Yeast Growth Rate Assay Method

Which Yeast Phase?

The purpose of quantifying insecticide growth rate inhibition is to determine which concentrations to select for the RNA-seq experiment. The RNA-seq experiment requires yeast cells to be in the log phase. This is to reduce background gene expression variation due to samples being in different phases of growth (Willis et al., 2017). To be able to compare insecticide driven differential gene expression to insecticide driven growth rate inhibition samples from the growth rate assay also have to be in the **log phase**. Log phase cells are created by dilution of an overnight culture and subsequent growth.

Maximum Growth Rate (MGR)

MGR is calculated by identifying the exponential growth phase of yeast, taking the natural logarithm to linearise the curve and calculating the gradient of this line through regression of ln(ODt/OD0) over time. Other studies that quantified MGR used either the gradient of ln(ODt) or ln(ODt/OD0) (Biesiadecka et al., 2020; Hall et al., 2014; Toussaint & Conconi, 2006). Both gradients are the same as the differential of both ln(ODt) or ln(ODt/OD0) is 1/ODt. I’ve chosen ln(ODt/OD0) because then all samples begin at 0, making visual comparisons of gradients easier.

How to Define the Exponential Phase

Hall et al., (2014) first linearised the growth curves (ln(OD)), then calculated the gradients of every possible 5 time point window. They then used time points associated with gradients ≥95% of the maximum gradient to calculate the overall gradient of the exponential phase, which is the MGR. For my experiment, doing the same but over three time points might be more sensible because from a brief visual inspection the exponential phase only spans approximately 5 time points. For ease, I could use the program *GrowthRates* to analyse plate-reader data or I could achieve this manually and alter the time window.

Background Variation in MGR

MGR will likely vary from sample to sample, even for yeast cells derived from separate wild-type, BY4741, colonies. This variation is accounted for by having separate controls for each replicate. The MGR from each insecticide concentration replicate will be divided by the MGR of the corresponding control replicate (MGRconc/MGR0). This will account for MGR background variation between controls by making insecticide concentration MGR a proportion of control MGR. This is similar to ODt/OD0 earlier. This will not affect the relationship the x axis (concentration) has with the y axis (MGRconc/MGR0), only alter the values on the y axis. For example, 80% maximum y value will have the same x value for both concentration vs MGRconc and concentration vs MGRconc/MGR0.

Variation in Media

Autoclaving media containing glucose leads to glucose degradation (Wang & Hsiao, 1995). The ratios of these degradation products are subtly different each autoclaving process. To avoid this, make up one 500ml bottle of media and use throughout. Aliquot 50ml of media stock into a 50ml sterile falcon tube to use day to day. This decreases the likelihood of the 500ml becoming contaminated. When making the stock media, autoclave everything apart from the glucose, which is filtered sterilised separately and then added.

How to Minimise Media Contamination

* Turn off the air conditioning before working with anything sterile.
* Don’t touch inside of media bottle.
* Decant into working media bottle by pouring next to flame to prevent main bottle from getting contaminated.
* Autoclave media then put filter sterilised glucose in so making new media doesn’t alter media composition. Avoids variable glucose degradation products.

**Stage 1: Prepare Media**

Follow Thorpe Lab standard protocol.

To make 500ml yeast, peptone, dextrose (YPD) media

350ml sterile H2O

5g Yeast Extract

10g Peptone

100ml H2O

Once mixed autoclave

Then add filtered sterilised 50ml 20% Glucose (w/v)

**Stage 2: Yeast Overnight Culture**

The stock wild-type yeast strain, BY4741, is stored as colonies on agar plates at 4oC. Innoculate 5ml of YPD culture with 1 yeast colony. Mix the cells and perform x5 serial dilution five times (i.e. 5x, 25x, 125x, 625x and 3125x dilutions). Place these overnight cultures in the orbital shaking incubator (225rpm) overnight at 30oC. Begin and end process at the same time every day (1200, 1000). By the following morning, I expect that one of the serial dilutions will be in log phase (between 0.4-0.6 OD) (x125). Use log phase overnight culture for subsequent experiments.

**Stage 3: Insecticide Stock Solutions**

0.5M stock solutions of insecticide are initially created. The highest concentration tested will be 5mM. Consequently, there will be a 1% final insecticide solvent concentration. To allow for comparison this solvent concentration has to be maintained for all insecticide concentrations. Insecticide solubility figures aren’t available for the same solvent. Instead they are split across DMSO and methanol, which are both miscible in water. To control for this variation 1% of both solvents will be present for each data point.

**Stage 4: Effects of 1% DMSO and 1% Methanol on BY4741 Growth**

* Measure OD of overnight culture in log phase. Calculate dilution required for final OD of 0.05. This will determine the volume of yeast culture added to each well.
* Treatments (5 replicates of each):
  + Control = yeast + media (n=5).
  + Treatment 1 = yeast + media + 1% DMSO (n=5).
  + Treatment 2 = yeast + media + 1% methanol (n=5).
  + Treatment 3 = yeast + media + 1% methanol + 1% DMSO (n=5).
* 96-well plate
* Make each replicate separately directly into well. For example, if OD of yeast culture was 0.5 then 1µL methanol + 10µL yeast culture + 89µL media for final volume of 100µL and 1% methanol concentration.
* Randomise plate using R script to control for pipetting error, temperature fluctuations etc.
* Add media first, then stressors and mix thoroughly, then finally yeast stock.
* Shake (vortex) yeast stock falcon tube before each yeast stock dispensing.
* Should I cover with parafilm to decrease evaporation?
* Quantify growth rate for 16h in spectrophotometer set at 30oC (600nm).
* Number of cycles = 193, number of flashes per well and cycle = 22, cycle time = 300s, shake time = 272s
* Shaking options = double orbital, 200rpm, 272s, before each cycle.
* Collect results the next day and analyse.
* Calculate log phase gradient (growth rate) for each treatment.

**Stage 5: Preparing Insecticide Stock Solutions**

* Thiacloprid – Worked Example
  + Solubility in DMSO = 150 g/l (Sieke, 2006).
  + Molecular Mass = 252.72 g/mol.
  + Mass of Thiacloprid = 0.1 g.
  + 0.1g / 252.72g/mol = 3.96 x 10-4 mol
  + For 0.5mol/l solution, 3.96 x 10-4 mol / 0.5mol/l = 7.91 x 10-4 L or 791.4 µL of DMSO added to 0.1g Thiacloprid.
  + 0.1g / 7.91 x 10-4 L = 126.4 g/l which is < 150 g/l solubility.
* Acetamiprid
  + Solubility in DMSO > 500 g/l (Zhao et al., 2019). Value given in mol fraction (425600). Used online calculator to convert to g/l (1694.6) (ToolBox, 2017).
  + For 0.1g acetamiprid add 898.2 µL DMSO for 0.5M stock solution.
* Imidacloprid
  + Solubility in DMSO > 200 g/l (Standing Committee on Biocidal Products, 2011).
  + For 0.1g imidacloprid add 782 µL DMSO for 0.5M stock solution.
* Clothianidin
  + No DMSO solubility figures available. Methanol 6.26 g/l, water 0.327 g/l (Standing Committee on Biocidal Products, 2014).
  + Methanol and water solubility figures comparable to imidacloprid (methanol 10g/l, water 0.51-0.6 g/l) (Bluestar, 2020; Standing Committee on Biocidal Products, 2011).
  + Shall I create 0.5M stock solution in DMSO and hope it’s soluble at that concentration? Yes and it appears to be soluble.
* Flupyradifurone
  + Solubility in DMSO > 250 g/l (Apvma, 2018).
  + For 0.1g flupyradifurone add 693µL DMSO for 0.5M stock solution.
* Sulfoxaflor
  + Solubility in methanol = 93.1 g/l (PPDB, 2007).
  + For 0.01g sulfoxaflor add 144 µL methanol for 0.25M stock solution (solubility not high enough for 0.5M stock solution).
  + For 1% methanol final max concentration is 2.5mM
* Malathion
  + Solubility in methanol > 250 g/l (PPDB, 2007).
  + For 0.1g malathion add 605 µL methanol for 0.5M stock solution.
* Chlorpyrifos
  + Solubility in methanol > 290 g/l (PPDB, 2007).
  + For 0.1g chlorpyrifos add 570 µL methanol for 0.5M stock solution.
* Cypermethrin
  + Solubility in methanol = 450 g/l (PPDB, 2007).
  + For 0.1g cypermethrin add 480 µL methanol for 0.5M stock solution.
* Tefluthrin
  + Solubility in methanol = 260 g/l (PPDB, 2007).
  + For 0.1g tefluthrin add 478 µL for 0.5M stock solution.
* Chlorantraniliprole
  + Solubility in methanol 1.714 g/l (Epa & of Pesticide Programs, 2008).
  + Slightly above 3 mM is max concentration solubility permits.
  + For 1% methanol final max concentration is 30µM.
  + For 0.025g chlorantraniliprole add 17.2ml for 3mM stock solution.
* Cyantraniliprole
  + Solubility in methanol 4.73 g/l (New Zealand Food Safety, 2013).
  + 9.99mM (10mM) is max concentration solubility permits.
  + For 1% methanol final max concentration is 0.099mM (0.1mM).
  + For 0.025g cyantraniliprole add 5.29ml for 9.99µM (10mM) stock solution.
* Spinosad (Spinosyn A and D)
  + Spinosyn D solubility in methanol = 2.52 g/l, Spinosyn A solubility in methanol = 190 g/l (Standing Committee on Biocidal Products, 2010).
  + Slightly above 3mM is max concentration solubility permits.
  + For 1% methanol final max concentration is 30µM.
  + For 0.05g add 22.34ml for 3mM stock solution.

**Stage 6: Effects of Insecticides on BY4741 Growth**

* Follow similar procedure to 4. n=5 too.
* Start with overnight culture in log phase. Dilute down to final well concentration of 0.05 OD (changed to 0.01OD theoretical starting well OD).
* Treat at room temperature. 16h growth quantification at 30oC.
* 96 well plate. 100µL final volume each well (altered to 200µl final volume).
* Instead of making replicate directly into well, make 500µL in Eppendorf first then aliquot 100µL into well. Why? Wasteful but more accurate.
* Remember to note and label well allocation for each insecticide.
* Randomise plate.
* 1% for both solvents.
* Maximum insecticide concentration is constricted by final solvent concentration of 1%. Limited by solubility in solvent. Raises issues for chlorantraniliprole, cyantraniliprole and spinosad.
* For imidacloprid, acetamiprid, thiacloprid, flupyradifurone, malathion, chlorpyrifos, tefluthrin and cypermethrin test at 5mM, 1mM, 100µM, 10µM and 1µM ( + control).
* For sulfoxaflor test at 2.5mM, 1mM, 100µM, 10µM and 1µM ( + control).
* For cyantraniliprole test at 100µM, 10µM and 1µM ( + control).
* For chlorantraniliprole and Spinosad test at 10µM and 1µM ( + control). Is there any point in this? Won’t be possible to construct a concentration-response curve from this. Could return these two insecticides.
* Example calculation for overnight yeast culture with 0.5 OD (final 0.05 OD). 1% final concentration for both solvents.

1. Create 200µL of 10mM insecticide solution.
   1. 4µL of 0.5M stock insecticide solution + 196 µL media.
2. Create 200µL of 5mM insecticide solution.
   1. 100µL of 10mM insecticide solution + 20µL yeast culture + 2µL other solvent + 78µL media.
3. Create 500µL of 1mM insecticide solution.
   1. 50µL 10mM insecticide solution + 50µL yeast culture + 5µL other solvent + 4µL insecticide solvent + 391µL media.
4. Create 500µL of 100µM insecticide solution.
   1. 50µL 1mM insecticide solution + 50µL yeast culture + 5µL other solvent + 4.9µL insecticide solvent + 390.1µL media.
5. Create 500µL of 10µM insecticide solution.
   1. 50µL 100µM insecticide solution + 50µL yeast culture + 5µL other solvent + 4.99µL insecticide solvent + 390.01µL media. <<<< (shall I do 5µL insecticide solvent + 390µL and accept the 1.002% insecticide solvent concentration?).
6. Create 500µL of 1µM insecticide solution.
   1. 50µL 10µM insecticide solution + 50µL yeast culture + 5µL other solvent + 5µL insecticide solvent + 390µL media.
7. Create control
   1. 5µL other solvent + 5µL insecticide solvent + 490 µL media.

* Above doesn’t work because the serial dilutions have yeast in them. Have to find another way. Type up new method tomorrow morning. See separate doc for dilution method.
* Same as above for sulfoxaflor except step 2 create 2.5mM instead of 5mM as 2.5mM is 1% methanol.
* Clothianidin don’t know what to do as I guess it is very soluble in DMSO. However, I can’t find a value.
* Currently 9 insecticides with 5 concentration values = 45 wells + 5 controls + 5 blanks = 55 wells. Can do this without using wells on perimeter of plate to avoid any potential edge effect.
* Repeat 5 times.
* What to do about clothianidin, chlorantraniliprole, cyantraniliprole and spinosad?
* With clothianidin try it. This would make it 10 insecticides so 60 wells. Still don’t have to use plate perimeter. Fill plate perimeter with blanks.
* Look at AUC instead of r because AUC captures total growth, where r is only maximal growth rate. r may be the same for two observations, but this would miss a difference in lag times, which could be an important effect of the insecticide. For looking at AUC, adding equal volumes of yeast stock into each well is extremely important.