Edvo-Kit # 304

Edvo-Kit #304

The Future of Biofuels: Alcohol Fermentation

Experiment Objective:

Ethanol fermentation is the most common method for biofuel production worldwide. In this kit, students will use small-scale flask fermenters to quantify ethanol production and sugar utilization by Saccharomyces cerevisiae. By controlling variables such as temperature and aeration, the students can compare the efficiency of the fermentations over a three day experiment..

See page 3 for storage instructions.

Version 304.161013

SAMPLE LITERATURE
Please refer to included weblink for correct version

EDVOTEK®

Table of Contents

	Page
Experiment Components	3
Experiment Requirements	3
Background Information	4
Experiment Procedures	
Experiment Overview	8
Laboratory Safety	9
Module I: Planning Your Fermentation Experiment	10
Module II: Production of Ethanol in the Fermenter	11
Module III: Residual Sugar Test	13
Analyze the Results & Study Questions	15
Instructor's Guidelines	
Overview	16
Pre-Lab Preparations	17
Experiment Results and Analysis	19
Study Questions and Answers	21
Appendix A - How to Use the Hydrometer	23
Appendix B - Instructions for Quantitative Analysis of Residual Sugar	24

Safety Data Sheets can be found on our website: www.edvotek.com/safety-data-sheets

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2013-2016 EDVOTEK, Inc., all rights reserved. 304.161013

Experiment Components

	Component	Storage	Check (✓)
A	Yeast	4° C	❑
B	Yeast Growth Media concentrate	4° C	❑
C	Glucose Concentrate	4° C	❑
D	Ampicillin	4° C	❑
E	Yeast Growth Media	4° C	❑
F	10% Glucose Solution	4° C	❑
G	Benedict's Reagent 1	Room Temp.	❑
H	Benedict's Reagent 2	Room Temp.	❑
I	Benedict's Reagent 3	Room Temp.	❑

Experiment #304 contains material for up to 5 lab groups.

REAGENTS & SUPPLIES

Store all components below at room temperature.

- Transfer pipets ❑
- Snap top microcentrifuge tubes ❑
- Screw top microcentrifuge tubes ❑
- pH paper ❑
- Hydrometer ❑
- 50 ml centrifuge tubes ❑
- 15 ml centrifuge tubes ❑

Requirements *(NOT included in this experiment)*

- Stir plate and stir bars
- Thermometer
- Graduated cylinders (100 and 500 ml)
- Erlenmeyer Flasks (one 250 ml and five 1000 ml flasks are recommended)
- 70% Ethanol
- Distilled water
- Waterbath (99° C) EDVOTEK® #539 highly recommended
- Air pump and flexible plastic tubing (optional)
- Shaker incubator (Optional)
- Autoclave or Oven (Optional)
- Centrifuge (Optional - use if quantifying Benedict's test)
- Spectrophotometer and Cuvettes (Optional - use if quantifying Benedict's test)

We recommend that students use 100 ml graduated cylinders for measuring alcohol content and a 500 ml graduated cylinder to set up the fermenters.

EDVOTEK and The Biotechnology Education Company are registered trademarks of EDVOTEK, Inc.

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2013-2016 EDVOTEK, Inc., all rights reserved. 304.161013

Background Information

For over 6000 years, the process of fermentation has been used for food preservation. However, it was not until the 1850s that microbiologists, including Louis Pasteur, demonstrated that microorganisms were the agents responsible for fermentation. Researchers have since learned that fermentation is the result of these microorganisms breaking the chemical bonds in sugar and starch molecules to create energy. The byproducts of this process, lactic acid, ethanol and acetic acid, produce staple foods including yogurt, sauerkraut and wine.

Current technologies have extended the utility of fermentation, which can now be exploited to manufacture products as diverse as biofuels, biopharmaceuticals and fine chemicals. Today, studies into the fermentation process continue to yield new and exciting advances. For example, microbial geneticists have identified new strains of microorganisms that grow faster and generate a wide variety of vitamins or antibiotics. Genetic engineering and recombinant DNA have allowed scientists to produce large amounts of important proteins, converting cells into living factories. Insulin, which is a hormone used to control diabetes, was the first medication for human use that was produced by genetic engineering. Recombinant medicines, such as antibiotics, interferon and blood clotting factor VIII, have helped save millions of lives and improved the quality of life for millions more.

Today, commercially relevant fermentation products generally fall into one of four groups:

1. Metabolites naturally produced by the microbial cells:
   a. Primary metabolites that are produced during the normal growth, development, or reproduction of an organism: e.g., ethanol, citric acid, lysine, vitamins, polysaccharides.
   b. Secondary metabolites that are produced by an organism, but are not necessary for its normal growth, development, or reproduction: e.g., antibiotic production.
   c. Enzymes naturally produced by the microbial cells: amylase, protease, pectinase, cellulase, lipase, lactase, streptokinase.
2. Recombinant proteins expressed by microbial cells: e.g., insulin, interferon, clotting factor VIII, the Hepatitis B vaccine.
3. Chemical compounds modified by microbes (bioconversion): e.g., steroid biotransformation.
4. The microbial cells themselves: e.g., whole cell yeast extracts, baker's yeast, *Lactobacillus*, *E. coli*, etc.

The demand for these products has encouraged the development of novel technologies for genetic engineering, fermentation, and biomolecule purification. In particular, ethanol biofuel production has become one of the fastest growing segments in industrial fermentation.

UNDERSTANDING MICROBIAL GROWTH

Fermentation requires growth conditions that provide cells with oxygen, water, essential minerals and sources of carbon and nitrogen. Because each organism has different physical and chemical requirements for growth, the formulation can vary greatly depending upon the organism and the process. In a natural fermentation the growth conditions are provided by the food source being fermented. Conversely, in a controlled fermentation a specific growth medium is used to optimize conditions and maximize the yield of the process.

Microbial growth does not occur immediately upon inoculation of the selected nutrient medium. A post-inoculation period, called the lag phase, allows the cells to adapt to the new environment by synthesizing factors necessary for growth and cell division. Once acclimated to the growing conditions, the microbes enter log phase, time during which cells grow and division occurs at an exponential rate. This is the optimal stage for bioprocessing applications, as the biological machinery within the cells is primed for rapid growth and protein expression. Eventually the rate of growth within a culture slows due to

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2013-2016 EDVOTEK, Inc., all rights reserved. 304.161013

decreased nutrient availability, and an increased concentration of toxic compounds causes some cells to die. When the rate of cell death equals the rate of cell growth, the culture has entered what is referred to as stationary phase. The culture will persist in stationary phase until the nutrients are exhausted or until the toxins in the culture result in cell lysis. At this point, the cells enter the death phase and die at an exponential rate (Figure 1).

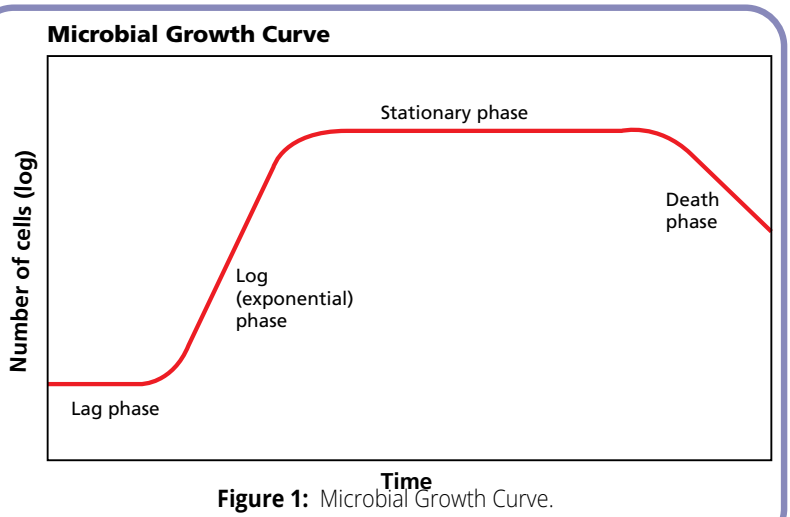


Figure 1: Microbial Growth Curve.

FERMENTATION VESSELS (BIOREACTORS OR FERMENTERS)

In practice, fermentation requires the careful selection of culture conditions to keep cells in a favorable state that allows for the production of the desired product. Cells are grown in a piece of equipment known as a fermenter (or bioreactor), which is fitted with sensors that continuously monitor the environmental conditions during the fermentation process (Figure 2). This information is used to optimize culture conditions. Some of the factors fermenters can control include temperature, oxygen levels, pH, antifoaming agents, and the rate of mixing.

Fermenters can be used to grow cultures on vastly different scales. While small cultures (1-10 liters) can be grown, fermenters are especially useful for very large culture volumes (> 1,000 liters). However, a large-scale fermentation reaction cannot be started in such a large volume. Instead, a very small "stock" culture (5-10 ml) of cells is grown, which is then used to inoculate a somewhat greater volume (200 to 1,000 ml) of fresh medium (Figure 3). When these cultures reach log phase growth, they are, in turn, used to inoculate an even larger volume (10-100 liters) in a seed fermenter. As its name suggests, the seed culture is then used to "seed"—or serve as the initial source of cells for—the final culture, grown in a production fermenter (1,000 to 100,000 liters).

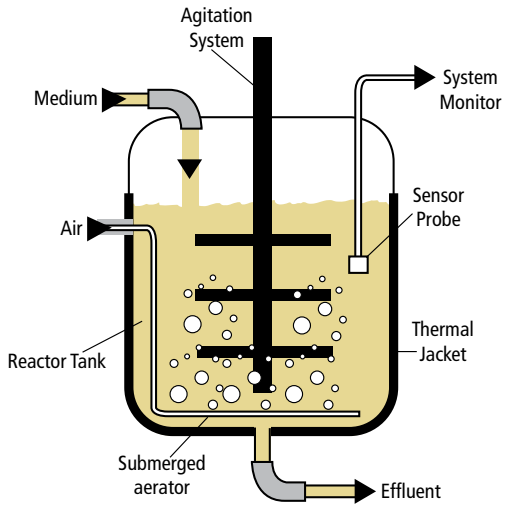


Figure 2: Example of Bioreactor.

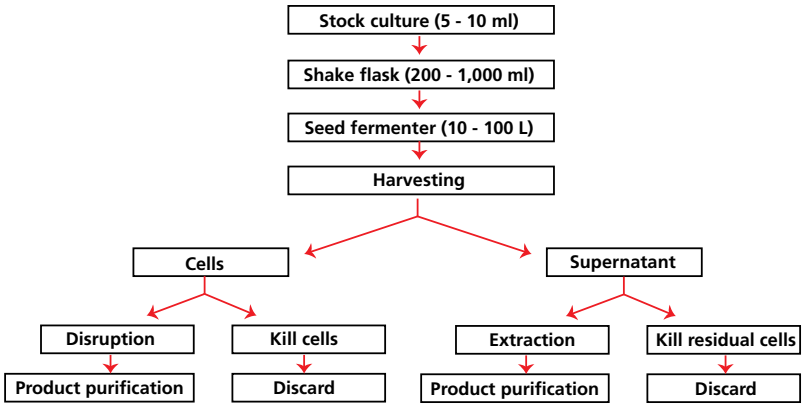


Figure 3: Schematic for large-scale fermentation process.

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2013-2016 EDVOTEK, Inc., all rights reserved. 304.161013

ETHANOL FERMENTATION

Ethanol is an organic compound produced as a metabolic waste product of sugar metabolism (Figure 4) that has been used since ancient times for its intoxicating effects. Arab and Roman communities learned to isolate ethanol for use as a solvent in the preparation of perfumes, cosmetics and medicines. Today, ethanol fermentation is commonly used for the production of alcoholic beverages, chemical solvents, baked goods, and ethanol fuel.

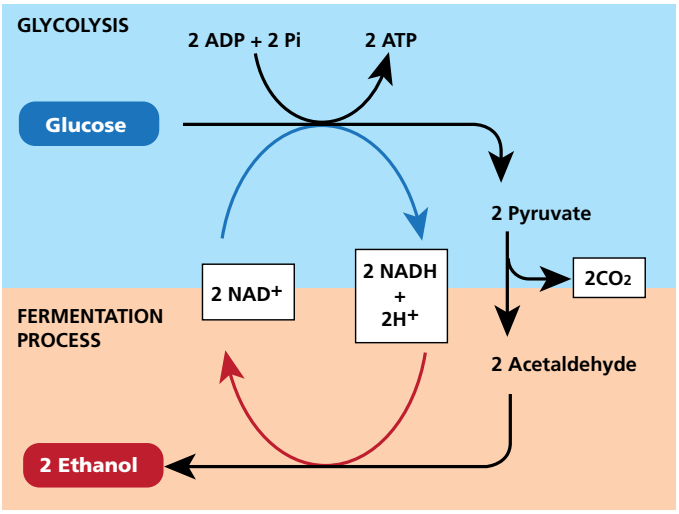


Figure 4: Overview of glycolysis and the fermentation process.

Fermentation is actually the second biochemical process in sugar metabolism. The preceding process, known as glycolysis, consists of two distinct phases: an "Energy Investment Phase" and an "Energy Pay-Off Phase".

During the investment phase, ATP is used to digest glucose into simple, three-carbon sugars. In the energy pay-off phase, these simple sugars are modified to produce ATP, pyruvate, and an energy-containing molecule called NADH (nicotinamide adenine dinucleotide). At the end of glycolysis, four molecules of ATP have been produced: two that recoup the initial ATP investment and two new ATP molecules.

Unlike plants and animals, which can use NADH to generate even more ATP, most microorganisms can only produce ATP using glycolysis. For glycolysis to continue, it needs a fresh supply of NAD+. Therefore, NADH must first be recycled to NAD+. The process of fermentation regenerates NAD+ by using NADH in the conversion of pyruvate into other molecules. For example, some microorganisms convert pyruvate into lactic acid, a feature that makes them useful to the dairy industry for production of yogurt and cheese. Other microorganisms, such as the *Saccharomyces cerevisiae* used in this experiment, can also convert pyruvate into ethanol.

S.cerevisiae is capable of utilizing many different sugars, including sucrose, fructose, and maltose, all of which are enzymatically converted to glucose before fermentation. Because ethanol fermentation is an important step for the production of baked goods and alcoholic beverages, *S.cerevisiae* is commonly known both as baker's yeast and brewer's yeast.

Each step of glycolysis and fermentation is tightly coupled to other metabolic activities so that the cell does not waste energy. Under normal circumstances, roughly 95% of the sugars consumed by microbes appear as ethanol and carbon dioxide after fermentation. The remaining 5% of the initial sugar carbon appears as other compounds, including building blocks for new cells.

IMPORTANCE OF ETHANOL IN THE ENERGY INDUSTRY

Our nation's quest for energy independence relies on the development of renewable energy sources, including ethanol, solar, wind and geothermal energies. Scientists and engineers are continually developing new technologies to make these energy sources more cost efficient and commercially competitive. These technologies allow us to create clean energy while reducing our dependence on non-renewal sources of energy like coal and petroleum.

Biofuels are combustible fuels derived from plant material like starches and cellulose. Ethanol is an example of a common biofuel produced by the fermentation of these plant materials. Unadulterated ethanol can be used as a fuel for vehicles; however it is usually used as a gasoline additive to increase octane rating and reduce vehicle emissions of carbon monoxide, nitrogen oxides and hydrocarbons. Over the past ten years, biofuels have become an increasing part of the world's planned energy mix. By 2022, 36 billion gallons of biofuels are slated for use in the United States, amounting to approximately 11% of the country's transportation fuel (Figure 5).

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2013-2016 EDVOTEK, Inc., all rights reserved. 304.161013

In this experiment, alcohol will be produced by growing yeast in a small-scale fermenter. Students will explore the contributions of temperature and pH on alcohol production. Over the course of the experiment, students will monitor the culture and harvest samples for analysis. The production of ethanol will be measured by using a hydrometer, while concentration of remaining sugars will be analyzed using the Benedict's residual sugar assay. Together, this data will allow students to measure the efficiency of the fermentation and determine the most successful fermentation conditions between groups.

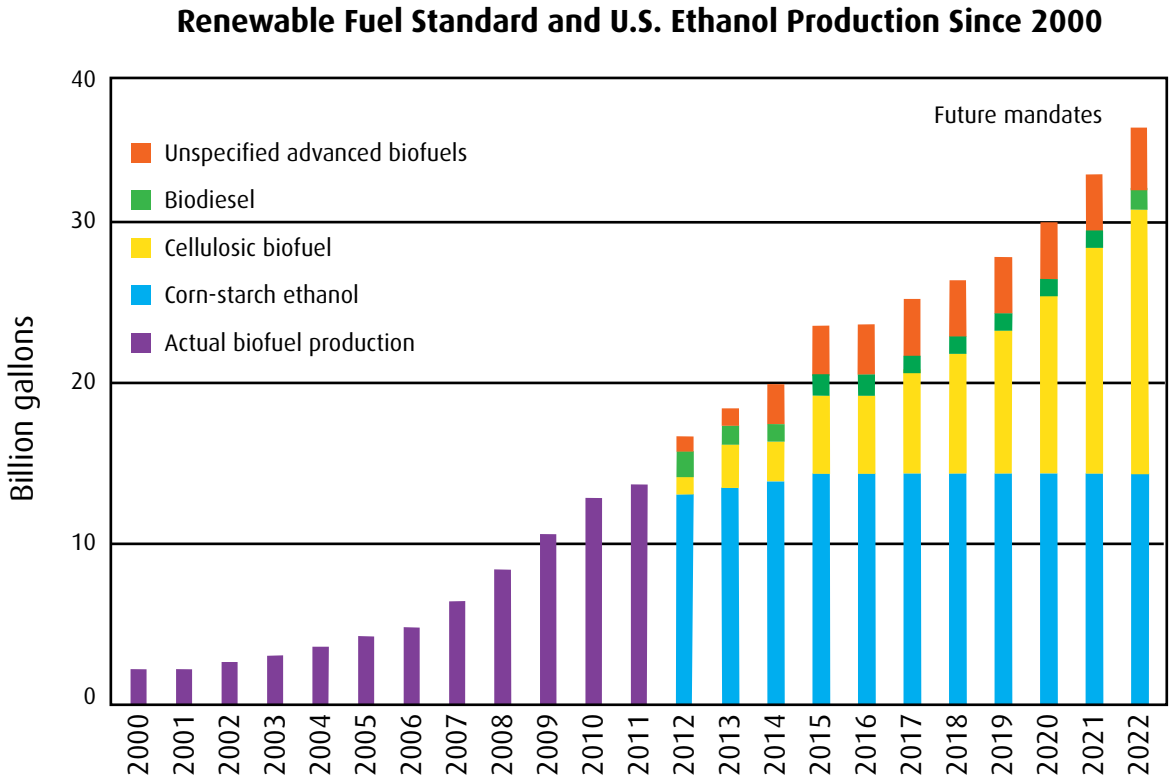


Figure 5:
Comparison of biofuel production in the US during the last 12 years.

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2013-2016 EDVOTEK, Inc., all rights reserved. 304.161013

Experiment Overview

EXPERIMENT OBJECTIVE

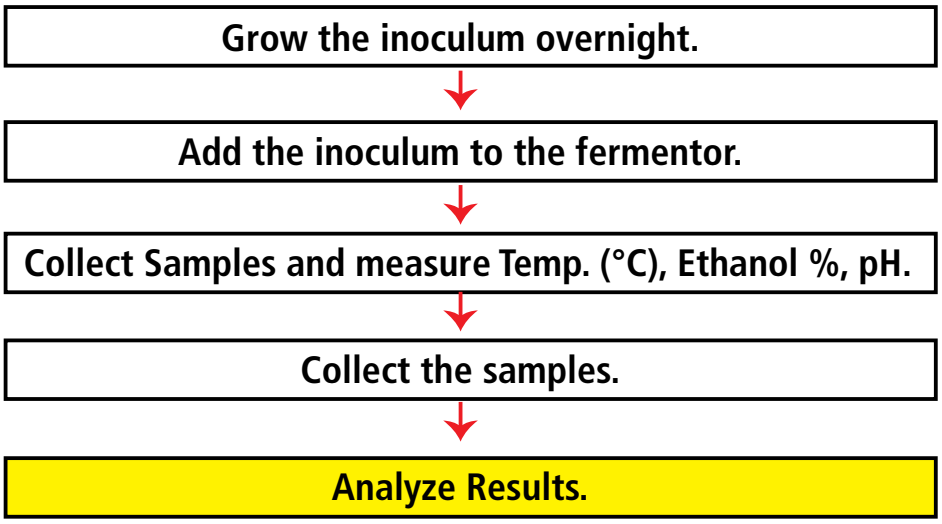
Ethanol fermentation is the most common method for biofuel production worldwide. In this kit, students will use small-scale flask fermenters to quantify ethanol production and sugar utilization by *Saccharomyces cerevisiae*. By controlling variables such as temperature and aeration, the students can compare the efficiency of the fermentations over a three day experiment.

BRIEF DESCRIPTION OF THE EXPERIMENT

In this experiment, alcohol will be produced by growing yeast in a fermenter. The production of ethanol will be measured by using a hydrometer to help to determine the optimal growth conditions for production of alcohol.

Over the course of the fermentation, students will monitor the process parameters (temperature, pH, and alcohol production) at regular intervals. Cells will be harvested from the fermenter at regular intervals for Ethanol quantification. Samples collected over the course of the experiment will then be measured for glucose content using the Benedict's residual sugar assay. Using an Excel spreadsheet, we will analyze the data to determine the relationships between these parameters and the efficiency of the fermentation.

EXPERIMENT OVERVIEW



Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2013-2016 EDVOTEK, Inc., all rights reserved. 304.161013

Laboratory Safety

IMPORTANT -- READ ME!

This experiment contains antibiotics that are used to keep cultures free of contamination. Students who have allergies to antibiotics, including **AMPICILLIN,** should not participate in this experiment.

It is good practice to follow simple safety guidelines in handling and disposal of laboratory materials.

Wear gloves and safety goggles

1. Wear gloves and goggles while working in the laboratory.
2. Exercise extreme caution when working in the laboratory – equipment used for heating and melting reagents can be dangerous if used incorrectly.
3. Do not mouth pipet reagents - use pipet pumps or bulbs.
4. Properly dispose materials after completing the experiment:
    a. Wipe down the lab bench with a 10% bleach solution or a laboratory disinfectant.
    b. All materials, including pipets, transfer pipets, loops and tubes, that come in contact with bacteria should be disinfected before disposal in the garbage. Disinfect materials as soon as possible after use in one of the following ways:
        - Autoclave at 121° C for 20 minutes.
          Collect all contaminated materials in an autoclavable, disposable bag. Seal the bag and place it in a metal tray to prevent any possibility of liquid medium or agar from spilling into the sterilizer chamber.
        - Soak in 10% bleach solution.
          Immerse open tubes and other contaminated materials into a tub containing a 10% bleach solution. Soak the materials overnight and then discard. Wear gloves and goggles when working with bleach.
5. Always wash hands thoroughly with soap and water at the end of each laboratory period.
6. If you are unsure of something, ASK YOUR INSTRUCTOR!

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2013-2016 EDVOTEK, Inc., all rights reserved. 304.161013

Module I: Planning Your Fermentation Experiment

LABORATORY NOTEBOOKS:

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. During this experiment, you will be documenting your experiment in a laboratory notebook or on a separate worksheet.

BEFORE STARTING THE EXPERIMENT

- As a group, determine which temperature and aeration conditions you would like to examine for your fermentation.

  Temperature (ie: Room temperature, 30˚C):_____

  Aeration (ie: Stir bar, shaker, air pump):_____

- Carefully read the introduction and the protocol. Use this information to form a hypothesis for the experiment and record it in your laboratory notebook or below.

_____

_____

_____

_____

- Familiarize yourself with the Hydrometer and determine which scale you will be using (Potential alcohol, Balling, or specific gravity). Record your choice below - it is important to use the same scale for each measurement throughout the experiment. See Appendix A for more information.

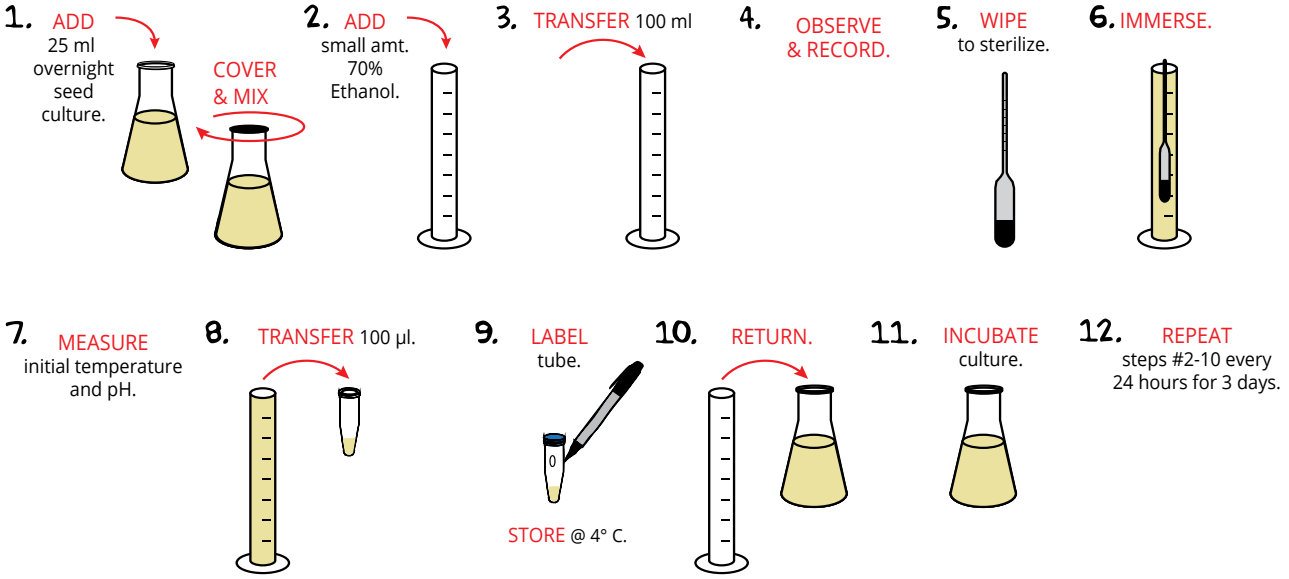
_____

EDVO-TECH Service
1.800.EDVOTEK
Mon. - Fri. 8am-5:30pm
Please Have the Following Info:
• Product Number & Description
• Lot Number on Box
• Order/Purchase Order #
• Approx. Purchase Date
Fax: 202.370.1501 • info@edvotek.com • www.edvotek.com

www.edvotek.com
• Online Catalog
• Order Products
• Experiment Protocols
• Tech Support
• Resources!

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2013-2016 EDVOTEK, Inc., all rights reserved. 304.161013

Module II: Production of Ethanol in the Fermenter



1. **INOCULATE** the media in your flask by adding 25 ml of the overnight seed culture. **COVER** the flask with a lid or aluminum foil to prevent contamination and gently swirl to **MIX**.
2. **ADD** a small amount of 70% Ethanol to a graduated cylinder to sanitize. ***NOTE: ROTATE the cylinder to ensure all surfaces are coated. Remove alcohol and allow the graduated cylinder to dry before continuing.***
3. **TRANSFER** 100 ml of your inoculated culture into a graduated cylinder.
4. Carefully **OBSERVE** the color, clarity, and smell of the culture. Record the observations in Table 1.
5. **WIPE** the alcohol hydrometer with 70% ethanol to sterilize.
6. **IMMERSE** the alcohol hydrometer in the culture and **RECORD** the gravity value in Table 1 or your lab notebook. For detailed instructions refer to Appendix A: How to Use a Hydrometer.
7. Using the thermometer and pH paper **MEASURE** the initial temperature and pH values and **RECORD** the values in Table 1 or your laboratory notebook. ***NOTE: In a typical culture the starting pH should be around 6.0 and the alcohol content should measure 0%.***
8. Using a sterile transfer pipet, **TRANSFER** 100 µl of the culture into a screw top microcentrifuge tube.
9. **LABEL** the tube with your initials and the time that the sample was taken. **STORE** the tube at 4° C for later analysis.
10. **RETURN** the culture from the graduated cylinder to the flask.
11. **INCUBATE** the culture using your experimental outline – temperature, aeration, etc.
12. **REPEAT** steps 2 through 10 every 24 hours for 3 days. You will collect four data points, including the initial data.

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2013-2016 EDVOTEK, Inc., all rights reserved. 304.161013

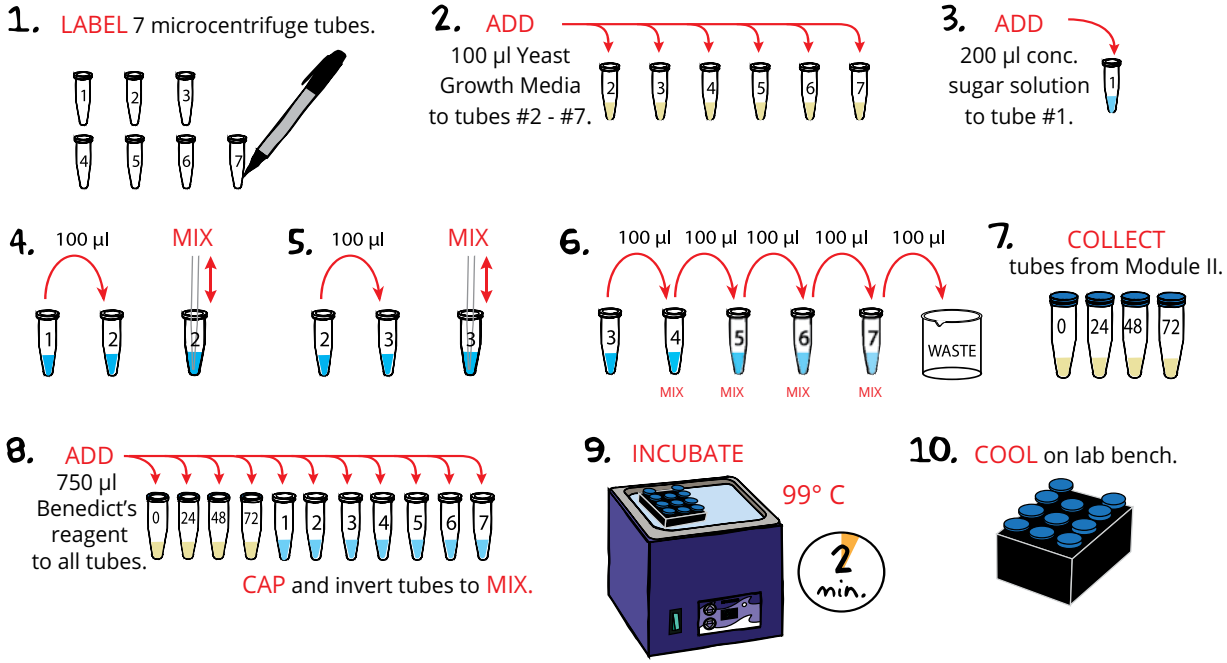
Module II: Production of Ethanol in the Fermenter, continued

Table 1 - Measurements from Module II - Production of Ethanol

	0 Hours	24 Hours	48 Hours	72 Hours
Odor				
Color				
Clarity				
Temperature				
pH				
Gravity				
% Alcohol				

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2013-2016 EDVOTEK, Inc., all rights reserved. 304.161013

Module III: Residual Sugar Test



The Benedict's test allows for a visual analysis of the concentration of reducing sugars in a solution. By creating a standard curve of known solutions, researchers can determine the approximate concentrations of unknown samples. To do this, you will perform a 2 fold serial dilution of sugar to water, creating seven samples of known sugar concentration. After performing the fermentation, these samples will be used to create a standard curve that establishes the relationship between sugar concentration and sample color intensity.

1. **LABEL** 7 screw top microcentrifuge tubes #1 through #7.
2. **ADD** 100 µl of Yeast Growth Media to tubes #2 through #7.
3. **ADD** 200 µl of the 10% sugar solution to tube #1.
4. **PIPET** 100 µl from tube #1 into tube #2. **MIX** the sample by gently pipetting up and down 5 times.
5. With a new pipet tip, **TRANSFER** 100 µl from tube #2 into tube #3 and **MIX** as in step 4.
6. Continue to serially **DILUTE** the remaining samples through tube #7. Discard 100 µl from tube #7 into a waste beaker.
7. **COLLECT** your experimental samples from 4° C.
8. **ADD** 750 µl Benedict's reagent to each screw top tube. **INVERT** each tube several times to mix.
9. **INCUBATE** the samples in a 99° C water bath for two minutes.
10. Carefully **REMOVE** the samples from the water bath and place them on your bench to cool.
11. **EXAMINE** the samples and **COMPARE** the experimental results with the standard curve. **RECORD** your results in Table 2.

NOTE:
A yellowish precipitate may appear in the samples. The tubes can be inverted to resuspend the precipitate while comparing colors to the standard curve.

Optional: The Benedict's test can be quantified by measuring absorbance at 735 nm. See Appendix B for the complete protocol.

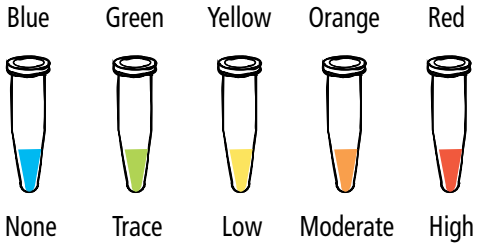
Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2013-2016 EDVOTEK, Inc., all rights reserved. 304.161013

Module III: Residual Sugar Test, continued

Table 2 - Results from Benedict's Solution test for sugar concentration

	Color	Sugar Concentration	Optional: Absorbance at 735 nm
Standard Curve #1			
Standard Curve #2			
Standard Curve #3			
Standard Curve #4			
Standard Curve #5			
Standard Curve #6			
Standard Curve #7			
0 Hours			
24 Hours			
48 Hours			
72 Hours			

COLOR OF SOLUTION



RESIDUAL SUGAR

Observation	Interpretation
No Color Change (Blue)	No non-reducing sugars present
Green	Trace amonts of non-reducing sugars present
Yellow	Low amounts of reducing sugars present
Orange	Moderate amount of reducing sugars present
Red	High amounts of reducing sugars present

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2013-2016 EDVOTEK, Inc., all rights reserved. 304.161013

Analyze the Results

Compile the classroom results in an Excel file. Plot time vs. pH, time vs. % ethanol, time vs. % residual sugar, and % ethanol vs. % residual sugar.

Study Questions

1. What variables need to be controlled during a fermentation to have a positive experiment?
2. When is the optimal growth phase for a fermentation? Why?
3. What are the main applications of ethanol fermentation in industry?
4. Why is there so much interest in expanding biofuel production in the US?
5. Upstream bioprocessing involves optimizing the microbial growth conditions in order to produce the maximum amount of a product. As a Bioprocess Engineer you have decided to change the temperature and the pH of your fermentation in order to increase the ethanol yield. You performed the following experiments and collected the following data.

   Using this data, calculate the average ethanol production and the standard deviation of each. Plot your results using graph paper or a computer graphing program.

   Which conditions would you use?

Ethanol (g/L)			
Temp. (°C)	Trial 1	Trial 2	Trial 3
25	16.1	15.4	16.2
30	19.6	20	19.5
35	18.1	17.9	17.4
40	14.3	14.1	13.4

Ethanol (g/L)			
pH	Trial 1	Trial 2	Trial 3
4.5	18.5	19.1	18.9
5	16.1	15.9	15.3
5.5	13.4	13	12.4
6	10.5	9.5	9

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2013-2016 EDVOTEK, Inc., all rights reserved. 304.161013

Instructor's Guide

IMPORTANT - READ ME!!

This experiment contains antibiotics that are used to keep cultures free of contamination. Students who have allergies to antibiotics, including AMPICILLIN, should not participate in this experiment.

ORGANIZATION AND IMPLEMENTING THE EXPERIMENT

Prior to starting this experiment, carefully check the list of Components and Requirements on page 3 to ensure that you have all the necessary components and equipment.

The guidelines presented in this manual are based on five laboratory groups. The experiment is divided into three modules which should take approximately one week to perform. The following are implementation guidelines, which can be adapted to fit your specific set of circumstances.

APPROXIMATE TIME REQUIREMENTS

Preparation for:	What to do:	When:	Time Req.:
Module II: Production of Ethanol in the Fermentor	Prepare and sterilize Yeast Growth Media	Up to one day before performing the experiment.	2 hours
	Prepare seed culture	One day before performing the experiment.	1 hour
	Gather materials for students	Anytime before performing the experiment.	10 min.
Module III: Residual Sugar Test	Prepare and aliquot reagents	Up to one day before performing the experiment.	20 min.
	Prepare the waterbath	One hour before performing the experiment.	10 min.

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2013-2016 EDVOTEK, Inc., all rights reserved. 304.161013

Pre-Lab Preparations

MODULE II PREPARATIONS

Preparation of the Fermenters

Each group will maintain one flask fermenter. We recommend using 1000 ml flasks with 500 ml of media, although smaller volumes can be used. Always ensure that the flask contains appropriate headspace; for best results we recommend filling flasks to no more than 50% capacity.

Sterilization of Lab Material

Successful ethanol fermentation depends heavily on keeping the yeast cultures free from contamination by microorganisms such as bacteria, fungi, and viruses. All materials that come into contact with the flask fermenters must be sterile, and manipulations must not allow any direct link between the cultures and the non-sterile surroundings.

To prevent contamination, the flasks, graduated cylinders, and stir bars used for this experiment must be sterilized. Many different techniques can be utilized to sterilize the equipment, please check with manufacturers to ensure heat or chemical resistance before selecting the method.

Autoclave: Cover the openings of the equipment with aluminum foil. Autoclave at 121° C for 20 minutes. ***NOTE: Autoclave indicator tape should be used to ensure that proper temperatures have been achieved.***

Dry Heat (Baking): Place components into a preheated oven at 170° C and bake for 60 minutes. Carefully remove the equipment and cover any openings with aluminum foil while still hot.

Cleaning with Alcohol: Rinse with 70% Ethanol, ensuring coverage of all surfaces. Allow equipment to air dry before covering any openings with aluminum foil.

NOTE:
We recommend you sterilize one 500 ml graduated cylinder, five 100 ml graduated cylinders, five 1000 ml flasks, one 250 ml flask, and magnetic stir bars per class.

Preparation of Yeast Growth Media

The yeast growth media can be prepared up to 48 hours before beginning the experiment. The Yeast Growth Media concentrate and Glucose concentrate provided in this kit are sterile. Distilled water can be purchased or briefly boiled to sterilize prior to preparing the final media.

1. Follow the table below to **PREPARE** the media you need for the experiment.
   NOTE: We recommend 5 groups with 500 ml cultures each, but smaller volumes can be used if necessary. Media can be mixed in individual volumes or as one large volume and then aliquoted.
2. **DISPENSE** 500 ml of media into 5 sterile flasks. Retain the remaining media to prepare seed cultures.
3. **ADD** 0.6 ml of sterile water to the tube of Ampicillin (Component D). Invert to mix.
4. **ADD** 0.1 ml of the Ampicillin solution to each 500 ml flask of media. Swirl to mix.

Final Volume	Yeast Growth Media Concentrate (Component B)	Glucose Concentrate (Component C)	Distilled Water
500 ml	80 ml	62.5 ml	357.5 ml
1000 ml	160 ml	125 ml	715 ml
2800 ml	450 ml	350 ml	2000ml

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2013-2016 EDVOTEK, Inc., all rights reserved. 304.161013

Pre-Lab Preparations, continued

Preparation of the Seed Culture

1. **ALIQUOT** 125 ml of prepared Yeast Growth Media in a sterile 250 ml Erlenmeyer Flask.
2. **ADD** 25 µl of Ampicillin to the Media.
3. **ADD** the entire contents of the Yeast vial (Component A) to the media. Gently swirl to mix, ensuring that the yeast is completely dissolved.
4. **INCUBATE** the flask for 24 hours in an incubated shaker at 30° C at 200 RPM (see note).
5. **ALIQUOT** 25 ml of culture in 50 ml conical tubes to each student group.

NOTE:
The seed culture can be grown at room temperature if an incubated shaker is unavailable. Aeration of the culture by shaking, stirring, or bubbling is highly recommended.

Fermenter Preparation

1. **PREPARE** one 1 L Erlenmeyer flask, one 100 ml graduated cylinder, 4 transfer pipets, and 4 screw top microcentrifuge tubes for each group.
2. **PREPARE** any optional equipment that will be used to incubate or aerate fermenters. This includes air pumps, stir plates, and shaker incubators.
3. **GATHER** the hydrometer and pH paper for the class.

For Module II, each group should receive:

- 1 Erlenmeyer flask
- 1 Graduated cylinder
- 4 Transfer pipets
- 4 Screw top microcentrifuge tubes
- 1 Tube of seed culture
- 70% Ethanol

MODULE III PREPARATIONS

Preparation of Residual Sugar test

1. **DISSOLVE** Benedict's Reagent 1 and 2 (Components G and H) in 50 ml distilled water in a 250 ml beaker.
2. **DISSOLVE** Benedict's Reagent 3 (Component I) in 25 ml distilled water in a small beaker.
3. Slowly **ADD** the entire volume of the Component I solution to the beaker containing the Component G/H solution and mix well. This is the completed Glucose Testing Reagent.
4. **DISPENSE** 10 ml of the Glucose Testing Reagent into 15 ml tubes for each group.
5. **ALIQUOT** 250 µl of the 10% Glucose Standard solution (Component F) into five snap top microcentrifuge tubes. Label tubes as "10% Sugar".
6. **ALIQUOT** 1 ml of Yeast Growth Media (Component E) into five snap top microcentrifuge tubes.
7. **GATHER** 10 transfer pipets and 7 microcentrifuge tubes for each group.
8. **PREPARE** a 99° C waterbath.

For Module III, each group should receive:

- 1 tube of Glucose Testing Reagent
- 1 tube of Yeast Growth Media
- 1 tube of 10% sugar
- 7 screw top microcentrifuge tubes

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2013-2016 EDVOTEK, Inc., all rights reserved. 304.161013

Experiment Results and Analysis

Below are results from Modules II and III from an experiment comparing a fermentation at room temperature with no aeration to a fermentation at 30˚C with a stir bar. Your results may vary depending on a number of factors, including the length of incubation, temperature, and accuracy of pipetting.

MODULE II

Flask #1 - Room Temperature, no aeration

	0 Hours	24 Hours	48 Hours	72 Hours
Odor	None	Light, bread	Strong, bread, sweet	Strong, bread, sweet
Color	Light brown	Light brown	Tan	Tan
Clarity	Clear	Hazy	Cloudy	Cloudy
Temperature	27˚C	27˚C	27˚C	27˚C
pH	6.0	6.0	5.0	5.0
Gravity	1.050	1.040	1.035	1.005
% Alcohol	0%	1.3%	1.9%	5.8%

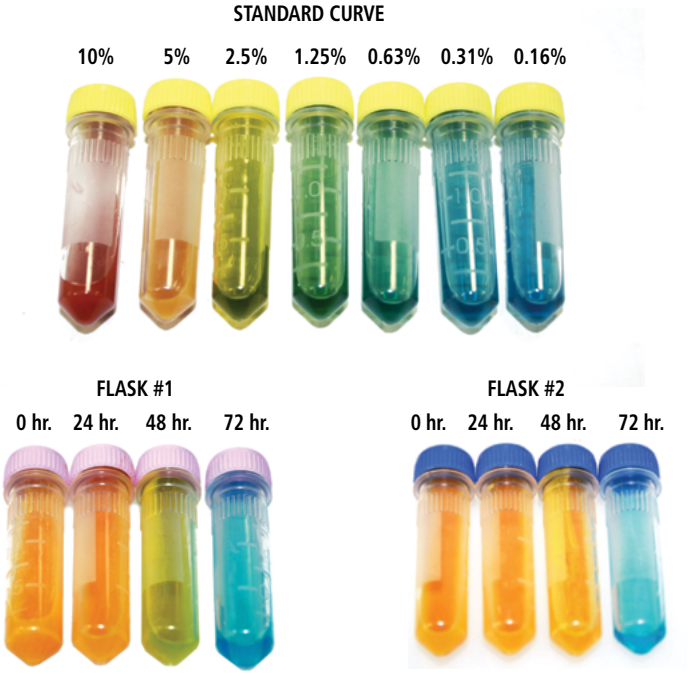
Flask #2 - 30˚C with aeration

	0 Hours	24 Hours	48 Hours	72 Hours
Odor	None	Light, yeasty	Strong, bread	Strong, bread, alcohol
Color	Light brown	Light brown	Tan	Tan
Clarity	Clear	Hazy	Cloudy	Cloudy
Temperature	30˚C	30˚C	30˚C	30˚C
pH	6.0	6.0	5.0	5.0
Gravity	1.050	1.040	1.010	1.000
% Alcohol	0%	1.3%	5.2%	6.4%

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2013-2016 EDVOTEK, Inc., all rights reserved. 304.161013

Experiment Results and Analysis

MODULE III



MODULE III - OPTIONAL RESULTS

Table 3 - Optional Results for Absorbance at 735 nm

Tube	Color	Abs at 735 nm	% Sugar
1	Red	0.0	10
2	Orange	0.0	5
3	Yellow-Green	0.947	2.5
4	Green	1.90	1.25
5	Green-Blue	2.533	0.63
6	Blue	3.135	0.31
7	Blue	3.135	0.16
Flask 1 - 0 hr.	Orange	0.0	4.59
Flask 1 - 24 hr.	Orange	0.0	4.59
Flask 1 - 48 hr.	Orange-Green	0.782	3.40
Flask 1 - 72 hr.	Blue	2.799	0.34
Flask 2 - 0 hr.	Orange	0.0	4.59
Flask 2 - 24 hr.	Orange	0.021	4.56
Flask 2 - 48 hr.	Orange	0.049	4.51
Flask 2 - 72 hr.	Blue	3.2	0

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2013-2016 EDVOTEK, Inc., all rights reserved. 304.161013

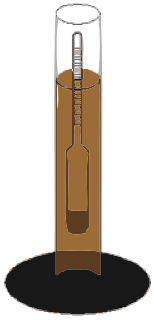
Please refer to the kit insert for the Answers to Study Questions

Please refer to the kit insert for the Answers to Study Questions

Appendix A

HOW TO USE THE HYDROMETER

Most of the hydrometers made today have three scales of measurement: potential alcohol, Balling, and specific gravity. In this experiment, we recommend using potential alcohol or specific gravity. Potential alcohol measurements indicate the maximum percentage of alcohol that could be produced from the solution in a perfect fermentation. Specific gravity measures the density of a substance relative to water. A simple calculation can be performed to convert specific gravity into percent alcohol.



1. **CLEAN** all equipment that will come in contact with your fermentation using 70% Ethanol. This includes the hydrometer and graduated cylinder.
2. **TRANSFER** 100 ml of the media into the graduated cylinder.
3. **PLACE** the hydrometer in the graduated cylinder.
4. **CHECK** that the hydrometer is not in contact with the sides of the cylinder.
5. With the sample at eye level, **OBSERVE** where the liquid crosses the markings.
6. **RECORD** the reading in your lab notebook.

NOTE: Typically the initial value (time 0) will be approximately 5% potential alcohol, or a specific gravity of 1.046.

To determine the amount of alcohol using the potential alcohol you subtract the second reading from the first. For example if your first reading was 5% and your current reading is 1%; take 5-1=4%, for a total of 4% alcohol in your solution

To determine the amount of alcohol using the specific gravity scale you need to subtract the second measurement from the first, then divide by 0.776 to convert to percent alcohol.

$$\frac{(\text{Specific Gravity 1} - \text{Specific Gravity 2})}{0.776}$$

For example if your first reading was 1.050 and your current reading is 1.010, take

$$\frac{(1.050 - 1.010)}{0.776} = \frac{0.04}{0.776} = 5.2\%$$ alcohol in your solution.

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2013-2016 EDVOTEK, Inc., all rights reserved. 304.161013

Appendix B

INSTRUCTIONS FOR QUANTITATIVE ANALYSIS OF RESIDUAL SUGAR

Using a spectrophotometer it is possible to use the Benedict's Residual Sugar Assay to quantify the percentage of sugar in your solution. By measuring the absorbance of solutions of known concentration (your standard curve) at 735 nm, we can establish a relationship between sugar concentration and absorption. This relationship is described by the equation of the standard curve. Using the equation we can then estimate the percentage of sugar in the fermentation samples.

1. **CENTRIFUGE** the cooled samples from Module III, step 11 at max speed for 2 minutes.

NOTE: It is important to fully remove any precipitate from the solutions. Suspended particles will interfere with the absorption measurements.

2. Gently **TRANSFER** the supernatant to a cuvette and **MEASURE** the absorbance at 735 nm.

NOTE: The spectrophotometer should be blanked with water before measuring your samples.

3. Create a standard curve.

    a. Plot the Percentage of residual sugar (x-axis) against the Absorbance at 735 nm (y-axis) for each concentration in your standard curve.

    b. Draw a best-fit curve through the points on the graph (for best results we recommend using graphing software). Record the equation for your curve for later use (Figure 6).

4. Determine the concentration of sugar in the fermentation samples.

    a. Find the absorbance values of the fermentation samples as in step 1 and 2.

    b. From the y-axis of the standard curve graph, extend a horizontal line from the absorbance vale to the standard curve. At the point of intersection extend a vertical line to the x-axis and read the corresponding concentration (Figure 7). Alternatively, use the equation of the best-fit curve to solve for x.

NOTE: Depending on your absorption readings the highest values of the standard curve might be saturated. In this case, absorption values that fall outside of the standard curve will be inaccurate.

Figure 6: Plotting a Standard Curve.

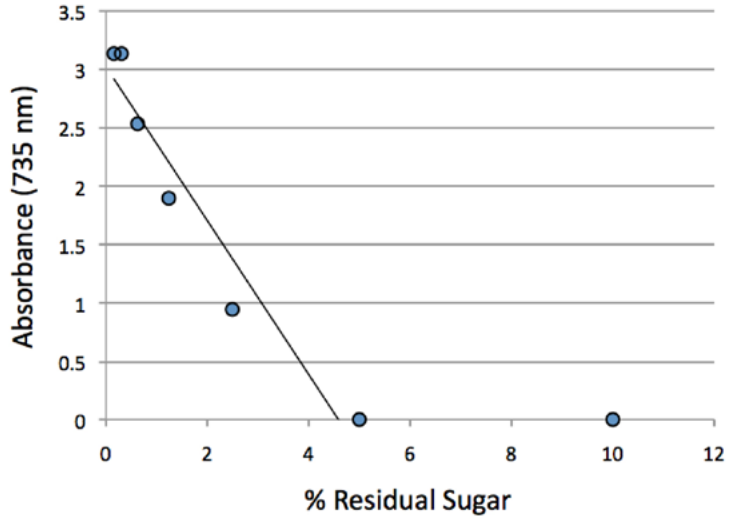
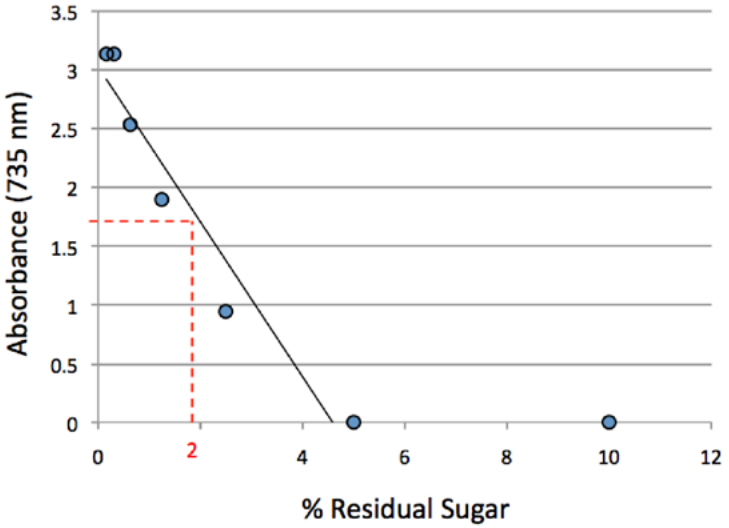


Figure 7: Determining Percent Residual Sugar



Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2013-2016 EDVOTEK, Inc., all rights reserved. 304.161013