ChE laboratory

BIOTECHNOLOGY LABORATORY METHODS

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THE PAST TWO DECADES have seen tremendous growth and interest in biotechnology. As a result of recent advances in molecular biology and genetics, many new pharmeaceutical, chemical, and agricultural products of microorganisms and cultured plant, animal, and insect cells are now available, or soon will be. The successful industrial-scale manufacture of these products will, in large part, depend upon new engineering initiatives in the development of high-rate bioreactors, efficient separation and purification techniques for bioproducts, and computer-interfaced instrumentation for optimal bioprocess control. These needs represent a major challenge to chemical engineering education—a challenge to provide students with appropriate training in both engineering and biology.

In order to help meet this challenge, many if not most chemical engineering departments now offer one or more elective courses in biochemical engineering, such as the course described by Bailey and Ollis [1]. In addition, interested students are encouraged to take technical electives from other departments in areas such as biochemistry, microbiology, and molecular biology. However, relatively few opportunities exist for these students to obtain direct laboratory experience in biotechnology methods, most likely because of the cost and effort associated with developing such opportunities. An exception is the biochemical engineering laboratory course described recently by Ng *et al.* [2]. At the University of Colorado, we have developed a course entitled "Biotechnology Laboratory" which introduces the students to a variety of laboratory methods associated with biotechnology and yet which has required only limited resources.

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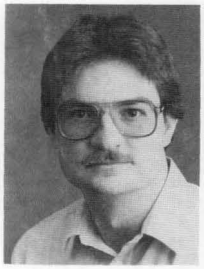

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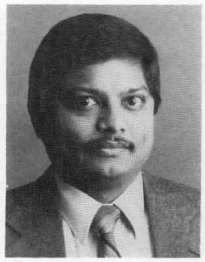

HISTORY OF THE COURSE

In the spring semester of 1984, our department initiated a lecture course entitled "Recent Advances in Biotechnology" [3]. This course, which is open to seniors and graduate students in both engineering and science, exposes the students to the many facets of biotechnology, including microbiology, molecular biology, biochemistry, biochemical engineering, and industrial applications. Guest lectures are given by faculty members from several disciplines and by local industry representatives. After our initial offering of this course, however, it became apparent that a laboratory component was an important missing ingredient.

In October of 1984, the five officers of our undergraduate Biomedical Engineering Society sent a memo to our then department chairman, Max Peters, stating:

> **At a recent BMES meeting, we discussed the possibility of the Chemical Engineering Department offering a "hands on" laboratory course for students who are planning careers in the biotechnology area. Although other departments offer**

laboratory courses in the bio area, there are no courses presently given that would fit the needs of an engineering student wishing to pursue a career in biotechnology.

We would like to suggest that the Department of Chemical Engineering offer a one hour credit optional laboratory course for students taking ChE 580, Recent Advances in Biotechnology . . .

In response to this request, Professors Robert Davis and Igor Gamow introduced a one credit hour course, "Biotechnology Laboratory," in the spring semester of 1985. The initial course offering was taken by 20 students—primarily undergraduates—and included experiments in microbiology, fermentation, genetic engineering, and ultrafiltration. With the aid of the latest addition to our faculty, Dhinakar Kompala, the course has been expanded to two credit hours. This expansion has led to a modest but welcome reduction in the class size. Unlike the associated lecture course, this laboratory course has been taken almost exclusively by chemical engineers. It is composed of graduating seniors who plan to attend graduate school or to work as biochemical engineers, and of graduate students who have chosen research projects in the biotechnology area.

COURSE CONTENT AND PHILOSOPHY

Experiments in biotechnology often require sophisticated equipment and can be quite expensive to undertake, and yet we were charged by our chairman to spend "a few thousand dollars at most" in developing this new laboratory course. Fortunately, we have been able to develop an effective course with available equipment in our undergraduate laboratory, borrowing of speciality items from our research laboratories, government surplus equipment, and a minimum of new purchases.

During the last few years, several new experiments have been introduced, and the original experiments have evolved with time. Our current repertoire of seven experiments is given in Table 1. Each year, typically four or five of these experiments are offered. Our philosophy is to choose the experiments to cover a spectrum of the subfields of biotechnology: microbiology, molecular biology, enzymology, microbial kinetics, biochemical reactor design, and bioseparations. A specific goal is to introduce the students to important laboratory methods such as sterile techniques, phenotype identification, fermentation,culture monitoring, electrophoresis, chromatography, ultrafiltration and microfiltration.

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In order to maintain our original objective of providing each student with hands on experience in biotechnology, the experiments are performed by teams limited to four or five students. Since most of the tasks, such as plating cultures and taking and analyzing samples, are repeated many times during the course of an experiment, this requires that all of the students participate fully. The students receive grades based upon their participation and the brief reports which they prepare on the experiments.

Because of the diversity of experiments and methods involved, we have drawn on expertise outside of our department to assist with the experiments. Ray Fall from the Chemistry and Biochemistry Department at the University of Colorado has assisted with the recombinant DNA and molecular cloning experiments, Dale Gyure from Coors Biotech Products Company has assisted with the mass transfer and filtration experiments, and Geoff Slaff from Synergen has assisted with the chromatography experiments.

TABLE 1
List of Experiments

MICROBIOLOGY AND MOLECULAR BIOLOGY
- Recombinant Bacterial Growth and Instability
- Recombinant DNA and Molecular Cloning

KINETICS AND FERMENTATION
- Enzyme Kinetic Measurements
- Mass Transfer Coefficients in a Stirred Fermentor
- Yeast Growth Kinetics and Fermentation

DOWNSTREAM PROCESSING–BIOSEPARATIONS
- Microfiltration and Ultrafiltration
- Column Chromatography

EXPERIMENT SUMMARIES

Recombinant Bacterial Growth and Instability

The excitement and interest in modern biotechnology are primarily generated by the ability to produce foreign proteins through recombinant DNA technology. To familiarize our students with some characteristics associated with the cultures of recombinant organisms, we have designed this experiment involving simple shake flask batch cultures of recombinant bacteria *E. coli* (RR1/pBR322). The equipment items required are a spectrometer (visible range) and a

water bath shaker (see Table 2 for a list of our source and estimated new purchase price for each item). The materials required include shake flasks, petri dishes, media, culture stocks, pipets, and antibiotics (total estimated cost of $50 per experiment).

Three shake flasks are prepared with appropriate media to culture the following: (i) plasmid-free host cells in M9 minimal medium (see Maniatis *et al.* [4] for media recipes); (ii) plasmid-bearing cells in M9 medium plus tetracycline, and (iii) plasmid-bearing cells in M9 medium with no antibiotics. By measuring optical density at regular intervals and plotting it versus time on a semi-log graph for the first two cultures, we determine μ^- and μ^+, the specific growth rates of plasmid-free and plasmid-bearing cells, respectively. For the third flask containing recombinant cells with no antibiotics, the fraction of cells containing the plasmids is also determined at two and five hours by plating the cells, after serial dilutions, on a petri dish containing a rich medium such as LB, and then replicating the grown colonies onto a dish with LB medium plus ampicillin, using a sterile nylon pad. The phenomenon of 'segregational instability' becomes obvious as the fraction of plasmid-carrying cells drops dramatically. Further, with all the measurements made here, plus the simple mathematic model proposed by Imanaka and Aiba [5] for the dynamics of these cultures, it is possible to estimate the average plasmid copy number for this host/plasmid system.

Recombinant DNA and Molecular Cloning

This experiment is designed to familiarize the students with some of the basic recombinant DNA methods such as bacterial transformation with extrachromosomal DNA, testing for gene expression, and restriction enzyme analysis. The equipment items required are a microcentrifuge, a small refrigerator, an incubator oven, and an electrophoresis unit. The materials required include culture stocks, media, plasmid stocks, petri dishes, restriction enzymes, pipets, antibiotics, microfuge tubes, and molecular weight markers (total estimated cost of $75 per experiment).

In the first part of the experiment, *E. coli* HB101 cells are grown in a rich liquid medium, centrifuged, chilled, and subjected to calcium chloride shock, according to the detailed protocol by Maniatis *et al.* [4], to make them 'competent' for taking up plasmid DNA. About 5 μL of pBR322 plasmid DNA at a concentration of 0.1 g/*l* is added to cold culture tubes containing 100 μL of competent cells. After incubating for 30 min at 4°C, the tubes are kept at 42°C for 2 min and then incubated at 37°C for 30 min with addition of 1 ml of LB medium. Finally, these cells are plated on culture plates with and without ampicillin or tetracycline to determine the transformation efficiency, since the pBR322 plasmid contains genes that render the transformed cells resistant to these antibiotics.

TABLE 2
Major Equipment Needed

	ITEM	SOURCE	EST. PUR. PRICE
1.	Spectrometer	Undergraduate Lab	$ 800
2.	Water Bath Shaker	Government Surplus	800
3.	Microcentrifuge	Research Lab	1,300
4.	Electrophoresis Unit	Research Lab	800
5.	Refrigerator	Purchased	200
6.	Incubator Oven	Government Surplus	200
7.	Fermentor	Undergraduate Lab	12,000
8.	Oxygen Electrode	Homemade	300
9.	Chart Recorder	Undergraduate Lab	800
10.	Microscope	Government Surplus	500
11.	Plate-and-Frame Filter	Manufacturer Demo	1,500
12.	Hollow-Fiber Filter	Purchased	600
13.	Peristaltic Pump	Research Lab	400
14.	Ultrafiltration Cell	Homemade	150
15.	Magnetic Stirrer	Government Surplus	150
16.	UV-vis Spectrophometer	Research Lab	7,000
17.	Chromatography Col.	Purchased	200

In the second part of this experiment, two plasmids, pBR322 and pSY426, a poorly characterized recombinant plasmid derived from pBR322 and containing an extra piece of DNA encoding for β-glucosidase, are digested with the restriction endonucleases Eco RI, Sal I and Bam HI in single, double, and triple digests. These digests are loaded and run on agarose gel electrophorsis units, along with known molecular weight markers. From the size of fragments on the gel and the known map of pBR322, a partial restriction map of pSY426 is then constructed.

Enzyme Kinetic Measurements

This experiment is designed to study the effects of competitive and non-competitive inhibitions, substrate and enzyme concentrations, thermal denaturation and pH on enzyme activity. The enzymes used are urease and amylase. These enzymes, their substrates, buffers, and other supplies may be obtained as an experimental enzymology kit from Carolina Biological Supply Company (estimated cost of $50 per experiment). A detailed laboratory procedure manual

is supplied along with these materials, which we modified slightly to provide a more quantitative study of each effect.

Urease is used to study the effect of inhibitions and concentrations. Mixtures of urea and thiourea at four different relative concentrations are used to measure the effect of competitive inhibition. Potassium iodide is used to demonstrate non-competitive inhibition. The enzymatic reaction is conducted in one-half of a divided circular dish, supplied by the vendor. Gaseous ammonia produced by the reaction diffuses to the other half of the dish and changes an acidic dye solution to a basic solution with a change in color. The same reaction may also be conducted in the presence of the dye solution in either a test tube or a small shake flask. The time of reaction before the color change is noted and used as a quick measure of the inverse of enzymatic activity. Similarly, the enzymatic activity for different substrate and enzyme concentrations are measured. The effects of pH and thermal denaturation are measured on the amylase activity using iodine-potassium iodide drops to indicate the complete degradation of amylase.

Mass Transfer in a Stirred Fermentor

One of the most important control variables in an aerobic fermentation is the dissolved oxygen concentration. The purpose of this experiment is to determine the effects of aeration and agitation on the rate of oxygen mass transfer from air bubbles to the fermentation medium, as quantified by the so-called $k_l a$ value [5]. The required equipment items for this exercise are a stirred vessel with an air supply (we used the fermentor described in the next experiment, although a homemade replacement could be made using a gallon jar and a motorized stirrer for about $200), a rotameter for gas flowrate, a dissolved oxygen electrode, and a strip chart recorder. In addition, a half-full nitrogen tank with a regulator is required (approximate cost of $15 per set of experiments).

The experimental procedure involves filling the vessel to its working volume with water or fermentation broth and then saturating the liquid with air. Once a steady electrode response is achieved, the air flow is suddenly replaced by nitrogen flow, which then strips the oxygen out of solution in a transient manner. From the decay characteristics of the voltage response of the probe, the corresponding $k_l a$ value may be inferred [6]. This procedure is then repeated for each desired stir rate and gas flow rate. Conclusions may then be drawn regarding the relative influence of agitation and aeration in order to provide an adequate dissolved oxygen supply for an actual fermentation.

Yeast Growth Kinetics and Fermentation

The goals of this experiment are to familiarize the students with the various steps involved in a typical fermentation run and to determine the fermentation characteristics of baker's yeast growing on carbohydrates with and without air. For this experiment, we have used an available twenty year old New Brunswick Scientific's Labroferm fermentor, which contains three 7-liter fermentation jars in a water bath. However, the less expensive alternative of three

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1-liter flasks on magnetic stirrers is equally appropriate (estimated cost of $600). Other equipment items required for this experiment are a water-bath shaker, a spectrometer, and a microscope with a hemacytometer slide. Materials required include shake flasks, test tubes, pipets, media, baker's yeast, and enzyme assay kits (estimated cost of $30 per experiment).

During the first class period, a rich medium containing yeast extract, malt extract, peptone, and glucose is made in three 250 ml flasks and sterilized in an autoclave. Using sterile techniques near a Bunsen burner or in a laminar flow hood, a previously grown inoculum is transferred into each of these flasks. The flasks are placed in a water bath shaker and allowed to grow overnight. Also using the previously cultured cells at various dilutions, a calibration curve is developed between the optical density measured with a spectrometer and the cell concentration measured with a hemacytometer. The three fermentor jars are filled with rich media and connected with tubing for air inlet, outlet, and culture sampling devices. In one of the jars, the 10 g/l of glucose in the rich medium is substituted with a mixture of glucose and fructose in the ratio of 1:4; in a second jar, the air inlet tube is clamped shut.

During the second class period, usually on a weekend morning, the three fermentors are inoculated with 100 ml of the cultures from the three small flasks freshly grown overnight. Samples are withdrawn every 30 min from each fermentor for measuring optical density. The experiment is continued for about 10-12 hours, until the optical density readings show no further increase. Occasionally, samples are

also collected for determining glucose and ethanol concentrations through enzymatic assay kits. Analysis of the data usually reveals a single growth phase in the fermentor with no air inlet, two growth phases in the fermentor with air and glucose as the only carbohydrate, and three growth phases with careful data collection in the other fermentor. Further analysis of the data, with a semilog graph of cell concentration versus time, will show identical specific growth rates during the first growth phases corresponding to the glucose fermentative pathway. The second and third growth phases will show decreasing growth rates.

Microfiltration and Ultrafiltration

Downstream processing in order to recover and purify a fermentation product from dilute solution often represents the largest cost in a biotechnology process. This experiment, which is carried out in two separate parts, uses membrane filtration for cell and protein separations. In the first part, the yeast cells from the previous experiment are concentrated using both a hollow-fiber and a plate-and-frame tangential-flow microfilter. This requires the filters and housings, a peristaltic tubing pump, a 4-liter feed jar, a pressure gauge, a graduated cylinder, and a stop watch. The filter is operated in a batch concentration configuration where the permeate is removed and the retentate is recycled to the feed jar. The purpose is to measure the decline in permeate flux due to fouling of the membrane as the cell concentration in the feed increases. Other process variables that may be studied include the pressure drop across the membrane and the flow rate at which the suspension is circulated through the filter.

The second part of the experiment uses an ultrafiltration cell with an internal magnetic stir bar in order to separate two proteins, lyzozyme (MW = 14,600) and bovine hemoglobin (MW = 64,000). A polymeric membrane with a specified molecular weight cutoff of 30,000 is used in the ultrafiltration cell. The proteins and disposable membranes cost approximately $15 per experiment. The ultrafiltration is carried out at a constant transmembrane pressure drop (40 psig), and the permeate flux as a function of time is measured in order to determine the effects of membrane fouling and concentration polarization. Concentrations of the proteins in the initial sample and in the final filtrate and retentate are measured by UV-vis spectrophotometry (hemoglobin absorbs strongly at 400 nm and so may be measured in the visible range, whereas lyzozyme requires a measurement at 280 nm in the ultraviolet range). These measurements are then used to determine rejection coefficients, which are important in scale-up calculations.

Column Chromatography

Column chromatography is the predominant technique used in industry for purifying biological compounds such as proteins, nucleic acids, and antibiotics. The goal of this experiment is to expose the students to the fundamentals of chromatography, in general, and to the separation of proteins based on their relative sizes using gel filtration chromatography, in particular. Equipment items required for this lab are two plastic columns, a peristaltic tubing pump, and a UV spectrophotometer. The disposable materials include sephadex G-75 resin, two proteins of different molecular weight (we use cytochrome c with MW = 12,400 and ovalbumin with MW = 43,000), and blue dextran as a void volume indicator (estimated cost of $40 per experiment).

During the lab, the students pack the columns with resin, load the columns with a mixture of the two proteins and the indicator, run the columns to separate the proteins, and collect frequent samples from the eluent streams. Total protein concentration in each sample is estimated by measuring the absorption in the ultraviolet range with the UV spectrophotometer. Two different sized columns are used to familiarize the students with the problems associated with scale-up. From the elution diagrams obtained, the students determine important parameters such as the distribution coefficient for each protein, the standard deviation of each of the elution peaks, and the resolution between the two peaks representing the larger and smaller proteins.

CONCLUDING REMARKS

The biotechnology laboratory course at the University of Colorado has been maintained, and indeed has flourished, as a cooperative effort. The industry representatives have volunteered their assistance, the faculty members have participated in addition to their normal teaching responsibilities, and the students have elected to take the course above and beyond the normal requirements. Because of time requirements, the experiments have been carried out during evening and weekend hours. Nevertheless, all involved feel that it is a worthwhile effort.

Finally, we have not required a published laboratory manual, although several manuals describing many of the general procedures used are available [4, 7-9]. Instead, we have prepared a handout for each of the experiments. Single copies of a compilation of these handouts may be obtained free of charge from the authors.

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tronic media (expert systems, video transmission, *etc.*) might be used to bring the most up-to-date technology and best practitioners within the reach of all interested engineering faculty.

CONCLUSION

As Nehari and Bender [22] suggest, it is not just the transmission of information, but the transmission of meaning that is important to vital teaching. Education succeeds when the student becomes intrinsically motivated to acquire the learning, and to look upon the activity as an end in itself. A teacher who sets an excellent role model for the students, both in terms of personal enjoyment and intellectual curiosity, has a good chance to teach the students similar enjoyment.

Before we can consider environments for encouraging faculty, either as teachers or as researchers, we must consider faculty as individual human beings, with human characteristics. At most, this paper may be considered to provide some clues, based on a larger engineering/science faculty sample, concerning the developmental stages and motivation on the formation of an environnent to encourage vital teaching.

Faculty see the opportunity and resources to improve their professional skills in somewhat the same light as unused computer availability; if opportunities are made available in a non-threatening manner, the faculty will naturally seek and use them.

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