METHAGONE

MSP-VECTOR

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POWER WEED

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Editors in Chief

Andres Lassus, a third year student at Maastricht Science Programme, Maastricht University.
Contact:
a.lassusmartinez@student.maastrichtuniversity.nl

Lotte Deneer, a third year student at Maastricht Science Programme, Maastricht University.
Contact:
ljm.deneer@student.maastrichtuniversity.nl

Saskia Rapp, a second year student at Maastricht Science Programme, Maastricht University.
Contact:
s.rapp@student.maastrichtuniversity.nl

Dear fellow-iGEMers and scientifically interested people,

MSP-MAASTRICHT, is an undergraduate team from Maastricht University, taking part in the 2021 iGEM competition. We strive to make a difference in society by solving a universal problem with the aid of synthetic biology. During this year's iGEM Competition we are publishing our very own MSP Vector Journal consisting of research articles and literature reviews from worldwide participating iGEM Teams.

As it was noticed that not every iGEM team has the privilege to receive academic writing training. Academic writing is a major part of science and academia. Thus, a very big focus this year's iGEM Team was to invest in education on scientific writing by creating Workshops on Scientific Writing that help to overcome possible difficulties. In total, the 5 Workshops cover the article structure, language use, citations and peer review. These workshops can be found on our website https://msp-igem-journal.steenredeker.net/.

This year, team MSP-Maastricht's project is about reducing methane emissions from cows. Climate change is caused by the greenhouse effect, where a specific group of gases trap the sun's radiation and heat within our atmosphere. Although is the most recognized greenhouse gas, arguably it is not the biggest contributor to global warming. Methane has shown to be 25 times more potent at trapping the sun's radiation compared to CO. Livestock alone, mainly cattle, is responsible for a quarter of annual methane emissions. Since our planet's future is dear to us, we chose to invest ourselves in finding an efficient way to reduce said emissions. Besides relieving pressure off our environment, reducing methane emissions can have another potential benefit. The production of methane within the cow's rumen leads to a loss of feed energy for the animal. By inhibiting methanogenesis the cows utilize this energy instead, which will save the farmers costs on feed.

MSP-Maastricht chose to say "moo" to methane by creating a microorganism that can produce bromoform for us. These microorganisms we design, will enable the addition of bromoform to the cow's food on a worldwide scale. Thereby actively contributing to a greener world.

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Antimicrobial peptides for bioengineering: applications, challenges and future perspectives

Inga Leske*, Erik Zimmer*, Adrián Vojtaššák*

* iGEM Team Tuebingen 2021, University of Tuebingen

***Abstract* - Antimicrobial resistance is an evolving problem worldwide, that needs to be addressed by researchers from various fields and backgrounds. One of the proposed solutions are antimicrobial peptides, which are part of the innate immune system of different species and might be utilized in the future as an alternative to common antibiotics. Great progress has been made in terms of recombinant production and stabilization of these peptides. Due to these promising prospects, it comes as no surprise that several iGEM teams focused on antimicrobial peptides in their projects in the past. Here we present some of the research iGEM teams have conducted in this area, summarize common issues they have faced, and provide an outlook on how these could be solved in the future.**

Index Terms - antimicrobial resistance, antimicrobial peptides, host-defense peptides, iGEM

I. INTRODUCTION

In 2019, the World Health Organization (WHO) declared antimicrobial resistance (AMR) one of the ten most severe global health threats to humanity (World Health Organization, 2021). AMR results in pathogens like bacteria resistant to common therapeutics and is caused by over- and misuse of those therapeutics. Given the significance of AMR, it is reasonable that many iGEM teams have focussed on projects concerning AMR over the past years.

A therapeutic alternative to conventional antibiotics are antimicrobial peptides (AMPs). These are short, amphipathic, often cationic peptides, which occur in most eukaryotes and in many prokaryotes. The mode of action of most AMPs involves disrupting the pathogen's cell membrane leading to cell lysis (Hancock & Sahl, 2006). Compared to conventional antibiotics, most AMPs have additional immunomodulatory properties indirectly promoting their antibacterial activity (Bowdish et al., 2005). Therefore, AMPs are less likely to lose their in vivo antibacterial activity (Abdi et al., 2019). These properties as well as their composition of amino acids (aa) make AMPs suitable candidates for bioengineering and synthetic biology projects. This review attempts to summarize past iGEM projects on AMPs originating from animals, plants and bacteria. The analyzed projects show some common features regarding their overall design and the problems they tried to solve. Finally, frequent challenges faced by the described iGEM teams and approaches to solve the problem of AMP's limited in vivo stability are addressed.

II. RESULTS AND FINDINGS

A. AMPs from animals

Antimicrobial peptides can be found in mammals and other animals as part of their innate immune system. Therefore, they are also called host-defense peptides. Apart from shared mode of actions, there is a great variety in AMPs among mammalian species (Hancock & Sahl, 2006).

Among the best studied cationic peptides from animals are the cathelicidins (Wang et al., 2011). Genes encoding cathelicidin have been found in many species of animals including invertebrates (Uzzell et al., 2003) and vertebrates such as mammals (Leonard et al., 2011) and reptiles (Zhang et al., 2010). The best studied cathelicidin LL-37 is simultaneously the only one found in humans (Larrick J W et al., 1995). This is probably one of the reasons why LL-37 is so popular among iGEM teams, who worked with AMPs in the past (iGEM team Slovenia, 2009; iGEM team Utah State, 2013; iGEM team Yale, 2014). For example, the team of Utah State University in 2013 aimed to develop a cost-efficient and large-scale recombinant expression platform for AMPs in *Escherichia coli* instead of inefficient isolation from natural sources. They focused on a variety of peptides from eight different species, among them LL-37 and WAM-1, a cathelicidin isolated from the tammar wallaby (*Macropus eugenii*) (iGEM team Utah State, 2013). WAM-1 is expressed in the mammary gland throughout lactation providing protection for the underdeveloped immunologically naïve young, that develop outside a sterile environment like a uterus and are therefore exposed to harsh pathogens. WAM-1 has proven highly effective against several Gram-positive and Gram-negative bacterial strains as well as the fungal pathogen *Candida albicans* (Wang et al., 2011). However, the activity against the desired expression host *E. coli* poses a problem as it must be avoided that the bacterial culture dies before a sufficient amount of the peptide has been produced. Team Utah therefore implicated a strong transcriptional promoter, which allows the bacterial culture to reach stationary growth phase before induction of high-level gene expression of the AMP (iGEM team Utah State, 2013).

B. AMPs from bacteria

Though it may be unintuitive at first glance, many Gram-positive and Gram-negative bacterial species are producing antibacterial compounds as well. Among these are bacteriocins, which are bacterial ribosomally synthesized AMPs. They can be separated into two classes depending on their degree of post-translational modifications. Many bacteriocins are expressed from gene clusters. Besides the bacteriocin encoding gene, these

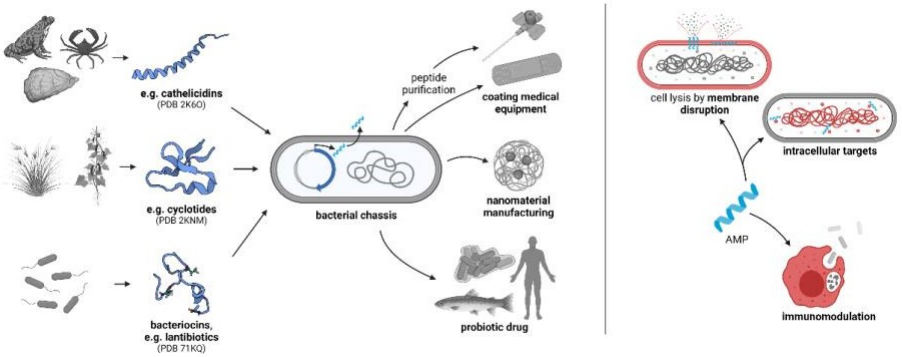

FIGURE 1: Antimicrobial peptides (AMPs) and their applications in iGEM. [A] Many different AMPs originating from animals, plants and bacteria have been used by iGEM teams to solve real-life problems. The most common project ideas include expression of one or multiple AMPs in a bacterial chassis. These engineered strains were either meant to be applied directly as a probiotic, or the expressed AMPs were meant to be purified and applied as a peptide drug. [B] Different modes of action of AMPs. Created with BioRender.com

gene clusters contain resistance genes rendering the producing strain immune against it (Cotter et al., 2013).

So far, several iGEM teams have worked with different bacteriocins from *E. coli* (iGEM team Cornell, 2015; iGEM team Oxford, 2015) and *Bacillus subtilis* strains (iGEM team LMU-Munich, 2014; iGEM team UI Indonesia, 2015). Most of these projects had the overall goal of creating an engineered bacterial strain producing a bacteriocin, to serve as a probiotic. This means that the living engineered bacterial strain was considered an antibacterial treatment. Compared to purifying the bacteriocins from the producing strains and using them as a treatment, this approach has several advantages. It enhances the potency of the AMPs, because they are constantly produced at the site of application. Furthermore, the peptide's selectivity can be increased, if expression of the bacteriocin by the producing strain is inducible by the target pathogen (Lamsa et al., 2012). A similar approach was followed by the iGEM team Munich in 2014. They tried to develop an engineered *B. subtilis* strain to selectively detect, attach to and kill *Staphylococcus aureus* and *Streptococcus pneumoniae*. This strain could then be applied in a probiotic therapeutic. As a killing factor, they planned on using Subtilin, a post-translationally modified lantibiotic bacteriocin originating from another *B. subtilis* strain (iGEM team LMU-Munich, 2014). A general difficulty of this design is the creation of an antimicrobial resistant bacterial strain, which would be released into the environment when applied in a drug. To tackle this problem, iGEM team Munich incorporated *B. subtilis* cannibalism toxin SDP as a second AMP (Lamsa et al., 2012). This should have served as a time-delayed kill-switch for the engineered strain (iGEM team LMU-Munich, 2014).

C. AMPs from plants

AMPs originated from plants have huge potential both in disease treatment and agriculture, as they have been shown not only to act as defense mechanism, but also playing a role in physiological processes such as regulating growth and development (Li et al., 2021). However, their full potential is yet to be exploited. This can be indicated by the fact that so far, only two iGEM teams used a plant AMP in their project (iGEM team Toulouse, 2014; iGEM team Utah State, 2013).

iGEM team Utah State 2013 was interested in using AMPs with different mechanisms. One of the used molecules was EcAMP-1, which is a 37 aa long peptide, folded into a helical structure held together by two disulphide bonds, with a unique hairpin structure. It belongs to the broad group of PR-proteins and possesses an antifungal activity inhibiting hyphae elongation (Rogozhin et al., 2018).

TABLE 1: Selection of the AMPs used by different iGEM teams over the years.

AMPs	Host organism	iGEM team	AMPs	Host organism	iGEM team
LL 37 - Cathelicidin	*Homo sapiens*	Utah State, Trieste, Slovenia, Yale, Linköping	Scygonadin	*Carcinoscorpius rotundicauda*	Utah State
EcAMP-1	*Echinochloa crus-galli*	Utah State, Toulouse	Granulysin	*Homo sapiens*	METU-Gene
Spheniscin-2	*Aptenodytes patagonicus*	Utah State	Latherin	*Equus*	Glasgow
Grammistin-Pp1	*Pogonoperca punctata*	Utah State	Protegrin-1	*Sus*	St. Andrews
CgDefh1	*Magallana gigas*	Utah State	Sufaction	*Bacillus subtilis*	Lyon INSA
WAM-1	*Macropus eugenii*	Utah State	Signiferin	*Crinia signifera*	TCU Taiwan
OH-CATH (3-34)	*Ophiophagus hannah*	Utah State	Epinecidin-1	*Epinephelus coioides*	TCU Taiwan
Subtilin	*Bacillus subtilis*	LMU Munich	Lactoferrin	*Homo sapiens*	Valencia UPV
sdp-canibalism toxin	*Bacillus subtilis*	LMU Munich	Pln1	*Lactobacillus plantarum*	Linköping
Magainin 1	*Xenopus laevis*	Melbourne	AAP2	*Arabidopsis thaliana*	WHU China
Microcin S	*Escherichia coli*	Oxford	EcnA	*Escherichia coli*	Cornell
Artilysin Art-175	*Escherichia coli*	Oxford	EcnB	*Escherichia coli*	Cornell
Artilysin Art-E	*Escherichia coli*	Oxford	DCD-L1	*Homo sapiens*	Aalto-Helsinki
Subtilosin-A	*Bacillus subtilis*	Indonesia	Tachyplesin-I	*Tachypleus tridentatus*	Duke

III. DISCUSSION

In last few years, iGEMers showed an incredible amount of imagination when coming up with different applications for AMPs and even though the range of possibilities is broad, most can be sorted into one of three categories: medical applications, material coating or nanomaterial manufacturing.

As AMPs have been proposed to act as an alternative to nowadays broadly used antibiotics, it is no surprise that many teams were interested in applying them to solve medical issues. An example would be the previously described project of LMU Munich 2014. There are also examples of more unusual approaches, demonstrated by Team Trieste 2012 who wanted to modify *E. coli* Nissle 1917 to create a platform for expression of different molecules in the intestinal tract (iGEM team Trieste, 2012). Another subcategory of medical applications, which overlaps with nanocoating solutions, is creating AMP infused bandages. An interesting example are fusion proteins of AMPs

and a thrombin cleavage site, attached to a polysaccharide bandage, leading to the release of the AMP directly to the infected wound (iGEM team Linkoping, 2019).

Aside from the medical field, AMPs have also been used for environmental issues, as demonstrated by Team Lyon 2012 arguing that biofilms cause problems in water systems, pipelines, and food industry (iGEM team Lyon-INSA, 2012). Furthermore, AMPs can be used for nanomaterial manufacturing. For example, Team Glasgow 2011 created a biomanufacturing platform controlled by light-inducible promoters fused to coding sequence of proteins influencing biofilms (iGEM team Glasgow, 2011).

As shown, AMPs have a great potential for solving a multitude of issues. There are however several aspects that should be considered in future research for these promising applications to become reality. Most of the AMPs used so far are coming from either *B. subtilis* or *E. coli*. We argue it is important to look further into other organisms in order to discover more molecules with similar modes of action, which could open up new possibilities. A good example would be plant-based AMPs called cyclotides, which are known for their stability. Here, grafting techniques might be used to enhance the stability of other AMPs, a problem that has to be solved for many of the above-described applications (Koehbach et al., 2021). Another issue involves manufacturing such peptides, as conventional bioreactors relying on *E. coli* could be rendered useless by the produced AMPs. We reckon that the solution could be again using plants for expression, as the transformation is rather simple and may be scaled up relatively easily, without the issue of the AMPs destroying the organism they are being produced in. Finally, one should not consider AMPs a miracle drug. Like with other antimicrobials, bacteria can acquire different resistance mechanisms against AMPs (Abdi et al., 2019), which is why new antimicrobial treatments will have to be researched constantly.

IV. CONCLUSION

All in all, we believe that no matter the problems that need to be solved first, AMPs are molecules with a very promising future and hope that we will see more and more iGEM teams as well as researchers in general become interested in experimenting with them to solve all sorts of problems we might face.

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AUTHORS

First Author – Inga Leske, B.Sc. Biotechnology, inga.leske@student.uni-tuebingen.de

Second Author – Erik Zimmer, B.Sc. Biochemistry, erik.zimmer@student.uni-tuebingen.de

Third Author – Adrián Vojtaššák, B.Sc. Biology, adrian.vojtassak@student.uni-tuebingen.de

Apprehending the Utilisation of Plant Viral Nanoparticles in Modulation of Stem Cell Differentiation: A Review

Harshil Jain*, Akshatha*, Neil Kashyap Dakshini*

* iGEM MIT_MAHE, Manipal Institute of Technology, Manipal 576104, India

***Abstract*- The ability of stem cells to differentiate into specialized cell types has stirred up research into their applications in regenerative medicine, tissue engineering, and diagnostics. However, the fate of stem cells is determined by different interactions of the cell such as its adhesion to extracellular matrix proteins, direct contact with neighboring cells, chemical exposure, and mechanical stresses that indirectly affect the regulation of stem cell differentiation. One of the potential modulators of stem cell differentiation currently being researched is virus nanoparticles(VNPs). VNPs are viruses of plants/animals which are considered natural nanoparticles as they are within the range of 1-100 nm. A VNP without a genome is a virus-like particle (VLP). VNP's and VLP's have huge potential in the fields of nanobiotechnology for imaging, immunotherapy, and delivery of drugs, etc. This review explores a combination of the principles of nanomaterials, stem cell research, and plant virology to moderate stem cell differentiation, using plant virus nanoparticles and thin films to occupy the extracellular matrix. Various studies and experiments pertaining to this line of research and future prospects of this technology are also discussed.**

***Index Terms*-** Plant virus, Nanoparticles, Stem cells.

I. INTRODUCTION

Bone marrow stromal cells or Bone marrow-derived mesenchymal stem cells (BMSCs), are capable of regeneration and differentiation into several cell lines such as chondrocytes, adipocytes, and osteoblasts (Nguyuen et al, 2018), making them the ideal therapeutic candidates for osteogenesis (T, L. et al, 2019). Their cellular processes are regulated by interactions with underlying substrates (G, K. et al, 2008). However, when analyzing the differentiation of BMSCs, the properties of the surface they are interacting with, such as the material topography and surface chemistry, also play an important role (T, L. et al, 2019).

Interactions between somatic stem cells and their immediate environment have given valuable insights into the various factors responsible for maintaining the existence of stem cells throughout life and controlling their differentiation (SJ, M. et al, 2008). This microenvironment is termed as a 'niche' (Ahmed et al, 2016). Research has found out that the extracellular matrix, previously considered as an "inert supportive scaffold", plays a very important role and is found to have distinct surface topologies, signaling mechanisms, and mechanical properties that maintain homeostasis and orchestrate stem cell differentiation (Gattazzo et al, 2014). This is mainly possible due to a vast array of specialised receptors such as integrins, expressed by stem cells in several combinations, which provide greater control over a variety of interactions with the microenvironment, effectively sensing topological and mechanical cues and even regulating the overall ECM composition (Ahmed et al., 2016).

This review is a compilation of significant data on the use of virus nanoparticles in existing research to conclude its potential use in controlling stem cell differentiation.

II. RESEARCH ELABORATIONS

A. Methods (Literature Search)

The literature search was done from the database PubMed using keywords "Plant Virus", "Nanoparticles" and "Stem Cells". Due to the lack of recent papers available, papers from a broader range (1999 to 2019) were used to obtain data.

III. RESULTS AND FINDINGS

A. The Role of the Extracellular Matrix in Stem Cell Differentiation

Extracellular matrices (ECMs) have complicated structures with varying compositions of collagens, glycoproteins, and glycosaminoglycans, depending on their anatomy (Hoshiba et al., 2015). Biochemical interactions controlling stem cell adhesion and proliferation are mediated by cell surface receptors like integrin (Giancotti et al, 1999).

ECMs also utilize the sensitive mechanotransduction mechanisms of cells, by fine-tuning their response to several mechanical stresses generated in the matrix and differences in stiffness and surface topography. Changes in the organization of integrin greatly influence the migration, shape, and fate ofthese stem cells, by mediating cell response to biochemical and mechanical cues and creating really fine gradients (DuFort et al., 2011).

B. Plant Virus Nanoparticles

Differentiation of stem cells is controlled based on signaling molecules present outside the cell (ECM), that are either taken in or converted into a cascade of other molecules at its membrane (e.g. Growth differentiation factor 5,6 for mesenchymal stem cells) (Clarke et al, 2014).

Nanoparticles of metals like gold and silver have been experimentally proven to regulate the movement of such transcription factors, proteins that induce differentiation in stem cells. This occurs due to some characteristics such as shape and surface chemistry, allowing these factors to easily enter the desired stem cell. (Dayem et al, 2016). The plant viruses are manipulated to incorporate more functional groups thereby changing their surface properties (Lee et al, 2012).

C. Results from Previous Studies

In a study based on tobacco mosaic virus (TMV) coated substrates influencing the differentiation of BMSCs, the virus TMV and its variant (TMV-RGD1), having cell adhesion motifs arginine-glycine-aspartic acid (RGD) with multivalent display, have a significant effect on the differentiation process of BMSCs. The process of experimental analysis commenced with the purification of the wild type and the variant of the virus, followed by characterization, and visualization under the microscope using different staining methods. Thereafter, the process of preparing the virus-coated substrate in a 12-well plate was carried out, followed by harvesting human BMSCs from healthy patients going through knee or hip arthroplasty and then isolating them. The cells were then transferred in a growth medium to expand. After an overnight halt, the culture was replaced by osteogenic media. They were cultured and the RNA was extracted to form cDNA, which was further multiplied using RT-PCR and observed under the microscope.

The substrate was nearly completely covered by the different viral particles (TMV and TMV-RGD1). The hBMSCs were cultured on these viral coated substrates and the result observed was a higher differentiation rate in the TMV-RGD1 culture and no significant difference in the cell morphology. OCN and RunX2, two specific osteogenic biomarkers, exhibited high levels of expression in the TMV-RGD1 cultured cells when compared to TMV and Tissue Culture Plastic (TCP) cultured cells. Finally, since osteogenic expression and adipogenic expressions are correlated, the study tried to determine whether adipogenesis and osteogenesis were opposite under the virus coated substrate and the outcome was consistent as per the literature, i.e., adipogenesis was downregulated whereas osteogenesis exhibited upregulation. (T, L. et al. 2019).

Another study explored the effects of TMV on the regulation of bone morphogenetic protein-2 (BMP2) (Sitasuwan et al., 2012). The methods used were similar to the previous study. Various parameters like the mRNA expression levels and osteogenic markers (like Integrin Binding Sialoprotein (IBSP)) were assessed over time, using RT- qPCR, ELISA, and immunofluorescence assays.

Rapid osteoinduction was triggered in BMSCs grown on TMV substrates, forming aggregates of bone-like nodules within 24 hours, proven by a substantial increase in BMP2 gene expression. However, the BMP2 levels were found to be lower in solutions containing TMV, and substantially lower in the TCP. This has been attributed to surface roughness in TMV substrates, which decreases focal adhesion, leading to a weaker cell-substrate interaction, increasing the chances of cell mobility leading to increased agglomeration, proven by the increased expression of IBSP genes. In contrast, cells were spread over the surface of the TCP, with very little chances of agglomeration.

An intermediate level of expression in solutions containing TMV shows that modulation of BMSC differentiation couldn't entirely be attributed to the properties of the virus alone, and the fact that it was immobilized to a rigid surface played a very important role.

IV. DISCUSSION

Stem cells are well known for forming a basis of cells in tissue engineering. However, certain limitations such as the slow rate of differentiation, a limited success rate of differentiating into osteocytes don't make it a viable option.

Based on various experimentations mentioned in this review, the use of plant VNP's in nanotechnology and tissue engineering has potential. The unique surface properties which can be genetically manipulated provide more control of the cell adhesion to factors required for osteocyte differentiation. With VNP's ability to enhance osteoinduction in stem cells by triggering the expression of vital genes, further enhanced by incorporation of cell adhesion motifs, bone tissue regeneration could be a future application of the virus nanoparticles. Further research on early interactions of stem cell components with plant virus substrates and the exact mechanisms involved in promoting the expression of BMP2 could provide valuable insights on modifying BMP2 levels in order to tailor stem cell differentiation for specific purposes, such as specialised biomaterials, with an increased accuracy. Saying that, the use of VNP's inside the human body has a long way to go. Certain risks have to be eliminated, such as any potential immunogenic reaction from the body. A high concentration of VNP's in a bloodstream is not a natural situation for the body cells. Neglecting these concerns, this method is more efficient and inexpensive to conventional methods indicating great future prospects.

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AUTHORS

First Author – Harshil Jain, B.Tech Biotechnology Engineering (ongoing – V semester), Manipal Institute of Technology. Mail –harshil.jain@learner.manipal.edu

Second Author – Akshatha, B.Tech Chemical Engineering (ongoing – V semester), Manipal Institute of Technology. Mail- akshatha.mitma2@learner.manipal.edu

Third Author – Neil Kashyap Dakshini, B.Tech Biotechnology Engineering (ongoing – V semester), Manipal Institute of Technology. Mail – neil.dakshini@learner.manipal.edu

Correspondence Author – Harshil Jain, B.Tech Biotechnology Engineering (ongoing – V semester), Manipal Institute of Technology. Mail - harshil.jain@learner.manipal.edu

Awareness about Reproductive Rights and Health in Indian Society

Niloufer Shanavas*

* Team IISER Tirupati iGEM 2021, Indian Institute of Science Education and Research (IISER), Tirupati

***Abstract*- In India, sexual health and rights are topics not widely discussed due to the cultural and traditional norms of society. Appropriate sexuality education and understanding its role in society enables an individual to make informed decisions on their Reproductive and sexual life and curtail the spread of misinformation and myths associated with it. This study aims to understand the prevalence of Reproductive Health and Rights related awareness in India through a survey. The survey evaluates whether there exists any correlation between education level and awareness about various aspects of Reproductive Health and Rights. Additionally, the study examines if gender inequality is reflected in knowledge about Reproductive Health and Rights. This study also aims to identify the most common sources of Sexuality education in Indian society and analyses the popular opinions of the public on Sexuality Education. Understanding the current status of reproductive rights and health-related awareness would help to identify the gaps and pitfalls in our Sexuality education system and make reforms in the curriculum.**

***Index Terms*-** Comprehensive Sexuality Education, Misinformation, Reproductive Health, Reproductive Rights, Sexually Transmitted Infection

INTRODUCTION

Sexual health is considered to be a state of physical, emotional, mental, and social well-being in relation to sexuality and not merely the absence of disease or infirmity as defined by the WHO (2006a). Provision of age-appropriate and scientifically accurate comprehensive education is important to allow individuals to make responsible decisions and protect themselves against sexually transmitted infection, unintended pregnancy and coercion. India has the third-largest population of HIV infected in the world and has one of the highest sexual crimes against women (UNAIDS, 2017). Providing Sexuality education can help individuals develop a healthy attitude towards members of the opposite sex and reduce indulgence in risky behaviour. Informing individuals of their Reproductive rights is equally important as it would help individuals protect against sexual violence, coerced pregnancy and enable them to seek medical and legal assistance. The introduction of sex education in the school curriculum was promoted by India's Ministry of Human Resource Development, but this initiative was met with much resistance with opponents claiming that it would corrupt youth and taint Indian values and consequently led to the ban of sex education in several states (Tripathi & Sekher, 2013). Even in schools that have introduced sex education, teachers often shy away from educating the younger generation about reproductive health. This leaves the young generation to find information by themselves without any proper guidance leading to dependence on unreliable sources of information and the spread of misconceptions. Understanding the state of Sexuality Education in India, our research studies if the extent of Sexuality education is influenced by personal background and examines the most used sources of Sexuality education.

I. RESEARCH ELABORATIONS

To determine the major sources of sexuality education and the extent of awareness, a questionnaire consisting of a total of 17 questions evaluating the age, educational level, gender, residential location (rural/suburban/urban) and the knowledge of Reproductive Health and Rights was prepared based on extensive literature reading. The questions were centred around sexually transmitted diseases, safe sexual practices, medical termination of pregnancy and reproductive rights. The data collected for this research was obtained through an online survey conducted using Google Forms. The survey participants included 633 Indian participants over 18 years of age (Table 1).

TABLE 1.
Population Composition.

Population	Percentage
Total	633 (in numbers)
Age	
18-25	92.7%
26-40	5.8%
40+	1.4%
Gender	
Male	28%
Female	57.3%
Inconclusive	14.5%
Residential location	
Rural	25.1%
Suburban	35.3%
Urban	40.6%
Educational Level	
Bachelor's degree or Higher	50.4%
Highschool	42.2%
Middle or secondary school	3.5%
Prefer not to say	3.9%

**Data entered as inconclusive includes people who were unsure of their gender identity or prefered not to disclose their gender identity*

II. RESULTS AND FINDINGS

1. SOURCES OF SEXUALITY EDUCATION

The sources of Sexuality education evaluated in the survey included: School/College, Movies, Books, Friends, Parents, Siblings, Cousins, Spouse, Pornography and other sources. Statistics revealed that the four major sources of Sexuality education were Friends, School/College, Movies, and Books, which were identified by 79%, 70%, 55%, and 52% of the survey sample, respectively, as their source of Sexuality education (Table 2).

TABLE 2.
Sources of Sexuality Education.

Source	Population
Friends	79%
School/College	70%
Movies	55%
Books	52%
Pornography	26.1%
Parents	23.5%
Cousins	14.4%
Siblings	10%
Spouse	4.3%
Other sources:	
Internet and social media	11%
Certified expert	0.2%
Documentary	0.2%
Partner	0.2%

We further evaluated if there is a difference in sources of Sexuality education based on gender. Our analysis revealed that the major sources of Sexuality education are different in males and females. While Friends, School/College and Books continued to be the major sources of Sexuality education for both genders, Parents were the next major sources for females and Movies and Pornography for the male participants. 62.1% and 54.23% of the male respondents were dependent on Movies and Pornography as sources of Sexuality education. In comparison, only 14.3% and 4.3% of the females identified pornography and movies as sources of Sexuality education. 30.39% of the female participants viewed parents as a source of Sexuality education, while only 10.2% of the males viewed parents as a source of Sexuality education.

2. OPINION ON THE RIGHT AGE TO INTRODUCE SEXUALITY EDUCATION IN THE ACADEMIC CURRICULUM

The participants were provided with the following alternatives to analyse the opinion of the survey sample on the right to age to introduce Sexuality education in the academic curriculum: Preschool, Class 1-5, Class 6-8, Class 9-10 and Sexuality education is not required. Of the 633 respondents, the majority of the participants, i.e., 52.1%, agreed that Class 6-8 was the right age to introduce Sexuality Education in the School curriculum (Table 3).

Of the remaining survey population, 27.9%, 13.9% and 5.7% believed that Class 9-10, Class 1-5, and Preschool respectively is the right age to introduce Sexuality education (Table 3). We further wanted to understand if the opinion on the right age to introduce Sexuality education in the academic curriculum is influenced by their residential location or education level. We found that irrespective of their educational background and residential location majority of the respondents agreed that Class 6-8 is the right age to introduce sexuality education.

TABLE 3.
Opinion on the right age to introduce Sexuality education in the academic curriculum.

Age	Population
Preschool	5.7%
Class 1-5	13.9%
Class 6-8	52.1%
Class 9-10	27.9%
No sexuality education is required	0.3%
No opinion	0.3%

3. UNDERSTANDING THE EXTENT OF REPRODUCTIVE HEALTH AND RIGHTS-RELATED AWARENESS

We wanted to understand how aware individuals are about various aspects related to Reproductive health and rights. This was analysed through an online survey, where the participants were asked ten questions related to Sexually Transmitted Infection, Safe Sexual Practices, Medical Termination of Pregnancy and Reproductive Rights, and the participants were scored such that the maximum score a participant can gain is 19, and the minimum is 0. The score obtained by the respondent is used as a measure of their knowledge and awareness of Reproductive Health and Rights. On analysis, the maximum score obtained was 19 and the minimum 4 (Fig 1). The values for average, median, and mode were consistent and were obtained to be 12 (63.3%), and the standard deviation of the data collected was calculated to be 3.26.

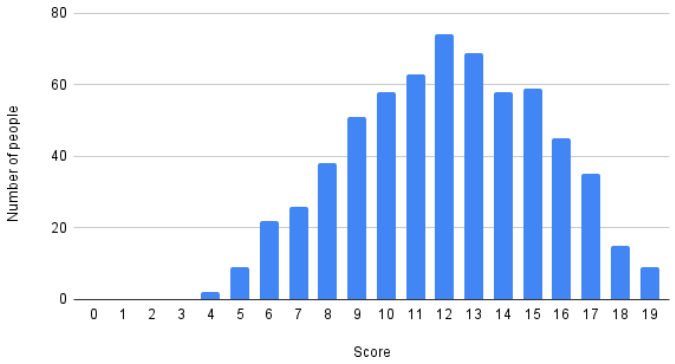

FIGURE 1.
Score Distribution.

We further wanted to analyse if there is any correlation between the scores obtained and the residential location, educational level or gender. On analysis, it was revealed that the distribution of the score in the population was similar irrespective of their residential location, education level or gender (Table 4). The mode, median, and average scores obtained were surprisingly similar, reflecting that the educational background, residential

location and gender did not correlate with the score in the sample population (Table 4). This result was unexpected; urban and semi-urban populations were expected to have higher scores due to the increased accessibility to sources of information in these regions compared to rural areas. It was also expected that people with a higher level of education had better awareness of Reproductive Health and Rights. A similar score could be due to improved accessibility to sources of information in rural areas due to the internet.

TABLE 4.
Comparison of score based on Residential location, Educational background and Gender.

Population	Score
Total Population Average Mode Median Standard Deviation	 12 12 12 3.3
Residential location:	
Rural Average Mode Median Standard Deviation	 11.7 11 11 3.1
Suburban Average Mode Median Standard Deviation	 11.9 12 12 3.4
Urban Average Mode Median Standard Deviation	 12.5 13 13 3.2
Education Level:	
Bachelor's or higher Average Mode Median Standard Deviation	 11.9 12 12 3.3
Highschool Average Mode Median Standard Deviation	 12.3 13 12 3.4
Gender:	
Female Average Mode Median Standard Deviation	 11.6 12 12 3.3
Male Average Mode Median Standard Deviation	 12.3 13 12 3.1

III. DISCUSSION

The aim of the study was to understand if the extent of awareness on Reproductive Health and Rights in society was influenced by gender, educational background and residential location. We also wanted to examine what were the major sources of information on sexuality. The results indicate that extent of awareness is similar irrespective of gender, residential location or educational background. The average test score is found to be 12/19 and shows the inadequacy of the current awareness and education programs. The major sources of information were found to be Friends, School/College, Movies and Books. The improved accessibility to these sources of information could be the reason for similar test scores irrespective of the personal background. School/college is one of the major sources of Sexuality education that contributes significantly to educating people on Reproductive Health and Rights. The curriculum could be revised and improved to emphasise the risks and means of protection from STI and make people aware of the Reproductive Rights they are entitled to. From the survey conducted, we made a surprising observation that only half of the respondents (55.2%) were aware that the Indian constitution guarantees Reproductive rights to its citizens. Revising the school curriculum to include Reproductive Rights would equip individuals to make informed choices and seek legal remedies in case of sexual abuse or coerced pregnancy.

IV. CONCLUSION

The extent of awareness in Reproductive Health and Rights is similar irrespective of gender, educational background and residential location (rural/suburban/urban). Most sources of Sexuality Education were identified to be Friends, School/College, Movies and Books. Friends School/College and books were major sources common to both genders. Male participants showed significantly higher dependence on Movie and Pornography as sources of Sexuality Education when compare to Female participants. Female Participants were seen to have a slightly higher dependence on parents as sources of Sexuality education than male participants.

APPENDIX

Link to survey questionnaire:
https://docs.google.com/document/d/1r1rZKIC8c7K4V33zdCtJYwCHo-wjHRxn6An8bylyTcQ/edit?usp=drivesdk

Link to additional questionnaire analysis:
https://docs.google.com/document/d/1RyLeYL-ZB6YiAeQHWxg84P4za-KlHouRCDsWY46fY80/edit?usp=drivesdk

ACKNOWLEDGEMENT

The author acknowledges Team IISER Tirupati iGEM 2021, especially the Human Practices Team (Shubhra Singhal, Fathima Aslaha T R, Shreyas Samir Parkhie) for their contribution in reviewing the questionnaire prepared for conducting the survey. The author particularly expresses her gratitude to Dibya Saha (IISER Tirupati alumni and an ex-iGEMer) for his guidance.

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AUTHORS

First Author – Niloufer Shanavas, Student at Indian Institute of Science Education and Research Tirupati, niloufershanavas@students.iisertirupati.ac.in

Correspondence Author – Niloufer Shanavas, Student at Indian Institute of Science Education and Research Tirupati, niloufershanavas@students.iisertirupati.ac.in

Birnam Wood: Empowering Kinetic Constant Estimation with Random Forest Modeling

Jonathan Chong*, Ramin Kahidi*, Cedric Acierto*, Rigel Tormon*,

Helen Zhang*, Andrew Symes*, Juan Sebastian Alvarez*

iGEM Calgary, University of Calgary

Abstract - **In the realm of pharmacology and biochemistry, detailed modeling of biological systems is essential for the growing practical applications of protein-ligand interactions. Of these interactions, understanding kinetic rate constants is crucial to properly leveraging the intermolecular interactions that propel biological processes into industry. However, getting accurate data requires arduous experiments, costly equipment and can be prohibitively expensive. Thus, we developed a random forest-based algorithm for predicting the k_{on} and k_{off} values of a given protein-ligand interaction and a nested cross-validation approach to features selection. Our program, *Birnam Wood*, aims to make k_{on} and k_{off} values more accessible by reducing computational requirements of the calculations.**

Index Terms- Kinetic Rate Constant, Machine Learning, Protein Interactions, Random Forest

I. INTRODUCTION

Kinetics of protein-ligand interactions are essential to characterizing biological systems and using them to their utmost potential. It has significant relevance to the early stages of drug discovery and can also be used to identify proteins that contain selective affinity for specific elements. Through kinetic rate constants, scientists are able to better understand and construct more comprehensive models. Due to their importance in evaluating interactions, there is a growing need to construct effective and accurate models for predicting kinetic rate constants. Current methods of getting these kinetic rate constants involve simulating the association process or characterizing the energy landscape, both of which are computationally expensive and timely (Tiwary et al., 2015). An esoteric method involves feature selection algorithms that predict kinetic rate constants based on structural and energetic properties derived from the structures of complexes and their unbound constituents (Moal & Bates, 2012). Thus, our software, Birnam Wood, improves on existing feature selection algorithms by introducing an ensemble learning method along with a resampling procedure to evaluate the effectiveness of the models. This ultimately reduces the runtime, the number of features needed for a prediction, and increases the accuracy of the results.

II. RESEARCH ELABORATIONS

A. Materials and Methods

We decided to use an ensemble learning method known as random forest (RF), a classification algorithm created by multiple decision trees. By looking at the bound and unbound states of protein-ligand interactions, we can collect structural and energetic parameters about the interaction (Moal et al., 2011). Starting with a list of 200 of these parameters, we trained our RF model and evaluated the accuracy of the model based on a mean squared error evaluation (MSE). From this, we used nested cross-validation to reduce the number of parameters and increase our precision.

Our nested cross-validation approach to feature selection relies on a concept known as multicollinearity, where an independent variable has a high correlation with one or more variables. Effectively, if one variable can be used to obtain another, then the model will over-bias those values, resulting in erratic changes in predictions in response to small changes in the model. Additionally, this overbiasing of protein descriptors can also decrease the model's accuracy. (Brownlee, 2021). Thus, we can find which combination of 200 parameters leads to the best results by reducing overlap and irrelevant features.

To implement nested cross-validation into the software, we ideally would have needed to produce an abundance of machine learning models each with its own unique set of features. Therefore, since we have 200 descriptors, we would have to create 200! machine learning models. This process was deemed to be too resource and time expensive, while also not being scalable to schemes with even larger datasets. Therefore we decided to produce 200 nests each beginning with the original 200 features. Each iteration uses the previous iterations model, the program then calculates and inverts the importance values of each parameter. These inverse importance values are then used in conjunction with monte carlo sampling to remove features pseudo-randomly with a bias towards removing low importance features. The program then runs a new RF model, repeating this process until the number of features in the nest reaches zero. Feature selection via a biased draw removes many of the ineffective models we would have seen through the initial brute

force method. This way we were able to significantly reduce the runtime and the number of machine learning models to 200^2 or 40 thousand. Run time was then further reduced through the use of a hash table that contained the results of each model which the program checks to prevent reruns of the same models.

As referenced before, we decided to evaluate the accuracy of each model through mean squared error; although, this evaluation neglects the number of features present in each model. Therefore, we decided to create our own evaluation criterion called J Score to evaluate models based on both MSE and the number of features. J Score evaluates each model by comparing its performance to the standard model with all 200 features, using MSE and the number of features (in this case *m* and *n* respectively).

$$J = \frac{m_{std} - m_{cur}}{m_{std}} * \frac{n_{std} - n_{cur}}{n_{std}} \quad (1)$$

Where "std" is the standard model, and "cur" is the current model

Using the formula for J Score shown in (1), the models which were less accurate (had a larger MSE value) than the original were assigned a negative score. While those which were both more accurate and had fewer features would result in a larger number with the most ideal model having a maximum value of 1. Therefore by looking at the largest J score values, we were able to find and filter out the best models from the monte carlo based nested cross-validation algorithm.

B. Research design

Training and test data were obtained from Moal et al. (2011), consisting of kinetic rate constants for 44 complexes compiled from literature. In addition to the kinetic rate constants, the dataset also contains a set of 200 molecular descriptors which describe a variety of different aspects of the interaction (Moal et al., 2011). These features were derived from a multitude of different bioinformatic programs that use energetic models, hydrophobic burial, Van der Waals terms, and four-body or two-body statistical potentials to gather descriptors (Moal et al., 2011).

III. RESULTS AND FINDINGS

After running over 200 nests, our program was able to produce results of all models for both k_{on} and k_{off} predictors as shown in Figure 1 and Figure 2. The final models selected due to their exceptional J Scores resulted in just 2 parameters for the k_{on} prediction, and 10 parameters for the k_{off} prediction. The k_{on} parameters were Change in Atomic Surface Area upon binding (DASA), and the General Four-Body Potential (GEN_4_BODY), with DASA coming from the NACCESS software suite (Hubbard & Thornton, 1993), and GEN_4_BODY coming from the Potentials'R'Us webserver (Feng et al., 2010). The referenced k_{on} model received a J Score of 0.936 including a K value of MSE value of 0.0225 which is a significant improvement compared to our standard model which received an MSE of 0.412. Our best k_{off} predictor had a J Score of 0.882 and an MSE of 0.00748. Unfortunately, because of the precision of this model, we deemed it as overfitted and decided against its use in our industrial application.

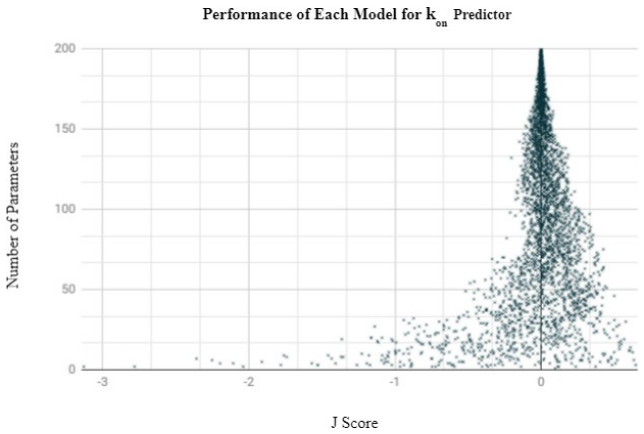

Figure 1: Correlation between number of parameters and J Score for each model in the k_{on} predictor

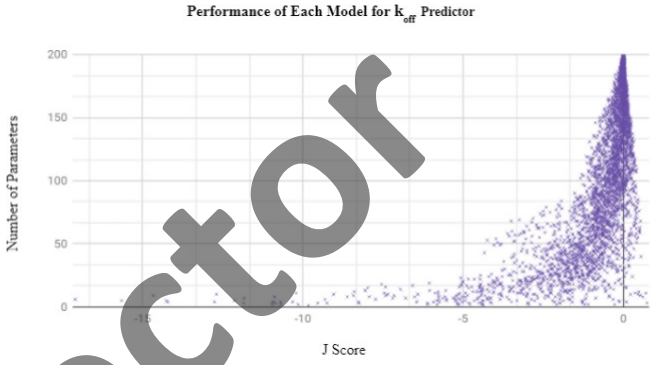

Figure 2: Correlation between number of parameters and J Score for each model in the k_{off} predictor

IV. DISCUSSION

Our models were successful in significantly reducing the computational burden of getting kinetic rate constants, showing a promising future for our nested cross-validation and scoring function. The next step in validating these findings is for the industrial application of the achieved models to obtain k_{on} and k_{off} parameters for our protein of interest, Lanmodulin. At this step, however, we ran into some limitations for this specific data set and a challenge that faces the field of bioinformatics as a whole; reproducibility. For instance, NACCESS was only accessible by directly emailing its author, and the Potentials'R'Us web-server was no longer active, and its authors did not reply to inquiries into other methods of accessing the software. In situations like this, aside from attempting to rebuild the entire software from the original paper, there is no convenient way of using these discontinued programs, making older research very difficult to work with and validate. Without many of our parameters being inaccessible due to discontinued software, using our model as intended was not feasible with this data set. In the future, we would want to either find a more recent dataset that uses programs that are still currently available, or choose our own parameters from a well-established software suite, like Pyrosetta (Chaudhury et al., 2010), that will have a greater chance of being accessible in the future.

Using a k_{on} predictor with accessible features, we were able to develop a kinetic rate constant prediction for lanmodulin and neodymium, a protein-ligand interaction and the primary focus of iGEM Calgary 2021's project. Using the value, we were able to calculate the estimated k_{off} value by using the complex's binding affinity from past literature (Cotruvo et al., 2018).

Finally, these values were then used for industrial-scale kinetic rate modeling and contributed to the development of an optimized bioreactor.

V. CONCLUSION

Birnam Wood presents a novel approach to predicting kinetic rate constants and significantly reduces the amount of time, and computational expenses required to make a prediction.

APPENDIX

Our code will be available on our team GitHub: https://github.com/iGEMCalgary

ACKNOWLEDGEMENT

We thank Dr. Anders Nygren and Dr. Mayi Arcellana-Panlilio for sponsoring our research. We thank the University of Calgary's Provost, Dru Marshall, and the Vice-President (Research) William Ghali. We thank the faculty of Science, the Cumming School of Medicine, and the Schulich School of Engineering who provide support to the iGEM program at the University of Calgary. We thank the PURE Program and the Schulich School of Engineering Activity Fund for sponsoring our research. We thank Tian Zhao (as our team's TA), and Tammy Lu (as the team's RA).

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AUTHORS

First Author – Jonathan Chong, Current Undergraduate Student in Software Engineering minoring in Biomedical Engineering, University of Calgary, Cumming School of Medicine, Email: jonathan.chong@ucalgary.ca

Second Author – Ramin Kahidi, Current Undergraduate Student in Bioinformatics, University of Calgary, Cumming School of Medicine, Email: ramin.kahidi@ucalgary.ca

Third Author – Cedric Acierto, Current Undergraduate Student in Software Engineering minoring in Biomedical Engineering, University of Calgary, Cumming School of Medicine, Email: johncedric.acierto@ucalgary.ca

Fourth Author – Helen Zhang, Current Undergraduate Student in Chemical Engineering minoring in Biomedical Engineering, University of Calgary, Cumming School of Medicine, Email: helen.zhang1@ucalgary.ca

Fifth Author – Rigel Tormon, Current Undergraduate Student in Chemical Engineering minoring in Biomedical Engineering, University of Calgary, Cumming School of Medicine, Email: rigel.tormon@ucalgary.ca

Corresponding Author – Andrew Symes, BSc Statistics concentrated in Nanoscience, Current Masters of Engineering Student with the Schulich school of Engineering, University of Calgary, Cumming School of Medicine, Email: andrew.symes1@ucalgary.ca

Corresponding Author – Juan Sebastian Alvarez, Current Undergraduate Student in Chemical Engineering, University of Calgary, Cumming School of Medicine, Email: juan.alvarez1@ucalgary.ca

Microfluidic Tools for Pushing the Boundaries of Synthetic Biology

Artem Belov*, Marc Saghiah*

* iGEM UParis_BME, Faculté des Sciences Fondamentales et Biomédicales, Université de Paris, France

***Abstract*- Modern synthetic biology is a rapidly expanding field that provides increasingly sophisticated solutions to the most acute problems around the globe. Genetic machines, therapeutic proteins, engineered bacteria, and other ingenious solutions offer immense potential for multiple industries, including healthcare. However, development and broad implementation of synthetic biology solutions often requires sophisticated equipment and trained personnel. This drastically limits the access to potentially lifesaving innovative science. Microfluidic devices, also known as lab-on-a-chip, rely on microchannel physics to downscale and streamline complex multi-step experiments. The unique hydrodynamic properties of narrow channels allow optimisation of the reagent use and product yield. A well-designed microfluidic device can convert a complex experimental procedure into an easy-to-use and affordable product that fits on a palm of your hand. Here, we review the advantages and tools provided by microfluidic technology, as well as examples that utilise these features to design an ultimate synthetic biology lab-on-a-chip.**

***Index Terms*-** microfluidics, modularization, synthetic biology, systematic design

I. INTRODUCTION

In the past few decades, the rapid advances in synthetic biology have made it possible to artificially design biological system features for various multidisciplinary purposes. Research, pharmaceutical, food, environment and energy solutions provided by genetic engineering already improve our daily lives, proving the great promise behind the emerging technology (Khan et al., 2016). Nevertheless, most of the innovative synthetic biology solutions prove complex to translate to "outside-the-lab" environments. The barriers for the immediate implementation can be summed up as two general scenarios: technology optimisation for a large-scale industrial production, and the protocol simplification for deployment in more remote and resource-limited settings. (Brooks & Alper, 2021) Both situations require engineers to optimise the involvement of complex machinery and trained laboratory personnel, which can prove challenging for a multistep experimental routine.

Counter-intuitively, the perfect solution to scale-up and simplify the technology can start with minimizing the experimental system on a finely manufactured microchip. Microfluidics is an umbrella term for describing the behaviour, precise control, and manipulation of liquids at a sub-millimetre scale, at which surface forces dominate volumetric forces. It can provide multiple solutions for streamlining multistep protocols, optimizing the reagent use and enhancing the analytical or productive performance of a system (Szita et al., 2010).

Microfluidics is a multidisciplinary field involving engineering, physics, biology, chemistry, nanotechnology and biotechnology. The utility of microfluidic chips is evident from the current multidisciplinary of this engineering approach. From simple laminar flow diagnostics, such as pregnancy tests, to sophisticated organs-on-a-chip, the miniaturization has proven useful in almost every area of biology (Azizipour et al., 2020). To help a newcomer navigate the microfluidics field, this review article describes several microfluidic solutions that have already been implemented for synthetic biology purposes, providing some technical detail and inspiring examples to adapt this technology into your experimental pipeline.

II. RESEARCH DESIGN

The relevant literature for this systematic review has been identified through the database search (PubMed, CrossRef, Google Scholar) utilizing the key words "synthetic biology", "microfluidics", "cell-free system", "lab-on-a-chip". The identified research and review papers were critically appraised ahead of their synthesis in the body of this manuscript.

III. RESULTS AND FINDINGS

A. Minimizing reagent quantity

Reagent availability and costs can pose a significant barrier for translating a novel solution into industry. While one-off experiments in an academic laboratory may tolerate some inefficiency in material consumption, product commercialization and its implementation in a resource-limited environment requires improved efficiency when utilizing the consumables. While simple minimization of the experimental workflow may rely on less reagents to complete the reaction, it can also result in reduced product yield. However, a microfluidic chip can drastically reduce reagent consumption while increasing the reaction efficiency and addressing multiple other problems encountered at the bench side.

A de novo DNA synthesis approach by Lee et al. (2010) perfectly showcases the benefits provided by microfluidic technology. Authors designed a chip that consumed ~100-fold less reagent per reaction, while producing sufficient oligonucleotide concentration to not require downstream amplification steps. Furthermore, low scale of the reaction allowed for direct synthesis without any concentration or pre-amplification, further reducing the cost and shortening the time required to perform the protocol. Finally, the individual reaction chambers separated by the valves allowed precise reaction

control and prevented cross-contamination, leading us to the second major advantage of microfluidic technology.

B. Chambers vs Single-droplet microfluidics

Whenever a pragmatic question is approached with an engineering mindset, multiple strategies can be employed to achieve similar results. Microfluidics is often no exception, as alternative chip designs and flow control methods can offer radically different outcomes with almost identical functionality. For instance, high-throughput cell-free protein expression systems can be realized through compartmentalisation with "conventional" chamber devices or with single-droplet systems.

As exemplified by the design proposed by Stech et al. (2014), laminar flow devices with multiple chambers can be used to generate continuous exchange systems, where the substrate (nucleotides, amino acids, ions, and energy resources) is continuously fed from a reservoir to a reaction chamber via a dialysis membrane. Unlike the batch-reaction methods (e.g. Kim et al., 2006), in which a single reaction chamber limits the reagent supply and caps reaction duration, this chip can generate high quantities of the product. Alongside extending reaction time and product yield per chip, such controlled flow systems allow fine-tuning of the reagent concentrations to optimise the efficiency of the enzymes.

An alternative approach was employed by Dittrich et al. (2005). Instead of chamber compartmentalisation with a semi-permeable membrane, the researchers built a device that generates single droplets while mixing the reaction components. The chip produces hundreds of "mini-reactions" in individual droplets. The result is a high throughput, yet compartmentalized system that provides the benefit of individual droplet analysis, characterisation and manipulation. Additional quality control and flexibility of the system offer the obvious benefits despite the seemingly higher complexity of the setup.

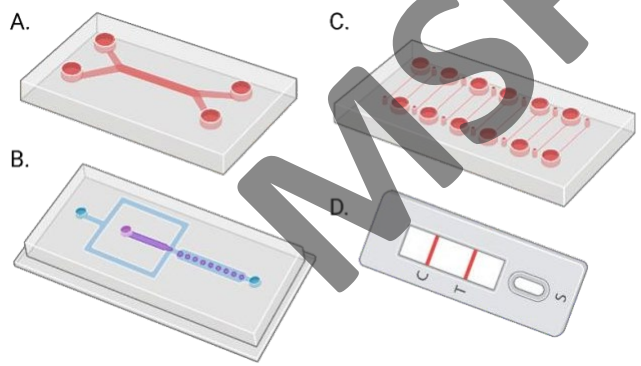

FIGURE 1.
A selection of exemplary microfluidic device designs. (A) a "standard" chip with 4 chambers connected by y-channels; (B) flow focusing droplet generation on a chip, inspired by Dittrich et al. (2005); (C) a chip design with multiple parallel channels for high-throughput experiments; (D) a lateral flow device with a window for sample loading (S), test (T) and control (C) strips. Figure generated with biorender.com

C. Automated workflow

Streamlining is another straightforward benefit behind implementing microfluidic technology. Every example cited above utilises some degree of experiment automation by controlling the substrate flow through the chip. Nevertheless, manual operation of the valves still introduces potential human error and limits potential of the unsupervised workflow.

Further automation of the microfluidic technology can be made possible via introduction of automated read-out that allows self-induction of the downstream protocol. Husser et al. (2018) introduced an Arduino-based optical density detection system. Their chip acts as a microbioreactor that can autonomously coordinate the semicontinuous mixing of a bacterial culture, ultimately reducing the labour intensity of bacteria transfection protocol. Authors automated and optimized the system using a RFP reporter gene and proved the validity of the method by introducing recombinant thermophilic β-glucosidase enzymes into their E. coli system.

Automation can also be done with the introduction of robotics. Robotic tools can execute complex tasks and allow the automation of many "conventional" experiments by themselves. The team of Langer & Joensson (2019) introduced a liquid-handling robot to produce cell spheroids. The robot performs all the steps from the experimental workflow independently, without requiring human help: sample and reagent loading, droplet generation, cell incubation, spheroid recovery and dispensing. Coupled with the streamlining and reagent handling efficiency provided by the microfluidic design, robotics proves to be a powerful tool for handling labour-intensive protocols and increasing result accuracy and reproducibility in research and industry.

D. Parallel processing

A major asset of microfluidics is parallel processing. Instead of sequentially repeating one experiment on your microfluidic chip, it is possible to independently run the same experiment multiple times on the same device, in parallel. This can increase the analyte throughput, while drastically saving the costs and time. Parallel processing can significantly benefit synthetic biology research, especially in the field of genetics.

Next-generation sequencing (NGS) has been relying on this technology for years now, as parallel processing allowed to sequence a gigantic amount of DNA in very little time by running parallel cycles of sequencing on a same device (Vyawahare et al., 2010). In NGS, hundreds of millions of DNA molecules can be arrayed on a single chip, allowing to sequence them in parallel (Abate et al., 2013), rapidly cutting the time needed to process a sample.

Since then, the applications of parallel processing have gone beyond simple DNA sequencing. It is also used for drug testing in microbiology. Kulesa et al. (2018) used parallel droplet processing in a microfluidic chip to predict the synergy between thousands of drugs and antibiotics against E. coli. This allowed them to significantly reduce the experiments time, reduce the degree of the human error and helped to discover new drugs and combinations for infectious bacterial diseases.

IV. CONCLUSION

Microfluidics, the study of liquid droplets and fine channels flows, has become a crucial multidisciplinary technology for multiple areas of the scientific research. Synthetic biology is no exception, as researchers design microfluidic devices to simplify, optimise and streamline their experimental routine. In this review, we have discussed the key advantages provided by minimisation of the protocol to a lab-on-a-chip. Microfluidics allows to minimise the reagent quality and offers additional control over the chambers/droplets to maximise the product yield, while allowing high degree of automation and parallelisation of the protocols. It offers multiple ways to accelerate lengthy and labour-intensive experiments, while also reducing the risk of contamination or human error. The list of potential advantages is not limited to what we discussed above. For instance, the material choice can significantly alter the properties and physics of the chip. Paper-based devices can provide a cheaper and flexible alternative to the commonly used polydimethylsiloxane (PDMS), as demonstrated by a SARS-CoV-2 diagnostic developed by Park & Lee (2021). Thus, the reader is advised to not limit their curiosity to this brief overview, but invited to further explored the various methods, technologies, and approaches behind microfluidic engineering.

ACKNOWLEDGEMENT

We would like to thank iGEM team MSP-Maastricht for inviting us to join this initiative. We also thank and acknowledge all the members from iGEM team UParis_BME who participated in this year's project. Additional thanks to our supervisors from Université de Paris and the BME Paris master's program for guiding us during our project.

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AUTHORS

First Author – Artem Belov, MSc Student in BioMedical Engineering, Faculté des Sciences Fondamentales et Biomédicales, Université de Paris, artem.belov@etu.u-paris.fr

Second Author – Marc Saghiah, MSc Student in BioMedical Engineering, Faculté des Sciences Fondamentales et Biomédicales, Université de Paris, marc.saghiah@etu.u-paris.fr

Correspondence Author – Artem Belov, MSc Student in BioMedical Engineering, Faculté des Sciences Fondamentales et Biomédicales, Université de Paris, artem.belov@etu.u-paris.fr

The Consequences of Inhibiting Methanogenesis in Ruminants

Tim Davalan[*+] , Anna Sennikova[*]

[*] iGEM MSP, Maastricht University
+ Corresponding Author: t.timnico@maastrichtuniversity.nl

***Abstract*- This literature review explores the consequences of methanogenesis inhibition in ruminants, with particular focus on adding *Asparagopsis taxiformis* to the feed. The main consequences are the accumulation of $H_2$and the possibility of this accumulation inhibiting fermentation. Furthermore, volatile fatty acids may be produced in an increasing trend, as methanogens stop competing for H_2. These volatile fatty acids may lead to an energy gain for the ruminants, as the energy is then neither belched out as methane or H_2, which is favourable for both animal and human alike, as this may lead to a weight gain for the animal, resulting in higher meat yield. In addition, feeding *A.taxiformis* can lead to bromoform exposure, which has been shown to possibly harm the animals and change their feeding behaviour.**

Index Terms- Methanogenesis Inhibition, Ruminants, H_2Redirection, *Asparagopsis taxiformis*

I. INTRODUCTION

From ancient times to the present day, cows and sheep have been a source of important resources for survival and dietary components for humanity. These animals belong to the suborder *Ruminantia*, meaning that they have a four chambered stomach, the first one of which is the rumen (Britannica, 2019). Within the rumen, microorganisms can digest cellulose taken up as grass, hay, straw etc., into compounds that can be taken up by the animal as an energy source. The main electron acceptors and donors in this rumen fermentation process are hydrogen (H_2) and carbon dioxide (CO_2) (Matthews et al. 2018), which therefore constitute the main end-products of this fermentation pathway (Danielsson et al. 2017). In a reduction pathway, these compounds are used by archaea in the rumen to produce methane (CH_4) (Kinley et al. 2020). CH_4 is a compound that can be seen as a loss of energy when it is formed and eructed, representing a loss of up to 12% of gross energy intake (Ungerfeld, 2021).

$CH4$ is a greenhouse gas with higher potency than $CO2$. This means that, although there is less of it in the atmosphere, its global warming potential is 86 times stronger per unit of mass than $CO2$over a timespan of 20 years, and 28 times stronger on a timespan of 100 years (Jackson et al. 2020). Current methane emissions contribute 17.3% to the 100-year global warming potential (Ritchie & Roser, 2021). With the goal of halting climate change, the emissions of methane from ruminants has been experimentally reduced in various ways. One example is the addition of canola oil to the cow feed, which can reduce methane emissions by up to 20%. Another method is adding 3-nitrooxypropanol as a food additive, which has even been proven to reduce methane emissions by up to 80% (Zhang et al. 2021). An alternative approach of reducing the methane output by ruminants is the usage of the red seaweed *Asparagopsis taxiformis*, which, depending on the feed amount, could reduce methane output by up to 98% (Kinley et al. 2020). This ability of *A.taxiformis* is due to its capability to generate halogenated compounds, amongst them bromoform (Machando et al. 2017), which is the major halogenated product of these red algae (Machando et al. 2016). Bromoform inhibits a cobamine-dependent methyltransferase, which is required for the synthesis of methyl-coenzyme-M, the key enzyme in the final part of methanogenesis (Machando et al. 2016). Thus, bromoform ultimately reduces methane production if present in the cow rumen (Kinley et al. 2020). These methyl-coenzyme-M are present exclusively in methanogens (Zhu et al. 2021), whereof the archaeal genus Methanobreviacter is the most abundant and most studied (Danielsson et al. 2017). Of the many ways of reducing methane emission, *A.taxiformis* shows the strongest dose-dependent CH_4 mitigating effects, while having the least impact on rumen fermentation (Chagas et al. 2019).

Although the mechanism of this form of methanogenesis inhibition is somewhat researched and understood, there is yet to be a consensus on what the consequences of such inhibition. This review will gather information on established positive and negative implications of inhibiting methanogenesis through feeding *A. taxiformis* to ruminants, amongst other techniques.

II. RESEARCH DESIGN

Databases such as 'Google Scholar', 'Web of Science' as well as direct Google searches were utilized, using terms such as '*Asparagopsis taxiformis*', 'Ruminants', and 'Methanogenesis Inhibition'. Papers were scanned for relevant information, which was then discussed with the review team.

Furthermore, several experts were contacted for personal correspondence, through either email or virtual meetings.

III. RESULTS AND FINDINGS

In the rumen, H_2 is produced from reducing equivalents released in glycolysis and pyruvate oxidative decarboxylation to acetyl CoA. Methanogenesis allows for a lower H_2 pressure and therefore shifts fermentation away from ethanol and lactate towards acetate, which increases ATP generation, as the electron transport-linked phosphorylation in methanogenesis itself and substrate level phosphorylation in the formation of acetate create ATP (Ungerfeld, 2013). Inhibiting methanogenesis can lead to an accumulation of H_2 (Duin et al. 2016; Kinley et al. 2020; Ungerfeld, 2013), which thermodynamically favours electron incorporation into propionate production. Yet this incorporation does not remove all the H_2 stoichiometrically, as is seen in Table
1 (Ungerfeld, 2013).

Inclusion Rate	High (0.2%)	Mid (0.1%)	Low (0.05%)
Methane	-98%	-38%	-9%
H_2	+1700%	+380%	+/- 0%

Table 1 Different inclusion rates of seaweed to feed of ruminants with measurements of methane and hydrogen compared to control. The feed intake percentages are of organic food intake (adapted from Kinley et al. 2020).

The excess H_2 may be belched out by the ruminant (E. Ungerfeld, *pers. comm.*), or even redirected into propionate biosynthesis, the main glucose precursor for ruminants (Li et al. 2016; Kinley et al. 2020; E. Ungerfeld, *pers. comm.*; Kinley et al. 2016; Roque et al. 2021). Indeed, alternative H_2 sinks, such as the volatile fatty acids acetate, butyrate and aforementioned propionate are in constant competition with methanogens for H_2. Yet their higher threshold of H_2 concentration than methanogenesis, results in them being outcompeted, as can be seen in equations (1) and (2) (Greening et al. 2019; Joblin, 1999).

Acetogenesis:

$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O\ \Delta G^0 = -104.6kJ$ (1)

Methanogenesis:

$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad \Delta G^0 = -135.6kJ$ (2)

Although reductive acetogenesis may release H_2, it does so in lower quantity than methanogenesis, making it a candidate as a H_2 sink when less methane is being produced (E. Ungerfeld, *pers. comm.*). Accordingly, when methanogenesis is inhibited, there is an observable shift from acetate to propionate production, as demonstrated in Table 2 (Ungerfeld, 2015).

Inclusion Rate	High (0.2%)	Mid (0.1%)	Low (0.05%)
Acetate	-20%	-14%	-4%
A/P	-35%	-29%	-14%

Table 2 Different inclusion rates of seaweed to feed of ruminants with measurements of acetate and the acetate/propionate (A/P) ratio compared to control (adapted from Kinley et al. 2020).

The A/P ratio stands at 2.97 when excess H_2 is redirected into reductive acetogenesis, and at 1.15 when excess H_2 is redirected to propionate production. The former increases the total VFA production, whereas there is no increase in VFA production in the latter case, as each mol propionate is one mol acetate that is not produced. The H_2: CO_2 ratio is 2:1 for reductive acetogenesis and 3:1 for propionate, meaning that there is a greater retention of Catoms in reductive acetogenesis (Ungerfeld, 2013). Yet neither would stoichiometrically incorporate all the H_2 that would be incorporated by methanogenesis (Ungerfeld, 2013). The most favourable alternative H_2 sinks upon inhibition of methanogenesis would therefore be as in following order: reductive acetogenesis, propionate and butyrate (Ungerfeld, 2015a).

Mild methanogenesis reductions of 20-40% may have no adverse effects on feed intake and animal performance without other mitigation (R. Mackie, *pers. comm.*). Even higher inhibition percentages have been suggested to not have adverse effects on the rumen fermentation, as the animal may be able to mitigate excess H_2 without additional mitigation (E. Ungerfeld, *pers. comm.*). This case has also been theorized by Roque et al. (2019), which found no negative effects on VFA production at 95% methane reduction. Yet the accumulation of $H_2$is energetically inefficient, as it inhibits the re-oxidation of cofactores, such as $Fd_{red}{}^{2} \rightarrow Fd_{ox}$, and therefore inhibits fermentation (Ungerfeld, 2015; Ungerfeld 2021). A tendency of reduced acetate and increased propionate is observable, whereas butyrate may not be affected (Kinley et al. 2020), or slightly stimulated (Ungerfeld, 2021; Kinley et al. 2016).

The in vitro digestibility of substrates was not negatively impacted by low doses of *A.taxiformis*, and fibre digesting microbes were not affected either (Kinley et al. 2016). Sheep were found to have ruminal mucosa consisting of granulomatous and keratotic overgrowth, as well as lower VFA and acetate concentrations, yet higher propionate amounts. The total VFA concentrations were also reduced (Li et al. 2016), yet this could also be related to dry matter intake changes. No bromoform or dibromochloromethane was found in any muscle or fat of the animals (Li et al. 2016). At any inclusion rate, Muizelaar et al. (2021) did not find any bromoform after 10 days (feces and organs) and 17 days (urine) of continuous administration of *A.taxiformis*. Yet upon inspection of the rumen of two animals, inflammatory cell accumulations were observed which formed ulcers and led to the loss of papillae and to the thickening of the rumen wall. Additionally, the underlying strome and muscle layers of the rumen were also inflamed, yet no other organs were found diseased. Furthermore, lower milk yields were reported, which may also correlate to reduced dry matter intake. Li et al. (2016) reported pathological changes of the mucosa with whitish tan discolorations. Interestingly, if given a choice, animals would choose against eating feed containing *A.taxiformis*.

IV. DISCUSSION

In general, it seems that the consequences of methanogenesis inhibition depend on the extent of this inhibition, which is mainly visible in the accumulation of $H_2$and the changing proportions of acetate and propionate. Yet there are a multitude of alternate H_2sinks besides methane, acetate and propionate, such as butyrate, formate and microbial biomass production (Kinley et al. 2020). The literature and experts do not necessarily agree on whether there is a need for redirecting excess H_2 upon methanogenesis inhibition, and whether accumulation of H_2would cause harm to the animal and discontinue fermentation.

One way of directing H_2 towards acetate production would be through the incorporation of phloroglucinol into the rumen (Ungerfeld, 2020). The reduction of phenolic compounds such as flavonoids, which generally form phloroglucinol, by using H_2 as an electron donor may mitigate H_2 accumulation, yielding acetate as a final product (Martinez-Fernandes et al. 2017). In cases where phloroglucinol has been added as a feed additive, weight increases of the animals have been observed, due to the increase in acetate, which is an energy source for the ruminant (Martinez-Fernandez et al. 2017). Yet due to large amounts of phloroglucinol that would be needed, this seems less feasible (P.H. Janssen, *pers.comm*). The approximate consensus is that redirection of H_2 towards reductive acetogenesis would benefit

the animal, as it would help with continuous fermentation as well as increase the animal's energy uptake (which would be lost if H_2 were belched out). Although bromoform is a known carcinogen for ruminants (Abbott et al. 2020), at low inclusion rates of *A.taxiformis*, cancerous lesions were not reported.

V. CONCLUSION

Although multiple studies have tried to evaluate the effects of inhibiting methanogenesis in ruminants, research results often contradict each other, indicating the novelty of the problem and the need for new experiments to be conducted. Although it has never been reported that methanogenesis inhibition proved to be lethal, the health effects reported vary. In general, according to the results of a remarkable amount of papers and the experts' estimations (Ungerfeld *pers. comm.* ; Janssen *pers. comm.*), the ruminants are quite able to tolerate the suppression of methanogenesis, yet further large scale necropsy studies are needed to determine tissue specific damage. Residual bromoform was reported as missing from any animal products

ACKNOWLEDGEMENT

We would like to thank Dr. Emilio Ungerfeld, Prof. Rod Mackie, Dr. Peter Janssen and Prof. Erik Steen Redeker for their valuable input for our project, which also helped in understanding the consequences of methanogenesis inhibition in ruminants.

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AUTHORS

Tim Davalan – undergrad student, Maastricht University, t.timnico@student.maastrichtuniversity.nl

Anna Sennikova – undergrad student, Maastricht University, a.sennikova@student.maastrichtuniversity.nl

AdAPTED: Augmenting dNTPs And Polymerase Through Enzymatic Design

Maria Ioanna Ioannidou*, Spyros Kanelopoulos, Eleftheria Kelefioti Stratidaki***, Kleoniki Pylarinou***, Vasilia Spyridaki***, Ilias Toumpe***, Orsalia Zoi Veloudiou******

* Team Athens, Agricultural University of Athens, Biotechnology Department
** Team Athens, National and Kapodistrian University of Athens, Biology Department
*** Team Athens, National Technical University of Athens, School of Chemical Engineering
**** Team Athens, National and Kapodistrian University of Athens, Medical School

***Abstract*- Nucleic acid amplification (NAA) techniques, such as Polymerase Chain Reaction (PCR), have been proven to be a powerful tool to detect and thus eliminate a pandemic, besides being a useful standard biological laboratory technique. The aim of this project is to make NAA techniques more accessible at non-centralized facilities. Two of the key components of PCR are a polymerase enzyme and the four types of deoxynucleotide triphosphates (dNTPs). However, the total cost of dNTPs' production is significant and they are often produced by complicated, low-yield, unsustainable chemical processes (Loan et al., 2019). Thus, the aim of this project is to make Nucleic Acid Amplification (NAA) techniques, and specifically Polymerase Chain Reaction (PCR), more accessible at non-centralized facilities. Problems of this nature can be generalized to all NAA techniques. Using the principles of synthetic biology, a high yield genetic circuit is designed to produce ribonucleotide reductase (RNR) and thymidylate synthase (TSase), two enzymes necessary to produce dNTPs. The produced dNTPs, along with thermostable Pfu DNA Polymerase, also included in the designed genetic circuit, allow for a low-cost and more eco-friendly PCR. This procedure thus enables the usage of PCR technique in point-of-need applications and remote settings.**

***Index Terms*-** Polymerase Chain Reaction (PCR), dNTP production, Ribonucleotide reductase (RNR), Thymidylate synthase (TSase)

I. INTRODUCTION

Since the standardization of PCR in 1987 (Process for Amplifying, Detecting, and/or Cloning Nucleic Acid Sequences Using a Thermostable Enzyme, 1987), the technique is used daily in applications ranging from biotechnological routine steps to diagnostics, identification protocols and other medical procedures. Especially during the COVID-19 outbreak, PCR tests are considered the golden standard of molecular diagnostics.

Two of the key components of PCR are a polymerase enzyme and the four types of deoxynucleotide triphosphates (dNTPs). However, the total cost of dNTPs' production is significant and they are often produced by complicated, low-yield, unsustainable chemical processes (Loan et al., 2019). Thus, the aim of this project is to make Nucleic Acid Amplification (NAA) techniques, and specifically Polymerase Chain Reaction (PCR), more accessible at non-centralized facilities.

Ribonucleotide reductase (RNR) and Thymidylate synthase (TSase) are the two main enzymes that are known to be vital for the de novo synthesis of dNTPs from NMPs/NDPs, which are naturally in abundance inside the cell. RNR is the enzyme responsible for the catalysis of the conversion of the four ribonucleotides triphosphates (NTPs) into their corresponding dNTPs. Out of all classified RNR enzymes, class Ia is the best studied, is naturally produced in *Escherichia coli*, has an aerobic oxygen dependence, and consists of two genes, nrdA and nrdB (Torrents, 2014). TSase has an essential role in catalyzing the reductive methylation of deoxyuridylate (dUMP) to thymidylate (dTMP), providing the sole intracellular de novo source of dTMP (Liu et al., 2002).

In this project, using the principles of synthetic biology, two high yield genetic circuits are designed to allow for a low-cost and more eco-friendly PCR, with components not requiring cold storage. The first one is designed to produce the two enzymes, RNR and TSase, which are needed to yield dNTPs. The second genetic circuit is designed to produce a thermostable and high fidelity Pyrococcus furiosus (Pfu) DNA Polymerase (Lundberg et al., 1991), to enable the performance of a PCR in a more accessible way.

II. RESEARCH ELABORATIONS

A. Genetic circuit design

In order for *E. coli* (strain BL21-D3) bacteria to be transformed and therefore to overexpress the enzymes RNR, TSase and Pfu Polymerase, two genetic circuits are designed. The genes encoding each protein are selected from the NCBI database, and are codon optimized based on *E. coli* bacteria. For each gene the appropriate regulatory elements are also selected. For RNR and TSase, two inducible promoters are chosen, each regulated by lactose and arabinose. This type of promoter is chosen to regulate the overexpression of the enzymes, preventing possible toxicity in the cell, due to mutagenesis caused by high concentrations of dNTPs. The two promoters are induced differently to study the activity of the two enzymes both in combination and separately. As for the promoter of Pfu Polymerase a constitutive promoter was chosen, as unlike dNTPs there is not any indication that Pfu overexpression will cause any toxicity to the cell and high amounts of the enzyme are needed. The terminator for all genetic circuits is a composite part consisting of two terminators derived from *E. coli* and T7

phage.

For all these elements, prefixes and suffixes were added during the design process, and restriction sites for the enzymes of the Type II S Golden Gate assembly were included. The aim of adding these sequences is the ligation optimization of all parts in a one pot reaction.

Lastly, some extra elements are added to each part. In the Pfu encoding gene, a histidine tag is added to isolate the protein for future applications. Purification of Pfu using polyhistidine affinity tags was selected as it is a rapid and efficient method, resulting in 100-fold enrichment and up to 95% purities in a single purification step (Bornhorst et. al. 2000). Also, GFP and RFP are added to the circuit of RNR and TSase respectively, to optimize the measurements required for the computational model.

After the connection of each gene with the appropriate regulatory elements, the transcriptional units (TUs) are prepared for insertion in a plasmid. Two plasmids were chosen: pSB1AK3 and pGGA Destination Plasmid. One containing RNR and TSase and the other containing only the Pfu encoding genes. All the BioBricks that were designed and used to assemble the above TUs are included in the Appendix A section.

B. Experimental procedure

The steps followed for the transformation of the *E.coli* bacteria are as follows. Firstly, each part is amplified using the universal primers for the additional prefix and suffix. After each PCR, the samples are cleaned using a DNA and PCR clean up kit (NEB) and then electrophoresed to confirm the amplification. After all elements of each TU are obtained, the ligation protocol is followed using the BsaI restriction enzyme and T4 DNA Ligase. After the ligation, each TU is once again amplified, cleaned, and electrophoresed. To obtain each fragment, a gel extraction kit is utilized. Lastly, using the same ligation protocol with enzymes SapI and T4 DNA Ligase, the RNR and TSase TU are ligated with pSB1AK3 and Pfu TU with pGGA Destination Plasmid. To isolate and purify Pfu polymerase, the transformed cells are grown in liquid cultures, centrifuged and broken with ultrasound and then gravity flow column chromatography with Nickel resin is used to collect the protein in imidazole. The protein is identified using sodium dodecyl sulfate polyacrylamide gel electrophoresis method (SDS-PAGE) and the concentration is calculated using Lambert-Beer law. For the isolation and purification of dNTPs, high-performance liquid chromatography is used with a C18 column. The four dNTPs are eluted at different times (dCTP at 16.9 min, dGTP at 23.8 min, dTTP at 24.8 min and dATP at 25.6 min).

C. Modeling

A kinetic model is developed, whose main purpose is to calculate the time needed to produce dNTPs. The rate equations used are based on Jackson (1984) and are qualitatively confirmed by Henderson et al. (1973). However, the system contains a set of kinetic parameters, whose values have not been measured for the WT E. Coli cell. To address this, all parameters were assigned a mean and standard deviation value and a series of models were created by parameter sampling from the assigned distributions to each parameter. The BRENDA enzyme database (Enzyme Database - BRENDA, 2021) was used to extract these mean and standard deviation values. Mean values were assigned based on measurements from E. Coli organisms and standard deviation values derived from all the measurements registered in the database for the given enzyme and substrate (different organisms and/or environment). In addition, rate equations describing the overexpression of RNR and TSase (Michaelis-Menten kinetics, where the substrate is IPTG/AraC and with a linear decay term) are used to inspect the effect, that elevated RNR and TSase levels have on the time course of the system. The rate differential equations and the parameter sampling were executed with the software application COPASI. Lastly, knowing that high levels of dNTPs increase mutagenic rates and the probability of loss of function of the plasmid, the model contains Monod equations with a stability-mutagenic factor, linearly proportional to the cell concentration of the culture. The explicit rate equations constructed for the modeling of the system can be found in Appendix B.

III. RESULTS AND FINDINGS

The amplification of all parts using the pair of universal primers was conducted in order to gain more than 100 ng of high purity DNA to use in the Golden Gate Assembly. Although this succeeded for the majority of the sequences, the purity and the quantity of nrdA BioBrick (the sequence encoding the first subunit of Ribonucleotide reductase) triggered determining difficulties for the progress of the current iGEM project. The most severe problem of these, being the presence of a different sequence with identical prefixes and suffixes, i.e. an unwanted part that could also stick with the genetic construction. More information for the troubleshooting that followed can be found in Appendix D. Each PCR sample was electrophoresed, and after the confirmation of the amplification, the concentration was measured using Nanodrop. Several DNA Cleanup & Gel Extraction Protocols were performed when needed.

The quantity of all the other parts was sufficient for the Golden Gate reaction protocol and therefore the parts underwent ligation to produce four TUs. Two of the TUs contain the RNR enzyme encoding genes with GFP as well as without GFP, and two TUs contain the TSase enzyme encoding genes with RFP as well as without RFP. The ligates were amplified using PCR and then electrophoresed. Although the results for both TSase TUs were as expected, the TUs for RNR did not ligate properly, resulting in many bands in the agarose gel of approximately 10ng of DNA, quantity insufficient to recover.

The TU of Pfu was successfully ligated and transformed, as well as identified. SDS-PAGE for Pfu purification solutions was performed three times from different colonies to confirm the results. The average protein concentration after chromatography was 0.312 (g of Pfu polymerase)/L that corresponds to 4.4 (mg of Pfu polymerase)/(L of liquid culture). The results can be found in Appendix C.

IV. DISCUSSION

A. Modeling significance

Based on the results of the kinetic model the user can have a good approximation of the time scale needed to reach the desired product quantities. Additionally, the system will be optimized from the relevant experiments and calculations of the plausible overexpression levels of RNR and TSase. The overall results can help to better understand the mechanisms involved in the project's proposed system. This model is expected to be used to develop software for autonomous hardware that could make the production of dNTPs even more streamlined. Moreover, the kinetic characterization of the proposed biological system is an essential first step to critically assess the possibility of scaling up the process to an industrial level.

B. Future applications

The aim of this project is to lyophilize the transformed bacteria that will have a high concentration of dNTPs as the final product. In this way, the lyophilized bacteria will be used for NAA techniques. The dNTPs concentrated lyophilized cells do not need cold storage and are non-chemically harmful or environmentally harmful. Also, the produced Pfu polymerase is to be combined with dNTPs for experiments and diagnostic tests that utilize PCR.

V. CONCLUSION

Enzymatic in vivo production of dNTPs is a more sustainable and accessible production method, compared to the established chemical production. Through this production method, NAA techniques are enabled in decentralized facilities, simplifying diagnostics routines by providing cheap reagents.

APPENDIX

The folder containing the detailed appendix, the model equations and pictures of experimental results, can be found in the following link.

ACKNOWLEDGEMENTS

We would like to thank Dr. Apostolos (Lee) Alissandratos for his fundamental contribution in providing theoretical and laboratory advice. Special thanks to Assoc Prof Evanglos Topakas, Asst Prof Vasiliki Koumandou, Assoc Prof Panagoula Kollia, lab technician Elena Pappa, PhD student Giorgos Taksidis, past iGEMers Nikolaos Kalavros and Anastasios Galanis for their support.

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AUTHORS

First Authors

M.I. Ioannidou – Agricultural University of Athens, Biotechnology Dept, marianna.ioannid@gmail.com.

S. Kanelopoulos – National and Kapodistrian University of Athens, Biology Dept, spyros889@gmail.com.

E. Kelefioti Stratidaki – National Technical University of Athens, Sch of Chemical Engineering, ekelefioti@gmail.com.

K. Pylarinou – National Technical University of Athens, Sch of Chemical Engineering, kleonikipylarinou@gmail.com.

V. Spyridaki – National Technical University of Athens, Sch of Chemical Engineering, siliaspr99@gmail.com.

I. Toumpe– National Technical University of Athens, Sch of Chemical Engineering, ilias12toumpe@gmail.com.

O.Z. Veloudiou – National and Kapodistrian University of Athens, Medical Sch, orsaliavel@gmail.com.

Correspondence Author

Kleoniki Pylarinou – kleonikipylarinou@gmail.com, igemathens2021@gmail.com, +306943075057

Affordable and Accurate Biosensing via Luminometry

Cedric Acierto*, Rigel Tormon*, Jonathan Chong*, Helen Zhang*,
Ramin Kahidi*, Andrew Symes*, Sebastian Alvarez*

*iGEM Calgary, *University of Calgary

***Abstract*- As we evolve into a more data-driven world, it has become more critical to design devices that offer instant and accurate data. To meet this demand, various biosensors have been developed for a wide variety of applications. Biosensors can be functionalized using electrochemical, physical, and optical principles. Luminometry was selected as a prime candidate for development as it offers high sensitivity and affordability compared to other measurement methods. Thus, we propose *Lumos*, a low-cost, modular luminometer with high irradiance responsivity and sensitivity. In order to provide instant and accurate data easily accessible to the users, *Lumos* features a mobile application that enables users to harness its reading in the field. Measurement methods in iGEM typically involve an Arduino Uno as the main microcontroller unit, which is sufficient for simple tasks. However, *Lumos* utilizes an ESP32 microcontroller unit which provides integrated Wi-Fi and Bluetooth capabilities, and 160-240 MHz clock frequency, which enables its processor to execute more instructions per second compared to Arduino. The *Lumos* device values modularity, which promotes the product's reusability and repairability.**

***Index Terms*-** luminometry, biosensor, ESP32, Bluetooth Low Energy, modularity

I. INTRODUCTION

Various measurement tools and methods have been developed to enable the collection of accurate and precise data for laboratory and on-site detection. Luminometry is an analytical technique used to measure the light produced by chemiluminescent and bioluminescent reactions. Compared to other analytical techniques, it offers rapid monitoring and greater sensitivity and selectivity (Meikle et al., 1992). This measurement technique has a wide variety of applications ranging from the estimation of postmortem interval of bone remains in forensic science (Sarabia et al., 2018) to rapid detection of heavy metal ions for water quality assessment (Lukyanenko et al., 2019).

The principles of luminometry can also be applied to optical biosensors, which measure biological reactions by generating signals directly proportional to the concentration of the sample or analyte. A typical setup for a biosensor consists of five parts: analyte, bioreceptor, transducer, electronics, and user interface. The analyte is the sample being measured, while the bioreceptor is what recognizes the analyte. In the case of optical sensors such as *Lumos*, the bioreceptor would be a luminescent/fluorescent protein, which creates a measurable glow. The transducer converts one form of energy into another. The electronics component receives that energy and processes it for the display to output. The TSL237 photodiode is a transducer which converts light into a square wave, with frequency directly proportional to the irradiance of the incident light on the diode's surface. The ESP32 microcontroller is an electronic component that measures the square wave's frequency in hertz (Hz) and outputs the data via a hardware interface like an LCD screen or a phone via Bluetooth. ESP32 microcontrollers have an advantage over Arduino Uno microcontrollers as they offer a higher clock frequency, as well as built-in Bluetooth functionality to enable wireless connection (specifically Bluetooth Low Energy) to a mobile phone. By making an accompanying mobile application, users can also save sensor readings to a database for future reference. The hardware design is centered around the principle of modularity, thereby providing users the ability to replace faulty parts with ease, and without the need to buy a new set. *Lumos* is a highly versatile and cost-effective measurement tool, with a price significantly lower than most luminometers (Boston Industries, Inc., n.d.).

II. RESEARCH ELABORATIONS

A. Materials and Methods

Cost was a major consideration in designing and developing the hardware for Lumos. We wanted to design an optical biosensor sensitive enough to low light levels, while still affordable enough for the average user. TSL237 is used as the transducer. ESP32 is the main electronics or microcontroller unit. An LCD1602 screen is used to display sensor readings in manual mode. Any Android phone with Bluetooth capabilities can be used to test the Bluetooth Low Energy mode. The photoresistor, an auxiliary component, is used to detect and notify the user about ambient light that may be leaking into the observation chamber. A capacitor and resistor are also used to provide stability to a few components. The estimated price for the entire system, including PLA filaments for the chassis and wires for connection, is around CAD $99.63. This is a significant reduction in cost compared to traditional optical biosensors and luminometers, which can reach upwards of a thousand dollars (Boston Industries, Inc., n.d.). The estimated prices per component are listed on the next page.

TABLE 1: Estimated price per material and total cost

Material	Price in CAD
ESP32	$18.99
TSL237	$2.95

LCD1602	$12.98
Photoresistor	$1.28
Capacitor	$0.32
Resistor	$0.14
PLA filaments (2)	$37.98
Wires	$24.99
Estimated Total Cost	$99.63

B. Research design

Once the analyte and bioreceptor solution is placed inside the observation chamber, the TSL237 photodiode receives the light produced by the sample and converts it into a square wave, which is then received and interpreted by the ESP32 microcontroller by counting the frequency of *interrupts* per second. The frequency value, in Hertz, is then displayed through the LCD1602 screen in real time. The photoresistor ensures that the observation chamber is perfectly sealed off from ambient light. Another option for display is to reroute the data to an Android application via Bluetooth Low Energy. A tool for mobile app development, Thunkable, was used to implement BLE in a faster and more efficient manner. All hardware components are controlled and programmed in Arduino, which is written in C++.

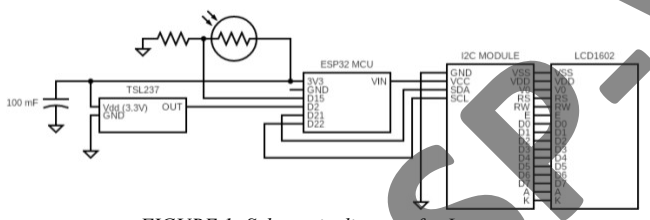

FIGURE 1: Schematic diagram for Lumos

Since the system's design is centered around the principle of modularity, the chassis was designed to have replaceable parts. The three main removable components are the observation chamber which houses the sample, the LCD screen, and the measurement module containing the TSL237 sensor. The ESP32 is designed to sit on a set of header pins for easy replacement in the event that it breaks or becomes faulty. Modularity will also be a major factor in facilitating future hardware updates as these modules can easily be replaced with newer components. The CAD model for the chassis is shown in the next figure. Internal components such as the observation module and ESP32 holder are shown.

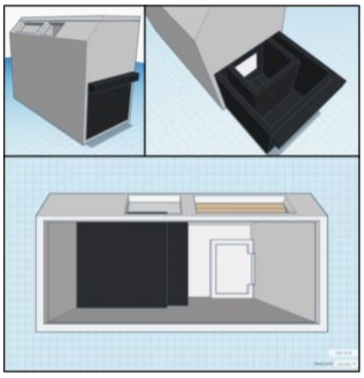

FIGURE 2: 3D model for the chassis

III. RESULTS AND FINDINGS

After testing the TSL237 sensor with a blue LED connected to a potentiometer, it was determined and confirmed that the interrupt frequency is directly proportional to the light intensity coming from a light source. It can be seen in the graph that the sensor does not start increasing until approximately 500 ADC units. This is mainly due to the LED not lighting up in the lower voltage settings.

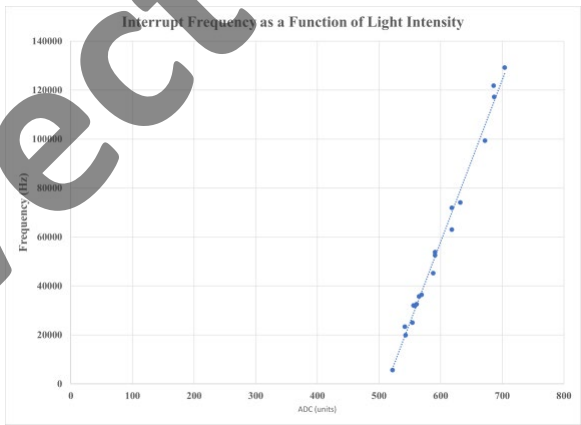

FIGURE 3: Interrupt Frequency vs Light Intensity Graph

BLE connection was successfully established between Lumos and the Android phone application. Bluetooth connection issues can occur occasionally and prevent data from being received, but this can be solved by either restarting the application or refreshing the microcontroller. A screenshot of the application interface is shown in the next figure.

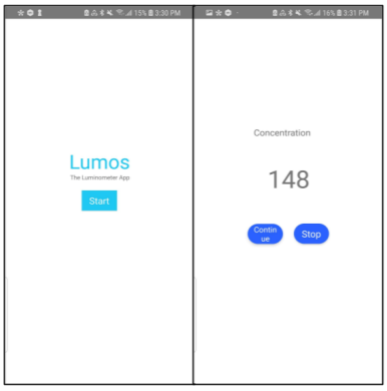

FIGURE 4: Screenshot of the Lumos app receiving data from the sensor

IV. DISCUSSION

The luminometer proves to be an effective tool for detecting light intensity even in low light conditions. Completing the calibration algorithm would be the next major step, as it will provide the connection between the measured interrupt frequency and the sample concentration. The data-saving functionality can be easily implemented as the app development tool has a built-in database for testing. This additional capability will be integral for environmental testing, specifically in situations where minute trends need to be measured. Certain software revisions have to be made to make the system more intuitive and user-friendly, especially on the Android application. Support for iOS devices will also be a future direction for Lumos.

Even though the TSL237 can effectively measure low light intensity, there are various alternatives that can provide better performance. Many electronic retailers are ending their supply of these sensors, and it may be difficult to order parts in the future. Thus, an update for this component is inevitable.

Future iterations of this luminometer will also feature a more compact chassis in order to save more on PLA filament for 3D printing.

V. CONCLUSION

Lumos proves to be a promising and effective tool for the detection of various luminescent enzymes, thus implying its usefulness in multiple areas in science, especially in synthetic biology. However, due to rapid advances in technology, some hardware components will need replacement and software will need to be constantly updated. Replacing and updating the parts with ease is made possible by following the principle of modularity. Thus, *Lumos* is flexible and constantly evolving to best meet the needs of both the iGEM community and the scientific community in general.

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AUTHORS

First Author – Cedric Acierto, Current Undergraduate Student in Software Engineering minoring in Biomedical Engineering, University of Calgary, Cumming School of Medicine, Email: johncedric.acierto@ucalgary.ca

Second Author – Rigel Tormon, Current Undergraduate Student in Chemical Engineering minoring in Biomedical Engineering, University of Calgary, Cumming School of Medicine, Email: rigel.tormon@ucalgary.ca

Third Author – Jonathan Chong, Current Undergraduate Student in Software Engineering minoring in Biomedical Engineering, University of Calgary, Cumming School of Medicine, Email: jonathan.chong@ucalgary.ca

Fourth Author – Helen Zhang, Current Undergraduate Student in Chemical Engineering minoring in Biomedical Engineering, University of Calgary, Cumming School of Medicine, Email: helen.zhang1@ucalgary.ca

Fifth Author – Ramin Kahidi, Current Undergraduate Student in Bioinformatics, University of Calgary, Cumming School of Medicine, Email: ramin.kahidi@ucalgary.ca

Corresponding Author – Andrew Symes, BSc Statistics concentrated in Nanoscience, University of Calgary, Cumming School of Medicine, Email: andrew.symes1@ucalgary.ca

Corresponding Author - Sebastian Alvarez, Current Undergraduate Student in Chemical Engineering, University of Calgary, Cumming School of Medicine, Email: juan.alvarez1@ucalgary.ca

Biological Detoxification of Gossypol in Cottonseed Meal

Ruchir Pratape[*], Purna Chandra Sekhar V[*], Maharshi Dixit[*]

[*] iGEM_NIT Warangal, National Institute of Technology Warangal, Telangana, India, 506004

***Abstract*- India is the second-largest cotton producer globally, contributing to about a quarter of the global cotton production. While the cotton bolls support India's giant cotton textile industry, the seed offers high value in livestock feed and oil production. Cottonseed meal (CSM), a by-product of the cotton oil industry, is an attractive source of feed enriched with high protein and vitamin E content. But its consumption is severely hindered by the presence of a polyphenolic compound known as "Gossypol". The current review elaborates on the biological routes of reducing the gossypol content to permissible limits and making the CSM a lucrative functional food ingredient, ready for the utilization of animal/human consumption.**

Index Terms- Cottonseed meal, Gossypol, Detoxification, enzymes, Protein source.

I. INTRODUCTION

In the present global scenario, malnutrition is a serious concern among the major countries of the Asian and African sub-continent. This has buzzed the alarm to find a sustainable solution to overcome the protein requirement among developing countries [1]. Cotton (*Gossypium* spp.) is a foremost cash crop belonging to the Malvaceae family. It is considered one of the primitive plants cultivated by humans at least 4000 years ago [2]. The global production of cottonseed is estimated to be 44.84 million metric tons (MMT). The cottonseed meal (CSM), which is a co-product of a cotton oil refinery, is projected to produce 10 MMT annually, which can serve the protein requirement of 590 million people every year [1,3]. However, the consumption of CSM is severely hindered by the presence of the polyphenolic compound known as gossypol.

Gossypol is produced by the pigment gland and is highly concentrated in seeds, with the concentration varying from 0.002-6.64%. The basic structure of gossypol comprises six phenolic hydroxyl groups and two aldehyde groups, making it a highly reactive compound [4]. Gossypol occurs in two vital forms, free and bound. Cottonseed may contain up to 14,000 mg/kg of total Gossypol and 7,000 mg/kg of free Gossypol, depending upon the species and weather conditions.

Previously, several attempts were made on de-gossypolization using the physical, chemical, and biological methods. The physical and chemical methods impose certain constraints in terms of their efficiency and cost economic point of view hence limiting its application on a large-scale platform. However, biological treatment has emerged as the most attractive and sustainable solution for reducing the gossypol content and improving the nutritional properties.

II. RESEARCH ELABORATIONS

A. Health implication of gossypol and its processing

Even though few reports are highlighting the medicinal properties of gossypol, a plethora of literature suggest that intake of gossypol has shown a deleterious effect on growth development and reproductive health of small ruminant and non-ruminant animals. The US FDA has set a limit of 450 ppm for free gossypol in human food products and 500 ppm for free gossypol in feed materials [1]. Thus, it is vital to reduce gossypol content below the prescribed limit for the safe utilization of CSM-based protein.

Generally, the gossypol occurs in the unbound free form, and the free gossypol binds to the ε-amino group of lysine, making it unavailable for intestinal absorption. Further, it also leads to health implications such as impaired body weight, respiratory distress, anaemia, weight gain, and reduced sperm motility/ concentration [3, 5–7].

To mitigate the toxicity of gossypol, several physical methods such as gland flotation, liquid cyclone, supercritical CO_2 extraction, and more recently, gamma and electron radiation methods have been employed. However, these methods tend to be expensive in their nature. Alternatively, chemical methods such as solvent extraction using green solvents have been attempted. These methods resulted in the reduction of gossypol content but are affected by parameters such as moisture content in seeds [1].

Despite the gossypol level reduction by physical and chemical methods the safety levels suggested by US-FDA are not met. Nevertheless, it is imperative to reduce gossypol cost-effectively to develop a circular bio-economy. Microbial cell factories offer a sustainable advantage over the former methods as they can degrade gossypol and also improve the nutrient content of CSM.

B. Research design

Studies on microbes belonging to the genera of *Bacillus, Aspergillus, Mucor, Candida,* and *Rhizopus have* displayed a tremendous reduction in gossypol content, but the exact mechanism of degradation is unclear. It is speculated that during microbial fermentation, either the microbes utilize gossypol as a carbon source, or it transforms free gossypol to bound gossypol contributing to the overall reduction in gossypol content in CSM [8]. To this end, various whole cell fermentation methods (solid (SSF)/submerged (SMF)) have been performed to detoxify the gossypol content. The preliminary step here is to isolate and screen the most robust strain, with the ability to degrade gossypol content followed by optimizing the process parameters

to maximize the degradation percentage without compromising protein content and other nutrients.

III. RESULTS AND FINDINGS

In the context of the biological route of de-gossypolization, Zhang *et.al*.[9] performed SSF for 48 hr using disinfected CSM as substrate. Among the microorganisms screened, C. *tropicalis* ZD-3, *S. cerevisiae* ZD-5 and *A. Niger* ZD-8 displayed an excellent detoxifying capacity with the percentage reduction of 94.57%, 88.51%, and 85.16%, respectively. Also, the crude protein content was improved by 10.76% and 22.24% with *C. tropicalis* ZD-3 and *A. Niger* ZD-8, respectively highlighting the significance of microbial fermentation.

On similar lines, Mageshwaran *et.al*.[10] isolated eight fungal strains using gossypol as the sole carbon/energy source and sequenced it using 18s rRNA. It was revealed that most of the strains belonged to the family of *Aspergillus* and *Fusarium* spp. When CSM was treated with *Fusarium thapsinum,* they observed a reduction of 65.2 of free gossypol.

The *Bacillus subtilis* GH38 isolated from the rumen of the fistulated cow have shown a reduction of 8.86% and 49% of free (FG) and total gossypol (TG), by maintaining the optimum condition of a temperature of 39℃, an inoculum of 10^7 cells/g, pH of 6.5 and incubation time of 72h [8].

Zhang *et.al*.[11] investigated the effect of carbohydrate, urea, mineral, and heat treatment on the reduction of free gossypol in the course of solid-state fermentation. The *C. tropicalis* ZD-3 strain has displayed an exceptional result in reducing FG. The supplementation of sucrose, starch, and minerals maximized the detoxifying percentage. Also, the fermentation resulted in the enhancement of crude protein and amino acid content. Kinetic studies performed under the impact of gossypol concentration on the growth of *Candida tropicalis* ZAU-1 in the inorganic salt glucose medium showed that the growth of the strain was unaffected even at the concentration of 1000 mg/L, and the degradation was about 94.12%.[12].

Rajarathnam *et al.* [13] reported that *Pleurotus Florida*, a white oyster mushroom grown on rice straw supplemented with cottonseed powder, showed a unique ability to degrade the gossypol content in cottonseed powder by using it as a nitrogen source and exhibited incredible growth ability. Furthermore, when the *Pleurotus Florida* was grown on synthetic media with gossypol as a nitrogen source, about 100 µg of gossypol was reduced with an inoculum load of 10 mg, temperature of 25℃, and incubation time of 10 days. There was no accumulation of gossypol in the budding fungi.

Cheng *et.al.*[14] has cloned and expressed *Helicoverpa armigera* CYP9A12 gene encoding cytochrome P-450 enzyme in *Pichia pastoris* under strong inducible Alcohol oxidase promoter (AOX). The authors adapted response surface methodology to optimize the process parameters and reported 40.91 mg/kg of FG by maintaining enzyme concentration of 2.5 ml, hydrolysis time of 2.5 h, and temperature of 35℃.

IV. DISCUSSION

Even though many microbial studies have been reported in the past to reduce the gossypol content, most of the organisms such as *Candida* and *Aspergillus* belong to the type II category and secretes toxins. The enzyme can be an attractive alternative as it is nontoxic, specific, and degrades with mild heat treatment, but only a few studies have reported on the enzymatic application. Since gossypol is a polyphenol content and is available abundantly in the lignin fraction of the cell wall, several enzymes such as Oxygenase, Phenolic Hydrolases, Manganese peroxidase, Laccase, Cytochrome p450, Hydrogenase, and methylase are frequently used in the treatment of lignin. Laccase has been shown to oxidize a wide range of phenolic/aromatic compounds [15]. Recently, Wang *et.al*.[16] showed oxidation of hydroxyl and aldehyde groups associated with gossypol, which decreases the toxicity level significantly. On the other hand, cytochrome P-450 plays a crucial role in the degradation of recalcitrant lignin-associated compounds [17]. The NIT-Warangal iGEM team is heading in this direction. We aim to screen competent enzymes through the computational tool and improve their stability/specificity via Insilco site-directed mutagenesis. Further, the enzyme will be cloned and expressed in the yeast system. The production will be enhanced using crude glycerol, which is a by-product of the biodiesel industry. Thus, addressing the problems associated with the food and biodiesel industry.

V. CONCLUSION

The association of gossypol in the cotton seeds is inevitable. But gossypol can be reduced via an appropriate processing strategy. To this end, several methods discussed in this review showcases the versatile approaches to tackle the gossypol problem in cottonseed meal. But none of these methods has yet been employed on an industrial scale. Biological techniques have precedence over other conventional methods used for gossypol pre-treatment and are sustainable in nature. However, the major drawback is, the majority of the ligninolytic producing strains are pathogenic in essence. Hence enzymatic production using a renewable carbon source is a possible solution for cost-effective reduction of gossypol and utilizing the CSM for food/feed purposes.

ACKNOWLEDGEMENT

We want to thank the 2021 iGEM Maastricht team for enabling us to participate in this initiative. A special thanks to our institute and Department of Biotechnology NIT Warangal for their support to the team. Sincere thanks to Dr. Ashish A Prabhu, PI, iGEM NIT_Warangal for his suggestions on the paper. Special thanks to Reticulum for sponsoring us. Special thanks to iGEM Foundation for enabling and supporting us in participating in iGEM 2021.

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AUTHORS

First Author – Ruchir Pratape, Undergraduate, Department of Biotechnology, National Institute of Technology Warangal. rp921878@student.nitw.ac.in

Second Author – Purna Chandra Sekhar V, Undergraduate, Department of Computer Science and Engineering, National Institute of Technology Warangal, vp932031@student.nitw.ac.in

Third Author –Maharshi Dixit, Undergraduate, Department of Biotechnology, National Institute of Technology Warangal. md83200@student.nitw.ac.in

Correspondence Author –Ashish A Prabhu, Assistant Professor, Department of Biotechnology, National Institute of Technology Warangal. ashishp@nitw.ac.in, ashishbiotec@gmail.com, +919582700174.

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Bio-Spire, Continuous Monitoring of Sepsis Biomarkers in Sweat

Amanda Adams*, Anca Frasineanu*, Blaine Dillingham*, Daniel Nakamura*, Ena Haseljic* , Irina Ter-Ovanesyan* , Jingyi Yan* , Maria Schapfel* , Muskaan Vasandani* , Nikol Pritsky* Tiana Salomon*, Tracey Moyston*, Helen Shammas*, Lynn Sidor*, Minseon Lee*, Alexis Stein*, Nancy Chen*, Anne S. Meyer*

*Team Bio-Spire, Department of Biology, University of Rochester, 500 Joseph C Wilson Blvd, Rochester, NY 14627

***Abstract* - Our team, Bio-Spire, is working on manufacturing a wearable biosensor device to diagnose sepsis using biomarkers found in sweat. Sepsis is a condition in which the body's response to an infection is too abundant. We are storing the receptors for sepsis-related biomarkers in *E. coli,* synthesizing them in-vitro and developing a sleeve-like biosensor that incorporates graphene, electrodes and microfluidics to monitor the changing levels of relevant biomarkers in patients. Additionally, mathematical modeling will be used to predict the concentrations of these biomarkers in blood based on the sweat samples, and a corresponding app will display a warning of the patient's elevated risk of sepsis. Our team is centering topics of safety, inclusivity, and ethics in our project, and we are doing outreach activities with a variety of local organizations to demonstrate basic scientific principles to elementary school-aged children.**

Index Terms- Continuous monitoring, iGEM, Sepsis, rGO

I. INTRODUCTION

Sepsis is caused by a dysregulated immune response of the body to a bacterial infection, eventually leading to dangerously low blood pressure and rapid deterioration of the patient (Teggert et al., 2020). According to the World Health Organization (WHO), sepsis accounts for roughly 20% of deaths worldwide, disproportionately affecting low- and middle-income countries. One of the reasons why sepsis is so fatal is the inability of health professionals to identify the onset of sepsis early enough to administer antibiotics. The most accurate test, a blood culture for bacteria, takes 24 hours and is often unreliable (Lever & Mackenzie, 2007). For this reason, we have come up with a method for accurately and quickly determining sepsis in patients through the continuous monitoring of a panel of inflammatory biomarkers that are more specific to sepsis. In blood, the most specific and studied biomarker of sepsis is procalcitonin (Pierrakos et al., 2020). However, to monitor the host's immune response and possible sepsis progression continuously, our device uses sweat, in which procalcitonin has not been found yet (Hussain et al., 2017). Therefore, we decided to use five other biomarkers that are present in sweat and are associated with sepsis: C-reactive protein (CRP), interleukin-6 (IL-6), interleukin-1β (IL-1β), tumor necrosis factor (TNF-α), and lactoferrin. CRP is an inflammatory marker elevated during infection, and IL-6 and IL-1β are associated with mortality rates of sepsis (Teggert et al., 2020, Bozza et al., 2005). TNF-α has proinflammatory effects caused by leukocytes activation and initiation of apoptotic, acute-phase responses (Sanchez-Munoz F Fau - Dominguez-Lopez et al.), and lactoferrin levels are elevated during infection (Kruzel et al., 2017). To detect these biomarkers, we decided to use ssDNA aptamers, which have higher specificity than antibodies, and they greatly improve the cost-efficiency of our device (Shatunova et al., 2020).

Since the device will be targeting sedentary post-operational patients, we must induce and transport their sweat. Microfluidics will collect, transport, and store small volumes of sweat (Reeder et al., 2019). To continuously induce sweat in resting patients, iontophoresis, which uses direct current to introduce ionized substance through intact skin, is considered. (Walia et al., 2000). Our device is a forearm sleeve with microfluidics channels inside. In patients, sweat will travel through the channels to sites where biomarkers bind to embedded aptamers, which are attached to reduced graphene oxide (rGO). This binding will induce a conformational change, thereby transmitting an electrical signal through the rGO-based electrodes (Aspermair et al., 2021). The readout of the change in electrical signal will be recorded as impedance and transmitted to a monitor that will distinguish between septic and healthy patients.

Reducing graphene oxide (GO) to rGO has traditionally been done with hydrazine, a highly toxic chemical (Lehner et al., 2019). The bacterial production of rGO from GO (using *Shewanella oneidensis* MR-1), is a growing field in synthetic biology, due to Shewanella's unique ability to export electrons into the surrounding media and reduce insoluble compounds (Lehner et al., 2019). This method of producing a conductive and versatile material is cost-effective, accessible, and safe. To more efficiently produce rGO, we plan to overexpress the riboflavin biosynthesis gene cluster - ribD, ribE, ribC and ribB/A to increase the production of riboflavin, which can shuttle electrons into the surrounding media to reduce extracellular molecules. We will also

express the porin oprF to increase the export of riboflavin from the cell. With higher amounts of riboflavin being made, we will overexpress ribF for the conversion of riboflavin to flavin adenine dinucleotide (FAD) and overexpress the transporter Bfe to export it out of the cell for reduction. Lastly, we plan to overexpress ydeH, diguanylate cyclase, in order to increase biofilm production to increase reduction through direct electron transfer (Liu et al., 2015). We hypothesize that with different combinations of the aforementioned genes we can get a safe, efficient alternative for production of rGO using microbial reduction with *S. oneidensis MR-1*.

To apply our method to the point of care setting, we consulted professionals from the medical field as well as experts on sepsis. Most notably, we discussed the most suitable location for our device, and have discovered that due to the fairly controlled setting of the post-surgical ICU, putting the sleeve on one forearm of the patient as they come out of the operation room is a good implementation. As our research suggests, there may be applications for our device in emergency rooms, with some modifications to the original design.

Our team has incorporated what we have determined to be the best practices and we have considered the cost of our device to make it as accessible as possible. Throughout the project, a wide range of educational and awareness efforts were extended to the public with the aim of improving early recognition of a critical condition.

II. RESEARCH ELABORATIONS

A. Materials and Methods

Materials
All oligonucleotide sequences were provided by IDT. Protein biomarkers were purchased from R&D. QuantiFluor® ssDNA System and dsDNA System were purchased from Promega. Monarch® PCR & DNA Cleanup Kit and Monarch® DNA Gel Extraction Kit were provided by NEB. Taq DNA Polymerase and dNTPs were purchased from Invitrogen.

Asymmetric PCR
Optimal forward to reverse primer ratios were used: 20:1 for TNF-α and lactoferrin, 30:1 for IL-1β, 15:1 for CRP and IL-6. For 220 µL PCR reaction master mix, 8.8µL 50mM $MgCl_2$(2 mM in PCR reaction solution), 4.4µL dNTP, 8.8µL DNA template purchased from IDT or miniprepped from *E. coli* culture, 22µL 10x PCR buffer, 2.2µL Taq Polymerase, 2.2µL forward primer, reverse primer concentration adjusted to proper ratio were mixed, with ddH2O until 220µL of final volume (Marimuthu et al., 2012).

PCR conditions were as follows: initial denaturation at 95°C for 1 min. Then, for the repeated steps, denaturation at 94°C for 45 seconds, annealing temperature at 53°C for CRP and IL-6, 63°C for TNF-α, 46°C for IL-1β, and 60°C for lactoferrin for 1 min were used, then elongation at 72°C for 1 min, repeated 20 times. Lastly, final elongation at 72°C for 5 min (Marimuthu et al., 2012). PCR products were analyzed on 5% agarose gel, with ethidium bromide.

Microbial Reduction of Graphene Oxide
Graphene oxide flakes were added to TSB to a concentration of 0.1 mg/mL. This solution was used to dilute an overnight culture of *S. oneidensis MR-1* to an OD600 of 0.1 in sterile 20 mL tubes. The tubes were spun in a drum at room temperature for 48 hours, with 2 mL samples being taken at 8, 24, and 32 hours. The samples were washed with 10 mL of DI water for 8 minutes at 6000 rpm three times, decanting and disposing of the supernatant each time. Finally, the samples were frozen until they could be characterized by Raman spectroscopy.

ssDNA Aptamer Purification
PCR products were purified using Monarch® PCR & DNA Cleanup Kit, oligonucleotides protocol (New England BioLabs).

ssDNA and dsDNA Quantification
Protocol was provided on the Promega website for QuantiFluor® dsDNA and ssDNA System. 200µL of fluorescent dye working solution was added into each well on a black 96-well plate. Then 10µL of each serial dilution (20ng/µL to 0.005ng/µL for dsDNA and 40ng/µL to 0.63ng/µL for ssDNA) were added in duplicates. 10µL of 1x TE buffer was added as blank control. 1µL of PCR cleanup samples were also added as duplicates. Mock PCR reaction without a DNA template was made for each aptamer to serve as a control. The fluorescence was measured using GEN5 software.

Aptamer Binding Assay
All aptamers were diluted in aptamer binding buffer (ABB: 20 mM HEPES, 2 mM $MgCl_2$, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM KCl, pH−7.4) and the biomarkers in their resuspension buffer (TNF-α in sterile PBS, CRP in sterile Tris-HCl at pH 8) . Then, the ABB was added to bring the volume to 300µL. Negative controls were as follows: ABB; ABB + aptamer (working concentration); ABB + target protein from R&D Systems® and Sigma-Aldrich (15 pM for CRP; 2 nM for TNF-α); ABB + aptamer + non-target protein (IL-6 at 100 nM). The assay was incubated for five minutes, and as another control, the target protein was added right before measurement as well.

Manufacturing a Microfluidic Device
The microfluidic device was first designed in COMSOL to simulate the fluid flow (smallest capillary 0.5mm wide, the largest 6mm, flow 0.001m/s). The device was composed of three different parts: an inverted Christmas tree-like structure, where sweat would be collected, a long channel for incorporating five electrodes and a reservoir with micropillars, where sweat evaporates. To make a master mold, a solid structure used to mold PDMS, a 3D design was created in Onshape with dimensions 169mm x 30.5mm x 5mm.. A Stratsys UPrint SE Plus 3D printer was used to print our master mold out of ABS plastic. PDMS was prepared by mixing PDMS base to curing agent in a 7:1 ratio (by weight). The mixture was vigorously stirred for 2-5 min, and poured onto the master mold. Then, the mold was incubated in a desiccator for an hour to remove all the bubbles. The master mold was incubated at room temperature for 48h to cure, and then removed with a scalpel (Blauch, 2019).

III. RESULTS AND FINDINGS

Synthesis of Aptamers

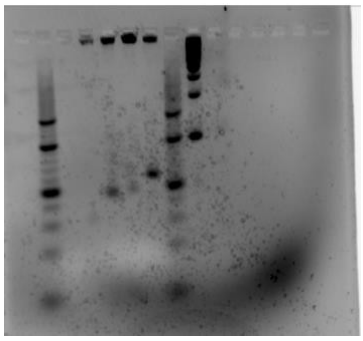

FIGURE 1.
5% agarose gel with following order: 10bp ladder, TNF-a, IL-1β, CRP, IL6, Lactoferrin, 10 bp ladder and 100bp ladder

Analysis of Aptamer Concentration.
Analysis of ssDNA and dsDNA concentration of each aptamer was done using fluorescence measurements. The standard curves equations are shown below: (1) for dsDNA and (2) for ssDNA.

$$[dsDNA]=\frac{average\ sample\ dsDNA\ fluorescence-average\ blank-408.41}{16428} \quad (1)$$

$$[ssDNA]=\frac{average\ sample\ ssDNA\ fluorescence-average\ blank+4928.9}{7438.3} \quad (2)$$

The quantification results in Table 1 indicated that the CRP PCR product had the highest ssDNA/dsDNA ratio of 7.92:1. Followed by IL-1β aptamer 4.54:1, and IL-6 4.53:1. Thus, generation of ssDNA with good yield was successful, and with ss:ds ratios similar or better to other reported values (3:2 in Marimuthu et al., 2012). Several replicates are needed for more thorough conclusions.

TABLE 1.
ssDNA and dsDNA quantification result from PCR

	ssDNA (ng/uL)	dsDNA (ng/uL)	ssDNA:dsDNA ratio
TNF-α	9.11	2.49	3.66:1
CRP	1.90	0.24	7.92:1
IL-1β	6.27	1.38	4.54:1
IL-6	7.83	1.73	4.53:1

Aptamer Binding Assay
Although currently the CRP binding assay has shown some information of binding, the aptamer binding assay is still in the process of improvement.

Design of a microfluidic device
The objective was to make a microfluidic design by using the master mold to make the final device out of PDMS. COMSOL was used to simulate a desired flow rate in channels and capillaries and Onshape was used to design our master mold for molding PDMS (Figure 2). Our first master mold was made out of ABS plastic, which was very rigid. As a result, capillaries almost broke, as their thickness was only 0.5mm.

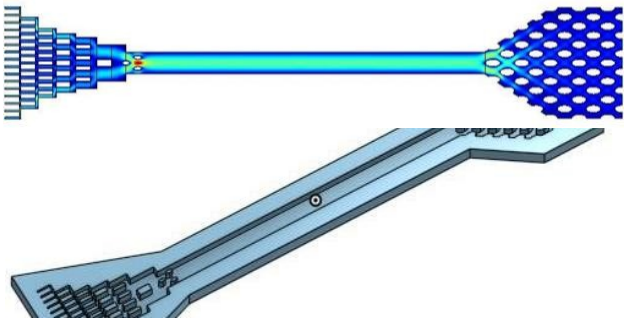

FIGURE 2.
COMSOL flow model (above) and Onshape 3D design of master mold (below)

IV. DISCUSSION

In Figure 1. we can observe successful synthesis of the aptamers, as CRP ran between 40 and 50 bp and the expected size is 44 nucleotides, and Lactoferrin ran between 50 and 60 bp and the expected size is 59 bp. IL-6 was also successfully synthesized, although in lower yield. For *IL-1*β, only the template is visible, which could be because the aptamer is too small (30 nucleotides) and low in the amount to be observed. A mistake was made for TNF-*a* and it didn't go inside the well.

For the binding assay (Figure 2), either certain improvements will be made to optimize it or a different assay will be tried. Since the K_D value for CRP aptamer was reported to be 6 pM, it was expected that, by using 1 pM aptamer concentration, protein-aptamer interactions would be observed at a wide range of concentrations, with different complex stabilities. We expected to see the typical 280 nm CRP protein UV-absorption peak being shifted to lower or higher wavelength as the complex formed. However, this shift was not clear and, to get better results, different concentrations will be tried. This way, the binding affinities of our aptamers will be calculated.

Our experiments showed that softer and more flexible material for a master mold was required for manufacturing the microfluidic device. To prevent capillaries from breaking when PDMS is taken out of the master mold, we are printing a new master mold made out of a flexible material.

V. CONCLUSION

Ultimately, the wearable device we are developing will aid in the rapid detection of sepsis using simple sweat collection and allow for the continuous monitoring of relevant biomarkers. This device incorporates microfluidics along with bacterially produced rGO and electrodes that will register and amplify the signal produced when binding of the aptamers occurs. We successfully expressed, purified, and quantified these aptamers in the lab and their binding was also assayed.

Throughout this process, our team considered human practices and inclusivity concerns, and we participated in outreach activities to educate others on scientific topics. We have also consulted with a

variety of medical professionals to ensure that our product will be useful in the early detection of sepsis.

ACKNOWLEDGEMENT

We acknowledge Dr. James L. McGrath and Jeffrey Beard for their help on the design of a microfluidic device, and Ram Gona for help with making rGO.

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AUTHORS

Correspondence Author – Dr. Anne Meyer, Faculty Advisor, anne@annemeyerlab.org

Chaperone Molecules: a Radioresistance Lead for Photosynthetic Organisms?

Maïa Henry*

* iGEM Sorbonne Université, Muséum National d'Histoire Naturelle de Paris

***Abstract*- Acute or chronic exposure to ionizing radiation (Ultraviolet C, gamma rays, X rays etc.) leads to the formation of reactive oxygen species (ROS), which affect the genome and the proteome of cells. Thus, although photosynthetic microorganisms, such as *Chlamydomonas reinhardtii,* constitute one of the main hopes for developing loop systems during long-term space travel, their use is called into question by the decrease of efficiency of the photochemistry and by the growth arrest caused by reactive oxygen species (ROS). Our project aims to make *Chlamydomonas reinhardtii* produce a peptide wich complex with the Mn^{2+} ion, inspired by a metabolite found in the radioresistant organism *Deinococcus radiodurans,* and act as a ROS scavenger. This study requires to demonstrate a decrease in ROS within the cell during production of the peptide and verify the growth of microalgae cultivated in minimum medium (photosynthesis dependent growth).**

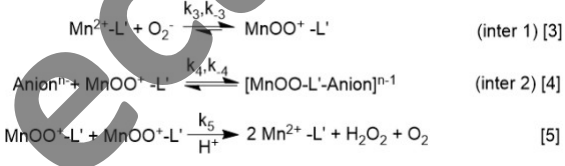
***Index Terms*-** *Chlamydomonas reinhardtii*, transgene, radioresistance, ROS scavengers.

I. INTRODUCTION

A space trip, such as an Earth-Mars/Mars-Earth journey, could last between one and three years (Horneck et al., 2006). The type and quantity of radiations absorbed by the crew would then be very different from those encountered in the shelter of the Earth's magnetic field: UVB, UVC but also X-rays and gamma rays, would reach doses close to 0.210 +/- 0.040 mGy/day (Hassler et al., 2014). The availability of food and breathable air resources are other limitations to long-duration space flights. To address these challenges, scientists are currently attempting to build loop systems that mimic the functioning of terrestrial ecosystems to convert crew waste into assimilable resources, including the use of photosynthetic organisms such as *Chlamydomonas reinhardtii* (Häder, 2020). However, the photochemistry and growth of this organism seem to be altered in several experiments, depending on the strain studied and the intensity and duration of radiation exposure, primarily due to the action of ROS (*Reactive Oxygen Species*) on its photosystem (Gomes et al., 2017).

ROS refer to chemical species that contain one or more unpaired electrons, such as hydroxyl radicals ($HO^{\cdot}$) or superoxide radicals ($O^{\cdot -}$), as well as species that do not possess a radical nature but can be converted to radicals, such as hydrogen peroxide (H_2O_2) (Munteanu et al., 2015). Depending on the environment or the situation, these radicals can behave as oxidants or as reductants and generate significant cellular damage (Munteanu et al., 2015): it is estimated that only 20% of DNA damage during radiation exposure would be directly due to radiation, while 80% would be due to indirect consequences of radiation such as ROS (Ghosal et al., 2005).

Radiation-resistant organisms typically have a significant manganese (II) oxide content: the cellular content of the *Deinococcus radiodurans*, for example, reaches up to 30mM of this compound, which is 15-150 fold higher than the total content of a radiosensitive organism (Peana et al., 2018). An Mn-Proteome interaction analysis performed *in silico* reveals that many Mn(II)-interacting biomolecules in *Deinococcus* play a more or less direct role in the defense against ROS. However, the action of Mn(II) is also direct: manganese-phosphate or manganese-carbonate complexes protect proteins by catalyzing the dismutation of superoxide ions via the formation of two intermediates during a second-order reaction (1).



(1)

(1) Catalytic mechanism for dismutation of a superoxide ion proposed by Barnese et al. (2012) The L represents phosphate and/or carbonate binding to manganese to form $MnHPO_4$ and/or $MnHCO_3^+$ and the n- anion an additional bond between the molecule and these compounds.

Previous studies by Berlett et al. (1990) already indicated the formation of such a complex but added a peptide component. These peptides act as ligands to activate Mn^{2+} and have a synergistic action with it. In 2016, a synthetic decapeptide (DEHGTAVMLK) was made by assembling some amino acids found in *Deinococcus radiodurans* lysate and was inoculated with Mn^{2+} and phosphate into mice. The mice survived an average of 30 days of 9.5 Gy radiation (LD70/30), while the control group showed lethality of nearly 63% at the end of this period. The team also demonstrated that their synthetic peptide was able to protect the activity of a T4 DNA ligase exposed to 60,000 Gy (Gupta et al., 2016).

Here we aim to reproduce this synthetic peptide in our photosynthetic model organism *Chlamydomonas reinhardtii* and verify its radioprotective action by toxicity tests (exposure to oxidants) *in vitro* and *in vivo* and by real exposure to gamma rays.

II. MATERIALS AND METHODS

A. Transformation of Chlamydomonas reinhardtii with undecapeptide (MDEHGTAVMLK)

The assembly method chosen during these experiments is the Golden Gate cloning method (Engler & Marillonnet, 2014). In addition, we used the MoClo Toolkit adapted to

Chlamydomonas reinhardtii made by Pierre Crozet's team (Crozet et al., 2018).

The double-stranded DNA sequence corresponding to the antioxidant peptide was suspended to reach a final concentration of 1ng/µL before ligation into the plasmid pAMG9121 (level 0 plasmid). Details of the ligation protocol are available in the appendix (appendix 1). Ligations were always performed using the restriction enzymes BbsI, BbsI-HF or BsaI-HF (NEB), CutSmart buffer (NEB) and a T4 ligase (NEB/Fisher). The transformation steps were performed on *E. coli* DH10β bacteria (New England Biolabs) rendered chemically competent by heat shock. Bacteria were grown on solid medium (Lysogeny Broth medium supplemented with 20% m/V agar, spectinomycin 50 µg/mL, X-gal 40 µg/mL from Sigma-Aldrich) overnight at 37°C. Colonies containing the plasmid of interest pL0-294 were re-grown overnight in a liquid medium. Plasmid DNA extraction was performed using the Macherey-Nagel "Nucleospin" kit following the manufacturer's instructions. The different ligation protocols for the L1 and LM levels are available in the appendix (appendix 1). Quality controls were performed at each step by monitoring the restriction profiles obtained for each insert using 1% agarose gel electrophoresis. The L0 level was also checked by sequencing. Transformation of *Chlamydomonas reinhardtii* by electroporation was set up following the Onishi Lab electroporation protocol (Onishi & Pringle, 2016).

B. Proof of concept of undecapeptide activity

The antioxidant activity of undecapeptide was tested on three oxidants: methyl viologen (1,1′-Dimethyl-4,4′-bipyridinium dichloride), hydrogen peroxide H_2O_2, and rose bengal (4,5,6,7-tetrachloro-3',6'-dihydroxy-2',4',5',7'-tetraiodo-3H- spiro-3-one). For this purpose, the LD50 of *Chlamydomonas reinhardtii* was determined for these three compounds.Increasing ranges of each oxidant were performed (appendix 2), and their activity on cells was monitored by UV-vis spectrometry and by cell counting after addition of 1% Evans blue by fluorescence microscopy. A concentrationrange was established for each oxidant in three conditions: a control, a first solution with manganese alone, and a solution with decapeptide or undecapeptide, for a total volume within each well of 50 µL (appendix 3).

III. PRELIMINARY RESULTS

A. Transformation of Chlamydomonas reinhardtii with undecapeptide (MDEHGTAVMLK)

All quality checks (sequencing of the level 0 plasmid, digestion and electrophoretic migration of the resulting plasmids) indicated that the intermediate plasmids pL0-294, pL1-226 and pM-156 were correctly assembled (see Figure 1 and 2).

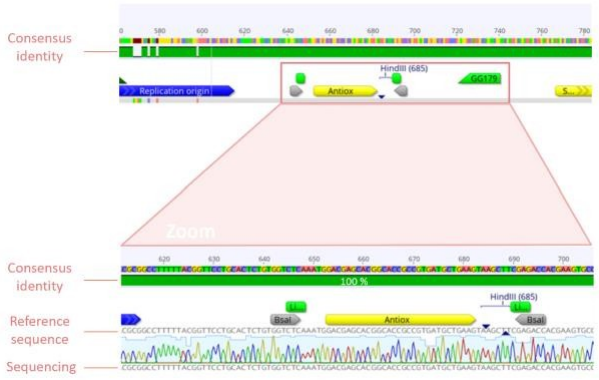


FIGURE 1.
Sequencing results of the first L0-level plasmid obtained. The results indicate a consensus identity between the expected sequence for pL0-294 and the sequence obtained.

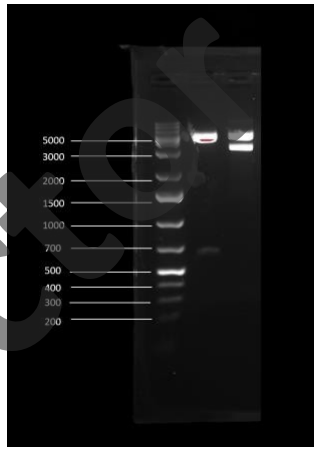


FIGURE 2.
Restriction profiles of the LM level plasmid. From left to right: scale: Generuler 1Kb Plus, pM-156 control (600bp band corresponding to LacZ and 4600bp band corresponding to the backbone), pM-156 (3175 band corresponding to the insert and 4600bp band corresponding to the backbone).

B. Proof of concept of undecapeptide activity

The cell death caused by the three oxidants can be measured qualitatively by the loss of colouration in the multi-well plates after several hours of exposure, or quantitatively by cell count or absorbance measurement (results not obtained). However, we do not yet have the results to establish the antioxidant activity of our undecapeptide.

IV. DISCUSSION

In this study, we had the photosynthetic model organism *Chlamydomonas reinhardtii* produce a peptide inspired by the amino acids of the radioresistant organism *Deinococcus radiodurans.*

We are currently awaiting preliminary results on the antioxidant action of our peptide. However, the observation of antioxidant activity would not confirm whether the increase in radioprotection comes from the formation of a Mn-peptide complex. In order to validate this hypothesis widely shared in the literature, and to rule out the less likely hypothesis of a direct radioprotective action of the metabolite, we would have to test the action of the peptide in isolation and to demonstrate the formation of a complex by NMR experiments coupled to X-ray

crystallography. These experiments would allow us to determine whether the peptide coupled to manganese indeed possesses antioxidant activity. However, the role played by this peptide would not yet be demonstrated: potentiation, activation, accumulation of molecules? To answer these questions, more detailed chemical and biochemical studies would be necessary. Finally, the demonstration of radioprotection at such scales (9.5 Gy) on photosynthetic organisms has not yet been studied to our knowledge. We want to test the efficiency of our undecapeptide in natural conditions, i.e. by exposing it to gamma and X-rays without having previously dehydrated the microalgae. We hope that understanding the mechanisms of action and the limitations associated with manganese could have many applications in space research, radiotherapy or nuclear industry.

V. CONCLUSION

Our preliminary results do not yet allow us to confirm the antioxidant activity of our peptide. Further research is necessary to understand the peptide's mechanism of action and investigate its role in inducing radioresistance in a photosynthetic organism. Additional *in situ* studies on the metabolism of *Chlamydomonas reinhardtii* under oxidative stress following actual exposure to ionizing radiation are also planned.

APPENDIX

Appendix 1: Ligation protocols.
https://docs.google.com/document/d/19KdRFd03ux3vvEVoAXQpSSpm3D_1ULWeYPkdYTZ_CAw/edit?usp=sharing

Appendix 2: Detailed protocol of toxicity test (LD50) of Chlamydomonas reinhardtii exposed to several oxidants.
https://docs.google.com/document/d/1WYR5KfO38dJTpr-DuFz8fBQpiUyMoCx04A0rDbsEJgI/edit?usp=sharing

Appendix 3: Detailed protocol for proof of concept of antioxidant activity of the peptide and synthetic undecapeptide.
https://docs.google.com/document/d/1dDL3pTD3P2ndFZg91gAbgsgecuofEXV3UKxxbXIoXK4/edit?usp=sharing

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ACKNOWLEDGEMENT

I want to thank Darine Ben Elbey for her proofreading and corrections and Théo Michielin for helping me with the scientific construction of this article.

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AUTHOR

First Author – Maïa Henry, student, Muséum National d'Histoire Naturelle, maia.henry@edu.mnhn.fr

Design of a bioaffinity-based system for rare earth element extraction from electronic waste

Rigel Tormon*, Helen Zhang*, Jonathan Chong*,
Ramin Kahidi*, Cedric Acierto*, Andrew Symes*, Juan Sebastian Alvarez*

iGEM Calgary, University of Calgary

***Abstract* - Due to the growing global demand for rare earth elements (REEs), there is great interest in recovering REEs from electronic waste; however, the compositional complexity of e-waste as a feedstock proves to be a challenge for the selective separation of REEs from other metal ions. Additionally, conventional methods are reagent-intensive and inefficient at low REE concentrations. Therefore, to produce a sustainable separation system with high throughput, lanmodulin, a lanthanide-binding protein, is immobilized on cellulose beads (CBs) using a cellulose-binding domain (CBD), and are ultimately packed in a fixed-bed adsorber column. A three-step modeling design workflow composed of determining the operational criteria, simulating the prototype design, and simulating the prototype performance is employed. This is utilized to determine preliminary values to initiate the design of a bench-scale prototype and the development of experiments needed to determine model fit parameters and to optimize the system.**

Index Terms - Adsorption, analytical modeling, protein immobilization, lanmodulin, electronic waste

I. INTRODUCTION

With the increased global dependency on information technology, there has been a corresponding global demand for rare earth elements (REEs). Recent attention has been revolving around electronic waste (e-waste) as an alternative feedstock, but the compositional complexity of e-waste presents a challenge for the selective separation of REEs from other metal ions. Standard processing methods such as solvent extraction and precipitation are reagent-intensive and inefficient at low ion concentrations, which is not ideal since e-waste typically has low REE concentrations. To address the existing process limitations of REE extraction, an alternative separation process is designed by using lanthanide-binding proteins and the throughput of fixed-bed adsorber systems.

The proposed extraction process immobilizes the protein, lanmodulin, on the cellulose beads (CBs), via fusion with a cellulose-binding domain (CBD) to enable high throughput lanthanide extraction. The design and optimization of an adsorber unit typically involves numerical modeling of interactions within the immobilization support of the fixed bed. However, this process involves identification of both kinetic and interaction parameters that require thorough experimental validation, thus limiting the determination of preliminary design parameters. Therefore, a robust analytical model is adapted into the design workflow to describe the process through simulating an adsorber unit prototype design and performance. This workflow for the adsorber unit consists of three principal steps: the determination of operational criteria, the simulation of the prototype design, and the simulation of the performance of the prototype design. The goal of this is to determine working values to begin development of a prototype and experiments needed to further optimize the system.

II. RESEARCH ELABORATIONS

A. Materials and Methods

All calculations were coded and performed in Python, while the graphs and figures were obtained with libraries such as NumPy, SciPy, Matplotlib on Google Colaboratory (https://colab.research.google.com/drive/1pYsr8Fi28PIN1gsxdRC2Yeh0IlZIoB9e?usp=sharing). The parameters were obtained from literature and used in the consequent prototype design simulation and prototype performance simulation.

B. Research design

The design workflow of the adsorber unit consists of three consequent steps, (1) determination of operational criteria, (2) prototype design simulation, and (3) prototype performance simulation.

a. Determination of Operational Criteria

The selection of appropriate operational criteria, which is made up of the treatment objective and process goal, allows for a target result and target process to be defined. The treatment objective identifies the quality of the final product, while the process goal outlines the rate at which the unit should operate to ensure a time-efficient system for potential industrial recovery of REEs from e-waste.

b. Prototype Design Simulation

The superficial velocity is approximated by standard flow velocities used in the chromatography column of which our proposed prototype is an analogue.

Then, the packed bed upflow velocities for varying flow rates and column diameters is determined and examined through graphing upflow velocity as a function of column feed flow rate. The flowrate Q, column diameter D, and upflow velocity v are related with the following formula.

$$Q = \frac{\pi D^2 V}{4} \qquad (1)$$

The velocity providing the maximum flow rate is evaluated for the pressure drop it generates. The effects of varying packed bed lengths on the column pressure drop are examined by graphing pressure drop across the column length as a function of flow rate, based on the Ergun relation (McCabe et al., 2014).

$$\frac{\Delta P}{Z} = \frac{150 V_0 \mu (1-\varepsilon)^2}{g_c \Phi_s^2 D_p^2 \varepsilon^3} - \frac{1.75 \rho V^2 (1-\varepsilon)}{g_c \Phi_s D_p \varepsilon^3} \qquad (2)$$

Where ΔP is the pressure drop, Z is the bed length, and V_0 is the superficial velocity, and all other variables are constants defined in Table 1.

Table 1. Process parameters descriptions and values obtained from literature

Parameter	Symbol	Value	Source
Diameter Particle	D_p	55 x 10^{-6} m	[5]
Dynamic viscosity	μ	1.0016 x 10^{-3} Pa*s	[9]
Minimum fluidization porosity	ε_m	0.45	[6]
Water fluid density	ρ	1000 kg/m3	-
Sphericity	Φ_s	0.6	[6]
Particle Density	ρ_p	1500 kg/m3	[7]
External void fraction	ε	0.4	[6]
Intraparticle porosity	ε_p	0.9	[3]
Adsorption Rate Constant	k	0.002 L/(min*mg)	[4]
Adsorptive Capacity	N_o	80 mg/L	[4]
Gravitational constant	g_c	9.81 m/s²	-
Superficial velocity	U_0	0.000100 m/s	[8]

Based on the graphical results of the computed process design parameters, appropriate sets of column diameters and packed bed heights are selected to align within the operational criteria defined in section I.

c. Prototype Performance Simulation

The Bohart-Adams model is used to determine the breakthrough curves through graphing relative concentration with respect to time. The Bohart-Adams Model gives the following equation: (Bohart & Adams, 1920)

$$\frac{C}{C_0} = \frac{exp(kC_0t)}{exp(kN\frac{Z}{U_0})-1+exp(kC\ t)} \quad (3)$$

From the set of possible design parameters in section II, the best set of parameters can be chosen that meets the process goal and treatment objective from section I.

III. RESULTS AND FINDINGS

A. Operation Criteria

a. Treatment Objective

Previous work has revealed that the extraction efficiency for REEs can be as high as 73% (Diaz et al., 2016). Therefore, the target treatment objective is designed to recover 80% of lanthanides in solution. By extension, the optimal operation time is defined as the point at which the process reaches a breakthrough concentration fraction (C/C_0) of 0.2.

b. Process Goal

Upon liaising with electronic waste processing facilities in Alberta, a standard facility is revealed to be capable of processing 500-600 metric tonnes of e-waste monthly. Using the material fraction in e-waste (Widmer et al., 2005) and the composition of a steel stream from electronic waste (Lister et al., 2016), the mass rate of lanthanides processed by the facility is estimated to be 6.02 kg/day.

The prototype unit is designed to have a capacity of a chromatography column. Hence, the process goal for the prototype performance is 602 g/day, which is based on a 90% scale-down of the estimated industrial processed lanthanide mass rate.

B. Prototype Design Simulation

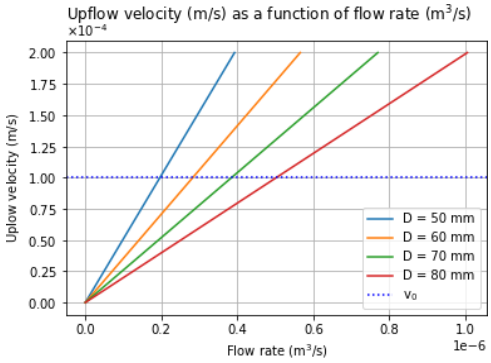

FIGURE 1: Upflow Velocity as a Function of Flow Rate at varying column diameters

The simulation results demonstrate how the upflow velocity is directly proportional to the flow rate. Maintaining a constant flow rate also shows how the upflow velocity is inversely related to the column diameter. To avoid fluidization of the packed bed, the maximum working velocity is set at the literature value of 0.0001 m/s (Swartz, 2007). A column diameter of 80 mm is chosen to accommodate the maximum operable flow rate.

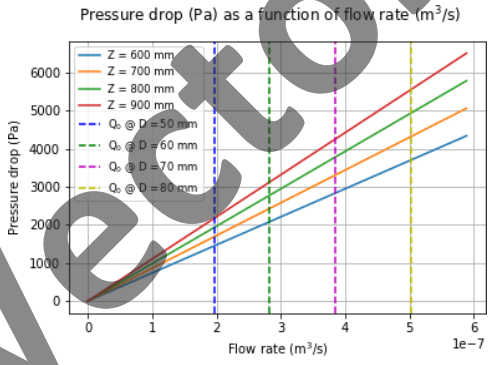

FIGURE 2: Pressure drop as a function of flow rate at different column lengths

Relationships between key design variables are displayed in Figure 2. The pressure drop across the column is directly proportional to the flow rate of the influent. At a constant flow rate, it is observed that pressure drop across the column increases with bed length. Minimizing pressure drop is desirable for the sake of integration into an existing processing facility. Furthermore, cellulose beads found at the column outlet may be compressed due to extreme pressures and swelling due to water retention. Using a column diameter of 80 mm, a bed length of 600 mm is concluded to minimize the pressure drop.

C. Prototype Performance Simulation

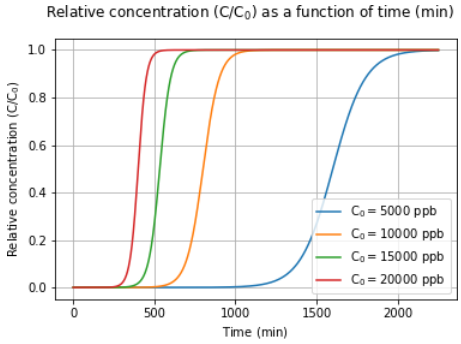

FIGURE 3: Relative concentration (C/C_0) as a function of time at varying influent concentrations

Using the selected values for the absorber column design (D = 80 mm; Z = 600 mm), the breakthrough behaviour of the column

is simulated. Figure 3 displays the dependence of the breakthrough curve on the influent concentration. It can be observed that higher influent concentrations correspond to decreasing breakthrough times. Furthermore, higher influent concentrations also lead to steeper breakthrough curves, minimizing the mass transfer zone width and indicating efficient utilization of the packed bed.

IV. DISCUSSION

Given the novelty of lanmodulin and the use of CBs as material supports for its immobilization, confirmatory experiments should be performed to accurately determine the parameters involved in predicting the efficacy of the adsorber unit's operation. Importantly, there is not a term within the model formalism that describes the parameters used in the prototype design and performance simulations as specific to the proposed system. To accommodate missing parameters describing CBs in adsorber columns and the activity of lanmodulin values for analogous systems are used when appropriate. Additionally, values extracted from the simulations are only relevant for the scale of a chromatography column used for bench-scale processing, which operates at a significantly lower throughput than that of industrial scales. Hence, caution should be taken when extrapolating data from the results. The workflow described for the design of a prototype unit would be suitable for characterizing the dimensionless groups and parameters required for scale-up analysis.

V. CONCLUSION

Based on the results from prototype design and performance simulation, a column design using a combination of the largest absorber diameter (80 mm), shortest adsorber bed length (600 mm), and the highest influent concentration (2×10^4 ppb) are ideal initial design parameters. Once the preliminary analytical model is performed, the next step would be the physical construction and assembly of the prototype. Experimentation with the prototype adsorber column would allow evaluation of the fit of the analytical model, as well as iterate assumed process parameter constants with experimentally derived values.

ACKNOWLEDGEMENT

We thank Dr. Anders Nygren and Dr. Mayi Arcellana-Panlilio for sponsoring our research. We thank the University of Calgary's Provost, Dru Marshall, and the Vice-President (Research) William Ghali. We thank the faculty of Science, the Cumming School of Medicine, and the Schulich School of Engineering who provide support to the iGEM program at the University of Calgary. We thank the PURE Program and the Schulich School of Engineering Activity Fund for sponsoring our research. We thank Tian Zhao (as our team's TA), and Tammy Lu (as the team's RA).

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AUTHORS

First Author – Rigel Tormon, Current Undergraduate Student in Chemical Engineering minoring in Biomedical Engineering, University of Calgary, Cumming School of Medicine, Email: rigel.tormon@ucalgary.ca

Second Author –Helen Zhang, Current Undergraduate Student in Chemical Engineering minoring in Biomedical Engineering, University of Calgary, Cumming School of Medicine, Email: helen.zhang1@ucalgary.ca

Third Author – Jonathan Chong, Current Undergraduate Student in Software Engineering minoring in Biomedical Engineering, University of Calgary, Cumming School of Medicine, Email: jonathan.chong@ucalgary.ca

Fourth Author – Ramin Kahidi, Current Undergraduate Student in Bioinformatics, University of Calgary, Cumming School of Medicine, Email: ramin.kahidi@ucalgary.ca

Fifth Author – Cedric Acierto, Current Undergraduate Student in Software Engineering minoring in Biomedical Engineering, University of Calgary, Cumming School of Medicine, Email: johncedric.acierto@ucalgary.ca

Corresponding Author – Andrew Symes, BSc Statistics concentrated in Nanoscience, Current Masters of Engineering Student with the Schulich School of Engineering, University of Calgary, Cumming School of Medicine, Email: andrew.symes1@ucalgary.ca

Corresponding Author - Juan Sebastian Alvarez, Current Undergraduate Student in Chemical Engineering, University of Calgary, Cumming School of Medicine, Email: juan.alvarez1@ucalgary.ca

DOPL LOCK: Creating a Comprehensive Biosafety System

Sebastiaan Ketelaar[*], Siheng Li[*]

[*] iGEM Leiden 2021

***Abstract*-A major challenge in synthetic biology is the question of bio-containment and safety. There are many different strategies for dealing with this issue. These include auxotrophy, kill-switches, cell-free systems and more. This year, the aim of team iGEM Leiden 2021 was to help with building a system for bio-containment that can help mitigate the environmental risk of using synthetic micro-organisms outside of the lab. In order to do this, a system was designed of two plasmids, which are mutually dependent. This was achieved by creating a double toxin-antitoxin cassette, where both plasmids contain a toxin and the antitoxin for the sister plasmid. A kill-switch is to be built into the system by placing the antitoxins downstream of a conditional promoter. In this way, both horizontal gene transfer and the spread of microbes containing synthetic genes can be prevented, helping future iGEM teams realize their own projects which involve the semi-contained use of genetically modified bacteria.**

***Index Terms*-** iGEM, toxin-antitoxin, synthetic biology, biosafety, biocontainment, iGEM

I. INTRODUCTION

A major challenge facing synthetic biology is the biological containment of GMOs. There are numerous safety risks and ethical objections to GMO release into the wild, largely since current systems have not been deemed to satisfyingly ensure that artificial genetic constructs will not escape confinement. We propose an easily-applicable system that prevents horizontal gene transfer (HGT), using a mutually dependent double plasmid system, called DOPL LOCK, or 'Double Plasmid Lock'.

DOPL LOCK is a set of plasmids, which carry two toxin/antitoxin (TA) systems. Both plasmids will carry the antitoxin for the toxin on the other plasmid. This ensures that each plasmid cannot exist in a cell without the other. We hypothesize that the chances of both plasmids being transferred to a wild-type bacteria is orders of magnitude smaller than the chances of HGT of just one plasmid.

One focus of our research is to find a balance between the different components of our system. Different origins of replication (Ori's) need to be used to prevent plasmid hybridization and segregation. This will however lead to a difference in copy-number, which in turn leads to differential expression (Plotka et al., 2017). Further, due to differences in toxicity and functionality, the expression of the TA's need to be balanced to exert enough selection pressure for both plasmids. Fluorescent proteins were used to compare relative expression levels of the genes under the promoters that will be incorporated in DOPL LOCK upstream of the TA systems.

II. RESEARCH ELABORATIONS

A. Materials and Methods

Plasmids

For making our system, the JUMP (Joint Universal Modular Plasmids) series of plasmids were used. Specifically, pJUMP26, pJUMP27, pJUMP28, pJUMP29 and pJUMP46, pJUMP47, pJUMP48 and pJUMP49 were used. The 20JUMP plasmids contain a kanamycin and the 40JUMP plasmids contain a spectinomycin resistance cassette. These plasmids are completely identical save for the selection marker and the origin of replication, which is determined by the second number in the row. There are 3 compatibility groups in the plasmids we selected, namely 6, 7 and 8/9. This gave us flexibility in determining which plasmids to use for our system.
Further, the standard iGEM plasmids pSB1A3, pSB1C3 and pSB1K3 were used for cloning purposes.

TA systems

4 different TA systems native to E. coli (Bernard & Couturier, 1992; Christensen et al., 2001; Marianovsky et al., 2001; Pedersen & Gerdes, 1999) were chosen for cloning. These systems were selected since the organism we will clone with is E coli and this made cloning the TA systems easier. The TA systems selected were ccdA/ccdB, HOK/SOK, MazE/MazF and RelB/RelE.

Promoters

In order to maintain equilibrium in the expression of toxin and antitoxin, choosing the right promoter-sequences for our system was essential. Therefore, we mixed conditional and constitutive promoters. pBAD was used for a conditional promoter, which can be induced with arabinose(Khlebnikov et al., 2001). This is particularly convenient when using E coli DH10b, also known as TOP10, since it is unable to metabolise arabinose and therefore will keep expression at a stable level.
For the constitutive promoters, biobricks from the registry were selected from Part:Bba_J23100, which is a series of constitutional promoters of different levels of expression. These promoters however, can be switched with others by future teams planning to use our system.

Cloning

For cloning, the standard bio-brick system was used. This system is very suitable for restricting and ligating DNA sequences in a modular way. All bio-brick contain flanks that have an EcoRI and XbaI restriction site upstream and a PstI and SpeI restriction site downstream. This means that you can clone 3-way ligations, by cutting your vector with EcoRI and PstI, your upstream insert with EcoRI and SpeI and your downstream insert with XbaI and PstI, and they will only ligate in the correct

orientation. All restrictions were performed at 37° C, while ligations were done at room temperature.

III. RESULTS AND FINDINGS

Co-transformation of JUMP plasmid

Different Ori's give plasmids with different copy numbers, which directly influences the expression of the inserted genes. To stabilize the TA system, we first investigated which Ori combination could be co-transformed easily and whether they are compatible.

To do this, an RFP cassette from the standard iGEM plasmid pSB1C3 was inserted into pJUMP40's. Combinations of pJUMP40's and pJUMP20's were co-transformed into DH5α and plated on agar with kanamycin and streptomycin. Successful transformants were picked out and their phenotypes were verified by fluorescent microscopy (Figure 1).

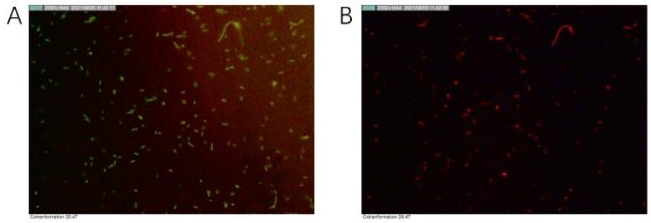

FIGURE 1.
Cotransformation of pJUMP26 and pJUMP 47. Most bacteria carried two plasmids simultaneously and showed sfGFP and RFP fluorescent at the same time.

Then, co-transformed strains were cultured until they reached optical density (OD) = 1. 5, replicates were diluted with LB to varying OD levels and their fluorescence was tested by plate reader. Single-plasmid transformed strains were used as control.

FIGURE 2.
Expression level of sfGFP and RFP in Cotransformed strain.

The results of the assay showed a complex interaction between the two series of JUMP plasmids (Figure 2). In general, fluorescent protein expression in single-plasmid strains was greater than most double-plasmid strains. High compatibility was observed between Ori p15A (in pJUMP26 and pJUMP46) and Ori pBR322 (in pJUMP29 and pJUMP49). Their combinations all showed high-levels of expression of both fluorescent proteins. The combination of pJUMP26 and pJUMP49 exhibited the most similar, high expression levels and was selected as the plasmid backbone of our double plasmid system.

Calibration of pBad inducible promoter

Calibration of pBad was carried out by constructing plasmid vectors of pBad-mCherry cassette and testing the expression of mCherry under different arabinose concentrations.

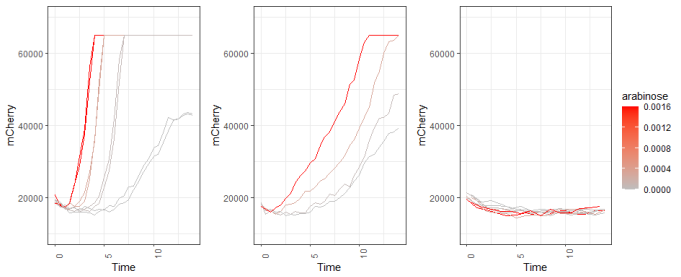

FIGURE 4.
Expression of mCherry under different inducer concentrations in strains carrying a pBad-mCherry cassette. left: mCherry expression in TOP10; middle: mCherry expression in DH5alpha; right: bacteria without pBad-mCherry as negative control. X-axis shows time, y-axis shows relative fluorescence

The result was shown in Figure 4. The induction efficacy of pBad promoter was greater in TOP10 than DH5alpha. In TOP10, leaky expression of pBad after 7h could lead to accumulation of mCherry that was detectable by plate reader. Interestingly, the arabinose concentration at 0.0016% already led to high-level expression of mCherry within 3 hours.

Toxicity assay

Four toxins, CCDB, HOK, MazF and RelE were tested for their toxic effect on E.coli. We constructed pBad-toxin cassettes in pJUMP via 3A assembly. The plasmids were transformed into the TOP10 strain and grown overnight. We diluted the overnight culture to OD=0.5 and added them to fresh LB medium with different inducer concentrations. The OD of these strains after 10h were tested and their growth curves were shown in Figure 3.

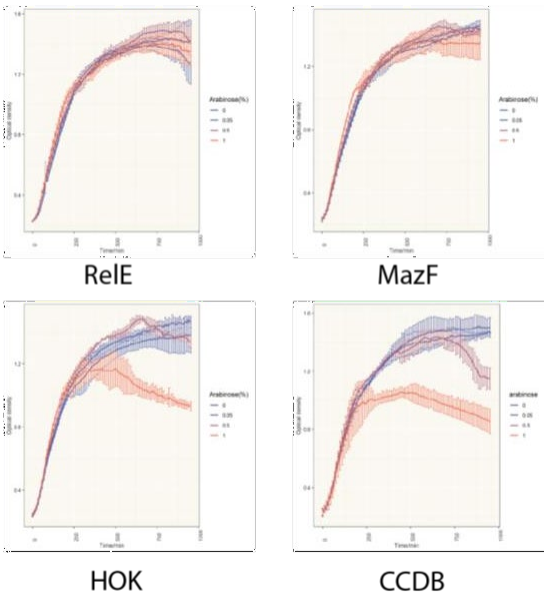

FIGURE 3.
Growth curves of pBad-toxin strains under different concentrations of arabinose. The colours of the line show the relative arabinose concentrations, ranging from 0 to 1% w/v.

HOK and CCDB exhibited considerable inhibitory effects on cell growth. However, their toxicity was not enough to kill all bacteria. According to Nathan Fraikin who we spoke to in an interview, the OD should have fallen to near starting value due to cell lysis (Kleinjan, Lisa). CCDB had the most pronounced effect in killing cells, showing very clear inhibition of cell growth. RelE and MazF however, have been shown to have a cytostatic, rather than cytotoxic effect, despite previous literature describing them as such (Pedersen et al., 2002).

IV. DISCUSSION & CONCLUSION

Unfortunately, due to difficulties in cloning, we have not been able to realize the full system. The time available in the lab was not sufficient to fully clone all constitutive and inducible promoters in front of the toxins and antitoxins, fuse it into one plasmid and co-transform them to *E. coli*. However, we have been able to make some advances in quantifying pBAD expression and relating comparing it to the expression of constitutive promoters (Li, 2021). In addition, after reviewing our results we consulted with experts on toxins and antitoxins due to our induction of pBAD::toxin gene fusions not killing most ofthe cells (Kleinjan, Lisa_. Nathan Fraikin was surprised upon learning this, but after some discussion hypothesized that one of the possible causes for this might be that the cultures were liquid rather than solid medium. This could mean that cell lines that get a mutation in the promoter-sequence might quickly out-compete the other cells. In the future, these experiments should be done on solid medium. Further, the full system needs to be constructed in order to validate the efficacy in vivo.

ACKNOWLEDGEMENT

Akos Nyerges for giving advice on the possible future experiments of our project. Amalia Kallergi for giving us insights into the problem of the validation of a biocontainment system. Barbara Brunnhuber and Stéfan Ellenbroek for sharing their knowledge in setting up a start-up and bringing us in contact with other companies. Braden Gilbert for sharing his expertise with us on the application of our system in bioremediation. Erik Vijgenboom for his help in designing the experiments. Nathan Fraikin for giving advice on the possible future experiments of our project. Renée Kapteijn for her help in designing the experiments and for sharing her expertise in our expert panel during the discussion evening.Tom Ellis for sharing his knowledge on biocontainment systems. Ellen de Waal and Helga van der Heul from the IBL for their help to order the necessary supplies for our experiments. Kees Koops from the IBL for allowing us to use his laboratory and machines over the summer. Gerda Lamers from the IBL for her permission and instruction on using confocal microscopy.

AUTHORS

First Author – Sebastiaan Ketelaar, Bsc Biology, Leiden University, s1715194@leidenuniv.nl

Second Author – Siheng Li, Bsc Chemical Biology, Xi'an Jiaotong University, s.li.16@umail.leidenuniv.nl.

Carola Feijt, Floor Stel and Tijn Delzenne from the IBL for their permission, help with booking and instruction on how to operate the plate reader, respectively.

Thomas van Dijk and Noortje Dannenberg from the IBL for their instruction and help to use the FACS machine.

Hein van der Meer from the IBL for his bright, positive attitude and warm welcome every morning at the Sylvius Laboratory during our summer lab work.

Lennart Schada von Borzyskowski for providing us with the E.coli Top10 strain.

Ivo Gariboldi for his help on the electrophoresis experiments. He provided us with a protocol and lent us cuvettes for the electroporation machine.

Jo-Anne Verschoor and Marjolein Crooijmans for providing us with feedback on our experiments.

Dennis Claessen for his critical comments on our data and the suggestion to use minimal medium in our experiments.

David Foschepoth for his critical questions on our data.

Floor Stel for her continuous support.

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Engineered *E.coli* Bacteria for Non-Invasive Monitoring of IBD with Ultrasound

Josephina Smits[*], Fleur Kalberg[*], Ingrid Kolen[*], Daniek Hoorn[*], Werner Doensen[*], Tessa Warmink[*], Naomi Span[*], Luke Rossen[*]

[*] Team iGEM Eindhoven 2021, Eindhoven University of Technology

***Abstract*- IBD is a chronic inflammation of the intestine. Worldwide, 6 to 8 million people suffer from this disease, and this number increases annually. Currently, monitoring of IBD is predominantly executed with a survey and endoscopy, of which the latter is invasive and unpleasant for the patient. Here, we propose an alternative non-invasive monitoring technique of new flare-ups of the disease. The proposed monitoring setup is based on the genetic engineering of *E.coli* to sense tetrathionate - an inflammatory marker - in the colon. Sensing is achieved by enriching the bacteria with the two-component TtR/S sensor system. TtrS, the receptor in the bacterial cell membrane, is phosphorylated (pTtrS) due to the presence of tetrathionate. Afterwards, pTtrS phosphorylates TtrR (pTtrR), which in turn activates the acoustic reporter genes (ARG) or a GFP signal. Once ARG is activated, twelve proteins should form tiny gas vesicles inside the bacteria. These vesicles can be measured using ultrasound technology, giving, in the future, quantitative non-invasive monitoring of flare-ups.**

***Index Terms*-** Non-invasive Monitoring, IBD, Two-component TtrR/S sensor system, Acoustic Reporter Genes, Ultrasound.

I. INTRODUCTION

Inflammatory bowel disease (IBD) is an umbrella term used to describe disorders that involve chronic inflammation of the digestive tract. The exact cause of IBD is unknown, however, it is thought to be the result of a defective immune system (Gajendran *et al*, 2018). Currently, the prevalence of IBD continues to increase globally (Kaplan & Ng, 2017). Nowadays, around 6 to 8 million people suffer from IBD worldwide (GBD, 2020).

The currently used monitoring consists of two components; a telemedicine system and a home faecal calprotectin test. Patients are requested to complete a survey monthly, which contains questions on all facets of this complex disease (de Jong *et al*, 2017). Next, the patient has to perform a home faecal calprotectin test, which is a point-of-care device based on the lateral flow that measures the level of calprotectin – an inflammatory marker mostly present in white blood cells – in patients' stools (Ricciuto & Griffiths, 2019; DiagnostiekvoorU, n.d.) If these two components indicate the presence of a flare-up, the patient has to undergo an endoscopy in the hospital for further validation. Out of conducted interviews with patients, it was shown that they experience an endoscopy as very intrusive, as they dread the laxative 2 day period beforehand.

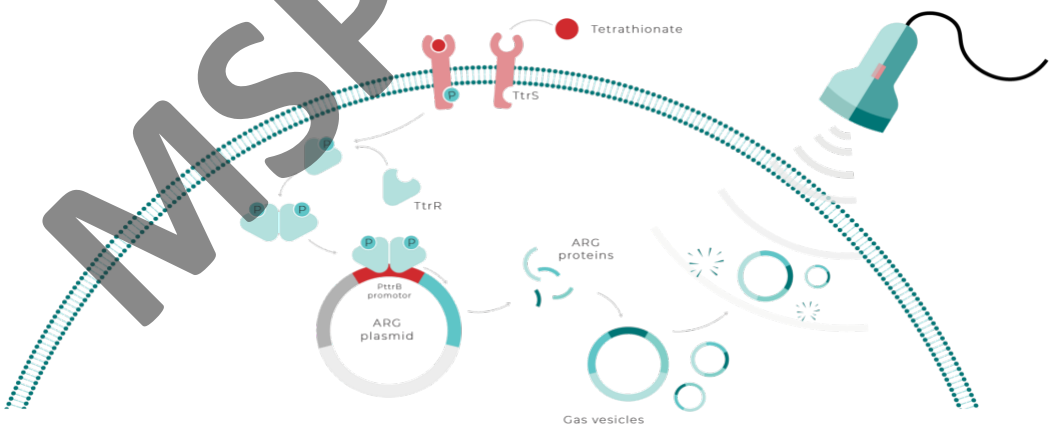

FIGURE 1: Overview of the full pathway, consisting of the combined two component TtrR/S system and the ARG plasmid resulting in versicles.

In this paper, a new way of monitoring will be researched to make the procedure for the IBD patients more specific and pleasant and less invasive and costly. Therefore, a design has been developed where a combination of the two-component system TtrR/TtrS for registering the tetrathionate, the inflammation marker, will be combined with an Acoustic Reporter Gene (ARG) (fig. 1). It is hypothesized that this design

can detect tetrathionate and produce gas vesicles that can be measured via ultrasound technology.

II. MATERIALS AND METHODS

A. *Bacterial strain, plasmids, and culture conditions*

The bacterial strain, plasmids, and antibiotics used are summarized in table S1. Standard heat shock co-transformation was used to implement the plasmids into the bacterial strain. The *E.coli* strain was grown routinely in Luria-Bertani (LB) agar plates with their corresponding antibiotics. Following this, a small and large culture with LB medium was performed to further grow the colonies before measurements took place. The *E.coli* cultures were induced with their respective competence for either their ARG protein expression or their GFP expression potential after the OD600 reached 0.6.

B. *GFP Measurement*

GFP measurement took place before and after the lysis of the cells. Measurements were taken with the Cary Eclipse Fluorescence Spectrometer (Agilent).

C. *Ultrasound Measurement*

Measurements were taken with a Vantage programmable ultrasound scanner system and L22-14v 128-element linear array transducer (Verasonics). E.coli was anchored in a gel consisting of Agarose - PBS (1%W/V), which is mixed with the induced large cultures in a ratio of 1:1.

D. *Nanodrop and SDS Page*

The *E.coli* strain with inserted ARG Plasmid after induction was purified and the concentration of protein was measured with Nanodrop 1000 (Thermofisher Scientific). To check if the right proteins were formed, a standard SDS Page was performed.

E. *Restriction and Ligation*

Two combinations of plasmids for ARG and TtrR will be made, using the restriction enzymes SgrAI and NheI.

III. RESULTS AND FINDINGS

A. *Co-transformation and Culture of TtrR/S and ARG Plasmids*

Co-transformation and subsequent culturing of the two-component system of TtrR/S took place simultaneously with co-transformation of ARG. Both were inserted in the *E.coli* strain as mentioned in table S1. Results can be found in Figures S1 and S2.

Co-transformation of the recombination of the TtrR and ARG Plasmids are not shown, as data was insufficient.

B. *GFP Measurement after Tetrathionate Induction*

After inducing the large culture with tetrathionate, samples were grown overnight. In the morning, samples were measured before and after the lysis of the cells. Results can be seen in Figure 2 (as well as supplementary figure 3) and suggest a dose-response starting from a tetrathionate concentration of 10 µM.

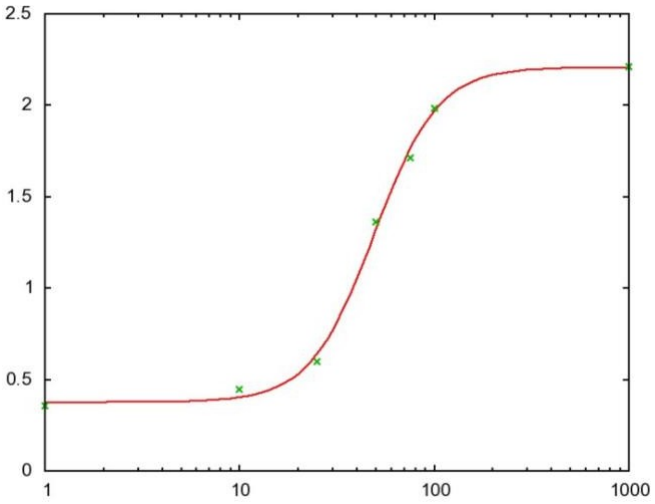

FIGURE 2: Dose-response curve of the normalized GFP emission for different concentrations of tetrathionate.

C. *Ultrasound Measurement*

After anchoring the induced *E.coli* strains with the ARG plasmid, the plates were measured with ultrasound (figure 3). Here, the vesicles could be seen in white on the black background. This suggests that potentially vesicles were made, as background noise is reduced utilizing subtracting pre- from post image after the collapse of the vesicles.

D. *Protein Measurement of ARG*

To check if the vesicles seen in figure 3 (and supplementary figure 4) were indeed the ARG proteins produced, protein concentrations were measured with an SDS-Page (supplementary figure 5).

IV. DISCUSSION

The above results suggest a dose-response with tetrathionate in the two-component system TtrR/S, although the presented data thus far only shows a promising result for the higher concentrations of tetrathionate (Figure 2). With regards to measuring in the intestine, further research needs to be done to see if optimization for smaller concentration measurements is necessary.

With regards to the setup for ARG proteins, an SDS Page was performed to see if proteins were formed as a control. This resulted in promising bands in the range of where the proteins

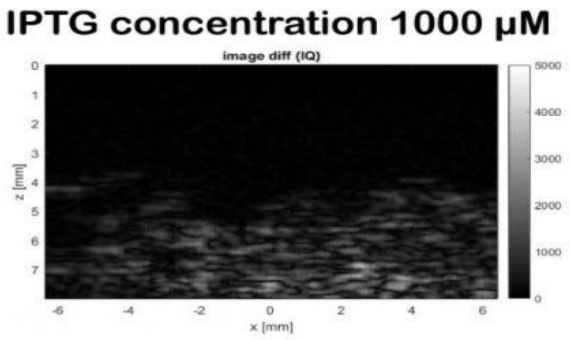

FIGURE 3: Ultrasound Images difference between pre-and post-images of an IPTG concentration of 1000 µM.

should be on the gel (Sup. Fig. 5). Although, one needs to keep in mind that in a cell also other proteins within the same range may be present. Also, it might be that the proteins interact with each other, and therefore form a band at a higher kDa as when they are single proteins (Völkner *et al*, 2020).

V. CONCLUSION

In this article, we proposed a new non-invasive monitoring setup for IBD patients based on the genetic engineering of *E.coli*, making them able to sense tetrathionate in the colon. We have proven that the implementation of the two-component TtrR/S sensor system was successful, as well as that the marker tetrathionate can be measured and results in a signal of GFP. Even more, we have succeeded in a dose-dependent GFP signal (fig. 2). Separate research on the ARG proteins has given sufficient results to believe that gas vesicles are being formed (fig. 3). Even more, the proteins which coagulate into the gas vesicles were seen on the SDS Page. Still, further research will need to be conducted, given the fact that the cotransfection of the combination of the two components was not successful yet.

APPENDIX

Supplementary figures of results can be found behind the link: https://docs.google.com/document/d/1NmGj3cKNtJYkoFvWQFWF3hYgK-0sVAEMjm8WiOWKxh0/edit?usp=sharing

ACKNOWLEDGEMENT

We thank our fellow team members for participating in the research presented here. We thank our daily supervisor, dr. Yan Ni, for her help in the lab and all her suggestions which made our laboratory work better. We would like to thank Ir. Hein de Hoop and Hans-Martin Schwab, PhD, for their help with the ultrasound code and setup. At last, we would like to thank our overall supervisor's prof. dr. Maarten Merkx, prof. dr. ir. Tom de Greef, and prof. dr. ir. Luc Brunsveld without their support and critical views this project wouldn't have even started.

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AUTHORS

First & Corresponding Author – Josephina Smits, BSc., Team iGEM Eindhoven 2021, Eindhoven University of Technology and igem@tue.nl.

Second Author – Fleur Kalberg, BSc., Team iGEM Eindhoven 2021, Eindhoven University of Technology and igem@tue.nl.

Third Author – Ingrid Kolen, BSc., Team iGEM Eindhoven 2021, Eindhoven University of Technology and igem@tue.nl.

Fourth Author – Daniek Hoorn, BSc., Team iGEM Eindhoven 2021, Eindhoven University of Technology and igem@tue.nl.

Fifth Author – Werner Doensen, BSc., Team iGEM Eindhoven 2021, Eindhoven University of Technology and igem@tue.nl.

Sixth Author – Tessa Warmink, BSc., Team iGEM Eindhoven 2021, Eindhoven University of Technology and igem@tue.nl.

Seventh Author – Naomi Span, Team iGEM Eindhoven 2021, Eindhoven University of Technology and igem@tue.nl.

Eight Author – Luke Rossen, BSc., Team iGEM Eindhoven 2021, Eindhoven University of Technology and igem@tue.nl.

Implementing a CRISPR/Cas Cascade towards a new Diagnostic Test for Endometriosis

Florent Poubanne*, Ombeline Rossier**

* iGEM GO-Paris-Saclay 2021, Faculté des Sciences d'Orsay, Université Paris Saclay
** Université Paris-Saclay, CEA, CNRS, Institute for Integrative Biology of the Cell (I2BC)

***Abstract*- Endometriosis, a disease that affects at least 10% of women worldwide, is a major health and social issue. This illness can cause extreme chronic pain, painful menstruations and infertility in affected women and can take up to 8 years to be diagnosed. One of the avenues for a faster diagnosis of Endometriosis could be the detection of certain microRNAs, small RNAs circulating in the blood whose concentration seems to be different in patients with endometriosis compared to healthy women. In this context, project "EndoSeek," of the iGEM GO-Paris-Saclay 2021 team, aims in developing a new diagnostic tool of high sensitivity to detect these miRNAs. This tool called "Cascade CRISPR/Cas" uses a sequence of two reactions, with each exploiting the properties of a Cas protein, Cas13a and Cas14a1, in order to detect the presence of a miRNA of interest in a sample.**

***Index Terms*-** CRISPR, Cas13a, Cas14a1, diagnosis, endometriosis, miRNAs

I. INTRODUCTION

A. Endometriosis

Endometriosis is a gynecological pathology whose prevalence in the world population is estimated to be around 10% of women of childbearing age. Although there are several theories as to the origin of the disease, it is accepted that the disease begins with the proliferation of tissue similar to that of the uterine lining outside of the uterus (Arafah et al., 2021). The most common effects of this pathology on the health of affected women are pain, increased menstruation, and decreased fertility. The "gold" standard diagnostic method for detecting endometriosis is by a surgical procedure called a laparoscopy (Rolla, 2019). The reluctance for invasive procedures, together with the taboo associated with gynecological diseases, contribute to the long delay in diagnosis, on average 8 years after the first visit to a health care professional (Greene et al., 2009).

B. miRNA production and endometriosis

First discovered in *C. elegans* as developmental regulators (Saliminejad et al., 2019), microRNAs (miRNAs) are small non-coding RNAs of about 18 to 24 nucleotides involved in the post-transcriptional regulation of the expression of many genes. These small RNAs can be transported from one cell to another via vesicles or associated with proteins, and modulate the translation or stability of mRNAs after complementary base-pairing. They are thought to be involved in the regulation of the expression of about 60% of mammalian genes. In addition, the differential expression of an increasing number of these miRNAs is associated with pathological conditions (Sohel, 2016). Thus miRNAs constitute potential biomarkers for certain pathologies such as prostate cancer (Arrighetti & Beretta, 2021) or endometriosis (Moustafa et al., 2020) (Bjorkman & Taylor, 2019).

C. The Cas13a system for RNAs detection

CRISPR-Cas systems are the adaptive immune system of prokaryotes. They allow bacteria and archaea to keep a trace in their genome of the phages they encountered and to specifically degrade their genetic material in case of infection. These systems, the most famous of which is CRISPR-Cas9, are now increasingly studied for their usefulness in genome editing and as diagnostic tools in biotechnology, agri-food, and health (Nidhi et al., 2021). Most recently, the RNA-cleaving protein Cas13a was used for miRNA detection (Gao et al., 2021).

D. Cascade CRISPR/Cas

To increase test sensitivity, most Cas13-dependent miRNA detection systems rely on target amplification by reverse-transcription recombinase polymerase amplification (Gao et al., 2021). In this project, Team iGEM GO-Paris-Saclay 2021 will test a signaling cascade using Cas13a and Cas14a1 in order to increase detection sensitivity in the absence of target amplification. Inspired by the "Cascade CRISPR/Cas" developed by Sha et al. (2021), our test will rely on the ability of the two Cas proteins to cleave different nucleic acids (Fig. 1): upon association with the target miRNA with the crRNA, the collateral activity of Cas13a will cut the phosphodiester bond between two uracil in the loop of a stable hairpin (ST-HP). The resulting duplex structure can interact with Cas14a1 through strand displacement with the single guide RNA (sgRNA). The resulting activated Cas14a1 nuclease can cleave a ssDNA probe linked to a fluorophore and a quencher, thereby releasing a fluorescence signal.

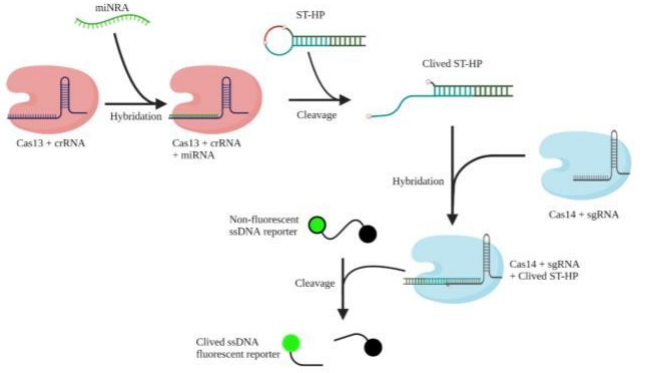

FIGURE 1.
Principle of the CRISPR/Cas Cascade for detection of miRNA" adapted from Sha et al., 2021. The target miRNA hybridizes with its complementary guide RNA (crRNA) associated with the Cas13a protein, which is then able to cleave the ssDNAstable hairpin (ST-HP) which carries two uracil ribonucleotides in

its loop (in red). One of the fragments released by ST-HP cleavage can then hybridize with sgRNA associated with Cas14a and activate it to cleave the ssDNA reporter, thereby separating the fluorophore (green circle) from the quencher (black circle) to allow fluorescence emission.

II. MATERIALS AND METHODS

A. Sequences of miRNAs and ST-HP

Two RNAs were chosen for the significance of the difference in their expression in the serum of women with endometriosis compared to healthy women : concentration of the miRNA miR-125b (UCCCUGAGACCCUAACUUGUGA) was greatly increased in the serum of women suffering from endometriosis compared to unaffected women, while that of miR-3613 (UGUUGUACUUUUUUUUUUGUUC) was reduced (Cosar et al., 2016) (Moustafa et al., 2020). The dual DNA-RNA molecule called ST-HP (GGCGTAGTTACATTCTCCCAGTTGA**UU**CTGGGAGAATGTA ACTACGCC) was synthesized by Eurofins.

B. RNA and DNA synthesis

Sequences of crRNAs of Cas13a or sgRNA of Cas14a1 allow the formation of a stem-loop structure. This is recognizable by the protein followed by a sequence complementary to the target miRNAs or to that of the segment released following ST-HP cleavage, respectively. Geneblock or complementary primers synthetized by IDT (Table 1) were used to generate dsDNA fragments that served as template for RNA synthesis using the T7 Ribomax express kit from Promega. RNAs were further purified with Promega ReliaPrep columns and checked by electrophoresis on urea-acrylamide gels. Reporter probes for Cas protein activity with fluorophore (FAM) and quencher (BHQ1) were ordered at Eurofins (Table 1).

Table 1.

Primers and geneblocks used in this study (T7 promoter sequence is indicated in italics).

Primer or Geneblock name	Sequence
miR-125b-5p-template-F	*GAAATTAATACGACTCACTATAGGG*TCCCTGAGACCCTAACTTGTGA
miR-125b-5p-template-R	TCACAAGTTAGGGTCTCAGGGACCCTATAGTGAGTCGTATTAATTTC
miR-3613-5p-template-F	*GAAATTAATACGACTCACTATAGGG*TGTTGTACTTTTTTTTTTGTTC
miR-3613-5p-template-R	GAACAAAAAAAAAAGTACAACACCCTATAGTGAGTCGTATTAATTTC
Cas13-crR-125b-F	*GAAATTAATACGACTCACTATAGGGGA*CCACCCCAAAAATGAAGGGGACTAAAACTCACAA GTTAGGGTCTCAGGGA
Cas13-crR-125b-R	TCCCTGAGACCCTAACTTGTGAGTTTTAGTCCCCTTCATTTTTGGGGTGGTCCCCTATAGT GAGTCGTATTAATTTC
Cas13-crR-3613-F	*GAAATTAATACGACTCACTATAGGGGA*CCACCCCAAAAATGAAGGGGACTAAAACGAACAA AAAAAAAAGTACAACA
Cas13-crR-3613-R	TGTTGTACTTTTTTTTTTGTTCGTTTTAGTCCCCTTCATTTTTGGGGTGGTCCCCTATAGT GAGTCGTATTAATTTC
Cas14-sgRNA-template-gblock	*GAAATTAATACGACTCACTATAGGG*TTCACTGATAAAGTGGAGAACCGCTTCACCAAAAGC TGTCCCTTAGGGGATTAGAACTTGAGTGAAGGTGGGCTGCTTGCATCAGCCTAA
Cas13a-reporter	FAM-UUUUUU-BHQ1
Cas14a1-reporter	FAM-TTTTTTTTTTTT-BHQ1

C. Protein production and purification

For the production of Cas13a and Cas14a1, *E.coli* Rosetta strain was transformed with plasmids pC0072 LbuCas13a His6-TwinStrep-SUMO-BsaI or pLBH531_His10-MBP-Cas14a1, respectively (Gootenberg et al., 2018; Harrington et al., 2018). The plasmids, which were ordered from Addgene, carry an ampicillin resistance gene and allow the constitutive production of the transcription inhibitor LacI. Expression of the gene of interest is under the control of the T7 polymerase promoter and the *lacO* operator. Transformed bacteria were grown overnight in LB supplemented with ampicillin (200 µg/mL), chloramphenicol (30 µg/mL) and glucose (0.2%). Cells were diluted in 1 L Terrific Broth supplemented with antibiotics to OD600=0.1 and grown at 37°C until the OD reached 0.5. Then, IPTG (0.5 mM) was added and the culture was shaken at 18°C for 16 h for protein production. The bacteria were harvested by centrifugation at 5000xg for 15 min and resuspended in40 mL LEW buffer (50mM NaH_2PO_4 300 mM NaCl pH 8.0) containing Complete protease inhibitor cocktail (Roche). Following lysis by sonication, the proteins carrying His-tags were purified using nickel columns (Protino Ni-IDA 1000, Machery-Nagel) and digested with SenP2 (Cas13a) or TEV protease (Cas14a1) in order to remove the histidine tags and solubilization domains they carried. Cas13a was then purified by FPLC on heparin column (1 mL, GE Healthcare) using a gradient using solution A (50 mM NaH_2PO_4 pH 7.5, 100 mM NaCl, 1 mM beta-mercaptoethanol (BME)) and solution B (50 mM NaH_2PO_4 pH 7.5, 1 M NaCl, 10% glycerol, 1 mM BME). For heparin purification of Cas14a1, solution B was 50 mM NaH_2PO_4 pH 7.5, 1.5 M NaCl, 10% glycerol, 1 mM BME. Proteins were then diluted 3-fold in 50 mM NaH_2PO_4 pH 7.5, 20 m M NaCl, 10% glycerol, 1 mM BME. Finally, a Millipore centrifugal device was used to concentrate the protein. Proteins were stored at -20°C after adding glycerol up to 50%. Proteins were analyzed by SDS-PAGE and Coomassie staining.

D. Cascade CRISPR/Cas

At the time of writing, the "Cascade CRISPR/Cas" has not yet been implemented, however the protocol used will be that described in the article "Cascade CRISPR/cas enables amplification-free microRNA sensing with fM-sensitivity and single-base-specificity," *Yong Sha et al, Royal Society of Chemistry, 2021*. The activity of the two proteins will first be tested individually using reporter probes to detect the activity of Cas13a and Cas14, once activated with their guide-RNAs.

III. EXPECTED RESULTS AND FINDINGS

We have successfully produced and purified Cas13a and Cas14a1, as well as the miRNAs and the crRNAs. We have not yet had the time to test their nuclease activity when associated to the crRNAs and corresponding target miRNAs. At the time of writing, the CRISPR/Cas Cascade has not yet been implemented. We will compare the sensitivity of the test using Cas13a alone (with a Cas13a reporter) to that of the test with the Cascade reaction. We expect that the Cascade reactions will increase the sensitivity of the test significantly to the sub-nanomolar range.

IV. DISCUSSION AND CONCLUSION

The most exciting results will be those to come as they will allow us to verify the efficiency of the "Cascade CRISPR/cas" system to detect the selected miRNAs.

Research on a correlation between the production of certain miRNAs and endometriosis is still in its infancy. The miRNAs that we propose to detect have been identified in reports with a small number of patients. Further studies are needed to confirm that they constitute biomarkers for the disease. However, it is

certain that in the future, many diseases, including endometriosis, could be diagnosed by detecting the fluctuation of the presence of certain miRNAs. The tool we have proposed constitutes a further step in this direction.

APPENDIX

https://2021.igem.org/Team:GO_Paris-Saclay

This is the link to the wiki presenting the Endoseek project, it contains the progress of the project after the writing of this article as well as the laboratory notebook and the protocols.

ACKNOWLEDGEMENTS

We thank IDT and Promega for providing us with gblocks, primers and reagents. We are grateful to the I2BC for lab space, equipment and material. We thank Carl Mann and Noureddine Lazar for kindly providing the SenP2 and TEV proteases, respectively, as well as Müge Senarisoy and Pascale Boulanger for their help with protein purification. LA DIAGONALE and the University Paris-Saclay are also acknowledged for funding. We also acknowledge Jingyi Yan and Maria Schapel from the iGEM team of Rochester who reviewed this article for their helpful comments.

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AUTHORS

First Author – Florent Poubanne, Microbiology master's degree student, florent.poubanne@gmail.com

Second Author - Associate Professor, Université Paris-Saclay, CEA, CNRS, Institute for Integrative Biology of the Cell (I2BC), ombeline.rossier@universite-paris-saclay.fr

Corresponding Author – Florent Poubanne, Microbiology master's degree student, florent.poubanne@gmail.com

MoClo-Mania
Facilitating the production of pharmaceutically relevant proteins by establishing the Modular Cloning System in *Leishmania tarentolae*

Authors: Nicolas Bayer*, Julia Spänle*
Team: Tabea Donauer*, Katrin Hieronimus*, Bastian Hinkel*, Salina Kibrom*, Jonas Klein*, Paul Moroka*, Benjamin Ziehmer*

* iGEM TU Kaiserslautern
Department of Biology, Technische Universität Kaiserslautern, Kaiserslautern, 67663, Germany

Abstract - **Large quantities of protein are needed every year for industry, research and medical purposes. Large-scale production of heterologous proteins in genetically modified microbial or cell culture expression hosts has long been established as a biotechnological standard. Still, the production of proteins destined for therapeutic application in humans often proves to be challenging for conventional microbial host organisms due to the complex post-translational modifications that most mammalian proteins undergo. Specific modification patterns, such as human-like glycosylation, cannot be replicated in microbial expression hosts, yet are essential to the protein's correct functionality and pharmaceutical potency. In recent years, the protozoan parasite *Leishmania tarentola*e has emerged as a favorable eukaryotic unicellular expression host because of its extensive, human-like glycosylation capabilities. To facilitate the use of *Leishmania tarentola*e as an expression host for biopharmaceutical proteins, we are establishing a library of genetic parts and vectors suitable for the usage of the Modular Cloning system (MoClo) within *Leishmania tarentolae*. This paves the way to a versatile and expandable basic part collection, making the expression of human-like glycosylated proteins more feasible and effective.**

Index Terms- Glycosylation, iGEM, *Leishmania tarentolae*, Modular Cloning

I. INTRODUCTION

Thanks to advances in modern biotechnology, large scale engineering and production of heterologous protein in transgenic expression hosts have long been established as an industry standard, greatly improving protein availability and advancing scientific and medical research. Protein therapeutics now represent a rapidly growing sector in the pharmaceutical industry (Otto, 2014), with biopharmaceutical proteins and monoclonal antibodies gaining importance as therapeutic applications in the treatment of various diseases, e.g. in auto immune disease and cancer therapy (Zhou et al., 2014). For recombinant proteins to be therapeutically applied within the human body, a high functional and structural resemblance of the recombinant protein to its native counterpart is imperative. Minor aberrations in amino acid sequence or protein folding may result in decreased or impeded functionality or cause severe immunological defense reactions in the patient (Mai, 2001).
The pharmaceutical applicability of a recombinant protein is furthermore dependent on its specific modification pattern. Most proteins expressed in mammalian cells are subject to complex enzymatic modifications, such as glycosylation, that not only facilitate protein folding and transport, but critically influence a protein's functionality (Kolarich et al., 2012). The type and order of carbohydrates attached to proteins during glycosylation greatly differs between species. Thus, common lab organisms such as *E. coli* struggle to recreate complex, human-like glycosylation patterns, whereas mammalian cell lines with preferable modification properties are more cost-intensive, less practical and may pose ethical concerns. Nonetheless glycoproteins (including monoclonal antibodies) are the most frequently approved recombinant therapeutic proteins (Walsh, 2010), and additional glycosylation can significantly improve the binding capabilities and average half-life of pharmaceutical proteins in the human body (Sola et al., 2009).

This raises the need for an easy method to cultivate microbial eukaryotic expression host with favorable protein modification patterns. In recent years, a genus of parasitic protozoan trypanosomes named ***Leishmania*** has gained popularity among researchers as a promising protein expression host due to its high content in glycoproteins and its glycosylation patterns closely related to those in mammals and higher vertebrates (Niimi, 2012).

Leishmania are unicellular parasites that are transmitted by phlebotomine sandflies and primarily infect vertebrates such as canids, rodents, reptiles and humans (Ryan & Ray , 2004*)*. They are most known for causing the infectious disease **Leishmaniasis** found in tropic and subtropic regions around the globe and affecting about 1.5 - 2 million people every year. (Torres-Guerrero et al., 2017). To avoid any possible biohazard and minimize safety concerns, all *Leishmania* strains used in conventional biotechnological research are nonpathogenic to humans (Klatt et al., 2019). A particular strain of *Leishmania* called *Leishmania tarentolae*, parasite of the common wall gecko *Tarentola mauritanica* (Elwasila, 1988), has been shown to successfully express functional mammalian antibody fragments and human glycoproteins (Langer et al., 2017; Klatt et al., 2012). We want to establish a modular cloning system that enables fast and variable gene design and allows for expression of the desired genetic constructs within *Leishmania tarentolae*.
Modular Cloning is a versatile cloning method first established by Weber et al. in 2011 and based on the principles of Golden Gate Cloning. This assembly method relies on the ability of type

IIS restriction enzymes to cut DNA outside of their recognition sequence. By placing these recognition sites at the opposite ends of any genetic sequence in inverse orientation, they will be removed during the cleavage process. This allows for seamless ligation of similarly structured genetic parts into a single construct. Since cleavage happens outside of the enzyme's recognition site and generates a four-nucleotide overhang, the sequence of this overhang can be chosen deliberately. This way the 5' and 3' ends of any fragments of interest can be equipped with unique overhangs, allowing for directional assembly of multiple genetic parts within one reaction. (Weber et al., 2011). By creating a set of different genetic parts such as signal peptides, purification tags and coding sequences, we establish a versatile genetic library that future researchers can draw on for expressing, detecting and purifying their proteins of interest utilizing *Leishmania tarentolae* as the expression host.

To demonstrate the functionality of our expression system, we chose the Sars-CoV-2 spike receptor binding domain (RBD) as an example protein. The recombinant production of RBD is of great importance regarding the current Covid-19 pandemic, yet the complex three-dimensional structure of RBD can also be difficult for common microbial organisms to recreate and poses an interesting challenge for *Leishmania*'s expression capabilities. The overall goal of our project is to observe successful transfection of the designed gene constructs into *Leishmania tarentolae*, as well as the purification of secreted, fully functional RBD out of the cell medium supernatant. Furthermore, every genetic part of the established MoClo collection ought to be tested for successful expression and functionality, verifying the quality of the parts for future users.

II. RESEARCH ELABORATIONS

Since the goal was to employ *Leishmania tarentolae* as an expression host, the generated genetic parts were designed to be consistent with the optimal codon usage in *Leishmania*. According to the Weber et al. nomenclature, the basic parts were labelled Level 0, meaning that they form the base unit of assembly for the Modular Cloning process. Cloning of multiple Level 0 (L0) parts into a composite DNA molecule, results in a so-called Level 1 (L1) construct. The genetic sequences used for creating L0 parts as well as vector backbones were partially drawn from online plasmid resources (Addgene) and partially purchased from the pre-existing Chlamy MoClo Kit of the Schroda working group (Crozet et al., 2018). After adaptation of codon usage, parts were synthesized (IDT, Sigma Aldrich) and built into according L0 vectors to obtain a stable and closed DNA structure for storage. All MoClo assembly reactions were performed according to standard protocols. For detailed information well as genetic sequences to all parts and vectors used, see appendix.

A. Establishing the Modular Cloning Kit

The first step in creating a functional expression system was to find a suitable DNA vector that would allow for stable insertion of the desired gene constructs and efficient transfection into *Leishmania tarentolae*. For this, we relied on the "LEXSinduce3 Expression Kit" distributed by Jena Bioscience GmbH, which is specially designed to be used for heterologous protein expression in *Leishmania*. The integrative vector contained in the kit is named pLEXSY_I-blecherry3 and carries bleomycin resistance for selection as well as conjugated mCherry for visible fluorescence of transfected cells (Jena Bioscience, 2021).

In order to utilize pLEXSY_I-blecherry3 as a plasmid backbone for Modular Cloning, any pre-existing BsaI recognition sites had to be removed from the plasmid to avoid fragmentation of the backbone during the cloning procedure. This was done by introducing single point mutations into the sites via PCR. New BsaI recognition sites at the 5' and 3' ends of the resulting PCR fragments should allow for their reassembly into a fully domesticated plasmid backbone via a standard MoClo reaction.

Aside from generating a suitable transfection vector, the main objective of this project was to create a set of different L0 parts available for assembly of variable genetic constructs. We decided on four different cloning positions (named B2-B5) which can be thought of as consequent sections of a single transcriptional unit: B2 presenting the most upstream and B5 the most downstream position. Thus, B2 parts may encode for N-terminal tags and B5 parts for C-terminal tags, whereas B3 and B4 are reserved for coding sequences of the desired proteins as well as additional tags. Different categories of tags were chosen to be realized in varying cloning positions, such as detection tags for live cell screening of target protein expression via fluorescence or bioluminescence as well as immunodetection of expressed protein from lysate or supernatant. Purification tags were included for efficient target protein purification from lysate or supernatant and coupled with TEV or Prescission™ protease motifs for easy retrieval of purified protein from respective column materials (see *FIG. 1*). A B2 secretion tag coding for the secreted acid phosphatase derived from *Leishmania mexicana* was added, causing *Leishmania* to secrete the desired proteins into the culture medium. This is a crucial step when it comes to the production of glycosylated protein, since glycosylation happens during passage of the secretory pathway (Roth, 2002).

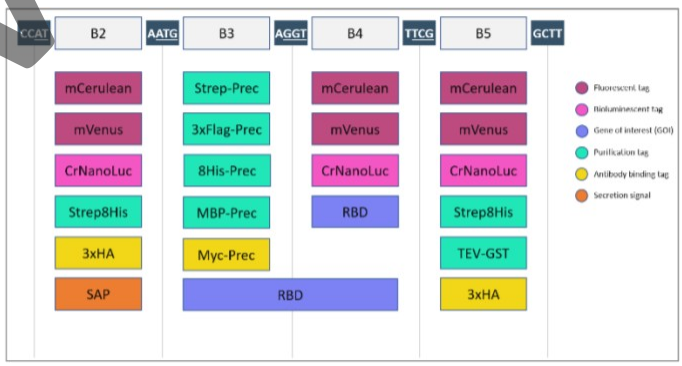

FIGURE 1.
Basic parts inventory. *Exemplary list of L0 parts to be generated for the Leishmania MoClo collection.* ***Red****. Fluorescent tag* ***Pink****. Bioluminescent tag* ***Green****. Purification tag* ***Yellow****. Immunodetection tag* ***Purple****. Protein coding sequence.* ***Orange****. Secretion tag. The dark blue boxes represent the respective nucleotide overhangs for every L0 position with the underlined letters indicating the transcription reading frame*

B. Transfection and expression in Leishmania tarentolae

By cloning basic parts into our *pLEXSY_dom* vector, we created an array of different L1 constructs ready for transfection into *Leishmania tarentolae* according to a standard electroporation protocol. After overnight incubation, the cells were grown on bleomycin agar plates, allowing only for growth of successfully transfected cells. After 7-10 days of incubation, colonies formed were inoculated overnight and grown on tetracycline plates. Tetracycline induces active gene expression of the gene sequences inserted into the LEXSY vector. Colonies formed after 3-5 days incubation were inoculated into culture medium.

Resulting liquid cultures could be grown to variable volumes and lysed or centrifugated to attain expressed intracellular or secreted protein. Protein kinetics ought to be performed to determine cell count and density at the moment of maximum protein expression. For detailed protocols on the handling of *Leishmania* in cell culture as well as composition of the media used, see appendix.

III. RESULTS AND FINDINGS

A. Establishing the Modular Cloning Kit

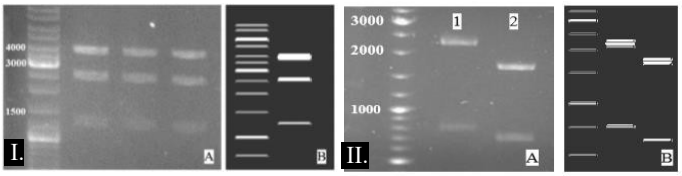

***FIGURE 2*. Restriction digests**
I. *of domesticated pLEXSY_I-blecherry3 using SacI in three samples*
II. *of exemplary L0-plasmids using BstEII + HindIII for*
1*. L0-RBD_B3_B4* ***2****. L0-SAP-B2*
A. *digest after gel electrophoresis* ***B****. in silico digest and gel*

B. *Transfection and expression in Leishmania tarentolae*

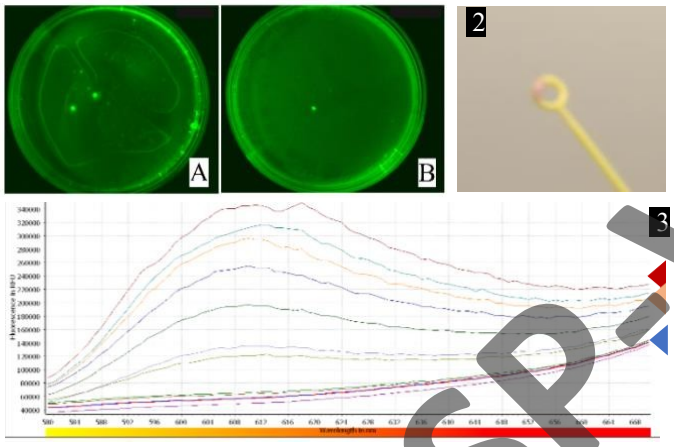

***FIGURE 4*. *Leishmania tarentolae* expressing mCherry**
1. *fluorescence detection of colonies grown on bleomycin plates after transfection with* ***A****. pLEXSY_I-blecherry3 original* ***B****. L1-SAP-RBD-Strep8His*
2. *picked colony with visible red coloration* ***3****. Emission spectrum (580-668 nm) for different cell culture suspensions and media, = purified mCherry in dilution, = transfected cell suspensions, = pure culture media*

IV. DISCUSSION

A. Establishing the Modular Cloning Kit

The domestication of pLEXSY_I-blecherry3 proved to be far more difficult than initially expected. In numerous attempts of PCR amplification, one of the fragments repeatedly turned out to be flawed, hindering successful ligation of the final vector. Analysis of the underlying genetic sequences revealed a highly repetitive region (labelled *FU*) within the fragment in question, suggesting possible difficulties of Q5 DNA polymerase with replicating such highly repetitive base pair sequences. Thus, a new strategy was employed, relying on standard insertion cloning of the *FU* region out of the original pLEXSY_I-blecherry3 into the otherwise domesticated vector.

In order not to re-introduce any BsaI recognition sites, only a specific set of restriction enzymes could be used for this, one of which was ClaI which is blocked by Dam-methylation. In order to bypass this issue, the relevant DNA fragments were transformed into CGSC5127, a *Dam- E. coli* strain. After preparing the non-Dam-methylated DNA fragments, the ensuing ligation resulted in correctly assembled plasmids which was proven by restriction digest (shown in FIG. 2) as well as sequencing to be the successfully domesticated and intact *pLEXSY_dom* vector.

Parallel to the generation of the transfection vector, the basic L0 parts were designed, synthesized and assembled into respective L0 backbone vectors. Many of them were successfully tested with the help of restriction digests as well as genetic sequencing of the resulting plasmids (see examples in FIG. 2, II.). Due to a lack in sufficiently expressed and purified protein, quality and functionality of the employed tags could not yet be verified via binding or activity assays.

B. Transfection and expression in Leishmania tarentolae

At first, the transfected colonies grown on bleomycin plates seemed to lack mCherry expression, which, according to LEXSY protocols, should have been observable as red coloration to the naked eye. However, expression of mCherry is genetically coupled to the expression of the bleomycin resistance gene, suggesting that the grown colonies had gained a bleomycin resistance prior to transfection. Thus, a new and not yet treated cell line was employed as a new transfection target. After transfection with several different L1 constructs, the resulting colonies displayed a weak red coloration that could be unambiguously ascribed to mCherry via fluorescence detection in the plates and photometric absorption analysis of the liquid cell cultures (see FIG. 4). This suggests that the domestication process did not have any negative effect on the expression quality of the pLEXSY_I-blecherry3 vector.

As of now, immunoblots have been made displaying the presence of tagged protein in cell culture supernatant after transfection and induction. However, the quantity and quality of expressed protein remains unclear since the cell cultures are currently grown in increasing volumes, and purification steps have not yet been carried out on supernatant or lysate of transfected cells.

V. OUTLOOK

Our main goal of expressing and purifying a functionally intact RBD has yet to be achieved. Thus, further efforts must be made in order to improve the transfection efficiency as well as the expression levels of *Leishmania tarentolae*. Resulting proteins would enable a further evaluation of the quality and functionality of the generated basic L0 parts.

Furthermore, expanding our set of example proteins beyond the RBD to actual glycoproteins in therapeutic use today (such as monoclonal antibodies, growth factors, hormones, etc.) would allow for a much broader and more practical evaluation of the viability of our genetic engineering toolbox and expression host. Additionally, the *Leishmania* MoClo collection could be extended with new L0 parts, allowing us as well as other future users to introduce new tags or coding sequences that are interesting and useful for protein production purposes.

APPENDIX

All protocols and parts used for Modular Cloning as well as for handling of *Leishmania tarentolae* cell culture and for protein purification can be found under the following link: *https://drive.google.com/drive/folders/1_hv5FDwhDdujQ1cG-YE3zPpbj5xuPM7S?usp=sharing*

ACKNOWLEDGEMENT

We want to thank Prof. Michael Schroda and Prof. Marcel Deponte, as well as their respective working groups, for their immense contribution and invaluable help in the planning and execution of this project. We also want to thank Nora Georgiev and Christina Huber from the microbiology working group for their great help and support with our protein purification. A special thanks goes out to our lab supervisors Anna Probst, Justus Niemeyer, Luzia Schneider and Anna-Maria Kiefer, who have accompanied and supported us on our lab journey every step of the way.

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AUTHORS

First Author – Nicolas Bayer, Bachelor student, TU Kaiserslautern, nbayer@rhrk.uni-kl.de

Second Author – Julia Spänle, Bachelor student, TU Kaiserslautern, spaenle@rhrk.uni-kl.de

Correspondence Authors iGEM Team TU Kaiserslautern 2021, igem@bio.uni-kl.de, 017682917488

Project Platylicious - A synthetic way to revolutionize dairy food production

Marius Schnutenhaus*, Lea Hellwig*, Luise Pinske*, Team RUBochum*

*RUBochum, Research Group Microbial Biotechnology

***Abstract* - Livestock farming, especially dairy and meat production, contributes to a large extent to CO2 emissions and thus to climate change. More and more people are questioning their diet in order to find new ways of contributing to the protection of the climate. The milk produced by fungi would offer an equivalent substitute for those people who do not necessarily want to switch to plant-based milk, but do not want to contribute and enforce global warming and animal exploitation. It is advisable to use yeast, *Pichia pastoris* in our case, as expression organisms, as they have a manageable genome that is simple to modify. In addition, the post-translational modifications of the yeast are capable of producing the correct products of our desired proteins.**

Index Terms - iGEM, *Pichia pastoris*, platypus milk,synthetic yoghurt

I. INTRODUCTION

Agriculture, especially livestock farming with milk and meat production, is a major contributor to environmental degradation and thus to climate change.
In Germany alone, 47% of the total area is used only for agricultural purposes (Umweltbundesamt, 2019). The use of antibiotics, fertilizers and pesticides also leads to a loss of biodiversity.
Milk production, which is dependent on the rest of the agricultural sector, is particularly characterized by heavy water consumption and methane emissions. 7.3% of all German emissions come from agriculture without fertilizer production (Umweltbundesamt, 2019). Due to environmental pollution, animal exploitation and animal suffering, more and more people are looking for alternatives to conventionally produced animal products.
Our project deals with the synthetic production of milk and flavors in yeast. The goal of "Project Platylicious" is to produce vanilla yogurt, which is based on both cow and platypus milk and does not differ from them in molecular structure.
Pichia pastoris is used as the expression organism, which has several advantages. Besides the post-translational modification, for the production of the different proteins e.g. caseins (αs1-, αs2-, β-, κ-caseins), the secretion allows an easier purification of the proteins (Gao et al., 2021). The lipids are synthesized due to the overproduction of the acetyl-CoA carboxyltransferase complex from *E. coli* K12 and a variety of acyl-CoA thioesterases.
The fascinating properties of the platypus, such as the "oozing" of the milk or its antimicrobial ingredients, can also create awareness for endangered species.
The Monotreme Lactation Protein (MLP) is tested regarding its antimicrobial activity. Hence it is suspected as an alternative for milk pasteurisation (Enjapoori et al., 2014).

II. RESEARCH DESIGN

The genes of interest are cloned into a secretion plasmid for *Pichia pastoris* (pPICZα A). Genes for the fatty acid synthesis and the MLP, however, are introduced without the secretion signal. The latter additionally features a 6x polyhistidine-tag and will be tested with various microorganisms in different concentrations. Furthermore, multiple tests are going to be performed with milk, yogurt and microbes of the humane gut flora.
Brazzein and the different caseins are produced with the secretion signal but without an affinity tag. A heterologous fermentation with multiple genotypes should allow the production of the ingredients in different concentrations for further optimizations.
Our second approach is to integrate the genes with promotor shuffling into *P. pastoris* genome via a Golden Gate assembly. Both results can be compared to enable an improved industrial process.
The cloning step is going to be performed in *E. coli* DH5α and the heterologous overexpression of the different proteins will be made during a “heterologous fermentation”.
Here, this means that multiple genotypes produce the different ingredients to get the final product.

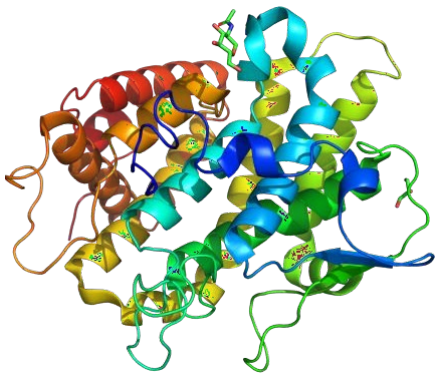

FIGURE 1: Molecular structure MLP

III. DISCUSSION

Food production has a huge impact on the environment. It is crucial to develop alternatives to the conventional and environmentally harmful farming processes. Dairy farming with its high water consumption, fertilizers, antibiotics and greenhouse gases is associated with climate change, loss of surfaces and the extinction of many species (Good & Beatty, 2011; Hagemann et al., 2012; Manyi-Loh et al., 2018; Shortall et al., 2018; Styles et al., 2018; Umweltbundesamt, 2019).
Milk contains different ingredients such as fatty acids, proteins e.g. caseins, lactose, water and calcium (Haug et al., 2007). For a high yield of fatty acids the enzyme of the rate limiting step acetyl-CoA carboxylase is overproduced (Connection Between Epigenetic State and Fatty Acid Synthesis, 2012). Many different kinds of fatty acids are included and differ in size and saturation state. The length of the fatty acids is controlled by thioesterases which terminate the elongation of the fatty acids at a particular length (Beld et al., 2015).
The common length of fatty acids in milk varies between 4-24 carbon atoms (Haug et al., 2007).
The production of the eukaryotic proteins is dependent on post translational modifications (Enjapoori et al., 2014; Simons et al., 1993). For affordable industrial synthesis, *Pichia Pastoris* was chosen. Due to its secretion mechanism the purification of the food molecules is simplified. The process of heterologous fermentation allows to optimize the processes to an industrial level. Plasmids and chromosomal integrations with promotor shuffling are getting compared. The aim is to reduce the number of genotypes by taking the most promising.
Besides the different caseins, vanillin and brazzein are produced as aroma compounds. Brazzein has a sweet taste but has the advantage of a protein (Ming & Hellekant, 1994).
The MLP is promising as an alternative antimicrobial agent and to common milk pasteurisation. Different tests with various bacteria types and the most common from the humane gut flora should give insight into the application.

IV. CONCLUSION

In Tasmania, homeland of the platypus, the milk production is located next to rivers due to its high water consumption and hence takes aquatic biospheres.
The massive use of fertilizer is promoting the eutrophication of those remaining habitats.
Platypus breeding places are destroyed and taken by bathing and drinking cows near the river due to erosion and missing vegetation.
The production of platypus proteins could raise awareness for this monotreme animal and stop the further threat on the platypus by the dairy industry (Bino et al., 2015; Bino et al., 2019; Geoff Williams, Luise Pinske, Lea Hellwig, Marius Schnutenhaus, 2021).

ACKNOWLEDGEMENT

First, we want to thank Prof. Dr. Dirk Tischler for giving us the opportunity to work on this project as the first iGEM-team from the Ruhr-University Bochum and for being our PI.
We want to thank the faculty of Biology and Biotechnology for supporting us with their resources.
Special thanks goes to Julia Beukert and Dr. Boas Pucker for their support and advice.

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AUTHORS

First Author – Marius Schnutenhaus, Research Group Microbial Biotechnology, Marius.Schnutenhaus@rub.de

Second Author – Lea Hellwig, Research Group Microbial Biotechnology, Lea.Hellwig@rub.de

Third Author – Luise Pinske, Research Group Microbial Biotechnology, Luise.Pinske@rub.de

"A Great Text is One that Moves the Heart" – Linguist Joop Hoekstra on the Importance of Academic Writing

Lotte Deneer*

* MSP-Maastricht, Maastricht University

***Abstract*- Communication is key, also within the scientific community. Being able to get good results within the lab is one, but almost just as important is being able to actually share these findings with others. In order to do so, one must have sufficient academic writing skills. During this interview linguist Joop Hoekstra, formerly part of the language centre within the Maastricht University, will share his journey within this ever so important field. Additionally, he will shed some light on some obstacles one might face along the road of becoming a scientist who can not only collect data, but is also capable of sharing these finding with other in a persuading and inspiring manner.**

Index Terms- Academic writing, publishing, iGEM, linguistics.

You have been active in the language centre of Maastricht university for some time, can you perhaps tell us a bit more about your journey in the world of academic writing?

To be honest, I do not consider myself as a writing expert *pur sang*; it would be nearer the truth to say I am a linguist, as I am by education. But even that label cannot fully explain why my interests, or my heart, took me in very different directions at different times in my professional life. It brought me in touch with other fields than education: health care, public administration, waste management, and even infrastructure; mostly in the role of the outsider who asks the critical questions. To and from language training I went back and forth. But let me caution you, before you get romantic ideas about such a career. Most people thrive under stable conditions, and I have regretted my choices more than once, despite the many, many moments of excitement.

Unlike what you might think, I was a lousy writer at school - so there is hope for you all. It was not until my student days that I discovered I could not only write decently, but that my writing could actually make others move. And that is where the practical linguist in me has played a role in all these fields I worked in: Using clear and persuasive language helped to open other people's eyes, sometimes for what went wrong in their work (and what went well!), and so fuel enthusiasm and new creativity. As a writing skills trainer at Maastricht university, my motivation is exactly the same. I wish to stir up an awareness in students of the power of language, to get clear messages across the footlight, definitely; but also, to convey beauty to readers and so gain their attention. Beauty you say? Are findings, facts, and theories, not the hallmark of scientific work? Yes and no. It is rare to find that facts convince on their own strength – as you can see around you, wherever you look. Facts and findings are like pawns you bring into a game. But the game is not won unless you manage to lead the reader to a point where facts and findings are willingly accepted. Never believe that academics are always rational and eager to take in exciting new information. Since Thomas Kuhn's famous work on the structure of scientific revolutions and the tendency in scientists to disbelieve what appears to clash with their own truths, we know all too well that convincing an academic requires more than the bare facts.

Considering the fact that you have been part of this field for several years now, what shifts have you observed in the process of writing and publishing scientific articles? What are some changes that you predict for the future?

During the past twenty years or so I have witnessed a tsunami of academic articles, many reflecting highly limited or even inadequate research, suffering from methodological shortcomings, blindness to alternatives in the interpretation of results and consequences, and oblivion to the work of others. That does no one any good, and language can never help to make better science out of what is essentially poor science. Politicians, and education authorities in their wake, have spread the message that it is numbers that count, not quality. Underneath is a commonly felt distrust in human nature, a belief that people will not work unless they are flogged, and so they are expected to turn out product after product, article after article. But creating value is often a slow process.

As a result of confusing quantity with quality, there has been a huge demand for publication space, giving rise to a flood of new journals, and journal editors that are sometimes easy-going when it comes to quality standards. I realize I am suggesting that there is a race to the bottom going on, and though a little exaggerated, there is truth in it. The good news is that universities are slowly turning away from this perverted system, as Maastricht University has now announced it will. One of the effects I have seen happening in the art of writing has been that reports and articles have become more standardized – even highly so - in length and composition. Predictability makes for easier reading and digesting, but the downside is that there is less room these days for personal touches or little asides, for instance on the potential a particular finding could have, if sustained by further research. Another feature that has gained weight is the role of peer reviewing. Although a quality instrument, and a necessary one, it is not always applied in fairness. Academia is a competitive world, and unwelcome findings are sometimes banned from publication. Language, it must be said, is not a major offensive aspect in peer reviewing. Though a manuscript may be turned down for lack of linguistic quality, this usually concerns reparable shortcomings, at least in my experience.

When it comes to writing good articles one can say "practice makes perfect". Speaking out of experience, what would you say are components of writing that students or even new scientists seem to struggle with a lot in particular?

Practice is indeed the road to perfection – or at least to a decent level of expression in writing, as is true for any skill. My own story is a reminder that you may start off as a mediocre sort of writer and still get better. Or worse than mediocre: I barely passed my writing exam at the end of my school days; they probably gave me the benefit of the doubt. But when you avoid the task, you will never get better. And so, you must take that straight and narrow road. There is much joy in gaining mastery. Mastery does not mean perfection. We are living in an individualized culture, have been so for nearly two centuries. Many of us rather die than ask for and accept help from others. If you feel your writing is more in your way than the quality of your work deserves, ask others to lend you a hand. There is nothing wrong in taking a shortcut at times.

Fear of writing is a problem in many, as I am sure it was once in me. Call it uncertainty. The blank page may stare back at you whilst you are staring at it, and cause mental paralysis. If the use of a non-native language, English for most of us, is too much in the way at first. Writing has the effect of making yourself visible to others, and stir up the feeling that you may show up as less than skilful, even stupid, for lack of words and phrases in English; one student expressed it once as a sense of stumbling about in shoes several sizes too small for his feet. He called it painful, for the lack of decorum it created.

If you could give a message to students who are struggling with scientific writing, what would you tell them?

For academic writing, fortunately, there are many resources available, some dealing with the vocabulary of academic English, its commonly used words and phrases. And on the style features of formal English. It is often helpful to realize texts never arise in one go, and that rewriting is part of the deal. Also keep in mind that writing may benefit from taking small steps at a time, each step being a small victory over yourself.

The main problem I find in students struggling with writing tasks is the feeling that writing is strictly linear: you sit down and start, introduction, main part, conclusion. But that is no way to go about it, and a recipe for trouble. First of all, sit down and draw up a plan. Think of the article or report as being composed of parts, each with its own function and subfunction. My group sessions always start with an overview of the functional units of a scientific report and article. When you write up the various parts and subparts, as in a table of contents, or in the form of a kind of skeleton, you see the text will and must hang together on the basis of logic, with premises, claim to be proved true, method used, and results and conclusion. This provides a framework which you may fill out, starting with the easy parts, usually the method and the results. Once you recognize the logic underneath, writing becomes easier. An added effect will be that it will tell you, whilst writing, where your thinking is not sufficiently clear, yet.

What would you consider to be the difference between an average and great scientific writer? Is there one aspect that stands out? Why?

A great text is one that moves the heart. The US Declaration of Independence is one such text. It is sincere, it is righteously proud, it embodies convictions which most people hold deeply true; and it speaks, if only between the lines, of wrongs to be set right. It is too much to ask that a science text should attain that level of persuasion, nor is it something to wish for. The convictions of science, its quest for truth, are of a different caliber. The conclusions of science are temporary at best, they can be overturned by later work, they are refutable. They do not echo eternity. It is too much to ask for brilliance in a scientific text, and what we do call brilliant are generally texts that report on work of rare excellence, on exceedingly clever thinking. But a text that is well-written, on top of being on sound work, may still have a strong emotional impact on the reader. In authoritative text books, the writer may string together a vast amount of detail in an all- encompassing paradigm. Taking details to a higher level of explanation, may be intoxicating. But these are mostly elements of content, the product of clever experimental designs, or of new explanatory perspectives.

The question is: how can writers achieve conviction in their readers, if they have nothing but some new facts and maybe no new view of their subject matter at their disposal, as most starting professionals do. In articles, which are only brief and restricted in scope, the options are limited. As a rule of thumb, a text is a good text if its shows strength of purpose. Its presentation of the facts obtained and its line of argumentation are flawless. This can be achieved by following the rules of logical composition to the letter. On top of that, a good article gives you all the detail needed for the reader to follow the text, and no more than that: articles are precise and concise. The same principle applies to the context of your work that you need to specify in order for the reader to understand where your work must be positioned. And again, for the objective your work was meant to achieve, and why that objective is at all relevant. Concise, precise, enough and no more than that. Cutting your text to the minimum will give it the ring of conviction. Your conviction will help convince others.

Within the scientific world, written articles are the main way of sharing one's findings with the rest of the community. However, these articles can be hard to grasp for someone outside of the scientific community. What would you consider to be suitable alternatives for sharing scientific findings with the general public?

It is a sad fact that scientists are little equipped to bridge the gap between their world and its self-evident certainties, and the public at large. Nuancing does not help to get a message across to unwilling ears. On the other hand, nor does activism: Climate change demonstrators are often seen as "probably right", but a bother at the same time, as they disturb people's dreams of an easy life.

Although some scientists have been trained, or have trained themselves, to transmit the gist of scientific development to the outside world, it remains difficult to step down to a readership, or audience, that wishes to see things happen before their very

eyes, rather than hearing somebody preach a new gospel. In fact, as far as I have seen, dissemination of results is a careful, step by step, and lengthy process which tends to reach a handful of interested forerunners, who may in turn persuade others by example. All this indicates that there is a need for specialists who make it their job to address the population at large, or play an influencer role in all sorts of relevant social and political settings. No university caters for such a specialisation, to my knowledge, and it would a wise move to set one up. Writing, as much as speaking at meetings or in smaller settings, would be important tools: using language that strikes the heart of key players in social change, showing awareness and respect for the position of those who find it hard to move. But I have seen examples, mostly in politics, where antagonistic key players suddenly realized how much their interests coincided – when differently worded. I would make a plea for such a mediating function between experts and the rest of the population, and a training programme to equip these students for this challenging and socially creative role.

How would these ideas then translate into ones writing style?

If you achieve this level of written presentation, you will be doing a good job. An even better job requires a mild form of persuasion: a bit of seduction without doing violence to the truth. Persuasion, seduction, requires putting yourself in the position of your readers and in what they may expect or what they may like to hear on your research. If you feel most of your readers will be surprised by what you are presenting, introduce your findings with the word *surprisingly*; at times, when your outcome is not particularly spectacular, you may introduce the findings by using *not surprisingly*. The one is meant to tickle, the other meant to soothe (and both are meant to bond at the same time). If you want to go one daring step further, you may say something like *in contrast to previous findings, in contrast to common assumption, in contrast to popular views on the matter*, each a little more provocative than its predecessor, and you may sit back and await the reactions, which are bound to come. Attention grabbing comes in subtle little phrases. If Dr. X is the man to be convinced of the quality of your work, Dr. X will have to make an appearance by your quoting his or her work, with a touch of approval and just a small critical note. I have seen it happen on a grand scale, time on time again. Dr. X will prick up his (or her) ears.

The message is that good work is forceful work: short in overall length, with short sentences that run like miniature statements, precise, and I may add, written in language that is formal but technical only where it cannot be avoided. Some texts are so burdened by jargon that you wish for a break after each sentence. You will not make yourself popular that way. The message is also that tiny bits of personal or evaluative coloring may help persuade others to pay attention, for whatever reason of their own, and respond. And yes, there are even better texts where the persuasive element takes the form of a stand for a particular point of view. Appeals to see reason on the basis of what science has to offer, appeals to common sense, to justice, tend to add urgency on top of an accumulation of data and arguments, taking into account what others may or may not like to hear. But as scientists go, their texts will still show restraint.

What are your thoughts on our team's concept of creating this journal in order to familiarise the other students in the competition with the process of publishing?

I think it is a wonderful idea, in that it familiarizes students hands on with what it is to handle findings in an academic environment and creates an awareness that this can be done at an early stage in one's career. It must be a truly transcendental experience for many to realize they have it all in them, given guidance and a framework to work in. Mind blowing, in a healthy sense of the phrase; I wish I had been given that sense of relevance, as a small person, when I was a student in my bachelor years. And the international aspect is crucial. When I was young, the world seemed immense; it was ungraspable. Today it is a small place, where we can only survive by reaching out and clasping each other's hands.

ACKNOWLEDGEMENT

On behalf of the entire MSP iGEM 2021 team I would like to thank Joop Hoekstra for all the time and effort he put into helping us to get a better inside into the world of academic writing. As well as the inspiriting stories he shared on how one can develop from not standing out, to persuading others with his words.

AUTHORS

First Author – Lotte Deneer, Bachelor student, Maastricht University, ljm.deneer@student.maastrichtuniversity.nl

Correspondence Author – Lotte Deneer, Bachelor student, Maastricht University, ljm.deneer@student.maastrichtuniversity.nl

AFTERWORD

„Science knows no country because knowledge belongs to humanity, and is the torch which illuminates the world.” – Louis Pasteur

iGEM provides an opportunity for teams from all over the world to connect with each other, exchange ideas, knowledge and advice. With the iGEM MSP-Vector, we enabled teams from around the globe to collaborate with each other by doing peer review on each other's articles, reflect upon different projects, and get to know each other. This would not have been possible without the teamwork and effort provided by each and every one of the participating teams.

Through the engagement and enthusiasm each member brought to this initiative; the excitement 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 to have used our platforms to educate the teams about scientific writing and emphasized the importance of such in a fun, engaging and informative manner! Most importantly, we hope the teams have enjoyed and benefitted from the collaboration. MSP-Maastricht says thank you!

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.

TEAM CONTRIBUTIONS

If any specific article in this Journal has sparked your interest, you can use the provided links below to receive more information on the team's wiki page. All their experimental data, detailed project elaborations, and more information on the team can be found on the following wiki pages.

This is the list of participating teams and the links to their wiki pages on the iGEM server:

iGEM Tübingen	https://2021.igem.org/Team:Tuebingen
iGEM MIT_MAHE	https://2021.igem.org/Team:MIT_MAHE
iGEM IISER Tirupati	https://2021.igem.org/Team:IISER-Tirupati_India
iGEM Calgary	https://2021.igem.org/Team:Calgary
iGEM UParis	https://2021.igem.org/Team:UParis_BME
iGEM MSP-Maastricht	https://2021.igem.org/Team:MSP-Maastricht
iGEM Athens	https://2021.igem.org/Team:Athens
iGEM NIT Warangal	https://2021.igem.org/Team:NIT_Warangal
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iGEM Eindhoven	https://2021.igem.org/Team:TU-Eindhoven
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