

Using Yeast Fermentation to Suggest and Then Challenge a Model

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Abstract: In the simplest version of this activity, students inoculate a yeast suspension into glucose solutions of several concentrations. The glucose disappears, and the amount of CO₂ produced is directly proportional to the initial amount of sugar in solution. This suggests a simple model of yeast fermentation. To challenge this model, students can perform parallel experiments in which the glucose concentration remains high but the *yeast* concentration is varied. Below approximately 25% of the standard yeast concentration, gas production appears to cease even though glucose disappears. The students then use data from past experiments to evaluate competing explanations for this phenomenon.

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Introduction for the Instructor

Overview

This laboratory contains two exercises: the effect of glucose concentration on the volume of CO₂ evolved by a “standard” yeast (*Saccharomyces cerevisiae*) concentration, and the effect of yeast concentration on the CO₂ evolved from a “standard” glucose concentration. Consequently, it could be used in two ways.

If a short, simple exercise in a laboratory with other respiration exercises is desired, then the students would only explore the effect of glucose concentration on gas evolution. In order to go this far, the students should perform steps 1-7 and then 12-17 in the Student Outline. This will allow them to observe fermentation and also use the amount of gas evolved as a simple, linear bioassay for the glucose concentration in an unknown. The “model” of CO₂ evolution in this case is that CO₂ produced is proportional to the glucose, so some constant fraction of the glucose in the mixture is released as CO₂. Glucose test strips will disclose that in the course of the exercise, all the glucose is used up.

If a more complex laboratory is desired, the students could also perform the experiment on the effect of yeast concentration. All yeast cultures here use the same glucose concentration. While the model above might make us think that a lower yeast concentration will produce the same amount of CO₂ as a higher one, albeit more slowly, this is not true. The lower the yeast concentration, the smaller the visible gas bubble is. Even after hours, the lowest yeast concentrations produce little or no visible CO₂ gas, even though the amount of glucose available is the same and the glucose does disappear from the culture. The exercise explores six possible explanations (discussed in detail in the Student Outline and Notes to the Instructor) and shows the students data from experiments attempting to test each explanation. The explanation favored by the authors is the rate hypothesis: CO₂ can only appear in a bubble if it is produced in a local area faster than it dissolves in the culture. Therefore, the faster the gas is evolved, the greater the percentage of it that appears in the gas phase. If the number of yeast is below a threshold, the gas production rate is less than the gas dissolution rate. Raising the culture temperature causes a large increase in the gas produced, even from less-concentrated yeast cultures, because it increases the rate at which CO₂ is produced. A more minor contributing factor may be the Pasteur effect, the fact that becoming anaerobic causes a substantial increase in the rate of glucose metabolism by a culture. Other hypotheses lack support from the experiments performed.

These observations are meant to challenge the simple glucose → CO₂ model of the first exercise by showing that while that outcome is real, it is only true for relatively high yeast concentrations that produce CO₂ quickly enough to force gas out of solution and into the gas phase. The exercise will teach students that they must look critically at models and understand the physical basis of the phenomena (such as gas production) they are examining.

Time Requirements

If all that is desired is the first exercise on gas production as a function of glucose concentration, the exercise keeps each student pair busy for only about 15 minutes at the beginning and 5 minutes at the end, with an additional 10 minutes for plotting of class data and discussion. The incubation period in between is 60-90 minutes. Therefore, the exercise should be incorporated into a larger laboratory on respiration and started early in the laboratory period. On the other hand, if the instructor desires to explore alternative explanations for the failure of gas production in the lower yeast concentrations, explanations of each of the hypotheses and discussion of the data given in the student outline could take about 45 minutes in addition to the times mentioned above. This discussion will be challenging, and

should probably not be attempted by freshman students. The senior author confessed during his presentation at the 2009 ABLE meeting, “This exercise is more like research than we ever wanted it to be.” However, the critical evaluation of competing hypotheses using data is the basic process of science, and is not fast and easy.

While the data presented here indicates that the rate hypothesis seems to be the best explanation for the yeast concentration effect, the rate hypothesis makes some false predictions too, and a better hypothesis is always possible. Probably the highest use of this laboratory will occur when students are able to use the data presented here as an inspiration for further fermentation experiments as part of student projects.

Student Outline

Background

Fermentation in Yeast. Oxygen is used as the final electron acceptor in aerobic respiration. That is, electrons and hydrogen ions from NADH and FADH₂ travel down the electron transport chain and then combine with oxygen to form water. Therefore, water is reduced oxygen. Because oxygen is very electronegative and holds its electrons tightly, the electrons release most of their energy as they merge with oxygen. This relatively complete energy release means that when oxygen is present, a maximum amount of ATP can be made from each glucose molecule. Another consequence of aerobic respiration is that when NADH and FADH₂ release their electrons to the electron transport chain, NAD⁺ and FAD are regenerated and can participate in glycolysis and the Krebs cycle again. One might express this as Eq. 1 for NADH:



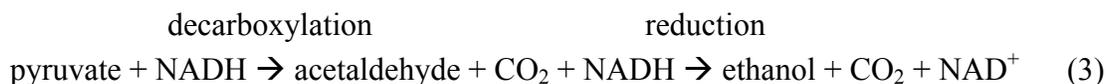
Note that NADH is oxidized in this reaction (it loses electrons and H⁺ ions) and oxygen is reduced.

The situation is different if oxygen is not present. Without oxygen to serve as a final electron acceptor, the cell cannot make any of the ATP made by the Krebs cycle and electron transport, and it must subsist on a net yield of only 2 ATPs per glucose (from glycolysis) rather than 38 ATPs in the case of aerobic respiration. Also, NADH cannot give up its electrons and hydrogen ion to regenerate NAD⁺. This seemingly minor problem could rapidly cripple the cell because the reactions of glycolysis and the Krebs cycle require NAD⁺ (*not* NADH). NADH is useless to glycolysis and the Krebs cycle.

Fermentation is a solution to the problem of regenerating NAD⁺ without the presence of oxygen. Eq. 1 shows that NADH can only be transformed back into NAD⁺ if it gives up its electrons and hydrogen to some electron acceptor. In fermentation, this electron acceptor is an organic molecule (Or) that becomes more reduced:



In human cells, “Or” in the equation above is pyruvate, and “HOr” is lactate. In yeast cells, fermentation is slightly more complicated. Pyruvate loses its carboxyl group to become acetaldehyde (decarboxylation of pyruvate). This carboxyl group is released as CO₂. Then acetaldehyde acts as the electron acceptor and is reduced to ethanol. Therefore, alcoholic fermentation produces ethanol and CO₂ as its two main products.



The Pasteur Effect. It is much more advantageous for yeast cells to use aerobic respiration rather than fermentation. One reason is that fermentation produces ethyl alcohol, which will poison the yeast if its concentration gets over about 10%. The other reason is that aerobic respiration produces 38 ATPs per glucose molecule, and fermentation only produces 2 ATPs. In 1861, Louis Pasteur observed that the presence of oxygen will stop fermentation, and that the rate of consumption of glucose *increases* dramatically when oxygen disappears. Both of these make sense in light of the facts above. It is advantageous for yeast to use oxygen if it is available, so there is strong selection pressure to turn off fermentation and respire aerobically if possible. If oxygen is *not* available, yeast have to use up much more sugar to get the same amount of ATP and maintain their growth. Therefore, cultures with low numbers of yeast are more likely to respire aerobically, produce CO₂ less rapidly, and not deplete their glucose as fast as crowded ones.

In this laboratory, you will observe both the effect of glucose concentration and of yeast population density on the rate of production of CO_2 .

Procedure

1. You should have three fermentation vials (each consisting of a thin tube and a thicker tube) at your station. Take these apart and put one of the three smaller tubes upright on the lab bench in front of you. Also note that you have a small flask labeled either 1%, 0.75%, 0.5%, 0.25%, or 0% glucose or "Glucose Unknown."
2. Your instructor has made a fresh yeast suspensions marked "1X." Take apart your plastic fermentation chamber and put 10 mL of "1X" yeast culture into the first of these smaller tubes.
3. Almost fill the smaller tube with about 10 mL of the sugar solution in "your" glucose bottle, but leave about 2-3 mm of space. There should be a wide-mouthed beaker at your place; do your pouring over the beaker to catch spillage. Immediately after filling up the smaller tube, mix it vigorously with the stirring rod and briefly dip a glucose test strip into the solution in the tube. Move the test strip up and down several times and then remove the test strip and put it aside. Then fill up the remaining space in the tube with your sugar solution.
4. After using the glucose test strip, put the larger tube over the smaller tube and quickly invert both tubes. The bottom of the smaller tube should now be pointing upward inside the large tube so that gas released will be trapped in the top of the smaller tube:

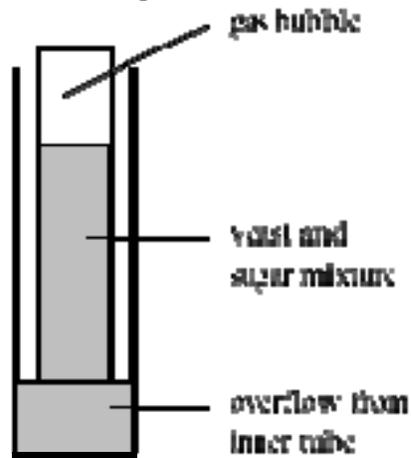


Figure 1. A working fermentation tube develops a bubble of CO_2 that forces yeast culture out into the larger, outer tube.

5. As soon as you have inverted your tube, measure the height of the gas bubble in mm. Measure from the roof of the smaller tube down to the liquid surface (*not* the surface of the foam or the level to which the yeast have settled). You may have to jiggle the tube a little to see this liquid surface. This initial reading will be subtracted from the final reading. In most tubes, it will be 2-5 mm. If spillage makes this measurement larger than 10 mm, make the tube again. Record this initial bubble height in Table 1. Also record the percent glucose you are using under the table.

Table 1. Glucose concentrations and bubble heights in the glucose concentration experiment.

	Approx. Glucose (mg/dL)	Bubble Height (mm)
Initial		
Final		
Change		

% Glucose Used _____

6. It takes two minutes for the full colors to develop on the glucose test strip, so the color should be developed by now. Record the initial glucose in Table 1.
7. While it is possible to measure the heights of the gas bubbles periodically, the final measurement (60-90 minutes after the start) is what matters. Therefore, you can ignore the tube and do other activities for at least 60 minutes. Put your tube somewhere where it won't be knocked over! Placing it in a 150 or 100 mL beaker would be a good idea to prevent it from toppling and spilling.
8. Now place the other two small tubes upright on the bench in front of you. Using the glucose concentration that was at your spot, use Table 2 to determine the amounts of "2X" yeast suspension and distilled water in your two tubes. *Don't* put any glucose in the tubes yet.

Table 2. Amounts of 2X yeast and distilled water to be placed in the two remaining tubes.

Glucose Flask	1%	0.75%	0.50%	0.25%	0%	Unknown
2X Yeast	10 mL	7.5 mL	5 mL	2.5 mL	1 mL	0.5 mL
DH ₂ O	0 mL	2.5 mL	5 mL	7.5 mL	9 mL	9.5 mL

Therefore, if you have 0.25% glucose at your position, you will put 2.5 mL of 2X yeast suspension and 7.5 mL of distilled water in both of your remaining tubes. If you had the "unknown" glucose bottle, you'll put in 0.5 mL of 2X yeast suspension and 9.5 mL of distilled water in both tubes.

9. Now one of the two tubes should be "topped off" with 1% glucose solution. Use the same procedure you used in steps 3-6. Work over the large beaker to contain spills. Note that all students will use 1% glucose, regardless of the glucose flask at your location. This 1% glucose will be in a larger flask on each bench. Almost fill up the tube, stir with the stirring rod, and top off again with the 1% glucose solution. Use the glucose test strip to estimate the glucose concentration.
10. The other tube should be topped off with distilled water (that is, it should have no glucose). Follow the directions in steps 3-6 again. Again, don't forget to mix and measure the initial glucose concentration. Write your initial bubble height and glucose in Table 3 for both the tube with 1% glucose and the tube with distilled water:

Table 3. Glucose concentrations and bubble heights in the yeast concentration experiment.

	Approx. Glucose (mg/dL) Glucose Tube	Bubble Height (mm) Glucose Tube	Approx. Glucose (mg/dL) DH ₂ O Tube	Bubble Height (mm) DH ₂ O Tube
Initial				
Final				
Change				

mL of 2X Yeast Used _____

- Now you will leave your two tubes undisturbed for 60-90 minutes. Again, put them out of the way so they don't get knocked over. A small beaker makes a fairly safe place for a single fermentation tube.
- The experiment on glucose concentration will probably end first. When your instructor tells you to terminate the experiment, measure the height of the bubble in your tube. Measure from the roof of the tube to the *surface of the liquid* (not the foam or to the settled yeast cells—see Figure 2).

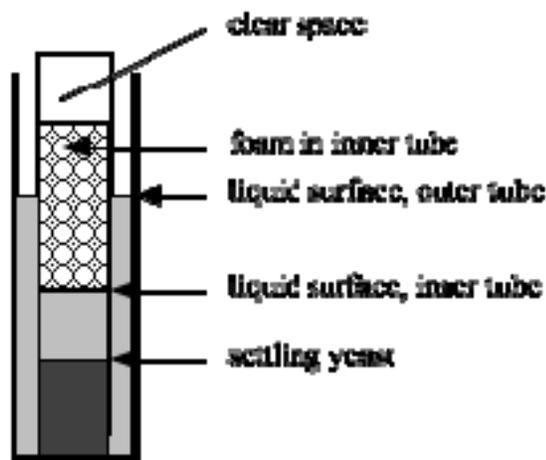


Figure 2. After fermenting for a period of time, the tube might contain a complex series of layers. The gas bubble is measured from the roof of the container to the *liquid surface in the inner tube*.

You may have to press the inner tube against the wall of the outer tube so you can see the liquid level in the inner tube. Jiggle the tube to see where the liquid surface is. Record this measurement in Table 1. Subtract the initial bubble height, and write down the change in bubble height in Table 1.

- Once the bubble measurements have been made, withdraw the smaller tube from the larger one, mix the contents with the stirring rod, and dip a new glucose test strip into the solution. Record the final glucose concentration in Table 1 also.
- Report the *change* in bubble height to your instructor. Your instructor will compile these results. Write the class results in the table below.

Table 4. Class results for the glucose concentration experiment. Two cells are provided to write down two observations for each glucose concentration.

Glucose Conc.	Bubble Change		Final Glucose Concentration	
0%				
0.25%				
0.50%				
0.75%				
1.00%				
Unknown				

15. Plot the results (except for the unknown) on the graph on the next page. Is there a predictable relationship between gas production and glucose concentration?

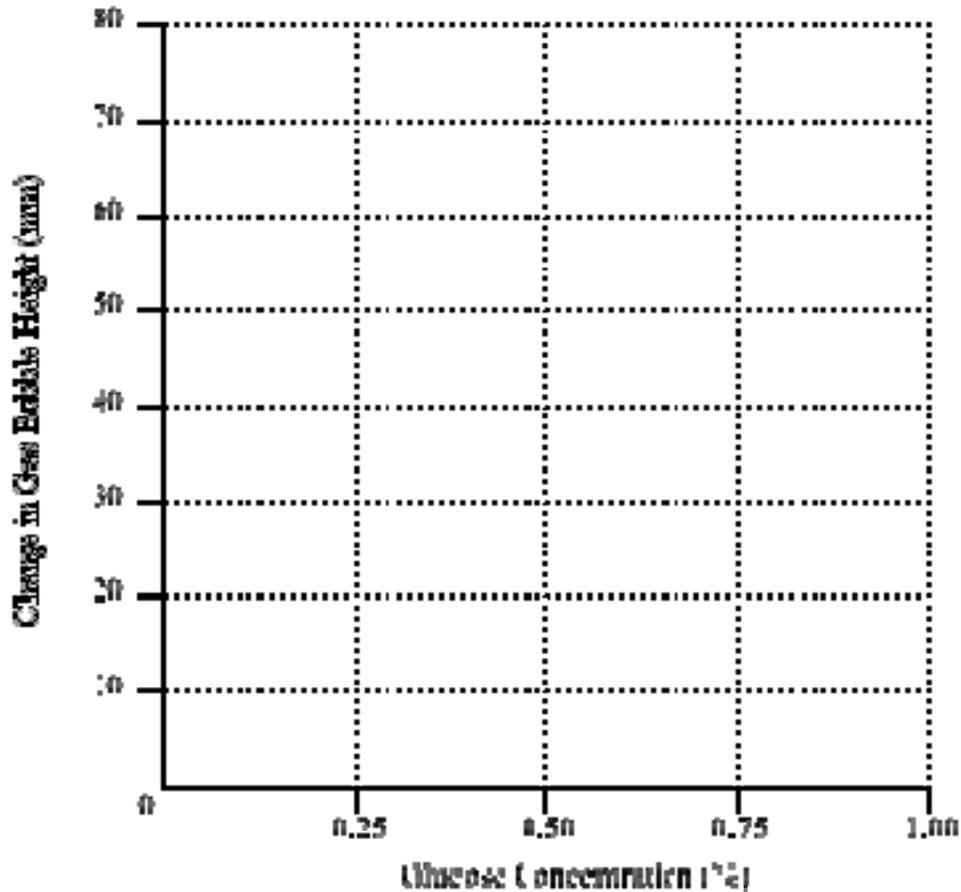


Figure 3. Gas production by yeast in different concentrations of glucose.

16. Use the data to estimate the glucose concentration in the unknown. After the class estimates the glucose concentration, your instructor will reveal the correct concentration.
17. How would you explain the relationship between the volume of gas evolved and initial glucose in the solution?
18. Now turn to the yeast concentration experiment. Using the techniques in steps 12, 13, and 14, fill in Table 3 with the final glucose concentrations and bubble heights in the tube with 1% glucose and the tube with distilled water. Compute the changes in bubble height. Report these to your instructor. When your instructor reports the class results, fill out the following table for the glucose-containing cultures only.

Table 5. Class results in the yeast concentration experiment for tubes using 1% glucose. Two cells are provided to write down two observations for each yeast concentration.

mL of 2X Yeast	Change in Bubble Height		Final Glucose Concentration	
0.5				
1.0				
2.5				
5.0				
7.5				
10.0				

19. Plot the bubble change vs. the mL of yeast used on the graph on the next page.

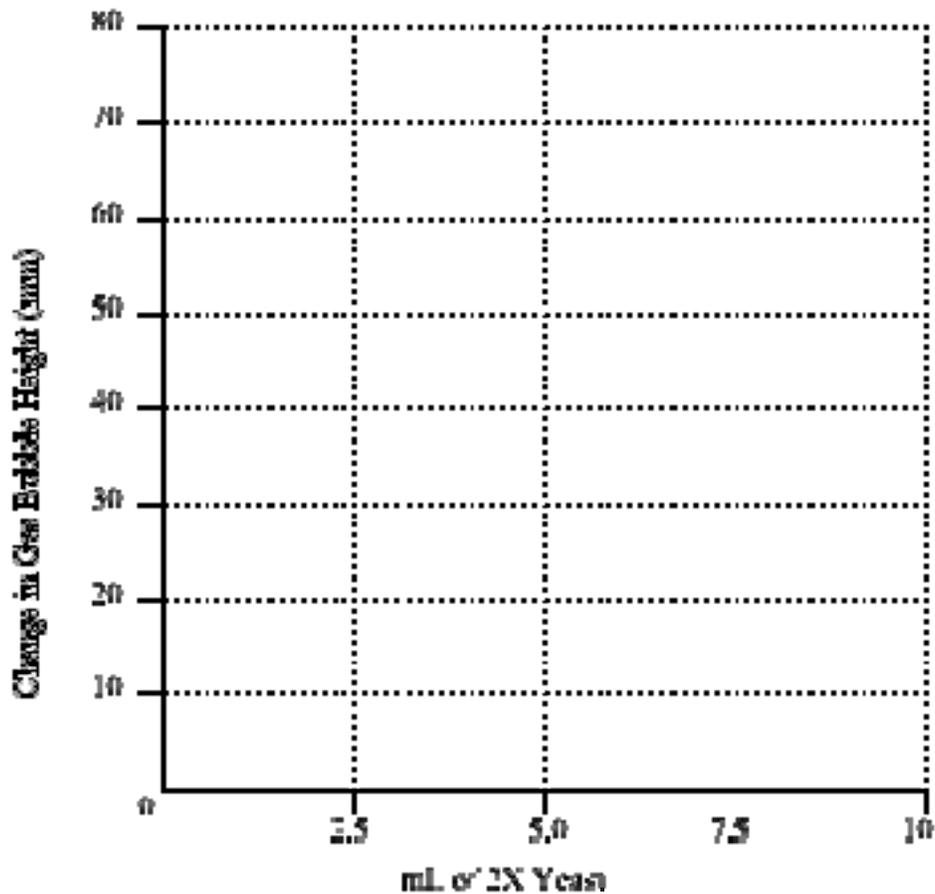


Figure 4. Gas production by different yeast concentrations in 1% glucose.

20. In the glucose concentration experiment, it seemed that a certain mass of glucose gave rise to a proportional volume of CO_2 . Now consider the graph above, in which all yeast concentrations started with the same mass of glucose. If a certain mass of glucose is turned into a certain volume of CO_2 , and all tubes had the same quantity of glucose, what pattern would you expect on the graph above?
21. Science is a comparison between observed results and the results expected if some hypothesis is true. If the observed and expected results are not consistent, we must modify our hypotheses. Some possible explanations for the lack of gas production in less concentrated yeast cultures are given below, along with experimental results testing the hypotheses. Consider the data, and then decide the best explanation for the results in Figure 4. In all of these explanations, “1X yeast” means one packet of yeast per 50 mL of water. This is a standard yeast concentration for fermentation experiments of this kind.
- a) **Hypothesis: We ended the experiment too soon.** Fewer yeast will produce gas and consume glucose more slowly than a dense yeast population. Since this is the simplest explanation, it should be considered first. Maybe our incubation was too short, so only the dense yeast populations had a chance to produce their full amount of CO_2 . Consider the data in Figure 5, which was obtained from an experiment like the one you did, except for the fact that it was allowed to run almost 9 hours:

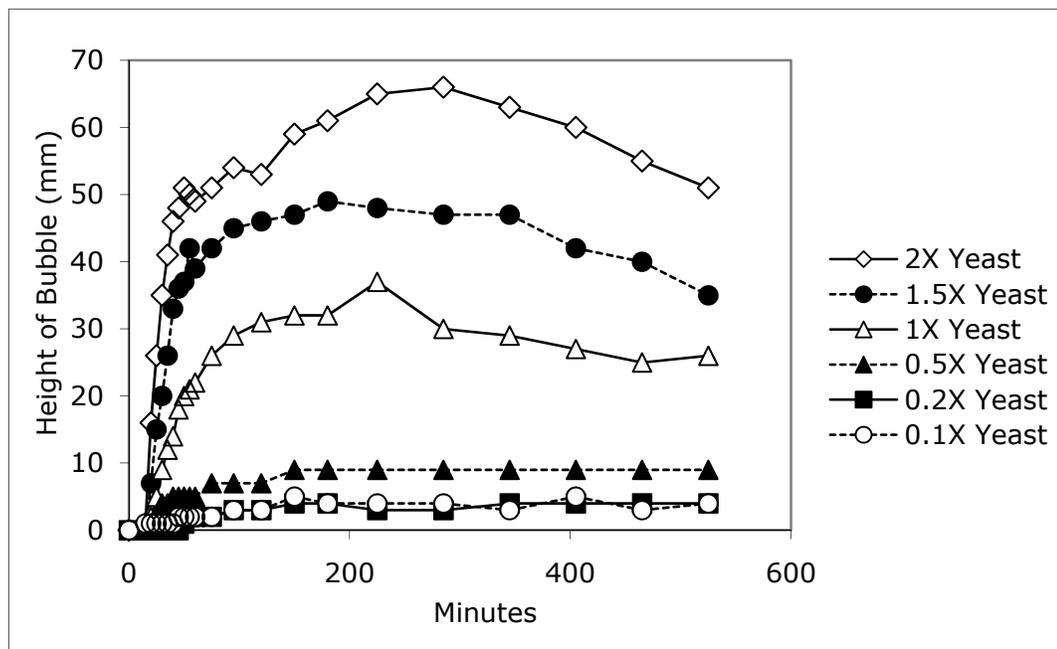


Figure 5. Gas production in an experiment using 1% glucose and varying yeast concentrations. 1X yeast = the “standard” yeast culture, with 1 pack of yeast per 50 mL of water.

Does it seem from these results that lower yeast concentrations would “catch up” to the heavier yeast concentrations if they had had more time?

- b) **Hypothesis: The Pasteur Effect.** Yeast that is very crowded uses up the oxygen, metabolizes anaerobically, and uses glucose 5-7 times faster than yeast that is still aerobic. Also, the presence of oxygen in the culture will inhibit this fast anaerobic metabolism. Therefore, if the lowest yeast populations are aerobic, they are producing gas much more slowly than their numbers alone would indicate.

Less concentrated yeast cultures use up their glucose more slowly than concentrated ones. Figure 6 shows typical results using 1% glucose and yeast cultures ranging from 200% of the standard yeast concentration (2X) down to 25% of the standard (0.25X). The glucose disappeared at 40 minutes in the 2X concentration, and at 60 minutes in the 1X concentration. If the rate of glucose use was the same in all cultures, we would expect that it would disappear at 120 minutes in the 0.5X culture and at 240 minutes in the 0.25X culture. However, the glucose took much longer than predicted to disappear in these cultures. This supports the assertion that individual cells in more concentrated cultures use glucose more rapidly.

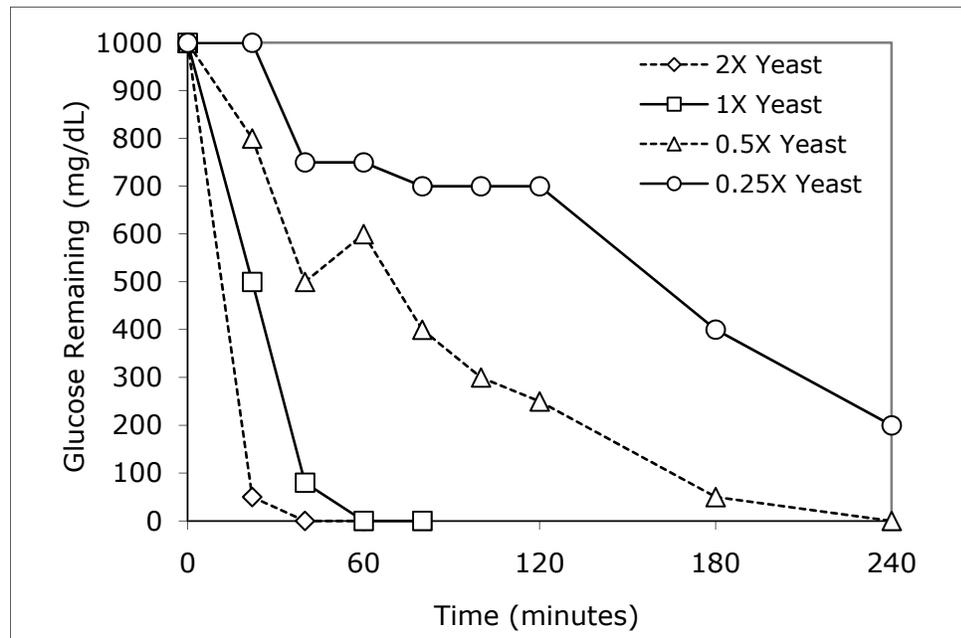


Figure 6. Decline of glucose in cultures with 1% glucose and four different yeast concentrations. “1X” = one packet of yeast per 50 mL water.

Another experiment addressing this question used “balanced” cultures, in which 2X yeast was given 2% glucose, 1X yeast had 1% glucose, and 0.5X yeast had 0.5% glucose. Note that the ratio of yeast to glucose is the same in each case. If each yeast cell were using glucose at the same rate, we would expect that all cultures would exhaust their glucose at the same time. Plotting the percent of initial glucose remaining, we find:

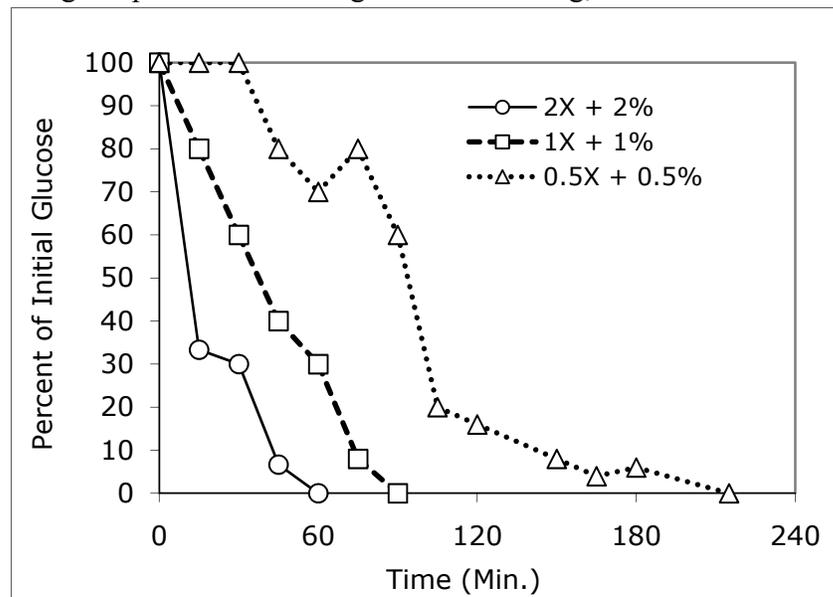


Figure 7. Percent of initial glucose remaining in a culture with 2X yeast and 2% glucose, 1X yeast and 1% glucose, and 0.5X yeast and 0.5% glucose.

It is clear that the yeast in the dilute cultures use glucose more slowly *per capita*. This is consistent with the Pasteur effect.

However, the Pasteur effect explanation depends on the assumption that more concentrated yeast cultures are anaerobic and less concentrated ones are aerobic. This is not true. All the yeast concentrations mentioned here become anaerobic within a few minutes. Yeast cultures down to 0.025X (far less concentrated than the ones used here) lose their oxygen within about 30 minutes. This casts doubt on the idea that concentrated cultures are respiring anaerobically but less concentrated ones are respiring aerobically.

- c) **Hypothesis: Lack of micronutrients and growth factors in dilute yeast cultures.** Packaged yeast contains micronutrients, vitamins, and other growth factors that help the yeast to grow. When we dilute the yeast, we also dilute the growth factors. Yeast without sufficient growth factors might not be able to respire as well. We can test this hypothesis by adding yeast extract to the cultures. Yeast extract is a powder derived from lysed, dried yeast cells. If we dilute the yeast with yeast extract solution rather than distilled water, the concentration of growth factors should remain high in all yeast concentrations. An experiment comparing yeast diluted with distilled water and yeast diluted with yeast extract solution is shown in Figure 8:

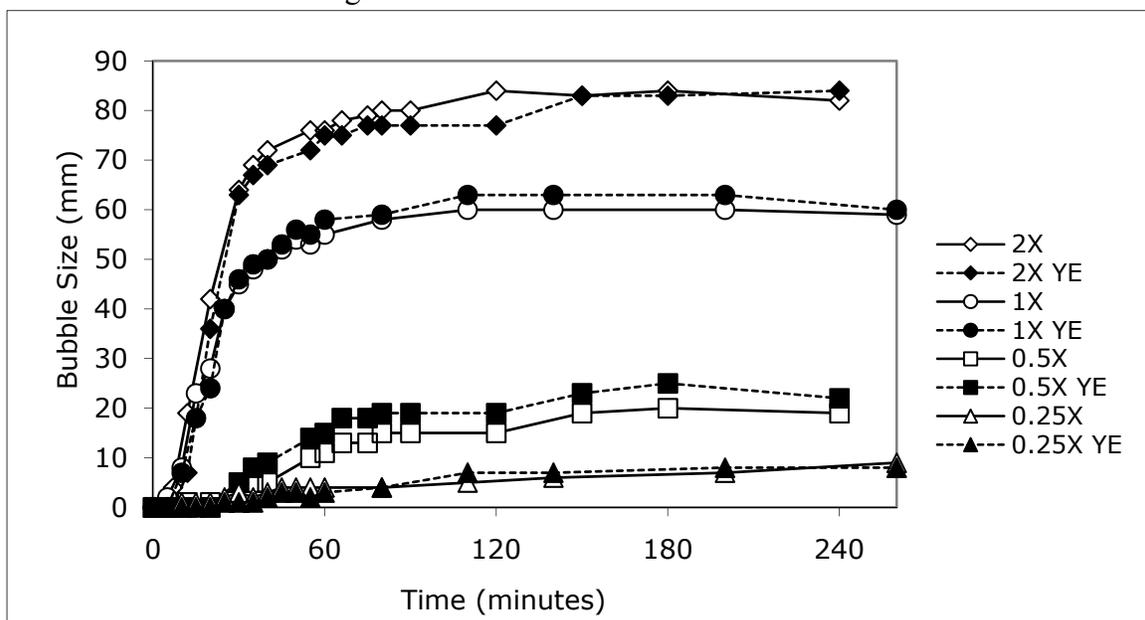


Figure 8. Gas evolution in yeast cultures with 1% glucose in which the yeast were diluted by distilled water or yeast extract (YE).

Does the addition of yeast extract seem to change the amount of gas evolved?

- d) **Hypothesis: the yeast are reproducing.** If the yeast are reproducing during the experiment, many more new yeast will be produced in the concentrated cultures than in the less concentrated cultures. These new yeast would probably have low glucose reserves and would be active in glucose uptake and respiration. Experiments were performed under standard conditions and with the addition of yeast extract as in Figure 8, and the yeast cells were counted. The results appear in Figure 9, below.

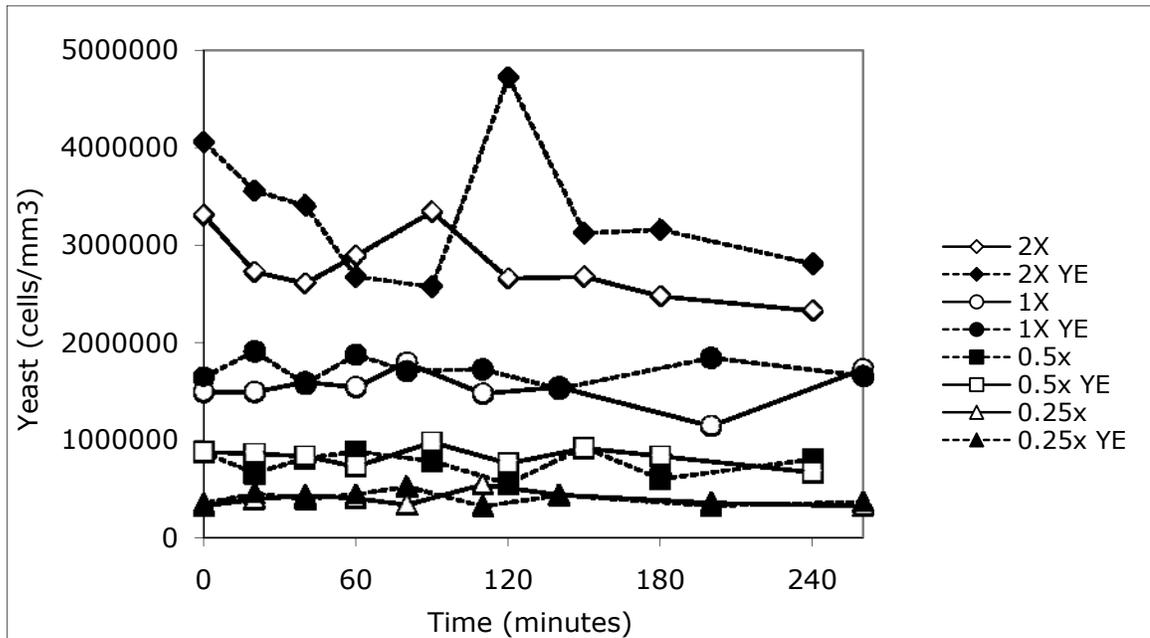


Figure 9. Numbers of yeast cells/mm³ in cultures with the yeast diluted with distilled water and with a yeast extract solution.

Are the yeast reproducing significantly during the first four hours of the experiment? The spike in “2X YE” at 120 hours is probably an erroneous count.

- e) **Hypothesis: CO₂ must “fill up” the liquid phase of the culture before it can bubble out.** A relatively large volume of CO₂ can dissolve in the liquid of the culture. For example, after 23 hours, the “2X” culture in Figure 5 had a bubble that had declined from a peak of 70 mm to only 18 mm. Perhaps CO₂ must saturate the culture before it can bubble out. This hypothesis might be presented as analogous to water in a sink:

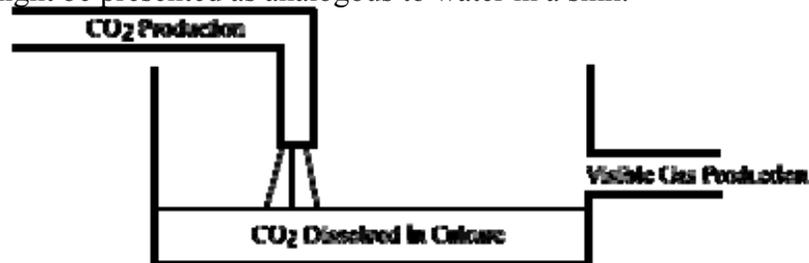


Figure 10. Water entering a closed sink must fill up the sink before it starts to flow out of the side drainpipe. Likewise, perhaps the culture liquid must saturate with CO₂ before gas can bubble out.

To test this hypothesis, both yeast cultures and 1% glucose solutions were placed in a covered flask, and the flask was then filled with CO₂ and left for an hour so the yeast suspension and glucose solution could become saturated with CO₂. Other yeast cultures and glucose solutions were left in air. Then yeast and glucose were mixed. The result is shown in Figure 11:

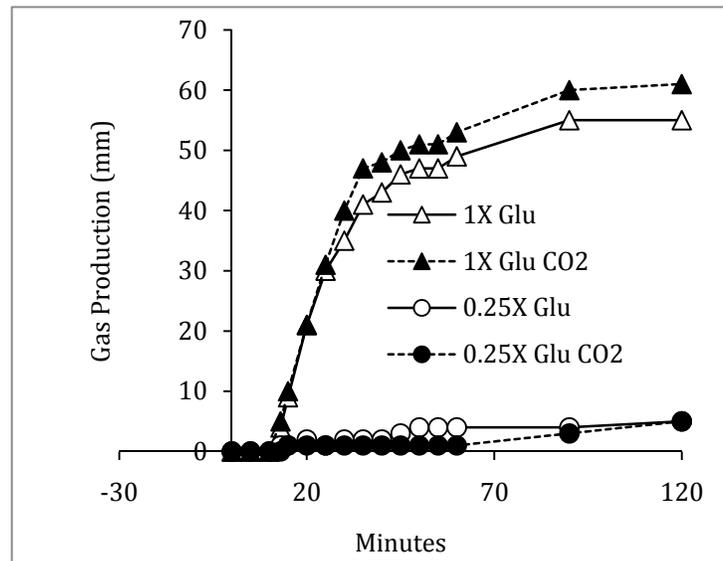


Figure 11. Gas production by 1X and 0.25X yeast cultures in 1% glucose solutions that had been left in air or in a CO₂ atmosphere for an hour before the yeast and glucose solutions were mixed.

Does it seem that saturation with CO₂ caused greater gas production or caused 0.25X yeast to have a gas production closer to that of 1X yeast?

- f) **Hypothesis: For CO₂ bubbles to form, the rate of CO₂ production must be above the rate of bubble dissolution.** As stated above, CO₂ tends to dissolve in the liquid phase of the yeast cultures, and will only enter the gas phase if its production is sufficiently rapid so that bubble formation in local areas exceeds the rate of dissolution of the bubbles. In other words, this hypothesis predicts that the 0.25X yeast concentration produces almost no bubbles because the smaller number of yeast cannot produce CO₂ fast enough to exceed the rate of bubble dissolution in the solution. This hypothesis can be visualized as a sink with a water flow into it and a very small drain in its bottom:

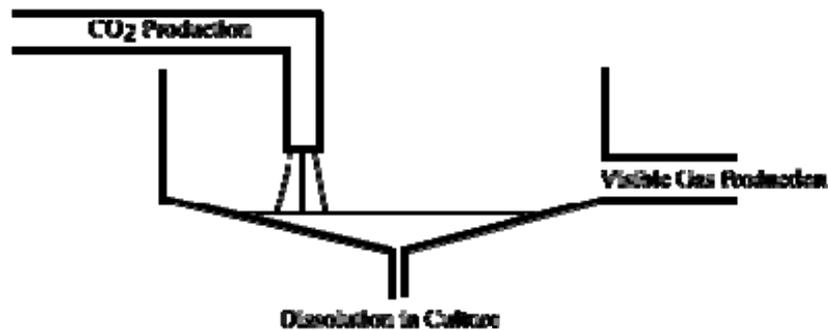


Figure 12. If the water (even a large volume of water) runs into the sink slowly, it will run down the small drain and none will be seen at the drainpipe to the side. If the water flow increases beyond the ability of the small drain to handle the flow, water will flow out of the side drainpipe. Likewise, the gas production rate must be above the rate of gas dissolution in the culture before a CO₂ bubble will form.

In this analogy, increasing the flow rate of water (even without increasing its total volume) will result in more water flowing out of the side drainpipe. Likewise, increasing the yeast rate of CO₂ production should result in more gas bubbling out of the solution. To test this rate hypothesis, the cultures were incubated at different temperatures. Figure 13 shows the dramatically different rates at which glucose disappears in cultures at 10°, 23° and 35° C.

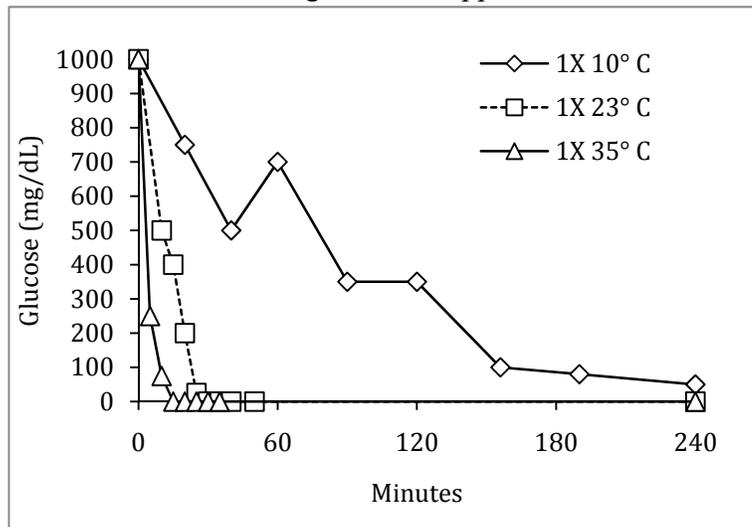


Figure 13. The disappearance of glucose from cultures using 1X yeast and 1% glucose at 10°, 23°, and 35°.

Figure 14 shows the final volumes of gas produced at three different temperatures.

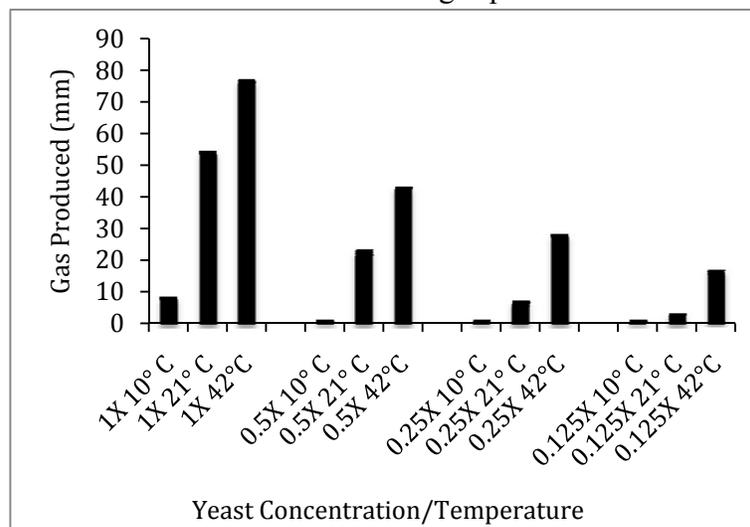


Figure 14. The volumes of gas produced at 3 hours by 1X, 0.5X, 0.25X, and 0.125X yeast cultures in 1% glucose at temperatures of 10°, 21°, and 42° C. All gas volumes have been corrected to their 21° C values.

The rate hypothesis would predict that the lower the rate of gas production, the less gas would appear in the bubble. The rate of gas production will be lowered either when the

yeast concentration is decreased (because there are fewer yeast producing CO₂) or when the temperature is lowered (because each yeast cell is producing CO₂ more slowly). Does it seem from these results that the rate of CO₂ production could explain the decline in bubble size as the yeast culture becomes less concentrated?

22. Discuss these and other alternatives with your instructor and come to a conclusion that explains the results in Figure 4.

Notes for the Instructor

Organization and Materials

Assuming a class of 24 distributed among four lab benches, the students would be organized into 12 pairs. A pair would work with one glucose concentration in the first exercise and with one yeast concentration (and a corresponding distilled water control) in the second exercise. The materials needed would be:

- 36 plastic fermentation chambers from Carolina Biological (Carolina item #202200, “Basic Fermentation BioKit,” \$53.60 in November 2009, contains 17 fermentation chambers).
- 36 100- or 150-mL beakers in which the students will place the assembled fermentation tubes (to keep the fermentation tubes from falling over)
- 4 100-strip packages of glucose test strips (Chemstrip uGK Urine Test Strips for Glucose and Ketones, \$16.99 in November 2009 for a package of 100)
- 8 packets of Fleischmann’s Active Dry Yeast, containing ¼ oz. of yeast each (available in most grocery stores). Fleischmann’s Rapid Rise Yeast probably produces similar results, but we have never used it.
- 2 small, labeled flasks containing 50 mL of distilled water
- 2 small, labeled flasks containing 50 mL of 0.25% glucose solution
- 2 small, labeled flasks containing 50 mL of 0.5% glucose solution
- 2 small, labeled flasks containing 50 mL of 0.75% glucose solution
- 2 small, labeled flasks containing 50 mL of 1% glucose solution
- 2 small, labeled flasks containing 50 mL of the “unknown” glucose solution; this should be about 0.7% glucose, or at any rate between about 0.2% and 1% glucose
- 4 larger, labeled flasks (one per lab bench), each containing 100 mL of 1% glucose solution
- 4 larger, labeled flasks (one per lab bench), each containing 100 mL of distilled water
- 4 1,000-mL beakers or other wide-mouthed vessel (one per lab bench) to contain spillage when fermentation vials are filled
- 4 100-mL beakers (labeled “Yeast”) for distributing yeast suspension to the four lab benches
- 4 widemouthed 10-mL pipets labeled “Yeast” (one per lab bench)
- 4 10-mL pipets labeled “DH₂O” (one per lab bench)
- 4 pipet pumps that fit the 10-mL pipets (one per lab bench)
- 2 250-mL beakers with volume gradations
- 12 rulers with a mm scale for measuring the size of gas bubbles
- 12 glass stirring rods
- 12 forceps for forcing the inner fermentation tubes against the outer ones when the bubble size is being measured

Distribute the flasks with the different glucose solutions to random pairs in the room. It is not necessary to place the two 0.25% flasks (for example) together; it would be better to separate them. Each pair should also have three fermentation vials, a ruler, a glass stirring rod, and a pair of forceps. Each table should have a 100-strip package of glucose test strips, a large 1% glucose solution flask, a distilled water flask, a yeast pipet, a distilled water pipet, a pipet pump, and a beaker to receive waste yeast suspension. Each bench will only use about 20 of the test strips, but it is necessary to have a

complete package on each bench because the students must compare the colors on the test strips with the color chart on the package.

Just before the class begins, the instructor should make up a “standard” or “1X” yeast suspension by mixing the contents of four yeast packets in 200 mL of room-temperature distilled water. Use a stirring rod to mix it thoroughly, and then let it hydrate for 5 minutes. Distribute $\frac{1}{4}$ of the suspension to each lab bench. The students will then follow the directions to perform the glucose concentration exercise.

When the setup of the glucose concentration exercise is nearing completion, the instructor should make a “2X” yeast suspension in the same way as above, but using 4 yeast packets in 100 mL of distilled water. Then collect the four 1X yeast beakers, clean them out, divide the 2X yeast suspension into the four beakers, and distribute the beakers to the lab benches.

Common Student Mistakes

When the students are measuring gas bubble sizes, the instructor should watch for two common mistakes. First, students should make their gas bubble measurements from the roof of the inner chamber to the *liquid* surface of the inner tube. They should not measure to the top of the foam layer in the inner tube, the liquid surface in the outer tube, or the level to which the yeast cells have settled in the inner tube. These layers are shown in Figure 2 in the student outline. Students should use forceps to press the inner tube against the outer tube so that the liquid surface in the inner tube is visible. Jiggling the tubes will help to find the liquid surface in the inner tube.

The other error is that students attempt to pull the inner tube up above the liquid in the outer tube so they can see where its liquid surface is, pull its mouth up above the liquid in the outer tube, and all the liquid drains out of the inner tube. If this happens, the chamber has been ruined for this experiment. Rather than lifting the inner tube, the students should use forceps to press the inner tube against the outer tube.

The remaining instructor considerations involve the interpretation of the results.

Interpretation—Glucose Concentration Experiment

When this exercise refers to a “1%” glucose concentration, for example, it means the concentration of glucose that was added to the fermentation vial. Because the inner tube of the Carolina fermentation vials holds 23 mL of liquid, 10 of which is yeast culture, the final concentration of glucose in the fermentation vial if 1% glucose is added is about 0.57%. Also, the instructor should not use more concentrated glucose than the recommended 1%. If this is done, the higher yeast concentrations will produce so much gas that the inner fermentation vial will be entirely filled, gas will escape, and the students will not be able to estimate the volume produced.

The results here are straightforward. An example is shown in Figure 15:

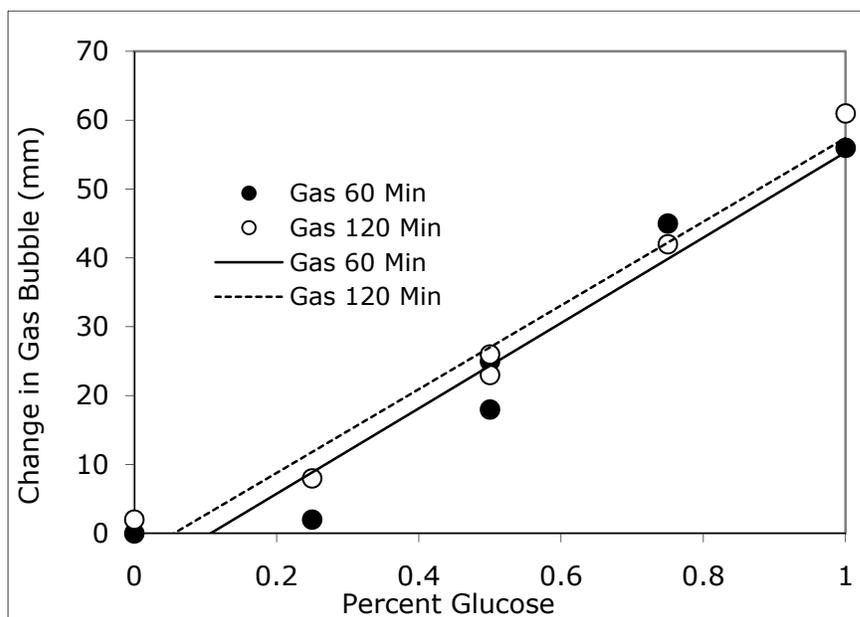


Figure 15. CO₂ bubble size (corrected for initial size) at 60 and 120 minutes using 1X yeast and several glucose concentrations.

The “standard” yeast concentration of 1 yeast packet per 50 mL of distilled water produced a vigorous gas evolution that lasted until the glucose was depleted. The volume of gas was proportional to the initial glucose concentration, and could easily have been used to estimate the glucose concentration in the unknown.

One misconception about this experiment that seemed to be startlingly universal among our teaching assistants was that yeast only produces CO₂ when it is anaerobic and fermenting. Of course, yeast produces 2 CO₂ per glucose when it is fermenting, but 6 CO₂ per glucose when oxygen is available. The reason that vigorous, foaming CO₂ evolution is so associated with fermentation may be the Pasteur effect (below).

At this point, the students may believe that they understand fermentation—glucose is converted (maybe completely converted?) to CO₂, and fermentation goes on until the glucose runs out. The purpose of the experiment on yeast concentration is to challenge these simple ideas with more complicated results.

Interpretation—Yeast Concentration Experiment

To the student with the ideas above, it seems to be easy to predict the results of the yeast concentration experiment. If glucose is converted to CO₂, and all the treatments use the same glucose concentration, then they all should produce the same amount of CO₂. The only difference will be that lower yeast concentrations will produce that amount of CO₂ more slowly. In other words, the predicted results resemble Figure 16.

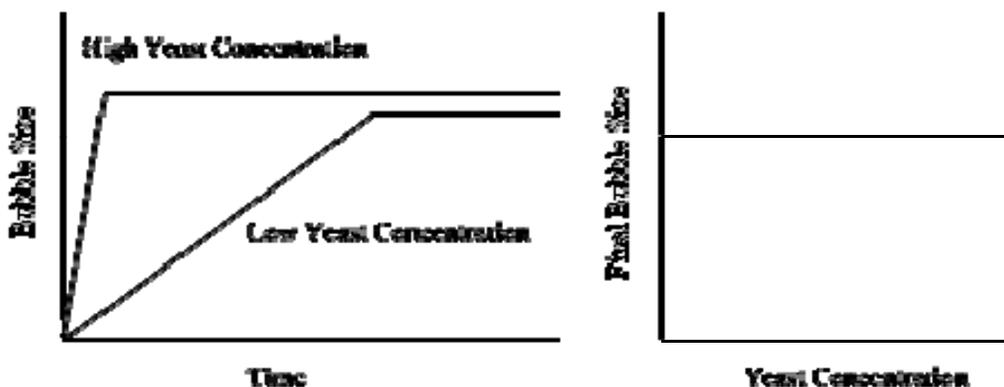


Figure 16. Left: hypothetical time course of CO₂ bubble size in experiments that use the same glucose concentration, but different yeast concentrations. Right: hypothetical relation between final bubble size and yeast concentration.

Actual results for the time course of bubble size are in Figure 5; the relation between yeast concentration and bubble size is shown in Figure 17.

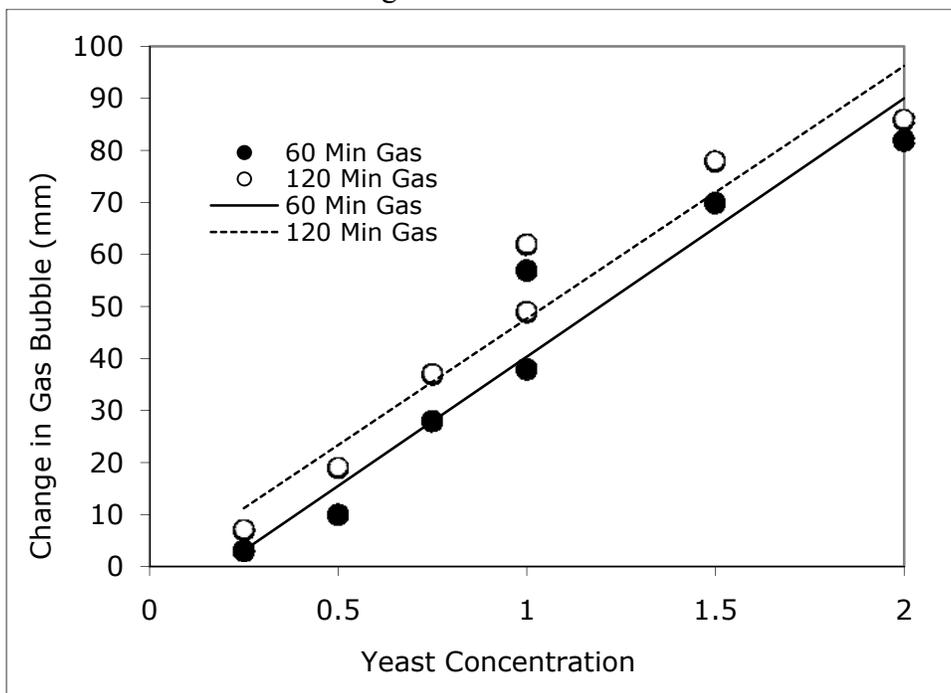


Figure 17. Relation between bubble size and yeast concentration. 1X yeast = 1 packet of yeast per 50 mL distilled water. All yeast concentrations used 1% glucose.

Figures 5 and 17 show that no matter how long we wait, the low yeast concentrations will never produce as much gas as the higher yeast concentrations, even though the amount of glucose is the same. The student outline considers the following hypotheses to explain this observation. Except where otherwise noted, the experiments described were performed in the fermentation vials in conditions as close to the student experiment as possible.

- a) The low yeast concentrations cannot produce their full amount of CO₂ in the short laboratory incubation, and if given enough time the low yeast concentrations would catch up with the higher yeast concentrations. Figure 5 shows that this is clearly not true. The low yeast concentrations never catch up. After 3-4 hours, particularly in the heavier yeast concentration, the amount of gas declines because it is resorbed into the culture.
- b) The Pasteur effect hypothesis was favored by the authors at first. The Pasteur effect is reviewed in Krebs (1972). Aerobic respiration produces 38 ATPs per glucose molecule, and fermentation only produces 2 ATPs. Therefore, yeast that can respire aerobically can capture much more energy from each glucose molecule. Pasteur noted that alcoholic fermentation was stopped by the presence of oxygen, and that the rate of glucose utilization by yeast *increases* dramatically when conditions become anaerobic (Krebs, 1972). In fact, the rate of utilization of sugar may increase seven-fold as oxygen disappears (Berg *et al.*, 2002, p. 846). Figure 7 shows that cells in heavier yeast concentrations do use glucose faster than cells in low yeast concentrations. In Figure 7, the ratio of yeast to glucose was the same in all treatments, and the glucose in the low yeast concentration persisted much longer than in the high yeast concentration. This is consistent with the Pasteur effect. However, measurement of dissolved oxygen with a YSI oxygen meter in a stirred 250-mL yeast culture flask using 1% glucose and yeast concentrations ranging from 2X to 0.0035X yeast showed that all the cultures using more than 0.03X yeast have oxygen concentrations of 0.5-1 mg/L. A stirred water control had an oxygen concentration of 8.36 mg/L, right at oxygen saturation. The authors still believe that the Pasteur effect may be causing the faster *per capita* use of glucose in the more concentrated yeast cultures because those cultures may be more intensely anaerobic.
- c) Biotechnologists we consulted hypothesized that the greater gas production in heavier yeast concentrations was caused by the fact that we were using unwashed Fleischmann's yeast. Commercial yeast is provided with growth factors, and these would be diluted when we diluted the yeast. However, when one set of yeast cultures was diluted with distilled water and another set was diluted with a yeast extract solution (final concentration of yeast extract was 0.13% in all yeast concentrations), there was no difference in gas production (Figure 8) or population growth (Figure 9).
- d) We suspected that the yeast cells were reproducing. Fermentation vials were made according to the student protocol, but then a portion of the yeast cells were removed at intervals shown in Figure 9 and preserved in 95% ethanol for later counting in a hemocytometer. The results showed no evidence of yeast population growth, even when yeast extract was added to the medium (final concentration 0.13% in all yeast concentrations). If anything, the yeast population both with and without yeast extract decreased over the course of a 4-hour experiment.
- e) Large volumes of CO₂ can dissolve in water. At 20° C, 0.17 g of CO₂ can dissolve in 100 g of pure water (Baird, 2009); this is about 92 mL of gas per 100 mL of water. An experiment starts with 23 mL of liquid (10 mL of yeast culture and about 13 mL of glucose solution). Given the dimensions of the inner fermentation tube, and assuming that the fermentation mixture has at least the same ability to dissolve CO₂ as water does, this means that this volume of liquid could dissolve a CO₂ bubble 81 mm tall. We also noted that in a long-term experiment, after CO₂ evolution had ceased, a bubble 70 mm high decreased to 18 mm over 18 hours. We reasoned that perhaps the dissolved CO₂ "compartment" must be filled up before the gas can leave the solution (Figure 10). To test this idea, we put small beakers containing both yeast cultures and

glucose solutions in a 2,000-mL beaker covered with aluminum foil. A tube connected the beaker with a 1,000-mL flask containing 400 mL of 0.5 N HCl (0.2 equivalents). Then we added 16.8 g of NaHCO₃ (also 0.2 equivalents) to the flask and piped the resultant 4.8 L (at 20° C) of CO₂ into the 2,000-mL beaker, where it overflowed around the aluminum foil cap, purging all the air from the beaker. After 1 hour, the pH of a beaker of distilled water in the beaker had decreased from 5.21 to 4.18 due to the dissolution of the CO₂. However, when the yeast and the glucose were mixed and fermentation began, this addition of CO₂ had no visible effect on gas evolution, especially in the 0.25X yeast concentration where it was expected to have the greatest effect (Figure 11).

- f) The rate of CO₂ production is a more complicated hypothesis than the dissolved CO₂ hypothesis above. In Figure 12, the rate at which water flows into the sink is the critical variable. If the flow rate is low, even a large volume of water can enter the sink and run down the drain, and no water will be seen at the side drainpipe. If the flow rate is high, even a small volume of water might cause some overflow into the side pipe. Likewise, if fermentation is fast due to either abundant yeast or high temperature, CO₂ will be formed so fast that bubbles will form in local areas and leave the solution. Once they leave, they will not tend to dissolve in the solution again during a 1-2 hour student experiment. Figure 14 shows that raising the rate of CO₂ production through elevated temperature is the only manipulation that caused less concentrated yeast cultures to produce significantly more gas. The effect of elevated temperatures also becomes more pronounced as the yeast concentration gets lower. The ratio of the 42° volume to the 21° volume in Figure 14 is 1.42 for 1X yeast, 1.87 for 0.5X yeast, 4.00 for 0.25X yeast, and 5.6 for 0.125X yeast. The rate of production theory would explain this by noting that if the yeast are abundant and most of the CO₂ is already being added to the bubble, elevated temperature will not have a dramatic effect on the final gas volume. However, if there are fewer yeast and almost all the gas is being lost by dissolution at 21° C, an increase in gas production rate at 42° C will have the greatest effect on the final size of the gas bubble.

Two other issues related to this hypothesis are the effect of temperature on gas volume and on gas solubility. The gas volumes in Figure 14 have been corrected to their volumes at 21° C using Charles' Law (Kotz *et al.*, 1987, p. 177), so temperature effects on gas volume are already taken into account. The solubility of CO₂ does decrease with temperature, from about 0.23 g/100 mL at 10° to 0.17 g/100 mL at 20° to 0.10 g/100 mL at 40° (Baird, 2009). Therefore, higher temperatures alone do tend to drive dissolved CO₂ out of solution and are part of the effect seen. However, for the lowest yeast concentrations, solubility effects are small compared to the increase in gas produced at elevated temperatures. The 5.6x increase in gas produced between 21° and 42° by the 0.125X yeast culture compares to a decrease in CO₂ solubility of only 41% over the same temperature range.

Finally, if CO₂ goes into solution at moderate and low temperatures and at low yeast concentrations, can we directly detect elevated CO₂ in the solution? We monitored pH of a fermenting culture and also estimated culture buffering capacity by titrating 1X and 0.25X yeast cultures containing distilled water rather than glucose solutions with 0.1 N HCl. The typical pattern of pH in the fermenting cultures is shown in Figure 18.

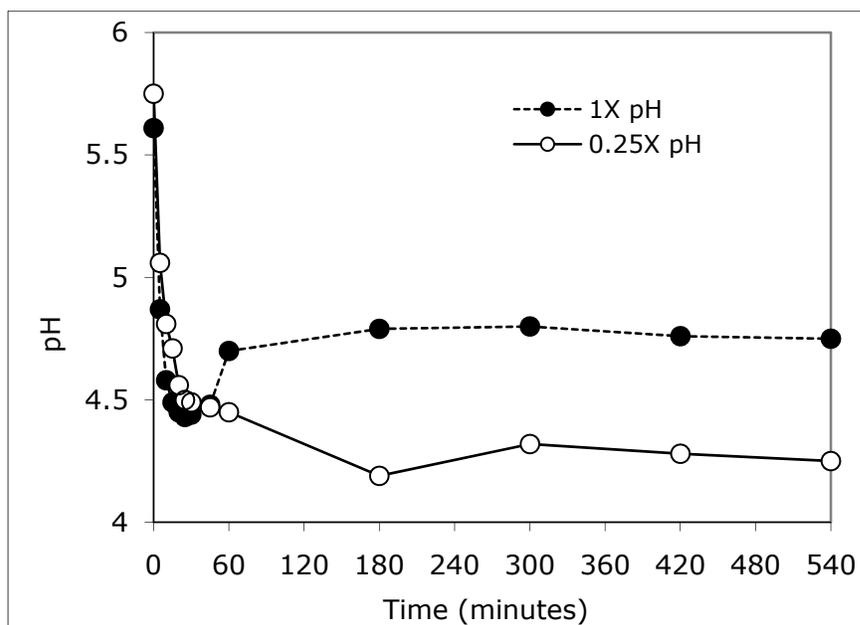


Figure 18. pH measured in cultures containing 1% glucose plus either 1X or 0.25X yeast concentrations.

The sharp “rebound” effect of the pH in the 1X culture was always seen in concentrated cultures, and suggests that the pH is increasing after 30 minutes because CO_2 is bubbling from the culture. Most gas production and glucose use did occur in the first 30 minutes in the 1X culture, and by 60 minutes glucose was exhausted and gas was no longer being evolved. The lower pH in the 0.25X culture implies there is more CO_2 in solution there. However, the 0.25X culture has less buffering capacity than the 1X culture. The 0.25X culture’s pH was lowered to 4.2 (its final pH) by 0.8 mL of 0.1 N HCl; 0.8 mL of HCl only lowered the pH of the 1X culture to 5.3. The true long-term pH of the 1X culture was 4.8. Therefore, it seems that the 1X culture has more CO_2 (or other acid-producing substance) in solution than the 0.25X culture. This is contrary to the predictions of the rate hypothesis, and the authors cannot explain it. However, they still favor the rate hypothesis because it was successful in other ways.

The overall message to students from the yeast concentration exercise is that scientific models are accepted as long as they explain the data. If they don’t explain the data, they must be revised. The first exercise produced a simple model that said that glucose is simply turned into CO_2 until it is gone. Figure 16 shows the results that this model would predict from the yeast concentration experiment. The serious lack of agreement between observed (Figure 17) and expected (Figure 16) results in the yeast concentration experiment caused model revision that touched on metabolic regulation and the physics of gas dynamics in cultures. If the students learn the lesson that revising a model often means that we will have to consult the literature more, perform more experiments, and think more deeply about the system, this exercise will have achieved its goal. We hope that upper-level students can use this exercise as a starting point for student projects that will repeat these experiments and develop still other hypotheses about fermentation.

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