Ethanol production by yeast fermentation

Experimental Concept

- Yeast cells are used to ferment glucose into ethanol. Measurements of glucose, cell concentration and carbon dioxide production are performed throughout the fermentation and analyzed in the context of the Monod kinetics.

Objective

- The objective of this experiment is to produce ethanol in a batch fermentor. This experiment provides familiarity with the dynamic behavior biological systems, and allows for the determination of the appropriate kinetic parameters to describe the fermentation process at hand.

INTRODUCTION

Fermentation processes are used extensively in the biotechnology, pharmaceutical, food and beverage industries. Typically, fermentations utilize microorganisms (bacteria, yeast) to produce a desired product from a substrate. Butyl alcohol, acetone, citric acid, hydrogen, glycol, fuel alcohol, and beer are examples of the hundreds of biochemicals produced by fermentation. In many cases, fermentation is the more cost effective means to manufacture products.

In this experiment, Saccharomyces cerevisiae yeast are used to convert glucose into ethyl alcohol. The yeast cell contains enzyme catalysts that provide an energetically favorable pathway for the reaction.

BACKGROUND

Currently, the most cost effective way to produce commercial ethyl alcohol is by the hydration of petroleum-derived ethylene. As petroleum becomes scarce and more expensive, this will probably change and ethanol, derived from an organic substrate, may become an important industrial feedstock. It is even possible that someday ethylene (a polymerizable molecule) will be made from ethyl alcohol commercially. The batch experiment conducted here illustrates fundamental kinetics and provides an introduction to fermentation engineering.

There are many environmental conditions that affect yeast cell growth and the kinetics of chemical reactions within living cells. These include the availability of major and minor nutrients, the temperature, pH, and dissolved oxygen concentration, and the possible presence of competing organisms.
Cell metabolism and growth are maintained by a large coupled set of simultaneous chemical reactions wherein essential cell nutrients are necessary for most reactions to occur. When one essential nutrient is absent or depleted (i.e., a limiting substrate), intracellular reactions are inhibited. The nutrient sources for this experiment are: glucose (a carbon source), ammonium chloride (a nitrogen source), potassium phosphate monobasic (a phosphorous source), magnesium sulfate (a sulfur source) and yeast extract, which provides all the essential trace minerals and growth factors. Since glucose is a preferred carbon source for microbial cell growth, it is often the limiting substrate.

The temperature also affects the growth and productivity of the cells. As S. cerevisiae approach their optimum growth temperature of 30°C their growth rate approximately doubles for every 10°C the temperature increases. Past the optimal growth temperature, the growth rate slows and metabolism may become faster than the diffusion rate within the bioreactor, making the diffusion of nutrients the rate limiting step.

The pH inside the bioreactor affects the enzymes inside the cells and changes the rates of reaction. Yeast cells have evolved so that they can thrive in more acidic environments than many competing organisms. As yeast cells consume their nitrogen source, hydrogen ions are released decreasing the pH of the solution. Buffers are often used to maintain the solution within the desired pH range.

The amount of dissolved oxygen in the fermentation broth has major implications for the reactions that occur in yeast. When oxygen is present, respiration occurs converting simple sugars to cell mass and carbon dioxide. Ethanol may be produced from glucose in the presence of oxygen by aerobic fermentation, under some conditions. Under other conditions, ethanol may be consumed by yeast cells. These and other reactions are quite complex.

For this experiment, anaerobic fermentation is the metabolic pathway of interest. Here glucose is converted to carbon dioxide and ethyl alcohol through a complex pathway. The overall reaction is shown here:

\[
1 \text{ C}_6\text{H}_{12}\text{O}_6 \xrightarrow{\text{yeast}} 2 \text{ CO}_2 + 2 \text{ CH}_3\text{CH}_2\text{OH}
\]

This reaction and those described above are performed to generate energy for life processes and for growth. This process of the breakdown of nutrients to obtain energy is referred to as catabolism. If oxygen is present, other more efficient catabolic pathways are available to the cell to obtain energy from glucose than the anaerobic reaction shown above.

For yeast cells to grow and to convert substrate to product, they must be free of other organisms that catabolize the substrate more quickly than the yeast cells and they must be free of disease organisms. In most cell culture work, absolute sterility is required and great effort is made to insure freedom from other organisms. A single contaminating cell can multiply rapidly resulting in a failed fermentation run.

In this experiment, although sterility will not be absolute, you should be able to achieve success with just thorough sanitation. The yeast cells chosen are robust and thrive at a low pH that is inhospitable for most other organisms. Additionally, the ethyl alcohol produced is toxic to many competing
organisms including bacteria. Finally, the bioreactor will be inoculated with a large charge of yeast cells to overwhelm any competing organisms that may be introduced, and improve productivity.

A typical batch growth curve for a liquid nutrient medium inoculated with a seed culture would include the following phases: (1) a lag phase, (2) a logarithmic or exponential growth phase, (3) a deceleration growth phase, (4) a stationary phase, and (5) a death phase.

(1) The lag phase occurs immediately after inoculation while the yeast cells adjust to their new environment. Low concentrations of nutrients can lengthen this phase. One way of shortening this phase is to activate the yeast in a nutrient rich environment before inoculating it into the reactor.

(2) The logarithmic or exponential growth phase occurs after the cells have adjusted to their environment. During this period, cell growth and substrate consumption are increasing exponentially with time.

(3) The deceleration phase immediately follows the exponential growth phase. In this phase the cell growth/metabolism decreases either due to the accumulations of toxins (e.g., ethanol) or the depletion of one or more essential nutrients.

(4) The stationary phase begins at the end of the deceleration phase when the cell growth rate and the cell death rate are equal. It is interesting to note that often when cells die they lyse (burst) releasing nutrients into the environment that other cells can utilize.

(5) Finally, the death phase occurs as the death rate is larger than the growth rate and the cell concentration decreases.

APPARATUS

The fermentation will take place in a one-liter round bottom flask, with heating and stirring.

PROCEDURE

First week

The first week, you should perform measurements on the yeast activation step, by preparing the yeast charge, thereby getting familiar with the carbon dioxide measurement apparatus and the Stanbio glucose test.

Measuring the carbon dioxide production by capture in the appropriate apparatus as it evolves from the fermentor is an indirect way to measure the production of ethanol (why?).

In order to prepare the yeast charge, proceed with the sterilization process of the bottom round flask (i.e. the fermentor). This step is performed by supplying ethanol and blowing it off with air in the hood. This process has to be performed at least two times.
Once the sterilization is complete, place the fermentor in the heating mantle, which is located on top of the stirring plate.

Prepare the yeast solution by measuring 20 g of yeast in an erlenmeyer flask and dissolving it with 50 ml of water using the vortex (to facilitate the dissolution), and pour this solution in the fermentor. Wash the erlenmeyer flask with another 50 ml of DI water (total volume in fermentor = 100 mL).

Place the thermocouple in the solution and turn on the stirring and the temperature controller. Allow for stabilization at 32 C.

**IMPORTANT: DO NOT TURN ON THE TEMPERATURE CONTROLLER BEFORE PLACING THE THERMOCOUPLE IN THE SOLUTION, OR YOU RISK OVERHEATING AND BURNING THE HEATING MANTLE.**

Prepare the glucose solution by measuring 60 g of glucose in a separate, clean, erlenmeyer flask and dissolve the solution in 100 mL of water using the vortex. Add two drops of a 1:10 dilution of the antifoaming agent.

Start your stopwatch (i.e. t=0) the moment you add the glucose solution to the yeast solution in the fermentor. Place the cap and start monitoring the carbon dioxide produced as a function of time and temperature (which eventually should stabilize at 32 C).

Equalize the water level in the PVC pipes by removing water through the valve located at the bottom of the apparatus. The volume of water removed should be measured after equalization has been reached, as that is the volume of carbon dioxide produced at room temperature and pressure conditions.

You should collect liquid samples from the fermentor every 20-30 minutes, and perform the collection after the water level in the PVC pipes has been performed. That way the carbon dioxide loss due to removal of the rubber stopper during liquid sampling is diminished.

**Glucose determination.**

Remove a vial of Stanbio reagent from the refrigerator, until it reaches room temperature.

Prepare glucose solutions in standard spectrophotometric cuvettes by dilution using DI water, until the final concentration reaches a maximum of 3 mg/mL.

Then apply the Stanbio procedure:

Pipette into a cuvette 2 ml of Stanbio reagent. Add 0.02 mL of the glucose solution. Incubate for 10 minutes at room temperature.

Measure the absorbance at 530 nm.
For calibration purposes, the blank is 2 mL of Stanbio reagent mixed with 0.02 mL of deionized water.

Following the procedure above, you can now measure the glucose concentration in the samples you collected from the fermentor using the calibration curve you obtained, by previously diluting the samples you extract from the fermentor by a factor of 100x.

**Yeast determination.**

A spectrophotometric procedure can also be applied to determine the yeast concentration, by performing absorbance measurements at 650 nm. A calibration factor has been determined that allows for a determination of the concentration of the yeast by using the following equation:

\[ X = 0.817A \]

, where A is the absorbance (in standard 1 cm path length cuvettes) at 650 nm, and X is the concentration of yeast (in g/L). This equation is valid for X<0.45 g/L so it is essential that the appropriate dilution of the samples be performed before absorbance measurement.

**Second week**

In contrast to the first week, the 200 mL solution of activated yeast will be prepared in an erlenmeyer flask placed in the constant temperature water bath. Other than this, the yeast activation procedure is identical to the one performed during the first week.

The fermentor will be used for the medium preparation:

After sterilizing the fermentor, the medium will be prepared by dissolving the following reagents in 800 mL of DI water:

<table>
<thead>
<tr>
<th>Medium</th>
<th>Reagent</th>
<th>Mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄ (monobasic potassium phosphate)</td>
<td>5.00</td>
<td></td>
</tr>
<tr>
<td>MgSO₄ (anhydrous)</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>NH₄Cl (ammonium chloride)</td>
<td>2.50</td>
<td></td>
</tr>
<tr>
<td>Yeast extract</td>
<td>3.00</td>
<td></td>
</tr>
<tr>
<td>Commercial antifoam</td>
<td>3 drops of 1:10 dilution</td>
<td></td>
</tr>
</tbody>
</table>

After the medium has been prepared, make sure you add a few drops of 2.0 N hydrochloric acid to decrease the pH of the solution to 4.6. If the pH goes too low, it may be necessary to add a few drops of sodium hydroxide solution.
Place the fermentor with the medium in the heating mantle, put the thermocouple and carbon dioxide collection stopper in place along with the central stopper, and turn on the temperature controller setting it to 32 C and wait.

Once the temperature has stabilized, add the yeast charge (t=0).

For optimal results, by the time the laboratory session is finished, the experiment should have run for at least 5 hours.

With the procedures you learned during the first week, you will perform measurements of glucose, yeast and carbon dioxide concentration as a function of time. It is suggested that you perform one measurement every 30 minutes.

CALCULATIONS

By the time you come to the lab, you should be able to fully derive and interpret the equations in the Monod model for bacterial cell growth rate$^{1,2,3}$. Also, using measurements of the rate of glucose disappearance, you should be able to demonstrate how you will obtain the production rate of ethanol. In your final report, you should quantitatively compare and determine whether your kinetics parameters match those published in the literature$^{4,5}$:

<table>
<thead>
<tr>
<th>Author</th>
<th>T (°C)</th>
<th>$\mu_{\text{max}}$ (h$^{-1}$)</th>
<th>$K_s$ (g/L)</th>
<th>$Y_{c/s}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pironti$^4$</td>
<td>30</td>
<td>0.26</td>
<td>15.5</td>
<td>0.47</td>
</tr>
<tr>
<td>Cysewski$^4$</td>
<td>35</td>
<td>0.58</td>
<td>4.9</td>
<td>0.44</td>
</tr>
<tr>
<td>Bazua and Wilke$^4$</td>
<td>35</td>
<td>0.64</td>
<td>0.24</td>
<td>0.52</td>
</tr>
<tr>
<td>Hoppe and Hansford$^4$</td>
<td>30</td>
<td>0.64</td>
<td>3.3</td>
<td>0.43</td>
</tr>
<tr>
<td>Badino and Hokka$^5$</td>
<td>30</td>
<td>0.32</td>
<td>0.63</td>
<td>0.35-.46</td>
</tr>
</tbody>
</table>

REFERENCES


Rev. 2/28/11 AP