

LABORATORY EXPLORATION

Anaerobic Metabolism in Yeast

In today's lab, you will investigate aspects of anaerobic respiration in a living model organism, Baker's yeast (*Saccharomyces cerevisiae*). As always, part of preparing for an investigation like this is educating yourself about the system and what it already known about it. (That's the "strong" part of strong inference.) To this end, you and your teammates should spend the weeks between now and the actual lab period researching what is known about yeast's ability to metabolize various sugars. Based on what is already known, can you come up with new hypotheses that you could test in this lab?

Alternatively, if you wish to explore an area of yeast metabolism that is not well known (good luck with that), you may wish to perform a pilot study to establish observations that will generate further inquiry. If your team chooses this direction, you should be prepared to pose competing hypotheses to explain your experimental observations, and to propose experiments that would allow you to test them.

In either case, your first goal is to read and educate yourself about anaerobic metabolism of sugars by yeast and come up with an interesting, relevant problem to solve. We will need to know **no later than one week before your lab** whether you will need any materials that are not already listed in this lab manual chapter. Online resources available from the Richter Library and Google Scholar (www.google.com) are both good sources of scholarly, peer-reviewed articles that will give you enough background to further explore fermentation in yeast.

I. Cellular Respiration: Freeing Stored Energy

Cells obtain the energy stored in sugars by breaking the molecules apart in a series of enzyme-mediated reactions. Energy is extracted most efficiently in the presence of oxygen via the process known as **aerobic respiration**. Under conditions where oxygen is scarce or absent, some cells are still able to split glucose via the process of **anaerobic respiration**, also known as **fermentation**--though far less energy per glucose molecule is extracted, since it cannot be fully broken down without oxygen.

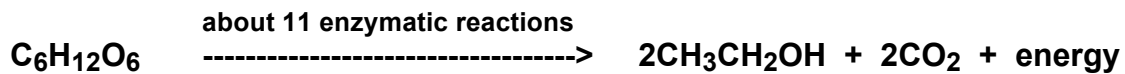
Objectives

After doing this lab, you should be able to:

- Design and perform an experiment to test the relative ability of yeast to ferment various potential nutrients.
- Graph and analyze rate curves on software such as Excel, and then graph and compare the resulting fermentation rates.
- Calculate mean fermentation rates for analysis via statistical tests.
- Devise further experiments designed to distinguish between competing hypotheses regarding yeast's ability (or inability) to ferment given sugars.
- Synthesize and explain your results on both the **proximate** (molecular) and **ultimate** (evolutionary) levels in your presentation, to be given in the next lab session.

Certain organisms can produce ATP by utilizing metabolic pathways that do not require participation of molecular oxygen. Usually, these are referred to as pathways of **fermentation** or **anaerobic glycolysis**. These pathways involve the sequential conversion of a carbohydrate into partially oxidized end product(s). The energy released during these reactions, and stored in the phosphate bonds of ATP.

One species of free-living (i.e., non-parasitic) unicellular fungus, commonly known as Brewer's yeast (*Saccharomyces cerevisiae*), can **ferment** various disaccharides and monosaccharides. For example, it can break down glucose:



Using appropriate equipment, we can estimate the rate of this pathway for any given sugar by determining the yield of CO₂ over time. Your observations should allow you to generate hypotheses about the fermentation pathway (using yeast as a model organism) and designing experiments to test them.

One might ask if yeast can ferment all sugars equally well. This is a question one can answer with a statistical hypothesis. But the next question is not so simple. If there is a significant difference in yeast's ability to ferment different sugars, then why? The second branch of the "hypothesis tree" brings us to the cellular and molecular level of this problem, which may be more challenging to address.

If you know something about yeast and its enzymes and enzymatic pathways, you can hypothesize about why yeast might not be able to ferment a particular sugar. This can lead you to target specific aspects of the enzymes, substrates, and pathways, and propose hypotheses about how they work.

If you know something about the sugars themselves (e.g., whether they are mono- or disaccharides, what type of bonds hold them together, what is their specific molecular structure), you might ask what it is about a particular sugar that makes it more or less suitable as an energy source for yeast. At this level, you are not searching directly for evolutionary explanations (e.g., yeast can't ferment sugar X because it is not present in yeast's natural environment). Rather, you are searching for the proximate causes of yeast's enzymatic "behavior" with respect to different sugars. After you have established the facts, you can hypothesize about the origin and evolutionary significance of your observations.

II. Setting up your problem and hypotheses

Once you and your teammates have gathered sufficient background knowledge about yeast's ability to ferment various types of sugars, you will be ready to formulate relevant, interesting hypotheses. Start with proximate questions. Given what you now know about this subject, consider the molecular structures and bonds of the sugars themselves. Might these be limiting factors? What aspects of sugar structure might affect the rate at which yeast can metabolize them, or whether they can metabolize them at all? What characteristics of yeast might affect the rate at which it can take up and metabolize a particular sugar. Do as much background research as possible so that you will be able to pose interesting, relevant, testable hypotheses.

In terms of the bigger, evolutionary picture (which you should consider once you have a proximate answer to your question), consider where yeast are found in nature. HINT: what's that "bloom" on the grape (Figure 4, and see Rossini *et al.* 1982)? How do you suppose prehistoric people discovered fermented fruit (a.k.a. wine) and fermented grain porridge (a.k.a. beer)? See Legras *et al.* (2007) for more about the history of symbiosis between yeast and humans.



Figure 4. Ripening Syrah grapes showing the characteristic powdery white coating, or 'bloom', on their surface that is composed of yeast (Hass 2008).

Now consider whether the various organic compounds available in the lab (monosaccharides and disaccharides) could be found in the microhabitat of yeast. Even if a potential nutrient molecule could be utilized, consider whether it is able to enter the cell, or if it would need to be enzymatically modified to enter the glycolysis pathway.

Compare the molecular structures of the compounds—size, shape, bonding, etc. Ask questions, share ideas, and—eventually—incorporate these larger ideas into the introduction of your presentation to help you explain why these questions are interesting and relevant.

By setting up an electronic communication network (via Blackboard, Facebook, or whatever venue your instructor chooses), your lab instructor will encourage the various teams to discuss their experiments well before lab begins. Each team should be prepared to give a brief explanation to their colleagues in order to generate feedback and improve the investigation. Many brains are often better than a few, once a framework has been established.

A Sample Problem: Carefully Wording Your Hypothesis

Let's say that your results have shown that yeast can ferment Sugar X (a monosaccharide) at a significantly greater rate than Sugar Y (a disaccharide). The next step is to hypothesize *why*--on a proximal, cellular/molecular level--this might be the case. One possible reason might be:

"There is a difference in the rate of fermentation between Sugar X and Sugar Y because yeast cannot break the glycosidic bond between Sugar Y's two monomer components."

(Remember: You should not stop at just one possible reason/hypothesis for your results. List *all* the competing hypotheses that could explain your observations. Competing hypotheses not only reduce bias in your experiments, but also prepare you for the next experimental step.)

Next, re-state the hypothesis in the form of mutually exclusive statements:

If it is the glycosidic bond alone that prevents yeast from fermenting Sugar Y as efficiently as it ferments Sugar X, then we predict that yeast will ferment Sugar W—a different disaccharide has the same type of glycosidic bonds as Sugar Y—at the same rate as Sugar Y.

If it is NOT the glycosidic bond alone that prevents yeast from fermenting Sugar Y as efficiently as it ferments Sugar X, then we predict that yeast will ferment Sugar W—a different disaccharide has the same type of glycosidic bonds as Sugar Y—at a different rate than Sugar Y.

Notice that with this careful wording, you cannot fail to be able to choose between these competing hypotheses once you have experimental results. Since your prediction is that the glycosidic bond is the limiting factor, you should predict that if you allow yeast to ferment Sugar W, it will be as ineffective at doing so as it is at fermenting Sugar Y.

Note that even here you must be cautious. Be sure to account for any other possible differences between Sugar Y and Sugar W that could confound your results.

In your presentation, you should present as many competing hypotheses as possible to explain your observations. Propose experiments to test these hypotheses, even if it is only to say, “Subjecting the yeast to a reagent that does XXX (e.g., opens a particular membrane channel) should allow us to determine whether this feature (the membrane channel’s permeability) is part of this complex system.” You get the idea.

Your Team’s Question and Hypotheses

Now that you have educated yourself about the various sugars available and also know a great deal about how yeast metabolizes sugars, your team should be ready to address an aspect of this system experimentally. Proceed with care, rigorous thinking, and as much background information about your system as possible, and you will succeed.

What is your observation/problem?

What is your hypothesis?

If you plan to use statistical methods (e.g., comparing rates of fermentation of two different sugars), then what are your null and alternative hypotheses for the particular experimental protocol you will be using?

H₀: _____

H_a: _____

rationale for H_a: _____

III. Equipment, Reagents, and Instructions

Rather than have you re-invent the wheel here in lab, we will teach you how to do certain experimental procedures by providing you with equipment and explaining its most effective use. However, you will be expected to decide how to use the equipment, mix your own reagents, and understand why you are manipulating the variable you have chosen.

The materials available to your team include:

- Different types of sugars (glucose, sucrose, fructose, lactose, galactose, maltose)
- aqueous suspension of live yeast
- four fermentation tubes in holding rack
- measuring equipment (graduated cylinders, syringes, beakers, etc.)
- thermometers

You will use **fermentation tubes** (Figure 1) to measure the rate of CO₂ generated by yeast as they ferment a nutrient source (sugar). Because our tubes are hand-blown and unmarked, you must calibrate them before you begin your experiment.

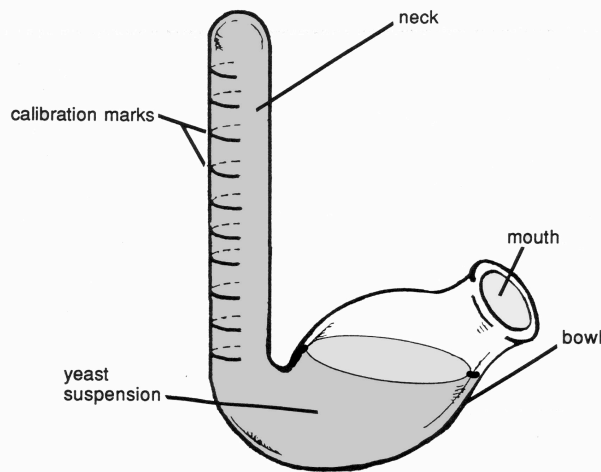


Figure 1. A fermentation tube.

A. Fermentation tube calibration

1. With a syringe, introduce 0.5cc of water into the tube
2. Tip the tube upside down (counterclockwise if you are holding the tube as it appears in Figure 1) so the water enters the neck of the tube
3. Observe the **meniscus** formed by the water. This is the curve formed by the upper surface of a liquid adhering to the surface of its container (Figure 2)
4. With a wax pencil (China marker), make a straight mark at the center of the meniscus. This will represent 0.5cc of volume in your tube.
5. Add more water, 0.5cc at a time, marking each meniscus in turn. Mark as far down the neck of the tube as possible.
6. Once you have finished calibrating your tube, replace it in the holding rack and repeat this process for all of your tubes.
7. You are now ready to perform your experimental fermentation runs.

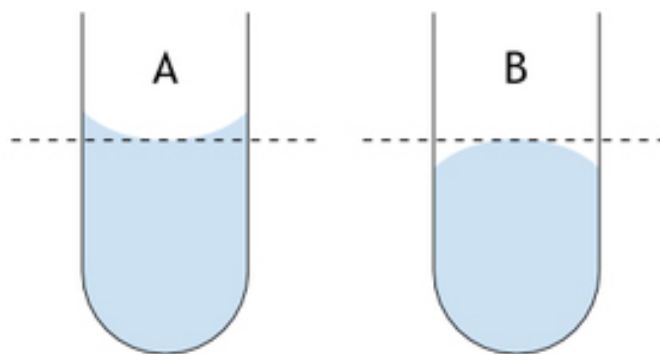


Figure 2. Concave (A) and convex menisci. The dotted line shows the appropriate level to use for measurement. Holding the vessel straight and at eye level, mark your tube at the bottom of the concave meniscus formed by the water.

B. Mixing Sugar Solutions

Available at the back table are several different types of sugars (Figure 3). The **molecular weight** of each sugar is listed on its container. As you may already know, the molecular weight is the mass, in grams, of 6.02×10^{23} molecules (this number is referred to as one **mole**) of any substance. Using this information, you should be able to calculate how many grams you will need to dissolve into pure water (also available at the back table) to obtain a solution of known **molarity** (moles per liter, or **M**). For example, if you know that the molecular weight of glucose is 180.2 g/mole, then you would dissolve 180.2 grams of glucose into one Liter (1000mL) of pure water to obtain a 1.0 M solution of glucose. To make a higher or lower concentration solution, adjust your quantities accordingly.

By now, your team should already know which sugars or other substances you will be using. We'll give you the hint that a 0.5M solution of most of these sugars will work well for your experiments, but you are free to vary the concentration of your sugars if you can defend why this is a relevant thing to do (and as long as you are careful to restrict your experimentation to only one variable at a time, of course!). Obtain the sugars your team will be using, and mix the appropriate solutions.

WE SHOULD NOT NEED TO TELL YOU THAT SUGARS CAN BE STICKY AND MESSY. KEEP YOUR LAB STATION CLEAN, AND BE SURE TO WIPE UP ALL LIQUID AND SOLID SPILLS! YOUR LAB INSTRUCTOR WILL CHECK YOUR STATION AND THE BACK TABLE FOR NEATNESS BEFORE YOU LEAVE, AND WILL DOCK POINTS IF THE LAB IS LEFT IN AN ANT-ATTRACTING MESS!

C. Yeast Suspension

Because your yeast needed to be metabolizing their stored carbohydrates for a while before you use them, the yeast suspension has been pre-mixed. The stock suspension contains 500g of live yeast/L of solution (0.05M pH 7.0 phosphate buffer).

The fermentation tubes we provide will nicely accommodate 2.0 ml of yeast suspension mixed with 10 ml of sugar solution for a yeast feast that will last about two hours. Your team will decide how long to run each experiment, but remember that all you need are enough data points to determine the rate of reaction. (Two hours is probably far longer than you need to collect data for any single experimental run.)

Once you have set up your experimental vessels, it may take about 30 minutes before you observe any generation of CO₂. Take this time to discuss possible outcomes with your teammates.

Keep an eye on your fermentation vessels. Once the process begins, it can proceed quite rapidly.

The buffer-suspended yeast have been incubated aerobically so that they will use up all their carbohydrate reserves. Why do you think this was necessary? _____

What is the difference between a **solution** and a **suspension**? Write it here:

Solution: _____

Suspension: _____

Remember that to compare mean rates, you must run more than one experiment with the same variable. You may do this by running four tubes, each with a different sugar, or by running the same experiment twice or more, and measuring gas generation for a shorter time in each experiments. We will leave it to individual teams to decide which is the more logical method.

1. List the materials your group will use

2. Briefly describe your experimental set-up and procedure. (Remember: you need not give a detailed explanation of equipment here, unless it is unique to your experiment and really requires an explanation for the educated reader to understand what you will be doing. Simply refer to the lab manual as a literature cited for equipment specifics.)

3. Do you think it is sufficient to run only one experimental run with each variable? Or should you run multiple trials? Explain your answer:

4. How many times will you replicate your experiment for each variable?

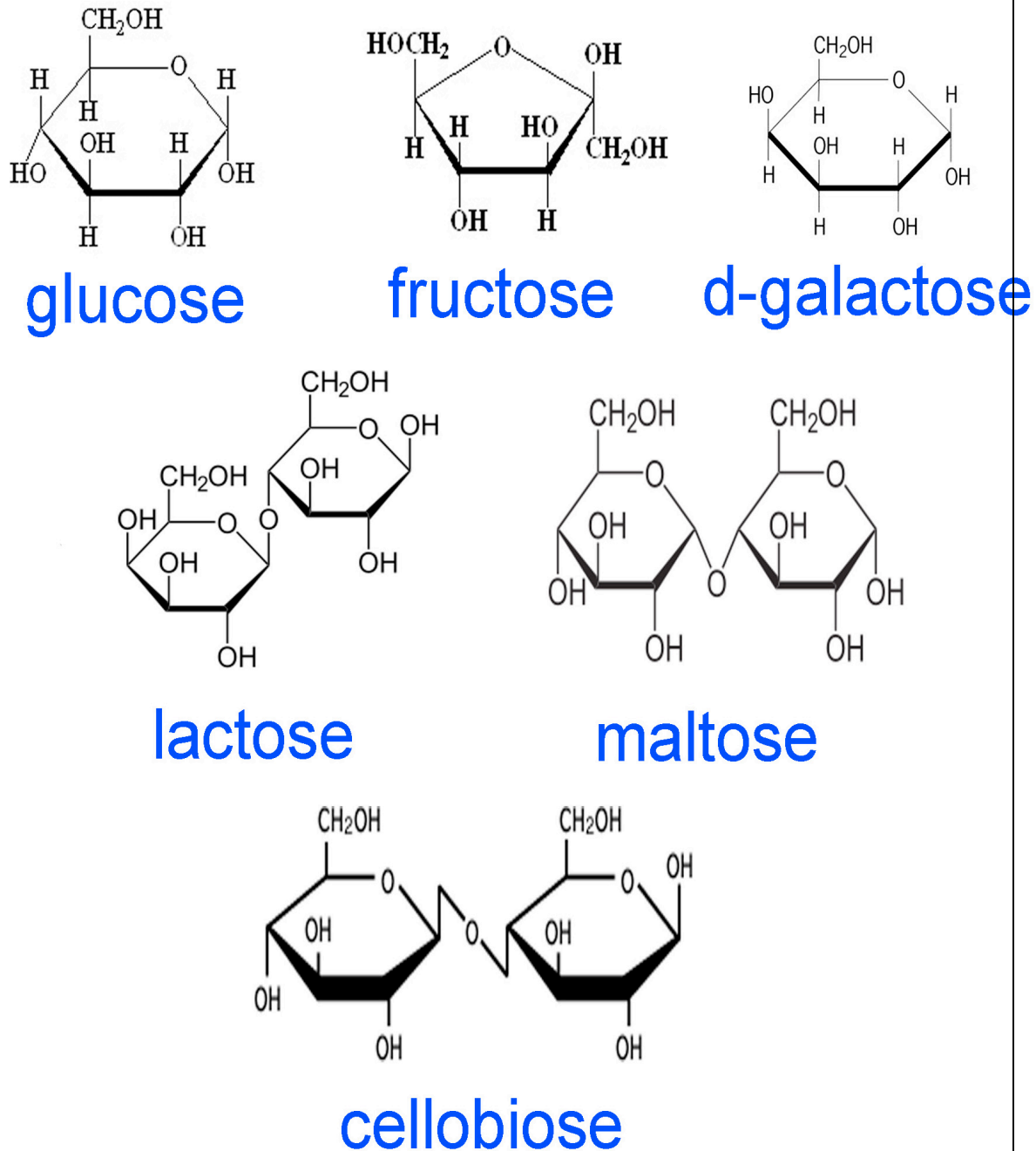


Figure 3. Molecular structures of selected monosaccharides and disaccharides that may be nutrients for yeast. Pictured are from top to bottom are the monosaccharides glucose, fructose, galactose and the disaccharides sucrose, lactose, maltose, and cellobiose. The first six will be definitely available for you in the lab.

If you plan to run multiple trials at each value of your variables—a wise choice—then you must calculate the **mean rate of reaction** of the runs. (For example, if you ran your experiment three times with a particular sugar, then you must calculate the mean

2. How will you analyze your data?

B. Predicting Outcomes

In the spaces below, list all possible outcomes of your experimental trials. (As before, we have given you more spaces than you might need for this.) Each outcome should be accompanied by an explanation of how it supports or refutes each hypothesis and its companion predictions. Remember that the result is not the same as the interpretation. The outcome is the summary of the results themselves, whereas the interpretation is the logical, reasoned *explanation* for the observed results. The explanation of your outcome can serve as the basis for the next level of hypotheses.

Outcome 1:

Explanation:

Outcome 2:

Explanation:

Outcome 3:

Explanation:

Outcome 4:

Explanation:

C. Data Collection

You may use the tables provided below to record your experimental results, or create your own if this one is not appropriate for your experiment. In either case, be sure to provide an appropriate legend.

Table _____

time (minutes)	tube 1 (cc CO ₂)	tube 2 (cc CO ₂)	tube 3 (cc CO ₂)	tube 4 (cc CO ₂)
zero				
10				
20				
30				
40				
50				
60				
70				
80				
90				

Table _____

time (minutes)	tube 1 (cc CO ₂)	tube 2 (cc CO ₂)	tube 3 (cc CO ₂)	tube 4 (cc CO ₂)
zero				
10				
20				
30				
40				
50				
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90				

Table _____

time (minutes)	tube 1 (cc CO ₂)	tube 2 (cc CO ₂)	tube 3 (cc CO ₂)	tube 4 (cc CO ₂)
zero				
10				
20				
30				
40				
50				
60				
70				
80				
90				

D. Data analysis

1. When you have completed the experiments, plot the raw data for each experimental run, and calculate reaction rate.
2. Calculate the mean reaction rate for each variable or treatment/control groups.
3. Create an appropriate figure, comparing the reaction rates for each of your experimental groups. Be sure to label your axes correctly, and provide an appropriate legend. If you don't remember how, then review Appendix 2.)

When discussing your results, consider the following. You may wish to integrate them into your presentation, or some of your ideas as a springboard to inspire the next level of investigation.

1. To what degree might the yeast cell membrane permeable to each of these sugars?
2. Even if a sugar passes through the cell membrane, it may not be fermented. Why might this be? HINT: Consider that glycolysis is mediated by many enzymes. Also consider the evolutionary history of *Saccharomyces cerevisiae*, a fungus that has evolved to obtain its nutrients from plant sugars. Where is each of the sugars you used found in nature?
3. Was CO₂ the only component of the gas produced in your experiment? What else might be there? How might this affect your analysis?
4. In using the fermentation tube, did you quantify all the CO₂ being generated? Does this affect your data analysis? Why or why not?
5. Can you state with confidence that the fermentation rates are significantly different? Explain.

IV. Your Team Presentation

Before you leave lab today, make sure every team member has a copy of the raw data and any analysis your team has done together, either on a laptop or flash drive. You should also arrange times and places to meet together during the next week so that you can finish analyzing your data and put together a PowerPoint presentation on your experiment. **DO THIS BEFORE YOU LEAVE LAB** so there will be no confusion and no lost souls unable to contact the other team members.

Instructions for creating an effective presentation can be found linked to your laboratory syllabus. Be sure to follow those instructions carefully, because—as always—you and your team will be graded not only on your results and interpretation, but also on the effectiveness with which you present your findings to your colleagues.

Rendering your data into clear, analyzable form is only the beginning of a scientific presentation or report. As before, remember that as long as you have performed your experiment with care and rigor, there are no "right" or "wrong" results—only valid and invalid explanations for the observations. The most important part of this lab is to explain your results logically, even if they are not what you expected. Think about your results and analyze them intelligently.

As before, you must go further than simply explaining the results of today's experiments. The next step is interpreting your results and posing the next question raised by your observations. In this stepwise fashion, scientists learn how and why a given system works.

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