INQUIRY & INVESTIGATION

Quantitation & Case-Study-Driven Inquiry to Enhance Yeast Fermentation Studies

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Abstract

We propose a procedure for the assay of fermentation in yeast in microcentrifuge tubes that is simple and rapid, permitting assay replicates, descriptive statistics, and the preparation of line graphs that indicate reproducibility. Using regression and simple derivatives to determine initial velocities, we suggest methods to compare the effects of experimental variables. This technique is straightforward enough to facilitate design of an inquiry lab based on a scenario that explores modifications to enhance the rate of fermentation.

Key Words: *Yeast; fermentation; inquiry; regression analysis; case studies.*

The demonstration of glucose fermentation by yeast in which carbon dioxide production is measured is a classic teaching experiment that utilizes a variety of techniques, including inverted pipettes (Yurkiewicz et al., 1989), inverted test tubes (BSCS, 1965; Collins & Bell, 2004), inverted graduated cylinders (Tatina, 1989), capillary tubes (Knabb & Misquith, 2006), and techniques that include direct CO_2 probes (Masterson & Holman, 2003). Each of these systems has its own advantages. One published method employing volume displacement in a novel design (Reinking et al., 1994) offered the advantages of simplicity of operation, use of inexpensive and common equipment, and ease of temperature control.

A simple experimental apparatus allows more time to be spent on other issues of importance to developing scientists. For example, the importance of investigative laboratories allowing students to design their own experiments has been emphasized (AAAS, 2009; Rissing & Cogan, 2009). In addition, the incorporation of more mathematics into introductory biology courses was urged by the National Research Council (2003).

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The goal in developing the present fermentation exercise was not only to demonstrate CO_2 production as a means of quantifying fermentation, but also to (1) simplify and shorten the procedure, allowing more replicates to be performed during the available time; (2) increase its accuracy and precision, enhancing its reproducibility and introducing descriptive statistics; (3) introduce a more mathematical approach to data processing that incorporates modeling of the data across the time frame of the experiment; and (4) use the simplified procedure as the basis for an inquiry-driven lab exercise.

Week 1: The Basic Procedure

We have modified the procedure of Reinking et al. (1994), which employs 15-mL conical tubes with holes punched in the screw-caps; a yeast–sugar mixture is added to the tube, inverted, and placed into a beaker of 40°C water for incubation. At various times throughout the incubation, the tube is removed and meniscus position marked.

Instead of 15-mL conical tubes, we use 1.5-mL microcentrifuge tubes with three holes (use 21-g or 23-g needles) in the cap (tubes are nominally 1.5 mL but will actually hold 1.8 mL). The volume is 10× lower and, thus, less expensive. To assay for change in volume, we weigh the tubes before and after incubation to the nearest 0.01 g on top-loading balances, the difference being taken as equal to the volume of sample displaced and, therefore, the volume of CO_2 produced, assuming the yeast solution to have a density of 1.0 g/mL (7% Fleischmann's Active Dry Yeast in water). This eliminates the need, as in the Reinking procedure, to estimate the volume of the yeast mixture, which has foam at the meniscus. To incubate the tubes at 40°C, we invert them into a microcentrifuge rack (Fisher catalog no. 14-809-166A), submerge the rack into a 1000-mL beaker with 40°C water, and then place the beaker into a 40°C water bath (see Figure 1). In the absence of such racks it is possible to make racks from 9-cm-diameter foam rubber circles with 5–8 mm holes for tubes and use appropriately sized bolts, nuts, and washers to submerge the rack. In addition, performing the assay in 5.0% glucose

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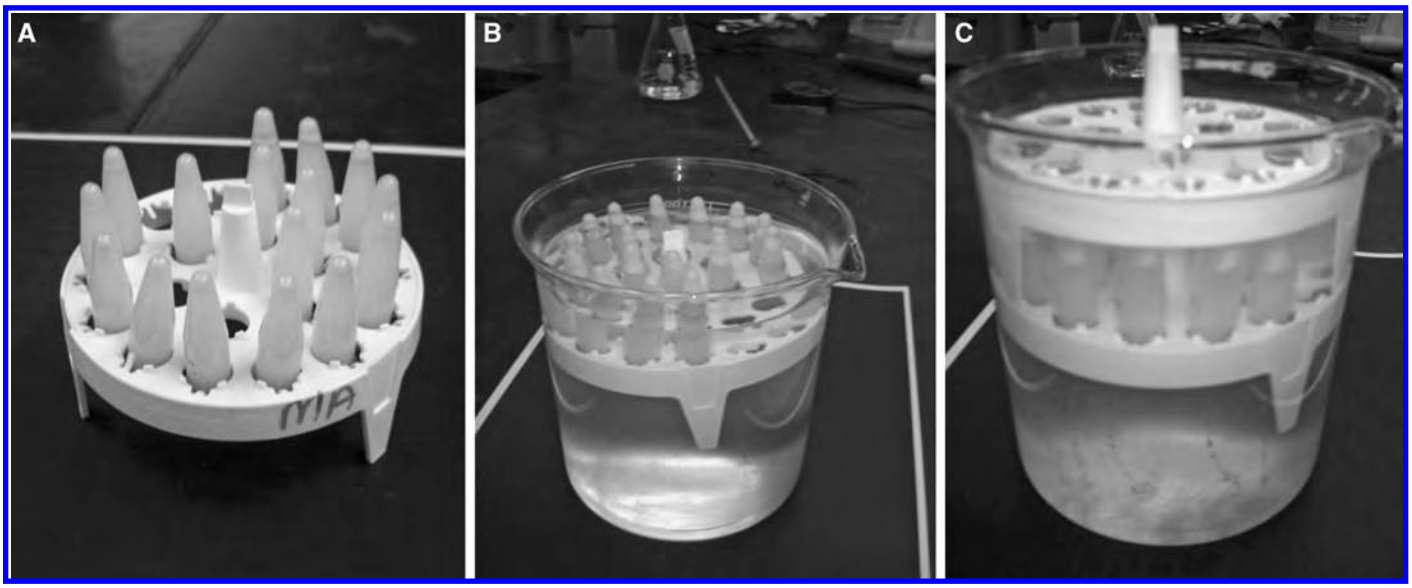

Figure 1. Assay setup. (A) Microcentrifuge tubes inverted into rack. (B) Rack placed into beaker with 40°C water. (C) Rack lowered into water by placing empty rack on top to completely submerge the tubes. Yeast–glucose mixture can be seen entering the water bath.

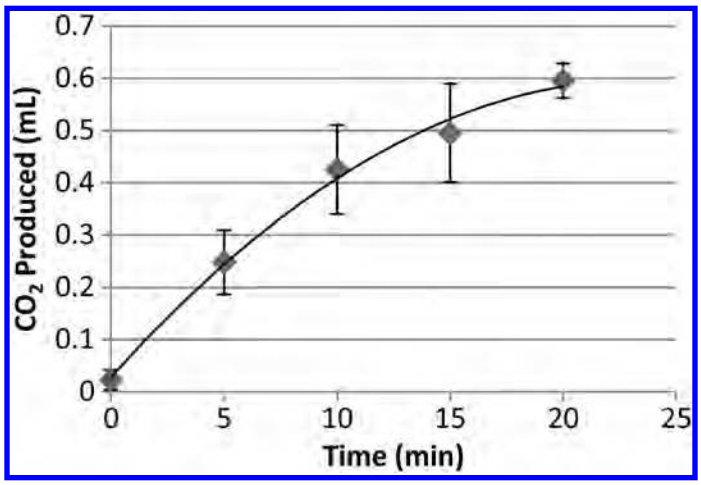

Figure 2. CO_2 production as a function of time. Performed with 7% yeast and 0.5% glucose at 40°C, the initial velocity was 0.0483 mL CO_2/min. Second-order polynomial regression, $R^2 = 0.9943$.

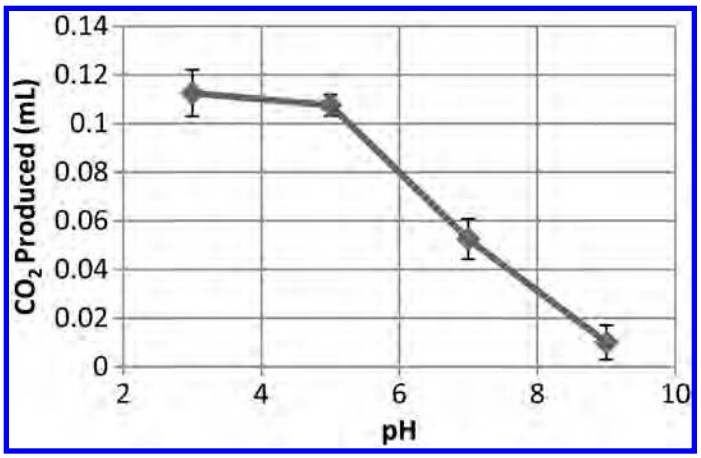

Figure 3. CO_2 production as a function of pH.

generates enough CO_2 that balances with only 0.1-g accuracy will suffice. The reproducibility is excellent, and students can complete the basic experiment in 2 hours.

As CO_2 forms in the top of the tube, yeast–glucose solution is pushed out (see Figure 1C), resulting in a decrease in weight of the tube and contents. We wash and reuse the tubes, providing each group of students with at least 16 prepunched tubes, allowing for four or more time points with n = 4 replicates.

A typical time dependency is shown in Figure 2, using 0.5% glucose as sugar source.

○ Week 2: Inquiry Exercise

The investigative portion occurs in the second week, the experiment being designed at the end of the lab period of the first week for instructor review. Students read the case study (see below) and choose which parameter potentially affecting fermentation rate they wish to test.

We emphasize the importance of positive controls. The inter-package variation in CO_2 production with commercial yeast is significant. In an examination of four different packages with four replicates each, CO_2 production varied from 0.35 to 0.55 mL and was not related to the actual weight of yeast in the package, which itself varied from 7.04 to 7.37 g.

Sample student results for the inquiry portion of the exercise are shown in Figures 3 and 4.

○ Data Analysis

Curve Fitting by Lines or Polynomials (Linear Regression)

In graphing the results of the basic experiment, students are asked to plot mean CO_2 production as a function of time and to show the standard deviation by error bars.

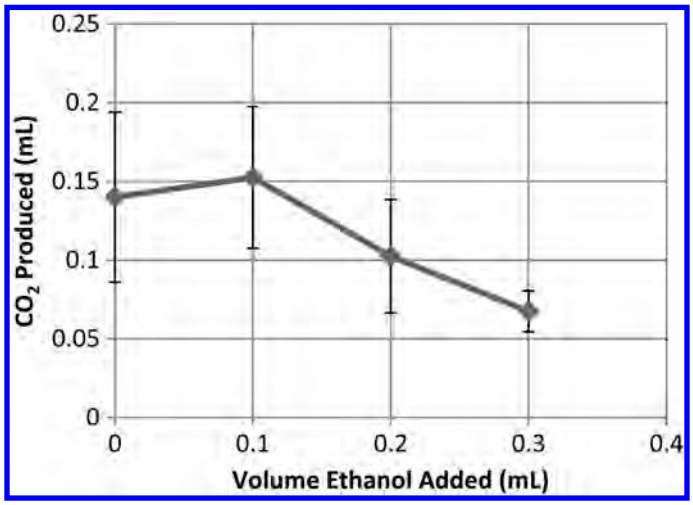

Figure 4. CO_2 production as a function of volume of ethanol added to the assay mixture.

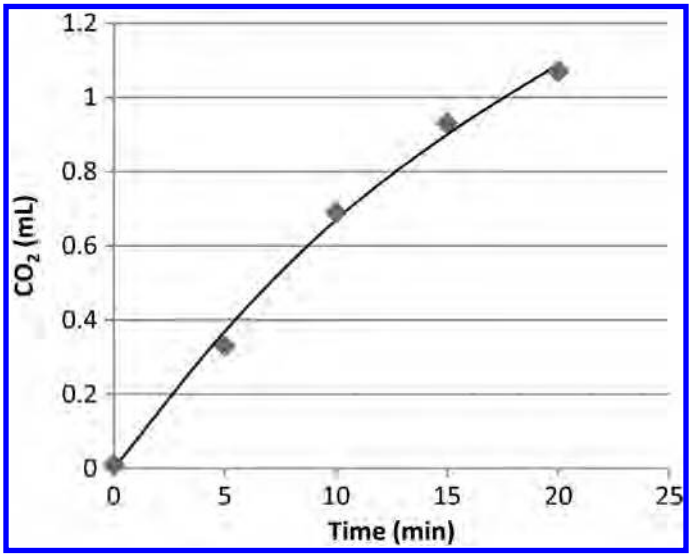

Figure 5. CO_2 production as a function of time. Performed with 7% yeast and 5.0% glucose at 40C. A_0 = 1.825, k = 0.0045.

When the data are plotted in Excel, a curve can be fit to the data using a linear or polynomial regression. The correlation coefficient (R^2) is used to determine how satisfactory the fit is (Figure 2). For later experiments in which parameters are varied, the initial velocity can be used for comparison. As suggested by Aitken et al. (2010, p. 137), the slope of the time dependency curve at time zero can be used for that initial velocity. If the regression curve is linear, the slope of the line of best fit is the initial velocity of the reaction. More generally, in a polynomial regression the initial velocity is the derivative of the equation of best fit evaluated at time zero. When fitting the data to a polynomial equation, the initial velocity is the coefficient of the linear term in the equation of best fit. Students who have had calculus will understand that the derivative of the second-order polynomial, $C(t) = at^2 + bt + c$, is given by $C'(t) = 2at + b$ and represents the slope of the curve at any time, t. Solving for the slope at time zero gives b. Students who have not had calculus can simply be taught that the initial velocity is given by the coefficient of the t term, b.

An appropriate adaptation of this exercise for less mathematically prepared students is to take time points at intervals of 2–3 minutes in 5.0% glucose and hand draw a line of best fit, from which the rate of fermentation can be estimated (rate = slope = rise/run).

Curve Fitting by Exponentials (Nonlinear Regression)

Fermentation is a series of linked biochemical reactions, and frequently, in such a series, there is a rate-limiting step (Aitken et al., 2010, pp. 403–405). In that case, the concentration of the final product as a function of time is given by $c(t) = A_0 (1 – \exp[–kt])$, where A_0 = initial concentration of the first reactant and k = reaction constant for the rate-limiting step.

That yeast fermentation, as measured by this technique, follows this equation is shown in Figure 5. The nonlinear curve fitting of these data was accomplished by the SOLVER add-in in Excel (Aitken et al., 2010, pp. 384–385). This approach is more complex and may not be within the abilities of most introductory biology students but might be useful for upper-level biology or biochemistry students.

○ Student Guide

Week 1: The Basic Experiment

Yeast cells undergo fermentation under anaerobic conditions, producing ethanol and carbon dioxide. This exercise quantifies the amount of fermentation by measuring carbon dioxide production. Thus, we can determine the effects of various environmental conditions on glycolysis and fermentation.

The reaction for alcoholic fermentation is 2 $CH_3COCOOH$ (pyruvic acid) + 2 NADH + 2 $H^+ \rightarrow$ 2 CH_3CH_2OH (ethanol) + 2 CO_2 + 2 NAD^+ (per glucose). CO_2 formation will be estimated by displacement of culture medium during incubation. Microcentrifuge tubes with holes in the lids will be used as reaction vessels. Yeast in 0.5% glucose will be incubated in inverted tubes. As CO_2 is formed, culture medium will be pushed out of the tube. Weighing tubes before and after specified incubation times will reveal loss of culture medium – and, therefore, CO_2 formation – as a function of time.

Materials & Methods

Equipment:
20 microcentrifuge tubes (punched)
Sharpie
Top-loading balance
Racks
Kimwipes
1000-mL beaker
1000-µL micropipette/tips or 1 mL pipette/pump

Solutions:
7% yeast in water
0.5% glucose

Table 1. Record of data.

Time (min)	Pre-weight (g)	Post-weight (g)	CO_2 produced (mL)	Mean CO_2 (mL)	SD
0A				–	–
0B				–	–
0C				–	–
0D					
5A				–	–
5B				–	–
5C				–	–
5D					
10A				–	–
10B				–	–
10C				–	–
10D					
15A				–	–
15B				–	–
15C				–	–
15D					
20A				–	–
20B				–	–
20C				–	–
20D					

Procedure

Zero minute time points:

1. Fill a 1000-mL beaker with 40°C water.
2. Number microcentrifuge tubes 0A-B-C-D, 5A-B-C-D, to 20D, representing intervals of 5 minutes and 4 replicates each.
3. To each microcentrifuge tube add 0.9 mL 0.5% glucose solution.
4. To the 4 zero tubes (0A-B-C-D), in turn, add 0.9 mL yeast, carefully close the cap (slight spillage may occur), dry thoroughly, weigh and record as pre-weight in Table 1. Then invert the tube into a rack, dip the rack into a 40°C beaker for 1 second, remove, and dry again thoroughly and reweigh. Record this as post-weight in Table 1 as "0A", "0B", etc.

All other time points:

5. To all remaining tubes quickly add 0.9 mL yeast.
6. Quickly and carefully (to avoid spillage) close the lids and thoroughly dry and preweigh the tubes, recording the data in Table 1. Immediately start the timer and the incubation at 40°C by placing tubes inverted into the racks. Then place the rack in a 1000-mL beaker of water at 40°C and place the beaker in the 40°C water bath (the beaker prevents fouling of the water bath by the expelled yeast).
7. Remove tubes at the appropriate times and weigh them. Record the data in Table 1 as "5A", "5B", "5C", and "5D" and continue in like manner for 20 minutes.

Week 1 Report

1. Calculate and plot the mean CO_2 production as a function of time on a graph.
2. What conclusions do you draw from the experimental results shown on your graph?
3. Describe the reproducibility observed. Could it be improved? How?

Pick one of the problems discussed in the following case study and, using the techniques you learned this week, design an experiment that will answer the question. You should have a clearly stated hypothesis, prediction, and methods.

Work in teams of two and *don't* get ideas from others.

4. A. What experimental parameter do you intend to vary in your inquiry lab? What solutions do you need? You will use 20 tubes. Describe your experimental design for all 20 tubes. Think about replicates, controls, and incubation times.
   B. What is your hypothesis regarding the effect of this variable on CO_2 production?
5. Think about the time dependency of CO_2 production you observed. Was it linear over 20 minutes or nonlinear? The initial velocity of the reaction is the best indicator of the effect of an experimental variable. For a linear reaction, that is the slope of your CO_2 production graph, but for a second-order polynomial it is the coefficient of the linear term.

The Case Study

Nicole Bailey has just taken over as chief operating officer for a small bakery. The bakery has had difficulty recently because several of the more experienced staff have retired or resigned. Nicole has called a meeting of the key bakery personnel to review current status and hear recommendations for future operations.

"OK, let's get started. Mike, tell me about our current situation in white bread production."

Mike Copeland, a new supervisor, begins, "Bread production has been declining in terms of quality. I've checked out the quality of the flour and sugar, and they seem OK. It could be the pH or the temperature, or it could be the supplements provided the yeast during fermentation. There are salts necessary for fermentation, and these have optimal concentrations, but I don't yet know what those are. Clearly fermentation is too low to get adequate CO_2 production for the dough to rise properly."

"Thanks, Mike. Mrs. Kinsley, what do you have to report from your investigation of the specialty bakery?" said Nicole.

Mrs. Kinsley was new to the bakery but also new to the bakery business itself. "It looks to me like the specialty bakery could use new ideas. We use the basic glucose for the yeast to ferment, but I've been wondering if we could add a different sugar – with a sweet flavor – and produce bread that not only rises, but also tastes sweet."

"Interesting idea," commented Nicole. "Do you know whether yeast can metabolize any other sugars than glucose? And what sugars do you have in mind?"

"Sucrose, I believe, is ordinary table sugar, and maltose and lactose I believe are also sugars."

"Those are disaccharides, but there are also the monosaccharides like fructose and galactose," said Larry Brinkwell, the lab chemist.

"And I don't know whether yeast can use any of these," Mrs. Kinsley replied.

"OK, thanks, Mrs. Kinsley," said Nicole. "Anybody else?"

"In response to Mike's problem, what I'm wondering," said Arthur McLain, the accountant, "is whether we can't get more production out of the yeast by simply fermenting longer."

"Perhaps so," responded Larry. "But the yeast will only do what they can do in a particular set of conditions. I've heard reports from the wine-making industry that yeast, when allowed to go too long, simply stop fermenting when the alcohol reaches some level. Remember that fermentation in yeast produces ethanol."

"Still thinking about the economics of the process," interrupted Arthur, "is it possible to achieve the same level of rising with less yeast, so that we could cut down on yeast costs?"

"OK, I think we have enough to go on here," said Nicole. "Let's meet in a week and see if we can bring in answers to the questions you've posed."

○ Week 2: The Investigative Experiment

Week 2 Report (Word document)

1. Insert an Excel graph of your Week 1 results, showing means, error bars, and Excel curve fit (data from the graph you made).
2. A. What experimental variable did you examine in Week 2?

   B. What was your hypothesis regarding its effect?
3. Experimental techniques: Describe the protocol you followed for the experiment.

4. Insert an Excel graph of your Week 2 results.
   A. Calculate the initial velocities shown with each experimental variable and report.
   B. Plot the initial velocity data (replicates should be shown as means ± standard deviation of the replicates). If your variables are qualitatively different (e.g., different sugars), use bar graphs to show your results. If the variables are quantitatively different (e.g., different pH values), use a line graph.
5. What conclusions do you draw from your experiment? Be sure to answer in terms of the case study.

Instructor Guide

Week 1

Per section:
40°C water bath
7% yeast in water
0.5% glucose

Per group:
16 microcentrifuge tubes (punched)
Sharpie
Top-loading balance
Rack
1000-µL micropipette/tips or 1-mL pipette/pump
Kimwipes

Week 2

Materials are essentially the same as for Week 1, except add the following.

Per section:
40°C, 60°C, and 80°C water baths
Dry yeast packets
0.5% solutions: sucrose, fructose, lactose, and maltose
20% solutions: NaCl, ethanol
Buffers of different pHs

Acknowledgment

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