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Molecular ecology of wild yeasts: Isolating yeast species from the local environment

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Abstract

Wild yeasts are everywhere, an invisible and understudied part of our planet's biodiversity. In this lab module, wild yeasts offer students an entry point into studying the ecology of their region. Over the course of 9 weeks (part time), students explore a natural area and choose their own samples of plants and insects, then culture their samples to isolate wild yeast strains. They DNA-barcode their strains and use the sequence data to identify what they have found. Finally, they look for patterns in pooled class data and present a research proposal for future experiments to identify associations between yeasts and their hosts. Students get a chance to collect genuinely novel data of interest to the scientific community while learning about their local environment. In addition, they practice important molecular biology and microbiology laboratory skills. They even have a chance of discovering a new species of yeast. Here, I provide materials to guide instructors through the some of the key experimental stages of the wild yeast project: collecting samples, inoculating liquid media, observing microbial growth in liquid culture, streaking strains to isolation, and studying colony morphology on agar plates.

Keywords: microbiology, ecology, biodiversity, yeast, fungi, CURE, inquiry-based learning

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INTRODUCTION

The Wild Yeast Biodiversity project invites students to participate in genuine discovery. Scientists know very little about the microbial ecology of the natural world, and neither the instructor nor the students know which species we will find. In this lab, we have repeatedly isolated strains that are not yet in GenBank and may represent new species. Because the students and the instructor are finding the answers together, students are inspired to take ownership of their strains and their data.

The data and strains from this class can generate authentically novel data of interest to the scientific community. I suggest contributing the results from your class to the national database of wild yeasts maintained by the Hittinger Lab at the University of Wisconsin-Madison where it can be used by other researchers. In addition, I freeze the yeasts that

students isolate indefinitely at -80°C, which means that doing this project across multiple years builds up a wild yeast strain collection and longitudinal data set that can be used to test ecological and evolutionary questions.

This project requires 9 weeks to complete (meeting twice each week) because the cultures take time to grow. However, the time required for the project on any given day varies across the 9 weeks. Some of the activities take up to 2 hours (sample collection) while others take only 10-15 minutes (re-streaking agar plate cultures). I provide time estimates for setting up and performing each individual activity below. In my course, I teach the Wild Yeast Biodiversity project along with two other part-time investigations that run in parallel throughout the term.

The Wild Yeast Biodiversity project is appropriate for undergraduate students at any level.

Learning goals for the activities described here:

- An understanding and appreciation of microbial biodiversity.
- Basic microbiology skills for liquid and agar plate culture.
- Aseptic technique in field and laboratory conditions.
- Natural history of the local area.
- Essential phenotypic characteristics of yeasts and bacteria in liquid and on agar plates.

Additional learning goals for the overall Wild Yeast Biodiversity project:

- Microbiology safety (because we culture unknown microorganisms, we use Biosafety Level 2 protocols and strongly emphasize lab safety).
- Basic molecular biology and bioinformatics skills.
- Generating hypotheses and designing experiments based on pooled class sampling data and wild yeast identifications.

Timeline: What students do for the project.

- Week 1: Learn background and aseptic technique.
- Week 2: Collect samples from local natural area (7 per student).
- Week 3: Inoculate samples in liquid media with antibiotics. Incubate up to 5 days.
- Week 4: Record observations of cultures; passage to new liquid media. Incubate up to 5 days.
- Weeks 5-7: Spread liquid cultures onto agar plates (YPD media). Pick colonies and re-streak as needed to isolate wild yeast strains.
- Weeks 8-9: Extract genomic DNA, PCR the barcoding locus, check results on a gel, send out for sequencing. Freeze each isolated strain for long-term storage at -80°C. Observe and document plate morphologies.
- Week 10: BLAST sequence results to identify isolated strains; research what is known about those strains. Pool everyone’s collecting/culturing/sequence/strain identification data into a shared spreadsheet. Students analyze the data to look for patterns and ecological associations, then write and present a research proposal to test those associations.

This paper focuses on some of the key experimental steps in the project:

- Activity 1: Collecting samples.
- Activity 2: Inoculating samples.
- Activity 3: Observing liquid cultures.
- Activity 4: Learning to pick and streak colonies.
- Activity 5: Observing agar plate cultures.

A full lab manual with details on all the steps is available by emailing the author.

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STUDENT OUTLINE FOR ACTIVITY 1: COLLECTING SAMPLES

Objectives

Collect samples of plants and other organisms that spark your curiosity.
Identify at least 7 organisms in our local habitat.
Use aseptic technique in field conditions to take wild yeast samples.

Introduction

Today, you will work with your partner to collect samples in the field, using the aseptic techniques you have learned. You will identify the organisms you sample using the Seek app. During the next several weeks, you will culture your samples to isolate and identify wild yeast strains.

Methods and Data Collection

Before setting out:

1. Tie long hair back so you don’t contaminate your samples.
2. Put a pair of gloves in your size into your collecting bag.
3. Start an entry in your field notebook. Record your name, group number, today’s date, and the collecting location.
4. Bring this page of your lab manual with you for reference, along with your bag of collecting materials.

How to take a yeast sample in the field:

1. Remember: no feces, eucalyptus, or soil. Don’t sample things that are lying on the ground, because they have soil on them. Please don’t sample from threatened species.
2. Identify the organism you are sampling using Seek. Make sure Seek records it as an observation. Take photos of the organism: one photo that includes the whole organism and, if it is a plant, a close-up photo of the part of the plant you are going to sample.
3. Wear gloves to reduce contamination and so you don't irritate your skin by touching the ethanol.
4. Pull the sample bag open to break the seal, then close it.
5. Take your tweezers out of the ethanol. Shake them to get rid of the excess ethanol. Let them dry in the air for several seconds (wet ethanol will kill the microbes you're trying to sample).
6. Pull the sample bag open using the wires. Pull the sample off the plant or other surface using the tweezers and place it in the bag. If at all possible, the volume of the sample should be the size of your little finger. Don’t touch the sample with anything but sterilized tweezers/scissors: not with your hands, not with the outside of the sample bag, etc. Note: If you have trouble getting the sample using just tweezers, sterilize your scissors by submerging them in the 70% ethanol for 15 seconds, then letting them dry. Then use them to cut off the sample while holding it with the tweezers. It’s usually easier to just pull off the sample with tweezers, so try that first before using the scissors.
7. Close the bag and fold it over a few times, then fold the wires inward to keep it closed. Each sample gets its own bag to avoid cross-contamination.
8. Once your sample is in the bag, wipe the tweezers/scissors off firmly with a kimwipe to remove plant sap etc. Return the tweezers to the ethanol tube and leave them there for at least 15 seconds to sterilize them before taking the next sample.
9. Write the sample number on the bag (1-7). Write your name and the date on the bag as well and place the bag in your quart-size zipperlock bag.
10. Write a note in your field notebook with the following information.
    a. Sample number (1-7).
    b. If a plant: The part of the plant you collected (leaf, flower, berry, seed, etc.) and whether it was fresh or dried.
    c. If a flower: whether the flower was open (i.e., accessible to pollinators) or a closed bud.
    d. A brief description of the area where you collected it.

e. The species name that Seek provided.
f. Notes to help you remember which of your photos go with this sample. A good strategy is to take a photo of the sample page itself, so you know that the photos immediately before the sample page photo go with this sample.

When we return to the lab:

1. Remove instruments from ethanol tube and let dry. Discard the ethanol in the ethanol waste container.
2. For each sample, record the collection sites and details in your lab notebook, based on the notes you took in the field. Allocate 3 lab notebook pages per sample, because you'll be recording additional notes about each sample as you culture it over the next several weeks.
3. Double-check that each sample bag/container is labeled properly.
4. Put all your bag samples into the quart-size zipperlock bag. Label the bag with your name and group number, and put it in the 4°C refrigerator.

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MATERIALS FOR ACTIVITY 1

For each student:

- A gallon-size zipper-lock bag containing the materials below.
- 7 sterile, sealable sample bags, at least 5x10 cm.
- 1 quart-size zipper-lock bag for carrying sample bags once they have materials in them. Students should write their names on these since they will be stored at 4°C until the class is ready to inoculate.
- 1 small field notebook or paper for taking notes.
- 1 pair of eyebrow tweezers (square tips) with no plastic/rubber coating. These are better than standard forceps for collecting because they are short enough to fit into the 50 mL conical tube with the cap on, and the tips are sturdy which allows students to tear pieces off plants with them.
- Sharpie pen for labeling bags and containers.
- 1 50ml conical tube, filled 2/3 full of 70% ethanol. Put eyebrow tweezers in the tube, tips down.
- Scissors for cutting off leaves/bark/twigs (can be shared by students working together).
- 7-10 kimwipes for wiping debris off tweezers/scissors.
- 2 pairs of disposable gloves.

NOTES FOR THE INSTRUCTOR FOR ACTIVITY 1

It is helpful for students to work in pairs while collecting so one student can hold the sample bag while the other student collects the sample. Collecting 7 samples takes students up to 90 minutes because I encourage them to explore the natural area and sample what catches their interest. Some students form ad hoc hypotheses in the field and design their sampling to test them, such as collecting fresh and dry material from each plant to see which will yield more yeast strains. You could shorten the collecting time by restricting students to a smaller area.

Students aren't allowed to collect feces, eucalyptus, or soil because in our area, those can harbor pathogenic yeasts. Most yeasts are harmless to healthy people when proper lab protocols are followed to avoid introducing them into the body, but I encourage you to talk to local experts about what potentially pathogenic yeasts may be present in your area. We use Biosafety Level 2 protocols when working with wild yeast cultures in the lab as an extra level of precaution. Those protocols require a few extra steps, such as using filtered pipet tips and disposable applicators to avoid aerosolization, but they are within the reach of most university teaching labs.

In order to reliably identify the organisms that students took samples from, I have students install the free "Seek by iNaturalist" app on their phone in advance. This app is surprisingly effective at using the phone's camera to identify the sample plant, animal, mushroom, etc. I have students upload the Seek ID plus photos of the organism. Seek makes a few mistakes with look-alike species, but with the photos plus knowledge of which plants are found in our area, I can correct the errors afterward and have high confidence in the resulting data.

Samples can be stored at 4°C for up to 3 weeks before inoculation (Sylvester 2015).

STUDENT OUTLINE FOR ACTIVITY 2: INOCULATING SAMPLES

Objectives

Inoculate wild yeast media with the samples you collected.
Use aseptic technique in laboratory conditions.

Introduction

Today, you will aseptically inoculate wild yeast liquid media with the samples you collected. Since your samples likely contain bacteria and mold in addition to yeast, we will culture them in conditions that encourage the growth of yeasts and discourage mold and bacteria. The media contains antibiotics as well as a high concentration of glucose to impose osmotic stress on bacteria. We will seal the tubes and will not shake them during culturing, which limits oxygen and thus mold growth.

Methods and Data Collection

Prepare to inoculate your cultures:

You will need:

- The bag of wild yeast samples that you collected, which have been stored at 4°C.
- 7 screw-cap plastic conical 15 mL tubes, each of which contains 9 mL of wild yeast media, 9 µL of 100 mg/mL ampicillin, and 9 µL of 30 mg/mL chloramphenicol.

Get out the 50 mL tube that you had 70% ethanol in while collecting. Put the 50 mL tube in a rack and fill it about 2/3 full of 70% ethanol.

You will use this to sterilize instruments during inoculation. Make sure you keep it at least 2 feet away from the burner at all times! When taking instruments in and out of it, be very careful not to knock it over or get ethanol on your fingers, which would make your fingers flammable!

Put both pairs of fine-point forceps from your equipment box into the 50mL tube of ethanol, points down. Make sure you can remove the forceps without getting your fingers in the ethanol. If necessary, pour out some of the ethanol into the ethanol waste carboy.

Do not use the eyebrow tweezers! They are too short, and if you use them you will get ethanol on your fingertips when you try to remove them from the ethanol. The ethanol on your fingers can then catch fire when you flame the tweezers. *For your safety, it is very important to keep your fingers free of ethanol.*

Get out a pair of scissors and put them on the bench to sterilize if needed. Generally it works better to pull samples apart with forceps rather than cut them — you have more control. However, if you need to use scissors for a particular sample, do the following:

- Dip them point-down in the ethanol with their blades open as far as will fit so the blades are exposed to the ethanol. Make sure the handles stay dry.
- Shake the excess ethanol off and flame them, points angled downward (see instructions for forceps below; handle scissors the same way.)
- Be especially careful when flaming scissors: make sure there is no ethanol on the handles, since it will burn your fingers. Dry the handles with a kimwipe if needed.
- Flame the blades of the scissors with the blades pointing slightly downward.

As you work, notice which plants have aromatic compounds (smell strongly). This is a common adaptation for chaparral plants. Record which samples/plants are aromatic in your lab notebook.

Do the following for each sample:

Find the sample bag/container with the sample ID to match the tube you plan to inoculate.
Shake the sample in the bag so that it's near the opening. Unfold the wires of the sample bag, but don't open it.
Take half a sterile Petri dish from the stack and place it face up in front of you near the flame. Keep the rest of the

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dishes closed or face down to preserve their sterility. This dish is your sterile work surface for this sample.

Take a pair of tweezers from the ethanol tube, holding them point down.

Away from the flame, shake them a bit to let excess droplets of ethanol fall off them.

Flame them, holding them horizontally with the tips pointing slightly downward. (Why? because heat rises and burning ethanol sinks.) Be careful not to let burning ethanol run down toward your fingers.

Prop the forceps on the side of the dish so the tips are in the air over the plate. Let them cool for at least a few seconds.

Pull the wire tabs on each side of the sample bag to open it. Hold the bag sideways with its mouth over the petri dish. Reach in with the sterile forceps and remove the pieces of sample, placing them on the dish.

Don't just pour the sample from the bag/container into the dish; it will tend to bounce out again and get contaminated. If a piece of sample falls outside the petri dish, discard it.

Close the bag.

Flame the second pair of forceps or scissors, remembering to shake off excess ethanol first and hold them horizontally with the tips angled downward.

Use the forceps/scissors along with the first pair of forceps to break the sample into pieces small enough to easily fit into the culture tube. When possible, use two pairs of forceps to pull samples apart rather than cutting them; you have more control and samples are less likely to fly away. One technique that works well is holding the sample with one pair of forceps and scraping pieces off it with the other pair of forceps. If you have a closed flower or seed pod, break it open so the inner parts will be exposed to the media. You don’t have to tear it into little pieces; just make sure it’s split open.

Aseptically open the conical tube containing the media for this sample. Place as much sample material in the tube as will comfortably fit, using the forceps. Try not to let the sample or forceps touch the rim of the tube, to avoid contamination.

Screw the cap tightly on the culture tube.

Wipe the inside and outside surfaces of the instruments off firmly with a kimwipe to get rid of detritus, sticky plant juices, etc. Return the forceps to the ethanol.

Tap the tube hard on the table to submerge the sample in the liquid and remove bubbles. If submerged bubbles remain in one of your samples, make a note of it in on that sample's page in your lab notebook so you can tell if bubbles you see in the future are new (from fermentation) or old (left over from inoculation).

If needed, use a kimwipe to brush debris from your bench into the used petri dish or lid. Throw away the used petri dish or lid, and any remaining material for this sample, in the regular trash can. Each sample gets a fresh one! Don't wipe down your bench with ethanol between samples, because you have an open flame.

Make a note in your lab notebook of anything special you noticed while inoculating (e.g., there was an insect inside the flower).

When all tubes are inoculated:

Make sure all the caps are on tightly. Yeast can ferment and emit carbon dioxide, and we don't want the tubes to leak from the pressure.

Parafilm each culture tube using half a square of parafilm per tube (ask an instructor to show you how). Make sure the parafilm seals and covers the place where the cap touches the tube: don’t just parafilm the cap!

Carry the tube rack in a secondary container to the front of the room. The instructor will put them in the 30°C incubator for you, where they will be incubated for 5 days without shaking.

MATERIALS FOR ACTIVITY 2

For the class as a whole:
- Roll of parafilm, scissors

For each pair of students:
- Bunsen burner
- Squeeze bottle of 70% ethanol
- Kimwipes
- 4 pairs of fine-tip forceps (not eyebrow tweezers)
- 1 pair of dissecting scissors
- 7 sterile, empty 10 cm Petri dishes, including bottom and lid.
- 500 mL of wild yeast media (enough for 2 rounds of liquid culture for 2 students). To make, combine in a large flask:
    - ~350 mL distilled water
    - 1.0 g Synthetic Complete Dropout Mix including all amino acids (e.g., US Biological D9515).
    - 40.0 g dextrose
    - 3.35 g Yeast Nitrogen Base (YNB) without amino acids, including ammonium sulfate (e.g., US Biological Y2025).

Stir without heat for ~20 minutes, until fully dissolved. Transfer to a graduated cylinder and add distilled water to a total volume of 500 mL. Filter-sterilize. For each student, aseptically aliquot 9 mL of media into each of 7 sterile, disposable screw-cap 15 mL conical tubes (Falcon tubes). Store at 4°C until ready to use. Shortly before inoculating, add 9 µL of chloramphenicol stock solution and 9 µL of ampicillin stock solution to each tube and invert to mix. Keep the antibiotics at -20C until ready to use. The ampicillin stock solution (1000x) is 100 mg/mL ampicillin in sterile milliQ/DI water. The chloramphenicol stock solution (1000x) is 30 mg/mL in 100% ethanol. Materials for preparing, sterilizing, aliquoting, and adding antibiotics to media are not listed below

For each individual student:
- Tube rack(s) with space for 8 15 mL conical tubes and 1 50 mL conical tube
- 50 mL screw-cap conical tube (can be the one they used for collecting samples)
- 7 15 mL conical tubes containing wild yeast media and antibiotics as described above.

NOTES FOR THE INSTRUCTOR FOR ACTIVITY 2

Inoculating 7 samples takes students about 1 hour, or 1.5 hours if students are sharing forceps with their lab partner. It helps to circulate and encourage them to simply pull samples apart to expose surfaces rather than dissecting them into minute pieces. Many insects can survive a week at 4°C so you will probably have some surprise guests in the plant samples; students can choose to inoculate these along with the plant sample or let them go.

It is helpful to demonstrate the inoculation process for students, especially the proper angle at which to hold the forceps to avoid being burned.

In my class, I have student make, filter-sterilize, and aliquot their own wild yeast media (~90 minutes). They tend to contaminate their media the first time they try this, so I have them practice the week before by aliquoting liquid LB media, which we can make in bulk cheaply. I incubate their LB aliquots and leftover media bottles so they can see contamination when it occurs. This improves their success rate when they later aliquot the more expensive wild yeast media. For the wild yeast media, I have students aliquot their 7 sample tubes plus an 8th tube, which will not be inoculated, as a control for contamination. I also have students add their own antibiotics to the tubes just before inoculating. Note that there are 2 rounds of liquid culture so each student will need 2 sets of tubes. The Materials list above does not include the materials for those activities.

The media and culturing conditions are based on protocols from the Hittinger lab (Sylvester 2015).

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STUDENT OUTLINE FOR ACTIVITY 3: OBSERVING LIQUID CULTURES

Objectives

Identify phenotypic characteristics of yeasts, molds, and bacteria in liquid culture.

Introduction

Today you will examine your tubes of wild yeast media and observe what, if anything, has grown from your samples. Later, when you spread these cultures on agar plates, compare the appearance of your cultures on plates to your observations today. Many microbial cultures look very different in liquid than they do on agar plates!

Methods and Data Collection

Double-glove and prepare your bench for aseptic work.

Make observations of your liquid culture tubes:

Handle the tubes gently. Some of them may be under pressure from carbon dioxide building up inside, and also you don't want to pop any bubbles before you get a chance to observe them.

Hold each tube up and examine it carefully, without opening it. Make a dated entry on that sample's page in your lab notebook and write down what you see.

Check for any of the following in your tube and describe them in your lab notebook:

Do you see pale sediment at the bottom of the tube that isn't dirt? Yeast cells can’t swim, unlike many bacteria, so they often settle at the bottom of the tube in a layer that looks a bit like sand. If the top of the sediment layer is a sharp line (the sediment is clearly distinct from the rest of the tube contents), that’s a good sign that it’s yeast. If you see sediment, describe its color, how deep it is (estimate in millimeters), and whether the sediment is clearly delineated from the rest of the tube contents.

Do you see fuzzy growth at the surface of the liquid? This is mold. Mold depends more on oxygen than yeast does, and its filamentous shape can help it form floating mats, so it’s often found at the surface of the liquid. It’s often gray, white, or black, but it can be other colors like green, yellow, or pink.

Do you see white puffballs in the liquid, floating or stuck to the sides of the tube? These are likely bacteria or mold. Sometimes they accumulate in a soft pile at the bottom of the tube. They are more diffuse and more buoyant than yeast sediment; if you gently tilt the tube, they may swirl or rise upward, while yeast will usually stay put at the bottom of the tube. Record what you see: color, size, shape, and where in the tube they are.

Do this last: Tap the tube firmly on the table, once. Don’t shake it! Then look closely. Do you see tiny bubbles rising? They can be a sign of yeast producing carbon dioxide as they ferment the sugars in the media. Turn the tube upside down and then right side up again, gently. Look again for tiny carbon dioxide bubbles rising.

Write down any other observations, such as the color of the media.

Check the negative control tube for cloudiness or other signs of contamination. If you see any, check with the instructor.

MATERIALS FOR ACTIVITY 3

Students' ROUND 1 or ROUND 2 liquid culture tubes, which have been incubated as described in the Notes for the Instructor.

NOTES FOR THE INSTRUCTOR FOR ACTIVITY 3

Students do 2 rounds of liquid culture. ROUND 1 is the tubes they inoculated in Activity 2 described above. After 5 days, they transfer 5µL of each ROUND 1 culture into a new tube of media with antibiotics, creating ROUND 2 cultures. After 5 more days, they observe the liquid ROUND 2 cultures and then spread a sample of each ROUND2 culture onto an agar plate. The observations here apply to both ROUND 1 and ROUND 2 liquid culture observations. They take students approximately 20 minutes.

If more than 3 days will elapse between students inoculating tubes and students passaging the resulting cultures (for either ROUND 1 or ROUND 2), check the tubes on day 3 to see if any yeasts are growing rapidly. If a tube has more than ~2 mm of pale sediment at the bottom and/or you see abundant bubbles rising when you invert the tube, move the tube to 4°C so the carbon dioxide pressure doesn't build up too much. If needed to accommodate the class schedule, all the liquid culture tubes can be moved to 4°C storage on day 5 until students are available to work with them.

The day that students open their ROUND 1 tubes to passage their cultures is the first time they work with Biosafety Level 2 (BSL2) cultures. I teach them BSL2 protocols in advance and we change some of the materials we work with at this point, primarily to avoid aerosolization: filter tips instead of regular pipet tips, sharps containers with closable tops, etc. We use disposable, screw-cap tubes for all procedures involving wild yeast cultures and pay extra attention to aseptic technique. Because liquid culture tubes can fizz when opened (especially after vortexing), I recommend opening the tubes with visible bubbles in a biosafety cabinet or other environment where the yeast aerosols are kept away from the user. For more details on BSL2 protocols, please email the author and contact your local Environmental Health and Safety officer.

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STUDENT OUTLINE FOR ACTIVITY 4: LEARNING TO PICK AND STREAK COLONIES

Objectives

Explain why it is useful to work with single colonies of microbes.

Pick and streak yeast cultures on agar plates using the specified streak pattern to obtain isolated colonies.

Introduction

In this activity, you will practice *picking a colony* and using it to make a *streak plate.* This is an essential microbiology skill which you will use many times in this course. You will use this skill to isolate your wild yeast strains on agar plates, but today we will practice with *Saccharomyces cerevisiae*, a harmless yeast that is used in the laboratory and also to bake bread and brew wine and beer.

A colony is a pile of cells on an agar plate that are all descended from a single cell. When we work with microbes, we try to begin our experiments with a single, isolated colony. This gives us the most consistent results because the cells in a colony are all copies of one original cell that was deposited on the plate. As a result, all the cells in a colony are approximately genetically identical (with the exception of new mutations) and thus may be expected to share very similar phenotypes.

Methods and Data Collection

Day 1: Streaking the plate.

To produce isolated colonies, we make streak plates. You will begin a streak by picking up a colony of yeast with a sterile instrument. (We will use sterile wooden applicators in this class.) This is called "picking a colony."

Then you will spread the cells out onto a new agar plate by making a series of zig-zags across the surface of the new plate. Initially, there will be so many cells on your applicator that your strokes across the agar will leave behind a continuous smear of cells. Those cells will grow into colonies that are so close together they will appear as a solid mass of cells. You want isolated, single colonies, so you will take a second sterile applicator and gently *drag it across the track of your first streak,* where it picks up some of the cells you laid down. Continue dragging the second applicator across the plate in a zig-zag pattern. Then repeat the process with a third sterile applicator crossing the second streak to spread the cells out even more.

The goal of a streak plate is to dilute the cells enough that at some point during the streaking, the applicator is depositing individual, spaced-out cells along its track. Each of those cells will grow into a single, isolated colony in which all cells are descended from a single original cell and are thus presumed to share the same genotype.

Thought questions: Why do you need to use a fresh sterile applicator for each new streak? How would the plate look different once the cells grow if you used the same applicator for each streak?

Study Figure 1 below. This a diagram of how to streak a plate with a single strain, as you are doing today. Some things to notice:

* Each streak goes in the direction of its arrow.
* The exact number or shape of the zigzags in each streak isn't important. If you're having trouble keeping your applicator from digging into the agar, you can make a series of parallel streaks instead of zigzags.
* The order of the streaks is important. The first streak carries the most cells, and spreads them out across a confined area of the plate with zigzags. Don't take up too much of the plate with the first streak, because it usually is too dense to yield single colonies.
* The second streak, made with a new sterile applicator, passes ONCE through some of streak 1's zigzags. Then it doesn't cross any additional parts of streak 1. That's important because streak 2 is diluting the cells it picked up, and we don't want to keep picking up additional cells by re-crossing streak 1.
* The third streak (again, made with a new sterile applicator) does the same as streak 2, but with even fewer cells because it crosses streak 2 instead of streak 1.
* The streaks fill the entire plate, but don't overlap each other after the initial crossings.

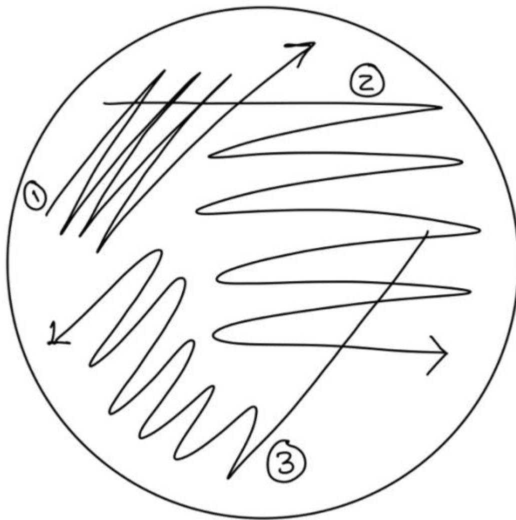

Figure 1: An illustration of the desired streak pattern on an agar plate.

Each individual student in your group should follow the instructions below to make a YPD streak plate of *Saccharomyces cerevisiae.*

1. Prepare your bench for aseptic work.
2. Pick up a new YPD plate and a packet of wooden applicators from the front of the room.
3. There are several pre-grown streak plates with isolated *S. cerevisiae* colonies for the class to pick colonies from. Get one from the front of the class. As soon as your group is done with it, return it to the front of the class for other groups to use.
4. Use a Sharpie to write your name and the date on one of the YPD plates.
5. Aseptically remove a new wooden applicator from your packet.
6. Examine the pre-grown streak plate without opening the lid. Choose a colony that is isolated enough that you're sure you can touch it with the applicator without touching any other colonies. Avoid picking extra-small colonies because they may be petites (cells that have lost their mitochondria).
7. "Pick" a colony from the pre-grown plate, as follows: Remove the lid. *Gently* scoop up part of the colony with the tip of your applicator. Don't gouge the agar! You don't have to scoop up the entire colony: if there are enough cells that you can see them on the tip of your applicator, you have enough.
8. Close the lid.
9. Open your YPD plate and make the first streak, following the diagram. Be gentle; don't dig into the agar. Make sure that the side of the applicator that has cells on it is what you're spreading on your plate.
10. Discard the applicator in the red sharps box on your bench.
11. Use *new* applicators to make the second and third streaks.
12. Turn the plate upside down (lid side down) and bring it to the front of the room. We keep streaked agar plates upside down so that if condensation forms on the lid, it doesn't drip down onto the agar and blur the colonies. We will incubate it at 30˚C until individual colonies are visible, which is usually 2 days.

Day 2: Observing your streak plate.

Today, you'll find out the results of the streak and spot plates you made last time. Pick up your group's streak plates from the front of the room. They have been incubated at 30°C to let the yeast cells grow.

Each individual student should record the following observations in your lab notebook about your streak plate:

1. Contamination: Without opening your plate, carefully examine it. Is the plate contaminated with mold or other microbes that don't look like yeast? If so, how could you improve your aseptic technique so that you don't get contamination in the future? Ask the instructional for coaching next time you do aseptic technique. Note: If the plate is contaminated, make the rest of your observations with the lid on. If not,

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take the lid off so you can see it more clearly. You don't need to worry about contaminating it because we will discard it after these observations.

2. Agar surface: Did you dig the applicator into the agar? If so, what happened to the cells/streak at that point?
3. Streak pattern: Compare your streak pattern to the diagram in the lab manual from last time.
   a. Did each of your streaks take up approximately the right amount of space on the plate?
   b. Do your streaks use up most of the space on the plate?
   c. Does streak 2 pass through streak 1's lines only once?
   d. Does streak 3 pass through streak 2's lines only once?
   e. Does each of the 3 successive streaks have fewer cells in it? How can you tell?
   f. Do you have isolated individual colonies on your plate?

If the answer to any of the lettered questions is "no," or if you have questions, talk to the instructor about your streaking technique. You will be streaking your wild yeast strains to isolation later in the course and it will save you time then if you improve your technique now.

When you write the Discussion section in your lab notebook, use your observations to reflect on how you can improve your streaking technique.

MATERIALS FOR ACTIVITY 4

For each student: 1 YPD plate, a packet of sterile wooden applicators, and a Sharpie pen.

Each pair of students will need access to a biohazardous waste sharps container for disposing of used applicators.

For the whole class: a set of YPD plates previously streaked with *Saccharomyces cerevisiae* and grown until they have isolated colonies (2-3 days at 30°C). Each student will need 1 colony to pick. Groups of students can take turns picking from the same plate.

NOTES FOR THE INSTRUCTOR FOR ACTIVITY 4

The only instructor setup is providing the materials and streaking the *S. cerevisiae* plates ahead of time. Part 1 of this activity takes students about 20 minutes, or less if you provide 1 plate to pick colonies from for each pair of students so they don't have to take turns. If your students are new to agar plates, you can precede this with an activity in which each pair of students practices "streaking" a shared plate with blue liquid (water and food coloring) until they get the feel of the applicator and stop cutting tracks into the agar.

Plates will need to grow for 2 days after Part 1 so colonies are visible. You can move plates from the incubator to 4°C storage after 2-3 days and keep them there until students are ready for Part 2. Part 2 takes students about 15 minutes.

There are many different streak patterns and you may have your favorite. I have found that for inexperienced students, the pattern above is the most reliable for getting isolated colonies. That tends to save time and plates in the long run when students are culturing their wild yeast strains, since they have to keep re-streaking onto new plates until they get isolated colonies. This is particularly true since different wild species grow at different rates, so if a student streaks two species on one plate, one may overgrow while the student is waiting for the other to have colonies large enough to pick.

Answer to the thought question in the student outline for Day 1: You use a new sterile applicator for each streak because if you use the same applicator for more than one streak, the subsequent streaks won't be diluted. A plate where you used 1 applicator for all 3 streaks probably wouldn't have isolated colonies; it would have three streaks of contiguous cells, all at roughly the same density.

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STUDENT OUTLINE FOR ACTIVITY 5: OBSERVING AGAR PLATE CULTURES

Objectives

Identify essential phenotypic characteristics of microbial colonies on agar plates.
Recognize dimorphic fungi such as *Aureobasidium pullulans.*

Introduction

By this point, you have streaked and re-streaked each of your yeast-like strains until you have a single agar plate containing isolated colonies of each strain. Today, you will closely observe the plate phenotypes of your strains.

Methods and Data Collection

Double-glove and prepare your bench for aseptic work.

For each of your strains, look closely at the isolated colonies and record their appearance for each numbered morphological characteristic below. Use the underlined terms, which are standard in microbiology, so other people looking at your data will know what you mean. If your isolated colonies are too small to observe clearly, you can base your observations on the denser growth elsewhere on the plate, but be careful and compare your observations to the isolated colonies wherever possible. For example, two circular colonies that have grown together can appear irregular.

1. Colony edge: What is the shape of an isolated colony when viewed from above? Is it circular, filamentous (fuzzy like mold), or irregular? Look at your colonies while your plate is on the lab bench, then hold your plate up to the light and examine the edges of the colonies closely.
   Note: If your strain is a dimorphic fungus like *Aureobasidium pullulans,* you may see young colonies that are circular with sharp, clean edges, but older colonies/growth that have little filamentous protrusions coming out of the edges. This is easiest to see when you hold the plate up to the light. These fungi can grow in either a yeast form (single-celled) or a filamentous, mold-like form – hence "dimorphic," meaning "two shapes." We often isolate *Aureobasidium pullulans* and its close relative *A. melanogenum* because they are extremely stress-resistant and can live in many different conditions. On plates, they tend to start out pink and then gradually darken as they produce melanin, which protects them against certain environmental stresses (Gostinčar et al. 2014). If you think your strain is dimorphic, record that in your lab notebook.
2. Surface appearance: Is the surface of the colonies glistening (shiny, smooth), dull (rough, matte), or wrinkled?
3. Elevation: What does the colony look like when viewed from the side? Is it flat, convex (low bump), pulvinate (tall, gumdrop-shaped), or umbonate (flat with a raised bump in the middle)?
4. What color are the colonies? If they appear white, try to describe the shade of white (yellowish-white? grayish-white? etc.).
5. Size: How big are the isolated colony/colonies? Estimate by eye and give your answer in millimeters.
6. Age of the plate: number of days between streaking the plate and today.
7. Did the colonies grow into the agar? If your strain is like this, you'll know, because you will have had trouble picking cells for re-streaking since the cells tend to be buried in the agar. If you're not sure, answer "no."
8. Variation: Record any obvious variation in color or surface appearance among the yeast on the plate. In many strains, older or denser colonies have a different color or surface appearance from smaller/younger colonies. For example, older/denser colonies may be wrinkled while newer colonies may be smooth.
9. Record any other observations such as texture that you have observed while culturing this strain.

Terms in this section adapted from Tindall et al. 2007.

MATERIALS FOR ACTIVITY 5

YPD (Yeast extract/Peptone/Dextrose) agar plates (10cm). YPD is a standard medium for culturing yeast in the lab. Example: BD Industrial/Difco catalog #242720. The number of plates each student will need for the plate culture process will vary, since students vary how many strains they have and how many times they need to re-streak a strain to get isolated colonies. For the entire process, starting with spreading 7 ROUND 2 liquid cultures onto plates and ending with isolated strains, the average student needs 16 YPD plates.

Students also need disposable sterile wooden applicators. We use disposable applicators rather than inoculating loops because flaming the loops can aerosolize the yeasts, creating a health hazard. Sterilized toothpicks are cheaper, but harder for students to use (they scratch the agar) and result in more rounds of re-streaking so you need more YPD plates; I have not found them to be worth it.

NOTES FOR THE INSTRUCTOR FOR ACTIVITY 5

After ROUND 2 liquid cultures have been incubated for 5 days, students spread 10 µL of each culture onto a YPD plate. They incubate the plate at 30°C for up to 5 days. Some plates show no growth, others are rapidly overtaken by mold, and some have visible colonies of yeast or bacteria. Many plates contain multiple species. Plates that have colonies but also visible mold should be opened in a biosafety cabinet to avoid dispersing mold spores into the classroom. Plates that contain only mold are discarded unopened.

Students pick the colonies and streak them onto new YPD plates. They re-streak as needed onto new YPD plates over the next two weeks until they have created a “stock plate” for each strain: a plate that has only 1 strain (at least as far as we can tell by colony morphology), no contamination, and at least 1 isolated colony.

There is no setup other than providing the materials. The time for students to do this activity varies depending on how many strains each student is currently propagating. Some students wind up with 0 strains and some get as many as 7-8, with an average of about 3. Re-streaking 1 plate takes a student about 5 minutes. I suggest scheduling other activities on these lab days, so students whose strains are already isolated or who had no successful strains can move on to other tasks. I emphasize to students that the number of strains they are able to isolate is due to luck, not technique, and that I will help students “adopt” extra strains as needed at the end so that each student has 3 strains to PCR.

During the re-streaking process, I consult frequently with students about their plates. Students often ask me whether a strain is yeast or bacteria. I give them my best guess based on the criteria described in the student outline above, but I cheerfully admit that I don’t know, because both yeast and bacteria have widely varying plate morphologies, so this is something we’ll discover together. (The PCR reaction will only succeed with fungi, because bacteria lack the ribosomal region we amplify, so the PCR results will have the final word.) This is an opportunity to emphasize that we are genuinely doing discovery science where no one knows the answers. I encourage students to propagate everything that could possibly be yeast; freezing strains is inexpensive and we have isolated a number of strains that looked like “typical bacteria” (shiny, flat colonies) but which turned out to be yeast based on the PCR and sequencing results.

The colony morphology descriptions and figures above are adapted from Tindall et al. 2007.

Next steps:

Once students have isolated their strains, they DNA barcode the strains via the following steps. For details on any of these steps, please email the author. Protocols adapted from Sylvester et al. 2015.

For each isolated strain:

Step 1: Extract the genomic DNA: Grow up the isolated strain in liquid YPD media overnight. Transfer 200 µL of the culture to a microfuge tube. Spin to pellet, discard supernatant. Add 50 µL 10mM NaOH to the microfuge tube, vortex to resuspend. Heat-shock at 99°C for 5.5 minutes, then put on ice. Optional: Combine 540 µL liquid culture and 240 µL sterile 50% glycerol in a cryotube to freeze at -80°C indefinitely.

Step 2: PCR the ITS/5.8S rDNA locus for barcoding:

Each 20µL PCR reaction contains the following:

0.8 µL 10µM ITS1 primer (TCCGTAGGTGAACCTGCGG)

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0.8 µL 10µM ITS4 primer (TCCTCCGCTTATTGATATGC)
10 µL 2X LongAmp Taq 2X Master Mix (New England Biolabs)
1 µL Cresol Red 0.1% sterile stock solution (helps students see their sample; optional)
6.4 µL Sterile MilliQ water
1.0 µL extracted genomic DNA sample (above)

PCR program:
94°C for 2 min (1x)
94°C for 30 s, 48°C for 30 s, 65°C for 2 min (40x)
65°C for 10 min (1x)
Hold at 4°C.

Step 3: Run PCR samples on a gel, then sequence: For each PCR reaction, transfer 4 µL to a new tube along with loading dye. Run samples on a 1% agarose gel stained with SybrSafe, along with a lane of 1 Kb Plus DNA Ladder (New England Biolabs). Samples showing a single clear band between 400-1000 bp are suitable for sequencing. Send the remaining 16 µL of each PCR reaction to an outside service for Sanger sequencing using the ITS4 PCR primer.

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