Notebook Week 4 (June 18-23)

Project: iGEM 2017
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Dates: 2017-06-18 to 2017-06-23

SUNDAY, 6/18

Plan:

- Inoculate overnight cultures for Lactobacillus and Bacillus Subtillus
- Liquid Worcester Tech Cultures

Both of these were completed.

MONDAY, 6/19

Plan:

- Worcester Tech 10:30-12
- Download MatLab SimBio
- Final NEiGEM Prep
- Interlab Prep
- Make Competent Cells
    - OD after 3 hours
    - 1- 0.648
    - 2- 0.440 (let incubate for 15 more minutes) -> 0.508
    - 3- 0.593
    - Continued to follow protocol: Electo-Competent Bacillus Subtilis

Competent B. subtilis: Electro-Competent Bacillus subtilis
Followed protocol
Spun at 4000 rpm @4C for 20 mins, added 20 mL glycine
Spun 4000 rpm @4C for 15 mins, added 10 mL glycine
Spun 4300 rpm @4C for 15 mins, added 5 mL glycine

- Growth Curve for Lactobacillus and Bacillus Subtillus Trial 2
    - This growth curve is different from Trial 1 before
    - B.subtilis was placed in a flask with 50mL of media autoclaved
    - Lactobacillus was kept in a 50mL conical tube
    - Both were incubated at 37 degrees celsius like normal but placed in a new shaker to accommodate flasks
        - higher shaking 250 rpm instead of previous at 220 rpm

Trial2GrowthCurve.xlsx

- Look @ Error Prone PCR Protocol
- Lead Assay
    - Lead assay #1 was set up as follows:
        - 41.5 uL 30 ppb lead spiked H20 --> 10.5 uL distilled H20 --> 34.6 uL AuNPs --> 1.5 uL GSH
        - 52 uL 30 ppb lead spiked H20 --> 34.6 uL AuNPs --> 1.5 uL GSH
        - 41.5 uL 15 ppb lead spiked H20 --> 10.5 uL distilled H20 --> 34.6 uL AuNPs --> 1.5 uL GSH
        - 52 uL 15 ppb lead spiked H20 --> 34.6 uL AuNPs --> 1.5 uL GSH
        - 52 distilled H20 --> 34.6 uL AuNPs --> 1.5 uL GSH
        - Adding additional lead-spiked water instead of distilled water seems to more accurately distinguish 30 ppb and 15 ppb
        - Results:

30 15.xlsx

- Lead assay #2 was performed with 52 uL of lead-spiked H20, based on results from assay #1. The lead-spiked water was added first then 34.6 uL AuNPs were added to the wells, in accordance with the results from 6/13/17. 1.5 uL of GSH was added last based on results from 6/14/17. Lead concentrations were 30 ppb, 15 ppb, 10 ppb, 5 ppb, 0 ppb.

lead small.xlsx

- Lead assay #3 was a repeat of assay #2, this time with a fresh stock of 5ppb lead-spiked water (we thought that the dilution may have been calculated incorrectly based on the data from assay #2)

lead small (2).xlsx

Everything on the "plan" list was completed except the final NEGEM prep. We are planning on going over our presentation with Farny tomorrow. The Worcester Tech students were very nice, and it was a lot of fun to get to run minipreps and make slides with them. They thought our iGEM project idea was great; their school is one of the ones in Worcester directly affected by lead pollution in its water. In preparation for their visit, we grew liquid cultures, spun them down to make the miniprep fit in the time frame, and we picked which liquid cultures would be used for the microscopic analysis. All of the solutions needed to be divided, and each lab station prepared for the students (pipets, tips, lab coats, safety glasses, etc). For more note on what was done, please see Outreach on 6/19.
The competent cells were made, and 3 lead assays were run. All of the results can be viewed in the attached excel links. The second trial of growth curves was also completed, and the error prone PCR protocol was reviewed. It will need to be looked at again when we know more details about exactly what needs to be done, how many errors we want, and how many the sequence would make under different conditions. In addition, Matlab was also downloaded to begin learning modeling for our project.

TUESDAY, 6/20

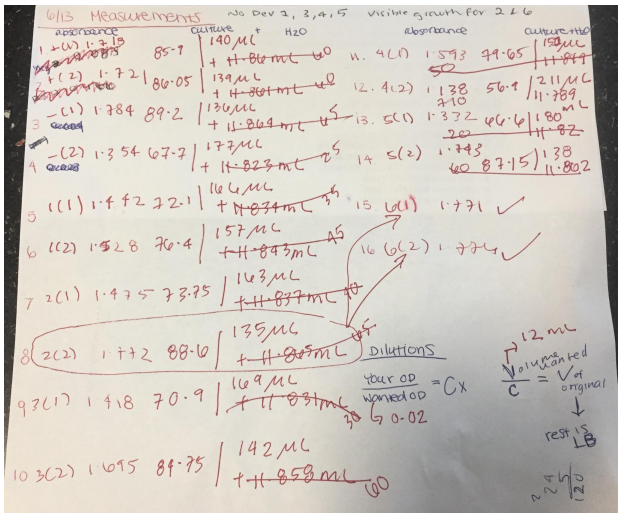
Plan:

- Interlab

Initial Abs600 measurments:

1. Positive control- Colony 1: 1.718
2. Positive control- Colony 2: 1.721
3. Negative control- Colony 1: 1.784
4. Negative control- Colony 2: 1.354
5. Device 1: J23101.BCD2.E0040.B0015- Colony 1: 1.442
6. Device 1: J23101.BCD2.E0040.B0015- Colony 2 : 1.528
7. Device 2: J23106.BCD2.E0040.B0015- Colony 1: 1.475
8. Device 2: J23106.BCD2.E0040.B0015- Colony 2: 1.772
9. Device 3: J23117.BCD2.E0040.B0015- Colony 1: 1.418
10. Device 3: J23117.BCD2.E0040.B0015- Colony 2: 1.695
11. Device 4: J23101+I13504- Colony 1: 1.593
12. Device 4: J23101+I13504- Colony 2: 1.138
13. Device 5: J23106+I13504- Colony 1: 1.332
14. Device 5: J23106+I13504- Colony 2: 1.743
15. Device 6: J23117+I13504- Colony 1: 1.771
16. Device 6: J23117+I13504- Colony 2: 1.774

interlab.jpg



Worcester_WPI_InterLab_2017_Measurements.xlsx

- Electroporation
  - Followed protocol Electroporation
  - Dilution- 8 µL of pet21b and 52 µL of comptent cells that were frozen in -80 freezer to get final volume of 60 µL
  - Cuvette 1: Time constant = 1.4
  - Cuvette 2: Time constant = 2.0
- Cloning for chromoproteins

Interlab study went really well. The readings looked a lot better than when we first completed the test. We believe the misreadings in plates 2 and 4 from the first trial were due to pipetting problems. The Google form for interlab will be submitted lated in the week.

Electroporation was also completed. The results for this will be seen later in the week. It will have to be done again if it does not work properly.

The cloning of chromoproteins was not started today; although, they did arrive in the mail. We first need to go over exactly what needs to be done with them, and how long it will take.

We did, however, complete our NEGEM presentation today. It took a long time to go over all of the edits and suggestions Professor Farny made to our original presentation. We also went over it a few more times once it was completed.

WEDNESDAY, 6/21

Plan:

- Boston NEGEM Trip
- Make cultures for Lactobacillus and Bacillus Subtillus

The Boston NEGEM Confrence was very beneficial. It was very important to get a feel for presenting, the kinds of questions we would be asked, and other teams' projects. We will go over the full experience, questions, potential collaborations, and any other concerns or comments tomorrow in a team meeting with Professor Farny.

The lactobacillus and bacillus subtillus cultures were set up for growth curves tomorrow morning.

Human Practices: Report from Frank R. Niles - DEP

LEAD REMOVAL BY PROBIOTICS (15).docx

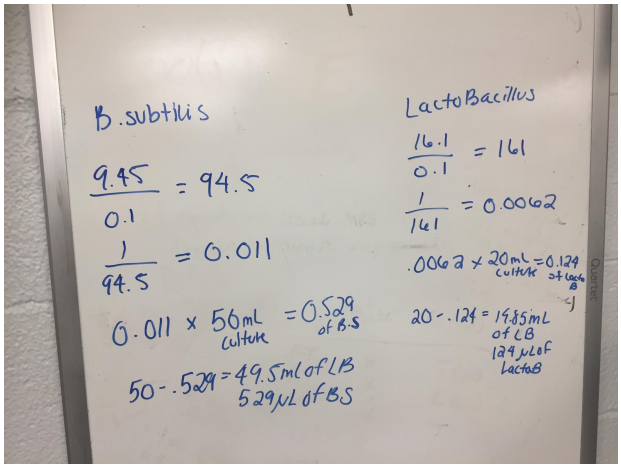
Contribution of Water Lead to Blood Lead Levels [56 FR 26469 (June 7-199....pdf

THURSDAY, 6/22

Plan:

- Growth Curve for Lactobacillus and Bacillus Subtillus Trial 3
  - Started at 6:30 am - 6:30 pm
    - Same as trial 2
    - 3 replicates from same parent culture of LactoBacillus and B.subtilis
    - B.subtilis in 50mL culture in flask
    - Lactobacillus in 20mL culture in 50 mL conical tube
    - Both shaking at 250 rpm at 37 degrees celsius
  - Lactobacillus starting OD ->
    - undiluted - 2.248
    - 1:10- 16.10
  - Bacillus subtitles starting OD ->
    - undiluted- 1.853
    - 1:10- 0.945
  - Calculate dilutions to start at 0.1

IMG_3152.JPG



  - OD after dilution calculation
    - Lactobacillus OD = 0.140
    - Bacillus Subtillis OD = 0.088

Growth Curves Analysis - Trial 3 with average of Trial 1 and 2

GrowthCurveTrial3.xlsx

- Lead Assay
  - Set up #1
    - Column 1: 41.5 uL 1000 ppb lead-spiked H20 --> 10.5 uL distilled H20 --> 34.6 uL 20 nm AuNPs --> 1.5 uL GSH
    - Column 2: 52 uL 1000 ppb lead-spiked H20 --> 34.6 uL 20 nm AuNPs --> 1.5 uL GSH
    - Column 3: 41.5 uL 1000 ppb lead-spiked H20 --> 10.5 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 1.5 uL GSH

- Column 4: 52 uL 1000 ppb lead-spiked H20 --> 34.6 uL 100 nm AuNPs --> 1.5 uL GSH
- Column 5: 52 uL distilled H20 --> 34.6 uL 20 nm AuNPs --> 1.5 uL GSH
- Column 6: 53.5 uL distilled H20 --> 34.6 uL 20 nm AuNPs
- Column 7: 52 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 1.5 uL GSH
- Column 8: 53.5 uL distilled H20 --> 34.6 uL 100 nm AuNPs

100 20 gold trial 1.xlsx

- Set up #2- GSH Optimization w/ 100 AuNPs
  - Column 1 (A-D): 41.5 uL 1000 ppb lead-spiked H20 --> 34.6 uL 100 nm AuNPs --> 12 uL GSH
  - Column 1 (E-H): 41.5 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 12 uL GSH
  - Column 2 (A-D): 41.5 uL 1000 ppb lead-spiked H20 --> 2 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 10 uL GSH
  - Column 2 (E-H): 43.5 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 10 uL GSH
  - Column 3 (A-D): 41.5 uL 1000 ppb lead-spiked H20 --> 4 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 8 uL GSH
  - Column 3 (E-H): 45.5 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 8 uL GSH
  - Column 4 (A-D): 41.5 uL 1000 ppb lead-spiked H20 --> 6 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 6 uL GSH
  - Column 4 (E-H): 47.5 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 6 uL GSH
  - Column 5 (A-D): 41.5 uL 1000 ppb lead-spiked H20 --> 8 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 4 uL GSH
  - Column 5 (E-H): 49.5 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 4 uL GSH
  - Column 6 (A-D): 41.5 uL 1000 ppb lead-spiked H20 --> 10 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 2 uL GSH
  - Column 6 (E-H): 51.5 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 2 uL GSH
  - Column 7 (A-D): 41.5 uL 1000 ppb lead-spiked H20 --> 12 uL H20 --> 34.6 uL 100 nm AuNPs
  - Column 7 (E-H): 53.5 uL distilled H20 --> 34.6 uL 100 nm AuNPs

GSH dilution 100nm.xlsx

- Set up #3 (repeat of Set up #1, with optimized GSH for each)
  - Column 1: 41.5 uL 1000 ppb lead-spiked H20 --> 10.5 uL distilled H20 --> 34.6 uL 20 nm AuNPs --> 1.5 uL GSH
  - Column 2: 52 uL 1000 ppb lead-spiked H20 --> 34.6 uL 20 nm AuNPs --> 1.5 uL GSH
  - Column 3: 41.5 uL 1000 ppb lead-spiked H20 --> 10.5 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> ***uL GSH
  - Column 4: 52 uL 1000 ppb lead-spiked H20 --> 34.6 uL 100 nm AuNPs --> *** uL GSH
  - Column 5: 52 uL distilled H20 --> 34.6 uL 20 nm AuNPs --> 1.5 uL GSH
  - Column 6: 53.5 uL distilled H20 --> 34.6 uL 20 nm AuNPs
  - Column 7: 52 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> *** uL GSH
  - Column 8: 53.5 uL distilled H20 --> 34.6 uL 100 nm AuNPs

- Set up #4
  - Column 1: 41.5 uL 30 ppb lead-spiked H20 --> 10.5 uL distilled H20 --> 34.6 uL 20 nm AuNPs --> 1.5 uL GSH
  - Column 2: 52 uL 30 ppb lead-spiked H20 --> 34.6 uL 20 nm AuNPs --> 1.5 uL GSH
  - Column 3: 41.5 uL 30 ppb lead-spiked H20 --> 10.5 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 1.5 uL GSH
  - Column 4: 52 uL 30 ppb lead-spiked H20 --> 34.6 uL 100 nm AuNPs --> 1.5 uL GSH
  - Column 5: 41.5 uL 5 ppb lead-spiked H20 --> 10.5 uL distilled H20 -->34.6 uL 20 nm AuNPs --> 1.5 uL GSH
  - Column 6: 41.5 uL 5 ppb lead-spiked H20 --> 10.5 uL distilled H20 -->34.6 uL 20 nm AuNPs --> 1.5 uL GSH
  - Column 7: 41.5 uL 5 ppb lead-spiked H20 --> 10.5 uL distilled H20 -->34.6 uL 20 nm AuNPs --> 1.5 uL GSH
  - Column 8: 41.5 uL 5 ppb lead-spiked H20 --> 10.5 uL distilled H20 -->34.6 uL 20 nm AuNPs --> 1.5 uL GSH
  - Column 9: 52 uL distilled H20 --> 34.6 uL 20 nm AuNPs --> 1.5 uL GSH
  - Column 10: 53.5 uL distilled H20 --> 34.6 uL 20 nm AuNPs
  - Column 11: 52 uL distilled H20 --> 34.6 uL 100 nm AuNPs --> 1.5 uL GSH

        - Column 12: 53.5 uL distilled H20 --> 34.6 uL 100 nm AuNPs
- LEAD IN LB and MRS TEST (control LB/MRS needs to be from same batch)
- NEED TO DO SAFETY FORMS BEFORE JUNE 30TH
- Research medal requirements

For the electroporation of B. Subtillus, both the 90ul and 10ul plates for the first transformation have a few colonies, but the second transformation was not successful. A test induction will be done next week.

The growth curve for lactobacillus and bacillus subtillus were completed today. The growth curves started well, and the data was concurred with what the expected growth was. The data from all of the trails is in the process of being complied, but this data will allow us to proceed with growing the lactobacillus and bacillus subtillus in the presence of lead. We need to measure how much lead the bacteria can actually bind, and find the threshold of how much lead will kill them. The growth curves with lead will be started next week.

The first and second trials of the lead assay were also done. The data was analyzed and posted. It is anticipated that next week we will be able to go over which concentration of GSH is optimum, and then proceed with set up #3, and a revised set up of #4. This will depend on the results. If the 100nm gold nanoparticles do not appear to benefit the assay, then the 20nm particles will be used. This would also mean that we would try to further optimize the GSH concentration to try to get more accuracy in the 5-30 ppb range. We will also be continuing to look into different ways the assay can be optimized or other methods could be used. For now we will be focusing on the accuracy of higher levels and use those to measure the uptake of lead from the probiotic. One thing that we failed to consider before trying to optimize our assay is that it must also work when LB and MRS are added, separately because those are the different kinds of media that the bacteria will be in. Those tests also need to be done.

For NEGEM review, our comments were mostly positive. We need to figure out how genetically engineered our probiotic will be considered, and we will need to try to figure out if it would be classified as a supplement or a drug by the USDA/FDA. We went over how our project relates to filters and the positives and negatives of both. We have decided to consistently update our presentation, and try to give weekly update presentations to faculty in the biology department weekly. We also want to practice answering questions and making sure that our answers are clear, concise, and accurate. We have decided that because we received so many questions about what would happen if the bacteria die, we will add testing to our project where we bind live bacteria to lead, kill them, and then measure if any lead is released by the death. It is expected that the lead binds to the surface of the bacteria and would not be released by death; however, it is a serious concern for our project and we must acknowledge that the bacteria may not do what we expect them too. We also expect that we will be able to collaborate with the BU Hardware team, because they think they might be able to build a microfluidics chip for our lead assay. This would make the process a lot easier and faster, especially because it is so time sensitive. The Tufts iGEM team is also working with probiotics. THier project is a lot different than ours, but there is still the opportunity due to enough similarity that we may collaborate. In addition, separate from NEGEM we have found several potential collaboration possibilities, including Germany, and Argentina, that we will need to go over as a team, likely next week.

Next week:

- Start adding lead to bacillus subtillus and lactobacillus
- Miniprep pet21a (Amp) and pet42a (Kan)
- Cloning for Chromoproteins
    - Amplify -> PCR from template DNA, run result on gel, purify gel (5kb site)
    - Vectors-> miniprep, digest
    - Gibson assembly rxn

FRIDAY, 6/23

Minipreps were done on pet21a and pet42a from the liquid cultures made the day before. Pet21a and pet42a are the vectors that the choromoproteins will be inserted into. pet21a has an ampicillin resistance whereas pet42a has kanamycin resistance. These will be used for making sure that the bacteria that grow will have both colors inserted, because the plate will have resistance to both amp and kan. The vectors were miniprepped so that PCR and gel purification can be done. The 5kb section of gel will be the target. A gibson assembly procedure will be followed and completed next week.

- pet21a yield = 78.3 ng/μL (given by Farny)
- pet42a yields

- 1= 92.1 ng/μL
- 2= 108.4 ng/μL
- 3 = 62.7 ng/μL

Work on the wiki, modeling research, human practices research, and the submission of the interlab study data was also completed. A general overview of our project was uploaded. This is important because the full description and thesis statement need to be posted by June 30th.

Final growth curve averages without Lead & error bars were created

Final growth Curve Analysis.xlsx

DIY Lead Assay

Introduction

Materials

- Gold Nanoparticles
- Lead Nitrate
- GSH
- NaCl (1 M)
- Phosphate Buffer (50 mM, pH=7)
- D.I Water
- 15 mL conical tubes
- 1.5 centrifuge tube

Procedure

Make Glutathione Liquid

1. Make 100mM solution of L-Glutathione

   30.72 g / 100mM
   Add 0.5 g of the Glutathione powder to 16.3 mL of D.I water
   Make 1 mL alaquotes and store in freezer

Make Phosphate Buffer

2. Make a 1 M stock of Phosphate Buffer
3. Add to a graduated cylinder 65.82 g/L of Sodium Monobasic and 93.1 g/L of sodium dibasic in 100 mL of water
4. Test pH with pH meter and adjust using 10 normal NaOH
5. Top graduated cylinder to 1 L

Make NaCl solution

6. Make 1 M stock of NaCl
7. Add 11.86 g into 200 mL of water into a bottle

Make GSH solution

8. Make dilute of the phosphate buffer to 50 mmol, add 1 mL of stock to 19 mL of D.I water
9. In a 15 mL conical tube add 110 µL of NaCl, 620 µL of Phosphate buffer, 480 µL of L-Glu liquid and 790 µL of D.I water.
10. Vortex thoroughly for 5 seconds

Prepare Lead Spiked Water Stock

11. Measure out 100 mg of Lead nitrate to get 1000 ppb
12. Dilute as necessary to achieve concentrations of 5 ppb, 10 ppb, 15 ppb, 30 ppb, 50ppb, and 100 ppb.

    5 ppb - 1 µL Pb in 20 mL of D.I
    10 ppb - 1 µL Pb in 10 mL of D.I
    15 ppb - 1 µL Pb in 6.67 mL of D.I
    30 pbb - 1 µLL Pb in 3.3 mL of D.I
    50 ppb- 1 µL Pb in 2 mL of D.I
    100 ppb - 1 µL Pb in 1 mL of D.I

Prepare Cadmium Spiked Water Stock

13. Measure out 100 mg of Cadmium nitrate tetrahydrate to get 1000 ppb
14. Dilute as necessary to achieve concentrations of 5 ppb, 10 ppb, 15 ppb, 30 ppb, 50ppb, and 100 ppb.

    50 ppb- 1 µL Cd in 2 mL of D.I
    100 ppb - 1 µL Cd in 1 mL of D.I

Well Preparation and Plate Reading

15. In wells A1-A7, place samples 5 ppb-control
16. In each well add 12 µL of GSH and 34.6 µL of AuNP along with 41.5 µL of the lead spiked water
17. Mix contents with pipette by pipetting up and down
18. Place replicates in B1-B7, C1-C7, and D1-7
19. In wells A, B, C, D8-9 add 12 µL of GSH and 34.6 µL of AuNP along with 41.5 µL of the cadmium spiked water (50-100 ppb)
20. Place in plate reader at Absorbance $A_6$10
21. Incubate for 10 min and read over period of time
22. Perform second well test and record color change every 1 mins f

Lead/Cadmium AuNP Assay 96-well plate

	A	B	C	D	E	F	
1	5 ppb Pb (1 uL Pb-H2O/20 mL DI H2O)	10 ppb Pb (1uL/10mL)	15 Pb (1uL/6.67mL)	30 Pb (1uL/3.33mL)	50 Pb (1uL/2mL)	100 Pb (1uL/1mL)	Cor
2	"	"	"	"	"	"	Cor
3	"	"	"	"	"	"	Cor
4	"	"	"	"	"	"	Cor
5							
6							
7							
8							

23.