Introducing Chemical Engineering Undergraduates to Bioprocessing through the Unit Operations Laboratory

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Abstract – In response to the recent biotechnology boom, many chemical engineering departments have recognized the need to incorporate biology, biochemistry, and bioprocessing into the undergraduate curriculum. Since curriculum changes can often be difficult to implement, departments must develop innovative problems, projects, and laboratory experiments illustrating bio-principles that can be incorporated into existing chemical engineering courses. The Dave C. Swalm School of Chemical Engineering has successfully implemented a bioprocessing experiment in the Unit Operations Laboratory. The key component to developing this experiment was utilizing a lab-scale fermentation unit to monitor and maintain system temperature, pH, agitation rate, and oxygen content. The goal of the lab was to utilize yeast to perform a glucose to ethanol fermentation. This paper will outline the methods used to develop the lab, the results obtained during the experiment, and provide information to other faculty regarding how to implement this experiment in their own chemical engineering lab.

Keywords: Bioprocessing, Fermentation, Unit Operations Laboratory

INTRODUCTION

One need only note the number of chemical engineering programs (approximately 20) which have been renamed chemical and biochemical, chemical and biological, or chemical and biomolecular engineering to realize the important role the fundamental biological sciences play in the advancement of this discipline. While not all find it necessary to change their department's name, nearly all of the remaining chemical engineering programs list some form of biotechnology as one of their key areas of research. Many departments have bridged the knowledge gap between the traditional biological sciences and engineering by requiring that undergraduates complete fundamental classes in biology, microbiology, or biochemistry. Others have successfully developed and implemented lower- and higher-level bio-engineering electives designed specifically for and taught by engineers. While enhanced knowledge of basic biology, biochemistry, and biotechnology related topics is the long-term goal, curriculum changes can often be difficult and time consuming to implement. Additionally, what separates engineers from many scientists is the engineer's ability to apply basic science principles to improve unit operations, troubleshoot production techniques, and improve or develop chemical processes. The challenge facing departments as they begin to explore this new area of chemical engineering is to find innovative problems, projects, and laboratory experiments illustrating bio-principles as they relate to the discipline that can be incorporated into existing core chemical engineering courses.

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The Dave C. Swalm School of Engineering at Mississippi State University has successfully implemented a bioprocessing experiment in the second semester Unit Operations Laboratory. The overall goal of the exercise was to give the students a hands-on opportunity to experiment with a unit operation that illustrates a basic bioprocessing concept, in this case fermentation. Key to developing this experiment was utilizing a laboratory-scale fermentor unit consisting of a reactor and controller to monitor and maintain system temperature, pH, agitation rate, and oxygen content. Specifically, the experiment involved utilizing yeast to perform a glucose to ethanol fermentation. This paper will outline the methods used to develop the lab, the results obtained during experimentation, as well as the bioprocessing principles the exercise conveys to students. It will also provide information and suggestions on how other educators might implement this experiment in their own chemical engineering lab.

THEORY

Chemical engineers pursuing a career in biotechnology or bioprocessing areas will likely encounter industrial processes that involve the growth of microorganisms for the production of fuels, specialty chemicals, and food items. Operation and optimization of these processes can be quite different from traditional unit operations typically investigated in the undergraduate curriculum. Industrial bioprocesses pose unique challenges, as microorganisms, or biocatalyst, often require strictly controlled environments with specific temperature, headspace gas, and nutrient requirements. These processes also tend to be highly sensitive to even small changes in operational conditions and are often more expensive to develop and maintain when compared to abiotic (non-biological) processes [Bailey, 1].

Probably the most common biological process utilized on an industrial scale is fermentation. In the physiological sense, fermentation refers to a cellular metabolic process in which the energy substrate is oxidized and degraded without the participation of an externally derived electron acceptor. Usually, the catabolic pathway produces an intermediate (such as pyruvate) that serves at the electron acceptor. Fermentation is typically an anaerobic (oxygen-free) process but in some instances can occur when oxygen is present. Fermentation is a much more general term when applied in the industrial sense, referring to the mass culture of microorganisms (or plant and animal cells), the production of alcoholic beverages, and the production of fuels, specialty chemicals, antibiotics, and amino acids. While the end products may be different, nearly all industrial fermentation processes face the same challenges involving nutrient media development, low product yields, and growing and maintaining a healthy population of the appropriate culture of microorganisms. Also key to successful industrial fermentation operations are the design of the chemical reactor, known as a fermentor, and the supplementary equipment required for processing, which are more traditional areas of expertise for chemical engineers [Prescott, 3].

This particular experiment involved the conversion of glucose to ethanol using a yeast culture, the pathway typically responsible for most beverage alcohol production. The biocatalyst used for this process includes strains of *Saccharomyces,* most notably *Saccharomyces cerevisiae*. The pathway for this conversion occurs in two stages: the breakdown of glucose to pyruvate and the fermentation process where pyruvate is converted to ethanol. The breakdown of glucose to pyruvate can occur by several different pathways, including the glycolytic pathway, the pentose phosphate pathway, and the Entner-Doudoroff pathway. While the details of these pathways will not be discussed here, the general purpose of each is to generate adenosine 5'-triphosphate (ATP), nicotinamide adenine dinucleotide phosphate ($NADP^+$), and nicotinamide adenine dinucleotide (NAD^+) for energy transport. More relevant to this experiment is the fermentation pathway, where in general, pyruvate acts as electron acceptor during oxidation of NADH to NAD^+ and ATP is formed in the absence of oxygen. Specifically during alcoholic fermentation pyruvate is decarboxylated to acetaldehyde, followed by reduction to ethanol by alcohol dehydrogenase with NADH as the electron donor. In addition to ethanol, the conversion of glucose to ethanol also produces carbon dioxide, as illustrated by Pathway 1 shown below [Prescott, 3].

$$\text{Pyruvate} \xrightarrow{\nearrow\ CO_2} \text{Acetaldehyde} \xrightarrow{NAD} \text{Ethanol} \qquad (1)$$

In order to generate sufficient amounts of biocatalyst, yeast must be first cultured in an aerobic growth process prior to beginning ethanol production. For this process to be successful, it is crucial that oxygen levels in the culture vessel not be limited. A continuous supply of air must be available, and oxygen transfer is often enhanced by

agitating the culture mixture. It is also important that the concentration of glucose remain relatively low during the growth process, as high sugar concentrations can induce ethanol production even under aerobic conditions. Both cell growth and ethanol production occur at moderate temperatures ranging from 25-38° C. *Saccharomyces* also prefers a slightly acidic pH range of 3.5-4.5, and like all living organisms, requires both nitrogen and phosphorus for cell growth and substrate metabolism. The enzymes responsible for the conversion of pyruvate to acetaldehyde and acetaldehyde to ethanol require a derivative of vitamin B_1 and Mg^{2+} in the production media. The maximum amount of alcohol produced by yeast is about 15% (vol), and approximately 55% of the glucose is converted to ethanol. Additionally, the ethanol production rate of *Saccharomyces* can approach 4.5 g per L-hr [Prescott, 3]. If a mixture of various sugars rather than pure glucose is used as the substrate, these yields may decrease, as not all sugars can be fermented by *Saccharomyces*.

The procedure required to complete this experiment illustrates a number of concepts to students. It is designed to provide experience in growing a culture of organisms and inoculating a reactor system, including understanding proper nutrient solution preparation and the process and purpose of autoclaving (steam-sterilizing) certain reactor components. These concepts have not traditionally been introduced in the ChE curriculum, but are important to biotechnology and bioprocessing education. The ethanol production and reactor operation portion of the lab follow more traditional chemical engineering laboratory experimentation, requiring students to collect operational data such as pH, temperature, and dissolved oxygen content and collect and evaluate samples using common analytical methods. Also illustrated are kinetic principles, including substrate conversion and production rates with respect to the amount of biocatalyst in the system. The data collected can be used to estimate reaction order, reaction rate, and ethanol production rate, allowing students to apply their knowledge of standard abiotic chemical processes to evaluation of biological processes.

MATERIALS AND METHODS

Equipment

Experimentation was carried out in a New Brunswick BioFlo 3000 bench-top fermentor with a working volume of 10 liters, as shown in Figure 1. The reactor headplate was equipped with ports for inoculation, media addition, and headspace gas supplementation; a thermowell for temperature detection; a sampling tube; an exhaust condenser; and dissolved oxygen and pH electrodes. The unit was also equipped with an agitation motor and impellers capable of providing agitation of 50 to 1200 RPM; however, due to equipment malfunction, this function was not utilized. The dissolved oxygen and pH electrodes were calibrated as described in the New Brunswick BioFlo 3000 Operations Manual. The dissolved oxygen probe was calibrated using two conditions: distilled water saturated with air and distilled water saturated with nitrogen. The dissolved oxygen probe is designed to report a unitless value in the range of 0-100, with zero corresponding to no dissolved oxygen in the liquid and 100 corresponding to complete saturation of oxygen in the liquid. The pH electrode required a two-point calibration using pH 4.0 and 7.0 buffer solution [New Brunswick Scientific, 2]. Figure 2 shows the entire experimental set-up, including the fermentation unit and compressed gas supply.

Inoculum Preparation

To perform the glucose to ethanol fermentation, a culture of *Saccharomyces* was obtained in the form of brewer's yeast (SuperStart Distiller's Yeast, Midwest Homebrewing Supplies, Minneapolis, MN). This particular dry yeast was selected due to its reported high alcohol tolerance (up to 22%). The dry yeast was reconstituted using tap water (5 g yeast per 10 mL total volume) and incubated at 30-40° C for 20-30 minutes, as recommended by the supplier. One liter of starter culture of yeast was grown for 3-5 days in a 0.5% glucose and mineral salts media at temperature of 25-30°C in a shaker incubator. Prior to adding the yeast, the sugar and MSM mixture and the necessary glassware were autoclaved and allowed to cool. The starter culture was housed in an Erlenmeyer flask fitted with a sponge stopper to facilitate oxygen transfer to the liquid. The ratio of starter culture to total volume was 5 g to 100 mL.

Reactor Operation

The media used for ethanol production consisted of sugar and MSM. This lab targeted a final ethanol concentration of 10% (vol), which, based on the accepted theoretical yield of ethanol production by *Saccharomyces*, translated to about 650 g of sugar in 3.5 liters of MSM. The glucose/MSM mixture was autoclaved prior to use in the bioreactor. An additional 1000 mL of starter culture was also added to the reactor, bringing the total working volume of the system to 4.5 liters. To acclimate the inoculum to the system, the reactor was first operated aerobically to promote additional cell growth. A compressed air bottle was connected to the reactor headplate and gently sparged air into the reactor liquid. After 18-24 hours, the air was disconnected and the reactor was placed under a nitrogen purge. Nitrogen gas was not bubbled directly into the liquid in order to prevent gas stripping of any ethanol produced during the experiment. Rather, a slight positive pressure of nitrogen gas was maintained in the reactor headspace in order to prevent oxygen from entering the system. The nitrogen purge remained on for the remainder of the experiment. The reactor temperature was maintained at approximately 30° C; however, successful fermentation can take place in a temperature range of 28-38° C.

Data Collection and Analysis

Since the conversion of glucose to ethanol is a relatively slow process, this experiment extended beyond the scheduled 4-hour laboratory periods. In addition to the two lab periods set aside for this experiment, students were required to collect samples for an additional 4 days beyond initialization of the experiment. Sampling consisted of collecting 5-10 mL samples of the liquid mixture for both ethanol analysis and biomass quantification using the sample port on the reactor headplate. Ethanol content was determined using a Reichert-Jung refractometer (Mark II ABBE model #10480) calibrated with mixtures containing known amounts of ethanol/glucose/MSM over the range (0% - 10% by volume ethanol). Each point on the calibration curve was the average of three individual analyses of the appropriate standard solution. All liquid samples were filtered using a syringe fitted with a 0.22 μm syringe filter before being placed on the refractometer. Filtering removed any biomass in the sample, which would interfere with the refractometer reading. An unfiltered portion of the liquid was reserved and used to quantify the amount of biomass present in the system. Biomass content was estimated via solids analysis using an Ohaus Moisture Analyzer (model #MB35). At least two samples were analyzed for solids content; an initial sample collected during reactor inoculation and setup, and a second sample sometime during anaerobic fermentation (day 3 or 4). The moisture analyzer required a sample size of approximately 1 g of liquid (about 1.5 mL) and reported the weight percent solids in the sample. Students also recorded the reactor temperature and system pH and dissolved oxygen concentration each time samples were collected.

TYPICAL RESULTS

This laboratory exercise was completed a total of 4 times during the Spring 2004 semester. With each subsequent experiment, slight modifications were made to the procedure; therefore, the procedure and results discussed in this paper reflect those changes. For consistency, the data shown in this paper was collected during experimentation using the procedure as described above. Additionally, the group that collected this data elected to allow the experiment to continue for 2 additional days beyond what is suggested in the procedure, providing additional information regarding ethanol production.

Operational parameters including pH, temperature, and dissolved oxygen content were observed to be in the appropriate range for this experiment. System pH decreased from 5.4 (pH of MSM and inoculum mixture) to 3.0 at the conclusion of the experiment. The temperature was controlled to a range of 25-30°C, and the dissolved oxygen during the aerobic phase just after inoculation was about 40 and decreased to 0 or less during the anaerobic ethanol production phase. The exact value of the dissolved oxygen content was not as important as the relative value on the scale of 0-100, allowing students to verify that anaerobic conditions were maintained in the reactor.

Figure 3 shows the best case results regarding ethanol production. As shown in the figure, when the experiment was allowed to progress for 6 days, the ethanol content in the fermentor increased to about 7% by volume. The

target ethanol concentration was approximately 10%; however, some sugar was likely consumed during the aerobic inoculation and acclimation phase on the first day of experimentation. As a result, the 7% concentration is not unreasonable considering the operational considerations, and this was the highest ethanol concentration produced of any of the four experiments. Due to limitations associated with the analytical equipment, low concentrations of ethanol (less than ~2%) produced negative percentage values that should not be considered in data analysis. Biological systems often exhibit considerable variations in behavior and production is typically expected to be less than theoretical. The biocatalyst concentration corresponding to this ethanol concentration was 13% by weight. Further analysis of the data yielded an ethanol production rate of 0.321 g/L-hr. This is a very low production rate as compared to literature values (up to 4.5 g/L-hr); however, the experiment was operated as a batch system and very likely not at steady state ethanol production. Additionally, the refractometer reported a negative level of ethanol production between days 1 and 2 of experimentation. This is certainly not a valid data point and is likely the result of the limitations of the refractometer at low ethanol concentrations as well as inaccuracies associated with the standards. As a result, no sound inferences can be made regarding ethanol production until day 3 of experimentation. Unfortunately, the motor used to operate the impellers in the unit was not working during experimentation, and the lack of agitation could have also contributed to a decrease in yield as a result of reduced contact between the media and the biocatalyst. Despite these inconsistencies, when considered in the overall context of the experiment, the ethanol production achieved in this experiment was reasonable, and the experiment was considered a success.

LESSONS LEARNED AND RECOMMENDATIONS

Overall, the laboratory exercise as described above was successful in achieving the stated goals. However, as the experimental procedure evolved over the 4 subsequent laboratory periods, several valuable lessons were learned that improved the quality of the laboratory experience. Listed below are those lessons and the corresponding recommendations.

- Anaerobic ethanol production should be allowed to occur for at least five days, but preferably 6-7 days. Experiments where the residence time was less than 5 days exhibited very low ethanol concentrations in the liquid phase, producing poor results.

- Baker's yeast is not an appropriate inoculum source, as it is not acclimated for the high levels of ethanol production achievable with brewer's yeast.

- For these experiments, the glucose source for preparation of the fermentation media was table sugar (due to the large amount needed, low cost, and ease of availability). However, table sugar is not pure glucose and contains other sugars that cannot be fermented to ethanol by *Saccharomyces*. The presence of these other sugars is one likely explanation for the lower yields observed during experimentation; therefore, to optimize the amount of ethanol produced, it is recommended that glucose be the sole substrate in the fermentation broth.

- Standards should be prepared and stored such that the same set can be used for all of the laboratory periods in a given semester. This would best be achieved by autoclaving the standards and stored them in a freezer when not in use.

- Ideally, ethanol content should be analyzed using gas chromatography (GC) or high performance liquid chromatography (HPLC), as the refractometer is unable to distinguish between ethanol and sugar and tends to yield erroneous results at very low ethanol concentrations.

- Additional analysis to measure glucose levels would provide further data for verifying rate calculations. This could possibly be achieved through use of a glucose monitor purchased from a local drug or discount store.

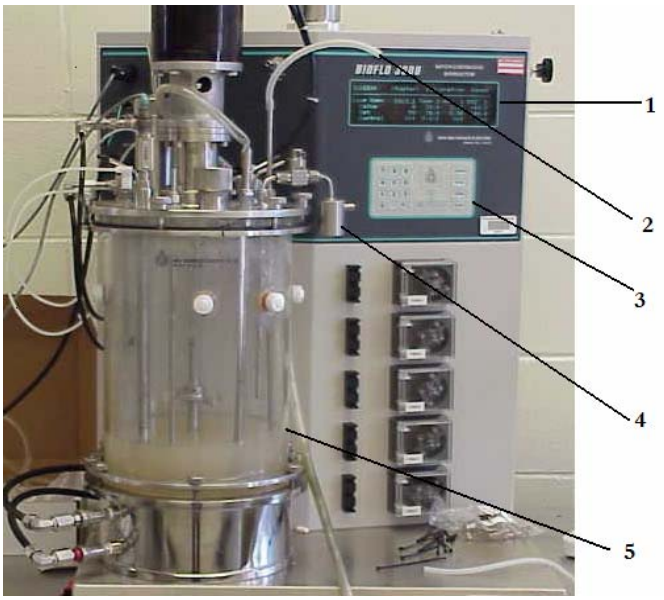
- The fermentation broth should not be sparged with nitrogen during the ethanol production phase, as this may result in gas-stripping the ethanol from the liquid.

- It is imperative that the fermentation unit be equipped with a working agitation device in order to collect well-mixed samples. The reaction mixture should be agitated at low rpm for the entire length of the experiment. Using reactor headspace gases to achieve this mixing is unreliable, difficult to control, and does not provide a well-mixed, homogeneous sample.
- Expand the data analysis requirements to include determination of kinetic constants with respect to ethanol production, time, and the dry weight of biocatalyst in the system.
- Be knowledgeable of university permitting and regulations regarding alcohol and ensure compliance with ATF rules.

SUMMARY

Despite the challenges, we found that with the proper supplies and equipment, this laboratory experiment is relatively easy to for students to set up and execute. Optimization of the laboratory procedures ultimately produced good experimental results, with ethanol production approaching about 7% by volume. With more refined analytical equipment, the data analysis portion of the laboratory report could be expanded to include advanced kinetic calculations. However, as described, the experiment combined with the supplementary reading material provided to the students was effective for illustrating basic bioprocessing principles [Bailey, 1; Prescott, 3]. The students also seemed very satisfied with the experiment, as expressed by the high level of cooperation and participation exhibited during the extended experimental period as well as the positive evaluations received at the end of the course. Laboratories that already have a fermentation unit similar to the one described in this paper should be able to successfully implement this experiment, even with the most basic analytical equipment and simplest chemical components and a few weeks of lead time. If a similar reactor system is not already available, units (new and refurbished) can be purchased for $10,000-$20,000 or more, depending on the size and complexity. If purchasing a unit, it is recommended that at least one semester of lead time be allowed for delivery and installation of the equipment.

FIGURES



1. Calibration and Display Screen
2. Gas Sparging Port
3. Operational Keypad
4. Liquid Sampling Port
5. Fermentation Liquid

FIGURE 1: New Brunswick BioFlo 3000 Fermentor

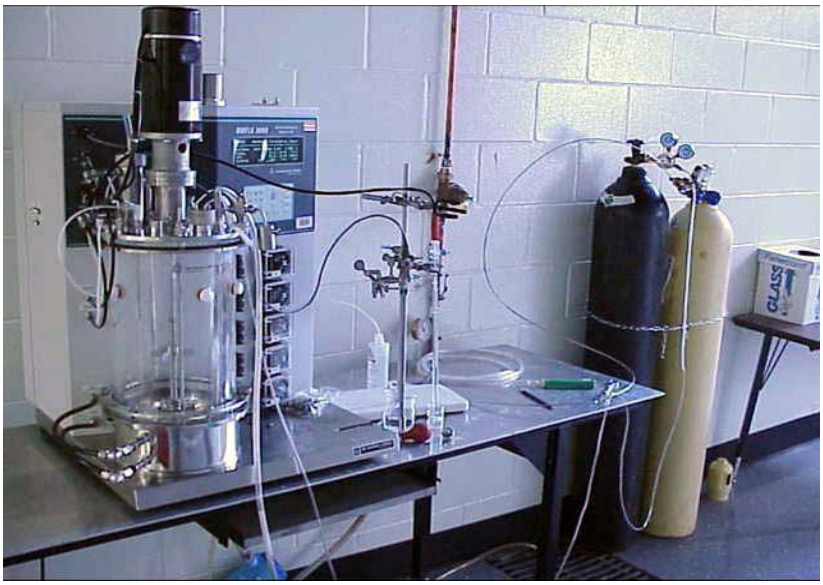


FIGURE 2: Complete Experimental Set-up

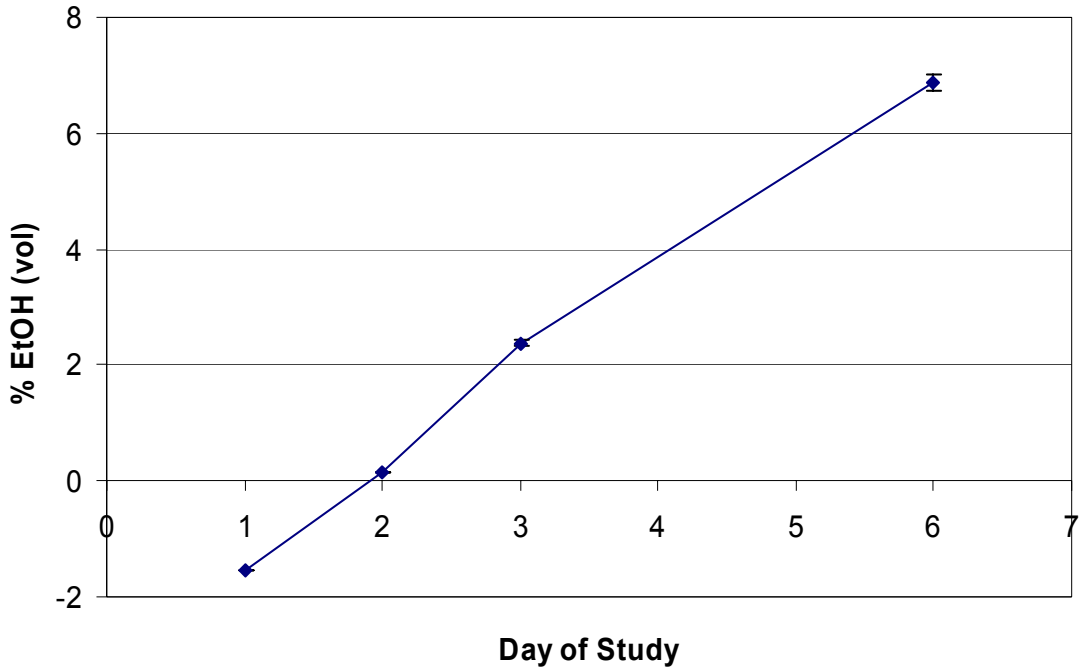


FIGURE 3: Experimental Ethanol Production (13 wt% Biocatalyst)

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