FabriFuel: Decomposing PET Using A Microbial Fuel Cell Containing Genetically Engineered Escherichia coli

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Abstract

With the ongoing growth in the fast fashion industry, millions of tonnes of plastic waste are dumped into landfills, negatively contributing to the environment. Current processes available for digesting polyester components in fabrics are time consuming and expensive, making them impractical for industrial use by landfills. Thus, it seems imperative to design a new system that can tackle the issue of fabric waste on a commercial scale. By inducing *E. coli* (BL21) to digest polyethylene terephthalate, a common plastic compound found in clothing, we aim to break down PET into products commonly used in metabolic processes, such as acetate. During this digestion process, a tri-chamber microbial fuel cell (MFC) will be implemented to produce electricity by way of hydrolysis.

Keywords: Microbial fuel cell (MFC), Poly(ethylene terephthalate), Mono(2-hydroxyethyl) terephthalate, Bis(2-hydroxyethyl) terephthalate (BHET), Terephthalic acid (TPA), Ethylene glycol, Enzymatic depolymerization

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Introduction

The fabric industry is the world's second largest contributor to plastic and water pollution, particularly through the production of synthetically-derived polyester-based fabrics (Portela, 2022). Out of 100 billion garments produced globally, 1.92 million tons of fabric waste are produced each year, and it is predicted that this number will increase to 134 million tons by the end of the decade (Igini, 2022). The fashion industry is also responsible for 20% of global waste water, and 10% of all global carbon emissions, making it a huge adversary in the global warming crisis. In Canada, only 9% of fabric waste is recycled, leaving 3 million tonnes of leftover fabric to be thrown away. The other 29, 000 tonnes end up scattering around the environment, 93% of which eventually makes its way into our oceans (Government of Canada, 2021). This adds up to nearly $8 billion worth of textile waste each year (Government of Canada, 2021). However, the Government of Canada has suggested that, through improving disposal techniques and managing plastic waste effectively, 1.8 megatonnes of carbon pollution can be reduced annually. Moreover, an increase in revenue for the textile industry is foreseen, with an approximate of 42,000 new jobs being created in this sector (Government of Canada, 2021). Thus, with the ongoing risks of leaving plastic waste unattended, and the predicted benefits that come with efficient disposal symptoms, discovering solutions to tackle fabric waste is paramount in today's time.

In 2019, 105 million metric tonnes of textiles were produced, yet only 1% of the material used to produce clothing was recycled (Damayanti et al., 2021). As of 2021, only 14% of polyester is recycled across Canada, namely due to the limitations present in current recycling methods (Environment and Climate Change Canada, 2021). There are two main methods of recycling polyester: mechanical and chemical recycling. Mechanical recycling is further split up into open-loop and closed-loop processes. The former pulls apart textile fibers that can be reused; however, the quality of the material declines during this process, and are often used solely for felting. The latter produces yarn or thread from textile fibers, yet is 30% more expensive than producing new polyester. In a 2021 study performed by Circle Economy, only 7 mechanical recyclers were found in use across Canada and the United States, demonstrating its limited application in the real world. Chemical recycling also produces lower-quality fabrics and is harmful to the environment due to volumes of microplastics released during the recycling process (Van Elven, 2018). The lack of sustainable and cost-effective methods for recycling polyester-based fabrics has led to an avoidance of recycling fabric materials. Thus, most waste ends up in our natural environment, polluting our oceans and filling up landfills.

In this paper, we discuss a potential strategy to address the issue of fabric waste: FabriFuel. Through this project, we aim to genetically engineer *E. coli* to break down polyethylene terephthalate (PET), the most common type of plastic found in polyester. We further investigate the use of a microbial fuel cell (MFC) to derive electricity from this digestion process. FabriFuel is innovative in that it combines multiple enzymes to fully degrade and metabolize PET through a 3-chamber system. Furthermore, no such study has paired this metabolism with electricity generation through a microbial fuel cell.

Solution and Implementation

Objectives

Our project has three specific aims. To begin, we aim to utilize genetically modified bacteria to fully digest PET. This includes harnessing an enzymatic system to break down PET into byproducts; further degrading the products of the PETaseMHETase chimera, TPA and EG, to be fully digested; and engineering bacteria without the use of plasmid transformation to digest ethylene glycol. Secondly, we aim to generate electricity from the metabolic processes of *E. coli* in an attempt to make the process greener. Lastly, we hope to optimize the combination of both systems to optimally degrade PET and generate electricity. This involves the use of a three chambered system to fully degrade polyethylene terephthalate and its byproducts so that they are completely metabolized by our engineered bacteria.

Identification of Biological Pathway of PET

The first reaction occurring with Bacteria 1 is the digestion of PET (Figure 1A). Poly(ethylene terephthalate) (PET) is degraded into mono(2-hydroxyethyl) terephthalate, bis(2-hydroxyethyl) terephthalate (BHET) and terephthalic acid (TPA) by the enzyme PETase. PETase also catalyzes the conversion of BHET into TPA and ethylene glycol (EG). MHETase produces TPA and EG from the reactant MHET (Yoshida, S. et al, 2016). PETase and MHETase are enzymes native to *Ideonella sakaiensis,* whose ability to degrade the plastic PET was first reported in 2016 by Yoshinda et. al in the report *A bacterium that degrades and assimilates poly(ethylene terephthalate).* Carboxylic acid reductase (CAR_{Mm}), an enzyme native to Mycobacterium marinum, can be introduced into *E. coli* in order to convert TPA into two aldehydes, 4-carboxybenzaldehyde (4-CBAL) and terephthalaldehyde (TAL), (Figure 1B)which are degraded further by endogenous enzymes in *E. coli* (Bayer, T., 2022). A pathway exists in Escherichia coli that assimilates the compound ethylene glycol(Figure 1C). However, ethylene glycol is not degraded by *E. coli* unless two enzymes, propanediol oxidoreductase and glycolaldehyde dehydrogenase are overexpressed. Propanediol oxidoreductase converts ethylene glycol into glycolaldehyde. Glycolaldehyde dehydrogenase degrades glycolaldehyde into glycolate, which is assimilated via endogenous enzymes (Pandit, A. V., Harrison, E., & Mahadevan, R., 2021). Based on a ResearchGate study written primarily by Brandon C. Knott, the performance of the PETaseMHETase biochemical reaction in chimeric proteins exhibits improved PET and MHET expression relative to the combination of two free systems. Chimeric proteins of MHETase_PETase not only statistically improve PET degradation but enzymatic hydrolysis rates as well. By using glycine-serine linkers covalently linking the C-terminal of MHETase to N-terminal of PETase, a higher catalytic activity of MHET was proven using methods of assaying. However, the process of protein expression by using the chimeric protein MHETasePETase requires more in depth research, as the study conducted above has not yet been replicated or achieved by any other labs. Even so, the hypothesis that working with a chimeric enzymatic system is more efficient and effective is based on the foundation that chimeric genes can be flexibly translated into new proteins with novel functions, such as depolymerizing PET and its subsequent byproducts.

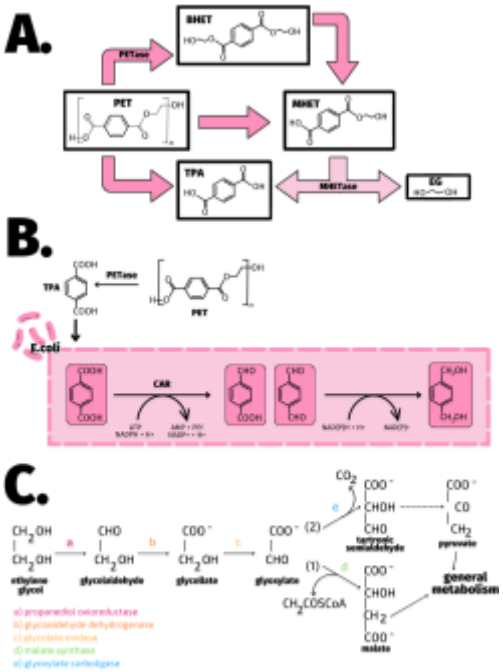

Figure 1: (a)Digestion of PET, ***(b)***Reduction of TPA, ***(c)***Metabolism of ethylene glycol.

Process of PET Degradation Within a Triple-Chambered MFC

To degrade PET into its component byproducts that can be digested and metabolized by the cell, three systems are being implemented to target

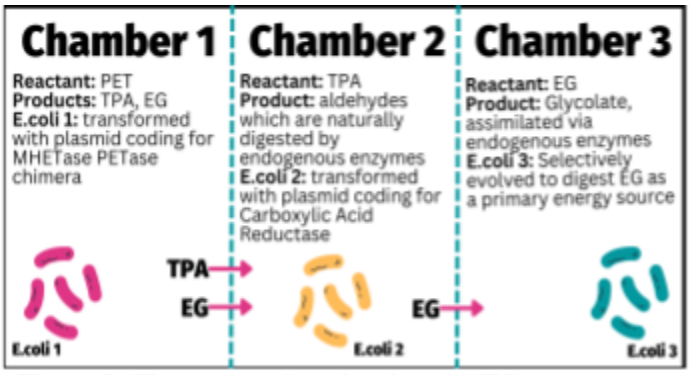

Figure 2: *Illustration of tri-chambered MFC.*

the breakdown of specific compounds. (1) We will use the *E. coli* strain BL21 transformed with a designed plasmid containing the genes for a PETaseMETase chimera to digest PET into TPA (Bacteria 1), EG, AND BHET. BHET will naturally be taken in by the cell, with very little amounts being present afterwards. (2) CAR_{Mm} will be secreted by a secondary BL21 bacteria (Bacteria 2) for the conversion of TPA into glycolaldehyde. (3) Finally, to digest EG, we propose the use of directed enzyme evolution in *E. coli* (Bacteria 3). Directed evolution will be used in order to engineer ethylene glycol utilization in Escherichia coli. If this is unsuccessful, we will introduce a plasmid coding for the enzymes necessary to degrade EG into compounds naturally integrated into the cell. A key feature of the plasmids we aim to create is that they will be self inducible, using the SILEX system (Briand et al., 2016) as a less-expensive way to make our final design compatible with real-world applications such as the frequent induction of gene expression (Bagheri, M. & Khani, M.-H, 2020). If the PET degradation using the MP12 chimera produces satisfactory results, this same protein will be used in the final design, a self-inducing plasmid that will secrete MP12 out of the cell. As PET is a larger molecule, it is unrealistic to assume that it will be naturally taken up by the cell. Therefore, PETase must be secreted from the cell in order to properly degrade PET. In order to degrade TPA, one of the products of the MHETase-PETase reaction, into compounds which can be naturally metabolized by the cell, the enzyme CAR_{Mm} will be utilized (Bayer, T., 2022). The protein will be secreted, because the electron transfer will occur in the next chamber of the MFC, so it is unnecessary for the *E. coli* in the chamber which degrades TPA to digest the aldehydes produced in the reaction. Our bacterial system will be placed into our MFC separated by three semi-permeable membranes with a pore size of less than 1µm. Our three bacterium will be separated to prevent conjugation while allowing for EG and TPA to move within the system.

Preliminary Results

Biological Part Design

(Reference to Figure 3.) A plasmid map of pCJ190 MP12, displaying a plasmid with a MHETase-PETase gene insert, combined by a 12 residue glycine-serine linker. The C-terminus of MHETase has been linked to the N-terminus of PETAse. It has been codon optimized for expression in *E. coli* K12, and has a His-tag at the C-terminus of PETase. This plasmid was chosen because the MP12 chimeric protein demonstrated a higher turnover rate of MHET than MHETase or any of the other chimeric proteins which were studied in (Knott, B. C., 2022). Protein expression is induced via IPTG and the protein is not secreted from the cell, so it must be purified.

Figure 3: Plasmid map of pCJ190 MP12.

Completed Experiments on E. Coli

The following experiments outlined are protocols conducted in our school's lab, with the intent of providing experimental data and corresponding hypotheses required to support our design's efficacy. However, the experiments and respective data collected serve as a proof of concept for the design and efficacy of our project.

Chemically Competent Cells

To ensure that our target plasmid DNA can be harnessed and replicated, we created chemically competent cells that allow us to sustain bacteria treated with target antibiotics to allow for uptake of exogenous plasmid DNA. The strain of bacteria used is DH5a *E. coli*, which is optimized for plasmid amplification. The creation of chemically competent cells allowed for successful completion of following experiments such as DNA replication using a maxiprep kit, and subsequent restriction digestion.

Maxiprep

A maxiprep experiment was performed in multiple scenarios in order to ensure our plasmid DNA is reliably extracted and isolated for future use. Through conducting our maxiprep, we noticed potential contamination in one of our samples due to an accumulation of unknown bacteria. While conducting the experiment once again, we ensured more strict sterilization, environmental exposure, and handling of our DNA samples and were successfully able to collect DNA which was then stored in the appropriate temperature. The DNA then is prepared for mapping whereby an analysis of breakage points are identified.

Restriction Digestion

A restriction digest is a procedure used to prepare DNA fragments for analysis, molecular cloning, or other subsequent processing. The procedure of a restriction digest allows fragments of DNA to be attached, or ligated, via a ligase enzyme during the process of transcription. By using specific restriction enzymes, Xbal and Pstl, we were successfully able to cleave DNA sequences at sequence specific sites to produce DNA fragments with the ability to be identified at both the 5' and 3' end. This is crucial for future steps with polymerase chain reaction (PCR) to amplify and clone our bacteria.

Restriction Analysis

Restriction analysis is a key component of proving the conceptual foundations of our project. It maps unknown segments of DNA by breaking it into fragments, allowing us to identify the location of breakages and their end sequences. This also allows us to create recombinant DNA, which theoretically proves the efficacy of our intention to create cells containing recombinant plasmid DNA. Through the mapping of the Pstl enzyme, breakage points were identified at base pairs 1144, 6188, and 6217. For Xbal, cuts were found at bps 5160 and 7313. By identifying the breakage points, we were successfully able to proceed by connecting our future plasmid design research to our proof of concept whereby certain breakage points were located and correlated to the respective endonucleases.

Gel Electrophoresis

DNA Electrophoresis is used to separate DNA fragments according to their size and charge, it is done in order to confirm that the right gene (DNA fragment) is cloned into the plasmid. It involves running a current through a gel containing molecules of interest. Based on the size and charge, the molecules will travel through the gel in different directions or speeds, which allows them to be separated from one another. In order to ensure a successful replication of our target plasmid DNA, the restriction digest (RD) maxiprep DNA was run though gels using the method of gel electrophoresis, which is used to separate DNA based on the molecular weight of each segment of base pairs. Through conducting a gel, we were able to derive confident results that our plasmid DNA from the RD maxiprep solutions was sound and precise to the corresponding separation of gene segments in a commercial PETaseMHETase DNA ladder. For the purpose of FabriFuel, we will be using DNA electrophoresis in order to ensure that we have cloned the right gene into the plasmid and the plasmid can carry out the digestion of PET.

Microbial Fuel Cell: Proof of Concept

Microbial fuel cells capture electrons from biochemical reactions in bacteria. Within the fuel cell are two chambers separated by a semipermeable membrane through which protons can pass, Figure 4. The anaerobic chamber (left) contains the bacteria and anode, acting as an acceptor of electrons produced by bacteria. The aerobic chamber (right) contains the cathode, where oxygen combines with electrons to produce water. When biomass such as PET is delivered to the bacteria, oxidation results in a loss of electrons. The ions immediately diffuse through the semipermeable membrane to the cathode, while the electrons are deposited onto the anode, and flow through an ionic bridge towards the cathode. The MFC takes advantage of this movement by acting as an electron acceptor, using the flow of electrons from the anode to the cathode to generate an electric current from the anode to the cathode to generate an electric current.

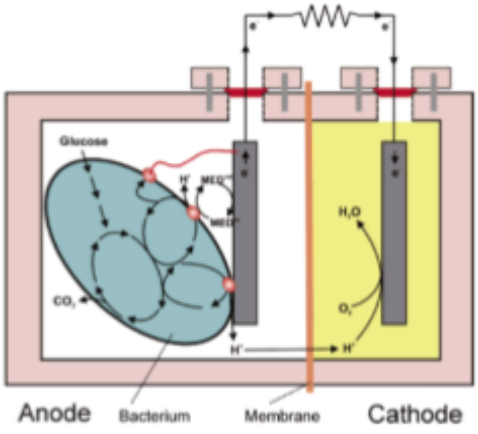

Figure 4: *(Logan et al., 2006).* Components of Microbial Fuel Cell and electron

MFCs are considered optimal for this project, as they are considered renewable sources of energy and never need to be recharged. They are able to generate a flow of electrons as long as biomass is provided, allowing them to run for long periods of time. The electrical output of the device is also sufficient, with the range of power production being similar to other sources as shown in Figure 5 (Slate et al, 2019).

Our team aims to address the problem of excess fabric waste by designing a MFC containing polyester-digesting *E. coli*. We intend to capture the

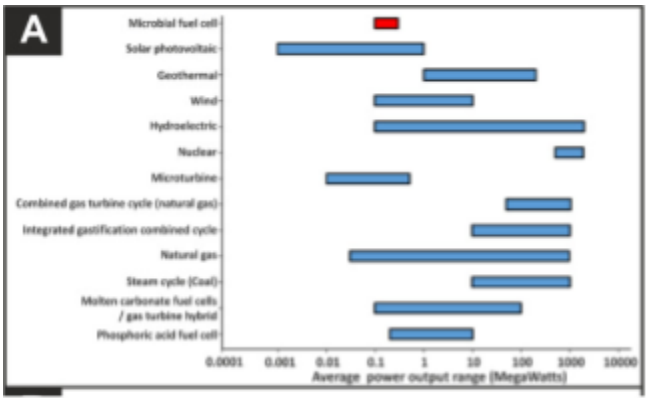

Figure 5: *(Slate et al., 2019).* Average power output of energy sources in comparison to MFC

energy from the fuel cell as a byproduct of cellular digestion. Currently, preliminary testing has been conducted, as we work towards testing the MFC that will house our genetic system while retrieving excess energy produced by *E. coli*'s metabolic pathways.

Microbial Fuel Cell Assembly:

We ordered the Flex-Stak Kit with the 5-cell stack configuration from the Fuel Cell Store of Education, Research and Fun. This open-air stack contains graphite plates and membrane electrode assemblies (MEA) as shown in Figure 6. The Flex-Stak nominal operating voltage varies depending on how aggressively the cell is being run, but normally ranges between 0.45 and 0.6 V per cell. The stack temperature and the fuel's humidity will both have a significant impact on the voltage and current values. This dismantable fuel cell allowed for our group to gain an initial understanding of different components and their function within the fuel cell.

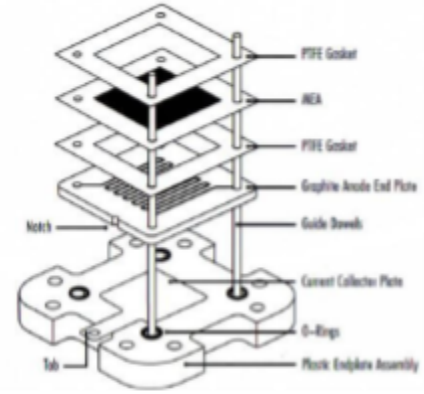

Figure 6: *(Fuel cell store, 2023)* Stacking of MFC components.

Theoretical Reactions:

The two reactions occurring in the fuel cell that generate electricity are at the cathode: $O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$ and at the anode: $C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^-$. These chemical reactions allow us to determine the theoretical electrical potential values. The theoretical electrical potential of the cathode reaction is 0.82 volts and the theoretical electrical potential value of the anode is -0.43 volts, which gives us a total electrical potential difference of 1.25 volts for the reactions (Schöder & Uwe, 2007). It is important to note that these reactions assume STP, pH = 7, and standard atmospheric concentration of oxygen.

We plan to conduct a series of examinations of the electricity produced by *E. coli* using a multimeter to measure the voltage and current outputs with different bacteria concentrations and resistors. Experiments have been completed in order to establish a proof of concept. In our experiment, we used 100mL of the control (LB without bacteria) and 100mL of the bacteria (BL21) in suspension (LB). As of now, preliminary testing has been completed, and through various challenges, we have identified areas for further refinement. In our subsequent experiments, we plan to test 100mL of the control (LB without bacteria) and 100mL of the transformed bacteria (PETase_MHETase) in suspension to determine if the transformed bacteria is also capable of generating electricity.

Bacteria Concentration	Voltage (mV)	OD
0	9.5mV	0
25	7.1mV	0.054
50	4.3mV	0.154
100	14.1mV	0.376

Figure 7: Table of voltage and OD measurements for differing bacterial concentrations in an open circuit.

Prior to experimentation with the microbial fuel cell, OD values (a relative measurement of concentration through the transmission of light through a medium) were measured using a spectrophotometer set to 600 nanometers. Results are shown in Figure 7. Starting with a baseline of OD=0 for 100% light transmission, the increased density of bacteria will cause less light transmission, resulting in a higher OD value. With a higher OD value, there is a concern of increased bacteria growth depleting nutrients too quickly, restricting the number of enzymes produced to catalyze electron transfer. If the obtained measurement was high, we diluted 50% of the sample for experimentation.

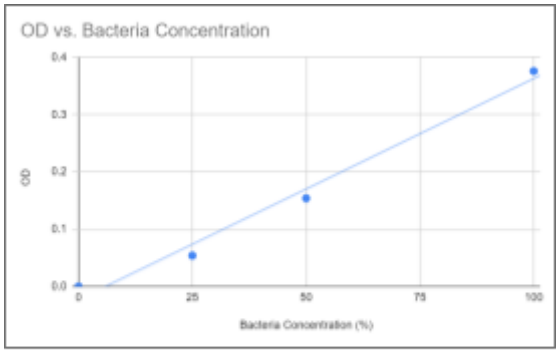

Figure 8: Comparison of optical density (OD) to bacterial concentration

We plan to use resistors, namely 470 Ω, 680 Ω, 1 kΩ, 2.2 kΩ, and 3.3 kΩ. These values are from other MFCs and previous tests and experiments. Through the use of resistors, we are able to determine the maximum power output of the MFC. We measure current and voltage to find the power the

MFC is capable of producing and then correlate them with expected values based on chemical reactions happening in each chamber of the MFC.

Future Experimentation

Our steps for future experimentation are carefully established based on hypotheses collected from various resources. The main experiments that are significant for our purposes are: (1) Determining rate of depolymerization of PET in vitro by PETase_MHETase Chimera, (2) Experimentation of *E. coli*, (3) Experimentation in MFC, and (4) Enzymatic efficacy. Other intermediary experiments include: PETaseMHETase expression, protein efficiency in chassis, and bacterial transformation with a plasmid containing genes for carboxylic acid reductase.

Determining the Rate of Depolymerization of PET in Vitro by the PETase-MHETase Chimera

The degradation of PET requires a PETaseMHETase enzyme complex, which then requires enzymatic degradation of TPA. In an absorbance method for analysis of enzymatic degradation kinetics of polyethylene terephthalate (PET) films, published in Nature in 2021, UV spectrophotometry was successfully used to estimate the formation of PET degradation products. MHET, TPA and BHET can be detected by UV spectrophotometry, most optimally between 240 and 260 nm, because of their aromatic rings. However, other than TPA, the presence of these products cannot be determined individually, as they can only be measured in bulk by this method. By determining the concentration of these products over time, the in vitro rate of reaction of the PETase-MHETase chimera can be determined.

Direct Evolutionary Experimentation on Escherichia Coli

One of the experiments to be conducted in the future is a directed evolutionary experiment with the aim of making *E. coli* primarily use ethylene glycol as a source of energy. In order to increase the likelihood of accomplishing this, the bacteria will be subjected to multiple rounds of UV radiation, which will significantly increase mutation rates (Shibai et al., 2017). Control tests will be run on well-understood strains of *E. coli* with known mutation rates. Environmental conditions will also be controlled. Bacteria will be exposed to ethylene glycol, and the most robust colonies will be selected for the next round of experimentation. If this experiment is unsuccessful, it will be repeated using propylene glycol, as suggested as a method in a study by Boronat et al. If successful, the mutants created on propylene glycol will be mutated with UV radiation. If the directed evolution experiment yields negative results, we will develop a plasmid which contains the genes for two enzymes, propanediol oxidoreductase and glycolaldehyde dehydrogenase, as studied by Microbial Cell Factories, 20(1). As propanediol oxidoreductase is oxygen sensitive (Lu et al., 1988) a variant of the gene coding for it, fucO, will be used. The mutations I7L and L8V have been demonstrated to at least double the activity of propanediol oxidoreductase (Lu et al., 1988). The genes would be expressed using the SILEX system, which has been shown to successfully promote the autoinduction of the expression of a diverse set of proteins in *E. coli* (Briand, L. et al., 2016). The SILEX system is crucial because it is a considerable alternative to various *E. coli* secretion systems for protein overexpression.

Enzymatic Efficiency

Through the processes described above, the cumulative data derived from the above research and experimentation will allow us to confidently test the enzymatic efficacy by performing methods of spectrophotometry, fluorescence and/or radiolabelling. Testing the efficiency of the enzyme is a key component we are confident in completing, on the basis that we will calculate rates of enzymatic activity by measuring how much product is formed or substrate is consumed in a set amount of time. The foundation of this experiment will come from our knowledge that: Rate of Reaction (s^{-1})=1/time taken (s).

Experimentation on Microbial Fuel Cell

We are in the preliminary stages of collecting data, and will need to run additional trials to perform statistical analysis. Through the Ethanol Fuel Cell Science Kit, we noted that a 70% C_2H_6O solution was used in place of 10% C_2H_6O solution when cleaning the MFC, causing degradation of ion exchange sheets (membrane electrode assembly). After switching out the ion exchange sheets, a 10% solution was used instead; however, damage to sheets may have impacted data outputs, warranting a re-trial of the experiments. Due to exposure to room temperature after refrigeration, our team recognized that the LB's temperature increased, resulting in different trials occurring under various temperatures. For this reason, our team has opted to let our bacterial cultures warm to room temperature before conducting experiments. A period of 15 mins was allocated for the bacteria in LB and MFC to warm, but we will continue to explore more reliable methods to control differing environmental factors. Other factors that may have contributed to the significant reduction of measured electric potential compared to theoretical predictions include excessive internal resistance, preventing current from being produced; or an electrode overpotential, which would require a larger input of electrochemical work within the MFC to effectively catalyze the reaction; or microbial irregularities. We will also ensure recording of baseline MFC variability with undesired values (noise) disturbing the general output. Listed potential errors are being further explored to prevent unreliable datasets and limit deviation from precise quantified data.

We are additionally considering the implementation of computer modeling in order to speed up the trial-and-error processes, as we can easily determine the effect of variable changes. We could further replicate these ideas in actual experiments to verify procedures.

Human Centered Design

The human practices portion of our project aims to create awareness regarding the problem of fabric waste and to connect with key stakeholders who will allow us to better target our project. In order to do so we established two objectives. First, identify and speak to relevant stakeholders to assess the effectiveness of implementing our project in the real world. Second, to raise awareness and understanding of the issue of fabric waste. The human practices team has contacted a number of individuals in the areas of textiles, plasmid design and MFC technology. We have created interview guidelines and questions for future stakeholders. Furthermore, we have found mentors who have aided significantly in our project. Dr. Robert Mayall and Dr. Emily Hicks, the co-founders of FredSense, have helped us predict and circumvent potential roadblocks in our prototype, and gave us advice on how we could best develop our idea. To fulfill our second objective, we have designed a survey meant to understand current knowledge of fabric waste. We have also done extensive research on the extent to which fabric waste is a global issue, current methods of fabric recycling and disposing of fabric, as well as how our local community deals with fabric waste. Through our project, we ultimately hope to spread awareness of the important issue of fast fashion and fabric waste and to use our project to incentivize individuals to invest in sustainable clothing and fabrics.

Conclusion

Through experimentation and research, a proof of concept on PET-degrading Escherichia coli in an MFC has been established. We have acquired plasmid Pcj190 encoding the PETase-MHETase chimera and BL21 (DE3) to assist in protein expression. Additionally, we have derived chemically competent bacteria from the aforementioned *E. coli* strain. Maxi preps and restriction analyses were completed to ensure that our plasmid will be successful in protein expression and PET degradation. Research and preparatory work has also been conducted on BL21 transformation with the chimera plasmid, which we will complete following the protocol of the PNAS paper titled, *Characterization and engineering of a two-enzyme system for plastics depolymerization,* by Brandon C. Knott and associates. Following this, we will purify and express the chimeric protein and validate our results through SDS-PAGE. Using a spectrophotometer, we will be testing PET pellet's degradation in-vitro, followed by incorporation in a 3-chamber process within the MFC. In the MFC experiments, a series of examinations were conducted to measure current and voltage outputs of varying *E. coli* concentrations. The experiments with the MFC have demonstrated that an electric potential difference is successfully generated and gives support for future testing. Future steps include completing the experiments once more with stable controls and ethanol concentrations, as well as researching other factors that affect voltage output. We will also look into the application of computer modeling to facilitate the testing process. Our ultimate goal is to design and implement a triple-chamber MFC with 2 separate anode for PETase and MHETase proteins; a proposal that still needs to be consolidated. Hence, we conclude that a synthetic-biology approach to digest PET within a MFC is possible, warranting future experimentation and research.

Biosafety

There is minimal concern regarding our project. Firstly, the materials are not aimed to be used for, or on any living organisms. Secondly, the enzymes being produced by modified bacteria will not harm living organisms if released into the environment, as our system utilizes processes that are specific to the digestion of polyethylene glycol. As such, organisms will not engage in other chemical reactions as far as research has suggested. Furthermore, if we succeed with large scale implementation of our project, we would focus on keeping the entire system and procedure contained within a risk level 1 facility. The two main substances that could potentially cause a disturbance to ecosystems would be terephthalic acid and ethylene glycol. Terephthalic acid is non-toxic to aquatic organisms at concentrations less than 15 mg/L, but naturally biodegrades in aerobic soil and water. Ethylene glycol is toxic to organisms in high concentrations, causing reproductive issues and fatality in animals, birds and fish, as well as killing or slowing the growth of plants. To prevent these chemicals from being leaked into the environment, we are using proper disposal methods, using proper PPE protection, and containing all reactions within our fuel cell.

Conflict of Interest

We would like to acknowledge that we are not affiliated with or thereby funded by any external organizations dealing with areas of synthetic and microbiology, or that are focused on concepts relating to our project. All funds and support is received directly from Renert School.

(Summarized) Materials and Methods

a. **Chemically competent cells:** Chemically competent cells were created by taking the BL21 (DE3) *E. coli* strain and culturing it in liquid broth (LB), further subculturing it in solutions of Magnesium sulfate (MgSO4) and Potassium chloride (KCl), while upholding a 1:50 ratio. Once chilled the subcultures were centrifuged at 3450 rpm, and the pellets were then resuspended in the supernatant. After repetition, aliquots of the substance were collected and stored at -80°C.
b. **Bacterial Transformation:** In order to establish our gene of interest which codes for MHETase_PETase, we used bacterial transformations to produce multiple copies of our recombinant bacteria. We selected the DH5a strain for this transformation due to its optimization for plasmid amplification. Transformed bacteria was then grown on agar plates. pCJ190 was a gift from Gregg Beckham (Addgene plasmid # 162667; http://n2t.net/addgene:162667; RRID:Addgene_162667)
c. **Miniprep and Maxiprep**: Maxipreps and Minipreps are completed to isolate plasmid DNA from bacteria and replicate the DNA for future use. For standard maxiprep experiments, preliminary transformation and bacterial cultures are created. Cultures centrifuged at 6000 rpm, 15mins, to retain pellets. Resuspend pellets in buffer, centrifugate, and use wash solution to separate cellular components. Elute DNA, incubate and filter supernatant multiple times. Measure concentration, dilute 100 times in dH_2O with dilution factor of 100, using $A_{260}1.0$= 50µg/ml pure dsDNA.
d. **Restriction Digestion:** Through the use of ligase enzymes, Xbal and Pstl, the DNA was cleaved at the desired sites, which allowed us to derive the desired fragment of DNA at both the 5' and 3' ends. Restriction digestion allows us to extract DNA from our plasmid.
e. **Restriction Analysis:** Through performing a restriction analysis, it allowed us to identify breakages in the plasmid sequence by using the Pstl and Xbal enzyme. The ability to pinpoint the breakages assists in our plasmid design research in order to ensure that the fragments produced by digestion are in agreement with the predicted sizes, confirming the identity and presence of the chimera gene in the plasmid we previously amplified by maxiprep.
f. **DNA Electrophoresis:** DNA Electrophoresis was used to separate DNA fragments according to their size and charge, and in order to validate that the correct gene (DNA) was put into the plasmid. The first DNA Electrophoresis was run using the maxi prepped DNA, this was followed by a second run where the agarose concentration was increased in order to adjust the pore size that the molecules traveled through the gel.
g. **Protein Expression/Gene Expression:** Protein expression is done to confirm that the plasmid created is capable of expressing the protein from the DNA held within the plasmid. For future purposes, we will be using IPTG in order to allow our plasmid DNA to express the protein that will be fully functional to degrade PET for the plasmid we have acquired, pCJ190 MP12. In the final design, gene expression will be self-induced via the SILEX system.
h. **Protein Purification:** Protein purification will be useful for our purposes in order to isolate necessary parts of the protein that are coded for the degradation of PET. To carry this out we will be doing a cell lysis, allowing the protein to bind to the matrix using nickel which will bind to the HIStag and the target molecule will bind to the immobilized ligand. After washing the non-bound components of the protein will be removed, allowing for the isolation of the bound target with the ligand. The elution will then allow for the target molecule to disassociate from the immobilized ligand, leaving us with the essential protein needed for the degradation of PET. Protein purification will be validade by spectrophotometry (yield) and SDS-PAGE.
i. **Enzyme Efficiency in Chassis :** In order to test the efficiency of the chimera and its ability to degrade PET, we will conduct spectrophotometry more specifically "kinetics of poly(ethylene terephthalate) films", where the recombinant protein will be incubated with PET pellets (optimal concentration to be established by concentration curves) and light absorbance will be measured.
j. **Testing Overall Efficiency in Microbial Fuel Cell:** Once the protein is confirmed to be able to degrade PET, it will be tested within the MFC by rate calculations based on enzyme activity as follows: rate = change/time. Once this step is completed, then the MFC will be able to produce energy from the breakdown of PET.
k. **Directed Evolution:** A cyclic system that transitions between gene variability and screening or selection of functional gene variants. To be completed by identifying a primary structure protein, diversifying its genome, expressing and screening, and repeating until sufficient performance level of enzymatic activity is reached.
l. **Performance of Chamber Systems in Cell:** Measured by a polarization curve which shows the chance of potential of the cell when current density changes. This will be our final step to determine the overall efficacy and systematic output of the MFC.

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