Yeast Lab Group Report

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Introduction

The goal of this experiment was to develop an assay that uses yeast to sense molecular environmental toxins to human health. Yeast is one of the most studied, inexpensive, and robust eukaryotic organisms with an easily tunable genome allowing for manipulation of behavior via addition of genetic circuits. Multiple reader and reporter strains of yeast with different additional genetic circuits that control growth in response to the presence of a molecule of interest can be used to create a competition assay. Specifically, a competition assay between a wild-type reporter strain and a Z4EV-S-Cup1-P modified reader strain was used to determine what concentration of added β-estradiol was able to rescue the cell death caused by added copper. Optical density measurements were used to quantify the amount of each strain combined for every trial, and final conclusions were drawn using qualitative visual analysis of all plated trials relative to the controls.

Methods and Materials

In preparation of the study, single yeast colonies of different strains were cultured in 3 mL synthetic complete broths overnight. They were stored in test tubes inside of shaking incubators at 30℃. Prior to the experiments, dilute aliquots of yeast (1:10) from culture were prepared and the optical density was measured and recorded. Next, master mixes containing reader and reporter strains were prepared. The reporter strain was wild-type with violacein and labeled strain 1. The reader strain was Z4EV-S-Cup1-P and labeled strain 4. With the desired optical density ratio of 1:5 for strain 1:4, and the requirement that the highest concentration strain should be at 0.1 OD, the following calculations were made to obtain the required stock volumes needed.

$$(Measured\ Strain\ 1\ OD)(V\,1) = (Desired\ Strain\ 1\ OD)(Desired\ Total\ Master\ Mix\ Volume)$$
$$(1.755)(V\,1) = (0.02)(6\ x\ 1mL)$$
$$V\,1 = Volume\ of\ Strain\ 1\ Stock\ Needed = 0.06838mL$$

$$(Measured\ Strain\ 4\ OD)(V\,4) = (Desired\ Strain\ 4\ OD)(Desired\ Total\ Master\ Mix\ Volume)$$
$$(1.673)(V\,4) = (0.1)(6\ x\ 1mL)$$
$$V\,4 = Volume\ of\ Strain\ 4\ Stock\ Needed = 0.35864mL$$

$$Volume\ of\ Media\ Needed = Total\ Master\ Mix\ Volume - V\,1 - V\,4$$
$$= 6mL - 0.06838mL - 0.35864mL = 5.574mL$$

After the appropriate volumes of the strain 1 stock, strain 4 stock, and media were added into a sterile test tube and well-mixed with a vortex mixer, the following test tubes representing different experimental conditions were prepared and well-mixed (Table 1).

Table 1. Recipe for test tubes containing 6 different experimental conditions including positive and negative control.

	Master Mix	**β-estradiol or Ethanol**	**Copper or Water**
Positive Control	1mL	10μL ethanol	10μL water
Negative Control	1mL	10μL ethanol	10μL copper
Experimental 1	1mL	10μL 92μM β-estradiol	10μL copper
Experimental 2	1mL	10μL 0.92μM β-estradiol	10μL copper
Experimental 3	1mL	10μL 0.092μM β-estradiol	10μL copper
Experimental 4	1mL	10μL 0.0092μM β-estradiol	10μL copper

Each of the experimental tubes were stored in a 30℃ shaking incubator overnight. The tubes were taken out of the incubator in the morning and placed in the refrigerator. In two labeled, gridded YPAD plates, 3 drops of 5μL of each control and experimental conditions were added to distinct locations on the plates. In summary, there were nine droplets representing 3 trials of 3 experimental conditions on each YPAD plate in a grid-like fashion. Pictures of the experimental setup can be found in Figure 1 below. The plates were left to dry before being covered, inverted, stacked and stored in 30℃ humid incubator for 30-50 hours. Observations were recorded and photographed before the plates were parafilmed and moved to the fridge for long term storage.

All of the equipment and supplies such as yeast, test tubes, plates and culture media were provided by the teaching team. In addition, personal protective equipment such as gloves, lab coats and goggles were worn by all team members as the group worked with yeast, β-estradiol, and other potential hazardous chemicals in the lab. Basic wet lab expectations were followed at all times. All waste were properly disposed either in the trash or taken care of appropriately by the teaching team. Specifically, the pipette tips were disposed in designated receptacles, and the liquid culture and plates were bleached and disposed as hazardous waste.

Results

Based on the experimental method described above, the positive control was expected to be predominantly white at the end of the experiment when the yeast were put in stasis. Because the white, Z4EV-S-Cup1-P strain was added in excess and there were no molecules affecting the

growth of either strain, the white reader strain should have out competed the purple wild type strain and grown in excess. The negative control was expected to appear mostly purple at the end of the experiment, due to the addition of copper, which inhibits the Z4EV-S-Cup1-P strain from growing due to the gene PPZ1 being expressed which inhibits growth. Experimental tests one through four, with decreasing concentrations of β-estradiol, were expected to show an inverse relationship with the ratio of reporter to reader strain. As concentrations of hormone decreased, it was hypothesized that the ratio of reporter (purple) to reader (white) would increase since the Z4EV-S-Cup1-P strain would not be able to inhibit PPZ1 gene which leads to inhibited growth.

After about two days after plating, all experimental tests and both controls appeared completely white, indicating the Z4EV-S-Cup-1-P strain was dominating growth (Figure 1). Because the Z4EV-S-Cup-1-P strain was originally added in excess, it cannot be determined whether or not this dominance is left over from the original strain ratio or is a result of the reagents added. The ratios of reporter to reader at six days after plating was observed as expected for the positive and negative controls, meaning that they were successful and can be used for comparison and analysis of the experimental trials. With no reagents added, the Z4EV-S-Cup-1-P strain (added in excess) dominated growth in the positive trial. With only copper added, growth of the Z4EV-S-Cup-1-P strain was inhibited and the wild type reporter strain dominated in growth for the negative control. However, the opposite of the hypothesized trend regarding the effects of varying β-estradiol concentration was observed for experimental tests one through four (Figure 1). Experimental test two, with an intermediate concentration of β-estradiol, appeared to have the highest ratio of reporter to reader of all conditions, as it appeared even more purple than the negative control. Experimental test one had the second highest ratio of reporter to reader, followed by experimental test three, both of which also appeared higher than the negative control. Experimental test four appears to have a very similar ratio to the negative control.

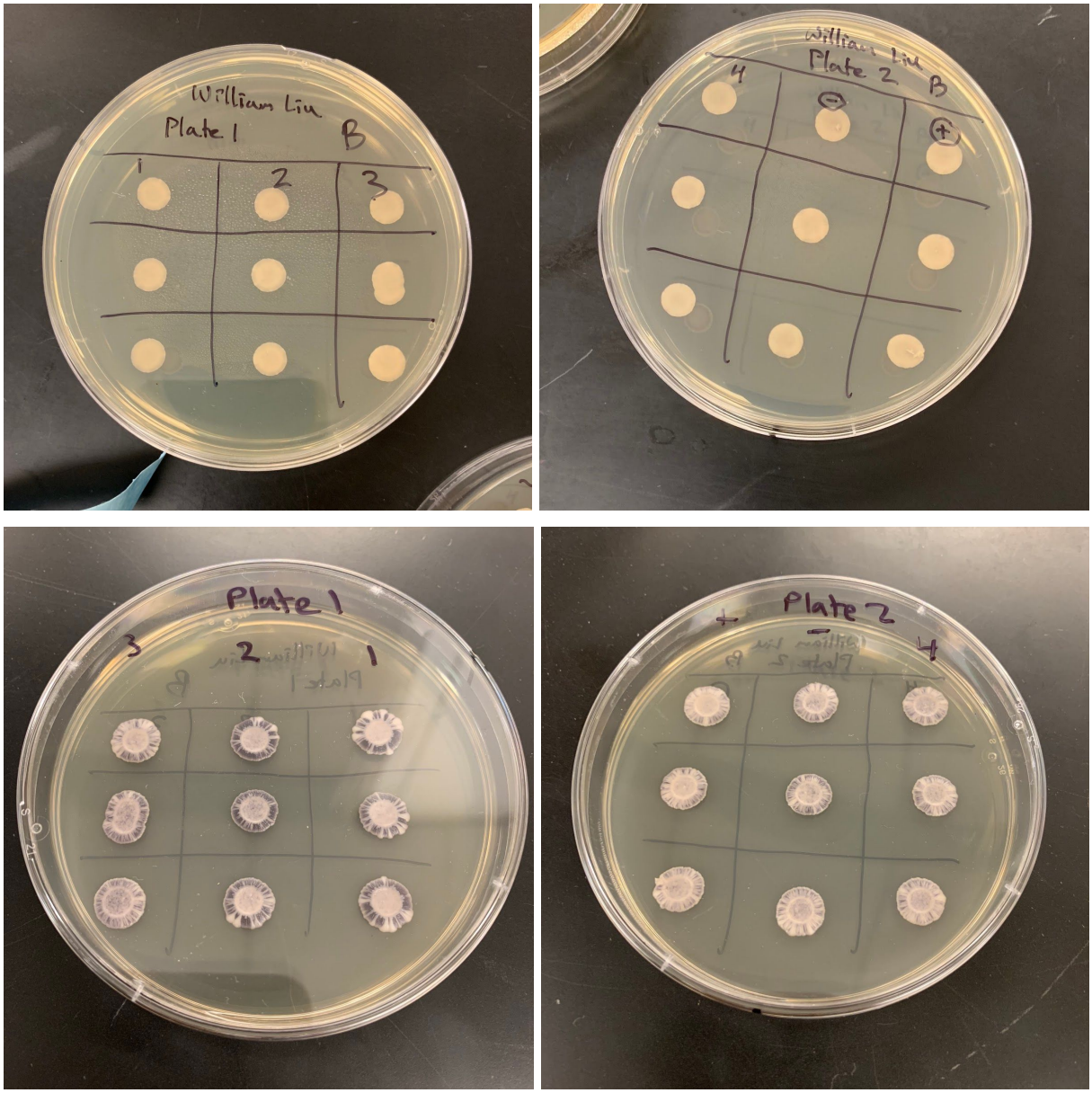

Figure 1. The top row shows the plates from the top two days after plating. The bottom row shows the plates from the bottom after being put in stasis at six days. Experimental tests 1-4 are labeled as such, and the positive and negative controls are labeled with a positive sign and a negative sign, respectively.

All data analysis was performed qualitatively by comparing the relative differences in the ratio of reporter to reader strain compared to the positive and negative controls at two days and six days after plating. Analysis of the two time points was included to provide a more complete picture of the competition between the two strains over time. Additionally, the experimental set up allowed for multiple images of the plated strains to be captured, which allowed for verification the experiment was progressing as intended. No quantitative measurements were

collected during the experiments, and no quantitative image analysis was done due to the unexpected and qualitatively obvious nature of the results.

Conclusions and Discussion

The results indicate that addition of different β-estradiol concentrations before plating did not have the hypothesized effect on the ratio of purple wild type strain (reporter) to Z4EV-S-Cup1-P strain (reader) growth within the observed time points. However, it is possible that the two day time point was too early to capture the effect of the added reagents, and likely that the six day time point was too late. The results of the experimental tests at day six are likely due to the reagents as well as other factors, or even just due to the other factors since the protocol did not include any readdition of the experimental reagents. Because the purple wild type strain appeared to dominate growth in all conditions tested, even tinting the positive control purple, it is likely that the wild type strain grows more robustly than the Z4EV-S-Cup1-P strain. This innate difference in growth ability would explain the observed results and indicate that imaging prior to day six would have been necessary to capture the effect of the varying β-estradiol concentration before the natural competition between the two strains took over. Therefore, there is a possibility that β-estradiol could rescue the inhibition of cell growth that copper causes, however, the experimental design issue of not frequently imaging the progress prevents a definitive conclusion from being drawn whether there is a concentration of β-estradiol that can recover growth.

This work fits into a larger context of understanding gene regulatory pathways and in general designing sensors that can track toxic molecules in any environment. Understanding these signaling pathways, allows people to design drugs and biotherapeutics that can change the expression of a gene and potentially inhibit a harmful process or stimulate a crucial process which may be deficient in one way or another in the body. Furthermore, as will be discussed in the future work section, being able to sense chemicals in the air will be able to save many lives from chemical toxins developed to target mass populations.

This lab illustrated the aspect of control theory and feedback inhibition that was taught in BIOEN 336. This experiment and the concept of signaling diagrams relates to control theory because multiple inputs are applied and then a certain output is identified. In this case, the inputs are copper which inhibits growth of the Z4EV-S-Cup1-P strain and β-estradiol which inhibits the gene that inhibits growth of that strain. The effects of the two inputs coincide in the signaling pathway and in turn cause an output which is either the growth of the Z4EV-S-Cup1-P strain or a lack of growth. This can possibly be modeled using a differential equation just like most control theory applications and the transfer function given for this experiments parameters could be generated for the Z4EV-S-Cup1-P strain. Through mathematical modeling of the genetic circuit for expression of PPZ1 (the gene that inhibits growth), the answer to whether or not β-estradiol could rescue cell growth would be obvious depending on the transfer function generated for specific concentrations of inputs. More research would have to be done however to accurately

model the differential equations. For example how does x amount of concentration affect the inhibition or promotion of the gene PPZ1.

As with all science experiments, complications always arise despite doing the best of trying to control for every possible factor that could affect the outcome of the experiment. One problem that could have affected the results could be due to accidental contamination from microorganisms in the general environment which could have affected the growth rate of the yeast. While the experiment was executed as sterile as possible, some contamination is a possibility. Some limitations in this particular experiment were lack of knowledge about how the different strains grew compared to each other and if one strain was more robust than the other in terms of growth under no toxic conditions. Additionally, there was a limitation on the number of plates that could be used to execute different experiments and more trials. If there had been more availability on resources, additional experiments could have been achieved to test the theories discussed throughout the paper on what went wrong in this first experiment.

Since the data collected was against the expected result for the experimental trials and to an extent the controls as well, another experiment to troubleshoot the issues would be to follow the same procedure, except scale up the amount of reagents used and check back on the progress of growth more frequently. Working with such small concentrations, it is very easy to lose some material or not have reagents thoroughly and equally mixed and so that is one possible way the result we received contradicted what was expected. Additionally, many more imaging time points would be included in these future experiments. This would ensure that the true effects of the reagents added are captured, not just the inherent differences in innate strain robustness, and would also allow for a greater understanding of the rate at which the strains compete and the actual time window in which the reagents have an effect. Additionally, experimental trials could be setup to determine how the Z4EV-S-Cup1-P reader strain competes with the growth of multiple different reporter strains, which would help eliminate the contribution of the inherent differences in growth ability to the experimental results and conclusions.

Future Directions

The large body of knowledge and research surrounding yeast makes it an ideal candidate when one wishes to develop a functional biosensor. It was shown in this lab that genetic circuits in yeast could be engineered such that their growth was modulated by the presence of environmental toxins; in this case beta-estradiol, copper, or both. Another class of molecules for which an assay would be extremely useful are the organophosphates. DNA, RNA, and ATP are all examples of organophosphates that most people are very familiar with, but this category of molecule also includes pesticides and nerve agents, which would be the main targets of the yeast biosensor. Although there are many different organophosphates that serve as nerve agents, paraoxon is considered the “model molecule”[1]. Like the other nerve agents, it functions as a acetylcholinesterase inhibitor which increases the intensity and duration of the neurotransmitter acetylcholine. Acetylcholine is released at the synapse between motor neurons, and when its

degradation is blocked by an acetylcholinesterase inhibitor, muscle contractions occur continuously. This is a very dangerous situation and can result in death by asphyxiation or cardiac arrest in as little as a few minutes[5].

Having a simple assay to detect nerve agents would be especially useful to maintain the safety of populations, especially as some very prominent chemical weapon attacks have been in the news over the past few years. Researchers were able to isolate more than 30 differentially expressed genes in yeast that responded to environmental paraoxon[7]. They did this by incubating yeast with or without paraoxon and analyzing which open reading frames (ORF) had transcripts induced by the presence of the toxin. Once they selected an ORF for its high sensitivity to paraoxon, they engineered the promoter site of the gene with green fluorescence protein so they could monitor transcription using a fluorescence assay.

While the fluorescence based assays are very common and offer accurate measurements, it would also be useful to engineer a paraoxon biosensor that worked on a similar principle to that of this lab; differential growth rate between the reporter and reader strains of yeast. The problem with the approach that Schofield and his team took was that they did not specifically engineer yeast to be responsive to the toxin, they simply found existing genes that were induced in the presence of paraoxon. For the relative growth assay it is necessary that there are two distinct strains of yeast that display differential growth when exposed to the toxin. To achieve this, we propose targeting the *erg6* gene which is plays a role in synthesizing the primary cholesterol found in yeast cell membranes, ergosterol[3]. It was shown that a mutation causing a null allele in the *erg6* gene had no effect on normal cell growth, but did make the cell membrane more permeable. Furthermore, Schofield showed that a ten hour incubation with 0.5mM paraoxon did no affect the growth of wild-type cells but it did have a drastic effect on the *erg6* mutant cells. This fact is the key to the proposed relative growth assay. The same violacein-modified yeast could be used as the reader strain as they grow like wild-type yeast, while the *erg6* modified yeast could be used as the reporter strain. A solution of both strains could be incubated with paraoxon for ten hours and then grown on a plate exactly like the process for this lab, making for a very accessible and cost-effective assay.

Being able to test for organophosphates easily is becoming more and more important in the current society. There are 36 organophosphate insecticides approved for use by the US government, all of which can cause severe health problems[6]. Additionally, the Novichok nerve agents, developed and used by Russia, are organophosphates that can cause death with only a ten milligram exposure to the skin[2]. Having an assay for this class of molecule is a necessity to protect public health, and making one that is simple to setup and read would allow more governments, especially those in low-resource settings, to perform this valuable test.

To take this application of yeast and make it even more useful, it appears possible to engineer it to be a biocatalyst such that it actively removes organophosphates from its environment. There is a bacterial enzyme called organophosphorus hydrolase (OPH) that does as its name suggests, it breaks down organophosphates by cleaving phosphorous-ester bonds[4]. It is

possible to make recombinant yeast cells that produce this enzyme, and thus have the ability to hydrolyze organophosphates. We suggest that engineering a yeast cell that had the null allele in *erg6* and produced OPH would make a very potent biocatalyst as it would allow more of the toxin into the cell to be broken down. This could prove to be a very useful yeast-based system that allows for the detection and removal of a toxin that has profound human-health implications, whether it is used as chemical weapon or found on the food we eat.

References

1. Adeniran, A., Sherer, M., Tyo, K., & Siewers, V. (2015). Yeast-based biosensors: Design and applications. FEMS Yeast Research, 15(1), 1-15.
2. Brunning, A. (2018, March 2). What We Do (and Don't) Know About Novichok Agents. Retrieved from https://www.americanscientist.org/article/what-we-do-and-dont-know-about-novichok-agents.
3. Gaber, R F, Copple, D M, Kennedy, B K, Vidal, M, & Bard, M. (1989). The yeast gene ERG6 is required for normal membrane function but is not essential for biosynthesis of the cell-cycle-sparking sterol. Molecular and Cellular Biology, 9(8), 3447-56.
4. Makkar, R., DiNovo, A., Westwater, C., & Schofield, D. (2013). Enzyme-Mediated Bioremediation of Organophosphates Using Stable Yeast Biocatalysts. Journal of Bioremediation & Biodegredation, 4(3), 1.
5. "Medical Management Guidelines (MMGs): Nerve Agents (GA, GB, GD, VX)". Agency for Toxic Substances and Disease Registry (ATSDR). U.S. Department of Health and Human Services. Archived from the original on 2018-01-25. Retrieved 2019-03-11.
6. Roberts, J. R., & Reigart, J. R. (2013). RECOGNITION AND MANAGEMENT OF PESTICIDE POISONINGS [Sixth Edition]. U.S. Environmental Protection Agency. Retrieved from https://www.epa.gov/sites/production/files/2015-01/documents/rmpp_6thed_final_lowresopt.pdf.
7. Schofield, D., Westwater, C., Barth, J., & Dinovo, A. (2007). Development of a yeast biosensor–biocatalyst for the detection and biodegradation of the organophosphate paraoxon. Applied Microbiology and Biotechnology, 76(6), 1383-1394.