

MASS TRANSFER AND CELL GROWTH KINETICS IN A BIOREACTOR

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Biototechnology is an increasingly important factor in the chemical process industries. The last decade has seen rapid growth in the resources committed to the development of biologically based processes. At the same time, the market value of new products generated by biological means has continued to grow at an accelerating rate. Accordingly, more and more chemical engineers are being employed in the development, design, and operation of bioprocesses for production of pharmaceuticals, foods, and specialty chemicals, with no indication that the demands and opportunities in this area will moderate in the future.

In recognition of this trend, we have developed a new "biotechnology experiment" for Northwestern's senior laboratory course.^[1] This experiment is aimed at giving our students an opportunity to become familiar with various factors involved in the implementation of bioprocesses and some of the attendant technologies. We hope this will introduce them to this broad field while they are still at Northwestern and also enhance their attractiveness to potential employers.

The experiment provides a means for studying two basic chemical engineering operations (mass transfer and cell growth kinetics) that occur in a three-liter stirred fermenta-

tion reactor. The initial part of the experiment involves the study of oxygen transfer rates from gas to liquid phases; transient dissolved oxygen profiles resulting from step changes in feed gas oxygen concentration are measured with a dissolved oxygen probe. The growth kinetics of *Escherichia coli* are then studied in the same reactor under standard conditions. Cell growth is monitored by spectrophotometric analysis of samples removed from the reactor at specific times. The complete experiment is normally run in two successive laboratory sessions, each about eight hours long, separated by one week. It is also necessary to perform some short preparative steps the day prior to the second laboratory session.

EXPERIMENT SETUP

Equipment • The principal apparatus used is an Applikon three-liter glass stirred bioreactor. It was obtained as part of a complete package that included a number of ancillary items, such as temperature, pH, and oxygen probes and control systems. Additional major items obtained for this purpose included an Innova 4200 shaken-cell incubator and a basic spectrophotometer (Spectronic 20+). The approximate cost of this equipment is indicated in Table I. Not included in the indicated cost, but of critical importance for this experiment, is a steam sterilizer large enough to accommodate the fermentation reactor. We had access to such a unit in our department (AMSCO Eagle 2300 Autoclave) and assume that similar equipment is likely to be available in chemical engineering or related departments at other institutions.

A sketch of the reactor is shown in Figure 1. It is stirred with dual turbine blade impellers on a single shaft, driven by an electrical motor with an adjustable speed control. The reactor top is a stainless steel disk equipped with multiple ports for sampling, introduction of inoculum, gas feed and outlet lines, and insertion of temperature, pH, and dissolved oxygen measuring probes. Additional specifications are indicated in the Appendix.

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Gas is fed into the reactor and dispersed into the liquid through an L-shaped sparger tube that has multiple holes along the horizontal section that is located near the bottom of the reactor vessel. Outlet gas passes through a small water-cooled condenser tube that serves to prevent evaporation of water from the normally warm liquid contents of the reactor.

Temperature in the vessel is sensed by a type-J thermocouple inserted through one of the reactor ports and controlled by a simple electronic control system. An electrically heated jacket provides required heat input, while cooling water can be simultaneously circulated through a small cooling coil immersed in the reactor liquid. Stable control of the reactor temperature at 37°C is easily achieved with this system.

The bioreactor can be fed with three different gases. Air is supplied by an air pump with an inlet microfilter; pure oxygen and nitrogen are provided from pressurized cylinders. The nitrogen is used in calibrating and spanning the dissolved oxygen probe and in the oxygen transfer-rate experiments. Air and oxygen are used in the cell-growth kinetics studies in conjunction with the dissolved oxygen (DO) controller. During a typical cell-growth experiment, air is continuously sparged into the liquid medium in the reactor with the controller set point at 70% of total saturation relative to pure air. Whenever the measured oxygen concentration falls below 70%, a three-way valve is actuated automatically to switch the sparging gas from air to pure oxygen. This control scheme is normally quite effective in returning the DO level back to the set point within a few minutes, except during the high oxygen uptake portion of the cell-growth curve (exponential

phase described below). At such times, the stirrer speed can be increased from 250 rpm (normal operating level) to 350 rpm in order to increase the gas-liquid interfacial area enough to permit increased oxygen transfer to the liquid phase. Operation at these stirrer speeds was found to be convenient and minimized foam formation during experiments (no anti-foaming agents were used).

Expendable Supplies • To perform the following experiments, a number of reagents and other expendable supplies are required. They include sodium chloride, Ampicillin, Tryptone, yeast extract, Agar, ethanol, deionized water, and bleach, as well as disposable gas-line filters.

DESCRIPTION OF THE EXPERIMENTS

(A) Determination of the Oxygen Transfer Coefficient

The first quantity measured with this system is the combined mass transfer coefficient for oxygen transfer from the gas to the liquid phase, $k_L a$. (Since the interfacial area available for mass transfer cannot be readily determined in these experiments, it has been incorporated in the definition of the coefficient in the usual fashion.) This simple experiment provides an opportunity for the student to become familiar with various parts of the apparatus while illustrating an important chemical engineering principle.

► The reactor is assembled and filled with 2 liters of deionized water. With the stirring speed set at 250 rpm, the temperature control system is activated and the system is allowed to reach a steady temperature of 37°C.

► The DO probe, having been previously polarized by operation for two hours in deionized water, is connected. The reactor is sparged with nitrogen at a rate of approximately 0.5 liters/minute until the DO signal has stabilized (normally about 30-45 minutes), at which point the zero of the DO controller is set to read 0% oxygen. The nitrogen flow is then replaced by air at the same volumetric rate and flow is maintained until the DO probe output remains constant. At this point the controller span is adjusted to read 100% (i.e., saturation with respect to the oxygen content of air).

► The feed gas is then rapidly switched back to nitrogen (step down in feed gas oxygen concentration), and the DO concentration is recorded every 30 seconds to 1 minute until it returns to 0%. The feed is then rapidly switched back to air (step up in feed gas oxygen concentration), and DO concentration is recorded every minute until it returns to 100%. These “step-up” and “step-down” data are then analyzed as indicated below to determine $k_L a$.

(B) Determination of Cell Growth Kinetics

This is the more difficult and demanding part of the experiment, especially for students unfamiliar with the protocols used in biochemical research. It involves two separate operations: the preparation of a stock culture of active cells and the subsequent measurement of cell growth kinetics.

TABLE 1
Major Equipment Needed for Experiment

■ Applikon 3-liter fermentor, with control system and oxygen, temperature, and pH probes	\$15,000
■ Innova 4200 Incubator	\$ 5,000
■ Spectronic Instruments 20+ Spectrophotometer	\$ 1,700
Total Cost	\$21,700

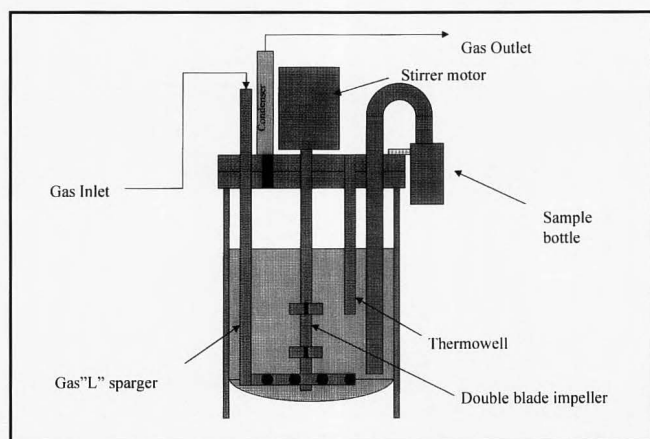


Figure 1. Fermentation reactor.

Throughout this portion of the experiment, emphasis is placed on the need to maintain sterility and cleanliness of the apparatus and the work area.

► **(1) Preparation of stock culture.** This part of the procedure is normally carried out during the first laboratory session along with the oxygen transfer measurements described earlier. Steps involved include:

- *Preparation of Luria-Bertani (LB) culture media* (see also the Discussion section).

Liquid LB medium is a mixture of sodium chloride, Tryptone, yeast extract, and deionized water (composition given in the Appendix).

Solid LB medium is a mixture of sodium chloride, Tryptone, yeast extract, Agar, and deionized water (composition given in the Appendix).

Each of these media is placed in an Erlenmeyer flask that is then covered with aluminum foil and autoclaved for 20 minutes in the sterilizer. The liquid medium can be used in the reactor as prepared.

The solid medium is used to prepare solid culture plates. After the initial sterilization, the solutions are allowed to equilibrate at 55°C and then antibiotic solution is added (see the Appendix for composition of antibiotic solution). The medium is then poured into sterile culture plates that are stacked and allowed to solidify in a sterile hood at room temperature (several hours).

- *Preparation of Cell Cultures.* The cells used in these experiments are from an *E. coli* strain, ER 2275, furnished by New England Bio Labs, Beverly, Massachusetts, and modified (pImP1) as described by Mermelstein, *et al.*^[2] A stock of *E. coli* on the solid medium is prepared by streaking a fresh solid medium plate with a colony of *E. coli* and then incubating the plate at 37°C overnight. If individual colonies of *E. coli* are then easily visible on the plate, it is placed in the refrigerator for storage. If not, another plate is streaked and incubated, as above. This process has proven to be easily reproducible.

- *Preparation of inoculum.* The inoculum is a solution containing living cells that is used to initiate the growth process within the bioreactor. It is prepared the day prior to the fermentation experiment. An individual colony from a stock plate is combined in a 250-ml. Erlenmeyer flask with 200 ml of liquid LB medium equilibrated at 37°C, antibiotic solution is added, and the inoculum is allowed to grow overnight (for approximately 12 hours) with shaking at 200 rpm in the incubator.

► **(2) Preparation of the Reactor for Growth Kinetics Studies.** The reactor vessel is assembled and filled with deionized water and then autoclaved for approximately 20 minutes along with a supply of liquid LB medium prepared as described above. After the reactor has cooled to room temperature, the water is pumped out and replaced by 1.8 liters

of the LB medium. The reactor is then allowed to come to thermal equilibrium at 37°C and the control systems are activated. (The DO probe must first be polarized and calibrated, as described above.)

► **(3) Growth Kinetics Studies.** When the system is ready, 200 ml of the inoculum solution is pumped into the reactor and the DO level is set to approximately 70%. A small sample (10-15 ml) of the reactor contents is then removed every 10-15 minutes and its turbidity measured in the spectrophotometer (at a wavelength of 600 nm). If the cell concentration gets too high, the sample is first diluted in order to keep it within the mid-range of the spectrophotometer. The experiment is concluded when the fermentation appears to have reached the stationary phase (see below). This normally requires 4 to 6 hours.

The final liquid medium still left in the reactor is autoclaved before disposal, and all equipment is carefully cleaned with bleach and soap.

DATA ANALYSIS

(A) Determination of Oxygen Transfer Coefficient

Typical data obtained in the “step-down” (nitrogen feed) and “step-up” (air feed) experiments described above are shown in Figure 2. These data were obtained with a reactor volume of 2.0 liters, a gas flow rate of 0.38 liters per minute, and a mixer rpm of 250. The data clearly exhibit an initial time lag that is the same for both experiments. This lag is apparently due to dynamic response of the dissolved oxygen probe itself. Since it was consistent and relatively small compared to the overall time scale of the experiment, the response data have been corrected by subtracting a lag of 1.5 minutes from the measured time in each transient experiment.

For either experiment, the oxygen transfer rate per unit volume of liquid (OTR) is given by the following equation, which also defined the volumetric liquid phase mass transfer coefficient:

$$\text{OTR} = k_L a (C^* - C) \quad (1)$$

where

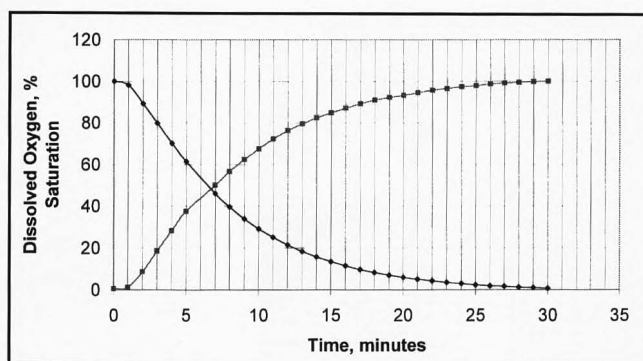


Figure 2. Typical oxygen transfer data: Dissolved oxygen concentration vs. time.

- C^* saturated dissolved oxygen concentration at the gas-liquid interface, mmol/L
- C dissolved oxygen concentration in the bulk liquid phase, mmol/L
- $k_L a$ liquid phase oxygen mass transfer coefficient, 1/minute
- OTR oxygen transfer rate, mmol/L/minute

The transfer coefficient typically depends on the gas flow rate, the bioreactor working volume, and the power input to the agitator (or stirrer speed). It may also depend on the parameters of the reactor design, such as impeller and sparger design and configuration, and the physical properties of the culturing medium, such as viscosity and interfacial tension.

A transient oxygen balance for the reactor volume is

$$\frac{dC}{dt} = \text{OTR} = k_L a (C^* - C) \quad (2)$$

Considering the experiment in which the initially oxygen-free solution is contacted with oxygen containing gas, Eq. (2) must be integrated with initial concentration = 0 and concentration C^* held constant. The well-known result is

$$\ln \frac{(C^* - C)}{C^*} = -k_L a t \quad (3)$$

For the reverse experiment in which the solution is initially saturated at concentration C^* and the gas concentration is = 0, the solution is

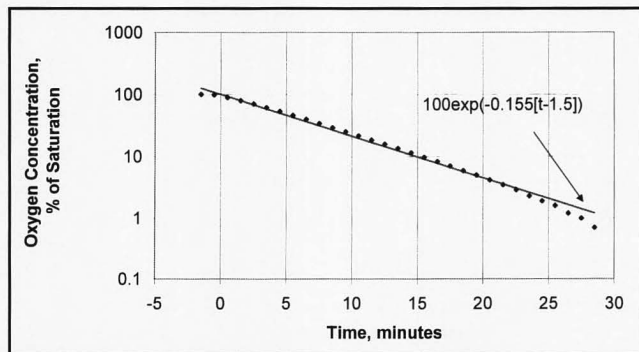


Figure 3. Typical Oxygen transfer data: Determination of $k_L a$ with nitrogen sparging.

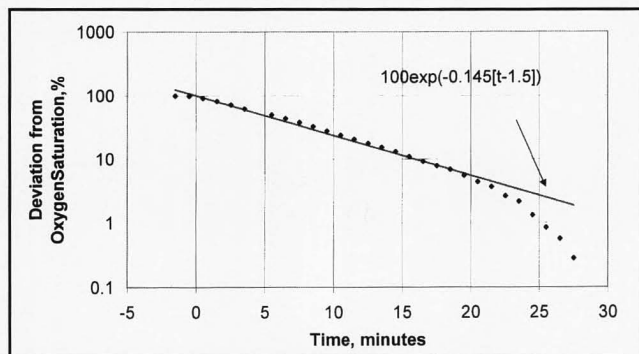


Figure 4. Typical oxygen transfer data: Determination of $k_L a$ with air sparging.

$$\ln \frac{C}{C^*} = -k_L a t \quad (4)$$

Logarithmic plots of the corrected step-down and step-up data according to Eqs. (3) and (4) are shown in Figures 3 and 4, respectively. It can be seen that the data conform quite well to the expected form, yielding the values for the mass transfer coefficient of 0.155 min^{-1} for the nitrogen sparging or step-up experiment, and 0.145 min^{-1} for the air sparging or step-down experiment, for an average value of 0.15 min^{-1} .

One other measurement of $k_L a$ was made with air sparging into the OB medium prior to the beginning of the cell-growth experiments. In this case, the mixer speed was set to 150 rpm while the other conditions remained as before. It was found that the data once again showed a time lag of 1.5 minutes and fit the expected exponential decay similar to Figure 4. The value of $k_L a$ determined, however, was 0.075 min^{-1} . Thus, it is clear that this mass transfer coefficient is a strong function of the degree of agitation in the vessel and the properties of the liquid.

It should be noted that Roberts, et al.,^[3] previously described a laboratory experiment to measure oxygen transfer in a 1-liter stirred fermentor. In that case, the stirring rate was considerably higher (500 to 700 rpm) and the method of determining $k_L a$ was different; those authors measured the quasi-steady-state rate of oxygen consumption by yeast in the absence of oxygen feed (the vessel contents were previously saturated with air). Although conditions were quite different in that experiment compared to the present case, the mass transfer coefficients reported were of the same order of magnitude—approximately 0.6 min^{-1} at a stirrer speed of 500 rpm. Using their exponent of 2.75 for the effect of mixer rpm, the expected value of $k_L a$ at 250 rpm would be 0.089 min^{-1} , which is unexpectedly close to the value of 0.15 min^{-1} found here under considerably different conditions.

(B) Determination of Cell Growth Kinetics

The immediate objective of the second part of the experiment is to measure the specific growth rate of the *E. coli* culture in the batch fermentation reactor system. Typically, such bacteria growing in a batch culture exhibit four distinct growth phases following inoculation with an active culture. As shown in Figure 5, growth usually begins with a very slow lag phase as cells introduced into the inoculum adjust to their new environment. This is followed by a rapid, exponential phase as acclimated cells reproduce via binary fission as quickly as nutrient and oxygen concentrations within the medium permit. This phase is followed by a stationary phase where the rate of oxygen supplied to the cells equals their rate of oxygen consumption. Finally, the cell concentration falls during the death phase due to the depletion of nutrients and the buildup of toxic byproducts.

The specific growth rate (μ) of the cells is determined during the exponential binary fission phase. This process is au-

tocatalytic and is usually represented as a first-order reaction, *i.e.*,

$$\frac{dX}{dt} = \mu X \quad (5)$$

Integration of this differential cell balance yields

$$X(t) = X_o \exp[\mu(t - t_o)] \quad (6)$$

where

- X cell concentration, number/volume
- t time, minutes
- μ cell specific growth rate, 1/minute
- o as a subscript refers to initial conditions

In the present experiments, cell concentration in the reactor is monitored at 10- to 15-minute intervals by measurement of the absorbance (at 600 nm) of a small sample of solution using the spectrophotometer. According to the usual Beer-Lambert law, the light transmitted through a solution is related to the incident light and the concentration of absorbing species, as shown in

$$\frac{I}{I_o} = \exp(-\epsilon cl) \quad (7)$$

where

- I/I_o fractional light intensity relative to incident intensity
- c concentration of absorbing species, number per unit volume
- l length of light path through solution
- ϵ extinction coefficient of absorbing species, area per number

Strictly speaking, for the present experiments ϵ should be regarded as an appropriate fitting parameter since changes in measured light intensity are no doubt due to a combination of absorption and scattering.

Since absorbance A is defined as $-\log_{10}(I/I_o)$, it follows from Eqs. (6) and (7) that

$$A = \frac{\epsilon lc}{2.303} = \frac{\epsilon l X_o}{2.303} \exp[\mu(t - t_o)] \quad (8)$$

Taking natural logs of Eq. (8) yields

$$\ln(A) = \mu(t - t_o) + \ln\left(\frac{\epsilon l X_o}{2.303}\right) \quad (9)$$

Thus, a plot of $\ln(A)$ against time should be linear with a slope equal to the specific cell-growth rate (μ) during the exponential growth phase. A cell doubling time, t_d , can be calculated once the growth rate is determined, according to

$$t_d = \frac{\ln(2)}{\mu} \quad (10)$$

Figure 6 shows typical data obtained over a 4-hour period following the experimental procedure described earlier. These data indicate an expected initial lag of 15 minutes, followed by an apparent exponential growth phase that levels off sometime after 200 minutes. When these data are plotted in accord with Eq. (9), a good fit to the exponential model is obtained, as shown in Figure 7. The corresponding specific growth rate

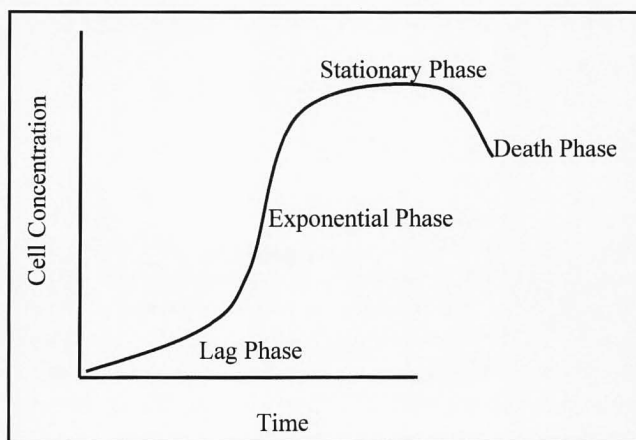


Figure 5. Typical batch culture growth phases.

of the *E. coli* in this experiment was 0.013 min^{-1} . This is equivalent to a doubling time t_d of 53 minutes. This relatively long doubling time confirms that the *E. coli* strain, while adequate for these experiments, is not particularly robust.

The only difficulty encountered in carrying out the cell-growth experiments has been maintaining the dissolved oxygen concentration at 70%. Large swings in the oxygen level (between 50% and 90% of saturation) have been observed even with increases in gas-flow rate and stirring speed. These variations, however, apparently do not have any significant effect on the observed growth rates.

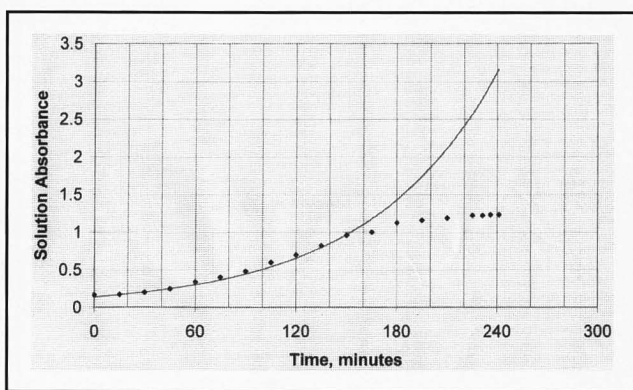


Figure 6. *E. coli* growth data: solution absorbance vs. time.

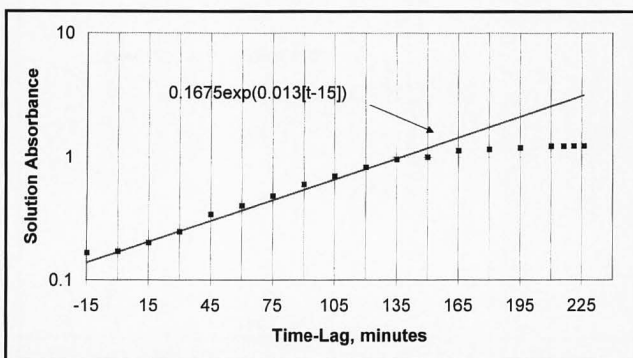


Figure 7. Determination of specific cell-growth rate.

DISCUSSION

The experiments described here have provided a means for introducing senior students to some aspects of bioprocessing. During the course of this experiment, students are exposed to standard procedures for preparing and handling a bacterial culture, including preparation of growth media, development of active bacterial colonies, and incubation and sterilization processes. They also become aware of the mass transfer processes involved, the underlying theoretical analysis, and relevant methods of data analysis, as well as the relatively long time scale of the experiments. The latter is not a serious problem in our laboratory since we are able to devote two 8-hour sessions to this experiment. Some compromises, such as more pre-lab preparations carried out by the instructors, would undoubtedly be necessary in order to perform similar experiments in a shorter laboratory session.

In designing this experiment, we have attempted to include as many of the preparative and analytical steps mentioned above as possible without unduly burdening the students, since our goal is to provide opportunities for "hands-on" experiences whenever possible. At the same time, we are not attempting to develop research-level competencies in our students by this means. Selection of LB culture media as opposed to chemically defined media is a case in point. While the former may yield somewhat less reproducible results from one student group to another, the LB media have proven to be robust and easy to use. Some lack of reproducibility was not considered to be a significant drawback in the present context.

A related laboratory experiment^[4] used the growth of yeast (*Saccharomyces cerevisiae*) and involved the simultaneous use of two fermenters. The rate of oxygen transfer to the liquid phase was studied with and without cell growth, and the rates of cell growth during the exponential phase were also measured under aerobic conditions with various concentrations of added ethanol. No performance data were presented, so a more direct comparison to the present experiment is not possible. It should be noted, however, that while the overall goals of these two experiments are similar, the systems of choice and the methods of data analysis differ somewhat.

Another experiment^[5] based on ethanol production using *Saccharomyces cerevisiae* yeast used 1 liter fermentors and measured CO₂ generated during fermentation to follow the course of the process. As in the above-mentioned case, the overall objective of the experiment is similar to the present case, although it is much more limited in scope.

We have now run this experiment successfully for two years, with increasing numbers of students and with very positive results. While the immediate and ancillary equipment required to mount such an experiment is not trivial or inexpensive, such equipment is becoming relatively common and is likely within reach of most chemical engineering departments interested in providing some direct introduction to biotechnology in their curricula. Of even greater importance than equip-

ment in the successful development of such an experiment are skilled and experienced people who can help in the early planning and implementation stages. We were particularly fortunate to be able to call on Professors E.T. Papoutsakis and W.M. Miller and some of their graduate students for technical assistance and encouragement.

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APPENDIX

1. Composition of Luria-Bertani liquid medium:
Per liter of solution:

NaCl	10 grams
Tryptone	10 grams
Yeast extract	5 grams
Deionized water	1 liter
2. Composition of Luria-Bertani solid medium:
Per liter of solution

NaCl	10 grams
Tryptone	10 grams
Yeast extract	5 grams
Agar	15 grams
Deionized water	1 liter
3. Composition of antibiotic solution:
Ampicillin 1 gram dissolved in 1 ml of deionized water
Added to LB medium at concentration of 100 micrograms/ml
4. Reactor dimensions
Type: 3 liter, dished bottom
Inside diameter: 130 mm
Impeller: Two 6-bladed Rushton turbines
Turbine diameter: 45 mm
Turbine distance from vessel bottom: 45 mm and 75 mm
Baffles: Three, equally spaced baffles, each 220 mm long