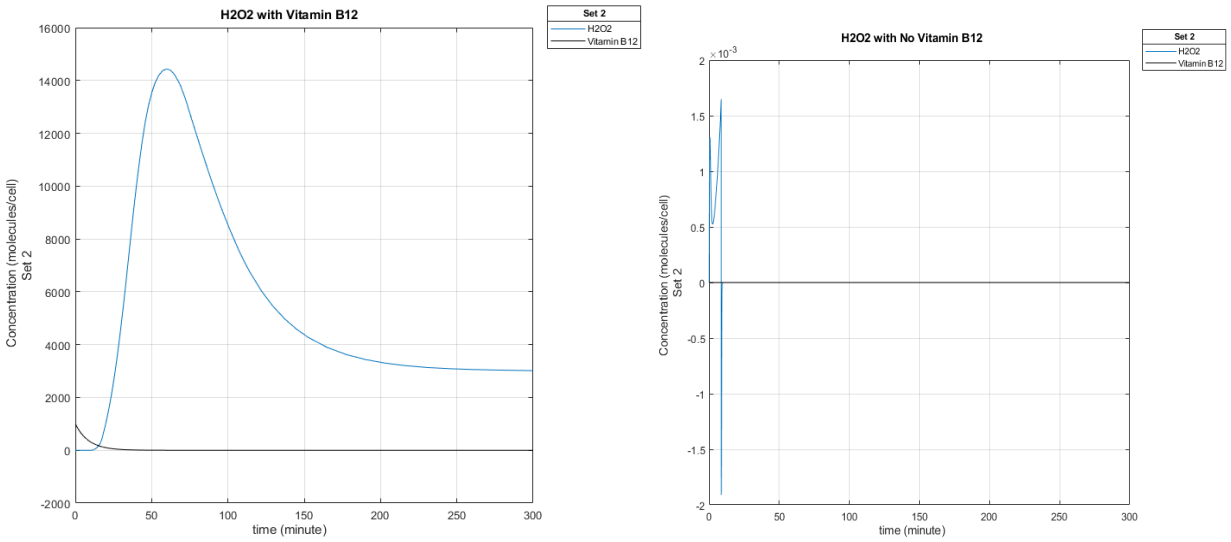


Figure 3a,b. As seen in *Figure 5a*, left, with high initial concentration of Vitamin B12, high levels of H2O2 are produced. Then as B12 is used up, H2O2 production falls, and as it degrades and gets used up by bromoperoxidase, H2O2 levels drop. In the absence of Vitamin B12 (*Figure 3b*, right), there is a negligible level of Hydrogen Peroxide (there is some erratic behavior due to the complications of modeling), as expected since transcription and translation of the enzyme producing it should not occur.

VFA-Dependant Bromoperoxidase Production Circuitry - MatLab

The VFA-dependent bromoperoxidase production circuit represents the in vivo detection of volatile fatty acids within the rumen, to indicate digestion is taking place. Within the circuit, VFA degradation was included to represent the natural progression of VFAs in the rumen, as they are used by the cow. The initial concentration of VFAs is rather high (1000 molecules per cell), as they are present in large amounts in the rumen. As in the ROS circuit, this rate was calculated by dividing ln(2) by the acetate half-life, as acetate is the most common VFA found in the rumen. A baseline concentration is included in the simulation, which is initially detected to begin transcription of bromoperoxidase. The transcription and translation rates of bromoperoxidase used in the circuit are included to accurately represent the in vivo conditions. Roughly 35 molecules of bromoperoxidase are produced when in the presence of VFAs. This is because bromoform has a half-life of 1-2 months, so limiting the amount of bromoperoxidase generated, ensures that large amounts of excess bromoform are not present in the rumen, and will limit the chances of exposing the environment to it. When not in the presence of VFAs, very little bromoperoxidase is produced, as VFAs are produced as the result of complete digestion within the rumen, thereby activating the promoter used in the VFA-dependent bromoperoxidase production circuit, as seen in Figure 4a.

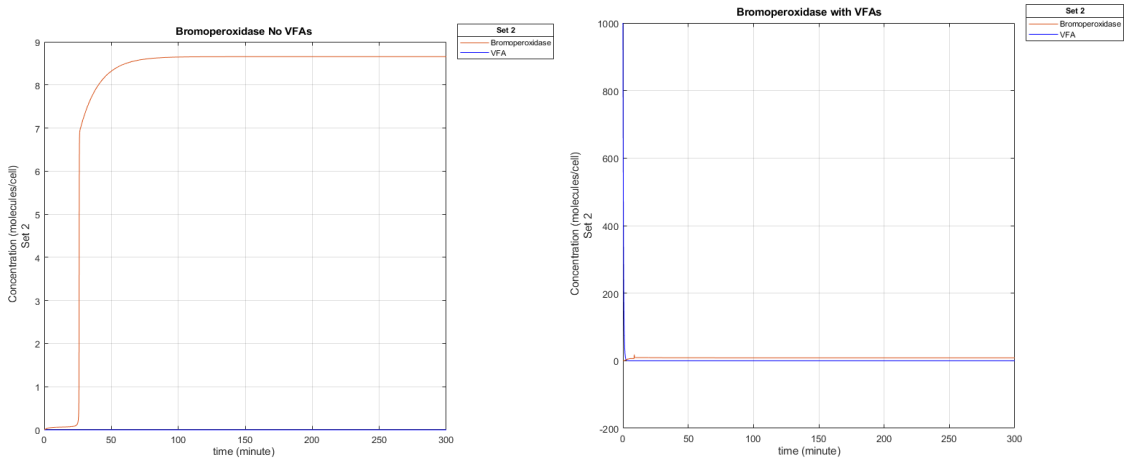


Figure 4a,b. Shown by *Figure 4a*, left, with zero VFAs present, very little Bromoperoxidase is produced due to the employment of hill-kinetics. With high initial levels of VFAs (*Figure 4b*, right), higher levels of bromoperoxidase are produced, as expected since VFA presence will allow for transcription and translation of the bromoperoxidase coding sequence.

Kill Switch Circuitry - MatLab

The kill switch circuitry is based on a model produced by F. Stirling et al., designed to induce apoptosis of the host under given environmental conditions [16]. At the ideal range (22-30 degrees Celsius) the antitoxin CCdA is being constantly transcribed at a low rate. Once the temperature is outside the ideal range, the transcription of the toxin CCdB commences at a much higher rate due to the strong promoter it proceeds. The simulated model accounts for the decay of the mRNA and proteins created during the circuit. The model also accounts for the difference in transcription rates in the translation efficiency value of CCdB which is at least double (59.94 for CCdB and 26.97 for CCdA, units in Transcripts/(promoter*min)) as required by the literature supporting the circuit. The TA complex depicted in the model represents the interaction of the antitoxin and toxin wherein the antitoxin CCdA acts to inactivate the toxic component of CCdB rendering it harmless to the cell. At the low concentrations of CCdA transcribed, this complex will only occur if there is a trace amount of CCdB. To model the temperature-dependent transcription a reflected hyperbolic tangent function was used, as such, it sets transcription to 0 after 22 degrees. This model assumes that the rate of transcription, translation, and decay rates of the mRNA and proteins are constant, specific values, and were taken from lecture slides and coursepack. To develop the most accurate model possible, in vitro testing would be performed to derive the proper values. The trends described in *Figures 5a,b* are expected as they show that an overpowering concentration of CCdB will be present when outside the ideal range. As well there are low, but present concentrations of CCdA being transcribed regardless of the temperature range.

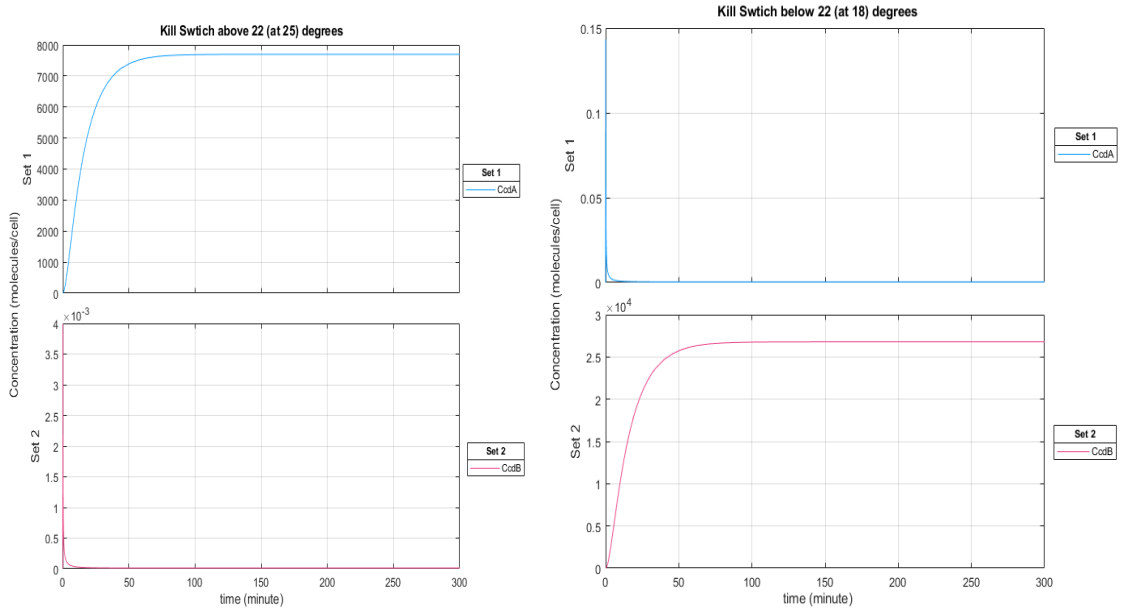


Figure 5a,b. Kill switch at the ideal (22-30℃) non-ideal (<22℃) temperature conditions. The results of *Figure 5a, (left)*, conclude that while trace amounts of transcribed CCdB are present the strong concentration of CCdA neutralizes the toxin and renders it harmless to the cell. *Figure 5b, (right)*, concludes that outside the ideal temperature range there will be a high concentration of CCdB transcribed and will therefore overpower CCdA and induce cell death.

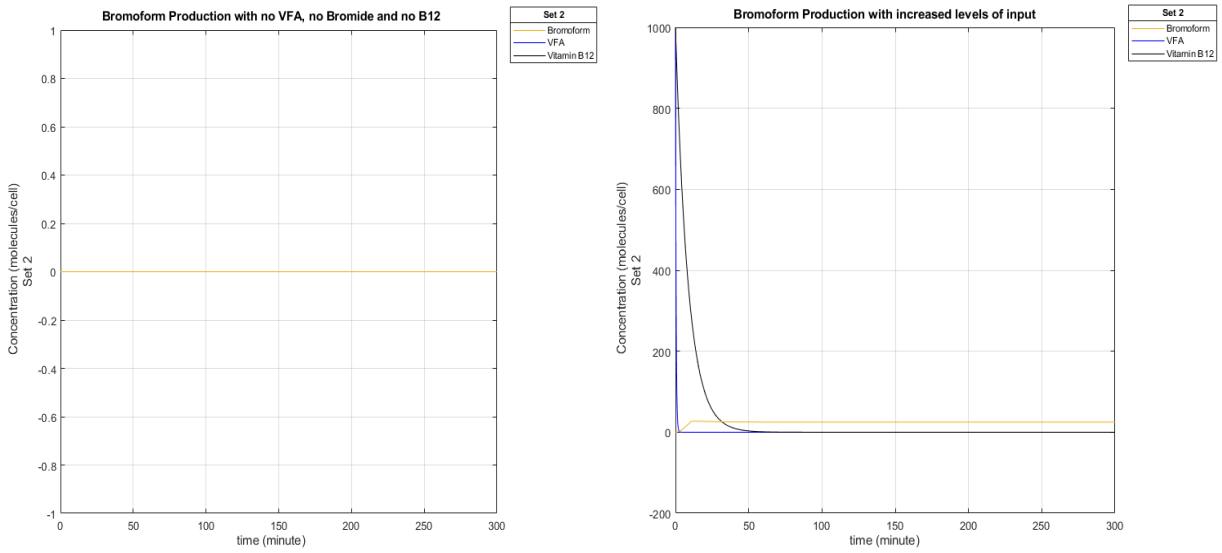


Figure 6a (left). Bromoform production without the necessary inputs (VFA, Bromide and B12). Without the necessary inputs it is expected that there would be no bromoform productions as its necessary inputs are not present. *Figure 6b (right)*. With maximum levels of all system inputs , Bromoform levels increase. Levels of Bromoform are not incredibly high as its half life is 1-2 months, resulting in very little degradation. Less bromoform production is desired so as to avoid any negative impacts on the cow and environment.

The modeling is limited by the values of cofactors that could be identified via the literature and what could be investigated using in silico models, having in vivo and in vitro models would aid in understanding the behavior of the model in greater detail. It was assumed that all cofactors are present and available in sufficient amounts,and that there wouldn't be other reactions that would prevent the pathway from being completed. The results indicate that bromoform is successfully produced, but not in large quantities. This implies that the model may not be producing sufficiently accurate results. However, the accuracy can only be properly determined through in-vitro or in-vivo experiments. Some values that were used were estimated using similar compounds as a reference, which may not have sufficed. The results indicate that more bromide is needed for this system to work, meaning the delivery of bromide to the target site is essential, however this process must be tested using further in-vivo experiments. Currently it is understood that the circuit is limited by the concentration of bromide that can be safely delivered to the rumen.

Conclusion

Given the results, it is visible that the recombinant bacterium functions in the idealized conditions inside an idealized simulation. However conducting in-vitro and in-vivo experiments is essential for the proper development of the product. These experiments would help in verifying the values for the constants found via other researching articles. It would also mean the development of appropriate vectors for delivering the recombinant E.coli to the rumen alongside the non-naturally occurring cofactors and determine how well the vector will sustain the function of the delivered system. It is paramount that the amount of bromoform released outside of the body is measured and reduced to avoid causing damage to the ozone layer, which would result in this solution's implementation creating another problem. This solution could also be further developed as a viable method of inhibiting methanogenesis in other ruminants, specifically those widely domesticated. Bringing this solution to market the approval of Food and Health inspection agencies would be necessary, as the intended market is the agricultural industry that utilizes ruminants in a large capacity. Governing bodies should be made aware of this solution, especially those in areas where ruminants are present in large populations. As in this way more impact in reducing methane emissions is made in communities that have a culture around taking care of ruminants.

Appendix

Plasmid Map of Biological Circuits:

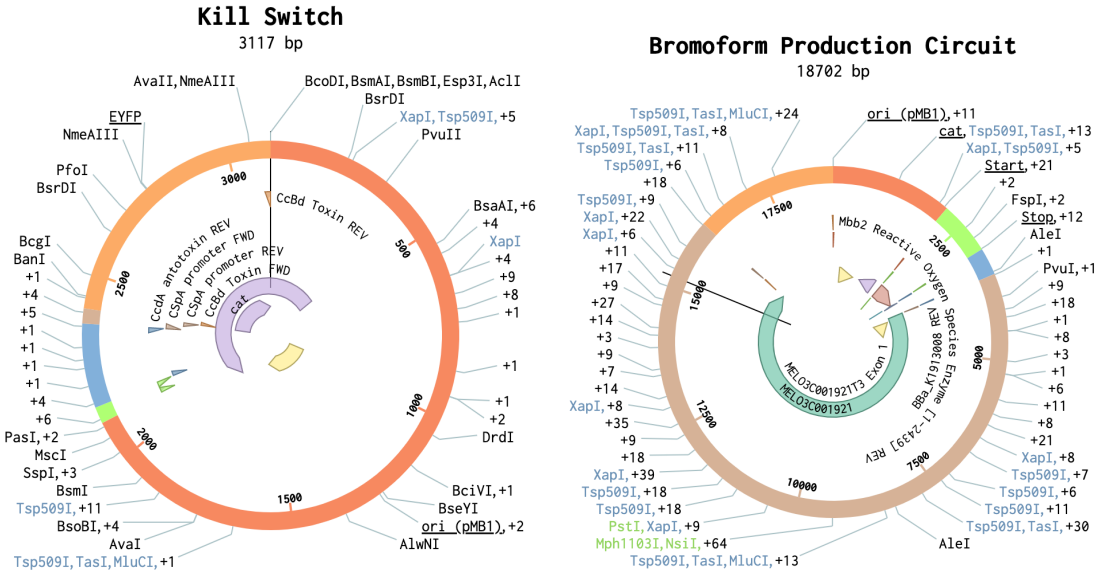


Figure 7. Plasmid diagram depicting bromoform production in an E Coli bacterial culture.

Figure 8. Plasmid diagram depicting the temperature-dependent kill switch used as a fail-safe to protect cow by-products from recombinant *E. Coli* cultures.

Table 1: List of Biological Parts

Part Number	Name/ Main Function	Description
BBa_K817002	Volatile Fatty Acid (VFA) Detection	With the presence of VFAs in the cow's rumen during digestion, this part will serve as a "start" condition for the transcription of bromoperoxidase while digestion is active. This part is vital for circuits' intended function as it will only promote transcription of bromoperoxidase during a period of digestion which is also the time when methane is produced [17].

BBa_K461002	Bromoperoxidase compound	Bromoperoxidase is a coding sequence that catalyzes the reaction of hydrogen peroxide into bromoform. Bromoform has been proven to inhibit the rumen's microbial layer, therefore leading to lower amounts of methane emissions. This coding sequence is crucial to the overall mechanism, as bromoform cannot be synthesized from hydrogen peroxide without the aid of bromoperoxidase [18].
BBa_K1913008	Vitamin B12 Detection	Vitamin B12 is present in the cow's rumen and is required to react with bromoperoxidase to form bromoform. This promoter detects when vitamin B12 is present to proceed to transcribing hydrogen peroxide [19].
BBa_K3947001	Hydrogen peroxide production	Hydrogen peroxide is required in the production of bromoform. This part contains the coding sequence for hydrogen peroxide production. The cofactors required are available due to the following reasoning. NAD(P)H is produced in pathways of reactions happening in animals, Heme and Flavin are organic compounds found inside animals [20].
BBa_K1319000	CcdB Toxin	This part plays a role in the cryo-death pathway by inhibiting DNA gyrase and inducing double strand breaks within E Coli [21].
BBa_K2282003	UP element followed by CspA Promoter	The transcription of CspA gene is believed to be regulated by the UP promoter at cold temperatures. The CspA promoter is also considered a strong promoter which works

		well in conjunction with the CcdB toxin that must be strongly promoted to overcome the antoxin effects of CcdA [22].
BBa_M36801	LacUV5 Promoter	This promoter will be placed before the transcription of CcdA, the antoxin to CcdB. The promoter will have no regulation and will consistently be transcribing CcdA at low concentration as it is considered a weak promoter [23].
BBa_K2142006	CcdA Antoxin	CcdA plays an important role in the switch as it will always be transcribed by LacUV5 (weak promoter) to inhibit the toxic effect of any CcdB that may be inadvertently transcribed [24].
BBa_B0036	Ribosome binding site	An RBS in the case of E.coli (the Shine-Dalgarno sequence) is necessary to begin transcription. This part is a constitutive (unregulated) RBS found in the iGem Standard Registry of Biological Parts from the Anderson RBS library family [25],[26].

BBa_B0010	Terminator	Terminators are required to stop transcription of a gene. This part is the most commonly used type; a forward terminator [27],[28].

Table 2: Primers Designed based on Biological Part Indicated

Part	Enzymes	Forward Primer	Reverse Primer
UP element followed by CspA Promoter	Gibson Method used	Acgagaacagggactggtg aaaaataattgttgcatcacc cgc	cagctcctcgcccttgctcaatt aagccacgcattggcgg
CcdB Toxin	Gibson Method used	ccgccaatgcgtggcttaatt gagcaagggcgaggagctg	tatgtttttcgtctcagccagtac agctcgtccatgccga
CcdA Antoxin	Gibson Method used	gaattgtgagcggataacaa atgaagcagcgtattacagtg	ggtgatgcaacaattatttttca ccagtccctgttctcgt
LacUV5 Promoter	Gibson Method used	aaactcacccagggattggc gcttccggctcgtataatgt	actgtaatacgctgcttcatttgt tatccgctcacaattc
Mbb2 Reactive Oxygen Species Enzyme (Hydrogen Peroxide Production)	Gibson Method used	GAATTCGCGGCCG CTTCTAGatggccaag cagaaaagcct	TACTAGTAGCGGCC GCTGCAGgctgaaatttt cctcccgaa
Volatile Fatty Acid Detection	Gibson Method used	gaggaactactatggccggc atgaatattcgtgatcttgagt ac	aggactgagctagccgtcaatt aaaccgcctgttttaaaact
Bromoperoxidase compound	Gibson Method used	acgctctggctgcttaataaT CCCTGTTTTCAAA CTTCTCC	CTAGAAGCGGCCG CGAATTCTTAAAA ATGTCCAAAAGAA ATTAGCA
Vitamin B12 Detection	Gibson Method used	ttttaaaacaggcggtttaatt gacggctagctcagtcct	GAGAAGTTTGAAA ACAGGGAttattaagcag ccagagcgt

MATLAB SimBiology Model Diagram

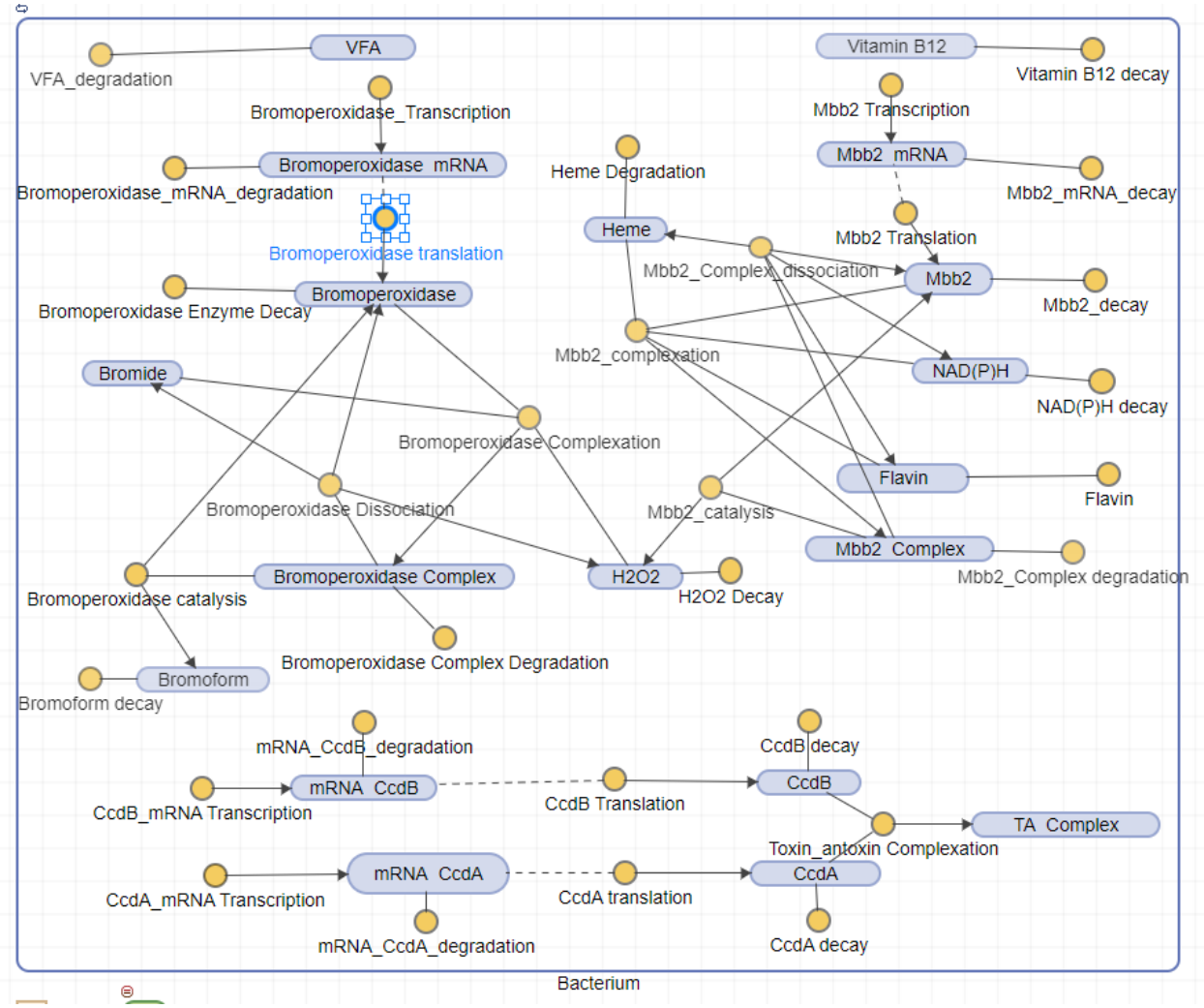


Figure 9. Screen capture of simulated cell with working circuitry to reduce methane production.

Table 3: SimBiology Parameters

Name	Value	Units	Taken from
kd_VFA (VFA decay rate)	2.40	mM/Minute	[29]
B12 half life	6	Day	[30]
Translation efficiency	20	Proteins/mRNA	DT3
tps_repr	5.0e-4	Transcripts/(Promoter* Second)	DT3

tps_active	0.5	Transcripts/(Promoter* Second)	DT3
Protein half life	10	Minute	DT3
n	2	Dimensionless	DT3
mRNA half life	2	Minute	DT3
Km	40.0	Monomers/Cell	DT3
aps	0.4000	1/(molecule*Second)	DT3
dps	1.2000	1/Second	DT3
Temperature	(variable)	Celcius	N/A
H2O2 half life	24	Hour	[31]
Bromoform half life	0.2	Day	[32]
NAD(P)H half life	2	Hour	This half life is based on the information for NAD [33]. Which is similar to NAD(P)H.
Kc	10	1/Second	The mode of the set of values is roughly 11, and the mean is roughly 177. The collection has a sample standard deviation of 797, meaning there is a high variance given the mean. This means it would be practical to have a low Kc (catalytic rate constant) in the range of 1-10. This is reasonable as the literature provides values that are proximal to 0 s^-1 for enzymes present in *E. Coli* [34]. This assumed value is applied to Bromoperoxidase and Mbb2 enzyme

			complexes as the values for these couldn't be clearly found in literature [35].
Flavin half life	75	Minute	The Flavin half life was based on Riboflavin Elimination half life, which is 66-84 mins (taken as 75 mins) [36].
Heme half life	1	Hour	[37]

Table 4: Required Materials for In Vitro Experiments

Material	Where to Obtain	Information
10 µm forward primer	Link	Custom forward and reverse primers will be ordered from ThermoFisher.
10 µm reverse primer		
List of biological parts (See appendix)	Link	Each part from the list of biological parts will be custom ordered from IDT.
Gibson Assembly kit	Link	The Gibson kit enables the assembly of multiple DNA fragments with little dependency on their respective lengths or end compatibility.
E. Coli bacteria	Link	*E. Coli* to be implanted with the recombinant plasmids in order to reduce methane gas production in a cow's rumen during digestion.
Nuclease-free water	Link	Nuclease-free water will be used in the procedures for creating the recombinant plasmids.
Centrifuge tubes	Link	Centrifuge tubes will be used during the PCR amplification stage of the biological parts.
Agar plate	Link	The agar plate will be a low nutrient medium with a neutral pH to mimic the conditions of a cow rumen during in vitro testing.
PCR kit	Link	PCR kits will be used as a method to amplify the genes of interest before ligation to the plasmid backbone.
Gel Electrophoresis kit	Link	Gel Electrophoresis will be used to determine if the plasmids were successfully inserted with the parts of interest.
Cow's rumen tissue	Link	The tissue from a cow's rumen will be used in the first experiment to test the

		efficacy of the recombinant *E. Coli* in disrupting the methane production pathway.
Volatile Fatty Acids (VFAs)	Link	VFAs will be used as one of the inputs to the circuitry during the in vitro testing of the recombinant *E. Coli.*
Vitamin B12	Link	B12 will be used as an input during the in vitro experiments in order to test the efficacy of the recombinant *E. Coli.*
Electrochemical Sensor for Methane Detection	Link	Sensor will be detecting methane levels during the in vitro testing of the recombinant *E. Coli* efficacy in reducing methane gas production.
Gas Chamber	Link	The gas chamber will be used in the first in vitro test as a method of detecting the production of methane gas.
Photon Technology International (PTI) QuantaMaster fluorometer	Link	The fluorometer will be used in the second verification experiment to determine the amount of fluorescein present, indicating the presence and function of the circuit-generated bromoperoxidase.
Polycarbonate Filter (2µm pores)	Link	A small, inert filter used to separate long macromolecules from smaller cellular constituents in the plankton cultures during the second efficacy experiment.
Zirconia Beads	Link	The beads will be used in the bead beating step of the second experiment to lyse cells.
Retsch Mixer Mill MM 400	Link	This will be used in the second experiment to carry out the bead beating to lyse cells.
Bead Beating Tube	Link	This will be used in the second experiment to hold the cultures, solutions, and beads during bead beating.
MES Buffer	Link	The MES buffer will be used in the second experiment to adjust the pH of solutions prior to bead beating to allow bromoperoxidase to reconstitute once cells are lysed.
Sodium Orthovanadate	Link	This will be used in the second experiment to ensure the active sites of bromoperoxidase are fully loaded.
Fluorescent Probe APF	Link	The APF will be used in the second experiment to validate the function of the bromoperoxidase, indicated through the fluorescence of this material.
Hydrogen Peroxide	Link	This will be used in the second experiment as a reactant in bromoperoxidase's oxidation of APF.
Navicula Plankton (CCMP 546)	Link	A negative control in the second efficacy experiment. This species of plankton does not naturally contain bromoperoxidase.
Nitzschia Plankton (CCMP 580)	Link	A positive control in the second efficacy experiment. This species of plankton naturally contains bromoperoxidase.

References

[1] “Cows and climate change | UC Davis.” https://www.ucdavis.edu/food/news/making-cattle-more-sustainable (accessed Jan. 23, 2022).

[2] B. M. Roque et al., “Red seaweed (Asparagopsis taxiformis) supplementation reduces enteric methane by over 80 percent in beef steers,” PLoS One, vol. 16, no. 3, p. e0247820, Mar. 2021, doi: 10.1371/JOURNAL.PONE.0247820.

[3] J .Enloe, “Billion-dollar weather and climate disasters,” Billion-Dollar Weather and Climate Disasters | National Centers for Environmental Information (NCEI). [Online]. Available: https://www.ncdc.noaa.gov/billions/. [Accessed: 07-Feb-2022].

[4] E. Quivet et al., “Underestimation of Anthropogenic Bromoform Released into the Environment?,” Cite This: Environ. Sci. Technol, vol. 2022, pp. 1522–1533, 2022, doi: 10.1021/acs.est.1c05073.

[5] “Addgene: Gibson Assembly Protocol.” https://www.addgene.org/protocols/gibson-assembly/ (accessed Feb. 27, 2022).

[6] “Say Goodbye to Genetic Scars with These Seamless Cloning Kits | Biocompare: The Buyer’s Guide for Life Scientists.” https://www.biocompare.com/Editorial-Articles/162406-Say-Goodbye-to-Genetic-Scars-with-These-Seamless-Cloning-Kits/ (accessed Feb. 27, 2022).

[7] “Addgene: Plasmid Cloning by PCR (with Protocols).” https://www.addgene.org/protocols/pcr-cloning/ (accessed Feb. 27, 2022).

[8] “Ligation Protocol with T4 DNA Ligase (M0202) | NEB.” https://international.neb.com/protocols/0001/01/01/dna-ligation-with-t4-dna-ligase-m0202 (accessed Feb. 27, 2022).

[9] “Addgene: Molecular Biology Protocol - Restriction Digest of Plasmid DNA.” https://www.addgene.org/protocols/restriction-digest/ (accessed Feb. 27, 2022).

[10] A. Froger and J. E. Hall, “Transformation of Plasmid DNA into E. coli Using the Heat Shock Method,” J. Vis. Exp., vol. 6, no. 6, Jul. 2007, doi: 10.3791/253.

[11] “Sanger Sequencing: Introduction, Principle, and Protocol | CD Genomics Blog.” https://www.cd-genomics.com/blog/sanger-sequencing-introduction-principle-and-protocol/ (accessed Feb. 22, 2022).

[12] S. D. Archer et al., “Fluorescent detection of bromoperoxidase activity in microalgae and planktonic microbial communities using aminophenyl fluorescein,” Frontiers in Marine Science, vol. 6, no. FEB, p. 68, 2019, doi: 10.3389/FMARS.2019.00068/BIBTEX.

[13] R. M. Moore, M. Webb, R. Tokarczyk, and R. Wever, “Bromoperoxidase and iodoperoxidase enzymes and production of halogenated methanes in marine diatom cultures,” J. Geophys. Res. C Ocean., 1996, doi: 10.1029/96JC01248.

[14] “Bacterial Cell Disruption by Bead Beating.” https://opsdiagnostics.com/applications/applicationtable/microorganism/bacteria.htm (accessed Feb. 27, 2022).

[15] G. Gelardi, S. Mantellato, D. Marchon, M. Palacios, A. B. Eberhardt, and R. J. Flatt, “Chemistry of chemical admixtures,” Sci. Technol. Concr. Admixtures, pp. 149–218, Jan. 2016, doi: 10.1016/B978-0-08-100693-1.00009-6.

[16] H. D. Knight and M. Reina Guerra, “Intoxication of cattle with sodium bromide contaminated feed,” Am. J. Vet. Res., vol. 38, no. 3, pp. 407–409, 1977.

[17] “BBa_K817002,” *Part*. [Online]. Available: http://parts.igem.org/Part:BBa_K817002. [Accessed: 07-Feb-2022].

[18] “BBa_K2461002,” *Part*. [Online]. Available: http://parts.igem.org/Part:BBa_K2461002. [Accessed: 07-Feb-2022].

[19] “Part:BBa K1913008 - parts.igem.org.” http://parts.igem.org/Part:BBa_K1913008 (accessed Feb. 08, 2022).

[20] “BBa_K3947001,” *Part*. [Online]. Available: http://parts.igem.org/Part:BBa_K3947001. [Accessed: 27-Feb-2022].

[21] “Part:BBa K1318000 - parts.igem.org.” http://parts.igem.org/Part:BBa_K1318000 (accessed Feb. 05, 2022).

[22] S. Phadtare and K. Severinov, “Extended -10 motif is critical for activity of the cspA promoter but does not contribute to low-temperature transcription,” *J. Bacteriol.*, vol. 187, no. 18, pp. 6584–6589, Sep. 2005, doi: 10.1128/JB.187.18.6584-6589.2005.

[23] “Part:BBa M36801 - parts.igem.org.” http://parts.igem.org/Part:BBa_M36801 (accessed Feb. 05, 2022).

[24] “Part:BBa K2142006 - parts.igem.org.” http://parts.igem.org/Part:BBa_K2142006 (accessed Feb. 05, 2022).

[25] “Ribosome Binding Sites/Catalog - parts.igem.org.” https://parts.igem.org/Ribosome_Binding_Sites/Catalog (accessed Feb. 08, 2022).

[26] “Terminators/Catalog - parts.igem.org.” http://parts.igem.org/Terminators/Catalog (accessed Feb. 08, 2022).

[27] “Part:BBa B0036 - parts.igem.org.” http://parts.igem.org/wiki/index.php?title=Part:BBa_B0036 (accessed Feb. 08, 2022).

[28] “Part:BBa B0010 - parts.igem.org.” http://parts.igem.org/Part:BBa_B0010 (accessed Feb. 08, 2022).

[29] “Measured parameters on acetate and acetate and glucose media,” *Measured parameters on acetate and acetate an - Bacteria Escherichia coli - BNID 108650*. [Online]. Available:https://bionumbers.hms.harvard.edu/bionumber.aspx?id=108650&ver=1&trm=acetate&org=. [Accessed: 28-Mar-2022].

[30] “Vitamin B-12,” Vitamin B-12 - Health Encyclopedia - University of Rochester Medical Center. [Online]. Available: https://www.urmc.rochester.edu/encyclopedia/content.aspx?contenttypeid=19&contentid=vitaminb-12. [Accessed: 28-Mar-2022].

[31] “US EPA-pesticides; hydrogen peroxide.” [Online]. Available: https://www3.epa.gov/pesticides/chem_search/cleared_reviews/csr_PC-000595_12-Jul-07_a.pdf. [Accessed: 29-Mar-2022].

[32] “Bromoform,” *National Center for Biotechnology Information. PubChem Compound Database*. [Online]. Available: https://pubchem.ncbi.nlm.nih.gov/compound/Bromoform#section=Metabolism-Metabolites. [Accessed: 28-Mar-2022].

[33] S. Srivastava, “Emerging therapeutic roles for NAD+ metabolism in mitochondrial and age-related disorders,” Clin. Transl. Med., vol. 5, no. 1, p. 25, Dec. 2016, doi: 10.1186/S40169-016-0104-7.

[34] “Search BioNumbers - The Database of Useful Biological Numbers.” https://bionumbers.hms.harvard.edu/search.aspx?trm=catalytic constant for bromoperoxidase&pi=2 (accessed Mar. 28, 2022).

[35] D. Davidia et al., “Global characterization of in vivo enzyme catalytic rates and their correspondence to in vitro kcat measurements,” Proc. Natl. Acad. Sci. U. S. A., vol. 113, no. 12, pp. 3401–3406, Mar. 2016, doi: 10.1073/PNAS.1514240113/-/DCSUPPLEMENTAL.

[36] C. A. Northrop-Clewes and D. I. Thurnham, “The discovery and characterization of riboflavin,” Ann. Nutr. Metab., vol. 61, no. 3, pp. 224–30, 2012, doi: 10.1159/000343111.

[37] D. Potter et al., “In vivo fate of hemopexin and heme-hemopexin complexes in the rat,” Arch. Biochem. Biophys., vol. 300, no. 1, pp. 98–104, 1993, doi: 10.1006/ABBI.1993.1014.