Ethanol from Fermentation

Learning Objectives:

- Enhance participant understanding of fermentation processes and chemistry.
- Enhance participant understanding of microbial growth processes.
- Provide opportunity for participants to gain hands-on experience with state-of-the art fermentor and fermentation monitoring equipment.
- Provide opportunity for participants to practice aseptic techniques.

Learning Outcomes:
Upon completion of this lab, participants will be able to:

- List what cells need to grow and the ways microorganisms use carbon.
- Describe the stages of typical cell growth.
- Prepare cell growth media and set-up a fermentation using aseptic techniques.
- Use analytical equipment to measure cell growth, and glucose and ethanol concentration over the course of the fermentation.
- Compare aerobic and anaerobic growth conditions.
- Present experimental data as graphs and yield factors.

Pre-Lab

Background

Yeast Fermentation

Fermentation is generally defined as the conversion of carbohydrates to acids or alcohols. The conversion of corn sugar (glucose) to ethanol by yeast under anaerobic conditions is the process used to make the renewable transportation fuel, bioethanol.

A fermentor is operated by inoculating a complex sugar medium with a microorganism. This microorganism is generally allowed to reproduce under aerobic conditions before the fermentor is switched to anaerobic conditions to produce secondary metabolites such as ethanol.

Carbon sources such as glucose ($C_6H_{12}O_6$) serve two purposes: material building blocks for biosynthesis and for energy. In this experiment, yeast uses the glucose in three ways:

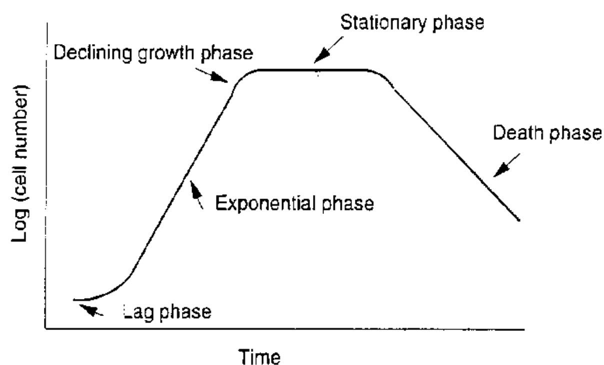
Energy: $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$ + energy
Growth (anaerobic or aerobic): $C_6H_{12}O_6$ (+ O_2) $\rightarrow$ biomass
Secondary metabolites: $C_6H_{12}O_6 \rightarrow$ 2 ethanol + $2CO_2$

This experiment will track three parameters throughout the fermentation: glucose concentration, ethanol concentration and yeast cell mass. Glucose and ethanol concentrations will be analyzed by the YSI biochemistry analyzer. Yeast cell mass will

be measured using the spectrophotometer to collect OD (optical density) readings. With these data, the amount of glucose consumed by each pathway can be calculated.

Growth Curves

In batch culture conditions, microbial growth typically follows a five phase cycle from inoculation to cell death. These phases can be seen below as an average over all *live* cells in the population. Growth curves based on OD readings will look slightly different using OD readings since live and dead cells are not distinguishable using absorbance.



Typical growth patterns of microorganisms in a batch reactor based on live cell counts
from Blanch, H.W., D.S. Clark, *Biochemical Engineering*, Marcel Dekker, New York (1996), 183.

The first phase, or *lag phase*, occurs directly after inoculation as the microorganism adapts metabolically to its new environment. The second phase is the *exponential* or *logarithmic growth phase* where cells exhibit *balanced growth*, meaning that cell mass and cell density grow exponentially with time at the same rate.

$$X = X_0 e^{\mu t}$$

X and X_0 are cell mass concentrations (g/L) at time t (hours) and μ (per hour) is the specific cell mass growth rate. The exponential growth period is characterized by a straight line on an *ln*(X) vs. time growth curve. The time to double cell mass, τ_d (hours), can be calculated from:

$$\tau_d = \frac{\ln 2}{\mu} = \frac{0.693}{\mu}$$

At some point during the exponential growth phase, the depletion of one or more essential nutrients, or the build-up of toxic growth by-products, reaches a point where rapid balanced growth is no longer possible. During this third phase, the *deceleration* or *declining growth phase*, cells have to again metabolically adapt, this time to increase cell survival potential rather than maximize cell growth. In batch cultures, this phase is generally very short.

The growth of the cells slows until the growth rate is zero (growth rate = death rate) and the cells enter the *stationary phase*. During this time, cells' metabolism shifts from producing primary growth-related metabolites (more cells) to secondary, non-growth, metabolites (ethanol). Cells must break down or *catabolize* their reserves for new energy and building-block monomers to keep up an energized cell membrane, nutrient transport, and cell structure repair. This process is called *endogenous metabolism* and provides *maintenance energy*. In the case of this fermentation, the start of the stationary phase occurs shortly after the reactor is switched to anaerobic conditions, increasing the production of ethanol, which is eventually toxic to the yeast.

The final phase is the *death* or *decline phase*. At this point, the depletion of nutrients or the build-up of toxins is so great that the cell death rate exceeds the growth rate.

Determining Yield

One way to track the fermentation is to calculate *yield factors*, written as a capital Y with two subscripts denoting product and substrate. For example, $Y_{X/S}$ is the yield factor for amount of cell mass (g of yeast) per amount of substrate (g of glucose consumed), and is equal to:

$$Y_{X/S} = -\frac{\Delta X}{\Delta S}$$

The yield factor can be calculated at the end of the fermentation to evaluate overall performance, but also for a specific time period during the fermentation to compare the rates of greatest growth. Typical yield factors observed for *S. cerevisiae* grown aerobically on glucose are around 0.5 g/g, with significantly lower yields under anaerobic conditions.

$Y_{P/S}$ stands for the amount of product (g of ethanol) produced per amount of substrate (g of glucose) consumed and is calculated the same way:

$$Y_{P/S} = -\frac{\Delta P}{\Delta S}$$

The amount of glucose used for cellular energy can be estimated by a simple mass balance equation and approximate stoichiometric equations relating the amount glucose consumed to the amount of ethanol or biomass produced:

$$\Delta S = \Delta S_{energy} + \Delta S_{biomass} + \Delta S_{ethanol} \qquad (9)$$

Aseptic Techniques

When working with any microorganism, avoiding contamination is vitally important. This prevents other organisms from depleting necessary nutrients and/or producing inhibitory metabolites.

Among the aseptic techniques that you will use in this lab are: disinfecting surfaces with 70% ethanol; sterilizing media and equipment under steam pressure in an autoclave; using disposable, radiation-sterilized syringes; and preventing airborne contaminants from entering the fermentor environment by clamping off tubing and attaching 0.2 μm filters, which is smaller than the size of all microorganisms, onto exhaust and inlet vents.

Cell Mass and OD Readings

In spectrophotometry, there are two ways to measure the amount of light that passes through a sample: transmittance and absorbance. Transmittance, T, is a ratio of the power of radiation exiting the sample, P_T, to the power of the radiation entering or *incident* to the sample, P_0, and is normally reported as a percent; 100% transmittance means that all the light passed through the sample and 0% transmittance means that no light passed through the sample.

The second way of measuring, absorbance, A, is related to transmittance:

$$A = -\log T = -\log \frac{P_T}{P_0}$$

Absorbance is more frequently used in analysis since it can be directly related to the concentration of the sample—a relationship known as *Beer's Law*:

$$A = abC \tag{2}$$

where a is the analyte's absorptivity in units of cm^{-1} $conc^{-1}$ (the most common is molar absorptivity, ε, with units of $cm^{-1}M^{-1}$), b is the length of the light path through the sample, i.e. the width or diameter of the cuvette in centimeters, and C is the analyte concentration. This linear relationship, however, is generally only dependable in the *linear range* (A = 1.0 for most analytes); to be on the safe side, fermentation samples with absorbances higher than 0.6 should be diluted.

The wavelength of light most commonly used for optical density (sometimes called *turbidity*) readings is 600-650 nm. Here, 620 nm is used, which is a yellow/orange color. Since other components in the medium can also absorb at 620 nm, it is important to make the blank as close to the actual samples as possible (i.e. same medium components, same size/material cuvette, etc.) For this reason also, OD dilutions should be made with blank medium rather than DI water.

This experiment uses a calibration curve to relate absorbance to dry cell mass. The calibration curve was constructed by measuring the absorbance of a sample, then taking a known volume of that sample, centrifuging it to remove the media supernatant, rinsing it with water and re-centrifuging it, then drying the cell pellet in an oven on a pre-weighed sample dish to get the dry cell weight.

Pre-Lab Tasks

1. In this fermentation, what are three ways that yeast use carbon (in the form of glucose)? Try to make some generalizations about what microorganisms need (chemically), keeping in mind that there are organisms that can fulfill their "building block" and energy needs using things like carbon dioxide, carbon monoxide, sulfur, ammonia, etc.
2. Find three other chemicals that can be produced from fermentation besides ethanol. Do these fermentations use yeast or some other organism? What are the substrates and the products?
3. In this fermentation, glucose is used as the substrate. As a pure sugar, however, it is rather expensive. List some other substrates that could be used, especially if they would typically be a byproduct of another process.
4. Brainstorm why yeast might produce ethanol during fermentation if ethanol is eventually toxic to them.
5. Why is it important to make a media blank for preparing OD dilutions? Why not just use water?
6. What aseptic techniques will be used in this lab? List some other disinfecting or sterilization processes used in everyday life.
7. Using the ethanol production equation, calculate the theoretical maximum yield factor, $Y_{P/S}$ = g/L ethanol / g/L glucose, if 20 g/L glucose was consumed.

$C_6H_{12}O_6$ (glucose)→ 2 C_2H_5OH (ethanol) + $2CO_2$

Molar masses: C = 12 g/mol, H = 1 g/mol, O = 16 g/mol

Fermentation Preparation and Monitoring

Safety Checklist

- o Proper attire is worn (long pants and closed-toe shoes).
- o Food and drinks are stored and consumed outside the laboratory.
- o Lab coat and safety glasses are worn.
- o Latex or nitrile gloves are used when handling samples and chemicals.
- o Insulated gloves are used when handling hot materials.

Problem Statement

Jill Saccharomyces and Bob Cerevisiae have decided to form a new business, *Saccharomyces Cerevisiae*, making both yeast and fuel ethanol. Jill has been making moonshine in her backyard and Bob has been making yeast to sell to brewers. Since Jill's past fermentations have been relatively slow processes, she has doubts about Bob's claim that yeast can grow and produce significant alcohol in an 8-hour day. She does not want to invest in equipment until she is sure. Bob has asked your group to demonstrate that this can be done using a small-scale fermentor and a sample of his brewers' yeast.

Preparing Media and Sterilizing Fermentor

Background

The fermentor used in this experiment is a 5L New Brunswick BIOFLO 310 fermentor. The components of the fermentor include:

- *Glass fermentation vessel* with stainless steel base containing a water heat exchanger and baffles insert
- *Stainless steel head plate* containing a drive shaft with impellers (agitation), a level sensor, an air sparger ring (aeration), an exhaust condenser, and ports for other components
- *RTD probe* in thermowell to monitor temperature
- *pH probe* to monitor pH
- *dO_2 probe* to monitor dissolved oxygen
- *Sterile sampling port* uses a vacuum created by a bulb to take samples aseptically from the fermentation broth
- *Acid, base and addition ports* for pH control and aseptic additions of nutrients
- *Peristaltic pumps* for addition of acid, base, and media components
- *Addition bottles* with filters and tubing
- *Primary control unit (PCU)* with touch screen for monitoring and adjusting fermentation conditions and cables for connecting to probes

Pre-experiment Checklist

- Media components, all fermentor parts, glassware, pH meter, stir/hot plates, acid and base solutions, and autoclave supplies are available.
- Autoclave is available to be used.

Media Composition

(per 1 L working volume)
20 g of peptone
10 g yeast extract
50 g glucose (dextrose)
Note: Glucose solution is prepared and autoclaved separately to prevent it from caramelizing; preparation volumes should give a combined volume of 3 L.

Media Prep Procedure

1. Place 4L Erlenmeyer flask on stir plate and add stir bar. Add 2000 ml of distilled water to the flask and turn on stirring.
2. Weigh out 60 g of peptone using beaker and bulk balance. Carefully add to flask. Peptone absorbs water and can become sticky on surface. Clean up any spills as soon as possible.
3. Weigh out 30 g of yeast extract and bulk balance. Carefully add to flask.
4. Rinse down side of flask by adding another 500 ml of distilled water. Stir until solution is no longer cloudy (several minutes).
5. Calibrate pH meter and then hang pH probe into flask so that the bulb on the end is just under the solution surface.
6. Adjust the pH of the solution to 4.5 (as read by the pH meter) by adding 10% sulfuric acid drop-wise with a plastic transfer pipette while stirring.
7. Empty flask of medium solution into fermentor. Add two drops of antifoam.
8. Using 1 L flask, prepare OD blank solution by adding 6 g peptone and 3 g yeast extract to 300 ml of distilled water. Stir until dissolved. Label and cover flask with aluminum foil.
9. Using another 1 L flask, slowly add 50 g glucose into 200 ml of DI-water *while stirring*. Heat the solution on a hot plate to dissolve completely.
10. Dilute glucose solution to 450 ml with distilled water and pour into labeled 500 ml addition bottle.
11. Cover a 125ml beaker with aluminum foil (used for yeast later).

Fermentor Preparation and Sterilization

1. Attach fermentor head plate:
    a. Apply thin layer of lubricant around top surface of glass vessel.
    b. Set head plate in place and secure sliding metal ring using four bolts.
    c. Tighten bolts evenly.
2. Calibrate the pH probe:
    a. Remove probe from 4M KCl storage bottle and rinse with DI water.
    b. Connect probe to PCU with cable.
    c. On PCU, select Calibrate button, then pH probe.

   d. Immerse pH probe in 7.00 buffer and allow Raw Input signal to stabilize.
   e. Enter the value 7.00 in box below Set Zero button and press enter.
   f. Press Set Zero button to accept value.
   g. Recap 7.00 buffer solution and rinse probe with DI water.
   h. Enter the value 4.00 in box below Set Span button and press enter.
   i. Press Set Span button to accept value.
   j. Recap 4.00 buffer and rinse probe with DI water.
   k. For best results, repeat calibration.
   l. Insert probe into fermentor carefully, making sure it has a tight seal and the end is covered in solution. (Do leave probe dry or in DI water!)
3. Prepare the fermentor for autoclaving:
   a. Insert dO_2 probe into fermentor.
   b. Place black rubber bearing cover on top of bearing housing to prevent steam damage.
   c. Clamp off the air sparger tube.
   d. Clamp off all tubes that have penetrations which go below the internal liquid level to prevent boiling over during autoclaving.
   e. Remove the RTD from the thermowell.
   f. Remove cables from pH and dO_2 probes; cover probe tops with shorting caps to prevent steam damage.
   g. Disconnect water lines from exhaust condenser and base heat exchanger.
   h. Loosen glass sample bottle on sterile sampler about ½ turn.
   i. *Make sure that at least one exhaust point is working* (such as the exhaust condenser) *to prevent pressure build-up.* It is also good to leave a spare port plug loosely set in its hole, which can be re-tightened immediately after autoclaving.
   j. Make sure all solutions are covered with aluminum foil, marked with indicator tape and labeled, and that the sugar addition bottle is clamped.
4. Autoclave the fermentor
   a. Get autoclave cart from BRL 1125.
   b. Set fermentor vessel upright on cart and all other solutions in the plastic autoclave pan.
   c. Move fermentor and solutions into autoclave in BRL using cart.
   d. Close autoclave doors by cranking wheel all the way counterclockwise.
   e. On touch screen, turn on jacket power.
   f. Press Select Cycle and enter “444” as the user ID.
   g. Select autoclave cycle for 45 minutes at 121°C.
   h. Autoclave will start automatically and cycle will take about 1 hour.
   i. After autoclaving, carefully remove vessel and solutions from autoclave using heat-resistant gloves and set-up in lab.
   j. Connect water and air lines; attach pH and dO_2 probes to PCU; replace RTD in thermowell; reattach motor drive.
   k. Turn on PCU power so probes can polarize overnight.

Fermentation

Background
Fermentation parameters to be used are:
- Agitation rate = 300 rpm
- Temperature = 32°C
- pH = 4.5-5
- Media initial glucose concentration = 50 g/L
- Yeast inoculum = 50 ml Old Main Lager yeast sample (may contain some ethanol)

Fermentation will be aerobic for 3-4 hours until noon, when air will be turned off and fermentation will e allowed to continue under anaerobic conditions for another 4 hours. Sample will be taken at time 0 and every hour thereafter to measure optical density, glucose concentration and ethanol concentration. Try to prepare samples for analysis as soon as possible after they are taken so that the fermentation does not continue in the sample vial before analysis. Base (NaOH) will have to be added to the fermentor periodically as fermentation byproducts acidify the media to keep the pH about 4.5.

Pre-experiment Checklist
- YSI biochemistry analyzer has fresh membranes, glucose standard, ethanol standard, ethanol buffer.
- Fermentor and additional solutions are prepared and sterilized.
- Probes have been polarizing for at least 12 hours.
- Yeast culture, all fermentor parts, sample vials, spectrophotometer curettes, micropipetter, plastic consumables, volumetric flasks, centrifuge, and NaOH solution are available.

Beginning Fermentation
1. Turn on spectrometer to warm up. Set absorbance dial to 620 nm and pull out filter pull until orange dot is showing.
2. Make sure water is connected correctly to base heat exchanger and exhaust condenser, then turn on the water by opening the valve on the wall just before the water regulator/filter system. Adjust flow with valve until a slow but steady flow drains into the sink.
3. Heat the fermentor to 32ºC by changing temperature control from Off to Auto, and setting Set Point to 32.
4. While vessel is heating, add glucose solution by threading addition bottle tubing through the lowest peristaltic pump (in the bottom and out the top) and connecting it to one tube on the addition tri-port. Remove the clamps.
5. On the Pumps screen, change the pump power setting to 100% until as much glucose solution as possible has been transferred into the fermentor. Re-clamp the addition tri-port tubing before disconnecting addition bottle.
6. After the glucose has been added and the fermentor is at temperature, set the aeration rate by opening up the air line (*turn the red dial on the wall counter*

clockwise until gas flows—do not adjust the regulator pressure) and selecting O2 Enrichment under the Air tab.

7. Set the agitation by changing the agitation control from Off to Auto, and entering the fermentation speed of 300 rpm into Set Point.
8. Calibrate the dO_2 probe:
   a. On PCU, select Calibrate, then dO_2 probe.
   b. Type 0 into the box below the Set Zero, but *do not press Set Zero yet*.
   c. Momentarily disconnect the cable from the dO_2 probe.
   d. When Raw Input stops decreasing, press Set Zero.
   e. Type 100 into the box below the Set Span button (this tells the controller that this is the max amount of oxygen available for the selected aeration rate, medium and agitation speed).
   f. When Raw Input stops increasing, press Set Zero button.
9. Check that pH is between 4.5 and 5. Thread the 4N NaOH addition bottle feed through the middle (base) peristaltic pump and connect to an unused addition tri-port tube.
10. Turn off air flow by pressing Off under the Air tab (this will keep air from bubbling into the NaOH addition bottle while the pump is being primed).
11. Prime the NaOH addition pump by removing the clamps, entering the Pumps screen and repeating pressing and holding the Prime button on the middle (base) pump's control. Continue priming the pump until NaOH is just about to drip into the fermentor.
12. Turn aeration back on.

Inoculation

13. Pour about 50 ml of Old Main yeast sample into sterilized beaker. Keep covered with foil as much as possible.
14. Remove sterile 60cc syringe from packaging and fill with yeast sample from beaker.
15. Remove foil covering inoculation port tubing and connect syringe to luer lock fitting.
16. Holding syringe plunger, unclamp inoculation tubing and slow push down on plunger until sample has been added.
17. Reclamp inoculation port tubing before removing syringe.
18. Repeat if not all of yeast sample fit into one syringe.
19. Let the fermentor rest (no agitation) for about 10 min while you clean up inoculation glassware, wipe down area with 70% ethanol solution, and fill "blank" spectrophotometer cuvette with blank solution.
20. Start fermentation: reset the agitation to 300 rpm. Under Trends menu, hit Clear button to reset data collection.
21. Immediately take the first sample by opening the red valve on the sterile sampling port, squeezing the red bulb to clear sample tube and create a vacuum, then slowly releasing the red bulb. Sample should flow up into the attached vial. Close red valve and replace full sample vial with an empty one.
22. Sample every hour. After last sample, export trend data with USB drive.

Sample Preparation for Analysis

1. OD analysis: fill glass spectrophotometer cuvette with sample.
2. Zero out spectrophotometer by placing blank cuvetter in spectrophotometer, closing the lid, and adjusting transmittance dial on the front until the instrument reads an absorbance of .00. Remove blank cuvette and set in accessible spot.
3. Wipe down outside of sample cuvette with Kim wipe and place inside spectrophotometer. If the absorbance reading is higher than 0.6 (most will be), use the micropipette and 10 ml volumetric flask to make a more dilute sample and reanalyze. (500µL to 10 ml is a 20x dilution and tends to work well.)
4. Record dilution factor and absorbance.
5. Glucose/ethanol analysis: pour about 1.5 ml of the remaining sample from fermentor into a microcentrifuge tube.
6. *Balance centrifuge* (with water blank of the same volume). Centrifuge for 60 sec at 10,000 rpm (yeast cells form a pellet at the bottom).
7. Remove 1 ml (2 x 500 µL) with the micropipette from the top of the microcentrifuge tube and add to 10 ml volumetric flask. Dilute with DI water. The maximum concentrations for glucose and ethanol on biochemistry analyzer are 9 g/L and 2.0 g/L, respectively.
8. Fill small test tube about 2/3 full with sample, cover with parafilm, label, and place on YSI analyzer turntable starting at position 1.
9. Have instructor demonstrate sample analysis. Record dilution factor, glucose concentration and ethanol concentration.

Clean-up

1. Disconnect fermentor:
    a. Set temperature control to OFF.
    b. Set agitation to OFF and remove drive motor.
    c. Disconnect fermentor from air sparger line, RTD probe, pH and dO_2 probe cables, base and addition lines.
    d. Remove, rinse and store the pH and dO_2 probes.
    e. Carefully remove NaOH addition bottle tubing from peristaltic pump. Disconnect tubing from addition tri-port such that excess NaOH in tubing drains into fermentor or back into addition bottle.
    f. Loosen nut holding sterile sampler in place and lift out assembly for easier cleaning. Wipe down metal tubing and rinse out with distilled water being careful that filter does not get wet.
    g. Disconnect exhaust condenser from head plate and set aside.
2. Clean fermentor:
    a. Rinse water through any used addition tubing to remove residue.
    b. Loosen four mounting bolts below head plate and disconnect metal ring.
    c. Lift off head plate and set rim on edge of sink.
    d. Gently wipe off the bottom of the head plate and penetrations with sponge and warm soapy water, then rinse with DI water.
    e. Set head plate on paper towels on counter to dry.
    f. Empty the contents of the glass vessel into sink.
    g. Set glass vessel in bottom of sink and wash out with warm soapy water.

   h. Rinse glass vessel with DI water several times and set upright on counter to dry.
3. Collect and discard disposables (pipette tips, microcentrifuge tubes).
4. Wash and rinse remaining glassware (cuvettes, test tubes, addition bottle). Set in racks or hang by sink to dry.
5. Wipe down work area with 70% ethanol solution, then water.

Data Analysis

1. Calculate the true absorbances and concentrations but multiplying the values measured by the dilution factors.
2. Covert optical density readings to dry cell mass using the calibration curve below.
3. Organize dry cell mass, glucose concentration, and ethanol concentration data in an Excel spreadsheet and create graphs of concentration vs. fermentation time.
4. Using trend data from the USB drive, make graphs of pH vs. time and dissolved oxygen concentration vs. time. Indicate when the reactor was switched from aerobic to anaerobic operation.
5. Calculate yield factors for cell mass, YX/S, and ethanol, YP/S, using the initial glucose concentration you measured.
6. How did growth and ethanol production change after aeration was stopped?
7. Did the results meet your expectations? What can you conclude about Jill's dilemma? If you did this again, what would you change?