Experimental evolution of multicellularity

Student handout

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Introduction. The evolution of multicellularity was one of a few events in the history of life that allowed for increases in biological complexity. All known multicellular organisms evolved from single-celled ancestors, most notably in the animals, land plants, and fungi. Take a moment to imagine the world without multicellular organisms. The most vibrant tropical rainforest would be reduced to little more than a barren open landscape encrusted with a slimy layer of photosynthetic bacteria and algae. The evolution of multicellularity resulted in radical changes in organismal size and complexity. Single cells, which for billions of years were organisms in their own right, give up this autonomy and become parts of new, more complex, higher-level organisms. These evolved cellular differentiation, allowing the multicellular organism to do things that were never possible before. And perhaps most remarkably of all, multicellularity has evolved not just once, or twice, but more than 25 times in different lineages (Figure 1)[1].

All known transitions to multicellularity are ancient. Even the most recent transitions (brown algae, such as kelp, and the volvocine algae) occurred more than 200 million years ago. Because of their ancient origin, early multicellular forms have largely been lost to extinction, making it hard for scientists to study the first steps in the evolution of multicellularity. Until now.

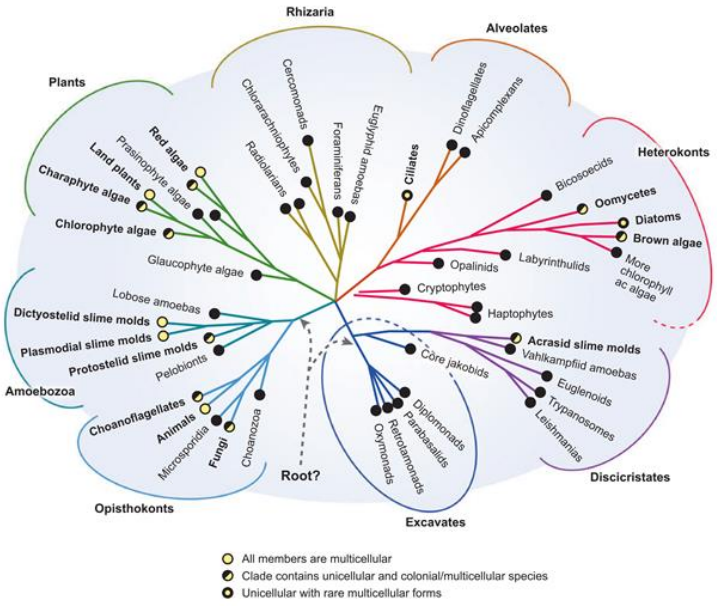

Figure 1- Phylogeny showing independent origin of multicellularity. All groups represented by yellow circles contain multicellular organisms. Figure reproduced from Grosberg and Strathmann, 2007[1].

In this lab we will use Baker's yeast to examine the very first step in the evolutionary transition to multicellularity- the evolution of cellular clusters. We will use the method of *experimental evolution* to select for cluster formation in large populations (roughly a billion yeast cells per tube). We will use gravity as our selective agent. Clusters of cells fall through liquid media faster than single cells, allowing us to quickly and easily separate single-celled yeast and any multicellular mutants that arise during the experiment. As a result, if a random mutation arises that results in cluster formation, these will have a *huge* competitive advantage over the wild-type unicellular yeast, ultimately driving them to extinction.

In addition, students will perform a *divergent selection experiment* on a multicellular 'snowflake' yeast isolated from 3 weeks of experimental evolution using the same protocol. The goal here is to select for either faster or slower settling on a multicellular yeast, and examine multicellular adaptation to these different environments. Every day that you evolve your yeast, they will go through about 7 generations. Over the course of the experiment, you're going to watch them go through 70-80 generations. The short generation time of microbes is a key reason that scientists use them in experimental evolution studies. How long would it take for us to do an 80-generation experiment with another organism, such as mice, dogs, chimpanzees or oak trees?

The evolution of multicellularity is a two-step process. First, clusters of cells must evolve from single-celled ancestors. In nature, this may be due to predation (predators can eat single cells, but not clusters), or benefits clusters gain over isolated cells (e.g., UV resistance, metabolic efficiency, etc.). Next, clusters of cells must possess the key ingredients required for Darwinian evolution: clusters must vary from one another, they must be capable of passing on this variation to their offspring, and this variation needs to affect fitness. If this is true, then mutations that affect the phenotype of clusters can be acted upon by natural selection, and clusters that possess beneficial mutations will outcompete those that do not. This process is known as adaptation (process illustrated in Figure 2, as well as in a short video on our website, http://www.snowflakeyeastlab.com). Over time, this can result in the evolution of multicellular complexity, such as cellular division of labor.

Objectives. Experimentally evolve single-celled yeast into multicellular 'snowflake' yeast. Examine multicellular adaptation through divergent selection for either faster or slower settling.

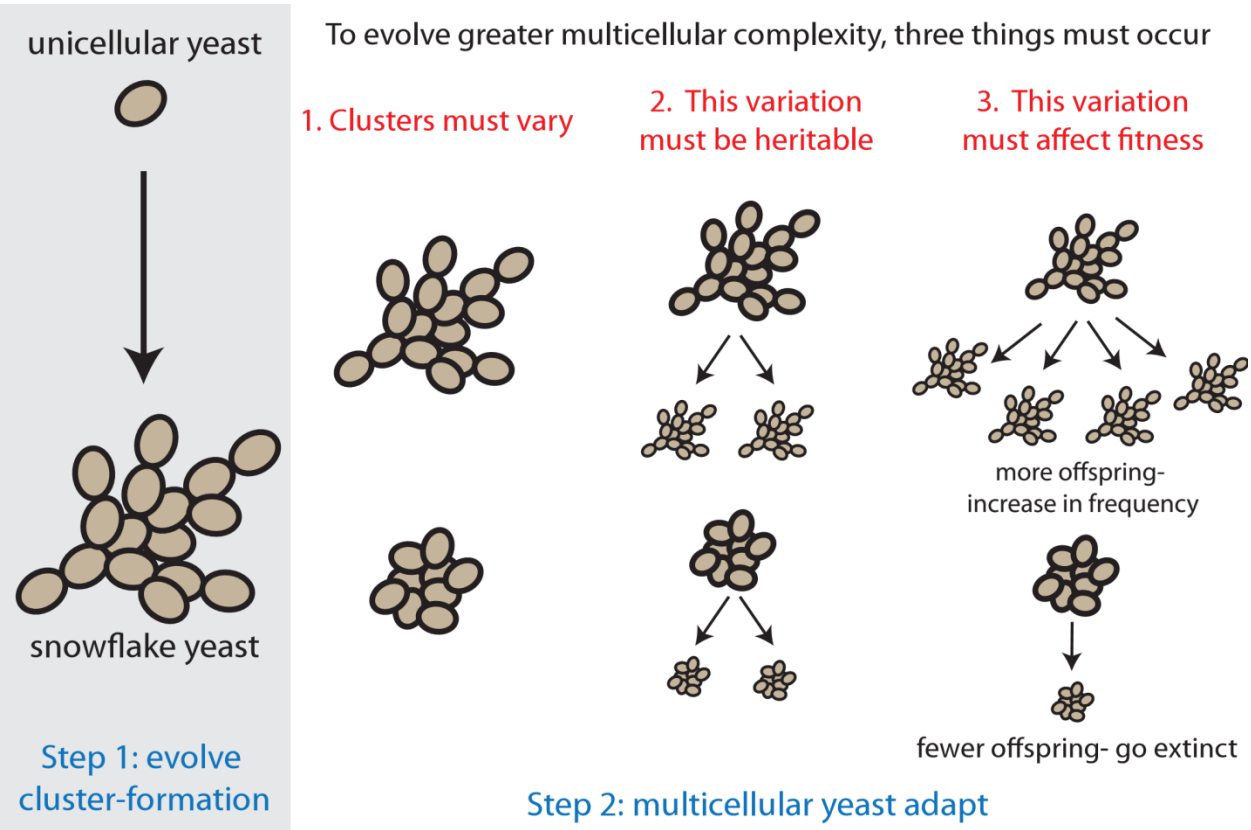

Figure 2- The evolutionary transition to multicellularity. First, clusters must evolve from single-celled ancestors. Next, clusters must adapt. To do this, there things must be true. First, clusters must vary from one another (shown are two different ways of forming a cluster, snowflake and clumpy). Second, this variation must be heritable. And finally, these cluster-level traits must affect fitness. If, for example, snowflake shaped clusters settle faster than clumpy clusters, then snowflake clusters will outcompete clumpy clusters and will take over the population. Similarly, if cellular division of labor evolved and improved a snowflake's ability to produce offspring or grow faster, then natural selection would result in an increase the frequency of this trait in the population. Like a ratchet, multicellular complexity can increase through time.

Baker's yeast are harmless, but in our experience it is difficult to maintain sterility in a student lab. Therefore, it is hard to know if something else besides yeast is growing in the media. *As a precaution, gloves and eyewear should be worn during the lab.*

In this lab, you will perform **settling selection**, in which you select for faster settling to the bottom of a pipette. To examine the evolution of both faster and slower settling in a snowflake yeast, you will perform **divergent selection** in which you will select for either faster or slower settling on otherwise identical populations of yeast. The protocols for this are described in the text below, and are illustrated in Figure 3.

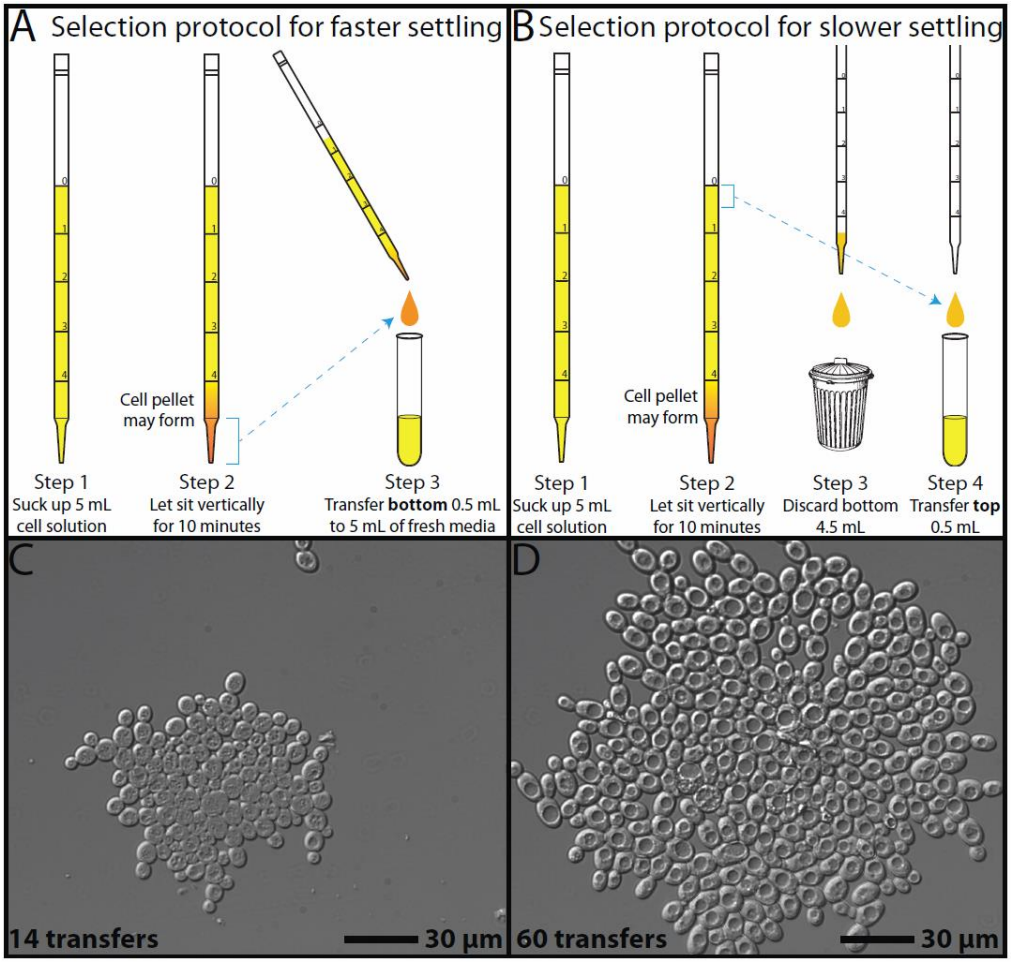

Figure 3. Settling selection protocols illustrated. Selection regime for faster settling (A), or slower settling (B). Snowflake yeast evolve faster settling by evolving larger size. Shown in (C) is an isolate taken from 14 transfers,

and in (D) 60 transfers. The cluster in (D) is larger both because there are more cells per cluster, but also because the size of individual cells has increased. Figure reproduced from Ratcliff et al (in press).[2]

Task 1: Inoculating Yeast Peptone Dextrose (YPD) media with yeast

1. Obtain three test tubes containing 5 mL YPD.
2. Label each tube using a permanent marker (tip: write on tape, then put tape on the tube) with the inoculation date, strain (Y55 or C1W3), and selection scheme (i.e., faster or slower settling).
3. Inoculate one tube with 100 µl of the Y55 stock culture, and two tubes with 100 µl of C1W3.
4. Incubate overnight (30ºC, shaking).

Task 2: Experimental evolution

Selection for faster settling

1. Obtain three test tubes containing 5 mL YPD. Label tubes with transfer number, date, strain, and selection scheme.
2. Obtain a 5 mL serological pipette with bulb.
3. Extract 5 mL of the turbid yeast culture from incubated tube into a sterile 5 mL serological pipette using a bulb or pipette.
4. Seal the bottom of the pipette with a piece of parafilm or hold firmly with the flat part of your thumb.
5. Allow the pipette to stand upright in a glass beaker or pipette rack for 10 minutes. Make sure the media is not leaking out.
6. Transfer the bottom 0.5 mL to sterile YPD.
7. Place all three tubes in the test tubes racks in either a dry shaking incubator or shaking water bath. Incubate 24 hours at 30°C.

Selection for slower settling

1. Obtain three test tubes containing 5 mL YPD. Label tubes with transfer number, date, strain, and selection scheme.
2. Obtain a 5 mL serological pipette with bulb.
3. Extract 5 mL of the turbid yeast culture from incubated tube into a sterile 5 mL serological pipette with a bulb or pipette.
4. Seal the bottom of the pipette with a piece of parafilm or hold firmly with the flat part of your thumb.
5. Allow the pipette to stand upright in a glass beaker or pipette rack for 10 minutes. Make sure the media is not leaking out.
6. Discard the bottom 4.5 mL of media in the pipette into waste beaker, transferring the top 0.5 mL to sterile YPD.
7. Place all three tubes in the test tubes racks in either a dry shaking incubator or shaking water bath. Incubate 24 hours at 30°C.

Data Collection

1. Daily record keeping. This is a lab, and you are a scientist doing an experiment. Accurate note keeping is critical. Record your observations and actions **daily.** Your observations should include what the test tube looks like (cloudy, presence/absence of a pellet, are tiny specks visible within the tube, or is it a homogenous haze?), what you see under the microscope, etc. Your actions should describe what you did (which transfers you conducted, if they made any mistakes, etc). Record your thoughts, too, because you'd be surprised how many good ideas get lost otherwise.

2. Microscopy. Unlike fruit flies or mice, you can't really tell what is going on with microscopic yeast unless you use a microscope. Every second or third transfer, you should examine your populations under

the microscope. You should draw a representative field of view at 10X, 40X and 100X magnification for each of the three populations you are transferring. A guide for drawing organization is suggested below.

Date

[insert]

Strain

[insert]

10x

40x

100x

3. At the end of the experiment, perform a comprehensive examination of your evolving populations.

View your yeast using both traditional flat slides and depression slides. Clusters get squished on traditional slides, so the depression slides can allow you to see them in their real 3D shape (like the clusters on the right). **Note: when viewing yeast on depression slides, dilute them 20-fold in water, otherwise they will be too dense to see clearly.** To do a 20-fold dilution, add 50 µL of yeast in YPD to 950 µL of water.

Answer the following questions in your lab notebook:

• Did any clustering evolve in your Y55 unicellular populations?

• Is it a snowflake yeast? The way to tell is through a characteristic branching pattern associated with its growth form: only snowflake yeast form clusters that are made up of long branches of cells (see picture at right).

• Did the snowflake yeast evolve to settle at different speeds in the divergent selection experiment? To measure settling speed, suck up 4 mL (make sure to leave some for microscopy!) of each of the C1W3 populations into two separate 5 mL transfer pipettes. Be sure to mix them well before pipetting. Allow them to stand upright for 5 minutes. Using a ruler, measure the height of the cloudy cell haze from the base of the pipette; faster-settling yeast will leave a smaller cell cloud.

How large was the cell cloud (in mm) for-

C1W3 faster settling: C1W3 slower settling:

• Did the C1W3 snowflake yeast evolve to be different sizes in the divergent selection experiment?

First, have get a good idea of what your yeast look like by viewing each population on both depression and regular slides. Low power microscope objectives (e.g., 4X) will give you the ability to view more clusters, but you will see less detail. Switch back and forth between the C1W3 faster-settling and C1W3

slower-settling selection lines. If a microscope camera is available, we recommend that you take pictures of each line. These images should be labeled with the strain and selection line. Do you see a difference in cluster size between the treatments?

It is usually hard to tell if there really is a difference in cluster size, especially if you know which tubes got the faster-settling treatment and which got the slower-settling treatment. Like it or not, we are biased by our knowledge of the treatment. One way to solve this problem is a procedure called **blinding.** You will conduct a simple, single-blind poll of your peers.

If you have a digital microscope camera- Take 2-3 representative images of the faster and slower settling C1W3 populations with a low power objective (so lots of clusters are visible) using both depression and flat slides. Now, conduct a poll of 5 of your peers **without telling them which treatments they came from.** Simply ask them to guess, based on size, which images are from the faster-selection line, and which are from the slower-selection line. It is critical that there are no easily visible labels on the photos that could tip off the respondents. You will record the results of the poll in the table below.

If you do not have a microscope camera- follow the above procedure, but have your respondents look through the microscope at the sample and make their determination. It is critical that there are no easily visible labels on the slides, otherwise you may tip off the respondents. You will record the results of the poll in the table below.

Data entry table. Record in your lab notebook (which should be closed when asking respondents about the identity of each image) the true identity of each image. Put a tally for each respondent's guess as to the identity of each treatment in the table below.

Image #	Image 1	Image 2	Image 3	Image 4	Image 5	Image 6	Image 7	Image 8
Faster settling								
Slower settling								
Treatment **[fill in only after completing Q&A]**								

For each treatment (faster or slower settling), sum up the number of correct and incorrect IDs.

C1W3 faster-settling- Correct identifications: Incorrect identifications:

C1W3 slower-settling- Correct identifications: Incorrect identifications:

Did your peers generally say that the faster-settling treatment was larger than the slower-settling treatment?

• Were there any changes in cell size in the C1W3 selected to settle faster in comparison to the C1W3 selected to settle slower (e.g., Figure 3)? Why might larger cells be adaptive under selection for faster settling?

Discussion

There are daily discussion questions for this lab. Depending on teacher preference, students will answer discussion questions in their lab notebooks or discuss these questions as a class. At the culmination of this lab, you will be asked to incorporate your thoughts and write up a full lab report.

References

1. Grosberg, Richard K., and Richard R. Strathmann. "The evolution of multicellularity: a minor major transition?" Annu. Rev. Ecol. Evol. Syst. 38 (2007): 621-654.

2. Ratcliff, W.C., A. Raney, S. Westreich, and S. Cotner. (in press the American Biology Teacher). A novel laboratory activity for teaching about the evolution of multicellularity.

Appendix 1. Protocols

Here you will find detailed procedures for all the techniques needed for these labs. List of materials and notes are also given. Videos of some procedures can be found on our website (http://www.snowflakeyeastlab.com).

Mixing and pouring YPD agar

Note: You will find a premixed bag containing YPD agar in your kit. This can be used to make a few Petri dishes, or pour solid media in a few culture dishes. This is enough media to streak out and grow spores, but not enough to do experimental evolution.

1. Add 150 mL distilled water to 250 mL or larger Erlenmeyer flask. Place on heated stir plate on medium.
2. Add packet of YPD agar (6.5g) to flask.
3. Autoclave for 20 minutes at 121degrees C.
4. Cool to the touch on stir plate.
5. On a clean bench (wipe down with ethanol prior to use), pour plates. You should add just enough agar to cover the bottom of the dish. Plates should set within 5 – 10 minutes. You can cover them immediately after the have been poured.

*Alternatively, if an autoclave is not available, sterile YPD media can be made using the same ingredients but following the directions below.

1. Add 150 mL distilled water to 250 mL or larger Erlenmeyer flask. Place on heated stir plate on medium.
2. Add packet of YPD agar (6.5g) to flask.
3. Swirl the flask to dissolve the media, and heat to **vigorous** boiling in the microwave or hot plate. (If using a hot plate be careful not to burn your media, swirl until dissolved.) Boil for 20 minutes.
4. Pour into plates as above.

Plating Yeast from Spores

Notes:

·The incubation of the yeast will take 2-4 days in an incubator set to 30°C, one week without.

·Enclosed in your reagent bag are dried spores of the yeast *Saccharomyces cerevisiae*, strain Y55 (unicellular) and strain C1W3 (multicellular). The latter is a 'snowflake' yeast, derived from Y55 with 3 weeks of settling selection.

·The yeast spores are dried onto filter paper. Use a different agar plate for each strain of yeast.

<u>Materials</u>

(2) 1.5mL microcentrifuge tube

Alternative: covered/capped test tube

(2) Sterile loop/stick

Alternative: pipette tip or sterilized toothpicks

Sterile water

Sterile forceps

Flame
70% ethanol
Saccharomyces cerevisiae unicellular yeast spores (strain Y55)
Saccharomyces cerevisiae multicellular yeast spores (strain C1W3)

Procedure
1. Carefully open the foil package labeled Y55.
2. Using flame-sterilized forceps, remove paper from foil package.
3. Unfold and place the filter paper onto a culture plate of YPD medium with sterile forceps. Label plate.
4. Moisten with 50 µL or so of sterile water or liquid YPD. Streak the paper around on the agar surface using sterile forceps. Leave the filter paper on the culture plate.
5. Incubate at 30°C. You should see colonies in a few days.
6. Repeat process with C1W3.
*When plating more than one yeast culture, be sure to flame sterilize forceps between each plate.

As an alternative, transfer the filter paper directly into a 10 mL tube of liquid Yeast Peptone Dextrose (YPD) medium with sterile forceps. Incubate these tubes at 30°C with vigorous shaking (requires shaking incubator). Within two days there should be plenty of growth. One can then extract 100 µL from these stock tubes for initial tube inoculation.

Suspending yeast into liquid *(in preparation for inoculating liquid YPD)*
Procedure
1. Fill 1.5 mL microcentrifuge tube with 1 mL sterile water
2. Scrape off a small clump of Y55 yeast with sterile loop or sterile "stick."
3. Insert sterile loop into microcentrifuge tube containing the water and twirl to release yeast. Label tube *Y55 Stock.*
4. Shake tube to suspend yeast.
5. Repeat steps 1-3 with C1W3 yeast. Label tube *C1W3 Stock.*

YPD liquid media preparation

Three test tubes of YPD media are needed for each group for every day of transfer (15 test tubes are needed for each group each week). This amount will make approximately 200 tubes of media. If you have 6 lab groups you will need approximately 270 tubes, make 300 as there will be mistakes and spills.

Ingredients:
- 20 g dextrose
- 20 g peptone
- 10 g yeast extract

1. Dissolve the above ingredients into 1 L distilled water in a 2 L or larger Erlenmeyer flask.
2. Bring the final volume up to 2 L.
3. Using the 25 mL serological pipette and pump, aliquot 5 mL YPD into each 25 mm X 150 mm test tube, and place into test tube racks. Test tubes of smaller diameter can be used, but you should increase the shaking speed of narrower test tubes to ensure adequate mixing during incubation.

4. Cap or cover tubes with foil. If using foil, make sure it covers the top 2" of the tube and is well-crimped.
5. Autoclave to sterilize at 123°F for 15 minutes.

*Alternatively, if an autoclave is not available, sterile YPD media can be made using the same ingredients but following the directions below.
1. Dissolve the above ingredients into 1L distilled water in a 2L or larger Erlenmeyer flask.
2. Bring the final volume up to 2 L.
3. Swirl the flask to dissolve the media, and heat to **vigorously** boiling in the microwave or hot plate. (If using a hot plate be careful not to burn your media, swirl constantly.)
4. Repeat heating and swirling about three times until all the media is dissolved (no more specks swirl around), but use caution not to burn yourself. Boil 20 minutes.
5. When media is dissolved, cover flask with foil and store the media in the refrigerator for up to 7 days.
6. Using a **sterile** 25 mL serological pipette and pump, aliquot 5 mL YPD into **sterile** 25 mm X 150 mm test tubes, and place into test tube racks.
7. Cap with **sterile caps** or cover tubes with **sterile** foil.
* Non-liquid items (e.g., caps) can be sterilized by baking in a standard oven for 45 minutes at 300°C. Small items like caps can be sterilized inside a large beaker covered in foil. Test a small number of items the first time you do this to make sure they don't melt.