Maximizing Acetyl-CoA Output by Genetically Engineering *E. coli* for the Overall Output of the Bioplastic PHB

Stakeholder Engagement in Pro-Sustainable Actions within Municipalities

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By

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On my honor as a University student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments.

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Introduction

Single-use or disposable plastics have become common use throughout the world leading to their accumulation in landfills and polluting the environment. It is estimated that about 300 million tons of plastic waste is being produced every year with about 60 percent of plastics produced since the 1950s ending up in landfills or the natural environment (UNEP, 2018). With the human race's continuing reliance on plastics, it is projected that 12 billion tons of plastic will be in landfills and the environment by 2050 (Plastic Soup Foundation, 2018). Biodegradable bioplastics, such as polyhydroxybutyrate (PHB), offer a sustainable alternative to petroleum-based plastics. They are natural byproducts of facilitated degradation of these synthetic plastics, such as styrene, through metabolic mechanisms found in microorganisms like *E. coli* (Kourmentza et al., 2017).

My current work in my Capstone project as an undergraduate biomedical engineer (BME) at the University of Virginia (UVA) utilizes such pathways found in the microorganism *E.coli* to produce bioplastics. *E. coli* possesses an endogenous intermediate phenylacetic (PAA) pathway that takes in phenylacetic acid to produce acetyl-CoA. Through genetically engineering *E. coli* to possess the styrene degradation pathway observed in *P. putida* (sty plasmid) with the PHB production pathway seen in *C. necator* (pha plasmid), the phenylacetic (PAA) pathway in *E. coli* can be utilized to produce a single-engineered device to produce the bioplastic PHB. The styrene degradation pathway degrades styrene into phenylacetic acid, which is the reactant for *E. coli*'s endogenous PAA pathway to produce acetyl-CoA. Lastly, acetyl-CoA feeds into the PHB production pathway to produce PHB. In particular, our team's main focus is on maximizing the output of acetyl-CoA in *E. coli* for the overall output of PHB.

However, in order for this engineering device to be effective, a market for this product is needed in order for plastic producing companies to adopt it. However, a study indicates that misconceptions about what makes bioplastics positive lead to unstable positive attitudes, which leads to negative misconceptions and misuses of bioplastics, such as littering (Zwicker et al., 2021). Fostering stakeholder (e.g. individual citizens, green technologies like composting facilities) involvement in pro-sustainable actions like recycling and composting can help engage and inform the public on green technologies like bioplastics and foster proper use and efficiency of such processes. Thus, the focus of the STS topic is to investigate how a municipality involves (or disregards) certain stakeholders in their development of pro-sustainable actions to identify how it promotes pro-sustainable behaviors like recycling (Wamsler, 2017). The municipality of focus will be on Charlottesville, Virginia due to it being home to a high density of biotech companies and its implementation of composting and recycling as waste management processes (City of Charlottesville, 2021d; CvilleBioHub, 2021).

Technical Topic

According to market data compiled by European Bioplastics in cooperation with the nova-Institute, the global bioplastics is expected to increase from around 2.11 million tons in 2020 to approximately 2.87 million tons in 2025 (EUBIO, 2021). This growing increase in bioplastics is motivated by the need to reduce the negative consequences of the plastic industry, such as the use of fossil fuels to produce the plastics and the toxicity of the products themselves. By maximizing the production of acetyl-CoA in *E. coli's* endogenous PAA pathway, it would optimize the production of PHB; thus, allowing it to compete with mass manufacturing of conventional plastics and reducing toxic emissions of synthetic plastics to the environment and gradually to humans. PHB is also non-toxic and completely biodegradable, degrading into carbon dioxide and water under aerobic conditions (Hankermeyer & Tjeerdema, 1999).

My role within our capstone team is to design a genome-scale metabolic model in order to simulate the metabolic reactions of our genetically engineered *E. coli*. Maximizing the production of acetyl-CoA would require the visualization of the response of our microorganism to different constraints and gene deletions. There are already existing GEMs for *E. coli*, such as the most recent version iML1515. This model has shown a 93.4 percent accuracy for gene essentiality simulation under minimal media containing 16 different carbon sources (Gu et al., 2019). Previous research was done on this topic by UVA's iGEM team in 2019 using the iML1515 GEM for *E. coli* K-12 with the COBRApy GEM reconstruction software (UVA iGEM 2019, 2019). The team manually edited this GEM to include metabolic pathways offered by the sty and pha plasmid and transport reactions for styrene across the cell and plasma membrane, since the bacterium will be using styrene as its carbon source for PHB production. They performed sensitivity analysis to determine the sensitivity of PHB production to certain metabolites in the system. Additionally, they attempted growth coupling to link the production of PHB to the growth of *E. coli*, which proved to be unsuccessful due to the main metabolite of acetyl-CoA being used for other cell metabolises, such as the TCA cycle. Based on this prior research, our team has decided to utilize the iML1515 model with the COBRApy software to simulate our genetically engineered microorganism.

Other team members are responsible for determining possible gene deletions and pathways to inhibit to help increase acetyl-CoA flux. One possible gene deletion our team has currently identified is the deletion of the ackA gene, which is used in the pta-acka pathway that consumes acetyl-CoA to produce acetate (Enjalbert et al., 2017). Deletion of this gene may result in a lower consumption of acetyl-coA; thus, a greater flux for PHB production. However, a large issue our team has discovered is that the enzyme B-ketothiolase (*PhbA*) is inhibited by high levels of CoA activity. This accumulation of CoA results in the dissimilation of acetyl-CoA through the TCA cycle, which is highly undesirable (Centeno-Leija et al., 2014). Thus, it is ideal to grow *E. coli* with glucose as its main carbon source before transitioning to phenylacetic acid to maximize PHB production.

My role is to determine how to configure the GEM to our modified *E. coli* and ensure its validity as a model. Otherwise, more research is needed to determine more gene deletions and pathways to consider. Additionally, research on assays and lab procedures to experimentally measure environmental conditions and acetyl-CoA production would be needed to determine if gene deletions provided the desired effect of increasing acetyl-CoA flux. By maximizing the production of acetyl-CoA for the overall production of PHB, our team optimizes the yield and cycle time of turning waste plastic into PHB. It allows for the creation of a more scalable and continuous process for the production of the sustainable plastic PHB.

STS Topic

From 1950 to 2019, it is estimated about one percent of the 368 million tonnes of plastic produced annually are made of bioplastics. According to market data compiled by European Bioplastics in cooperation with the nova-Institute, the global bioplastics is expected to increase from around 2.11 million tonnes in 2020 to approximately 2.87 million tonnes in 2025 (EUBIO, 2021). With this growing increase in bioplastic production, it generates the question of where the need for it originates from. Bioplastics are told to be more advantageous with characteristics like conventional petroleum-based plastics while reducing its impact on global warming and harming the environment. However, how much do citizens of the general public understand the term bioplastics and its characteristics? A study generated in Australia investigated how much the public knew, what they knew, and their perceptions were about bioplastics. Their results discovered that their perception of bioplastics were positive but their knowledge about them was low. This led to a discovery of potential issues related to the role that governments and local councils play in driving the development of waste management standards for bioplastics, such as what bioplastics are deemed ‘recyclable’ (Dilkes-Hoffman et al., 2019). Furthermore, a study found that misconceptions about bioplastics could end up being harmful, such as all bioplastics being automatically biodegradable, whereas that is not always the case. They indicate that such misconceptions about what makes bioplastics positive lead to unstable positive attitudes, which leads to negative misconceptions and misuses of bioplastics, such as littering (Zwicker et al., 2021). Thus, investigating how municipalities engage stakeholders in developing pro-sustainable actions can provide insight on how to properly inform and engage stakeholders (e.g. individuals, green technologies like composting facilities) in promoting proper pro-sustainable behaviors involving biopolymers and waste infrastructures.

A framework proposed by Christine Wamsler (2017) provides a guide in investigating municipal development of pro-sustainable action and its attempts in co-production through stakeholder involvement (e.g. future research and policy recommendations) to foster continuous and transformative adaptation processes. It examines the why and how different stakeholders are involved (or excluded) in the development of municipal strategies and how it is reflected in process outcomes (Wamsler, 2017). The focus of this research paper is to investigate the extent to which municipalities engage their internal and external stakeholders (e.g. individual citizens, proximity to the decision-making body, staff, outstanding individuals) to provide insight into how stakeholder engagement affects the outcomes of pro-sustainable actions and its process. The process timeline is illustrated by Wamsler in 4 main parts: (1) the trigger (i.e. the context), (2) the starting point / set-up (i.e. organizational and regulatory set-up, responsibilities, resources, goals), (3) development (i.e. assessing risks and knowledge, identifying and selecting adaptation options, and designing), and (4) the output (i.e. measures and strategies). This framework allows for assessment and comparison of developing adaptation and their outcomes with respect to the issue of collaborative governance arrangements.

Using Wamsler's (2017) framework on investigating how municipalities engage their stakeholders (internal and external) in developing pro-sustainable strategies, I will be studying how the City of Charlottesville, Virginia, and the University of Virginia (UVA) promote pro-sustainable behaviors and policies that include biopolymers and waste infrastructures to include (or disregard) stakeholders. Charlottesville is home to one of the highest density of biotech companies in the Virginia Commonwealth (CvilleBioHub, 2021). Additionally, Charlottesville has multiple options for drop-off composting and recycling collection of household compostable and recyclable materials (City of Charlottesville, 2021d). UVA is one of the largest institutions in Charlottesville that implement recycling and composting processes working with green technologies like Eco-Products and Black Bear Composting. Furthermore, the company our Capstone project is working with (called Transfoam LLC) is a biomanufacturing platform founded by students and professors at UVA (Transfoam LLC, 2021). This makes Charlottesville a rich municipality to investigate how their decision-making bodies in pro-sustainable actions involve (or disregard) internal and external stakeholders to promote green initiatives and behaviors.

One of the large focuses of the City of Charlottesville's government are its sustainability initiatives, such as emphasizing recycling and reuse (City of Charlottesville, 2021). One of the main ways the government wishes to achieve this is through sustainable waste management. The Public Works department within the government is responsible for waste management and provides recycling, composting, and trash service for residents and businesses. Waste collection at public locations is managed by the Department of Parks and Recreation, Charlottesville Area Transit, and Charlottesville City Schools (City of Charlottesville, 2021d). However, the government chooses to engage individual stakeholders (e.g. residents and businesses) in their recycling and composting processes by providing curbside recycling collection and source-separating recycling drop-off at Rivanna Solid Waste Authority's McIntire Recycling Center (City of Charlottesville, 2021b). In terms of composting, only drop-off or backyard composting is available for residents (City of Charlottesville, 2021a). The Sustainable Waste Management and Landfill Diversion practices are integrated in the City's Solid Waste Management Program and are supported by the City Council's Strategic Plan and Vision Statement (City of Charlottesville, 2021c). The Charlottesville City Council serves as the city's legislative and governing body. Thus, the internal stakeholders for sustainable waste management can be identified as those in the City of Charlottesville's City Council and the Public Works department.

External stakeholders involved in pro-sustainable processes of recycling and composting include residents, businesses, and composting and recycling facilities in Charlottesville. One of the main external stakeholders that is featured within the CIty of Charlottesville's sustainability initiatives pages is UVA, where they attach an interactive guide for sustainability projects happening on the university's grounds (City of Charlottesville, 2021). UVA helps to push the city's waste management sustainability initiatives by implementing their own recycling and composting programs (UVA Sustainability, 2021c, 2021a). UVA's mixed plastic is recycled by a contracted company in a material recovery facility in Raleigh, NC. Any plastics placed in the City of Charlottesville single stream recycling containers get sent to clean material recovery facilities and sold to end-users and processors (UVA Sustainability, 2021b). UVA also has a single-use plastics reduction strategy that plans to phase-out the use of all other single-use plastics by 2025 and has begun such by providing more sustainable alternatives, such as paper straws and compostable Eco-Products, such as cups and containers (UVA Sustainability, 2021d).

Next Steps

A few internal and external stakeholders were identified in the development of pro-sustainable actions of recycling and composting in the City of Charlottesville as well as a few initiatives to achieve these actions by such stakeholders. To apply Wamsler's (2017) framework in investigating external and internal stakeholder engagement in current adaptation strategies in plastic waste management in Charlottesville, the next steps would be to identify a certain plastic waste management process being developed (or was developed) in the City of Charlottesville in order to apply the four main parts of the process timeline within the framework. Then, the chosen internal and external stakeholders roles, behaviors, and interactions with one another should be described and analyzed to determine how they were engaged throughout each step in the process timeline.

For the technical portion of the project, a “Capstone Proposal” will be approved and signed by our team’s advisor by November 10th, 2021 that fully outlines our team’s Capstone project to provide direction on our research and provide preparation for in-lab experiments in the Spring 2022 semester. A final “Fall Progress Update” will be turned in to our advisor by Friday, December 10th depicting our project process at that time. Otherwise, our team’s main focus will be to continue research on which genes to edit within *E. coli* to maximize the output of acetyl-CoA and which experimental procedures to use to measure the output of acetyl-CoA. My role within the Capstone team is to have a fully designed GEM by the start of the Spring 2022 semester to allow for the simulation of our genetically-engineered *E. coli* before our team begins experimental testing.

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