Notebook Week 6 (July 2-7)

Project: iGEM 2017
Authors: Locke Bonomo , Haylea Northcott, Mike Savioe, Edith Sawyer, Aylin Padir, Catherine Sherman
Dates: 2017-07-02 to 2017-07-07

SUNDAY, 7/2

Researched other methods of lead water testing and cost, also found a Massachusetts site about lead in water:
http://www.mass.gov/eea/agencies/massdep/water/drinking/is-there-lead-in-my-tap-water.html
http://www.mwra.com/04water/html/testinglabs.html

Found that lead testing can cost between $12-$80, and take about 4-14 days. They appear to use Flame Atomic Absorption Spectrometer (AA), and/or Inductively Coupled Plasma Spectrometer (ICP). One of the previously posted links has a list of places that test in Massachusetts that could be helpful to contact for more specific details.

MONDAY, 7/3

Data Analysis for MRS & LB Standard Curves

Standard Curve.xlsx

THURSDAY, 7/6

Plan:

- Skype Call with BU @ 10am
- Contact Lead water testing companies in Massachusetts
    - Make Questionnaire Sheet for Calls: Questionnaire
- Go over responses from FDA
    - Our probiotic would be considered a recombinant LBP, and therefore a IND
    - Used to start filling in "Questions" Page
    - https://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/General/UCM292704.pdf
    - https://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm153222.pdf
- Research more into the Lead Growth Curves
    - How to isolate lactobacillus colonies on MRS agar?
        - Dilute to an OD 0.1 and then plate liquid cultures on MRS agar to try and get isolated colonies to use for growth curves instead of Culturelle pill
    - Grow the bacteria in lead overnight? Why are they not dying with exposure to lead within 8 hours?
    - Alter pH? Paper (here) says that B.subtilis absorbed 97.68% within 48h of incubated at an optimum pH 4.5 and 40 degrees celsius
- Go over lead assay results, try to figure out what to do next
    - Try assay row by row on 7/7/17, start with 5,000ppb then decrease to 2,000ppb, 1,500ppb, and 1,000ppb, make sure to time how long Pb and AuNP's are together before GSH is added.
- Go over questions/ start answering them
- Look at:

https://www.epa.gov/ground-water-and-drinking-water/national-primary-drinking-water-regulations
https://www.epa.gov/dwreginfo/strategic-plan-targeted-outreach-populations-affected-lead
https://www.epa.gov/dwlabcert/contact-information-certification-programs-and-certified-laboratories-drinking-water

- Work on presentation
- Overnight cultures for Growth Curve
    - All lead spiked comes from large bottle 300 ppm stock
    - Lactobacillus
        - L(1) 1 parent culture (20 mL MRS & Culturelle pill)
        - L(2) 1 culture with (20 mL MRS, Culturelle pill, 6.68 mL of DI H20)
        - L(3) 1 culture with 50 ppm lead (20mL MRS, Culturelle pill, 3.34 mL h20, 3.34 mL Lead spiked)
        - L(4) 1 culture with 100 ppm lead (20mL MRS, Culturelle pill, 6.68 mL lead spiked water)

    - B.subtilis
        - B(1) 1 parent culture (20mL Lb & 3 colonies)
        - B(2) 1 culture with (20mL LB, 3 colonies, 6.48 mL H20)
        - B(3) 1 culture with 50 ppm lead(20mL LB, 3 colonies, 3.24 mL H20, 3.24 mL lead spiked)
        - B(4) 1 culture with 100 ppm lead (20mL LB, 3 colonies, 6.48 mL lead spiked)
- PCR of chromoproteins Chromoprotein PCR
    - Primer prep - reconstitute primers to 100 uM stock:
      - spin down dry primers at max speed (13.2 rpm) 5'
      - Check quantity (25.9 nmol, 26.5 nmol), then: X nmol x10 uL of NE elution buffer (e.g. 25.9 nmol + 259 uL water) -> 100 uM stock
      - Need to dilute: 2 uL of 100 uM stock + 18uL DI water -> 10 uM stock -> 1.25 uL in PCR rxn
    - Setup PCR reaction: 1 uL template (chromoprotein), 1.25 uL of each primer, 25 uL 2X OneTaq MM (green), 22.5 uL DI water
      Parameters:
        95C 1'
        45C 1'
        72C 1'
            X32 cycles
        72C 10'
    - Once done: Gel confirm PCR products - run 5 uL of each product on a gel to check size and purity (should be one band of the size of the template. If gel good, do PCR cleanup on remaining 45 uL. (doing this tomorrow)
    - After cleanup -> do gibson again (once enzymes come in)
        - Prep more vector (pET21a -- whole miniprep digest, 50 uL miniprep and digest all, gel purify. have to get restriction enzymes before can prep vector)
        - Using pET21a miniprep from this March, which has a DNA concentration of 57.5 ng/ul, which was read this afternoon on the nanodrop
- Tomorrow: Prep for B. subtilis IPTG - grow E. coli expressing same plasmid in parallel to make sure it's not IPTG/plasmid/electroporation/whatever that caused subtilis to not glow last time.
    - Next electroporation - do known plasmids, such as RFP and/or Hi/Lo GFP plasmids - is expression variable/constitutive or what? Can B. subtilis express the plasmid? - Plasmids: pET21b-GFP, RFP construct (100pg/ul from iGEM 2017 Comp cell test kit)
    - Transform some E. coli w pET21b, already have FF E. coli for RFP - E. coli = controls to check fluorescence.

FRIDAY, 7/7

Plan:

- Lead Assay
    - Try row by row, starting with 5,000ppb, use one column of control with 4 replicates
    - Start with 5,000ppb, then 2,000ppb, 1,500ppb, and 1,000ppb for LB and MRS
        - 41.5 µL of lead spiked LB, same for MRS
        - 2 µL of control LB, 4 µL of control MRS
        - 34.6 µL AuNPs, same for MRS
        - 10 µL GSH, 8 µL GSH
    - Control: 43.5 µL for LB, 45.5 µL for MRS with GSH, 53.5 µL for control without GSH

The dilutions needed to be remade. Cat did not get to finish making the MRS dilutions, but all that needs to be done is that 5mL of MRS needs to be added. It also needs to be labeled because the MRS claimed came right from the autoclave. The assay was done row by row for LB, and the 5,000ppb was done twice because too much gold may have been added to the first one. The second one was done to double check. Also the extra 2 µL of LB control was not added to the samples containing lead, but the data will be analyzed to see if the extra volume and the time differences made an impact.

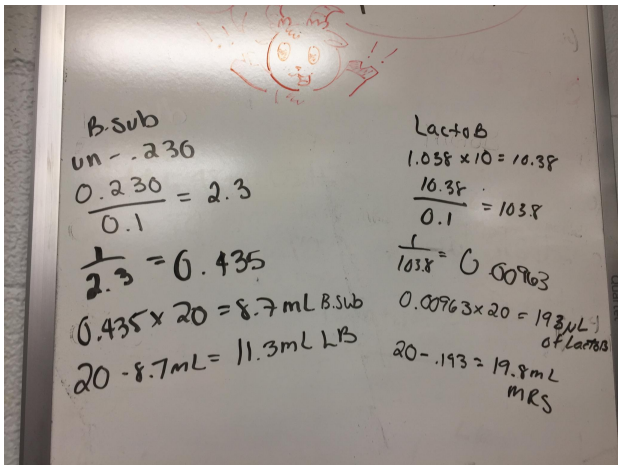
LB Lead Assay by row.xlsx

The 595nm wavelength looked promising before the 5:30 time point. Only one trial was done so error bars are not included, but the 3:30 graph has an R^2 value of .9768 which is really exciting.

- Electroporation Trial 2, make sure to use both B. Subtillus and E. Coli
- Research Culturelle Clinical Trials and if other probiotics are used as LBPs or recombinant LBPs

- Redo Vector Prep:
    - minprep of pET21a
    - Digest
    - Gel Purify
- Growth curves:
    - Run 2 different experiments
    - Experiment 1: lead exposure overnight
        - Using cultures 2, 3,4 take OD every hour starting at 6am
        - Incubate shaking at 37 degrees in 50 mL conical tubes
        - Note: No bubbles were seen in any tube B.subtilis containing lead overnight, bubbles were seen in both the parent culture and culture containg just DI Water
    - Experiment 2: Lead exposure after dilution when in lag phase
        - Make 3 cultures from overnight parent culture and dilute to an OD of 0.1 in 20mL culture
            - Culture 1- Control with added H20
            - Culture 2 - 50 ppm with added H20 and Lead
            - Culture 3 - 100 ppm with added H20 and Lead
        - Incubate at 37 degrees shaking and take OD every 1.5 hours in 50 mL conical tubes
        - Parent cultures OD
            - LactoBacillus- 10.38 - (1.038 .. 1:10)
            - B.subtilis- 0.238
            - Dilution math in picture below to get a subculture of a desired OD of 0.1

IMG_3236.JPG



    - B.subtilis didn't grow as expected too, this is a problem that has happened more than once. Are they not getting enough air? (aerobes)
        - Needed 8.7mL of parent culture for experiment 2 to reach an OD of 0.1 in subcultures.. so chose to not perform 50 ppm lead and only go through with 0ppm as a control and 100 ppm.
        - literature says that B.subtilis can survive and bioabsorb up to 800 ppm
- Serial dilutions from left over parent culture of Lactobacillus and plate each 10 fold to get individual colonies on MRS agar
- Leftover B.subtilis and Lactobacillus cultures you don't need(from parent culture) -> MAKE GLYCEROL STOCKS
    - 500 uL of culture, and 500 uL of 50% glycerol in screw cap cryotubes
    - 3 or 4 tubes of each one
    - Put in -80 freezer
- IND Application Info
    - Long Process involving interaction with the FDA before clinical trials, animal testing of some sort would need to be completed before send in approval for clinical trials, clinical trials would need to be with humans. Our project would be considered a drug because it is intended to prevent lead poisoning from drinking water, would also be considered genetically engineered because it does not say how the genetic material is altered, only if it is (which it is). We can request a formal meeting, but it would be better to contact them to try to arrange something different because the meetings are much more formal and meant for industry. We would also need to get WPI to sponsor us, and the meeting probably wouldn't be approved without the intent of involving industry right away.

    - https://www.fda.gov/downloads/drugs/guidances/ucm079744.pdf
    - https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070568.pdf
- Continue Outreach to Water Testing Companies
    - Left message for Aaron Benoit at Con-Test Analytical
    - Left message (with secretary) at Northeast
    - Need to Email Timothy Begley at Analytical Balance on Tuesday: tim@h2otest.net
    - Talked to Steve Hartman at STL Westfield
    - Need to call SCILAB-Boston Inc at 9:30 to talk to Mark
- Presentation Prep
    - Made copy of Updated presentation, added slides to talk about small things that were done
- B. sub electroporation - followed Electroporation of Bacillus subtilis
    - Two transformations, two plasmids (to test if cells are competent again) - pET21b-GFP, RFP construct (100 pg/ul, from iGEM comp cells test kit)
    - Need to get to DNA concentration of 10 ng/ul plasmid in final volume of 60 ul, but RFP plasmid is too low (100 pg/ul), so will just add 10 ul of RFP plasmid with 50 ul of cells for a final concentration of ~0.017 ng/ul (can't use too much DNA bc of salt and electricity physics things). pET21b has a concentration of 106.1 ng/ul, so used 6 ul plasmid + 54 ul cells -> final concentration of 10.61 ng/ul
    - Time constants:
        - RFP: 2.4
        - GFP: 2.0
- E. coli transformation - followed second part of Gibson Plasmid Assembly and Transformation into E. coli
    - Transformed pET21b into cells twice (two transformations)
- PCR products check of Chromoprotein PCR done yesterday
    - Run 5 uL of each product on a gel to check size and purity (should be one band of the size of the template. If gel good, do PCR cleanup on remaining 45 uL.
    - Ran the PCR products on a gel, all bands (except gene 3 = efored) were clear and of the correct size (~700bp), there were no other bright bands in any of the samples - PCR product is pure and is most likely the correctly-amplified fragment. Cleanup of the 5 successful products will be performed next week.