Design Brief

Catalysis of methane to methanol by methane monooxygenase with engineered *E. coli*

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Methane is a potent, heat-absorbing greenhouse gas and a major contributor to human induced climate change. While there are many natural sources of methane (such as emissions from oceans and wetlands), human related activities (such as agriculture and energy production) are playing a major role in the dramatic increase of the concentration of methane in the atmosphere. So far, it has been difficult to reduce methane emissions because they are so widely produced. A group of bacteria called methanotrophs could aid in reducing methane emissions. These bacteria are able to catalyze methane to methanol, a promising biofuel. This reaction is catalyzed by an enzyme known as methane monooxygenase (MMO). However, methanotrophs require certain conditions to survive and have a low rate of reproduction, two factors which have lowered their industrial viability. By inserting the gene encoding for MMO into the chassis *E. coli* the growth challenges could be mitigated while also efficiently catalyzing the conversion of methane to methanol. Of the two types of MMOs, soluble MMO appears to be most viable as it is more well-documented and does not rely on copper co-factors. Thus, we would insert an engineered version of the gene for soluble MMO into *E. coli*. Transformed *E. coli* would be contained in a bioreactor from which methanol can be extracted. The objective of our design is to reduce greenhouse gas emissions from human activity and to provide a source of a promising and sustainably produced biofuel.

Keywords: Methane monooxygenase, methane, methanol, *Escherichia coli*

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Background

In 2018, methane made up approximately 10 percent of greenhouse gas emissions in the US (Environmental Protection Agency 2020). Over a period of 100 years, its impact was at least 25 times greater than that of carbon dioxide due to its high heat absorbance (Environmental Protection Agency 2020). Since 1750, the atmospheric concentration of methane has risen about 150%, largely caused by human activity (UCAR Center for Science Education 2012). The highest human-related emissions are from waste treatment, rice production, raising cattle, and energy generation- especially from natural gas, crude oil, and coal mining (Environmental Protection Agency 2020). Natural sources of methane include emissions from wetlands and oceans, as well as melting arctic permafrost (which is being exacerbated by global warming in a vicious cycle).

Methane, being the chief component of natural gas, is commonly burned to generate energy. However, the large amounts of methane are still released into the atmosphere during the production, processing, storage, transmission, and distribution of natural gas (Environmental Protection Agency 2020). Many of the current techniques for reducing methane emissions

rely on capturing methane and using it for energy. However, methane in gas form is difficult and expensive to transport, so in less connected locations it is not practical to keep it in gas form. In addition, current methods using methane as a feedstock for liquid fuels have harmful effects on humans and the environment, in addition to large capital expenditure and risk (Dry 2002, Cartwright 2013).

Methanotrophs are prokaryotes that rely on methane as a carbon source, and have biological pathways for methane metabolism. The common first step of methane metabolism is the oxidation of methane to methanol, which is catalyzed by enzymes known as methane monooxygenases (MMOs). MMOs are capable of selectively oxidizing methane into methanol at ambient temperature and pressure, a sustainable solution to the main challenge in current methane gas-to-liquid processes (Sirajuddin and Rosenzweig 2015, Arakawa, Aresta, Armor, et al. 2001, Tinberg and Lippard 2011). However, methanotrophs grow very slowly and are difficult to maintain, requiring large amounts of methane just to survive, which can be dangerous and is economically unfeasible on an industrial scale. Thus, if MMOs can be expressed in other, fast growing bacteria, then their production can be upscaled greatly and they could be used for the large-scale conversion of methane to methanol.

Methanol is a compound with a variety of industrial uses. Methanol also degrades quickly (relative to petroleum fuels) and is a high octane, clean burning substance, meaning that it produces fewer greenhouse gases when combusted and would be a cleaner alternative to conventional fuels (Petruzzello 2020). There have even been proposals to wean the world off fossil fuels by establishing a methanol based economy, due to its clean burning nature and ability to fit into existing infrastructure for fuel (Bullis 2006). One caveat about methanol, however, is its high toxicity. More precautions will have to be taken to prevent exposure to humans and the environment if methanol becomes more widely used.

Systems Level

Our proposed system is a device that will oxidize methane into methanol, thereby reducing atmospheric concentrations of a potent greenhouse gas and using it to create a valuable resource (Figure 1). The chassis, *E. coli* BL21(DE3), will be modified to perform the function of methane oxidation. Waste methane, which is collected from the site, is bubbled through the bioreactor, and some is taken up by the recombinant *E. coli* and converted into methanol. Methanol can then be extracted from the *E. coli*. At the systems level, we have identified nutrients and methane as the input and methanol as the output. A good candidate for the growth medium is NMS medium, which is commonly used with methanotrophy (Whittenbury, Phillips and Wilkinson 1970).

Cells will be grown in liquid media containing ampicillin, which will help select for the transformed cells. The engineered plasmid will not be expressed during this stage because the promoter must be induced by IPTG, which allows them to replicate more comfortably. The cells will then be transferred to the bioreactor (Figures 1), which contains IPTG, and the sMMO will begin to be expressed. Methane is bubbled through the bioreactor and taken up by the modified bacteria.

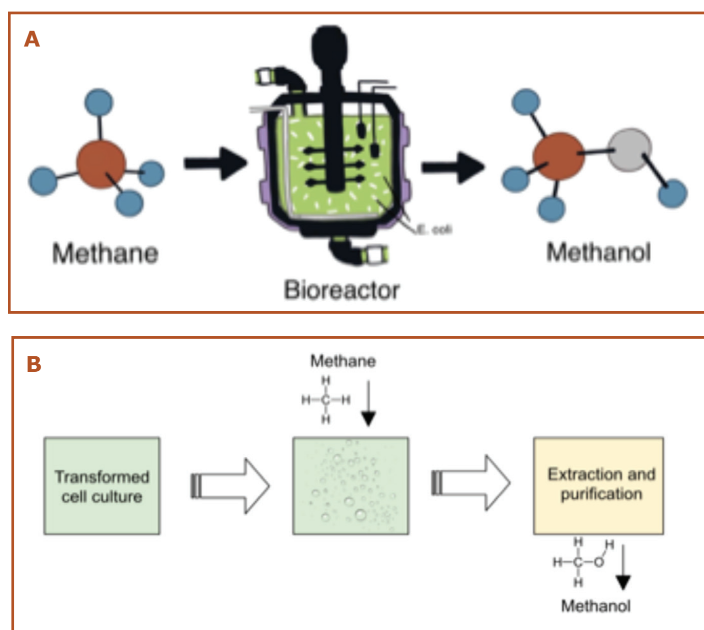
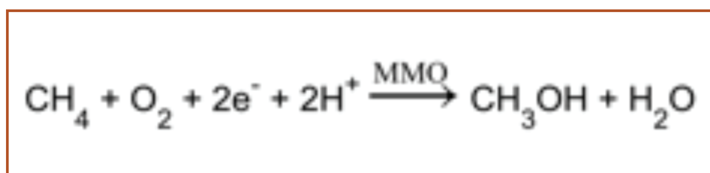


Figure 1. *(A) Conceptualization of our design. Actual design of the bioreactor will vary. (B) Depiction of systems level in greater detail. Transformed cells will be cultivated to desired density and transferred to a container into which collected methane is bubbled through, after which methanol will be extracted from the cells. The second container (bioreactor) will have IPTG to activate the promoter and begin transcription for sMMO.*

Finally, we will use lipid extraction and purification techniques to isolate the methanol from the *E. coli*. Because this process involves lysing the cell membrane, it is less pertinent to keep the cells healthy and alive. In addition, this alleviates the concern of methanol toxicity. Thus, for the purposes of this design, we can overexpress the plasmid so that more sMMO can be produced, and accordingly more methanol.

Device Level

We chose *E. coli* as our chassis because it is a model organism, commonly used in research and biotechnology.



$$CH_4 + O_2 + 2e^- + 2H^+ \xrightarrow{MMO} CH_3OH + H_2O$$

Figure 2. *Oxidation of methane to methanol catalyzed by methane monooxygenase (Source: Lawton and Rosenzweig 2016).*

More specifically, we will use the BL21(DE3) strain, which is widely used for high levels of recombinant protein expression and is compatible with our desired promoter. Our design utilizes the first step of the methane metabolism pathway common to all methanotrophs, which is the oxidation of methane to methanol (Figure 2).

In our design, this reaction will be catalyzed by soluble methane monooxygenase (sMMO), an enzyme found in a subset of methanotrophs. Although there is another more common variety of methane monooxygenase, particulate MMO (pMMO), its exact shape and nature is still an ongoing area of research, while that of sMMO is better understood (Sirajuddin and Rosenzweig 2015). In addition, sMMO has a higher carbon uptake rate than pMMO under methane saturated conditions (Petruzzello 2020). Thus, for this project, we chose to work with sMMO.

Parts Level

For our design, we will use a T7 promoter. This promoter is recognized by T7 RNA polymerase, which is not usually found in *E. coli*; however, the BL21(DE3) strain is able to produce the T7 RNAP (Rosano and Ceccarelli 2014). The T7 promoter system we chose is part of pRSET A, an expression vector that we will use for high-level expression in *E. coli* (Schoepfer 1993). pRSET A also encodes ampicillin resistance and is IPTG inducible.

The sMMO protein from Methylococcus capsulatus (Bath) is made up of three main bodies: MMOH (hydroxylase), MMOR (reductase), and MMOB (regulatory protein). MMOH is further divided into three subunits α,β, and γ, which are coded by the genes mmoX, mmoY, and mmoZ respectively. MMOR is coded for by mmoC; MMOB is coded for by mmoB, and increases sMMO expression by ten-fold (Meyer, Keller, Hartl, et al. 2018, Grosse, Laramee and Wendlandt 1999). In addition, the sMMO operon encodes the genes mmoD, mmoQ, mmoR, mmoS, and mmoG. These coding sequences were successfully isolated from M. capsulatus (Bath) by iGem team Braunschweig (Narsimhan, Iyoki, Dinh, et al. 2016, iGem Team Braunschweig 2014). Although there are more genes involved in the sMMO cluster, they may play a role in downregulation and were thus excluded (Csáki, Bodrossy, Klem, et al. 2003). A medium RBS, BBa_B0032, precedes each coding sequence (Mahajan, Marinescu, Chow, et al. 2003) (Figure 3). Finally, the double terminator (Mahajan, Marinescu, Chow, et al. 2003) will stop transcription of the genes.

In this design, the aim is to overexpress sMMO while ensuring that it is functional and minimizing the formation of aggregate bodies. Thus, the protein folding process will be assisted by chaperone proteins groEL-ES, which have been shown to enhance folding accuracy in bacteria (Goyal and Chaudhuri 2015). GroEL-ES will be coexpressed in the plasmid. This will increase the rate of expression as well, because the rate of protein folding limits the rate of transcription/ translation.

Even with all of these parts, we estimate that the plasmid size will still be less than 15 kb. The vector backbone size is 2897 bp; the complete sMMO sequence is about 5403 bp.

Safety

As methane is a highly flammable gas and methanol in different phases can be flammable and toxic, safety precautions must be taken. Careful attention must be kept on the pressure of the gas containment. The place where our device is constructed, tested, and used will be a well-ventilated area in order to avoid accumulation of methanol and vapors in a confined space (Canadian Centre for Occupational Health 2020, Methanol 2020). To avoid fire hazards, the area must be temperature-controlled and clear of any forms of ignition sources, heat sources, combustible and flammable materials, and incompatible materials.

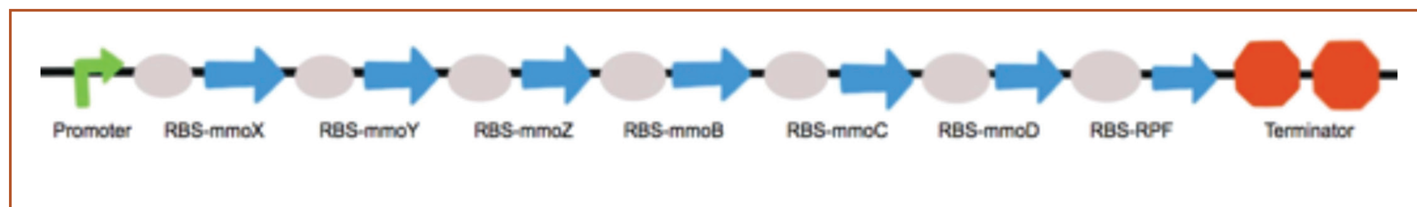


Figure 3. *Depiction of the genes encoding soluble methane monooxygenase.*

The resulting methanol will be put away into containers to be used in the future. The containers must be secured in upright positions to solid structures in storage, and they must be moved with suitable hand-trucks in transportation (Canadian Centre for Occupational Health 2020).

Developers and users must be careful to wear proper Personal Protective Equipment: chemical safety goggles and face shield when contact is possible and insulated protective clothing if contact with refrigerated gas is possible (Canadian Centre for Occupational Health 2020).

In the case of the bacterium released into the environment, they will no longer be able to survive in an environment other than the one provided in the bioreactor and without essential nutrients. An optogenetic kill switch could be added if necessary.

Discussions

As methane gas becomes increasingly available, it would be advantageous to convert it into methanol, an easily transportable and more efficient fluid fuel (Khirsariya and Mewada 2013). Traditionally, the methane to methanol conversion has been performed by a two-step process of first converting the methanol to syngas, which is then converted to methanol via an indirect route (Khirsariya and Mewada 2013). This method is known to be cost and energy intensive with a low conversion efficiency of 25%, and damaging to the environment (Li and Ge 2016, Khirsariya and Mewada 2013). Thus, there is lots of interest in developing a better conversion method. Finding new methods of methane to methanol catalysis is still an active area of research. One such method is a route of partial oxidation, which has been studied already for decades, yet has only reached a methanol yield of 5% (Khirsariya and Mewada 2013). Other promising recent progress includes methane oxidation over a Cu-zeolite catalyst which "catalyzes the direct oxidation of methane into methanol in the gas phase, at low temperature, and using only molecular oxygen and water" (Park, Park and Ahn 2019, Meyer 2014). However, this method is inefficient in that there are many specific conditions that must be met. For example, the Cu-zeolite is only activated for several hours at a temperature of 450☐ in a specific oxygen atmosphere treated with inert gases such as helium, when the actual extraction of methane must be done at a temperature of 200☐ in order to prevent the methane from oxidizing to CO or CO2 (Meyer 2014). Thus, utilizing methane monooxygenase is very much an option with high potential (Ross and Rosenzweig 2016).

Our device can be altered and improved in many ways. In order for it to be industrially feasible, the rate of methane metabolism and methanol production must be maximized. There are many factors that affect these rates, including the growth rate of bacteria, strengths of the promoter and/or RBS, the combinations of genes in the sMMO operon, as well as the folding rate of the protein, among others.

While researching for this project, we thought of several other system level possibilities: the secretion system of the bacteria could be utilized to secrete the recombinant sMMO into extracellular medium, moving the methane to methanol conversion outside of the cell, and simplifying the process of isolating the methanol. However, this design has its caveats; namely, the fact that methanol above a certain threshold is toxic to *E. coli*, and that the sMMO has low stability and its lifetime outside of the cell is unknown (Lawton and Rosenzweig 2016). While the first problem could be addressed by determining that threshold, and maintaining a methanol concentration below it, this requires rigorous testing and the final threshold could be hard to maintain. It is also possible to increase *E. coli's* tolerance by engineering it to incorporate methanol into its metabolic activities (Meyer, Keller, Hartl, et al. 2018); however, this would also reduce the efficiency of methanol production. One could also experiment with a chassis that is more tolerant to methanol; in particular, Bacillus subtilis may be a good candidate because it has efficient secretory machinery and higher methanol tolerance (Calero and Nikel 2019). There may also be advantages to using pMMO instead of sMMO (Lawton and Rosenzweig 2016).

Researchers such as Lawton and Rosenzweig (2016) have pointed out that, while methane-oxidizing enzymes such as methane monooxygenase are a promising area of research and innovation, there is still a lot to uncover in order to realize that potential. This project can be seen as a stepping stone on the road to introducing sMMO for industrial use, as a more sustainable alternative to current methods of gas-to-liquid conversion. Our prototype will impart a better understanding of sMMO function in transformed bacteria. However, as we learn more about the nature of these enzymes, we can better apply them to designs such as this, lowering cost and increasing efficiency (and thereby industrial viability)--and one step closer to a more sustainable world.

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