

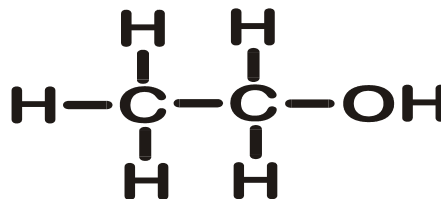
BioFuel Production through Yeast Fermentation

Ethanol offers a valuable energy alternative to fossil fuels, which are both nonrenewable and contribute significantly to atmospheric pollution. Ethanol is often referred to as a 'biofuel' since it can be manufactured by fermenting grains, plant biomass and other organic materials using microorganisms, such as yeast cells. Ethanol can be used as a fuel directly, but most often it is blended with gasoline (at about 5-10%) to yield gasohol. Since ethanol contains oxygen atoms (Figure 1), gasohol burns 'cleaner' than regular gasoline, reducing emissions of carbon monoxide, nitrogen oxides and hydrocarbons. Although the raw materials used to produce ethanol are generally considered to be a renewable resource, there are practical and ethical issues that must be considered before society becomes too dependent upon this energy source (see Ethics of Biofuel Use below). Nevertheless, ethanol is already making a significant contribution to world energy needs, and its use will certainly be increasing.

Currently, ethanol production is measured in the billions of gallons. Canada and Brazil are major producers and users of this energy source, much of it derived from grains such as corn and wheat. To convert grain to ethanol, the grain is first milled into a fine powder and boiled to liquify the starch. The starch is then enzymatically broken down to release smaller sugars that can be readily fermented by microorganisms.

The ethanol released during the fermentation process is later distilled from the liquid and further purified. Yeasts are the type of microorganism most commonly used to ferment grain into ethanol. Yeast have been used in fermentation processes for millennia, and their use is still the foundation of the baking and beverage brewing industries. Fermentation is part of the energy producing process called **cellular respiration** that is shared by essentially all cells. In this laboratory exercise, you will learn about yeast fermentation and the factors that affect its rate, and learn some of the basic principles of proper experimental design.

Figure 1. The molecular structure of ethanol. The presence of the oxygen atom makes ethanol a cleaner burning fuel than gasoline.



Objectives

This lab exercise has the following objectives:

1. To learn about the use of ethanol as an alternative energy source.
2. To further develop your ability to properly design an experiment and interpret the results.
3. To improve your understanding of cellular respiration and fermentation

Exercise Synopsis

Week 1: read assigned materials; run preliminary fermentation experiment.

Week 2: read assigned materials; plan student-designed experiment; use Excel for graphing.

Week 3: perform student designed experiment; graph and interpret results for week 4.

Week 4: students present experiment, results and interpretations to class.

Week 1

Before coming to lab for the first week of this lab exercise you should read the following sections on the bioethics of biofuel production and review the basic experimental methodology. You will graph the results and calculate fermentation rates, and the class will compare results and discuss sources of experimental error.

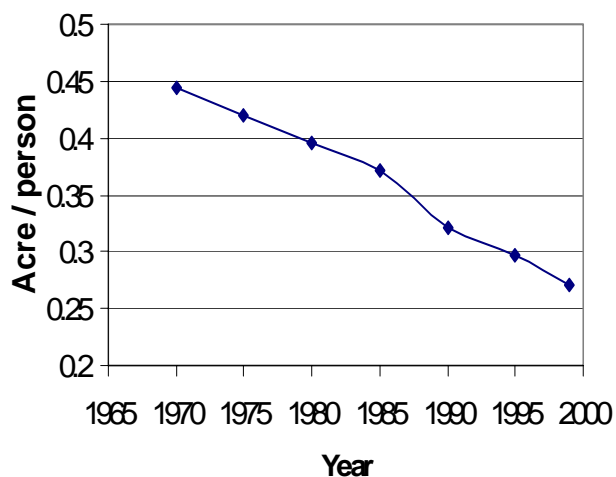
The Ethics of Biofuel Production

Should grains be used as an energy source? Currently, approximately 6% of the U.S. corn crop is used for ethanol production. There are arguments for and against this practice. A number of indicators suggest that petroleum supplies will begin to dwindle during the 21st century, and this would seem a sufficient incentive for us to identify alternative (and ideally renewable) energy sources. Grains and plant matter (biomass) commonly used in biofuel production are generally considered to be renewable, and thus ethanol production could help to meet our energy needs. The environmental qualities of ethanol are better than those of petroleum. The presence of the oxygen atom allows ethanol to burn more cleanly – releasing less nitrogen oxides, carbon monoxide, hydrocarbons and particulate matter – than gasoline. Gasohol, the ethanol/gasoline blend, also burns more cleanly than gasoline alone. Thus there are indirect benefits of using ethanol fuel that cannot be measured directly in energy content and cost.

How much ethanol can the U.S. produce? Quite a lot. The US Energy Bill of 2005 mandated production of about 8 billion gallons of ethanol by 2012. [By comparison, gasoline consumption in 2004 was about 1.4×10^{11} (140 billion) gallons (EIA, 2005, Table 5A).] Each bushel of corn (approx. 56 lbs) can yield approximately 2.5 gallons of ethanol, and at a corn yield of 125 bushels per acre (Shapouri, et al., 1995), an acre of farmland would yield enough corn for 310 gallons of ethanol. Thus, production of 8 billion gallons of ethanol would require about 2.5 million acres of land.

For this and other reasons, some people suggest that it is wrong to use grain for energy production instead of food. The World Watch Institute (Gardner, 2000, 44) reports that the amount of harvested (farmed) land in the world has declined over the last quarter century, while over the same time period world population has increased 50%. The result has been a dramatic decrease in the area of harvested land per person (Figure 2). This has been partially offset by improvements to grain yield per acre; however, there has been little tangible evidence of this improvement for the 70% of the world's population that live in or near poverty. Ultimately, society must decide whether the virtues of ethanol as an energy source outweigh the potential consequences on available food supply of using grain for biofuel. Furthermore, there is debate also over the energy balance of

Figure 2. World grain harvest area. This graph is created by dividing the total area used for grain production by the world population. Data adapted from Gardner, 2000, 45.



ethanol production, with different studies showing either a net gain or loss when the energy inputs to ethanol production are compared to the energy yield (for example, compare Shapouri et al., 1995 and Pimentel, 2003).

Development of alternative energy sources is not the only way to meet future energy needs. There are other root causes to potential future energy shortages, including overpopulation, increased energy consumption by developing nations, and excessive and wasteful energy usage by developed nations such as the U.S. We are unlikely to meet future energy needs by merely seeking to replace petroleum resources – other changes in energy consumption habits must also occur.

EIA (Energy Information Administration). 2005. Short-Term Energy Outlook. November 2005. US Department of Energy Publication.
Gardner G. 2000. Grain Area Shrinks. In: Brown LR, Renner M, Halweil B, editors. Vital Signs 2000. New York: WW Norton and Co. p 44 - 45.
Pimentel, D. 2003. Ethanol Fuels: Energy Balance, Economics, and Environmental Impacts are Negative. *Natural Resources Research* 12:127-133.
Shapouri H, Duffield, JA and Graboski MS. 1995. Estimating the Net Energy Balance of Corn Ethanol. USDA Agricultural Economic Report Number 721.

The procedure for measuring yeast fermentation

As grain is and will continue to be used for biofuel production, it is appropriate that we should seek to maximize the yield of ethanol. One way to do this is to study the effects of various factors on the rate of fermentation. There are a number of ways to measure the rate of yeast fermentation. Since we are interested in ethanol production, measuring the amount of ethanol produced over time would be the most direct and useful. Unfortunately, measuring ethanol released by the yeast cells is too involved for an intro biology lab. However, CO₂ production can be measured quickly and accurately, and it affords an acceptable means of studying the effects of different variables on yeast fermentation.

The experiments you will perform will be carried out using a respirometer apparatus (Figure 3). The principles of its operation are very simple: the yeast suspension is placed into a syringe, and the CO₂ released by the cells pushes a small water droplet up a pipet, which allows the volume of CO₂ to be measured at different times over a period of 20 minutes.

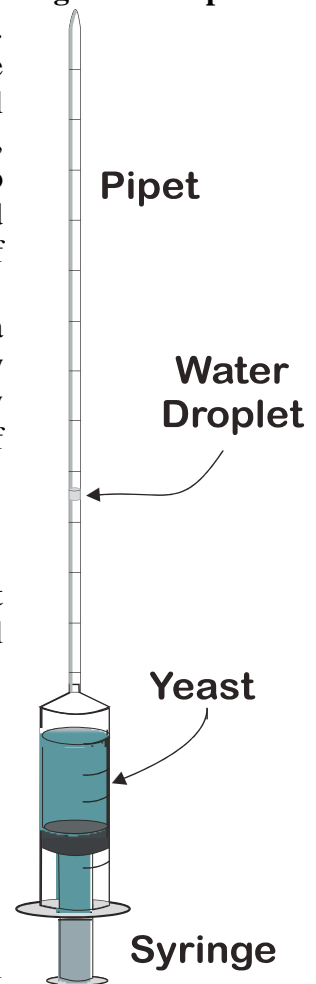
Experimental Procedure

In this week's lab you will study the rate of cellular respiration by yeast cells in the presence of a 5% (w/v) glucose solution. The procedure you will use is described below.

*** Each group member should perform his or her own experiment. ***

1. Add 10 ml of dH₂O to a 50 ml flask.
2. Weigh out 1.0 g of glucose, place it in the flask and gently swirl until the carbohydrate is completely dissolved.
3. Using a 10 ml pipet, transfer 10 ml of the yeast suspension into the flask.
4. Allow the yeast suspension to incubate for 5 minutes, with occasional swirling.

Figure 3. Respirometer



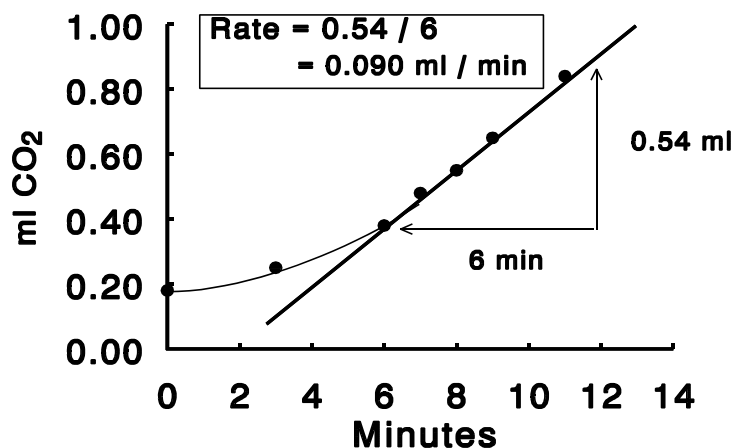
5. Take up exactly 3 ml of the yeast suspension into a syringe. Invert the syringe and draw 1 ml of air above the liquid.
6. Complete the assembly of the respirometer (don't forget the water droplet), and place it into your test tube rack.
7. As soon as the water droplet reaches the 0 ml mark, begin taking measurements at 1 minute intervals and record the data in the tables on page 89. You can mark measurements using either the top *or* the bottom of the droplet, but be consistent. You may need to decrease the time interval between measurements as the rate of CO₂ production increases.
8. Plot the raw data on graph paper, and draw a line through the linear part of the curve (Figure 4). Using only the **linear part of the data** calculate the slope of the line as change in volume ÷ change in time. This yields the rate of respiration in units of ml CO₂/ minute.

Table 1. Sample raw data

<u>Time</u>	<u>Reading</u>
0 min	0.18 ml
3	0.25
6	0.38
7	0.48
8	0.55
9	0.65
11	0.84

rate: 0.09 ml/ min
(calculated from graph)

Figure 4. Graphing fermentation raw data. The raw data (Table 1, the actual CO₂ volume measurements) is graphed as shown below. Note that the rate of fermentation is calculated from the slope of the line drawn through the linear part of the curve.



Analysis of results

The fermentation rate obtained by each student should be listed on the board. Each group should also calculate an average rate, and place this value on the board as well. These data will be used for a class discussion of the importance of replication in experimental design and potential sources of experimental error.

Cellular Respiration and Fermentation

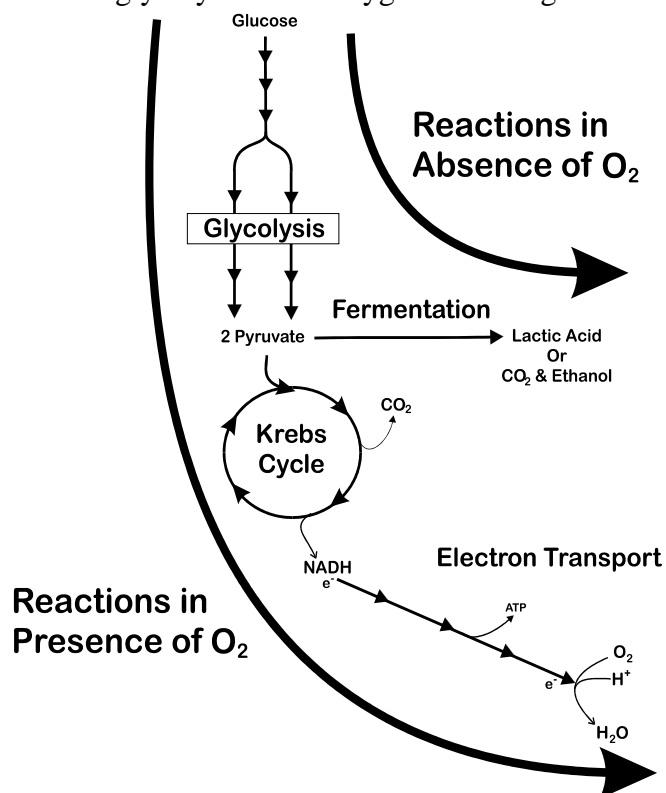
Before coming to the first lab of the Biofuel exercise, you should read the following sections on cellular respiration.

There are many factors that contribute to the economics of biofuel production, including cost of the raw material, efficiency of the conversion processes, and market value of ethanol. Economic conversion of starch to ethanol depends to a large extent upon the speed and efficiency of the yeast fermentation process, and biofuel industries are continuously modifying their processes to increase the speed and efficiency of the yeast fermentation process. The scientists and engineers responsible for improving industrial processes also must have a detailed knowledge of the cellular processes that are ultimately responsible for the conversion of sugar into ethanol. Fermentation is intimately associated with the energy yielding process called cellular respiration that occurs in essentially all cells, and you also must have some knowledge of this process to understand the results of this exercise.

What is cellular respiration?

Cellular respiration is a sequence of enzymatic reactions that extract energy from molecules, such as carbohydrates and fats, and make that energy available in the form of ATP. In most higher organisms, cellular respiration is an aerobic (oxygen requiring) process. The three principal stages of cellular respiration are called **glycolysis**, **Krebs cycle**, and the **electron transport chain** (Figure 5). These processes are an example of an ‘enzymatic pathway’, a sequence of chemical changes catalyzed by a series of different enzymes. During these processes, electrons are removed from the organic molecules (a process called ‘oxidation’) and the energy held in the electrons is eventually transferred to ATP. The breakdown of carbohydrates begins with glycolysis, and the oxidation is completed during the Krebs cycle. The energy held in the electrons is subsequently used to produce ATP during the electron transport process. Ultimately, the energy-depleted electrons are deposited on an oxygen atom, the ‘terminal electron acceptor.’

Figure 5. Overview of cellular respiration. Glycolysis, Krebs cycle and electron transport proceed in the presence of oxygen, but fermentation follows glycolysis when oxygen is lacking.



What is fermentation?

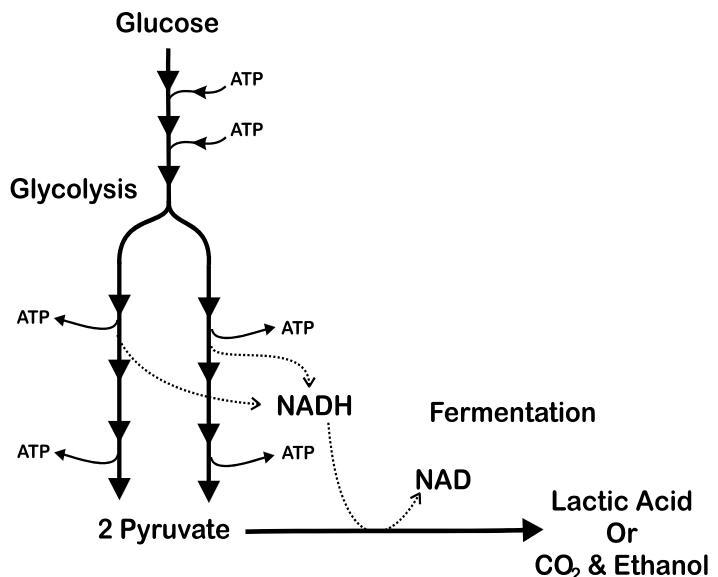
Fermentation is an enzymatic process that allows cells to obtain energy from carbohydrates even in the absence of oxygen. As described above, in most organisms (and all higher plants and animals) cellular respiration requires the presence of oxygen (such organisms are said to be **aerobic** – requiring of oxygen). If oxygen is not available to serve as the terminal electron acceptor, the electron transport pathway and then the Krebs cycle will shut down, just as an electric power plant that could not dispose of its waste products would eventually cease operations. (Each day a coal-burning electric power plant produces tons of flyash – the powdery ash collected from its smoke emissions – which must be hauled away continuously for disposal in landfills. If the dump trucks that haul away this waste were to stop running, the power plant would soon become so inundated with flyash that it would have to shut down.) In a similar manner, if oxygen is not available to carry away the electrons moving through the electron transport pathway, the whole process of cellular respiration will grind to a halt.

For aerobic organisms, the Krebs cycle and electron transport pathways do indeed cease to function in the absence of oxygen. However, glycolysis can continue if pyruvate is diverted to the fermentation pathway (Figure 6). The fermentation pathway provides a way for the cell to dispose of the pyruvate that otherwise would build up when the Krebs cycle is not functioning.

During the fermentation process, the NADH produced during glycolysis is converted back to NAD. Thus, the fermentation pathway utilizes NADH that the cell would otherwise use to produce ATP, but it does allow glycolysis to continue in the absence of O₂. Compared to aerobic respiration, the energy yield of glycolysis operating through fermentation is quite low, with a net yield of only 2 ATP per glucose.

Indeed, this energy yield is too low to sustain the life of higher organisms, and plants and animals cannot live on glycolysis and fermentation alone. Some cells can function for a while on the energy provided by fermentation; for example, during strenuous activity, muscle cells continue to function for a short period of time in this manner. However, the amount of ATP produced anaerobically is too low to support the tremendous energy demands of our bodies, and this is why higher organisms suffocate in the absence of oxygen.

Figure 6. Overview of the reactions that occur during fermentation. The NADH produced during glycolysis is converted back into NAD to allow conversion of pyruvate into ethanol or lactic acid. The result is a net yield of only 2 ATP.



How do yeast cells survive via fermentation?

In the presence of oxygen, yeast cells will carry out the complete pathway of cellular respiration. However, yeast cells can survive and reproduce via fermentation alone. The ethanol and CO₂ that are produced diffuse readily out into the environment surrounding the yeast cells. Single celled organisms can survive on fermentation alone for several reasons. On one hand, the energy needs of a single cell are much lower than those of a complex, multicellular organism. Also, yeast cells can import and metabolize carbohydrates very rapidly – in effect compensating for the inefficiency of fermentation by processing greater quantities of carbohydrate. As a result, the yeast release great quantities of CO₂ and ethanol during fermentation, which makes these organisms particularly useful to the biofuel industry.

WEEK 2

Before coming to the second lab session of the Biofuel exercise, you need to read the Biofuel Lab Internet Resource Page about the different variables that will affect yeast fermentation. During this lab period your group will discuss the variable your group will investigate during week 3, and also turn in a detailed experimental design and procedure. You will also learn how to use Excel to graph the data you obtained during week 1.

Some Considerations in Experimental Design

A scientist wishing to increase biofuel ethanol production might evaluate the effects of many different factors (or **variables**) on the rate of yeast fermentation. There are many variables that could potentially affect the rate of yeast fermentation. Indeed, an infinite number of factors (e.g., the alignment of the planets) might *potentially* affect the outcome of an experiment; however, a scientist seeking to optimize biofuel fermentation must use judgement to decide which variables are *most likely* to significantly affect the process. Some of these variables would include the type of yeast that is used, type of sugar available, concentration of the sugar, temperature, and pH of the solution.

A well designed experiment tests the effect of only ONE variable at a time, the one described in the hypothesis. As you have already learned, a hypothesis is an educated guess of the outcome of the experiment. Often we have little knowledge by which to formulate a hypothesis, but there is always some knowledge that can be applied. Some examples of hypotheses would include:

- Yeast fermentation is fastest in the presence of glucose.
- Fermentation rates decrease as temperature is increased.
- The presence of calcium will increase the rate of fermentation.
- etc..

Often, a single experiment will test a range of **conditions of the variable**. For example, a study of the effect of temperature may test the effects of three or more different temperatures, and an investigation of sugar concentration may test the effect of a range of different values.

The results of such experimental tests are always compared to a **control**. *A control is a test that is identical in every way to the other tests, EXCEPT in the variable that is being tested.* Thus, if one wanted to determine the effect of calcium on the rate of fermentation, the control would be a sample that lacked calcium but otherwise was the same. Sometimes more than one control test must be included to rule out other alternative explanations for the results. For example, someone might argue that the CO₂ released during this experiment is the result of chemical reactions that do not require living yeast cells (as was argued by some organic chemists in the early 1800s). What control would you suggest to ‘control for’ (rule out) this possible explanation?

What are some variables that affect the rate of fermentation in yeast cells?

Before leaving lab the first week, your group will select or be assigned a yeast fermentation parameter to investigate. The choices are listed below. Groups will be given the opportunity to select a topic, but some adjustment may need to be made to assure that each group in the class investigates a different topic. Further information about these factors is available in the web resource page, which can be accessed from <http://marietta.edu/~biol/introlab/index105.html>

Type of carbohydrate. What are the rates of CO₂ production when fermentation is allowed to proceed in the presence of glucose, fructose, lactose or sucrose?

Concentration of carbohydrate. What is the effect of glucose over a range of 0% to 2% on the rate of yeast fermentation?

Concentration of salt. How does varying the salt (NaCl) concentration over a range of 0 - 10% affect the rate of yeast fermentation?

Ethanol concentration. What are the effects of varying the ethanol concentration in the fermentation mixture from 0% to 10% on yeast fermentation?

pH. What is the effect of varying pH in the basic range (from pH 7 to pH 10) or in the acidic range (pH 3 to pH 7) on yeast fermentation?

Temperature. What are the effects on yeast fermentation of temperatures over a range of 0°C to 60°C?

Calculating Concentration

For all of these experiments you must calculate the amount of a material dissolved in the fermentation mixture. 'Concentration' is the most meaningful way to express this quantity. Concentration indicates the amount of a substance per unit volume of the liquid in which it is dissolved, and units of '% w/v' (percent weight/volume) or '% v/v' (percent volume/volume) are acceptable for this lab. When the substance being measured is added as a dry powder, '% w/v' should be used and is calculated by dividing the grams of the substance added by the final volume of the solution and multiplying by 100:

$$\% \text{ w/v} = \frac{\text{grams of substance added}}{\text{Final volume (ml) of solution}} \times 100$$

For example, in the first week's exercise you measured fermentation by yeast cells provided with 1 gram (g) of carbohydrate dissolved in a total volume of 20 milliliters (ml), which represents a concentration of 5% w/v.

When the substance being measured is added as a liquid, ‘% v/v’ should be used and is calculated by dividing the volume (ml) of the substance added by the final volume (ml) of the solution and multiplying by 100:

$$\% \text{ v/v} = \frac{\text{ml of substance added}}{\text{Final volume (ml) of solution}} \times 100$$

For example, to make a 5% solution of ethanol in a mixture with a final volume of 20 ml, you would need to combine 1 ml of ethanol + 19 ml of the other components.

Week 3

Before coming to lab this week you should read the following sections on writing a lab report and graphing results. During week 3 of this lab exercise you will perform the experiment that you planned. (The results should be graphed prior to coming to lab next week.)

Writing your lab report

You will prepare a lab report for the investigation that you designed and performed during the third week of this exercise. Each student must write an independent report and prepare his or her own graphs. The report will include an Introduction, Hypothesis, Procedures, Results, Discussion and Literature Cited sections. **General information about writing lab reports is provided in the Document entitled “Guidelines for Writing Lab Reports”.** Some specific information pertaining to this report is provided below and on the Web Resource Page.

Introduction

You should use information from at **least five of the literature sources** on reserve in the library for background information (see the web resource page for a list of these literature sources), since one of the goals of this exercise is to improve your ability to use library resources in an effective and meaningful way. In addition to these five literature sources, you can use your textbook, lab manual and web resource page as literature sources. The Background Information section should include information about fermentation, yeast, biofuel production, and the variable that your group is studying.

Procedures

You will thoroughly describe the experiment that your group performed this week, including the procedure for measuring yeast respiration described above. Remember, the procedure must be written in essay format, in the third person passive voice, and in enough detail for some else to repeat the experiment and obtain the same results. You are encouraged to include a picture of the respiration apparatus (with a figure legend).

Results

Tables and Figures

Note: all tables and graphs must be formatted (Figure numbers, title, axes labels, etc.) as described in the Lab Report Guidelines.

Your lab report should include:

1. Graphs of all raw data, prepared on Excel, with trendlines drawn through the linear region of the data. To conserve paper, plot the raw data for all conditions of your variable for a given experimental replicate in a single figure; all lines must be distinguishable and clearly labeled. **Do not include tables of the raw data.**
2. A table of calculated respiration rates for each replicate and the averages.
3. A summary graph showing average respiration rates vs your independent variable. You should use the graph format appropriate to your variable.

Description of Data

1. Describe the raw data for one typical replicate, but only describe any unusual aspects in the raw data from other replicates.
2. The summary graph that presents average fermentation rates represents the most important results of the experiment, and these results should be fully described.

Graphing Results — Independent vs dependent variables

It is not sufficient in a lab report to include only tables or graphs of the “raw data” (such as that shown in Table 1 and Figure 4). You must also summarize the calculated results in a suitable graphical form that reveals relationships between the variable you studied and the rate of fermentation. The results of your experiments will show the effect of some factor over a range of conditions (e.g., temperatures) on the rate of yeast fermentation. The results must be presented in an appropriate graphical form that best reveals any relationships that may exist. To do so, you must select the proper type of graph and plot the data correctly.

The “independent variable” must be plotted on the X-axis (abscissa) and the “dependent variable” must be plotted on the y-axis. The independent variable is the one that you controlled during the experiment (e.g., time, pH, temperature, or type of carbohydrate). The dependent variable is the value that was measured or calculated during the experiment. In this case the dependent variable was either CO₂ volume (ml) or the calculated respiration rate (ml CO₂/min). In a properly designed experiment, the measured value is dependent upon the value of the independently controlled variable.

The two types of graphs you are most likely to use are a “bar graph” and a “line graph”. **A bar graph is appropriate when there is not a mathematical relationship between the values of the independent variable.** For example, the results of a study of fermentation in the presence of different types of carbohydrates should be presented in a bar graph (see Figure 7) because there is no “mathematical” relationship between ‘types of sugars’.

Figure 7. Fermentation rate in presence of different types of sugars.

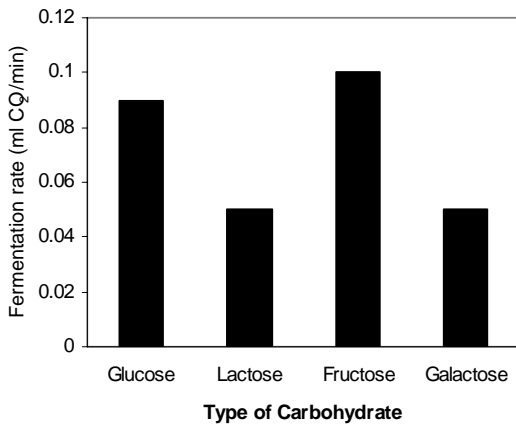
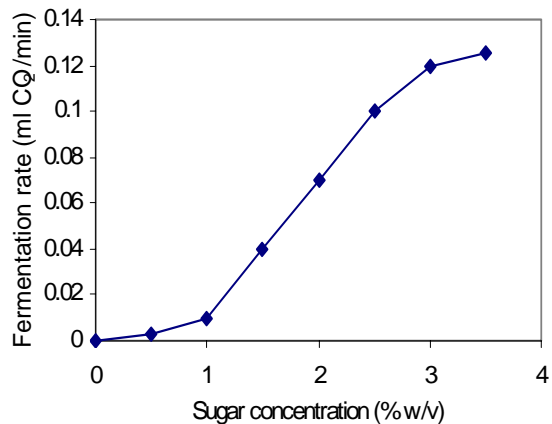


Figure 8. Rate of respiration in presence of different amounts of sugar.



However, a **line graph is only used when there is a mathematical or numerical relationship between the values of the independent variable.** Examples include amount of sugar added, temperature of incubation, incubation time, etc. (see Figure 8). Notice that the data points are clearly marked and connected by a line. This type of graph helps to reveal trends and relationships in the data that can be described and interpreted in the report.

Week 4

Prior to coming to lab for week 4 you should read the following section on interpreting results. You should also consult reference materials about your research topic provided in the library. Each student should independently graph the results obtained during the previous week on Excel. A copy of all graphs will be turned in. During the lab period each group will work together to interpret the results of the experiment and prepare a 10 minute oral presentation describing the procedure, results and interpretations.

Discussing and explaining your results

What conclusions and interpretations can be drawn from the results of your experiment? Certainly, the most important conclusion is whether the results support or contradict the hypothesis. You should always begin by describing how the results would appear if they support the hypothesis, and then explain how the actual results compare to these expectations. Remember, your *results will never prove* that your hypothesis is correct, but provide support for it. What should you do if your results are ambiguous and do not allow a basis for a firm assessment? In this case it is important that

you explain how the results are ambiguous. If the results contradict your hypothesis, then you should offer an appropriate alternative conclusion.

There are other issues that should be addressed as you interpret the results. Your interpretations for this experiment should assess whether the variable you studied showed an optimum or maximal value. The effect of quantitative variables (a factor that can be varied by a quantitative amount, e.g., temperature, amount of sugar, etc.) on a process generally show one of two patterns. When the rates from a series of experiments where the variable differs quantitatively are plotted, sometimes the rate of the reaction decreases at values above and below a particular **optimal value** (Figure 9). Other variables may show a **maximal value**, above which the rate remains constant (Figure 10). You should interpret results based upon your knowledge of cellular and/or enzymatic processes. Why might the variable you studied show an optimal or maximal pattern? What processes in the cell (properties of membranes, enzyme, pathways, etc) would account for the results you obtained?

Figure 9. Sample results showing an optimal value. These data show that the rate of fermentation is highest when the independent variable has a value of 5 (the optimal value).

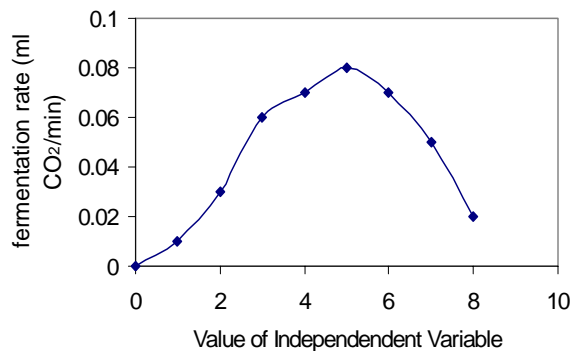
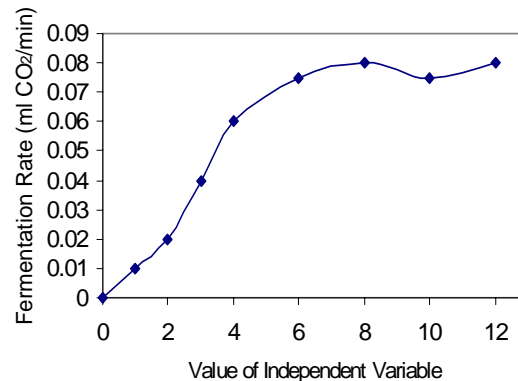


Figure 10. Sample results showing a maximal value. These data show that the rate of fermentation does not increase when the value of the independent variable exceeds 8 (the maximal value).



You should also explain how your results compare with the results of similar studies available in literature sources. You should consult the Biofuel Lab Internet Resource Page and reference material available in the library (some are on reserve).

You should also describe the implications of your findings in context of biofuel production. How would you apply your findings if you were actually attempting to improve the process? What are the implications for a biofuel production process when your variable shows a optimal or maximal value? Do you believe that your findings afford a means to significantly improve biofuel production? Why or why not?

Even if your results support your hypothesis, you should also propose a future experiment (a single, well described experiment is best); for example, you might suggest an experiment to refine your data, or possibly control for another variable. If your results contradicted your hypothesis or were ambiguous, you might propose an experiment that would test an alternative hypothesis.

Experimental Plan

Group members:

Each group will turn in a plan describing the experiment you will perform during week 3 of the Biofuel fermentation lab. The group should work together to plan the experiment and answer the questions. You should feel free to consult with the lab instructor as necessary.

1. What question are you seeking to answer? What variable will you be testing?
2. What will be your hypothesis?
3. What will be your control(s)?
4. Describe in detail the procedure you will use. (Write it neatly on the back.) Include a table that lists the components (and concentrations, where appropriate) that will go into each flask. What range of conditions will be tested; i.e., range of pHs, temperature, sugar concentration, etc.? Explain why this range was selected. Identify the controls(s) that will be included. State how many replicates will be performed. Consult with your instructor if necessary when answering these questions. **Do not include the procedure that is provided in the lab manual, include only those procedures that your group designs.**
5. How do you expect the results to appear if your hypothesis is correct? (Draw a hypothetical graph summarizing the expected relationship between your variable and rate of fermentation.)

