INCREASING THE PRODUCTION OF ENCAPSULATED DSRNA FOR BIOLOGICAL PESTICIDES

GENTIC ENGINEERING'S COMPLICATED ROLE IN FUTURE SOCIETY

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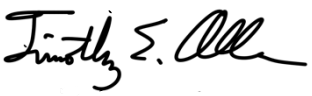
On my honor as a University student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments.

Signed  Date: 11/24/20

Approved:  Date: 11/24/20
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One of the most world-changing scientific discoveries came in the year 1953 with the model of the double helix structure of DNA (Pray, 2008). At last, the molecule responsible for every feature of every form of life was defined. Later in 1976, Herbert Boyer and Robert Swanson founded the company Genentech and were able to use recombinant DNA in the genome of *E. coli* (National Human Genome Research Institute, 2013). This revolutionary method of drug production sparked the creation of the biotechnology industry, now set to be worth $727.1 billion by the year 2025 according to Grand View Research (2017). Today, constant improvements to biotechnology are bringing scientists closer to the God-like abilities of manipulating life and matter. Yet while buying into this God-complex is tempting, it is imperative scientists realize their human fallibility, holistically anticipate emerging biotech's role in society, and learn from past mistakes of attempting genetic perfection in order to build an industry which will benefit all of humanity.

The overall goal of this thesis project is to understand the technical and societal operation of biotechnology. More specifically, this thesis will feature tightly coupled projects which deal with optimizing a biopesticide manufacturing process and evaluating the role Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), one of the most influential artifacts in the biotechnology industry, in society. The technical project is currently ongoing, where baseline data is being obtained and improvements to the system will be made and subsequently observed in the spring semester. The STS project will also be conducted through the spring semester, with more in depth research into the topic to provide a better understanding for how CRISPR interacts with the different actors present in society.

Currently, the agriculture industry relies heavily on the use of chemical pesticides to protect crops. About 5.6 billion pounds of pesticides are used globally each year, and 1 billion pounds are used annually by the United State (Alavanja, 2009). However, the widespread usage of chemical pesticides has produced signıtıcant negative impacts on human health, the environment, and crop protection itself. As the world population continues to grow, agriculture will also have to scale up to feed increasing numbers of people, amplifying pesticide use even further. Therefore, it is crucial to create alternative biological pesticides to address the myriad of problems associated with chemical pesticides.

Chemical pesticides have been linked to a number of serious health conditions in humans. This includes disruptions and problems relating to endocrine, respiratory, reproductive, neurological, and gastrointestinal function (Nicolopoulou-Stamati et al., 2016). The World Health Organization reports that many scientific studies have classified chemical pesticides as carcinogenic, neurotoxic, and teratogenic substances (World Health Organization, 2016). In addition, there are multiple ways that humans can be exposed to the harsh chemical pesticides including inhalation, ingestion or skin contact (Nicolopoulou-Stamati et al., 2016). Residues on food, in water, and in the air all contribute to the accumulation of pesticides within the human body (Nicolopoulou-Stamati et al., 2016). Pesticide poisonings have also become a public health problem, with an average of 20,116 cases requiring medical treatment in the United States every year (Langley & Mort, 2012). Exposure to these chemicals is toxic to the human body, causing both short- and long-term effects.

Numerous environmental effects also result from the continued use of chemical pesticides. Since many of these pesticides are synthetic, they persist in the environment since

microorganisms have not evolved enzymatic mechanisms to break these chemicals down (Gavrilescu, 2005). Persistence becomes a more global environmental issue when coupled with the transport of pesticides through the air, soil, and water through emission, leaching, and runoff (Gavrilescu, 2005). These methods of environmental pesticide transport cause these chemicals to spread away from farms, where one example case study found a total of 26 pesticide products in the Guayas river basin (Deknock et al., 2019). Toxic pesticides eventually accumulate in adipose tissue of animals which causes health problems as the accumulation increases (Gavrilescu, 2005). With the persistent expansion of the agricultural industry to feed the growing population, it is clear that the continued use of chemical pesticides will in fact exponentially exacerbate the destruction of the environment.

Furthermore, many organisms targeted by current pesticides have evolved genetic resistances to the chemicals used, rendering them ineffective. Resistance is a result of a pest evolving an enzyme or other physical mechanism to overcome and survive the effects of a pesticide (Buhler, 2015). When a pesticide is consistently applied, a population of pests will eventually develop resistance over time as an evolutionary response to the continued exposure (Buhler, 2015). As a result, resistance shortens shelf life which requires the expensive development of new pesticides. While some pesticides have longer shelf lives than others, with excessive application it is only a matter of time before a pest develops resistance which causes an endless cycle of pesticide development (National Research Council (US) Committee on Scientific and Regulatory Issues Underlying Pesticide Use Patterns and Agricultural Innovation, 1987).

Suitable biopesticides must be developed to replace chemical pesticides and mitigate their harmful effects. RNA interference (RNAi) is a technology that causes gene knockdown in

organisms (Mamta & Rajam, 2017). RNAi uses double stranded RNA (dsRNA) sequences to bind to mRNA transcripts, therefore blocking translation of mRNA into proteins within the cell (Kim & Rossi, 2008). Standard chemical pesticides are dangerous for human consumption because they mostly target pests' enzymes which are often homologous to human enzymes (Coman et al., 2013). Because pest mRNA sequences are not as homologous with human sequences due to variable codons, RNAi pesticides are safer for human consumption (Koonin & Galperin, 2003). In addition, RNA breaks down easily in the environment and poses no risk of persistence or contamination (Fletcher et al., 2020). RNAi is also an effective method for overcoming genetic resistances pests have evolved to current chemical pesticides. This is because unique genetic sequences are targeted in these organisms for which genetic resistances have not yet been evolved. This makes RNAi biopesticides significantly more effective in eliminating pests than many chemical pesticides. Despite the many advantages of using RNAi biopesticides, significant barriers exist that have prevented widespread adoption by the agriculture industry.

One major obstacle in creating a viable RNAi biopesticide is the tendency of RNA to degrade rapidly in the environment, leaving crops vulnerable (Fletcher et al., 2020). This means that topical field applications of RNAi biopesticides would have to be performed much more often, which is inefficient for farmers. RNAi biopesticides must also be successfully scaled up to match the industrial production capabilities of chemical pesticides.

The biotechnology company AgroSpheres has made significant strides in developing an RNAi biopesticide technology that is both commercially feasible and more stable in the field. AgroSpheres utilizes a unique RNAi delivery mechanism that protects dsRNA from rapid degradation in the field. Delivery of the biopesticide is facilitated by bacterial minicells. The

membranes protect the dsRNA inside so the biopesticide can remain effective in protecting crops. In order to increase the efficacy of AgroSpheres' platform RNAi biopesticide technology, the capstone team will focus on improving both the dsRNA binding protein expression and the fermentation production processes.

The dsRNA binding protein is expressed along with the dsRNA itself, and acts to increase stability of the dsRNA within the minicell. However, preliminary investigations within AgroSpheres showed that a significant portion of the dsRNA binding protein was being expressed in the insoluble protein fraction as inclusion bodies, meaning that it cannot bind to the dsRNA expressed in the soluble fraction. Therefore, increasing the concentration of dsRNA binding protein expressed in the soluble fraction will prevent degradation of dsRNA within the minicells, increasing overall dsRNA yield. In order to optimize protein expression in the soluble fraction, several variables will be tested. Temperature of cell growth and protein expression, protein expression induction timing, and inducer concentrations will be tested to determine the best dsRNA binding protein expression conditions on a small scale. In addition, new genetic alterations to the plasmid with the dsRNA binding protein will be implemented to improve expression in the soluble fraction. Currently, AgroSpheres has a plasmid that fuses Green Fluorescent Protein (GFP) to the double stranded RNA binding protein. A new protein quantification methodology will be developed that uses both GFP fluorescence levels and protein concentration measured using a Nanodrop machine to determine the amount of protein expressed in both the soluble and insoluble fractions, and the total protein in general.

Optimizing fermentation is important in ensuring and improving the scalability of production, and several aspects of the fermentation process will be targeted for improvement. Fermentation optimization will primarily be conducted using lab-scale bioreactors which is

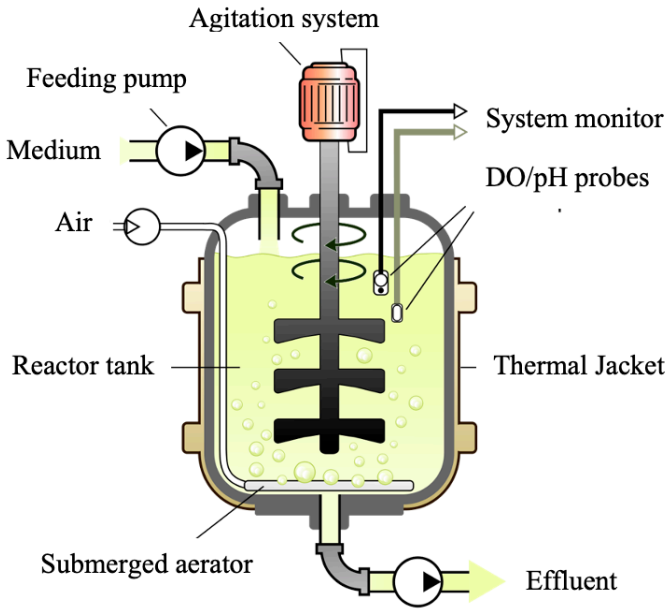

Figure 1: Components of a Lab Scale Bioreactor: Component either sense or control medium specific factors such as dissolved oxygen (DO), pH, and available substrate (Adapted by Evan Biederman, 2020 from Y. Mrabet, 2009)

diagrammed in Figure 1. By utilizing the DO and NewBrunswick Biocommand software, an exponential feed rate will be implemented which will result in a higher level of cell growth by providing nutrients proportional to the exponentially growing population. In addition, adjusting batch phase minimal media components through small-scale growth studies in shake flasks will optimize growth at this stage of production. Most importantly is optimizing the conditions around induction, since this is a step in the bioprocess which shifts the metabolism of the cells from growing to producing protein and dsRNA (Wechselberger et al., 2012). An analysis of substrate uptake before and after induction is needed since studies show this parameter to be essential for increasing the production of recombinant protein products (Wechselberger et al., 2012). Implementing these methods will be an innovative approach to characterize protein expression patterns at a larger scale.

The intended effect of this project, which will be presented as a scholarly article, will be to lower the production costs for manufacturing biological pesticides. Characterizing the expression of dsRNA binding protein will lead to increased stability of the dsRNA encapsulated within the bioparticle. In addition, improvements to the fermentation process will contribute to making the technology easier to scale up in production. By optimizing the production of AgroSphere's biopesticides, the company will be able to more effectively compete with the

harmful chemical pesticides produced by the current leaders of the agriculture industry. Therefore, the outcome of this project will be a translational RNAi biopesticide that is safer, more sustainable, and commercially scalable.

CAN HUMAN ENHANCEMENT BE DONE ETHICALLY?

In the Fall of 2020, the Nobel Prize in chemistry was awarded to Jennifer A. Doudna and Emmanuelle Charpentier for their work developing the revolutionary gene editing technology known as CRISPR-Cas9 (Gaurino, 2020). This award is historic not only for being the first Nobel Prize in chemistry awarded to two women, but also by marking the paradigm shift CRISPR-Cas9 has had on field of genetic engineering. The genetic editing system is composed of two parts: CRISPR, an enzyme which functions in archaea and bacteria to target and remove viral DNA, and Cas9, a protein which works with CRISPR to carry a guide RNA strand which specifically targets a complementary DNA piece for extraction (Ishino, Y., Krupovic, M., & Forterre, 2018). This simple enzyme-protein couple which was first identified as the immune system for bacteria and archaea in microbiology labs now allows engineers to edit the genome of most organisms.

The significance of the CRISPR-Cas9 system is in its simplicity. Before CRISPR, gene editing was a laborious and time-intensive process. Molecular widgets such as zinc-finger nucleases could take up to a year to design for a target gene (Bleicher, 2018). Proteins designed to specifically cleave strands of DNA would cost more than $1,000 to develop (Schwartz, 2018). Stanford bioethicist Hank Greenly eloquently denotes CRISPR's impact by comparing its development to the invention of the Model T, saying:

> The Model T was cheap and reliable, and before long everybody had a car and the world changed. CRISPR has made gene editing cheap, easy and accessible, and therefore more common (Schwartz, 2018).

CRISPR ultimately lowers the economic and expertise barriers which previously hindered genetic engineering research, priming biotechnology for an explosion of innovation in the next decade.

Currently, genetic engineering with CRISPR-Cas9 is being used to develop gene therapy treatments for a few genetic diseases. For example, researchers at Stanford University are using CRISPR to alter the genes which cause sickle cell anemia (Dusheck, 2016). This application of CRISPR is on human somatic cells, which are cells which are not involved in reproduction. While this treatment has yet to receive FDA approval, somatic cell gene editing does not generate significant ethical concerns in the medical community and promises to be the source of many useful therapies in the future (Dusheck, 2016).

The controversy surrounding CRISPR-Cas9 emerges when researchers turn their attention to germ-line cells, cells involved in reproduction. In 2018, researchers in China made this controversy more than just a hypothetical concern when they announced they had successfully used CRISPR to edit a gene in unborn human germ-line cells to make unsusceptible to HIV (Raposo, 2019). While this study was unethical for a number of reasons, the main issue which caught the world's attention was that this was the first successful experiment to effectively enhance a human genome. The issue of human enhancement is a controversial subject in the field of biology, mainly for its historical connection to the fundamental ideas of the eugenics movement.

The eugenics movement was first put forth by Francis Galton in 1901 for the purpose of population control of the lower classes in England (Boulter, 2017). Through the early 20th century, the ideas gained popularity across Europe and in America. Though now considered a movement based on pseudoscience for the lack of statistical or genetic evidence to back up their

claims, the ideas of eugenics received wide spread acceptance in social and even scientific circles (Paul, 2016). Yet eugenics eventually fell out of favor with the American public after WWII, mainly because the Nazis used its foundational beliefs of enhancing the human race to motivate the implementation of the Holocaust (Paul, 2016). However, eugenics eventually came back in the 1960's in the form of family planning and birth control (Goering, 2014).

The later form of eugenics had some marked differences from the movement in the early 20th century. Instead of restricting an individual's control over their reproductive rights through horrific methods such as forced sterilization, this form of eugenics focused on enhancing an individual's control over their reproduction rather than restricting the individual's control (Goering, 2014). Nonetheless, the goal of these new practices was to ensure that human reproduction could be controlled to produce people with less genetic disorders, thus this movement is still considered a form of eugenics. Scholars started to differentiate this later movement of eugenics, labeling it "liberal eugenics" as opposed to the earlier version of "authoritarian eugenics" (Goering, 2014).

While at face value, liberal eugenics may seem more morally acceptable than its authoritarian counterpart, some bioethicists still note several serious issues these ideas have in society. One main critic is known as the *expressivist argument*, which takes issue with terminating pregnancies when it is known that the child will have a disability (Goering, 2014).

To further explore the literal existential questions surrounding the use of CRISPR, an analysis of the different social groups who shape and are shaped by this technology is necessary.

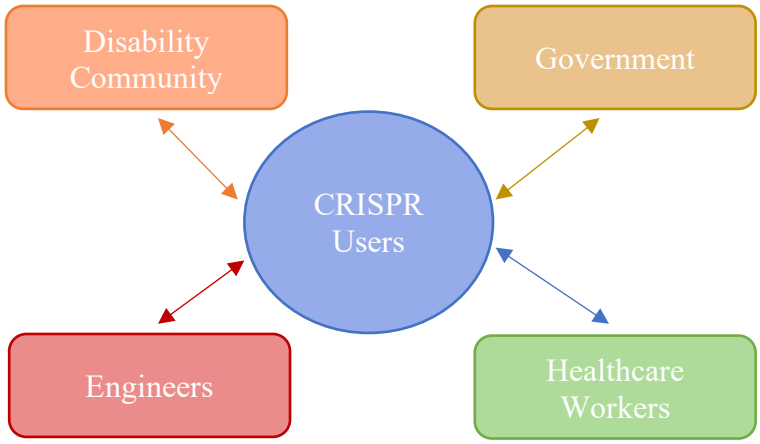

Figure 2: CRISPR SCOT Model: The user of CRISPR at the center of the social construction influences the different actors in society by the use of the technology (Biedermann, 2020).

This research paper will attempt to understand how CRISPR will interact with society by applying the broader Social Construction of Technology model (SCOT) (Johnson, 2009). The broader SCOT framework is useful for analyzing how aspects of society work to influence the development of a technological artifact and how that artifact in turn changes society (Johnson, 2009). As a result, this STS framework provides the ideal methodology for understanding the societal implications of an artifact surrounded by a lot of moral ambiguity such as CRISPR. As seen in Figure 2 above, the actors most directly involved with the CRISPR user are the government, the disability community, healthcare workers, and the engineers developing CRISPR technology. It is important to consider how the potential enhancements CRISPR may have in people would affect government systems, where it is quite likely that it may concentrate power to wealthy people who have the means to afford enhancements.

The other component of the of the project will be to examine the gatekeepers of the CRISPR technology. As seen in Figure 3, the current gatekeepers of CRISPR are the government and scientific community who either have regulations or codes of ethics which prevent the use of

CRISPR on humans. The desired outcome of this research paper will be to identify methods these actors should take to ensure CRISPR is ethically used to bring about a better future. While this research project will inevitably identify tradeoffs brought about by the use of CRISPR, this paper will aspire to determine the most ideal socio-technical position for the further use of CRISPR.

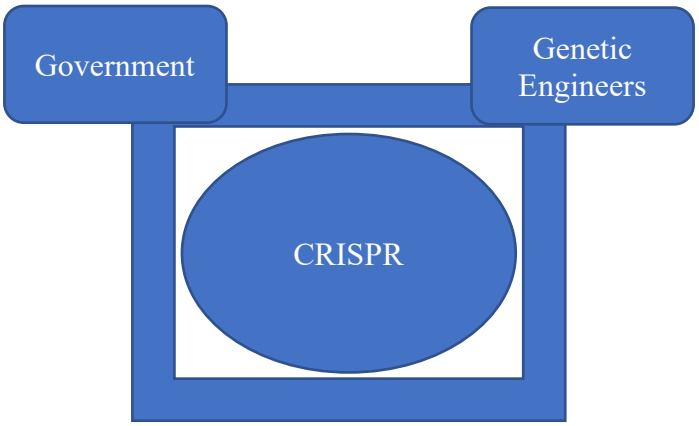

Figure 3: The Gatekeepers of CRISPR: Currently government regulation and scientific codes of ethics are the gatekeepers for the use of CRISPR (Biedermann, 2020).

THE MATTER REVOLUTION

Society has been through many technological revolutions since the middle of the 19th century. The industrial revolution saw the invention of the steam engineer and large-scale factory production which brought about new technologies such as automobiles in large quantities. The information revolution came about with the invention and expansion of the internet, and the effects of this revolution are still unfolding today as society learns to cope with a constant stream of information available through the internet. With the advances in biotechnology over the past decade, society is on the brink of the next revolution: The Matter Revolution. By increasing the ability to engineer biology, humanity is now reaching the point where we can design with specificity down to the molecular level.

With continuing innovation, the future will likely be shaped by biotechnology. This industry has the potential to define better methods for agriculture, sustainability, and healthcare. However, this technology is also powerful enough to also bring humanity to new levels of oppression and disaster. With stakes this high, it is essential to evaluate exactly what

biotechnology's role should be in society, because just assuming technological determinism is gambling with humanity's fate.

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