Biodegradability

Project Journal

Biodegradability of cellulose:

- **5/28/2014**
    - http://www.acetateweb.com/pdf/Degradation_of_Cellulose_Acetate-Based_Materials_%20A%20Review.pdf
        - 2 mechanisms for degrading cellulose acetate: use of acetylesterases for partial deacetylation then the rest is easily degraded on its own or using photodegradation (sunlight not strong enough therefore use of TiO2 along with sunlight to degrade cellulose acetate)
            - combining both mechanisms would increase degradation rate
            - commercial has DS of 2.5
        - http://link.springer.com.revproxy.brown.edu/article/10.1023%2FA%3A1021869530253/-
            - anaerobic biodegradation of cellulose acetate
            - http://www.daicel.com/cell_ac/en/
                - (cellulose acetate being heat resistant, chemical resistant, burning resistant?)
        - Determining degrees of substitution
            - “Characterization was performed with Thermogravimetric Analysis (TGA), Fourier Transform Infrared Spectroscopy (FTIR), retrotitration (known as back titration) to determine the degree of substitution of AC and atomic force microscopy (AFM).”
            - http://www.scielo.br/scielo.php?pid=S0104-14282013000100013&script=sci_abstract
        - http://pdf.lookchem.com/pdf/22/4723f226-387e-4f0d-81ab-248719ba82ad.pdf
            - use of cellobiose octaacetatease (DS of 0.76)

- use of pseudomonas paucimobilis (DS of 2.5 'commercial')
- can we potentially use P. fluorescens to degrade cellulose acetate?

- It would be important to put this project into two phases/subgroups:
  - Degrading cellulose acetate in general using biological means- The industrial cellulose is 2.5 substituted (90% acetylated) and this causes it to be less biodegradable. The degradation of this highly substituted cellulose requires deacetylation process to occur first since cellulase enzyme cannot act on acetylated cellulose. Links above show how the biodegradation occurs. To improve the biodegradability of industrially made cellulose:
    - Are there microbes which produce deacetylases or which are capable of breaking down cellulose acetate? http://www.tandfonline.com/doi/abs/10.1080/10601329308021259#preview/ - cellulose acetate buried in moist soil loses 70% of its original weight due to a fast substitution of cellulose acetate with components which are easily degraded. Possibly deacetylation process is carried by some micro-organisms in soil? We can possibly find these organisms and find the proteins/enzymes they use for biodegrading cellulose
      - How can we increase the photo-degradability of cellulose without using Titanium oxide?
    - Degradation of the drone made by cellulose acetate:
      - When, how and under what conditions do we need the drones to bio-degrade?
      - How will the cross-linkers on the drone affect the biodegradability of cellulose?
      - Can we put lambda/genetic switches which under specific conditions (probably collision etc) will turn on the bio-degradability switches?
- Using hell cell biofilm to secrete acetylesterases and cellulase enzyme:

        - What if the hell cell could be engineered in such a way it will produce acetylesterases and cellulases (important for biodegradation) in certain conditions (eg upon collision)
    - http://onlinelibrary.wiley.com/doi/10.1002/app.1993.070471001/abstract/- Aerobic degradation of cellulose acetate and the number of days it takes to degrade cellulose di-acetate/triacetate or just cellulose acetate
    - **http://www.ncbi.nlm.nih.gov/pubmed/8987659**
        - **Biodegradation of cellulose acetate by Neisseria sicca.** (use gene only)
    - **http://www.ncbi.nlm.nih.gov/pubmed/20834142**

06/09/14

- Both sequences have been optimized and checked for restriction enzyme sites. They will be ordered soon.
    - Further research needed on WHEN degradation should occur. Some ideas:
        - 1) Finding a pressure-sensitive promoter (so that the drone can degrade upon impact)
            - High pressure-sensitive gene expression in Lactobacillus sanfranciscensis. (these are hydrostatic pressure-sensitive genes!)
                - http://www.ncbi.nlm.nih.gov/pubmed/16082466
            - http://www.pnas.org/content/84/8/2297.full.pdf
                - Pressure-sensitive ion channel in Escherichia coli (spheroplast membrane channels were opened by positive pressure (blowing) problem is that opens ion channels under low pressures)
        - 2) Linking the death of the power source running the drone to degradation (This idea would first require research on possible power sources for our drone and some research on electrical/mechanical engineering to see if we can link it to biological activity)
            - Cambridge tried to create an electrical biological interface but was unsuccessful in their attempts
                - http://2008.igem.org/Team:Cambridge/Voltage
        - 3) Time (a biological clock of some sort so that you can tune the drone to degrade after a certain time)
- 6/10/2014

    - More research on a promoter needed to induce complete degradation of cellulose:
        - 1. Pressure sensitive promoters
            - http://www.ncbi.nlm.nih.gov/pmc/articles/PMC129069/- using ionic regulation of a mechanosensitive channel from E. coli
        - 2. Time induced promoters
        - 3. Vibration sensitive promoters
    - The constructs for both esterase and endo 1,4-beta glucanase had to be supplemented with primers, T7 promoter, RBS and a terminator. Thanks to Kosuke, that has been done!
- **6/11/2014**
    - Researching more into assays for purification of both esterase and endo 1,4-beta glucanase
- **6/12/2014**
    - The signal sequence for Neisseria sicca was taken out of the initial cellulase and esterase constructs and replaced with a signal sequence (sec-tag) specific to E. coli. The sec-tag that was put into the constructs will allow E. coli to secrete the proteins into the extracellular environment and begin the degradation of the cellulose acetate.
    - http://www.ncbi.nlm.nih.gov/pubmed/19013157/- the tat signal amino acid sequence for E.coli is MNNNDLFQASRRRFLAQLGGLTVAGMLGPSLL. The nucleotide sequence optimized for E.coli is ATGAATAACA ATGACCTGTT TCAGGCTTCC CGCCGTCGCT TCCTGGCACA GTTGGGCGGG TTAACAGTGG CCGGGATGCT CGGTCCTAGT CTGCTG
- **6/13/2014**
    - Finding assays for detection of deacetylation of cellulose acetate by an esterase enzyme and for detection of breakdown of cellulose by endo 1,4-beta glucanase
        - Esterase
            - To test the activity of an esterase enzyme, three possible assays can be done: detection of the presence of cellulose in the liquid culture, or detection of acetate groups in the liquid mixture
            - %acetylation of cellulose acetate using a titration assay http://lib.njutcm.edu.cn/yaodian/ep/EP5.0/16_monographs/monographs_a-c/Cellulose%20acetate.pdf

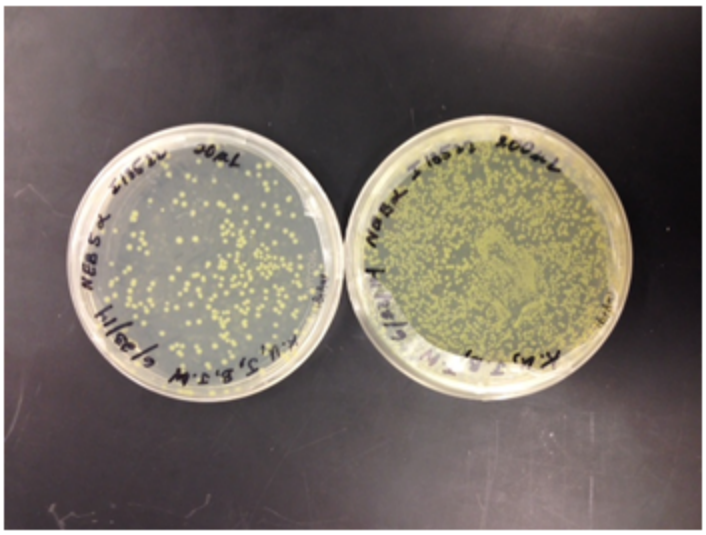
        - Endo 1,4 beta-glucanase
            - To detect the activity of this enzyme, a liquid mixture could be tested for the presence and increase of glucose, or for the decrease of cellulose concentration in the mixture
                - http://www.ncbi.nlm.nih.gov/pubmed/7139920/
                    - Four methods for glucose assay compared for various **glucose concentrations**
- **6/17/2014**
    - Finding a proper promoter to initiate biodegradability
        - http://www.scielo.br/scielo.php?pid=S0100-879X2005000800013&script=sci_arttext/- High pressure sensitive gene expression in Lactobacillus Sanfranciscensis used for food packaging
- **6/19/2014**
    - A specific Outer Membrane Protein is over-expressed upon high pressure induction. ompH is particularly increasingly expressed as pressure is increased.
      http://www.ncbi.nlm.nih.gov/pmc/articles/PMC176696/ - ompH is regulated by other environmental cues in addition to high pressure in SS9 barophilic bacteria
    - There is a pressure-inducible operon found in barophilic marine organisms http://www.uniprot.org/uniprot/Q56736/
    - Analysis of a pressure-regulated operon from the barophilic bacterium strain DB6705. www.uniprot.org/uniprot/Q56736/

6/23/2014

First day of lab!

- Transformation of a ptet pressure sensitive constitutive promoter from 2012 distribution kit to amp-competent E.coli cells was done
    - ➢ Promoter position : 8A, plate 2 from 2012
    - ➢ Registry number K783080
    - ➢ A regular transformation protocol was followed

- ➢ 2 amp-plates were used. In one plate, 20ul o



f cells was plated, in the second plate, 200ul of cells was plated.
- ➢ Plates were kept at 37 C from 2.00pm on 6/23

- More research on ways of exerting and quantifying pressure was done. Some ideas suggested include:
  - ➢ Use a centrifuge. The force exerted on the liquid culture will be given by the centrifuge scale, and pressure on the surface of the culture will be calculated from the force exerted and the surface area of the culture
  - ➢ Physically exerting known weights on the solid culture. If we exert a specific known weight (e.g. 200g) on a 0.01squared cm, we can get a considerably high pressure exerted on the surface

6/24/2014

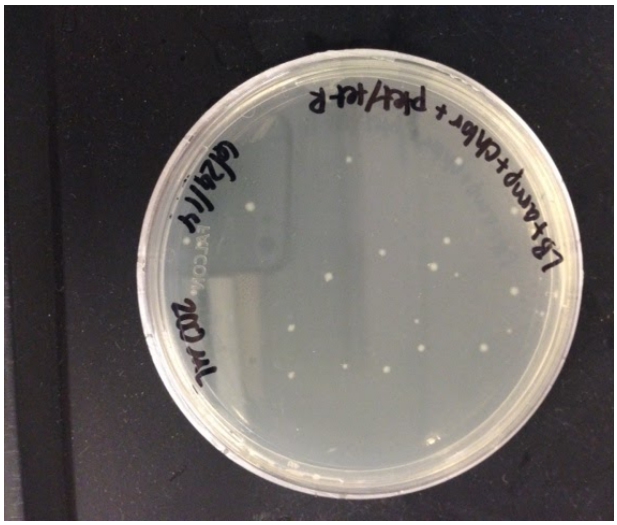
- When we checked the plates made on 6/23, the E. coli were expressing GFP, even though they were only under atmospheric pressure, so we decided to find a repressor of the ptet promoter that would decrease its sensitivity. The picture below shows how green the cells looked at atmospheric pressure

  - Found tetR gene that produces tetR protein which represses ptet
    - Position: 14K, plate 3 from 2014
    - Registry number K145201
  - Using a regular transformation protocol, we simultaneously transformed both the ptet promoter and the tetR gene (on two separate plasmids) into chemically competent E. coli cells.
  - Plated 20/200µL of cells onto LB+amp+chlor plates

        - By using plates with both antibiotics, we were able to select for only the cells that contained both plasmids
    - Plates were kept at 37°C from 4pm on 6/24

6/25/2014

- Fortunately, the repressor worked well in repressing the ptet promoter, hence the cell colonies we transformed on 6/24 were white under fluorescent light, showing that there was no significant GFP expression. The photo below shows how the colonies with both plasmids looked after night-long incubation



- Today we selected a single colony from the 200µL LB+amp+chlor plate and put it in LB+amp+chlor media to grow a liquid culture overnight. We did the same with a colony from the 20µL LB+amp plate containing E. coli with just the ptet promoter (not repressed), and grew this liquid culture to use as a control during pressure experiments
- Discussion with Dr. Shih about pressure testing:
    - Need to do literature search on the normal pressure conditions a UAV would face in flight (take-off, landing, normal flight, etc), so that we can make sure our drone does not start to degrade just from facing these normal pressures
    - Need to do field research/experiments on pressure experienced by a drone upon crashing
        - We can do this using a force plate and weighted paper airplanes. We will throw the airplane at the force plate from different angles/heights and use the collected data to calculate the expected pressure experienced when crashing

- Need to find methods for creating pressure to test our constructs
  - Use a centrifuge to create pressure
  - Use an ultra-centrifuge if the normal centrifuge does not cause enough pressure
  - Use a pressure chamber filled with air instead of hydrogen gas

6/26/2014

- The liquid culture with 2 antibiotics is growing slowly and needs to stay in the incubator longer
- We did a trial with the centrifuge and the flourometer to find out how much GFP was expressed before and after the centrifuge.
  - ➢ From the graph comparison between the cells containing Ptet promoter without tetR gene and cells containing both ptet and tetR plasmids, it was clear that there was more GFP expression in cells without the tetR plasmids.
  - ➢ Centrifugation force value of 8000g was used for 30 seconds as a trial
  - ➢ The cell samples which had been centrifuged and those which had not been centrifuged were tested for fluorescence under a flourometer using liquid culture as a blank

6/27/2014

- Today we developed a proper protocol for testing the effect of pressure on the fluorescence value of GFP.
  - ➢ There were two groups of cells: a group with ptet promoter alone (ptet-RBS-GFP) and a group with both ptet promoter and tetR repressor.
  - ➢ For each group of cells, samples were made for centrifugation under different speeds. 5 different rpms were tested on: 1000rpm, 2000rpm, 5000rpm, 10000rpm and 14000rpm.
  - ➢ For each speed, 3 samples of each of the two groups of cells were tested. This allowed us to calculate the average of fluorescence to get a more precise value
  - ➢ Since we expected the time of centrifugation to have an effect on the amount of pressure applied to the cells, we decided to submit the two cell groups in two sets of time: 5 mins and 30 mins.
- Some literature has shown that E.coli cells can die under very high pressures. We needed to test if centrifugation would cause death of E.coli cells. To do this, we made serial dilutions (1/100, 1/10000, 1/1000000) for

both cell groups and for each centrifuge speed. Serial dilution was done on un-centrifuged cell cultures too.

- ➢ To make 1/100, 10ul of the respective cell culture was mixed with 990ul of the respective lb+ antibiotic medium. Same procedure was done for the other serial dilutions
- ➢ The diluted cell cultures were plated on a square grid plate and incubated at 37C overnight

- Since the number of cells present in the cell culture can affect the amount of fluorescence, we measured OD (optical Density) of all the cell cultures that were sent to the centrifuge so as to correlate the amount of fluorescence to the number of cells

First pressure assay (design):

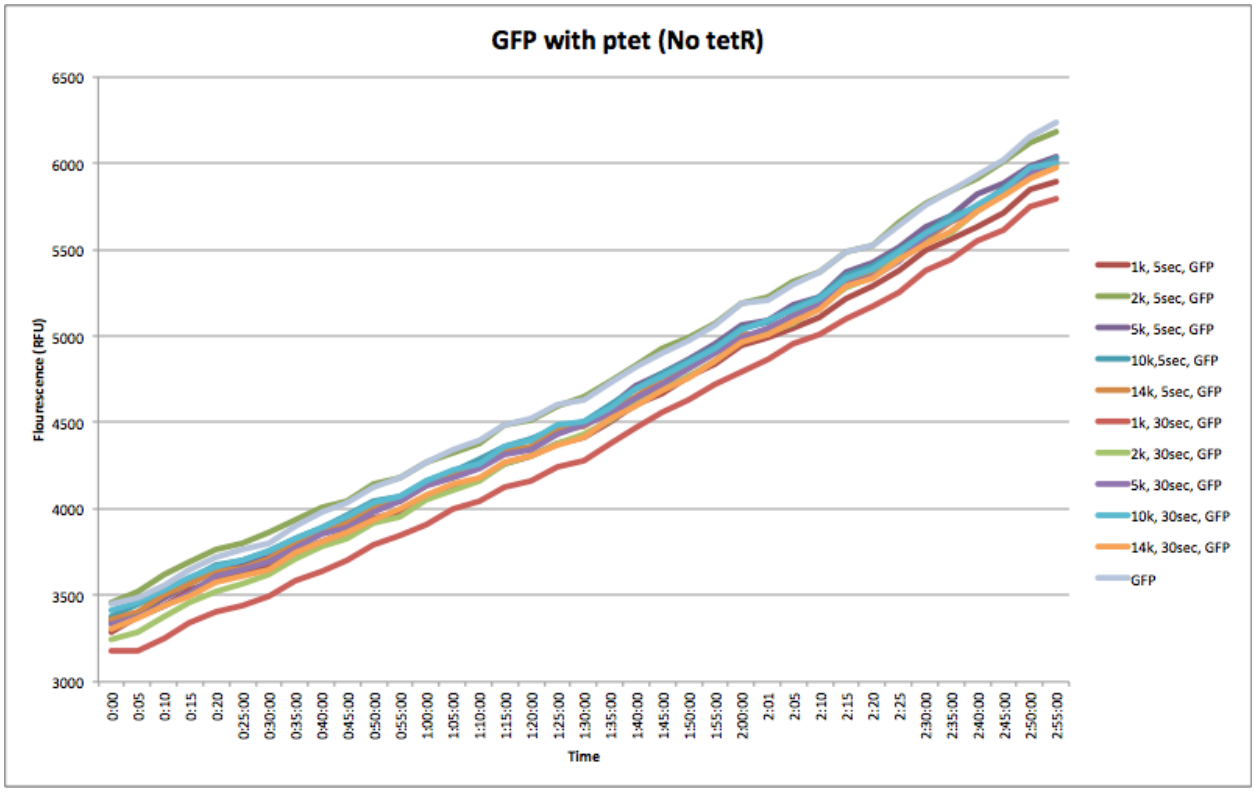
	1	2	3	4	5	6	7	8	9	10	11	12
A			1k	2k	5k	10k	14k	1k	2k	5k	10k	14k
B												
C												
D												
E			1k	2k	5k	10k	14k	1k	2k	5k	10k	14k
F												
G												
H			GFP Control (NC)				Rep Control (NC)					

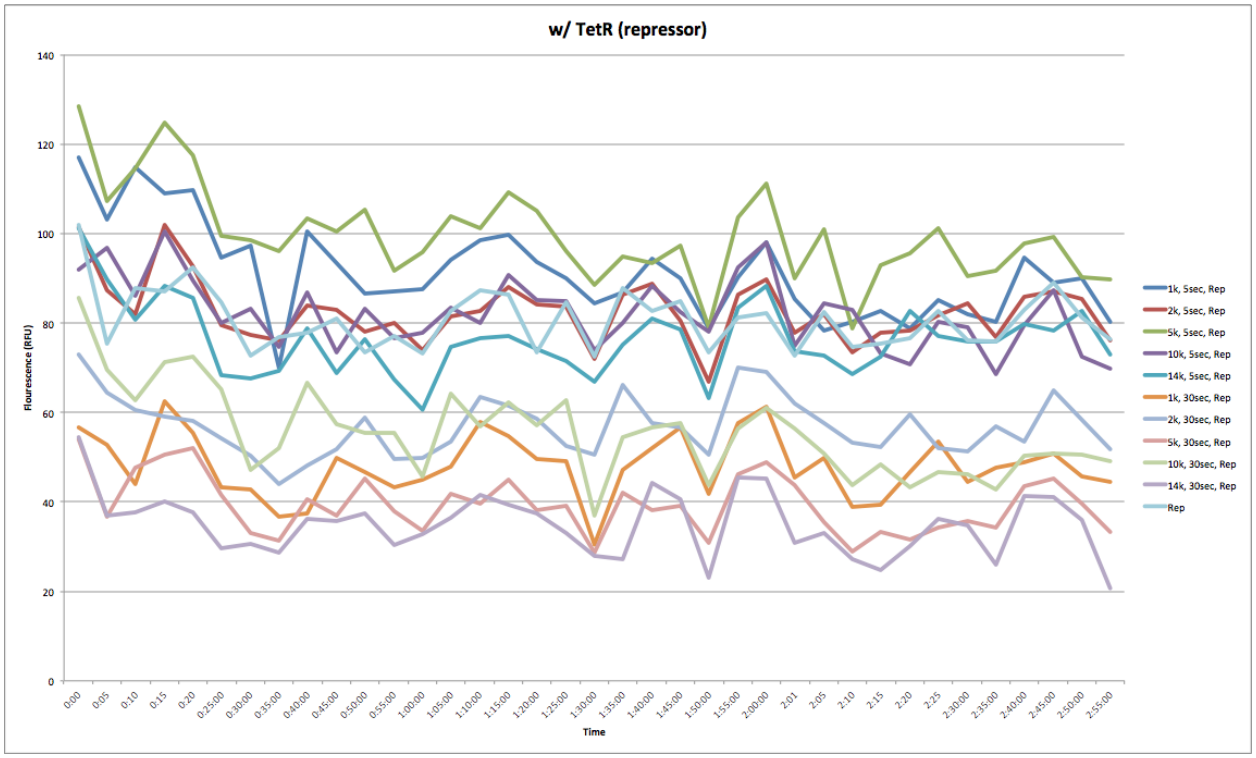
5 seconds
30 seconds

GFP (No tet repressor)
With tetR (tet repressor)

1k-14k denotes the rpm amounts

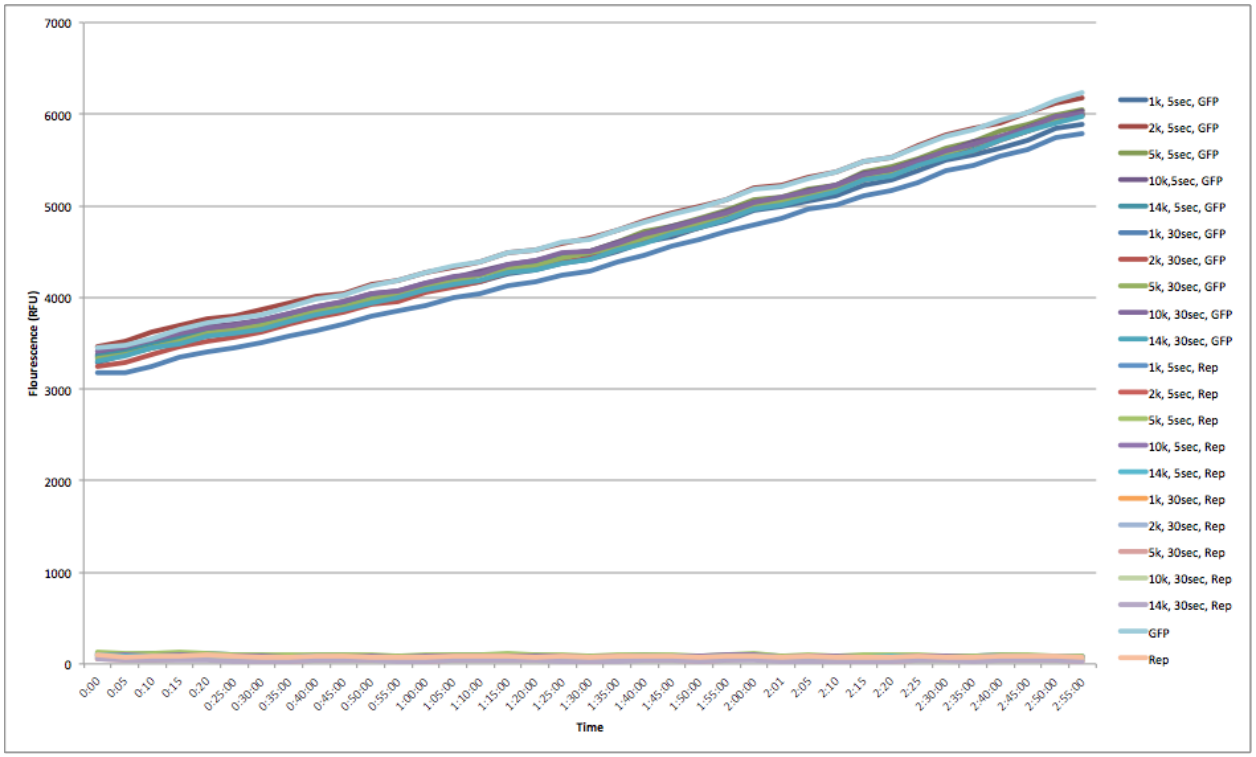
Results from assay:





(There seems to be stronger fluorescence for the samples centrifuged for 5 seconds than 30 seconds)

Both graphs together:



6/30/2014

- Cellulose Acetate Esterase (CAe) arrived on Friday 6/27, hence we had to carry out PCR to amplify the gene
    - ➢ the powder form DNA was trolled down and diluted by 20 milQ water
    - ➢ The liquid extract was vortexed and then trolled down
    - ➢ 1ul of liquid construct was mixed with 1ul of primers, 25ul of PCR master mix and 23ul of water
    - ➢ The primer melting temperature used was 60C
    - ➢ 30 PCR cycles were carried out
- PCR products were viewed on 1% agarose gel with gelred die
    - ➢ Expected band: 1.6kbp
    - ➢ Viewed band: 300bp (probably it is
    - ➢ Conclusion: PCR did not work!
- Another PCR was carried out following the same procedure. After viewing it on the gel, it did not work too!

7/1/2014

- Today, we re-ran the PCR using a different PCR master mix and a lower primer annealing temperature (57C)
    - ➢ The PCR was viewed on 1% Agarose gel
    - ➢ The expected band appeared at 1.6kb!
- DNA cleanup was done following the procedure in the cleanup kit:
    - ➢ 50ul of the PCR product was added to 500 ul of PX
    - ➢ The mixture was transferred to 1.5ml tube and with a collection tube on it
    - ➢ The mixture was centrifuged at 5000rpm for 45s. The flow-through was discarded
    - ➢ The column was washed with 500ul of WN buffer, centrifuged at 5000rpm for 45s and its supernatant discarded
    - ➢ The column was again washed with 500ul of WS buffer, centrifuged at 5000rpm for 45s and its supernatant discarded
    - ➢ The column was centrifuged again at 13000rpms for 3 more mins to remove ethanol. Then column tube was transferred to 1.5ml tube, and 25ul of elution buffer was added
    - ➢ After 3 mins, it was centrifuged at 13000 rpm for 2min to elute DNA

7/2/2014

- The stored esterase gene and a straight backbone construct from distribution kit were nano-dropped to measure their concentration.
    - ➢ Esterase: 44ng/ul
    - ➢ Backbone: 25ng/ul
- Digestion of both the backbone and the esterase genes were done so as to allow their ligation to make a circular plasmid:
    - ➢ Restriction enzymes: 0.5 ul of EcoRI and 0.5 Pst1
    - ➢ 1ug of esterase and the backbone were added.
    - ➢ 10X NE buffer was added. Total reaction volume was 50ul
    - ➢ Both of the digestion mixtures were incubated at 37C for 15 mins
- PCR cleanup was done in order to get rid of the restriction enzymes and unwanted buffer. The protocol followed was the same as that used on 7/1/2014
- The mixtures were nano-dropped to find out the concentrations of the esterase and the backbone after DNA clean-up:
    - ➢ Esterase: 9.4ng/ul

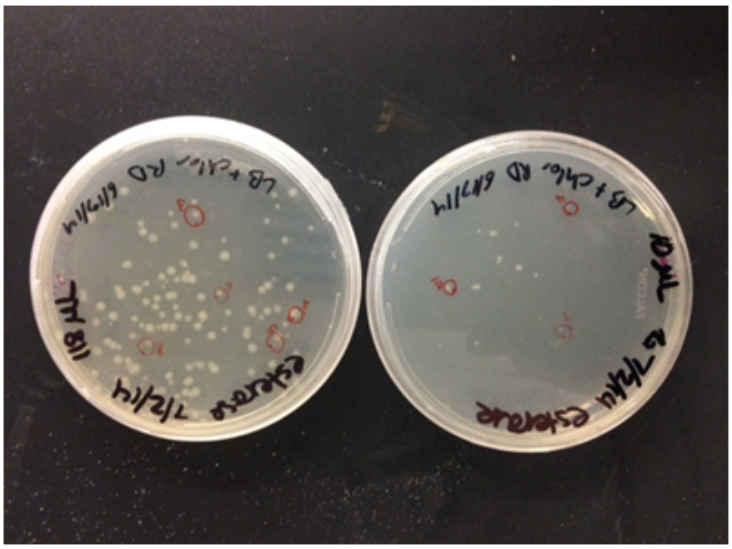
    - ➢ Plasmid: 3.2 ng/ul
- After clean-up, ligation was done using ligase:
    - ➔ 1ul ligase
    - ➔ 2ul 10X buffer
    - ➔ 17ul of the backbone
    - ➔ 5ul of the Esterase
- The ligase was heat-killed before transformation was done so as to increase the efficiency of the transformation.
- The esterase plasmid was then transformed into E.coli at 5.00pm
    - ➢ 2 plates were plated: 1 with 10ul volume and another with 118ul
    - ➢ The cells were Incubated at 37C overnight

Experimenting with cellulose acetate:

- Cellulose acetate present in our lab was ordered in powder form with following specifications:
    - ➔ Average Mn : 50000 by GPC
    - ➔ Density: 1.3g/ml at 25C
    - ➔ n20/D: 1.475
- A procedure for preparing sheets and films of cellulose acetate was found online: http://www.terrificscience.org/lessonpdfs/PolymerLab21.pdf

7/3/2014

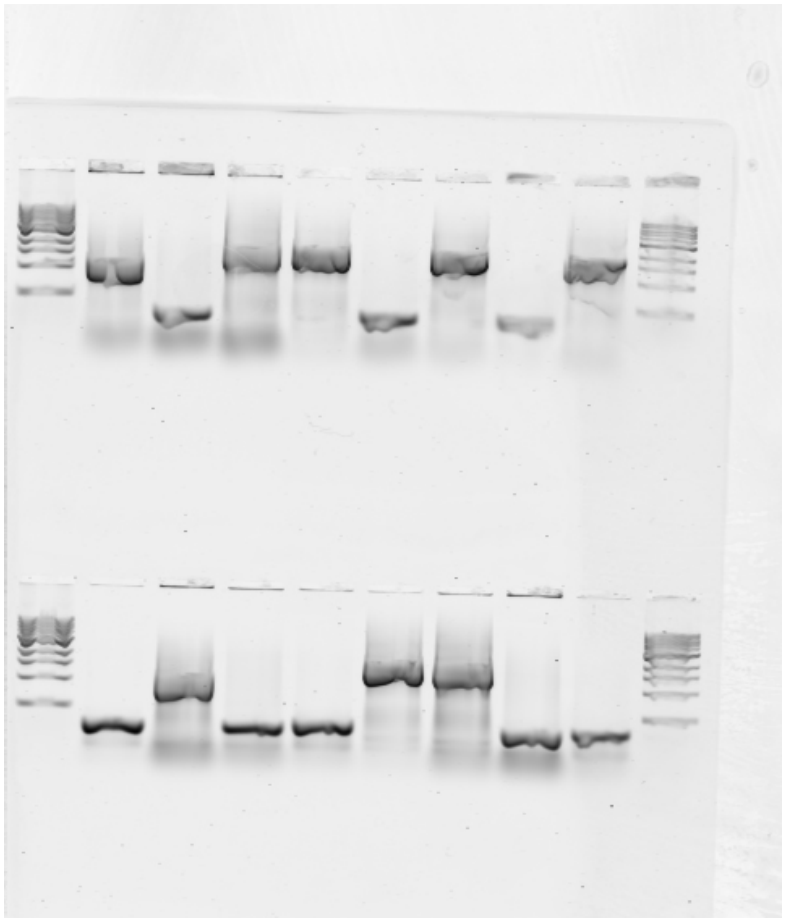
- The transformed E.coli cells grew! The colonies looked white and circular



- Colony PCR was done so as to choose the colonies that have the inserted esterase gene in the plasmid:
  - ➢ 8 colonies were chosen from both the 10ul and 118ul
  - ➢ A total of 25ul was prepared: 1ul of primers, 12.5ul of mastermix, 10.5 of milQ water and a trace of each chosen E.coli colonies
  - ➢ PCR cycles: 32
  - ➢ Primers annealing temperature: 58C
- 0.75% agarose gel was prepared in order to visualize the plasmid with the esterase gene
- Unfortunately, the bands expected (1.6kb in length) were not found in any of the colonies selected. Since the primers used were for the insert, it was hard to decide whether the PCR did not work due to

7/7/2014

- Since the colony PCR did not work on 7/4, we conducted another colony PCR using two sets of primers:
  - ➢ 0.5ul of VR (V-reverse)
  - ➢ 0.5ul of VF2(V-forward)
  - ➢ 25ul of PCR master mix
  - ➢ 23ul of miliQ water
  - ➢ 16 different colonies
- After the PCR was done, we ran the products on 1% agarose gel. We were able to detect successful transformation of esterase in colonies
  3, 4, 6, 8, 13, 14



- Our cellulase gene construct (Endo 1,4 Beta-glucanase) arrived today. We performed PCR in order to amplify our gene.

7/8/2014

- Today, we did a mini-PREP so as to extract the esterase plasmid from the liquid culture of E.coli
  - ➢ Colonies used: 3, 4, 13, 14
  - ➢ The liquid culture was centrifuged at 13000RPM to get a pellet of cells
  - ➢ Cells were resuspended in 20ul of MXI buffer by vortexing
  - ➢ 250ul of MX2 buffer was added and mixed gently in order to lyse the cells
  - ➢ 350ul of MX3 was added in order to neutralize the lysate. A white precipitate was formed
  - ➢ After centrifuging for 5-10mins, the supernatant was transferred to columns

    - ➢ A procedure similar to DNA clean-up was followed to extract the plasmid from the lysed cells
- The PCR done on 7/7 to amplify the endo 1,4-beta glucanase was viewed on 1% agarose gel. The band appeared around 800bp instead of 1.5kb. This could be caused by the fact that the DNA polymerase we used amplified only a region of the DNA. The PCR will be repeated tomorrow using Q5 polymerase
- We also went to Dr. Shih's Dry Lab at Stanford today to work with a force plate.
    - Procedure:
        - Created makeshift drone weighing approximately 571 g
        - Dropped the drone on the force plate from various heights and angles to simulate a drone crashing while out in the environment
    - Data Analysis:
        - Data analysis showed that even if the drone fell straight down from 10 meters in the air, the crash would only cause approximately 0.5 MPa of pressure. (I will insert the graph for this analysis once my computer starts working again).
        - The ptet promoter only increases its activity when subjected to greater than 30 MPa of pressure, which is unlikely to happen with a drone as low-flying and small as ours will probably be
    - Conclusions/Next Steps:
        - We need to look for a much more sensitive pressure promoter
            - This may or may not work depending on the natural pressures the drone would experience in flight
        - We need to start researching other options such as apoptosis sensors and potentially heat sensors that could respond to the heat dissipated during the crash
        - We may also consider experimenting with a mechanical method of releasing biodegradability enzymes from a small container that would burst upon crashing
- We need to start thinking about quorum sensing and ordering quorum sensing parts ASAP so that the parts arrive early enough to give us plenty of time for experimentation

7/9/2014

- Today, we are trying to develop assays to verify the action of esterase gene. Since the esterase enzyme deacetylates cellulose acetate, the detection could be done by a titration assay to detect the presence of acetic acid in solution.
  https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=9&cad=rja&uact=8&ved=0CFUQFjAI&url=https%3A%2F%2Fscilearn.sydney.edu.au%2Ffychemistry%2FLabManual%2FE10.pdf&ei=soa9U8PhEIOkyATenIDgDw&usg=AFQjCNHIaE0cOrPW0-HPXChru8V6VEmICg&bvm=bv.70138588,d.aWw/ - volumetric analysis by titration using phenolphthalein indicator

  https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=9&cad=rja&uact=8&ved=0CFUQFjAI&url=https%3A%2F%2Fscilearn.sydney.edu.au%2Ffychemistry%2FLabManual%2FE10.pdf&ei=soa9U8PhEIOkyATenIDgDw&usg=AFQjCNHIaE0cOrPW0-HPXChru8V6VEmICg&bvm=bv.70138588,d.aWw/- titration of acetic acid with NaOH
- The esterase plasmids extracted using the mini-prep method on 7/8/2014 were prepared for sequencing. The procedure needed us to prepare 500ng of each of the colonies mixed with specific forward and reverse primers to make a 15ul solution:
  - ➢ Colony 3 concentration: 50/7ng/ul, Volume used to make 500ng: 9.86ul. 1ul of primers and 4.1 of water were added
  - ➢ Colony 4: Concentration: 57.7ng/ul, volume used: 8.66ul
  - ➢ Colony 13: Concentration: 81.7ng/ul, volume used: 6.12ul
  - ➢ Colony 14: Concentration: 17.1ng/ul, volume used: 14ul
- Since the PCR we did to amplify the cellulase gene produced two bands, we repeated the PCR using the Q5 polymerase expecting that the polymerase will preferably amplify the whole DNA and produce a band at 1.5kb
- Once the PCR was done, the products were run in 1% agarose gel to visualize the DNA. Again, two bands appeared: one at 800bp and another at 1.5kb. The best approach was to carry out gel extraction to extract the 1.5kb DNA
- Gel extraction was done by cutting the 1.5kb band under UV light. After that, a procedure was followed in order to extract the DNA from the gel following DNA extraction kit procedures.

7/10/2014

- Today we carried out a digestion of both the PSC13 backbone and the cellulase before ligating the two together. For the digestion of PSC13 backbone, the following components were added to make a 50ul digestion mixture:
    - ➢ 4ul of linearized backbone
    - ➢ 5ul of 10X NE buffer (Cutsmart)
    - ➢ 40ul of miliQ water
    - ➢ 0.5 EcoRI
    - ➢ 0.5 PstI

  For the digestion of cellulase, the following components were added:
    - ➢ 10ul of cellulase DNA
    - ➢ 5ul of 10X NE buffer (Cutsmart)
    - ➢ 34ul of miliQ
    - ➢ 0.5 EcoRI
    - ➢ 0.5ul of PstI

  Both mixtures were incubated at 37C for 15 minutes for the restriction enzymes to work.
- After the digestion was done, both mixtures were cleaned up following the PCR cleanup procedure so as to remove the buffers and the restrictions enzymes
- Following the DNA clean up, the PSC13 backbone and the cellulase gene were ligated together following the following procedure:
    - ➢ 1ul of ligase
    - ➢ 2ul of 10X ligation buffer
    - ➢ 17 ul of PSC13 backbone
    - ➢ 5ul of cellulase gene insert
- In order to express the esterase gene, it was necessary to link the gene to a constitutive promoter. To do this, a plasmid with a consitutive promoter and RBS were taken from the registry kit, diluted and transformed in Amp plates. The colonies that will grow will be used to carry out a colony PCR so as to find out if they have the promoter-RBS construct in their plasmids
- Since we realized that pressure might not be the best option for initiation of the biodegradability cascade, we decided to do more research on other promoters that will work well in our drone. Light sensitive promoters seemed as the best option: **Article describing cyanobacteria promoters for night/day detection**

7/11/2014

- The transformed E.coli cells grown on Amp-agar plates on 7/10 grew. Two colonies were selected to be tested if they have the promoter-RBS construct by carrying out a colony PCR:
  - ➢ 2 colonies were selected and labelled colon 1 and 2
  - ➢ 0.5ul of both forward and reverse primers were added (VF2 and VR)
  - ➢ 25ul of master mix
  - ➢ 24ul of miliQ water
  - ➢ A trace of colony 1 and 2
- The PCR products were visualized on 1% agarose gel. The bands appeared at 300bp which was the expected length of the backbone
- Unfortunately, we realized that the digestion and ligation work we did on the cellulose gene on 7/10 was useless since we used the cellulase gene ordered from IDT and not the cellulase we got from gel extraction. We had to repeat the digestion and ligation, but before that we needed to verify that the cellulase we extracted from the gel was still present.
  - ➢ Cellulase concentration: 25.2ng/u
  - ➢ The cellulase was visualized on 1% agarose gel using 0.5ul of loading dye with 2.5of DNA2
  - ➢ After 30 minutes of running the gel with 100V, no DNA bands were observed. Seems like there was no DNA around

7/14/2014

- Since no cellulase DNA band (1.5kb) was visualized on 1% agarose, we carried another PCR to amplify the cellulase gene, so as it can be run in a gel and extracted:
  - ➢ 1ul of primers
  - ➢ Q5 DNA polymerase 25ul
  - ➢ MiliQ water 23ul
  - ➢ Cellulase DNA 1ul
- The cellulase PCR products were visualized on 1% agarose gel, and two bands were observed: 800bp and 1.5kb. The 1.5kb band is the correct length of the cellulase gene, and in order to extract this DNA, we did a gel extraction under UV light:
  - ➢ The 1.5kb band was carefully cut from the agarose gel and put in a 1.5mil tube
  - ➢ 600GEX was added on the gel

- ➢ The mixture was incubated at 55C for 10 mins until the gel was dissolved
- ➢ 700mil of the dissolved DNA and agarose mixture was placed on a column
- ➢ The solution was centrifuged at 5000rpm for 30s. The flow through was discarded
- ➢ 500ul of WN was added to the column and the mixture was centrifuged at 5000rpm for 30s. The flow through was discarded. Same procedure was repeated using WS solution
- ➢ After removing the excess alcohol by centrifugation, the extracted DNA was suspended in 30ul of elution buffer.

- The gel extracted cellulase was digested with EcoRI and PstI. The digested products were stored in -20C fridge
- In order to express the esterase gene, the constitutive promoter-RBS construct had to be ligated into the plasmid with the esterase:
  - ➢ The tet plasmid was digested with EcoRI and PstI
  - ➢ The Promoter+RBS (with amp resistance) was digested with EcoRI +SpeI
  - ➢ The esterase plasmid (with chloro resistance) was digested with XbaI + PstI

  For the promoter + RBS construct and the esterase gene, the following volumes were used:
  - ➢ 0.5ul of each of the respective restriction enzymes shown above
  - ➢ 10ul of DNA construct
  - ➢ 5ul of 10X NE buffer (cut smart)
  - ➢ 34ul of MiliQ water

  For the tet-plasmid backbone, the following volumes were used:
  - ➢ 0.5ul of EcoRI and PstI
  - ➢ 4ul of the backbone plasmid
  - ➢ 5ul of 10X NE buffer (cut smart)
  - ➢ 40ul of miliQ water
- Since the promoter-RBS- construct was less than 100bp, its restriction enzymes and buffers used for digestion could not be cleaned up following a normal cleanup procedure. For this reason, the EcoRI and SpeI were heat-killed at 80C for 20 mins
- The concentrations of all digested products were determined by the nano-drop technique:
  - ➢ Promoter+ RBS: 2.7ng/ul
  - ➢ Tet plasmid backbone: 1.7ng/ul

    - ➢ Esterase gene: 2/3ng/ul
- More literature research on the light sensitive promoter/protein: http://www.nature.com/nature/journal/v438/n7067/abs/nature04405.html

7/15/2014

- The digested cellulase was ligated with the linearized Chloro backbone (PSC13):
    - ➢ 10ul of the cellulase DNA
    - ➢ 5ul of PSC13 backbone
    - ➢ 1ul of ligase
    - ➢ 2ul of buffer (10X water)

  The mixture was incubated at 37C for 20 mins.
- After ligation was done, the cellulase was heat-killed at 65C for 20 minutes. The ligated products were then transformed into NEB competent cells:
    - ➢ 5ul of ligated product was added into 50ul of competent cells.
    - ➢ 2 plates of LB+ agar + chloro were prepared
    - ➢ 1 plate was plated with 20ul of transformed cells, and another plate was plated with 200ul of transformed cells
    - ➢ The plates were incubated at 37C overnight
- The promoter-RBS construct, the esterase gene, and the tet plasmid were ligated together by mixing the following volumes:
    - ➢ 0.5ul of promoter-RBS DNA
    - ➢ 6l of esterase gene
    - ➢ 10.5ul of tet-plasmid
    - ➢ 1ul of T4 DNA ligase
    - ➢ 2ul of T4 ligase buffer
- After the ligation was done, the ligated products were heat-killed at 65 for 20 minutes. The ligated products were then transformed into NEB competent cells and incubated on tet+lb+ agar plates at 37C overnight

7/16/2014

- The cells transformed with the cellulase gene did not grow. Also, the cells transformed with promoter-RBS-esterase plasmid did not grow too. Lack of growth for both colonies could either be caused by failure of ligation or failure of transformation itself
    - ➢ To test whether the ligation was successful, the ligated products were run on 1% agarose gel

    - ➢ The cellulase plasmid (around 4000bp) was seen observed on the scanned gel
    - ➢ The esterase+promoter plasmid was not seen on the gel
- Since the cellulase plasmid was observed, the problem was most likely the transformation. The transformation was hence repeated with newly made competent NEB cells
- For the esterase-promoter-RBS plasmid, the ligation was repeated again following the same procedure done on 7/15. Transformation was repeated too

7/17/2014

- The cells transformed with cellulase grew. Colony PCR was done to find out if the colonies had the cellulase gene in them:
- After running the gel, different bands of different lengths (500bp, 1kb and 1.5kb) were observed
- For promoter-RBS plasmid, few colonies grew. Colony PCR was done to find out if the right DNA construct was present in the transformed genes

7/18/2014

- Even though the gel done after cellulase PCR showed multiple bands, we decided to carry out a mini-prep to extract the plasmids from transformed E.coli and send them for sequencing
- 2 cellulase colonies were mini-preped : colony 2 and colony 3. They were prepared for sequencing by mixing around 500ng of the respective plasmid with the forward and reverse primers

7/21/2014

- The sequencing data for cellulase was available online today. The sequencing data did not match very well with the cellulase gene construct we had ordered. There were a lot of undetermined nucleotides. The problem could be caused by the forward and reverse primers we had used, or by the DNA sequence not being readable:
    - ➢ If the problem is caused by primers, it could either be due to an incorrect annealing temperature or the wrong primers (e.g. contaminated primers).
    - ➢ To find out the exact annealing temperature for the VF2 and VR primers, we carried out a gradient pcr using temperatures of 72-57C. Both colonies and extracted plasmids of the cellulase gene were used for the gradient PCR

    - ➢ The best annealing temperature was 59C. However, three bands were observed: 500bp, 1kb and 1.5kb bands
- For the esterase gene, the ligation of the promoter+RBS and esterase is still not working.
- We received the primers for the construct needed for the light sensitive construct: the PcyA and hol1

7/22/2014

- In order to get the construct we need for the light sensitive construct, we had to carry out a PCR to cut out the unwanted gene from the promoter-RBS-PcyA-RBS-hol1-RBS-cph8 construct. The cph8 plasmid was ordered differently, hence the PCR was needed to cut off the cph8 and get the PcyA and hol1 gene
    - ➢ Biobrick number: BBa_M30109
    - ➢ Well 14 H 2013 kit plate 3
    - ➢ Total length of the plasmid is 6.4kb (6403)
    - ➢ The cph8 plasmid is 2263bp
    - ➢ The dry DNA was diluted with 10ul of miliQ water. 1ul of DNA was used for the PCR, with 1ul of primers, 25ul of master mix and 23ul of miliQ water
- The PCR products for PcyA&hol1 were visualized on 1% agarose to find out if the 4kb band would be observed. There were no bands observed. This could be due to incorrect primers or failure in PCR
- The PcyA-hol1-cph8 construct was transformed in NEB 5alpha E.coli competent cells and left overnight at 37C for growth
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7/23/2014

- Today, the PCR was repeated using the same set of primers (newly made) and following the Q5 polymerase protocol so as to amplify the light sensitive complex. After visualizing the PCR products on the gel, nothing was observed. We figured out that the problem could be due not having enough dNTPs for such a long sequence (4kb).
- For cellulase, another colony pcr was done using a new set of VF2 and VR primers, and a thin band was observed at 1.5kb. This is a good sign for cellulase, and for this reason, we prepared a liquid culture so as to extract the DNA tomorrow and send it for sequencing

- During the lab meeting it was suggested that we attempt the esterase ligation using a backbone that already contains an insert (esterase or PRBS). We will try this tomorrow.
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7/24/2014

- The liquid culture for cellulase did not grow due to use of wrong selection antibiotic. Another liquid culture was grown for sequencing the next day
- The esterase was ligated in two ways → PRBS ligated into esterase backbone and esterase ligated into PRBS backbone. The ligation mixture was transformed and plated.
- An old esterase ligation plate left in the incubator showed colony growth, so colony PCR was done and a band appeared at 1.6kb, resulting in Poorwa running a victory lab around the building. A liquid culture of the colony was grown for mini-prepping/sequencing the next day.

7/25/2014

- A mini-prep was done for promoter-RBS-esterase to extract the plasmid for sequencing. the promoter-RBS-esterase was prepared for sequencing and sent for sequencing
- The liquid culture of the cellulase on lb+amp grew. A mini-prep was done to extract the plasmid which was then sent for sequencing
- A colony PCR was done on light sensitive complex (PcyA-hol1-cph8) so as to cut off the cph8 part. 2ul of dNTPs were added so as to amplify the whole complex:
    - ➢ 25 ul of master mix
    - ➢ 2ul of dNTPs
    - ➢ 0.5ul of VF2 and 0.5ul VR
    - ➢ 22ul of miliQ water
- The light sensitive complex PCR failed indicating a problem on primers

7/28/2014

- A gradient PCR was done on the light sensitive complex so as to determine the best annealing temperature. Both sets of primers were used: the newly made primers and the old VF2 and VR.
    - ➢ The newly made primers failed.

    - ➢ The VF2 and VR produced results at 59C. A band of 4kb was observed on 1% agarose gel
- Due to the bacillus contamination which occurred in the lab, the cellulase PCR was repeated from the beginning using gblock amp:
    - ➢ 1ul of cellulase DNA
    - ➢ 1ul of primers
    - ➢ 25ul of Q5 master mix
    - ➢ 23ul of water
- The cellulase DNA was visualized at 1% agarose and two bands were observed: 1 at 800bp and another at 1.5kb
- The 1.5kb cellulase DNA gel was extracted using a gel extraction method
- We found out that the esterase gene has had a T7 promoter in front of it the whole time, so the miniprepped esterase was transformed into T7 cells and plated on LB + chlor + IPTG plates, to hopefully express the esterase enzyme.

7/29/2014

- The LB+chlor+IPTG plates contained a lawn of cells from the esterase transformation because T7 cells are naturally resistant to chloramphenicol. The original construct from IDT was ligated into an ampicillin backbone and plated on LB+amp as well as LB+amp+IPTG plates.
- The cellulase and an Amp backbone were digested using EcoRI and PstI. They were then ligated together and later transformed in T7 cells

7/30/2014

- The esterase T7 cell plates showed no growth, regardless of the presence of IPTG. It is possible that the production of the esterase is toxic to the cells, so the plates were left in the incubator to see if there will be growth by tomorrow.
- The cellulase T7 LB+Amp showed a bit of growth in one plate, and no growth at all in IPTG. A colony PCR was done to find out if the plasmid has the right 1.5kb band. The cellulase gene was not seen
- The cellulase which was transformed on alpha-5 cells and plated on LB+chloro on a previous day was used in running a colony PCR so as to

amplify the construct. Simultaneously, a liquid culture of the cellulase colony grown on this plate was prepared. Hopefully, if the cellulase construct is detected after PCR, the DNA can be mini-preped and used for digestion and ligation into Amp backbone

7/31/2014

- A gel was run on the colony PCR run on 7/30 so as to see if the colonies have cellulase construct in them. The gel showed that colony 1 had the cellulase construct
- We mini-preped the liquid culture of colony 1 prepared on 7/30 so as to extract the plasmid with the cellulase construct
- The Amp Backbone, cellulase and esterase were digested using EcoRI & PstI restriction enzymes:
  For Amp backbone digestion: 7ul of BB
  - ➢ 5ul of cutsmart
  - ➢ 0.5ul of EcoRI
  - ➢ 0.5ul of PstI
  - ➢ 37ul of water

  For cellulase & esterase digestion
  - ➢ 10ul of cellulase /esterase
  - ➢ 5ul of cutsmart
  - ➢ 0.5ul of EcoRI
  - ➢ 0.5ul of PstI
  - ➢ 34ul of water
- The cellulase and the esterase were ligated on Amp backbone and then transformed in T7 cells. Later they were plated on LB+Amp plates and LB+Amp+IPTG

8/1/2014

- The esterase cells grown on LB+Amp and LB+Amp + IPTG grew. this could indicate that the construct is not toxic to the cells. A colony PCR was done to find out if we have the right constructs in the esterase colonies. Among 10 colonies selected for colony PCR, colony 1 and 8 from the LB+ Amp plates had the right construct, while colony 17 from LB+Amp +IPTG was the only colony which showed the right band. Liquid cultures were prepared so as the plasmids can be mini-preped on Monday to send them for sequencing

- The transformed T7 cells with cellulase construct in them did not grow in both LB+Amp and LB+Amp+IPTG. It was not clear if the failure was due to a failed transformation or a failed ligation.

8/4/2014

- The liquid cultures of esterase prepared on 8/1/2014 were mini-preped to extract the DNA and the extracted plasmids were sent for sequencing
- More liquid cultures were prepared so as to start testing the esterase activity. The following are sub-groups of assays we can use to test functionality:
    - ➢ E.coli with Cellulose Acetate - negative control for the necessity of esterase construct for degradation
    - ➢ Water with Cellulose acetate- negative control for necessity of living cells with esterase constructs in them
    - ➢ T7 E.coli transformed with esterase and plated on LB+amp without IPTG - negative control for the necessity of IPTG for T7 RNA polymerase action
    - ➢ T7 E.coli transformed with esterase and plated on LB+Amp + IPTG - expected expression of esterase
- The cellulase was PCR amplified, digested, ligated and re-transformed again

8/5/2014

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