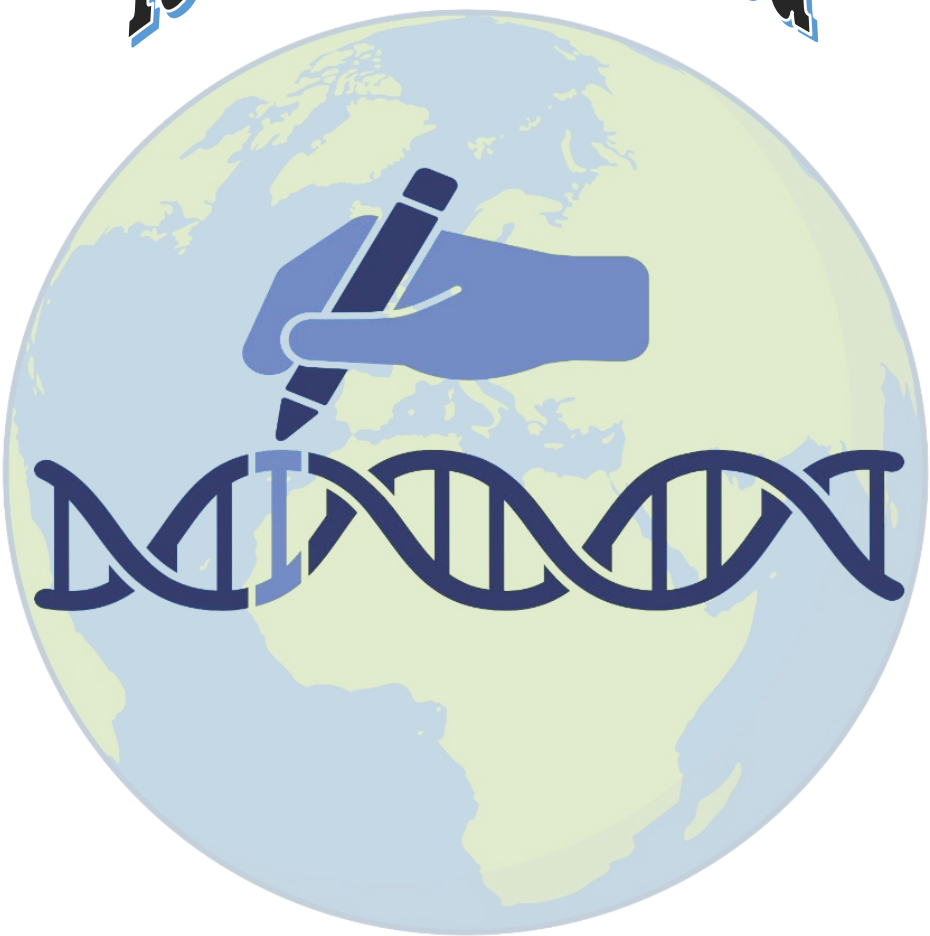
SYNTHETIC BIOLOGY for Global Good



Volume I

Synthetic Biology for Global Good: Volume I

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Contents

Preface

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Science discovery relies on exploring the primary literature to summarize what is already known, and identify gaps in understanding. Fundamentally grasping the role that primary literature plays in conceiving experiments and proposing new strategies to address these gaps is critical for the execution of scientific inquiry. Disseminating new results and ideas to the scientific community is accomplished (and valued) via original contributions to the primary literature, which continues the cycle of discovery. In emerging and expanding fields such as synthetic biology, these contributions are particularly important and impactful.

Teaching our students as nascent scientists how to navigate the existing literature, and ultimately contribute to the growing knowledge in their field, is a critical yet often overlooked skill in undergraduate life sciences education. Upper-level courses can provide students with an opportunity to learn to read journal articles and explore the primary literature. Less frequently within a course or across a curriculum, this exploration is coupled with explicit instruction in how to navigate the literature to find the most important and original works. Rarer still is teaching undergraduates the skills necessary to *contribute* to the primary literature by defining students as authors in the process of writing journal articles suitable for archiving and publication.

This collection of articles represents the outcomes of our approach to engaging students as direct contributors to the synthetic biology primary literature. BB4260:Synthetic Biology is a seven-week, three-credit course, and uses a medley of mini-lectures, case studies, discussion, and active learning to explore current primary literature in the field. Thirty-one students majoring in life sciences (biology and biotechnology, biochemistry, or biomedical engineering), primarily juniors and seniors, enrolled in and completed the course. As part of this course, we created a project designed to engage students deeply in the process of creating biological literature by writing a mini-review style article, and engaging in the process of peer review. We chose to have students write in the style of a “Forum” piece for the review journal *Trends in Biotechnology*. These articles are a maximum of 1200 words, 12 references, and up to two additional features (figures/tables/text boxes); recent examples of synthetic biology-focused Forum articles were provided via the course website. This format maps well to the scale and scope of the articles we expected as an output of the writing project.

For this course and the project, we defined the process of scientific writing to include:

- finding primary articles and relevant sources;
- summarizing understanding of current knowledge as well as identifying gaps in knowledge;

- identifying global problems where synthetic biology may offer a unique approach or solution;
- creating an original contribution to the synthetic biology literature.

The learning outcomes for this project were defined as follows:

- Students will understand how the practice of science is related to solving global societal challenges.
- Students will be able to write about science at a professional level, using the formats, vocabulary, and established practices for the creation of peer-reviewed literature.
- Students will be able to synthesize new knowledge on a scientific topic by broadly reading the literature and then integrating multiple research findings into a coherent and original thesis.
- Students will understand how to work effectively as a team, will be able to negotiate the terms of authorship with their collaborators, and will understand the relevance of those negotiations to the process of creating scientific literature.
- Students will gain an appreciation for the process of generating scientific knowledge through peer-reviewed publication.

To define the theme of their article, and to help students place their projects within the context of important global challenges, we prompted each student to review the 17 Sustainable Development Goals identified by the United Nations (https://sdgs.un.org/goals) and rank the goals according to their personal interests. We then used these rankings to create five project groups of students with like interests. From there, students searched the primary literature to refine their topic ideas within their groups. The students iterated on their projects by writing and revising a total of three drafts. As a final assignment, each student wrote a peer review of another group's article.

For us as instructors, this project was an experiment to see if it was even possible to engage undergraduate students as authors within a very condensed time and limited context. The creation of this collection of articles determines unequivocally that undergraduates are capable of making important contributions to their discipline, by delving into the primary literature to identify unsolved global problems for which synthetic biology may offer creative and novel solutions.

The student authors have made important contributions in the areas of human well-being, equity, sustainability, and environmental health, through their insightful synthesis of the literature into the following articles in this collection: "Carbon Capture by Transgenic Plant Greenspaces in Urban Communities" by Katherine Stratton, Hannah Shell, Anna Wix, William Miller, Hayley Wigren, and Priscilla Anand; "Water Quality Biosensing Using Engineered Microbial Fuel Cells" by Erik Breiling, John Gabelmann, Rachel Grandmaison, Caitlin Guifoyle, Taylor Johnson, and Adam LaBombard; "Improving Inequalities: Protein-Based vs. mRNA Vaccines" by Shelby Tweedie, Maire Murphy, Bethany Atwood, Jocelyn Hinchcliffe, Samantha Lopez, and Andrew Voronin; "Hot & Bothered: Engineered Microbes to Treat GI Inflammation" by Hope Hutchinson, Lauren Abraham, Alyssa Carta, Gabrielle Paquette, Kaitlyn Bergeron, and Kaleigh Caserta; "Enhancing Algae Biomass from Biofuel Production as an Alternative Feed for

Livestock” by Gianluca Panza, Mira Kirschner, Aashi Akare, Dylan Mackisey, Mikayla Raffin, and Komlavi Touglo.

We sincerely thank our student authors for their creativity, their dedication, and their collaboration in this process.

-N.G.F and L.A.R

Carbon Capture by Transgenic Plant Greenspaces in Urban Communities

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Hayley Wigren, Priscilla Anand

Abstract

Greenspaces have been indicated as a potential strategy for reducing carbon pollution in urban environments. We highlight synthetic modifications to increase the carbon capturing capabilities of *Arabidopsis thaliana* for use in temperate urban greenspaces. These modifications can be applied to other plant species for use in phytoremediation in other climates.

Keywords

Carbon capture, phytoremediation, urban sustainability, greenspaces, pollution

Urban CO_2 Emissions and Greenspaces

Carbon dioxide (CO_2) emissions have been increasing since the early 2000s (European Commission, 2022). This is largely due to urbanized areas such as the Beijing-Tianjin-Hebei region, the largest urbanized region in northern China. The region uses coal as its primary fuel source, and is responsible for more than 10% of the country's total energy consumption (Han et al., 2020). Beyond replacing urban fuel sources, a potential method to combat increased CO_2 emissions is phytoremediation through urban greenspaces — open urban patches of local flora in forms such as parks, gardens, road verges, and rooftops. Urban greenspaces help reduce carbon emissions via photosynthesis, removing atmospheric CO_2 and releasing oxygen (BON et al., 2017).

Glossary

Climate Change - long term shifts in temperatures and weather patterns

Greenspace - Open-space area reserved for plant growth. In cities, they are often used to mitigate the effects of pollution.

Carbon Concentrating Mechanism (CCM) - an important process that maximizes the efficiency of inorganic carbon uptake and carbon dioxide fixation in organisms

Cytochrome P450 - protein family that plays a crucial role in the detoxification and drug metabolism in mammals, and synthesis of fatty acids and hormones in plants

Rubisco - enzyme that catalyzes the incorporation of carbon dioxide with ribulose bisphosphate in photosynthesis (crucial and rate limiting step in carbon fixation)

Photosynthesis - process by which plants and other organisms use light energy to synthesize sugars from carbon dioxide and water

Phytoremediation - the treatment of pollutants or waste in the air, water and soil using plants and associated soil microbes

Nonnative - not indigenous to a particular region

C3 plant - A plant in which carbon dioxide is first fixed into a compound with three carbon atoms before entering the Calvin cycle in photosynthesis (most common carbon fixation pathway)

PEP carboxylase - enzyme found in plants and some bacteria that catalyzes the addition of bicarbonate to phosphoenolpyruvate to form acid oxaloacetate (key role in photosynthesis)

As beneficial as urban greenspaces are, introducing genetically modified plants through synthetic biology can magnify their impact. Specifically in temperate locations like Beijing, adding genetically altered thale cress (*Arabidopsis thaliana*) to urban greenspaces could prove beneficial. *A. thaliana* has potential to be engineered as an effective carbon sink to reduce greenhouse gases in temperate urban environments due to its preference for temperate climates and ability to adapt to unfavorable conditions (Krämer, 2015).

Figure 1. Schematic for application of modified *A. thaliana* for urban carbon capture. Map of species distribution (top), generated via mapchart.net using data released by the Royal Botanic Gardens, Kew (Diazgranados et al., 2020). Urban environments (CC0, middle right) within the *A. thaliana* species distribution will be targeted for planting modified *A. thaliana* (middle left). Modifications for increased carbon capture focus on molecular pathways within *A. thaliana* leaves, including stomatal density modifications, cyanobacterial CCMs, and MOG pathways utilizing PEP Carboxylase (bottom).

Genetic Manipulation of Stomatal Density

One method to increase *A. thaliana*'s carbon fixation is to modify the endogenous mechanisms already present in the leaves, such as by genetically enhancing stomatal density.

The stomata are a characterized plant structure in the epidermal layers of plant leaves, responsible for control of gas and water exchange through the opening and closing of a stomatal pore on the leaf surfaces (Wang et al., 2022). One study found that overexpression of STOMAGEN, a positive regulator of stomatal development in *A. thaliana*, could increase the photosynthetic rate (and therefore the plant's ability to take in and fix CO_2) by 30% compared to wild type (Tanaka et al, 2013). STOMAGEN is a gene encoding the secretory peptide EPFL9, a member of the epidermal patterning factor family that regulates production and differentiation of stomatal lineage cells by competing with negative EPF regulators for interaction with cell-surface receptor-like protein TMM (Sugano et al., 2010). Tanaka et al. found that their transgenic plant with overexpressed STOMAGEN displayed increased stomatal density per square millimeter and had enhanced water use and photosynthetic rate due to improved gas diffusion into the leaves from the higher stomatal density. This allowed for increased gas uptake in the plant in constant well-watered, ambient CO_2 conditions (Tanaka et al., 2013).

Integration of Cyanobacteria Carbon-Concentrating Mechanism into C3 Plant Leaves

In addition to the modification of endogenous carbon fixation mechanisms, incorporation of nonnative genes to improve CO_2 fixation in *A. thaliana* is also possible. One example would be adding cyanobacterial carbon-concentrating mechanisms to the plant's photosynthetic pathway. Carbon-concentrating mechanisms (CCMs) facilitate the elevation of CO_2 levels near the active site of Rubisco. CCMs are found in a range of photosynthetic organisms, including cyanobacteria and algae (Kupriyanova et al., 2023). Current research of CCM incorporation in various plant species focuses on agricultural plants. A simulation of cyanobacterial carboxysome-based CCM addition to C3 plant leaves through integration into the plants' genomes yielded a 60% improvement in net CO_2 uptake (McGrath & Long, 2014). Cyanobacterial CCMs may be promising for effective C3 plant integration due to higher plant chloroplasts and cyanobacteria's common ancestry, allowing the addition of CCM components without changes in leaf anatomy (McGrath & Long, 2014). *A. thaliana*, a C3 plant, is therefore a desirable candidate for the modifications modeled in this study due to photosynthetic pathway similarities. With CCMs integrated into *A. thaliana*, the plant could drastically increase greenspace removal of CO_2, making the greenspace more efficient and effective.

Transformation of Cytochrome P450 and Pollutant Resistance

If *A. thaliana* greenspaces capable of increased CO_2 capture are to be integrated in polluted urban environments, they must be able to survive other pollutants common in those spaces. Cytochrome P450s (CYP) play an important role in detoxification in mammals and biosynthesis of hormones and fatty acids in plants (Munro et al., 2006). In a study involving a transgenic cross-bred species of poplar trees (*Populus tremula x Populus alba*), mammalian cytochrome P450 2E1 (CYP2E1) was introduced to the poplar's genome, allowing the trees to survive in environments polluted by volatile organic compounds including trichloroethylene (TCE), chloroform, carbon tetrachloride, and benzene. They found the transgenic poplars metabolized TCE 45 times more than the control and did not display adverse reactions to TCE or its metabolites. The plants also removed 79% TCE and 36-46% of the benzene from the air in two separate experiments (Doty et al., 2007).

This P450 mutation could be integrated into transgenic *A. thaliana* possessing the carbon sequestering mammalian transgene suggested above. Atmospheric chemicals like benzene can inhibit plant growth by displacing CO_2, decreasing photosynthesis rates (Pacheco-Valenciana et al., 2022). While not key to photosynthesis, the study shows that incorporation of mammalian CYP2E1 into a plant's genome confers resistance to non-CO_2 pollutants, increasing transgenic *A. thaliana*'s potential to survive in environments containing other urban pollutants (Doty et al., 2017). *A. thaliana* is already capable of growth in hostile conditions such as extreme hot or cold temperatures and extra protection from atmospheric pollutants could only benefit its purpose to reduce carbon (Krämer, 2015).

Design and Analysis of Carbon Fixation Pathways

Carbon capture in *A. thaliana* can also be increased by engineering synthetic carbon fixation pathways optimizing the kinetics, thermodynamics, energy efficiency, and topology of the pathways. One study assessed natural and synthetic pathways based on these criteria, identifying malonyl-CoA-oxaloacetate-glyoxylate (MOG) pathways as the most efficient. MOG pathways use the PEP carboxylase, which has the highest carboxylation rate of all known enzymes. This results in systems that are three times more efficient than the Calvin cycle and could result in a highly productive, low waste carbon capture system (Bar-Even et al., 2010). Implementation of these synthetic pathways will likely require modification of the endogenous carbon fixation pathways in *A. thaliana* and minimizing interference between exogenous material and the plant's natural metabolism.

Future Applications and Considerations

Measuring the growth of genetically modified plants in terms of total dry biomass relative to the wild type would be the most direct method to measure the effectiveness of these plants for greenspaces. The modifications described in this paper could also be applied to capture particulates characteristic of other urban pollutants, which would require more direct analysis of particulate matter uptake in the leaves to measure effectiveness. It is important to note that the methods of genetic engineering described in this paper are in reference to *A. thaliana*, the model plant chosen to best support urban greenspaces in temperate climates. Key considerations when choosing a model plant include plant size and aesthetics, location implementation, and biomass accumulation. *A. thaliana* is a small, weed-like plant that would be ideal for small patches of naturalized areas (such as highway dividers) and could be composted, but the techniques applied to our model can also be applied to other plants that likely have homologous genes or mechanisms. These modifications, while difficult to optimize, could be applied to a wide variety of plant species adapted to their respective urban environments, possibly utilizing native species to support ecosystems.

Author Contributions

Conceptualization: K.S., H.S., W.M., P.A.; Investigation: K.S., H.S., A.W., W.M., H.W., P.A.; Writing - Original Draft: K.S., H.S., A.W., W.M., H.W.; Writing - Review & Editing: K.S., H.S., A.W., H.W., P.A.; Project administration: K.S., P.A.; Visualization - figure creation: K.S., H.S., H.W., P.A.

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Water Quality Biosensing Using Engineered Microbial Fuel Cells

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Keywords
microbial fuel cells, biosensor, clean water, synthetic biology

Abstract

Microbial fuel cells (MFCs) can be engineered as biosensors to detect water contaminants. MFCs have quick response times and instantaneous read-outs; however, better selectivity and changes to materials are needed to make it a long-term solution. Synthetic biology can enhance contaminant detection by genetically engineering host cells, for improved selectivity.

Water Quality Biosensing

In 2019, it was estimated that around 2.2 billion people lack access to safely managed drinking water [1]. Universal access to clean water for hydration, sanitation, and hygiene is a fundamental human need essential for health and well-being. Current challenges with providing access to clean water and sanitation include water scarcity, overuse and poor management, and contamination of freshwater supplies. The United Nations has outlined 17 sustainable development goals, including the goal to “ensure access to water and sanitation for all” [2]. To ensure access to clean and safe water, an effective yet inexpensive system must be put in place to monitor water quality. Recently, developments in the field of synthetic biology offer a potential solution- the use of biosensors to monitor water quality and detect contaminants. Biosensors exist naturally within microbes, and are used to detect and respond to changes in the environment, including the detection of chemicals and specific compounds [3]. Microbial fuel cells (MFCs), a specific type of biosensor that converts the chemical energy within organic substrates to electricity, can be used to monitor water quality [4]. MFCs have been investigated as potential biosensors for detecting heavy metals and other toxic compounds including lead, arsenic, mercury, cyanide, chromium (VI), and cadmium [4].

Synthetic Biology

By using synthetic biology and genetically engineering the host cells to detect one or more toxins, MFCs can detect and report if a water source becomes contaminated [5]. To construct specific biosensors, microorganisms can be engineered to respond to the presence of a contaminant. *Shewanella oneidensis,* a bacterium, is often incorporated into MFCs as it can use a variety of contaminants as electron acceptors or donors. To create an arsenic specific MFC, a plasmid containing an arsenic-responsive genetic circuit was transferred into *Shewanella oneidensis.* When arsenic enters the cell, ArsR dissociates from the operator and allows the transcription of genes that increase current production [6]. Currently, field deployable

MFCs are limited by sensor selectivity; however, synthetic biology offers potential solutions to create sensors capable of detecting contaminants within acceptable ranges. This article will discuss reporter methods used by MFCs as well as advantages and current limitations with MFCs design.

Fuel Cell Function and Reporter Methods

Microbial fuel cells (MFCs) are a class of biosensor reporter that uses microbes to detect and report water contaminants. MFCs in the context of biosensors function by utilizing microbes that respond to a specific contaminant through altered metabolism, either by utilizing the contaminant as food [7] or having the contaminant stunt metabolism [8]. Synthetic gene circuits can also be employed to conditionally alter electron output in response to the contaminant [9]. Electroactive bacteria have been used in the design of MFC biosensors by modifying these bacteria to produce an electric current in response to a specific chemical contaminant in a synthetic electron transfer pathway [9]. Microbes conduct oxygenation reactions in the anode chamber and transfer electrons to the anode (see Figure 1B for a model of the functional components). These electrons then travel to the cathode, where they function to reduce oxygen. As contaminants affect the rate of bacterial metabolism, they can be reported via altered rate of the reactions (Figure 1A). Through reading changes to the microbe's electron transfer to the reporter cathode due to the contaminant, the system can report the presence and amount of the contaminant [8].

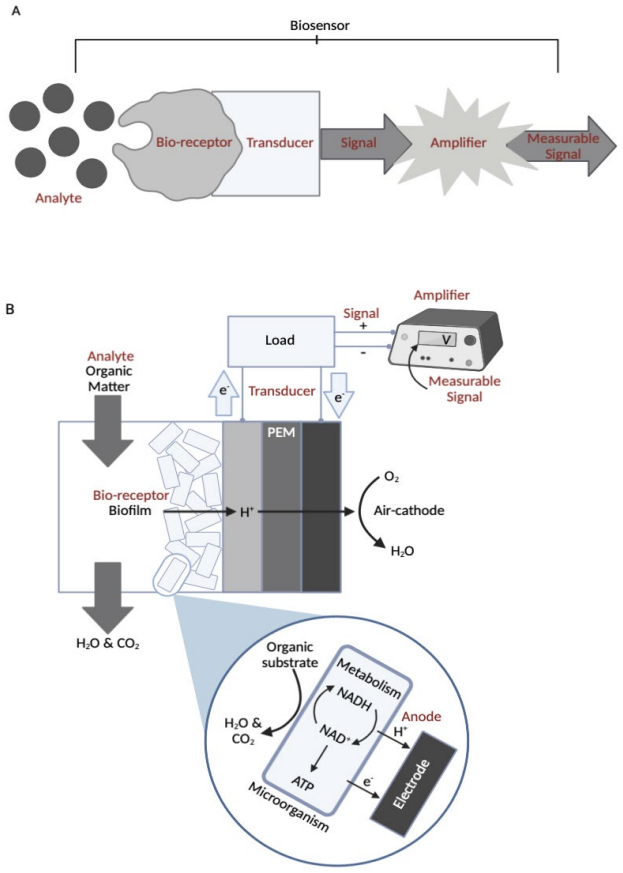

Figure 1. Engineered microbial fuel cell biosensor set-up. (A) The overall biosensor parts, from recognizing an analyte to creating a measurable signal. **(B)** Set-up of an engineered microbial fuel cell biosensor [7]. Organic matter is recognized by the biofilm, where a microorganism metabolizes the organic substrate to produce electrons that are recognized in a measurable signal. Image was created using BioRender.

Advantages of Engineering MFCs as Biosensors

The development of an inexpensive, reliable, and rapid-detection device remains a challenge for academic and industrial scientists. Microbial fuel cells could prove to be a viable solution to counteract the challenges of traditional time-consuming and expensive methods of detecting contaminants in water. Additionally, MFCS can function to detect general toxicity [8]. Through testing fuel concentration, a study incorporating monosaccharides as fuel kept the feeding rate of a MFC at 0.53 mL/min and it obtained the shortest response time, described below, among all fuel feeding rates [10]. This study showed that MFCs as biosensors could shorten the response time from 2.1 to 1.4 h as well as scaling down the anode compartment was able to dramatically decrease the response time from 36 min to 5 min [10]. Another study showed that MFCs can have quick response times as low as 2.8 minutes [11], and can be instantaneous, when compared to other common biosensors due to the quickness of the changes in the bacteria. With this idea, the MFC can detect the sudden presence of target compounds, as microorganisms in the MFCs are fast to respond to changes in their surroundings [8]. MFCs also require little maintenance as the microbes can sustain themselves while being fed [8], and can even be used to power the reporter signal to make the MFC act completely autonomously [7]. Furthermore, MFC biosensors offer the advantage of correlating biological activity of electroactive microorganisms with the chemical composition of the feedstock via signal frequency [7]. Shown in Figure 1A, MFCs allow for long-term monitoring of water quality due to their ability to self-repair and self-sustain while measuring signals using voltage. This instantaneous read-out of the targeted compounds in addition to the other advantages, shown in Table 1, proves engineered MFCs as biosensors to be a great success for improving water sanitation.

Table 1 - Advantages and Limitations of Engineered MFCs as Biosensors

Advantages	Limitations
● Respond quickly to changes ○ Signal production can be as soon 4 minutes after target contact ● Wide variety of target compound detection ● Self sustaining; require little maintenance once implemented ○ Can power reporter autonomously ● Better than other biosensors at long term monitoring	● Limited signal generation capabilities (mainly electric signal and metabolic compound detection) ● Cost prohibitive components ● Can be very sensitive to environmental changes ○ Can falsely express signal in absence of target compound

Limitations and Future Directions

MFCs have a promising future but several aspects of the process need to be adjusted before widespread use. One concern is selectivity. MFCs measure electricity changes from metabolism meaning that edible contaminants aside from the target molecule can provide false readouts [12]. In order to try and resolve this issue, one lab found that including digestates of the target in the cathode somewhat increased selectivity in a volatile fatty acid sensor [12]. Other approaches have instead elected to use MFCs as power sources for more selective sensors, such as one that measures temperature and humidity [13]. Neural networks and reference channels can also be used to account for background noise [14, 8]. Synthetic

avenues also exist, with one lab modifying an electron transporter to only function in the presence of estrogen antagonists, allowing the detection of specific metabolites despite lower power yield [9].

Another important concern is sensitivity, with small concentrations of target molecules not producing enough electricity to be measured. One approach to solve this is to increase the power output of MFCs. By increasing the expression of the phmZ gene in *Pseudomonas aeruginosa* MFCs, one lab was able to increase MFC current by 4-fold compared to wild type [15]. Another lab was able to increase power generation by 9-fold with a recombinant strain of *S. elongatus* [16]. Changes to the biofilm and cell adhesion have also been achieved by altering pili extension mechanism, further increasing power consistency and output [16].

Combining increased sensitivity and selectivity should allow the creation of effective MFC biosensors, but this theory needs to be tested further in the laboratory. Design changes will also be important in this process, with a need to address issues with the existing proton exchange membranes, the oxygenation catalyst, and low proton circulation [17, 18, 19]. This has been done successfully in some designs, with a BOD sensor permitting detection at 5 mg/L, the upper limit for BOD in clean water [20]. Overall, there are many alterations and optimizations that must be done before MFCs can be practically used, but both synthetic biology and design approaches should help MFCs approach practical use.

Author Contributions

Conceptualization: T.J. (lead); E.B., J.G., R.G., C.G., A.L. (equal). Investigation: T.J. (lead); E.B., J.G., R.G., C.G., A.L. (equal). Project Administration: T.J. (lead), E.B., J.G., R.G., C.G., A.L. (equal). Visualization: T.J. (lead); E.B., J.G., R.G., A.L. (equal). Writing – Original Draft: E.B., R.G., C.G., A.L. (equal); J.G., T.J. (supporting). Writing – Review and Editing: T.J. (lead), E.B., R.G., C.G., A.L. (equal); J.G. (supporting).

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Improving Inequalities: Protein-Based vs. mRNA Vaccines

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Keywords
mRNA vaccines, Protein vaccines, Inequalities, Global Distribution, Storage, Manufacturing

Abstract

The COVID-19 pandemic highlighted many inequities in global mRNA-based vaccine accessibility and distribution. Protein-based vaccine approaches using recombinant and conjugate proteins demonstrate the potential to reduce these inequalities. Using synthetic biology, the feasibility of manufacturing and storage can be improved with protein-based techniques creating inexpensive, accessible options for widespread distribution.

Introduction

Beginning in March of 2020, the world was stunned by the COVID-19 pandemic. As a result, the world saw the rapid production of vaccines to combat this lethal disease. Both protein and mRNA vaccines were produced to prevent the spread of disease. However, the mRNA vaccines produced by companies such as Pfizer and Moderna were most effective against the virus and proved to be life-saving during the COVID-19 pandemic [1]. mRNA vaccines function by introducing a segment of RNA that codes for a viral protein [1]. Cells can then use the mRNA to translate the viral protein, and the immune system can produce antibodies to protect the body against infection [1]. Antibodies will then remain in the body and can help fight against the infection if the body is exposed to the pathogen again [1].

However, mRNA vaccines were and still are not equally distributed worldwide. Vaccine doses have been highly distributed in high and upper-middle-income countries, while developing countries, particularly African countries, have far less access to the critical vaccines [2]. The COVAX global initiative was started in April of 2020 to help make vaccines more accessible to less developed countries [2]. However, this initiative has yet to successfully make vaccine distribution equal to all countries. Studies have shown that less-developed countries, which have lower life expectancies and higher cardiovascular-related deaths, also have lower vaccination rates among the population [2]. For example, Africa has the lowest vaccination rate of any other continent, with only about 10% of the population fully vaccinated as of October 2022 [2]. Synthetic biology can therefore help combat global health crises by ramping up vaccine development processes to reduce inequalities in vaccine distribution [3].

Of particular interest to reduce global inequalities are protein-based vaccines, which are composed of recombinant or purified antigens from a pathogen [4]. These vaccines can induce immune responses against specific viruses or bacteria. Protein-based vaccines can be produced through recombinant bacteria, yeast, insect cells, or mammalian cells [4]. Multiple recombinant protein vaccines are now used clinically worldwide. Examples include vaccines against hepatitis B and the pneumococcal polysaccharide vaccine [4]. Synthetic biology can help make the production of recombinant protein vaccines more efficient and inexpensive and

can increase their distribution span. This review discusses how the growing field of synthetic biology can improve global vaccine accessibility. Furthermore, by comparing mRNA and protein-based vaccines, we highlight how protein-based vaccines are best-suited to improve inequalities in distribution and increase global accessibility.

Table 1: Comparing mRNA Vaccines and Protein-Based Vaccines.

	mRNA Vaccines	Protein-Based Vaccines
Components	mRNA strand encodes a pathogen's protein in the body to stimulate an immune response [1]	Whole/parts of a pathogen's protein are used to stimulate the immune response [4]
Synthesis Production	Produced by synthesizing mRNA using the sequence information of a pathogen's protein, which is a quick process once the sequence information is known [1]	Produced with recombinant DNA by inserting a gene encoding the protein of interest into a host cell (bacteria, yeast, mammalian cells), which is a slow process requiring protein expression, purification, and formulation [4]
Storage	Require very low freezing temperatures to maintain stability, limiting distribution ability [1]	Can be stored at normal refrigerator temperatures [5]
Level of Immune Response	Induce a strong and specific immune response due to their ability to mimic the natural infection [1]	May not produce a robust immune response because the body may not recognize the pathogen as foreign or the pathogen may degrade before reaching immune cells [4]
Safety Risks	Considered to be "safer" as the living virus is not contained in the vaccine [1]	Considered to have a higher risk of contamination with live viral particles [4]
Established Date	New technology, where safety and efficacy has not yet been well-established as the first mRNA vaccine brought to the market was in **2020** during the COVID-19 pandemic [6]	Safety and efficacy have been well-established, as the first protein-based vaccine was brought to the market in **1986** for Hepatitis B [4]
Application	Could be applicable for non-infectious diseases, such as cancer [1]	Protein antigens can only be obtained from the pathogen that causes an infection [4]

Cost Effective Development of mRNA vs Protein-Based Vaccines

Costs of mRNA Vaccine Synthesis

A significant barrier to vaccine accessibility is the price of vaccine formulation for both mRNA and protein-based vaccines. For mRNA vaccines, there is a lack of cost-effective options to utilize their manufacturing processes in large scale production. The general process for mRNA vaccine formulation includes *in-vitro transcription* (IVT) of DNA templates followed by a series of degradation and purification steps to achieve the desired mRNA product [7]. However, increases in price result from scaling up many of these processes to meet global demand. One particular example is the synthesis of chemically modified cap analogs that place 5' caps onto synthesized mRNA [7]. These analogs add expense when used in large quantities due to their synthesis requirements and their unpredictability when introducing new cap substituents [7]. Likewise, isolation and purification of mRNA can introduce further development costs, specifically for chromatography techniques that introduce costly complications or are not

feasibly scaled up [7]. Ion exchange chromatography (IEC) is one prominent example, as it is applicable to larger scale mRNA purification and it produces high yield results [7]. However, because this chromatography requires denaturation conditions, it requires strict temperature control, including that of the mobile phase, which introduces additional costs to the purification process [7]. With a lack of current cost-effective alternatives, mRNA vaccines are less ideal than protein-based technology for solving present global vaccine distribution problems.

Bioconjugation as a Strategy to Reduce Costs of Protein Vaccines

As such, further developing cost-effective protein-based vaccines shows promise to reduce global inequalities in vaccine accessibility. For protein-based vaccines, emerging efforts to apply synthetic biology to vaccine development challenges have proven successful – particularly with bioconjugate vaccines, an alternative to traditional conjugate vaccine technology. Synthetic biology can be exploited to simplify conjugation processes and eliminate the complex chemical synthesis of traditional protein conjugate vaccines in favor of *in vivo* bacterial conjugation. Such bacterial conjugation joins carrier proteins and antigen polysaccharides with conjugating enzymes [8]. Bioconjugate vaccines are currently in various stages of testing against a range of diseases, including pneumococcal disease and shigellosis, among others. For example, one bioconjugate vaccine candidate against *Shigella flexneri* 2a, Flexyn2a, entered Phase 2b of clinical trials in 2021 [9]. The trial results showed that Flexyn2a was well-tolerated by participants and provided some protection against shigellosis using a controlled infection model [9]. In addition, the vaccine alleviated disease symptoms in participants, although it could not reach the target efficacy outlined by researchers [9]. Nonetheless, this trial provided insight into what kinds of modifications may be needed for more successful vaccine prototypes in the future.

Cost-Effective Innovation in Bioconjugation Protein Vaccine Technology

Researchers in another study examined how bioconjugation could be exploited to simplify the synthesis of polyvalent pneumococcal vaccines. Researchers used bioconjugation with a novel conjugating enzyme, an O-linking oligosaccharyltransferase (OTase), to join a pneumococcal polysaccharide to a carrier protein [8]. Expressing the OTase and a conventional protein carrier in E.coli, such experiments were the first to prove that conjugating enzymes could naturally act on polysaccharides with a glucose-reducing end [8]. This glucose structure is present in approximately 75% of *S. pneumoniae* species, making this a significant finding for pneumococcal disease [8]. These findings also display commercial potential to create more comprehensive pneumococcal vaccines that require less costly synthesis in an *E. coli*-based system [8]. Such methods could also be applied to an expanding number of serotypes using the same conjugating enzymes, which is significant for microorganisms like *S. pneumoniae* that have over 90 different strains [8]. Thus, these techniques can reduce costs for a range of vaccines.

Manufacturability and Storage of mRNA Vaccines vs. Protein-Based Vaccines

Vaccine Manufacturability

Recent developments in mRNA vaccines have shown that producing and purifying mRNA is generally quicker and simpler than producing proteins for vaccines [10]. However, synthetic biology techniques show promise in improving the production of recombinant protein vaccines. A 2013 study by Chen et al. exemplifies a cost-effective, reproducible, mass production of ribosome binding domain recombinant proteins of the spike protein from the SARS virus using the yeast *Pichia pastoris* [11]. These proteins contain ribosome binding domain mutations found to incite immunity to the SARS virus without causing undesired immunopathologic effects that can occur by including the unaltered spike protein [11]. This yeast is readily available and easy to grow, making this vaccine's synthesis simple and cost-effective. Using organisms such as yeast to produce proteins for vaccines can improve protein-based manufacturing capabilities.

Vaccine Storage

One of the greatest limitations of mRNA vaccines currently is their instability. Most mRNA vaccines must be stored at freezing temperatures which limits the distribution of these vaccines for financial and practical reasons. Lyophilization of mRNA and protein vaccines is the current method for efficiently transporting vaccines; however, there are inherent limitations of shipment quantity and vaccine efficacy over time.

A 2021 study by Stark et al. confronts this issue of storage and shipment by presenting a cell-free *E. coli*-based lysate solution that can produce conjugated proteins for vaccines [12]. This study has two novelties: the ability to lyophilize functional metabolic pathways of a ruptured cell for reactivation by water at a later time, and the versatility of this process to create proteins with different types of modifications [12]. The ability to freeze dry the biological machinery, transport it at ambient temperatures, and then rehydrate it to begin production once again up to 3 months later means that production can occur anywhere and match fluctuations in demand [12]. This is a more equitable way to distribute vaccines that is also time and cost-efficient by sending the vaccine machinery, not just a finished product. Figure 1 illustrates the methods of production of thermostable cell lysates for on-demand vaccine production.

The other notable achievement of this lysate technique is that non-endogenous biological components can be added to the reaction mixture to synthesize and modify many different conjugated proteins, which can be used in vaccines [12]. Unlike living cells which can resist external changes such as temperature or chemicals, the lysate only contains intact organelles and pathways of the central dogma [12]. Stark et al. successfully showed that their lysate incorporated known protein modification tools to produce diverse synthetic protein vaccines [12]. The lysate product is safe to use in vaccines because the researchers developed a strain of *E. coli* that contains a safe form of the endotoxin penta acylated, monophosphorylated lipid A [12]. The researchers publicly disclosed the genes they knocked out and altered within the strain, which provides other groups the ability to produce this safe form of *E. coli* in the future [12].

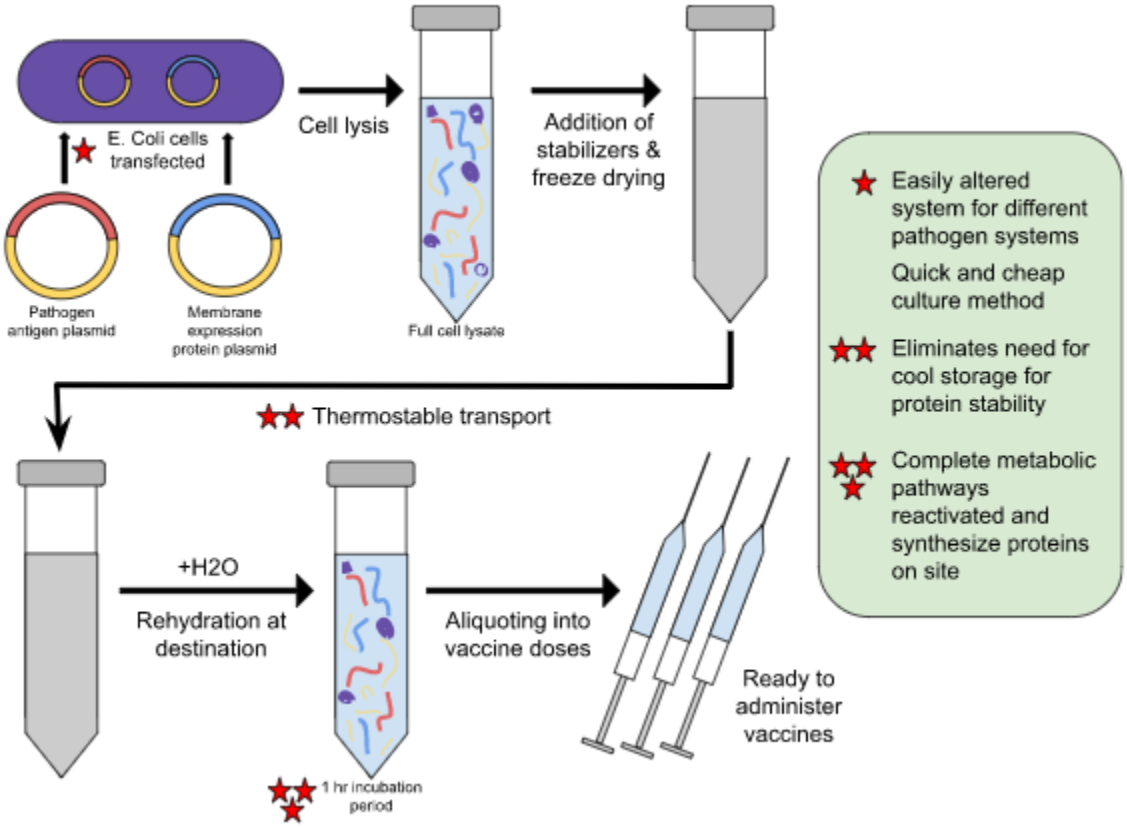

Figure 1. Production of thermostable cell lysates for on-demand vaccine production. This system eliminates the need for cold storage at -80℃, which is expensive and not always feasible in many regions of the world. The process begins with plasmids for the target pathogen and their corresponding antigen which are transformed into *E. coli*, where the cells are grown in large batches to produce and express the antigen. The cells are then lysed and purified to isolate the antigen in vitro, where stabilizing agents such as carrier proteins are added to the solution. All of the components are then freeze-dried and transported to the desired locations. Once the freeze-dried solutions arrive, they are rehydrated with water, which initiates *in vitro* transcription and translation of the proteins. An hour after rehydration, the protein vaccines are ready to be prepared and administered (Figure adapted from Stark et. al. [12]).

Conclusion

With the rapidly growing field of synthetic biology, mRNA and protein-based vaccines both demonstrate the potential to address inequalities in global distribution and accessibility. However, synthetically produced protein-based vaccines display a greater capacity for improved manufacturability and storage for widespread distribution at a reduced cost. Effective methods have been implemented to simplify the manufacturing processes of protein-based vaccines via recombinant proteins. Advances in preservation strategies have also been proven to improve storage and distribution of protein-based vaccines by freeze drying the biological machinery for transport, allowing for on-site production. In addition, with the use of bioconjugate technology, the cost of protein-based production can be reduced. In conclusion, the growing field of synthetic biology has ongoing potential to continue to improve the manufacturing, storage, and distribution of protein-based vaccines in a cost-efficient manner to reduce inequalities in global vaccine accessibility.

Author Contributions

Conceptualization: S.T., M.M., B.A., J.H., S.L., A.V., M.F. (equal). Investigation: S.T., M.M., B.A., J.H., S.L., A.V., M.F. (equal). Project Administration: J.H. (lead); S.T., A.V., S.L. (equal); M.F., B.A. (supporting). Visualization: M.F., S.T., S.L. (lead); A.V., M.M. (equal), J.H. (supporting). Writing – Original Draft: S.T., M.M., B.A., J.H., S.L., A.V., (equal); M.F. (supporting). Writing – Review and Editing: S.T., A.V., S.L. (lead); M.F., B.A., J.H., M.M. (equal).

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Hot & Bothered: Engineered Microbes to Treat GI Inflammation

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Table 1: Glossary

Term	Explanation
AvCystatin	An immunomodulator that helps to alleviate IBD inflammation
BMDC	Murine bone marrow-derived dendritic cells
eATP	Extracellular ATP that causes inflammation
E. Coli Nissle 1917 strain	First strain of *E. Coli* to be used as a probiotic
Escherichia Coli	A gram negative, anaerobic bacteria commonly found in the lower intestines
IBD	Inflammatory bowel disease
Interleukin 10	An anti-inflammatory cytokine
i-ROBOT	Intelligent responsive bacteria for diagnosis and therapy
KanR	Kanamycin (antibiotic) resistance gene; selectable marker
sfGFP	Superfolder GFP; reporter gene
Thiosulfate	Inflammatory marker

Keywords

Inflammatory bowel disease, engineering gut microbes, gut microbiome, synthetic biology, probiotic, prebiotic

Abstract

Synthetic biology is being applied to treat Inflammatory Bowel Disease (IBD) using two main approaches: engineering native gut microbes to treat IBD, and engineering non-native microbes to survive in the gut microbiome. We discuss examples of each approach and propose suggestions for future engineered microbe treatments for IBD.

Introduction

The gut microbiome is composed of diverse microbes, including bacteria, fungi, and viruses. It is also responsible for aiding digestion, absorption of nutrients, and more [1]. **Inflammatory Bowel Disease (IBD)** (Table 1) is a chronic inflammatory gastrointestinal disease that is characterized by abdominal pain, diarrhea, and weight loss. IBD subsets include Crohn's disease and ulcerative colitis, which affect roughly 1.6 million Americans [1]. Although IBD is incurable, treatments to reduce symptoms include medication, surgery, and diet and lifestyle changes. Synthetic biology is also being explored for treatment options.

Systems for Detection and Relief of Inflammation

Approach 1: Engineering Organisms Native to the Gut Microbiome to Treat IBD

The yeast *Saccharomyces cerevisiae* is well established within the gut microbiome of humans. Researchers engineered a sensor-effector strain of *S. cerevisiae* by inserting plasmids containing a gene for a P2Y2 receptor with increased sensitivity to **eATP** [2]. When eATP binds P2Y2, the MAPK pathway is activated and the enzyme apyrase is produced. Apyrase degrades eATP, resulting in suppression of intestinal inflammation and recruiting CD4+ T cells that produce **interleukin 10** (IL-10) in mice [2]. Supplementation with wild-type, heat-killed *S. cerevisiae* was also found to reduce inflammation through this mechanism in **BMDCs** [3].

Limitations of Approach 1

Approach 1 (Figure 1a) involves engineering native gut microbes to reduce IBD-associated inflammation. In this way, species that possess the ability to colonize specific regions of the GI tract can be engineered to treat inflammation at its source. IBD predominantly affects the colon, so bacteria native to this region, such as *Bacteroidaceae*, *Prevotellaceae*, *and Ruminallaceae*, could be engineered and colonized within the colon to detect and treat inflammation [4]. Similarly, *Lactobacillus* and *Enterobacteriaceae*, which are native to the small intestine, could be engineered and colonized there to treat inflammation [4]. These microbes may be more difficult to grow outside of the gut microbiome and therefore possess more nuances for engineering; however, once engineered, these microbes are predicted to colonize and persist within the gut microbiome without disrupting microbial balance [5]. Additionally, these therapies are most likely to require only a single administration as the engineered microbes should be able to colonize and persist. As described above, *S. cerevisiae* is one such native microbe that has been successfully engineered to treat IBD.

Maintaining microbial balance in the gut is essential to avoid infection from microbial supplementation. Introducing yeast to a gut microbiome where gut bacteria has been diminished from antibiotic use or from being immunocompromised can cause uncontrolled growth. Yeast overgrowth in the gut can lead to yeast infection in other parts of the body such as the skin or the mouth [5]. Other challenges posed by engineered microbes include increased susceptibility to mutation and decreased population expansion rates due to the energy demand to replicate engineered genetic components [6].

Approach 2: Engineering Existing Lab Strain IBD Systems to Persist in the Gut

Multiple studies have looked at engineering lab strains of ***Escherichia coli*** to monitor and treat IBD. Studies have shown that ***E. coli* Nissle 1917**, a non-native microbe, can colonize both mouse and human intestines [7]. One such system is the ***E. coli* Nissle 1917** system known as **i-ROBOT**, which detects high **thiosulfate** levels and activates a base-editing system, which generates a heritable DNA sequence and colorimetric signal that fluctuates and triggers the release of **AvCystatin**, thus alleviating IBD-associated inflammation [7]. This low-copy sensor plasmid (pWT-A) contains two components; First, thiosulfate binds the constitutive promoter P_{j23100} and activates transcription of the thsR gene, which produces the thsR protein [7]. Next, ThsR protein binds the PpysA promoter and activates transcription of the reporter gene **sfGFP**, the selectable marker **KanR,** and the Hly-AvCystatin gene, which produces AvCystatin [7].

In a recent study, *E. coli* Nissle 1917 was engineered to metabolize phenylalanine into non-toxic metabolites within the gut [8]. PKU is a metabolic disorder caused by mutations in the PAH gene, which encodes the enzyme that catalyzes the conversion of phenylalanine to tyrosine, resulting in an inability to convert phenylalanine to tyrosine [8]. A human clinical trial utilized oral treatment and produced positive results for disease treatment with no severe side effects, resulting in FDA approval. In the study, no signs of colonization were reported, mitigating concern for the use of non-native microbes [5, 7].

Limitations of Approach 2

Approach 2 (Figure 1b) involves engineering non-native microbes containing existing IBD treatment systems to survive the gut microbiome. Microbes that are commonly engineered in the lab include *E. coli*, *Lactobacillus*, and *Salmonella*. While these microbes pose fewer engineering challenges, they are not natively found in the gut and therefore pose issues of survival, colonization, and overgrowth risk. *E. coli* Nissle 1917 is one such non-native microbe that can survive the gut microbiome but cannot colonize. Similar to yeast overgrowth described above, treatment with antifungals can result in bacterial dysbiosis, which can alter the proportion of bacterial species in the gut and result in disease [5]. To bypass the need for colonization, which increases bacterial dysbiosis risk, treatments containing these microbes could be developed into daily supplements to bypass the need for colonization and persistence [7].

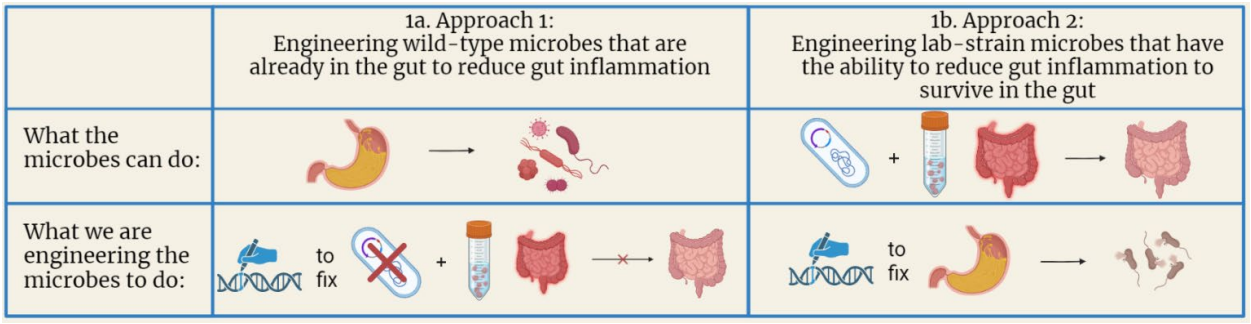

Figure 1: Two approaches to synthetic modification of microbes to aid in gut inflammation. The top row of the figure clarifies what a microbe is able to do: for example, survive in the gut or reduce gut inflammation. The bottom row of the figure clarifies what a microbe would be engineered to do; for example, to reduce gut inflammation or survive in the gut. **(1a). Approach 1.** In this approach, wild-type microbes that are already in the gut are engineered to reduce gut inflammation. **(1b). Approach 2.** In this approach, lab-strain microbes that are able to reduce gut inflammation are engineered to survive in the gut. Figure created using Biorender.

Future Advancements in Synthetic Biology Treatments for IBD

Limitations and Additional Considerations

Currently, there are many research gaps in synthetic biology treatments for IBD. Some microbes cannot survive within the gut microbiome, leading to functional limitations when engineering IBD treatment systems. To study microbial survival in the gut, researchers have created synthetic gut microbial communities, where two or more native microbial species are transplanted into the gut microbiome of a diseased individual and study their interactions. This

can be used to determine the functional, ecological, and structural concepts of the microbiome [9]. These can be used as a model for testing these treatments to see if the microbes can survive in the gut microbiome.

There are additional considerations in producing human therapeutics, such as delivery methods and FDA approval. Oral delivery is the most convenient and least invasive delivery method for therapeutics. A major challenge presented in synthetic biology is protecting engineered microbes to survive the harsh journey through the GI tract to their site of action as most microbes cannot survive the drastic pH changes, whether native or non-native to the gut [10]. Polymers for probiotic encapsulation, including pH-sensitive and enzyme-sensitive polymers, are currently being investigated [10]. The use of nanoparticle coatings for oral delivery could allow for non-invasive treatment. Upon FDA approval for public consumption, these nanoparticle-coated probiotics could be dispensed through pharmacies, allowing patients with a prescription to access this noninvasive treatment. The PKU treatment described above utilized polymer coatings and received FDA approval [8].

Conclusions

Many synthetic biology advancements have been made to improve human health and both approaches show promise in IBD treatment. While Approach 1 has only been tested in animal models, Approach 2 has advanced to human trials and received FDA approval for PKU [8], suggesting validity in human therapeutics. Similar to the PKU treatment, the use of nanoparticle coatings on the *E. coli* Nissle 1917 IBD treatment systems described above should be considered for advancement to human clinical trials.

Author Contributions

Conceptualization: H.H., L.A., A.C., G.P., K.B., K.C (equal). Investigation: H.H., L.A., G.P., K.C (equal). Project Administration: L.A. (lead); A.C., G.P., K.C. (equal). Visualization: H.H., L.A., G.P. (lead); K.B., K.C., A.C. (supporting). Writing – Original Draft: K.C., H.H. (lead); L.A., A.C., G.P., K.B. (equal). Writing – Review and Editing: K.C., H.H. (lead); L.A., A.C., G.P., K.B. (equal).

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Enhancing Algae Biomass from Biofuel Production as an Alternative Feed for Livestock

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Keywords
Algae, Biomass, Animal Feed, Sustainable Consumption and Production, Economics

Abstract

Biofuels promise to provide a more sustainable and eco-friendly alternative to traditional fossil fuels. However, the amount of biofuel and byproducts produced is limited by photosynthetic factors. Opportunities towards optimizing the manufacturing process of these biofuels may be required in order to address production limitations all while achieving responsible rates of consumption. In this review article, we discuss the benefits and opportunities of turning biofuel production waste products into a sustainable feed for livestock.

Introduction

Algae are rapidly emerging as a promising source of renewable biofuels due to their remarkable ability to grow quickly, capture solar energy and carbon dioxide, and their simple genetic makeup. Through synthetic techniques, algae can be fermented and anaerobically digested to produce biofuels. Additionally, algae biomass can be utilized as feedstock. While the lipid content of algae is used to create biofuels, the proteins can be processed and used as animal feed, with high-value components being extracted for use in nutraceuticals and food supplements. The use of algae biofuels reduces food competition and the need for arable land. However, since algae biomass is limited, specific engineering techniques, such as upregulating photosynthesis factors, are necessary to enhance biomass production.

Fourth Generation Biofuels

Biofuels are produced by renewable feedstocks such as wood chips, crops, and algae which have the potential to be renewable resources unlike fossil fuels. Biofuels can be categorized into four different generations based on the raw materials being processed. Fourth generation biofuels utilize bioengineered microorganisms to produce a variety of products (Nidal et al., 2022).

Algae has a high lipid content, high growth rate, and is well understood, making it an attractive host for biofuel production. Furthermore, the carbohydrates from algae can be fermented to create additional biofuels which can then be used for combined heat and power generation. At the same time, there is also biomass left over after the algae is processed. The exploitation of algae's byproducts can be used in several applications such as fertilizer, fuel, biomaterials, and bioremediation.

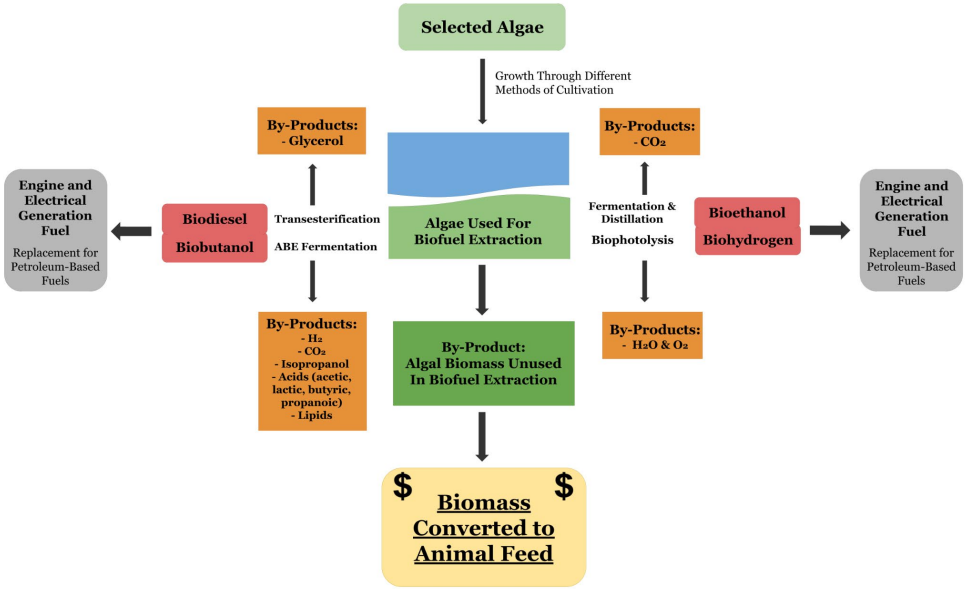

Figure 1: From the process of extracting organic material used to create biofuels, there are many other by-products produced that are not directly used in the biofuels. There are many by-products that come from the different extraction methods used to isolate the material needed for the four main types of biofuel. These are either materials released into the atmosphere or used in different commercial industries. The main by-product from these different extractions is the leftover algal biomass that did not consist of the extractable biofuel materials. One of the most popular and economically effective uses for the biomass by-product of biofuel production is using it as animal feed in the farming industry. Information was adapted from the following sources: Epidemiology, 2018, Afzaal et al., 2022, Anyanwu et al., 2022, Bala et al., 2023, Energy.gov n.d., Dahmen et al., 2019, Jolliff, 2017, Lipnizki, 2017, Mahmood et al., 2022, Nawkarkar et al., 2022, USDA n.d., 2008, Vignesh & Barik, 2019, Wukovits & Schnitzhofer, 2009, and Xu & Jiang, 2011.

Photosynthetic Mechanisms to Improve Algal Biomass Production

Algal biomass growth is usually limited by photosynthetic factors (Kumar et al., 2020). In essence, the more energy the algae has available to it, the more it grows and reproduces. Researchers usually target aspects of the Calvin cycle, which is responsible for carbon fixation in the algae. Upregulating important enzymes within the cycle or modifying them to be more efficient are common ways to increase photosynthetic efficiency (Kumar et al., 2020). Additionally, molecules that aid in the concentration of CO2 outside of the cycle, such as CCMs, can also be modified to increase photosynthesis. All genetic engineering that increases photosynthesis efficiency also aids in production of biofuels. Therefore, increasing biomass production via photosynthesis engineering will not interfere negatively with biofuel production (Kumar et al., 2020).

With photosynthesis playing a significant role in biomass production, it is necessary to optimize this process. RuBisCO is a major photosynthetic protein and a key enzyme involved in initiating the carboxylation step of photosynthesis. RuBisCO activase (RCA) is a catalytic chaperone of RuBisCO in algae. When this activase gene is overexpressed (Figure 2), it has increased biomass accumulation in algae by 46% (Wei et al., 2017). Although this is an improvement, there would still need to be a steeper increase to be financially viable. Furthermore, RCA can be modified into thermostable RCA through DNA shuffling or single amino acid substitutions to improve the instability of the gene under stress conditions. By

introducing thermostable RCA in algae grown in such conditions, there will still be optimized photosynthetic activity and enhanced biomass production in face of global climate change (Ignacio et al., 2023, Wei et al., 2017). This increase in biomass will then lead to an increase in the amount of sustainable animal feed available, as well as biofuel produced.

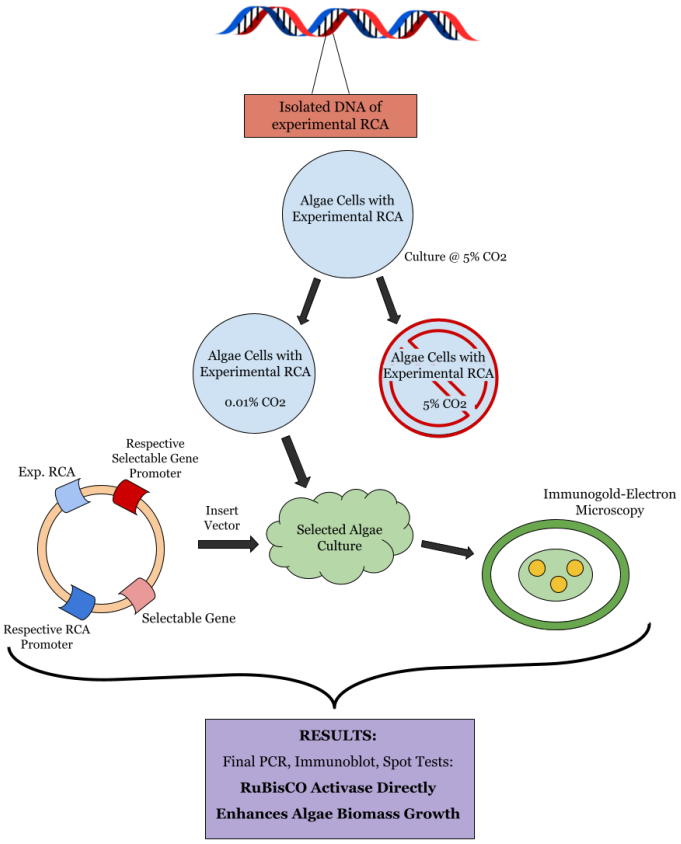

Figure 2: To determine if RuBisCO Activase (RCA) enhances algae biomass growth several experimental tests were performed. Starting with isolating the DNA of the experimental RCA and inserting it into a selected algae to then culturing it in different CO2 conditions, allowing for the determination of the most effective growth condition which was found to be air with no added CO2. This was due to the fact that this specific RCA was found to be transcriptionally upregulated in lower levels of CO2 with less translational regulation in higher levels of CO2. The next experiment performed was inserting a transformation vector through electroporation that had the experimental RCA gene with its respective promoter and an additional selectable gene with its respective promoter. The cells were allowed to grow, then samples were taken at different time points and PCR was performed to determine the overexpression levels of the RCA. From the PRC results, it was found that there was significant overexpression of the RCA gene which is representative of an increase in RuCisCO's carbon fixation allowing for increased algae growth. The additional experiment of immunogold electron microscopy revealed that the experimental RCA was found in the chloroplast of the algae cells which is the same location that known RCAs can be found in; again showing the experimental RCA is properly functioning and causing enhanced growth. Finally to further prove RCA's efficiency in enhancing algae growth, additional PCR along with spot testing, and immunoblotting were performed. These tests would show overexpression of the RCA genes, and the growth rate and biomass dry weight of the algae grown with the experimental RCA showed the RCA enhanced growth (Wei et al., 2017).

The genetic blueprint for the photosynthetic mechanism is highly conserved across photoautotrophs, which is conducive to heterologous expression for beneficial modifications of algae. A study that heterologously regulated a psbA gene engineered from various photoautotrophs showed successful expression of the encoded D1 protein of photosystem II, exemplifying the applicability of heterologous photosynthetic coding sequences (Gimpel and Mayfield, 2013). In one study, biomass production is compared between low-light and high-light absorbing D1 protein isoforms extracted from the cyanobacteria *Synechoccocus sp.* and expressed in *C. reinhardtii*. The *C. reinhardtii* expressing the low-light isoform revealed a substantial 11% increase in dry biomass compared to the high-light isoform and unmodified algal strains (Vinyard et al., 2013).

Genetic transformation of the algae strain *P. celeri* has shown similar potential for robust microalgae production. To enhance environmental stress resistance, *P. celeri* was successfully transformed with an engineered clonNAT resistance marker gene (Genbank ARQ80408.1), endogenously driven by two photo-dependent promoters, pPSAE and pRBCS2, and two photo-independent through electroporation at 25 ms and an optimal field strength range of 6500 Vcm^{-1}. The construct pGAPDHNAT displayed the greatest enhanced transformation efficiency with ~ 50 “pickable” colonies per 10^8 cells after 14 day incubation periods. The transformed *P. celeri* show promise for large-scale outdoor growth, especially considering they are the only noted strain that yields biomass at high productivity in 50 PPT marine salts level environments at the time of the experimentation (Krishnan et al., 2021).

Potential Benefits of Adopting Algae for Livestock Feed

Growing algae for commercial use not only produces several valuable products, but also helps combat climate change. According to the United Nations, livestock feed production is the second largest source of total emissions, taking up 41% of all global emissions in 2015. For reference, energy consumption is only 5% of global emissions (United Nations, n.d.). One ton of algal biomass removes roughly 1.83 tons of CO_2 from the atmosphere (Ighalo et al., 2022), while one ton of traditional feedstock production releases about 3.37 tons of CO_2 into the atmosphere (United Nations, n.d.; Alltech, 2015). This means that for every ton of traditional feedstock replaced with algal feedstock, about 5.2 tons of CO_2 is removed from the atmosphere.

One ton of CO_2 released into the atmosphere is estimated to cost society $168 due to climate change exacerbation (Rennert et al., 2022). Therefore, one ton of algae biomass saves $873.60, while one ton of traditional feedstock costs $566.16 in CO_2 emissions. Considering the 2015 global production of feedstock was 980 million tons (Alltech, 2015), this costs society approximately $554 billion. Replacing all traditional feedstock with algae feedstock would save society roughly 1.4 trillion US dollars in carbon dioxide emissions while removing 5 billion tons of carbon dioxide from the atmosphere (61% of total 2015 emissions).

Unfortunately, the cost of producing one dry ton of algae is estimated to be $1,137 (Zhu et al., 2018), while the cost of producing one dry ton of traditional feedstock is around $100 (Salassi et al., 2017). Additionally, switching to algae feedstock production would require the construction of many new photobioreactors or algae farms, which when combined would likely cost hundreds of billions of dollars. Therefore, current algae biomass production is not as cost efficient, at least to the company producing it, as traditional feedstock. The cost of algae

production can be split into two categories: production and logistics. Production refers to growing algae, while logistics refers to turning algae into valuable products. When using algal biomass as feedstock, logistic costs are the main reason algae products are so expensive (Barros et al., 2015). Synthetic biology can be used to dampen the high logistical cost by improving production. If algal feedstock and traditional feedstock become price competitive, then the initial costs of creating algae farms could be subsidized by the government. To the company producing the feedstock, the price is competitive and may yield the same returns. To the government, however, the hidden social savings by reducing carbon emissions makes algae more competitive. Therefore, governments could cover the cost of switching over to algae feed production, while potentially saving money via carbon emissions reduction.

Concluding Remarks

In recent years, much attention has been paid to 4th generation biofuel production for its promise to deliver sustainable and environmentally friendly fuel alternatives, however, if we are to attain responsible consumption and production through biofuels, efforts should be made towards optimizing every part of the production process. Most research regarding algal biofuel has been paid towards generation of novel microbial strains to increase the production yield. While this research is encouraged and necessary for the advancement of biofuel production, efforts should also be made towards optimizing every aspect of production, including the management of waste products including leftover algae biomass.

We believe there is untapped potential in the 4th generation biofuel industry regarding the use of leftover algae biomass after biofuel production. Specifically, we suggest the use of this biomass to be made into feed for livestock, which has the potential to benefit the environment in a variety of ways. We suggest taking advantage of algal growth pathways to increase total biomass production, to increase potential use of algal biomass as feed for livestock. Doing so would help move biofuel production towards a zero-waste process, positively impacting the environment and potentially increasing revenue for biofuel producers. This may increase the viability of commercial scale biofuel production, which has yet proven to be financially sustainable.

Author Contributions

Conceptualization: M.K. (lead); G.P., A.A., D.M, M.R., K.T. (equal). Investigation and Data Analysis: K.T., A.A., (lead); G.P., M.K., D.M, M.R. (equal). Project Administration: M.K. (lead); G.P., A.A., K.T. (equal); D.M, M.R. (supporting). Visualization: M.R. (lead); G.P., D.M. (supporting). Writing – Original Draft: K.T., A.A., (lead); G.P., M.K., D.M, (equal). Writing – Review and Editing: K.T., A.A., (lead); G.P., M.K., D.M, (equal); M.R. (supporting).

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