Manipulation of Yeast Respiration Using Acetic Acid to Demonstrate the Scientific Method

Michael J. Keller[1] and Curtis Gilliam[2]
College of Chemical & Life Sciences
1222 H.J. Patterson Hall (Bldg. 073)
University of Maryland
College Park, MD 20742
[1]*kellermi@umd.edu*
[2]*gilliam@umd.edu*

Abstract

The application of the Scientific Method to realistic scenarios using authentic experiments should be an important component of the teaching laboratory. As part of a large introductory biology lab course, the manipulation of yeast respiration has proven to be a reliable, engaging exercise showcasing hypothesis generation and testing. Recent research on *Saccharomyces cerevisiae* cells has demonstrated that at low pH acetic acid interferes with glucose metabolism and can induce apoptosis. In the teaching lab, we have implemented protocols for testing hypotheses addressing the possible effects of acetic acid on CO_2 production by yeast as measured by a simple respirometer, including (1) suppression of glucose metabolism, (2) reduced cell proliferation, and (3) enhanced cell death. Students generate hypotheses based on a fictitious scenario, frame alternative and null hypothesis, test their hypotheses, and draw conclusions based on class data. This exercise provides beginning students experience with hypothesis testing and introduces them to basic data analysis and experimental error. These lessons are reinforced throughout the semester through a series of exercises explicitly stressing different components of the Scientific Method.

Introduction

The Scientific Method is a formal process for answering questions about the world objectively, efficiently, and clearly. Understanding the Scientific Method is a core objective of our course, and students are expected to apply this process in the laboratory throughout the semester. Most lab exercises are presented with a ''scenario'' that establishes context and provides a basis for meaningful hypothesis testing. We employ glucose metabolism by yeast as an experimental model in part of a two-session introduction to the Scientific Method. Here we describe procedures used to test hypotheses addressing the influence of acetic acid, a metabolic product of fermentation, on CO_2 production by yeast.

Background

Yeast cells actively transport glucose from the extracellular environment into their cytoplasm where it is metabolized to produce ATP, with CO_2 as a waste product. In the presence of oxygen the pyruvate from glycolysis is used generate ATP via oxidative phosphorylation, while under anaerobic conditions, ATP is produced entirely by glycolysis coupled to fermentation. Yeast cells also exhibit the usual phenomenon of glucose repression ("Crabtree effect") where high concentrations of glucose inhibit the oxidative phosphorylation pathway even in the presence of oxygen (see Raghevendran et al., 2005).

Glucose repressed yeast cells use glycolysis coupled to fermentation almost completely to generate ATP. Both processes produce CO_2 although in different ratios: the oxidative phosphorylation pathway yields six moles of CO_2 per mole of glucose, while the fermentation pathway yields only two moles of CO_2 per mole of glucose. However, fermentation consumes glucose at a much faster rate than does aerobic respiration as a result of the Pasteur effect (see Reinking et al., 1994).

We take advantage of the ability to manipulate yeast glucose metabolism using acetic acid as a basis for a multiple hypothesis testing lab. Acetic acid has several advantages over other solutions: first, it is not toxic to students like metabolic poisons (eg. Tatina, 1989); second, students do not see it as an obvious "cheat" like using different amounts or types of sugars, or altering environmental conditions (eg. Collins and Bell, 2004; Pigage et al., 1998); and third, it reflects a real problem faced by yeast during fermentation (Pinto et al., 1988) and fits a realistic scenario.

Acetic acid is a normal end product of fermentation by *Saccharomyces cerevisiae*, or baker's yeast, that is not metabolized in glucose repressed cells. Acetic acid is 30 times more toxic than ethanol, inhibiting glycolysis and fermentation and eventually triggering cell death (Ludovico et al., 2001; Pampulha and Loureiro-Dias, 1990; Pinto et al., 1988). Acetic acid does not affect glucose transport, but inhibits glycolytic enzymes and NADH dehydrogenase (Pampulha and Loureiro-Dias, 1990; Zhao et al., 2008).

Student Outline

Scenario

The Norganic Baking Company is a manufacturer of bread products distributed world-wide. You are part of the research and development team making a sourdough-like product with the goal of extending shelf life. You decide to add acetic acid to the dough, as it is a major product of sourdough fermentation and has known preservative properties. The result is a loaf of bread with triple the normal shelf life, but it is dense and does not fare well in taste tests. Your boss wants to know precisely why the bread is dense so your team can try to fix it without losing the benefits of the new formula.

Observations

The first step in the Scientific Method should always be to make objective observations about a phenomena or process of interest. Record all observations made in the Scenario above.

Most bread recipes included flour, sugar, yeast, water, and salt as basic ingredients, any of which could potentially be affected by an additive. As with most problems, this means that you can probably come up with a large number of valid hypotheses, so how do you decide which ones to test first? What kind of experiments should you perform? Consider the following questions and discuss them with the class:
   • Which ingredients are *most likely* to influence the texture of bread?
   • Which elements are *most likely* to be altered by the presence of the additive?
   • Given the likely effects of the additive on the different ingredients, and the equipment and methods available in your lab, which hypotheses can you *reasonably* test?

Given what you discussed about the components of bread and in light of your observations, what *single ingredient* do you think is most likely influenced by the addition of acetic acid?

Background

After much discussion, your research team at Norganic Baking concludes that the most sensitive part of bread making, and the most important determinant of density, is the leavening process. Leavening is the process by which dough rises prior to baking, and poorly risen dough will make dense bread that would almost certainly have a different texture. In yeast breads leavening is a result of CO_2 production by yeast cells as they metabolize sugars through aerobic respiration and fermentation (Fig. 1).

In the presence of oxygen, yeast cells actively take up and metabolize glucose using aerobic respiration, producing ATP through oxidative phosphorylation and giving off CO_2 as a waste product. Under anaerobic conditions yeast depend on the production of ATP through substrate phosphorylation of ADP during glycolysis. The ability to sustain glycolysis depends on the recycling of NADH to NAD+ by fermentation, which produces secondary metabolites such as ethanol, lactic acid, and acetic acid.

Yeast cells are special because they have an unusual trick called "glucose repression," where they switch to using glycolysis coupled to fermentation even in the presence of oxygen if there is abundant glucose available. Why would glucose repression have evolved given that aerobic fermentation produces a lot more ATP per glucose molecule than glycolysis? It turns out that glucose can be metabolized at a far greater rate through glycolysis coupled to fermentation, and this more than makes up for the difference in productivity. The ethanol and organic acids store energy that can be used later on when the glucose bonanza is used up.
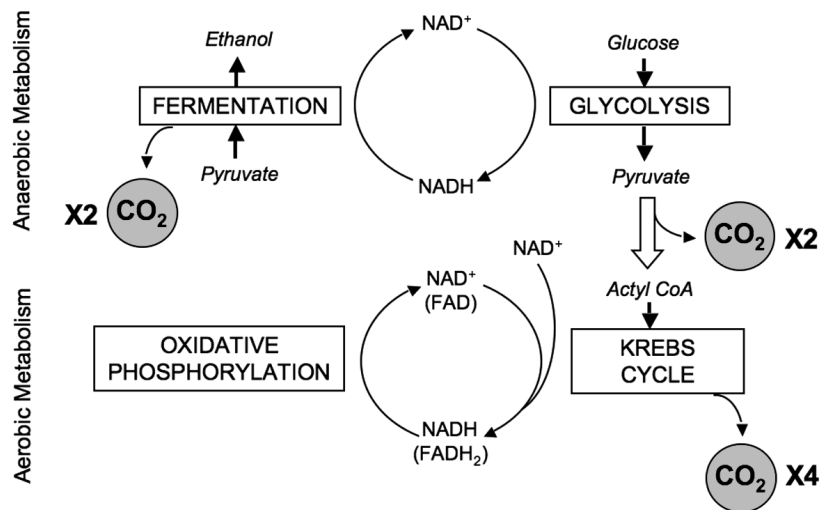

Figure 1. NAD cycling and CO_2 production by alternate pathways during glucose metabolism in yeast.

Hypotheses

Given what you know about cellular respiration and fermentation, cells in general, and your own common sense, you should be able suggest at least three general hypotheses for the affect of acetic acid on glucose metabolism by yeast. Compare your suggestions to the rest of the class, discuss them, and together develop a consensus as to three reasonable, testable hypotheses you should address.

Methods
Your team must assess the level of glucose metabolism by bakers yeast and compare untreated and acetic acid treated cultures. Two possible variables you could measure would be (1) glucose concentration is the media, or (2) production of CO_2 as a product of glucose metabolism. As a class discuss the advantages and disadvantages of each approach. Why are we going to measure CO_2 production and calculate glucose utilization rates rather than measuring changes in the amount of available glucose directly?

Hopefully one of your hypotheses has something to do with acetic acid interfering with glucose metabolism by yeast cells. Two alternative hypotheses that seem obvious once you think of them have nothing to with metabolism per se, but rather the cells themselves. What would happen if there were differences in the number cells (why might they differ)? What if the acetic acid kills some cells - what would that do to CO_2 production?

A Quick Review: The Ideal Gas Law
In Procedure I you will measure the rate of CO_2 production by yeast in a respirometer, but ideally we would like to know the rate of glucose utilization through cellular metabolism. The Ideal Gas Law is used to convert volume of a gas, such as CO_2, to moles of molecules in that volume. Given moles of CO_2 the conversion to moles of glucose is simply a matter of multiplying by two (fermentation) or six (aerobic respiration).

$$\text{moles = PV/RT}$$

The Ideal Gas Law works equally well on volumes (V) expressed as rates. Most of the time, we assume that **P** = 1 atmosphere (atm), and **T** = 295° Kelvin (K). The gas constant **R** = 0.08206 L atm/mole K. Note that volume should be expressed in *liters*, or for rates as L/minute.

Procedure I: Measuring yeast metabolism via CO_2 production
Each group will set up two respirometers (Fig. 2), one containing "control" yeast suspension and the other "treated" yeast suspension. Two group members should setup and monitor each respirometer, being careful to begin the experiment and record data at the same time for both conditions.
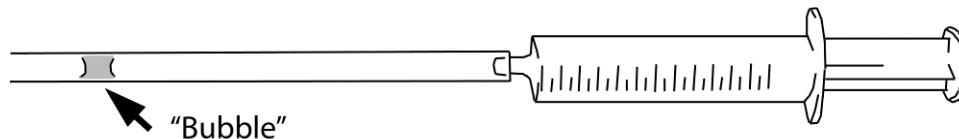

"Bubble"

Figure 2. Diagram of an assembled respirometer.

1. Your instructor will suspend 3 g baker's yeast in 75 mL yeast buffer, stirring vigorously for 15 minutes. Pipette 5 mL yeast suspension into each of two 50-mL beakers, labeled "control" and "treated."
2. *Simultaneously*, pipette 5 mL of either "control" or "treated" glucose solution into the appropriate beakers. The control is 5% glucose in yeast buffer, pH 5.0, and the treatment is 5% glucose + 1% acetic acid in yeast buffer, pH 5.0. Incubate at room temperature for 5 minutes, swirling occasionally.
3. Draw exactly 3 mL yeast/glucose suspension into each of two 10-mL syringes. Invert and draw 1 mL air into syringe above yeast. *Reserve the remaining yeast suspension in the beakers for Procedures II and III!*
4. Add a small "bubble" of water to a 2-mL pipette, cotton removed, letting it fall to about the zero mark. *Simultaneously,* firmly place a pipette on the tip of each syringe.

5. Place the sealed syringes in a 100-mL beaker, upright, and record the position of the bubble at time zero. Record the subsequent positions of the bubbles at 2-minute intervals for at least 20 minutes, or until the end of the pipette is reached.

6. Plot cumulative CO_2 production as a function of time and draw a line of best fit through the linear portion of the data to determine the slope or rate of gas production (mL CO_2/minute).

7. Convert the rate of CO_2 production to the rate of glucose utilization (moles/minute) using the Ideal Gas Law. Enter your rates in the spreadsheet provided and record the class means and standard errors.

Procedure II: Determining relative cell number

Yeast are single-celled organisms with cell walls that absorb visible light in proportion to the number or *density* of cells suspended in media. The spectrophotometer can measure the optical density of a yeast suspension as an index of cell density. Assuming cell size does not differ radically, cell densities are good measures of relative cell number.

1. Make a blank with 3 mL yeast buffer in a clean cuvette. Blank the spectrophotometer at a wavelength of 630 nm.

2. Dilute 300 µL of reserved control and treated yeast suspensions in 3 mL yeast buffer in two additional cuvettes and measure absorbances at 630 nm.

3. Enter your data in the spreadsheet provided and record the class means and standard errors.

Procedure III: Determining relative cell viability

Living cells actively exclude certain solutes in the environment, but when cells begin to die these mechanism fail and they rapidly accumulate solutes. We can take advantage of this property to develop "live-dead" cell assays based of the adsorption of dyes by dead cells.

1. Mix 40 µL of reserved control and treated yeast suspensions each with 960 µL methylene blue solution in two microcentrifuge tubes. Centrifuge the samples for 5 minutes to pellet cells.

2. Gently pipette your two solutions into clean cuvettes and dilute each one with 1 mL water. Using 2 mL water in a third cuvette as a blank, measure the absorbance of each solution at 550 nm.

3. Enter your data in the spreadsheet provided and record the class means and standard errors.

Procedure IV: Comparing sample means

Plot bar graphs for each procedure above using the class means and standard errors. Are there significant differences between the control and treated yeast suspensions in any of the three procedures? What conclusions can you draw about your three hypotheses? Can more than one hypothesis be supported at one time? Given what you know, is there a reasonable way for Norganic Baking to compensate for the affect of acetic acid on bread texture?

Instructor's Notes

Hypotheses

Given what they know about bread making and the information in the scenario, students can generate many hypotheses and must be guided toward the importance of yeast in the leavening process. After realizing the most obvious candidate for the cause of dense bread is a lack of CO_2 production, the class converges on three general, testable hypotheses:

(H1) The bread is dense because of reduced CO_2 production resulting from inhibition of yeast

glucose metabolism in the presence of acetic acid.
(H2) The bread is dense because of reduced CO_2 production resulting from slower growth of yeast in the presence of acetic acid.
(H3) The bread is dense because of reduced CO_2 production resulting from increased cell death in presence of acetic acid.

Procedure I: Measuring CO_2 production and estimating glucose utilization

These procedures are conducted in acidic media, pH 5.0, for two reasons: (1) yeast metabolism is many times greater at low pH (3-5) than at near-neutral pH (≥ 6), and (2) only undissociated acetic acid can be transported into cells, and this form occurs predominantly under acidic conditions (Mollapour and Piper, 2007).

Students work in groups of four; each group sets up two respirometers to measure CO_2 production, with pairs responsible for either the control (untreated) or experimental (acetic acid) treatment. Students need to take measurements for approximately 20 minutes, graph their data, determine the *rate* of CO_2 production (Fig. 2), and finally use the Ideal Gas Law to infer the rate of glucose metabolism. There is an initial lag in CO_2 production of 5-10 minutes before glucose repression causes the switch to fermentation. Students enter their data into a spreadsheet that calculates class means and standard errors for the two treatments.
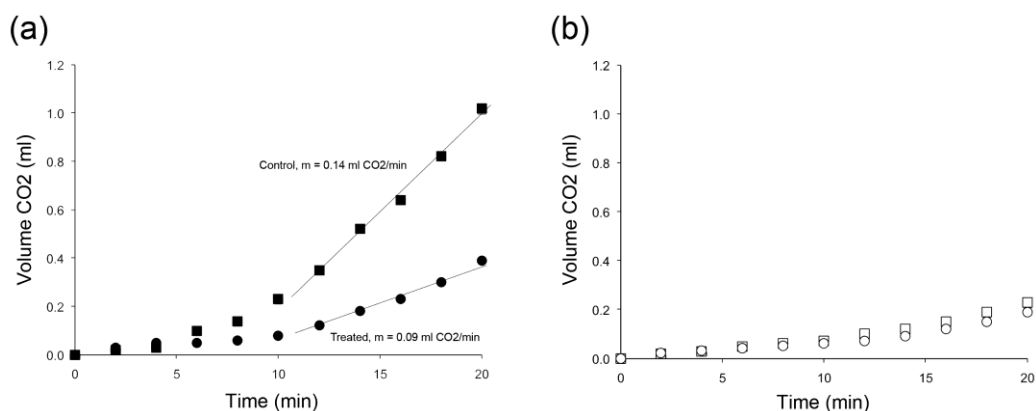

Figure 3. Sample data showing time typical time courses for CO_2 production by 3 mL of yeast suspension under control and treatment conditions. (a) pH 5.0, with best-fit-curves drawn through linear portion of each curve; (b) pH 7.0. Cell density, 0.5 g/25 ml; Control media, YPB + 2.5% glucose; Treatment media, YPB + 2.5% glucose + 0.5% acetic acid.

Procedure II: Estimating cell density

Yeast absorb light at 630 nm in proportion to the number (or density) of cells in the same way bacteria do, so the spectrophotometer can be used to measure the optical density of yeast suspensions as an indicator of relative cell density. A yeast suspension of 0.5 g/25 mL should give absorbance values near 0.50 OD. No differences are expected in this exercise because there is not enough time for the treatment to influence cell number in the population. When differences are observed the instructor should discuss the contribution of experimenter error to distributions of data.

Procedure III: Determining relative cell viability

Living cells exclude many solutes, while dead cells rapidly accumulate solutes in the cytoplasm. "Live-dead" cell assays measure the uptake of dyes by dead cells. In yeast, relative cell viability is determined using spectrophotometry to measure adsorption of methylene blue (Boranzi and Vairo, 1958). Live cells adsorb some dye in their cell walls (~50%), but adsorption is greater for dead cells (~75%). Following centrifugation of the yeast cells, the residual dye in the methylene blue solution is measured by absorbance at 550 nm, giving a value that is inversely proportional to cell viability (ie. higher OD = fewer dead cells). No differences are expected because the induction of apoptosis in glucose repressed yeast takes at least 90 minute. Cell death can be demonstrated if low glucose concentrations are used.

Procedure IV: Graphing and interpretation

Students are asked to graph the means and standard errors for untreated and treated yeast from Procedure I as a bar graph and to infer significance based on overlap of error bars. Aggregate data from ten lab sections (Figure 4, Table 1) show the procedures yield consistent results supporting Hypothesis 1 (acetic acid interferes with metabolism), but not H2 (difference in cell number) or H3 (difference in viability).
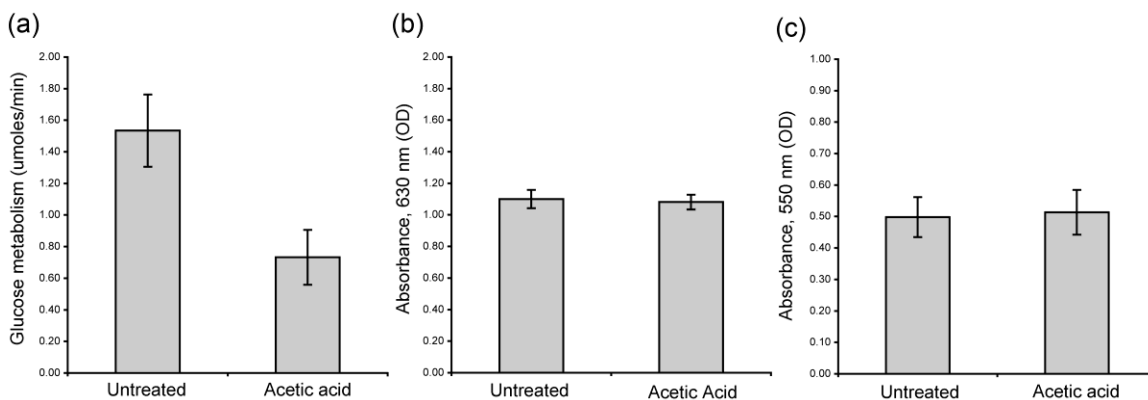

Figure 4. Student data support a significant reduction in glucose metabolism as the principle effect of acetic acid on yeast suspensions. Means and standard deviations are for data from (a) 45 or (b, c) 29 lab groups.

Table 1. Comparisons of paired data by group for ten lab sections showing frequency of experimental outcomes.

Test	Outcome of treatment observed (# Groups)		
	Increase	No change	Decrease
Glucose utilization	5	1	39
Cell Density	15	0	14
Cell viability	15	2	12

Time frame

This lab is intended for a three-hour session. We introduce the scientific method in the preceding session and use this exercise as the "wet lab" demonstration of hypothesis testing. All groups do Procedure

I, but Procedures II and III are divided among groups and run concurrently, leaving time to discuss error and distributions and to graph the class means. We have also run an abbreviated version where we introduce the scientific method and run only Procedure I in a single session, but find students are more engaged by the multiple hypothesis version because it seems less "cookbook."

Acknowledgements

Literature Cited

Boranzi, W., and M. L. R. Vairo. 1958. Quantitative adsorption of methylene blue by dead yeast cells. The Journal of Bacteriology, 76:251-255.

Collins, L. T., and R. P. Bell. 2004. How to generate understanding of the scientific process in introductory biology: a student-designed laboratory exercise on yeast fermentation. The American Biology Teacher, 66:51-53.

Keller, M. J., and P. J. 2009. Introduction to experimental biology. Hayden-McNeil Publishing, Plymouth, MI, 166 pages.

Ludovico, P., M. João Sousa, M. T. Silva, C. Leão, and M. Côrte-Real. 2001. *Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid. Microbiology, 147:2409-2415.

Mollapour, M., and P. W. Piper. 2007. Hog1 mitogen-activated protein kinase phosphorylation targets the yeast Fps1 aquaglyceroporin for endocytosis, thereby rendering cells resistant to acetic acid. Molecular and Cellular Biology, 27:6446-6456.

Pampulha, M. E., and M. C. Loureiro-Dias. 1990. Activity of glycolytic enzymes of *Saccharomyces cerevisiae* in presence of acetic acid. Applied Biotechnology and Microbiology, 34:375-380.

Pinto, I., H. Cardoso, C. Leão, and N. van Uden. 1988. High enthalpy and low enthalpy death in *Saccharomyces cerevisiae* induced by acetic acid. Biotechnology and Bioengineering, 33:1350-1352.

Pigage, H. K., M. C. Neilsen, and M. M. Greeder. 1998. BioLab: using yeast fermentation as a model

for the scientific method. Pages 109-123, *in* Tested studies for laboratory teaching, Volume 19 (S. J. Karcher, Editor). Proceedings of the 19th workshop/conference of the Association for Biology Laboratory Education (ABLE), 365 pages.

Raghevendran, V., J. Nielsen, and L. Olsson. 2005. Teaching microbial physiology using glucose repression phenomenon in baker's yeast as an example. Biochemistry and Molecular Biology Education, 33:404-410.

Reinking, L. N., J. L. Reinking, and K. D. Miller. 1994. Fermentation, respiration & enzyme specificity: a simple device & key experiments with yeast. The American Biology Teacher, 56:164-168.

Tatina, R. 1989. Apparatus & experimental design for measuring fermentation rates in yeast. The American Biology Teacher, 51:35-39.

Zhao, J., Z. Wang, M. Wang, Q. He, and H. Zhang. 2008. The inhibition of *Saccharomyces cerevisiae* cells by acetic acid quantified by electrochemistry and fluorescence. Bioelectrochemistry 72:117-121.

About the Authors

Michael Keller earned a B.S. from the State University of New York at New Paltz, an M.S. from Villanova University, and a Ph.D. from the University of Missouri, Columbia. He was a post-doctoral fellow at the National Institute of Child Health and Human Development before accepting his current position as Lecturer and Lab Coordinator at the University of Maryland.

Curtis Gilliam earned a B.S. from Virginia State University and a Ph.D. in Pharmacology from the University Maryland, Baltimore. He also holds a B.S. in Management Studies and an M.S. in Biotechnology from the University of Maryland University College. He is currently a Lab Manager at the University of Maryland.

Appendix A
Materials

Per lab
- Magnetic stirrer
- Microcentrifuge
- Micropipettors (P-1000, P-200)
- Micropipette tips (1000-µL, 200-µL)
- 10-mL Serological pipettes (2)
- Pipette pump
- 250-mL Beaker
- 100-mL graduated cylinder
- Rapid Rise Yeast
- Yeast Phosphate Buffer (YPB)
- 5% Glucose/YPB
- 5% Glucose/1% Acetic acid/YPB
- 50 mg/L Methylene blue in water
- Water with food coloring
- Distilled water

Per group
- Spectrophotometer
- 2-mL Pipettes, cotton removed (2)
- 10-mL Syringes (2)
- Beakers: 50-mL (2), 250-ml
- 1.5-mL Microcentrifuge tubes (2)
- 10-mL Test tubes (2)
- Cuvettes (3) (Good Pyrex tubes work)

Solutions

1x Yeast Phosphate Buffer (YPB or "Yeast Buffer")

KH_2PO_4	13.6 g/L	(100 mM)
Na_2HPO_4	11.4 g/L	(80 mM)
KCl	7.5 g/L	(100 mM)

- Dissolve in 800 mL dH_2O; adjust to pH 5.0
- Bring to 1000 mL total volume

5% Glucose/1x YPB ("Glucose, control")

Glucose	50 g
1x YPB solution	1000 ml

5% Glucose/1% Acetic acid/1x YPB ("Glucose, treated")

YPB solution	800 ml
Glacial acetic acid	10 ml
Glucose	50 g

- Mix YPB with acetic acid; adjust to pH 5.0
- Bring to 1000 mL with YPB; check final pH
- Dissolve glucose in YPB/Acetic acid solution

Methylene Blue Solution

Methylene blue	5 mg
dH_2O	100 ml

- Solution may be filtered, but it is not necessary for this application