BioCapture: Enhancing Polyhydroxybutyrate (PHB) Production Through the Electrolysis and Fermentation of *Cupriavidus necator*

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Abstract

Currently, three hundred fifty million metric tonnes of plastic annually accumulate in landfills, taking up to five hundred years to degrade naturally. This has an adverse impact on the health and biodiversity of ecosystems. This paper proposes a method to enhance the capture of atmospheric carbon and production of Polyhydroxybutyrate (PHB), a common bioplastic, using the *Cupriavidus necator* H16 strain and transform PHB into plastic packaging that is commercially viable. Optimizing the existing biohybrid system, developed by Lim et al. in 2023[1], we plan to enhance carbon dioxide electrolysis along with subsequent PHB production through bacterial manipulation within a fermenter and develop a more environmentally friendly and cost-effective method for PHB extraction.

Keywords**:** *Cupriavidus necator* (*C. necator*), polyhydroxybutyrate (PHB), carbon dioxide electrolysis, fermenter, plastic, carbon emissions

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Introduction

With the onset of climate change, many research efforts have been directed toward the achievement of carbon neutrality through the development of bioplastics to replace the current modes of plastic production. Plastic pollution has become a prevalent concern in recent decades, with three hundred fifty million metric tonnes of plastic accumulating in landfills and bodies of water each year[2]. As worldwide plastic consumption proliferates, so does generated waste. Although plastics have lifespans of approximately ten years, they can take up to five hundred years to decompose naturally, posing both environmental and social sustainability risks to populations. From an environmental perspective, plastic pollution can impact ecosystems and harm wildlife, especially aquatic populations. According to the International Union for Conservation of Nature, 80% of marine debris can be attributed to plastics[3]. The most prominent impacts of plastic marine debris are the ingestion, suffocation, and entanglement of various marine species, including seabirds, whales, fish, and turtles. Oftentimes, plastic is mistaken for prey, resulting in internal lacerations, infections, and injuries of species. Additionally, Canadian Geographic advances that plastic is a part of the carbon cycle, contributing to the growing issue of climate change[4]. Approximately one ounce of carbon dioxide is produced for every ounce of polyethylene produced, making the emission of carbon dioxide through plastic production comparable to annual amounts of carbon emitted by 19 million vehicles[5]. Carbon fixation is the process by which atmospheric CO_2 is converted from a gas into biomass. In addition, humans can be adversely impacted by plastic accumulation, leading to reduced health and longevity[6]. Microplastics have been found in tap water and drinking water, which could impact the health of individuals due to the carcinogenic properties of plastics that can interfere with the endocrine system[3]. Health risks from plastic production in developing nations have increased, as the accumulation of discarded plastic impacts countries' infrastructure, land, and bodies of water. A World Wildlife Fund study[3] highlights that plastic pollution causes up to a million deaths each year in developing countries due to the release of toxic chemicals emitted from discarded plastic.

In this paper, we discuss a potential strategy to address the issue of plastic waste through the production of biological plastic. We aim to genetically engineer *Cupriavidus necator* to enhance bioplastic (Polyhydroxybutyrate (PHB)) production and develop a commercially viable system for extracting PHB

Solution and Implementation

Objectives

This project entails two branches of investigation: (a) to enhance Polyhydroxybutyrate (PHB) production using the *C. necator* H16 strain through sucrose utilization genes insertion, focusing on scaling up PHB production and (b) to optimize PHB recovery for increased yield and purity and transform PHB into materials suitable for plastic packaging. Through both a biological and engineering lens our comprehensive approach seeks to create a sustainable and economically viable solution for carbon capture and bioplastics production.

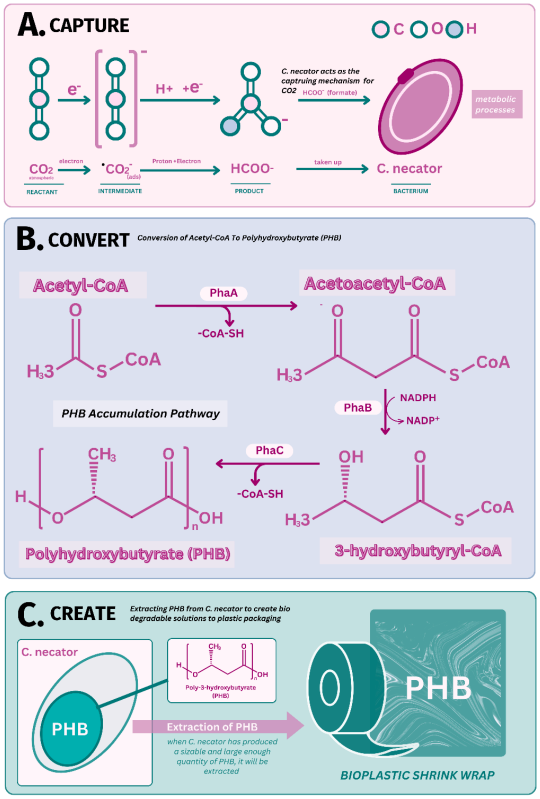

Figure 1. (A) Conversion of CO2 to Formate By C. necator; (B) Conversion of Acetyl-CoA to Polyhydroxybutyrate; (C) Extraction of PHB to Create Biodegradable Plastics

Biohybrid System: Proof of Concept

To transform CO_2 into PHB, two systems are being implemented, inspired by the principles discussed in *Biohybrid CO_2 electrolysis for direct polyester synthesis from CO_2*[1]: (1) the electrochemical conversion of CO_2 to formate, utilizing a gas diffusion electrode (GDE) within an electrolyzer, and (2) the subsequent conversion of formate to PHB using genetically modified *C. necator* cells within a fermenter. Between these chambers, a continuous medium circulation system is established through interconnected tubing, beginning in the electrolyzer, where formate is carried into the fermentation chamber, before returning to the electrolyzer. To maintain the integrity of the system, a hollow fibre filter is added (ref. Figure 2) to prevent bacteria from entering the electrolyzer, ensuring their retention within the fermenter. With this system, one culture medium can be recycled throughout the system, whilst ensuring high PHB production. As a separate step, *C. necator* cells will be harvested from the fermentation chamber, and the extraction of PHB will be carried out using the previously described solvation method.

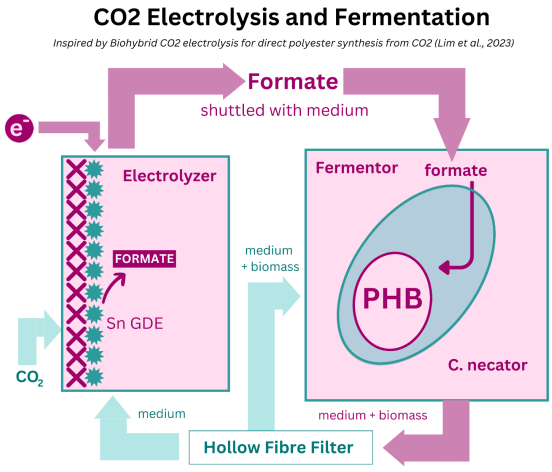

Figure 2. Biohybrid Electrolysis and Fermentation Chambers

Advantages of utilizing C. necator bacteria in PHB production

Several groups have identified the biodegradable material, poly(3-hydroxybutyrate) (PHB), as a promising means for bioplastic production[7]. Among the millions of bacteria capable of producing PHB, *Cupriavidus necator*, a gram-negative bacterium, is widely used by researchers due to its ability to grow in both aerobic and anaerobic environments. In nutrient-deficient environments, *C. necator* accumulates trace amounts of PHB and other polyhydroxyalkanoates as a means of reserving carbon, making it a particularly viable platform for the biosynthesis of PHB, and eventually bioplastics[8]. Moreover, *C. necator*'s metabolism is highly flexible due to its ability to act as both a chemolithotroph and heterotroph. As noted by Morlino et. al in their 2023 review[8], *Cupriavidus necator* as a platform for polyhydroxyalkanoate production: An overview of strains, metabolism, and modelling approaches, a particular strain of *C. necator*, H16, can utilize either reduced organic compounds or hydrogen as electron donors, leading to heterotrophic or chemolithotrophic lifestyles, respectively. In heterotrophic metabolism, the reduced organic compounds serve as the primary carbon source, while chemolithotrophic metabolism relies on 1-carbon molecules.

In particular, in *C. necator* H16, chemolithotrophy can occur with carbon dioxide or formate as carbon sources. With these key considerations in mind, in developing this project, it was decided that *C. necator* H16 would serve as an ideal bacterium for the production of PHB in a nutrient-deficient environment. In 2023, Lim et. al designed a biohybrid system utilizing a fermentation chamber with formate acting as the primary carbon source for *C. necator*-based production of PHB. Within this project, Lim et. al produced 1.38 g PHB with a PHB content of 83.0% by running the biohybrid system consistently for 120 hours[1]. Essentially, with *C. necator*'s ability to grow in both aerobic and anaerobic environments along with its extensively studied ability to produce PHB in nutrient-deficient environments, it serves as a ready biological component for the aims of our project.

Identification of the primary Metabolic Pathways of C. necator

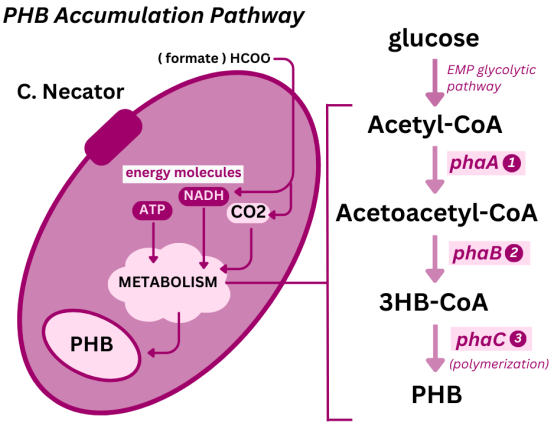

Figure 3. PHB Accumulation Pathway

As stated earlier, *C. necator* H16 possesses a unique ability to grow in both aerobic and anaerobic environments, and is frequently denoted as either a chemolithotroph or heterotroph depending on the specific bacterial strain being utilized in culture. In addition to this behaviour, *C. necator* H16 has displayed an ability to act mixotrophically[9]. The final electron acceptor in the aerobic growth of *C. necator* H16 is oxygen, however, in the absence of oxygen, the bacterium reduces nitrate through a complete denitrification pathway[8]. The heterotrophic growth of *C. necator* H16 is driven by numerous carbon sources including, but not limited to, fructose, PHA precursors, gluconate, and amino acids. However, a key omission from the viable substrates of *C. necator* H16 growth is glucose. In their 2011 paper, Raberg et. al[10] suggested that the bacterium lacked the transport system for glucose intake. However, the paper discusses how mutant strains such as, *C. necator* H16 G+1, glucose uptake is enabled by point mutations in the transporter for N-acetyl-glucosamine, providing sufficient glucose affinity and allowing for its uptake in the bacterium[8]. As a chemolithotroph, *C. necator* H16 conserves energy with the reduction of hydrogen through the action of Ni-Fe hydrogenases, namely a soluble hydrogenase and a membrane-bound hydrogenase[11]. In *C. necator*, carbon fixation occurs through the Calvin-Benson-Bassham cycle, in which RuBisCO acts as the key enzyme, facilitating the fixation of CO_2 molecules to a molecule of Ribulose 1,5-bisphosphate (RuBP) to form 3-phosphoglycerate. While most studies into carbon fixation in *C. necator* H16 are centred on the use of carbon dioxide as the primary carbon source, formate can also serve as a carbon source in the chemolithotrophic lifestyle of *C. necator* H16 as it is first oxidized to CO_2 by a soluble formate dehydrogenase, and then enters the CBB cycle. In terms of PHB biosynthesis, the ability of *C. necator* to be developed in anaerobic environments is not widely used due to the extended lag phase and limited biomass productivity that the lack of oxygen causes in the bacteria[12]. However, within the design of this particular project, the heterotrophic lifestyle of *C. necator* H16 serves as the most viable method for the production of PHB as most biohybrid systems that use *C. necator* operate with batch reactor systems only compatible with heterotrophic organisms[1].

In its production of PHB, *C. necator* utilizes a three-step mechanism first detailed by Peoples and Sinskey in their 1989 paper, *Fine structural analysis of the Zoogloea ramigera phbA-phbB locus encoding β-ketothiolase and acetoacetyl-CoA reductase: nucleotide sequence of phbB*[13]. In the first step, two molecules of acetyl-CoA are condensed by 3-ketothiolase to form acetoacetyl-CoA with the action of the gene PHAa. In the second step, acetoacetyl-CoA reductase with the action of PHAb converts the metabolite into 3-hydroxybutyryl-CoA, with the consumption of NADPH. Finally, the molecule is added to an existing polymer by PHA synthase PHAc[13]. The key precursor in the process of PHB production by *C. necator* is acetyl-CoA, and due to its prevalence in various metabolic pathways involving carbon, the PHB cycle in *C. necator* can be linked to the TCA cycle, beta-oxidation pathway, and fatty acid synthesis. The rate of PHB synthesis is directly in competition with the activity of the TCA cycle based on the direction in which acetyl-CoA flux is directed; either towards the TCA cycle or towards the condensation reaction catalyzed by PhaA[14]. However, in nutrient-limiting conditions, the environment in which *C. necator* is grown is deficient in nitrogen, prompting acetyl-CoA to favour the PHB production cycle over the TCA

cycle. The Embden-Meyerhof-Parnas (EMP) pathway for glycolysis is a widely studied glycolytic pathway, the progression of which is a significant aspect of intracellular PHB production in a myriad of bacteria. In this pathway, glycolysis occurs to produce pyruvate, which is eventually oxidized through the Pyruvate Oxidation process into Acetyl-CoA. This process is aided by the activity of the enzymes hexokinase, phosphohexose isomerase, phosphofructokinase-1, fructose bisphosphate adolase, glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, phosphohexose mutase, enolase, and pyruvate kinase[15]. From acetyl-CoA, in nutrient-deficient environments, *C. necator* intracellularly accumulates PHB, and with its previously described metabolism, it can be retrieved to eventually produce bioplastics. In short, acetyl-CoA in nutrient-deficient environments leads to the intracellular collection of PHB in *C. necator*, which can be retrieved to eventually produce bioplastics.

System Optimization

The Wet-Lab component of our project is aimed at enhancing the production of PHB using *C. necator*. We will enhance this PHB production by evaluating the effectiveness of implementing three methods: genetic engineering, feeding strategy optimization, and fermenter environment optimization.

Genetic Engineering

In the first phase of our experimentation, we will be genetically engineering the bacteria by inserting the csc gene from *Escherichia coli* W into *C. necator* H16's genome. Reported by Arikawa, et. al in 2017[16], the addition of this gene allows *C. necator* to use sucrose in its production of PHB allowing the bacteria to increase its PHB production in the PHB cycle. The use of sucrose in the biosynthesis of PHB through *C. necator* is particularly of interest due to sucrose's widespread availability and low cost of production. In their development of a catholyte compatible with the biohybrid system, Lim et. al[1] identified three primary issues with previously developed catholyte solutions: low ionic conductivity, metal ion contamination, and nutrient limitation in regards to enhanced PHB production. In our proposed modification in which we will introduce sucrose to the catholyte solution, the aforementioned issues of metal ion contamination and nutrient limitation will not be exacerbated. However, in terms of ionic conductivity, sucrose may add to the ohmic resistance due to its propensity to form aquated polar molecules in solution. However, Lim et. al successfully lessened the effects of ohmic resistance with addition of phosphate salts to the catholyte, and thus, with the addition to sucrose. Despite this potential issue with sucrose's presence in the catholyte, it should be noted that the presence of phosphate salts will likely offset this issue. However, most strains of *C. necator* are unable to use sucrose as a carbon source due to a lack of the necessary mechanisms for the assimilation of sucrose and its monomers. Instead, food wastes, lignocellulosic materials, and glycerol, serve as the most common carbon sources for PHB production in *C. necator*[7]. In light of this pitfall, we aim to genetically modify the makeup of *C. necator* to allow for greater sucrose fermentation based on the work of Ariwaka et. al in their 2017 paper[16]. Furthermore, the fermenter environment will also be optimized to promote bacterial growth. Temperature will be kept at 30°C, and a pH of 6.8.

Environment optimization

As mentioned previously, *C. necator*'s surrounding environment plays an important role in determining the metabolic pathways that are active at any given moment. The rate of PHB synthesis is directly in competition with the activity of the TCA cycle based on the direction in which acetyl-CoA flux is directed; either towards the TCA cycle or towards the condensation reaction catalyzed by PhaA[14]. The competition of the two pathways inhibits the biosynthesis of PHB in *C. necator*, and in order for PHB production to be optimized, the environment in which fermentation occurs should be deficient in the nutrients necessary for *C. necator* growth. To this end, this project aims to establish a fermenter environment with a carbon to nitrogen ratio of 20, due to its proven effectiveness in maximizing intracellular accumulation of PHB in *C. necator*. As detailed by both Yang et. al in their 2010 work[17] and Park and Kim in their 2011 paper[18], the C/N ratio of 20 resulted in optimal PHB content[19].

Feeding strategy optimization

Next, in our production of PHB through microbial fermentation of *C. necator*, we will use a multi-stage fermentation process in which cell growth is initially promoted through a nutrient-balanced environment. Then, in the second stage, the nutrient limiting condition is applied to the fermenter so that PHB accumulation starts to speed up, while biomass production is restricted[7]. As opposed to the single-stage method used by Lim et. al[1] in their

development of a biohybrid system for PHB production, the multi-stage fermentation method described above will lead to greater *C. necator* growth, while also promoting the intracellular biosynthesis of PHB by the bacterium.

PHB Extraction

Numerous studies have suggested various extraction methods to enhance both the yield and purity of PHB while minimizing manufacturing costs related to recovery. Among these methods, the common industrial approach employs solvent extraction, known for its efficiency, minimal biopolymer degradation, and effective removal of bacterial toxins from the polymer[20]. Currently, the majority of solvent extraction methods rely on halogenated organic solvents, namely chloroform. Nonetheless, chloroform presents challenges due to its high cost, environmental toxicity, polymer degradation, and safety concerns for commercial use arising from the emission of toxic fumes. Thus, a safer, practical, environmentally friendly and cost-effective method for PHB recovery using non-halogenated solvents is necessary.

In extracting PHB from *C. necator*, three non-halogenated solvation methods demonstrate promising results: extraction using 1,2-propylene carbonate, ethylene carbonate, and butyl acetate.

The method involving 1,2-propylene carbonate has been widely adopted based on the technique outlined by Lafferty and Heinzle[21]. The separation of cell mass from nutrient solution is achieved through decanting, filtering or centrifuging. The isolated cell mass is then suspended in a cyclic carbonic acid ester, specifically 1,2-propylene carbonate. Following this, the suspension undergoes heating with stirring, where the extraction rate and molecular weight of the extracted PHB can be altered by adjusting temperature and stirring duration. The resultant hot extract is separated from the extracted cell mass by means of decanting, filtering, or centrifuging. Pure PHB is subsequently extracted from the obtained liquid through cooling or by adding a small amount of water, and the precipitate is separated. The solvent, once recovered, can be reused iteratively for additional extractions of PHB from fermentation masses. To purify, the heated solution containing PHB in solution is gradually cooled to room temperature and mixed with an equal volume of ethanol. The resulting precipitate was separated by filtration, washed with ethanol, and dried overnight at 110°C before being weighed. In a separate experiment conducted by Fiorese and colleagues[22], optimal recovery conditions were assessed. A 95% yield was obtained with a purity of 84% with conditions of 130℃ combined with a suspension period of 30 minutes and a precipitation period of 48 hours. The PHB's molecular weight under these conditions was 7.4×10^5.

The method employing ethylene carbonate as a solvent is also widely used and was initially discussed in the same study by Laffety and Heinzle[21]. The experimental process is analogous to that of 1,2-propylene carbonate, but uses ethylene carbonate in the carbon ester. To purify, the PHB-ethylene carbonate solution is cooled to 60°C and diluted with an equal volume of water. The resulting precipitate is filtered, washed with water, and dried overnight at 110°C before being weighed. A separate study, conducted by Aramvash and colleagues[20], saw the most optimal results under conditions including a temperature of 150°C and 60 minutes of suspension time. The highest grade of PHB purity obtained was 98%, with a 98.6% recovery percentage. The recovered PHB's average molecular weight was 1.3×10^6. Furthermore, ethylene carbonate is recognized for its biodegradability, low volatility, and lower toxicity in comparison to other substances, making it a strong candidate for a solvent.

Another efficient and environmentally friendly method of extracting PHB uses butyl acetate. In a study conducted by Aramvash and colleagues[23], the following experimental method was outlined. Approximately 1 g of wet biomass was suspended in 100 ml of solvent and transferred into a tube immersed in a thermostatic water bath while stirring. The suspensions underwent stirring and heating at different times and temperatures. Once the suspension reached the set temperature, it was maintained for testing at different incubation times. Subsequently, the suspension was left at room temperature for 24 hours and then centrifuged under appropriate conditions. The supernatant was collected and precipitated with acetone, and the resulting biopolymer was washed with distilled water. Finally, the biopolymer was dried at room temperature. A purity of up to 99% and recovery level of 96% was achieved at a suspension temperature of 103°C and a heating incubation time of 30 minutes. By keeping the suspension temperature below butyl acetate's boiling point (126°C), the procedure can be safely scaled up. To attain a recovery yield equivalent to conventional techniques (e.g., recovery by chloroform), *C. necator* strain ATCC 17699 was cultivated in batches. The molecular weight of PHB ranged from 3×10^5 to 1.4×10^6.

Table 1: Performance Metrics Across Solvents

	% PHB Recovery	% PHB Purity	Molecular Weight	Toxicity	Optimal Temperature (°C)
1,2-propylene carbonate	95%	84%	7.4×10^5	Low	130°C
Ethylene Carbonate	98.6%	98%	1.3×10^6	Low	150°C
Butyl Acetate	96%	99%	Min. 3×10^5	Moderate	103°C

Between the three solvents, it is important to consider the percentage of PHB recovery, environmental friendliness, and practicality on an experimental and commercial scale. Our team has presently selected butyl acetate as the solvent for PHB, notably due to its high recovery rate and purity (ref. Table 1)[20][22][23]. Despite its lower molecular weight, butyl acetate has an advantageous lower temperature, making it practical in both a high-school laboratory and on a commercial scale. While deemed minimally toxic, the chemical does possess the capacity to contribute to air pollution when in vapour form. However, in the experimental design, as previously outlined, we intentionally maintain it below the boiling point to ensure it remains in a liquid state.

Bioplastic Formation

Following the extraction of PHB, our project aims to utilize this biopolymer in the development of biodegradable transportation packaging. Approximately 49% of plastic waste comes from the packaging industry, which includes transportation wraps[2]. The most common plastic, PVC, contains chemicals such as phthalates, lead, cadmium or organotins, which are harmful to the environment when exposed; moreover, it is generally non-recyclable and left in landfills. Of the plastics that could be recycled, 91% are also left in landfills, contributing to the global climate crisis that impacts our world today[2]. In light of this, it is necessary to develop an eco-friendly solution to replace traditional shrink-wrap packaging. In addition to its eco-friendly properties, PHB stands out as an ideal choice, as the primary requirement is to prevent dust accumulation on merchandise rather than possessing exceptional strength. Given the typical strength limitations of biopolymers, PHB serves as an effective solution for eco-conscious dust-cover packaging. To achieve this, a process called injection moulding can be employed, where plastic pellets are melted and injected into a mould cavity, and cooled to form a desired shape. In a study conducted to examine PHB packaging within the food industry[24], several key points were brought up in altering the injection moulding process tailored for PHB. Firstly, the study revealed excessive polymer degradation in the initial phases, resulting in the production of defective packaging samples. This degradation was attributed to the lower molecular weight of PHB compared to standard polypropylene (PP). Secondly, temperature-dependent performances between PHB and PP were highlighted. Under normal freezing and refrigeration conditions, PHB exhibited inferior characteristics compared to PP, whereas in elevated temperatures, PHB outperformed standard polymers. The study also tested dynamic compression resistance, crucial for intricate mould filling, characterizing PHB as a more rigid and less flexible material. Thus, specific adjustments to the moulding process, particularly injection processes and temperature conditions, are required to accommodate PHB's unique characteristics. In the context of transportation packaging, while injection moulding remains a viable option for PHB transformation, certain modifications to the process are essential. As a high school team, we anticipate challenges in obtaining a sufficient volume for PHB for thorough experimentation, which is why we consider both the design of the injection mould, and subsequent conversion into bioplastic, areas of improvement, warranting further consideration for future projects.

Future Experimentation

Fermenter optimization for PHB production

As detailed in many previous works, the limitation of key nutrients in the fermentation and growth of *C. necator* has maximized the production of intracellular PHB. Our primary focus within this experiment is to extend the optimization strategies used to improve PHB yields. The amount of sucrose utilized in the fermenter will be increased, and minimal amounts of nitrogen will be reintroduced into the fermentation chamber to establish a carbon-to-nitrogen ratio of 20, the optimal composition for PHB production identified by Yang et. al in 2010[17]. The introduction of nitrogen in the fermenter environment of the biohybrid system is rooted in the work of Zhang et. al.[7], in which they explain that in cases of 0% nitrogen supply, "pH control is monitored through NaOH instead of NH_4OH to achieve a 0% nitrogen supply". However, it was also proved by several studies that PHB production in *C. necator* could be negatively affected due to the accumulation of Na+ ions when NaOH is used[25]. By making the aforementioned modifications to the fermenter environment, in addition to the formate produced by the amount of PHB produced by the metabolism of *C. necator* in the biohybrid system should increase.

Implementation of the CSC gene

C. necator genetic modifications discussed previously will be done through the construction of various plasmids with the sucrose intake CSC genes. Genetically engineered *C. necator* H16 was successfully demonstrated for uptaking sucrose as a carbon source by Arikawa et al. in 2017[16], through the introduction of the expression vectors, intracellular sucrose hydrolase gene (cscA) and sucrose permease gene (cscB) of *E. coli* W strain, *C. necator* was capable of hydrolyzing glucose and utilizing it as a carbon source. These enzymes will allow the cell to uptake and hydrolyze sucrose and use it as a carbon source for the production of PHB. By applying Arikawa's methods, we hope to integrate the sucrose assimilation capabilities of genetically modified *C. necator* H16 -

Predicted Impacts

Human-Centered Design

The innovative aspect of this project comes from scaling the discussed biohybrid system to a commercial level, where it can be used in various industries, workspaces, office buildings, and schools. As of the date this paper was written, the specific construction methodology remains under consideration. However, the predicted impacts of this scalable model will allow for increased sustainability on a scale that is not industrialized. As a result, the solution aims to be accessible to all individuals, limiting plastic waste on commercial levels in order to promote the enhancement of plastic sustainability on a grander scale. The human-centred design approach will promote both sustainable and inclusive practices.

Conclusion

This paper outlines the issue of plastic accumulation and its connections to limited environmental and social sustainability. Through the proposed solution of BioCapture, we aim to enhance Polyhydroxybutyrate (PHB) production using the *C. necator* H16 strain, optimize PHB recovery, and transform PHB into materials suitable for plastic packaging. Using the biohybrid system developed by Lim et. al.[1] as a guide, future experimentation for this project includes optimizing the fermenter and constructing various plasmids with the sucrose intake csc genes in order to yield greater PHB production. Thus far, we have a proof of concept for the biohybrid model and have substantial research to support future PHB production and extraction plans.

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 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.

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 are received directly from Renert School.

(Summarized) Materials and Methods

In their biohybrid system, Lim et. al.[1] obtained the seed culture of *C. necator* H16 from the Leibniz Institute and prepared a 25-mL test tube containing 10 mL Luria–Bertani (LB) medium (10 g L−1 tryptone, 5 g L−1 yeast extract, and 5 g L−1 NaCl; ≥ 99.0%, Samchun Chemicals). After overnight shaking by a rotary shaker, 0.5 mL of the culture was used to inoculate a 250-mL baffled flask containing 50 mL LB medium. After cultivation for 24 h, the *C. necator* cells were harvested by centrifugation and washed twice with autoclaved distilled water. Then, the cells were transferred to a fresh modified catholyte (6.67 g L -1 KH_2PO_4, 4 g L -1, $(NH_4)2HPO_4$, 0.8 g L-1 citric acid, 0.8 g L-1 MgSO4·7 H2O, 5 mL L-1 TMS, and 30 g L -1 of fructose to act as the carbon source) for injection into the developed biohybrid system. 40 mL culture broth was collected to measure PHB production. The collected cells were washed with DI water 3 times and dried overnight in an 80 °C oven. The strains, plasmids and carbon sources used by Arikawa et. al in their 2017 paper[16] are as follows: *C. necator* (H16, 005dZ126, 005dZG, 141SR, 142SR, 143SR, and 144SR), *E. coli* (JM109 and S17–1), plasmid vectors: pCUP3, pCUV5-cscA, pCTRC-cscA, pCTRC-cscB, pCUV5-cscAB, and pCTRC-cscAB, and 0.129% (w/v) $(NH_4)_2SO_4$ and 1.5% (w/v) sucrose, fructose, or glucose as the sole carbon source.

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We would like to acknowledge the following people for their tremendous support with this project. Firstly, the research from Lim et al., 2023[1] provided us with a proof of concept for a biohybrid system that we could modify in our project. Secondly, we would like to acknowledge Mindfuel for continuing to mentor and support our team in our endeavours. Finally, a great depth of gratitude goes to our teachers, Dr. Iaci Soares, Ms. Kim O'Keefe, Mx. Dallas Mythril, and Ms. Alyssa Crispin for providing direct guidance and support throughout this project.

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