ChE *laboratory*

A BIOREACTOR EXPERIMENT FOR THE SENIOR LABORATORY

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Many chemical engineering students are not exposed to biology through the standard curriculum. But the use of concepts related to biological reactors may be required of students since they apply not only to the bioprocess industries but also to problems in waste treatment and remediation of sites that are environmental hazards. One approach for introducing these concepts is to incorporate new examples within the existing curriculum, and the senior laboratory course offers an excellent opportunity to do just that. A biologically based experiment also provides a forum for introducing material that reinforces traditional chemical engineering principles.

The goals of our senior laboratory are given in Table 1 (based on a handout prepared by George Scheele). We will show that these goals can be met through experiments that test the oxygen transfer capabilities of two New Brunswick Scientific Bioflow III fermenters during one class period; we then examine the kinetics of yeast growth and the effects of ethanol inhibition for a batch reactor during a second class period.

STRATEGICAL APPROACH

In Cornell's senior laboratory course there are lectures, briefings before a new laboratory, and a report session. Experiments span two separate three-hour periods. Student groups consist of three students each, and a group comes to the laboratory the same days of the week for two consecutive weeks.

Because of the relatively slow response time of biological systems, designing experiments that will fit within two separate three-hour blocks is difficult. Bioprocess laboratory experiments at most universities require an extended one-day

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experience or make use of continuous culture devices requiring sampling over a period of many days. Our approach requires considerable prelaboratory preparation by the instructor and teaching assistant to circumvent this difficulty. By using two fermenters in batch mode, with cells already in exponential growth, and concentrating on transient responses to specific stimuli, students can acquire sufficient data to explore several important concepts.

Although we require that students practice good aseptic technique when taking samples from the reactor, we wanted a system that was sufficiently robust to minimize the consequences of any mistakes. Consequently, we chose a common yeast, *Saccharomyces cerevisiae* for the experiments. It grows well at low pH (4.0), and if inoculated at high cell density, it will dominate when another organism accidentally enters the reactor.

The goal in the first laboratory period is to determine volumetric mass transfer coefficients (k_La) for oxygen as a function of air-sparge rates and agitator speed. Two methods are used: unsteady-state with no cells and dynamic method with cells. During this first period, students also determine aerobic growth kinetic parameters and develop calibration curves for measuring glucose and ethanol.

In the second lab period, the effects of ethanol inhibition

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on aerobic (oxygen present) growth kinetic parameters are tested. Four levels of ethanol are added to the fermenters: 15 g/L, 30 g/L, 45 g/L, and 60 g/L. Aerobic conditions are used since confounding effects from endogenously generated ethanol can be avoided. If this system were being used for ethanol production, it would be conducted under anaerobic conditions (no oxygen present). The three-hour period makes the use of anaerobic conditions impractical.

To rationalize the use of aerobic conditions, the students are given a scenario in which a two-stage process is envisioned. The first stage of the proposed process is to build up cell mass as quickly as possible using aerobic growth conditions, followed by a second-stage anaerobic fermentation to ethanol. One common method of scale-up is to hold k_La constant. This aspect justifies the first laboratory period; the second period is justified by requiring the students to suggest a kinetic expression that would account for the inhibitory effects of ethanol. The presumption is that such a kinetic expression could be applied to both aerobic and anaerobic growth situations.

A general introduction to biochemical engineering is given in two lecture periods focusing on oxygen mass transfer in fermenters and summarizing kinetic models for growth and inhibition. Students are given copies of a general review article[1] on biochemical engineering written for physical scientists, an article on inhibition kinetics,[2] and a part of a textbook describing oxygen transfer in fermenters.[3] The topics covered in these two lectures are given in Table 2.

TABLE 1
Objectives for a Senior Chemical Engineering Course

A. *In operating equipment, how to*
- Judge response time: *i.e.,* bigger is slower to respond
- Understand special properties or conditions: *i.e.,* flooding of a packed column
- Develop an intuitive feeling for the behavior of chemical engineering equipment and the magnitude of process streams
- Develop a feel for data that are reliable and data that aren't

B. *In interpreting the behavior of equipment, how to*
- Apply the theoretical and empirical equations discussed in the lecture courses to real systems
  - ¤ Develop a sense of appropriateness
  - ¤ Recognize the limitations of theories
  - ¤ Unify material from previous courses
- Treat data
  - ¤ Recognize the difference between independent and dependent variables; between theoretical and empirical equations
  - ¤ Develop the ability to arrange equations to analyze data
  - ¤ Develop the ability to interpret results

C. *In telling others what you have done, how to*
- Use generally accepted conventions of written reports
  - ¤ Know the format for a good technical report, magazine article, or journal paper
  - ¤ Know the conventions of writing, such as consistent tense and person, proper paragraphing, pronoun-antecedent agreement, and correct spelling
  - ¤ Search for vague, overblown, or unnecessary words and phrases, and to rectify problems
- Use graphs and tables
  - ¤ To analyze data—to fulfill your needs
  - ¤ To convey ideas—to fulfill others' needs
- Learn to synthesize the sometimes conflicting data and ideas at your disposal into a clear, logical report

TABLE 2
Lecture SubTopics

• The cell as a chemical reactor	• Genetic engineering
• What are proteins and enzymes?	• Batch growth kinetics
• Simple enzyme kinetics	• Kinetics of inhibition
• Major intracellular components	• Experimental methods to determine cell mass and number
• The genetic code and the Central Dogma	• Scale-up problems in bioreactors
	• How to determine k_La

APPARATUS AND PROCEDURE

Bioreactor System

A diagram for the primary apparatus is shown in Figure 1. We use two New Brunswick Scientific Bioflow III fermenters with 3.3 L culture vessels, Phoenix Polarographic (Houston, Texas) dissolved oxygen electrodes, and Ingold (Wilmington, Massachusetts) pH probes. An IBM PS/2 Model 30 286 computer controls the two fermenters, and an Epson FX-850 (Torrance, California) printer prints out data at the end of the experiment. To simultaneously control the two fermenters and for data logging, we use the New Brunswick Scientific Advanced Fermentation Software Package. Standard set points are pH at 4.0, temperature at 30°C, and agitation at 250 or 350 rpm. Antifoam C (Sigma Chemical Company, St. Louis, Missouri) was added automatically. The working volume for each fermentor is 2.3 L.

We added a check valve in the gas-supply lines to allow

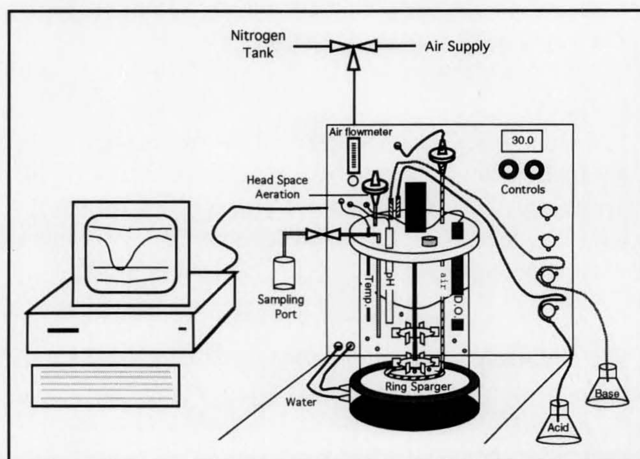

Diagram of the Bioflow III with computer-data capture. Note the three-way valve to change the gas supply. The filtered gas supply can be directed to the head space or the ring sparger, as desired. Temperature is controlled by the water bath on the base, and acid or base is added automatically to control pH. The computer monitors agitation rate, dissolved oxygen, pH, and temperature.

for a rapid switch from air to nitrogen gas. Also, a "Y" after the flow meter and the use of clamps allowed the gas stream to be directed to either the sparger ring submerged in the liquid medium or to the headspace. For measuring k_La (with no cells present), nitrogen gas is sparged through the medium to remove oxygen; after oxygen is depleted the check valve can be used to switch to air only. Measurement of the dissolved oxygen upon re-aeration allows k_La to be calculated.[3] A plot of $\ln(C^* - C_L)$ versus time (t) yields a slope equal to $-k_La$. C^* is the saturated level of dissolved oxygen, and c_L is the value of dissolved oxygen in the medium.

Cells are present for the dynamic method. In this method, air flow is stopped for a short period of time (the length of time depends on cell concentration and initial level of dissolved oxygen) and is then restarted before the dissolved oxygen drops low enough to alter cellular metabolism (*e.g.*, below 10% saturation). The rate of oxygen uptake (or specific respiration rate) can be calculated from an oxygen balance during the period when no air is being supplied, and the k_La can be calculated from the re-aeration part of the response by including a correction for oxygen consumption by the cells.[3]

A plot of C_L versus $q_{O_2}X + dC_L/dt$ has a slope of $-1/k_La$ and is plotted from the data from the re-aeration part of the experiment. Here q_{O_2} is the specific rate of oxygen uptake, while X is the total biomass concentration. The product, $q_{O_2}X$, is determined from the rate of oxygen depletion when the air is off. Since oxygen transfer by surface aeration would invalidate these calculations, it is important to displace air in the headspace with nitrogen as quickly as possible. The check valve and "Y" in the gas line make it easy to switch from air sparging through the medium to nitrogen flow to the headspace to displace headspace air and to provide a nitrogen "blanket." Clearly, nitrogen sparging in the medium could not be used since oxygen removal from the medium would then be due to both cellular respiration and gas stripping.

Other pieces of equipment used in this laboratory include a spectophotometer (Milton Ray, Spectronic 301; UV-visible wavelengths) and a bench-top shaker (New Brunswick Scientific Company, Edison, New Jersey; G24 Environmental Incubator Shaker).

Organism and Medium

The organism used was *Saccharomyces cerevisiae* Cuy8, obtained from Dr. Tim Huffaker's lab at Cornell. Most strains of *S. cerevisiae* (a yeast) would be acceptable. The composition of the growth medium was

- 10 g/L glucose
- 1.5 g/L yeast extract
- 4.8 g/L $(NH_4)_2SO_4$
- 0.75 g/L KH_2PO_4
- 0.24 g/L $MgSO_4 \cdot 7H_2O$
- 0.036 g/L $CaCl_2 \cdot 2H_2O$ pH to 4.0

Medium was autoclaved at 121°C for 50 minutes in four 2L batches.

Start-Up Procedures

For the experiment to be completed in the allocated time, the fermentation must already be under way when the class begins. The inoculum is prepared by inoculating 100 mL of sterile medium in a 250 mL Erlenmeyer flask with silicon closure (for good gas transfer) using a loop of yeast from a colony on a Petri plate. Three flasks are used; they are incubated on the Model G24 shaker for 16 hours at 30°C and 350 rpm. The bioreactors were inoculated 4 hours prior to student arrival with 300 mL of inocula and 2 L fresh medium. This procedure circumvents the lag phase and ensures that the culture is in the early exponential growth phase. This procedure is critical if students are to complete the experiment within three hours.

Although the reactor itself should, in principle, be sterilized by autoclaving each day, we autoclaved the vessel for Monday's laboratory but did not autoclave for subsequent laboratory sessions. Rather, we emptied the vessel after the laboratory and cleaned and disinfected it with 70% (by volume) ethanol acidified to pH 2 with HCl. The low pH of the medium provides protection against significant contamination, and using a disinfection solution reduces the time and labor involved in laboratory preparation.

Another aspect of laboratory preparation is calibration of the dissolved-oxygen and pH probes using manufacturer protocols. The dissolved-oxygen probes are particularly sensitive to electrical interference and fouling by proteins and medium components.

Assays: We used two enzyme-based assay kits (glucose and ethanol) and directions for both were supplied by the manufacturer (Sigma Chemicals, St. Louis, Missouri). The assay requires a spectrophotometer capable of working at a wavelength of 340 nm. We immediately filtered samples from the reactor through a 0.22 μm filter (Millipore) at the end of a 10 mL syringe, and removed cells to prevent glucose consumption before the assay was complete.

We made optical density measurements at 660 nm, using unfiltered samples. Because of non-linearities in the relationship of optical density to dry weight, samples with O.D. > 0.300 were diluted with sterile medium. Sterile medium was used as the blank.

LAB PERIOD OBJECTIVES

The objectives of the first period are to measure k_La and base-line (*e.g.*, zero ethanol) growth parameters. One fermenter is used exclusively for unsteady-state k_La measurements. Suggested conditions are two agitator speeds (250 and 350 rpm) and three air-flow rates (1.0, 2.0,

and 4.0 L/min) at each agitator speed. Of course, this fermenter is not inoculated with cells.

We inoculate the second fermenter and use it to determine the maximum specific growth rate, substrate utilization rate, and specific respiration rate under aerobic conditions with no ethanol added. Additionally, we determine dynamic k_La values for selected values of agitator speed and gas-flow rate corresponding to two of the conditions used in the unsteady-state experiments.

We inoculate both reactors in the second lab period, and we determine growth substrate utilization and respiration rates upon the addition of known amounts of ethanol. One reactor is challenged at 15 g/L ethanol, then 45 g/L, and the second reactor is challenged at 30 g/L and then 60 g/L.

BRIEFINGS AND REPORTS

For the briefing, we give the students a general statement of the problem along with the goals for laboratory periods one and two. We also provide selected instructions from the manuals for the New Brunswick Scientific Bioflow III fermenters, the enzyme assay kits for glucose and ethanol, and the Spectronic Model 301 spectrophotometer. A standard curve relating optical density at 660 nm to dry weight of the culture (developed by the teaching assistant) is also provided. We then introduce the students to the equipment and demonstrate the use of the software for fermenter control and data logging. We also demonstrate the use of Eppendorf pipetters since many of the students have not previously used them.

Based on this information, we then ask the student group leader to formulate an experimental plan and to assign duties to the other group members. Without this kind of good preparation it is impossible for the group to complete the laboratory on time.

We discuss problems in the experiments and the format of the report (written in some cases and oral in others) during the report session. We ask the students to calculate or to provide the following:

1. Calibration curves for ethanol and glucose
2. Sample calculation for k_La from the unsteady-state method[3]
3. Sample calculations for k_La from the dynamic method[3]
4. Calculation of μ, the specific growth rate (h^{-1}), which is defined as

$$\mu = \frac{dX / dt}{X} \tag{1}$$

during the exponential phase of culture growth[1] where X is the dry-weight concentration of cells (g/L)

5. Calculation of q_{O_2} specific respiration rate (mgO_2/g cells-h) for oxygen consumption. A value for $q_{O_2}X$ is found using the procedure for dynamic k_La[2] and dividing this value by X yields q_{O_2}.
6. Calculation of the specific glucose uptake rate, q_{glu} (g.glucose consumed per g cells per hour). Note that

$$q_{glu} = \frac{dS / dt}{X} = \frac{dS / dt}{dX / dt}\mu = \frac{\mu}{Y_{X/S}} \tag{2}$$

where S = glucose concentration in the growth medium (g/L), t = time (h) and $Y_{X/S}$ is the yield coefficient or mass of cells formed per mass of substrate (glucose in this case) consumed.

7. Calculation of $Y_{X/S}$.

Items 4 through 7 above are calculated from all experiments (0 to 60 g/L of added ethanol).

Based on these calculations, each member of the group is expected to develop a correlation of k_La with agitator speed and air-flow rate, and this correlation is compared to expected dependency of k_La on agitator speed and air-flow rates. The expected dependency can be found from the combining expressions[1,3,4] such as

$$k_La \propto Pg^{0.4} Q^{0.5} N^{0.5} \tag{3}$$

where N = agitator speed (rpm), Q is volumetric gas-flow rate (L/min), and Pg is the power requirement in the aerated fermenter, with constant impeller diameter

$$Pg \propto \left(\frac{P^2 \cdot N}{Q^{0.56}} \right)^{0.45} \tag{4}$$

where P is the power required in the unaerated fermenter, and with

$$P \propto N^3 \tag{5}$$

where Eq. (5) applies in the turbulent region.[4]

For the analysis of kinetics, the students test by fitting several possible kinetic expressions to the five available data points for m, q_{O_2}, and q_{glu}. We also asked them to comment on the possible effects, if any on the yield coefficient.

Many equations have been suggested to describe product inhibition.[2] Three examples are

$$\mu = \mu_{max}\left[1 - \left(\frac{P}{P_{max}}\right)\right]^n \tag{6}$$

$$\mu = \mu_{max}\left(e^{-k_iP}\right) \tag{7}$$

$$\mu = \mu_{max}\left(\frac{K_{ix}}{K_{ix} + P}\right) \tag{8}$$

In the above equations, μ_{max} is the maximum specific growth rate (h^{-1}) and can be determined from exponential growth at zero ethanol concentration; P is the extracellular ethanol concentration. Students evaluate μ_{max} from growth data during the first period (for the yeast used in these experiments, μ_{max} is about 0.4 h^{-1}). For these equations, we assume that the

glucose substrate is present at sufficiently high concentrations that growth is zero order with respect to glucose. In Eq. (6), P_{max} is a semi-empirical parameter corresponding to the highest ethanol concentration which will allow growth. This value can vary significantly from one strain of yeast to another; values of 90 to 120 g/L are typical. The exponent, n, is empirical, but often a value of 0.45 is used. In Eqs. (7) and (8), k_i and K_{ix} are empirical parameters. For the yeast strain we used, Eq. (6) was the most satisfactory.

CONCLUDING REMARKS

Equipment malfunctions, particularly with the dissolved oxygen electrodes, should be anticipated, and the techniques for preparing inocula should be followed consistently. Under some stress conditions, this strain of yeast can develop a pinkish pigment that can invalidate the optical density versus dry weight relationship.

We obtained generally good results for determining k_La, particularly with the unsteady-state method. The kinetic data are more problematic since few data points are available due to the laboratory's time constraints. Further, the requirement for a high level of precision in biomass and glucose measurements is not met by every student group. Although the actual techniques are straightforward, careful attention to detail and sample handlings are required. Groups with fewer than three students are unworkable as the students are too rushed for time to complete the assays carefully.

In summary, we believe that it is possible to introduce a meaningful, challenging but doable, bioreactor experiment to chemical engineering seniors who lack any prior exposure to biology or bioprocessing. The use of two fermenters is necessary if these goals are to be accomplished in two separate laboratory periods of three hours. The student response to the experience has been very positive.

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REVIEW: Oscillations and Instabilities

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tionary states are calculated and analyzed, showing that steady-state multiplicity can occur also in this case. The effects of different kinetic mechanisms and boundary conditions on the multiplicity pattern and the stability of the steady-states are discussed.

In Chapter 10, the formation of stationary spatial patterns, the so-called Turing structures, is considered for the thermokinetic model, *e.g.,* non-isothermal first-order kinetics. First, the homogeneous steady-state is evaluated and its stability character is determined. In the case where it is stable to spatially homogeneous perturbations and the ratio of mass and thermal diffusivities is sufficiently large, it is demonstrated that stable spatial patterns can form due to spatially inhomogeneous disturbances. On the contrary, when the uniform steady-state is unstable to spatially homogeneous perturbations (*e.g.,* the corresponding well-stirred system exhibits limit cycle behavior), diffusion processes have no stabilizing effect. Spatial patterns can form and survive for a finite time, but eventually they decay to spatially homogeneous oscillations.

Chapter 11 deals with chemical traveling waves in a one-dimensional space domain. The case of constant speed of propagation is considered and its limitations are discussed. It is shown how quadratic and cubic autocatalysis produce traveling waves.

In Chapter 12, the broad issue of heterogeneous reactions is addressed. The aim of this chapter is only to show that also in this case, steady-state multiplicity and instability can occur due to non-linearities in the model equations. How these non-linearities can arise is discussed in some detail. In particular, the cases of activated adsorption, multi-site reaction mechanism and competitive chemisorption are considered. The chapter includes some examples of steady-state multiplicity and oscillations.

The last chapter of the first part of the book, Chapter 13, introduces the reader to the world of chemical chaos. After clarifying that more than two state variables are required for a continuous system to exhibit deterministic chaos, the authors turn to simple discrete mappings to illustrate the striking phenomenon of the Feigenbaum cascade. Then the periodic forcing of oscillatory systems is considered by illustrating some techniques for their analysis and some examples of their behavior. Subsequently, complex oscillations and chaos in autonomous systems are dealt with and the determination of the stability of limit cycles through the computation of Floquet multipliers is described. Examples are provided by a modified version of the autocatalator in an isothermal batch reactor and by two consecutive exothermic