Antibiotic Resistance Assay

Experiment Derived from Ampicillin Titration on Microplate from Alyssa.

Media

Strains

P. putida pWW0 – Located in -80 C freezer in IGEM box cell 6

Culture Media

1. M9 Minimal Media (200 mL)
    1. 40 mL 5x M9 Salts
    2. 200 uL 1M MgSO4
    3. 40 mL 25% (w/v) glucose
    4. 2 mL ATCC sodium benzoate media
2. ATCC Sodium Benzoate Media
    1. 3g/L sodium benzoate solution

Antibiotics

Kanamycin – Located in the -20 °C freezer in the Lin Lab.

Chloramphenicol – Located in the -20 °C freezer in the Lin Lab.

Autoclave

800 mL 5x M9 Salts (see M9 Minimal Media)

500 mL DIH2O

Antibiotic Resistance Assay on a Microplate Protocol

Goal: The microplate based antibiotic is designed in order to determine the concentration of a specific antibiotic required to slow, stop, or prevent growth over a 24 hour incubation period. The absorbance of 600 nm light in a microplate will be used to determine cell density and growth rate over a time period of about 24 hours. An increase in OD_{600} over a period is evidence of antibiotic resistance at the antibiotic concentration. A decrease in $OD_{600,}$ no growth, or the eventual elimination of cells is evidence of antibiotic effectiveness. Good results will show a small standard deviation among similar wells and a statistically significant difference between the OD_{600} of plates of differing concentrations of antibiotics.

Growing Overnight Seed Cultures

1. An overnight culture of the strains to be measured is taken from -80 °C fridge via pipette tip and grown up overnight at 30 °C in a solution of:
    - 2 mL of M9 Minimal Media
    - 2 mL ATCC Sodium Benzoate Media (see Dose Dependant Swim Assay)

Prepare Media for Cultures

1. Calculate and then mix the total amount of media and antibiotic stock using the excel sheet found below located on my T60 at:

   C:/Desktop/IGEM/ExperimentalProtocols/AntibioticEffectivenessAssay(p.putitas)

Antibiotic effectiveness assay

Stock Concentration Multiplier	1

	CHL	stock (µg/mL)	35000								**KAN**	stock (µg/mL)	50000							
Final Antibiotic concentration (µg/mL)	50	75	100	150	200	250	300	500	700	1000	75	100	125	150	200	250	300	500	700	1000
Final Volume (mL)	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
M9 Sodium Benzoate Media (mL)	4.993	4.989	4.986	4.979	4.971	4.964	4.957	4.929	4.900	4.857	4.9925	4.99	4.9875	4.985	4.98	4.975	4.97	4.95	4.93	4.9
Antibiotic Stock (uL)	7.1	10.7	14.3	21.4	28.6	35.7	42.9	71.4	100.0	142.9	7.5	10	12.5	15	20	25	30	50	70	100
Final Volume (mL)	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

2. Dilute Overnight Culture
    1. Find the OD_{600}
        1. Vortex overnight culture to ensure it is well mixed.
        2. Load 2 wells of the microplate spectrophotometer with 200 uL overnight culture and 2 wells with 200 uL blank media.
        3. Find OD_{600} by subtracting the average blank absorbance from the average sample absorbance.
    2. Dilute the overnight culture.
        1. Centrifuge 500 uL of overnight culture at 1400 rpm for 2 min.
        2. Remove supernatant and resuspend in calculated amount of media
            1. Volume (uL) for resuspension = 250 x OD_{600}
            2. Note: I must have at least 200 uL of resuspended cell culture for this experiment

3. Prepare 20 eppendorf tubes
    1. Add 990 uL of desired media and label with the antibiotic and concentration used.
    2. Harvest 10 µL of diluted cell culture, and innoculate the 990 uL of media in one eppendorf tube. Mix with pipette by pipetting up and down. Record the culture type.
    3. Vortex immediately before adding media to plates.
4. Add media to multi well plate
    1. Place 200µl of inoculated, well mixed media into each well of the plate. Also place 200µl of M9SB and 200µl of antibiotic plus M9SB in the blank wells as listed below in the layout.
    2. Follow the layout in the following format

Layout:

	Pww0 Chlor 50	Pww0 Chlor 75	Pww0 Chlor 100	Pww0 Chlor 150	Pww0 Chlor 200	Pww0 Chlor 250	Pww0 Chlor 300	Pww0 Chlor 500	Pww0 Chlor 700	Pww0 Chlor 1000	
	Pww0 Chlor 50	Pww0 Chlor 75	Pww0 Chlor 100	Pww0 Chlor 150	Pww0 Chlor 200	Pww0 Chlor 250	Pww0 Chlor 300	Pww0 Chlor 500	Pww0 Chlor 700	Pww0 Chlor 1000	
	Pww0 Chlor 50	Pww0 Chlor 75	Pww0 Chlor 100	Pww0 Chlor 150	Pww0 Chlor 200	Pww0 Chlor 250	Pww0 Chlor 300	Pww0 Chlor 500	M9 SB	M9 SB Pww0	
	Pww0 Kan 75	Pww0 Kan 100	Pww0 Kan 125	Pww0 Kan 150	Pww0 Kan 200	Pww0 Kan 250	Pww0 Kan 300	Pww0 Kan 500	M9 SB	M9 SB Pww0	
	Pww0 Kan 75	Pww0 Kan 100	Pww0 Kan 125	Pww0 Kan 150	Pww0 Kan 200	Pww0 Kan 250	Pww0 Kan 300	Pww0 Kan 500	Pww0 Kan 700	Pww0 Kan 1000	
	Pww0 Kan 75	Pww0 Kan 100	Pww0 Kan 125	Pww0 Kan 150	Pww0 Kan 200	Pww0 Kan 250	Pww0 Kan 300	Pww0 Kan 500	Pww0 Kan 700	Pww0 Kan 1000	

Key:

M9 SB = M9 Media with ATCC sodium benzoate concentration

Pww0 = Inoculated with p. putitas strain Pww0

Kanxxx or Chlorxxx = Antibiotic at the concentration (ug/mL) following the Kan or Chlor

3. Tape all 4 edges with a little space at the edges
4. Read wells that contain media and blanks (B2-G11 for above example)
5. Turn on incubator to 30 °C (for p. putitas)

Results:

No growth in any plates!!! This indicates that the culture was not viable or that the spectrophotometer was incorrectly zeroed. Literature review for pWW0 antibiotic resistance is the suggested course of action.