Biochemical Engineering Laboratory Course for Chemical Engineering Students

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Abstract – Several initiatives have been implemented to provide hands-on experiences for undergraduate chemical engineering students with a concentration or emphasis in biochemical engineering. At San José State University, a laboratory course was developed that introduces molecular biology methods through a five-week subcloning experiment in addition to sessions on fermentation, protein purification, and protein handling. The purification of green fluorescent protein, that has been produced in the fermentation, includes hydrophobic interaction and ion exchange chromatographic methods. A session on enzyme kinetics involves the measurement of Michaelis-Menten kinetic parameters of the hydrolysis of a colorimetric substrate of trypsin. In addition, this year, two inquiry-based lab periods have been included for the students to explore a question of their choice and learn to design and execute an experiment.

Index Terms - Bioprocess Engineering Laboratory Course, chemical engineering, green fluorescent protein, inquiry-based learning.

BACKGROUND

The chemical engineering curriculum has been rapidly evolving in recent years to address the needs of current graduates. More and more graduates are entering the biotechnology and pharmaceutical industries, in response to the growth of those industries[1]. Until recently, chemical engineering curricula included lecture classes and some unit operations laboratory courses to give students practice with chemical process units. While lecture courses are less costly to offer and require less time input by the faculty, there is a consensus that students should be given opportunities to learn engineering with a hands-on approach, preferably in an inquiry-based format[2]. The San José State University (SJSU) College of Engineering is committed to help students to be well prepared to begin working in industry following completion of the bachelor's degree. Thus, the lecture course alone was determined to be inadequate to educate the students in biochemical engineering.

In order to facilitate students learning the principles of biochemical engineering, a one-semester laboratory course (Biochemical Engineering Laboratory, CHE 194[3]) was developed to accompany the one-semester lecture course (Introduction to Biochemical Engineering, CHE 192) offered at SJSU. Both of these courses are three-unit elective courses and constitute the upper division elective courses that are the foundation of the Biochemical Engineering Emphasis. Students in their senior year or beyond of chemical engineering may take the lecture course. If they have taken an upper division biochemistry course they may also take the lecture course even if they are not yet in their senior year. With regards to the laboratory course, senior chemical engineering students or juniors who have completed the CHE 192 lecture course may take the laboratory course. In addition, students from the biochemistry or biology departments may also take the laboratory course, although they find it challenging. In fact, students from other departments are recruited for the laboratory course to foster the multidisciplinary environment that is prevalent in biotechnology companies.

The laboratory course was fashioned as an adaptation of the ECH 161L Bioprocess Engineering Laboratory Course developed by Professor Karen McDonald in the Chemical Engineering Department at the University of California, Davis[4]. To support the laboratory course development at SJSU, an NSF Course, Curriculum and Laboratory Improvement grant was obtained[5]. The funds supported the purchase of two computer-controlled fermentors and a gas analyzer for the offgas. An Agilent University Philanthropy grant was awarded that included a HPLC/MS and a 2100 Bioanalyzer. With the help of some small grants and donations of equipment by local biotechnology companies, the laboratory was fully operational.

COURSE OVERVIEW AND GENERAL SKILLS

The thematic thread of the course is green fluorescent protein. There are several reasons why this is a good protein for students to work with to learn basic laboratory techniques. The protein is very stable and can be exposed to different buffers and pH environments without being denatured. It does not need to be kept cold to prevent degradation. Likewise, since you can detect it with a naked eye by shining a long-range ultraviolet (UV) light on it you can quickly identify where it is. For example, in performing ultrafiltration, you can see immediately whether it is in the retentate, permeate or both. Of course this does not model the real situation, but it facilitates that the students be introduced to many new techniques without requiring additional steps to quantify the protein concentration. The concentration of GFPuv can be determined using a spectrophotometer at 397 nm where the extinction coefficient is 30,000 liter mol⁻¹/cm⁻¹[6]. Students can also use a simple fluorimeter to detect and quantify the
protein when it is in the bacteria. The absorbance measurement requires lysing the cells and removing the cell debris, which results in some loss of the protein, but this method works well for the protein purification labs.

Although all the bacteria handled during the course are generally recognized as safe (GRAS) organisms, there are still safety issues for the students to consider. During the molecular biology labs, ethidium bromide is used to identify DNA bands on the gels. Students are cautioned to wear gloves and thoroughly clean any spatulas or other tools used to handle the gels. The binders of MSDS in the lab are available for all chemicals handled and students are advised to consult those reports prior to working with any chemicals. Gloves and safety goggles are worn at all times in the lab.

Students are expected to develop certain basic skills during the laboratory course. The list of learning objectives pertaining to general procedures is:

**LEARNING OBJECTIVES: GENERAL PROCEDURES**

1. Explain any term on the syllabus to a lay person.
2. Carry out bioprocess and molecular biology experiments taught in the course in a safe manner.
3. Document an experiment in a laboratory notebook format.
4. Determine the total protein in a solution of proteins.
5. Transfer volumes of liquid accurately using micro-pipettors.
6. Prepare buffer solutions at the appropriate pH.
8. Prepare nutrient agar and nutrient agar plates.
10. Measure optical density of bacteria and estimate the concentration of bacteria in a sample.
11. Streak a nutrient agar plate with a solution of bacteria appropriately to achieve single colonies.
12. Measure fluorescence and optical density in the linear region of the instrument.

**SUBCLONING EXPERIMENT**

During the first five weeks of the course, students perform a subcloning experiment. Students are split into five groups with a maximum of 4 students per group. The gene for green fluorescent protein (GFPuv developed by Crameri [7]) is amplified from the pBAD-GFPuv plasmid with polymerase chain reaction, cut with two restriction enzymes, and inserted into a pET vector for high protein expression levels[8]. Students learn basic molecular biology methods as well as sterile techniques. The goal of this experiment is to solidify some of the biology content they learn in the lecture course by actually performing the experiments. The students learn about bacterial transformation and the process for designing PCR primers, among other topics. It is not the intent that students be prepared to take a job after graduation that is primarily molecular biology, but it is helpful for the chemical engineering students to understand the molecular biology in order to communicate effectively with biologists and biochemists in the companies where they take employment.

The final class period is dedicated to comparing the expression levels of the GFPuv in the Bl21(DE3)-pET-GFPuv host with the levels in the HB-101-pGLO bacteria. The level of fluorescence in the Bl21(DE3) pET strain is about 50% higher than with the pBAD-transformed strain.

As this is the first time that many of the students are doing molecular biology, it normally happens that some of the students get very faint or no bands on the gels. For example, after the PCR step, often there are bands that are too faint for the students to realistically complete the remaining steps with adequate DNA to insert, transform, and successfully obtain colonies. This situation is handled by having students with very bright bands share some of their DNA with the students without adequate DNA. The principle technique that has not yet been mastered by the students is pipetting, and the novices need to practice for a few lab periods. So far, every year there are students who complete all the steps and successfully obtain colonies expressing the GFPuv. The specific learning objectives for the subcloning experiment are listed below:

**LEARNING OBJECTIVES: SUBCLONING EXPERIMENT**

13. Transform competent *E. coli* cells with a plasmid.
14. Cut DNA using a restriction enzyme.
15. Estimate the annealing temperature of a PCR primer.
16. Estimate the size of DNA using gel-electrophoresis.
17. Purify a band of DNA from an agarose gel using a commercial kit.
18. Purify plasmid DNA using a mini-prep kit.
19. Identify ribosome binding site, start and termination locations of gene transcription, location of PCR primer binding, promoter and other key features of a plasmid.
20. Estimate the volumes of insert and vector solutions necessary for a ligation.
21. Ligate an insert into a vector using a 5-minute ligation kit.
22. Identify the bases on sticky ends of DNA cut with known restriction enzymes.
23. Determine whether an insert is present after ligation and transformation.

The remainder of the course is split into three three-week modules. The class is divided into three groups with a maximum of 6 students per group. Students carry out fermentation, protein handling – including enzyme kinetics and ultrafiltration, and chromatography. Two labs are included, as well, for students to propose, design and execute an experiment of their own interest.

**CHROMATOGRAPHY MODULE**

Many biochemical engineers working in industry focus on downstream processing. One of the principle methods used for the purification of proteins is chromatography. In the lab class, a Fast Performance Liquid Chromatograph (FPLC) is used to purify GFPuv from cell extracts (Pharmacia LKB-501 Plus Controller with FPLC Direktor software). Chromatography steps comprise two of the three weeks of the module, and the third week is set for one of the inquiry lab sessions. The procedure for the purification of GFPuv is
adapted from Chalfie and Kain [6]. Students are provided with approximately 1.5 mL of concentrated cell extracts to start the purification. The objective is to perform first a hydrophobic interaction chromatography (HIC) step followed by concentration of the fractions with ultrafiltration and then to perform an anionic exchange (DEAE) step. Typical plots of the elution profile of the HIC and DEAE steps can be seen in Figures 1 and 2, respectively.

Finally the students concentrate again the pooled fractions and measure the final purification factor and total GFP present. The purification factor is the ratio of GFP to total protein compared with the same ratio determined in the original cell extracts. The yield of GFP is the fluorescence times the volume of product of the purified solution relative to the same value calculated for the original cell extracts. Students report both the purification factor and the yield after each step of the purification process. Typical values of the purification factors and yields are listed in Table I.

Students are also asked to consider how the process could be scaled up to purify the cell extracts from a fermentation run. The learning objectives for the chromatography lab include:

**LEARNING OBJECTIVES: CHROMATOGRAPHY**

24. Partially purify GFP using an FPLC.
25. Analyze the purity of a protein of interest throughout the steps of a purification process.
26. Identify the fractions of eluate that contain the protein of interest.
27. Predict the elution time of a column based on experimental data.
28. Perform a HIC chromatography step to partially purify a protein.
29. Perform an ion-exchange chromatography step to partially purify a protein.

**PROTEIN HANDLING MODULE**

Proteins are a common product in biotechnology companies and thus, their special handling requirements merit time in the bioprocess engineering laboratory. The first week of the module is dedicated to determining the kinetic parameters of an enzyme. The lab was designed to be as inexpensive as possible, which presented a challenge because purified enzymes are often high priced. Trypsin, however, is a common enzyme because it is used for many applications in tissue culture and digestion of protein samples. The source of trypsin is a 500 gram bottle of Fisher laboratory grade trypsin, and a colorimetric substrate was identified so the kinetics can be easily measured in a spectrophotometer. The substrate is benzoyl DL-arginine p-nitroanilide hydrochloride, which is hydrolyzed to produce p-nitroanaline (DL-BAPA) in the presence of trypsin[9]. This enzyme/substrate combination has some interesting educational advantages, namely, the autohydrolysis of trypsin and the precipitation of the substrate together expose the students to some typical challenges of determining enzyme kinetic parameters. Because of both the autohydrolysis and the precipitation, the data is highly scattered (see Figure 3) and the students need to thoroughly discuss the sources of error in their reports.

The first year the class was given, the enzyme kinetics lab was run as an inquiry experiment. Students were presented with the enzyme and substrate and told which pH to use and what the extinction coefficient of p-nitroanaline was, but they were to determine the proper concentration of enzyme to use and what range of substrate concentrations to use. The result was the students spent the lab period testing various solutions and each time measuring not enzyme kinetics but rather the
kinetics of precipitation of the substrate in the cuvettes. Thus, for this experiment, it was determined that a “cookbook” strategy was the most effective to enable them to collect the necessary data to determine the kinetic parameters. The procedure for the experiment is available on the course website [3]. The limited solubility of the substrate prevents the determination of the rate close to the $V_{\text{max}}$, but a typical plot (Figure 2) of the kinetics enables the estimation of $k_{\text{cat}}$ and $K_M$. It can be seen that the error in the estimations of $k_{\text{cat}}$ and $K_M$ are very high (standard error about 50%).

The second week of the module is an experiment on cross flow ultrafiltration. The apparatus consists of a pump, a small tank, a pressure meter and the hollow fiber membrane module with two simple valves, one upstream and one downstream from the membrane, to allow for control of the cross-membrane pressure drop. The students are to determine the rejection coefficient ($\sigma$) of the membrane as a function of pressure for milk protein according to (1),

$$\sigma = 1 - \frac{C_P}{C_R}$$

where $C_P$ and $C_R$ are the concentrations of protein in the permeate and retentate, respectively. Protein concentrations are determined using the Pierce Coomassie-Plus reagent. Additionally, the concentration factor as a function of pressure is determined by (2) as

$$CF = \frac{V_R + V_F}{V_R}$$

where $V_R$ and $V_F$ are the final volumes of retentate and permeate. The learning objectives for the protein handling module include:

**LEARNING OBJECTIVES: PROTEIN HANDLING**

30. Measure the $k_{\text{cat}}$ and $K_M$ of an enzyme for a colorimetric substrate.

31. Design an experiment to confirm the $k_{\text{cat}}$ and $K_M$ of an enzyme.

32. Design and experiment to measure the $k_{\text{cat}}$ and $K_M$ of an enzyme for a non-colorimetric substrate.

33. Measure the rejection coefficient of a membrane in a cross-flow apparatus.

**FERMENTATION MODULE**

The first week students prepare the fermentor with a semi-minimal media. The media used is described by Reisenberg [10] with the addition of 0.1 g/l of yeast extract. The wildtype *E. coli* are able to grow in the minimal media, but the transformed cells only grow with the addition of the yeast extract. The strain grown in the fermentor is the BL21(DE3) pET-GFPuv E. coli that the students prepared with the subcloning experiment. As the fermentation experiment is much longer than 5 hours, the TA or instructor must tend to

the process for the beginning of the run until the students come to class. Frequently, students participate outside the regular class time in order to learn the entire procedure well.
Typical data from the fermentation can be viewed in Figures 4-5. Note that the fluorescence shown in Figure 5 is related to the expression level of the GFP and thus provides a simple and fast way to identify the levels of recombinant protein expression. The fluorescence is normalized to the optical density to show that the amount of protein expressed per cell increases after induction. The learning objectives for the fermentation module include:

**LEARNING OBJECTIVES: FERMENTATION**

34. Prepare a fermentor for autoclaving and autoclave it.
35. Prepare a semi-minimal media for an E. coli fermentation.
36. Perform a carbon balance on a fermentation as the material balance for the process.
37. Perform an ATP balance on a fermentation as an estimate of an energy balance.
38. Sample a fermentor.
39. Measure the optical density of a fermentation sample using a UV/Vis Spectrophotometer.
40. Measure the glucose concentration of a fermentation sample using a YSI Glucose Analyzer.
41. Estimate the oxygen uptake rate, carbon dioxide evolution rate and mass transfer coefficients using measurements of O2, CO2 and total gas flow of the offgases of a fermentor.
42. Estimate the glucose consumption rate in a fermentor based on glucose concentrations.
43. Identify the phase of a fermentation run based on the dissolved oxygen data.
44. Determine if a contaminant organism is influencing a fermentation run.
45. Estimate \( Y_{XOS} \) and \( Y_{XO} \) in a fed-batch fermentation run.

**INQUIRY-BASED LEARNING**

Two lab periods are open for students to address a question of their own choice in the laboratory. The details of this assignment are described in this proceedings [11]. The students are to submit a proposal for their experiment at least two weeks prior to the class period when they conduct the experiment. The proposal should state the objective of the experiment and demonstrate the significance of the experiment. This allows the students to learn more on the particular topic they are interested to pursue. The learning objective for the inquiry experiments is:

46. Students can design and execute an experiment in the bioprocess lab.

**LABORATORY REPORTS**

The weekly laboratory reports and one major report constitute 50% of the grade for the course. The emphasis on report writing is to help the students learn to carefully document their data in their experiments as well as to be able to discuss the outcome of their experiments. The discussion has proven to be the most difficult part of the report for the students. They are often inclined to simply repeat general information about a particular technique or repeat the typical sources of error from one report to another, without reflecting on the meaning of the data they gathered. Well written reports include a thorough evaluation and interpretation of the results in the discussion section that lead to the statement of conclusions from the experiments. This is a process that requires critical thinking skills that students must possess to be effective engineers. The report format is described below.

**LABORATORY REPORT FORMAT**

The report is broken into two parts. The Objective, Materials and Procedure are to be turned in at the beginning of the class period when the experiment is performed. In this way, students are prepared to carry out the experiment. The Results, Discussion and Conclusion are turned in a week after the experiment is performed. Each section is described for the students. Examples are presented on the course website to assist the students with writing their reports.

**Objective:** One or two sentences describing the purpose of the experiment. You should be able to articulate this prior to doing the experiment. Ask yourself, "Why are we doing this?" and let the answer to the question guide your objective. The objective should be more focused than "to use a particular piece of equipment" such as "to run a fermentation" or to do a "type of experiment" such as "to do a miniprep." These do not answer the question, "Why?"

**Materials Used:** Since this portion should be done prior to the actual experiment, it is recommended that you work on it during the previous week while you are in the lab.

**Chemicals:** Note the brand, catalog number (if available on the packaging) and reference to any relevant hazardous information (only seriously hazardous chemicals need to be described - e.g. ethidium bromide).

**Equipment:** list the equipment used for the experiment including make and model. Be careful to note if there are perishable solutions as part of the piece of equipment and note the date prepared. Some equipment include several parts, e.g. the Pharmacia FPLC has detachable columns, pumps, detectors, fraction collector, computer and software. Note changes relevant to each experiment, e.g. you changed the column, or the column media, etc.

**Procedure:** The steps of the experimental process are listed. You should include details here of how to set up the equipment, if it needs to be set up. You should include relevant times, such as how long it takes to warm up, if it needs to warm up, etc.

1. Include details of the order chemicals are put into solutions, listing the exact amounts added and also the molar concentrations. Note the order to add the components, if it is necessary.
2. Accuracy is more important than length. For the report, include adequate information for another person to repeat the experiment, assuming they already have run similar experiments.
3. Procedures carried out earlier may be referenced by stating the date of the lab.

**Results:** Describe the outcome of your experiment. Be as clear and complete in your presentation as possible. Results...
should be appropriate to the experiment. For most straight line plots, just include the equation with the relative variance ($R^2$).

Discussion: Discuss your experiment. The discussion should not include merely restating the procedure that was followed or the general significance of the technique such as could be found in a textbook. Include all your observations about the experiment here. Anything you put into conclusions should be described here first. If there was previous work in other labs, list references here.

Conclusions
1. A simple and concise statement is appropriate if there are conclusions.
2. Do not jump to conclusions that you have not demonstrated.
3. The conclusion should have technical content, not a summary of the actions performed in the experiments. If the objective of the experiment was to determine some parameter, the parameter should be presented together with the standard error.

Summary
A laboratory course to provide hands-on experience in bioprocess engineering was developed for chemical engineering students. Students learn fermentation, chromatography, determination of enzyme kinetic parameters, ultrafiltration and some molecular biology. The goal of the course is to prepare students to work in biotechnology companies upon graduation with their chemical engineering degrees. A strong emphasis is put on proper report writing in the course to help hone their written communication skills. Procedures for the experiments are all available on the course website.

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References