iGEM VECTOR

The Unofficial iGEM Proceedings Journal 2020

"Ideas shape the course of history."

J. M. Keynes

History of iGEM and Some Exciting Projects of the Past.
Team
MIT_MAHE

Pages 2-4

SARS-CoV-2

COVID-19: A Current Review On Pathology, Progression, and Intervention
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Novel Diagnostics

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Not a normal iGEM year…..

iGEM can be challenging. Even in a normal year. You need to come up with a great project, do your research, navigate obstacles, work as a team in- and outside of the lab and deal with all the challenges along the way. The wrong bands on the gel, the trouble to get actual funding money instead of five packs of free polymerase, hours upon hours upon hours of work, not just in the lab, but also in meetings and at home, and in the end, the last bit of sleep-deprived cramming to fit everything you did into a great wiki. Even in a normal year those things are not easy, in a normal year when you do not have to wonder when you can finally go to the lab again, when you do not have to expect and adjust to new regulations and when you do not have to worry about the health of your loved ones. But this is not a normal year.

This year everything was more difficult, some things were just a little bit more difficult, like doing more meetings online instead of in person, others were a lot more difficult, like not being able to enter the lab for months while the time was slowly running out. This year was full of countless challenges no other iGEM team ever faced before. But from the experience we made working together with you and creating this journal, we know one thing for sure, you faced those new challenges head on and you kicked ass doing it.

You managed all the obstacles and all the hardships, you created ways to make lockdowns productive, shifted from wet-lab to dry lab, held meetings online, used every online-tool you could to get ahead and worked as hard as you could to overcome the uncertainties this pandemic threw upon you, relentlessly. And when the day finally came and the lockdown ended and you could start getting back to the lab, you did not stop, you charged right ahead and worked even harder to make up for the lost time. And it was worth it, because now you are here, you made it to the end. And you can be proud of it, because a lot of groups did not. The year started with around 450 groups and only 257 ended up here. Be proud. You deserve it!

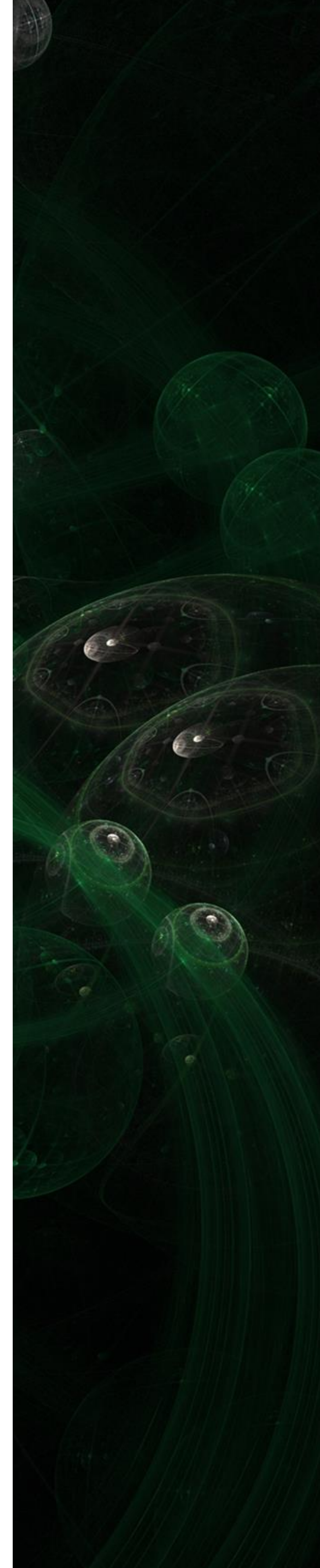
We would like to thank you for putting all this hard work in, for all the great collaborations, and the great online meetings and the occasional in-person meetings when it was permitted. And of course especially a big thanks to the teams that took part in this journal. It was an amazing experience for us to organize this and to put everything together, and we hope it was for you writing and peer-reviewing it just as much. All of the 34 Teams that contributed to this very first iGEM proceedings journal have our greatest gratitude.

What you hold in your hands now would not have been possible without the great work of you, the many talented young scientists that contributed to this project, in this definitely not normal igem year. Thank You!

Larissa and Juliette, Editors in Chief.

Quote of the Year :

> *Improvise. Adapt. Overcome.* - Bear Grylls



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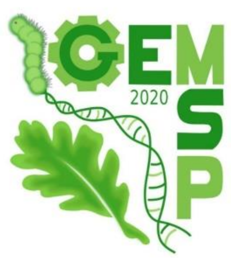

Dear Fellow-iGEMers and scientifically interested people,

We are the MSP-Maastricht iGEM team consisting of 13 aspiring scientists from Maastricht University. Apart from one biomedical sciences bachelor's student and one student pursuing a master's in systems biology, we are all students of the Maastricht Science Programme and together we have a very broad background on all natural sciences. All together, we have nine different nationalities and speak twelve languages.

This year, team MSP-Maastricht's project is about fighting an invasive insect pest, the oak processionary caterpillar, which poses a local and continental threat. The oak processionary caterpillar not only defoliates trees and entire oak forests, feeding on their leaves. It also poses a serious health hazard especially in urban areas, as its toxic bristles can spread over hundreds of meters and cause skin rashes, eye complaints as well as respiratory issues for both humans and animals. Current methods and pesticides used to manage this pest are relatively inefficient, expensive and unspecific, so that a broad use of the pesticide is not possible due to its harmful effects on the ecosystem. This is why our team's project is to develop a safe biological pesticide that specifically targets the oak processionary and is therefore environmentally-friendly and presumably more efficient. Our solution, OakShield, is a bacterial pesticide that expresses shRNA to exploit the caterpillar's own RNA interference mechanism. By targeting essential gene sequences unique to the oak processionary, we want to effectively reduce the pest species' growing population while remaining highly specific and thus ecologically harmless.

Team MSP-Maastricht decided that it is not only the general public that needs to learn more about synthetic biology and research, but also members of the iGEM community. It is incredibly important to not only educate the general public about the new discoveries in synthetic biology, but also the scientists and researchers working in the field. In these fast changing times, it is hard to keep the overview of all great inventions that are made in synthetic biology and all of them should be acknowledged, as they can inspire new ideas in other people. For this reason we decided to create a Proceedings Journal for the 2020 iGEM competition composed of as many team project articles as possible.

We hope you enjoy reading all these amazing texts from the 2020 iGEM teams and again a big thank you to all participating teams!

The MSP-Maastricht iGEM team 2020

The History of iGEM and Some Exciting Projects of the Past

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Abstract- **iGEM (International Genetically Engineered Machines) is a premier undergraduate and high school level Synthetic Biology competition that began at MIT in 2004. iGEM began in January 2003 with a month-long course during MIT's Independent Activities Period (IAP) where students designed biological systems to make cells blink. This university design course then grew to a summer competition with five teams in 2004. Fifteen years later, iGEM has grown to 350 teams from over 40 countries. With such a vast and diverse history, iGEM's history cannot be scaled down to just a single article, but we will try to do iGEM justice. Through these 15 years, the competition has seen a lot of new and innovative projects, and this article will also compile our choices for some of the most exciting and interesting ones.**

Index Terms- iGEM, Engineered Bacteria, Synthetic Biology, BioBrick

INTRODUCTION

The International Genetically Engineered Machine (iGEM) Foundation is an independent, non-profit organization fostering the growth of synthetic biology, education, and competition. The platforms they host consist of the iGEM competition, Lab program, iGEM Registry of Standard Biological Parts and After iGEM, making it a budding place for enthusiasts both as a workbench and a showcase for synthetic biology. In 16 years, over 40,000 undergraduate, postgraduate, and high school students from multidisciplinary backgrounds have participated in the competition annually making it one of the largest of its kind.

In January 2003, iGEM began as a month-long course at MIT's Independent Activities Period (IAP). By 2019, it had grown to have 352 teams from over 45 countries and continues to expand with every edition. This article explores the different facets of iGEM - how they have grown over the years and what they do.

Figure 1: Logo of the iGEM foundation

I. HUMAN PRACTICES, BIOSAFETY AND BIOSECURITY

As Peter Carr - the Director of Judging said, "Human Practices is the study of how your work affects the world, and how the world affects your work." iGEM has been a framework for increasing interest in humane practices with approaches such as biosafety and biosecurity (Cristina Vilanova, 2014). It involves the iGEM teams considering the safety, sustainability, and environmental impact of their project beyond the lab while developing solutions to real-world problems ethically.

In the evolving field of synthetic biology, biosafety and biosecurity play a crucial role. iGEM emphasizes every team to consider the risk factors by monitoring and helping alleviate risks involved ensuring teams follow all relevant rules on safety and security while updating their guidelines regularly.

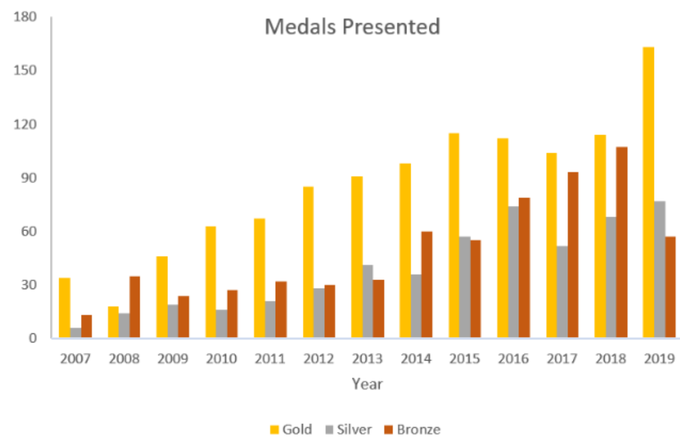

Figure 2: Gold, silver and bronze medals presented

II. THE GIANT JAMBOREE

Every year iGEMers have their very own meet up called "The Giant Jamboree" in Boston to present projects, take part in workshops, poster sessions and a career fair. There exists a judging committee of dedicated volunteers and executive judges to evaluate the projects based on all-inclusive excellence. There are grand prizes, first and second runner ups, medals (Figure 2) and various track and best category awards. The tracks include diagnostics, therapeutics, energy, environmental, manufacturing, food and nutrition, foundational advance, open,

software and new application. Wikis (the website describing the team projects) were introduced in 2006 and have been assessed since then.

III. DIVERSITY

A study performed by the 2013 Paris Bettencourt iGEM team showed that women were underrepresented at all levels of iGEM. Inspired by the study, the iGEM Diversity & Inclusion Committee since 2013, has fostered the participation of women, the LGBTQ community, and members representing other diverse communities. In 2018, 42% of participants were female or non-binary, 44% of advisors and instructors were female and non-binary, 50% of judges were female and non-binary. This shows the growth in gender parity over the years.

IV. REGISTRY

The iGEM Registry of Standard Biological Parts with a motto of "Get, Give & Share" is a collection of various biological parts that together make a 'Biobrick,' which can be used to construct synthetic biology devices and systems. Starting from 2010, pSB1C3 was used for shipping of all the parts submitted until 2019, as iGEM's partners facilitated DNA synthesis. By 2020, the registry expanded to have over 25,000 parts (Figure 3). The tested and characterized parts help in the systematic building of the biological systems. They contain information on sequence, functionality, and usage of all parts such as Promoters, Ribosome binding Sites, Protein domains, Protein-coding sequences, Translational units, Terminators, DNA, Plasmid backbones, Plasmids, Primers, and Composite parts.

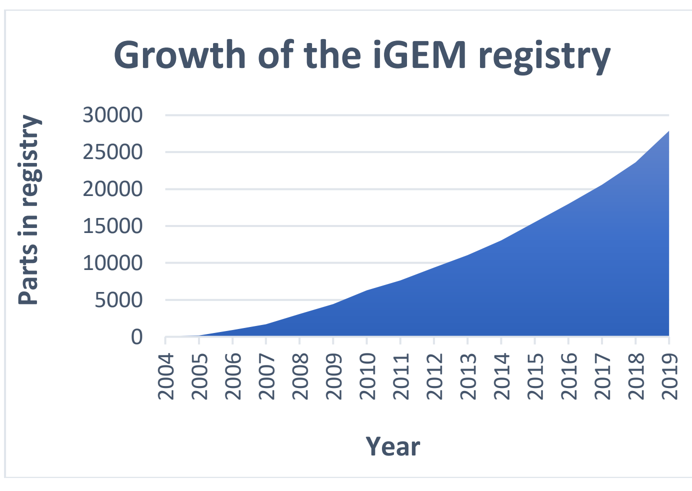

Figure 3: Growth in the Registry

V. AFTER iGEM, START-UPS AND ENTREPRENEURSHIP

The After iGEM program enables iGEMers globally to continue to be a part of the iGEM community post the competition, supporting a 30,000+ member community and an international network of academics and industry professionals beyond the competition. Over 150+ projects have advanced to start-ups today. The Entrepreneurship Program and Innovation Community (EPIC) helps SynBio business ventures to find co-founders, investors and corporate partners. Some of the notable start-ups are Gingko Bioworks, Opentrons and Puraffinity.

VI. PROJECTS

Calgary 2019's team, designed a protein-based emulsion system where chlorophyll-binding proteins designed with genetic algorithms were used to remove excess chlorophyll from green canola oil and convert it to pheophorbide (Calgary, 2019). With interaction and homology modelling a novel spacer was made to purify enzymes for pheophorbide production. Their goal was to bring better solutions for canola seed production, grading, selling, oil production and converting chlorophyll to a useful market product pheophorbide, a photosensitizer.

NCKU Tainan 2019, came up with **CreSolve**, a living therapeutic drug consisting of engineered E. coli which converts tyrosine - a precursor of p-cresol, to an antioxidant p-Coumaric acid to reduce the accumulation of uremic toxins, the root cause of Chronic Kidney Disease which is otherwise filtered out by dialysis (Tainan, 2019). They built a biosensing device CreSense which reads blood p-cresol levels with the help of live bacteria.

CascAID - a tool to help avoid unnecessary prescription of antibiotics was developed by team Munich 2017 (Munich, 2017). The point-of-care in vitro device comes with hardware and software components to detect and distinguish a wide range of viral and bacterial pathogens with Cas13a proteins and different CRISPR RNA (crRNA) molecules to identify the target.

Team Wageningen 2019, used genetically engineered phages for an enhanced bacteriophage therapy for *X. fastidiosa* called **Xylencer** (Wageningen, 2019). It encompasses an automated in-situ detection device that can detect the presence of X. *Fastidiosa.* In their insect vectors, a phage delivery bacterium with a similar spreading mechanism as X. *fastidiosa* so that it can get to more bacteria through the host and an artificial trigger to the plant's immune response.

Imperial College's 2016 team engineered a genetic circuit and a software tool for designing them - to support co-culture population control employing three modules - A communicator utilizing two orthogonal quorum sensing systems allowing E. coli populations to detect their own and the other's population density, a comparator linking quorum sensing signals to RNA logic, so the bacteria can compare all cell line populations and a growth module allowing cell lines to respond to the signal relayed by the comparator (Imperial, 2016).

TU Delft's 2015 team made **BioInker** - a 3D printer, forming layers of designed bacterial Bioink printed with the help of a

flexible scaffold hydrogel which was later dissolved for biofilm printing (Delft, 2015). The modified bacteria were mixed with sodium alginate and calcium chloride to keep the structure fixed, creating a stable gel. This hydrogel-bacteria mixture was induced with rhamnose, a sugar specific for their promoter, which triggered synthesis of CsgA, the linking protein that helps in polymerization giving a 3D structure.

Kaiserslautern 2019's team in their project **Chlamy Yummy** used a modified *Chlamydomonas* to produce the enzymes PETase and MHETase for recycling Polyethylene terephthalate (PET) by degrading it and using the by-products as it sole carbon source (Kaiserslautern, 2019).

Making use of the 7 minutes doubling time of Vibrio natriegens **Marburg 2018**'s team made one-day-cloning a reality by building and characterizing a flexible golden-gate-based part collection to enable creation of complex pathways in a short amount of time (Marburg, 2018). They engineered two V. natriegens strains for cloning and protein expression applications, respectively.
Team Heidelberg 2014 introduced **The Ring of Fire**, a method of heat stabilization of proteins by circularization with catalytically active internal polypeptide, to alter the functionalities of proteins in ways post-translationally (Heidelberg, 2014). They built a software and a toolbox to purify, fuse, regulate, oligomerize and circularize, the protein using a linker without changing the natural conformation of the protein.

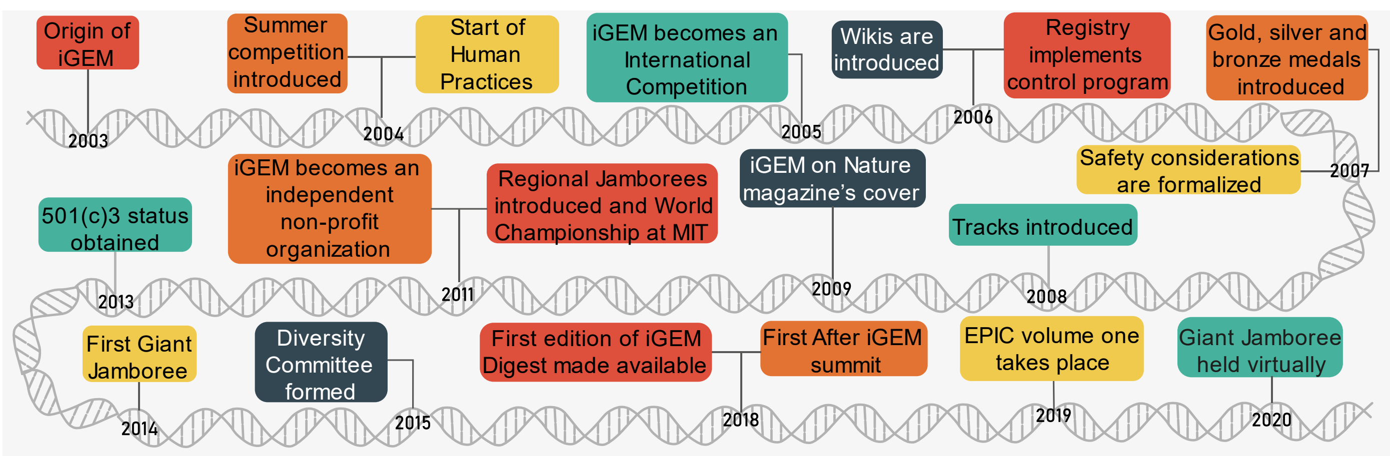

Figure 4: Timeline

VII. DISCUSSIONS

The iGEM competition serves as an incubator to test out what works and what doesn't. They have enabled systematic engineering, promoted open and transparent tools and constructed a society that can productively and safely apply biological technology. There has been a drastic expansion in iGEM's community over the years as a result of their persistent efforts in endorsing and building upon various segments of synthetic biology. It has been a place for people and ideas to flourish and grow.

REFERENCES

Calgary. (2019). Retrieved from https://2019.igem.org/Team:Calgary
Cristina Vilanova, M. P. (2014). *iGEM 2.0—refoundations for engineering biology.* Nature America.
Delft, T. (2015). Retrieved from http://2015.igem.org/Team:TU_Delft
Heidelberg. (2014). Retrieved from http://2014.igem.org/Team:Heidelberg
Imperial, C. (2016). Retrieved from http://2016.igem.org/Team:Imperial_College
Kaiserslautern. (2019). Retrieved from https://2019.igem.org/Team:TU_Kaiserslautern
Marburg. (2018). Retrieved from http://2018.igem.org/Team:Marburg
Munich. (2017). Retrieved from http://2017.igem.org/Team:Munich
Tainan, N. (2019). Retrieved from https://2019.igem.org/Team:NCKU_Tainan
Wageningen. (2019). Retrieved from https://2019.igem.org/Team:Wageningen_UR

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Bio-Startups: From iGEM to the Worldwide Industry

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***Abstract*- With the onset of the 21st century, synthetic biology has established itself as an effective science field to design biological systems, as an increasing solution. Numerous synthetic biology companies now offer tools, products and services to sustainably cope with global health, medicine, food and environmental issues. The iGEM competition is a showcase for different synbio projects and prototypes. In this article, the iconic startups born in iGEM are detailed with an emphasis on those that aim to improve accessibility to science. The exponential increase of bio-startups and applications derived from iGEM is evident, so it is likely that in the near future this foundation will facilitate the growth of the bioeconomy. That is why, we present starups developed in Latin America, describing the panorama of synthetic biology in the region, through the analysis of its limitations.**

***Index Terms*-** entrepreneurship, iGEM teams, innovation, synthetic biology.

I. INTRODUCTION

In the 21st century, society is facing problems in health, energy and food security. Diverse fields of science, one of them synthetic biology, have emerged as critical tools to face these challenges (Kelwick et al. 2015). The European Comission defined Synthetic Biology as "the application of science, technology and engineering to facilitate and accelerate the design, manufacture and/or modification of genetic materials in living organisms" (Delebecque and Philp 2015)

Research in synthetic biology has become a priority for some developed countries such as the United States (Delebecque and Philp 2015). This is verified by the amount of money invested in this science, in which the United Kingdom stands out with an initial capital fund of £ 10 million for the development of new startups in synthetic biology (Baker 2017). Such level of effort is understandable because synthetic biology has been perceived as a branch that directs the bioeconomy by generating biomass for industry avoiding traditional sources such as crude oil (Delebecque and Philp 2015).
Undoubtedly a source of startups in the area of synthetic biology has been the International Engineered Machine (iGEM) competition (Hung 2020). In this contest it is proposed to use biological systems (microorganisms, plants, etc.) to propose a project that offers a solution or product that satisfies a problem in society. The dynamics are very reminiscent of robotics competitions for engineers (Ochoa Cruz 2015).

Here, we illustrated the impact of this competition describing 3 of the best known iGEM-related startups in terms of providing accessibility to science: Ginko Bioworks, Genspace, and Labster, as shown in figure 1. Then, we explored some of the initiatives that have emerged in Latin America in the area of synthetic biology and identify the limitations faced by this region. The aim is to captivate the reader with diverse applications and demonstrate how synthetic biology is capable of offering innovative products to solve large-scale problems.

II. RESEARCH ELABORATIONS

A. *Materials and Methods*

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We searched the terms "iGEM" & "startup" in Scholar Google, considering relevant articles from 2015 onwards. Based on this information, we went to the websites of the iGEM teams, as well as interviews and notes made to the same teams. As well as the analysis of databases such as Elsevier and Pubmed. Anyone interested in learning more about other initiatives can visit: https://igem.org/Startups

III. RESULTS AND FINDINGS

A. *Iconic bio-startups*

Since its beginning, iGEM has been a competition where innovative projects, based on synthetic biology, are presented with the aim to solve relevant problems. Novel projects are presented based on innovative ideas from each participating team. The organization and entrepreneurial spirit of the groups, which continues even after the competition, has led to the creation of successful companies that turn their prototypes into innovative products (Wright, 2020).

Among the startups that have emerged from iGEM, Ginkgo Bioworks, a synthetic biology company located in Boston, United States, stands out. Its five co-founders participated in IGEM 2006 with the "Eau d'e coli" project, where *Escherichia coli* bacteria were genetically modified to produce aromas of wintergreen and banana, using only endogenous metabolites. Today Ginkgo Bioworks designs customized organisms for different clients. The team's representatives indicate that the biggest obstacles they faced at the beginning were the availability of capital and the low acceptance of synthetic biology by the society of that time; however, this was not an impediment for the development of their ideas (Feldman 2019).

Genspace, a U.S. participating group in iGEM 2014, gave a boost to facilitate access on synthetic biology and biotechnology with its OpenLab project, focused on creating a website that includes full knowledge and resources needed to develop a community-based biological laboratory. Their work included the design of liquid handling robots, the registration of genes of fluorescent proteins, genes free of intellectual property, and a platform for the development of visual laboratory protocols (Genspace 2014). From this project Opentrons was born, a company dedicated to the creation of robots for the automation of laboratory procedures (iGEM 2019).

Denmark also has a startup from iGEM, Labster, a company that designs 3D virtual labs and science games to revolutionize the way science is taught to students around the world. Its labs are being used by universities such as Harvard, MIT, and Stanford internationally. Its founder Mads Tvillinggaard together with five other biotechnology students participated in iGEM 2009 with the project "The Redoxilator & the user fusion standard". Their work contributed to the improvement of the registration of standard biological parts that are still used by other iGEM teams (Technical University of Denmark 2009). An schematic summary of iGEM-derived startups is presented on Figure 1.

B. SynBio is developing in Latin America

Seventeen teams from five Latin American countries participated in the iGEM 2019. Within Latin America, iGEM has had a great impact on the development of synthetic biology, but participation is low when compared to other regions, with an average of only 5.3% of Latin American teams in the last 10 years (Ochoa Cruz 2015). The low participation is mainly produced by a consecuence of economic difficulties faced by the region, as well as the lack of investment in scientific research projects. In 2020 the COVID-19 pandemic has worsened the synbio development, where only 4 teams from Mexico, Puerto Rico and Perù are participating in iGEM 2020.

The startups in this region face excessive and time-consuming bureaucratic procedures and permissions to carry out a study or project. Also, importing reagents and materials can be very expensive. However, initiatives to incubate synthetic biology startups are just emerging and many of the nascent startups seek to improve accessibility to synthetic biology for the community and thus limit existing barriers.

Scintia is a startup founded in 2017, whose objectives are to facilitate the way of learning, teaching and undertaking synthetic biology in Latin America. The founders Camilo Chávez, Minerva Castellanos, Alejandra Garza and Alejandro Guzmán help to reduce the biotechnology industry's dependence on imports, a problem that is holding back its development in the region. Since its inception, Scintia's vision has been proposed to be developed in several phases. The first phase is currently executed, which consists of building simple equipment for laboratory work, and over time they plan to develop more advanced technologies (Red Syn Bio MX, 2018).

Cibus 3.0, the first Central American iGEM derived startup was founded after participating in 2012. The company designs and transforms organisms to convert dairy industrial waste into biodiesel available for transportation, machinery functioning, etc. The team addressed the waste of whey resulting from the manufacture of cheese as a massive environmental problem. By modifying *Rhodococcus opacus* and *Escherichia coli*, they obtained encapsulated lipase enzymes, capable of producing biofuel, then mixed them up with ethanol and thus produced biofuel. This system is a profitable alternative due to the large amount of whey waste and the generation of environmentally friendly energy. Their main obstacles were delays in delivery times of reagents and the lack of financial support (iGEM, 2012; Jimenez, 2016).

Grid Exponential is another Latin American company with a different idea of innovation as it helps to develop and promote biotechnology startups. It was born in 2016 as an idea of Matías Freire, former iGEMer, when he observed multiple visionary projects in the competition with large possibilities of successful companies. The startups range from companies based on food engineering (Einsted) to those dedicated to the creation of cosmetics based on the modification of body microbiomes to improve people's well-being (Cryosmetics) (Grid exponential,2016).

FIGURE 1: Schematic summary of derived-iGEM startups in this review

IV. CONCLUSION

The iGEM Program promotes the opportunity of seeking solutions for different problems that the world faces, through the use of synthetic biology. By generating proof of concepts and prototypes inside the iGEM competition, it is an international well-founded incubator for bio-startups and bio-industries. Although the presence of Latin American iGEM teams is relatively low, several ideas have been consolidated as startups or non-profit organizations to support biotechnology initiatives. Therefore, significant growth in the region's participation in the bioeconomy field is expected in the coming years

Since 2003, iGEM's projects have directly contributed to more than 150 startups and this program provides successful industry leaders and entrepreneurs around the world. In recent years, there has been an increase in the participation of Latin American teams, and it is expected that this will be accompanied by the generation of more successful startups, and support for the development of the biotechnology industry.

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REFERENCES

Baker, Beth. (2017). «Synthetic Biology and the Marketplace». BioScience 67(10): 877-83.

Delebecque, Camille, y Jim Philp. 2015. «Training for Synthetic Biology Jobs in the New Bioeconomy». Science. http://sciencecareers.sciencemag.org/career_magazine/previous_issues/articles/2015_06_02/caredit.a1500143 (30 de julio de 2020).

Feldman, Amy. 2019. «Ginkgo Bioworks: hacer realidad la ciencia ficción». https://www.forbes.com.mx/ginkgo-bioworks-hacer-realidad-ciencia-ficcion/.

Genspace. 2014. «What Is the Open Lab?» Open Lab. http://2014.igem.org/Team:Genspace/Project.

«Grid exponential main page». 2016. GridX. https://gridexponential.com/.

Hung, Chia-Suei. 2020. «International Genetically Engineered Machine (IGEM) Jamboree Integrates Materials Research». MRS Bulletin 45(3): 170-72.

iGEM. 2019 «Opentrons». iGEM 2019. https://2019.igem.org/Sponsors/Special_Offers/Opentrons..

iGEM. 2012. «Costa Rica-TEC-UNA». iGEM 2012. http://2012.igem.org/Team:Costa_Rica-TEC-UNA

Jimenez G. (2016). «Investigadores graduados del TEC buscan producir biodiesel a partir de suero de leche». https://www.tec.ac.cr/hoyeneltec/2016/11/17/investigadores-graduados-tec-buscan-producir-biodiesel-partir-suero-leche.

Kelwick, Richard, Laura Bowater, Kay H. Yeoman, y Richard P. Bowater. (2015). «Promoting Microbiology Education through the IGEM Synthetic Biology Competition» ed. Beatrix Fahnert. FEMS Microbiology Letters 362(16): fnv129.

Ochoa Cruz, Edgar Andres. 2015. «Participation in IGEM Competition; Education toward Synthetic Biology Innovation». Journal of Biotechnology & Biomaterials 05(01). https://www.omicsonline.org/open-access/participation-in-igem-competition-education-toward-synthetic-biology-innovation-2155-952X-1000170.php?aid=47202 (30 de julio de 2020).

Red Syn Bio MX. (2018). Perfil emprendedor: Scintia. Obtenido de https://synbiomx.org/2018/03/06/perfil-emprendedor-scintia/

Technical University of Denmark. 2009. «Dtu Denmark». Dtu Denmark' 09. http://2009.igem.org/Team:DTU_Denmark.

Wright, W. (2020, Marzo 4). The iGEM Entrepreneurship Program. Retrieved from The iGEM Blog: https://blog.igem.org/blog/2020/3/4/the-igem-entrepreneurship-program

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Promoters in International Genetically Engineered Machine (iGEM)

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Abstract- **Promoters are an integral part of synthetic biology as they control gene regulation, protein expression and cellular growth. They are the key to all synthetic biology projects, and iGEM has a vast repository of promoters. Every year, iGEM teams choose to work with and add data to the existing or add new promoters to the iGEM repository, which makes it a sizable dynamic reservoir of different types of promoters. These promoters have been categorized into six major types based on their function; constitutive, cell signalling, metal sensitive, phage promoters, Indian Institute of Technology (IIT) Madras stress kit and University of Science and Technology Chittagong (USTC) logic promoters. Here, we review all the major promoter types used in iGEM, which might help readers choose appropriate promoters for future projects.**

Index Terms- iGEM, Gene expression, Gene regulation, Promoters

I. INTRODUCTION

The promoter architecture largely determines the expression pattern of a gene[1]. Biotechnology has seen the use of hybrid promoters to achieve goals that otherwise would not have been gained by natural promoters. The strategy of selection of promoters for experiments depends not only on the level of expression required but also on the consequences of gene expression to the cell[2]. New iGEM teams either choose pre-existing promoters from the iGEM repository or modulate, design and synthesize new ones to fit their requirements. Some of the reasons driving the careful choice of promoters in synthetic biology are- maximum expression in the growth or stationary phase, constitutive expression, induced expression, controlled expression and temporal expression. Such work over the years has led to the development of a vast repository of promoters in iGEM. They have been divided into six major categories based on their function. Let us revisit some of these promoters, look at the consensus sequences and how teams have tweaked them over the years.

iGEM PROMOTERS: A REVIEW OF CURRENT STATUS

II. CONSTITUTIVE PROMOTERS

1.1. CONSTITUTIVE PROKARYOTIC PROMOTERS

All bacterial RNA polymerases require sigma factors to recognize the promoter. This recognition determines the specificity and efficiency of transcription[2][3][4]. The prokaryotic constitutive promoters in iGEM have been categorized based on the type of sigma factor recognized. The iGEM repository has *E. coli* promoters recognizing σ^{70}, σ^{38}, σ^{32}, and *B. subtilis* promoters recognizing σ^{A} and σ^{B}. Table 1 gives a brief overview of *E. coli* and *B. subtilis* sigma factors and some of their regulated promoters. The σ^{70} recognizing Anderson promoters are of the most popular choice (J23100-J23119). This popularity could be attributed to the fact that most teams use *E. coli* as their chassis organism. These promoters are part of a small combinatorial library, and the part J23119 is the wildtype "consensus" promoter and the strongest promoter of the family. The consensus promoter regulates housekeeping genes and is maximally expressed during the growth phase of the bacteria. John Anderson of iGEM 2006_Berkeley designed this family[18]. Since then, numerous teams have adapted these promoters and tweaked them with regulatory sequences to meet their requirements.

Table 1. E. coli and B. subtilis sigma factors in iGEM[19]

Organism	Factor	Gene	Genes regulated	Some commonly used promoters
E. coli	σ^{70} (σ^{D})	*rpoD*	Housekeeping	Anderson family, β-lactamase (I14018), Kanamycin resistance (I14034)
	σ^{38} (σ^{S})	*rpoS*	Stationary phase	*osmY* (J45992, J45993)
	σ^{32} (σ^{H})	*rpoH*	Heat shock	*dnaK* (K1895002), *htpG* (K1895003)
B. subtilis	σ^{A} (σ^{43}, σ^{55})	*sigA*, *rpoD*	Housekeeping/ early sporulation	*liaG* (K823000), *veg* (K823003), *lepA* (K823002)
	σ^{B} (σ^{37})	*sigB*	General stress response	*ctc* (K143010), *gsiB* (K143011)

1.2. CONSTITUTIVE BACTERIOPHAGE PROMOTERS

The T7 RNA Polymerase (T7 RNAP) is a widely preferred tool for recombinant gene expression owing to its high transcriptional activity and easily tunable promoter. These parts have been derived from the T7 bacteriophage[5]. The orthogonality of the T7 RNAP to most host transcription machinery and high specificity to the T7 promoter allows broad applications in protein production. However, the high enzyme activity also results in increased metabolic burden and affects the host physiology[6]. The iGEM repository has a collection of T7 promoters mutated at specific sites in the T7 consensus site leading to altered strengths which were submitted by Barry Canton from MIT in 2005[19] (Table 2).

1.3. CONSTITUTIVE YEAST PROMOTERS

Promoters of several genes like *CYC1*, *ADH1*, *STE5* and *GPD1* form *Saccharomyces* have been reported in iGEM. The strength of these promoters varies as P(*GPD1*)> P(*ADH1*)>> P(*CYC1*)[8]. The *CYC1* promoter is of medium strength which allows for basal transcription. The team ESBS-Strasbourg in 2008 submitted different mutants of the minimal *CYC1* promoter having different strengths. These had mutations in the TATA box leading to an alteration in their strengths (Table 3).

Table 2. Sequence and relative strengths of engineered T7 RNA polymerase promoters in iGEM[19]

Part Number	Nature	Sequence* (Runs from -17 to +6)	Relative strength[7]
R0085, I719005	Consensus (wildtype)	TAATACGACTCACTATAG GGAGA	1.00
R0180	Mutated	TTATACGACTCACTATAG GGAGA	0.72
R0181	Mutated	GAATACGACTCACTATAG GGAGA	0.50
R0182	Mutated	TAATACGTCTCACTATAG GGAGA	0.30
R0183	Mutated	TCATACGACTCACTATAG GGAGA	0.09

*nucleotides represented in red are the mutations

Table 3. Variants of the minimal CYC1 promoter and their relative strengths in iGEM[19]

Promoter	Part number	Nature	TATA box*	Relative strength
Minimal *CYC1* Promoter	K105027	Consensus (wildtype)	TATATAAA	1.00
	K105028	Mutated	TATATAAC	0.70
	K105029	Mutated	TATATAGA	0.43
	K105030	Mutated	TATATTAA	0.28
	K105031	Mutated	TATATGAA	0.16

*nucleotides represented in red are the mutations

1.4. CONSTITUTIVE PROMOTERS FROM EUKARYOTES

The CMV (I712004) and the *UBC* promoter (K076017) are used for stable constitutive expression in mammalian cells[9][10]. The promoter of the gene *gpdA* (K1021010) has been isolated from *Aspergillus nidulans* and is used for strong constitutive expression in fungal chassis. Several synthetic constitutive promoters have also been created for *Aspergillus niger* as part of the Library of Engineered Aspergillus Promoters (LEAP) and were submitted by DTU-Denmark in 2019[19]. These promoters have different strengths in different growth phases (Table 4).

Table 4. Submitted LEAP promoters in iGEM[19]

Promoter name	Gene	Part number	Relative nature	Growth phase
PLEAPglaA_2	*glaA*	K3046001	Strong	Exponential
PLEAPsonB_1	*sonB*	K3046002	Medium	Independent*
PLEAPgpdA_1	*gpdA*	K3046003	Strong	Lag
PLEAPgpdA_1	*gpdA*	K3046004	Medium	Independent*
PLEAPmstA_1	*mstA*	K3046005	Very strong	Exponential
PLEAPunk_1	-	K3046006	Weak	Independent*
PLEAPgfaA_1	*gfaA*	K3046007	Strong	Late exponential
PLEAPhfbD_1	*hfbD*	K3046008	Strong	Stationary

*Expression by the promoter is independent of the growth phase of the bacteria

III. CELL SIGNALLING PROMOTERS

The lux operon of *Vibrio fischeri* is naturally involved in quorum sensing[11]. The promoters of this operon have been extensively used with regulatory modulations by several teams over the years. This operon has two promoters, both of which give a weak constitutive expression of genes downstream in opposite directions. The promoter to the 'right' (R0062, R1062) transcribes the *luxI* gene, whereas the one to the 'left' (R0063) transcribes *luxR* gene. The LuxR repressor protein downregulates the activity of the left promoter. It forms a homodimer and binds two molecules of 3-oxo-hexanoyl-l-homoserine lactone (3-oxo-C6-HSL), an auto-inducer produced by LuxI, to create an activator complex for the right promoter[11][12]. Promoters from *Pseudomonas aeruginosa* which are controlled by RhlR and N-butyrylhomoserine lactone (R0071), and by LasR and N-acylhomoserine lactone (R0079) have also been exploited in iGEM[20].

IV. METAL SENSITIVE PROMOTERS

Metal ions are necessary components of many physiological reactions in a cell, but an excess of such ions could be toxic. Bacteria have developed several regulatory mechanisms to maintain cellular metal ion homeostasis[13]. The iGEM repository has several promoters which are sensitive to a range of metal ions (Table 5). These parts might have been modified or used as such from the source to meet project requirements.

Table 5. Metal sensitive promoters in iGEM[21]

Source	Gene regulated	Part number	Metal	Promoter Nature
R. metallidurans	*pbrABCD*	I721001	Pb	Lead inducible
E. coli	*aceB*	K1163101	Fe	Iron repressible
E. coli	*fes*	K1163107	Fe	Iron repressible
E. coli	*yncE*	K1163110	Fe	Iron repressible
E. coli	*yodA*	K896008	Cd	Cadmium inducible
B. subtilis	*cadA*	K174017	Cd, Zn, Co	Cadmium, Zinc and Cobalt inducible
E. coli	*copA*	K1980006	Cu	Copper inducible
E. coli	*cusABCF*	I760005	Cu	Copper inducible
S. flexneri	*merPTAD*	K346002	Hg	Mercury inducible

V. PHAGE PROMOTERS

Apart from the T7 constitutive promoters documented in section 1.2, the repository also has the T3 promoter (K2084000) which is dependent on the T3 RNAP, isolated from the T3 bacteriophage[14]. The 2017 iGEM Edinburgh team improved the inducible T7-*lacO* promoter (R0184). They fused a *lacO* site downstream to the promoter and upstream to the T7 RNAP thus making the resultant system (K2406020) less "leaky" and IPTG inducible. They also integrated this system to their recombinase protein expression units and created five inducible expression generators (K2406080-K2406084)[22].

VI. IIT MADRAS STRESSKIT PROMOTERS

The Lutz and Bujard *lacO* promoter contains two LacI binding sites and responds to the σ^{70} factor[15][16] (K086017). The IIT Madras iGEM team in 2008 engineered the classic Lutz and Bujard to generate the stresskit promoters in which the σ^{70} boxes were altered to respond to other stress associated sigma factors like σ^{24} (K086018-K086021), σ^{28} (K086022-K086025), σ^{32} (K086026-K086029) and σ^{38} (K086030-K086033). They created four promoters responding for each of the stress associated sigma factors thus making a total of sixteen. All the promoters were IPTG inducible in the absence of stress. The σ^{24} promoters showed heat shock response while the σ^{28} promoters responded to starvation[23].

VII. USTC LOGIC PROMOTERS

The *lac*UV5 promoter is transcribed by the *E. coli* RNAP and is dependent on the σ^{70} transcription initiation factor[17]. Using this promoter the USTC iGEM team in 2007 generated a collection of highly specific repressible promoters with one or two repressor binding sites which were suitable for performing several logical operations like NOT (I732200- I732207), NAND (I732301- I732306) and NOR (I732351- I732352) at the level of transcription. These logic promoter models are highly simplified with reduced number of parameters making them easy to construct, predict expression patterns and test experimentally[24].

VIII. CONCLUSION

Choice of a correct vector, promoter and ribosome binding site is a major step towards achieving successful transgene expression. The promoter could be natural, minimal or synthetic. There are several techniques available to assemble synthetic promoters and their use has seen a steady increase over the years. The promoter repository of iGEM is huge and robust. Selection of appropriate promoters is one among the many challenges faced by new iGEM teams every year. We believe that this review might ease up the selection of appropriate promoters from the iGEM repository for upcoming synthetic biology projects.

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REFERENCES

[1] Rushton PJ. What Have We Learned About Synthetic Promoter Construction?. *Methods Mol Biol*. 2016;1482:1-13. doi:10.1007/978-1-4939-6396-6_1

[2] Goldstein MA, Doi RH. Prokaryotic promoters in biotechnology. *Biotechnol Annu Rev*. 1995;1:105-128. doi:10.1016/s1387-2656(08)70049-8

[3] Burgess, R. R. (2001). "Sigma Factors," in *Encyclopedia of Genetics*, eds S. Brenner, and J. H. Miller (Cambridge, MA: Academic Press), 1831–1834. doi: 10.1006/rwgn.2001.1192

[4] Haldenwang WG. The sigma factors of Bacillus subtilis. *Microbiol Rev*. 1995;59(1):1-30.

[5] Tabor S. Expression using the T7 RNA polymerase/promoter system. *Curr Protoc Mol Biol*. 2001;Chapter 16:Unit16.2. doi:10.1002/0471142727.mb1602s11

[6] Kar S, Ellington AD. Construction of synthetic T7 RNA polymerase expression systems. *Methods*. 2018;143:110-120. doi:10.1016/j.ymeth.2018.02.022

[7] Imburgio D, Rong M, Ma K, McAllister WT. Studies of promoter recognition and start site selection by T7 RNA polymerase using a comprehensive collection of promoter variants. *Biochemistry*. 2000;39(34):10419-10430. doi:10.1021/bi000365w

[8] Lee KS, Kim JS, Heo P, et al. Characterization of Saccharomyces cerevisiae promoters for heterologous gene expression in Kluyveromyces marxianus. *Appl Microbiol Biotechnol*. 2013;97(5):2029-2041. doi:10.1007/s00253-012-4306-7

[9] Norrman K, Fischer Y, Bonnamy B, Wolfhagen Sand F, Ravassard P, Semb H. Quantitative comparison of constitutive promoters in human ES cells. *PLoS One*. 2010;5(8):e12413. Published 2010 Aug 26. doi:10.1371/journal.pone.0012413

[10] Bäck S, Dossat A, Parkkinen I, et al. Neuronal Activation Stimulates Cytomegalovirus Promoter-Driven Transgene Expression. *Mol Ther Methods Clin Dev*. 2019;14:180-188. Published 2019 Jul 3. doi:10.1016/j.omtm.2019.06.006

[11] Haseltine EL, Arnold FH. Implications of rewiring bacterial quorum sensing. *Appl Environ Microbiol*. 2008;74(2):437-445. doi:10.1128/AEM.01688-07

[12] Qin N, Callahan SM, Dunlap PV, Stevens AM. Analysis of LuxR regulon gene expression during quorum sensing in Vibrio fischeri. *J Bacteriol*. 2007;189(11):4127-4134. doi:10.1128/JB.01779-06

[13] Chandrangsu P, Rensing C, Helmann JD. Metal homeostasis and resistance in bacteria [published correction appears in Nat Rev Microbiol. 2017 May 12;15(6):379]. *Nat Rev Microbiol*. 2017;15(6):338-350. doi:10.1038/nrmicro.2017.15

[14] Rong M, He B, McAllister WT, Durbin RK. Promoter specificity determinants of T7 RNA polymerase. *Proc Natl Acad Sci U S A*. 1998;95(2):515-519. doi:10.1073/pnas.95.2.515

[15] Lutz R, Bujard H. Independent and tight regulation of transcriptional units in Escherichia coli via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements. *Nucleic Acids Res*. 1997;25(6):1203-1210. doi:10.1093/nar/25.6.1203

[16] Zamora-Chimal CG, Zeron ES. A simple model for Lutz and Bujard's controllable promoters and its application for analyzing a simple genetic oscillator. *In Silico Biol*. 2015;12(1-2):69-82. doi:10.3233/ISB-150465

[17] Brodolin K, Zenkin N, Mustaev A, Mamaeva D, Heumann H. The sigma 70 subunit of RNA polymerase induces lacUV5 promoter-proximal pausing of transcription. *Nat Struct Mol Biol*. 2004;11(6):551-557. doi:10.1038/nsmb768

[18] *Promoters/Catalog/Anderson*. (n.d.). Registry of Standard Biological Parts. Retrieved September 28, 2020, from https://parts.igem.org/Promoters/Catalog/Anderson

[19] *Promoters/Catalog/Constitutive*. (n.d.). Registry of Standard Biological Parts. Retrieved September 28, 2020, from https://parts.igem.org/Promoters/Catalog/Constitutive

[20] *Promoters/Catalog/Cell_signalling*. (n.d.). Registry of Standard Biological Parts. Retrieved September 28, 2020, from http://parts.igem.org/Promoters/Catalog/Cell_signalling

[21] *Promoters/Catalog/Metal_sensitive*. (n.d.). Registry of Standard Biological Parts. Retrieved September 28, 2020, from http://parts.igem.org/Promoters/Catalog/Metal_sensitive

[22] *Promoters/Catalog/Phage*. (n.d.). Registry of Standard Biological Parts. Retrieved September 28, 2020, from https://parts.igem.org/Promoters/Catalog/Phage

[23] *Promoters/Catalog/Madras*. (n.d.). Registry of Standard Biological Parts. Retrieved September 28, 2020, from https://parts.igem.org/Promoters/Catalog/Madras
[24] *Promoters/Catalog/USTC*. (n.d.). Registry of Standard Biological Parts. Retrieved September 28, 2020, from https://parts.igem.org/Promoters/Catalog/USTC

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Biological Lasers: A Review of The Past iGEM Projects

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***Abstract*- Biological laser is an emerging technology which harnesses the power of a micro cavity most of the time filled with fluorescent proteins or synthetic dyes which creates a gain medium. This material allows the laser to amplify. On pumping with sufficient laser pulses, lasing is obtained from these cavities within the cells. Such a method can be employed in sensing, labelling, tracking and imaging on molecular, cellular and tissue level within different organisms. Two iGEM teams, TU_Delft 2016 and UiOslo_Norway 2017, have designed biological lasers using *E. coli* and *S. pombe* for better microscopy modalities, respectively. Although both teams have not been successful in obtaining lasing from their designs, they bring new tools and applications into the iGEM community. Here, we review the projects of both teams in detail while proposing our solutions on the problems they face during building a functional biological laser.**

Index Terms- biological laser, tagging, tracking, live imaging, biological sensors, lasers, fluorescence

I. INTRODUCTION

A typical laser consists of three main components: gain medium, resonator/cavity, and excitation source/pump. One can use biological materials such as fluorescent proteins or synthetic dyes as gain mediums whereas spherical or disk shaped structures made of biological materials can be employed as resonators in order to create biological counterparts of lasers. The biological laser is an emerging concept in biological research due to its potential applications in diagnostics and basic research [1, 6-8].

In principle, the biolaser concept is very similar to the fluorescence microscopy technique where fluorescent molecules start emitting photons when they are excited by an outside laser. However there are strong advantages of the light obtained from biolasers compared to fluorescence emission since biolasers emit laser light which have special properties [2].The laser light is a coherence optical source which provides a high signal to noise ratio, consists of a very narrow spectrum of light, and can focus on a small spot.

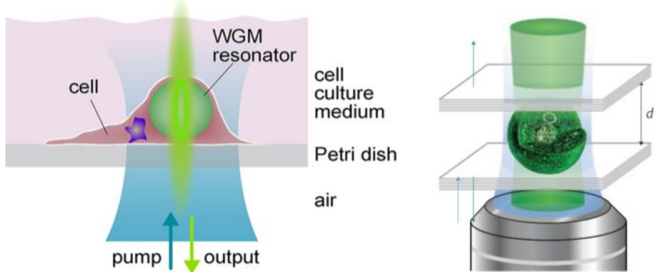

FIGURE 1: *A whispering gallery mode microresonator engulfed by a HeLa cell [5]. A HEK293 cell sandwiched between two parallel mirrors [2].*

Biological lasers can be employed in detection of various molecules and changes in physical properties of the gain medium environment [3], tagging and tracking of single cells [4, 5], and they can be implanted in tissues for biomedical applications [6-8].

In this review, we have compared the projects of TU_Delft 2016 (Delft team) and UiOslo_Norway 2017 (Oslo team) teams [9, 10]. We pointed out the problems in these two projects, and proposed some solutions for further development of these projects.

II. RESEARCH ELABORATIONS

There are three main components of a biolaser system, a gain medium, a resonator, and excitation energy. Every working system needs energy to run. For biolasers, the pump/excitation energy provides us that. It feeds the organic luminescent gain medium (such as a fluorescent protein), the protein along can emit light. However, mirror-like reflective surfaces/resonators must be used to enhance the emitted light. The total internal reflection inside the resonator confines the emitted light and helps the amplification process of it to turn the biological system into a lasing system.

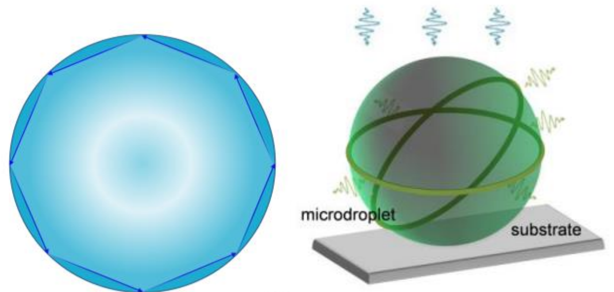

FIGURE 2: *left: The light ray undergoes multiple total internal reflections along the surface. The closed beam path defines the whispering gallery modes (WGM) [original work by Özgür Can]. right: The dimensional schematic of a spherical droplet resonator demonstrating WGM [15].*

i. Gain Mediums

The Delft team employed fluorescent proteins (FPs) expressed in *Escherichia coli* (*E. coli*) as gain medium. They have expressed four different FPs (GFP, mVenus, mKate and mCerulean) and investigated whether overexpression of these proteins affect the cell growth since high concentrations of FPs are needed inside the cell for a cell laser. Although they found out that overexpression of mCerulean and mVenus might be harmful for cell growth, they continued to use these proteins for further steps.

The Oslo team employed super folded fluorescent protein (sfGFP) expressed in *Schizosaccharomyces Pombe* (*S. pombe*) as the gain medium. They have also expressed sfGFP in *E. coli* and purified it to test their laser setup with the protein solution outside the cell. They have expressed sfGFP instead of regular GFP because of its resistance to denaturation and improved folding kinetics [14].

ii. Resonators

The Delft team developed a new concept for a resonator. They planned to use the cell membrane itself as a resonator, so they have coated *E. coli* cells with a layer of polysilicate by transformation of the silicatein-α gene originating from two different organisms: *Suberites domuncula* and *Tethya aurantia.* They have characterized the properties of polysilicate covered *E. coli* cells by several methods. First, they have stained cells with the dye Rhodamine 123 to check whether a polysilicate shell is present or not, then these cells are observed under a scanning electron microscope and transmission electron microscope to further examine the effects of the polysilicate layer on the cell itself. Also they have examined the cells under an atomic force microscope to determine the physical properties of the polysilicate layer. Finally, they have performed a viability test to investigate whether the cell covered with a polysilicate layer can grow as in its natural environment.

The Oslo team used two parallel mirrors as a resonator. They sandwiched the S. pombe cells between these mirrors. Mirrors confine the light waves into the gain medium environment between mirrors. They mentioned that their proof of concept did not work because of the lack of feasible equipment. In further studies, they are planning to use concave mirrors to achieve “short pass dichroic mirrors” effect.

iii. Optical Setup

The Delft team designed their custom optical setup with generous support by photonics companies around Europe. They have used a blue laser (peak: 405 nm) as an excitation source for the gain medium. The emitted light from the biolaser is collected by a CCD camera and a spectrometer. CCD camera is used to image the sample, and spectrometer is used to analyze the spectrum of the emission. They have used a dichroic mirror to filter out the pump laser from the emission laser. Also, a beamsplitter was used to couple the emission laser to the CCD camera and spectrometer (see *Figure 3*).

The Oslo team wanted to excite the gain medium with a blue LED which emits broadband light and has lower intensity compared to a laser. They have managed to excite sfGFPs by this LED but they could not measure emitted light with a CCD camera possibly because of the low intensity.

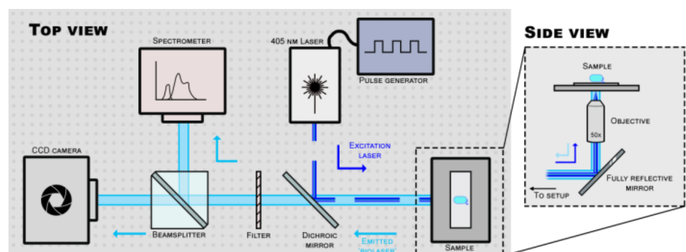

FIGURE 3: *Optical setup of TU_Delft 2016 Team [9].*

III. RESULTS AND FINDINGS

The Delft team successfully expressed FPs, and covered the cell with a polysilicate layer but they did not observe lasing from their setup which may be caused by the following problems (see *Figure 4*):

- Dimensions of *E. coli* are not suitable for sustaining laser modes, the size of the cell may be too small to support whispering gallery modes (WGM).
- Although they have transformed the cells to make them near spherical, there may be some surface irregularities since a WGM laser requires a perfect symmetry inside the resonator.
- Protein concentration required for lasing is higher than the achievable limit in *E. coli* so concentrations may be another problem.

The Oslo team successfully expressed FPs inside *S. pombe* and *E. coli* but they did not observe lasing from their setup although they observed fluorescence emission by excitation of FPs with LED.

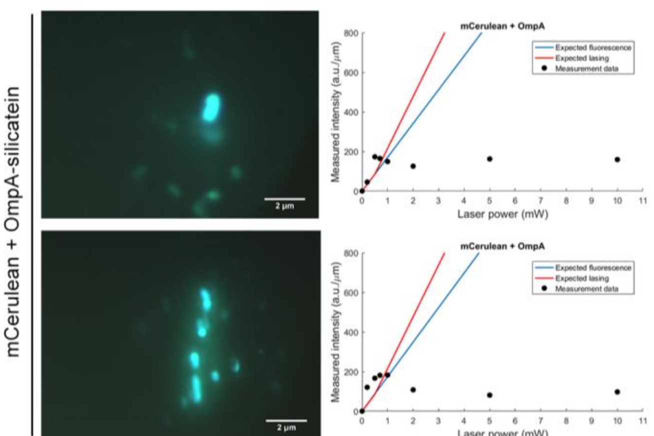

FIGURE 4: *Intensity measurements of cells transformed with mCerulean and OmpA-silicatein [9].*

IV. DISCUSSION

Overall the plan and execution by the Delft team was very successful. However there are some points which need to be altered to make the best out of such a biolaser. First of all, *E. coli* cells are too small to obtain lasing, so the Oslo team used bigger cells but they were not successful in constructing the setup. Thus, using bigger cells to create a biolaser is yet to be tested by further teams.

The Delft team used silicatein proteins to coat the cells. Their computational model confirmed the lasing modes of such a setup however they assumed *E. coli* cells as perfect spheres in their computational model which was a huge drawback of their model. So a more realistic computational model should be developed by future teams. Also cells can be coated with different proteins such as reflectins which have a similar refractive index as silicatein [11, 12]. One can coat cells with either of these proteins or both of these proteins at the same time resulting in some irregularities in the refractive index of the membrane [9]. However it should not be much of a concern because cell membranes are already composed of molecules with different refractive indices.

The fluorescent protein concentrations are another major problem for both teams. Since a high concentration is needed to obtain lasing, bigger cells should be used for further studies [9, 13].

Obtaining lasing is really hard in such complex environments, but the Oslo team could not even collect emitted light on a CCD camera which points out that sample concentrated more solvent of sfGFP to prove lasing. The other problem was, they used the same sample for a long time, this sample started to decay and they recorded some remitted red light which was not emitted before.

V. CONCLUSION

Although we can not count these two projects as biological lasers since they did not obtain lasing from their samples, they are still important for the iGEM community because they bring new tools by biological laser concept. Future teams working on this concept should consider the drawbacks faced by both teams while checking out the possibility of using LEDs to excite FPs. The biological lasers open a new class of tools for diverse applications from sensing to diagnostics, so there should be more research conducted on this topic within the iGEM community.

REFERENCES

[1] Chen, Y. C., & Fan, X. (2019, September 1). Biological Lasers for Biomedical Applications. *Advanced Optical Materials*. Wiley-VCH Verlag. https://doi.org/10.1002/adom.201900377

[2] Gather, M. C., & Yun, S. H. (2011). Single-cell biological lasers. *Nature Photonics*, *5*(7), 406–410. https://doi.org/10.1038/nphoton.2011.99

[3] Armani, A. M., Kulkarni, R. P., Fraser, S. E., Flagan, R. C., & Vahala, K. J. (2007). Label-free, single-molecule detection with optical microcavities. *Science*, *317*(5839), 783–787. https://doi.org/10.1126/science.1145002

[4] Martino, N., Kwok, S. J. J., Liapis, A. C., Forward, S., Jang, H., Kim, H. M., … Yun, S. H. (2019). Wavelength-encoded laser particles for massively multiplexed cell tagging. *Nature Photonics*, *13*(10), 720–727. https://doi.org/10.1038/s41566-019-0489-0

[5] Schubert, M., Steude, A., Liehm, P., Kronenberg, N. M., Karl, M., Campbell, E. C., … Gather, M. C. (2015). Lasing within Live Cells Containing Intracellular Optical Microresonators for Barcode-Type Cell Tagging and Tracking. *Nano Letters*, *15*(8), 5647–5652. https://doi.org/10.1021/acs.nanolett.5b02491

[6] Song, Q., Xiao, S., Xu, Z., Liu, J., Sun, X., Drachev, V., … Kim, Y. L. (2010). Random lasing in bone tissue. *Optics Letters*, *35*(9), 1425. https://doi.org/10.1364/ol.35.001425

[7] Chen, Y.-C., Chen, Q., & Fan, X. (2016). Lasing in blood. *Optica*, *3*(8), 809. https://doi.org/10.1364/optica.3.000809

[8] Humar, M., Dobravec, A., Zhao, X., & Yun, S. H. (2017). Biomaterial microlasers implantable in the cornea, skin, and blood. *Optica*, *4*(9), 1080. https://doi.org/10.1364/optica.4.001080

[9] TU_Delft iGEM (2016). Team Wiki http://2016.igem.org/Team:TU_Delft/

[10] Ui_Oslo_Norway iGEM (2017). Team Wiki http://2017.igem.org/Team:UiOslo_Norway

[11] Chatterjee, A., Cerna Sanchez, J. A., Yamauchi, T., Taupin, V., Couvrette, J., & Gorodetsky, A. A. (2020). Cephalopod-inspired optical engineering of human cells. *Nature Communications*, *11*(1). https://doi.org/10.1038/s41467-020-16151-6

[12] Müller, W. E. G., Boreiko, A., Wang, X., Belikov, S. I., Wiens, M., Grebenjuk, V. A., … Schröder, H. C. (2007). Silicateins, the major biosilica forming enzymes present in demosponges: Protein analysis

and phylogenetic relationship. *Gene*, *395*(1–2), 62–71. https://doi.org/10.1016/j.gene.2007.02.014

[13] Hall, J. M. (2017). *Biological Cell Resonators*. University of Adelaide.

[14] Fisher AC, DeLisa MP (2008) Laboratory Evolution of Fast-Folding Green Fluorescent Protein Using Secretory Pathway Quality Control. PLoS ONE 3(6): e2351. https://doi.org/10.1371/journal.pone.0002351

[15] Nizamoğlu S, Lee K.B, Gather M.C, … Seok-Hyun Yun (2015) A Simple Approach to Biological Single-Cell Lasers Via Intracellular Dyes. Advanced Optical Materials: 3, 1197–1200 https://doi.org/10.1002/adom.201500144

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Like-Particles Systems as Molecular Carriers in The iGEM Competition: Where It Has Gone and What Is Ahead?

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Abstract- **The need to carry molecules, drugs or proteins of interest from one place to another within the body has led to the production of hollow protein bodies, with or without decorations, that serve as delivery systems and molecular carriers. These systems are particularly similar with some forms that we can find in nature: such as viruses, vesicles, organelles and magnetosomes, among others. The iGEM competition has fostered the development of multiple like-particles. Over the years, multiple teams have been awarded medals for their work in this branch of nanotechnology and synthetic biology. This review summarizes the most influential projects in this area from 2010 to 2019, the like-particles seen in the competition until this year, some perspectives of what remains to be done in the future, and where the Ciencias_UNAM team wants to make its contribution.**

Key words: Like-Particles, iGEM, delivery systems, molecular carriers, synthetic biology.

I. INTRODUCTION

The diversity of the teams that are formed for the iGEM (International Genetically Engineered Machine) competition is always vast. The number of registered teams at the beginning of 2010 was 117, and now in 2019 the number of participants increased to 359 teams, which constitutes a significant grow in the number of teams, countries, and very interesting topics like the use of like-particles.

Although the description of these particles dates back many years, since they were discovered with the electron transfer microscope (Kim, 2016), the term like-particle was originally only used to describe unidentified particles that could be viruses because of their similar size and shape. Therefore they were named Viral Like-Particles (VLPs), which are currently recognized as viral protein multimeric complexes produced with recombinant DNA technologies. Despite being the most recognized for being candidates for vaccines, the viral proteins are not the only like-particles in nature that can effectively transport and deliver molecules and drugs, or serve as a compartment inside a cell.

Bacterial microcompartments (BMCs) are large, protein-based assemblies present inside many bacterial cells. They were first found in the late 1950s inside cyanobacteria as electron dense, polyhedral shaped bodies, reminiscent of viruses or phage capsids. BMCs are now recognized to be metabolic compartments that carry out specific series of reactions in their interiors. (Tsai, 2011).

This article provides a review of the iGEM teams of the past decade (2010-2019) who developed a project and promoted the use of any kind of like-particle as delivery system, focusing on the analysis of how many teams from which regions and countries following which track worked with like-particle, with the goal of evaluating what has been made and what can be done in future competitions.

II. A BRIEF HISTORY OF THE LIKE-PARTICLES THAT THE COMPETITION HAS SEEN IN THE PAST DECADE

The 2010 Lethbridge team created a microcompartment with a catechol degrading enzyme in an attempt to develop a way of easily removing the useful hydrocarbon product from the tailings ponds; Minnesota demonstrated the potential of BMCs as nano-bioreactors, and TokioNoKogen created EcoTanker, a BMC that can collect and deliver target compounds, also Team Freiburg worked on a functional modular Virus Construction Kit specifically targeting and killing tumor cells, making the beginning of the decade a great promise in this area of nanotechnology and synthetic biology.

The next five years introduced us to like-particles with a lot of different natures: in 2011 TokioNoKogen made a comeback and created EcoLion for the collection of heavy metal ions. In 2012 the Wageningen UR team tackled the problem of site specific drug delivery using VLPs, while team Technion created a Trojan Phage that can kill the bacteria using a AND logic gate system. In 2013 Team UC Chile motivated us by creating their own functional bacterial organelle, a platform for in vitro metabolic engineering; VesiColi by Team NTNU showed us the outer membrane vesicles (OMVs) of an *E.coli* with a protein cargo inside, while in 2014 Hong Kong HKU constructed a flexible plasmid for the customized expression of a BMC. From 2015 to 2017 we have more projects focusing on evading the side effects of drug delivery, like the Freiburg Team which produced engineered spores from *Bacillus subtillis* to serve as a carrier, the Lethbridge team which, in 2018, used Protein nanocompartments (PNCs) to design a delivery strategy, and the HS Lethbridge team which used a copper-binding protein of a bacteriophage capsid to bind the copper in order to help clean up the environment. Team HAFS showed us that a minicell (achromosomal cell that does not reproduce) could be created and engineered to secrete insulin in the intestine, and that year's competition set the most teams working in these topics with a

total of 10. By 2019 there have been a lot of creative projects: UANL created a *E.compa,* a synthetic organelle for toxicity reduction and optimized biosynthesis of compounds, UCL engineered encapsulins as a drug delivery vehicle for cancer treatment, and TU_Darmstadt created the "real MVP" (Modular Virus like-particle), a platform to decorate a cargo on the exterior of a VLP, demonstrating that one just has to be creative in order to use whatever kind of like-particle because the potential of these tiny (a few nanometers big) compartments is a lot bigger than we think.

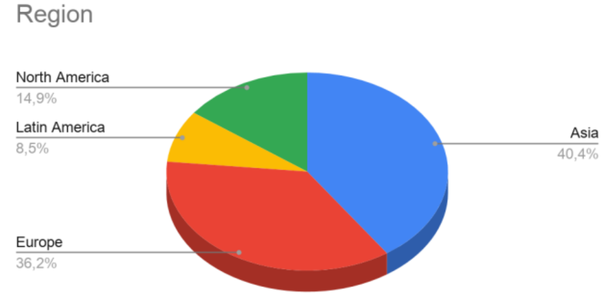
III. RESEARCH ELABORATIONS

A search for projects about like-particles systems as molecular carriers by year from 2010 to 2019 was performed on the iGEM official website and the official notebook of the giant jamboree by year, using the key words: like-particle, delivery systems, molecular carriers, microcompartment, virus, vesicles, vehicle, particles, organelle. The projects in which the main goal of their work was the construction of a like-particle or the use of one were selected for review and used for the creation of a database of the listed teams, year by year, from 2010 to 2019.

IV. RESULTS AND FINDING

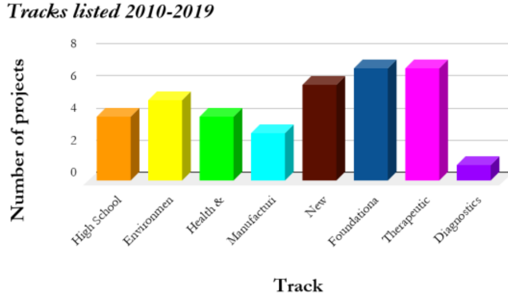
Map 1: Region and country map of the teams that have used a like-particle in the iGEM competition (2010-2019). Country predominance in a color scale, in which the green color represents the country with most teams, while red and dark red represents the countries with less teams or just one

It was found that the teams that participated the most were in Asia (18 teams) and Europe (17 teams) out of the total of 47 teams (Graph 1), and the most frequent countries were China with 13 teams, followed by Canada with 5 teams, and Japan with 4 teams.



Graph 1: Graphic representation of Regional predominance of teams working with like-particle systems, 80% of teams stem from Asia or Europe

As seen in *Graph 2* the teams that participate using a like-particle, tend to focus their projects on the track of Therapeutics and Foundational advance, with seven teams each track. Followed by the New application track with six teams, and the environment track with five teams. The least frequent track was the Diagnostics track with just one team.



Graph 2: Tracks and number of projects of the teams that have used a like-particle in the iGEM competition (2010-2019)

V. DISCUSSION

The number of teams throughout the decade was not constant, but every year there was at least one team with a related project. There has been an increment of the teams in the competition from 2017 to 2019, achieving an impact in the number of teams that in these three years presented a related project, being 2018 and 2019 the years with the most number of teams, with a total of 10 and 8 respectively.

The track with most teams using like-particles was Foundational Advance and Therapeutics with 7 teams in each one, then, New Applications with six teams. This can be attributed to the fact that many LPs have demonstrated to be a possible replacement to the conventional vaccines, and can be a potential revolution to medicine with more specific and designed therapies. Something that attracts attention is the very low number of projects in the Diagnostics track, and the Manufacturing track, where only five teams have directed their work. And as an example, Zurich's team in 2018 obtained a high number of prizes competing in the Manufacturing track that year, suggesting that upcoming projects should choose the tracks with low concurrency, covering a wider range of problems to fix.

Asia and Europe are the regions where more teams have made contributions; Zurich and Tokyo have directed their projects to

this topic for 2 consecutive years. A total of 82% of all teams in Like-Particle projects have come from these regions, and that is not a coincidence because every year most of the teams originated there. This presents an opportunity for the whole region of North America and Latin America to make their presence at upcoming competitions, as the UANL in Mexico did recently in 2019, as well as Toronto and the HS team of Lethbrindge who, in different years, have managed to carry out projects with LPs as their topics in 2018.

VI. PERSPECTIVES

The Ciencias_UNAM team has the objective to generate an impact that improves the healthcare of Mexico with a molecular vehicle VLP of VP2 protein domain of the parvovirus B19 with accessory decorations (Cayetano, 2019) that increases the efficiency of internalization for the CRISPR-Cas9 system and its entry into the cell nucleus. This technology can be used to edit the founding genetic error causing a disease in hepatocytes liver cells, contributing to the list of Mexican and Latin-American teams that use these kind of particles to solve a problem in their community.

VII. CONCLUSION

Like-particles systems have become a recurrent topic in iGEM year after year, and probably in the future it will become an indispensable research in every competition. The development of Biobricks for this interesting topic will serve as inspiration for many other teams to innovate and get amazing ideas, not just for the competition but also for startups projects. This work may serve any team to identify what has been done before in this area, use our database in their bibliographic search, and note that like-particles can make a very important impact in other tracks of the competition.

APPENDIX

In this link you will find the database used for the elaboration of the graphs for this paper, feel free to contact us if there is any suggestions.

https://docs.google.com/spreadsheets/d/1hN6NvdhgOEWrheBgSOLi-xaFegBhsMH9E5X4H5Dwv5Y/edit?usp=sharing

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REFERENCES

Cayetano C., Valadez G., Mendez C. and Bustos J.. (2019). Virus-like Nanoparticles Derived from Parvovirus B19 Efficiently Internalized in Human Hepatocytes: A Biocompatible Delivery System for Peptides and Proteins. ACS Applied Nano Materials, 2, pp 6178–6186

Jamboree for iGEM Championship (2010 - 2019) [Results]. Retrieved from https://igem.org/Results?year=2010&name=Championship&division=igem

Kim H.J.(2016) Yeast as an expression system for producing virus-like particles: what factors do we need to consider? Letters in Applied Microbiology 64, 111--123 doi:10.1111/lam.12695.

Sophia J. Tsai. (2011)Bacterial Microcompartments: Insights into the Structure, Mechanism, and Engineering Applications. Progress in Molecular Biology and Translational Science, vol 103, 1--20 https://doi.org/10.1016/B978-0-12-415906-8.00008-X

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Current State, Developments, and Future Perspectives of Bioprinting

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Abstract- **3-Dimensional (3D) printing can be used to produce biomaterials for numerous purposes in a process called bioprinting. Bioprinting approaches such as biomimicry and autonomous self-assembly are used to form mini-tissues which can later be macro-assembled into intact organs. The 3D bioprinting method also shows promise for future advancements in complex tissue engineering and drug delivery with complex profiles including recent SARS-CoV-2 research. Materials for bioprinting are often challenging to determine with biocompatibility requirements. Inkjet, microextrusion, and laser-assisted are the most common bioprinting technologies. Despite its advantages and applicability, the concept of bioprinting is not fully developed to address many of the limitations such as biocompatibility, cell viability, cell sources, vascularization, and scale handicaps. This review will discuss numerous studies that attempt to alleviate some of these intrinsic limitations in the process and implementation of bioprinting including in situ cross-linking and novel precursor hydrogel compounds.**

Index Terms- *Bioprinting, biomaterials, tissue engineering, biocompatibility*

I. INTRODUCTION

Bioprinting is a type of additive manufacturing process that uses biomaterials and living cells to create structures that have various functions (Murphy & Atala, 2014). Currently, 3D bioprinting is mainly utilized for creating complex systems that can imitate natural tissues. Bioprinted artificial tissues can be used for medical and experimental purposes. The two main approaches to bioprinting are: biomimetics and autonomous self-assembly. The biomimetic approach aims to mimic the natural tissue's function by creating identical structures. This requires an in-depth understanding of the tissue's cells and internal processes. Both the microenvironment of the tissue and the macroenvironment must be accurately "mimicked" for an accurate imitation (Ingber et al., 2006). The second of the two main approaches, autonomous self-assembly, uses living cells to first create a structure which changes after printing. The differentiation and development of the cells are directed, which causes the tissue to gain various structural and molecular attributes, such as generation of an extracellular matrix (ECM), cell signaling components and secretion of various substances. This approach requires a fundamental understanding of the differentiation and development processes of tissues and cells (Jakab et al., 2010). In most situations, both of these approaches are applied in unison to 3D bioprinting projects.

In 3D bioprinting, materials used in the process need to be coherent with the process of printing and maintain certain mechanical and functional properties for aimed tissue constructs (Tappa & Jammalamadaka, 2018). Therefore, materials have to be based on naturally acquired or synthetic polymers. Examples of naturally derived polymers include alginate, gelatin, collagen, chitosan, fibrin, and hyaluronic acid (HA), which are often isolated from animal or human tissues; whereas, an example of synthetic molecules is polyethylene glycol (PEG) and polyvinyl alcohol (PVA). Naturally derived materials are important due to their similarity to human ECM in terms of compatibility and bioactivity. Synthetic polymers, on the other hand, can be adapted to certain processes by altering some of their physical properties. These materials have poor biocompatibility, toxicity, and also when degrading, they lose some of the mechanical properties. These properties are significant for bioprinter deposition. Especially, these materials have to be suitable in terms of cross-linking mechanisms such as gelatin linked with more polymeric chains forming chemical bonds for transplantation and also enable cellular attachment, proliferation, and additional functionality (Gungor-Ozkerim, Inci, Zhang, Khademhosseini, & Dokmeci, 2018). To create personalized models for any diseases, stem cells derived from patients, which are induced pluripotent stem cells (iPS cells) or mesenchymal stem cells, are used. To mimic the extracellular matrix environment and to generate the final shapes of relevant tissue structure, bioinks including living cells and biomaterials are used as cross-linkers and as stabilizers during or after bioprinting (Pan, Bruyas, & Yang, 2016). In short, depending on the application, a variety of different materials, methods, and cells can be used to achieve the desired tissue structure in the bioprinting process.

There are three noteworthy technologies associated with depositing and forming complex patterns with biological materials to enable the bioprinting mechanism: inkjet, microextrusion, and laser-assisted.

Inkjet printers can utilize living cells to "print" individual particles of biological materials (including hydrogel beads) through small nozzles in precise and arbitrary positions to form biological tissues with scales varying from micro- to macro-scale in high resolution (Nakamura, Nishiyama, & Henmi, 2008). Several layers of beads may be printed atop each other to form a larger structure in a layer-by-layer approach. It is further possible to engineer spatially defined cell microenvironments with inkjet bioprinting (Phillippi et al., 2008). Thermal,

piezoelectric, and electromagnetic methods are prevalent in inkjet bioprinting (Angeloupoulos, Allenby, Lim, & Zamorano, 2018). Thermal inkjet bioprinting utilizes heat to eject material through the printer's nozzle which exposes cells being printed to heat and stress, but still ensures a low apoptotic ratio (Cui, Dean, Ruggeri, & Boland, 2010). The bioink is locally heated with a voltage pulse, causing the formation of a vapor bubble which generates pressure and overcomes the surface tension at the nozzle during ink ejection (Gudapati, Dey, & Ozbolat, 2016). Piezoelectric is similar to thermal, except the voltage pulse causes a change in the shape of the ejection chamber for the bioink's ejection (Gudapati et al., 2016). Electromagnetic inkjet printers are also similar and eject the bioink through an expansion of their chamber size (Gudapati et al., 2016).

Microextrusion bioprinting forms a 3D structure through depositing gel-filaments of bioink in a layer-by-layer approach (Ouyang, 2019). A microneedle is present instead of a nozzle which is responsible for the deposition of the bioink.

Laser-assisted bioprinting is a high-speed (kHz range) bioprinting method with microscale resolution (Guillotin et al., 2010). Aspects of a laser may be altered in this process to manipulate the ejection of droplets of bioink.

II. DISCUSSION

Currently, 3D-bioprinted tissues are not only used for transplantation but also for drug discovery, toxicology, and in research test platforms, promising to replace the requirement of animal testing partially in many applications. However, the field is far from perfect. Bioink components are only selected from known-compatible materials (e.g. collagen, hyaluronic acid) with feasible growth, function, cross-linking, and extrusion characteristics. Often, the problem is the intrinsic limitations of such materials (Murphy & Atala, 2014).

One present solution to this problem is the possibility of developing tailor-fit materials adapted to a specific context by reprogramming shape, properties, or functionality when stimulated by external input. This novel approach, 4D bioprinting, provides dynamicity to 3D-bioprinting with the ability to possess intrinsic shape memory and recovery (Wan et al., 2020). Furthermore, if the biomaterial is designed to respond to physiological stimuli, engineered biomaterials can be subject to natural tissue regulation post-deposition (Murphy & Atala, 2014). Improving construct vascularization is essential for the long-term viability of bioprinted tissues. Several base studies have demonstrated vascular formation on bioprinted organs in vitro (Norotte et al., 2009; Visconti et al., 2010). Often, documented maturation/assembly time for such vascular networks exceeds the cell survival time-frame. Currently, bioreactors are used to maintain tissue viability during post-processing steps preventing immediate usage of constructs (Murphy & Atala, 2014).

On another lane, in vivo bioprinting is a paradigm shift from the traditional in vitro bioprinting approaches. In this approach, cells and materials are directly deposited in the final destination of the construct. This presents the opportunity of maintaining the compatible cells and materials, and directly delivering them to a patient in real-time. Nevertheless, bioprinter technology is still not developed to facilitate deposition of biomaterials in real-life applications (i.e. surgery) (Murphy & Atala, 2014). With the increasing speed and resolution of 3D bioprinters, in vivo bioprinting may become available during surgery. One expected advancement is integrating 3D bioprinters into "minimally invasive, robotic surgical tools" to automate the administry process (Murphy & Atala, 2014). Moreover, advances in small-molecule-assisted cell proliferation/differentiation suggest a future of total external control of cell processes (Okumura et al., 2009; Yu et al., 2012). In a 2019 review, the possibility of future advancements on the rheological properties of bioinks has been proposed (e.g. decreasing extrusion pressure). Bioinks capable of withstanding nozzle pressure to preserve viability is crucial. Another technical area of concern, enabling different printing-geometries "not feasible by traditional layer-by-layer fabrication", may be overcome by hydrogel scaffolds (Galarraga et al., 2019). Significant advancements in the production of hydrogels such as supramolecular guest-host hydrogels printed on top of supporting gelatin facilitate different printing-geometries (Murphy & Atala, 2014).

Bioprinting has been previously studied in constructing organoids. Prior research on the development of specific bioinks for viral infectivity of human organoid models has been conducted by many including Berg et al. (2018). Human induced pluripotent stem cell (iPSC) formed organoids, self-organized and standardized with similar structure and function to real organs, were proposed by Zhou et al. (2020) as superior models to currently used cell lines and model organisms in long-term ex vivo viral cycle mimicry and drug screening. Human intestinal organoids have already been used as models to study the SARS-CoV-2 replication in these sites where intestinal epithelium was successfully identified as a potential replication center (Clevers, 2020).

One of the major contributors to high pressure build-up in nozzles is the viscosity requirement of bioinks for post-deposition cross-linking. Future advancements allowing non-viscous bioinks may increase cell viability. Currently, light exposure as an *in situ* cross-linking method is developed and implemented on mesenchymal stromal cells decreasing bioink viscosity (Galarraga et al., 2019). In 2020, optical 3D printing (O3DP) utilizing light to initiate polymerization was used in producing bio-based polymers. As these are biological molecules, they may be suitable candidates for bioink solutes providing low pressure with a light activated post-deposition cross-linking and biocompatibility (Skliutas et al., 2020).

III. CONCLUSION

Human tissues have a 3D shape which can be replicated via computer-aided design (CAD) and computer-aided manufacturing (CAM) tools as an application of 3D bioprinting. It might play an important role in future clinical applications of

the 3D bioprinting technology (Gillispie et al., 2019). However, there is a limitation for printing tissues. Although the technology works for printing organs and tissues that are relatively small and simple, they are printed as avascular, aneural, thin, and nourished by diffusion from host vasculature. It creates the problem of limited oxygen diffusion when the thickness of tissue is over 200 micrometers in the complex and thick transplantation organs such as kidney, liver, and heart. In order to maintain metabolic functions of organs when they are transplanted and being able to produce complex, well-vascularized tissue structures for clinical use, bioprinting aims to print functional vascular structures (Gillispie et al., 2019; Ventola, 2014).

3D printing technology plays an important role for the purpose of drug development and delivery. The technology aims for high reproduction of personalized medications by precise control of droplet size and dosage. Thereby pharmacists could analyze patients' characteristics, pharmacogenetic profiles, be able to print personalized drugs in an optimal dose, and even adjust the dose afterwards. 3D printing technology also has the potential to produce drugs in a multidose form that includes multiple ingredients for patients with multiple chronic diseases (Ventola, 2014). In general, multidose and personalized drug development using automated 3D printing systems is a future goal for 3D printing technology, and it provides efficient drug delivery.

REFERENCES

Angelopoulos, I., Allenby, M. C., Lim, M., & Zamorano, M. (2019). Engineering inkjet bioprinting processes toward translational therapies. *Biotechnology and Bioengineering, 117* (1), 272-284. doi:10.1002/bit.27176

Berg J, Hiller T, Kissner MS, et al. Optimization of cell-laden bioinks for 3D bioprinting and efficient infection with influenza A virus. Sci Rep. 2018;8(1):13877. Published 2018 Sep 17. doi:10.1038/s41598-018-31880-x

Clevers H. COVID-19: organoids go viral. *Nat Rev Mol Cell Biol*. 2020;21(7):355-356. doi:10.1038/s41580-020-0258-4

Cui, X., Dean, D., Ruggeri, Z. M., & Boland, T. (2010). Cell damage evaluation of thermal inkjet printed Chinese hamster ovary cells. *Biotechnology and Bioengineering, 106* (6), 963-969. doi:10.1002/bit.22762

Galarraga, J. H., Kwon, M. Y., & Burdick, J. A. (2019). 3D bioprinting via an in situ crosslinking technique towards engineering cartilage tissue. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-019-56117-3

Gillispie, G. J., Park, J., Copus, J. S., Asari, A. K. P. R., Yoo, J. J., Atala, A., & Lee, S. J. (2019). Three-dimensional tissue and organ printing in regenerative medicine. In *Principles of regenerative medicine* (pp. 831-852). Academic Press.

Gudapati, H., Dey, M., & Ozbolat, I. (2016). A comprehensive review on droplet-based bioprinting: Past, present and future. *Biomaterials, 102*, 20-42. doi:10.1016/j.biomaterials.2016.06.012

Guillemot, F., Souquet, A., Catros, S., Guillotin, B., Lopez, J., Faucon, M., . . . Amédée, J. (2010). High-throughput laser printing of cells and biomaterials for tissue engineering. *Acta Biomaterialia, 6* (7), 2494-2500. doi:10.1016/j.actbio.2009.09.029

Guillotin, B., Souquet, A., Catros, S., Duocastella, M., Pippenger, B., Bellance, S., . . . Guillemot, F. (2010). Laser assisted bioprinting of engineered tissue with high cell density and microscale organization. *Biomaterials, 31* (28), 7250-7256. doi:10.1016/j.biomaterials.2010.05.055

Gungor-Ozkerim, P. S., Inci, I., Zhang, Y. S., Khademhosseini, A., & Dokmeci, M. R., (2018). Bioinks for 3D bioprinting: an overview. Biomaterials Science, 6(5), 915–946. https://doi.org/10.1039/c7bm00765e

Ingber, D. E., Mow, V. C., Butler, D., Niklason, L., Huard, J., Mao, J., Yannas, I., Kaplan, D., & Vunjak-Novakovic, G. (2006). Tissue engineering and developmental biology: going biomimetic. *Tissue engineering*, *12*(12), 3265–3283. https://doi.org/10.1089/ten.2006.12.3265

Jakab, K., Norotte, C., Marga, F., Murphy, K., Vunjak-Novakovic, G., & Forgacs, G. (2010). Tissue engineering by self-assembly and bio-printing of living cells. *Biofabrication*, *2*(2), 022001. https://doi.org/10.1088/1758-5082/2/2/022001

Murphy, S. V., & Atala, A. (2014). 3D bioprinting of tissues and organs. *Nature Biotechnology*, (32), 773–785. https://doi.org/https://doi.org/10.1038/nbt.2958

Nakamura, M., Nishiyama, Y., & Henmi, C. (2008). 3D Micro-fabrication by Inkjet 3D biofabrication for 3D tissue engineering Publisher: IEEE. *2008 International Symposium on Micro-NanoMechatronics and Human Science,* 451-456. doi:10.1109/mhs.2008.4752495

Norotte, C., Marga, F. S., Niklason, L. E., & Forgacs, G. (2009). Scaffold-free vascular tissue engineering using bioprinting. *Biomaterials*, *30*(30), 5910–5917. https://doi.org/10.1016/j.biomaterials.2009.06.034

Okumura, N., Ueno, M., Koizumi, N., Sakamoto, Y., Hirata, K., Hamuro, J., & Kinoshita, S. (2009). Enhancement on Primate Corneal Endothelial Cell Survival In Vitro by a ROCK Inhibitor. *Investigative Opthalmology & Visual Science*, *50*(8), 3680. https://doi.org/10.1167/iovs.08-2634

Ouyang, L. (2019). *Study on Microextrusion-based 3D Bioprinting and Bioink Crosslinking Mechanisms*. Singapore: Springer Singapore. doi:https://doi.org/10.1007/978-981-13-9455-3

Pan, C., Bruyas, A., & Yang, Y.P. (2016). Bioprinting for Tissue Engineering and Regenerative Medicine. *Material Matters,* 11(2), 49-55.

Phillippi, J. A., Miller, E., Weiss, L., Huard, J., Waggoner, A., & Campbell, P., Ph.D. (2008). Microenvironments Engineered by Inkjet Bioprinting Spatially Direct Adult Stem Cells Toward Muscle- and Bone-Like Subpopulations. *Stem Cells, 26* (1), 127-134. doi:10.1634/stemcells.2007-0520

Skliutas, E., Lebedevaite, M., Kasetaite, S., Rekštytė, S., Lileikis, S., Ostrauskaite, J., & Malinauskas, M. (2020). A Bio-Based Resin for a Multi-Scale Optical 3D Printing. *Scientific Reports*, *10*(1). https://doi.org/10.1038/s41598-020-66618-1

Tappa, K., & Jammalamadaka, U. (2018). Novel biomaterials used in medical 3D printing techniques. Journal of Functional Biomaterials, 9(1), 17. doi:10.3390/jfb9010017

Ventola C. L. (2014). Medical Applications for 3D Printing: Current and Projected Uses. *P & T : a peer-reviewed journal for formulary management*, *39*(10), 704–711.

Visconti, R. P., Kasyanov, V., Gentile, C., Zhang, J., Markwald, R. R., & Mironov, V. (2010). Towards organ printing: engineering an intra-organ branched vascular tree. *Expert Opinion on Biological Therapy*, *10*(3), 409–420. https://doi.org/10.1517/14712590903563352

Wan, Z., Zhang, P., Liu, Y., Lv, L., & Zhou, Y. (2020). Four-dimensional bioprinting: Current developments and applications in bone tissue engineering. *Acta Biomaterialia*, *101*, 26–42. https://doi.org/10.1016/j.actbio.2019.10.038

Yu, Z., Liu, M., Fu, P., Xie, M., Wang, W., & Luo, X. (2012). ROCK inhibition with Y27632 promotes the proliferation and cell cycle progression of cultured astrocyte from spinal cord. *Neurochemistry International*, *61*(7), 1114–1120. https://doi.org/10.1016/j.neuint.2012.08.003

Zhou H, Liu LP, Fang M, Li YM, Zheng YW. A potential ex vivo infection model of human induced pluripotent stem cell-3D organoids beyond coronavirus disease 2019 [published online ahead of print, 2020 Apr 27]. Histol Histopathol. 2020;18223. doi:10.14670/HH-18-223

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Machine Learning Applications in iGEM

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***Abstract- Abstract-* In recent years, computational advances, and massive amounts of data, which in some cases are free for the scientific community, have had a great impact on the development of machine learning methods. High-level tools such as Tensorflow and PyTorch have allowed the development of more complex neural network architectures. Such methods have proved to be efficient enough for cancer detection applications through images, natural language processing, among others. The use of machine learning and deep learning has been very well received in projects aimed at Synthetic Biology at iGEM. For example, to develop cancer detection methods based on the analysis of fragments of tumor material, to find specific cancer markers for hepatocellular carcinoma, and to design diagnostic assays to detect methylated DNA at expected levels.**

Index Terms- iGEM, synthetic biology, liquid biopsy, TensorFlow, datasets, Tooth decay

I. INTRODUCTION

Machine learning has become an immensely powerful tool,that has applications in many different fields. One of these fields is synthetic biology and it has become evident in several teams that participated in the iGEM competition.

For the configuration, programming and training of machine learning, the following tools have been used: TensorFlow, Keras, tidyverse and DNAshapeR. TensorFlow should be written together with Google. It would be useful to give examples of these packages (Tensorflow, 2020).

Keras is a neural network design library, applying best practices to reduce cognitive load: consistent and simple code, minimizes the number of actions required for common use cases and provides clear and actionable error messages. It also has extensive documentation and developer guides. (Keras, 2020).

Tidyverse is a stubborn collection of R packages designed for data science. All packages share a design philosophy, syntax and underlying data structures. Tidyverse helps in the whole process of importing, transforming, visualizing, modeling and communicating all the information that we normally use in data science processes (Tidyverse, 2020). DNAhapeR is an R / BioConductor package for ultra-fast, high-throughput predictions of DNA shape characteristics. The package allows predicting, visualizing and encoding of DNA shape features for statistical learning (Bioconductor, 2019).

II. RESEARCH DESIGN

In 2018, the AFCM-Egypt team implemented a classifier to predict the possibility of TLR binding of various DNA oligonucleotides through the characterization of binding motif sequences and CpG content. In order to achieve that, one million ODN (oligodeoxynucleotides) were analyzed, which classified either immunomodulators or non-immunomodulators with which the model was built in TensorFlow (iGEM AFCM-Egypt, 2018) .

In 2019, the William_and_Mary team used web scraping and machine learning tools to create a database of different tools and projects that the iGEM teams have carried out, which is automatically updated, and with the aim of the database is to provide an efficient registry so that new teams can be inspired by previous projects to better serve their communities (iGEM William_and_Mary, 2019).

In 2020, the Heidelberg team aims to use different machine learning techniques for the rational design of functional RNAs. The project will carry out the design of the secondary structure of RNA, learning by imitation for the design, design of trans-splicing ribosomes, generation of protein-RNA binding pairs and modeling of dynamic regulatory networks (iGEM Heidelberg, 2019).

However, this year is not the first year when team “Heidelberg” has utilized machine learning in their project. In the “DeeProtein” project, made in 2017, it was required to analyze a large amount of data about the proteins studied in the project. To carry out this analysis, the team developed their own neural network to predict protein function based solemnly on their sequence. To train the neural network, SwissProt and Uniprot datasets were used, comprising around 7 million proteins with their respective sequences, being able to generate a total of 886 protein functions with an average of 1.3 labels assigned per sequence. The code for the neural network is available in the GitHub repository (iGEM Potsdam, 2019).

The "SYSU-Software" team presented their project that consisted of a system that makes recommendations to users

based on their interests. To make these recommendations, a database developed by NLP (natural language processing) and Random Walk was used for training. The search is carried out by consulting similar keywords in the database of the unknown word offered by users and then recommending the genetic parts that are highly related to keywords entered by users. To search for similar keywords efficiently, the k-d tree algorithm, a fast binary tree-based algorithm, was used to implement the k-nearest neighbors' strategy (iGEM SYSU-Software, 2017).

In 2018, the "SKLMT-China" team established a useful software tool named "DePro" based on wet lab results to help people easily find and predict a suitable promoter to adjust gene expression in the synthetic study. DePro is a promoter search and force prediction website based on Deep Learning and Python. With the expansion of the promoter data, the strength of the new promoter can be quickly calculated with the help of the proposed model, after entering the central sequence, the program written in Python will calculate the promoter's strength. So, researchers can exchange their data and share their results, then, the software is further enriched with the new data entered (iGEM SKLMT-China, 2018).

III. RESULTS AND FINDINGS

To begin to analyze it is important to begin by detailing that in the branch of synthetic biology the use of bioinformatics tools has increased, which is the use of programs for the analysis of results.

In the AFCM-Egypt team project.MiRNAs were used as master keys to restore the balance of deregulated pathways in cancer. We begin with the construction of a disease miRNA network by selecting potential miRNAs that have an apoptotic effect against colorectal cancer cells.

The team has developed their algorithm based on convolutional neural networks (CNN). In the first instance, a training phase is carried out, where the model is trained with 7 million amino acid sequences, in order to predict the optimal growth temperature of the organism. The process is carried out until the analyzed structure reaches thermal stability.

After the training phase, the previous networks are recycled through pre-trained models, and the use of cross-validation techniques and Gradient Boosting. The information obtained is used to introduce new mutations in a certain protein sequence of interest, in order to find new stable variables.

As described above, the influence and use of machine learning in iGEM projects has been growing more and more. For this reason, it is important to detail how this interaction between machine learning and synthetic biology takes place for future iGEM projects.

This will allow us projects to be increasingly sophisticated in data analysis and can use them to generate more benefits for society, which will be a fundamental pillar so that in the coming years the use of machine learning will be an obligatory tool in the iGEM projects

With the use of machine learning tools, technology has advanced in large fields such as artificial vision, medicine, security, etc. Therefore, it is important that synthetic biology is related to machine learning tools so that it can continue to develop in a better way.

As we have seen, several Igem teams have already implemented these tools obtaining outstanding results, which is why it is essential that the use of machine learning tools be encouraged in future iGEM projects.

IV. DISCUSSION

The "AFCM-Egypt" team have created a consistent database of pragmatic databases with a corresponding label to generalize the data set or ODN (Optical Data Network). They used a deep learning model to classify DNA oligos on the basis of TLR binding. This model is not perfect, and they still need to develop their models to avoid the over fluctuations of complex deep learning models during the process of parameter adjustment.

The "DeeProtein" team has developed an application based on CNN for the creation of thermophonic sequences, protein sequences are not of a size that varies depending on the protein. At this point the results of the different experiments were acceptables. However, there is a lot of work going on in the predictions and the stability of the protein structure.

As is known, convolutional neural networks have a greater impact than deep neural networks on the extraction of features from images. For this reason, data that is not in accordance with the sequence of the proteins, can be increased and it cannot have a greater precision in the use of CNN for this type of application since. There are other neural network approaches that allow working with sequential values (protein sequence), such as LSTM and GRU neural networks, which are a type of neural network with long-term memory units.

After the training phase, the previous networks are recycled through Transfer Learning which consists of storing the knowledge acquired while solving a problem and applying a different but related problem.

Through the use of cross-validation techniques and Gradient Boosting, the information obtained is used to introduce new mutations in a certain protein sequence of interest, in order to find new stable variables.

V. CONCLUSIONS

The flexibility of the syntax of the Python programming language and the application and development of new libraries of it, have allowed great exploration in the different areas of data management through the application of machine learning algorithms.

Machine learning algorithms oriented towards the development of synthetic biology such as DNAhapeR, have allowed iGEM teams to use these tools as alternatives for a better performance of their projects, such as synthesizing the idea in a small device, a better analysis of data and better image processing.

The development of these tools requires collaboration between different areas of engineering. Therefore, multidisciplinary work is encouraged, in order to find new fields where machine learning algorithms can improve the performance of a process or address a problem from another approach. Although multidisciplinary work has brought many benefits, it also presents new complications. One of these and the most obvious is the integration or synergy of these disciplines for a final project.

It has been shown in the analysis of the AFCM-Egypt project that there are several data classification methods from machine learning algorithms to obtain a result. Many of them have not been used optimally, so there is a possibility of creating new projects by optimizing the classification methods.

REFERENCES

iGEM AFCM-Egypt (2018). Project description. http://2018.igem.org/Team:AFCM-Egypt

iGEM William_and_Mary (2019). Project description. https://2019.igem.org/Team:William_and_Mary/Description.

iGEM Heidelberg (2019). Project description. https://igem-heidelberg.com/.

iGEM Potsdam (2019). Project description. https://2019.igem.org/Team:Potsdam/Improve

iGEM SYSU-Software (2017). Project description. http://2017.igem.org/Team:SYSU-Software/

iGEM SKLMT-China (2018). Project description http://2018.igem.org/Team:SKLMT-China/SoftWare_Overview

iGEM UC_San_Diego (2018). Project description http://2018.igem.org/Team:UC_San_Diego/

TensorFlow (2020). TensorFlow. Available https://www.tensorflow.org/

Keras (2020). Keras. Available https://keras.io/

Tidyverse (2020). Tidyverse. Available https://www.tidyverse.org/.

Bioconductor (2019). Bioconductor. Available https://www.bioconductor.org/packages/release/bioc/

DNAshapeR (2018). DNAshapeR. Available https://bioconductor.org/packages/release/bioc/html/DNAshapeR.html

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Disasters and Successes of Genetic Engineering

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Abstract-
Genetic Engineering provides clues to solve various problems that humanity faces in the medical, environmental and energy fields. All organisms produced by genetic engineering are by essence Genetically Modified Organisms (GMOs). These GMOs often made the headlines as their status is still in debate. However, should we reject all the possible advances promised by genetic engineering or, on the contrary, try to develop them? In this review article, we first explore the solutions proven to be conclusive, now established worldwide, and then the disasters caused by genetic engineering. Then, we discuss its use while respecting ethical limitations.

Index Terms- Ethics, GMOs, Genetic engineering

I. INTRODUCTION

Genetic engineering (GE) '*is the set of methodologies for altering the genetic material*' of an organism (C. Rey, 2017). The aim is to provide it with new skills to serve a useful purpose. Hence, this field is affecting not only science in general, but also our daily lives and our society. Controlling microorganisms has been a long-envisioned dream that became more reachable in the 1990s with the genomic revolution and the rise of systems biology (i.e. studying biological objects as a whole, at a system level) (Fig. 1). This has resulted in the establishment of a new discipline, genetic engineering, that has in recent years grown considerably and is already transforming biotechnology and medicine. The timeline (Fig.1) shows some breakthroughs-notably the synthesis of insulin (Vecchio et al., 2018) as well as the elaboration of tangible GMOs such as golden rice (Swamy, B. et al., 2019) and HIV resistant babies (Cameron et al., 2014). In this review we discuss and analyze different products of GE as well as their consequences on our society, whether they be positive or negative. We also explore what can be done in order to regulate this discipline and set ethical boundaries.

II. SUCCESSES AND HOPES OF GENETIC ENGINEERING

Today, approximately 100 million people around the world need insulin, which has been used in the treatment of diabetes for over 90 years (Wirtz V. et al., 2016). Without insulin, people living with type 1 diabetes have a bad vital prognosis and many suffer from diabetes-related complications (e.g. blindness, amputation, and kidney failure) and ultimately, die prematurely.
Human insulin was previously extracted from pigs, which was an issue not only because of its ethical complications, but also because of its instability. Today insulin is genetically engineered with a very high stability. Since 2010, many patients treated with it are at some point no longer dependent on insulin (Fig. 1). Another advantage of producing insulin with genetic engineering is that it permits a much higher production rate that was previously not reachable.

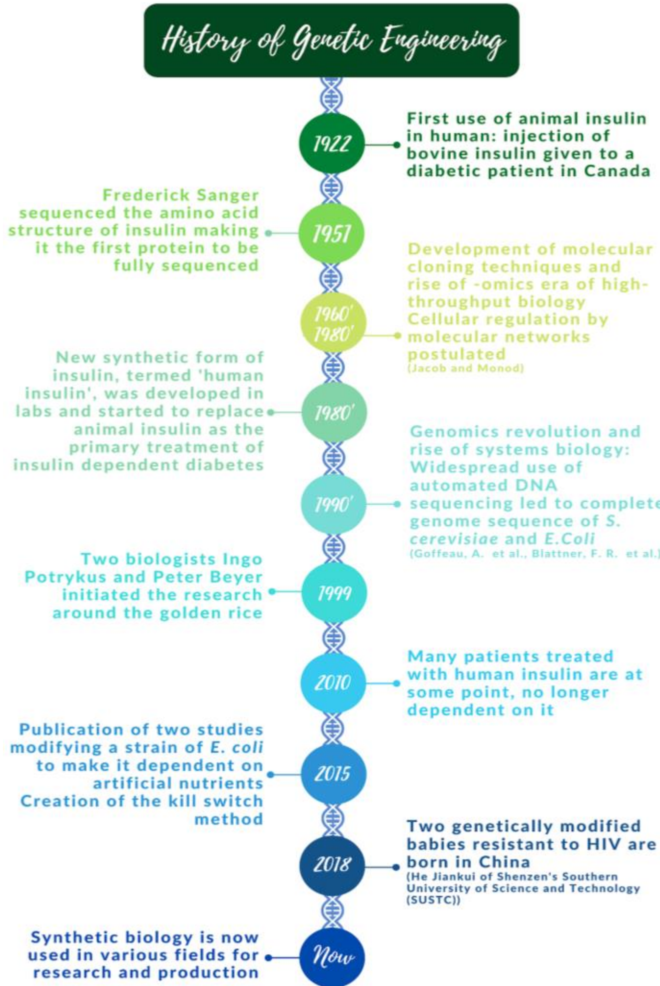

FIGURE 1: Examples of genetic engineering advances

The first injection of animal insulin in humans was given in 1922 to a diabetic patient (Fig. 1). The researchers involved in this breakthrough, Frederick Grant Banting and John James Rickard Macleod, with the help of Charles Best, were honored with the Nobel Prize of Medicine in 1923. At this time, animal insulin caused swellings and pain at the injection sites because it contained multiple contaminants. Between 1930 and 1950, new animal insulin was developed with different action peaks to better stabilize blood sugars (Fig. 1). In 1951, Frederick Sanger sequenced insulin, which made it the first protein to be fully sequenced (Stretton et al., 2002). In the 1970s, purification of animal insulin from bacteria helped reduce allergic reactions that had previously been associated with animal insulin. In the

early 1980s, the synthetic form of insulin was developed in a laboratory and started to replace animal insulin as the primary treatment of insulin dependent diabetes.

The number of people with diabetes increased from 108 million in 1980 to 422 million in 2014; thus, the need for insulin is rising as well. Today, insulin is the most important medication needed in the basic health system. More and more solutions are being developed (e.g insulin pump, artificial pancreas) which would not have been possible without genetic engineering. Indeed, this field of application makes possible the development of new, much more effective treatments and tools (WHO, Diabetes, 2020). The advantages of genetic engineering are numerous, especially with the possibility of large volume production and a better health security. The most used technique is cell culture in a closed environment. The DNA coding sequence for the protein of interest is introduced in bacteria, yeast, or other kinds of cells. The organism will then produce the compound as if it was its own. Nowadays, more than 80 products used in medicine are generated with this method.

Apart from the medical field, this technique can also be applied to the preservation of the environment for example. Within the framework of our project, which lies between public health and environment, we want to introduce DNA coding for enzymes known to be able to degrade antibiotics and pesticides in the *Chlamydomonas reinhardtii* alga. The alga will then be able to degrade those pollutants. Our goal is then to build a filter from this genetically modified alga and integrate it in wastewater treatment plants. However, producing GMOs raises the question: what happens if they end up in the wild? If we take back the example of our project, the bioepuration, which is the purification of pollutants by living beings by fixation, degradation or absorption processes, happens in closed facilities. But there is always a risk of contamination if the microalgae get out of the water tanks. Researchers have been and still are searching for ways to biologically isolate GMOs, so that if they were accidentally released, they would not be able to survive. In 2015, two studies modifying a strain of *E. coli* to make it dependent on artificial nutrients provided in a controlled environment were published (Mandell, D. et al., Rovner A. et al., 2015). Thus allowing much safer usage of the GMOs. This method is called “kill switch.” Since then, other techniques have been developed and the one we have chosen to use for our project is inspired by the iGEM team TU-Munich 2013. Their “kill switch” relied on making the survival of their transgenic moss dependent on a specific infrared wavelength that was absent in the lab (or wastewater tank). Escape from this environment would lead to exposure to red light and activation of the dying process. We plan to use the same principle with the *Chlamydomonas reinhardtii* alga using ultraviolet light.

Therefore, it is essential to take safety into account while designing GMOs to avoid any potential risks they may pose. This allows the use of GMOs broadly and for multiple purposes (medicine, agriculture, research).

III. CONTROVERSIES AND DISASTERS OF GENETIC ENGINEERING

Some GMOs have now become part of our everyday life, such as insulin for diabetics. It goes without saying that many projects are being developed, but some of them are quite controversial--for example, the golden rice, genetically modified with two newly implanted genes that enable the production of beta-carotene, a precursor of vitamin A. According to the World Health Organization (2019), “*In infants and children, vitamin A is essential to support rapid growth and to help combat infections. Inadequate intakes of vitamin A may lead to vitamin A deficiency which can cause visual impairment in the form of night blindness and may increase the risk of illness and death from childhood infections*.” Conventional rice is the staple diet in many countries in Asia where severe vitamin A deficiencies have been found.

Ingo Potrykus and Peter Beyer, two biologists, initiated the research around the golden rice in December 1999 (Potrykus, 1999). They focused on beta-carotene since it is the major carotenoid present in the human diet and an effective source of vitamin A. It is also considered “virtually nontoxic” (Hathcock, J. et al., 2019) So why is it controversial despite there being no apparent danger? The ethical questions raised do not attack the purely scientific aspect of these crops. Indeed, the environmental impact is difficult to assess, since gene transfers are inevitable from one plantation to another, which can compromise food security. Moreover, once approved, there is a risk that plantations will overgrow and thus reach a stage of monoculture. This would not only be problematic for biodiversity, but also it could lead to new deficiencies among the consumers. The challenge will therefore be to communicate with the public about public health. The intervention of various organizations will therefore be necessary, which will prevent the countries concerned from becoming autonomous on this subject. Finally, the risk pointed out by associations such as GreenPeace (Cotter, J., 2013) is that once introduced into people's eating habits, golden rice will set a precedent and give way to other genetically modified foods or organisms.

In fact, now that GMOs have been introduced in food, (genetically modified corn and soy in the US have indeed been used for years), one can expect that it will extend to other domains. Transhumanist currents are becoming more and more widespread and progress in genetic engineering could serve them.

For example, researcher He Jiankui from the Shenzen's Southern University of Science and Technology (SUSTC) announced in 2018 that he had succeeded in modifying the DNA of twin girls to make them more resistant to human immunodeficiency virus as their father was a carrier, thus preventing them from getting AIDS. To do this, he used the CRISPR/Cas9 technique to introduce a 32-base-pair deletion in the CCR5 gene. This gene encodes a HIV receptor located at the surface of T lymphocytes. Unfortunately, very few data on the experiment are known which makes it not fully reliable (Greely, H. T., 2019). What we know is that both babies are what we call “mosaic”: some cells had modified CCR5 genes while other cells did not. This means that the CRISPR “cut” did not do exactly what it intended to do. In fact, it caused changes that had never been observed before. Some papers claim that there are further mutations in the rest of their genome because of off-target edits. The scientific community agrees on the fact that this technology is still too far from being perfect to be used for therapeutic purposes. In this example of GMOs, the main problem raised is that the alteration

took place in the germline, which means that it is inheritable. This treatment therefore affects the human lineage. Moreover, the drift of this affair leaves us doubtful of the limit not to be crossed by international ethical rules. Jiankui carried out his research in spite of these regulations. What will happen if other scientists were to break these rules? It will no longer be a question of modifying the genome for preventive purposes, but of making changes, targeted improvements to the lifestyle we want for our descendants. This is the case of the company Genomic Prediction, which is already seeking to select embryos for implantation that are not at elevated genetic risk for known disorders. The co-founder of the company, Stephen Hsu, says, "*I think people are going to ask for it. If we do not do it, someone else will.*" But is this a valid and sufficient reason?

IV. DISCUSSION

Genetic engineering can bring many effective solutions to our world, but it is also a source of potential problems and raises multiple ethical questions. Several associations and committees have been created over the years to set the limits of what is acceptable and to prevent as much drifts as possible.

In 1964, after the experiments carried out by the Nazis during the Second World War, the World Medical Association wrote the Declaration of Helsinki. This document codified the uses and ethical views on human experimentation. It is addressed primarily to physicians but invites anyone working in medical research to follow its principles. Unfortunately, as a non-legally binding document, the Declaration of Helsinki only has power when it is cited in national regulations (Curran, K., 2020). Its effectiveness and weight therefore remain very limited. In 1993, The International Bioethics Committee of UNESCO was created featuring 36 independent experts from different disciplines (mainly medicine, genetics, law, and philosophy). Their role was to follow the progress in life sciences and its applications to ensure respect for human dignity as well as human rights. To this end, it coordinates the drafting of reports promoting the exchange of knowledge, international cooperation and pushing the ethical and legal reflection of the subjects addressed in order to advise governments. Other structures have also emerged such as the World Commission on the Ethics of Scientific Knowledge and Technology created in 1998 also by UNESCO. Like the previous committee, this one does not have legal weight. It only aims to formulate ethical principles that could provide decision-makers with criteria that extend beyond purely economic considerations.

Even if these entities have not been able to prevent certain disasters like He Jiankui from continuing its research and putting it into action, they remain a means of inducing individual and collective responsibility (IBC, 2019).

V. CONCLUSION

The imbalance of legal power between governments, organizations, and committees does not facilitate international regulations concerning genetic engineering. Especially when the countries concerned do not take the advice of these entities in order to draft binding standards. To establish standards followed by all countries, regulatory entities need to have more legal weight worldwide. This is not possible in today's society because of its political system: each country has its own legislative, executive and judicial powers. Hence, in order to prevent future disasters and create new successes, we must always keep in mind the ethical aspect of every scientific question.

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REFERENCES

Benner, S., Sismour, A. Synthetic biology. *Nat Rev Genet* 6, 533–543 (2005). https://doi.org/10.1038/nrg1637

Cameron, D. E., Bashor, C. J., & Collins, J. J. (2014). A brief history of synthetic biology. *Nature Reviews Microbiology*, 12(5), 381-390. https://doi.org/10.1038/nrmicro3239

Cotter, J., (2013), Golden Illusion, *GreenPeace International,* Amsterdam, The Netherlands

Curran, K., How on earth are we currently regulating human genetic modification?, (2020) *Rising Tide Biology.*

Diabète. (2020, June 8). World Health Organisation OMS https://www.who.int/fr/news-room/fact-sheets/detail/diabetes

Greely, H. T., (2019), CRISPR'd babies: human germline genome editing in the 'He Jiankui affair', *Journal of Law and the Biosciences*, Volume 6, Pages 111–183, (October, 1st 2019) https://doi.org/10.1093/jlb/lsz010

Hathcock, J. N.; Hattan, D. G.; Jenkins, M. Y.; McDonald, J. T.; Sundaresan, P. R.; Wilkening, V. L. Evaluation of vitamin A toxicity. *Am. J. Clin. Nutr.*1990 10.1093/ajcn/52.2.183

"Les enjeux de la biologie de synthèse" Rapport n° 378 (2011-2012) de Mme Geneviève FIORASO, député, fait au nom de l'Office parlementaire d'évaluation des choix scientifiques et technologiques, déposé le 15 février 2012

Les 90 ans de la découverte de l'insuline | Fédération Française des Diabétiques. (2020).

Mandell, D., Lajoie, M., Mee, M. et al. Biocontainment of genetically modified organisms by synthetic protein design. *Nature* 518, 55–60 (2015). https://doi.org/10.1038/nature14121

Mayer, N., Bientôt des bébés sur mesure nés d'une FIV ? (2018) *FuturaSanté*.

Model List of Essential Medicine. (2013, April) World Health Organisation OMS https://apps.who.int/iris/bitstream/handle/10665/93142/EML_18_eng.pdf?sequence=1

Potrykus, I., (1999) Vitamin-A and Iron-Enriched Rices May Hold Key to Combating Blindness and Malnutrition: A Biotechnology Advance, *Nature Biotechnology*, 10.1038/70401

Rovner, A., Haimovich, A., Katz, S. et al. Recoded organisms engineered to depend on synthetic amino acids. *Nature* 518, 89–93 (2015). https://doi.org/10.1038/nature14095

Stretton A. O. (2002). The first sequence. Fred Sanger and insulin. *Genetics,* 162(2), 527–532.

Swamy, B., Samia, M., Boncodin, R., Marundan, S., Rebong, D. B., Ordonio, R. L., Miranda, R. T., Rebong, A., Alibuyog, A. Y., Adeva, C. C., Reinke, R., & MacKenzie, D. J. (2019). Compositional Analysis of Genetically Engineered GR2E "Golden Rice" in Comparison to That of Conventional Rice. *Journal of agricultural and food chemistry*, 67(28), 7986–7994. https://doi.org/10.1021/acs.jafc.9b01524

UNESCO, (n.d.), International Bioethics Committee (IBC) (2020)

UNESCO, (n.d.), Report of the IBC on the Principle of individual responsibility as related to health (2019). https://unesdoc.unesco.org/ark:/48223/pf0000367824

Vecchio, I., Tornali, C., Bragazzi, N. L., & Martini, M. (2018). The Discovery of Insulin: An Important Milestone in the History of Medicine. *Frontiers in endocrinology,* 9, 613. https://doi.org/10.3389/fendo.2018.00613

Veronika Wirtz: global leader in improving access to medicines *The Lancet* 2017 Richard Lane 10.1016/s0140-6736(16)32137-7

Vitamin A supplementation in infants and children 6–59 months of age, (2019) eLENA, World Health Organization, OMS

Website of the company Genomic Prediction : https://genomicprediction.com

World Medical Association, (2018), WMA Declaration of Helsinki - Ethical Principles for medical research involving human subjects.

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Synthetic Biology and Sustainable Development Goals

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***Abstract* - The 2030 Agenda for Sustainable Development, adopted by all United Nations Member States in 2015, provides a shared blueprint for peace and prosperity of the people and the planet, now and in the future. At its heart are the 17 Sustainable Development Goals (SDGs), which are an urgent call for action for all countries - developed and developing - in a global partnership. Synthetic biology is a developing technology of the 21st century, which can be moulded and developed by the UN member states to attain the different goals. Through this article, we highlight previous instances of the utilization of Synthetic Biology in SDGs and all the current plans in progress. We have also included plans currently in development and our opinions on the possible areas of expansion**.

Index Terms - Synthetic biology, Sustainable Development Goals, United Nations, Engineering.

I. INTRODUCTION

Synthetic biology applies engineering principles in biology for designing and developing biological systems or engineering the existing ones. It has embarked on its advancements in various fields which include Agriculture, Health care, Food technology, Biomaterials, Bioremediation, Industrial applications, Cosmetics, Biomanufacturing, and more. Synthetic biologists relentlessly work to deliver risk-free, affordable, eco-friendly, and sustainable products. With innovative projects and an array of countless opportunities, synthetic biology has paved a way towards the achievement of the Sustainable Development Goals. In this article, we have reviewed some works in the field of synthetic biology that have contributed to the achievement of various SDGs.

II. SUSTAINABLE DEVELOPMENT GOALS

Sustainable Development Goals are a set of 17 global goals with 169 targets that intend to achieve a better and sustainable future for everyone, everywhere. Announced in 2015 by the United Nations General assembly, SDGs are to be accomplished by 2030. The objective of SDGs revolves around the four P's: People, Planet, Peace, and Prosperity. All the SDGs are interrelated and balance the three dimensions of sustainable development: economic, social, and environmental. These integrated and indivisible SDGs help in making the world a better place,leaving no one behind. Every SDG has an average of ten targets.(*United Nations Development Programme,* n.d.)

FIGURE 1: The proposed goals

III. WHAT IS SYNTHETIC BIOLOGY?

"What I cannot create, I do not understand."- Richard Feynman

Synthetic biology is an interdisciplinary field that intersects biology, genetics, computational biology, molecular biology, engineering, computer science, and technology. It has the potential to shape the future by providing plausible solutions to numerous real-world problems. With its wide range of application and boundless potential, it is now emerging to be a promising field in solving global issues. Synthetic biologists build or engineer biological systems to produce any desired product, perform any desired function, possess new abilities and more. Advancements in DNA synthesis, microfluidics, and genetic engineering tools have allowed synthetic biologists to develop sensational projects over the years.

"It's much easier to take things apart than put them back together."- Dan Fletcher

Although synthetic biology has seen many ground-breaking works, the field also has its bottlenecks. Starting from understanding the natural complexity of an organism to scaling up the process, synthetic biologists encounter several challenges in every step of the process. Questions of bioethics and biosafety arise as the field deals with living organisms that involve manipulating them or even intaking them for multiple purposes. It demands immense effort to reach out to the public to educate and break the stereotype of using transgenic products. Assembling, testing, and measuring the biological parts used to build biological devices is a time-consuming process as it involves a lot of trial and error. Characterization of the long-term performance, behaviour, and stability of the synthetic circuits is required (Cheng & Lu, 2012). Sustainable funds and willingness to embrace the role of engineering biology in solving societal challenges are much needed (Fletcher, 2018).

IV. SYNTHETIC BIOLOGY AND SUSTAINABLE DEVELOPMENT GOALS

A. *No poverty, zero hunger, good health and well-being, and life on land.*

Synthetic biology plays a vital role in achieving the targets of "Zero Hunger". Farm input, production of more potent fertilizers, plant growth treatments and pesticides that respond to specific conditions or targets are formalized with its aid. Genetically modified crops help in increasing food production and yields, nutritional value, and enhanced taste. Systems metabolic engineering (SysME) has been contributing to the production of food and feed supplements through industrial fermentation of engineered microbes (Yang et al., 2017). The use of synthetic fertilizers over the decades has silenced the ability of the bacteria to naturally fix nitrogen in the soil. Pivot Bio (*Pivot Bio*, n.d.) uses proprietary technology of genetically modified microbes that fix nitrogen and deliver it directly to the roots of the corn plant by creating a symbiotic relationship, thus helping corn growers in better production.

The use of synthetic biology for making the food industry better is increasing day-by-day. It is used to produce safe and high-quality food for humans, feed for animals, bioactive compounds, flavours, colours, additives, processing aids, sensors, and supplements. Synthetic biology, in combination with systems biology, is now being explored to understand and reprogramming of gut micro-flora (van Passel et al., 2011). With synthetic biology, food is being made healthier and better.

The potential of synthetic biology in solving global health issues and contributing to "Good Health and well-being" is multi-fold. Its tools are utilized in developing novel drugs, efficient production of drugs, building diagnostic tools, synthetic DNA vaccine, sustainably and affordably. It also has its application in Synbiotics, a combination of probiotics and prebiotics that act as cost-effective measures against a variety of human ailments by providing various health benefits to the host (Gurry, 2017). With the advancements in gene editing tools such as CRISPR(clustered regularly interspaced short palindromic repeats), synthetic biology enables advancements in many clinical applications. The Chimeric Antigen Receptor (CAR)-T cells are genetically engineered T cells that bind to specific proteins. This is a unique approach used extensively in cancer therapy, targeting an array of cell surface tumour antigens (Sadelain et al., 2013). Commercial production of semi-synthetic artemisinin, an anti-malarial drug, is the first success of synthetic biology in the production of pharmaceutical agents. Semi-synthetic Artemisinin Project engineers S. cerevisiae to produce an artemisinin precursor at high yields, followed by chemical conversion to artemisinin (Paddon & Keasling, 2014). This kind of semi-synthetic approach to produce desired products without any environmental impact on the plant source will also help in reaching the targets of "Life on Land".

B. *Clean water and sanitation, and life below water.*

Probiotic-based sanitation can consistently decrease surface pathogens up to 90% more than conventional disinfectants (Caselli, 2017). Synthetic biology principles are applied to develop biosensors that can detect various chemicals, pathogens, toxins, and induce the production of specific proteins that offset the harmful effect of the toxin. Puraffinity (*Puraffinity*, n.d.) has developed a bio-based Customised Granular Media (CGM), explicitly designed to eliminate poly-fluoroalkyl substances (PFAS) from wastewater. This has applications in various sectors such as Point of use systems, Groundwater remediation, Run-off water from military/air force bases, oil and gas industry, and Industrial manufacturing. Bacterial Biofilms that form on various surfaces that are resistant to antibiotics can cause numerous problems in the industrial and natural environment. To control biofilms, CSIRO (*CSIRO*, n.d.) aims to use synthetic biology to engineer bacteriophages targeted against these biofilms. The field is contributing to the accomplishment of the goal "Clean water and Sanitation" by several means.

Synthetic biologists harness algae and other microbes as an alternative source for fish feed, which helps in regulating overfishing (Oakes & Haydon, 2018). Plastic pollution is reduced using bioengineering principles by upcycling plastic waste into harvestable lipids which could also be used in sustainable aquafeeds.

C. *Affordable and clean energy, industry, innovation and infrastructure, responsible consumption and production, and climate action.*

Synthetic biology helps in achieving sustainability in chemical industries as it uses renewable feedstock. Bio-based production of materials from renewable resources is essential due to climate change and limited resources. Pili (*Pili*, n.d.) has developed an efficient and sustainable process to produce tailored colours adapted to various applications using enzymes that are integrated into a bacteria that uses renewable raw materials. Modern Meadow (*Modern Meadow*, n.d.) delivers biobased materials that are made by growing the cells that are engineered to produce proteins tailored for optimal material attributes, into billions of protein-producing cell factories, the building blocks of the materials.

Global emission of carbon dioxide exceeds 40 billion tons annually. The increase in carbon dioxide concentration causes irreversible climate change. Employing synthetic biology, carbon capture, and reduction of atmospheric carbon can be done by engineering plants, bacteria, algae, and other photosynthetic organisms in several ways. The application of synthetic biology concepts can potentially increase the efficiency of gas fermentation and expand the production of high-value products and commodity chemicals (Liew et al., 2016). Making use of its ability to grow under high osmotic pressure and pH, *Halomonas* strain is engineered to produce chemicals, biofuels, and other valuable compounds (Lorenzo et al., 2018). Oakbio's (*Oakbio - Technology*, n.d.) proprietary biotechnology platform can convert CO_2 from flue gas and other carbon sources into food and feed products, bioplastics, and

chemicals. The bioreactor containing proprietary microbes consume the feed and produce specific products depending on the process.

V. DISCUSSION

Despite all the hurdles, synthetic biologists continue to contribute to the accomplishments of SDGs and will continue to tackle pressing global challenges. Achievements in the last few years and the developing projects assure a promising future for synthetic biology.

VI. CONCLUSION

Synthetic biology is an emerging field with exciting implications for sustainable development and the future of our planet. New projects and technologies further our understanding of biology at the fundamental level every day and help develop innovative solutions to global issues like hunger, health, poverty, and pollution.

REFERENCES

Caselli, E. (2017). Hygiene: microbial strategies to reduce pathogens and drug resistance in clinical settings. *Microbial Biotechnology*, *10*(5), 1079–1083. https://doi.org/10.1111/1751-7915.12755

Cheng, A., & Lu, T. K. (2012). *Synthetic Biology An emerging.pdf*. *April*, 155–178. https://doi.org/10.1146/annurev-bioeng-071811

CSIRO. (n.d.). https://research.csiro.au/synthetic-biology-fsp/research/projects/

De Vos, W. M. (2011). Systems solutions by lactic acid bacteria: From paradigms to practice. *Microbial Cell Factories*, *10*(SUPPL. 1), 1–13. https://doi.org/10.1186/1475-2859-10-S1-S2

Fletcher, D. (2018). Which biological systems should be engineered? *Nature*, *563*(7730), 177–179. https://doi.org/10.1038/d41586-018-07291-3

Gurry, T. (2017). Synbiotic approaches to human health and well-being. *Microbial Biotechnology*, *10*(5), 1070–1073. https://doi.org/10.1111/1751-7915.12789

Liew, F. M., Martin, M. E., Tappel, R. C., Heijstra, B. D., Mihalcea, C., & Köpke, M. (2016). Gas Fermentation-A flexible platform for commercial scale production of low-carbon-fuels and chemicals from waste and renewable feedstocks. *Frontiers in Microbiology*, *7*(MAY). https://doi.org/10.3389/fmicb.2016.00694

Lorenzo, V., Prather, K. L., Chen, G., O'Day, E., Kameke, C., Oyarzún, D. A., Hosta-Rigau, L., Alsafar, H., Cao, C., Ji, W., Okano, H., Roberts, R. J., Ronaghi, M., Yeung, K., Zhang, F., & Lee, S. Y. (2018). The power of synthetic biology for bioproduction, remediation and pollution control. *EMBO Reports*, *19*(4), 4–9. https://doi.org/10.15252/embr.201745658

Modern Meadow. (n.d.). http://www.modernmeadow.com/our-technology/

Oakbio - Technology. (n.d.). http://www.oakbio.com/technology-2/

Oakes, C., & Haydon, I. (2018). *Protect the ocean: Applying synthetic biology based on systems thinking*. Synbiobeta. https://synbiobeta.com/protect-the-ocean-applying-synthetic-biology-based-on-systems-thinking/%0A

Paddon, C. J., & Keasling, J. D. (2014). Semi-synthetic artemisinin: A model for the use of synthetic biology in pharmaceutical development. *Nature Reviews Microbiology*, *12*(5), 355–367. https://doi.org/10.1038/nrmicro3240

Pili. (n.d.). https://www.pili.bio/

Pivot Bio. (n.d.). https://www.pivotbio.com/

Puraffinity. (n.d.). https://www.puraffinity.com/

Sadelain, M., Brentjens, R., & Rivière, I. (2013). The basic principles of chimeric antigen receptor design. *Cancer Discovery*, *3*(4), 388–398. https://doi.org/10.1158/2159-8290.CD-12-0548

United Nations Development Programme. (n.d). Sustainable Development Goals. https://www.undp.org/content/undp/en/home/sustainable-development-goals.html

van Passel, M. W. J., Kant, R., Zoetendal, E. G., Plugge, C. M., Derrien, M., Malfatti, S. A., Chain, P. S. G., Woyke, T., Palva, A., de Vos, W. M., & Smidt, H. (2011). The genome of Akkermansia muciniphila, a dedicated intestinal mucin degrader, and its use in exploring intestinal metagenomes. *PLoS ONE*, *6*(3). https://doi.org/10.1371/journal.pone.0016876

Yang, D., Cho, J. S., Choi, K. R., Kim, H. U., & Lee, S. Y. (2017). Systems metabolic engineering as an enabling technology in accomplishing sustainable development goals. *Microbial Biotechnology*, *10*(5), 1254–1258. https://doi.org/10.1111/1751-7915.12766

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COVID-19: A Current Review On Pathology, Progression, and Intervention

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***Abstract* - COVID-19 possesses a unique immunopathology that scientific efforts from around the globe are currently charting in order to better care for the millions of lives infected by SARS-CoV-2. Here, we review established therapeutics and their mechanisms of action with respect to the infection cycle of the SARs-CoV-2 virus, as well as their immunological implications for a lethal outcome of COVID-19: the cytokine storm.**

Index Terms - COVID-19, cytokine storm, immunology, inflammation

I. INTRODUCTION

The COVID-19 pandemic has changed the rhythm of millions of lives, from the structure of iGEM to hospital protocol to human interaction. As of August 2020, worldwide cases number over 25 million.

The signs of COVID-19 parallel flu-like symptoms with classic symptoms being fever, shortness of breath, and dry cough, and in some cases a loss of sense of smell or taste—the body's response to a viral pathogen. Yet COVID-19 has been more lethal than the flu: one assessment provides statistics that statistics on counted deaths that suggest that the number of COVID-19 deaths for the week ending April 21 was 9.5-fold to 44.1-fold greater than the peak week of counted influenza deaths during the past 7 influenza seasons in the US, with a 20.5-fold mean increase (Faust & Del Rio et al.). One immune phenomenon that is responsible for mortality in severe COVID-19 is the cytokine storm, an excessive misregulation of signalling by intercellular immune messengers collectively known as cytokines. The COVID-19 cytokine storm is characterized by a clinical presentation of overwhelming systemic inflammation, hyperferritinemia, hemodynamic instability, and multi-organ failure; and if left untreated, it may lead to death.
This Review seeks to provide teams with a broad overview of current understanding of existing and possible therapeutic interventions for such immune conditions at each stage of the infection, as well cytokine storm physiology and progression in COVID-19.

II. RESEARCH ELABORATIONS

This Review approaches understanding emerging observations about COVID-19, based upon many sources in the existing literature as well as patterns seen in SARS-CoV, MERS, and other immune-dysfunction diseases. As a disclaimer, many papers published on this topic have not yet been peer reviewed due to the critical nature of the research; furthermore, the details presented here may change with the course of the rapidly developing topic of study.

III. RESULTS AND FINDINGS

SARS-CoV-2 first binds (Fig. 1) to the ACE2 receptor, present mainly on lung and gut cells, with its spike protein. Once bound, it enters the cell via endocytosis or transports its RNA into the cell via membrane fusion.

Hydroxychloroquine and chloroquine, two small, basic molecules first used in the treatment of malaria and rheumatic autoimmune diseases, have been explored as treatments for malaria, rheumatic autoimmune diseases, and until its emergency use authorization (EUA) was revoked in the US in June 2020, in COVID-19 (Schrezenmeier et al.). These molecules have been shown to accumulate in the endosome and lysosome compartments, where their alkalinity is thought to interfere with Toll-like receptor activation, as well as antigen presentation in lymphocytes and the subsequent inflammatory immune response–desirable qualities in preventing the body from overworking. However, the compounds were not able to show sufficient antiviral activity *in vivo* at the quantity prescribed for COVID-19. The compounds were suspected to interfere with potassium levels and ion channel function, with several COVID-19 patients reporting cardiovascular abnormalities, including arrhythmias and other conduction disorders, after treatment with hydroxychloroquine (Nguyen et al.). Further research is needed before administering such compounds with minimized risk.

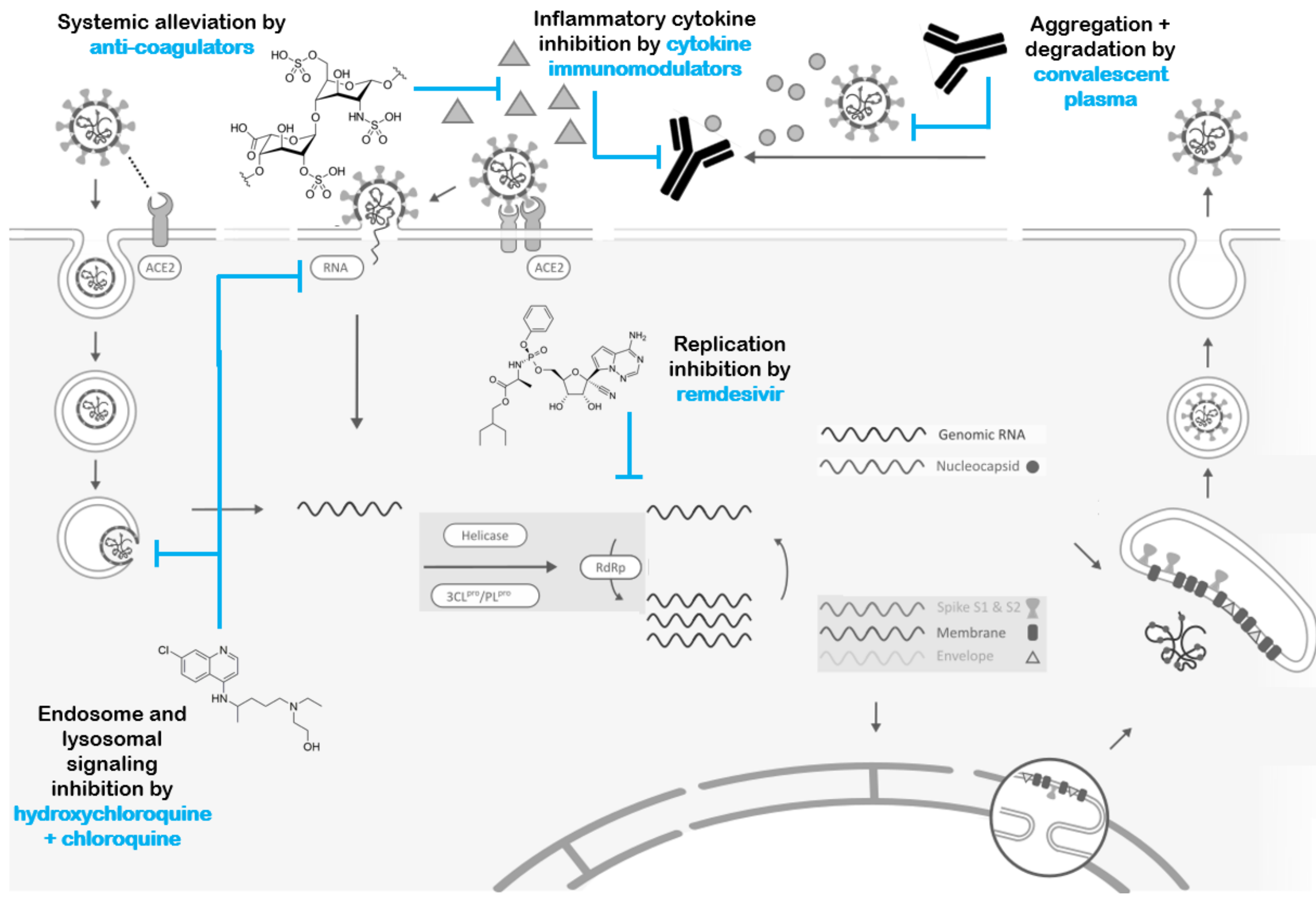

FIGURE 1. Overview of various therapeutic intervention mechanisms for COVID-19 to date. Adapted from viral life cycle diagram by Pampel, J., 2020

When entering the cell, the SARS-CoV-2 viroporin 3a protein activates the NOD-like receptor protein 3 inflammasome, causing IL-1β production which contributes to the cytokine storm (Song et al.). Downstream cytokine activation will be described in the last section.

The viral genetic material begins its replication once the virus enters the cell. Replication of SARS-CoV-2 depends on the viral RNA-dependent RNA polymerase (RdRp), which is likely the target of remdesivir (RDV), a nucleotide analogue that shows broad-spectrum antiviral activity against RNA viruses, such as MERS and Ebola virus. Studies show that incorporation of the active triphosphate form of RDV (RDV-TP) has almost identical results with SARS-CoV, MERS-CoV, and SARS-CoV-2 RdRp whereas RDV-TP was less efficiently incorporated by the distantly related Lassa virus RdRp, thus demonstrating RNA synthesis termination with high target specificity in coronaviruses (Gordon et al). A clinical study published in the *New England Journal of Medicine* in May 2020 compared RDV to a placebo in more than 1,000 hospitalized COVID-19 patients and found that patients who received RDV recovered more quickly than those taking a placebo (a median of 11 days for RDV compared to a median of 15 days for placebo). This was a statistically significant difference. However, RDV was less effective in sicker COVID-19 patients, including those on a ventilator or on a heart-lung machine, raising the question of whether remdesivir reduces the risk of dying from COVID-19 (Beigel et al) .

Upon rounds of successful viral replication, the natural immune response of many individuals (including seniors, the immunocompromised, or those with underlying conditions/ comorbidities) is insufficient to overcome viral infection. This lends to high viral titers within systemic circulation. On August 23, 2020, the FDA authorized an EUA for convalescent plasma to treat COVID-19 in hospitalized patients. Convalescent plasma contains SARS-CoV-2–specific antibody (IgG). In an uncontrolled case study, administration of convalescent plasma improved the clinical status of five patients (Shen et al.). However, there has not yet been a large scale clinical trial for this treatment and it remains controversial.

Sustained viral infection poses another concern: the systemic immune response. Upon each step of the viral cycle detailed above, body cells secrete signals of foreign invasion through both inflammatory and anti-inflammatory cytokines to counteract. Precise regulation of cytokine levels is critical to patient outcome; thus, many studies have been and continue to

be undertaken in regards to understanding how individual cytokines play into the cytokine storm phenomenon. Analysis of cytokine levels in plasma of 41 COVID-19 confirmed cases in China revealed elevated levels of IL-1β, IL-7, IL-8, IL-9, IL-10, FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1A, MIP1-B, PDGF, TNF-α, and VEGF in both ICU and non-ICU patients (Ragab et al). In another study, positive COVID-19 patient outcomes were correlated to early secretion of IFN-1 (Acharya et al.). If cytokine storms are untreated, they often cause sepsis conditions in which systemic resources are depleted in over-inflammation. In many cases in COVID-19, this manifests as acute respiratory distress syndrome (ARDS).
One potential treatment for severe COVID-19 patients is heparin, an anticoagulant that has been shown to decrease mortality in patients who have met the sepsis induced coagulopathy criteria of ≥ 4 or patients with markedly elevated D-dimer (Thachil et al.). D-dimer is a biomarker that correlates with elevated pro-inflammatory cytokine levels. Additionally, activation of the coagulation system has been shown to contribute to the pathogenesis of ARDS.

IV. DISCUSSION

The pathology of COVID-19 is multifaceted, still being pieced together through immunological advances of the past, efforts from interdisciplinary biologists, and current events.
Possibilities for future COVID-19 research include the long term effects of COVID-19, what dictates and differentiates asymptomatic patients from symptomatic, and how long antibodies last. Additionally, although an excess of pro-inflammatory cytokines in the cytokine storm is considered to be a major contributing factor, the exact mechanism of ARDS in COVID-19 is not yet fully understood.

V. CONCLUSION

The COVID-19 pandemic has led to significant disruptions in daily life and affected millions of people globally. Because of the urgency of this issue, there have been many experimental treatments that target SARS-CoV-2 at different stages of its entry, signaling, and proliferation inside the cell. Some potential therapeutics focus on calming the cytokine storm, rather than the virus itself, in order to reduce hyperinflammation. There has also been discussion about the cause, mechanisms, and effects of the cytokine storm on ARDS and other symptoms of COVID-19 patients. These issues, along with the safety and efficacy of potential treatments for COVID-19, are still under critical investigation and development.

APPENDIX

ARDS - Acute Respiratory Distress Syndrome
EUS - Emergency Use Authorization
FDA - Food and Drug Administration (USA)
MERS - Middle Eastern Respiratory Syndrome
RDV - remdesivir

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REFERENCES

Acharya, D., Liu, G. and Gack, M., 2020. Dysregulation of type I interferon responses in COVID-19. Nature Reviews Immunology, 20(7), pp.397-398.

Beigel, J. H., Tomashek, K. M., Dodd, L. E., Mehta, A. K., Zingman, B. S., Kalil, A. C., ... & Lopez de Castilla, D. (2020). Remdesivir for the treatment of Covid-19—preliminary report. *New England Journal of Medicine*.

Faust, J. S., & Del Rio, C. (2020). Assessment of deaths from COVID-19 and from seasonal influenza. *JAMA Internal Medicine*.

Gordon, C. J., Tchesnokov, E. P., Woolner, E., Perry, J. K., Feng, J. Y., Porter, D. P., & Götte, M. (2020). Remdesivir is a direct-acting antiviral that inhibits RNA-dependent RNA polymerase from severe acute respiratory syndrome coronavirus 2 with high potency. *Journal of Biological Chemistry*, *295*(20), 6785-6797.

Nguyen, L., Dolladille, C., Drici, M., Fenioux, C., Alexandre, J., Mira, J., Moslehi, J., Roden, D., Funck-Brentano, C. and Salem, J., 2020. Cardiovascular Toxicities Associated With Hydroxychloroquine and Azithromycin. Circulation, 142(3), pp.303-305.

Pampel, J. (2020). SARS-Cov-2 Replication Cycle and Inhibitors [JPG]. Antibodies Online. https://www.antibodies-online.com/resources/18/5410/sars-cov-2-life-cycle-stages-and-inhibition-targets/

Ragab, D., Salah Eldin, H., Taeimah, M., Khattab, R., & Salem, R. (2020). The COVID-19 cytokine storm; what we know so far. *Frontiers in immunology*, *11*, 1446.

Schrezenmeier, E., Dörner, T. Mechanisms of action of hydroxychloroquine and chloroquine: implications for rheumatology. Nat Rev Rheumatol 16, 155–166 (2020).

Shen C, Wang Z, Zhao F, et al. Treatment of 5 Critically Ill Patients With COVID-19 With Convalescent Plasma. JAMA. 2020;323(16):1582–1589. doi:10.1001/jama.2020.4783

Song, P., Li, W., Xie, J., Hou, Y., & You, C. (2020). Cytokine storm induced by SARS-CoV-2. Clinica chimica acta; international journal of clinical chemistry, 509, 280–287. https://doi.org/10.1016/j.cca.2020.06.017

Thachil, J. (2020). The versatile heparin in COVID-19. *Journal of Thrombosis and Haemostasis*, *18*(5), 1020-1022.

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An Intertwined Approach to Synthetic Biology and Synthetic Chemistry for Health and Welfare

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***Abstract*- Over the years, natural products have become a vital basis for synthesising diverse compounds constituting drugs, nutrient supplements, pigments, cosmetics, etc. Combinatorial biosynthesis and mutasynthesis based approaches have played a key role in the efficient biosynthesis of essential natural products and their complex analogues for their commercial exploitation. Here we provide a brief overview of a few instances of the successful implementation of these approaches vis-à-vis four major classes of natural products - terpenoids, aminocoumarins, nonribosomal peptides and polyketides.**

Index Terms- Aminocoumarins, combinatorial biosynthesis, mutasynthesis, nonribosomal peptides, polyketides, terpenoids

I. INTRODUCTION

The remarkable structural and chemical diversity and the wide range of biological activities of natural products, especially secondary metabolites, have led to an indisputable interest in their commercial utilisation in the fields of scientific research, lifestyle, health and welfare. However, despite their great potency for specific functions, the commercial exploitation of these compounds is limited by the setbacks in terms of their other characteristics such as solubility, stability, potential off-targets, toxic effects, etc. Also, while intricately assembled and modified via complex biosynthetic pathways of host organisms, the extraordinarily complex structures of these natural products make their chemical synthesis very challenging due to the involvement of countless steps, diverse and harsh conditions, toxic reagents or solvents, waste byproducts and elaborate purification procedures, made worse by the poor yield and huge expenses incurred in their production. These concerns have led to the advent of cheap, high yielding and eco-friendly approaches, at the interface of synthetic biology and synthetic chemistry, for the efficient biosynthesis of a wide range of natural products and their analogues, with synthetic biology for the cheap and bulk generation of stereochemically complex structures and synthetic chemistry for producing analogues and introducing non-biological functional groups.[1,2]

Combinatorial biosynthesis is a process that utilises genetic engineering to manipulate existing biosynthetic pathways for natural products to obtain new, altered and a diverse range of bioactive structures. It may be employed at the level of precursor modifications, enzymatic alterations, complete pathway-level heterologous recombinations or a mix of all.[1,3]

Another approach is that of mutasynthesis, which involves the use of a mutant microbial strain, with a random or site-directed mutation, to eliminate a key aspect of its biosynthetic pathway. This enables the synthesis of the respective natural product (or its analogues) only on supplementation of the eliminated precursor (or its analogues), providing scope for the generation of modified bioactive compounds.[2]

Both the aforementioned methods have come to be of immense interest in the last one and a half decades for the biosynthesis of essential natural products and their analogues. In this review, we attempt to elucidate how exactly are these approaches implemented sophisticatedly by citing examples of their past successful applications with regards to four key classes of natural products - terpenoids, aminocoumarins, nonribosomal peptides and polyketides.

II. TERPENOIDS

Terpenoids (isoprenoids) are the largest and the most structurally diverse class of secondary metabolites in plants and lower invertebrates, with widespread and crucial applications in therapeutics, cosmetics, vitamins, food, etc.[4,5] The obvious interest in their commercial exploitation is limited by their poor yield and high cost of extraction via chemical processes. This has stimulated the microbial production of terpenoids via much more efficient mechanisms like combinatorial biosynthesis.

Terpenoids are biosynthesised and made diverse by three steps: condensation of isoprene subunits isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP), their cyclisation, and finally, the addition of functional groups at various positions.[6] IPP and DMAPP are mainly synthesised by the dominant mevalonate (MVA) pathway (cytoplasm of eukaryotes and plants) and the non-mevalonate based 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (bacteria and plant plastids) [Appendix, Fig. 1].[7]

Combinatorial biosynthesis of terpenoids has proved to be extremely advantageous over their chemical synthesis with efficient and cheap production of IPP, DMAPP and other key substrates/precursors using heterologous pathways. This was made possible by extensive and elaborate studies directed towards the understanding of complete terpenoid biosynthesis pathways in various organisms to identify measures for enhanced production.

Despite them being the building blocks, IPP and DMAPP accumulation can retard cell growth and hence also the

terpenoid production. Thus, an optimum supply of IPP and DMAPP is necessary and can be achieved by:[7]

1. manipulating the central metabolic pathway - directing the carbon flux towards the primary substrate (acetyl-CoA, G3P, pyruvate) formation
2. blocking the formation of non-essential metabolic intermediates which are not involved in the MVA and MEP pathways
3. overexpressing the enzymes involved in the two pathways
4. enhancing interaction between the two pathways via product-substrate based exchange
5. overproducing and regulating enzymes downstream of IPP and DMAPP to attenuate their toxic accumulation

Variability inducing cyclisation by terpene synthases has inherent benefits over the chemical synthesis of terpenoids. Hence, identifying terpene synthases that can permit substrates of variable lengths and generating suitable polyisoprenoid diphosphate substrates for these enzymes would lead to efficient combinatorial biosynthesis of terpenoid skeletons.[6]

Zhu *et al*.[8] attempted a targeted metabolic approach and reconstructed certain enzymes of the MVA pathway from *E. coli* and *S. cerevisiae in vitro*. Proteomic and metabolic analyses were done to acquire information on factors that significantly influence terpenoid biosynthesis, like the determination of the best concentrations of IPP, isomerase (Idi) and DMAPP required, identification of optimum ratios of components, alteration of the rate-limiting steps, estimation of the expression of key enzymes and the accumulation of intermediates and co-factors for terpenoid overproduction. The hence obtained information was then used to perform an extremely successful *in vivo* engineering in *E. coli* for an enhanced lycopene (a tetraterpenoid) production of 1.23 g/L (34.3 mg/gDCW) in a 100-L fermenter. Similar targeted approaches have been used for successful combinatorial biosynthesis of terpenoids in other organisms.[9]

III. AMINOCOUMARINS

Aminocoumarin is a family of antibiotics derived from *Streptomyces* species. They compete with ATP for binding irreversibly to the GyrB subunit of heterotetrameric DNA gyrase, essentially inhibiting the ATP-dependent DNA supercoiling catalysed by the gyrase and, thereby, attenuating bacterial division.[10,11]

Three major aminocoumarin antibiotics are known:[10]

1. Novobiocin
2. Coumermycin A1
3. Clorobiocin

Simocyclinone D8, although structurally distinct with a highly complex hybrid structure, is also classified as an aminocoumarin.[12,13]

The narrow target spectrum (ineffectiveness against gram-negative bacteria), slow blood distribution and unfavourable pharmacokinetics due to poor water solubility, and certain eukaryotic off-target effects at higher doses (like on human topoisomerase II) of aminocoumarins led researchers and pharmaceutical companies to shift their focus towards identification and development of other potential antibiotic classes in the early years of discovery of antibiotics.[12,14]

The unregulated use and inadvertent misuse of antibiotics in healthcare and as growth promotional agents in animal husbandry over the last five decades have led to a global antibiotic resistance crisis, necessitating the identification of new antibiotic classes or reappraisal of the previously discontinued classes such as aminocoumarins. This has resulted in a huge boon of scientific efforts redirected at biosynthesising analogues of aminocoumarin compounds with enhanced antibacterial activities and reduced functional limitations.[14]

The renewed interest in developing new aminocoumarin-based antibiotics has largely been addressed through mutasynthesis, which was made possible with the identification of biosynthetic gene clusters of the three aminocoumarin compounds and the generation of diverse mutants of producing strains.[15] For instance, clorobiocin has three principal components: an aminocoumarin moiety, a noviose sugar and a 3-dimethylallyl-4-hydroxybenzoic acid (DMAHB) moiety. The understanding of the importance of the prenyltransferase CloQ in the catalysis of a key step in DMAHB biosynthesis led the researchers to identify a strain of the clorobiocin producing organism *Streptomyces roseochromogenes*, with an inactivated *cloQ* gene, which was unable to synthesise clorobiocin unless supplemented with DMAHB. DMAHB was then substituted with synthetic analogues, which got successfully incorporated into the biosynthetic pathway, resulting in the formation of new and active clorobiocin analogues [Appendix, Fig. 2]. While among the most active compounds synthesised, none could prove to be as effective as clorobiocin, these clorobiocin derivatives exhibited 50-200% of the novobiocin's inhibitory activity on DNA gyrase, indicating scope for improvement.[14]

IV. NONRIBOSOMAL PEPTIDES

Nonribosomal peptides are bacterial and fungal secondary metabolites synthesized by specific nonribosomal peptide synthetases and often having cyclic and/or branched structures, non-proteinogenic amino acids and other diverse modifications. They are used as toxins, siderophores, pigments, antibiotics, immunosuppressants, etc. due to their broad range of biological activities and pharmacological properties.[16]

Certain nonribosomal peptide-based antibiotics, being highly effective against multidrug-resistant bacteria, have also been produced by employing mutasynthesis. For instance, the non-proteinogenic C-terminal amino acid (S)-3,5-dihydroxyphenylglycine (DPg, now DHPG) is a key component of the glycopeptides vancomycin and the related balhimycin. Elimination of its biosynthesis by disruption of the *dpgA* gene in *Amycolatopsis balhimycina*, followed by supplying DPg analogues to the disrupted strains, resulted in the production of novel and active glycopeptide antibiotics [Appendix, Fig. 3].[17]

V. POLYKETIDES

Polyketides are a large group of bioactive secondary metabolites synthesised by the stepwise condensation of diverse coenzyme A ester units of short-chain fatty acids by polyketide synthases (PKSs). They have a broad range of commercial applications as

antibiotics, antifungals, immunosuppressive agents, animal growth promoters, etc.[18]

A variation of mutasynthesis was developed specifically from a comprehensive understanding of the enzymology of modular PKS systems. The method involves site-directed mutagenesis of a multienzyme PKS to inactivate a particular enzymatic function, followed by supplying a thioester as a substrate analogue, leading to its incorporation into the final polyketide.[19]

The normal biosynthesis of the erythromycin macrolide 6-deoxyerythronolide B (6-dEB) [Appendix, Fig. 4] is mediated by the PKS 6-deoxyerythronolide B synthase (DEBS), consisting of three large polypeptides with two complete modules in each, wherein the growing polyketide chain passes from module to module while remaining bound to DEBS through thioester linkages. When DEBS was inactivated by mutating the active site cysteine of the ketosynthase of module 1 to alanine and an N-acetylcysteamine (SNAC) thioester was fed to the strain as a diketide analogue, in place of a diketide itself (which normally binds to the enzyme as a thioester as the natural substrate of module 2), the analogue was processed by the mutant DEBS PKS leading to the formation of the usual product 6-dEB [Appendix, Fig. 4]. The potential of this method was then exhibited for biosynthesis of erythromycin analogues by generating 6-dEB analogues, even the aromatic and ring expanded ones unattainable by synthetic chemistry, using diketide analogues as thioester substrates.[19]

VI. CONCLUSION

The extreme challenges in the chemical synthesis of essential natural products and their analogues have necessitated the use of sophisticated approaches exploiting the pros of a combination of principles from synthetic biology and synthetic chemistry. Combinatorial biosynthesis has been widely used for the generation of terpenoids, facilitated by a comprehensive understanding of the respective microbial biosynthetic pathways. Mutasynthesis has found its applications in the biosynthesis of aminocoumarins and nonribosomal peptides via random mutagenesis-based manipulations of the natural pathways in microbes. A special variation of the mutasynthesis approach, employing a site-directed mutation to specifically attenuate a key function of an essential biosynthetic pathway associated enzyme, arose especially with the interest of generating polyketide analogues using PKS substrate analogues. While synthesising analogues using the aforecited techniques requires designing a new genetic construct with every attempt, both combinatorial biosynthesis and mutasynthesis have proven extremely successful for cheap, high yielding and eco-friendly biosynthesis of a diverse range of bioactive structures, and have paved the way for their efficient commercial exploitation.

APPENDIX

Link for the document containing the cited figures: https://drive.google.com/drive/folders/14qUAPIqiKSskymxdQ9jwtUI7Xkur6sQi?usp=sharing

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REFERENCES

1. Sun, H., Liu, Z., Zhao, H., & Ang, E. L. (2015). Recent advances in combinatorial biosynthesis for drug discovery. Drug design, development and therapy, 9, 823–833. https://doi.org/10.2147/DDDT.S63023
2. Kennedy J. (2008). Mutasynthesis, chemobiosynthesis, and back to semi-synthesis: combining synthetic chemistry and biosynthetic engineering for diversifying natural products. Natural product reports, 25(1), 25–34. https://doi.org/10.1039/b707678a
3. Floss H. G. (2006). Combinatorial biosynthesis--potential and problems. *Journal of biotechnology*, *124*(1), 242–257. https://doi.org/10.1016/j.jbiotec.2005.12.001
4. de las Heras, B., Rodríguez, B., Boscá, L., & Villar, A. M. (2003). Terpenoids: sources, structure elucidation and therapeutic potential in inflammation. Current topics in medicinal chemistry, 3(2), 171–185. https://doi.org/10.2174/1568026033392462
5. Shagufta Perveen (November 5th 2018). Introductory Chapter: Terpenes and Terpenoids, Terpenes and Terpenoids, Shagufta Perveen and Areej Al-Taweel, IntechOpen, DOI: 10.5772/intechopen.79683. Available from:https://www.intechopen.com/books/terpenes-and-terpenoids/introductory-chapter-terpenes-and-terpenoids
6. Bian G, Han Y, Hou A, et al. Releasing the potential power of terpene synthases by a robust precursor supply platform. Metab Eng. 2017;42:1-8. doi:10.1016/j.ymben.2017.04.006
7. Wang, Q., Quan, S., & Xiao, H. (2019). Towards efficient terpenoid biosynthesis: manipulating IPP and DMAPP supply. Bioresources and Bioprocessing, 6, 1-13. DOI: 10.1186/s40643-019-0242-z
8. Zhu, Fayin & Lu, Lei & fu, Shuai & Zhong, Xiaofang & Hu, Mengzhu & Deng, Zixin & Liu, Tiangang. (2014). Targeted engineering and scale up of lycopene overproduction in Escherichia coli. Process Biochemistry, 50(3), 341-346. https://doi.org/10.1016/j.procbio.2014.12.008
9. Bian, G., Deng, Z., & Liu, T. (2017). Strategies for terpenoid overproduction and new terpenoid discovery. Current opinion in biotechnology, 48, 234–241. https://doi.org/10.1016/j.copbio.2017.07.002
10. Heide L. (2014). New aminocoumarin antibiotics as gyrase inhibitors. International journal of medical microbiology : IJMM, 304(1), 31–36. https://doi.org/10.1016/j.ijmm.2013.08.013
11. Anderle, C., Stieger, M., Burrell, M., Reinelt, S., Maxwell, A., Page, M., & Heide, L. (2008). Biological activities of novel gyrase inhibitors of the aminocoumarin class. *Antimicrobial agents and chemotherapy*, *52*(6), 1982–1990. https://doi.org/10.1128/AAC.01235-07
12. Team iGEM Tuebingen 2017 http://2017.igem.org/Team:Tuebingen/Team
13. Bilyk, O., Brötz, E., Tokovenko, B., Bechthold, A., Paululat, T., & Luzhetskyy, A. (2016). New Simocyclinones: Surprising Evolutionary and Biosynthetic Insights. *ACS chemical biology*, *11*(1), 241–250. https://doi.org/10.1021/acschembio.5b00669
14. Galm, U., Heller, S., Shapiro, S., Page, M., Li, S. M., & Heide, L. (2004). Antimicrobial and DNA gyrase-inhibitory activities of novel clorobiocin derivatives produced by mutasynthesis. *Antimicrobial agents and chemotherapy*, *48*(4), 1307–1312. https://doi.org/10.1128/aac.48.4.1307-1312.2004
15. Weist, S., & Süssmuth, R. D. (2005). Mutational biosynthesis--a tool for the generation of structural diversity in the biosynthesis of antibiotics. *Applied microbiology and biotechnology*, *68*(2), 141–150. https://doi.org/10.1007/s00253-005-1891-8
16. Martínez-Núñez, M.A., López, V.E.L.y. (2016). Nonribosomal peptides synthetases and their applications in industry. Sustain Chem Process 4, 13. https://doi.org/10.1186/s40508-016-0057-6
17. Weist, S., Kittel, C., Bischoff, D., Bister, B., Pfeifer, V., Nicholson, G. J., Wohlleben, W., & Süssmuth, R. D. (2004). Mutasynthesis of glycopeptide antibiotics: variations of vancomycin's AB-ring amino acid 3,5-dihydroxyphenylglycine. Journal of the American Chemical Society, 126(19), 5942–5943. https://doi.org/10.1021/ja0499389

18. Shang-Tian Yang, Xiaoguang Liu, Yali Zhang. (2007). Chapter 4 - Metabolic Engineering – Applications, Methods, and Challenges. *Bioprocessing for Value-Added Products from Renewable Resources,* 73-118. https://doi.org/10.1016/B978-044452114-9/50005-0
19. Hartung, I. V., Rude, M. A., Schnarr, N. A., Hunziker, D., & Khosla, C. (2005). Stereochemical assignment of intermediates in the rifamycin biosynthetic pathway by precursor-directed biosynthesis. Journal of the American Chemical Society, 127(32), 11202–11203. https://doi.org/10.1021/ja051430y

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Synthetic Biology Based Medicine Projects from Latin American iGEM Teams

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***Abstract-* Synthetic biology is a biotechnology tool that incorporates engineering principles to harness and enhance biological systems. In biomedicine, it allows the generation of constructs for diagnostics or the modification of biological mechanisms and processes related to disease. The International Genetically Engineered Machine (iGEM) Foundation is one of the main organizations leading the advancement of synthetic biology, which is a competition focused on the development of synthetic biology projects to solve everyday issues. Latin American (LATAM) iGEM teams represent 5.17% of the registered teams worldwide. Among them, 15% have worked on issues related to health and medicine, of which about 70% of these projects have had a great impact in these areas. This work aims to highlight the synthetic biology projects developed by the Latin American teams in biomedicine focused on prevention, diagnosis, and treatment of high-incidence diseases.**

Index terms: Synthetic biology, diseases, diagnostics, therapeutics.

I. INTRODUCTION

Synthetic biology is revolutionizing the world, modifying biological systems for the benefit of the humankind in different areas like biomedicine (Church et al., 2014). An analysis of the distribution of grants for biomedical research by 12 major funders of health research, using data in the World RePORT platform, showed that in 2018 almost 1.62% of grants were awarded to Latin Amerian contries (NIH, 2020).
Since 2006, the "International Genetically Engineered Machine" (iGEM) competition has driven synthetic biology around the world and establishing strategical tracks such as: diagnostic, therapeutic, energy, environment, hardware, food & nutrition, among others. Health-related tracks have allowed the development of projects focused on innovative therapies, diagnostics and treatments systems in Latin America, promoting the advancement of bio-entrepreneurship in the region (Bajak, 2013). This work highlights the synthetic biology projects developed by Latin American iGEM teams in health and medicine applications.

II. RESEARCH ELABORATIONS

Data on iGEM teams between 2004 and 2019 from around the world were obtained from the database on the official iGEM Foundation website (https://igem.org). The criteria for a project selection were: (1) Region "Latin America"; (2) Tracks "Therapeutics", "Diagnostics", "Health & Medicine"; (3) Status "Accepted". Project information was obtained from every team's website.

III. RESULTS AND FINDINGS

Until 2019, 154 Latin American teams have participated in iGEM, representing 5.17% of the total number of registered teams worldwide per each year; 15% of all Latin American teams have worked on projects related to the health area. Besides, it can be noted that about 70% were awarded gold and silver medals (Fig. 1). Latin American iGEM teams' projects are shown in Table 1, and some of them are detailed below.

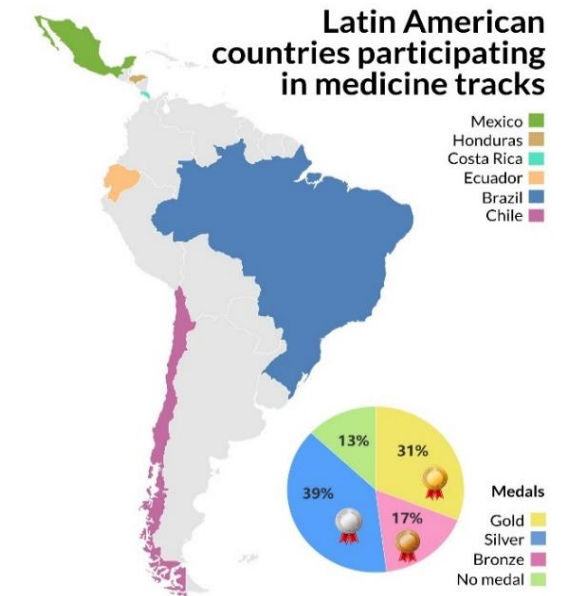

FIGURE 1: iGEM teams from Latin American that have participated in medicine tracks. Pie chart medals and awards obtained (2006-2019).

D. Diagnosis and treatment of cancer

Cancer represents the second cause of death in the world (Bray et al., 2018). An iGEM project involved the production of

bacteriophages capable of interacting cancer cells with apoptine gene and small interfering RNA (siRNA) directed against the survivin gene (apoptosis inhibitory protein) (iGEM Tec-Mty, 2014). On the other hand, Igem LIKA-CESAR (2014), developed a biosensor to detect breast cancer using synthetic biology and robotics. They built a system that can extract DNA/RNA with a quality control named ColiAlert that accounts for the process success.

TABLE 1. Projects of Latin American iGEM teams in the field of health and medicine (2004-2019).

Track/approach	Project summary	Reference
Diagnostics	Design of *Escherichia coli* that responds to changes in glucose concentration. It consists of a biotint encapsulated whole-cell biosensor to detect diabetes.	(iGEM TecMonterrey _GDL, 2019)
	Detection system for Acute Coronary Syndrome based on the identification of biomarkers of ischemia and thus predict a myocardial event to provide rapid treatment.	(iGEM UFMG, 2013)
	Modified *Saccharomyces cerevisiae* that detects L-DNA molecules as colorectal cancer biomarkers in feces using a chimeric protein.	(iGEM UFMG, 2014)
	Printed and modified electrodes with conductive nanocomposites to detect microRNA 155 found in blood samples from breast cancer patients.	(iGEM LIKA-CESAR, 2014)
	Cell surface microchip for early detection of CKD using Cystatin C as a biomarker and a QS system.	(iGEM Brasil-SP, 2014)
	Non-active Cas9 endonuclease (dCas9) to detect the prostate cancer biomarker pCA3 in urine samples.	(iGEM TEC-Costa_Rica, 2016)
	Lactobachilli, a bacteria capable of secreting soluble peptides to inhibit the signaling of IL-6, a molecule associated with depressive disorders.	(iGEM TecMonterrey _GDL, 2018)
	E. coli strain as probiotic, for production of catelicidin LL-37 (an antimicrobial peptide) when it detects a QS marker produced by pathogenic bacteria.	(iGEM UNAM, 2013)
	***Lactobacillus casei* to detect *Clostridium difficile* in the virulence phase and inhibit its growth by expressing a specific endolysin.**	(iGEM Costa_Rica, 2019).
Therapeutics/ Diabetes	*Bacillus subtilis* strain capable of producing and releasing insulin directly into the intestine of patients with diabetes, according to glucose levels from the diet.	(iGEM Unesp_Brazil, 2018).
	Modification of *Lactococcus lactis* to produce an insulin analog capable of being absorbed in the intestinal epithelium.	(iGEM AQA_Unesp, 2017)
	Biocompatible device composed of bacteria capable of generated a protein that detects glycemia and delivering insulin according to the patient's needs.	(iGEM UNAM-CU, 2015)
Therapeutics/ Cancer	Probiotic to colonize tumors in response to tumoral microenvironment and to express α-hemolysin media (HlyA), a therapeutic protein.	(iGEM Amazonas-Brazil, 2019)
	Expression of therapeutic apoptin and death receptor 4 proteins bound to HlyA peptide to induce cancer cell apoptosis.	(iGEM TEC-MTY, 2013)
	Transfection of cancer cells mediated by bacteriophages modified to produce apoptine and silence surviving.	(iGEM TEC-MTY, 2014)
Therapeutics/ Regeneration	**Biomaterial for bone regeneration based on bacterial cellulose and the bone regeneration recombinant protein BMP-2 produced by *E. coli*.**	(iGEM Ecuador, 2018)
	Extracellular matrix made of collagen, heparin, chitosan, and leptin B to reduce wound healing time in people who have suffered burns.	(iGEM TecCEM, 2018)
Therapeutics/ Immunotherapy	*Leishmania* strain optimized to secrete interferon β (IFN-β) to specific macrophages associated to inflammatory joint diseases.	(iGEM UFMG, 2015)
Therapeutics/ Genetic terapy	A preventive gene therapy for atherosclerosis, based on improving the ability of macrophages to enzymatically degrade 7-cholesterol.	(iGEM ITESM-CEM, 2014)
Therapeutics/ Bacteria infection	*E.coli* as a Trojan horse to weaken and break up arginate, component of the biofilm generated by *Pseudomonas aeruginosa,* a pathogen in cystic fibrosis infection.	(iGEM Mayor-Chile, 2014)
Therapeutics/ Vaccine	Peptide-based vaccine produced against the 4 reported serotypes of Dengue virus.	(iGEM UNA_ Honduras, 2015)

E. *Diagnosis and treatment of diabetes*

Diabetes is a disease that is the fourth cause of death in America (Tabish, 2007). One of the iGEM silver medal projects developed a biocompatible device using bacteria capable of generating a chimeric protein that interacts with the glucose-galactose binding protein (GGBP) at the molecular level. GGBP detects glucose concentration in blood and then it delivers insulin according to the patient's needs (iGEM UNAM-CU, 2015).

F. *Tissue regeneration*

Regenerative engineering provides a temporary scaffolding for cells to proliferate and tissue regeneration (Frassica & Grunlan, 2020). C-lastin was a project focused on bone regeneration in case of a human fracture. C-lastin is a biomaterial made from bacterial cellulose that carries the protein BMP-2, responsible for bone fast regeneration. This was a silver medal project proposed by the first Ecuadorian team (iGEM Ecuador, 2018).

G. *Probiotics*

Next-generation therapies are likely to involve beneficial microorganisms in the gastrointestinal tract that can produce and administer therapy within the human body when an abnormality is detected (Pedrolli et al., 2018). DiffEASY, a 2019 project, is a probiotic (*L. casei*) capable of detecting *C. difficile* and

releasing a specific endolysin that inhibits the growth of *C. difficile* (iGEM Costa_Rica, 2019).

H. Other approaches

Some teams have focused on other diseases such as rheumatoid arthritis (RA), an autoimmune disease that manifests as chronic synovitis. In Latin America, there is limited access to early diagnosis of RA (Meier et al., 2013; da Mota et al., 2015). A project that aimed to shed light on this matter involves the use of a visceral *Leishmania* strain to secrete IFN-β on specific macrophages associated to inflammatory diseases. A kill switch system was designed for the genetic control of the 3'-nucleotidase/nuclease enzyme to prevent the parasite spread (iGEM UFMG-Brasil, 2015). Another problem is the lack of a Chronic Kidney Disease (CKD) early-diagnostic; on this regard iGEM Brasil-SP (2014) team designed a biosensor and a Quorum-Sensing (QS) system transducer that is able to diagnose CKD on its early stages, using Cystatin C as a biomarker.

IV. CONCLUSION

Although Latin America represents a low percentage of the total iGEM participating teams, they have developed important projects focused on health and medicine for the diagnosis, prevention and treatment of severe or mortal diseases such as diabetes, cancer, CKD and others. These proposals act as foundations of new and further iGEM projects, as well as the start-point of bio-entrepreneurship promoting the development and the improvement of healthcare and medical areas in the region, and eventually around the world.

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REFERENCES

Bajak, A. (2013). *La ingeniería de la vida en América del Sur*. Retrieved from: http://latinamericanscience.org/spanish/2013/10/igem-en-america-del-sur/

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: *A Cancer Journal for Clinicians*, *68*(6), 394–424. doi: 10.3322/caac.21492

Church, G., Elowitz, M., Smolke, C. et al. (2014). Realizing the potential of synthetic biology. *Nat Rev Mol Cell Biol, 15*(4), 289–294. doi:10.1038/nrm3767.

Da Mota, L. M. H., Brenol, C. V., Palominos, P., & da Rocha Castelar Pinheiro, G (2015). Rheumatoid arthritis in Latin America: the importance of an early diagnosis. *Clinical Rheumatology, 34*(S1), 29–44. doi: 10.1007/s10067-015-3015-x

Frassica, M. T., & Grunlan, M. A. (2020). Perspectives on Synthetic Materials to Guide Tissue Regeneration for Osteochondral Defect Repair. *ACS Biomaterials Science & Engineering*, *6*(8), 4324–4336. doi:10.1021/acsbiomaterials.0c00753

iGEM Amazonas-Brazil team. (2019). Project: Description and Inspiration. https://2019.igem.org/Team:Amazonas-Brazil/Description

iGEM AQA Unesp team. (2017). Insubiota: treating diabetes with genetically engineered probiotic. http://2017.igem.org/Team:AQA_Unesp/Description

iGEM Brasil-SP team. (2014). Project description. http://2014.igem.org/Team:Brasil-SP

iGEM Costa Rica team. (2019). Project description. https://2019.igem.org/Team:Costa_Rica/Description

iGEM Ecuador. (2018). Project description. http://2018.igem.org/Team:Ecuador

iGEM ITESM-CEM team. (2014). Project overview. http://2014.igem.org/Team:ITESM-CEM/Project

iGEM LIKA-CESAR-Brasil team. (2014). Project description. http://2014.igem.org/Team:LIKA-CESAR-Brasil

iGEM-UMayor-Chile team. (2014). Project description. Troy Horse. http://2014.igem.org/Team:UMayor-Chile

iGEM UFMG-Brazil team. (2013). Project Design. http://2013.igem.org/Team:UFMG_Brazil/Ourdesign

iGEM UFMG-Brazil team. (2014). Project description. http://2014.igem.org/Team:UFMG_Brazil

iGEM UFMG-Brazil team. (2015). Project description. http://2015.igem.org/Team:UFMG_Brazil

iGEM UNAM team. (2013). Project description. http://2013.igem.org/Team:Ciencias-UNAM/Project

iGEM UNAM-CU team. (2015). Project Description. http://2015.igem.org/Team:UNAM-CU/project

iGEM UNA Honduras team. (2015). Project description. http://2015.igem.org/Team:UNA_Honduras

iGEM Unesp Brazil team. (2018). Project Description. http://2018.igem.org/Team:Unesp_Brazil/Description

iGEM TecCEM team. (2018). Project Description. http://2018.igem.org/Team:TecCEM/Description

iGEM TEC-Costa_Rica team. (2016). Project description. http://2016.igem.org/Team:TEC-Costa_Rica

iGEM TecMonterrey_GDL team. (2018). Project Description. http://2018.igem.org/Team:TecMonterrey_GDL/Description

iGEM TecMonterrey_GDL team. (2019). Project Description. https://2019.igem.org/Team:TecMonterrey_GDL/Description

iGEM TEC-MTY team. (2013). Project Description. https://2013.igem.org/Team:TecMonterrey/Project.html#BKG

iGEM Tec-MTY team. (2014). Project Overview. http://2014.igem.org/Team:Tec-Monterrey/ITESM14_project.html#tab_overview

Meier, F. M., Frerix, M., Hermann, W., & Müller-Ladner, U. (2013). Current immunotherapy in rheumatoid arthritis. *Immunotherapy, 5*(9), 955–974. doi:10.2217/imt.13.94

NIH. (2020). World RePORT. Bethesda: National Institutes of Health. https://worldreport.nih.gov/.

Pedrolli, D. B., Ribeiro, N. V., Squizato, P. N., de Jesus, V. N., Cozetto, D. A., Tuma, R. B., Gracindo, A., Cesar, M. B., Freire, P. J. C., da Costa, A. F. M., Lins, M. R. C. R., Correa, G. G., & Cerri, M. O. (2018). Engineering Microbial Living Therapeutics: The Synthetic Biology Toolbox. *Trends in Biotechnology*, *37*(1), 100–115. doi: 10.1016/j.tibtech.2018.09.005

Tabish, S. A. (2007). Is Diabetes Becoming the Biggest Epidemic of the Twenty-first Century? *International Journal of Health Sciences, 1*(2), V–VIII.

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A Review of Coral Bleaching Resistance Genes in *Symbiodinium*

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GA_State_SW_Jiaotong, Georgia State University & Southwest Jiaotong University

***Abstract*- Coral bleaching, or the loss of corals' symbiotic algae, is the result of environmental stressors such as excessive heat, light, acidity, and pathogenic microorganisms. This phenomenon causes coral death and may lead to irreversible environmental damage in the form of habitat loss for a quarter of all marine life. Recent studies suggest that thermal stress may impair algal processes such as photosystem I&II, protein phosphorylation, oxidation counseling and programmed cell death, which could all be factors driving algal, or *symbiodinium*, expulsion. Some genes identified in thermally stressed algae were differentially expressed compared to non-stressed algae. These genes include heat shock proteins and antioxidant enzymes such as superoxide dismutase, which indicates they may play a major role in the bleaching process. Recently, an increasing number of coral and algal symbiont genomes have been sequenced. This, along with advances in transformation techniques such as Crispr/Cas9 genome editing, will advance our understanding of coral bleaching and bring remediation closer to success than ever before.**

***Index Terms*-** Coral bleaching, Resistance genes, Symbiodinium, Synthetic Biology

I. INTRODUCTION

The most diverse marine habitats in the world, coral reefs, are suffering. Models have predicted that within this century, most coral reefs will be severely damaged or lost due to coral bleaching (loss of their algal symbionts) unless novel approaches to restore corals are established (Levin et al. 2017). Many stressors contribute to coral bleaching, including extreme temperatures, ocean acidification, high irradiance, heavy metals, and pathogenic microorganisms (Warner et al. 1999, Douglas 2003). *Symbiodinium*, the obligate symbiont of coral, produces nutrients via photosynthesis, which are translocated to the host and can provide up to 100% of coral's required energy in nutrient-deprived tropical waters (Davies et al. 2018). Correspondingly, coral provide algae with access to light, inorganic nutrients, and dissolved inorganic carbon all within a protected microenvironment (Davies et al. 2018). However, with global temperatures on the rise, this symbiotic relationship has been threatened (Douglas 2003). Coral bleaching affects not only the host-symbiont system, but also the reef fishes that rely on these structures (Pratchett et al. 2011). Pratchett et al. suggested that up to 75% of reef fishes heavily rely on coral reefs for food, shelter, and settlement. Loss of coral cover would eventually lead to loss of abundance and biodiversity in these reef environments, which would threaten human food security and lead to billions of US dollars in economic damage (Pratchett et al. 2011). Levin et al. and other researchers are exploring synthetic approaches to coral bleaching by performing genomic analyses and identifying genes that could be linked to bleaching events in the coral and algae (Levin et al. 2016). There are different strains of *Symbiodinium* with varying resistance to bleaching. Differential expression between sensitive and resistant strains may indicate that the genes associated with thermal-sensitive strains can be up or down regulated in order to prevent coral bleaching. Furthermore, new advances in biotechnology such as the CRISPR/CAS-9 system and biolistics show promise in genetic modification of coral's symbiotic algae. These new genetic manipulation techniques for coral reef management could not only promote ecological sustainability, but could also benefit human health and food security. (Levin et al. 2017).

II. RESEARCH ELABORATIONS

A. *Research design*

Articles were compiled by several GSU iGEM members in order to structure a review of studies related to coral bleaching resistant genes within the genus Symbiodinium.

Database
https://docs.google.com/spreadsheets/d/e/2PACX-1vRzSPKnMx0KjTpqEuGQGvVPmyQiDpq_GOyPbsiGoZVWTyiNanwEn8BkjweupikgcPf237OPRYWlPt1c/pub?output=pdf

III. RESULTS AND FINDINGS

A. *Causes and Effects of Coral Bleaching*

High temperatures have been shown to damage the symbiont's photosystem II (PS II) (Warner et al. 1999). Damage to the symbiont's PS II will cause a decline in the D1 protein reaction

center of that photosystem (Warner et al. 1999). The balance between the rate of light-induced damage to photosynthetic proteins and the rate of subsequent cellular repair, including reinsertion of the DI subunit, can be broken by high thermal stress (Weis 2008, Mcginley et al. 2012). This could then result in a loss of transfer of photosynthate to the coral host, thereby adversely affecting the host-symbiont relationship (Warner et al. 1999). Another potential consequence of PS II damage is the overproduction of reactive oxygen species (ROS) (Mydlarz et al. 2010). Reactive oxygen species such as superoxides and peroxides are produced when excessive heat reduces the consumption of ATP and NADPH by the enzyme rubisco, which, in turn, results in a buildup of excitation energy in the PS II (Weis 2008). ROS leakage can oxidatively damage both coral and algal membranes, proteins, and DNA, which is correlated to the expulsion of the algal symbiont from the host cells (Mydlarz et al. 2010). With the increase of ROS concentration and the destruction of photosynthetic function, the antioxidant system in the symbiote collapses and cannot detoxify ROS. The resulting positive feedback loop of accumulating ROS causes further damage to the photosynthetic cells and eventually the expulsion of the algae from the host (Weis 2008).

B. Target Genes

Although thermally tolerant strains of *Symbiodinium* already exist in nature, they tend to have slow growth rates compared to thermally sensitive ones (Karim et al. 2008). The optimal situation would be to have thermally tolerant strains of fast-growing algae. Coral bleaching could potentially be solved by taking advantage of endogenous genes in thermally tolerant *Symbiodinium* that are correlated to bleaching resistance by either up or down regulating them in fast-growing thermally sensitive strains. Analysis of critical target genes during a stress period allows for the identification of bleaching resistance sources. Two transcriptional analyses (Levin et al., 2016 and Gierz, Forêt, & Leggat, 2017) of *Symbiodinium* in response to heat stress revealed the importance of genes related to antioxidant defense, photosynthesis, fatty acid desaturase, meiosis, and RNA binding. In Gierz, Forêt, & Leggat's study comparing the expression levels of thermally stressed *Symbiodinium* clade F to the same clade at control temperatures, HSP70, HSF, UCH (ubiquitin carboxyl-terminal hydroxylase), chlorophyll A-B binding protein, VDE (violaxanthin de-epoxidase), fer4_17 (4FE-4S di cluster domain), ICL (isocitrate lyase family), MORN, SAE2 (DNA repair protein endonuclease), and FATC were upregulated. On the other hand, FHA, DNA photolyase, fer2 (2FE-2S di cluster domain), VDE, cty-B559, cyt-B5 (cytochrome b5-like heme/steroid binding domain), pkinase (protein kinase domain), rad51, TBPIP (tat binding protein), RRM_2 (RNA recognition motif) were all downregulated (Gierz et al. 2017). However, there did not seem to be any pattern with respect to downregulation versus upregulation of these various categories.
Levin et al. compared differentially expressed genes (DEGs) between thermally sensitive and thermally tolerant clade C1 and revealed that Hsp70 and Hsp90 were upregulated in only the thermal tolerant strain on day 13 (Douglas 2003, Levin et al. 2016). Heat shock proteins (Hsps) are molecular chaperones that partake in protecting cellular functions related to protein folding. In the same transcriptomic analysis, DEGs responsible for metabolism, biosynthesis, oxidoreductase activity, and motile cilium were upregulated and appeared to give *Symbiodinium* adaptive mechanisms against heat stress. Upregulated genes including Fe-Sod, Ccpr, Gpx, Txn, Cyp450 reveal the importance of ROS scavenging for thermal tolerance. Recent research by Krueger et al. into antioxidant genes Fe-SOD, Mn-SOD, APX and KatG suggests that the enzyme superoxide dismutase (SOD) acts as the first line of ROS defense in *Symbiodinium*, as it can catalyze the disproportionation of O_2- into H_2O_2 and O_2 to reduce the damage of superoxide to cells (Krueger et al. 2015, Polle 2001).
Although Levin et al. stated that Fe-SOD is a major contributor to thermal tolerance in *Symbiodinium,* another study by Goyen et al. suggested that Mn-SOD was more common than Fe-SOD among *Symbiodinium.* Fe-SOD had little to no expression in some *Symbiodinium,* while both secretory and mitochondrial pathways in *Symbiodinium* contain Mn-SOD. In addition, Mn-SOD may be located in peroxisomes, which produce molecules that readily react to form ROS. So, Mn-SOD may be a valuable target gene to induce bleaching resistance in *Symbiodinium*.

IV. CONCLUSION

Based on the information gathered, the most relevant target genes for coral bleaching resistance are related to ROS scavenging and protein folding in response to heat stress. The genes that stood out the most were those that coded for heat shock protein, heat shock factor, and superoxide dismutase. Synthetic biologists can now take these genes and introduce them into the genomes of thermally sensitive strains of *Symbiodinium.* Hopefully, this will confer bleaching resistance and prevent the destruction of one of the most biodiverse ecosystems on the planet.

REFERENCES

Davies, S.W., Ries, J.B., Marchetti, A., & Castillo, K.D. (2018). Symbiodinium Functional Diversity in the Coral Siderastrea siderea Is Influenced by Thermal Stress and Reef Environment, but Not Ocean Acidification. Frontiers in Marine Science, 5(150).

Douglas, A. E. (2003). Coral bleaching—how and why?. Marine pollution bulletin, 46(4), 385-392.

Gierz, S. L., Forêt, S., & Leggat, W. (2017). Transcriptomic Analysis of Thermally Stressed Symbiodinium Reveals Differential Expression of Stress and Metabolism Genes. Frontiers in Plant Science, 8.

Goyen, S., Pernice, M., Szabó, M., Warner, M. E., Ralph, P. J., & Suggett, D. J. (2017). A molecular physiology basis for functional diversity of hydrogen peroxide production amongst Symbiodinium spp. (Dinophyceae). Marine Biology, 164(3).

Karim, W., Nakaema, S., & Hidaka, M. (2015). Temperature Effects on the Growth Rates and Photosynthetic Activities of Symbiodinium Cells. Journal of Marine Science and Engineering, 3(2), 368-381.

Krueger, T., Fisher, P. L., Becker, S., Pontasch, S., Dove, S., Hoegh-Guldberg, O., . . . Davy, S. K. (2015). Transcriptomic characterization of the enzymatic antioxidants FeSOD, MnSOD, APX and KatG in the dinoflagellate genus Symbiodinium. BMC Evolutionary Biology, 15(1).

Ladner, J. T., Barshis, D. J., & Palumbi, S. R. (2012). Protein evolution in two co-occurring types of Symbiodinium: An exploration into the genetic basis of thermal tolerance in Symbiodinium clade D. BMC Evolutionary Biology, 12(1), 217.
Levin, R. A., Beltran, V. H., Hill, R., Kjelleberg, S., Mcdougald, D., Steinberg, P. D., & Oppen, M. J. (2016). Sex, Scavengers, and Chaperones: Transcriptome Secrets of Divergent Symbiodinium Thermal Tolerances. Molecular Biology and Evolution, 33(11), 3032-3032.
Levin, R. A., Voolstra, C. R., Agrawal, S., Steinberg, P. D., Suggett, D. J., & Oppen, M. J. (2017). Engineering Strategies to Decode and Enhance the Genomes of Coral Symbionts. Frontiers in Microbiology, 8.
Mcginley, M. P., Aschaffenburg, M. D., Pettay, D. T., Smith, R. T., Lajeunesse, T. C., & Warner, M. E. (2012). Transcriptional Response of Two Core Photosystem Genes in Symbiodinium spp. Exposed to Thermal Stress. PLoS ONE, 7(12).
Mydlarz, L. D., Mcginty, E. S., & Harvell, C. D. (2010). What are the physiological and immunological responses of coral to climate warming and disease? Journal of Experimental Biology, 213(6), 934-945.
Polle, A. (2001). Dissecting the superoxide dismutase-ascorbate-glutathione-pathway in chloroplasts by metabolic modeling. Computer simulations as a step towards flux analysis. Plant physiology, 126(1), 445-462.
Pratchett, M.S., Hoey, A.S., Wilson, S.K., Messmer, V. & Graham, N.A. (2011). Changes in Biodiversity and Functioning of Reef Fish Assemblages following Coral Bleaching and Coral Loss. Diversity, 3, 424-452.
Richardson, L. E., Graham, N. A., Pratchett, M. S., Eurich, J. G., & Hoey, A. S. (2018). Mass coral bleaching causes biotic homogenization of reef fish assemblages. Global Change Biology, 24(7), 3117-3129.
Warner, M. E., Fitt, W. K., & Schmidt, G. W. (1999). Damage to photosystem II in symbiotic dinoflagellates: A determinant of coral bleaching. Proceedings of the National Academy of Sciences, 96(14), 8007-8012.
Weis, V. M. (2008). Cellular mechanisms of Cnidarian bleaching: Stress causes the collapse of symbiosis. Journal of Experimental Biology, 211(19), 3059-3066.

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The Potential for Combining Microbial PET Degradation with Microbial Fuel Cell Technology in Order to Improve Water Security, Remediate Plastic Pollution and Produce Renewable Energy

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***Abstract* - The global challenges of water security, plastic pollution and renewable energy are pressing threats that will require innovations in order to tackle them. Fortunately, evolution has developed a rich tapestry of biological technologies. The discovery of naturally occurring plastic-degrading enzymes such as PETases in 2016 represented a huge leap forward in efforts to remediate plastic pollution, whilst the invention of microbial fuel cells in the last century has opened the door to harnessing electron flow produced by exoelectrogens. Since then, microbial fuel cell technology has been adapted for new uses such as microbial desalination cells, which use the electricity to desalinate seawater. These two technologies are now significantly developed individually, but their combination into one device is yet to be thoroughly researched. Through a two-year iGEM project, the team aims to combine these biological technologies by coupling plastic degradation microbial desalination in a single device. Here, we summarise the advancements in both areas and explore the potential for their combination.**

Index Terms - PET degradation, microbial fuel cell, exoelectrogens, microbial desalination cell,

I. INTRODUCTION

Throughout the last century, modernisation has changed the face of global commerce. Advances in industry have generated ever-lighter and more durable materials to keep up with the demand from global economies. As a consequence, plastics such as polyethylene terephthalate (PET) have become a ubiquitous part of 21st century life, appearing abundantly in packaging products, clothing and single-use bottles (Austin et al., 2018). However, the drawbacks of global plastic use are well-documented, especially the impact made on marine ecosystems (Worm, Lotze, Jubinville, Wilcox, & Jambeck, 2017). This impact is compounded by the fact that PET is highly resistant to environmental degradation (Palm et al., 2019), posing a serious threat to ecosystems and biodiversity. By isolating bacteria from a bottle-recycling facility, Yoshida et al. (2016) identified a strain of *Ideonella sakaiensis* that was capable of using two enzymes (PETase & MHETase) to degrade PET into the monomers ethylene glycol and terephthalic acid (Yoshida et al., 2016).

The concept of using microbes to produce electricity dates back to 1911 but was not implemented into the context of microbial fuel cells (MFCs) until late in the 20th century (Santoro, Arbizzani, Erable, & Ieropoulos, 2017). MFCs predominantly rely on exoelectrogenic bacteria that can oxidise a wide range of organic compounds to produce a flow of electrons (Zuo, Xing, Regan, & Logan, 2008). In their native environment, this flow of electrons travels through the respiratory chain to reach external electron acceptors such as Fe(III) oxide (Zue, Xing, Regan, & Logan, 2008) . This process is termed exoelectrogenic electron transfer (EET). MFCs harness this EET by constructing a circuit between an anode and a cathode, each placed in the anodic and cathodic chamber, respectively (Tharali, Sain, & Osborne, 2016). When provided with a suitable organic compound, the exoelectrogen grows as a biofilm on the anode and produces a flow of electrons that travels through the circuit to produce electricity (Tharali, Sain, & Osborne, 2016).

The development of MFCs stimulated further research into novel applications, resulting in the invention of microbial electrolysis cells (MECs) and microbial desalination cells (MDCs) (Santoro, Arbizzani, Erable, & Ieropoulos, 2017). The former uses the electricity to produce hydrogen, whilst the latter uses the electricity to desalinate saltwater (Santoro, Arbizzani, Erable, & Ieropoulos, 2017). This review aims to answer is whether microbial PET degradation can effectively be coupled to MDC technology in order to tackle global challenges. We hypothesise that with the correct genetic engineering and co-culturing approaches, these two technologies can be combined to produce an effective solution. Specifically, we hypothesise that *E. coli* can be engineered to degrade PET into constituent monomers, whilst *P. putida* can be engineered to convert these monomers into lactate which will can supply *S. onediensis* with a carbon source from which to perform EET.

II. RESEARCH ELABORATIONS

Research design

This review article has been written as a systematic literature review, where relevant research is identified and critically appraised, and data from this research is analysed to judge its impact. Relevant research will be accessed through online publication databases such as Pubmed and Web of Science.

III. MICROBIAL PET DEGRADATION ADVANCEMENTS

The crystallinity of PET poses a real problem, because increasing crystallinity negatively impacts on enzymatic PET degradation (Kawai, Kawabata, & Oda, 2019). The initial discovery of the PETase enzyme in 2016 observed its action on PET of 1.9% crystallinity, which is much lower than the crystallinity of PET found in bottles and textiles (Austin et al., 2018). To remedy this, considerable scientific effort has been aimed at elucidating PETase and MHETase structures, in the hopes that the insight gained could lead to improved enzyme activity. X-ray crystallography has been used to identify the active site of the PETase and MHETase enzymes, (Austin et al., 2018; Palm et al., 2019), as well as revealing the enzyme's mechanism to follow the induced-fit model (Palm et al., 2019).

Fortunately, these structural insights have led to rational improvements in PETase and MHETase activity. The same X-ray crystallography studies incorporated rational mutation experiments into their study design, which resulted in a PETase mutant capable of degrading 15% crystalline PET (Austin et al., 2018) and a MHETase mutant displaying roughly twice the MHET turnover rate as the wild type MHETase (Palm et al., 2019). Crucially, PETases have been heterologously expressed in *E. coli*, allowing for the use of PETases in various experimental conditions.

IV. MICROBIAL DESALINATION CELL ADVANCEMENTS

As an adaptation of the MFC technology, MDCs are a relatively recent invention, with the first appearance in the literature dating back only to 2009 (Cao et al., 2009). In this paper, a laboratory-scale MDC was constructed and achieved a salinity removal rate of 90% (Cao et al., 2009). Subsequent research built on this to produce MDC configurations that achieved even higher levels of salinity removal, such as the upflow MDC that achieved over 99% salinity removal in 2011(Jacobson et al., 2011). As of 2018, more than ten different configurations of MDCs have been put forward conferring advantages in different areas such as increased cost effectiveness, reduction in electrode contamination and improved pH control (Al-Mamun, Ahmad, Baawain, Khadem, & Dhar, 2018).

However, these studies have all been conducted on laboratory-scale MDC configurations. These improvements in various aspects of laboratory-scale MDC performance do not yet translate to scaled-up versions of MDCs required for effective implementation. This is because studies suggest that issues encountered by scaled-up MDCs would be harder to remedy, such as the projected difficulty in controlling pH (Saeed et al., 2015) and reducing membrane fouling (Al-Mamun, Ahmad, Baawain, Khadem, & Dhar, 2018).

V. POTENTIAL FOR COMBINING PET DEGRADATION WITH MDC TECHNOLOGY

The first question that must be answered is whether bacteria suitable for both PET degradation and EET processes can be co-cultured successfully. This is because a proposed strategy for coupling these two processes requires the bacterial populations to be in close proximity, so that the PET monomers produced by *E. coli* can diffuse towards the *P. putida* population, and the lactate produced by *P. putida* can diffuse towards the *S. onediensis* biofilm on the anode. Fortunately, *E. coli* and *P. putida* co-cultures have been successfully achieved (Molina-Santiago, Udaondo, Cordero, & Ramos, 2017), as well as with *P. putida* and *S. onediensis* (Wang et al., 2014) and with *E. coli* and *S. onediensis* (Wang et al., 2015).

The next question in need of answering is whether enzymatic degradation of PET would be possible in the anodic chamber of an MDC. The major factors that affect PET biodegradeability are as follows: molecular size, crystallinity, surface topography and hydrophobicity (Kawai, Kawabata, & Oda, 2019). With increased molecular size, PET-degrading enzymes would have access to less surface area and degradation rates would therefore decrease. However, this could be avoided by seeding the anodic chamber with pulverized PET. The high PET crystallinity of bottles and textiles presents a significant challenge, but recent research into UV treatment of PET has been shown to significantly remedy this issue (Falkenstein et al., 2020).

VI. DISCUSSION

From the conducted literature research, the most significant barriers to success for this novel technology seem to be the resistance of highly crystalline (30-40%) PET to degradation by PETase enzymes, along with difficulties in the scale-up of MDC technology. The rational improvements to PET-degrading enzymes show real promise for tackling the first barrier, as mutant variations of PETase have been shown to degrade 15% crystalline PET (Austin et al., 2018). Given that bacterial PETases were only discovered as recently as 2016 (Yoshida et al., 2016), this shows significant progress has been made in a short amount of time. Therefore, one could argue that consistently- enzymatic degradation of highly crystalline PET may be realistically achievable in the next few years.

The research on MDCs presented here is promising. Unfortunately, the field remains undeveloped to argue that the combination of these technologies holds great potential in tackling the aforementioned global challenges. This is because there is no example in the literature of an effectively scaled-up MDC system, making it difficult to stake such a claim.

VII. CONCLUSION

In this review paper, we have summarized the current state of enzymatic PET degradation, its future challenges and possible routes for solving these challenges. The development of MDC technology and difficulties in its scale-up has also been described, identifying this as the major barrier to the potential of combining these technologies to tackle impending global challenges. Using this review, researchers will be able to identify areas of enzymatic PET degradation suitable for further research as well as targeting MDC research towards improving its scale-up. When these two areas have progressed, researchers will be able to return to this review to assess the opportunities in the combination of these technologies.

REFERENCES

Al-Mamun, A., Ahmad, W., Baawain, M. S., Khadem, M., & Dhar, B. R. (2018). A review of microbial desalination cell technology: Configurations, optimization and applications. *Journal of Cleaner Production*, *183*, 458–480. https://doi.org/10.1016/j.jclepro.2018.02.054

Austin, H. P., Allen, M. D., Donohoe, B. S., Rorrer, N. A., Kearns, F. L., Silveira, R. L., … Beckham, G. T. (2018). Characterization and engineering of a plastic-degrading aromatic polyesterase. *Proceedings of the National Academy of Sciences*, *115*(19), E4350–E4357. https://doi.org/10.1073/pnas.1718804115

Cao, X., Huang, X., Liang, P., Xiao, K., Zhou, Y., Zhang, X., & Logan, B. E. (2009). A New Method for Water Desalination Using Microbial Desalination Cells. *Environmental Science & Technology*, *43*(18), 7148–7152. https://doi.org/10.1021/es901950j

Falkenstein, P., Gräsing, D., Bielytskyi, P., Zimmermann, W., Matysik, J., Wei, R., & Song, C. (2020). UV Pretreatment Impairs the Enzymatic Degradation of Polyethylene Terephthalate. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.00689

Jacobson, K. S., Drew, D. M., & He, Z. (2011). Efficient salt removal in a continuously operated upflow microbial desalination cell with an air cathode. *Bioresource Technology*, *102*(1), 376–380. https://doi.org/10.1016/j.biortech.2010.06.030

Kawai, F., Kawabata, T., & Oda, M. (2019). Current knowledge on enzymatic PET degradation and its possible application to waste stream management and other fields. *Applied Microbiology and Biotechnology*, *103*(11), 4253–4268. https://doi.org/10.1007/s00253-019-09717-y

Molina-Santiago, C., Udaondo, Z., Cordero, B. F., & Ramos, J. L. (2017). Interspecies cross-talk between co-cultured *Pseudomonas putida* and *Escherichia coli*. *Environmental Microbiology Reports*, *9*(4), 441–448. https://doi.org/10.1111/1758-2229.12553

Palm, G. J., Reisky, L., Böttcher, D., Müller, H., Michels, E. A. P., Walczak, M. C., … Weber, G. (2019). Structure of the plastic-degrading Ideonella sakaiensis MHETase bound to a substrate. *Nature Communications*, *10*(1), 1717. https://doi.org/10.1038/s41467-019-09326-3

Saeed, H. M., Husseini, G. A., Yousef, S., Saif, J., Al-Asheh, S., Abu Fara, A., … Aidan, A. (2015). Microbial desalination cell technology: A review and a case study. *Desalination*, *359*, 1–13. https://doi.org/10.1016/j.desal.2014.12.024

Santoro, C., Arbizzani, C., Erable, B., & Ieropoulos, I. (2017). Microbial fuel cells: From fundamentals to applications. A review. *Journal of Power Sources*, *356*, 225–244. https://doi.org/10.1016/j.jpowsour.2017.03.109

Tharali, A. D., Sain, N., & Osborne, W. J. (2016). Microbial fuel cells in bioelectricity production. *Frontiers in Life Science*, *9*(4), 252–266. https://doi.org/10.1080/21553769.2016.1230787

Wang, V. B., Chua, S.-L., Cai, Z., Sivakumar, K., Zhang, Q., Kjelleberg, S., … Yang, L. (2014). A stable synergistic microbial consortium for simultaneous azo dye removal and bioelectricity generation. *Bioresource Technology*, *155*, 71–76. https://doi.org/10.1016/j.biortech.2013.12.078

Wang, V. B., Sivakumar, K., Yang, L., Zhang, Q., Kjelleberg, S., Loo, S. C. J., & Cao, B. (2015). Metabolite-enabled mutualistic interaction between Shewanella oneidensis and Escherichia coli in a co-culture using an electrode as electron acceptor. *Scientific Reports*, *5*(1), 11222. https://doi.org/10.1038/srep11222

Worm, B., Lotze, H. K., Jubinville, I., Wilcox, C., & Jambeck, J. (2017). Plastic as a Persistent Marine Pollutant. *Annual Review of Environment and Resources*, *42*(1), 1–26. https://doi.org/10.1146/annurev-environ-102016-060700

Yoshida, S., Hiraga, K., Takehana, T., Taniguchi, I., Yamaji, H., Maeda, Y., … Oda, K. (2016). A bacterium that degrades and assimilates poly(ethylene terephthalate). *Science*, *351*(6278), 1196–1199. https://doi.org/10.1126/science.aad6359

Zuo, Y., Xing, D., Regan, J. M., & Logan, B. E. (2008). Isolation of the Exoelectrogenic Bacterium Ochrobactrum anthropi YZ-1 by Using a U-Tube Microbial Fuel Cell. *Applied and Environmental Microbiology*, *74*(10), 3130–3137. https://doi.org/10.1128/AEM.02732-07

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Development of a Synthetic Probiotic Bacterium Capable of Converting Methylmercury to Elemental Mercury to reduce its Toxic Potential

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***Abstract* - The primary route for human exposure to methylmercury (MeHg) is widely considered as absorption through the gastro-intestinal tract. MeHg is a potent neurotoxin which is produced by marine anaerobic microbes and can naturally bioaccumulate to highly toxic levels through the aquatic food chain. In this study, we aim to genetically engineer a probiotic bacterium – *Escherichia coli* Nissle 1917 that will be capable of converting methylmercury to elemental mercury in conditions prevalent inside the human gut. This exploits the fact that elemental mercury is much less toxic in the gut than MeHg hence providing a proof of concept for the use of such engineered probiotic bacteria as a therapeutic intervention to prevent mercury poisoning and its allied health effects in humans. A novel probiotic bacterium will serve as a long term and efficient solution to this problem of global importance and can be made accessible to many people, in terms of cost as well as usability. This project will also tackle gut inflammation which may otherwise be a deterrent to the positive effects of the project.**

Index Terms – Mercury, Gut inflammation, iGEM, Methylmercury, Probiotics

I. INTRODUCTION

Marine and freshwater animals, fish in particular, form a crucial part of many people's diets. It is one of the most important sources of high-quality protein, providing nearly 17% of animal protein consumed by the world's population (FAO, 2020). According to the Food and Agriculture Organization (FAO, 2020), about 3.3 billion people around the world population rely on fish as a significant source (>20%) of animal protein. As one of the world's primary sources of food, any drastic change to the population or nutritional value of fish will have a considerable impact on the health of humans directly, and indirectly on the global economy. Methylmercury is a highly toxic organometallic compound, and the primary source of exposure of this heavy metal for humans is the consumption of food (primarily but not limited to fish) contaminated with it. (Thackray & Sunderland, 2019). Mercury is a vital component in industries, used to manufacture electrical devices and electronics such as batteries, industrial chemicals. (Nogara et al., 2019). Unfortunately, improper disposal techniques create massive problems that can lead (and has previously led) to catastrophes. Mercury in water bodies (from different sources) is converted by aquatic anaerobic bacteria (such as sulphate-reducing bacteria and iron-reducing bacteria) and fungi into methylmercury and other organic mercury compounds via methylation, which makes the mercury circulating in the environment bio-available (Nogara et al., 2019). These compounds are then absorbed by planktons, considered to be the entry-point of MeHg in the aquatic food web (Wu & Wang, 2011). Fishes and other aquatic animals that depend on these for food, also ingest the toxic organic mercury compounds with them. At each level, the amount of toxic methylmercury dangerously increases, and thus the end consumer can be severely affected (Gilmour & Riedel, 2009). This is called biomagnification and has been well studied (Campbell, Chumchal, Jardine, Lavoie, & Kidd, 2011). As MeHg is fat-soluble, it continues to reside inside the fatty tissues of organisms, resulting in bioaccumulation (Chan, 2011). It is no coincidence that this was responsible for one of the world's environmental disasters, i.e. the Minamata Disease. Also referred to as Chisso-Minamata disease, it is a neurological syndrome caused by severe mercury poisoning (Grandjean et al., 2010; Hong et al., 2012). Mercury poisoning can damage hearing and speech, induce numbness in the hands and feet owing to general muscle weakness. Symptoms also include ataxia, narrowing of the field of vision while in extreme cases - paralysis, coma, insanity, and death can occur (Nogara et al., 2019; Puty et al., 2019). There are studies that suggest that MeHg can pass the blood-placenta barrier and affect foetuses (Aaseth et al., 2020). In this paper, we propose a genetically engineered probiotic bacterium capable of demethylation of methylmercury, which is considered an effective solution to prevent methylmercury poisoning (Syversen & Kaur, 2012). The elemental mercury thus released, is much safer as less than 0.01% of it is absorbed by the intestines (National Research Council, 2000). However, since the release of elemental mercury has the potential to disturb the gut microbiota and induce inflammations, we also explore a mechanism to tackle that problem.

II. DESIGN OF PLASMIDS

Our project includes a dual plasmid system -

A. *Plasmid 1 (Composite BioBrick 1)*

Plasmid 1 will be responsible for the transport of methylmercury inside the bacterial system, production of mercury (II) reductase

enzyme and alkylmercury lyase, along with its regulation, and the production of SoxR which controls the release of IL-10 from Plasmid 2. It features a modified Mer operon with an SYFP2 reporter downstream to all coding regions and control elements in a pSB1C3 vector.

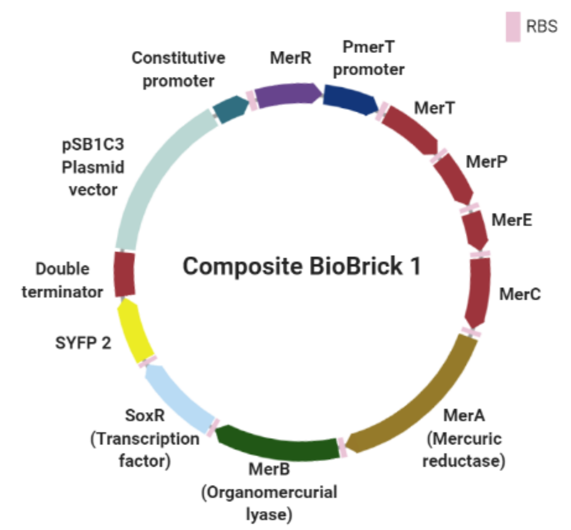

FIGURE 1: Representative Diagram of a successful construct of BioBrick 1

B. Plasmid 2 (Composite BioBrick 2)

Plasmid 2 will have the anti-inflammatory IL-10 and the associated transport and regulatory system.

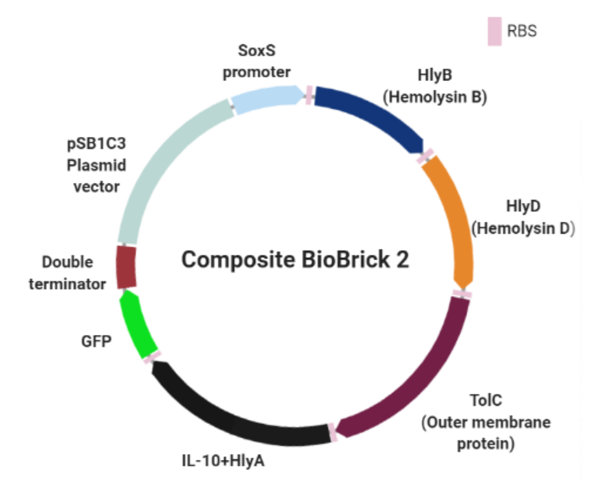

FIGURE 2: Representative Diagram of a successful construct of BioBrick 2

III. WORKING PRINCIPLE

A. For composite BioBrick 1

- A constitutive promoter ensures continuous transcription of MerR (Brown, Wilkie, & Hobman, 2005).

- When translated, MerR produces a weak repressor molecule that can bind to PmerT region, preventing the transcription of genes downstream to it. In the presence of mercury (II) cation, the repressor molecule would not bind to PmerT but instead bind to mercury (II) cation and reactivate the transcription of all downstream elements (Brown et al., 2003; Nakaya et al., 1960; Park et al., 1992; Ralston & O'Halloran, 1990).

- MerP, MerT, MerE, MerC deal with the production of transport proteins that will help transport of methylmercury inside the bacterial system (Barkay et al., 2003; Hamlett et al. 1992; Rossy et al., 2004; Sone et al., 2013; Sone, Nakamura, Pan-Hou, Sato, et al., 2013; Steele & Opella, 1997).

- MerA and MerB produce our dual enzymes – mercuric (II) reductase and alkylmercury lyase required for the conversion of MeHg to elemental Hg (Mathema et al., 2011; Parks et al., 2009).

- The plasmid will also contain the gene for the transcription factor SoxR, which will be useful in activating the anti-inflammatory system present in Plasmid 2 hence allowing for the anti-inflammatory signals to be produced only during the presence of a Hg (II) compound. (Hidalgo et al., 1998; Miki et al., 2008).

- SYFP2 is to assess the functioning of Plasmid 1 components, mainly the MerR regulation. (Gadella Jr., Goedhart, Kremers &, Munster, 2006).

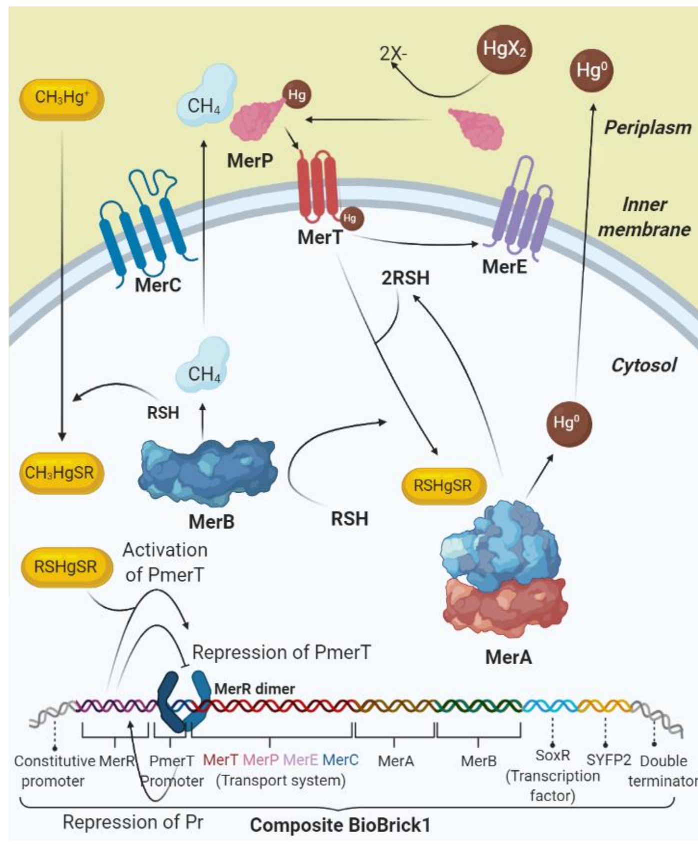

FIGURE 3: Schematic diagram showing the working of BioBrick 1

B. For composite BioBrick 2

- This plasmid will be responsible for the production of IL-10 cytokine (which is an anti-inflammatory cytokine) fused with Hly-A protein; the Hly-A protein will allow for the transport of the cytokine (Elenkov & Chrousos, 1999; Hess et al., 1990; Steidler et al., 2000; Sung et al., 2006)
- This plasmid will also have the Hly-B, Hly-D, TolC producing genes, these proteins help in the transport of any protein that is fused with Hly-A, which in our case is IL-10 (Bavro et al., 2008; Delepelaire, 2004; Gentschev et al., 2002; Schmitt et al., 2003).
- This entire system has a promoter SoxS which is activated by SoxR (produced by the plasmid 1) when it is bound to NO (which is a pro-inflammatory signal) (Demple, Hidalgo & Leautaud, 1998).
- GFP is to assess the functioning of Plasmid 2 components, mainly the SoxS regulation. (Remington, 2011).

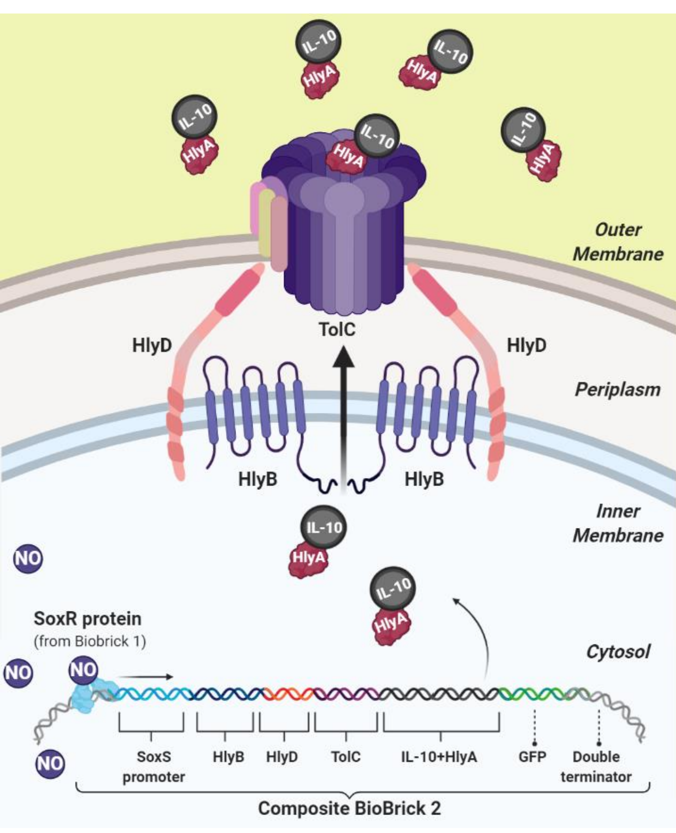

FIGURE 4: Schematic diagram showing the working of BioBrick 2

IV. DISCUSSION

The component parts can be assembled using Gibson Assembly and transformed to *Escherichia coli DH5α* (control chassis) and *Escherichia coli nissle 1917* (probiotic chassis). There is enough evidence to support the use of probiotics as therapeutic interventions (Brüssow, 2019; Jiang et al., 2018; Reid et al., 2014), including *Escherichia coli nissle 1917* (Ou et al., 2016). Data produced from this study can be used to demonstrate that a probiotic bacterium may be developed to look into the issue of methylmercury poisoning or other forms of organic mercury poisoning in humans. The probiotic can have other assisting protective excipients such as naringenin, mangiferin to further tackle the neurotoxic effects of methylmercury compounds (Christina, Das, Krishna Chandran, Mumbrekar & Rao, 2019).

V. CONCLUSION

Methylmercury poisoning is a problem of global importance, and no treatment specific to MeHg exists so far. The success of such a therapeutic intervention will be both cost-effective and efficient. The concept can also be expanded by making use of genetically engineered probiotic bacterium to tackle other forms of heavy-metal poisoning or any other health hazard whose primary form of absorption is through the gastro-intestinal tract. Since our project also briefly tackles inflammation of the gut caused by produced elemental mercury leading to the death of gut microbiota – an engineered probiotic bacterium might also serve as a solution for inflammatory diseases such as Crohn's disease. The final results for this project shall be presented at iGEM 2020.

APPENDIX

http://bit.ly/apdxmit - Details of Parts used in BioBrick 1 and BioBrick 2

REFERENCES

Aaseth, J., Wallace, D. R., Vejrup, K., & Alexander, J. (2020, January). Methylmercury and developmental neurotoxicity: A global concern. *Current Opinion in Toxicology*, *19*, 80–87. https://doi.org/10.1016/j.cotox.2020.01.005

Barkay, T., Miller, S. M., & Summers, A. O. (2003). Bacterial mercury resistance from atoms to ecosystems. *FEMS Microbiology Reviews*, *27*(2–3), 355–384. https://doi.org/10.1016/S0168-6445(03)00046-9

Bavro, V. N., Pietras, Z., Furnham, N., Pérez-Cano, L., Fernández-Recio, J., Pei, X. Y., ... & Luisi, B. (2008). Assembly and Channel Opening in a Bacterial Drug Efflux Machine. *Molecular Cell*, *30*(1), 114–121. https://doi.org/10.1016/j.molcel.2008.02.015

Brown, N. L., Stoyanov, J. V., Kidd, S. P., & Hobman, J. L. (2003). The MerR family of transcriptional regulators. *FEMS Microbiology Reviews*, *27*(2–3), 145–163. https://doi.org/10.1016/S0168-6445(03)00051-2

Brüssow, H. (2019). Probiotics and prebiotics in clinical tests: An update [version 1; peer review: 2 approved]. *F1000Research*, *8*, 1–9. https://doi.org/10.12688/f1000research.19043.1

Campbell, L. M., Chumchall, M. M., Jardine, T. D., Kidd, K. A., & Lavoie, R. (2011). *Biomagnification rate of mercury in aquatic food webs: a world-wide meta-analysis.*

Chan, H. M. (2011). Mercury in Fish: Human Health Risks. *Encyclopedia of Environmental Health*, 697–704. https://doi.org/10.1016/B978-0-444-52272-6.00459-1

Delepelaire, P. (2004). Type I secretion in gram-negative bacteria. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1694*(1-3 SPEC.ISS.), 149–161. https://doi.org/10.1016/j.bbamcr.2004.05.001

Elenkov, I. J., & Chrousos, G. P. (1999). Stress hormones, Th1/Th2 patterns, pro/anti-inflammatory cytokines and susceptibility to disease. *Trends in Endocrinology and Metabolism*, *10*(9), 359–368. https://doi.org/10.1016/S1043-2760(99)00188-5

FAO. (2020). The State of World Fisheries and Aquaculture 2020. In *The State of World Fisheries and Aquaculture 2020*. FAO. https://doi.org/10.4060/ca9229en

Gentschev, I., Dietrich, G., & Goebel, W. (2002). The E. coli α-hemolysin secretion system and its use in vaccine development. *Trends in Microbiology*, *10*(1), 39–45. https://doi.org/10.1016/S0966-

842X(01)02259-4
Gilmour, C., & Riedel, G. (2009). Biogeochemistry of Trace Metals and Mettaloids. *Encyclopedia of Inland Waters*, 7–15. https://doi.org/10.1016/B978-012370626-3.00095-8
Grandjean, P., Satoh, H., Murata, K., & Eto, K. (2010). Adverse effects of methylmercury: Environmental health research implications. *Environmental Health Perspectives*, *118*(8), 1137–1145. https://doi.org/10.1289/ehp.0901757
Hamlett, N. V., Landale, E. C., Davis, B. H., & Summers, A. O. (1992). Roles of the Tn21 merT, merP, and merC gene products in mercury resistance and mercury binding. *Journal of Bacteriology*, *174*(20), 6377–6385. https://doi.org/10.1128/jb.174.20.6377-6385.1992
Hess, J., Gentschev, I., Goebel, W., & Jarchau, T. (1990). Analysis of the haemolysin secretion system by PhoA-HlyA fusion proteins. *MGG Molecular & General Genetics*, *224*(2), 201–208. https://doi.org/10.1007/BF00271553
Hidalgo, E., Leautaud, V., & Demple, B. (1998). The redox-regulated SoxR protein acts from a single DNA site as a repressor and an allosteric activator. *EMBO Journal*, *17*(9), 2629–2636. https://doi.org/10.1093/emboj/17.9.2629
Hobman, J. L., Wilkie, J., & Brown, N. L. (2005). A design for life: Prokaryotic metal-binding MerR family regulators. *BioMetals*, *18*(4), 429–436. https://doi.org/10.1007/s10534-005-3717-7
Hong, Y. S., Kim, Y. M., & Lee, K. E. (2012). Methylmercury exposure and health effects. *Journal of Preventive Medicine and Public Health*, *45*(6), 353–363. https://doi.org/10.3961/jpmph.2012.45.6.353
Jiang, X., Gu, S., Liu, D., Zhao, L., Xia, S., He, X., ... Ge, J. (2018). Lactobacillus brevis 23017 relieves mercury toxicity in the colon by modulation of oxidative stress and inflammation through the interplay of MAPK and NF-κB signaling cascades. *Frontiers in Microbiology*, *9*(OCT), 1–12. https://doi.org/10.3389/fmicb.2018.02425
Kremers, G. J., Goedhart, J., Van Munster, E. B., & Gadella, T. W. J. (2006). Cyan and yellow super fluorescent proteins with improved brightness, protein folding, and FRET förster radius. *Biochemistry*, *45*(21), 6570–6580. https://doi.org/10.1021/bi0516273
Krishna Chandran, A. M., Christina, H., Das, S., Mumbrekar, K. D., & Satish Rao, B. S. (2019). Neuroprotective role of naringenin against methylmercury induced cognitive impairment and mitochondrial damage in a mouse model. *Environmental Toxicology and Pharmacology*, *71*(July), 103224. https://doi.org/10.1016/j.etap.2019.103224
Mathema, V. B., Thakuri, B. C., & Sillanpää, M. (2011). Bacterial mer operon-mediated detoxification of mercurial compounds: A short review. *Archives of Microbiology*, *193*(12), 837–844. https://doi.org/10.1007/s00203-011-0751-4
Miki, K., Watanabe, S., Kita, A., & Kobayashi, K. (2008). Crystal structure of the [2Fe-2S] transcriptional activator SoxR bound to DNA. *Acta Crystallographica Section A Foundations of Crystallography*, *64*(a1), C89–C89. https://doi.org/10.1107/s0108767308097122
Nakaya, R., Nakamura, A., & Murata, Y. (1960). Resistance transfer agents in Shigella. *Biochemical and Biophysical Research Communications*, *3*(6), 654–659. https://doi.org/10.1016/0006-291X(60)90081-4
National Research Council. (2000). Toxicological Effects of Methylmercury. In *Toxicological Effects of Methylmercury*. National Academies Press. https://doi.org/10.17226/9899
Nogara, P. A., Oliveira, C. S., Schmitz, G. L., Piquini, P. C., Farina, M., Aschner, M., & Rocha, J. B. T. (2019). Methylmercury's chemistry: From the environment to the mammalian brain. *Biochimica et Biophysica Acta - General Subjects*, *1863*(12), 129284. https://doi.org/10.1016/j.bbagen.2019.01.006
Ou, B., Yang, Y., Tham, W. L., Chen, L., Guo, J., & Zhu, G. (2016). Genetic engineering of probiotic Escherichia coli Nissle 1917 for clinical application. *Applied Microbiology and Biotechnology*, *100*(20), 8693–8699. https://doi.org/10.1007/s00253-016-7829-5
Park, S. J., Wireman, J., & Summers, A. O. (1992). Genetic analysis of the Tn21 mer operator-promoter. *Journal of Bacteriology*, *174*(7), 2160–2171. https://doi.org/10.1128/jb.174.7.2160-2171.1992
Parks, J. M., Guo, H., Momany, C., Liang, L., Miller, S. M., Summers, A. O., & Smith, J. C. (2009). Mechanism of Hg-C protonolysis in the organomercurial lyase MerB. *Journal of the American Chemical Society*, *131*(37), 13278–13285. https://doi.org/10.1021/ja9016123
Puty, B., Leão, L. K. R., Crespo-Lopez, M. E., Almeida, A. P. C. P. S. C., Fagundes, N. C. F., Maia, L. C., & Lima, R. R. (2019). Association between methylmercury environmental exposure and neurological disorders: A systematic review. *Journal of Trace Elements in Medicine and Biology*, *52*(November 2018), 100–110. https://doi.org/10.1016/j.jtemb.2018.12.001
Ralston, D. M., & O'Halloran, T. V. (1990). Ultrasensitivity and heavy-metal selectivity of the allosterically modulated MerR transcription complex. *Proceedings of the National Academy of Sciences of the United States of America*, *87*(10), 3846–3850. https://doi.org/10.1073/pnas.87.10.3846
Reid, G., Jass, J., Sebulsky, M. T., & Mccormick, J. K. (2014). *Potential Uses of Probiotics in Clinical Practice Potential Uses of Probiotics in Clinical Practice*. *16*(May), 658–672. https://doi.org/10.1128/CMR.16.4.658
Remington, S. J. (2011). Green fluorescent protein: A perspective. *Protein Science*, *20*(9), 1509–1519. https://doi.org/10.1002/pro.684
Rossy, E., Sénèque, O., Lascoux, D., Lemaire, D., Crouzy, S., Delangle, P., & Covès, J. (2004). Is the cytoplasmic loop of MerT, the mercuric ion transport protein, involved in mercury transfer to the mercuric reductase? *FEBS Letters*, *575*(1–3), 86–90. https://doi.org/10.1016/j.febslet.2004.08.041
Schmitt, L., Benabdelhak, H., Blight, M. A., Holland, I. B., & Stubbs, M. T. (2003). Crystal structure of the nucleotide-binding domain of the ABC-transporter haemolysin B: Identification of a variable region within ABC helical domains. *Journal of Molecular Biology*, *330*(2), 333–342. https://doi.org/10.1016/S0022-2836(03)00592-8
Sone, Y., Nakamura, R., Pan-Hou, H., Itoh, T., & Kiyono, M. (2013). Role of MerC, MerE, MerF, MerT, and/or MerP in resistance to mercurials and the transport of mercurials in escherichia coli. *Biological and Pharmaceutical Bulletin*, *36*(11), 1835–1841. https://doi.org/10.1248/bpb.b13-00554
Sone, Y., Nakamura, R., Pan-Hou, H., Sato, M. H., Itoh, T., & Kiyono, M. (2013). Increase methylmercury accumulation in arabidopsis thaliana expressing bacterial broad-spectrum mercury transporter MerE. *AMB Express*, *3*(1), 1–8. https://doi.org/10.1186/2191-0855-3-52
Steele, R. A., & Opella, S. J. (1997). Structures of the reduced and mercury-bound forms of merP, the periplasmic protein from the bacterial mercury detoxification system. *Biochemistry*, *36*(23), 6885–6895. https://doi.org/10.1021/bi9631632
Steidler, L., Hans, W., Schotte, L., Neirynck, S., Obermeier, F., Falk, W., Fiers, W., & Remaut, E. (2000). Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. *Science*, *289*(5483), 1352–1355. https://doi.org/10.1126/science.289.5483.1352
Sung, I. Y., Logsdon, N. J., Sheikh, F., Donnelly, R. P., & Walter, M. R. (2006). Conformational changes mediate interleukin-10 receptor 2 (IL-10R2) binding to IL-10 and assembly of the signaling complex. *Journal of Biological Chemistry*, *281*(46), 35088–35096. https://doi.org/10.1074/jbc.M606791200
Syversen, T., & Kaur, P. (2012). The toxicology of mercury and its compounds. *Journal of Trace Elements in Medicine and Biology*, *26*(4), 215–226. https://doi.org/10.1016/j.jtemb.2012.02.004
Thackray, C. P., & Sunderland, E. M. (2019). Seafood methylmercury in a changing ocean. In A. M. Cisneros-Montemayor, W. W. L. Cheung, & Y. B. T.-P. F. O. Ota (Eds.), *Predicting Future Oceans: Sustainability of Ocean and Human Systems Amidst Global Environmental Change* (pp. 61–68). Elsevier. https://doi.org/10.1016/B978-0-12-817945-1.00006-X
Wu, Y., & Wang, W. X. (2011). Accumulation, subcellular distribution and toxicity of inorganic mercury and methylmercury in marine phytoplankton. *Environmental Pollution*, *159*(10), 3097–3105. https://doi.org/10.1016/j.envpol.2011.04.012

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Pollutant Biosensor with *Shewanella oneidensis* MR-1 MFC

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***Abstract*-** **Water is indispensable to all life on earth, still ocean and water pollution continues to be an issue. To cure our water, the exact issues need to be diagnosed. The current detection process of water pollution is taking a water sample and analyzing it chemically in a lab. This causes issues when trying to monitor continuously. PCB and PFOS are particularly problematic pollutants since PFOS can be dangerous in very small amounts and PCBs accumulate in biological material. To monitor these pollutants, iGEM Stockholm 2020 is trying to create a biosensor (E. coli module) connected to a Microbial fuel cell will Shewanella oneidensis Mr-1 that has metal-reducing capabilities. Shewanella oneidensis Mr-1 can produce an electrical output that would correspond to the amount of pollutants detected by the biosensor. The MFC has been partially successful with biofilm forming on the anode which produces a voltage. The biosensor is still under construction.**

Index Terms - MFC, Fuel cell, iGEM, Shewanella oneidensis., Biofilm.

I. INTRODUCTION

The Baltic sea is an indispensable part of the Nordic and European ecosystem. It is almost completely landlocked and therefore more exposed to industrial and municipal waste. To protect the ecosystem, it is necessary to be aware of what is polluting it. Some pollutants are more difficult to measure and require lengthy and expensive tests to quantify. In the Baltic Sea persistent organic pollutants have proven especially difficult to manage and these substances can be detrimental even in small quantities. PCB (polychlorinated biphenyl) was banned by United States federal law in 1978, and by the Stockholm Convention on Persistent Organic Pollutants in 2001, but it remains a problem. Mutagenic and Toxic effects stem from PBC interfering with hormones in the body. PFOA (perfluorooctanoic acid) was banned within the EU in 2020 - it can hinder the reproductive system and a possible carcinogenic (Swedish chemical Agency, 2020). Currently, the only way to quantify the amount of environmental pollutants is by collecting water samples and chemically analyzing them in a lab. Continuous sampling is needed to monitor the levels of pollutants increasing the possible errors in measurements limiting our knowledge of what is in our seas (Diersing, 2009).

An MFC, microbial fuel cell, is a bio-electrochemical system. An electric current is driven by utilizing bacteria and high energy oxidants for example O_2, that then mimics natural bacterial interactions (Schmidt-Rohr, K. 2020). Generally, there are two types of MFC, mediated and unmediated. In an unmediated MFC, the bacteria usually have electrochemically active redox proteins such as cytochromes on their outer membrane that can transfer electrons directly to the anode. Mediated MFCs, uses a mediator (chemical compound) that transfers the electrons. (Venkata et.al, 2008) (Mohanakrishna et. al., 2008)

Shewanella *oneidensis* MR-1 is known as a" Dissimilatory Metal-Reducing Bacteria (DMRB)" due to their metal reducing ability coupled with their metabolism. Dissimilatory metal reduction is a process used by microbes that conserves energy through oxidation of organic or inorganic electron donors and reduction of metals and metalloids. This process creates a electrochemical gradient which provides the chemical energy for growth (Akob, 2008). A microbial fuel cell is constructed with Shewanella *oneidensis* MR-1 biofilm grown on the anode. If implanted into a functioning system, Shewanella *oneidensis* will produce an electrical output. A biofilm should be established for Shewanella *oneidensis* to perform optimally as a metal reducing bacteria as this increases the anode area exposed to the bacteria. The MtrB operon is a vital part of the electron transfer on the outer membrane and when knocked out Shewanella produce smaller voltages (Wiatrowski et.al. 2006).

A biological sensor or biosensor is an analytical device used to detect a chemical substance. The device combines a physicochemical detector in a biological component(bio-receptor). (Turner, 1987) The bio-receptor can be biomimetic or biologically derived material e.g. enzymes, nucleic acids, cell receptors, organelles, or microorganism. (Dincer, 2019) The bio-receptor binds, interacts, or recognizes the analyte. A biologically sensitive element can also be bioengineered to sense the analyte. Generally, there are two major parts in a biosensor, the detector element (transducer) preceded by the bio-receptor (Wang, J. 2008). The transducer transforms the biological signal to an optical or electrochemiluminescence signal etc. that can be more easily quantified.

A modular system subdivides a system into smaller parts (modules). These modules can be independently modified, created, or changed between different systems. (Bănică et. al., 2012)

Our iGEM project we will combine a biosensor module that produces the quorum sensing molecule (AHL). AHL activates the MtrB operon that aids the cytochrome on the outer

membrane which then creates an electrical output. This output would correspond to the amount of pollutants sensed by the E. coli module. The entire system would work as a biosensor with an electrical output. And a modifiable E. coli sensing module that could sense specific compounds.

II. RESEARCH ELABORATIONS

MFC setup

A hybrid fuel cell was utilized. The cathode side is exposed to air, while the anode side is liquid. Both electrodes are made of carbon paper that has been thermally treated to be hydrophobic. This treatment is to minimize any clogging in the carbon paper that might disturb the flow. Sandwiched between the anode and cathode is a proton membrane to facilitate exchange. The catholyte, inflow of air and the analyte inflow of liquid media (LB) are inoculated with wildtype *Shewanella oneidensis*. The bacteria naturally form a biofilm on the electrode. Since it is in a continuous flow cell, we are unable to disassemble it during runs. When a biofilm forms on the electrode there is going to be an increased voltage output. In similar MFC setups the external resistance ranged between 200-10kΩ. Due to limited available equipment an external resistance of 1kΩ was deemed most appropriate.

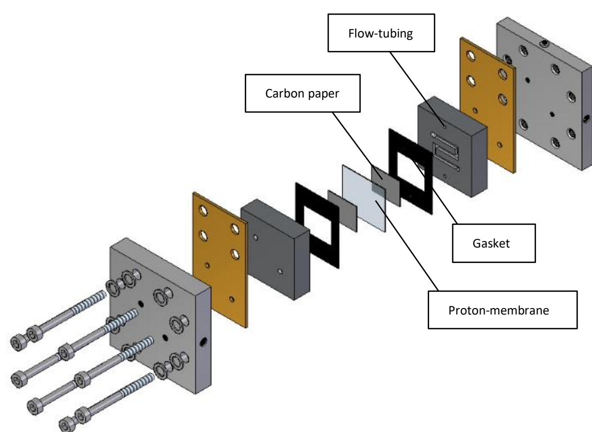

Figure 1: fuel cell

Modular biosensor

The bio-receptor for PFOS detection part is, prmA, a promotor with 3-fold up-regulation of the stress-associated gene, prmA, in Rhodococcus jostii RHA1. The bio-receptor for the PCB part is a combination of bphR1, a positive regulator for bphR1 protein and bphR2. In the absence of biphenyl, small amounts of bphR2 protein binds to bphR2 operator to repress bphR2 transcription (autorepression) and activate bphR1 weakly. When there is biphenyl in the media, bphR2 protein binds to bphR1 operators to strongly activate their transcription.

The bio-receptor part starts the transcription of the QS-molecule (AHL) produced by LuxI which can be measured with a sfGFP fusion to the protein. This allows for measurement of LuxI produced and relation to the production rate of AHL.

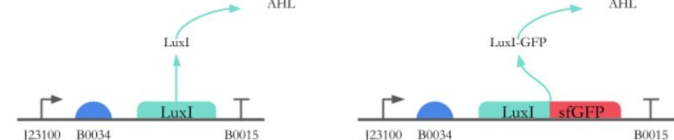

Figure 2, bio-receptor LuxI system

To insert the desired parts into E. coli heat shock transformation and restriction digest/ligation was performed, following NEB standard protocols). To check that the correct inserts have been introduced in the correct loci western blot, gel electrophoresis and sanger sequencing was utilized.

III. RESULTS AND FINDINGS

Due to issues brought on by COVID-19 experiments with the modular biosensor are still in its infancy and no results can be presented yet.

The MFC has been more established, voltage and a viable biofilm has established in the flow cell. The voltage gradually increased and after ca 275h of running, liquid media was changed to fresh LB media without inoculated bacteria. An increase in voltage is illustrated in figure 3.

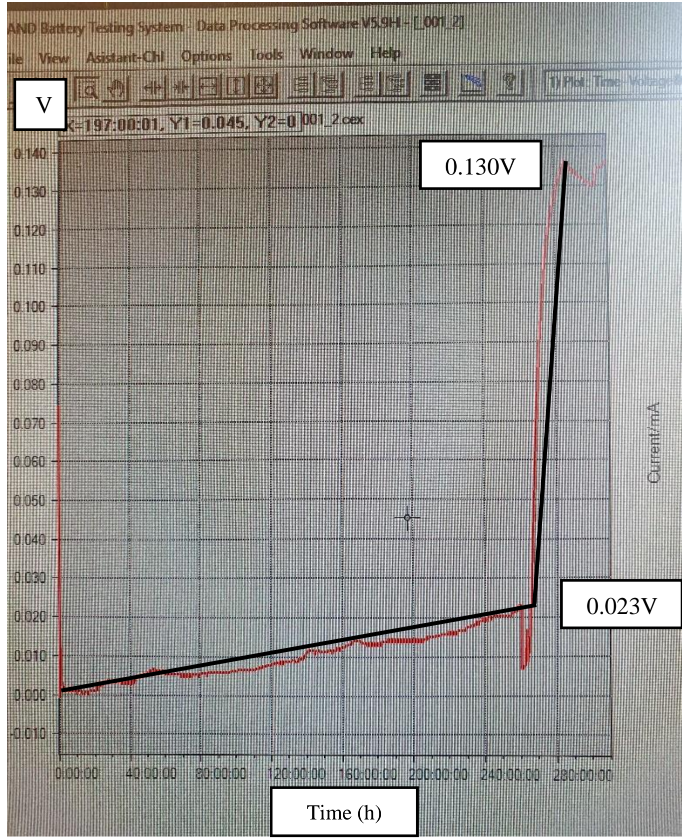

Figure 3:Y-axis voltage, X-axis, time in hours

This spike in voltage is due to the established *Shewanella O.* biofilm getting a spike in nutrients from the media change. With these nutrients the redox reaction by the electrode is positively promoted.

To calculate the electric current in Ampere, Ohms law was utilized. (1)

$$I = \frac{U}{R}. \qquad (1)$$

IV. DISCUSSION

After the media was exchanged a significant increase in voltage production was recorded. This shows that the biofilm has been established on the anode and that Shewanella can produce a measurable voltage. The flow cell used so far has been a hybrid unmediated MFC, the original plan was to also test a non-hybrid system with a mediator. Due to time constraints these experiments have not yet been performed. Previous studies have shown that the efficiency can be significantly increased in a liquid-liquid flow cell that has ferric-ion to aid the redox reaction. Future runs will test Shewanella oneidensis with the Mtrb-operon knocked out. The operon will be activated by a promotor that is activated by AHL.

With the lack of results regarding the biosensor there's little that can be discussed but with more time spent in the lab there is a possibility that the modular E.coli pollutant detection can be utilized for continual monitoring. With the limited time of this study the assembly of the genetic parts is still underway. For further testing of the biosensor emphasis should be put on the optimization of the promoter's specificity. With continual monitoring this system in tandem with the MFC, can be used for more than aquatic pollutants.

V. CONCLUSION

The voltage output produced by the MFC shows promising results, after the MtrB-knouckout strain can be tested proof of concept can be properly established. The modular biosensor needs further work to properly show proof of concept.

VI. REFERENCES

Akob, Denise M., Mills,Heath J., Gihring,Thomas M., Kerkhof, Lee, Stucki, Joseph W.,Anastaio, Alexandre S., Chin, Kuk-Jeong, Kusel, Kirsten, Palumbo,Anthony V.,Watson, David B and Kostka, Joel E. "Functional Diversity and Electron Donor Dependence of Microbial Populations Capable of U(VI) Reduction in Radionuclide-Contaminated Subsurface Sediments." Applied and Environmental Microbiology, 2008, DOI:10.1128/AEM.02881-07

Bănică, Florinel-Gabriel (2012). Bănică, Florinel-Gabriel (2012). Chemical Sensors and Biosensors:Fundamentals and Applications. Chichester, UK: John Wiley & Sons. p. 576. ISBN 9781118354230.

Dincer, Can; Bruch, Richard; Costa-Rama, Estefanía; Fernández-Abedul, Maria Teresa; Merkoçi, Arben; Manz, Andreas; Urban,Gerald Anton; Güder, Firat (2019) 0198547242"Disposable Sensors in Diagnostics, Food, and Environmental Monitoring". Advanced Materials. 31 (30): 1806739. doi:10.1002/adma.201806739. hdl:10044/1/69878. ISSN 0935-9648. PMID 31094032

Diersing, Nancy (2009). "Water Quality: Frequently Asked Questions." Florida Brooks National Marine Sanctuary, Key West, FL.

Mohanakrishna, Venkata Mohan, S; G; Reddy, B. Purushotham; Saravanan, R; Sarma, P.N (2008). "Bioelectricity generation from chemical wastewater treatment in mediatorless (anode) microbial fuel cell (MFC) using selectively enriched hydrogen producing mixed culture under acidophilic microenvironment". Biochemical Engineering Journal. 39: 121–30. doi:10.1016/j.bej.2007.08.023.

Venkata Mohan, S; Veer Raghavulu, S; Sarma, P.N (2008). "Biochemical evaluation of bioelectricity production process from anaerobic wastewater treatment in a single chambered microbial fuel cell (MFC) employing glass wool membrane". Biosensors and Bioelectronics. 23 (9): 1326–32. doi:10.1016/j.bios.2007.11.016. PMID 18248978.

Schmidt-Rohr, K. (2020). "Oxygen Is the High-Energy Molecule Powering Complex Multicellular Life: Fundamental Corrections to Traditional Bioenergetics" ACS Omega 2221–2233. http://dx.doi.org/10.1021/acsomega.9b03352). Barge, E. G.,

Swedish Chemical Agency, 2020 Highly fluorinated substances (PFOS, PFOA and others) https://www.kemi.se/en/chemical-substances-and-materials/highly-fluorinated-substances

Turner, Anthony; Wilson, George; Kaube, Isao (1987). Biosensors:Fundamentals and Applications. Oxford, UK: Oxford University Press. p. 770. ISBN 978

Wang, J. (2008). "Electrochemical Glucose Biosensors". Chemical Reviews. 108 (2): 814–825. doi:10.1021/cr068123a. PMID 18154363.)'

Wiatrowski HA; Ward PM; Barkay T. (2006). "Novel reduction of mercury (II) by mercury-sensitive dissimilatory metal reducing bacteria". Environmental Science and Technology. 40 (21): 6690–6696. Bibcode:2006EnST...40.6690W. doi:10.1021/es061046g. PMID 17144297.

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Mossphate: Yesterday's Wastewater can Fuel Tomorrow's Crops

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***Abstract* - Our goal is to accumulate phosphate from wastewater and reuse it for the agricultural sector using the moss *Physcomitrella patens*. The phosphate molecules will be stored intracellularly in the form of polyphosphate granules, which are formed by polyphosphate kinases. Both homologous and heterologous expression will be used to ensure maximal activity.**

Overexpression of phosphate transporters will allow the chassis to import more phosphate, thus improving the phosphate accumulation.

With these modifications, we hope to provide a sustainable way to filter phosphate from wastewater and grow phosphate-rich moss plants. These mosses can be directly used as fertilizer to provide crops with recycled phosphate.

Index Terms: Physcomitrella patens, phosphate, polyphosphate, flux balance analysis, photobioreactor

I. INTRODUCTION

Phosphate is an essential element for all living organisms. It is involved in diverse cellular functions and it is a key limiting factor of plant growth. Phosphate is used in the production of fertilizers for higher crop yields, but the usage of these fertilizers has resulted in the increase of the phosphate concentration in inland water, being the main cause of algal blooms and eutrophication (McGrath and Quinn et al. 2003).

The increasing world population has triggered greater food demands, and therefore an increasing demand for phosphate fertilizer to grow crops. But phosphate rock deposits, the main source of phosphate for industrial use, are predicted to run out in the next 50-100 years, should the use of fertilizers continue in the way it is now (Cordell et al. 2009).

To produce enough fertilizer for crops, while preventing eutrophication, a sustainable solution to recycle phosphate is vital.

Current technologies in phosphate removal consist of the classical chemical precipitation process and the so-called Enhanced Biological Phosphate Removal (EBPR). The former is quite expensive and produces large amounts of waste, thus research has shifted to the more novel and efficient EBPR – the details of which can be read in the article by McGrath et al (McGrath and Quinn et al. 2003).

We present a new approach by focusing on the polyphosphate kinase and transporters.

II. RESEARCH ELABORATION

We aim to improve multiple parts of *Physcomitrella patens (P. patens)*. For a lack of time we will test every change by using transient expression.

We will compare the native *P. patens* PPK, one from *E. coli* K12 in a wild-type and mutated form. This mutated form was shown to have a higher rate of throughput (Rudat et al. 2018). The comparison will be done using DAPI staining on the transformed *P. patens* cell to visualize the polyphosphate content.

After we have determined which of these is the most effective PPK we would then test if it is more effective to store the polyphosphate in either the cytosol or the vacuole. This would be done by overexpressing the respective transport proteins.

III. PHOSPHATE METABOLISM AND POLYPHOSPHATE

III.1 *Phosphate metabolism in plants*

Phosphate is a constituent of ATP and thus it is involved in a majority of biological processes, most importantly energy metabolism (Carstensen et al. 2018), as well as carbohydrate, nucleic acid and phospholipid synthesis (Bieleski and Ferguson 1983). Phosphate is the major non-metabolic/storage form and is stored inside the vacuole, while polyphosphate is the minor constituent in the cytosol (Bieleski and Ferguson 1983). The basic concept can be seen in FIGURE 1.

III.2 *Polyphosphate*

Polyphosphate (PolyP) is a long linear chain of condensed (ortho)phosphate molecules. Its main purpose in prokaryotes is as a phosphate storage, being synthesized during times of phosphate surplus and used in times of phosphate deprivation (Kulaev et al. 2004). It is synthesized with two exceptions by the enzyme ATP:polyphosphate phosphotransferase, also called PPK (Kulaev et al. 2004), (Kulakovskaya and Kulaev 2013). ATP is used as a donor to add phosphate groups to the polyphosphate chain. While higher plants do possess PPK's, they lack the necessary enzymes for bioenergetic degradation and build up polyphosphate only in small quantities and during certain developmental stages (Kulaev et al. 2004).

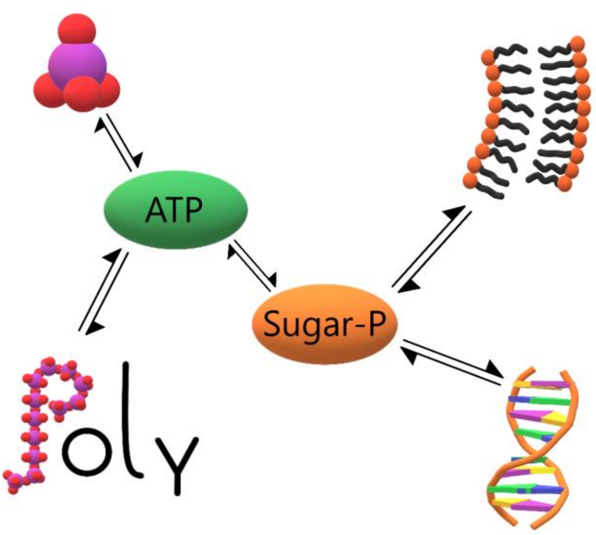

FIGURE 1: Basic concept of Phosphate metabolism. Phosphate enters the metabolism through ATP and 'travels' through sugar-phosphates to the main phosphate pools, being nucleic acids and phospholipids.

IV. ENHANCING PHOSPHATE ACCUMULATION

Plants have a fine tuned Pi homeostasis in which many different types of transport mechanisms play a role. In order to use these mechanisms for an enhanced phosphate accumulation, multiple concepts are possible.

IV.1 *General concept*

An expression of genes from the PHO1;1-PHO1;7 family is necessary. These transporters import Pi through the extracellular matrix into the cytosol.

IV.2 *First concept*

Overaccumulation of PolyP inside the cytosol. This will be achieved by overexpressing PPK.

IV. 3 *Second concept*

Vacuolar storage of PolyP to avoid magnesium starvation, as it complexates with PolyP (Rudat et al 2018). Translocation and synthesis is simultaneously achieved via the vacuolar transport chaperone (VTC) (Gerasimaite et al 2014). 5-InsP7, an inositol-diphosphate, stimulates VTC (Wild et al 2014). Overexpression of the InsP6-Kinase can thereby result in a higher amount of PolyP.

IV.4 *Third concept*

Also vacuolar storage of PolyP, but in contrast to concept 3, phosphate transport and synthesis occur separately. Translocation of PPK will be achieved via C- and N-terminal addition of signal peptides (Schaaf et al 2003). Expression of phosphate exporters like PHO91 (Hürlimann et al 2007) will be downregulated.

V. MODELING

Genome-scale model assisted metabolic engineering has proven to be an efficient and promising novel approach in synthetic biology (Feist and Palsson 2008). Constraint-based models like Flux Balance Analysis (FBA) belong to those models and were successfully tried and tested on numerous occasions to accurately predict metabolic flux through an organism (Feist and Palsson 2008). To our knowledge, no metabolic model exists for *P. patens* with the exception of automatically generated models. Since the manual curation of those seemed far higher than creating a model from scratch, we decided to go with the latter option. We used much of the reactions from a metabolic model of *Chlamydomonas reinhardtii* (Kliphuis et al. 2012) with some adjustments. Currently we are in the process of troubleshooting. In the future, the model will allow us to simulate the effects of a maximization of the polyphosphate synthesis on the whole metabolism. It has been observed that polyphosphate overproduction leads to phosphate deficiency (Wei et al. 2020), therefore we will also add mechanisms to cope with this stress to explore whether those modifications to the metabolism could further improve phosphate accumulation.

VI. ENGINEERING

We aim to build our own prototype of a (photo-) bioreactor (PBR) with which we can cultivate our strains of *P. patens*. There are a multitude of PBRs which differ not only in scale but the exact way of cultivating and utilizing the organisms (igv-GmbH 2011). Most PBRs can be used for algae, cyanobacteria, and moss interchangeably.
PBRs can be roughly separated into two categories, the open systems and the closed systems. As the names suggest, the difference is whether the respective organism is isolated from the environment or not.
After guidance from Dr. Holger Klose and Dr. Ladislav Nedbal from Research Center Juelich we decided to build the open system referred to as a “turf scrubber”. The main reasons for it are 1) the facile construction and 2) the ease with which we are able to access the moss for harvest/maintenance. In the turf scrubber, the moss is anchored to a lattice, making harvesting and exchanging an older lattice with a new one trivial.
We aim to model our system after the example found at (Research Center Juelich 2018).
An open system like this would call for a kill switch in the moss if it were to be used with the enhanced moss.
Our changes to the system comprise of a constant amount of water beneath the lattice to better the moss's take up of nutrients from the water. The water itself however will be fed into the system in a similar way to the example.

VII. INTENDED USE

In the end, our proposal could be used to create a new last-stage in wastewater treatment facilities (WWF). A bioreactor array with bioengineered *P. patens* would reduce the phosphate content to a hypotrophic level, which current WWF fail to accomplish (McGrath and Quinn et al. 2003). The efflux

wastewater would no longer cause algal blooms and at the same time the phosphate-rich moss can be dried and used as a fertilizer.
Such upgrades to existing WWF would require a significant investment due to its necessary size alone, and therefore a joint operation with state authorities seems to be unavoidable.
This concept would provide a considerable enhancement to the phosphate cycle, as the phosphate would constantly be removed and put back to its source. This recycling would potentially reduce the consumption of phosphate rock by a large margin, thus sparing the environment from the adverse effects of phosphate mining (de Boehr et al. 2019).

VIII. CONCLUSION

In conclusion, our approach to the current phosphate consumption would provide a more sustainable and environmentally-friendly use of phosphate fertilizer and at the same time, contribute to the establishment of *P. patens* as a biotechnological chassis.

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REFERENCES

Bieleski, R. L., Ferguson, I. B. (1983) Physiology and Metabolism of Phosphate and Its Compounds. In Läuchli, A. & R. L., Ferguson, I. B. (Eds.), *Inorganic Plant Nutrition* (pp 422-449). Encyclopedia of Plant Physiology (New Series), Vol 15. Berlin, Heidelberg, Germany: Springer, Berlin, Heidelberg. /doi.org/10.1007/978-3-642-68885-0_15

Carstensens, A., Herdean, A., Birkelund Schmidt, S., Sharma, A., Spetea, C., Pribil, M., Husted, S. (2018). The Impacts of Phosphorus Deficiency on the Photosynthetic Electron Chain. Plant Physiology, 177(1): 271-287. doi: 10.1104/pp.17.01624

Cordell, D., Drangert, J.-O., White, S. (2009). The story of phosphorus: Global food security and food for thought. Global Environmental Change, 19(2): 292-305. doi: 10.1016/j.gloenvcha.2008.10.009

de Boer, M., Wolzak, L., Slootweg, J. C. (2019). Phosphorus: Reserves, Production and Applications. In Ohtake, H. & Tsuneda S. *Phosphorus Recycling and Recovery* (pp 75-100). Singapore. Springer Nature Singapore Ltd. doi: 10.1007/978-981-10-8031-9

Feist, A. M., Palsson, B.Ø. (2008). The growing scope of applications of genome-scale metabolic reconstructions using Escherichia coli. Nature Biotechnology, June 2008, 26(6): 659-667. doi: 10.1038/nbt1401

Gerasimaite, R., Sharma, S., Desfougères, Y., Schmidt, A., Mayer, A. (2014). Coupled synthesis and translocation restrains polyphosphate to acidocalcisome-like vacuoles and prevents its toxicity. Journal of Cell Science, 2014(127): 5093-5104. doi: 10.1242/jcs.159772

Hürlimann, H. C., Stadler-Weibel, M., Werner, T. P., Freimoser, F. M. (2007). Pho91 Is a Vacuolar Phosphate Transporter That Regulates Phosphate and Polyphosphate Metabolism in *Saccharomyces cerevisiae*. Molecular Biology of the Cell, 18(11). doi: 10.1091/mbc.e07-05-0457

Kliphuis, A. M. J., Klok, A. J., Martens, D. E., Lamers, P. P., Janssen, M., Wijffels, R. H. (2012). Metabolic modeling of *Chlamydomonas reinhardtii*: energy requirements for photoautotrophic growth and maintenance. Journal of Applied Phycology, 2012(24): 253-266. doi: 10.1007/s10811-011-9674-3

Kulaev, I. S., Vagabov, V. M., Kulakovskaya, T. V. (2004). The Functions of Polyphsosphates and Polyphosphate-dependent Enzymes. In Kulaev, I. S., Vagabov, V. M., Kulakovskaya, T. V. (Eds.) *The Biochemistry of Inorganic Polyphosphates, Second Edition* (pp 91-123). Hoboken, USA: John Wiley & Sons Ltd. doi: 10.1002/0470858192

Kulakovskaya, T. V., Kulaev, I. S. (2013). Enzymes of Inorganic Polyphosphate Metabolism. In Müller W., Wang X., Schröder H. (Eds.) *Biomedical Inorganic Polymers* (pp 39-63). Progress in Molecular and Subcellular Biology, vol 54. Berlin, Heidelberg, Germany: Springer, Berlin, Heidelberg. doi: 10.1007/978-3-642-41004-8_3

McGrath, J. W., Quinn, J. P. (2003). Microbial phosphate removal and polyphosphate production from wastewater. Advancements in Applied Microbiology, 2003(52): 75-100. doi: 10.1016/s0065-2164(03)01003-7

Research Center Juelich (2018). DIY Algal Turf Scrubber VIDEO. Retrieved from https://www.algal-solutions.eu/?page_id=907

Rudat, A. K., Pokhrel, A., Green, T. J., Gray, M. J. (2018). Mutations in *Escherichia coli* Polyphosphate Kinase That Lead to Dramatically Increased *In Vivo* Polyphosphate Levels. Journal of Bacteriology, Feb. 2018, 200(6). doi: 10.1128/JB.00697-17

Schaaf, A., Reski, R., Decker, E. L. (2004). A novel aspartic proteinase is targeted to the secretory pathway and to the vacuole in the moss *Physcomitrella patens*. European Journal of Cell Biology, 2004(83): 145-152. doi: 10.1078/0171-9335-00371

Wei, R., Wang, X., Zhang, W., Shen, J., Zhang, H., Gao, Y., Yang, L. (2020). The improved phosphorus utilization and reduced phosphorus consumption of *ppk*-expressing transgenic rice. Field Crops Research, 1 March 2020(248). doi: 10.1016/j.fcr.2020.107715

Wencker, T. (2011). Photobioreactor Design Principles. Retrieved from http://www.submariner-project.eu/images/stories/events/algae-trelleborg/presentations/wencker.pdf

Wild, R., Gerasimaite, R., Jung, J.-Y., Truffault, V., Pavlovic, I., Schmidt, A., Saiardi, A., Jessen, H. J., Poirier, I., Hothorn, M., Mayer, A. (2016). Control of eukaryotic phosphate homeostasis by inositol polyphosphate sensor domains. Science 14 Apr. 2016. doi: 10.1126/science.aad9858

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Microdestruction-When Painkillers Hurt: Diclofenac-Inactivating Laccases Produced by Modified *C. reinhardtii* as an effective Wastewater Treatment Option

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Abstract- **Diclofenac is a known anti-inflamatory drug and a primary example of the type of micropollutants which accumulate in wastewater due to overuse, thus contaminating the environment. Laccase has been shown to chemically deactivate Diclofenac, leading to functional degradation. By cloning two different laccase genes (MarLac and BaLac) into both our control bacterium *Escherichia coli* and primary organism, the green algae *Chlamydomonas reinhardtii*, we hope to produce laccase which could then be incorporated into a bioreactor within an existing sewage treatment plant system. The enzymes must withstand the prevailing conditions in the sewage treatment plant, such as a certain pH range, to be able to begin oxidation. With our current experimental design, we seek to demonstrate the optimal conditions which would be needed for our laccase to effectively inactivate Diclofenac in a wastewater system. The first step of this is to confirm our laccase activity against a positive control (*Trametes versicolor*) to produce a standard to compare by. It is our vision to create a cost-effective and efficient approach to a cleaner and healthier environment.**

Index Terms- *Chlamydomonas reinhardtii,* Diclofenac, *Escherichia coli,* iGEM, wastewater, pH, *Trametes versicolor,* BaLac, MarLac

I. INTRODUCTION

Micropollutants like pharmaceutical residues or personal care products can be found in the aquatic environment and pose a risk to environmental and human health. Wastewater treatment plants can only partially remove these compounds and their degradation products. These degradation products can also pose a greater risk to the environment than the initial compound. The bioavailability of micropollutants is dependent on their structure and their affinity to the environment. Furthermore, bioaccumulation can increase with exposure over time and biomagnification can severely impact predatory species as concentrations become higher the more exposed prey they consume, leading to collapsing food webs.

In addition, many of these micropollutants are not covered by legal restrictions and interactions between compounds are not taken into consideration. One of the most problematic micro-pollutants in Germany is Diclofenac, a common anti-inflamatory drug in use since the 1960's which has been linked with the near extinction of Indian vultures.

Laccases are multicopper-oxidases that oxidize substrates like polyphenols, methoxylated phenols, aromatic amines, and inorganic materials. It results in a direct one-electron substrate oxidation, an electron transported to other copper domains where up to four electrons can be stored, and finally a four-electron reduction of O_2 to H_2O. This allows it to inactivate substrates like Diclofenac.

We chose the bacterium *Escherichia coli* as our control organism as it has been used in previous studies and is a common and easily replicated organism. We chose the green algae *Chlamydomonas reinhardtii* as our final model for two reasons; the first is it is highly studied optional phototroph, making it an unbelievably valuable and versatile research organism, with a collection of more than 300 plasmids and over 3000 strains. Our second reason for the choice was due to our previous iGEM team's work with a genetically modified *C. reinhardtii* which could degrade microplastics, with our end goal as making one final organism with multiple wastewater treatment facets.

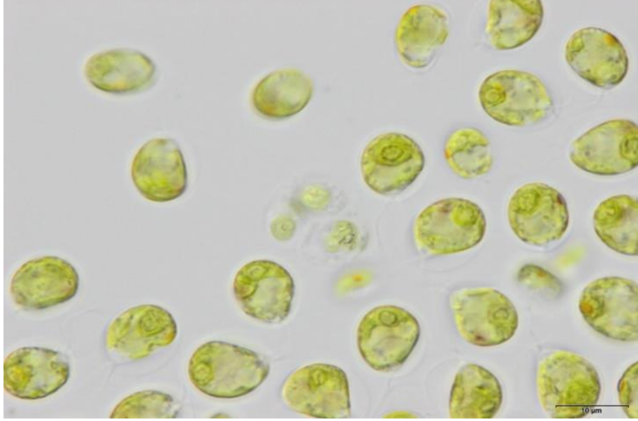

Fig. 1: Green algae mutant under light microscope with 100x magnification

The aim of this work is to test the laccases from the ascomycete fungi *Botrytis aclada* (BaLac) and an unknown marine bacteria (MarLac) and integrate them into our model organisms to find the optimal production conditions for each laccase

(Scheiblbrandner *et al*., 2017; Yang *et al.,* 2018). With this information we want to find out which of the laccases shows the best activity and stability at conditions found within a wastewater treatment plant. Furthermore, we want to examine the kinetics of the micropollution degradation.

II. RESEARCH ELABORATIONS

Diclofenac is a member of the acidic antipyretic analgesics group (pain agents). Like all representatives of this class, Diclofenac inhibits cyclooxygenases (COX) and thus reduces prostaglandin synthesis, which influences peripheral pain formation (Aktories *et al.*, 2005). Because it is an active ingredient in pain-relieving ointment, Diclofenac is highly consumed among the German population, having been amongst the most popularly sold drugs in Germany for many years (Glaeske, 2017).

Fig. 2: Chemical structure of Diclofenac

In addition, Diclofenac has an anti-phlogistic anti-rheumatic effect and is counted among the non-selective non-steroidal anti-inflammatory drugs (non-selective NSAID) due to its inhibitory effect on both COX isoenzymes (COX-1 and COX-2). In Germany, Diclofenac is the most common administered active ingredient used to treat rheumatic arthritis (Aktories *et al.*, 2005).

The wide application, primarily within Germany, generates a consumption of about 90 tons per year in Germany. This high utilization has costs however, as only about 30 % of the active substance is metabolized by the human body, the rest being excreted unchanged through urine. This leads to an estimated 63 tons of Diclofenac per year that enter the Germany water systems (Meißner, 2008b).

Due to its high stability and water solubility, Diclofenac is poorly filtered out in conventional wastewater treatment plants. Although Diclofenac does not pose an acute danger to humans in the concentrations for which it is found in German waters, it is highly toxic to aquatic organisms. More modern sewage treatment plants are trying to solve this problem by additional purification (ie. nanofiltration, ozone or activated carbon). In addition to the high costs, the lack of experimental data on emerging products in the application of non-specific degradation methods (ie. ozone) make these far from ideal. Recent work, including our project, have set themselves the objective of investigating the use of enzymes as catalysts for the degradation of micropollutants such as Diclofenac (Bilal *et al.,* 2019; Meißner, 2008a).

We hope to establish a self-sustaining, enzyme-based system, which is characterized by high turnover rate, low maintenance, and low costs. In addition, the use of enzymes and the specificity associated with them should make it predictable which reaction products are produced during the degradation of Diclofenac, so that a disruption of environmental homeostasis by possibly toxic products can be avoided.

Laccases (*p*-benzenediol: oxygen oxidoreductase EC 1.10.3.2) are enzymes which catalyze the oxidation of a wide variety of aromatic and non-aromatic molecules (Agrawal *et al.,* 2018). They contain multiple copper atoms in their active site to store and transport electrons (Zerva *et al*., 2019).

The laccases have three copper types which have multiple differing arrangements. The mononuclear copper-binding site found in Type 1 copper is located near the protein´s surface. Here the substrate one-electron oxidation takes place. In addition, a trinuclear copper-binding site (TNC) which is built by one T2 copper and two T3 copper atoms are located here. The TNC can bind oxygen in the fully reduced state of the protein and is responsible for reducing O_2 to H_2O. Therefore, it takes the electrons from the substrate oxidation after a cycle of four of the previously detailed reactions (Agrawal *et al.,* 2018; Zerva *et al*., 2019).

The reaction cycle consists of 4 single-electron transfers from a reducing substrate to the copper atoms in the active site. This forms a radical cation from the substrate (Fig. 3). In its reduced form, the laccase is then able to reduce molecular oxygen into two-electron transfer reactions to form water (Agrawal *et al.,* 2018; Zerva *et al*., 2019).

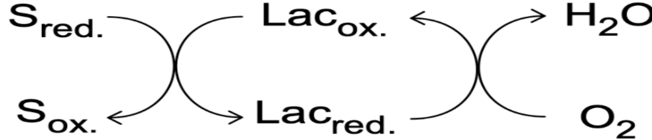

Fig. 3: Schematic representation of the reaction catalyzed by laccases. S: substrate; Lac: laccase.

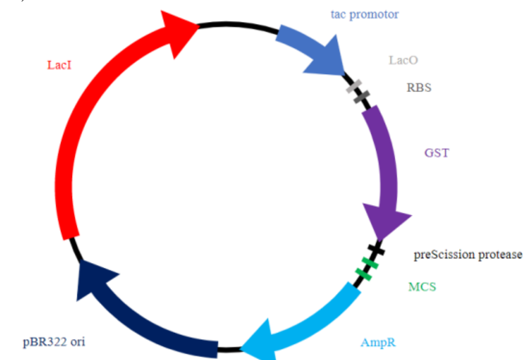

Fig. 4: pGEX-6P-1 Vector

Our vector pGEX-6P-1 contained the tac promotor combining the strong sigma-factor binding site from trp and the controllability with IPTG from lac. The Ampicillin-resistance is

needed for selection. To isolate the protein, we have the GST Tag.

III. RESULTS AND FINDINGS

Because ABTS interacts with laccase in a similar way to the laccase-diclofenac interaction, we preformed a series of ABTS assays to determine reaction strength. To begin, we performed an assay to visualize our positive control laccase *Trametes versicolor* at the two optimal pH's documented for BaLac (pH 4) and MarLac (pH 7), as well as its own optimal pH 5. In a 96 well plate, varying concentrations of our positive control laccase at different pH levels were added to the substrate. Enzyme activity for the different pH's was calculated from 4 technical replications with 2 biological replicates for each concentration. From the data collected from this plate it was confirmed pH 5 is optimal for *T. versicolor's* laccase, and absorption graphs were created to visualize our positive control activity at each of our created enzyme's optimal pH levels.

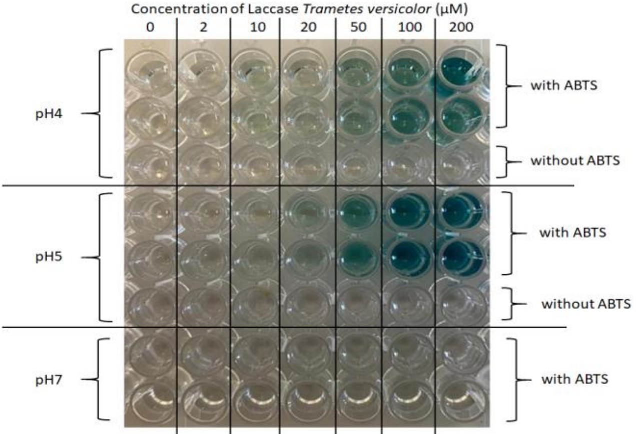

Fig. 5: 96 well plate set up

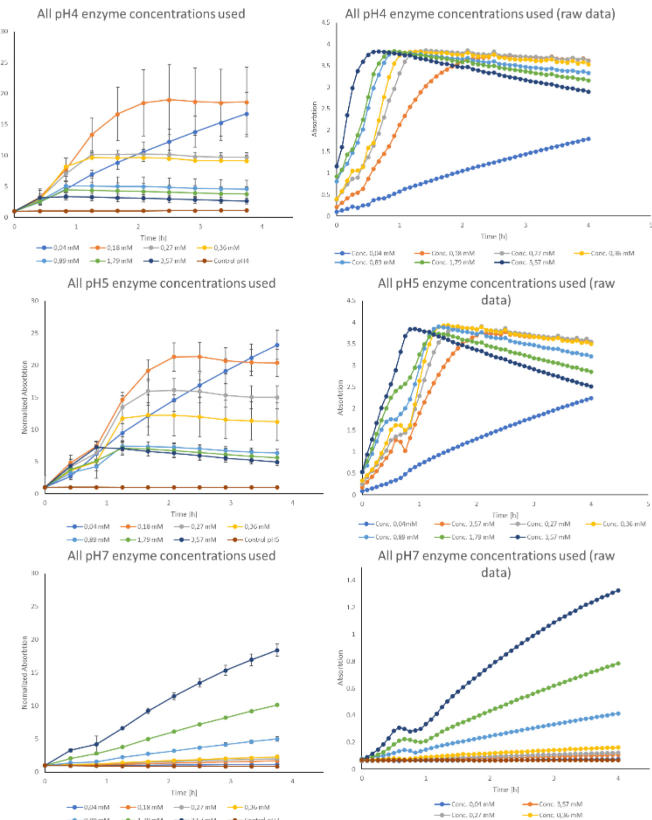

Fig. 6: Enzyme absorbtion graphs used for later ABTS assay comparison standards with our produced laccase BaLac and MarLac

IV. DISCUSSION

We plan to express our chosen laccases from *C. reinhardtii* and *E. coli*. Once the laccase is succusfully expressed we will complete assays to determine the activity and functionality. The assays with the organism's laccase will be compared with the purchased standard of *T. versicolor* using the created activity standards. We also hope to perform HPLC to see the final products produced to verify their inactivity and reduction in harm for the environment.

Once the organism is creating functional enzymes to degrade Diclofenac, we will create a bioreactor to separate the genetically modified organisms from the distributed enzymes within the wastewater. We chose to have two different enzyme constructs, BaLac and MarLac, due to their wide range in pH activity, giving us multiple options for placement within the astewater treatment facility. This is the first step will provide us standards to test their effectiveness and decide where the final placement of the bioreactor will go within the treatment plant (towards the front of the system for the pH 7 thriving MarLac, near the end for the more acidic pH 4 BaLac) and whether an acidification step will be required for effectiveness. Eventually we would like to test our enzyme functionality with other pollutants beyond Diclofenac and hope to implement bioreactors for many pollutants, not only in Germany, but across the world.

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Chemical structure of Diclofenac created in MarvinSketch, version 20.2.0, developed by ChemAxon, https://chemaxon.com/products/marvin, 2020

REFERENCES

Agrawal, K., Chaturvedi, V., & Verma, P. (2018). Fungal laccase discovered but yet undiscovered. *Bioresources and Bioprocessing*, *5*(1), 4. https://doi.org/10.1186/s40643-018-0190-z

Aktories, K., Förstermann, U., Hofmann, F., & Starke, K. (2005). *Allgemeine und spezielle Pharmakologie und Toxikologie* (9. Auflage). Urban & Fischer Verlag.

Bilal, M., Adeel, M., Rasheed, T., Zhao, Y., & Iqbal, H. M. N. (2019). Emerging contaminants of high concern and their enzyme-assisted biodegradation – A review. *Environment International*, *124*, 336–353. https://doi.org/10.1016/j.envint.2019.01.011

Glaeske, G. (2017). Medikamente 2015 – Psychotrope und andere Arzneimittel mit Missbrauchs- und Abhängigkeitspotential. *Jahrbuch Sucht*.

Meißner, M. (2008a). Arzneimittel in der Umwelt: Natur als Medikamentendeponie. *Dtsch Arztebl International*, *105*(24), A-1324.

Meißner, M. (2008b). Arzneimittel in der Umwelt: Natur als Medikamentendeponie. *Dtsch Arztebl International*, *105*(24), A-1324.

Scheiblbrandner, S., Breslmayr, E., Csarman, F., Paukner, R., Führer, J., Herzog, P. L., Shleev, S. V., Osipov, E. M., Tikhonova, T. V., Popov, V. O., Haltrich, D., Ludwig, R., & Kittl, R. (2017). Evolving stability and pH-dependent activity of the high redox potential Botrytis aclada laccase for enzymatic fuel cells. *Scientific Reports*, *7*(1), 13688. https://doi.org/10.1038/s41598-017-13734-0

Yang, Q., Zhang, M., Zhang, M., Wang, C., Liu, Y., Fan, X., & Li, H. (2018). Characterization of a Novel, Cold-Adapted, and Thermostable Laccase-Like Enzyme With High Tolerance for Organic Solvents and Salt and Potent Dye Decolorization Ability, Derived From a Marine Metagenomic Library. *Frontiers in Microbiology*, *9*, 2998. https://doi.org/10.3389/fmicb.2018.02998

Zerva, A., Simić, S., Topakas, E., & Nikodinovic-Runic, J. (2019). Applications of Microbial Laccases: Patent Review of the Past Decade (2009–2019). *Catalysts*, *9*(12), 1023. https://doi.org/10.3390/catal9121023

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A^3 Project: A New Look on Algae Revalorization

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ABSTRACT

The accumulation of green macroalgae *(Ulva spp.)* responsible for green tides leads to the production of hydrogen sulfide gas (H_2S). Our main goal is to promote the degradation of algae by the sulfate-reducing bacteria (SRBs), and thus accelerate the H_2S produced by those bacteria, before transforming this gas into our final product – sulfuric acid. For this purpose, we aim to develop a bioreactor to enable bacterial growth which will produce sulfatases and other degradation enzymes that will destroy the main component of the cell-wall of algae: ulvan. Once produced, the enzymes will be added to a tank filled with algae taken directly from the beach. Since SRBs are naturally found in green tides, they will also be collected along with the algae. The degradation gas produced in the tank will serve as a base for sulfuric acid production a useful compound for many industries such as the production of detergents, textiles and many others products

Index terms- Green macroalgae, Ulva spp, Green tides, SRBs, sulfuric acid, hydrogen sulfide

I. INTRODUCTION

Green macroalgae (*Ulva spp.*) have been poisoning coast sides for decades. Rising temperatures and eutrophication of coastal waters due to nitrogen fertilizer pollution are mainly responsible for their proliferation. These green seaweed blooms are called "green tides". This accumulation of algae affects many ecosystems in the world including those in the north-west of France. This phenomenon is getting worse each year and raises many health, economic and environmental concerns.
The significant accumulation of green macroalgae and their degradation causes the formation of anoxia zones, which leads to the production of hydrogen sulfide (H_2S) by the SRBs. Hydrogen sulfide can be an extremely toxic and harmful gas, and its asphyxiation of flora and fauna is just one of the side effects this gas has. Just a few minutes of inhalation might become lethal to humans and animals.
In the past few years, this gas has been the cause of several animal and human casualties.
Nantes iGEM team decided to promote green algae, responsible for green tides on the coasts of French Brittany. Our project, therefore, revolves around the development of these algae. Our final goal is to produce sulfuric acid, a compound used in many industries such as the production of detergents, textiles and many other products.
In this article, we will have a look at the methods and pathways for algae degradation by using a specific mix of enzymes.

II. MATERIALS AND METHODS

Enzymes

Our project will be using a total of seven enzymes from the bacterium *Formosa agariphila*. There are three degradation enzymes and three sulfatases.
In order for the sulfatases to work, an additional enzyme will be used – Formylglycine-generating enzyme (FGE). This last enzyme is used to activate the sulfatases.

Enzymes choice
The ulvan degradation cascade described by Reisky *et al.* (2019) shows us that the degradation enzymes P30_PL28, P10_Plnc and P31_GH39 have a significant effect on ulvan. Based on this research, Nantes team decided to use those three enzymes.
Regarding the sulfatases, we based our decision on the same article. The P18_S1_7, P32_S1_8 and P36_S1_25 enzymes show an important activity on ulvan.

Plasmids design
To achieve the goals we set, our project will use two types of plasmids.

- **pET 11 plasmid**

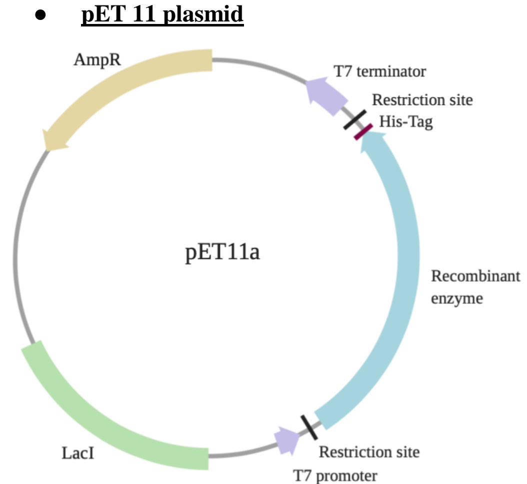

Fig. 1 pET11a plasmid containing each one of the following enzymes : degradation enzymes P30_PL28, P10_Plnc and P31_GH39 and sulfatases P18_S1_7, P32_S1_8 and P36_S1_25.

The pET11a plasmid in Fig. 1 will be used for the expression of the three so-called degradation enzymes and the three sulfatases.

- **pEVOL-1 plasmid**

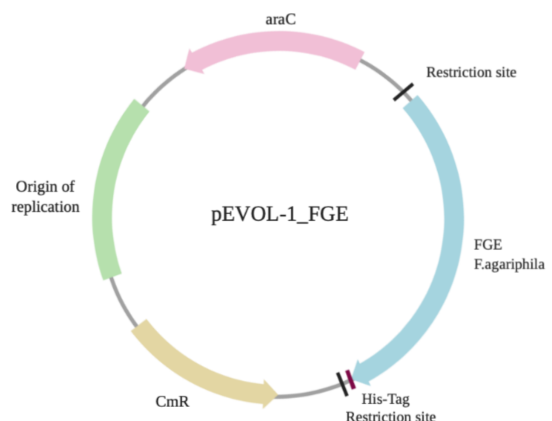

Fig. 2 pEVOL-1 plasmid containing FGE.

The pEVOL-1 *(Fig. 2)* plasmid will be used for the expression of the FGE. FGE and sulfatases will be either co-expressed or put together once produced.
The His-Tag sequence will allow us to purify the enzymes once they are produced, by using an immobilized metal affinity chromatography (IMAC) with nickel resin.

Expression of enzymes
The strain *E.coli* BL21 DE3 was chosen for the expression of our enzymes. This is a popular strain used to express recombinant proteins.

III. BIOREACTOR

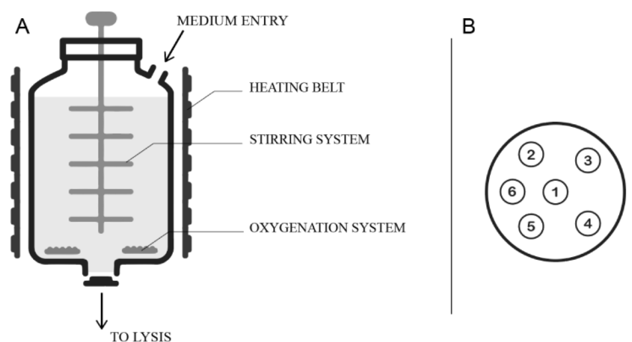

Fig. 3 A Diagram of the bioreactor. **B** Details of the cap: 1. agitation system; 2. sampling catheter; 3. pH probe; 4. temperature probe; 5. overpressure valve; 6. injection catheter.

Our bioreactor will follow the continuous flow method, which means that our BL21 DE3 bacteria will be kept in a constant exponential phase. To do this, an X quantity of medium and bacteria will be taken out and the same X quantity of the medium (sterile) will be added in the bioreactor through the designated inputs and outputs (Fig. 3 A). A stable quantity of degradation enzymes and sulfatases will be produced. The bioreactor is connected to a reservoir containing ulvan or green algae that need to be degraded. The produced enzymes will be poured into this tank and the degradation will take place in this compartment. Hydrogen sulfide will also be produced in the same tank.
At the end, the hydrogen sulfide (H_2S) that results from the degradation will be collected via a special system.

IV. DISCUSSION

In order to achieve the absolute valorization of green algae and produce sulfuric acid, two groups of enzymes will be produced in an *E. coli* chassis. A group of enzymes composed of three degrading enzymes will allow an accelerated degradation of the ulvan. The second group of enzymes consists of sulfatases which will afterwards promote the release of the sulfates attached to the ulvan. This will therefore enable the production of H_2S by the SRBs. Then, from this released gas and by chemical conversion, sulfuric acid will be obtained in another compartment of the bioreactor.

V. CONCLUSION

The degradation of green algae causes many issues on a global scale. Considering the negative effects it has on human health, on local ecology but also on the touristic field, a solution for valorizing *Ulva spp.* proves useful on many levels. For this purpose, we will put the algae collected in a bioreactor and accelerate their degradation by targeting the ulvan, a sulfated polysaccharide found in the wall of these algae (38% to 54% of the dry weight of the ulvae). Thus, this degradation of the ulvan will allow the release of hydrogen sulfide into the bioreactor.

REFERENCES

Smetacek, V., & Zingone, A. (2013). Green and golden seaweed tides on the rise. Nature, 504(7478), 84-88.
Ménesguen, A., & Piriou, J. Y. (1995). Nitrogen loadings and macroalgal (Ulva sp.) mass accumulation in Brittany (France). Ophelia, 42(1), 227-237.
Reisky, L., Prechoux, A., Zühlke, M. K., Bäumgen, M., Robb, C. S., Gerlach, N., ... & Song, T. (2019). A marine bacterial enzymatic cascade degrades the algal polysaccharide ulvan. *Nature chemical biology*, *15*(8), 803-812.
Bobin-Dubigeon, C., Lahaye, M., Guillon, F., Barry, J. L., & Gallant, D. J. (1997). Factors limiting the biodegradation of Ulva sp cell-wall polysaccharides. *Journal of the Science of Food and Agriculture*, *75*(3), 341-351.
Dominguez, H., & Loret, E. P. (2019). Ulva lactuca, A source of troubles and potential riches. *Marine drugs*, *17*(6), 357.

Dorr, K. H., Grimm, H., Sander, U., Peichl, R., & Tacke, M. (1976). *U.S. Patent No. 3,944,401*. Washington, DC: U.S. Patent and Trademark Office.
Fleurence, J., Le Coeur, C., Mabeau, S., Maurice, M., & Landrein, A. (1995). Comparison of different extractive procedures for proteins from the edible seaweeds Ulva rigida and Ulva rotundata. *Journal of Applied Phycology*, *7*(6), 577-582.
Fleurence, J. (1999). The enzymatic degradation of algal cell walls: a useful approach for improving protein accessibility?. Journal of applied phycology, 11(3), 313-314.
Hoffman, M. R., & Edwards, J. O. (1975). Kinetics of the oxidation of sulfite by hydrogen peroxide in acidic solution. *J. Phys. Chem*, *79*(70), 2096-2098.
Jones, A. G., Quillien, N., Fabvre, A., Grall, J., Schaal, G., & Le Bris, H. (2020). Green macroalgae blooms (Ulva spp.) influence trophic ecology of juvenile flatfish differently in sandy beach nurseries. *Marine Environmental Research*, *154*, 104843.
Konasani, V. R., Jin, C., Karlsson, N. G., & Albers, E. (2018). A novel ulvan lyase family with broad-spectrum activity from the ulvan utilisation loci of Formosa agariphila KMM 3901. *Scientific reports*, *8*(1), 1-11.
Lahaye, M., Cimadevilla, E. A. C., Kuhlenkamp, R., Quemener, B., Lognoné, V., & Dion, P. (1999). Chemical composition and 13C NMR spectroscopic characterisation of ulvans from Ulva (Ulvales, Chlorophyta). *Journal of Applied Phycology*, *11*(1), 1.
Mann, A. J., Hahnke, R. L., Huang, S., Werner, J., Xing, P., Barbeyron, T., ... & Glöckner, F. O. (2013). The genome of the alga-associated marine flavobacterium Formosa agariphila KMM 3901T reveals a broad potential for degradation of algal polysaccharides. *Applied and environmental microbiology*, *79*(21), 6813-6822.

Muyzer, G., & Stams, A. J. (2008). The ecology and biotechnology of sulphate-reducing bacteria. *Nature reviews microbiology*, *6*(6), 441-454.
Peu, P., Sassi, J. F., Girault, R., Picard, S., Saint-Cast, P., Béline, F., & Dabert, P. (2011). Sulphur fate and anaerobic biodegradation potential during co-digestion of seaweed biomass (Ulva sp.) with pig slurry. *Bioresource technology*, *102*(23), 10794-10802.
PIÉPLU, A., Saur, O., Lavalley, J. C., Legendre, O., & NÉDEZ, C. (1998). Claus catalysis and H2S selective oxidation. *Catalysis Reviews*, *40*(4), 409-450.
Ray, B., & Lahaye, M. (1995). Cell-wall polysaccharides from the marine green alga Ulva "rigida"(Ulvales, Chlorophyta). Extraction and chemical composition. *Carbohydrate Research*, *274*, 251-261.
Renuka, N., Guldhe, A., Prasanna, R., Singh, P., & Bux, F. (2018). Microalgae as multi-functional options in modern agriculture: current trends, prospects and challenges. *Biotechnology advances*, *36*(4), 1255-1273
Robic, A., Rondeau-Mouro, C., Sassi, J. F., Lerat, Y., & Lahaye, M. (2009). Structure and interactions of ulvan in the cell wall of the marine green algae Ulva rotundata (Ulvales, Chlorophyceae). *Carbohydrate Polymers*, *77*(2), 206-216.

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Engineering *Synechococcus CB0101* to Improve Iron Uptake and Processing

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***Abstract-* In 1/3 of the world's oceans, the iron concentration limits phytoplankton growth. Iron is required for photosynthesis and is a critical micronutrient for the base of the marine food web. A better ability to capture iron could increase phytoplankton populations which would have benefits such as reducing atmospheric carbon dioxide. Experiments have been conducted to supplement iron in the ocean as a way of improving phytoplankton populations which then act as a carbon sink. Although continuous iron supplementation is possible, improving the organisms' ability to capture iron is a more sustainable way of addressing the issue. In choosing a phytoplankton to engineer, we decided upon *Synechococcus* (cyanobacteria) because it consumes high levels of CO2, has a high replication rate, and has been used by many iGEM teams in the past. Our project will engineer cyanobacteria to transport iron into cells and reduce it to the bioavailable Fe(II) form. The increased iron utilization will increase photosynthesis and growth of phytoplankton. To prevent harmful phytoplankton blooms, a kill switch will also be added to the cells to prevent overgrowth of cells if iron concentration were to increase significantly. This modification will stabilize the food supply for the marine food chain and absorb CO2 from the atmosphere.**

***Index Terms*-** Carbon sequestration, Cyanobacteria, Global warming, Phytoplankton

I. INTRODUCTION

Phytoplankton populations are declining worldwide due to increased surface temperature and limited iron availability. This absence resonates up the food chain, from tiny krill to the massive whales that feed on them (Ryabov, 2017). Iron is a critical micronutrient for the base of the marine food web since it is required for photosynthesis (Schoffman, 2016).

Phytoplankton have evolved to consume the Fe(II) form of iron. However, the Fe (II) concentration in the ocean is low and most iron is in the Fe(III) form. Phytoplankton have evolved a variety of ways to acquire iron in the Fe(II) form they require (Schoffman, 2016). One such way is through the use of siderophores and reductases. Siderophores are proteins secreted by phytoplankton which capture Fe(III) from the water (Ahlgren, 2019) These ligands have an extremely strong affinity for Fe(III), so they are effective in capturing the ion from the water. Once inside the cell, phytoplankton must separate the iron from the siderophore using reductases. Reductases separate Fe(III) from the siderophore by converting it to Fe(II) which has a low affinity for siderophores. Through this process, organisms can capture the iron they need and convert it to a usable form.

As a global trend, the biomass of phytoplankton is decreasing by ~1% per year (Boyce, 2010). However, in iron deficient regions of the ocean, phytoplankton have evolved a variety of ways to better use iron. Phytoplankton in these regions exhibit higher expression rates of genes coding for proteins such as ferritin, flavodoxin, iron uptake proteins, and siderophores (Ahlgren, 2019). Our project will modify cyanobacteria, *Synechococcus CB0101*, taking inspiration from the naturally evolved isolates described in Ahlgren et. al. We reason that bringing together the mutations that have evolved separately into one strain will enhance the ability of phytoplankton to grow in varying ocean conditions that are low in iron. The increase in available iron will increase photosynthesis in phytoplankton. This growth of phytoplankton will stabilize the marine food chain as well as absorb CO_2 from the atmosphere.

II. MATERIALS AND METHODS

Characterization of Cyanobacteria Growth in Varying Concentrations of Iron

In order to test the importance of iron to the phytoplankton population, *Synechococcus CB0101* at an initial OD of 0.05 was put into 5 conical tubes containing SN growth media with different iron concentrations: No iron, 0.01X, 0.1X, 1X (0.023 mM), and 10X normal iron concentration. Stock concentrations were based on the UTex Culture Collection of Algae. Growth was measured by optical density (O.D.) after 2-3 weeks. *CB0101* was grown under light intensity measuring 1000-1700 Lux and either at room temperature or at 30°C.

Improving Iron Consumption Efficiency in Cyanobacteria

Ahlgren *et al.* describes several genes related to iron consumption that have evolved to be expressed at a higher level in iron-deficient environments. Several of these coding regions were synthesized with promoter, RBS, and terminator and cloned into pSB1C3. Separate samples of *E. coli* cells were modified with each of these genes. These genes and their functions can be found in Table 1.

TABLE 1: *List of low* Fe^{2+} *adaptation genes and function (modified from Ahlgren et al.)*

Gene Names	Function
feoA	Transition metal binding ion, works in complex with *feoB*
feoB	Transmembrane transporter of a GTP-driven Fe^{2+} uptake system
isiB	encodes for Flavodoxin, which functions as an electron donor in redox reactions
idiA	Metal binding ion, protects against oxidative damage
pcopM	Encodes for Ferritin, an Iron storage protein
tonB	Siderophore uptake across the membrane
zupT	Mediates uptake of divalent cations and Fe^{2+}

Designing an Iron Sensitive Kill Switch

In order to prevent phytoplankton blooms, a kill switch was engineered using the pAceB promoter that will be implemented as a regulator upstream of the previously mentioned iron consumption genes. The pAceB promoter contains a binding site for FUR, a ligand that binds iron at concentrations of 10^{-7}-10^{-4} Mol.L^{-1}. However, as those values are above oceanic iron levels we wanted to modify the promoter to be sensitive at iron concentrations found in the ocean.

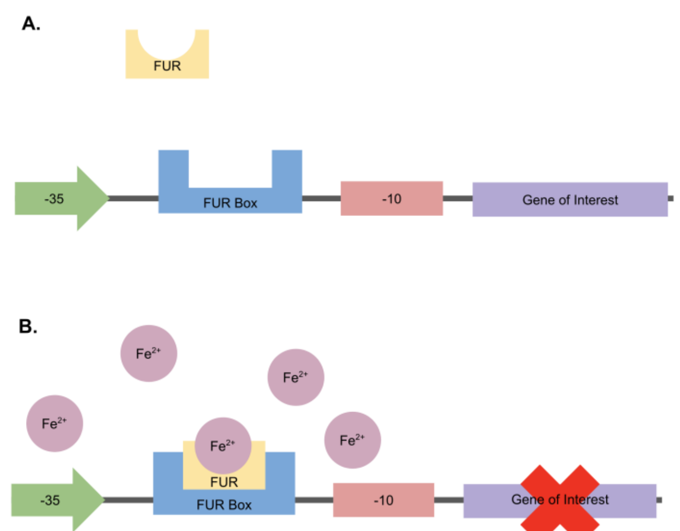

FIGURE 1: *Diagram showing the inhibition of the pAceB promoter. (A) With the absence of Fe(II), FUR cannot bind to the FUR Box, and gene expression is uninhibited. (B) The Fe(II) molecule binds to the FUR ligand, which binds to the FUR Box, inhibiting the promoter system.*

When bound to FUR, pAceB downregulates gene expression. Seven promoter constructs, PFur-1, PFur-2, PFur-3, PFur-1-2, PFur-1-3, PFur-2-3, and PFur-1-2-3, were designed and titled for the respective positions of their fur boxes. To determine the sensitivity of the promoters, separate plasmid constructs containing mCherry fluorescent protein in place of the iron uptake genes will be generated. Fluorescence will first be measured in E. coli cells and then the promoter that expresses the least mCherry will be used in subsequent studies with *CB0101*.

III. RESULTS AND FINDINGS

In this part of the investigation, we aimed to characterize the growth of Cyanobacteria in the presence of iron and transform *E. coli* with our iron uptake genes and kill switch constructs to test their viability in a living system.

From measuring the O.D. of the growth of *Synechococcus CB0101* in varying concentrations of Fe(II) (*Figure 2)* it can be observed that as the concentration of Fe(II) increases in the media there is an increased growth of *Synechococcus CB0101*.

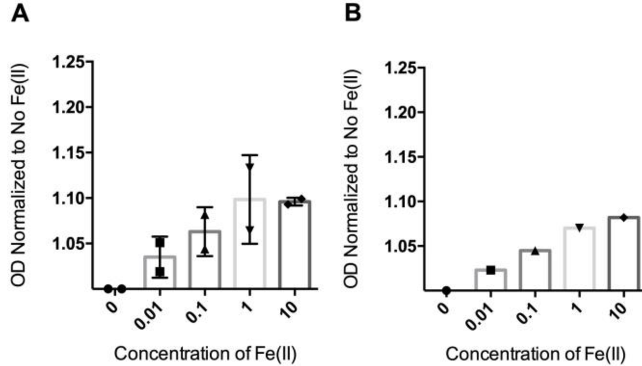

FIGURE 2: *Optical density of Synechococcus CB0101 at varying concentrations of Fe(II). All values are normalized to no Fe(II) control. (A) O.D. for samples 1 and 2 were measured following 3 weeks of growth at 30°C. (B) O.D. for sample 3 was collected after 1 week of growth at 20°C.*

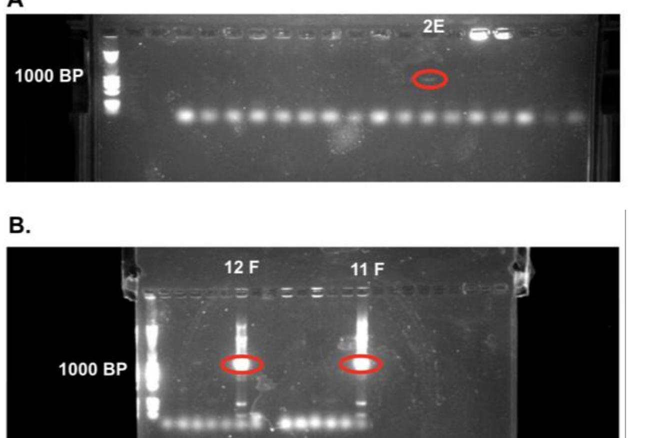

FIGURE 3: *Results of gel electrophoresis for E. coli transformations. (A) Gel containing PCR product from E. coli samples transformed with Kill Switch constructs. Amplification was observed for sample 2E, a colony transformed with construct PFur1-3. This construct contained two separate FUR binding sites. (B) Gel containing PCR product from E. coli samples transformed with Iron Consumption genes. Amplification was observed for samples 11F and 12F, both samples that were transformed with the undigested vector backbone. No amplification was observed for experimental plates*

It can be seen from *Figure 3A* that the construction of pFur1-3-mCherry was successful. The amplification of the undigested vector in *Figure 3B* validates the transformation and PCR, but the lack of amplification in experimental samples implies further testing of additional bacterial colonies is required.

IV. DISCUSSION

In our experiments, we are using a relatively new strain of Synechococcus, CB0101 (Reference), so we wanted to verify that iron is a limiting nutrient for them. The strain grew relatively slowly during the first round of growth characterization at 30°C so growth conditions were optimized to 20°C and will be used throughout the remainder of our project.

From the results observed in *Figure 2*, it can be seen that the growth of *Synechococcus CB0101* is limited by iron concentration. This validates our hypothesis that by increasing the amount of Fe(II) available to phytoplanktons it should lead to increased growth. Future work will consist of transforming *Synechococcus CB0101* with each iron consumption gene and testing whether this enhances the iron uptake ability of the newly transformed strain of *Synechococcus*.

As observed in the gel electrophoresis depicted in *Figure 3A,* pFur1-3 appears to be the most viable construct for testing changes to the promoter since it was the only construct that yielded amplification, however, it is necessary to repeat the transformation so that additional promoter systems can be prepared. Once this step has been repeated, each promoter system's ability to downregulate the expression of mCherry in the presence of iron can be compared. The construct that yields the least expression of mCherry will be used in the final construct containing the modified pAceB promoter, a Lac Operon acting as an inverter, and a CCDB suicide gene. The Lac operon will block transcription of CCDB under normal conditions. With the presence of iron, expression of Lac will be downregulated, leading to expression of CCDB. Once the viability of this construct has been proven, it will be adapted for *Synechococcus CB0101.* This will allow for iron mediated apoptosis to avoid the possibility of a phytoplankton bloom.

The autofluorescence of cyanobacteria can lead to difficulties when genetically modifying them with fluorescent proteins. Therefore, different reporter proteins will be characterized in *CB0101* to see what can be best detected even with autofluorescence.

V. CONCLUSION

The results of the preliminary tests in this study prove the validity of improving phytoplankton growth in iron deficient environments through enhanced consumption of iron. It was shown that iron is vital to the growth of phytoplankton, and our literature review has revealed that consumption can be improved in low iron environments. To make such an improvement safe, a kill switch was engineered to address the possibility of overgrowth. The enhancements described in this study will have positive implications on the aquatic ecosystem as phytoplankton sit at the bottom of the marine food web.

REFERENCES

Ahlgren, N., Beslile, B., et al (2019). Genomic mosaicism underlies the adaptation of marine Synechococcus ecotypes to distinct oceanic iron niches. Environmental Microbiology. doi:10.1111/1462-2920.14893

Boyce, D., Lewis, M. & Worm, B. (2010). Global phytoplankton decline over the past century. Nature 466, 591–596 https://doi.org/10.1038/nature09268

Marsan, D, et al (2017) Toxin-Antitoxin Systems in Estuarine Synechococcus Strain CB0101 and Their Transcriptomic Responses to Environmental Stressors. Front. Microbiol. doi: 10.3389/fmicb.2017.01213

Ryabov, A., de Roos, A., Meyer, B. et al. (2017). Competition-induced starvation drives large-scale population cycles in Antarctic krill. Nat Ecol Evol 1, 0177 doi: 0.1038/s41559-017-0177

Schoffman H, Lis H, Shaked Y and Keren N (2016) Iron–Nutrient Interactions within Phytoplankton. Front. Plant Sci. 7:1223. doi: 10.3389/fpls.2016.01223

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Targeting the Oak Processionary Caterpillar (*Thaumetopoea processionea*) Pest by Means of Bacterium-mediated RNA Interference

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***Abstract*- The Oak Processionary Caterpillar is an invasive species that affects the flora in which it resides and presents a health risk for humans and animals due to the allergenic protein contained in its bristles. The research aim is to genetically engineer a bacterial pesticide, which targets specific and essential sequences in the Oak Processionary caterpillar using siRNA to reduce their growing population. siRNA sequences were identified by using nucleotide BLAST and would be delivered through genetically modified bacteria. To evaluate the effect of the gene silencing, a metabolic model for the oak processionary caterpillar using the IMAT algorithm was created. Creation of the engineered bacterial strain and a series of experiments are planned for future research.**

***Index Terms*-** iGEM, Oak Processionary Caterpillar, Pest control, RNAi, *Thaumetopoea processionea*

I. INTRODUCTION

The Oak Processionary Caterpillar (OPC) is the larvae of the Oak Processionary Moth (OPM) (*Thaumetopoea processionea*), which is a major invasive species, spreading rapidly from its native parts of Central and South Europe due to global warming (Groenen & Meurisse, 2011). These include significant populations in in western and northern areas of Europe, including France, the Netherlands, Germany, and the UK (van Oudenhoven, van Vliet & Moraal, 2008). It owes its name to the larvae's behavioral trait of forming long processions when in search of food during the night (Koppert Biological Systems, 2020).

There has been a growing interest in the application of RNA interference (RNAi) in pest control and in the development of biological pesticides (Niu et al., 2018). RNAi is widely applied to control gene expression. It refers to a mechanism of gene silencing, where translation of target mRNA is inhibited by homologous, double-stranded siRNAs (Petrova, Zenkova & Chernolovskaya, 2013). There has been promising research for the use of RNAi as a new pest control approach in protecting food crops (Fishilevich et al., 2016; Palli, 2014). Another potential and significant target for the implementation of RNAi is the control of invasive species and nature conservation.

The larvae are present from mid-April until June, during which they feed on the leaves of different oak species; large populations have been observed to cause severe defoliation of the trees (Wagenhoff & Veit, 2011). This may have further negative consequences for tree health, including increased vulnerability to disease and other pests (EFSA, 2009). In addition, the OPC poses several health risks to humans and other animals in areas near cities and inhabited areas. From the third to sixth instar in their caterpillar life cycle, the larvae start growing and shedding setae containing thaumetopoein (Rahlenbeck & Utikal, 2015). This is an allergenic protein found in the bristles of the caterpillar, which causes several adverse reactions when it encounters humans, such as skin rashes, respiratory problems, and eye irritation (Maier et al., 2003; Rahlenbeck & Utikal, 2015).

Due to the health risks to humans and animals as well oak trees, several measures have been taken to control the speed and damage caused by the OPC. Preventative methods, such as the use of pesticides at the early stages of the caterpillar life cycle (J. Sondeijker, personal communication, May 7, 2020), are favored as they are considered to be the most effective (Straw, Williams & Tilbury, 2013). Physical methods to remove the OPC from infested trees, such as special vacuums, are also commonly used (J. Sondeijker, personal communication, May 7, 2020). However, the current methods are non-specific, ineffective, expensive and harmful to the infested oak trees and other insects. Furthermore, the currently used pesticides have great negative impact on the whole ecosystem and not just the OPC. Henceforth, these pesticides cannot be used in the forest, where the ecosystem could be severely disturbed and damaged (J. Sondeijker, personal communication, May 7, 2020). With an effective and specific control method the human and environmental impact could be greatly reduced.

To combat this major deficiency in the OPC control, the MSP-Maastricht iGEM 2020 project aims to develop a biological, species-specific and more effective alternative than the current control measures. This will either mitigate the effects caused by the allergenic protein of the OPC, or cause cellular aoptosis. To achieve this, a genetically engineered bacterial pesticide is designed to target specific and essential sequences in the OPC using RNAi.

II. RESEARCH ELABORATIONS

C. Materials and Methods

Target research and siRNA design

In order to identify target genes specific to the OPC for the siRNA inhibition, NCBI sequence data was used by performing Blast N searches of barcode genes to find unique regions. After these target genes were identified, twenty-one nucleotide sequences were identified within the genes according to siRNA design guidelines (Thermo Fisher Scientific, n.d.) which include 30-50% GC content, absence of stretches with 4 or more A's or T's in a row and an AA nucleotide beginning. These siRNA sequences were selected at different target sites for the same gene to potentially increase the effectiveness of RNA interference. These short sequences were run through BLAST again in order to assess their specificity. Finally, only siRNA that were most specific to OPC were chosen.

Network Modeling

To evaluate the effect of the gene silencing, a metabolic model for the OPC using the IMAT algorithm was created (Zur, Ruppin & Shlomi, 2010). The model was used to identify the effects of gene silencing using a single gene deletion analysis. This worked by using the expression of certain genes to model the fluxes to their respective reactions to create a model that finds a steady state from their flux balance analysis. To check whether the silencing of the chosen gene has lethal effects, a replica BIOMASS reaction was added into the model that requires materials the cell needs to survive and duplicate. The model was set to optimize for the BIOMASS reaction. Expression data from *Drosophila Melanogaster* was used in the Network Modeling. This is generally considered an appropriate model species in insect experiments, and has similar expression levels of essential housekeeping genes among different insects. The model does not directly model the siRNA but rather the expected effect, namely a gene knockout.

III. RESULTS AND FINDINGS

C. Target research and siRNA design

After the target research and siRNA design steps, four main OPC target genes were identified; Tha p2, Pro2, Wg and EF-1a. Tha p2 is the gene for the subunit 2 of the allergenic protein that can be found in the urticating setae of the caterpillars (Berardi, Battisti & Negrisolo, 2015). Pro2 photolyase gene is involved in proline metabolism, which plays an important role in insect energy metabolism (Arrese & Soulages, 2010) and EF-1a gene for elongation factor-1 alpha which takes part in the protein synthesis elongation phase. Finally, the gene for wingless protein (Wg) is thought to be involved in numerous processes through development (Simonato et al., 2013). Based on these findings specific siRNAs were identified (Table 1).

TABLE 1: OPC genes with their respective, specific siRNAs
For Tha p2, 5 DNA sequences were identified as target sites for the siRNA. 3 DNA sequences were identified for Wg and 2 for the Pro2 partial gene. For EF-1a gene 1 DNA sequence was found.

GENE	Sequences	Position in the gene	# of Off-target hits (*Thaumetopoea* Hits and other species)
Tha p2	aaatcctcgtaatccatggtc	193-213	0 and 0
	aatggctgttggagttgcggc	351-371	0 and 0
	aaggagcctaagctcgagctg	423-443	0 and 0
	aaaggcgttcttcaagtcctg	593-613	0 and 0
	aacttcttgcactccgactgt	618-638	0 and 0
Pro2 (partial)	ttgagcctagtacaacagtc	-	2 and 0
	tttcctcacaagaggagatt	-	3 and 4
Wg	aacgactccagtatgaaagt	316-335	2 and 2
	aaggcttgtctctcctacaaac	370-391	0 and 0
	aacaacacctctctacattgac	389-410	1 and 4
EF-1a	aagttcgaaactggcaaatat	91-111	4 and 1

D. Network Modelling

To evaluate the effect of the gene silencing, a metabolic model for the OPC using the IMAT algorithm, COBRA toolbox and Gurobi optimization was created (Zur et al., 2010). This led to the creation of a model that contained 5407 reactions, and 2248 genes. In this model, the knockdowns of 23 genes had a biomass reduction greater than 10%, meaning a 10% decline in metabolic function. This is generally considered lethal (Brunk et al., 2018). One out of the four previously proposed genes were in that list: Pro2 knockdown is lethal with respectively a reduction of 16% in biomass production.

The Wg gene was not lethal according to the model, as there seemed to be many backup genes and alternative pathways. so This enabled it to sustain high metabolic function despite gene knockdowns. The model was not able to test translation protein EF-1a as the model does not consider translation. Tha p2 was not tested in this model as no analogous gene was found as it is unique to the OPC.

IV. DISCUSSION

It was found that focusing on four genes specific for the OPC would enable the production of a pesticide specifically for the OPC. According to the metabolic model, the knockdown of one of the target genes would be lethal. The model, however, has limitations, because it is based on expression data of *Drosophila Melanogaster* and therefore does not include the Tha p2 gene. It can be expected that the knockdown of this gene could limit the allergenic effects on humans and other species. Furthermore, the knockdown of the transcription factor EF1a1 could lead to OPC death, as the amount of proteins produced decreases while there is interference in protein translation. Therefore, both of these genes will be included in further experiments. Wg deletion is also generally accompanied by severe to lethal developmental defects, but has back-up genes that could potentially interfere with a complete gene silencing.

In future research, two experiments should be conducted. Firstly, a PCR run to check the presence of selectd gene regions in the OPC DNA. Secondly, to prove the silencing effect of the selected siRNAs, the gene expression of the target genes should be analyzed by RT-PCR before and after siRNA treatment.

Naked siRNA sequences can be used as a negative control in this experiment.
Finally, a bacterium will be designed to produce the preselected siRNAs. In order to create the bacterial pesticide
Utilizing the RNAi mechanism, L4440 plasmid with two T7 promoters would be used as a vector and transfected into the HT115(DE3) RNase III-deficient E. coli strain. When the bacteria are modified to synthesize the preselected siRNA, its expression should be checked in order to ensure the effectiveness of genome editing.
Additionally, the team is working on the creation of an OPC outbreak model based on the model described by D. Ludwig in his famous paper: "Qualitative analysis of insect outbreak systems: the spruce budworm and forest" (Ludwig, Jones & Holling, 1978). This model will help to determine when an outbreak of the OPC will occur based on environmental factors and the mathematical approximation of these factors. The adaptation of D. Ludwig's model can be done with only minor modifications due to the similarity between the organisms. One of these modifications for example is the reduction of the impact of predation, as the oak processionary caterpillar has almost no natural predators in North Western Europe. By using this model one could determine in which areas outbreaks would occur in the future and thus preemptively act to prevent this. It could also help determine the most important factors causing the caterpillar outbreaks (Ludwig, Jones & Holling, 1978).

V. CONCLUSION

The OPC is a major environmental and public health concern. The OPC also affects a great part of the Netherlands as well as many other countries in Europe; effective interventions are needed to control its rapid spread. Current control methods are nonspecific and ineffective to use for a long period of time. However, a siRNA pesticide delivered by the engineered *E. Coli* bacteria is specific to the OPC species and could stay on the oak trees for a longer time period. Using this method of control, it is possible to lower health issues in the affected areas, restore the oak tree populations and protect the ecosystem.

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REFERENCES

Arrese, E. L., & Soulages, J. L. (2010). Insect fat body: energy, metabolism, and regulation. Annual review of entomology, 55, 207–225. https://doi.org/10.1146/annurev-ento-112408-085356

Baker, Richard., et al. (2009). Evaluation of a pest risk analysis on Thaumetopoea processionea L., the oak processionary moth, prepared by the UK and extension of its scope to the EU territory. EFSA Journal, 7(6), 1–64. doi:10.2903/j.efsa.2009.1195

Berardi, L., Battisti, A., & Negrisolo, E. (2015). The allergenic protein Tha p 2 of processionary moths of the genus Thaumetopoea (Thaumetopoeinae, Notodontidae, Lepidoptera): Characterization and evolution. Gene, 574(2), 317–324. doi:10.1016/j.gene.2015.08.019

Brunk, E., Sahoo, S., Zielinski, D. C., Altunkaya, A., Dräger, A., Mih, N., … Palsson, B. O. (2018). Recon3D enables a three-dimensional view of gene variation in human metabolism. Nature Biotechnology, 36(3), 272–281. doi:10.1038/nbt.4072Fishilevich, E., Vélez, A. M., Storer, N. P., Li, H., Bowling, A. J., Rangasamy, M., … Siegfried, B. D. (2016). RNAi as a management tool for the western corn rootworm,Diabrotica virgifera. Pest Management Science, 72(9), 1652–1663. doi:10.1002/ps.4324

Bursell, E. (1981). The role of proline in energy metabolism. In: Downer R, (Ed.). Energy Metabolism in Insects (pp. 135–54). New York: Plenum.

Groenen, F., & Meurisse, N. (2011). Historical distribution of the oak processionary moth Thaumetopoea processionea in Europe suggests recolonization instead of expansion. Agricultural and Forest Entomology, 14(2), 147–155. doi:10.1111/j.1461-9563.2011.00552.x

Koppert Biological Systems (2020). Oak processionary moth. Retrieved August 25, 2020, from https://www.koppert.com/challenges/caterpillars/oak-processionary-moth/

Ludwig, D., Jones, D. D., & Holling, C. S. (1978). Qualitative Analysis of Insect Outbreak Systems: The Spruce Budworm and Forest. The Journal of Animal Ecology, 47(1), 315–332. doi:10.2307/3939

Maier, H., Spiegel, W., Kinaciyan, T., Krehan, H., Cabaj, A., Schopf, A., & Honigsmann, H. (2003). The oak processionary caterpillar as the cause of an epidemic airborne disease: survey and analysis. British Journal of Dermatology, 149(5), 990–997. doi:10.1111/j.1365-2133.2003.05673.x

Niu, J., Taning, C. N., Christiaens, O., Smagghe, G., & Wang, J. (2018). Rethink RNAi in Insect Pest Control: Challenges and Perspectives. Advances in Insect Physiology Crop Protection, 1-17. doi:10.1016/bs.aiip.2018.07.003

Palli, S. R. (2014). RNA interference in Colorado potato beetle: steps toward development of dsRNA as a commercial insecticide. Current Opinion in Insect Science, 6, 1–8. doi:10.1016/j.cois.2014.09.011

Petrova, N. S., Zenkova, M. A., & Chernolovskaya, E. L. (2013). Structure - Functions Relations in Small Interfering RNAs. In A. O. Andrade, A. Alves Pereira, E. L. M. Naves, & A. B. Soares (Eds.), Practical Application In Biomedical Engineering (pp. 127–228). London, UK: IntechOpen. doi:10.5772/53945

Property and Environment Research Center. (2007, Winter). Less is more when it comes to packaging. Retrieved from http://perc.org/articles/less-more-when-it-comes-packaging

Rahlenbeck, S. & Utikal, J. (2015). The oak processionary moth: a new health hazard? Br J Pract, 65(637), 435-436. doi:10.3399/bjgp15X686341

Simonato, M., Battisti, A., Kerdelhué, C., Burban, C., Lopez-Vaamonde, C., Pivotto, I., … Negrisolo, E. (2013). Host and Phenology Shifts in the Evolution of the Social Moth Genus Thaumetopoea. PLoS ONE, 8(2), e57192. doi:10.1371/journal.pone.0057192

Straw, N., Williams, D. & Tilbury, C. (2013). Monitoring the oak processionary moth with pheromone traps. Forestry Commission. FCPN020, 15(2), 126-134) doi: https://doi.org/10.1111/afe.12005

Thermo Fisher Scientific. (n.d.). SiRNA Design Guidelines: Technical Bulletin #506: Thermo Fisher Scientific - US. Retrieved from https://www.thermofisher.com/de/en/home/references/ambion-tech-support/rnai-sirna/general-articles/-sirna-design-guidelines.html

Van Oudenhoven, A.P.E., van Vliet, A.J.H. & Moraal, L.G. (2008). Climate change exacerbates the oak processionary caterpillar problem in The Netherlands. Gewasbescherming. 39(6), 236-237.

Wagenhoff, E., & Veit, H. (2011). Five Years of Continuous Thaumetopoea processionea Monitoring: Tracing Population Dynamics in an Arable Landscape of South-Western Germany. Gesunde Pflanzen, 63(2), 51–61. doi:10.1007/s10343-011-0244-z

Zur, H., Ruppin, E., & Shlomi, T. (2010). iMAT: an integrative metabolic analysis tool. Bioinformatics, 26(24), 3140–3142. doi:10.1093/bioinformatics/btq602

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Light-triggered Knockdown of the *WUSCHEL* Gene in *Nicotiana benthamiana*

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Abstract: **Gene flow may reduce genetic dissimilarity between wild-type and genetically modified (GM) crops, leading to decreased biodiversity in the environment. Decreased biodiversity can have potentially harmful consequences for the natural environment. For example, invasive GM crop variants are often resistant to extreme environmental conditions and are the most prolific carriers of pathogens. To stem gene flow from transgenic crops, a solution is proposed wherein an optogenetic transcription control system is introduced into *Nicotiana benthamiana* leaves, preventing plant development upon exposure to UV-B light. This optogenetic transcription control system utilizes ULTRAVIOLET RESPONSE LOCUS 8 (UVR8) and its binding partner CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) to control the transcription of synthetic trans-acting small interfering RNAs (syn-tasiRNAs) upon exposure to UV-B light (~311 nm). These syn-tasiRNAs are highly mobile and will be transported to the shoot apical meristem (SAM) via phloem, form an RNA-induced Silencing Complex (RISC), and bind to WUSCHEL (WUS) mRNAs. The resulting knockdown of the *WUS* gene will interfere with the CLAVATA-WUSCHEL signaling pathway, causing stem cells within the SAM to differentiate. This stem cell depletion ultimately prevents growth of the whole plant.**

Index Terms- CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1), RNAi, Shoot Apical Meristem (SAM), ULTRAVIOLET RESPONSE LOCUS 8 (UVR8)

I. INTRODUCTION

For decades, the agricultural industry has utilized genetically modified (GM) crops to improve crop yields and eliminate losses due to pests and pathogens. However, gene flow—the transfer of genetic material between individuals within and among populations—may threaten agrobiodiversity (Van Deynze et al. 2016). One example of this is cross pollination, which enables GM crops to out-compete their wild-type counterparts. Today, 7,000 plant species are available for human consumption, but just four crops (wheat, maize, rice and potato) provide half of the global plant-based energy intake and another 15 contribute two-thirds (Carpenter et al. 2011).

Currently, indoor farming works to mitigate the risk of gene flow by providing some degree of control over the plants being grown. However, it has no inherent mechanism preventing the escape of transgenes. In order to issue direct control of plant growth, manipulation of the shoot apical meristem (SAM) may be desired. SAMs are the source of above-ground organs and can be classified into different zones based on cytology (Somssich et al. 2016). The central zone (CZ) contains a pool of pluripotent stem cells which divide slowly and replace the daughter cells in the peripheral zone (PZ) (Figure 1). These daughter cells, which have a higher rate of cell division, form the organ primordia on the flanks of the SAM. A small group of cells underneath the CZ, the organizing center (OC), expresses the transcription factor WUSCHEL (WUS).

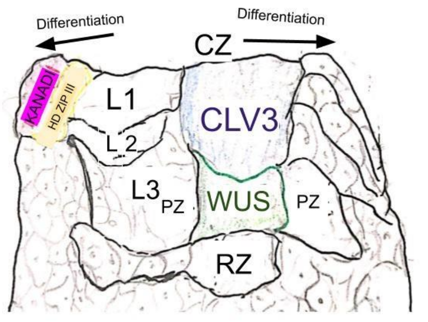

Figure 1: Structure and composition of the shoot apical meristem (SAM).

The CLAVATA-WUSCHEL signaling pathway is responsible for maintaining the meristematic stem cell population in the SAM. In *Arabidopsis thaliana*, the coordination of cell proliferation and differentiation is achieved through an autoregulatory negative feedback loop composed of the genes *WUSCHEL (WUS)* and *CLAVATA3 (CLV3)* (Figure 2). *CLV3* encodes a signaling peptide that interacts with plasma-membrane localized receptor-like kinases (RLKs) such as CLV1 and CLV2. This triggers a signalling cascade that ultimately downregulates *WUS* transcription (Adibi et al. 2016). Further research is necessary to accurately characterize the CLAVATA-WUSCHEL pathway in *N. benthamiana*, but the conservation of the negative feedback loop in plant species such as *Arabidopsis*, *Solanum lycopersicum* (tomato), *Oryza sativa* (rice), and *Zea mays* (maize) suggest that it is relatively conserved in *N. benthamiana* (Fletcher 2018).

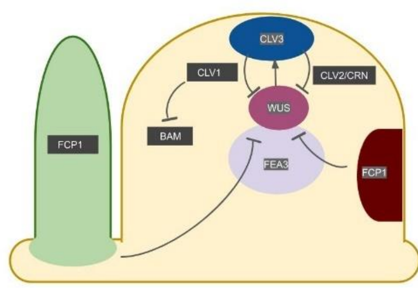

Figure 2: Representation of the CLV-WUS pathway

When *WUS* expression is reduced, stem cell differentiation is promoted and the SAM stem cell population is depleted. This allows for stem cells to differentiate but prevents them from being replaced, preventing growth of the whole plant. Knockdown of the *WUS* gene is accomplished through the production and proliferation of synthetic trans-acting small interfering RNAs (syn-tasiRNAs). Syn-tasiRNA biogenesis begins with the transcription of a syn-tasiRNA precursor with RNA Polymerase II. This single-stranded precursor undergoes Argonaute 1 (AGO1)-mediated cleavage guided by a co-expressed miRNA, miR173. The cleaved syntasi-RNA is then acted upon by RNA-dependent RNA polymerase 6 (RDR6), forming a double-stranded RNA which is cleaved by Dicer-like 4 (DCL4). This results in the formation of a mature, double stranded, 21-nt long syntasi-RNA (Allen et al. 2010). This mature syntasi-RNA is then loaded into Argonaute 2 (AGO2), which cleaves the syntasi-RNA passenger strand. The complex of the guide strand and AGO2 then forms the RNA-Induced Silencing Complex (RISC) with the guide strand and mRNA. Within the RISC, the guide strand is used for Watson-Crick base pairing to target mRNA transcript and AGO2 functions as a riboendonuclease that cleaves target mRNA (Carthew et al. 2009). Cleavage of the mRNA transcript obstructs translation, "silencing" the gene. This silencing is not isolated to the cells which produce interfering RNAs. Ta-siRNAs, si-RNAs, and mi-RNAs are highly mobile and can affect gene silencing in distal plant tissues through movement via the phloem.

The transcription of the *WUS* syn-tasiRNA is controlled by a light-inducible promoter activated by a UVR8-COP1 optogenetic pair. UVR8 is a plant photoreceptor responsible for regulating UV-B-triggered signaling pathways (Yang et al, 2015). Its binding partner, COP1, is a key regulator of photomorphogenesis. UVR8 perceives light in the UV-B region using tryptophan residues (Trp 233/285) as chromophores. Upon UV-B irradiation UVR8 undergoes a conformational change and completely dissociates, exposing a 27 residue C-terminal extension (C27), facilitating UVR8-COP1 interaction (Figure 4).

II. RESEARCH DESIGN

It is necessary to produce a UVR8-COP1 pair that does not interact with existing components of UV-B signaling pathways. To mitigate these interactions, a truncated COP1 was employed. This truncated COP1 contains only the WD40 domain critical for interaction with components of the UV-B signaling pathway, including UVR8. This abolishes the E3 ubiquitin ligase activity of COP1 and with it a portion of its downstream signalling capacity. Further modifications are being investigated but, due to a lack of full-length crystal structures of UVR8, have been confounded. To that end, homology models of the full length UVR8 were produced on I-TASSER.

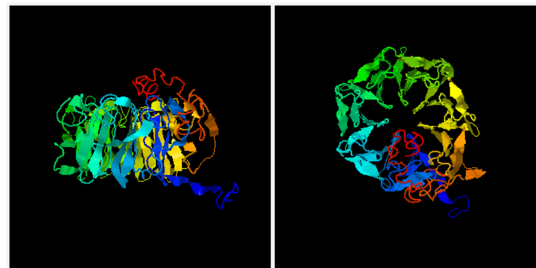

Figure 3: Full Length UVR8 Homology Models

These homology models will be employed to examine how key motifs, namely the VP motif on the C27 extension and the corresponding residues on COP1, may be manipulated to preclude interaction with further components of the UV-B signaling pathway.

pCOP1-UVR8 and pAtTASI were produced from pFGL815, an empty backbone designed for *Agrobacterium*-mediated transformation (Yang et al. 2014). pCOP1-UVR8, and pAtTASI inserts encode the optogenetic transcription control system and a syn-tasiRNA directed against *WUS* mRNA, respectively. *N. benthamiana* is then transformed through *Agrobacterium*-mediated infiltration. Upon controlled UV-B exposure, the UVR8-COP1 pair associates, activating expression of syn-tasiRNA.

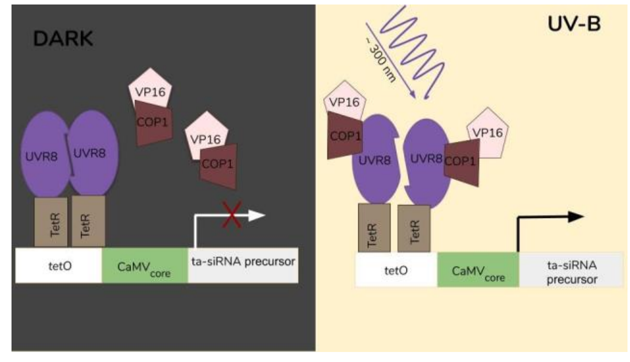

Figure 4. UV-B light inducible gene expression system. UV-B light-induced monomerization of UVR8 recruits COP1-VP16 to the CaMV core promoter, thus activating the expression of the tasi-RNA precursor.

III. RESULTS AND FINDINGS

A proof of concept model in *N. benthamiana* through transient expression of UVR8-COP1 induced transcription of our syn-tasiRNA would have been most effective to demonstrate the efficiency of this optogenetic system. However, due to the unavailability of an in-person laboratory setting, the above experiment was unable to be performed.

Future research will include the following procedures conducted in a wet lab setting. For transformant genotyping, mRNA will be extracted from the SAM and subjected to qRT-PCR. qRT-PCR primers will prime *WUS* mRNA; if the UV-B-triggered syn-tasiRNA production mechanism is functional, agarose gel electrophoresis will not yield an amplicon. For phenotyping, putative transformants will be selected by using the appropriate antibiotic in media. In this case, kanamycin and hygromycin are used to select for successful double transformants. A Western blot will be performed to detect the presence of UVR8 and COP1 fusion proteins and a Northern blot will be performed to detect the presence of syn-tasiRNA transcripts.

IV. DISCUSSION

The use of optogenetic switches in plants is a relatively new area of research. However, these switches provide distinct advantages in their spatiotemporal precision and reversible control over cellular signaling. In addition, they overcome many limitations of chemically induced systems, such as toxicity to host cells. However, optogenetics is infrequently used in plant research because of its tendency to produce undesirable system activation from ambient light. Ambient light refers to system activity affected by exposure to a non-excitatory wavelength. However, one optogenetic tool, Plant Usable Light-Switch Elements (PULSE) (Ochoa-Fernandez et al. 2020), allows for reversible control of plant gene expression without background stimulation from ambient light. Furthermore, undesirable protein-protein interactions (PPIs) with homologous proteins are one factor to consider since UVR8 and COP1 are native to *N. benthamiana*. As stated previously, key residues within UVR8 or COP1 could be modified to minimize undesirable PPIs.

V. CONCLUSION

The threat of gene flow is a major concern when it comes to preserving agrobiodiversity. Environments with an absence of UV-B light, such as indoor farms, may be utilized as part of the solution. Accordingly, a UV-B light-activated killswitch to implement in GM crops cultivated in a controlled environment has been proposed. This killswitch exploits RNAi to silence the WUSCHEL gene, a key player in plant development, ultimately preventing further growth.

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REFERENCES

Adibi, M., Yoshida, S., Weijers, D., & Fleck, C. (2016). Centering the Organizing Center in the Arabidopsis thaliana Shoot Apical Meristem by a Combination of Cytokinin Signaling and Self-Organization. Plos One, 11(2). doi:10.1371/journal.pone.0147830

Allen, E., & Howell, M. D. (2010). MiRNAs in the biogenesis of trans-acting siRNAs in higher plants. Seminars in Cell & Developmental Biology, 21(8), 798-804. doi:10.1016/j.semcdb.2010.03.008

Carpenter, J. E. (2011). Impact of GM crops on biodiversity. GM Crops, 2(1), 7–23. doi: 10.4161/gmcr.2.1.15086

Carthew, R. W., & Sontheimer, E. J. (2009). Origins and Mechanisms of miRNAs and siRNAs. Cell, 136(4), 642-655. doi:10.1016/j.cell.2009.01.035

Deynze, A. V., Bradford, K. J., Daniell, H., Ditomaso, J. M., Kalaitzandonakes, N., Mallory-Smith, C., . . . Acker, R. V. (2016). The Science of Gene Flow in Agriculture and Its Role in Coexistence. The Coexistence of Genetically Modified, Organic and Conventional

Fletcher J. C. (2018). The CLV-WUS Stem Cell Signaling Pathway: A Roadmap to Crop Yield Optimization. Plants (Basel, Switzerland), 7(4), 87. https://doi.org/10.3390/plants7040087

Ochoa-Fernandez, R., Abel, N. B., Wieland, F.-G., Schlegel, J., Koch, L.-A., Miller, J. B., Engesser, R., Giuriani, G., Brandl, S. M., Timmer, J., Weber, W., Ott, T., Simon, R., & Zurbriggen, M. D. (2020). Optogenetic control of gene expression in plants in the presence of ambient white light. Nature Methods, 17(7), 717–725. https://doi.org/10.1038/s41592-020-0868-y

Somssich, M., Je, B. I., Simon, R., &; Jackson, D. (2016). CLAVATA-WUSCHEL signaling in the shoot meristem. Development, 143(18), 3238-3248. doi:10.1242/dev.133645

Deynze, A. V., Bradford, K. J., Daniell, H., Ditomaso, J. M., Kalaitzandonakes, N., Mallory-Smith, C., . . . Acker, R. V. (2016). The Science of Gene Flow in Agriculture and Its Role in Coexistence. The Coexistence of Genetically Modified, Organic and Conventional Foods, 13-37. doi:10.1007/978-1-4939-3727-1_2

Yang, F., & Naqvi, N. I. (2014). Sulfonylurea resistance reconstitution as a novel strategy for ILV2-specific integration in Magnaporthe oryzae. Fungal Genetics and Biology, 68, 71-76. doi:10.1016/j.fgb.2014.04.00

Yang, X., Montano, S., & Ren, Z. (2015). How Does Photoreceptor UVR8 Perceive a UV-B Signal?. Photochemistry and photobiology, 91(5), 993–1003. https://doi.org/10.1111/php.12470

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The Uptake of Neuropeptides by Nematodes and its Implications for Pest Control

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***Abstract-* Crops all around the world are negatively affected by plant parasitic nematodes. Plant parasitic nematodes feed on the roots of the crop-plants and ultimately impair their growth. Current efforts to protect the plants are either insufficient or have detrimental effects on the biodiversity of the soil. The use of neuropeptides may provide a new alternative to conventional crop protection. Neuropeptides are signalling molecules used by the nervous system to modulate the activity of neuronal networks. It is widely believed that the neuropeptides are majorly responsible for the relative complex behaviour of the nematodes when we compared it to the primitive anatomy of their nervous system. This makes the use of neuropeptides a highly promising alternative to combat plant parasitic nematodes, as their impact on a nematode's functioning may be large. In our current iGEM project, we are developing *RootPatch*, a community of bacteria that colonizes the roots of potato plants. RootPatch will be engineered to produce and secrete specific neuropeptides which repel the harmful nematodes from the plants, thereby preventing their infiltration and parasitic effect. This review briefly summarizes the uptake mechanism of neuropeptides by nematodes and how they could be utilized by a method such as RootPatch.**

Index Terms- iGEM, nervous system, neuropeptide, plant parasitic nematode

I. INTRODUCTION

Plant parasitic nematodes (PPNs) are a major problem in agriculture, accounting for crop losses as large as 80 to 118 Billion USD worldwide (Nicol *et al*., 2011). Conventional methods of combating PPNs, such as fumigants and carbamate, have been withdrawn from the European market due to environmental and human health concerns (Council of the European Union, 1991). Breeding of resistant strains has often been challenging and not always effective, and genetically modified plant varieties face very stringent regulations in the EU (Nelson et al., 2018). Neuropeptides present a new and promising alternative to these existing methods of crop protection. Neuropeptides are a large group of neuroactive molecules that play an important role in modulating nematode behaviour and functioning (Li & Kim, 2008). Neuropeptide antagonists, which prevent neuropeptides from binding to their receptors, have already been discovered to act as an insecticide in several insect species (Alstein, 2001). However, just changing their levels in the nervous system may be sufficient to control parasitic insects as well. In a pioneering study of Dalzell and colleagues in 2017, they were able to show that by presenting neuropeptides in their environment, thereby changing the internal levels of the same neuropeptides, a nematode's chemotaxis behaviour can be altered leading to the avoidance of certain external stimuli such as the exudate of a crop. Utilizing these findings, we could potentially come to a new way of crop protection. In this review, we will highlight the pathway of neuropeptide uptake by a nematode and how this leads to altered neuropeptide levels in the nervous system. In addition, we will elaborate on RootPatch and how this technology could potentially open the door for protection methods against many different parasitic nematodes.

II. METHODS

Extensive Literature Survey was conducted using Google Scholar. Initially, the latest research papers (last 5 Years) were studied followed by a survey of the back references.

Neuropeptides and their uptake

Neuropeptides are highly fundamental to the functioning of nematodes. They are involved in motor function, reproduction and sensing of environmental stimuli. To support such a wide variety of functions, nematodes produce a wide array of neuropeptides, all affecting the nervous system in a different way. (Li & Kim, 2008)

Neuropeptides are different from the classical neurotransmitters in two ways: their binding activity and location. First of all, a neuropeptide can be recognized by multiple G-protein coupled receptors, having therefore a diverse effect on different types of neurons. In addition, they are capable of traveling between synapses whereas neurotransmitters are fixed to single synapses, a process called volume transmission (Li & Kim, 2008). Together, these properties result in a much broader effect of neuropeptides on the neuronal signalling and this has made it difficult for researchers to find out the function of each neuropeptide.

Small peptides are usually not considered as useful pesticides since they are easily broken down and are not readily taken up by the cell. However, the application of giving neuropeptides to nematodes may be an exception since nematodes are highly effective in taking these compounds up by their amphidia. These are sensory organs which are open to the environment and readily take up molecules.

The amphidia are located on the head of the nematode and consist of a pore and a duct which lead to a pouch that houses sensory neurons (**Figure 1**). This anatomy is broadly conserved

among nematode species (Bumbarger *et al*., 2009). The sensory neurons are organized in groups called sensilla (Ward *et al*., 1975). Different sensilla can sense different stimuli, e.g. chemical, thermal or mechanical.

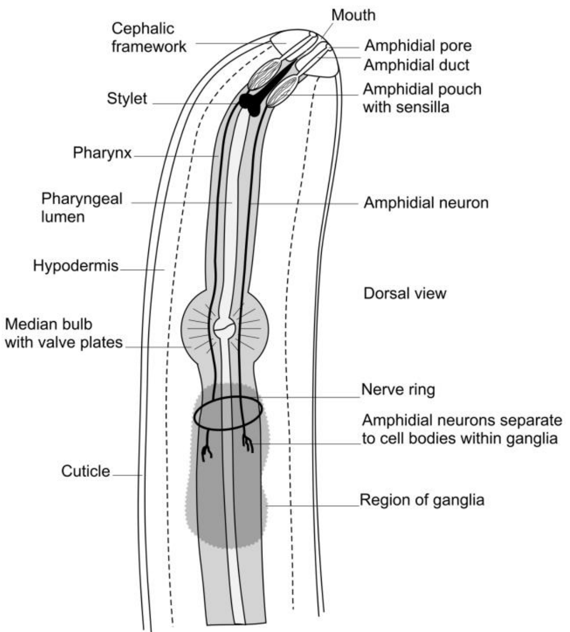

Figure 1: *Schematic representation of the head of the nematode with sensory neurons. From Wang et al. (2011)*

The sensory neurons that are packaged in the sensilla contain cilia with which the nematodes gather molecules that provide information about their surroundings. The shape of the cilia determines what kind of information they gather (Doroquez *et al*., 2014).

These neurons are the entry point of the neuropeptides. Using volume transmission, the neuropeptides travel along the axons to new synapses posterior in the nervous system. Eventually, they will come as far as the nerve ring which is connected to many other neurons in the nervous system (Ware et al., 1975). Through this route, neuropeptides can modulate the neuronal activity of many neurons and ultimately shape the nematode's behaviour (Wang et al., 2011).

Potential of neuropeptides in crop protection

Because of the ubiquitous nature and indispensable function of neuropeptides in nematodes, they have been eyed with keen interest as prospective nematicides or insecticides (Geary & Maule, 2011). Recent efforts have shown that secreted neuropeptides can effectively prevent PPNs from locating their host (Warnock *et al*., 2017). In the study of Warnock and colleagues, they tested out a wide range of different neuropeptides by applying them in the parasitic nematode's environment and found that several of them reverse their attraction towards the root exudate of their host plant. They specifically focused on a subclass of neuropeptides called neuropeptide-like proteins (NLPs). This class is hardly studied before but shows great potential since their diversity in sequence and therefore its contained effect on specific nematode species (Li & Kim, 2008) (**Table 1**). In addition, the production of this peptide on a large scale is relatively easily achievable compared with other neuropeptide classes since it is the only one that doesn't require post-translational modifications.

However, because it is not very well studied, there are still a lot of uncertainties in the possible application of these neuropeptides. Essential information such as binding receptors, stability and long-term effects still have to be explored. In addition, although all the current information implies a very high target specificity of the neuropeptide-like proteins, this still has to be investigated further to prevent any possible side-effects on other non-parasitic organisms in the soil.

Table 1: *Table with the sequences of several neuropeptide-like proteins. As the sequences point out, there are large differences between different neuropeptide-like proteins supporting the idea that each neuropeptide-like protein will have a different effect and potential in affecting different parasitic nematodes. Data from Warnock et al. (2017).*

Neuropeptide-like protein	Sequence
NLP-8A	FSDDELAAMPLNDLYLSSPYAFGPF
NLP-8B	SFDRLEESAFFGQ
NLP- 14A	ALDILESDDFGGF
NLP- 14B	ALDVMDGDGFGSFE
NLP- 14C	ALDTLEGDDFMGL
NLP- 14D	LNELEGDGFMGLD
NLP- 14E	ALDILDGDDFTGFS
NLP- 14F	ALDALEGNSFGF
NLP- 15A	SFDSLTGPGFTGLDT
NLP-15B	SFDSFTGPGFTGLD
NLP-15C	SFDSFTGSGFTGLD
NLP-15D	AAFDTDFTNYD
NLP-15E	FEPFDGYGFNGFE
......	......

III. ROOTPATCH: application of neuropeptides in crop protection

The 2020 iGEM team of the university of Groningen aims to utilize the potential of NLPs in crop protection by developing genetically modified microbes capable of producing the necessary peptides. These GMOs work together in RootPatch, a community of bacteria which coats the roots of crops that are normally affected by PPNs. The microbes in RootPatch are normally abundant in the plant's environment giving it a competitive advantage in the soil, an approach that has been proven effective several times before with genetically modified microbes in nature (Glandorf, 2019). By letting the bacteria in RootPatch produce high amounts of NLPs, an NLP-rich environment will surround the root system of the plant. Whenever a nematode comes in close proximity to the roots, it will take these NLPs up via the pathway described in this review. The uptake of these NLPs will change the balance of the neuropeptides in the nervous system, leading to a changed chemotaxis behaviour of the nematode and, therefore, an avoidance of the roots of the plant. RootPatch will initially be developed for the *Globodera pallida,* the parasitic nematode that is affecting the potato plant. However, because of the diverse nature of the NLP class, RootPatch could potentially be re-

developed for different kinds of parasitic nematodes that affect different kinds of food crops around the world. To ensure safety, the bacteria of RootPatch are dependent on molecules that are specific to the crop that it is protecting. This way, RootPatch will be contained at the roots and bacteria that leave the root environment will not be able to survive.

REFERENCES

Altstein, M. (2001) Insect neuropeptides antagonists. Biopolymers, 60(6), 460-473. https://doi.org/10.1002/1097-0282(2001)60:6<460::AID-BIP10181>3.0.CO;2-Y

Bumbarger, D. J., Wijeratne, S., Carter, C., Crum, J., Ellisman, M. H., & Baldwin, J. G. (2009). Three-dimensional reconstruction of the amphid sensilla in the microbial feeding nematode, Acrobeles complexus (Nematoda: Rhabditida). The Journal of comparative neurology, 512(2), 271–281. https://doi.org/10.1002/cne.21882.

Council of the European Union (1991). Council Directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market.

Doroquez, D.B., Berciu, C., Anderson, J.R., Sengupta, P., Nicastro, D. (2014). A high-resolution morphological and ultrastructural map of anterior sensory cilia and Glia in Caenorhabditis elegans. eLife. doi: 10.7554/eLife.01948.

Geary, T.G., Maule, A.G. (2011). Neuropeptide Systems as Targets for Parasite and Pest Control. Advances in Experimental Medicine and Biology, 692: v–vi.

Glandorf, D. C. M. (2019). Re-evaluation of biosafety questions on genetically modified biocontrol bacteria. *European Journal of Plant Pathology, 153(1), 243-251.* https://doi.org/10.1007/s10658-018-1598-1

Li, C., Kim, K. (2008). Neuropeptides. WormBook. doi/10.1895/wormbook.1.142.1.

Nelson, R., Wiesner-Hanks, T., Wisser, R., & Balint-Kurti, P. (2018). Navigating complexity to breed disease-resistant crops. Nature Reviews Genetics, 19(1), 21–33. https://doi.org/10.1038/nrg.2017.82.

Nicol, J.M., Stirling, G.R., Turner, S.J., Coyne, D.L., de Nijs, L., Hockland, S., Maafi, Z.T. (2011). Current nematode threats to world agriculture. Genomics and Molecular Genetics of Plant-Nematode Interactions, pp. 21–44.

Wang, D., Jones, L.M., Urwin, P.E., Atkinson, H.J. (2011). A Synthetic Peptide Shows Retro- and Anterograde Neuronal Transport before Disrupting the Chemosensation of Plant-Pathogenic Nematodes. PLoS ONE, 6 (3).

Ward, S., Thomson, N., White, J.G., Brenner, S. (1975). Electron microscopical reconstruction of the anterior sensory anatomy of the nematode Caenorhabditis elegans. J Comp Neurol. 160, 313–337.

Ware, R., Clark, D., Crossland, K., Russell, R.L. (1975). The nerve ring of the nematode Caenorhabditis elegans: Sensory input and motor output. J Comp Neurol. 162, 71–110.

Warnock, N.D., Wilson, L., Patten, C., Fleming, C.C., Maule, A.G., Dalzell, J.J. (2017). Nematode neuropeptides as transgenic nematicides. PLoS Pathog, 13(2). https://doi.org/10.1371/journal.ppat.1006237.

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Production of a Protein-based Antifreeze Product for Crops in the Peruvian Highlands

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***Abstract*- In the Peruvian highlands, frosts during the winters are a severe problem for agricultural communities, due to the harsh exposure and lack of protection. Frost damage on crops forces these communities to stop their working activities on fields, causing significant economic losses. Therefore, developing a solution to this issue will improve the quality of life for one of the most vulnerable sectors in the country. In nature, there is a significant population of organisms adapted to freezing temperatures (insects, plants, fish, bacteria, etc.), whose tolerance is given by Antifreeze Proteins (AFPs). Because Peruvian legislation prohibits the use of transgenic crops, we propose an indirect approach consisting of the production of an antifreeze protein. Our team intends to use *Pseudoalteromonas nigrifaciens* in order to produce AFPs in a low-tech environment that will allow production in the affected region. The final goal is to provide farmers with the ability to protect their crops from ice formation by spraying an AFP solution to plant leaves.**

Index Terms- AFPs, agriculture, frosts, *Pseudoalteromonas nigrifaciens*

I. INTRODUCTION

Environmental stressors include biotic and abiotic factors that affect plant development (*Rihan, Al-Issawi, & Fuller, 2017*). Freezing is an abiotic factor that limits plant distribution, growth, and productivity *(Yadollahpour, Bagheri, & Rahimina, 2016*). This occurs during frosts, a time in which environmental temperatures drop down to 0ºC or less (*Senamhi, 2010*) and causes ice crystal formation in the intercellular space of plant cells, leading to dehydration (*Wei et al., 2017*).

Every year, the agricultural sector in Peru faces many challenges related to frost season, which generally occurs in the Andean region and generates crop losses of up to 180 thousand hectares (*Instituto Crecer, 2018*). To prevent this, Andean communities schedule their sowings so they do not coincide with frost season (May, June and July) (*Senamhi, 2010*). Additionally, they preferably sow frost-tolerant native seeds in hillside areas and set up tall plantations on the perimeter of their properties as a protective barrier against frost (*Senamhi, 2010*). However, no particular product is being used to prevent or lessen frost damage on crops (*Senamhi, 2010*).

Due to agriculture being composed of small landowners in its majority, crop losses translate into the perpetuation of poverty in the rural population (*Keller & Echevarria, 2013*). Thus, developing a solution to this problem will improve the quality of life of one of the most vulnerable sectors of the country.

Given that, in nature, different organisms produce antifreeze proteins (AFPs) (*Chattopadhyay, 2007*), we propose to develop a product based on recombinant expression of antifreeze proteins in a useful presentation for Peruvian farmers, in order to mitigate frost damage on crops. We plan to express three types of AFPs from *Lolium perenne* and *Tenebrio molitor* (LpAFP, LpIRI3, TmAFP) in *Escherichia coli* and *Pseudoalteromonas nigrifaciens*.

P. nigrifaciens, an antarctic marine bacterium (*Baumann, Baumann, Bowditch, & Beaman, 1984*), was selected as one of the chassis, in order to make our protein production system appropriate for low-tech environments, such as possible lack of sterile conditions and standard incubators. Due to the lack of studies regarding this species, we evaluated the most suitable medium for bacterial growth, confirmed its morphological characteristics, and tested its medium selectivity and antibiotic susceptibility.

To optimize protein expression in this new chassis, our project aims to test different promoters with a GFP-reporter construct. We expect that *P. nigrifaciens* ability to grow at low temperatures and in high-salinity conditions will facilitate a high production of the AFP, and reduce medium contamination by other bacteria. This is important because we envisioned this product to be produced in low-tech environments such as the Andean region. Currently, we are working on the first stage of the project, which focuses on experiments using *E. coli*.

II. RESEARCH ELABORATIONS

A. *Materials and Methods*

P.nigrifaciens strain 217 characterization

We used *Pseudomonas bathycetes* (PB) medium for the growth of *P.nigrifaciens*. The medium contained 1% (wt/vol) proteose

peptone, 0.3% (wt/vol) yeast extract, 2.4% (wt/vol) NaCl, 0.07% (wt/vol) KCl, 0.53% (wt/vol) $MgCl_2$ and 0.7% (wt/vol) MgSO4 · $7H_2O$. To prepare this medium, we used the composition of the LB medium and added the missing salts. Plate incubation was made at 25°C overnight and kept at 4°C. Morphological characterization was made by regular procedure for Gram staining. Medium selectivity in PB for *P. nigrifaciens* was compared using *E. coli*, *Salmonella spp*., and *Pseudomonas spp.* inoculation. The antibiotic sensitivity test for *P. nigrifaciens* was made in a plate titer with exposure to different antibiotics such as ampicillin (50 µg/ml), chloramphenicol (34 µg/ml), gentamicin (50 µg/ml), kanamycin (10 µg/ml), tetracycline (5 µg/ml) and spectinomycin (100 µg/ml). Bacterium's growth curve was characterized in a TECAN spectrophotometer at 600 nm.

Genetic design

Bacterial strains, construct inserts (promoters, AFP genes) and plasmids used for AFP expression are listed in Supplementary Table 1. The designed constructs have been synthesized as gBlocks and their sequences have been optimized for the expression organisms. For *E. coli*, the construct inserts contain the restriction sites NcoI and HindIII; for *P. nigrifaciens* the restriction sites will be added with primers.

Construction of plasmids

The gBlocks assembly for the vectors will be different depending on the chosen expression organism. For *E. coli*, the parts will be digested with restriction enzymes (NcoI and HindIII) at 37℃ for 16 h. The fragments will be ligated using T4 ligase at 16°C for 16 h. For *P. nigrifaciens*, the Gibson assembly method will be used to assemble the parts of the gBlocks. All the possible combinations of each part of the *P. nigrifaciens* gBlocks are listed in Supplementary Table 2. The primers required for the Gibson assembly have yet to be defined.

Plasmid transformation

The plasmids will be transformed in *E.coli* and *P.nigrifaciens* by electroporation. One microliter of the mixture will be electroporated in electrocompetent *E. coli* BLR using 1800 V. After the pulse, the bacteria will be resuspended in SOC medium and allowed to recover under stirring for 1h at 37℃, after which it will be plated on LB agar with Kanamycin and incubated overnight. Colony PCR will be done to confirm the presence of the desired plasmids. The primers are listed in Supplementary Table 3.

Protein expression

For *E. coli*, protein expression will be induced using 0.5mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at different temperatures (16, 30, 37°C). For *P. nigrifaciens,* expression will be induced depending on the promoter, except the constitutive Pasp promoter. Galactose will be added for pMAV and L-malate for pSHAB.

Protein purification

For *E. coli*, the AFP purification will be performed using a nickel column. The protein purification protocol for *P. nigrifaciens* is not established yet.

Protein antifreeze activity test

The protein antifreeze activity will be tested in aqueous solutions, as well as in the presence of a nucleating agent (plating bead) at different temperatures (0, -1, -2, -3, -4, -5, -6, -7, -8, -9 and -10 °C) and protein concentrations (0.00, 0.10, 0.20, 0.50, 0.75 y 1.00 mg/mL). One plating bead will be placed in each test tube and 5 mL of water or the AFP solutions will be added. These solutions will be prepared in triplicate. The tubes will be immersed in a recirculating water/ethanol bath at each of the temperatures mentioned above. After 30 minutes, the tubes will be shaken vigorously and ice formation will be observed in certain tubes.

Expression optimization

Bacterial strain, construct inserts and the plasmid used for the expression optimization are listed in Supplementary Table 4. The induction of expression will be performed following the steps explained in "Protein expression".

III. RESULTS AND FINDINGS

A. *Growth and characterization of Pseudoalteromonas nigrifaciens strain 217*

P. nigrifaciens can either grow in PB or Marine medium at 25°C, but the former showed optimal growth (Figure 1). Staining characteristics confirm that it is a gram-negative bacillus (Figure 2). PB medium's selectivity test showed only *P.nigrifaciens* growth (Figure 3), which suggests that this medium is selective for this particular species. We observed that strain 217 is highly resistant to kanamycin and gentamicin but weakly resistant to spectinomycin, tetracycline and ampicillin (Figure 4). Its susceptibility to chloramphenicol was clear (Figure 4).

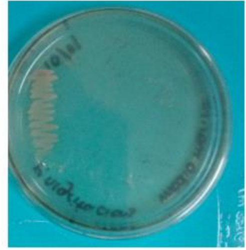

FIGURE 1: Growth of P.nigrifaciens in PB medium (left) and in Marine Medium (right)

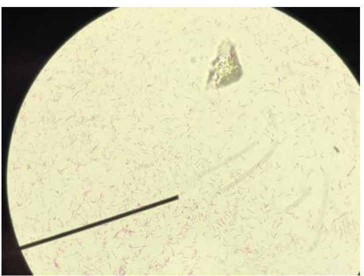

FIGURE 2: Gram-negative rods of P.nigrifaciens under a light microscope at 1000x magnification.

FIGURE 3: Growth of P.nigrifaciens, E.coli, Pseudomonas spp., and Salmonella spp. in PB medium.

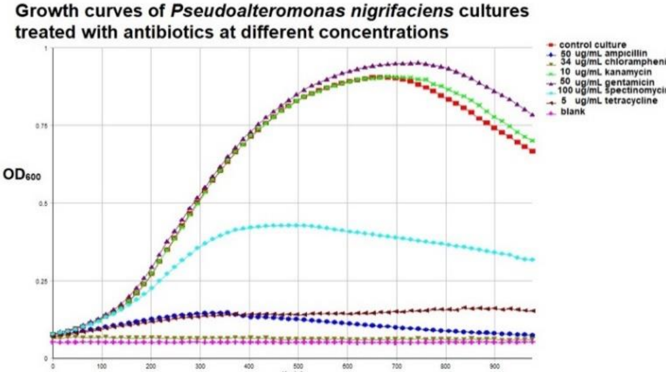

FIGURE 4: Growth curves of P.nigrifaciens in PB medium with different antibiotics. Control: No antibiotic

B. Genetic design

We decided to work with the following AFPs: LpAFP, LpIRI3 and TmAFP, because they have a high antifreeze activity characterized by their properties of inhibiting ice recrystallization (IRI) and thermal hysteresis (TH) (*Bredow, et. al., 2017; Middleton, 2011*). Two of them, LpAFP and LpIRI3, have a high IRI activity and come from a grass species, *Lolium perenne* (Figure S1). On the other hand, TmAFP has a high TH activity and comes from the beetle *Tenebrio molitor* (Figure S2). Additionally, three promoters were chosen for the production of these proteins in *P. nigrifaciens*; one promoter is constitutive (Pasp) and two of them are inducible (pSHAB and pMAV).

C. Construction of plasmids

The recombinant plasmid containing LpAFP gene (591 bp) was successfully transformed into *E. coli* BLR (Figure 5). We expect to obtain LpAFP in the next few weeks.

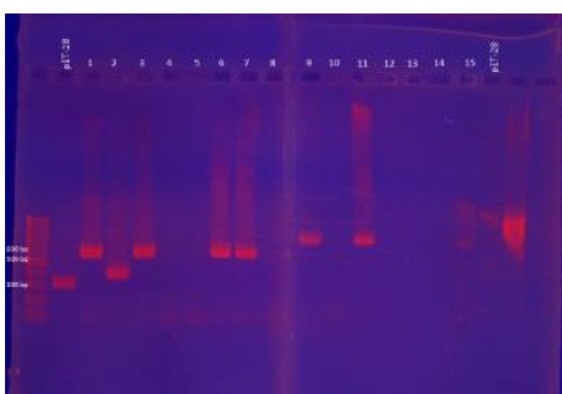

FIGURE 5: Visible bands between 500-600 bp in wells 1, 3, 6 and 7 corresponding to the recombinant plasmid containing LpAFP gene. Wells 1-15: colony PCR products from 15 transformed colonies. First well: ladder.

IV. DISCUSSION AND CONCLUSION

So far, we have successfully characterized the bacterium that will be used to produce the antifreeze proteins. We determined that the PB medium allowed better bacterial growth compared to the Marine Medium used in other studies (Figure 1) (*Ivanova et al., 1996; Ivanova et al., 1998; Ivanova et al., 2002*). Regarding antibiotic susceptibility, we determined that *P. nigrifaciens* strain 217 is highly resistant to kanamycin and gentamicin, which differs from what was found in other strains of this bacterium (*Gorshkova & Ivanova, 2001*). This confirms that the strain of *P.nigrifaciens* we plan to use for the production of the antifreeze proteins is likely to be different from strains previously studied.

The characterization we have made of *P.nigrifaciens* helps us continue with the transformation and AFP expression experiments. Although the *Pseudoalteormonas* genus has been widely used for recombinant proteins expression (*Duilio et al., 2004; Marino, 2004; Medigue, 2005; Margesin et al., 2008; Wilmes et al., 2010; Wang et al., 2015*), *P. nigrifaciens,* paticularly, has been scarcely explored at a morphological and genetic level (*Baumann, Baumann, Bowditch, & Beaman, 1984*). Therefore, all the experiments we are conducting with this species will open a future line of research around this bacterium.

At the molecular level, gBlocks construction for *P. nigrifaciens* will allow us to characterize the promoters we plan to use, which will optimize the expression of the proteins in this new chassis. Our current work in *E.coli* will help us test the antifreeze activity of the AFPs before producing them in *P.nigrifaciens*.

As part of the long-term implementation, we aim to design an accessible low-tech growth system that allows AFPs production in the Andean region. Thereby, agricultural communities will obtain the antifreeze product in a confined space, as well as in the amount and time frame required. This antifreeze product will consist of a solution of purified AFPs, which we plan to test, through the use of a spray, on the leaves of the most damaged Peruvian crops, such as potato and corn.

We still need to define the activity test to determine both the independent and joint antifreeze activity level of the AFPs. Moreover, we have to evaluate the AFPs half-life to establish the potential adjuvants for the solution.

With the development of this protein-based antifreeze product, we will be able to mitigate crop damage caused by frosts, thus helping the Peruvian high Andean population to sow and harvest all year round. In addition, through the use of synthetic biology, we hope to encourage Peruvians to trust science as an alternative solution to local problems.

APPENDIX

Supplementary Tables 1-4 and Supplementary figures (S1 and S2) can be found in the following link: https://drive.google.com/drive/folders/164J3SpFO1O9EjAReKbDv8Ah5yItklk9-?usp=sharing

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REFERENCES

Baumann, P., Baumann, L., Bowditch, R., & Beaman, B. (1984). Taxonomy of Alteromonas: A. nigrifaciens sp. nov., nom. rev.; A macleodii; and A. Haloplanktis. International Journal of Systematic Bacteriology, 34(2), 145-149. doi: 10.1099/00207713-34-2-145

Bredow, M., Vanderbeld, B., & Walker, V. K. (2017). Ice-binding proteins confer freezing tolerance in transgenic Arabidopsis thaliana. Plant Biotechnology Journal, 15(1), 68–81. doi: 10.1111/pbi.12592

Chattopadhyay, M. K. (2007). Antifreeze proteins of bacteria. *Resonance*, 12, 25–30. doi: 10.1007/s12045-007-0122-2

Duilio, A., Tutino, M. L., & Marino, G. (2004). Recombinant Protein Production in Antarctic Gram-Negative Bacteria. *Recombinant Gene Expression,* 225-238. doi:10.1385/1-59259-774-2:225

Gorshkova, N.M. & Ivanova, E.P. (2001). Antibiotic Susceptibility as a Taxonomic Characteristic of Proteobacteria of the Genera Alteromonas, Pseudoalteromonas, Marinomonas, and Marinobacter. *Russian Journal of Marine Biology,* 27, 116-120. doi: 10.1023/A:1016655516417

Instituto Crecer. (2018). Del frío de la burocracia a las heladas de la Sierra. *Noticias Gestión Perú.* Recuperado de: https://gestion.pe/blog/innovacion-sinergias-y-crecimiento/2018/08/intervenciones-del-gobierno-frente-a-las-heladas-en-el-sector-agrario.html/?ref=gesr

Ivanova, E.P., Kiprianova, E.A., Mikhailov, V.V., Levanova, G.F., Garagulya, A. D., Gorshkova, N. M., Vysotskii, M.V., Nicolau, D. V., Yumoto, N., Taguchi, T., & Yoshikawa, S. (1998). Phenotypic diversity of Pseudoalteromonas citrea from different marine habitats and emendation of the description. *International Journal of Systematic Bacteriology*, 48(1), 247–256. doi: 10.1099/00207713-48-1-247

Ivanova, E.P, Kiprianova, E.A., Mikhailov, V.V., Levanova, G.F., Garagulya, A.D., Gorshkova, N.M, Yumoto, N., & Yoshikawa, S. (1996). Characterization and Identification of Marine Alteromonas nigrifaciens Strains and Emendation of the Description. *International Journal of Systematic Bacteriology*, 46(1), 223-228. doi: 10.1099/00207713-46-1-223

Ivanova, E.P., Matte G.R., Matte M.H., Coenye, T., Huq, A., & Colwell, P.R. (2002). Characterization of Pseudoalteromonas citrea and P. nigrifaciens isolated from different ecological habitats based on REP-PCR genomic fingerprints. *Systematic and Applied Microbiology*, 25(2), 275-283. doi: 10.1078/0723-2020-00103

Keller, M., & Echeverria, D. (2013). Climate risk management for agriculture in Peru: Focus on the regions of Junin and Piura. Retrieved from www.undp.org

Margesin, R., Gerday, C., Marx, J., & Schinner, F. (2008). Heterologous Protein Expression in Psychrophilic Hosts. In *Psychrophiles: From Biodiversity to Biotechnology* (pp. 365-379). Berlin, Heidelberg: Springer-Verlag Berlin Heidelberg. doi:https://doi.org/10.1007/978-3-540-74335-4_21

Marino, G. (2004). Novel expression systems for recombinant protein production at low temperatures. Retrieved from: http://www.academia.edu/31715551/Novel_expression_systems_for_recombinan t_protein_production_at_low_temperatures

Medigue, C. (2005). Coping with cold: The genome of the versatile marine Antarctica bacterium Pseudoalteromonas haloplanktis TAC125. *Genome Research, 15*(10), 1325-1335. doi:10.1101/gr.4126905

Middleton, A., Walker, V. K., Davies, P. L., Lauersen, K. J., & Brown, A. (2011). Expression and characterization of an antifreeze protein from the perennial rye grass, Lolium perenne. Cryobiology. doi: 10.1016/j.cryobiol.2011.03.003

Rihan, H. Z., Al-Issawi, M., & Fuller, M. P. (2017). Advances in physiological and molecular aspects of plant cold tolerance. *Journal of Plant Interactions*, 12(1), 143-157. doi: 10.1080/17429145.2017.1308568

Senamhi. (2010). Atlas de heladas del Perú. Retrieved from: http://idesep.senamhi.gob.pe/portalidesep/files/tematica/atlas/helada/atlasheladas.pdf

Yadollahpour, A., Bagheri, N., & Rahimina, H. (2016). INA gene inactivation in isolated strains from frozen leaves and its effects on plant freezing. *Cercetari Agronomice in Moldova*, 3(167), 63–70. doi: 10.1515/cerce-2016-0026

Wang, P., Yu, Z., Li, B., Cai, X., Zeng, Z., Chen, X., & Wang, X. (2015). Development of an efficient conjugation-based genetic manipulation system for Pseudoalteromonas. *Microbial Cell Factories, 14*(1), 11. doi:10.1186/s12934-015-0194-8

Wei, C., Huang, J., Wang, X., Blackburn, G. A., Zhang, Y., Wang, S., & Mansaray, L. R. (2017). Hyperspectral characterization of freezing injury and its biochemical impacts in oilseed rape leaves. *Remote Sensing of Environment*, 195, 56–66. doi: 10.1016/j.rse.2017.03.042

Wilmes, B., Hartung, A., Lalk, M., Liebeke, M., Schweder, T., & Neubauer, P. (2010). Fed-batch process for the psychrotolerant marine bacterium Pseudoalteromonas haloplanktis. *Microbial Cell Factories, 9*(1), 72. doi:10.1186/1475-2859-9-72

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SPARKLE: Solar Potentiated Artificially Knitted Lipid Enclosures

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***Abstract*- Due to an increased anthropogenic pressure on the environment, the use of eco-friendly and renewable energy sources has become essential. Thus, we focused on creating a yeast cell factory for lipid production as an alternative to plant-, animal-, and chemically derived sources. However, industrial-scale bioproduction is not competitive due to its high energy demand and laborious product extraction. To boost the efficiency, we engineered yeast cells to utilize light to start lipid production, as well as to drive lipid synthesis. This approach allows us to segregate biomass accumulation and expression of lipid synthesis enzymes. The cells are coated with nanoparticles that absorb light and provide electrons for the formation of NADPH, a reductant in lipid synthesis. This results in higher yields of triacylglycerols and promotes lipid droplet formation.**

Index Terms- bioproduction, nanoparticles, TAG, yeast.

I. INTRODUCTION

Lipids are key components of the cell with functions in signaling, energy storage and cell structure. Neutral lipids (a subgroup of lipids lacking charged groups), like triacylglycerols (TAGs) and sterol esters (SE) are stored in lipid droplets (LDs) - specialized organelles emerging from the endoplasmic reticulum (ER). Neutral lipids comprise the core of LDs, which is surrounded by a phospholipid monolayer covered with perilipins (Olzmann & Carvalho, 2019).
The main function of LDs is lipid and energy homeostasis. LDs also prevent lipotoxicity by sequestrating free fatty acids that may act as detergents (Olzmann & Carvalho, 2019). Additionally, TAG in LDs is a promising feedstock for biodiesel production and could also be used in the food and pharmaceutical industries.
For LD production, we decided to use *Saccharomyces cerevisiae*, as it is a well-studied organism, whose genome can be easily modified and which tolerates extreme fermentation conditions (Ma et al., 2019); Zhou et al., 2016). However, *S. cerevisiae* natively produces lipids only at low levels (Johnson et al., 1972). Therefore, genetic manipulation is necessary to reach high TAG amount (Ferreira et al., 2018).

There are different approaches to increase TAG content. One of them is inactivation of lipid-hydrolyzing enzymes. The deletion of TAG lipases (Tgl3/4/5; Fig. 1) increases TAG's abundance (Ferreira et al., 2018).
Another approach is increasing the carbon supply for lipid biosynthesis. Acetyl-CoA carboxylase (Acc1; Fig.1) catalyzes the conversion of acetyl-CoA into malonyl-CoA, a fundamental precursor for lipid synthesis. Mutation of protein kinase Snf1 phosphorylation sites in Acc1 leads to elevated Acc1 activity which causes an increase in lipid production (Chen et al., 2018).
Overexpression of lipid-synthesizing enzymes and improving capacity for LD formation can further enhance lipid biosynthesis. Overexpression of PAH1 (converts PA, phosphatidic acid, to DAG, diacylglycerol), DGA1 (acyl-CoA: diacylglycerol acyltransferase, which converts DAG to TAG) and PLIN3 (covers phospholipid monolayer of LDs) (Fig.1) has been shown to increase the accumulation of TAG in *S. cerevisiae* (Teixeira et al., 2018).

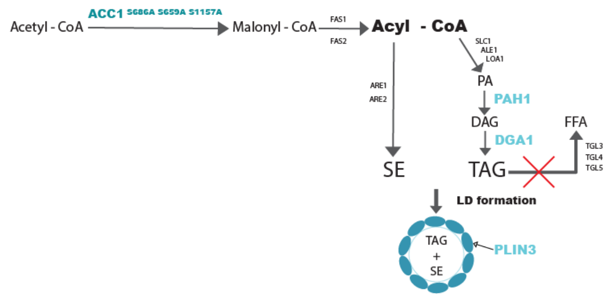

FIGURE 1: Schematic representation of TAG biosynthesis in S. cerevisiae. Acc1 is subjected to three phosphorylation site mutations shown in blue. Overexpressed proteins are highlighted with light blue. Red X - TAG hydrolysis to fatty acids is prevented by deletion of TAG lipases (Tgl3/4/5).

TAG accumulation depends on *de novo* fatty acid biosynthesis which is catalyzed by Fatty Acid Synthase (FAS) that uses malonyl-CoA and NADPH (Ma et al., 2019). The primary source of NADPH is the pentose phosphate pathway (PPP). During its oxidative phase, per each glucose molecule two NADPH are produced and one CO_2 is released. By inhibiting the PPP oxidative phase it is possible to enhance carbon flux

towards TAG production if an alternative way of NADPH generation is used.
To do this we can use the biological hybrid system created by (Guo et al., 2018). It combines the deletion of the gene *ZWF1* (which encodes glucose-6-phosphate dehydrogenase) to disrupt the oxidative portion of the PPP, and use of surface-bound indium(III) phosphide nanoparticles. The nanoparticles will donate electrons to regenerate NADPH for lipid production.

In SPARKLE, we aimed to develop a light-powered yeast cell factory that would serve as a platform for efficient and adjustable lipid production in the form of TAGs. To promote TAG build-up, we will delete *TGL3/4/5* genes, and replace ACC1 with constitutively active $ACC1^{S686A,S659A,S1157A}$. Also, we construct an overexpression cassette, where *PAH1*, *DGA1*, and human *PLIN3* genes are under the control of a light-inducible transcription factor VP-EL222 described in (Benzinger & Khammash, 2018).
Furthermore, to reduce carbon loss and maximize lipid production, we are planning to utilize a yeast - indium phosphide hybrid system for NADPH generation (Guo et al., 2018). Finally, we aim to improve inducible yeast autolysis to ease the product extraction by overexpression of glucanases ("Team:Tartu TUIT - 2019.igem.org," n.d.).

II. RESEARCH ELABORATIONS

We used mathematical modeling to assess the feasibility of our project and to predict the final TAG yields. We modelled the expression of *PAH1* and *DGA1,* which are under control of light-inducible promoters 5xBS-CYC180pr and 5xBS-GAL1pr, respectively. The expression of *PLIN3* was not considered, since perilipin, its product, is a structural protein in lipid droplets and does not directly affect TAG production (Teixeira et al., 2018).
The following set of ordinary differential equations (ODEs) describes VP-EL222-mediated expression (Benzinger & Khammash, 2018):

$$(1)\ \frac{d[TF_{on}]}{dt} = I \cdot k_{on} \cdot ([TF_{total}] - [TF_{on}]) - k_{off} \cdot [TF_{on}]$$

$$(2)\ \frac{d[mRNA]}{dt} = k_{basal} + k_{max} \cdot \frac{[TF_{on}]^n}{K_d^n + [TF_{on}]^n} - k_{degR} \cdot [mRNA]$$

$$(3)\ \frac{d[Protein]}{dt} = k_{trans} \cdot [mRNA] - k_{degP} \cdot [Protein]$$

Here we assume constitutive expression of TF at the level of 2000 molecules/cell, as reported by Benzinger (2018). In the first equation, parameter I denotes the blue light input. Further description of parameters, their values, and sources can be found in Supplementary Table 1. MATLAB ode23t function was used to solve this ODE system.
To assess the accuracy of our model, we modelled TAG production using Michaelis-Menten equation and compared the yields with published values. Michaelis-Menten model describes a reaction involving an enzyme E, which binds a substrate S to form a product P and the enzyme E. Under the quasi-steady-state approximation, the rate of product formation can be expressed as Michaelis-Menten equation (Briggs & Haldane, 1925):

$$\frac{d[P]}{dt} = \frac{k_{cat}[E]_0}{K_d + [S]}$$

This allows us to find the rate of change of product at time t, given the concentrations of enzyme and substrate.
We first modelled the accumulation of TAG precursor DAG. TAG formation is spatially constrained to certain regions of ER (Athenstaedt & Daum, 2006). Therefore, our model assumes the formation of DAG and TAG from microsomal PA (i.e., PA found only in ER). We estimated the concentration of microsomal PA given 24.2 mg phospholipids/g CDW and that 0.2% of phospholipids is microsomal PA (Grillitsch et al., 2011; Zinser et al., 1991). To account for the expression of endogenous *PAH1* and *DGA1*, we consider the median abundance of their products (2607 and 1431 molecules/cell, respectively).

III. RESULTS AND FINDINGS

To test the dynamics and tunability of the VP-EL222-mediated expression, we modelled the expression of *PAH1* and *DGA1* under light intensities ranging from 0 to 400 μW cm^{-2} (Fig. S1). When *PAH1* expression was induced under maximum light intensity, concentration of its product PAP reached half of its steady-state concentration in under 50 min.

TAG concentration reached the value of about 100 mg/gCDW (Fig. 2). A similar study reported yields of around 80 mg/gCDW of TAG for a strain with overexpressed *DGA1*, *PAH1*, deletions of lipase genes *TGL3/4/5*, and $ACC1^{S659A,S1157A,S686}$ mutation (Teixeira et al., 2018). Both modeled and literature yields at the end of 72 h cultivation are reported.

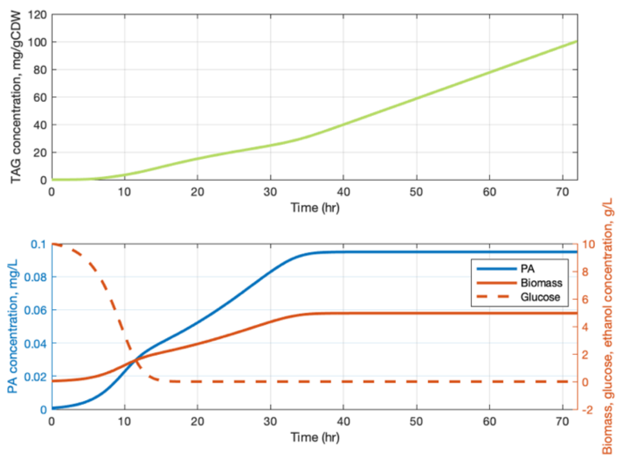

FIGURE 2: Top panel: TAG production under constant illumination with a light intensity of 420 μW cm^{-2}. Bottom panel: estimated PA, biomass and glucose concentrations.

IV. DISCUSSION

We constructed a mathematical model of TAG production in lipid droplets that takes into account overexpressed *PAH1* and *DGA1* genes as well as deleted TAG lipases *TGL3/4/5*. The

latter is reflected in the model by the fact that degradation of TAG is not considered. Our model allows us to predict the final yield of TAG at the end of a fermentation period of any given length and under different light intensities, which makes it a useful tool during the design of the production process.

Our model, however, does not consider the effect of electron generation by nanoparticles or the efficiency of our improved yeast autolysis method. The electron transfer from semiconductor to cytosol, and eventually to the enzyme, is a complex process which is not yet fully understood (Guo et al., 2018). Although these aspects will affect the final lipid yield, they are difficult to model mathematically using available tools.

Our modeling results indicate that the light-regulated expression has fast response and low basal activity, enabling to achieve the desired gene expression. Compared to other induction systems that require changes in growth medium, the light induction is inexpensive and does not require the use of chemicals that may have off-target effects (Johnston & Davis, 1984; Mountain et al., 1991). It allows precise control of protein activities in the cell factory, which is necessary to maximize the efficiency of the production process.

In SPARKLE, we have designed a fast, easy, and adjustable platform for lipid production in the model organism *S. cerevisiae*. It is a well-studied organism widely used for industrial applications that gives it an advantage over the use of other yeasts in terms of developing the system for more sustainable large-scale lipid production.

APPENDIX

Supplementary data is available here.

REFERENCES

Athenstaedt, K., & Daum, G. (2006). The life cycle of neutral lipids: Synthesis, storage and degradation. In *Cellular and Molecular Life Sciences*. https://doi.org/10.1007/s00018-006-6016-8

Benzinger, D., & Khammash, M. (2018). Pulsatile inputs achieve tunable attenuation of gene expression variability and graded multi-gene regulation. *Nature Communications*. https://doi.org/10.1038/s41467-018-05882-2

Briggs, G. E., & Haldane, J. B. S. (1925). A Note on the Kinetics of Enzyme Action. *Biochemical Journal*. https://doi.org/10.1042/bj0190338

Chen, X., Yang, X., Shen, Y., Hou, J., & Bao, X. (2018). Screening Phosphorylation Site Mutations in Yeast Acetyl-CoA Carboxylase Using Malonyl-CoA Sensor to Improve Malonyl-CoA-Derived Product. *Frontiers in Microbiology*, *9*, 47. https://doi.org/10.3389/fmicb.2018.00047

Denomme, J., Stark, K. D., & Holub, B. J. (2005). Directly Quantitated Dietary (n-3) Fatty Acid Intakes of Pregnant Canadian Women Are Lower than Current Dietary Recommendations. *The Journal of Nutrition*, *135*(2), 206–211. https://doi.org/10.1093/jn/135.2.206

Domergue, F., Lerchl, J., Zähringer, U., & Heinz, E. (2002). Cloning and functional characterization of Phaeodactylum tricornutum front-end desaturases involved in eicosapentaenoic acid biosynthesis. *European Journal of Biochemistry*, *269*(16), 4105–4113. https://doi.org/10.1046/j.1432-1033.2002.03104.x

Enzyme Explorer Key Resources: Lysing Enzymes | Sigma-Aldrich. (n.d.).

Ferreira, R., Teixeira, P. G., Gossing, M., David, F., Siewers, V., & Nielsen, J. (2018). Metabolic engineering of Saccharomyces cerevisiae for overproduction of triacylglycerols. *Metabolic Engineering Communications*, *6*, 22–27. https://doi.org/https://doi.org/10.1016/j.meteno.2018.01.002

Grillitsch, K., Connerth, M., Köfeler, H., Arrey, T. N., Rietschel, B., Wagner, B., Karas, M., & Daum, G. (2011). Lipid particles/droplets of the yeast Saccharomyces cerevisiae revisited: Lipidome meets Proteome. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*. https://doi.org/10.1016/j.bbalip.2011.07.015

Guo, J., Suástegui, M., Sakimoto, K. K., Moody, V. M., Xiao, G., Nocera, D. G., & Joshi, N. S. (2018). Light-driven fine chemical production in yeast biohybrids. *Science*, *362*(6416), 813 LP – 816. https://doi.org/10.1126/science.aat9777

Inokuma, K., Kurono, H., den Haan, R., van Zyl, W. H., Hasunuma, T., & Kondo, A. (2020). Novel strategy for anchorage position control of GPI-attached proteins in the yeast cell wall using different GPI-anchoring domains. *Metabolic Engineering*, *57*, 110–117. https://doi.org/https://doi.org/10.1016/j.ymben.2019.11.004

Johnson, B., Nelson, S. J., & Brown, C. M. (1972). Influence of glucose concentration on the physiology and lipid composition of some yeasts. *Antonie van Leeuwenhoek*, *38*(1), 129–136. https://doi.org/10.1007/BF02328084

Johnston, M., & Davis, R. W. (1984). Sequences that regulate the divergent GAL1-GAL10 promoter in Saccharomyces cerevisiae. *Molecular and Cellular Biology*, *4*(8), 1440 LP – 1448. https://doi.org/10.1128/MCB.4.8.1440

Liu, J., Chen, H., Ma, L., He, Z., Wang, D., Liu, Y., Lin, Q., Zhang, T., Gray, N., Kaniskan, H. Ü., Jin, J., & Wei, W. (2020). Light-induced control of protein destruction by opto-PROTAC. *Science Advances*, *6*(8), eaay5154. https://doi.org/10.1126/sciadv.aay5154

Ma, T., Shi, B., Ye, Z., Li, X., Liu, M., Chen, Y., Xia, J., Nielsen, J., Deng, Z., & Liu, T. (2019). Lipid engineering combined with systematic metabolic engineering of Saccharomyces cerevisiae for high-yield production of lycopene. *Metabolic Engineering*, *52*, 134–142. https://doi.org/https://doi.org/10.1016/j.ymben.2018.11.009

Malone, J. P. (2012). The Systems Theory of Autistogenesis: Putting the Pieces Together. *SAGE Open*, *2*(2), 2158244012444281. https://doi.org/10.1177/2158244012444281

Mountain, H. A., Byström, A. S., Larsen, J. T., & Korch, C. (1991). Four major transcriptional responses in the methionine/threonine biosynthetic pathway of Saccharomyces cerevisiae. *Yeast*, *7*(8), 781–803. https://doi.org/10.1002/yea.320070804

Olzmann, J. A., & Carvalho, P. (2019). Dynamics and functions of lipid droplets. *Nature Reviews Molecular Cell Biology*, *20*(3), 137–155. https://doi.org/10.1038/s41580-018-0085-z

Orlean, P. (2012). Architecture and Biosynthesis of the Saccharomyces cerevisiae Cell Wall. *Genetics*, *192*(3), 775 LP – 818. https://doi.org/10.1534/genetics.112.144485

Stewart, G. G. (2017). *The Structure and Function of the Yeast Cell Wall, Plasma Membrane and Periplasm BT - Brewing and Distilling Yeasts* (G. G. Stewart (Ed.); pp. 55–75). Springer International Publishing. https://doi.org/10.1007/978-3-319-69126-8_5

Teixeira, P. G., David, F., Siewers, V., & Nielsen, J. (2018). Engineering lipid droplet assembly mechanisms for improved triacylglycerol accumulation in Saccharomyces cerevisiae. *FEMS Yeast Research*, *18*(6). https://doi.org/10.1093/femsyr/foy060

Zhao, E. M., Zhang, Y., Mehl, J., Park, H., Lalwani, M. A., Toettcher, J. E., & Avalos, J. L. (2018). Optogenetic regulation of engineered cellular metabolism for microbial chemical production. *Nature*, *555*(7698), 683–687. https://doi.org/10.1038/nature26141

Zhou, Y. J., Buijs, N. A., Zhu, Z., Qin, J., Siewers, V., & Nielsen, J. (2016). Production of fatty acid-derived oleochemicals and biofuels by synthetic yeast cell factories. *Nature Communications*, *7*(1), 11709. https://doi.org/10.1038/ncomms11709

Zinser, E., Sperka-Gottlieb, C. D., Fasch, E. V, Kohlwein, S. D., Paltauf, F., & Daum, G. (1991). Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote Saccharomyces cerevisiae. *Journal of Bacteriology*, *173*(6), 2026–2034. https://doi.org/10.1128/jb.173.6.2026-2034.1991

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The Magnetic ATP Recycling System

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***Abstract-* The demand for safe, eco-friendly and cost-efficient ways of energy generation and supply is not only of great relevance in the macroscopic world of everyday life, but also in relation to the molecular level. As one of the most important energy sources for metabolic reactions and enzymatic pathways, Adenosine triphosphate (ATP) plays a vital role in various fields like production of medical drugs and fine chemicals. It currently takes several time-consuming and costly steps to meet the need for ATP in all these different areas of application. Here, we present and establish M.A.R.S., short for magnetic ATP recycling system, an innovative strategy to create light-powered, mitochondrion-like protocells and a bioreactor that will recycle those cells by magnetism, while simultaneously feeding it into a cell-independent, product-oriented enzyme cascade. The results of this project give new insights into the industrial production of ATP.**

Index Terms- ATP, energy, iGEM, light driven, recycling

I. INTRODUCTION

The complex regeneration of biochemical energy sources represents a cost-intensive hurdle for many production and research processes. A corresponding energy source is also required when exporting such enzyme reactions into a system that is independent of living cells, for example to produce pharmaceuticals or fine chemicals. The generation of these molecular energy source has so far been very expensive, not always eco-friendly and often requires a higher effort than the enzyme reaction itself.
Adenosine triphosphate (ATP), a nucleotide and complex organic molecule, serves in the cells of all living things as a universal transport and storage material for energy and is therefore produced and consumed in large quantities every day. Due to the hydrolytic cleavage of its high-energy anhydride bonds, it supplies the necessary energy for the work of many enzymes by releasing up to 50 kJ/mol (Conrath, 2004). As a result, however, ATP is converted into a less potent energy source, namely adenosine diphosphate (ADP), and must be regenerated in metabolic cycles as the respiratory chain or phototrophy to make it available again (Berg, Tymoczko, & Stryer, 2014). ATP is mainly recycled with the help of ATP synthase (ATPase). This enzyme complex uses a proton gradient built up over a membrane to synthesize ATP from its individual components ADP and phosphate (P_i) by means of phosphorylation. In many cases, the proton gradient is built up by an electron transport chain. Protein complexes in the organelle membrane use the energy of the electron flow to translocate protons from one side of the membrane to the other. The resulting uneven distribution of protons creates a pH gradient and an electrical transmembrane potential, which ultimately generates the proton motor force required to drive the ATPase (Berg et al. 2014). Instead of the electron transport chains, a few bacteria and archaea use various alternative ways to generate the proton gradient. These include *Halobacterium salinarum*, a marine, obligately aerobic, extremely halophilic archaea species, which requires a minimum salt content of 20% to grow (Becher et al. 1975). Being a phototroph, it can maintain its metabolism with the help of light energy in the event of a lack of oxygen. For that purpose, it produces the purple pigment bacteriorhodopsin (BR) and embeds it in newly synthesized membrane segments, the so-called purple membrane (PM). While absorbing a photon of light, the retinal chromophore of BR is isomerized, which leads to a proton shift by pumping protons from the cytoplasmic to the extracellular side of the protein pore and thus creating a proton gradient (Becher et al. 1975).
Like many other enzymes, the mitochondrial ATPase has specific regulatory mechanisms. One of them is the mitochondrial F_1-ATPase inhibitor protein (IF_1). It inhibits the hydrolytic but not the synthetic activity of the ATPase and thus prevents the consumption of cellular ATP. Due to its pronounced specificity, the IF_1 is also suitable for highly selective affinity chromatography for the purification of the enzyme complex from the mitochondria (Runswick et al. 2013).
The production of synthetic cells is being researched with increasing success within synthetic biology. Easily applicable methods such as those from Göpfrich et al. (2019) may soon enable a standardized procedure. In addition to the demonstration of the function of BR or ATP synthase through the coreconstitution into polymersomes or liposomes, as well as the investigation of their kinetics and their influence on each other, the possibility of cell-independent energy generation crystallized from the experiments of the last decades (Runswick et al. 2013; Van der Bend et al. 1985; Berhanu et al. 2019). So far, however, there is no knowledge of how long synthetic cells with these membrane proteins can be kept and stay functional, let alone a method of reusing them.
The aim of project "M.A.R.S." is to use already successful and easily reproducible research to establish an innovative energy supply for enzymatic reactions of all kinds. Therefore, it will be possible to combine ATP-dependent turnover reactions directly with their necessary energy source. For this purpose, simple protocells will be enabled to recycle ATP only through exposure to light by incorporating BR and ATP synthase. By connecting these "chassis" with magnetic particles via anchor peptides, the reuse of the protocell system in a specially designed reactor will be made possible. This should avoid the need to produce new

recycling cell systems and thereby reduce production costs in the long term.

II. RESEARCH ELABORATIONS

The ATP synthase is obtained from mitochondria of *Saccharomyces cerevisiae*. For this purpose, fresh medium (peptone, yeast extract, 3% (v/v) glycerol, adenin, antifoam 204) was inoculated with cells after two previous preculture steps to produce a 500 mL main culture, which was then incubated at 30 °C on the shaker. To harvest the mitochondria, the cells were lysed using the ultrasonicator and the French press. Afterwards, they were separated from cell debris and remaining organelles using several centrifugation steps. After subsequent washing steps and resuspension in buffer (20 mM Tris–HCl, pH 8.0, and 10 per cent glycerol(v/v)), the mitochondria were initially stored at -20 °C (Runswick et al. 2013). For the purification of the ATP synthase, a single chromatography step will be used with the aid of the highly selective IF_1 with a C-terminal glutathione-S-transferase (GST) domain. For this purpose, IF_1 will be expressed in *Escherichia coli* C41 (DE3) by means of plasmid cloning and purified by affinity chromatography with a Hi-Trap nickel sepharose column equilibrated in buffer (20 mM Tris–HCl (pH 7.4), 10% (v/v) glycerol, 25 mM imidazole, and 0.1 M sodium chloride) and subsequent dialysis against buffer (20mM Tris-HCl, pH 7,4) (Runswick et al. 2013). The inhibitor protein is then concentrated by centrifugal concentrators (Runswick et al. 2013).
BR was extracted from *H. salinarum* R1 by purifying the PM. For this purpose, a 500 mL main culture was incubated by inoculating fresh medium with a preculture for eight days at 37 °C on a shaker under constant lightning. To induce the expression of the PM, the incubation vessel was closed and thus an increasing oxygen deficit was created. The cells were then lysed by osmotic shock with the aid of Aqua Bidest and the PM was separated from cell debris by several centrifugation steps. For a gradual separation of the PM according to purity, a sucrose density gradient centrifugation with subsequent dialysis of the purest fraction was carried out. The PM was then resuspended in buffer (0,01 M Potassium-Phosphate-Buffer at pH 7) stored at -20 °C (Becher et al. 1975).
The formation of liposomes was carried out by rehydrating a dry lipid film. To achieve this, the liquid lipid mixture was evaporated using a rotary evaporator and the resulting lipid film was rehydrated using Sodium-Bicine-Buffer. The result was checked using fluorescence microscopy (Zhu et al. 2013).
By mixing liposomes, green fluorescent protein (GFP), magnetic particles and various anchor peptides, binding tests were performed using a microtiter plate photometer (TECAN).

III. RESULTS AND FINDINGS

Polymersomes were synthesized as a proof of concept using rehydration methods. Liposomes were synthesized with soybean lipids via film rehydration method. The liposomes were used for further lab work. The gene coding for the F_1-ATPase inhibitory protein was extracted.
The binding capabilities of several anchor peptide candidates to magnetic polystyrene beads were evaluated via confocal microscopy. LCI, MBP-1, CG-DEF and TA all successfully showed binding behavior to the magnetic particles, the best binding was observed for MBP-1 (Figure 1). MBP-1 was tagged with GFP before the coupling.
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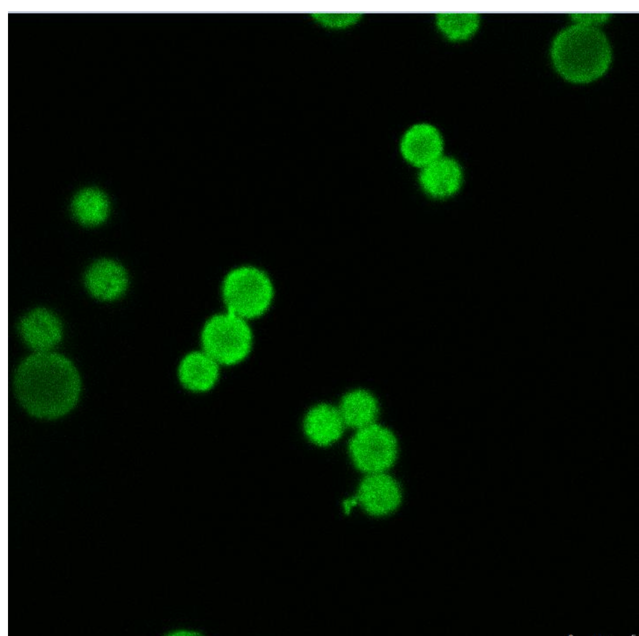

FIGURE 1: Confocal Microscopy Image of MBP-1 (GFP-Tagged) coupled to magnetic polystyrene particles

All candidates did not bind to the synthesized liposomes. Zecropin-A showed promising binding behavior to the liposomes. The bound liposomes could not be separated by centrifugation.
The integration of BR into the liposomes for activity testing, the recombinant expression of the IF_1 and further binding tests for the liposome anchor peptides were started in the wet lab.

IV. DISCUSSION

Since the integration of membrane proteins into polymeric membranes is a delicate and time-consuming process, liposomes were used in the wet lab to conduct further testing and to construct our chassis. The expectation is to observe the same capabilities with liposomes, except the estimated stability. To deliver results with high confidence in similarity, a calcein stability test will be conducted with the produced liposomes. The binding tests with MBP-1 and Zecropin-A both showed promising results. Zecropin-A will be tested further. A fitting linker peptide must be evaluated to finish the development of the binding mechanism between the chassis and the magnetic beads. The isolation of the purple membrane of *H. salinarum* remains challenging. The experiment is currently repeated with a different centrifugation setup. After the recombinant expression of F_1-ATPase inhibitory protein and purification of BR from the purple membrane, the chassis can be fully

synthesized by insertion of both membrane proteins. The current progress in the planned experiments shows great potential for the achievement of the envisioned main goal, the completion of magnetically coupled chassis. The final goal is the completion of a M.A.R.S. bioreactor, which uses the constructed chassis and its magnetic abilities to produce ATP continuously for any ATP-dependent process. The team's hardware group is close to conceptual completion and modelling of a prototype batch process bioreactor, which will enable testing the chassis in large quantities.

V. CONCLUSION

The presented innovative idea of M.A.R.S., short for magnetic ATP recycling system, ranks at the forefront of scientific research in industrial biotechnology. The vision, to use synthetic biology and combine some of the most interesting methods available, enables to power the fifth industrial revolution, a revolution of biotechnological engineering. Scientists all over the world envision great ideas and concepts to make industrial production of bulk, fine and medical components more efficient, eco-friendly and precise. Many of them lack a convenient and efficient way to power their turnover reactions. This is where M.A.R.S. comes into play, by taking the first step in a new direction of cofactor regeneration. Through the research and development of this unique project, it is possible to get all of us and the whole biotechnological industry energized.

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REFERENCES

Becher, B. M. & Cassim, S. Y. (1975). Improved Isolation Procedures for the Purple Membrane of Halobacterium Halobium. Preparative Biochemistry, 5:2, 161-178, DOI: 10.1080/00327487508061568

Berg, J. M., Tymoczko, J. L., Stryer, L. (2014). Biochemie, Kapitel 18 – Die oxidative Phosphorylierung.

Berhanu, S., Ueda, T., Kuruma, Y. (2019). Artificial photoynthetic cell producing energy for protein synthesis. *Nat Commun* 10, 1325 (2019). https://doi.org/10.1038/s41467-019-09147-4

Conrath, U. (2004). Einführung in die Biochemie – Metabolismus.

Göpfrich, K., Haller, B., Staufer, O., Dreher, Y., Mersdorf, U., Platzman, I., Spatz, J. P. (2019). One-Pot Assembly of Complex Giant Unilamellar Vesicle-Based Synthetic Cells. ACS Synth. Biol. 2019, 8, 937−947.

Michel, H., Oesterhelt, D. (1976). Light-induced changes of the pH gradient and the membrane potential in *H. halobium.*

Mosin, O., Ignatov, I. (2015). The Improved Method for Isolation of Photochrome Transmembrane Protein Bacteriorhodopsin from Purple Membranes of Halobacterium *Halobacterium Halobium ET 1001*.

Oesterhelt, D. (1976). Bacteriorhodopsin as an Example of a Light-Driven Proton Pump. https://doi.org/10.1002/anie.197600171

Runswick, M. J., Bason, J. V., Montgomery, M. G., Robinson, G. C., Fearnley, I. M., Walker, J. E. (2013). The affinity purification and characterization of ATP synthase complexes from mitochondria. Open Biol 3: 120160. http://dx.doi.org/10.1098/rsob.120160

Van der Bend, R. L., Petersen, J., Berden, J. A., Van Dam, K., Westerhoff, H. V. (1985). A critical appraisal of evidence for localized energy coupling. Kinetic studies on liposomes containing bacteriorhodopsin and ATP synthase. *Biochem J* (1985) 230 (2): 543–549. https://doi.org/10.1042/bj2300543

Zhu, T. F., Budin, I., Szostak, J. W. (2013). Preparation of fatty acid or phospholipid vesicles by thin-film rehydration.

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Bellatrix: Software Providing a Novel Protein Representation Method

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***Abstract*- In a machine learning world, data is an essential currency. For proteins, the Protein Data Bank offers a large collection of experimentally verified protein structures, providing lots of helpful data. However, these file types are often arduous to work with and do not easily integrate into machine learning methods. Ideally, these files and the structural phenomena they portray would be represented by a large scale matrix, as this allows for efficient data manipulation. As a step towards this utopia, the Star data structure was born. Star files are large matrices composed of distance vectors between atoms and every other atom present in a protein. Bellatrix, a software capable of creating standardized Star files was developed with Python using numerous packages. Computationally, this work and its implications within deep learning open up applications within the field of homology modelling. Bellatrix has generated numerous protein Star file libraries with sub-minute read and write times per protein. Bellatrix hopes to provide a novel protein representation method to ignite new perspectives, hypotheses, and practices with impacts that stretch beyond the world of biochemistry.**

Index Terms- Protein Engineering, iGEM, PDB, Python

I. INTRODUCTION

Databases are the cornerstone for computational biochemistry, providing access to data that is both reliable and accessible (Xu & Xu, 2004). The Protein Data Bank consists of over 160,000 files, each containing detailed structural information on proteins. Protein Data Bank files, known as PDB files, are a treasure trove of high-quality structural data and provide invaluable information without the demand for expensive laboratory equipment and expertise. PDB files and the information they provide have fingerprints across the field of computational biology. Popular methods include homology modelling, ligand docking, drug discovery, and molecular dynamics, all made possible through the data provided by PDB files.

One strength that arises from the use of PDB files is that it simplifies the task of non-traditionally representing these protein structures. These novel representations breathe new life into the field of computational protein analysis, allowing it to continue growing with a new set of user friendly tools. Eliminating some of the roadblocks associated with using and manipulating PDB files can also help facilitate this growth. The use of a novel, refined filetype also has a potential application in the emerging field of machine learning. These can then be leveraged to assist in other biological industries such as pharmaceutics and agriculture.

To capitalize upon the plethora of information PDB files contain, we intend to create a process that extracts and organizes protein structural data from PDB files into a form readily available for machine learning and modelling applications.

II. RESEARCH ELABORATIONS

In order to create a new protein library in parallel with the rigid one available with PDBs, a new method for representing protein coordinates and structures was necessary. To appease this need, we developed "Star" files. A Star file is a matrix with dimensions *i,j* where the *ij* th element is the vector from amino acid i to amino acid j, as numbered in the protein sequence. Along with this, the Star file includes important metadata on the protein such as its method of structural characterization, the chi-angles of each side chain, and the amino acids where no coordinates were given. This matrix and metadata are then written to a comma-separated file for users to apply as needed. Figure 1 illustrates the anatomy of a Star file.

Meta Data	Protein Name	Experimental Collection Technique	Missing Residues in the PDB file		
Star File	Amino Acid	Ala1	Gly2	Ser3	Thr4
	Ala1	0,0,0	Ala1 → Gly2	Ala1 → Ser3	Ala1 → Thr4
	Gly2	Gly2 → Ala1	0,0,0	Gly2 → Ser3	Gly2 → Thr4
	Ser3	Ser3 → Ala1	Ser3 → Gly2	0,0,0	Ser3 → Thr4
	Thr4	Thr4 → Ala1	Thr4→ Gly2	Thr4 → Ser3	0,0,0

FIGURE 1: Star file anatomy. Cells in green represent the coordinate matrix. The red cells indicate the amino acid sequence. Blue cells represent metadata collected from the PDB. Yellow cells are for figure interpretation and are not included in Star files. "→" denotes a vector from one amino acid to another.

The generation of Star files requires structural data. To fulfill this requirement, Bellatrix was developed to harness the structural information presented within PDB files. Bellatrix is a translational tool operated via Python 3.7.6, that takes data in a PDB file format and converts it into accessible Star files (Van Rossum, 2019). The following Python packages were integral to Bellatrix's development; csv, pandas, numPy, tkinter (GUI), urlLib, and biopandas. (McMaster et al., 2020) (Walt, Colbert, & Varoquaux, 2011) (Lundh, 1999) (Open Source, 2020) (Raschka, 2017). PDB files were queried and supplied from the Research Collaboratory for Structural Bioinformatics (RCSB) data bank. After PDB translation, the Star files were read and displayed through Microsoft Excel. Development and testing of the Bellatrix software was conducted using PDB files for cellulases from multiple organisms. These PDB files were selected due to their abundance, and possible uses in synthetic biology.

Some special considerations needed to be made while working with PDB files. The inconsistent numbering of residues and variation in the header and periphery sections were dealt with by a custom text reader that parsed this data by finding keywords and executing logic. In crystallography files, a residue is often represented as multiple coordinates due to an experimental inability to exact its location. To simplify working with Star files, it was decided to include coordinates of the residue at its most probable location, thereby making the Star files as representative of structure as possible. In addition, an aberrance detection function was created to notify the user of anomalous data. All these techniques were then conglomerated to form a PDB troubleshooting algorithm that was integrated to run automatically during the creation of every Star file.

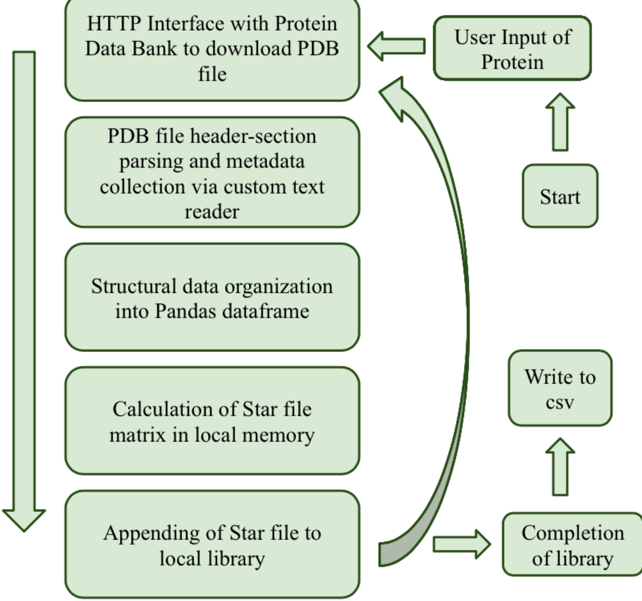

FIGURE 2: Bellatrix circular workflow diagram

Bellatrix was then expanded to be conducted not only on a single protein, but on a user-defined interrogation set. Interrogation sets are text or comma-separated files that include a list of proteins. From this interrogation set a list of proteins can then be translated in series and aggregated to form Star libraries (a CSV file composed of multiple Star files). Local cache optimization was implemented to enable the generation of large libraries without straining the executing computer's functionality while simultaneously allowing those with large scale computing architecture to best utilize their RAM resources.

III. RESULTS AND FINDINGS

When Bellatrix was carried out on a single protein it generated a Star file in 59.32 seconds. The star files were then verified empirically using Microsoft Excel. Working on an interrogation set of 20 identical 1EG1 proteins (Kleywegt et al., 1997), Bellatrix was able to construct a Star file library on this set in 18.16 minutes, averaging 54.5 seconds per protein. This result was shared when conducted on a set of dynamically obtained PDBs of similar size. PCA, a common and powerful machine learning technique, has been successfully run on Stars as a proof of concept illustrating the potential of these files in machine learning.

Bellatrix has undergone user testing within a diverse group of undergraduate students, from varying backgrounds. Through testing and tweaking of the user interface, the accomplished product has been successfully used by users of different backgrounds, who identified plausible implementation into their workflows. This testing was instrumental in file formatting and for intuitive program documentation

IV. DISCUSSION

Bellatrix provides a way to quickly compile structural data on an interrogation set of proteins. The true power of Bellatrix lies not in the Star files it generates, but in the imagination and function awarded to it by its users. One of the most potent use we foresee is clustering through machine learning. Star files enable the direct comparison between structural relationships in proteins and therefore provides a way for structurally based clustering. Coordinate matrices are already currently utilized in structural protein-based clustering methods, such as k-medoids (Polychronidou, *et al.,* 2018). Therefore, Bellatrix can supply scientists with new data architectures that can be extorted.

Another promising utilization of Bellatrix is the potential for a stability criterion of proteins that have undergone molecular dynamic simulation. From dynamic data, multiple PDBs can be generated at instantaneous time points. These files can then be sorted temporally and run through Bellatrix as an interrogation set. The resulting Star Library can then be manipulated to

observe the entire protein's movements with respect to any amino acid. The result can be quantified by a variety of different methods. The results then can used as a potent alternative to the commonly used root-mean-square deviation, allowing for more specialized indicators of protein stability. Star files can be applied in homology modelling by highlighting differences between similar proteins.

V. CONCLUSION

A novel file type for representing PDB files, known as a Star file, and a method for creating them has been proposed. Large Star files have been successfully constructed in sub-minute times, with low computational demand and high accessibility. The reach and impact of Star files have been elucidated earlier, but the true scope of Bellatrix is yet to be realized.

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REFERENCES

Xu, D., & Xu, Y. (2004, November). Protein databases on the internet. Retrieved August 24, 2020, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3265122/

Van Rossum, G. (2019, Winter). Python 3.7.6[Computer software]. Retrieved from https://www.python.org/

McMaster, A., Saxton, D., Goddard, E., Li, F., Virshup, I., Van den Bossche, J., . . . S. (2020). Pandas (Version 1.1.1) [Computer software]. Retrieved 2020, from https://pandas.pydata.org/

Travis E, Oliphant. *A guide to NumPy*, USA: Trelgol Publishing, (2006).

Walt, S. V., Colbert, S. C., & Varoquaux, G. (2011). The NumPy Array: A Structure for Efficient Numerical Computation. *Computing in Science & Engineering, 13*(2), 22-30. doi:10.1109/mcse.2011.37

Lundh, F. (1999). An introduction to tkinter. *URL: Www. Pythonware. Com/Library/Tkinter/Introduction/Index. Htm*.

Open Source. (2020). Urllib. Retrieved 2020, from https://docs.python.org/3/library/urllib.html

Sebastian Raschka. Biopandas: Working with molecular structures in pandas dataframes. *The Journal of Open Source Software*, 2(14), jun 2017. doi: 10.21105/joss.00279. URL http://dx.doi.org/10.21105/joss.00279.

Polychronidou, E., Kalamaras, I., Agathangelidis, A., Sutton, L., Yan, X., Bikos, V., . . . Tzovaras, D. (2018). Automated shape-based clustering of 3D immunoglobulin protein structures in chronic lymphocytic leukemia. *BMC Bioinformatics, 19*(S14). doi:10.1186/s12859-018-2381-1

Open Source. (2020). Time. Retrieved 2020, from https://docs.python.org/3/library/time.html

Berman, H. M. (2000). The Protein Data Bank. *Nucleic Acids Research, 28*(1), 235-242. doi:10.1093/nar/28.1.235. *URL: http://www.rcsb.org/*

Kleywegt, G. J., Zou, J., Divne, C., Davies, G. J., Sinning, I., Ståhlberg, J., . . . Jones, T. (1997). The crystal structure of the catalytic core domain of endoglucanase I from Trichoderma reesei at 3.6 Å resolution, and a comparison with related enzymes 1 1Edited by K.Nagai. *Journal of Molecular Biology, 272*(3), 383-397. doi:10.1006/jmbi.1997.1243

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Guide for Using Rosetta when Designing Ligand Binding Sites

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***Abstract*- During the past years, computational methods and tools for biology have been developed at an unprecedented speed. These methods allow scientists to carry out preliminary studies before working in the lab, saving both time and resources. One of the most commonly used tool for protein studies is Rosetta, a software that includes algorithms for computational modelling and analysis of protein structures. Rosetta allows enzyme design, *de novo* protein design, ligand docking, as well as structure prediction of biological macromolecules and complexes, among others. Although extremely useful, Rosetta and other software may lack suitable documentation for unexperienced computational biologists. Here, we present a guide on how to use Rosetta for the design of ligand binding sites.**

Index Terms- Computational methods, iGEM, Ligand binding site(s), Rosetta.

I. INTRODUCTION

We present a step-by-step guide on how to design ligand binding sites with the Rosetta software. We decided to use this case for elaboration since in our iGEM project we had to design ligand binding sites of the MphR transcription factor so it had a higher affinity for its ligands, macrolide antibiotics. This guide is based on the article *Rosetta and the Design of Ligand Binding Sites* published by Moretti and their colleagues (2016). Importantly, this guide is only addressed to users that want to design ligand binding sites of proteins that bind to one or more ligand molecules, as long as the molecules bound are the same. We acknowledge that Moretti's article is already concrete and useful, but non-computational biologists might have some trouble following the steps and understanding what is being done, specially in the docking and designing phase itself. We hope this guide will help to bridge this gap. We will discuss considerations when designing a ligand binding site, the material and software required, the necessary steps and, ultimately, how to interpret scores in Rosetta. Rosetta gives these scores together with the output pdb files, so the user can interpret how "good" is the design of the protein.

II. RESEARCH ELABORATIONS

The first piece of information that non-computational biologists need to know is that Rosetta is a non-graphical software exclusively run in a Unix-like environment, which can be new for beginners.

Before starting the design of a ligand binding site, there are several important considerations regarding the receptor that need to be taken into account. First of all, the receptor and the ligand structures to be modelled need to be in a database in order for the user to be able to work with the files (see more in the supplementary material "Material and Software" section). It is of great importance that the user knows the structure of the receptor, especially the number of chains (named A, B, C…), the amount of ligand molecules to which it binds, if it binds to more than one ligand, and whether the molecules are the same. This will be of special relevance when preparing the scripts for the modelling of the binding sites.

D. *Material and Software*

In order to use Rosetta for the design of ligand binding sites, several materials and software are required.

Material

1. The structure of the protein to be re-designed, in PDB format. The main database of tridimensional protein structures is Protein Data Bank (PDB).
2. The structure of the ligands that bind to the protein that wants to be re-designed. Examples of chemical databases where ligands can be found are PubChem, ChemSpider or Zinc, among others.

Software

1. **A *Unix*-like operating system such as Linux.**
2. **Access to a computer cluster.** A computer cluster is a set of connected computers with combined computational power, which allows to cut significantly the calculation time for heavy tasks. Rosetta's simulations are computationally heavy and normally the users' computers take a lot of time to run the jobs or even do not have enough memory or CPU's (central processing units) to run them. Thus, access to a computer cluster is needed if results are wanted to be obtained in a reasonable time-frame.
3. **Rosetta.** A license is needed to download Rosetta. There are two types of licenses: academic or commercial. After requesting it, the software can be downloaded on the following page:

https://www.rosettacommons.org/software/license-and-download.

4. **PyMOL.** PyMOL is an open-source molecular visualization software used to study the structure of proteins and molecules. The software is free to download on the following website: https://pymol.org/2/. Another existing software with the same purpose is Chimera (https://www.cgl.ucsf.edu/chimera/download.html).
5. **OpenBabel.** OpenBabel is a software used to study chemical data. In our case, it is used to introduce some modifications to the ligands of the protein that is going to be re-designed. The download and installation instractions can be found on https://openbabel.org/docs/dev/Installation/install.html.
6. **MGL tools (AutoDock 1.5.6).** AutoDockTools is a graphical front-end for setting up and running AutoDock - an automated docking software designed to predict how small molecules, such as substrates or drug candidates, bind to a receptor of known 3D structure. The software can be downloaded from here: http://mgltools.scripps.edu/downloads.
7. **For MacOS: XQuartz.** XQuartz is needed to run the AutoDockTools. The XQuartz project is an open-source effort to develop a version of the X.Org X Window System that runs on MacOS. Together with supporting libraries and applications, it forms the X11.app that Apple shipped with MacOS versions 10.5 through 10.7. It can be downloaded from here: https://www.xquartz.org/.
8. **AutoDock Vina.** AutoDock Vina is an open-source program for doing molecular docking, which can be downloaded here: http://vina.scripps.edu/download.html.

III. STEP-BY-STEP GUIDE

In this step-by-step guide we provide the user with the steps and commands they have to run in order to re-design the ligand binding sites of the protein of interest. Notes for the reader: (i) the text in italics should be changed according to the user's preference and (ii) to use the Rosetta files specified for the commands here provided, the user needs to know the file and the path to it. Here we provide the paths to the files at the date June 2020. However, depending on the Rosetta version, these paths may change. If that is the case the user will see an error message when running the command. In order to find the path to the file the command find -name *name_of_the_Rosetta_file_to_be_used* can be used. Then, they will have to change the path to the file in the commands that we provide.

1. **Access to the computer cluster.** Depending on the computer cluster used, it might be necessary to load certain modules before starting the design itself. Importantly, these modules have to be uploaded every time the user accesses the computer cluster. Also, the user might need to load different modules depending on the computer cluster being used. To know which modules are needed the user needs to read the warning and error messages that will pop-up when running Rosetta. The modules we have used for our design are:

```
module load openmpi #Module to load MPI
module load sqlite #Module needed for
relaxing the protein
module load gcc #Module needed for
running Rosetta
```

Moreover, it is possible that the user needs to add some libraries to the path where they are working. Once more, to know the paths needed, the user should read the warning messages. To add the libraries the command needed is:

```
LD_LIBRARY_PATH=$LD_LIBRARY_PATH:PATH TO
THE LIBRARY
```

2. **Preparation of the Protein.** Before starting the design, the protein file has to be pre-processed. This pre-processing consists of two steps:

   2.1. **Clean the PDB file.** PDB files normally contain water molecules among others, which need to be removed before starting the design. This is done with the following command:

```
rosetta/main/tools/protein_tools/
scripts/clean_pdb.py
protein_file_name protein_chains
```

   Note that the user has to specify the chains that the processed protein has (e.g. A, B, C…), which can be previously visualized in PyMOL. In the case the protein has two chains (A and B), instead of *protein_chains* the user should write AB. The output files should be one pdb file, which is the clean protein file, and as many fasta files as chains the protein has.

   2.2. **Relaxing the protein structure.** The protein structure has to be "relaxed" so the designing of the ligand binding sites can be done. When the user relaxes a protein, basically they sample conformations of a given structure in 3D space to find the lowest-scoring variant. It is recommended to relax the structure many times (at least 10). This will give the user different output files with different scores. The user should work with at least more than 2 of these relaxed files. Also, the user has to consider this job can take some time to finish.

In these cases, it is worth creating a .sh script (*protein_relaxing.sh*) to launch it and run things in the background. This means the user can close the session in the computer-cluster and close their computer and the job will still run. Note that depending on the cluster used, the user might have to write a different script. In our case, the cluster used has the workload manager Slurm, which helps to manage resources between different users on the same computer. The script written had the following structure:

```
#!/bin/bash

#SBATCH --time=05:00:00
#SBATCH --mem=4G
#SBATCH --output=relaxing.%j.out

srun mpiexec
rosetta/main/source/bin/relax.mpi
.linuxgccrelease -database
rosetta/main/database -s
path_to_clean_file/clean_file -
nstruct
number_of_relaxed_structures
```

Importantly, the user can specify different flags (options in command-line programs) to be performed when running the command. Specific flags can be consulted in the official Rosetta documentation (https://www.rosettacommons.org/docs/latest/application_documentation/structure_prediction/relax). To run the above script in the background the following command should be run:

```
sbatch protein_relaxing.sh
```

The output files should include as many pdb files as relaxed structures the user wants to get and a scoring file with the scores of all the relaxed structures. For more information on scoring files please refer to the section "Scoring in Rosetta" (Table 1) found in the Appendix. After performing the relaxation, the protein residue numbers might have changed as a result of this step. The user can look at the differences in residue numbers between the original file and the clean and relaxed file using PyMOL. This change in residue numbers is really important when the user wants to re-design specific residues from the protein. In order to do so, they will have to check what are the numbers of the residues they want to change in the new clean and relaxed file.

3. **Preparation of the Ligand.** Preparation of the ligand is also needed before starting the design. For this preparation, the user will need OpenBabel and Rosetta.

3.1. **Convert the ligand to SDF format and add hydrogens if needed.** In this step, the ligand file is converted to an appropriate format to work with Rosetta and the user can also add hydrogens to the molecule if needed. The hydrogens can also be added depending on the pH the user is expected to work with. To know more about the possible flags that can be used with Obabel visit https://openbabel.org/docs/dev/Command-line_tools/babel.html.

```
obabel ligand_file.format -flags
-O output_ligand_file_1.sdf
```

The output file should be one sdf file with the hydrogens added. The user can use PyMOL or another visualisation software to see the new file.

3.2. **Generate a library of ligand conformers.** In order to do this, the user needs to run:

```
path_to_bcl/bcl
molecule:ConformerGenerator -
ensemble_filenames
output_ligand_file_1.sdf -
conformers_single_file
ouput_ligand_file_2.sdf
```

The output file should be one sdf file with the different conformations of the ligand. The user can use PyMOL or another visualisation software to see the output file.

3.3. **Conversion of the conformer library into a Rosetta-formatted parameters (params) file.** The previous output file cannot be read by Rosetta, that is why the user needs to transform it so it can be used with the Rosetta software in the next steps.

```
rosetta/main/source/scripts/pytho
n/public/molfile_to_params.py -n
output_ligand_file_3 -p
output_ligand_file_3 --
conformers-in-one-file
output_ligand_file_2.sdf
```

The user should get three output files: *output_ligand_file_3.params*,

output_ligand_file_3.pdb and *output_ligand_file_3_conformers.pdb*. These three files are needed during all the design process and are the ones that the user should be working with from now on.

4. **Docking.** The next step is to manually dock the ligand into the binding pocket of the protein. The files used here are the pdb protein file obtained from the preparation of the protein and the pdb ligand file obtained from the preparation of the ligand. Since the docking is performed manually, a different software than Rosetta is used. There are different programs to do this, such as PyMOL or AutoDock. Another software to consider is SwissDock, which does not require any downloads. From our experience, we recommend AutoDock Vina, which is the newest version of the AutoDock software. Note that when docking multiple ligands, separate configuration files and dockings need to be performed. The docked ligands will not be combined in the same file until the end.

For the docking, additional preparation of the files is needed. The ligand needs to be converted to .pdbqt file format. This can be accomplished with OpenBabel in the terminal with the following command:

```
obabel /path/output_ligand_file_3.pdb -O
/path/ligand.pdbqt
```

Note that with very large ligands it is harder to achieve good results and these are computationally heavier.

Preparation of the receptor is done in the graphical user interface of Autodock 4.2.

1. Right-click “All molecules”, choose read molecule, choose your receptor file e.g. “*protein.pdb*”, click “Open”.
2. Click “Edit” and “Delete waters” (this is just an extra step, the waters should be removed already from your relaxed protein file).
3. Click “Edit”, choose “Hydrogens” and “Add”.
4. Click “Edit”, choose “Hydrogens” and “Merge non-polar”.
5. Click “Edit”, choose “Charges” and “Compute Gasteiger charges”.

Next, the grid box needs to be set up. The grid box defines in which space the dockings for the ligand will be searched for. If the exact binding site of the ligand is known, try to fit the grid box around that space. The general rule is to have it as small as possible, but not too small. If the binding site of the ligand is not known, blind docking is performed by setting the grid box around the whole protein. However, this is much more difficult and will not give as good results. Additionally, the larger the grid box is, the longer the computations will take. Autodock 4.2 GUI is used to set the grid box and get the coordinates to use while preparing the Autodock Vina configuration file. To open and set the gridbox click “Grid” and then choose “Grid box”. The size and orientation of the grid box can be adjusted so that it covers the binding site or ligand. Make sure to set the spacing to 1 Angstrom before you start adjusting the grid box.

Before running AutoDock Vina the configuration file in .txt format needs to be prepared (*conf.txt*). The easiest way is to define the receptor, ligand, output file, coordinates, and all other parameters in this file. The file could look like this:

```
receptor = protein.pdbqt
ligand = ligand.pdbqt

out = out.pdbqt

center_x = 24.332
center_y = 1.255
center_z = 24.103

size_x = 16
size_y = 18
size_z = 18

exhaustiveness = 10
energy_range = 25
num_modes = 25
weight_hydrogen = -2.4
```

There are a number of parameters that can be defined depending on how many outputs and scores the user wishes to get. More information about the different parameters can be found in the AutoDock Vina documentation (http://vina.scripps.edu/manual.html).

To run AutoDock Vina in the terminal you need the following command (note that all the needed files need to be in the same folder):

```
/path/vina --config conf.txt --log
log.txt
```

When ready, results will be in the predefined output file (in this example *out.pdbqt*). To visualize results in PyMOL, the molecules need to be converted to .pdb format. This can be done in Open Babel as mentioned above. When looking at docking results the most vital thing to consider is the visual positioning of the ligand. If you know the binding site of the ligand, you can compare and make sure that the docked ligand is as well aligned as possible (Fig. 1). In the *log.txt* the scores of the run are also available. The binding affinity is displayed for all the conformations and if the binding affinities are too high (> -6 kcal/mol), you might want to consider adjusting the docking parameters.

However, always mainly choose the ligand based on how well it is aligned in the known binding site. Multiple runs might be required and it is natural to get different results even when not adjusting the parameters. After choosing the best conformation save it in a separate .pdb file. Then perform all the multiple steps above for all the ligands (separate preparation of the same receptor is not needed). When all ligands are docked, the ligands can be combined into one .pdb file by clicking “Action” and “Copy to Object” in PyMOL.

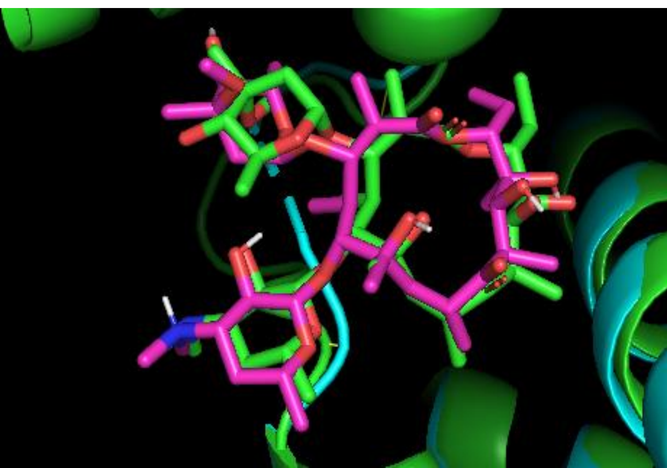

Figure 1. *Erythromycin docked to MphR(A) protein with AutoDock Vina (pink) compared to the crystallized structure of erythromycin bound to MphR(A) protein retrieved from PDB (green) (Olson A., 2009).*

5. **PyMOL and revising the Docked Files.** After the docking step, the user can revise the docked files to make sure the docking has been correctly performed. This step is especially important if the protein binds to more than one ligand molecule, since the ligand file will need some modifications. When doing the docking, the ligands are automatically assigned to a chain name (e.g. X, A, B…). If there is only one ligand, the name of the chain it is placed on is X. If there are two, the first ligand is placed in chain X and the second one in chain A and so on. The chain names can be visualized in PyMOL when looking at the sequence: /name of the file/segi(segment-identifier-list)/chain/residue. If the user is only placing one ligand, they do not have to do any modifications to the ligand docked file. On the other hand, when there is more than one ligand molecule, the user has to change the names of the different segi(s) and chains so all the ligands are placed under the same segi and chain names. This chain name will be ideally X, since it is, by default, the first chain name that is always assigned. In order to do this, the user has to open the docked ligand file in PyMOL and do the following steps:

   5.1. Select one of the ligands by clicking on its name in the sequence viewer.

   5.2. Change the residue number of the ligand selected with the following command: `alter (sele), resi=2`

   5.3. Change the residue chain of the ligand selected running the following command: `alter (chain` *`chain_to_change`*`), chain='X'`

   5.4. Remove the segi of the ligand selected with the following command: `alter (segi` *`segi_to_change`*`), segi=''`

   5.5. Save the file as a .pdb file: `save` *`file_name`*`.pdb`

   In this way, the output file should now be a pdb file with the two or more ligands placed under the same segi and chain names and each one of them having a different residue number.

6. **Design.** This is the main step where the ligand binding sites are re-designed. For this to be done, there are several steps needed.

   6.1. **Prepare a residue specification file (*mutations.resfile*).** In this file, the user specifies which residues should be re-designed. The user can allow all residues to mutate or only some of them. It is important to remember that the residues have been renumbered when processing the protein file. The structure of a resfile is the following:

```
Command applied to all residues
not specified in the body
AUTO #Use the default behaviour
start #After this command the
body starts
Residue_Number Chain_in_Protein
Command applied
```

   Next, you can see an example of a resfile in which only the natural amino acid is allowed for all the residues not specified in the body (NATAA command) and where the amino acids 52, 59, 85, 86, 93, 116, 137, 144, 145, 234, 241, 267, 268, 275, 298, 319, 326, 327 are allowed to change to all the amino acids except cysteine (ALLAxc). Amino acids 52-145 are in chain A of the protein and amino

acids 234-327 in chain B. The alterations to cysteine are not allowed, because cysteine tends to form stable sulphur bonds. Allowing for changes to this amino acid would lead to almost all residues being changed to cysteine, even if it would not be the most optimal mutation. To know more about the possible options when designing a resfile, the user can visit the following website: https://www.rosettacommons.org/manuals/archive/rosetta3.5_user_guide/d1/d97/resfiles.html.

```
NATAA
AUTO
start
52 A ALLAxc
59 A ALLAxc
85 A ALLAxc
86 A ALLAxc
93 A ALLAxc
116 A ALLAxc
137 A ALLAxc
144 A ALLAxc
145 A ALLAxc
234 B ALLAxc
241 B ALLAxc
267 B ALLAxc
268 B ALLAxc
275 B ALLAxc
298 B ALLAxc
319 B ALLAxc
326 B ALLAxc
327 B ALLAxc
```

6.2. **Prepare a docking and design script (*design.xml*).** This script will optimize the location of the ligand in the binding pocket, re-design the surrounding sidechains and refine the interactions in the designed context among others. The following script is based on the script provided by Moretti and colleagues (2016). The modifications added are needed for the script to be run in the last release of Rosetta at date June 2020. Between <!-- --> the user can find explanations of some of the commands. Notes for the user: (i) the resfile has to be specified in the body of the script where indicated; (ii) it is important the user knows which scoring function is being used when performing the design, since each Rosetta score function scores the structures (for more information on scoring functions visit https://www.rosettacommons.org/). The scoring functions used here can be found in the command ScoreFunction of the next script:

```
<ROSETTASCRIPTS>
  <SCOREFXNS>
    <ScoreFunction
name="ligand_soft_rep"
weights="ligand_soft_rep"/>
    <ScoreFunction
name="hard_rep"
weights="ligandprime"/>
  </SCOREFXNS>
  <TASKOPERATIONS>
    <DetectProteinLigandInterface
name="design_interface"
cut1="6.0" cut2="8.0" cut3="10.0"
cut4="12.0" design="1"
resfile="mutations.resfile"/>
  </TASKOPERATIONS>
  <LIGAND_AREAS> <!--Describes
parameters specific to each
ligand-->
    <!--cutoff: the distance in
angstroms from the ligand an
amino-acid’s C-beta atom can be
and that residue still be part of
the interface-->
    <!--add_nbr_radius: increases
the cutoff by the size of the
ligand neighbor atom’s radius
specified in the ligand .params
file-->
    <!--Calpha_restraints:
Calpha_restraints greater than 0,
backbone flexibility is enabled--
>
    <LigandArea
name="docking_sidechain"
chain="X" cutoff="6.0"
add_nbr_radius="true"
all_atom_mode="true"
minimize_ligand="10"/>
    <LigandArea
name="final_sidechain" chain="X"
cutoff="6.0"
add_nbr_radius="true"
all_atom_mode="true"/>
    <LigandArea
name="final_backbone" chain="X"
cutoff="7.0"
add_nbr_radius="false"
all_atom_mode="true"
Calpha_restraints="0.3"/>
  </LIGAND_AREAS>
  <INTERFACE_BUILDERS> <!--
Describes how to choose residues
that will be part of the protein-
ligand interface. These residues
are chosen for repacking, rotamer
trials, and backbone minimization
during ligand docking-->
    <!--ligand_areas: list of
strings matching Ligand Area
names-->
    <!--extension_window:
surrounds interface residues with
residues labeled as ‘near
interface’. This is important for
```

```
backbone minimization, because a
residue's backbone cannot really
move unless it is part of a
stretch of residues that are
flexible-->
    <InterfaceBuilder
name="side_chain_for_docking"
ligand_areas="docking_sidechain"/
>
    <InterfaceBuilder
name="side_chain_for_final"
ligand_areas="final_sidechain"/>
    <InterfaceBuilder
name="backbone"
ligand_areas="final_backbone"
extension_window="3"/>
  </INTERFACE_BUILDERS>
  <MOVEMAP_BUILDERS> <!--
Constructs a movemap: A movemap
is a 2xN table of true/false
values, where N is the number of
residues of your protein/ligand
complex. The two columns are for
backbone and side-chain
movements-->
    <MoveMapBuilder
name="docking"
sc_interface="side_chain_for_dock
ing" minimize_water="true"/>
    <MoveMapBuilder name="final"
sc_interface="side_chain_for_fina
l" bb_interface="backbone"
minimize_water="true"/>
  </MOVEMAP_BUILDERS>
  <SCORINGGRIDS ligand_chain="X"
width="25">
    <ClassicGrid grid_name="vdw"
weight="1.0"/>
  </SCORINGGRIDS>
  <MOVERS>
    <FavorNativeResidue
name="favor_native"
bonus="1.00"/>
    <Transform name="transform"
chain="X" box_size="5.0"
move_distance="0.1" angle="5"
cycles="500" repeats="1"
temperature="5" rmsd="4.0"/>
    <HighResDocker
name="high_res_docker" cycles="6"
repack_every_Nth="3"
scorefxn="ligand_soft_rep"
movemap_builder="docking"/>
    <PackRotamersMover
name="design_interface"
scorefxn="hard_rep"
task_operations="design_interface
"/>
    <FinalMinimizer name="final"
scorefxn="hard_rep"
movemap_builder="final"/>
    <InterfaceScoreCalculator
name="add_scores" chains="X"
scorefxn="hard_rep"/>
    <ParsedProtocol
name="low_res_dock">
      <Add
mover_name="transform"/>
    </ParsedProtocol>
    <ParsedProtocol
name="high_res_dock">
      <Add
mover_name="high_res_docker"/>
      <Add mover_name="final"/>
    </ParsedProtocol>
  </MOVERS>
  <PROTOCOLS>
    <Add
mover_name="favor_native"/>
    <Add
mover_name="low_res_dock"/>
    <Add
mover_name="design_interface"/>
    <Add
mover_name="high_res_dock"/>
    <Add
mover_name="add_scores"/>
  </PROTOCOLS>
</ROSETTASCRIPTS>
```

6.3. **Run the design application.** In this step, the user runs the design of the binding site itself. It is recommended to do from 1000 to 5000 designs of the protein, which means the user will obtain from 1000 to 5000 pdb files with modifications with respect to the original clean and relaxed pdb file. This is computationally heavy and can take several hours even when using a computer cluster. For this reason, we recommend starting with making a script (*launch_design.sh*) that will be run in the background. Note that depending on the cluster used, the user might have to write a different script. In our case, the cluster used has Slurm, and the script written had the following format:

```
#!/bin/bash

#SBATCH --time=20:00:00
#SBATCH --array=0-49
#SBATCH --mem=3100M
#SBATCH -n 1
#SBATCH --nodes=1
#SBATCH --
output=path_where_the_output_is_w
anted/output_job_name.out

NUM=20

srun mpirun
rosetta_scripts.mpi.linuxgccrelea
se -ex1 -ex2 -linmem_ig 10 -
restore_pre_talaris_2013_behavior
-parser:protocol design.xml -
extra_res_fa
output_ligand_file_3.params -s
```

```
"clean_and_relaxed_protein.pdb
docked_ligand.pdb" -nstruct $NUM
--out:prefix $SLURM_ARRAY_TASK_ID
-out:file:scorefile
/path_where_the_output_is_wanted/
output_design_file.out
```

In the above script, the job has 20 hours to run, and it is assigned to 50 arrays (0-49), each one of which will perform 20 designs. Therefore, the user obtains 1000 pdb files (20x50=1000). To run the script the user has to type the following command:

```
sbatch launch_design.sh
```

After the run, the user should get 1000 pdb files and one score file with the scores of all the pdbs. To see more about scoring go to the section "Scoring in Rosetta" (Table 2) found in the Appendix.

7. **Filtering.** After running the design, the user needs to filter the 1000 pdb files and choose the ones with the score that is more convenient for them. There are two main filtering steps.

7.1. **Prepare a metrics file (*metric_thresholds_1.txt*).** This file specifies the thresholds to use when filtering the output of the design run. It will filter the 1000-5000 pdb previous files. It is important to take into account that this filtering can be done for different parameters. Here we present the parameters we have used in our protein design. To see other parameters that can be used for filtering, refer to Table 2. From our experience, we recommend to use the average values as the cutoff. In order to obtain these values, the user needs to run the command in step 7.2.

```
req total_score value < -1606.71
req if_X_fa_atr value < -50.60
req fa_rep value < 197.15
req if_X_fa_rep value < 14.29
req ligand_is_touching_X value >
0.8
output sortmin interface_delta_X
```

7.2. **Filter on design metrics.** Next, the user uses the previous file to filter the pdb files with the following command:

```
perl
$WRKDIR/rosetta/main/source/src/a
pps/public/enzdes/DesignSelect.pl
-d <(grep SCORE
output_design_file.out) -c
metric_thresholds_1.txt -
tag_column last >
filtered_designs.sc
```

When the user runs this command, in the terminal there will appear the average values for the score metrics. Once the user has these ones, they can be specified in the *metric_thresholds_1.txt* file and run this same command again. The user will get a *filtered_designs.sc* file, which has a list of the scores and the name of the files that have passed the filtering. Next, the user needs to create a file consisting of a list with only the names of the files that have passed the filtering plus the .pdb extension. This is done with the following command:

```
awk '{print $NF ".pdb"}'
filtered_designs.sc >
filtered_pdbs.txt
```

7.3. **Calculate additional metrics.** Once the first filtering step has been done, the user needs to calculate additional metrics that are focused on the protein-ligand interface. These metrics are needed to do the second filtering step and calculating them also requires a lot of computational power. Therefore, we recommend making another script (*launch_interfaces.sh*) to be able to run this step on the background of the computer cluster. Note again, that depending on the cluster used, the user might have to write a different script. In our case, the cluster used has Slurm, and the script is the following:

```
#!/bin/bash

#SBATCH --time=08:00:00
#SBATCH --mem=4000M
#SBATCH --
output=path_where_the_output_is_w
anted/output_job_name.out

srun mpirun
InterfaceAnalyzer.mpi.linuxgccrel
ease -interface AB_X -
compute_packstat -pack_separated
-score:weights ligandprime -
no_nstruct_label -
out:file:score_only interfaces.sc
-l filtered_pdbs.txt -
extra_res_fa
output_ligand_file_3.params
```

Importantly, in the previous command the user needs to specify the interface where the additional metrics will be calculated with the flag -interface. The user has to specify the interface of both the protein and the ligand. To do it, they have to write the chain names of the protein (in this case, A and B) followed by an underscore and the chain name of the ligand, which is, by default, X. Once the script is done, the user can launch it with the command:

```
sbatch launch_interfaces.sh
```

The user should obtain one output file, *interfaces.sc*, with additional metrics of the files that passed the first filter. To know more about these additional metrics, please refer to the section "Scoring in Rosetta" (Table 3) found in the Appendix.

7.4. **Prepare a metrics file (*metric_thresholds_2.txt*).** This file specifies the thresholds to use when filtering the output of the interfaces run. It will filter the already filtered pdb files. It is important to take into account that this filtering step, like the last one, can be done for different parameters. Here we present the parameters we have used in our protein design. To see other parameters that can be used for filtering, please refer to Table 3. From our experience, we recommend to use the average values as the cut-off. In order to obtain these values, the user needs to run the command in step 7.5.

```
req packstat value > 0.58
req sc_value value > 0.48
req delta_unsatHbonds value <
17.88
req dG_separated/dSASAx100 value
< -1.53
output sortmin dG_separated
```

7.5. **Filter on interface metrics.** Next, the user does the second filtering step using the file created in 7.4.

```
perl
$WRKDIR/rosetta/main/source/src/a
pps/public/enzdes/DesignSelect.pl
-d <(grep SCORE interfaces.sc) -c
metric_thresholds_2.txt -
tag_column last >
filtered_interfaces.sc
```

When the user runs this command, in the terminal there will appear the average values for the score metrics. Once the user has these ones, they can specify them in the metric_thresholds_2.txt file and run this same command again. The output file should be the *filtered_interfaces.sc* file, with a list of the scores and the names of the files that have passed the filtering. If the user wants to have a file with only the names of the files that have passed the filtering, they can run the following command:

```
awk '{print $NF ".pdb"}'
filtered_interfaces.sc >
filtered_pdbs_final.txt
```

8. **Check the filtered results and re-run.** Once the user has filtered the results two times, they will obtain a reduced list of filtered pdbs. It is recommended that the user chooses at least the three best files according to their purposes and analysing the scores obtained. After that, the user can manually inspect the pdb files and compare them with the clean and relaxed protein file with PyMOL to check (i) if there are any modifications in the desired residues and (ii) if the structure of the protein is correct. Also, apart from doing the design of the binding sites with the three best protein files obtained after the preparation of the protein, it is recommended to re-apply the design protocol for at least the three final best pdb files obtained. So, after choosing these files, this protocol needs to be re-applied from point 6 onwards. In this case, when running the script *launch_design.sh*, the names after the flag `-s` should be changed to the name of the selected file. It is recommended to do this re-run from 3 to 5 times. Figure 2 shows a scheme of the steps that should be followed.

9. **Extract the selected protein sequences into fasta format.** Once the design has been run 3-5 times, the user might one to obtain the mutated protein sequence in fasta format. This can be done by opening the file in PyMOL and running the following command:

```
save file_name.fasta
```

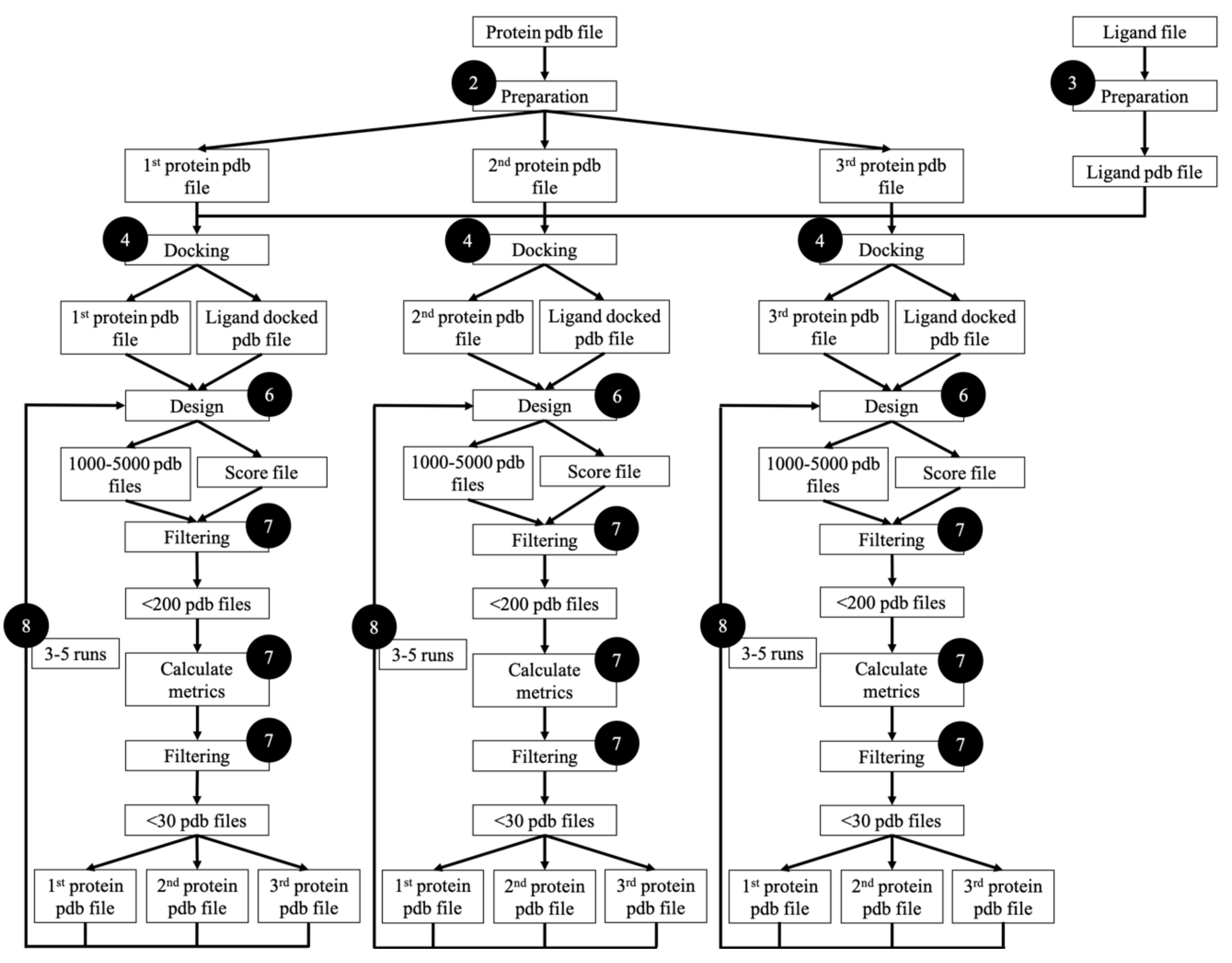

Figure 2. *Steps required when following the Rosetta Guide here presented*

IV. CONCLUSION

Designing ligand binding sites is a complex process that includes several steps. All the previous existing guides for designing ligand binding sites are complete, but we have found they require some basic computational knowledge the user might lack. With this guide, we hope to help users who are less experienced in the computational field to be able to design ligand binding sites. Due to the difficulties when working with Rosetta, we recommend starting a project with these characteristics in advance.

APPENDIX

Information about Scoring in Rosetta can be found in the following link https://drive.google.com/file/d/103y4aJplsX-Gcp7yzjFzk2qYSBt5hlDK/view?usp=sharing.

REFERENCES

AutoDock Vina - molecular docking and virtual screening program. (2020). Retrieved 26 August 2020, from http://vina.scripps.edu/

Computational Design of Ligand Binding Proteins. (2016). Methods In Molecular Biology. doi: 10.1007/978-1-4939-3569-7

License and Download | RosettaCommons. (2020). Retrieved 26 August 2020, from https://www.rosettacommons.org/software/license-and-download

MGLTools Website - Welcome — MGLTools. (2020). Retrieved 26 August 2020, from http://mgltools.scripps.edu/

Moretti, R., J. Bender, B., Allison, B., & Meiler, J. (2016). Rosetta and the Design of Ligand Binding Sites, *1414*, 47–62. https://doi.org/10.1007/978-1-4939-3569-7

Olson, A. (2009). AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. Journal Of Computational Chemistry, NA-NA. doi: 10.1002/jcc.21334

Open Babel. (2020). Retrieved 26 August 2020, from https://openbabel.org/

PyMOL | pymol.org. (2020). Retrieved 26 August 2020, from https://pymol.org/2/

Team:Technion Israel/Modifications/Rosetta - 2016.igem.org. (2020). Retrieved 26 August 2020, from http://2016.igem.org/Team:Technion_Israel/Modifications/Rosetta

Team:TU-Eindhoven/Modeling/Rosetta - 2016.igem.org. (2020). Retrieved 26 August 2020, from http://2016.igem.org/Team:TU-Eindhoven/Modeling/Rosetta

The Rosetta Software | RosettaCommons. (2020). Retrieved 26 August 2020, from https://www.rosettacommons.org/software/ Trott, O., &

UCSF Chimera Home Page. (2020). Retrieved 26 August 2020, from https://www.cgl.ucsf.edu/chimera/ XQuartz. (2020). Retrieved 26 August 2020, from https://www.xquartz.org/

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Amalthea: A Modular Platform for Monitoring Gastrointestinal Health

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Abstract- **Malnutrition is a modern and global health challenge, affecting one in three people. Imbalanced intake of essential nutrients, like dietary fibers, and the lack of personalized patient-centered care can lead to Inflammatory Bowel Diseases (IBDs). IBDs are a multifactorial family of interrelated diseases that affect the human gut, that affects 10 million people worldwide in 2020. Dysregulation of the human gut microbiome has been proven to lead to inflammation in the gut intestine. However, current diagnosis and treatment practices are highly invasive, as they can cause discomfort and side effects, respectively. To tackle this major issue, we are developing a complete, personalized, modular platform, which provides diagnosis, evaluation of the gut flora, and treatment of IBD. A non-invasive encapsulated detection module, consisting of a genetically engineered bacteria-based system and an electronic system, will identify metabolite deficiencies directly correlated to IBD. This exploits a bio-electronic interface to enable real-time monitoring on the patient's smartphone. Based on this personalized data, a synthesis module will respond with selective production of the missing metabolites, thus eliminating the nutritional deficit and relieving the patient from the symptoms. Our product is designed in conjunction with healthcare experts and according to international standards, to ensure biosafety. With this work, we aspire to provide cost-efficient and innovative solutions for detecting intestinal deformities and improving the gut microbiome, while facilitating one of humans' essential needs – to enjoy one's food.**

Key Words- Malnutrition, IBD, Gut microbiota, Capsule, Evaluation, Real-Time Monitoring.

I. INTRODUCTION

Malnutrition is one of the common problems that afflict the poor in low- and middle-income countries, presenting as a major concern in the era of Sustainable Development Goals (SDGs) in which achieving the goals is imperative. (Adebisi et al., 2019). According to a World Health Organization (WHO), nearly one in three people globally suffer from at least one form of malnutrition: wasting, stunting, vitamin and mineral deficiency, overweight or obesity and diet-related non-communicable diseases (WHO, 2016). Evidence suggests that nearly half of all deaths in children under 5 are attributable to undernutrition, which puts children at greater risk of dying from common infections, increases the frequency and severity of such infections, and delays recovery (UNICEF, 2020). On a global level, almost 13.6 million children die annually from undernutrition, while approximately 151 million (22.2%) under-five years of age were found to be stunted in 2016. (Boah et al., 2019).

Another aspect of malnutrition-induced immunological deficiency is the comorbidity with Inflammatory Bowel Diseases (IBDs), being prevalent in up to 70% of patients with active disease and up to 38% of patients in remission and having been associated with increased hospitalizations and poor clinical outcome (Balestrieri et al., 2020), as well as being in conjunction with changes in gut microbiota (Lim et al., 2018). In IBD patients, malnutritional deficits are mostly manifested through vitamin A, B, D, K, zinc and iron deficiencies (Jayawardena & Dudeja, 2020). IBDs incorporate a heterogenous and multifactorial class of chronic, relapsing inflammatory disorders that affect the Gastrointestinal (GI) tract, with Crohn Disease (CD) and Ulcerative Colitis (UC) being the principal representatives of this family of syndromes (Balestrieri et al., 2020).

To combat this issue, a range of drugs, natural products and treatment options have been employed by physicians, even though the multi-dimensionality of the disease and uncertainty of the severity of the disease present challenges (Seyedian et al., 2019). Alternative IBD treatment options, have led to the use of effective natural-based products (Day et al., 2019). The so-called "nutraceuticals", comprise any food-derived natural products, like bioactive peptides or fatty acids, that appear to have health-promoting features Additionally, recent research provides evidence that many nutrients and food elements can cure IBD symptoms (Al Mijan & Lim, 2018).This health-

promoting effect is likely based on the fact that the human body interacts with and depends on the microbiome, a wide range of probiotic bacteria that have established a symbiotic relationship, as they perform some very vital roles regarding the host's health. The products of probiotics and their energy source, prebiotics, belong to the "nutraceutical" umbrella (Thursby et al., 2017).

Probiotics encompass a broad category of bacteria and their metabolic byproducts, that promote well-being, especially gut health and intestinal homeostasis (Day et al., 2019). Despite these health-promoting effects, our project revolves around the ability of certain probiotics to produce Short Chain Fatty Acids (SCFAs) (Markowiak-Kopeć et al., 2020). SCFAs are a category of carboxylic acids (acetic, propionic and butyric acid) that exert important immunoregulatory and physiological roles. These metabolites are produced by intestinal bacterial fermentation of luminal carbohydrates and proteins. They contribute to intestinal health, by lowering the pH level in the colon, thus limiting the growth of pathogens (Parada et al., 2018).

IBD diagnosis and treatment are complex, as the disease's etiology and pathophysiology still are not fully understood. To complicate matters further, IBD can manifest in organs other than the gut. One such organ is the oral cavity. A clinically important biomarker is calprotectin, a protein that is associated with inflammatory diseases (Manceau et al., 2017).

A different approach on this matter is the potential use of the salivary calprotectin to reflect disease activity and treatment response using serum concentrations as a positive control. Investigating effects on salivary calprotectin showed that the level of calprotectin in saliva is elevated in IBD patients and is related to IBD activity and treatment (Majster M, et. al., 2019).

In recent years, the science community has turned its focus on the gut microbiota in search for a different approach to the diagnosis of IBDs; that search has led to SCFAs. SCFAs have been utilized as metabolites possessing both diagnostic and therapeutic value. The gut microbiota ferments indigestible carbohydrates and the major end-products thereof are the SCFAs (Markowiak et al., 2020). Among them, the fermentation of undigested dietary components is of paramount importance for the physiology and metabolism of the host. The subsequent microbial released metabolites have a key role in the interplay between bacterial producers and other gut inhabitants as well as with the host cells (Rios-Covian et al., 2020). As they are normally a byproduct of the intestinal microbiome metabolism, any alteration or depletion of the gut microbiota lead to lower concentrations of SCFAs, making them potential biomarkers (Parada et al., 2019).

II. RESEARCH DESIGN

The Amalthea project proposes a complete method of confronting IBDs, containing the diagnosis of IBDs, the evaluation of the gut microbiota and the personalized treatment according to the gathered data. It also enables wireless and real-time evaluation of the microbiome, which is accomplished through the cooperation of the biological system and the electronic devices. Combining sensing, computation and communication in a non-invasive manner, the capsule leverages the advantages of each approach.

The first step in the diagnosis of IBDs can be accomplished with a diagnostic tool consisting of engineered aptamers and toehold switches (Chau THT et al., 2020), that can detect salivary calprotectin, providing a cell free diagnostic test. These measurements will be used to determine the stage of the inflammation and the category of the disease, CD or UC. Calprotectin was chosen as a biomarker for inflammation, while SCFAs as biomarker for the function of gut flora. For the validation of the precision of the biomarkers for predicting and monitoring response to treatment, we will combine the results of the calprotectin levels with the evaluation of the gut microbiota, by measuring the levels of SCFAs.

The second step is the manufacturing of the capsule and its bioelectronic system, which is separated in two channels. In the first channel, the bacteria are contained, bordered from the electronics, that consist the second channel. One critical parameter to take into consideration, when building an ingestible device is the capsule size, so the capsule's proposed dimensions are 21mm x 12 mm (length x diameter). The electronics are coated with 1 mm thin Parylene-C membrane and then the whole device is casted into a PDMS capsule that is 12 mm in diameter. This procedure ensures its protection from the caustic gastrointestinal environment (Mimee et al., 2018). Concerning the power supply, a silver oxide button battery that provides the device 3V is equipped. This device meets the requirements on size and power, while also giving a good balance between range and human tissue penetration. (Kourosh et al., 2017) (Figure 1.).

The device employs semipermeable membrane to allow small molecules, such as SCFAs, to penetrate and react with the bacteria. This bacteria-based system possesses a NOT-gate genetic circuit, acting as a negative feedback loop. The NOT-gate system performs a logical negation on its input, inverting the initial signal and representing the opposite logic-level to the input (Singh V., 2014). This circuit is composed of two modules, a diagnostic and a therapeutic one.

The diagnostic module has the form of a Tango-GPCR system coupled to a downstream reporter system. GPCR receptor, that can be activated by endogenously present SCFAs, used for this system has tethered a repressor with a linker that contains a cleavage site for a specific protease. Activation of the receptor recruits a signaling protein fused to the protease that then cleaves and releases a repressor to alleviate the expression of the reporter gene. The absence of SCFAs allows the expression and activation of the reporter system, allowing the physician to diagnose gut microbiota distortion (Barnea et al., 2008). The reporter system is an electrochemical module that transforms the biological signal into an easily detectable electrical one, enabling real-time monitoring (Vanarsdale et al., 2020).

To provide a proof-of-principle, we tried to reduce this sophisticated system to its basic logic gate and organic structure, by respectively designing a simple NOT-gate circuit based on the tet-off system and a simplified promoter system, the Prom Assay. This stimulates the circumstances of the logic gate behind the Tango Assay. The prom Assay consists of three basic parts, the promoters that can recognize the SCFAs, a repressor protein and a reporter gene. The presence of SCFAs leads to the expression of the repressor protein provoking a series of consecutive events, which concludes to the suppression of the signal. On the contrary, the lack of SCFAs obstructs the expression of the repressor protein, which allows the activation of the reporter gene.

The therapeutic module has the form of a bacteria-based system capable of producing SCFAs. As mentioned before SCFAs can be used in multiple ways, including therapeutics, considering their anti-inflammatory properties and their effect on the gut microbiota. A crucial aspect of our project is to create personalized treatments for the patients by replenishing the missing amount of SCFAs, that will be provided by probiotics, which will initiate their production (Markowiak-Kopeć et al., 2020).

In the second channel our device utilizes the compilation and transmission of bio-electronic information from the gut. The biological signal is transformed into an electrochemical one by the tyrosine-tyrosinase system. This occurs through a redox reaction, that results in the conversion of tyrosine to oxidative derivatives, a process that produces electric current, that can be detected with the use of electrodes attached below each channel. When it flows through the electrodes, the electrical signals generated, are processed by the microcontroller, and transmitted wirelessly to an external receiver. A simple RF transmission device operating in the 433 MHz commercial communication band - ideal for biomedical applications- is selected for the transmission (Caffey et al., 2015).

The bio-electronic information generated will be instantly stored to the cloud on the user's smartphone or computer, for convenient readout. It will be accessible, at any given time, to patients and to professionals, who will use it to evaluate the microbiome and visualize the functionality of the intestinal flora. This information is then used to provide a personalized treatment based on the needs of each patient, to achieve relief of symptoms and to design a daily diet without dietary restrictions.

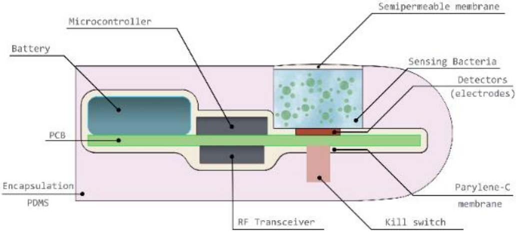

Figure 1. Side view of the capsule. The capsule includes a semipermeable membrane, sensing bacteria, detectors (electrodes), Parylene-C membrane, kill-switch, RF transceiver, encapsulation PDMS, PCB, Battery, Microcontroller.

III. CONCLUSIONS

Project Amalthea aims to provide a complete, personalized, modular platform, which provides full monitoring of the GI tract's health by accomplishing diagnosis, evaluation of the gut microbiota, and treatment of IBD. The non-invasive encapsulated detection device consists of two modules, a genetically engineered bacteria-based module, and an electronic module. Succeeding in the communication between our inner and outer world, through the understanding of our body's needs. This will be possible by identifying metabolite deficiencies directly correlated to IBD, exploiting a bio-electronic interface to enable real-time monitoring on the patient's smartphone. All in all, the micro-bio-electronic ingestible device will support physicians in better assisting and accommodating to what their patients' bodies require, primarily those who suffer from IBDs. This way, Amalthea contributes to the improvement of their well-being and quality of life.

REFERENCES

Adebisi, Y. A., Ibrahim, K., Lucero-Prisno, D. E., Ekpenyong, A., Micheal, A. I., Chinemelum, I. G., & Sina-Odunsi, A. B. (2019). Prevalence and Socio-economic Impacts of Malnutrition Among Children in Uganda. Nutrition and Metabolic Insights, 12, 117863881988739. https://doi.org/10.1177/1178638819887398

Al Mijan, M., & Lim, B. O. (2018). Diets, functional foods, and nutraceuticals as alternative therapies for inflammatory bowel disease: Present status and future trends. World Journal of Gastroenterology, 24(25), 2673–2685. https://doi.org/10.3748/wjg.v24.i25.2673

Balestrieri, P., Ribolsi, M., Guarino, M. P. L., Emerenziani, S., Altomare, A., & Cicala, M. (2020). Nutritional aspects in inflammatory bowel diseases. Nutrients, 12(2). https://doi.org/10.3390/nu12020372

Barnea, G., Strapps, W., Herrada, G., Berman, Y., Ong, J., Kloss, B., Axel, R., & Lee, K. J. (2008). The genetic design of signaling cascades to record receptor activation. Proceedings of the National Academy of Sciences of the United States of America, 105(1), 64–69. https://doi.org/10.1073/pnas.0710487105

Boah, M., Azupogo, F., Amporfro, D. A., & Abada, L. A. (2019). The epidemiology of undernutrition and its determinants in children under five years in Ghana. PLoS ONE, 14(7), 1–23. https://doi.org/10.1371/journal.pone.0219665

Chau THT, Mai DHA, Pham DN, Le HTQ, Lee EY. Developments of Riboswitches and Toehold Switches for Molecular Detection-Biosensing and Molecular Diagnostics. Int J Mol Sci. 2020;21(9):3192. Published 2020 Apr 30. doi:10.3390/ijms21093192

Day, R. L. J., Harper, A. J., Woods, R. M., Davies, O. G., & Heaney, L. M. (2019). Probiotics: Current landscape and future horizons. Future Science OA, 5(4). https://doi.org/10.4155/fsoa-2019-0004

Jayawardena, D., & Dudeja, P. K. (2020). Micronutrient Deficiency in Inflammatory Bowel Diseases: Cause or Effect? Cmgh, 9(4), 707–708. https://doi.org/10.1016/j.jcmgh.2019.12.009

Kourosh Kalantar-zadeh, Nam Ha, Jian Zhen Ou, and Kyle J. Berean, (2017), Ingestible Sensors, ACS Sensors 2017 2 (4), 468-483 DOI: 10.1021/acssensors.7b00045

Lim, H.-S., Kim, S.-K., & Hong, S.-J. (2018). Food Elimination Diet and Nutritional Deficiency in Patients with Inflammatory Bowel Disease. Clinical Nutrition Research, 7(1), 48. https://doi.org/10.7762/cnr.2018.7.1.48

Majster M, Almer S, Boström EA. Salivary calprotectin is elevated in patients with active inflammatory bowel disease, 2019, Arch Oral Biol.,107:104528. doi:10.1016/j.archoralbio.2019.104528

Markowiak-Kopeć P. , Śliżewska K. The Effect of Probiotics on the Production of Short-Chain Fatty Acids by Human Intestinal Microbiome. Nutrients. 2020;12(4):1107. Published 2020 Apr 16. doi:10.3390/nu12041107

Manceau H, Chicha-Cattoir V, Puy H, Peoc'h K. Fecal calprotectin in inflammatory bowel diseases: update and perspectives. Clin Chem Lab Med. 2017;55(4):474-483. doi:10.1515/cclm-2016-0522

Mimee MNadeau P, Hayward A, et al. (2018) An ingestible bacterial-electronic system to monitor gastrointestinal health. Science. 2018;360(6391):915-918. doi:10.1126/science.aas9315

Parada Venegas D, De la Fuente MK, Landskron G, et al. Short Chain Fatty Acids (SCFAs)-Mediated Gut Epithelial and Immune Regulation and Its Relevance for Inflammatory Bowel Diseases [published correction appears in Front Immunol. 2019 Jun 28;10:1486]. Front Immunol. 2019;10:277. Published 2019 Mar 11. doi:10.3389/fimmu.2019.00277

Rios-Covian D, González S, Nogacka AM, et al. An Overview on Fecal Branched Short-Chain Fatty Acids Along Human Life and as Related With Body Mass Index: Associated Dietary and Anthropometric Factors. Front Microbiol. 2020;11:973. Published 2020 May 27. doi:10.3389/fmicb.2020.00973

Seyedian, S. S., Nokhostin, F., & Malamir, M. D. (2019). A review of the diagnosis, prevention, and treatment methods of inflammatory bowel disease. Journal of Medicine and Life, 12(2), 113–122. https://doi.org/10.25122/jml-2018-0075

Singh, V. (2014). Recent advances and opportunities in synthetic logic gates engineering in living cells. Systems and Synthetic Biology, 8(4), 271–282. https://doi.org/10.1007/s11693-014-9154-6

Thursby E, Juge N. Introduction to the human gut microbiota. Biochem J. 2017;474(11):1823-1836. Published 2017 May 16. doi:10.1042/BCJ20160510

UNICEF, Malnutriton, New York, March 2020

Vanarsdale, E., Hörnström, D., Sjöberg, G., Järbur, I., Pitzer, J., Payne, G. F., Van Maris, A. J. A., & Bentley, W. E. (2020). A coculture based tyrosine-tyrosinase electrochemical gene circuit for connecting cellular communication with electronic networks. ACS Synthetic Biology, 9(5), 1117–1128. https://doi.org/10.1021/acssynbio.9b00469

World Health Organization. (2016). The double burden of malnutrition: policy brief. World Health Organization. https://apps.who.int/iris/handle/10665/255413. License: CC BY-NC-SA 3.0 IGO

World Health Organization, Malnutrition, key facts (1 April 2020). https://www.who.int/news-room/fact-sheets/detail/malnutrition

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HORMONIC: An Artificial Close-loop System for Hormonal Homeostatic Regulation

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Abstract- **The project consists on developing a device that can regulate thyroid hormone imbalances. This device will be designed to sense triiodothyronine (T3) at skin level through our engineered *Escherichia coli (E. coli)*, which will contain an intein-mediated protein splicing linked to eGFP expression. A DIY turbidostat will be considered to maintain the cellular culture conditions constant. eGFP luminescence will be electronically sensed and , based on this measurement, a PDI control system will generate a feedback response so as to self-regulate and restore the normal levels of T3 hormone in the human body.**

Index Terms- AHL, Close-loop, eGFP, Feedback, iGEM, Intein, Lactone, sfGFP, T3 & Thyroid.

I. INTRODUCTION

The endocrine system is responsible for controlling the balance of hormones in the bloodstream by the use of intrinsic feedback loops, which can be disrupted leading to disease. Among these disruptions, thyroid disease is one of the most common, affecting millions of individuals throughout Europe, 5% of the general population [1]. Over 99% of affected patients suffer from hypothyroidism and are mainly prescribed levothyroxine: a medication that has a narrow range of doses at which is effective without adverse effects [2]. As a consequence, one-third of the patients who receive this treatment still exhibit symptoms.

Therefore, several studies have concluded that there is a clear need for patient-specific dosage optimization [1]. However, current medications mainly offer 11 possible dosages that cover the range of 25 μg to 200 μg [3] and are administered once a day, which causes difficulties when adjusting the levels of thyroid hormones with respect to the real homeostatic conditions.

Consequently, the aim of this study is to restore the feedback system by focusing on the development of a medical device similar to the insulin pump. Since T3 is a thyroid hormone, more active than thyroxine, sensing it constantly will give us a lot of information on how the feedback is operating, something that cannot be done regularly by conventional blood tests. Therefore, our reporter bacteria will sense T3 by producing a recombinant protein that contains a mini-intein domain (ΔI-SM). Inteins allow for protein splicing, and when activated, they produce a ligation of C-terminal and N-terminal external proteins [4]. This allows that in presence of T3, the mini intein will fold to attach the two halves of an enhanced Green Fluorescence Protein (eGFP), thus creating a functional eGFP which, when excited, will emit a fluorescence that can be captured by the use of a photodiode. The obtained value can be then compared to a reference value (that could be previously defined by an endocrinologist) and the system will inject levothyroxine until the optimal concentration is reached.

II. *RESEARCH ELABORATIONS*

2.1. Intein mediated T3 sensing

To quantify the amount of T3 in the body we need to express a marker with an intensity proportional to the quantity of T3 present at the skin level. It has been proven that the previously engineered ΔI-SM mini-intein, derived from the Mycobacterium tuberculosis RecA wild-type intein (Mtu recA), can be modified so that it is activated by the T3 hormone [4]. This is possible by replacing residues 110-383 of the Mtu recA sequence by the thyroid receptor $\beta 1$ (TR$\beta 1$) sequence, that will be acting as a ligand binding domain [5].

Considering the wide usage of the green fluorescence protein (GFP) as a reporter and its capabilities to be linked to an optogenetic circuit makes GFP a great tool for our purposes. Given the splicing activity of the inteins to fuse the two subunits of a protein linked to its terminals, and the usage of eGFP for this purpose, eGFP was spliced at the 70 residue, so that the residue at the N terminal linked to the modified intein is a cysteine, as it seems to give better results [6].

Having such a large protein complex when eGFP is fused to the intein can result in the formation of inclusion bodies and, therefore, eGFP could not be visible [7]. The novel Fh8 system used as a solubility tag can help to avoid the formation of these inclusion bodies when added to the terminals of the protein complex [8] (figure 1).

As the intein activity happens at the post-translational level, the constitutive promoter BBa_K880005 was added to the previous construct (splitted eGFP + modified ΔI-SM mini-intein + Fh8 tag), so that the expression of eGFP is only dependent on the activation of the intein by the fusion of the T3 hormone to the TR$\beta 1$ ligand binding domain.

Finally, the Flagx3 tag was added at the end the end of the construct to be able to inmuno detect its expression afterwards and to see if the splicing is being done correctly depending on the molecular weight of the tagged protein.

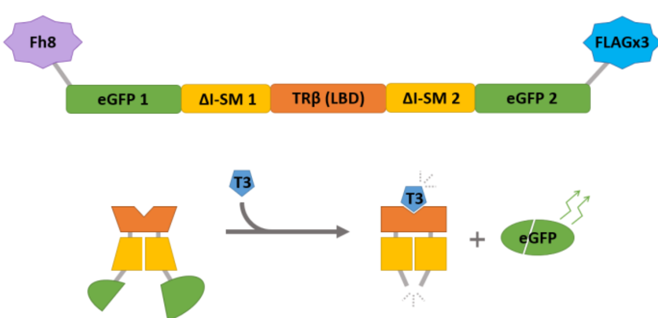

FIGURE 1: Construct of the T3 reporter cell containing the thyroid receptor (TRβ) in the middle, the 2 halves of the mini intein (ΔI-SM) and the 2 halves of the eGFP that will be spliced into a functional eGFP.

2.2. T3 sensing modelling

$$\frac{d[T3]}{dt} = m - \beta\,[T3][P] - \delta_{T3}[T3] \quad (1)$$

$$\frac{d[P]}{dt} = \alpha_p mRNA - \beta\,[T3][P] - \delta[P] \quad (2)$$

$$\frac{d[GFP]}{dt} = \alpha_{GFP}\,\beta\,[T3][P] - \delta_{GFP}[GFP] \quad (3)$$

In order to model the T3 reporter cell, a system of ordinary differential equations (ODE), that took into account the underlying dynamics of the T3 sensing construct was designed (equations 1-3). Because our construct (Figure 1) is produced at a constant rate the mRNA levels could be considered as in a steady state.

As T3 is a small hormone it can cross through the cell membrane via simple diffusion. This implies that the T3 concentration inside and outside is always the same, and thus the constant (m) represents the rate of entry of T3 into the system (1). In equation 2, T3 is consumed in contact with the construct protein (P) at a constant rate (β) and T3 is degraded at at a constant rate (δ_{T3}).

The construct protein (P) is produced at a stable rate (α_P) when in contact with mRNA (2) and is also consumed in contact with T3 at a constant rate (β). As with T3, the construct protein (P) is degraded at at a constant rate (δ).

In equation 4 the mini intein folds to form GFP at a stable rate (β) when T3 and the construct protein (P) are in contact. Due to the fact that the folding of the intein, to produce the splicing of the two halves of the GFP is not 100% reliable, a probability that the folding of the intein generates a functional GFP has been introduced (α_{GFP}).GFP is degraded at at a constant rate (δ_{GFP}).

2.3. Turbidostat

To prove that our feedback system can work for extensive amounts of time, a turbidostat had to be built to ensure that cells are able to survive long enough. This device is based on the eVOLVER turbidostat designed by Wong B. et al. [9]. To do so, we counted with the help of the PhD Guillermo Nevot, who already built an imitation of the eVOLVER turbidostat achieving great results and could advise us during the whole process.

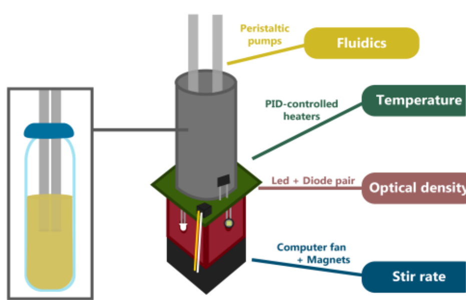

FIGURE 2: Turbidostat currently in development (circuit not included in the image).

The structure of the turbidostat is compounded by five pieces: a 12V computer fan, a double methacrylate layer, a 3D printed integrated sleeve, an aluminum tube and a PCB (printed circuit board) or breadboard.

The 12V computer fan is used to rotate a stirring magnet placed into the vial where the cells are contained, thus we can keep the medium homogenous, which is a key aspect when measuring the OD (optical density) of the contained culture. To do so two small magnets are stuck in opposite blades of the fan. The methacrylate layer is used in order to separate the blades of the fan from the bottom part of the vial. The aluminum tube due to its thermal conductivity was used to keep the cells at an optimal temperature.

The 3D printed piece that covers part of the aluminum tube includes holes that are used to introduce the LED and the photodiode used to obtain the OD. This allows to avoid as much external visible light as possible, as it can interfere in the photodiode measures. These measures are crucial since they will determine what the turbidostat will do to reach a desired value of OD set by the user. Thus, the turbidostat can either wait for the cells to reproduce so that the OD increases or dilute the culture with LB (lysogeny broth) medium to decrease the OD. Fixing the OD of the culture at a certain level ensures that a long-term in vitro experiment can be performed without being influenced by the cells concentration.

Finally, a PCB or breadboard is used to provide a structure where all the components that synchronize the activity of the turbidostat are included: which are resistors, capacitors, transistors, LEDs for the OD and the GFP excitement.

Nevertheless, although a detailed description of the circuit is out of the scope of this summary article, it has to be stated that all the control of the electronic components of the turbidostat is carried out with Arduino, an open-source electronic prototyping platform, and through a sophisticated software called Firmata Node-Red. This software was programmed to control the main

parameters of the turbidostat by some proportional-integral-derivative (PID) controller. The basic idea behind a PID controller is to read a sensor, then compute the desired actuator output by calculating proportional, integral, and derivative responses and summing those three components to compute the output.

III. RESULTS AND FINDINGS

3.1 T3 Sensing

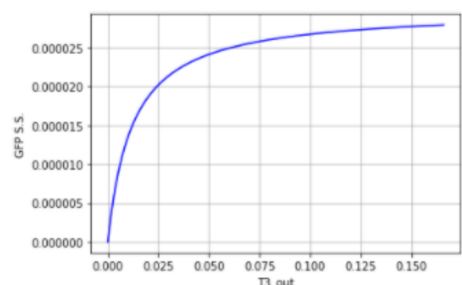

FIGURE 3: Final GFP steady states of with respect to T3_out

$$GFP = \frac{\alpha_{GFP}\,\beta\,T3\,\gamma_P}{\delta_{GFP}(\beta T3+\delta_p)} \quad (5)$$

In order to evaluate the viability of the T3 reporter cell as a sensor the transfer function (5) of the ODE system was evaluated (figure 3).

3.2 Turbidostat

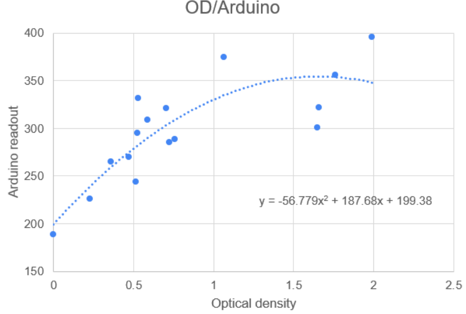

FIGURE 4: Transfer function between our arduino OD sensor and the optical density measured in a spectrophotometer

As our turbidostat needs to maintain a constant optical density (OD) in time a transfer function must be drawn between our arduino OD sensor and the optical density measured in a spectrophotometer (figure 4). This allows us to measure the optical density of the culture in the turbidostat using our arduino OD sensor, thus allowing an implementation of a fast PID controller in the Arduino to control the OD by pumping out old media with cells and pumping in new fresh media, from an Erlenmeyer, at the turbidostat.

IV. DISCUSSION

This current project constitutes the first steps towards continuous in vivo hormone autoregulation; developing a small electronic device located on the skin that gives your body the regular levothyroxine it needs. The obtained first results from the plate reader showed that it is possible to correlate T3 hormone levels in an external medium to GFP fluorescence, however, reading this value with the electronic circuit incorporated in the proposed DIY turbidostat is still in progress. The implemented PID code on the turbidostat has experimentally shown to provide a robust, fast and accurate response over the control of the temperature and the management of the peristaltic pump responsible for delivering the right T3 hormone value. These findings enable progress towards developing a revolutionary therapy to hormonal imbalances using the demonstrated fully automatic feedback system.

Even though initial results seem promising this study has encountered some difficulties and limitations. On the one hand, the specificity and sensibility of our biosensor need to prove to be high enough not to cause a big error in the T3 hormone administration pump system. In other words, this study must demonstrate that the GFP luminescence is capable of providing a reliable indirect measurement of the level of T3 hormone in the body. Since this project aims at finding a revolutionary therapy applied to the complex human body, it needs to be highly secure and accurate. On the other hand, since this study deals with engineered bacteria as biosensors for a future human therapy, a reliable and practical method for biocontainment needs to be contemplated.

Further work to be done in this area constitutes a deeper characterization of the inteins and the study of strategies for making them more specific. This project also considers the use of the inteins design pipeline for making it specific to not only to T3, but also to other hormones. Moreover, a deeper study is proposed on the implementation and optimization of the final design, that is, the miniaturization of the final medical device; as well as developing a user friendly GUI to allow clinicians to remotely control the settings of the device.

V. CONCLUSION

All in all, ‘Hormonic’ device would be able to sense the patient’s levels of T3 and regulate its concentration in order to reach normal levels of this hormone. This would mean a patient-specific treatment for the patient and thus, and an improvement in the quality of life. Nevertheless, this project constitutes the first steps into this kind of therapy for thyroid imbalances, and so, further advances in its development will be finished at the end of the iGEM 2020 project.

REFERENCES

[1]: Chiovato, L., Magri, F. & Carlé, A. Hypothyroidism in Context: Where We've Been and Where We're Going. Adv Ther 36, 47–58 (2019). https://doi.org/10.1007/s12325-019-01080-8

[2]: Fliers, E., Demeneix, B., Bhaseen, A., & Brix, T. H. (2018). European Thyroid Association (ETA) and Thyroid Federation International (TFI) Joint Position Statement on the Interchangeability of Levothyroxine Products in EU Countries. European thyroid journal, 7(5), 238–242.

[3]: Gottwald-Hostalek, U., Uhl, W., Wolna, P., & Kahaly, G. J. (2017). New levothyroxine formulation meeting 95-105% specification over the whole shelf-life: results from two pharmacokinetic trials. Current medical research and opinion, 33(2), 169–174. https://doi.org/10.1080/03007995.2016.1246434

[4]: Skretas, G., & Wood, D. W. (2005). Regulation of protein activity with small-molecule-controlled inteins. Protein science : a publication of the Protein Society, 14(2), 523–532. https://doi.org/10.1110/ps.04996905

[5]: Gierach, I., Li, J., Wu, W. Y., Grover, G. J., & Wood, D. W. (2012). Bacterial biosensors for screening isoform-selective ligands for human thyroid receptors α-1 and β-1. FEBS open bio, 2, 247–253. https://doi.org/10.1016/j.fob.2012.08.002

[6]: Tornabene, P., Trapani, I., Minopoli, R., Centrulo, M., Lupo, M., de Simone, S., Tiberi, P., Dell'Aquila, F., Marrocco, E., Iodice, C., Iuliano, A., Gesualdo, C., Rossi, S., Giaquinto, L., Albert, S., Hoyng, C. B., Polishchuk, E., Cremers, F., Surace, E. M., Simonelli, F., … Auricchio, A. (2019). Intein-mediated protein trans-splicing expands adeno-associated virus transfer capacity in the retina. Science translational medicine, 11(492), eaav4523. https://doi.org/10.1126/scitranslmed.aav4523

[7]: Gangopadhyay, J. P., Jiang, S. Q., & Paulus, H. (2003). An in vitro screening system for protein splicing inhibitors based on green fluorescent protein as an indicator. Analytical chemistry, 75(10), 2456–2462. https://doi.org/10.1021/ac020756b

[8]: Costa S, Almeida A, Castro A and Domingues L (2014) Fusion tags for protein solubility, purification, and immunogenicity in Escherichia coli: the novel Fh8 system. Front. Microbiol. 5:63. doi: 10.3389/fmicb.2014.00063

[9]: Wong, B., Mancuso, C., Kiriakov, S. et al (2018). Precise, automated control of conditions for high-throughput growth of yeast and bacteria with eVOLVER. Nat Biotechnol 36, 614–623. https://doi.org/10.1038/nbt.4151

Synthetic Mammalian Circuitry for Graded Treatment of COVID-19 Cytokine Storms

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***Abstract* - The COVID-19 pandemic, caused by SARS- CoV-2, has afflicted millions of people, with one prominent feature of its lethality being an overactive immune response, or cytokine storm. We aimed to design a synthetic mammalian network to alleviate cytokine storms using powerful, switchlike endoribonucleases. By sensing changes in concentration of two biomarkers indicative of cytokine storms, our system will respond with graded output of a cytokine-sequestering single-chain variable antibody fragment in order to differentially treat patients with varying levels of disease severity. We computationally constructed a cellular and plasma-level immune response to COVID-19 through an ODE-based SimBiology model to inform the design of our sensor specifications, network topology, and tailored treatment response. This engineered system, once experimentally verified *in vitro*, can be used to further our current understanding of COVID-19 immunopathology, with a particular focus on IL-1.**

Index Terms- COVID-19, cytokine storm, endoribonucleases (ERNs), immunomodulation, mammalian synthetic biology

I. INTRODUCTION

COVID-19 has affected millions in the world by storm–that is, a cytokine storm. Cytokines are a family of secreted small proteins used in cell-cell signaling; of this family, uncontrolled levels and signalling of interferons (IFNs), interleukins (ILs), chemokines, and tumor-necrosis-factors (TNFs), result in an immune system overreaction often worsening patient outcome–this is a cytokine storm, the phenomena linked to lethality in COVID-19 (Ragab et al.).

Much of COVID-19 pathology is still being unraveled; remotely, through the power of computational experimentation, we saw the opportunity to contribute with minimal risk. We thus studied literature pointing to unique biomarker patterns found in COVID-19 cytokine storms (CCSs), as well as underlying mechanisms of other overactive immune disorders, such as rheumatoid arthritis and psoriasis. Based on statistical analyses by Yang et al., IP-10 and MCP-3, two cytokines secreted by the IFN-γ cascade, when present together in elevated concentrations, served as promising biomarkers for severe CCSs.

Additionally, quantifiable levels of cytokines and other immune signatures vary greatly between patients' unique health profiles. Here, we realized a critical need: mitigation of the inflammatory response, catered to the individual patient's immune response to SARS-CoV-2 infection. This challenge was one we believed synthetically engineered mammalian cells were well suited for: their ability to continually monitor state, implement dosage-feedback regulation, and utilize native biological parts pose many advantages over traditional pharmaceuticals.

Synthetic biology requires the engineering of nonlinear biology into predictable, digital behaviors; our goal was to employ effective digital molecular mechanisms to construct an analog, or "graded", response which is tailorable to individual patients using already available synthetic biology-designed tools. Such an approach would ensure maintenance of systemic immune homeostasis.

II. EXPERIMENTAL DESIGN

Our team was interested in AND gates because we wanted to respond to the presence of both of our chosen CCS biomarkers. We iterated through three versions of AND logic for the two primary cytokine biomarkers: firstly, cascades (Schukur et al.), secondly, logarithmic-scale (Daniel et al.), and lastly, a PERSIST-based system (DiAndreth et al.). Schukur et al.'s cascade method employs two receptors with distinct inputs: upon binding of the first input to the receptor, an intermediate necessary for the second transduction pathway is produced; this second pathway eventually transcribes an output gene. Daniel et al.'s synthetic analog gene circuits produced through variable copy number AraC-based plasmids were shown to logarithmically transform a wide dynamic range of input inducer concentrations into output protein levels.

When trying to apply this analog circuit design to mammalian cells, we ran into difficulties finding a system mechanistically analogous to the bacterial Arabinose activator protein. Programmable Endonucleolytic Scission-Induced Stability Tuning (PERSIST), a CRISPR-based RNA regulation system developed in the Weiss lab that can produce switchlike "ON" and "OFF" responses, offered promising tunable behavior for our circuitry with digital specificity at the post-transcriptional mRNA level.

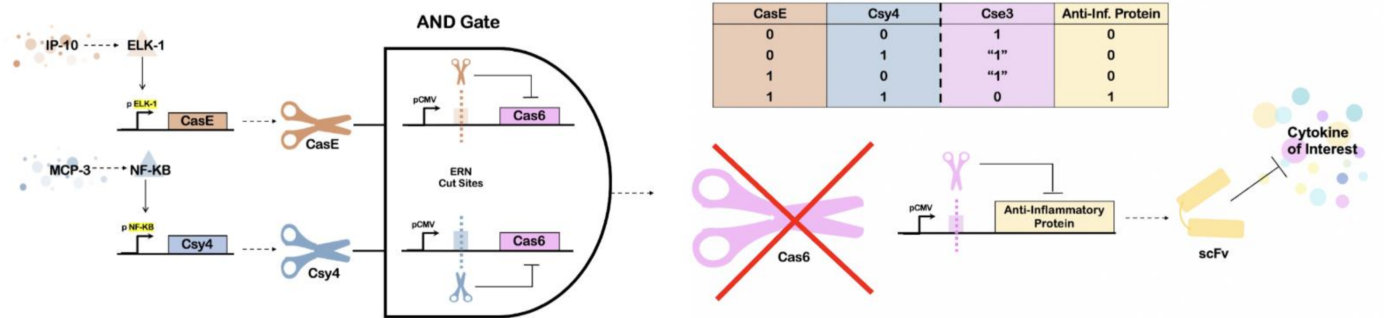

FIGURE 1: Full network circuit design, responsive to COVID-19 cytokine storm biomarkers' (IP-10 + MCP-3) nuclear transcription factors (ELK-1 + NF-κB) , using digital PERSIST "OFF" logic (CasE, Csy4, Cas6), and producing an individualized anti-inflammatory, single chain antibody fragment output (scFv)

We considered various options for anti-inflammatory actuation upon cytokines integrated in the CCS: competitive agonists, receptor antagonists, and soluble decoy receptors that could prevent native receptor signal transduction. Based on the specificity and efficacy of drugs like tocilizumab (IL-6 antibody) for alleviating overactive immune disorders, we implemented single-chain variable fragments of antibodies (scFv) as the AND gate output, due to their ease of translation and structural integrity. Additionally, as some of the whole antibodies against cytokine targets in our CCS system have not been clinically well-characterized in humans, scFvs' reduced immunogenicity was appealing in our system's context.

III. RESULTS AND FINDINGS

We developed an AND gate with five PERSIST-based plasmids (Fig. 1). IP-10 and MCP-3 signal downstream phosphorylation pathways to activate nuclear transcription factors (nTFs) ELK-1 and NF-κB, respectively. Two minimal constitutive (here, CytoMegaloVirus, CMV) promoters are then induced by nTF-responsive element binding, upstream of ERN genes. In choosing our ERN components for translation, we evaluated the sensitivity with the relative abundance of the biomarker in plasma. In CCSs, MCP-3 has been observed about 10^3 fold lower than IP-10; Csy4, an experimentally stronger "OFF" ERN, was thus placed downstream of the NF-KB-responsive promoter, while CasE, a slightly weaker ERN, was used for IP-10's path. Cas6, the weakest ERN of the Cas family evaluated, when produced by either of two CMV-constitutively-expressed transcripts, represses translation of anti-inflammatory soluble scFv with a slight buffer to allow for IP-10 and MCP-3 elevation to be reached. To further enhance mechanism dynamics by hastening steady state, a PEST degradation tag can be fused to the Cas6 sequence. Our AND gate thus is engineered to powerfully and dose-responsively secrete output when there is both enough IP-10 and MCP-3 to result in the degradation of Cas6 transcripts.

From this design, we created a compartmental ODE-based model in MATLAB©'s SimBiology to simulate intracellular reaction dynamics, utilizing both mass action and repressor Hill function equation parameters from established biological phenomena (Mishra et al.).

As a representation, (1) illustrates the rate of change of the AND gate Cas6 mRNA transcripts:

$$\frac{d(mRNACas6)}{dt} = \beta_{CMV} - \delta_{mRNA}[mRNACas6] + \frac{\varepsilon}{1+\left(\frac{\kappa}{[ERN]}\right)^{n}} \tag{1}$$

where β_{CMV} represents the basal transcription rate of the CMV promoter, δ_{mRNA} is the systemic mRNA degradation rate, ε is the ERN's cut rate, κ is the ERN binding dissociation constant, and n is the Hill coefficient.

To gauge the effect of AND gate output on systemic plasma, we built an extracellular cytokine network map (Fig. 2) based upon principal component analysis of cytokine interactions clinically observed in plasma of a population undergoing cytokine storms (Yiu et al.). Rate constants for interactions are coupled concentration coefficients from the study.

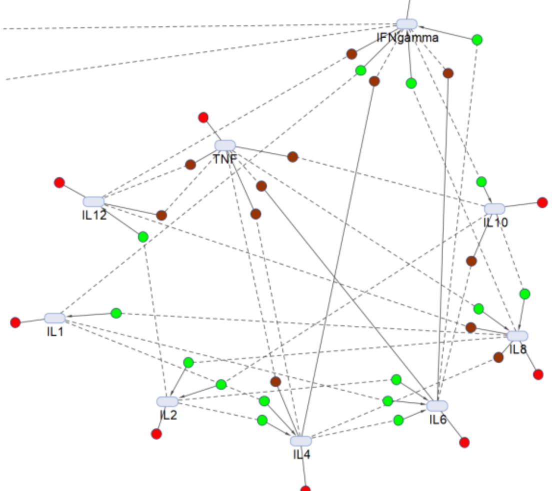

FIGURE 2: Systemic cytokine storm interactions in MATLAB©'s SimBiology, developed based on findings from Yiu et al. Cytokine species are marked as pale blue ovals. Solid lines are one-way reactions, dotted lines indicate the presence of reactants also being products. Red dots indicate self-attenuation interactions, green are upregulation, and brown are downregulation.

ELK-1 was sufficient as our observed output of cytokine sequestration as its level was representative of if our cytokine modulator was able to act as a negative feedback loop, reducing its own inputs. Additionally, in our model, it is produced at a scaled, otherwise identical, rate to NF-KB.

Our results showed two distinct features: changes in the peak ELK-1 observed and the steady state value of ELK-1 (Fig. 3).

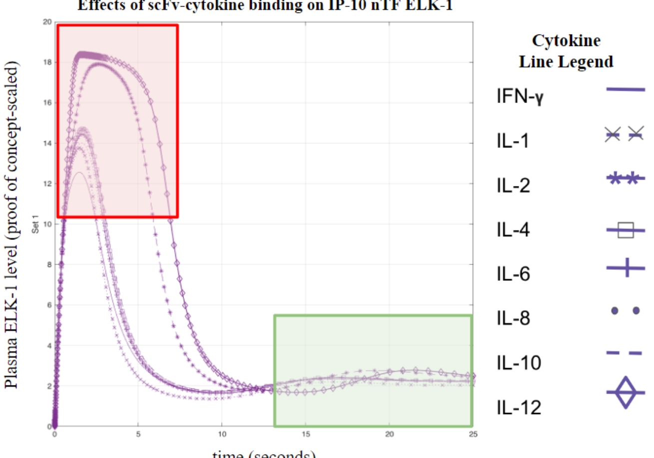

FIGURE 3: scFv-cytokine binding affects IP-10 nTF levels in two regimes: in red, peak, and in green, steady state.

From our model, it is apparent that IFN-γ most directly modulates ELK-1. Though IFN-γ thus may seem like the optimal candidate for sequestration, targeting this cytokine, which takes a critical role in the antiviral immune response could be dangerous for a CCS patient (Acharya et al. 2020). Thus, since IL-1 generated the second lowest peak and the lowest steady state value of ELK-1, it appears to be a better scFv target. Though IL-1 is also a key player in the immune response, based on studies that have shown clinical efficacy of IL-1 inhibition (Cavalli et al., 2020), we postulated that IL-1 could still be a critical target for our immunomodulator system.

IV. DISCUSSION

The finding of IL-1 to be most effective in reducing scFv production without potentially severely compromising the antiviral response points to IL-1 having a critical role in cytokine storm pathology in our model. Such insight could pave future studies guided by experts in experimental immunology, in which special focus is given to understanding the interactions of IL-1 in CCS pathology.

Given our network development thus far, there are a number of fine-tuning measures we would be interested in pursuing as more unfolds about CCS in research efforts like ours. Implementing a multi-input refined AND gate based upon sensing other biomarkers and mechanisms not emphasized yet, reshaping input-output relationships through adding multimodal regulation at the pre-transcriptional level, and characterizing + iterating strengths of promoters, ERNs, & other network parts in the context of an integrated transfection of HEK-293 cells will translate our computational findings into a working, living system.

V. CONCLUSION

In this project, we aimed to better understand and treat COVID-19 cytokine storms. Our engineered system uniquely composes the power of digital PERSIST architecture into a graded output that can access a broad range of situation-specific values. Having characterized our network computationally, we are more informed in our next steps of observing system behavior in cells *in vitro*. Our work not only develops a methodology to comprehend and intervene in uncharted COVID-19 cytokine storm immunopathology, but also the underexplored cases of other similar immune disorders to come.

APPENDIX

Our MATLAB© SimBiology model code may be found at: https://www.dropbox.com/sh/v7w2uccp23oaxtw/AAAwQdwyPj2YZZS-GNVL8AtLa?dl=0

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REFERENCES

Acharya, D., Liu, G., & Gack, M. U. (2020). Dysregulation of type I interferon responses in COVID-19. *Nat Rev Immunol* 20, 397–398 doi:10.1038/s41577-020-0346-x

Cavalli, G., Luca G. D., Campochiaro C., Della-Torre E., Ripa M., Canetti M., … Dagna L. (2020). Interleukin-1 blockade with high-dose anakinra in patients with COVID-19, acute respiratory distress syndrome, and hyperinflammation: a retrospective cohort study. *The Lancet Rheumatology*, *2*(6), 325-331. doi:10.1016/S2665-9913(20)30127-2

Daniel, R., Rubens, J., Sarpeshkar, R., & Lu, T. K. (2013). Synthetic analog computation in living cells. *Nature 497*, 619–623 doi:10.1038/nature12148

DiAndreth, B., Wauford, N., Hu, E., Palacios, S., & Weiss R. (2019). PERSIST: A programmable RNA regulation platform using CRISPR endoRNases. *bioRxiv*, 12(15) 1-17. doi:10.1101/2019.12.15.867150

Mishra, D., Rivera, P., Lin, A., Vecchio D. D., & Weiss R. (2014). A load driver device for engineering modularity in biological networks. *Nature Biotechnology*, 32, 1268–1275. doi:10.1038/nbt.3044

Ragab, D., Salah Eldin, H., Taeimah, M., Khattab, R., & Salem, R. (2020). The COVID-19 Cytokine Storm; What We Know So Far. *Frontiers In Immunology*, 11, 1446.doi:10.3389/fimmu.2020.01446

Schukur, L., Geering, B., Charpin-El Hamri, G., & Fussenegger, M. (2015). Implantable synthetic cytokine converter cells with AND-gate logic treat experimental psoriasis. *Science Translational Medicine*, *7*(318), 318ra201. doi:10.1126/scitranslmed.aac4964

Yang, Y., Shen, C., Li, J., Yuan, J., Wei, J., Huang, F., Wang, F., Li, G., Li, Y., Xing, L., Peng, L., Yang, M., Cao, M., Zheng, H., Wu, W., Zou, R., Li, D., Xu, Z., Wang, H., Zhang, M., … Liu, Y. (2020). Plasma IP-10 and MCP-3 levels are highly associated with disease severity and predict the progression of COVID-19. *The Journal of allergy and clinical immunology*, *146*(1), 119–127. doi:10.1016/j.jaci.2020.04.027

Yiu, H. H., Graham, A. L., & Stengel, R. F. (2012). Dynamics of a cytokine storm. *PloS one*, *7*(10). doi:10.1371/journal.pone.0045027

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Yeast-based Biosensor for Detection of Interleukins in Human Sweat

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Today, chronic inflammatory diseases (CID) are determined to be the leading cause of deaths worldwide. People suffering from CIDs need to monitor their disease progression regularly. Current inflammation detection methods are invasive and require frequent hospital visits. We aim to utilize the advances in synthetic biology to design a sweat collecting skin patch as a non-invasive, use-at-home biosensor for CIDs. The patch will contain genetically modified yeast capable of responding to interleukins (IL) in sweat. The yeast will be cloned with a modified human IL-receptor and upon ligand-receptor binding, a signal will be translated into the yeast pheromone pathway via a modified G-alpha mechanism. The pheromone pathway will induce the transcription of a visible color pigment. We envision our design as a model platform for future development of non-invasive monitoring devices for CIDs as well

Index Terms- iGEM, chronic inflammatory diseases, engineered yeast, interleukins, biosensor, G-alpha, colour change

I. INTRODUCTION

Chronic inflammatory diseases (CIDs) are determined to be the leading cause of deaths worldwide [1]. Estimations attribute over 50% of premature deaths to CIDs and this number is predicted to increase according to WHO [2,3]. They are life-long, debilitating illnesses, where patients can suffer from pain, fatigue, swelling and fever. Some examples of CIDs are Rheumatoid Arthritis, Crohn's Disease, and Inflammatory Bowel Disease.

Treatment of CIDs focus on the alleviation of symptoms. However, due to the complication of the disease profiles, treatments do not always work and usually there is a long searching process for the right treatment. Monitoring tools to follow the diseases and assess treatment are essential but current tests are time consuming and not always available or accessible, causing a reduction in the quality of life.

Diagnosis of CIDs is very complicated due to the difference in biomarker profiles for each individual patient. However, in all CIDs, the level of inflammation is high, which is commonly treated with various medications. Similar biomarkers, such as interleukins (ILs), are found in many illnesses. It is only the level of these interleukins that vary among the different CIDs. Making a diagnostic tool from their levels require extensive research and pricesness. However, for all CIDs, the monitoring of these *general* inflammatory markers is of interest for following disease progression and treatment efficacy. Some testing methods exist, such as specialized blood tests. However, home-safe non-invasive test are still new to the field of inflammation monitoring. Research regarding the existence of biomarkers in sweat demonstrate the utility for using sweat biosensors [4]. Inflammatory biomarkers are present in sweat and correlates to the levels found the bloodstream [5]. Thus, sweat is a promising tool for non-invasive testing. Here we will explore the utility of GMOs as a sweat-based biosensor tool, by engineering yeast cells to respond with a color signal to interleukins in human sweat.

II. RESEARCH ELABORATIONS

In order to detect the inflammatory biomarkers in sweat, the endogenous human interleukin receptors are cloned into yeast as the source of specialized detection [6]. Our research provides the necessary information for succesfull integration of human IL receptors into genetically modified yeast cells and outlines the functionality of the organism as a biosensor tool.

E. Research Design

Interleukin receptor modification

Most interleukin receptors fall into the category of receptor tyrosine kinases, and as such, many interleukins require the binding of both a primary and an accessory receptor for signal relay. We will test this dimerization of the two receptors by using the tried and tested yeast two-hybrid method, using split-ubiquitin to see how our extracellular domains interact. To this end, we will fuse the extracellular domain of a human interleukin receptor and its accessory protein to endogenous yeast transmembrane proteins, and then again to a one part each of a split TEV protease intracellularly.

Hijacking the pheromone pathway

The amplification and transduction of the signal to our receptors will happen through modification of the regular pheromone pathway in yeast. Normally, it is the beta/gamma complex that relays the receptor signal. We will inhibit the beta/gamma complex in the resting cell by using a modified G alpha protein, which will have TEV recognition sites inserted into its sequence. Upon reception of interleukins, our extracellular receptors will bind to their ligand, and the intracellularly fused split TEV-protease will reconstitute. This reactivated TEV protease can then recognize the cleavage sites in G alpha and cut this inhibition away, thus, rendering the remaining beta/gamma

complex able to serve as a starting point for the pheromone pathway.

Signal transduction via color expression

Triggering the pheromone pathway will result in the activation of a transcription factor, which will promote transcription of a gene encoding a pigment (betanin). Through this, our biosensor will produce a clear, visible color when the inflammation levels are high.

III. METHODS AND MATERIALS

In order to accomplish the development of a biosensor design with the capacity to relay a significant signal from the interleukin concentration in sweat, we have designed two methods of signal propagation. Here, we use computer simulations on MATLAB and Rosetta to compare the applicability of the different designs.

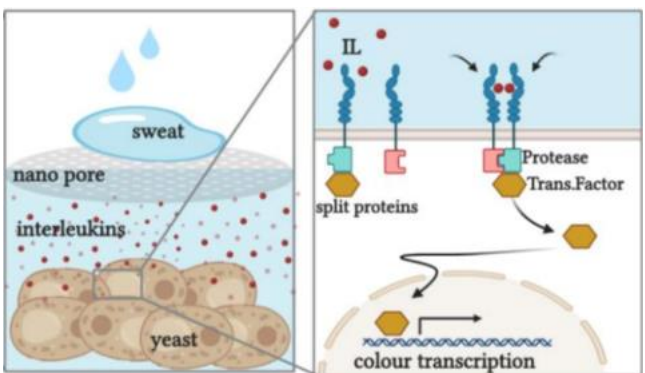

Figure 4: Illustration of split-protein design

In the first design, the receptor-ligand binding causes a split-ubiquition or TEV protease to release a transcription factor (*Figure 1*). This will be accomplished by cloning the yeast with the receptors and making a split ubiquitin assay. Modeling techniques based on ODE (ordinary differential equations) showed that these two designs are very similar in terms of dynamic ranges. However, from our ODE studies, we found that these designs do not have the necessary sensitivity to respond to physiological concentrations of interleukines. In other words, in order for the biosensor to be functional, a different design is needed.

Our second design incorporates the signal amplification from the endogeneous pheromone pathway. The cascade that is part of this pathway leads to a phenomenon known as hypersensitivity, i.e. very high levels of response even with very small initial concentrations.

The simplicity of the split-ubiquitin design makes it extremely useful for initial assays analyzing the functionality of our cloned interleukin receptor. However, after the success of this assay, the pheromone pathway design will be necessary for sufficient signal transduction.

IV. RESULTS AND DISCUSSION

The presence of inflammatory biomarkers in sweat makes it a promising tool for monitoring of chronic inflammatory diseases. *Figure 2* shows the dynamic range of this design, and the correspondence with physiological concentrations of interleukins can be seen (i.e. nanomolar concentrations).

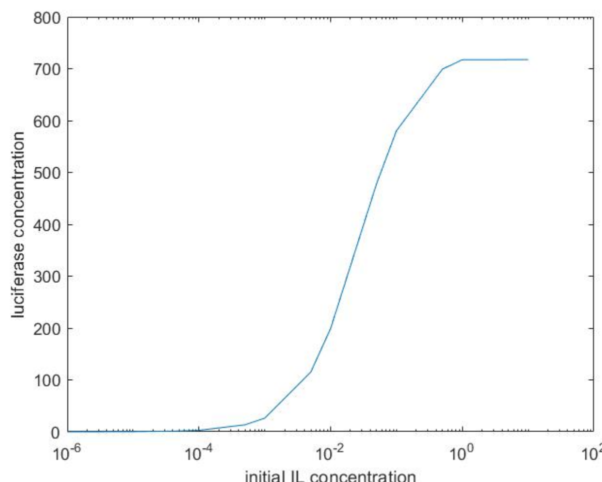

Figure 5 : Dynamic range of the first two designs (split ubiquitin and split TEV protease).

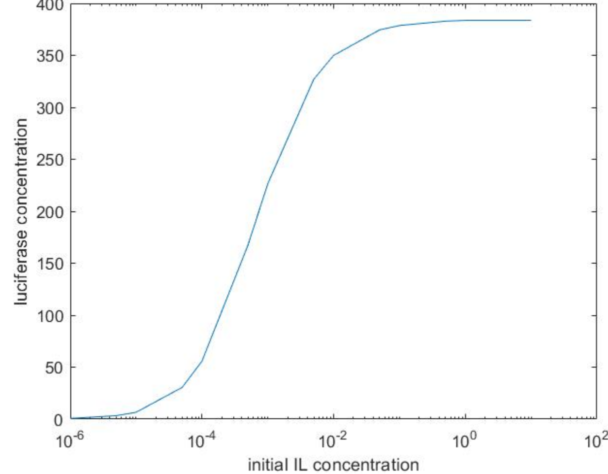

Figure 6 : Dynamic range of the design based on the pheromone pathway.

V. CONCLUSION

Our modified yeast can not only be used for a sweat biosensor but is a valuable tool for research in diagnostics and management of diseases related to inflammation, both to biomedical researchers and future iGEM teams. As a cheap, sustainable source of biomarker detection, it can provide the basis for research into CIDs across the world and potentially aid in the development of new medical discoveries within the field. All this is enabled by engineering signaling pathways that provide significant signal amplification, and based on our simulations, the pheromone pathway seems to be a good candidate for achieving functional biological implementation.

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REFERENCES

Furman D, Campisi J, Verdin E, et al. (2019). Chronic inflammation in the etiology of disease across the life span. *Nat Med*. 25(12):1822-1832. doi:10.1038/s41591-019-0675-0

World Organization of Health. P*reventing chronic diseases: a vital investment.* 2005. Geneva, Switzerland. JoAnne Epping-Jordan, Robert Beaglehole, Catherine Le Galès-Camus, et al.

Pahwa R, Goyal A, Bansal P, et al. 2020. *Chronic Inflammation*. StatPearls. Retrieved from: https://www.ncbi.nlm.nih.gov/books/NBK493173/

Katchman BA, Zhu M, Blain Christen J, Anderson KS (2018). Eccrine Sweat as a Biofluid for Profiling Immune Biomarkers. *Proteomics Clin Appl*. 12(6):e1800010. doi:10.1002/prca.201800010

Marques-Deak A, Cizza G, Eskandari F, et al. (2006) Measurement of cytokines in sweat patches and plasma in healthy women: validation in a controlled study. *J Immunol Methods*. ;315(1-2):99-109. doi:10.1016/j.jim.2006.07.011

Vollmer P, Peters M, Ehlers M, et al. (1996). Yeast expression of the cytokine receptor domain of the soluble interleukin-6 receptor. *J Immuol Methods*. 199(1):47-54. doi:10.1016/s0022-1759(96)00163-9

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A Synthetic Biology based solution for combating Antimicrobial Resistance

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***Abstract*- For decades, multiple varieties of antibiotics have been used for therapeutic purposes and across industries such as agriculture and animal husbandry. However, in recent times both the use and inadvertent misuse of antibiotics have led to the advent of multi-drug-resistant strains. Through literature screening, it was identified that poultry excreta is one of the various sources that significantly contribute to antibiotic pollution. This, in turn, increases the chances of the formation of mutant strains that are resistant to antibiotics thus leading to antibiotic resistance. Also, sulfonamides which are extensively used as growth promoters in poultry farms are reported to have high recovery percentages. To address this concern, through our iGEM 2020 project Coli-Kaze, we propose a synthetic biology-based model that would help in reducing the steady-state level of antibiotics present in poultry waste. Here, in this article, we present our model design and preliminary modeling analysis which was carried out to ensure that the proposed model works effectively.**

***Index Terms*-** Antibiotic resistance, Biosafety, Conjugation, Kill-switch, Sulfonamides

I. INTRODUCTION

Antibiotics are the 'wonder drugs' used to combat and treat a variety of bacterial infections. However, the extensive usage of antibiotics has caused multiple microbes to resist its effects and this has resulted in the emergence of antibiotic-resistant bacteria, ultimately contributing to antimicrobial resistance (AMR)[2]. These resistant pathogens pose a huge risk to human and animal health, with their potential to develop into ferocious superbugs and cause widespread epidemics with seemingly no cure. Addressing this immense global threat of antimicrobial resistance is one of the most urgent priorities, especially with regard to public health and welfare. In our project, we propose a proof-of-concept model of genetically engineered *E.coli* harnessing sulfonamide degrading genes. These *E.coli* would degrade the sulfonamides present in poultry waste below the Predicted No Effect Concentrations (PNEC)[3] when incubated together in a closed tank. This would render the poultry waste sulfonamide-free and would make it safer to dispose or use as manure as there is no selection pressure for resistant bacteria or co-existence of resistant bacteria with sensitive strains. Surface exclusion and complement resistant genes would also be used alongside the bacteria to reduce horizontal gene transfer. A 'kill-switch' with a DNA degrading mechanism would be engineered into the bacteria to ensure biosafety, degrade the bacterial DNA, and prevent their escape into the environment. Although the proof-of-concept model is for the degradation of sulfonamides in farm waste, this model can be extended for other antibiotics by switching the antibiotic degrading genes involved.

II. RESEARCH ELABORATIONS

A. *Research design*

Our model has three individual modules which are designed to accomplish each of these specific goals:

Module 1: Degradation of antibiotics

To degrade the excess antibiotics present in poultry waste, the combination of antibiotic degrading genes *sulX* and *sulR*[4] would be cloned into the bacteria and optimized to degrade large quantities of antibiotics quickly.

Module 2: Reduction of conjugation

To reduce horizontal gene transfer, the genes *traT* and *traS* would be used which help in complement resistance and surface exclusion[5][6].

Module 3: User modulated 'kill-switch'

To ensure biosafety, a user modulated tightly regulated 'kill-switch' involving the gene *DNASEI*[7] would be engineered in the bacteria such that the bacteria deteriorates itself and also its DNA when stimulated by an inducer.

B. *materials and methods*

To ensure our research design is rational a few preliminary mathematical models were developed.

a. To predict the structure of our enzyme SulX, the I-TASSER server was used. The predicted structure was aligned with HsaA[4] as this was the protein used to homology model our enzyme in the literature[8][9][10] (PDB ID: 3AFF).
b. Protein-ligand docking for the same protein was performed for sulfonamide, FMNH2, degradation product (4-aminophenol), and FMN using Autodock VINA[11]. The pose (orientation) with the highest

affinity and closest to the site predicted in the literature was used.

c. Molecular Dynamics simulations were performed for the SulX sulfonamide complex.
d. To find the time taken to degrade the antibiotics, enzyme kinetics was used where an ordered bi-mechanism was considered for modeling.

III. RESULTS AND FINDINGS

Computational methods were used to predict the structure of the protein (SulX) which has not been solved before. The protein structure that was predicted using the I-TASSER[8][9][10] server was observed to have a good overlap with HsaA with RMSD = 1.4. The corresponding substrate binding sites in the sequence were used to determine the grid box for docking studies and the predicted structure was used for molecular dynamics (MD) simulations.

a) Docking Studies

Docking was done using Autodock VINA[11]. The pose with the highest affinity and closest to the site predicted in the literature was used.

TABLE 1: Affinity value for the substrates and the the degradation product of the drug towards SulX

Substrate	Pose	Affinity (kcal/mol)
Sulfamethazine	1	-7.4
4-aminophenol	1	-4.6
FMNH2	4	-7.7
FMN	4	-6.8

TABLE 2: SASA (Solvent Accessible Surface Area) values for the two substrate-binding sites of SulX

Substrate	SASA value
FMNH2	135.298 Å^2
Sulfonamide	85.035 Å^2

b) Molecular Dynamics

MD was performed using DESMOND[12] for ligand and protein for 1.16ns where sulfonamide was taken as ligand and SulX was taken as protein. The position of sulfonamide used as predicted by the docking studies.

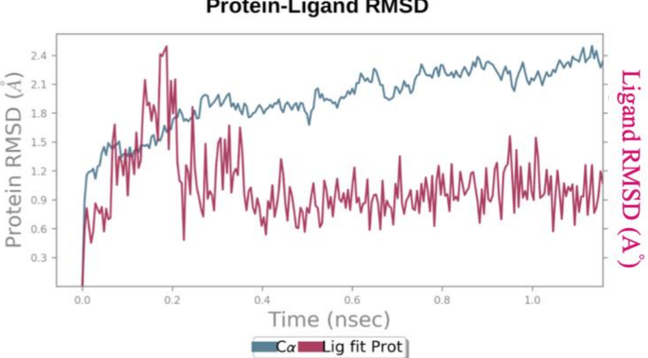

FIGURE 3: protein-ligand rmsd plot.

IV. DISCUSSION

1. *Module 1: Degradation of antibiotics*

This module uses a double gene system to degrade antibiotics. The advantage of using a double gene mechanism is the tremendous reduction in horizontal gene transfer through transduction as it is very unlikely for a virus to transfer both the genes into the same organism. The two-component monooxygenase system, sulfonamide monooxygenase (SulX), and flavin reductase (SulR), are key enzymes in the cleavage of sulfonamides. Monooxygenases are oxidoreductase enzymes. This two-component monooxygenase system was identified from the sulfonamide dependent gene cluster of a species of Actinobacteria called *Microbacterium sp* CJ77. These bacteria can survive in sulfonamide-rich environments and are dependent on them for their carbon source. Both these genes would be constitutively expressed in the engineered bacteria. The flavin reductase helps to reduce FMN to $FMNH_2$ which is used up by the monooxygenase in catalysis of the sulfonamides.[4] An initial High-performance Liquid Chromatography and Mass spectrometry (HPLC-MS) analysis were carried out for the poultry excreta collected from farms following the methodology already reported in the literature for sulfa drugs[15]. The plots obtained have shown the presence of the sulfadiazine drug thus validating the literature studies on sulfonamide recovery rates.

1.1. *Docking and Molecular Dynamics:*

Docking and molecular dynamics studies have shown that there is a clear decrease in the affinity of the active site of the sulfonamide for the degradation product 4-aminophenol (Table 1). This suggests that the degradation product is not held as tightly as the drug itself. The SASA values obtained were found to be significantly higher for $FMNH_2$ than sulfonamide (Table 2). We hypothesize that the bi-substrate mechanism which SulX follows is most likely an ordered bi-substrate mechanism as $FMNH_2$ has more access to its active site than the drug. However, further studies will be performed to confirm the reaction mechanism. The ligand RMSD values from the simulation (Figure 3) are significantly lesser than the protein RMSD values for most of the simulation indicating that the ligand has not diffused away from the active site.

2. *Module 2: Reduction of conjugation*

This module is incorporated in the engineered bacteria to reduce bacterial conjugation and thus reduced horizontal gene transfer. The two anti-conjugation genes *traT* and *traS* are used for this purpose. Both the genes would be expressed downstream to constitutive promoters ensuring biosafety throughout. TraT is a complement resistant protein while TraS is a surface exclusion protein [10]. Both *traS* and *traT* are found naturally in the F plasmid of *E. coli*. TraT is found in the outer membrane of the bacteria and it is responsible for preventing the formation of stable mating pair aggregates. TraS protein is found in the inner membrane of the bacteria and it prevents DNA replication of the F plasmid in conjugal donors. Thus, to be precise, TraT prevents conjugation by preventing donor bacteria (Coli-Kaze bacteria) from forming stable mating pairs and this is achieved by the prevention of pili attachment. TraS, on the other hand, is involved in inhibiting DNA replication of the F-plasmid which is involved in conjugal transfer thus preventing conjugal acceptance even if stable mating pairs are formed[5][6]. These proteins when overexpressed reduce the conjugal frequency of the clone.

3. *Module 3: User modulated 'kill switch'*

This module ensures that the DNA of the engineered *E.coli* bacteria is degraded after the antibiotics in the waste are remediated. To achieve this, bovine pancreatic *DNASEI* (*bpDNaseI*)[7] is placed downstream to the P*BAD* promoter[14] which is regulated by the AraC protein and L-arabinose. In the absence of arabinose, AraC protein binds to specific regions near the promoter forming a loop and thus prevents the RNA polymerase from binding with the promoter and inhibits expression of the downstream gene. On adding arabinose, it binds with the AraC protein, releasing the DNA loop, letting the RNA polymerase bind to the promoter and express *bpDNaseI*, which degrades the bacterial DNA.

V. CONCLUSION AND FUTURE DIRECTIONS

Through our project, we aim to reduce the steady-state level of antibiotics in the poultry waste by degrading them before they are released into the environment. Each of the modules stated in this article was designed after a thorough screening of literature and all possible scenarios were considered to ensure the biosafety of our system.
The mathematical model data presented here is based on the preliminary work conducted by our team. For future analysis, we will build upon these initial results. Docking and molecular dynamics studies were conducted to predict the enzyme kinetic mechanisms. This will be used for further enzyme kinetics calculations i.e estimating the time required by our enzyme for degrading the sulfonamides. Apart from this we also plan to look for mathematical models to predict the effect of the double gene system, the genes *traS* and *traT,* and the kill switch. All this modeling data will then be coupled and will be used for designing the final tank model that can be set-up in poultry farms. Further, we are also looking for ways to degrade the final byproducts obtained to ensure they do not cause any hazards when released into the environment.

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REFERENCES

1. Meek, R. W., Vyas, H., & Piddock, L. J. (2015). Nonmedical Uses of Antibiotics: Time to Restrict Their Use?. *PLoS biology*, *13*(10), e1002266. https://doi.org/10.1371/journal.pbio.1002266
2. Martínez-Carballo, E., González-Barreiro, C., Scharf, S., & Gans, O. (2007). Environmental monitoring study of selected veterinary antibiotics in animal manure and soils in Austria. *Environmental pollution (Barking, Essex : 1987)*, *148*(2), 570–579. https://doi.org/10.1016/j.envpol.2006.11.035
3. Bengtsson-Palme, J., & Larsson, D. G. (2016). Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. *Environment international*, *86*, 140–149. *b*https://doi.org/10.1016/j.envint.2015.10.015
4. Kim, D. W., Thawng, C. N., Lee, K., Wellington, E., & Cha, C. J. (2019). A novel sulfonamide resistance mechanism by two-component flavin-dependent monooxygenase system in sulfonamide-degrading actinobacteria. *Environment international*, *127*, 206–215. https://doi.org/10.1016/j.envint.2019.03.046
5. Achtman, M., Kennedy, N., & Skurray, R. (1977). Cell-cell interactions in conjugating Escherichia coli: role of traT protein in surface exclusion. *Proceedings of the National Academy of Sciences of the United States of America*, *74*(11), 5104–5108. https://doi.org/10.1073/pnas.74.11.5104
6. Gago-Córdoba, C., Val-Calvo, J., Miguel-Arribas, A., Serrano, E., Singh, P. K., Abia, D., Wu, L. J., & Meijer, W. (2019). Surface Exclusion Revisited: Function Related to Differential Expression of the Surface Exclusion System of *Bacillus subtilis* Plasmid pLS20. *Frontiers in microbiology*, *10*, 1502. https://doi.org/10.3389/fmicb.2019.01502
7. Yamaguchi, Y., & Inouye, M. (2011). Regulation of growth and death in Escherichia coli by toxin-antitoxin systems. *Nature reviews. Microbiology*, *9*(11), 779–790. https://doi.org/10.1038/nrmicro2651
8. Yang, J., Yan, R., Roy, A., Xu, D., Poisson, J., & Zhang, Y. (2015). The I-TASSER Suite: protein structure and function prediction. *Nature methods*, *12*(1), 7–8. https://doi.org/10.1038/nmeth.3213
9. Roy, A., Kucukural, A., & Zhang, Y. (2010). I-TASSER: a unified platform for automated protein structure and function prediction. *Nature protocols*, *5*(4), 725–738. https://doi.org/10.1038/nprot.2010.5
10. Zhang Y. (2008). I-TASSER server for protein 3D structure prediction. *BMC bioinformatics*, *9*, 40. https://doi.org/10.1186/1471-2105-9-40
11. Trott, O., & Olson, A. J. (2010). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of computational chemistry*, *31*(2), 455–461. https://doi.org/10.1002/jcc.21334

12. Kevin J. Bowers, Edmond Chow, Huafeng Xu, Ron O. Dror, Michael P. Eastwood, Brent A. Gregersen, John L. Klepeis, István Kolossváry, Mark A. Moraes, Federico D. Sacerdoti, John K. Salmon, Yibing Shan, and David E. Shaw, "Scalable Algorithms for Molecular Dynamics Simulations on Commodity Clusters," Proceedings of the ACM/IEEE Conference on Supercomputing (SC06), Tampa, Florida, November 11–17, 2006. https://doi.org/10.1145/1188455.1188544
13. Ippen-Ihler, K. A., and E. G. Minkley Jr. (1986) "The conjugation system of F, the fertility factor of Escherichia coli." *Annual review of genetics* 20.1: 593-624.3
14. http://parts.igem.org/Part:BBa_K2442101
15. Tolika, E. P., Samanidou, V. F., & Papadoyannis, I. N. (2011). Development and validation of an HPLC method for the determination of ten sulfonamide residues in milk according to 2002/657/EC. *Journal of separation science*, *34*(14), 1627–1635. https://doi.org/10.1002/jssc.201100171

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In silico Design and Analysis of Peptide Inhibitors Against *P. falciparum* Malaria

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Abstract- **Malaria, a mosquito-borne infectious disease caused by the parasite *Plasmodium sp*. is responsible for over 200 million cases and 400 thousand deaths per year. Current methods of treatment against the malarial parasite *Plasmodium falciparum* have started to fail because of the increasing drug resistance in the parasite population. One way to tackle this problem is to generate a peptide drug library which will have multiple peptide-based inhibitors against essential host-parasite interactions. Once the parasites develop resistance against a drug chosen from this library, a new one can be selected to combat the disease. We used homology modelling, saturated mutagenesis and molecular dynamic simulations to design inhibitory peptides against two such interactions (PfEMP1-ICAM1 and CIDRa-CD36). These peptide inhibitors are small (~10 amino acids) and thus can be grafted into a cyclotide (KalataB1) which is an ideal drug delivery scaffold due to its unique ability to remain stable at high temperatures and resistant to chemical and biological degradation.**

Index Terms**-** Cyclotide, Drug library, Drug resistance, Malaria, Peptide inhibitors, *Plasmodium falciparum.*

I. INTRODUCTION

Malaria, a mosquito-borne infectious disease caused by the *Plasmodium* parasite is responsible for a high mortality rate throughout the developing world with India alone contributing 77% of the total malaria cases in South-East Asia [1]. The most dangerous form of malaria is caused by *Plasmodium falciparum* which replicates quickly if not diagnosed and treated, results in a high rise of infection levels in a short period of time [2]. Over the past five decades, Plasmodium falciparum has gained resistance against drugs like chloroquine, sulfadoxine, quinine, and mefloquine, especially in Southeast Asia. Latest genomic surveillance has revealed that resistance to first-line antimalarial drugs is spreading in South-East Asia with the first case of artemisinin-resistant parasites reported in West Bengal, India in 2018 [3]. The parasites have increasingly become resistant through selection pressure and evolution [4]. In recent times, protein-protein interactions (PPI) have emerged as potential and effective targets for medicinal and therapeutic studies [5]. PPI studies and modulation has helped to get a better understanding of host-pathogen interactions and in building novel peptide drugs that could inhibit these host-parasite protein interactions. To tackle the problem of malaria in an efficient and innovative manner, we are designing a library of inhibitory peptide molecules for various host-parasite interactions. Using two such interactions, we show that relatively small peptide inhibitors can be designed (~10 amino acids long) and characterised. These peptides can then be grafted in a stable drug-scaffold like cyclotides which can be orally administrable, robust, cost-effective and resistant to degradation [6], [7].

II. MATERIALS AND METHODS

Selection of suitable interactions:
After an advanced search with 444hits from the RCSB database, PfEMP1-ICAM1 and PfEMP1-CD36 interactions were finalised due to their wild type nature. The interaction complexes were obtained from the Protein Data Bank [8][9]. Missing regions and residues in the obtained complexes were filled in by homology modelling techniques with the help of MODELLER software and the SWISS-MODEL server [17].

TABLE 1: The host-parasite protein interactions, chosen from amongst hundreds of interactions in malaria databases (PlasmoDB, PDB). Availability of the crystal structure of the host-parasite protein complex was an important parameter in choosing candidate interactions.

Parasite Protein	Human Protein	Function	PDB ID
PfEMP1 (Plasmodium falciparum Erythrocyte Membrane Protein 1)	ICAM-1 (Intercellular Adhesion Molecule 1)	PfEMP1s, predicted to bind to ICAM-1, is associated with increased risk of developing cerebral malaria [12].	5MZA
CIDRa domain of PfEMP1 variant 1 of strain MC	CD36 domain of Platelet glycoprotein 4	PfEMP1 proteins maintain the ability to tether to the endothelium and avoid splenic clearance by interacting with CD36 region.	5LGD

Identification of interacting regions:
Potential inhibitory sequences were determined with the help of the software Chimera [10]. Hotspot regions of the host protein which were at a distance less than 3.5-5.0 Å from the parasite protein were identified using knowledge based methods. All the amino acids that fall within this threshold distance were identified as the different wild type inhibitory peptide sequences.

Computational Saturation Mutagenesis and Scoring of Inhibitors:
After identifying hotspot regions, saturated mutagenesis was performed on inhibitory sequences to **establish** a combinatorial

mutation library [11]. We wrote a script that does this based on the functionality of UCSF Chimera [18]. This gave multiple outputs by changing amino acid residue to a different side-chain conformation (Rotamer) or replacing it with other amino acids. For selecting the best inhibitors obtained from the saturation mutagenesis library we scored each model using **FoldX,** which is an empirical force field [12]. The 'RepairPDB' function within FoldX was used to perform a quick optimisation of native structures [13]. Hybrid peptides were made from the mutants by selecting the residues for each position having the least binding energy at that position.

MD simulation of PfEMP1 inhibitors:
After scoring the peptide inhibitors, the best scored mutants and hybrids were selected for further characterisation by Molecular Dynamic (MD) Simulations using Gromacs version 2019.1 [14]. We used AMBER99SB-ILDN force field for the simulations with the protein-peptide inhibitor complex inside a cube with a minimum distance of 1nm from the edges [16]. The cube was solvated with SPC/E water molecules. The first phase of equilibration using the NVT ensemble (constant number of particles, Volume and Temperature) was conducted for 100 ps by using the velocity rescaling thermostat. The target value of the temperature was 300 K. In the second phase, pressure was equilibrated using the NPT ensemble where the number of particles, pressure and temperature was kept constant. This was run for 100ps for 1 bar pressure. Pressure coupling was done using the Parrinello-Rahman barostat. MD simulations were run for each inhibitory peptide-protein complex from 5MZA and 5LGD for a duration of 80 and 100 ns respectively with a time step of 2 fs on the PARAM Brahma supercomputer (https://parambrahma.iiserpune.ac.in/). The MD simulation for each complex was repeated to ascertain the results obtained.

Analysis of MD simulation results:
Atom specific Root Mean Square Deviation (RMSD) calculations were done relative to the structure present in the minimised, equilibrated system as well as on the crystal structure and were plotted. The radius of gyration of the complex was also analysed to study the compactness of the structure. To visualise the simulations, snapshots of the simulations at a time interval of 0.5 ns for the entire simulations time were taken. Using these snapshots the distance between the centroid of the protein and the peptide was calculated and plotted. We calculated the number of intermolecular Hydrogen bonds between the parasite protein and peptide over the entire duration to quantify its relative abundance.

III. RESULTS AND FINDINGS

Identification of Interacting regions:
Peptide inhibitors for PfEMP1 were found using two complexes: PfEMP1-ICAM1 (5MZA) and PfEMP1-CIDRa (5LGD) (Fig. 1). We identified interacting peptide sequences for ICAM1 and CIDRa (Table 2).

Scoring of mutants:
For 5MZA [Appendix 1.2, 1.3] the most negative interaction energy was obtained when serine 16 was mutated to isoleucine (ILPRGGIVL, -8.64 kcal/mol) while for 5LGD [Appendix 2.2, 2.3] mutating serine 160 to methionine yielded the same (NQFVQMILNM, -18.82 kcal/mol). These were greater than the interaction energies of the wild types. The interaction energy is the free energy of binding, thus negative interaction energy means spontaneous protein-peptide binding. For both 5LGD and 5MZA the mutants with the least interaction energy scores were selected for MD simulations.

TABLE 2: Chosen Host-Parasite protein interactions.

Interaction	Sequence (Wild Type)	Residue Number	Interaction Energy (Kcal/mol)
5MZA (wild type)	ILPRGGSVL	10-18 Chain B	-7.45405
5LGD (wild type)	NQFVQMILNS	151-160 Chain A	-16.5112

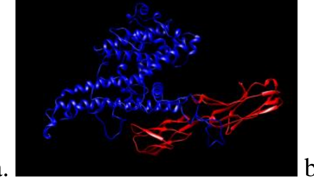
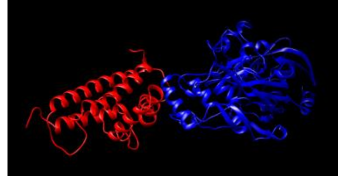
a.  b. 

FIGURE 1. (a) PfEMP1-ICAM1 peptide interaction. Blue represents PfEMP1 and red represents ICAM1 [Appendix 1.1]. (b) PfEMP1-CD36 peptide interaction. Blue represents the CIDRa domain and red represents the CD36 domain [Appendix 2.1].

MD Simulation:
RMSD graphs of 5MZA stabilise over time showing stable binding [Appendix 1.4-1.7] but that of 5LGD indicates conformational changes showing unstable binding [Appendix 2.4-2.7]. Protein backbone was used for both the least-squares fit and the group for RMSD calculation. The radius of gyration of the complexes were analysed and we see that for 5MZA complex it remains stable, in its compact (folded) form over the course of 100 ns at 300 K [Appendix 1.8,1.9] but for 5LGD there are peaks indicating unstable binding [Appendix 2.8,2.9]. The distance between the protein and the peptide from 5MZA has a standard deviation of 0.4-0.5 A with a mean around 30 A [Appendix 1.16] while that from 5LGD is large, around 6A [Appendix 2.14-2.15]. Two hydrogen bonds in 5MZA were found to be retained in almost all of the trajectories, the number of hydrogen bonds fluctuated between 1-10 with an average of 4 hydrogen bonds [Appendix 1.10-1.13]. For 5LGD two hydrogen bonds were found to be retained in almost 50% of the trajectories, the number fluctuates from 0-7 hydrogen bonds with an average of two. The difference in the number and nature of the hydrogen bonds can explain the trends in other graphs [Appendix 2.10-2.13].

IV. DISCUSSION

Towards designing peptide drugs against candidate host-parasite interactions in malaria, the relevant crystal structures were retrieved from the RCSB database and analysed for interacting host epitopes. 9mer and 11mer peptides were

respectively identified in this manner for 5MZA and 5LGD, which were then subjected to *in-silico* saturation mutagenesis. The mutant peptides obtained in this manner were screened for high affinity towards the parasite PfEMP1 protein. For the 5MZA (PfEMP1-ICAM1) interaction, the most efficient inhibitor (S16I) is found to form two additional hydrogen bonds *in silico* with the *P. falciparum* protein than the wild type. For 5LGD, a mutant CD36 peptide (S160M) yielded the greatest interaction energy. These results must be experimentally confirmed, with Circular Dichroism spectroscopy. The high affinity host-mimetic peptides were subjected to MD simulations. Further steps will involve mining databases and analysing interactions like PfRH5-Basigin, an important stage in blood stage of Malaria. To test the efficacy of these inhibitors, we plan to graft them into cyclotides and express them using plasmid vectors and standard biobricks. The circularisation of the cyclotide will be achieved with native chemical ligation (NCL) using the Split-Intein approach.

V. CONCLUSION

We have described the *in silico* designing of peptide inhibitors against two candidate *Plasmodium falciparum*-human protein interactions. Also the various steps involved in the processing and analysis of peptide interactions- from retrieving structures from the PDB to obtaining the desired inhibitors were discussed. This approach may also be used to generate peptide inhibitors for other interactions, thus further contributing to the generation of the final peptide drug library. Furthermore, one can easily envision that these inhibitors can be developed into orally ingestible drugs using cyclotide scaffolds, for which animal and clinical trials would be necessary.

APPENDIX

Reference graphs and plots can be found here.
Direct link: https://drive.google.com/drive/folders/11n_gymFqMGhczV2hbLOOqOMKrPAZZfNb

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REFERENCES

[1] Kumar A, Valecha N, Jain T, et al. Burden of Malaria in India: Retrospective and Prospective View. In: Breman JG, Alilio MS, White NJ, editors. Defining and Defeating the Intolerable Burden of Malaria III: Progress and Perspectives: Supplement to Volume 77(6) of American Journal of Tropical Medicine and Hygiene. Northbrook (IL): American Society of Tropical Medicine and Hygiene; 2007 Dec. Available from: https://www.ncbi.nlm.nih.gov/books/NBK1720/

[2] Q&A, M. (2012, March 1). Dangerous. Retrieved from http://www.malaria.com/questions/dangerous

[3]Hamilton, W. L., Amato, R., van der Pluijm, R. W., Jacob, C. G., Quang, H. H., Thuy-Nhien, N. T., Hien, T. T., Hongvanthong, B., Chindavongsa, K., Mayxay, M., Huy, R., Leang, R., Huch, C., Dysoley, L., Amaratunga, C., Suon, S., Fairhurst, R. M., Tripura, R., Peto, T. J., … Miotto, O. (2019). Evolution and expansion of multidrug-resistant malaria in southeast Asia: a genomic epidemiology study. *The Lancet Infectious Diseases*, *19*(9), 943–951. https://doi.org/10.1016/s1473-3099(19)30392-

[4] Fairhurst, R. M., & Dondorp, A. M. (2016). Artemisinin-Resistant Plasmodium falciparum Malaria. Microbiology Spectrum, 4(3). https://doi.org/10.1128/microbiolspec.ei10-0013-2016

[5]- 1. M. R. Arkin and J. A. Wells, Nature reviews. Drug discovery, 2004,3, 301-317.

[6] Sancheti, H., & Camarero, J. A. (2009). "Splicing up" drug discovery.☆Cell-based expression and screening of genetically-encoded libraries of backbone-cyclized polypeptides. Advanced Drug Delivery Reviews, 61(11), 908–917. https://doi.org/10.1016/j.addr.2009.07.003

[7] Gould, A., & Camarero, J. A. (2017). Cyclotides: Overview and Biotechnological Applications. ChemBioChem, 18(14), 1350–1363. https://doi.org/10.1002/cbic.201700153

[8] Hsieh, F.-L., Turner, L., Bolla, J. R., Robinson, C. V., Lavstsen, T., & Higgins, M. K. (2016). The structural basis for CD36 binding by the malaria parasite. Nature Communications, 7, 12837. doi:10.1038/ncomms12837

[9] Lennartz, F., Adams, Y., Bengtsson, A., Olsen, R. W., Turner, L., Ndam, N. T., … Jensen, A. T. R. (2017). *Structure-Guided Identification of a Family of Dual Receptor-Binding PfEMP1 that Is Associated with Cerebral Malaria. Cell Host & Microbe, 21(3), 403–414.* doi:10.1016/j.chom.2017.02.009 sci-hub.tw/10.1016/j.chom.2017.02.00

[10] Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., & Ferrin, T. E. (2004). UCSF Chimera?A visualization system for exploratory research and analysis. *Journal of Computational Chemistry*, *25*(13), 1605–1612. https://doi.org/10.1002/jcc.20084

[11] Chronopoulou, E. G., & Labrou, N. E. (2011). Site-saturation Mutagenesis: A Powerful Tool for Structure-Based Design of Combinatorial Mutation Libraries. *Current Protocols in Protein Science*, *63*(1), 26.6.1-26.6.2https://doi.org/10.1002/0471140864.ps2606s63

[12]Schymkowitz, J., Borg, J., Stricher, F., Nys, R., Rousseau, F., & Serrano, L. (2005). The FoldX web server: an online force field. *Nucleic Acids Research*, *33*(Web Server), W382–W388. https://doi.org/10.1093/nar/gki387

[13]Xiong, P., Zhang, C., Zheng, W., & Zhang, Y. (2017). BindProfX: Assessing Mutation-Induced Binding Affinity Change by Protein Interface Profiles with Pseudo-Counts. *Journal of Molecular Biology*, *429*(3), 426–434. https://doi.org/10.1016/j.jmb.2016.11.022

[14] Abraham, M. J., Murtola, T., Schulz, R., Páll, S., Smith, J. C., Hess, B., & Lindahl, E. (2015). GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX*, *1–2*, 19–25. https://doi.org/10.1016/j.softx.2015.06.001

[15]Pronk, S., Páll, S., Schulz, R., Larsson, P., Bjelkmar, P., Apostolov, R., Shirts, M. R., Smith, J. C., Kasson, P. M., van der Spoel, D., Hess, B., & Lindahl, E. (2013). GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit. *Bioinformatics*, *29*(7), 845–854. https://doi.org/10.1093/bioinformatics/btt055.

[16]Lindorff-Larsen, K., Maragakis, P., Piana, S., Eastwood, M. P., Dror, R. O., & Shaw, D. E. (2013). Correction: Systematic Validation of Protein Force Fields against Experimental Data. *PLoS ONE*, *8*(4), e32131. https://doi.org/10.1371/annotation/8301b5d4-1ba3-40e7-8fcd-3e169b967044

[17] https://swissmodel.expasy.org/interactive

[18] Rao, A. (2020). *Anantha-Rao12/Peptides-against-Cerebral-Malaria.* GitHub. https://github.com/Anantha-Rao12/Peptides-against-Cerebral-Malaria

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Engineering the Probiotic *Escherichia coli* Nissle 1917 for Oscillatory Colorectal Cancer Therapy

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***Abstract-* Colorectal cancer (CRC) incidence and mortality have increased over the past decades, and CRC is now the third-most common cancer worldwide. Whilst many new therapies are being explored, the standards remain chemotherapy, radiotherapy and surgery. Studies suggest that chronotherapy can reduce side effects without loss in efficiency. We aim to use chronotherapy as the basis of our project by introducing a repressilator into the probiotic E. coli Nissle 1917, with which we intend to deliver the anticancer protein azurin in an oscillatory manner. We aim to modify the repressilator to produce azurin and to test the full system on Caco-2 cells, our chosen in vitro model. For the end of the competition we hope to provide proof of concept for our engineered system.**

Index terms- CRC, iGEM, azurin, probiotics, repressilator, synthetic biology

I. INTRODUCTION

Colorectal cancer (CRC) is a growing concern, affecting more and more patients each year. It is the third most common cancer, and accounts for a little under 10% of all new cancer diagnoses every year (Kuipers et al., 2015). The most typical treatments are surgery, followed by radiotherapy. Chemotherapy is also often recommended alongside the surgical removal of tumours, in order to prevent reoccurrences (Kuipers et al., 2015). However, these therapies often lead to multiple side effects. Due to this, different avenues of treatment are being explored. One of them is chronotherapy, wherein the effect of an anticancer drug can vary depending on the timing of its administration (Eriguchi et al., 2003; Lévi, 2006). It has been found to be effective when using 5-fluoruracil, a common chemotherapeutic drug for CRC (Mormont & Levi, 2003; Ye et al., 2018).

Another advance in the domain of cancer therapy has been the proposed use of bacterial anticancer peptides to replace more commonly used chemotherapeutic molecules (Chakrabarty, Bernardes, & Fialho, 2014; Thundimadathil, 2012). One such peptide is azurin, a blue copper protein involved in electron transfer during denitrification in *Pseudomonas aeruginosa* (van de KAMP et al., 1990). Azurin has elicited interest thanks to its specificity for cancer cells and cytotoxic activity have been demonstrated (Gao, Zhou, Su, & Huang, 2017; Huang et al., 2020). The main mechanism through which azurin acts appears to be by stabilising p53, leading to its accumulation in the cell, thereby inducing apoptosis (Yamada et al., 2009).

Based on these aspects of cancer research, we propose to engineer *Escherichia coli* Nissle 1917 Δclb, a probiotic strain shown to preferentially target and colonise tumours present in mice (Stritzker et al., 2007) to produce azurin at time specific intervals. To be able to obtain the production and secretion of azurin in an oscillatory manner, we manipulated the repressilator system described by Potvin-Trottier et al., which has been shown to retain its oscillations once in the gut of mice (Riglar et al., 2019).

II. MATERIALS AND METHODS

A. *Strains & plasmids*

All strains and the corresponding plasmids used for the repressilator can be found in the *Appendix*. Nissle 1917 Δclb contains a mutation that removes its genotoxicity towards mammalian cells (Olier et al., 2012). The pLPT41 plasmid is used as a P_{tetO1} molecular sponge to stabilise the oscillations generated by the repressilator plasmids. The pLPT119 plasmid is the repressilator plasmid, containing CFP and mVenus, with no degradation tags and so can be used in Nissle 1917.

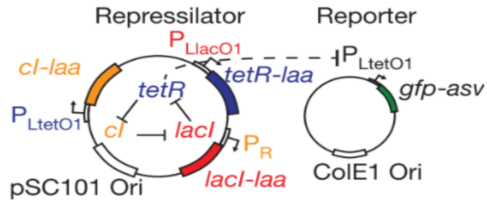

FIGURE 7: Repressilator and reporter/sponge plasmids. Figure from Potvin-Trottier et al., 2016

Those used for protein purification are in Table 2 in the *Appendix*. The azurin sequence was taken from the genome of *P. aeruginosa* PAO1 and codon optimized for *E. coli* ATCC8739 using Genome Calligrapher. The azurin(ETH) sequence refers to the export signaling peptide truncated azurin sequence used by the 2017 ETH Zurich iGEM team (part BBa_K2500001). pelB is a secretion tag, which has been shown to lead to secretion of azurin (Zhang et al., 2012).

B. Detection of fluorescence

For the detection of fluorescence, the flask experiment protocol from Potvin-Trottier et al., 2016 was adapted to be used with a plate reader. Overnight cultures of the strains carrying the repressilator plasmids were done in 5 mL imagining medium (see *Appendix*) with either isopropyl β-d-1-thiogalactopyranoside (IPTG) or anhydrotetracycline (aTc) to synchronise the repressilator system of the cell population at

37°C under shaking. The overnight cultures were then diluted to OD_{600} 0.05 in 96-well plates to measure fluorescence. The cells were then diluted every hour to keep them in exponential phase to be able to visualise the oscillations. Fluorescence was measured in a plate reader using the wavelengths detailed in Table 1.

Table 1: Excitation and emission wavelengths of fluorescent reporter genes

Reporter	Excitation	Emission
mKate2	588nm	633nm
mVenus	515nm	527nm
CFP	456nm	480nm

C. Protein purification

Purification of azurin was done following the standard Ni-pull down protocol. More details can be found in the *Appendix*.

III. RESULTS AND FINDINGS

A. *Recreating the repressilator*

We were able to successfully reproduce the oscillation experiment in the DHL708 strain using pLPT119 with pLPT41 (Potvin-Trottier et al., 2016, **Figure 1**). We introduced the repressilator system in Nissle 1917 Δclb and obtained results similar to those with DHL708 (**Figure 2**).

B. *Modifying the repressilator*

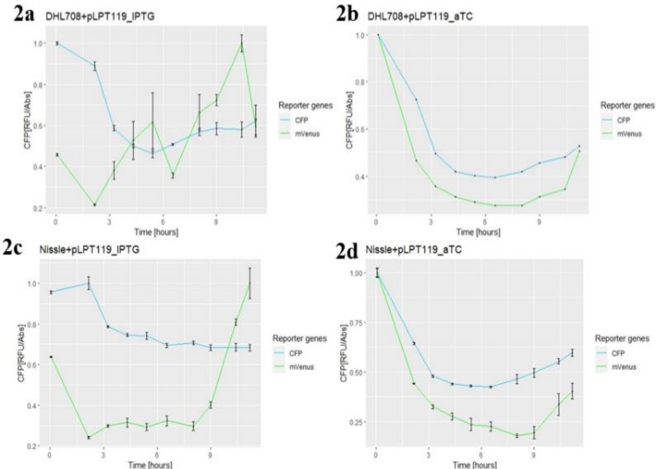

FIGURE 2: Fluorescence measurements of mVenus (green) and CFP (blue) in Nissle 1917 and DHL708. Measurements were taken every hour over 10h and kept at an OD_{600} 0.2-0.3. 2a and 2c were synchronised overnight with IPTG, 2b and 2d with aTc.

We inserted azurin into pLPT41 under the P_{LtetO1} promoter, so that its expression would coincide with that of **mVenus** on pLPT119. To test this co-expression, we first inserted mKate2 under the P_{LtetO1} of PLPT41, and tested the construct with pLPT119 in Nissle 1917. The measurement of mKate2 and CFP did not show the expected oscillatory behaviour (**Figure 3a**). We then tested the repressilator with the pLPT41-pelB-azurin construct and obtained results similar to those with pLPT119+pLPT41 (**Figure 3b, 3c**).

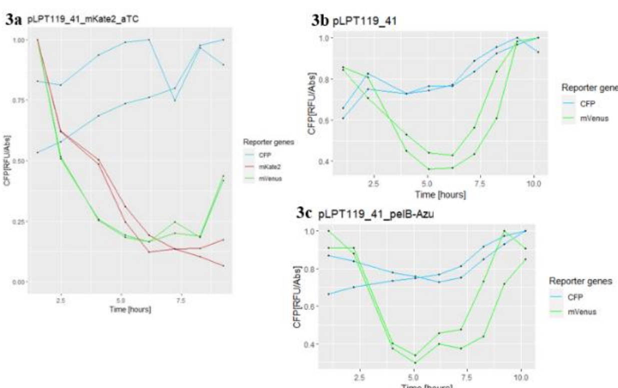

FIGURE 3: Fluorescence measurements of repressilator constructs over 10h: CFP in blue, mKate2 in red and mVenus in green. OD_{600} kept between 0.2-0.3. All constructs in Nissle 1917 and synchronised overnight with aTc. 3a shows pLPT119 + pLPT41-mKate2, 3b pLPT119 + pLPT41 and 3c pLPT119 + pLPT41-pelB-azurin.

C. *Azurin purification*

The third aim was to produce azurin from the protein expression strain *E. coli* BL21. We were unable to introduce our plasmids into this strain, so we purified directly from Nissle 1917. None of the purifications were successful as no bands at expected size of 16kDa were clearly visible (see *Appendix.)*

IV. DISCUSSION

As we started our experiments, we soon realised that, in contrast to our expectations, we observed a constant level of fluorescence for CFP. After inspection of the plasmid sequence, we noticed that this gene is in fact under the control of a constitutive promoter in pLPT119 and so its expression follows the OD_{600} of our cultures (see *Appendix*)**.** Despite this, mVenus appeared to be acting in the way we expected. However, the fluorescence measurements of mKate2 did not act in the expected way originally – we believe this is due to its low expression and detection as we first kept the OD_{600} of our cells too low. Moving forward, we plan to let the cells grow more to avoid being too close to the detection threshold of our plate reader & to use mVenus as our fluorescent marker instead.

Modifying the repressilator also proved to be a challenge, as the size and repetitive sequences of the pLPT119 plasmid complicated its amplification by PCR. This difficulty is why we decided to insert azurin and mKate2 into pLPT41. We were assured that the insertion of mKate2 or azurin downstream of the P_{LtetO1} promoter in the sponge plasmid doesn't disrupt the function of the repressilator.

Finally, for the production of azurin it appears that the pelB tag does not lead to secretion into the supernatant (as there is no visible band at the expected size), or if it does, it is in very low quantities which are undetectable through SDS-PAGE. In order to validate this hypothesis, we plan to perform more sensitive assays such as immunoblotting to detect if azurin is present or not. We also are testing cell-free synthesis to obtain pure azurin. In the next stage of our project, we plan to test *E. coli* Nissle 1917 Δclb transformed with pLPT119 + pLPT41-azurin (and variants thereof) directly with Caco-2 cells, the cell line we chose to model colorectal cancer *in vitro*.

V. CONCLUSION AND OUTLOOK

Despite the fact that we encountered multiple challenges in the implementation of our project, most notably with the production and purification of azurin, moving forward we intend to provide proof of concept by testing the cytotoxicity of our engineered Nissle 1917 on Caco-2 cells, our chosen *in vitro* model. To increase the biosafety of our system, we intend to implement a kill-switch to constrain the activity of our system to the colonic environment. In addition, the kill-switch will also allow us to remove the cells once the therapy has concluded.

APPENDIX

Link to protocols, further results and sequences: https://drive.google.com/drive/folders/1utQ4W5X_HZJgLIhpx_ihHxJzcFL4Rhk4?usp=sharing

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REFERENCES

Chakrabarty, A. M., Bernardes, N., & Fialho, A. M. (2014). Bacterial proteins and peptides in cancer therapy: Today and tomorrow. *Bioengineered*, *5*(4), 234–242. https://doi.org/10.4161/bioe.29266

Eriguchi, M., Levi, F., Hisa, T., Yanagie, H., Nonaka, Y., & Takeda, Y. (2003). Chronotherapy for cancer. *Biomedicine and Pharmacotherapy*, *57*(SUPPL. 1), 92–95. https://doi.org/10.1016/j.biopha.2003.08.012

Gao, M., Zhou, J., Su, Z., & Huang, Y. (2017). Bacterial cupredoxin azurin hijacks cellular signaling networks: Protein–protein interactions and cancer therapy. *Protein Science*, *26*(12), 2334–2341. https://doi.org/10.1002/pro.3310

Huang, F., Shu, Q., Qin, Z., Tian, J., Su, Z., Huang, Y., & Gao, M. (2020). Anticancer Actions of Azurin and Its Derived Peptide p28. *Protein Journal*, *39*(2), 182–189. https://doi.org/10.1007/s10930-020-09891-3

Kuipers, E. J., Grady, W. M., Lieberman, D., Seufferlein, T., Sung, J. J., Boelens, P. G., … Watanabe, T. (2015). Colorectal cancer. *Nature Reviews Disease Primers*, *1*, 1–25. https://doi.org/10.1038/nrdp.2015.65

Lévi, F. (2006). Chronotherapeutics: The relevance of timing in cancer therapy. *Cancer Causes and Control*, *17*(4), 611–621. https://doi.org/10.1007/s10552-005-9004-7

Mormont, M. C., & Levi, F. (2003). Cancer chronotherapy: Principles, applications, and perspectives. *Cancer*, *97*(1), 155–169. https://doi.org/10.1002/cncr.11040

Olier, M., Marcq, I., Salvador-Cartier, C., Secher, T., Dobrindt, U., Boury, M., … Oswald, E. (2012). Genotoxicity of escherichia coli nissle 1917 strain cannot be dissociated from its probiotic activity. *Gut Microbes*, *3*(6), 501–509. https://doi.org/10.4161/gmic.21737

Potvin-Trottier, L., Lord, N. D., Vinnicombe, G., & Paulsson, J. (2016). Synchronous long-term oscillations in a synthetic gene circuit. *Nature*, *538*(7626), 514–517. https://doi.org/10.1038/nature19841

Riglar, D. T., Richmond, D. L., Potvin-Trottier, L., Verdegaal, A. A., Naydich, A. D., Bakshi, S., … Silver, P. A. (2019). Bacterial variability in the mammalian gut captured by a single-cell synthetic oscillator. *Nature Communications*, *10*(1), 1–12. https://doi.org/10.1038/s41467-019-12638-z

Stritzker, J., Weibel, S., Hill, P. J., Oelschlaeger, T. A., Goebel, W., & Szalay, A. A. (2007). Tumor-specific colonization, tissue distribution, and gene induction by probiotic Escherichia coli Nissle 1917 in live mice. *International Journal of Medical Microbiology*, *297*(3), 151–162. https://doi.org/10.1016/j.ijmm.2007.01.008

Thundimadathil, J. (2012). Cancer Treatment Using Peptides: Current Therapies and Future Prospects. *Journal of Amino Acids*, *2012*, 1–13. https://doi.org/10.1155/2012/967347

van de KAMP, M., SILVESTRINI, M. C., BRUNORI, M., Van BEEUMEN, J., HALI, F. C., & CANTERS, G. W. (1990). Involvement of the hydrophobic patch of azurin in the electron-transfer reactions with cytochrome c551 and nitrite reductase. *European Journal of Biochemistry*, *194*(1), 109–118. https://doi.org/10.1111/j.1432-1033.1990.tb19434.x

Yamada, T., Mehta, R. R., Lekmine, F., Christov, K., King, M. L., Majumdar, D., … Das Gupta, T. K. (2009). A peptide fragment of azurin induces a p53-mediated cell cycle arrest in human breast cancer cells. *Molecular Cancer Therapeutics*, *8*(10), 2947–2958. https://doi.org/10.1158/1535-7163.MCT-09-0444

Ye, Y., Xiang, Y., Ozguc, F. M., Kim, Y., Liu, C. J., Park, P. K., … Han, L. (2018). The Genomic Landscape and Pharmacogenomic Interactions of Clock Genes in Cancer Chronotherapy. *Cell Systems*, *6*(3), 314-328.e2. https://doi.org/10.1016/j.cels.2018.01.013

Zhang, Y., Zhang, Y., Xia, L., Zhang, X., Ding, X., Yan, F., & Wu, F. (2012). Escherichia coli Nissle 1917 targets and restrains mouse b16 melanoma and 4T1 breast tumors through expression of azurin protein. *Applied and Environmental Microbiology*, *78*(21), 7603–7610. https://doi.org/10.1128/AEM.01390-12

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The Viability of Engineering a Clostridium to Produce DBHB and Enhance Neuroprotection

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Abstract- **Neurodegenerative diseases provide a unique challenge in a world with an increasingly ageing population. More than 850,000 people have been diagnosed with Alzheimer's disease in the UK. A further 140,000 will experience the hardship that accompanies Parkinson's disease. These neurodegenerative diseases currently have no cure and limited knowledge of causes; with treatments focusing on alleviating symptoms rather than preventing further neurodegeneration. Even if a curative treatment was available for diagnosed patients, fifty percent of the neurons in the substantia nigra are incapacitated by the time Parkinson's disease can be officially diagnosed. This leads to the conclusion that treatments need to focus on preventing neuronal damage before it can occur. D-Beta-Hydroxybutyrate (DBHB) is a ketone body that is usually produced during periods of fasting when glucose levels are low. This molecule has been linked with reducing reactive oxygen species and increasing the transcription of antioxidant genes as well as providing an alternate energy source for neurons. Our project aims to ameliorate the growing restriction on aging by using a Clostridia-based probiotic to increase the amount of DBHB reaching the brain which will help prevent neurodegeneration.**

Index Terms- C.*sporogenes*, DBHB, Ketone bodies, neuroprotection, therapeutic.

I. INTRODUCTION

Our general research goal for this project was to find out if it was possible to engineer a Clostridium to produce DBHB. The DBHB molecules would then reach and generate a positive effect in the brain. Therefore, the hypothesis was that an engineered Clostridium (C. sporogenes) could make enough DBHB to provide significant neuroprotection and delay the onset of neurodegenerative diseases. Research has already shown multiple positive effects of DBHB and ketones in general. This includes its role in neuroprotection and migraine prevention to its promotion of brain-derived neurotrophic factor (BDNF) expression (Yang et al., 2019) (Gross et al., 2019) (Hu et al., 2018). The current landscape for treating neurodegenerative diseases is far from ideal. Diagnosis can only occur after significant neuronal death leading to treatments that help with symptoms rather than a proactive neuroprotectant that reduces the risk of developing a neurodegenerative disease in the first place (Oertel et al, 2016).

II. RESEARCH ELABORATIONS

Unfortunately, we could not perform any lab work this year. Various online resources were utilized to make the project as scientifically sound as possible as well as expertise from our supervisors. This includes reviewing applicable papers on pubmed, building models, and using tools such as snapgene and BLAST.

III. RESULTS AND FINDINGS

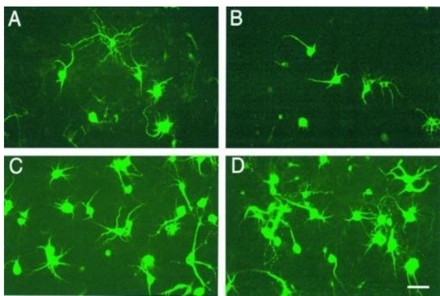

Figure 8: Anti-TH stain on day 7 of a rat mesencephalic neuronal culture. (A) Control culture. (B) Culture after addition of 5 µM MPP+.(C) after addition of MPP+ and 4 mM ketone bodies. (D) after addition of 4 mM ketone bodies alone. The anti-TH stains tyrosine hydroxylase which is an enzyme present in dopaminergic neurons such as the ones affected in Parkinson's disease.

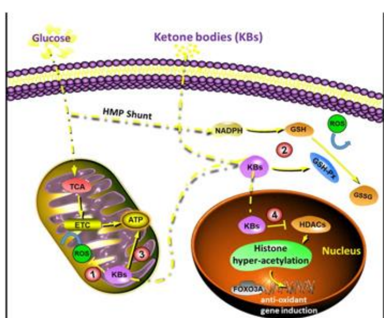

Figure 9: (1) DBHB reduces NAD couples, decreasing ROS production (2) KBs activate glutathione peroxidase, this increases rate of ROS elimination (3)ATP concentration increased (4) Increase anti-oxidant gene expression.

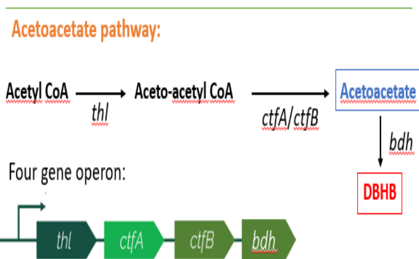

Figure 10: The pathway that will be used to produce DBHB in C.sporogenes as well as the genes that will be transfered to our species. Thl (thiolase), ctfA/ctfB (CoA-transferase subunit A/B) will be utilised from C. Acetobutylicum. Bdh (beta-hydroxybutyrate dehydrogenase) will be taken from S.dysgalactiae subsp. equisimilis.

IV. Discussion

During this project, we wanted to answer 3 main questions: **(1)** Does this ketone body act as a neuroprotectant? **(2)** Could our species produce DBHB? **(3)** Would our therapeutic provide a risk to those who ingest it?

(1) Figure 1 shows the effect that DBHB has on dopaminergic neurons especially in the presence of a toxin (MPP+) that is known to cause neuronal death (Kashiwaya et al., 2000). It is important to notice the difference in parts A and D of Figure 1. The sole addition of ketone bodies to these neurons leads to a greater number of neurons. Comparing B and C also sheds light into the ability of DBHB to protect against this toxin which causes Parkinson's like symptoms. This work on rat mesencephalic neurons shows the potential that DBHB could have in providing neuroprotection. We also must note that rat neurons and brains in general will have differences to human neurons and therefore a future focus should be on the ability to translate this work through to human neurons. Despite this, further papers have shown how a ketogenic diet improves cognitive function in Alzheimer's patients leading to the assumption that DBHBs neuroprotective effects are not just limited to the rodent brain and can be translated effectively (Ota et al., 2019).

Figure 2 highlights the mechanisms by which DBHB could provide increased neuronal survival (Yang et al., 2019). A large proportion of its actions focus on anti-oxidation. These actions counter the oxidative stress that is a common cause of neuronal death (Kim et al., 2015). DBHB works in the neuronal cells to decrease the concentration of reactive oxygen species (ROS') via the activation of glutathione peroxidase, reduction of NAD couples and the increase of anti-oxidant gene expression. Reactive oxygen species are produced naturally in the metabolism of oxygen in mitochondria. A large concentration of ROS' can be toxic to the cell - an excess of these is called oxidative stress. ROS' can cause damage to DNA, deactivate enzymes via the oxidation of their cofactors and oxidise certain amino acid residues in proteins (Kim et al., 2015). Their presence in the cell, especially in high concentrations, can cause apoptosis and are thought to contribute to many neurodegenerative diseases (Nunomura et al., 2006) (Ramalingam & Kim, 2012). Therefore, DBHB could have great potential as a neuroprotectant if it focuses on preventing the oxidation of neurons which would normally lead to excessive neuronal death. Neurodegenerative diseases present themselves due to lack of available neurons able to perform certain tasks. If there was less neuronal death, then there would be a lower likelihood of neurodegenerative diseases overwhelming the brain.

(2) C. *sporogenes* has many useful pathways that could lead to DBHB production. We created a structural model to ascertain the most suitable pathway to produce DBHB. It came down to two pathways in the native C. *sporogenes* Acetone-Butanol-Ethanol (ABE) fermentation pathway (Cooksley et al, 2012). Figure 3 shows the chosen pathway, it is a manipulation of the acetoacetate pathway. During the process of choosing a pathway we examined various complications such as increased ethanol production. These factors were considered giving us a pathway that we believe is suitable to exist in the human gut. DBHB will be produced in the gut and reaches the brain via the bloodstream. DBHB can travel through the blood brain barrier. Further research is needed to ascertain the amount of DBHB being produced and reaching the brain as well as if this creates a worthwhile protective effect. This project is in the very early stage of drug development and if it were to follow that route, further work in C. *sporogenes* and animal models would be used to look at the effectiveness of our therapeutic.

(3) As we progressed through the project, it became clear that a method of control was needed. This control would prevent our bacteria from escaping into the environment or evolving to threaten the health of the patient. Various methods have been thought through. Our vision is to use sporulation as a control mechanism. In short, we would prevent our anaerobic bacteria from sporulating after it reaches the gut. This would prevent its survival outside of this anaerobic environment. We would insert an inducible (tetracycline) promoter just prior to our target genes (Dembek et al, 2017). During production of the bacteria in the factory, anhydrotetracycline would be present so that sporulation could occur. When C. sporogenes is outside of this factory environment and not in the presence of anhydrotetracycline, it will not be able to express our target genes as the promoter cannot be induced. Our target genes are SpoIIIAA, SpoIVA and SpoIID. These were chosen as they are essential to the sporulation process and the lack of their expression prevents sporulation. They are also spread throughout the genome to prevent a horizontal gene transfer event from wiping the control mechanism out at once. This control mechanism limits the ability of our species to survive outside of our target environment preventing any unforeseen damage to the environment or organisms.

V. CONCLUSION

Our work throughout the project has furthered the belief that this solution would be effective. The project is entirely dry lab based and the lack of experiments prevents numerous avenues of exploration that we would have liked to go down. The future of this project would include proof of concept experiments for our

control mechanism and DBHB production. This would involve experiments on E. coli and then C. sporogenes. In the long term, a therapeutic like this would be a regular treatment from early adulthood through to old age giving neuroprotection throughout the lifetime and ultimately decreasing the risk of neurodegenerative diseases.

Our project focuses on a problem that does not yet have a solution. Given the heterogeneity of brain composition in the population; one solution may not be applicable to all. However, using DBHB to increase neuroprotection from early adulthood would diminish this problem as its effects are applicable to neurons in general rather than focusing on one possible cause of neurodegenerative diseases. An example of this are treatments that focus on reducing amyloid beta plaque formation in Alzheimer's patients. Current treatments for Parkinson's disease and Alzheimer's are prohibitive in that they are reactive to when symptoms start presenting themselves rather than being proactive. This means that treatments only focus on alleviating symptoms. A solution like ours would work on preventing neurodegeneration in the first place with the goal of increasing the age of onset of neurodegenerative diseases by decreasing the rate of neuronal death. This delay of onset would not only improve the lives of those that are potentially affected but also decrease the economical and societal burden that these diseases provide.

REFERENCES

Cooksley, C.M., Zhang, Y., Wang, H., Redl, S., Winzer, K., Minton, N.M. (2012) Targeted mutagenesis of the Clostridium acetobutylicum acetone–butanol–ethanol fermentation pathway. Metabolic Engineering 14(6); 630-641. https://doi.org/10.1016/j.ymben.2012.09.001

Dembek, M., Willing, S.E., Hong, H.A., Hosseini, S., Salgado, P.S., Cutting, S.M. (2017) Inducible Expression of spo0A as a Universal Tool for Studying Sporulation in Clostridium difficile. Frontiers in Microbiology 8: 1793 doi: https://dx.doi.org/10.3389%2Ffmicb.2017.01793

Gross, E., Klement, R.J., Schoenen, J., D'Agostino, D.P., Fischer, D. (2019). Potential Protective Mechanisms of Ketone Bodies in Migraine Prevention. Nutrients, 11(4): 811. doi: 10.3390/nu11040811

Hu, E., et al. (2018). Beta-hydroxybutyrate Promotes the Expression of BDNF in Hippocampal Neurons under Adequate Glucose Supply. Neuroscience, 386: 315-325. doi: 10.1016/j.neuroscience.2018.06.036

Kashiwaya, Y., Takeshima, T., Mori, N., Nakashima, K., Clarke, K., Veech, R.L. (2000). d-β-Hydroxybutyrate protects neurons in models of Alzheimer's and Parkinson's disease. PNAS 97(10); 5440-5444. Retrieved from https://www.pnas.org/content/97/10/5440

Kim, G.H., Kim, J.E., Rhie, S.J., Yoon, S. (2015) The Role of Oxidative Stress in Neurodegenerative Diseases. Experimental Neurobiology, 24(4): 325–340. doi: 10.5607/en.2015.24.4.325

Nunomura, A. et al. (2006). Involvement of oxidative stress in Alzheimer disease. Journal of Neuropathology and experimental neurology 65(7):631-41. Retrieved from https://academic.oup.com/jnen/article/65/7/631/2646707'

Oertel, W., Schulz, J.B. (2016) Current and experimental treatments of Parkinson disease: A guide for neuroscientists. https://doi.org/10.1111/jnc.13750

Ota, M. et al. (2019) Effects of a medium-chain triglyceride-based ketogenic formula on cognitive function in patients with mild-to-moderate Alzheimer's disease. Neuroscience letters, 690: 232-236. Retrieved from https://www.sciencedirect.com/science/article/pii/S0304394018307304

Ramalingam, M., Kim, S.J. (2012). Reactive oxygen/nitrogen species and their functional correlations in neurodegenerative diseases. Journal of Neural Transmission 119:891–910. Retrieved from https://link.springer.com/article/10.1007/s00702-011-0758-7#citeas

Rovner, A.J. et al. (2015) Recoded organisms engineered to depend on synthetic amino acids. Nature 518, 89–93. Retrieved from https://www.nature.com/articles/nature14095

Yang, H., Shan, W., Zhu, F., Wu, J., Wang, Q. (2019). Ketone Bodies in Neurological Diseases: Focus on Neuroprotection and Underlying Mechanisms. Frontiers in Neurology. doi: 10.3389/fneur.2019.00585

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Creation of a Novel, Noninvasive Diagnostic Method for Endometriosis using Menstrual Effluent

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***Abstract* - We sought to create a novel, noninvasive diagnostic for endometriosis using menstrual effluent. Endometriosis is a chronic disease that causes aberrant endometrial-like tissue growth outside of the uterine cavity and affects 10% of women worldwide. Currently, there are no commercially available, noninvasive diagnostic methods available for this disease. Our wet lab team collaborated with experts in the field of endometriosis to create lateral flow assays that can both qualitatively and quantitatively measure biomarkers of endometriosis in menstrual effluent. This work was supported by the modeling team, who created predictive models to judge the predictive value of our diagnostic design. Additionally, our hardware team created a specialized menstrual cup for the collection of menstrual effluen. Using synthetic biology to produce our desired antibodies, we were able to create a simple, inexpensive diagnostic for endometriosis that can be employed in a variety of clinical settings and be used to resolve the knowledge gap in female reproductive healthcare.**

Index Terms- Endometriosis, Diagnosis, Antibody Production

I. INTRODUCTION

Endometriosis is a chronic disease in which endometrial tissue grows outside of the uterine cavity (Mutter, 2014). Endometriosis affects approximately 10% of women of reproductive age across the world (Cramer & Missmer, 2009). Symptoms of endometriosis include menstrual cramps, pain with intercourse, pelvic pain, and infertility (Mutter, 2014). Despite the intense discomfort experienced by those suffering from endometriosis, the current method for diagnosis relies upon exploratory surgery, laparoscopy, which has been associated with negative outcomes (Biacchiardi et al., 2011). These factors have contributed to a diagnostic delay of greater than ten years for endometriosis patients following the onset of their symptoms (Ballard et al., 2006). As such, there is a dire need to develop novel, noninvasive diagnostic methods for endometriosis.

Menstrual effluent has been an underutilized sample for the diagnosis and characterization of reproductive disorders in women (Greaves et al., 2017). Recent research has brought to light its potential in identifying certain characteristics of endometriosis, specifically indications of altered immunity in endometrial tissue (Sabbaj et al., 2011). Of particular interest to researchers are interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor (TNF-). Their elevated levels in endometrial tissue are suspected to be a cause of infertility in endometriosis (Nematian et al., 2017). Therefore, the quantification and comparison of these biomarkers in endometriosis patients' menstrual effluent to those of healthy individuals may provide useful future directions for managing endometriosis symptoms. Furthermore, insulin growth factor-binding protein 1 (IGFBP-1) concentrations have been quantified in menstrual effluent and shows high sensitivity and specificity for the diagnosis of endometriosis, indicating its favorability as a biomarker (Nayyar et al., 2020). Lastly, CA125 has been found to be significantly elevated in the peripheral blood of patients suffering from either endometriosis or ovarian cancer (Karimi-Zarchi et al., 2016). However, the explicit connection between many of these biomarkers and the pathophysiology of endometriosis has not been explored in-depth.

We sought to create a noninvasive diagnostic for endometriosis using menstrual effluent as our sample source. We created a lateral flow assay (LFA) that could detect our desired biomarkers for endometriosis in menstrual effluent with high sensitivity and has practical applications as a point of care diagnostic. We used computational modeling to predict optimal test line placement for signal production and created models to test the sensitivity of our design. Lastly, to permit the inexpensive production and accessibility of this assay, we used synthetic biology to allow for the efficient synthesis of the therapeutic antibodies we chose to implement in this sandwich-style immunoassay in *Escherichia coli (E. coli)* SHuffle. The creation of this immunoassay using therapeutic antibodies will create an easier method of diagnosis for endometriosis and facilitate a personalized medicine approach in the future for the treatment of symptoms.

II. RESEARCH ELABORATIONS

F. *Research Design*

Sample Preparation

Specialized menstrual cups were designed for the collection of menstrual effluent using Onshape software.

3D-Modeling of Antibody-Antigen Interactions

Three-dimensional modeling of our antibody and antigen interactions was performed using Rosetta software (Appendix B).

Synthetic Production of Antibodies

All parts for antibody production were designed following all current BioBrick compatibility standards. Sequences were optimized for use in *E. coli* using Reverse Translate Software from The Sequence Manipulation Suite.

Determination of Threshold Values

Threshold values, sensitivities, and specificities for the diagnosis of endometriosis were identified through previous literature (Bedaiwy, 2002; Galo et al., 2005; Hirsch et al., 2016; Juul et al., 1997; Malutan et al., 2015; Ohata et al., 2008; Warren et al., 2018).

TABLE 1: Literature values for the diagnostic thresholds, sensitivities, and specificities of our biomarkers.

Biomarker	Diagnostic Threshold	Sensitivity	Specificity
IL-1□	2.26E-04 pM	0.751	0.851
IL-6	9.52E-05 pM	0.900	0.670
IL-8	2.98E-03pM	0.810	0.714
TNF-□	1.73E-03pM	0.633	0.744
CA125	30 units/mL	0.520	0.930
IGFBP-1	1.60 pM	0.875	0.917

Lateral Flow Assay

LFA test strips for all six of our desired biomarkers were developed using standard methods for gold nanoparticle (GNP)-enhanced LFAs as previously described (Teerinen et al., 2014). A competitive binding design was used for measuring the concentration of IGFBP-1 by detecting the amount of free, unbound IGF-1. Samples were allowed to air dry prior to quantification using visual imaging. Optimization of test line placement for the lateral flow assay was carried out using mathematical modeling equations (Appendix A).

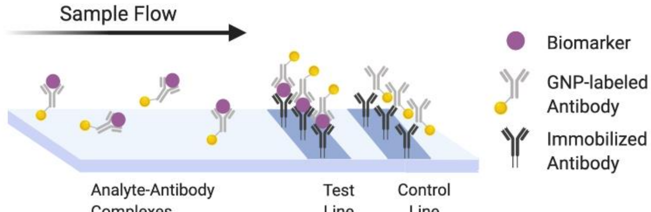

FIGURE 1: Diagram of a lateral flow assay.

Calculations of Diagnostic Probability

Using the sample size and reported sensitivities and specificites of twelve candidate biomarkers (IL-6, IL-1□, IL-8, CA-125, INF-gamma, glycodelin A, prolactin, monocyte chemotactic protein-1 and VEGF), the combination of biomarkers with highest combined log of the odd ratio (log(OR)) was determined. A clinical predictive model was created using a subset of a patient dataset including 378 control patients and 378 endometriosis patients. We then built the model using the randomforest library in R on a training set made from a random sample (70%) of the balanced dataset. The model was tested on the remaining 30% of the dataset,

Cost Analysis of Diagnostic Assay

The average cost of each reagent used in our LFA was determined using commercial websites. The per unit price was then determined and used in our final price calculation (Appendix C). The cost of required equipment was not taken into consideration for these calculations, assuming laboratories using this method would already be equipped with standard equipment.

III. RESULTS

A. 3D Modeling of Antibody-Antigen Interactions

3D modeling of our antibody-antigen interactions revealed that there was no significant overlap between the binding portions of the sensing antibody and immobilized antibody in our LFA (Appendix B). The lack of overlap indicated that the selected antibodies could be used together in an LFA.

B. Synthetic Production of Antibodies

Plasmids were designed to permit for the expression of the various portions of the immunoglobulins used in the lateral flow assay. These plasmids were created to allow for efficient expression of antibodies in *E. coli* SHuffle.

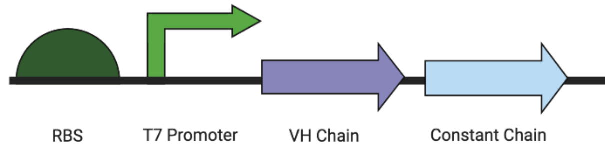

FIGURE 2: Plasmid insert for the full-length sequence of a well-characterized antibody for IL-6 (Siltuximab).

C. Lateral Flow Assay

The mathematical model indicated placement for the test line 20 mm from the sample pad based upon the reagent concentrations provided in the LFA protocol to allow for the greatest visualization of signal at the test line. The signal concentration at the test line indicated the strength of the colorimetric signal produced, and the test line was placed at the location that exhibited the highest concentration of our GNP-antibody-antigen-immobilized antibody complexes.

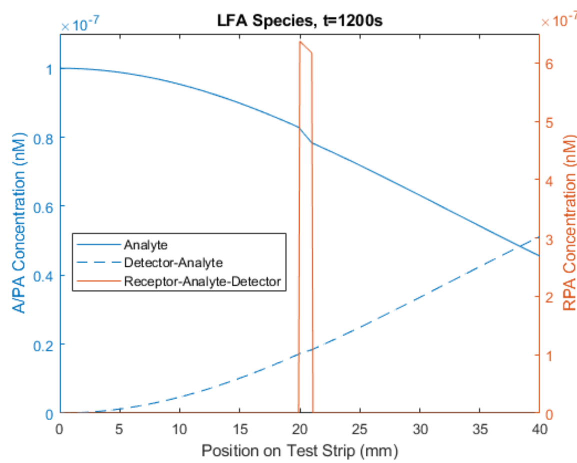

FIGURE 3: Analyte (biomarker), detector-analyte (GNP-antibody and biomarker) complex, and receptor-analyte-detector (immobilized antibody, GNP-antibody, and biomarker) complex concentrations depicted as a function of test strip position over time. This model starts at an analyte concentration of 0 ng/mL and a distance of 0 mm from the sample pad. The greatest increase in receptor-analyte-detector complex occurs at

x = 20 mm, indicating the optimal placement of the test line 20 mm past the sample pad for the greatest colorimetric signal (accumulation of receptor-analyte-detector complex at the test line).

D. Diagnostic Accuracy

The combined log of the odd ratio for our diagnostic 4.76, showing that our selected panel of biomarkers has predictive value. Our clinical predictive model yielded a sensitivity and specificity of 90% and 95%, respectively.

E. Cost Analysis of Diagnostic Assay

The cost of our diagnostic was calculated to be $299.32 per unit using commercially purchased antibodies. However, we hypothesize that the cost of our LFA will be brought down by the biosynthetic production of antibodies in *E. coli*. The price of our diagnostic design is markedly less than the cost of a definitive diagnosis via laparoscopy, which can cost $4,852 in the United States (Soliman et al., 2016).

IV. DISCUSSION

We were able to produce a sensitive lateral flow assay that could be used in combination with a clinical predictive model to provide an accurate diagnosis for endometriosis. We were also able to lower the cost of creating an antibody-based assay (immunoassay) by synthesizing our own antibodies for employment in our diagnostic, thus removing financial barriers for the implementation of immunoassays in clinics and laboratories worldwide. The design of plasmids for antibody production allows for future teams to utilize this method in other applications and helps break down financial barriers of access to reproductive healthcare testing in clinics and laboratories worldwide. Additionally, since we selected therapeutic antibodies for the biomarkers in our design, the inexpensive production of these antibodies allows for the potential of targeted immunotherapies for treatment of endometriosis symptoms. In particular, the modified constant chain region used provides future researchers and clinicians with the opportunity to prescribe these *E. coli*-synthesized antibodies for *in vitro* use due to its modified regions for improved immune system effects, pending FDA-approval of these therapies.

V. CONCLUSION

Our design allows for the creation an inexpensive point of care diagnostic for endometriosis. This improvement in diagnostic methods in part due to the use of synthetic biology to synthesize antibodies with promising application in immunoassay designs. Further interest remains in raising awareness and educating the public and physicians on topics including female reproductive healthcare. Education is an important component of detecting endometriosis early and improving female reproductive healthcare. Additionally, detection and diagnosis of this chronic disease is not synonymous with treatment. Limited treatment options are available to endometriosis patients and the few treatments that are available have been associated with undesirable side effects that can exacerbate endometriosis symptoms. The prolonged delay in diagnosis and lack of reliable therapeutic options demonstrate that there is a need for greater research funding into the pathophysiology, diagnosis, and treatment of this disease.

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REFERENCES

Ballard, K., Lowton, K., & Wright, J. (2006). What's the delay? A qualitative study of women's experiences of reaching a diagnosis of endometriosis. *Fertility and Sterility*, *86*(5), 1296–1301.

Biacchiardi, C. P., Piane, L. D., Camanni, M., Deltetto, F., Delpiano, E. M., Marchino, G. L., Gennarelli, G., & Revelli, A. (2011). Laparoscopic stripping of endometriomas negatively affects ovarian follicular reserve even if performed by experienced surgeons. *Reproductive BioMedicine Online*, *23*(6), 740–746.

Cramer, D., & Missmer, S. (2009). The Epidemiology of Endometriosis. *Annals of the New York Academy of Sciences*, *955*(1), 11–22.

Greaves, E., Critchley, H. O. D., Horne, A. W., & Saunders, P. T. K. (2017). Relevant human tissue resources and laboratory models for use in endometriosis research. *Acta Obstetricia et Gynecologica Scandinavica*, *96*(6), 644–658.

Hirsch, M., Duffy, J. M. N., Davis, C. J., Nieves Plana, M., & Khan, K. S. (2016). Diagnostic accuracy of cancer antigen 125 for endometriosis: a systematic review and meta-analysis. *BJOG: An International Journal of Obstetrics and Gynaecology*, *123*(11), 1761–1768.

Karimi-Zarchi, M., Dehshiri-Zadeh, N., Sekhavat, L., & Nosouhi, F. (2016). Correlation of CA-125 serum level and clinico-pathological characteristic of patients with endometriosis. *International Journal of Reproductive Biomedicine (Yazd, Iran)*, *14*(11), 713–718.

Mutter, G. L. (2014). Endometriosis. In *Pathology of the Female Reproductive Tract* (pp. 487–508).

Nayyar, A., Saleem, M. I., Yilmaz, M., De Franco, M., Klein, G., Elmaliki, K. M., Kowalsky, E., Chatterjee, P. K., Xue, X., Viswanathan, R., Shih, A., Gregersen, P. K., & Metz, C. N. (2020). Menstrual Effluent Provides a Novel Diagnostic Window on the Pathogenesis of Endometriosis. *Frontiers in Reproductive Health*.

Nematian, S. E., Mamillapalli, R., Kadakia, T. S., Majidi Zolbin, M., Moustafa, S., & Taylor, H. S. (2017). Systemic Inflammation Induced by microRNAs: Endometriosis-Derived Alterations in Circulating microRNA 125b-5p and Let-7b-5p Regulate Macrophage Cytokine Production. *The Journal of Clinical Endocrinology & Metabolism*, *103*(1), 64–74.

Sabbaj, S., Hel, Z., Richter, H. E., Mestecky, J., & Goepfert, P. A. (2011). Menstrual blood as a potential source of endometrial derived CD3+ T cells. *PLoS ONE*, *6*(12), 4–12.

Soliman, A. M., Yang, H., Du, E. X., Kelley, C., & Winkel, C. (2016). The direct and indirect costs associated with endometriosis: a systematic literature review. Human Reproduction, 31(4), 712–722. https://doi.org/10.1093/humrep/dev335

Teerinen, T., Lappalainen, T., & Erho, T. (2014). A paper-based lateral flow assay for morphine. *Analytical and Bioanalytical Chemistry*, *406*(24), 5955–5965.

Warren, L. A., Shih, A., Renteira, S. M., Seckin, T., Blau, B., Simpfendorfer, K., Lee, A., Metz, C. N., & Gregersen, P. K. (2018). Analysis of menstrual effluent: Diagnostic potential for endometriosis. *Molecular Medicine*, *24*(1), 1–12.

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Bullshit Detection in Times of Corona

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Abstract- This research focused on evaluating the factors which influence the perception and spread of disinformation regarding COVID-19. It is hypothesized that psychology plays an important role when it comes to one's susceptibility to false information. It is also hypothesized that 'new media' is one of the largest platforms used to communicate information, and thus should contribute towards the spread of fake news. Thus, the means of mitigating the spread are noted to see plausible ways of reducing the spread of false information. Results were concluded from both primary data, collected from a questionnaire, as well as from a thorough literature review. This study shows that during times of crisis, the public seeks to find 'truth,' due to several psychological factors. With the aid of social media, it is possible to share disinformation with others rapidly, causing a rise in conspiracy theory spread, and an increase how credible the information appears to be. Future studies should include further statistical analysis and deeper investigation regarding the effects of demographics and human behavior, due to their importance within this field.

Index Terms- false information, disinformation, COVID-19, conspiracy theory

I. INTRODUCTION

The flow of incorrect information has drastically increased with the increased use of new media, leading to drastic consequences. The main issue with false information being spread is that it is rarely acknowledged as incorrect; nor is it removed from being viewed, making it more prone to susceptibility (Newman & Fletcher, 2017). A study conducted by Chao et al (2020) noted how several factors including age, socioeconomic background, and type of platform used can heavily influence people's emotional response, leading to the further spread of misinformation. When the COVID-19 outbreak in Wuhan occurred in December 2019, a copious number of infected and deaths followed on with a lack of understanding of the necessary protective measures to evade contraction. Wu, Leung & Leung (2020) stated that as of January 29th, there have been 5993 cases confirmed in mainland China and 132 deaths and grew exponentially. So, because of this, the information shared and received must be of the best quality and factually credible. However, if altered, misinformation and disinformation regarding the virus can pose a threat since they can negatively affect the health and safety of the public. Therefore, the article serves as a brief overview of the full research report which aims to answer the question: *To what extent has bullshit regarding COVID-19 influenced the public and how has it been communicated?* Knowing this, with regard to the literature review, it is hypothesized that *there are different psychological factors influencing one's susceptibility to conspiracy theories and that during a global crisis human behavior is influenced by several factors*. With regard to the survey, it has been hypothesized that *new media is the most used platform for spreading false information about COVID-19 rather than traditional media which aids the spread of conspiracy theories.*

II. RESEARCH ELABORATIONS

Research design:

The data collected in this research was obtained from both primary and secondary sources. A literature review as well as a questionnaire, which gathered empirical data, were employed to make conclusions.

Literature Review:

The credible database Google Scholar & JSTOR was used as the search engine. An article was deemed relevant if it contained keywords from this paper's research question (false information, disinformation, COVID-19, conspiracy theory). The credibility of the paper was ascertained by confirming the authors and the institution's credentials. In total, three trustworthy peer-reviewed papers were investigated

Questionnaire:

A survey, consisting of 19 questions, evaluating 'background information', 'general news', and 'news regarding COVID-19' was completed by a total of 209 people from various educational backgrounds, globally. From those, 57,9% were female, 42,1% were male and 0,9% preferred not to disclose; the majority (68,3%) being between the ages of 18 and 25. The survey was conducted in an attempt to gain more insight on how or where people obtain their information, as well as if they believed in conspiracy theories.

Statistical Analysis

The analysis of the survey involved forming bar charts quantize the answers for each category and compare the data. This created a visual display of the results for further deciphering. At the same time, three t-tests were computed using the SPSS Version 25 software, at a 95% confidence interval. These tests focused on assessing the relationship between specific data from the questionnaire.

III. RESULTS AND FINDINGS

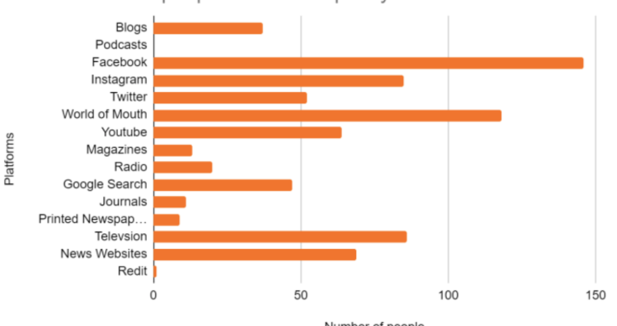

FIGURE 1: Where conspiracy theories regarding COVID-19 were detected.

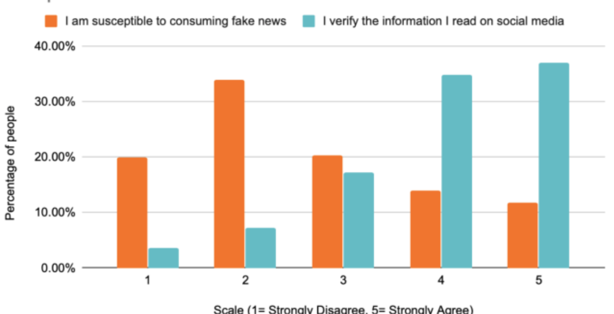

FIGURE 2: Subjective evaluation of one's susceptibility to fake news in contrast to information verification.

IV. DISCUSSION

The aim of this study was to determine the reasoning behind an individual's belief in conspiracy theories, their origin, and their general effects on society. The results procured have shown that false information with respect to COVID-19 has a biological and psychological foundation and has implications for the general population. The research has indicated that 'new media,' mass communicating using digital technology, is a common platform for spreading conspiracy theories.

On one hand, results obtained from the questionnaire/survey showed that false information and conspiracy theories were primarily found on social media sites and via word of mouth (see *Figure 1*). Moreover, the relationship between the susceptibility of fake news and information verification is investigated (Figure 2). Based on the results collected (see Figures 1 & 2) as well as the findings of paired t-tests conducted, between the variables, one is more likely to be susceptible to fake news when one does not verify the information they see on social media. Another conclusion drawn from the test is that there is a correlation between one's verification practices and one's ability to detect false information. Those who feel they are able to identify false information also claim to actively verify the information. However, these findings are subject to the Dunning-Kruger effect, meaning individuals who took part in the questionnaire may have overestimated their ability at verifying information and detecting false information. Overall, this study showed that the more active and conscientious someone is towards validating their information, the better they should be at detecting false information.

Complimentary to this, a study by Chao et al (2020) investigated the influence of media on people who were indirectly exposed to COVID-19, during its initial outbreak phase within China (2020). The study looked at the hierarchical regression analyses of media use types, media content, and media engagement in relation to psychological outcomes. With media use type, the study concluded that new media associates with negative effects; anxiety, stress, and depression. However, no significant association was found with traditional media (Chao et al, 2020). With media content, the study shows that individuals who encounter heroic responses and speeches from experts lead to an increase in more positive feelings. Finally, with media engagement, the study indicated that the level of engagement has a significant association with negative psychological effects. New media users who posted more information regarding the COVID-19 outbreak were more active in the search for updates surrounding the virus (Chao et al, 2020). Overall, users of new media experienced negative feelings when faced with uncertainty about the crisis and when informed by acquaintances on the crisis.

V. CONCLUSION

In conclusion, susceptibility towards disinformation during the COVID-19 pandemic has been high due to several psychological and behavioral factors (Chao et al, 2020). Establishing a level of susceptibility for the general public is difficult as it should acknowledge the factors associated with the individual's psychology and general background. Moreover, it is evident through empirical research and the conducted literature review, that disinformation has been found to be most prominent within new media. This study is of high relevance, since its paramount for people to detect false information as it can negatively impact behavior regarding health and safety (Erceg, 2020).

APPENDIX

https://docs.google.com/spreadsheets/d/14kNlwZT4PXrf4bFGwXN97yGHkqhvsHXJVRNbrMmfHDY/edit?usp=sharing

- Link to Questionnaire Results

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Both Figure 1 & 2 was made using data collected from primary research surveying students and/or other people within and outside of Maastricht.

REFERENCES

Chao, M., Xue, D., Liu, T., Yang, H., & Hall, B. J. (2020). Media use and acute psychological outcomes during COVID-19 outbreak in China. *Journal of Anxiety Disorders*.

Erceg, N., Galic, Z., Ruzojcic, M. (2020). Misbehaving in the corona crisis: the role of anxiety and unfounded beliefs. Department of psychology, Faculty of humanity, and social sciences. 10.31234/osf.io/cgjw8.

Newman, N., & Fletcher, R. (2017). *Bias, Bullshit and Lies: Audience Perspectives on Low Trust in the Media*. Retrieved from Reuters Institute for the study of Journalism website: https://www.reutersagency.com/wp-content/uploads/2019/03/nic-newman-and-richard-fletcher-bias-bullshit-and-lies-report.pdf

Wu, J. T., Leung, K., & Leung, G. M. (2020). Nowcasting and forecasting the potential domestic and international spread of the 2019-ncov outbreak originating in wuhan, china: a modelling study. *Lancet (London, England)*, *395*(10225), 689–697. https://doi.org/10.1016/S0140-6736(20)30260-9

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Interview: Prof. Dr. Kremsner Evaluates the Current Situation of the Corona Crisis

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Abstract- **Over the last months, 24.000.000 people worldwide have been infected with the virus SARS-CoV-2, which is the causative agent of the disease COVID-19. Of those people, 800.000 have lost their lives. As a reaction to this, governments and public as well as private institutions from all over the world established numerous vaccine programs. In Tübingen, the infection biologist and Director of the Institute for Tropical Medicine Tübingen Prof. Dr. Peter Kremsner is the head of a phase 1 clinical trial, testing the mRNA-based vaccine developed by the company CureVac. In this interview, Prof. Dr. Kremsner evaluates the current situation of finding a cure, talks about the different approaches of designing a potential drug and gives personal insight into his academic career.**

Index Terms- COVID-19, vaccine, clinical trial, interview

You are currently involved in three major vaccine studies. One that is an mRNA-based vaccine, another one that is based on the use of viral vectors, and a third one that uses virus-like particles. Could you explain the basic differences between the approaches to us a little more?

The first CVnCoV vaccine study has been in the 1st clinical phase since June 18. It is a so-called mRNA-based vaccine. The sponsor is the Tübingen company CureVac. In contrast to conventional vaccines, in which the patient or the test subject is given the antigen for immunization, this vaccine injects mRNA (the building instructions for a virus protein), in this case for the virus's spike protein. The mRNA then penetrates the cells and is translated by the cell's own translation machinery into a spike protein that the human body recognizes as foreign and then triggers an immune response. This reaction begins as soon as fragments of the S antigen are presented on the MHC complexes of the affected cells. The difficulty with mRNA-based vaccines is that the mRNA is a chemically unstable molecule that can - in vivo - be broken down by RNases. However, CureVac's research has managed to address these issues. At the end of the study, the subjects should show an increased level of nCov spike protein antibodies and nCov neutralizing antibodies.

In autumn, we want to start another vaccine study with Danish colleagues. This is called COUGH-1 and has the EU as a financial sponsor. In this study, our work will be based on a previously researched malaria vaccine. At that time, we incorporated the malaria antigen, which is used for immunization, into virus-like particles. Now for nCov this will be done analogously, using the antigen of the coronavirus. The study will begin with a small phase 1 that will be continued if promising results are shown. In accordance with the mRNA study, the blood concentrations of the nCov-specific antibodies will be examined fourteen days after the first and second vaccination. The third approach is based on Modified-Vaccinia-Ankara (MVA) virus vectors in which the S antigen is incorporated.

Which of the approaches do you think is more promising?

All of these approaches generally have a very high potential. Overall, we receive a large number of international requests for cooperation. Large, medium-sized, and small companies worldwide are currently trying to develop a vaccine. Globally, there are around two hundred vaccine programs right now, most of which are in preclinical studies until further notice. This means that these are still tested in a test tube or animals. There are about 30-40 serious programs at the moment, and 10-20 vaccine candidates that are likely to enter the final testing phase. Of course, we try to work on the most promising approaches, granting us conceptual freedom in our research and testing. For example, in the mRNA study we collaborated with CureVac in developing the clinical protocols.

Have you noticed a difference in working with other scientists since the pandemic has started? Did it bring all of you closer together or are you rather fighting in the search for an active compound?

Especially in times like these, we as scientists have to move closer together. At the same time, we also have to work more ambitiously. We will not be able to succeed if we all work individually. It usually takes 10 or even 20 years before a possible vaccine is designed and approved for selling. Decreasing this time to one to two years is only possible in a very close and well-coordinated cooperation. This is because despite the high-pressure work, no reduction in the very high standards of development, security testing, and especially in the work of the ethics committee can be made. At the moment, many groups are working together in the university area. We are in close contact with colleagues from the University Hospitals in Ghent, Hanover, and Munich when testing the CureVac vaccine. CureVac coordinates who takes which steps and when. But we are also currently in active contact with colleagues from the Netherlands, Denmark, Finland, and France.

Especially with regard to the CureVac vaccine, the work of the groups involved interferes very closely. Therefore, considering the central coordination by CureVac, it is more a friendly cooperation than a reckless conflict. However, you are of course also happy if, as in our case, you can work on the most promising projects. I am therefore very optimistic that we will find a vaccine soon. However, I also believe that the CureVac vaccine will not be the only one that will soon receive preliminary approval because, as I said, there are numerous companies and working groups that are currently in the running. But that's good, because it means that there will be a wide range of corona vaccines in the future, which can offer more effective protection against COVID-19 in all parts of the world than protective masks and hand hygiene currently do.

When do you think the first COVID-19 vaccine will be available?

The studies are currently running smoothly. Right now, we are in phase 1 of the drug trial which means that we are administering the drug in the lowest dose to 168 healthy human subjects for the first time. The aim is to find out whether the vaccine is safe and does not do any harm. What is pleasing about this study is that we are in the luxurious position of being able to choose our test subjects due to the massive excess of applications, which is not common in many studies. It is not yet possible to make any statements about the concrete results, but the study is running very rapidly, which is promising for a phase 1 study. In fact, we aim to get the first results in the next two months, so that we can move on to phase 2 and, if possible, to phase 3 before the beginning of the next year. In the phase 2 tests, healthy 18 to 60 year olds will be vaccinated, as in the first phase, but risk groups will also be included. In the risk group, mainly those over 65 year of age and those with previous illnesses will be included.

It is then to be examined whether the vaccine still has the desired effect and to determine the amount of the vaccine that needs to be administered for one person to build up an immunity. As of right now, the phase 3 study, aka the approval study, will very likely become a classic placebo-controlled, blinded, randomized, multicentre study, in which, in addition to the safety and tolerability of the vaccine, its effectiveness will also be tested for countries that were heavily affected by the virus. The extension to other countries is important because we can then test the vaccine in many different patient groups and later possibly even offer it worldwide.

I believe that the CureVac vaccine will be approved by the end of next winter, mainly because of the joint efforts, and can then be administered to a broader population. In fact, many companies are already adjusting and increasing their current production of drugs and other vaccines to be able to meet the demand in the following years.

Recently there were news reports that cured COVID-19 patients show a measurable decrease in neutralizing antibodies in the blood. This is seen as an indication that the permanently acquired immunity to SARS-CoV-2 may decrease. Do you see the vaccine effort at risk?

Of course, this is a phenomenon that still needs to be investigated, but the immune system and in particular the acquired immunity cannot simply be characterized by measuring the antibody concentration. The immune system is a very complex system in which cellular components such as lymphocytes play an important role. To evaluate the chances for the effectiveness of an active ingredient, one must examine the dynamics of the entire immune system. There are other viral diseases in which immunity can be acquired that is not necessarily detectable by an increased antibody concentration. Therefore, despite these reports, I do not consider efforts to get a vaccine at risk.

Teams of young students compete against each other at the iGEM competition. In contrast to us, you can look back on a very successful and long scientific career. If you had yourself in front of you as a student, what advice would you want to give him for his career as a scientist?

The most important qualities in my experience are hard work, perseverance, and self-organization. I can remember how I always got up at six a.m. in my first semester to study as much as I could before the lectures started. This made it possible for me to spend my free time however I liked it. For example, I enjoyed going to the opera even when I was still a student. You will not be successful without discipline. But it is also important to specialize in what interests you most as early as possible in your studies. During my studies, I specialized in the field of infectiology as early as I could and worked on corresponding projects. This enabled me to gain experience as a young student and gain insight into current research, which helped me a lot for my future career.

ACKNOWLEDGEMENTS

Thank you, Prof. Dr. Kremsner, for taking the time to answer our questions about the SARS-CoV-2 vaccine development and your insights into the scientific community in these testing times.

D.K. interviewed P.K. K.S. translated the interview into English and was involved in the review process. L.W., L.V., A.A.R. reviewed the article.

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Interview: PHOCUS: Target Locusts from Within

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[*] Interviewer, team MSP-Maastricht
[**] Team TUDelft

Abstract- **This year, an outbreak of massive locust swarms has been categorized as the worst in recent decades. Locust swarms threaten croplands and food security in East Africa, Asia, and the Middle East. Records of locust plagues afflicting cities date as far back as ancient Egypt. However, this isn't just an issue of the past. This years' TU Delft iGEM team, Phocus, have chosen to concentrate their efforts on finding a solution to the locust crisis. We have invited them to answer some questions and bring awareness of this problem.**

We have invited them to answer some questions and bring awareness of this problem

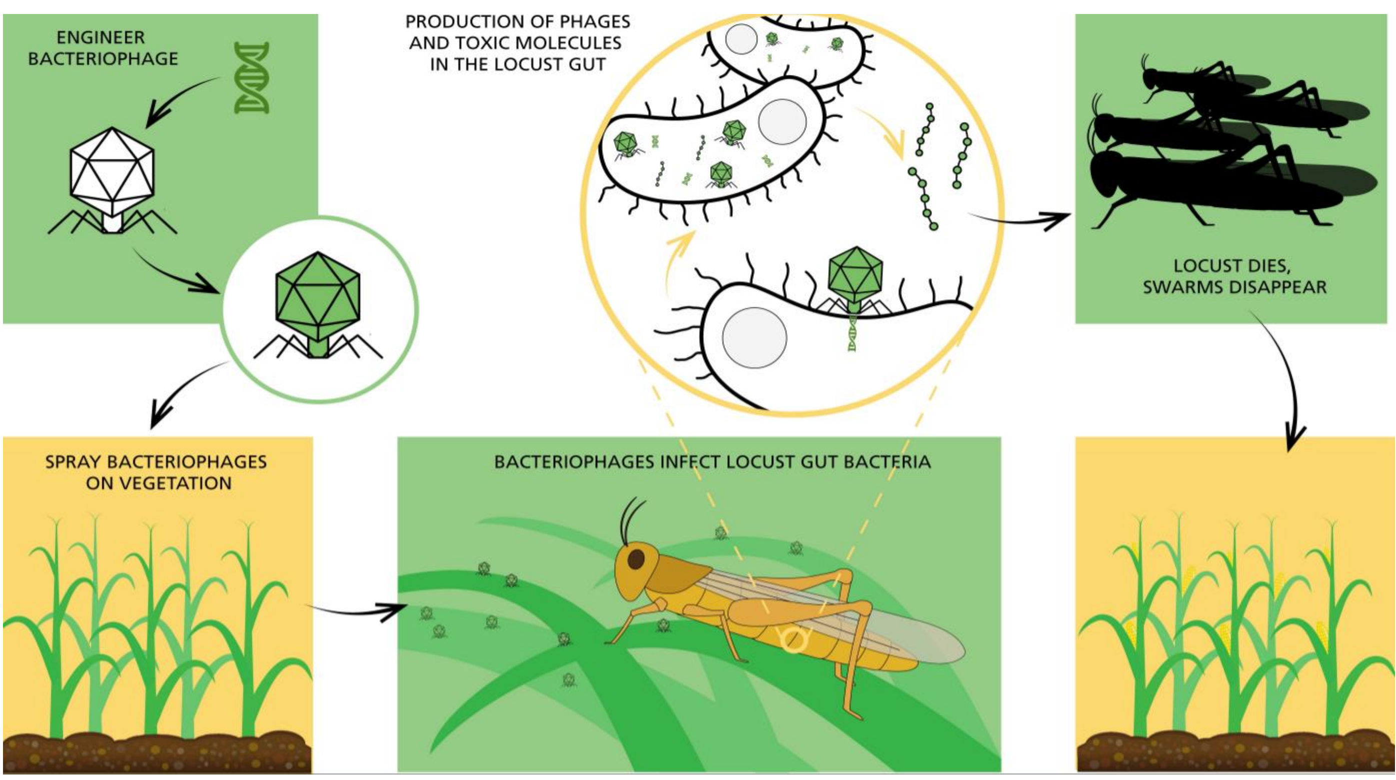
Hi guys! Can you introduce yourself? Who are you (people that answer the questions) and what is your role in the team?
Thanks for having us, we are excited to have the opportunity to partake in your journal! My name is Gabriela van Leersum and I am one of the eleven enthusiastic students making up Phocus. Within the team we are all involved in developing the scientific aspects of the project, personally I fill the role of Outreach and PR manager.

Now let's get to the real questions:
How did you reach this topic and decided it would be the focus of your project?
At the beginning of the competition we had many different ideas about potential projects. We realised that it would be important to take enough time to properly consider them all, before making a decision. After many interesting discussions, we found a topic that we were all motivated to work on; the locust crisis. The idea first came to us after reading a small news article on the damage locust swarms were causing in the Arabian Peninsula. From our discussions we learned that we wanted to make an impact and decided to contribute to finding an answer to the locust crisis.

What can you tell us about the locust itself? and why is it so devastating for agriculture?
When you think about an individual locust, they may seem quite harmless, which is true. As is often said, there is strength in numbers. In the right conditions, as occurred in the past year, locusts gather in specific locations and form swarms. These swarms can grow to the size of Paris and eat the equivalent of half of France. In addition to swarming, they undergo a physiological change which allows them to develop wings, allowing them to travel large distances and grow in size. This year the United Nations' Food and Agriculture Organisation have estimated that the food security of 10% of the world's population is threatened by locusts.

What are some causes of these infestations?
This is a good question, however also difficult to answer. After speaking to multiple experts, including members of the FAO, it was clear that there is no consensus on one explanation. The process of swarm formation is known to be linked to weather patterns – namely due to drought after extended periods of rainfall. Yet the reason why the current upsurge is so large is still unclear. Some scientists have associated the changing weather cycles with the climate crisis, although there is no hard evidence to verify this.

What is the approach your team has worked on to provide a solution to the problem and how does it apply the concepts of genetics and synthetic biology?
Our aim is to design a specific, fast working and safe bio-pesticide. Specifically, we are using bacteriophage as a delivery method and engineering them to encode for toxic molecules. The phage is applied on vegetation in the affected areas and is ingested by the locusts. Once in the locusts' guts, the bacteriophages encounter bacteria into which they inject their DNA. This allows them to "hijack" their machinery and produce the encoded toxin. The bacterium eventually bursts, releasing the toxin into the gut and kills the locust from within.



Our mission is to tackle the locust crisis by developing a sustainable bio-pesticide through responsible innovation and collaboration

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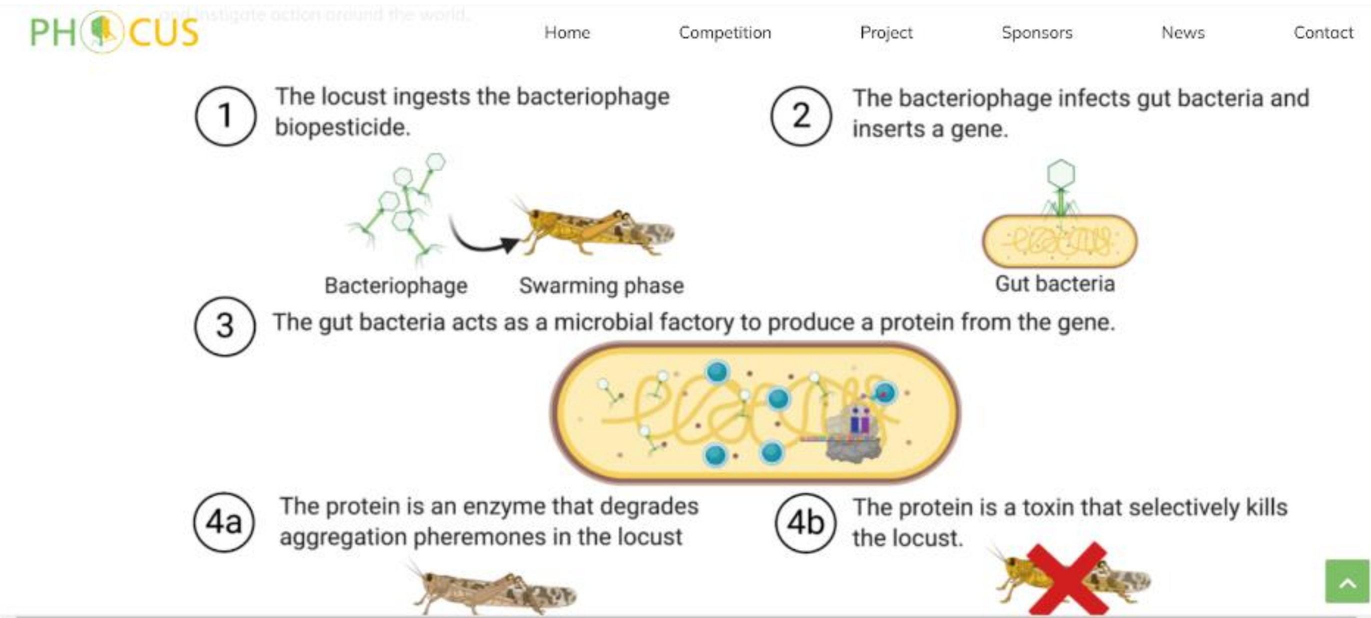

What challenges have you encountered while working on this approach?
Similar to many of the other iGEM teams in the rest of the world, the restrictions associated to covid-19 have had a big impact on how we work. Especially, working with the restricted lab access has proven to require extensive planning and preparation. Nonetheless, we are confident that we will be able to present interesting results at the Jamboree (the final event of the competition).

This project has a direct effect on the global community, what human practices has your team worked on to engage with the public?
Our human practices involve engaging with a diverse range of stakeholders and we are proud to say that it has grown to be a substantial part of our project. We have talked to multiple experts and gained valuable insights into the reality of the current crisis. One of these discussions lead to our participation in the Mondial desert locust management Conference, hosted by TheWaterChannel. With a problem as widespread as the locust crisis, we have also been working hard to engage with the general public. Ranging from young to old, we have created material for *all*. An example would be the children's book about phages or the multiple lectures that we have presented at elderly homes.

Regarding iGEM itself, what is your team looking forward to the most?
Until now, we have already made many memories together that I know we will all look back on with pleasure. I think something that we are still looking forward to in the future would have to be the Jamboree. It's really exciting to think about the fact that we will come together with teams from all over the world to present our projects and learn from each other.

What has your team learned from participating at this competition and working on a research project like this?
iGEM is not a project that compares to a lab practical or a workshop at the university. The competition itself is incredibly broad and consists of merging skills from multiple disciplines into one project. This can be hard work however at the same time it is very fulfilling.

What other problems do you think could be tackled with synthetic biology and genetic engineering?
I speak for the whole team when I say that we believe in the potential for synthetic biology to come with solutions for many of the world's problems. To get a better idea of how broad this really is, I'd like to invite you to watch our synthetic biology video on YouTube!

https://www.youtube.com/watch?v=Yuj46jG4_Q4&t=4s

Last but not least. Is there anything that you as a team would like to share with the audience?
Partially due to the covid-19 epidemic, the locust crisis is not getting quite as much attention as it otherwise might. If you can, we would like to invite you to take 5 minutes to have a quick browse through google and see what you can learn about the areas that are currently dealing with locust swarms. Mention it to your friends and family, and together we can make sure that the people affected are heard!

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A Ground-breaking Sense of Community

Team Msp-Maastricht, Team Aachen, Team Uulm, Team Nantes, Team Stockholm

Abstract- **Feedback is taking time to analyse one's performance and then thinking of the best possible solution to perform better the next time. It provides positive critic and allows to see what everyone can change to improve their focus and results. We , Team Maastricht also want to improve ourselves, our organisation and our Journal for the next year. Therefore, we decided to ask a few teams for feedback on how the experienced the Journal initiative, if it was interesting and educational for them. We are thankful for all the effort every single team put into this Initiative. We grew together as a community and learned from each other more than we could have ever learned by ourselves. All teams were very invested in their collaboration, expressed their thoughts and provided feedback but some wrote a longer feedback for us that showcases just how much this Journal initiative inspired every participating team. We greatly appreciate the hard work and time the teams invested in this Initiative and into writing this Feedback. Thank you!**

TEAM UULM

Creating a platform where university students from all around the world can come together, work together and discuss science together is an essential part of the iGEM community. As first-time participants in the iGEM competition, experiencing this sense of community was ground-breaking to us.
The Journal Initiative from the iGEM MSP team aims to combine the efforts of all the teams by giving them the opportunity to publish their work. In this way, they reflect the diversity and flexibility of this year's projects and engage us in an interdisciplinary communication.

To make the writing process as easy and clearly as possible, a well-designed template was provided. After submitting the first draft of an article, the peer review phase began. Two articles were sent to the participating teams for revision. In addition to the article, a detailed guide and form was provided, the instructions were clear and well worked out. Even with little experience, it was no problem to implement the requirements. Even though we had internal difficulties in our team and were not able to submit a final paper, the iGEM MSP team reacted understandingly and continued to support us in this collaboration. Despite our difficulties, the MSP team gave us the opportunity to participate in the final voting phase and cast our votes for the articles that were to be published in the end.
Overall, sufficient time was planned for the individual sub-steps during the collaboration. The communication between the iGEM MSP team and the participating teams was direct, fast and clear. They were open to every request from our side and always tried to support the participating teams in the process.

The MSP team gave us more than enough time to plan and write a paper, the registration deadline was a few months before the actual submission deadline. Additionally, the deadline for the submission of the first draft was late enough in the iGEM year so that first lab results could be implemented in the article. Reviewing two papers didn't only give us the opportunity to get to know other teams and their projects better, we also got the chance to look "behind the scenes" of the judging process. Where lies the focus when critically looking at a scientific article? Apart from the content, how important are form and layout of an article? We also received very useful feedback on our own article, which we implemented in our work regarding the wiki pages. Also, the provided guideline made the reviewing time efficient. The articles could be thoroughly reviewed, and the form easily directed the focus on the different aspects of judging a scientific paper. The final voting process was fair, transparent, and uncomplicated. We appreciated that the iGEM MSP team shuffled the articles for every team on the website, so that there was no unfair advantage for the articles on top of the page. In addition, voting for one's own article was not allowed.
All in all, our participation in the collaboration was a very instructive experience. In addition to new skills in scientific writing, we were able to experience first-hand the teamwork and support that make up the iGEM community.

We are grateful for the opportunities the collaboration has given us, even if we did not cross the finish line in the end. We also admire the hard work and time effort the iGEM MSP team has invested in this project and are looking forward to collaborating again in future competitions.

TEAM NANTES

The Journal initiative done by MSP was a really interesting collaboration! The Framework we had that helped us write our article was really well done and made it easier for us. It was fun to create an article based on our project.

The review phase was fun to do - reading other team's articles was inspiring and the framework we had was extremely useful! We think it was an interesting experience since it was the first time we actually wrote a whole article and the first time we did a peer review on one!
We want to thank MSP Maastricht for the opportunity and for their commitment!

The team would like to personally thank Larissa for her friendliness, for her willingness and for always answering to all of the questions we had via WhatsApp!

TEAM AACHEN

First: thank you very much for this great opportunity! It was a very interesting experience to write a paper about our project "M.A.R.S.". The entire initiative seemed well planned and the corresponding instructions were very extensive and helpful, but still left enough space for independent development. Due to the intensive work within a processing time, which was of course rather short for typical paper, we got an initial idea of what it means to want to publish something – including the eager wait to see whether others are also so exciting about the topic. The double review also gave us a good insight into the concept of peer review. All in all, we are very happy that we could participate in this great collaboration!

TEAM STOCKHOLM

The journal initiative was a great driving force to begin describing and highlighting the important aspects of our project. By having the peer review of other teams, we got diverse feedback on our project as well as a perspective on what other iGEM teams had been working on. The entire process was well structured and even though some issues arose the Maastricht team empathetically supported us through the process in writing our journal submission. The printed journal will be treasured reminder of the blood, sweat, tears and joy spent on iGEM as a whole.

"Alone we can do so little, together we can do so much." – Helen Keller

The first, unofficial iGEM Proceedings Journal exists because of the teamwork and effort provided by each and every one of the participating teams. This would not have been possible without the prolific engagement and enthusiasm each member brought to this initiative; excited to experience what is like to work on a research paper, the authors put forth their best effort, their most valuable content, and all those months of hard work, into a concrete article, to share with the world what they have contributed to the scientific community. The teams have also dedicated the time to help and support other participants by peer-reviewing each other's work, ensuring the quality of the content as well as providing advice to improve their final paper, a key process in the publishing experience that was only feasible because of their commitment to our initiative. We want to thank every team for helping us make our proposal a reality, and allowing us to reach the final product of a collaboration we hope establishes as a tradition in the iGEM competition every year, we truly appreciate your effort.

Furthermore, our iGEM journey would not have been possible without the support of our sponsors, who provided the means necessary for our team to work on the project throughout all these months. Because of them, our team got the opportunity of participating in this year's competition and materialize all the ideas we came up with. Thank you to Maastricht University, MaCSBio, Restore and Revive, and DSM, for believing in our mission and carrying us to the final stages.

If any specific article in this Journal has sparked your interest, you can check out the teams Page under the provided link. All their experimental data, detailed project elaborations, and more information on the team can be found on the following wiki pages.

List of participating teams and links to their wiki pages on the iGEM server:

IISER-Tirupati_India	https://2020.igem.org/Team:IISER-Tirupati_India
UCopenhagen	https://2020.igem.org/Team:UCopenhagen
Aachen	https://2020.igem.org/Team:Aachen
IISER-Pune-India	https://2020.igem.org/Team:IISER-Pune-India
Calgary	https://2020.igem.org/Team:Calgary
Stony_Brook	https://2020.igem.org/Team:Stony_Brook
Rochester	https://2020.igem.org/Team:Rochester
KU_ISTANBUL	https://2020.igem.org/Team:KU_ISTANBUL
UNILausanne	https://2020.igem.org/Team:UNILausanne
UPF Barcelona	https://2020.igem.org/Team:UPF_Barcelona
Duesseldorf	https://2020.igem.org/Team:Duesseldorf
TU_Kaiserslautern	https://2020.igem.org/Team:TU_Kaiserslautern
Groningen	https://2020.igem.org/Team:Groningen
Sorbonne_U_Paris	https://2020.igem.org/Team:Sorbonne_U_Paris
Stockholm	https://2020.igem.org/Team:Stockholm
Estonia TUIT	https://2020.igem.org/Team:Estonia_TUIT
MSP-Maastricht	https://2020.igem.org/Team:MSP-Maastricht
Tuebingen	https://2020.igem.org/Team:Tuebingen
Thessaly	https://2020.igem.org/Team:Thessaly
MIT_MAHE	https://2020.igem.org/Team:MIT_MAHE
MIT	https://2020.igem.org/Team:MIT
Nottingham	https://2020.igem.org/Team:Nottingham
GA_State_SW_Jiaotong	https://2020.igem.org/Team:GA_State_SW_Jiaotong
IISER_Bhopal	https://2020.igem.org/Team:IISER_Bhopal
UPCH_Peru	https://2020.igem.org/Team:UPCH_Peru
Nantes	https://2020.igem.org/Team:Nantes
Baltimore_BioCrew	https://2020.igem.org/Team:Baltimore_BioCrew
UULM	https://2020.igem.org/Team:UUlm
Aalto-Helsinki	https://2020.igem.org/Team:Aalto-Helsinki
UCL	https://2020.igem.org/Team:UCL
TUDelft	https://2020.igem.org/Team:TUDelft

Furthermore, for teams outside the 2020 iGEM competition, their contact information is provided below:

Bilkent UNAMBG	unambgigem@gmail.com
Ciencias_UNAM	ciencias.igem2020@gmail.com
Ecuador	igemecuador@gmail.com
Maastricht Independent	stevenvantrooijen@gmail.com

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For more information: www.maastrichtuniversity.nl

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