Introducing Biochemistry and Cellular Biology to Chemical Engineering Students by Cultivating a Bacterial Pathogen in a Bioreactor

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Abstract

Maximizing student retention and matriculation in STEM degree programs requires the use of active and collaborative learning techniques and exposure to enriching educational experiences. Studies have shown that interactive instructional methods enhance student engagement and increase student performance overall. The goal of this work was to install a bioreactor lab station in the University of Tennessee at Chattanooga's chemical engineering laboratory, thereby improving student engagement and success through exposure to state-of-the-art research methods in biochemistry and cellular biology. The bioreactor station has been installed and deployed in the junior and senior level chemical engineering laboratory courses, and is also currently being used in undergraduate and graduate level research projects. Initial experiments have focused on studying the bacterial pathogen *Vibrio cholerae* in collaboration with the UTC Department of Biology, Geology, and Environmental Science. The work presented in this paper details the chosen apparatus and its capabilities, the results of the students' work with initial feasibility trials using *Vibrio cholerae* samples and the bioreactor, and how we intend to further integrate innovative bioreactor experiments into engineering curricula and outreach programs. The chosen equipment allows students to monitor and control temperature, pH, and dissolved oxygen concentration, as well as agitation control and real-time sampling capability. During initial feasibility trials, students found that *Vibrio* could be grown in the bioreactor and that data such as growth, membrane permeability, and motility could be collected in real-time over a range of environmental conditions (e.g. temperature, pH, salinity). Future work will be focused on improving the capabilities of this apparatus and expanding the slate of experiments for further integration into engineering curricula and outreach programs.

Keywords

Education, Biochemistry, Cellular Biology, Bacteria, Pathogen

Introduction

Bioengineering is the application of engineering principles to solve real-world problems related to the life sciences. It combines the concepts of biology and chemistry with the methodologies and practicality of engineering to advance applications and create biotechnology. The complexity and biological diversity of organisms provides ample opportunity for innovation, and the field of bioengineering has pioneered technological breakthroughs in agriculture, chemicals processing, and human health and the environment [1]. While there are many different avenues to explore in such a large field, one particular area of interest in bioengineering is the study of the biochemistry and cellular biology of microorganisms, such as bacteria, in order to advance applications in biotechnology.

The first goal for this work is to provide an apparatus that will be utilized in a number of undergraduate chemical engineering laboratory courses and graduate and undergraduate research projects similar to programs employed by Northwestern and Cornell Universities [2, 3]. Through interaction and experimentation using the apparatus, students will be taught the principles of biochemistry and how they can be applied to study microorganisms in a way that can advance the knowledge base of biotechnology. This apparatus will provide a means for students to cultivate microorganisms under controlled conditions, as well as the ability to conduct biochemical assays, collect useful biological data, and learn about bioreactor systems.

In any industrial bioengineering application, bacteria need to be grown for observation, testing, or extraction. A bioreactor lab station suits these needs very well. Bioreactors can produce large amounts of microbial cultures, which would be similar to microbial-based medicinal or industrial processes. A bioreactor can also grow bacteria in a wide range of controllable growth parameters, which is ideal for classroom experimentation and scientific research. Additionally, a bioreactor can be used to demonstrate the use of control systems, which are important tools for a broad range of industrial processes. Finally, the measurement tools and instrumentation of a bioreactor are widely used throughout many industries.

The need for a self-contained and easy to use apparatus, as well as a desire for students to be able to conduct experiments with minimal training, drove the selection of equipment that was as user-friendly as possible. This led to the selection of the New Brunswick Bioflo 3000 Benchtop Fermentor, a microprocessor controlled batch or continuous bioreactor capable of temperature, pH, dissolved oxygen (DO), agitation, pump feed, antifoam, and vessel level control (Figure 1). There is also software available for remote connection to the Bioflo 3000. This computer connection allows for the acquisition of real-time probe measurement data and access to the

Figure 1. Bioflo 3000 5 L Benchtop Bioreactor.

Figure 2. Yamato Scientific SQ810C Autoclave.

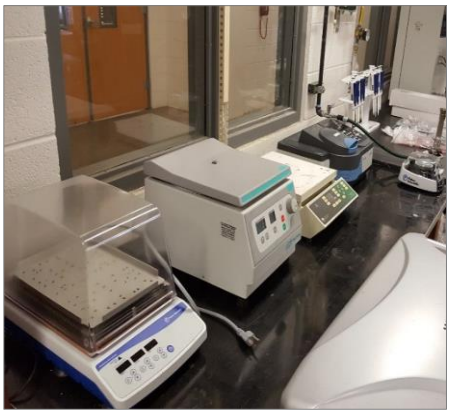

Figure 3. (From Left to Right) Incubator/Shaker, Centrifuge, Heated Stir Plate, Spectrophotometer, Mini Centrifuge, and Pipettes.

control systems settings from virtually anywhere. One such program produced by Foxy Logic, called the Fermentor Control Program, has been used as an interface tool in published research with bioreactor systems [4]. This remote-control capability will enable future projects aimed at K-12 outreach and industry collaborations.

The Bioflo 3000 can support one of four glass fermentor vessels, which come in 2.2, 5, 7.5, and 14 L volumetric sizes. The vessel also has a stainless-steel jacket which supports dramatic temperature shifts, a process that is especially useful in protein production with temperature induced expressions or temperature shock treatments. The vessel is designed with fitted ports at the top allowing for instrumentation such as probes to be inserted, and it has a sample port allowing an easy way to extract samples from the closed vessel. Auxiliary laboratory equipment selected for the bioreactor apparatus includes: a visible range spectrophotometer, two centrifuges (standard and mini), and an autoclave. The autoclave is used for medical grade sterilization of lab equipment, growth media, and the bioreactor vessel (Figure 2). The spectrophotometer can be used for a variety of biochemical assays including sample growth and membrane permeability (Figure 3).

The initial test case for this apparatus was a series of experiments on the Gram-negative bacterium *Vibrio cholerae*, the pathogen responsible for the acute intestinal infection known as cholera. A highly adaptive organism, *V. cholerae* normally inhabits aquatic environments, but upon ingestion by a human host, it is able to survive the acidic environment of the stomach and colonize the small intestine [5-7].

Methodology

The objective of the first set of experiments was to have students demonstrate that *V. cholerae* could be grown aseptically in the bioreactor under controlled conditions, and that relevant biological data such as optical density, membrane permeability, and motility could be collected over the course of a growth cycle. This set of experiments involved five main tests: temperature ramp, temperature shock, pH ramp, salinity shock, and aquatic-to-host transition.

The students aseptically streaked a pure culture of *V. cholera* O1 El Tor C6706 onto a Luria broth (LB) agar plate from a frozen glycerol stock. The inoculated plate was left to proliferate

overnight in an incubator. A colony was then transferred to 5 mL LB in a test tube and incubated overnight. 100 mL LB media was prepared in an Erlenmeyer flask and sterilized in the autoclave, and was inoculated with the overnight culture. The inoculum was then allowed to proliferate for another day, creating a starter culture for the bioreactor. 3 L of LB or minimal salts (M9) media was prepared in the bioreactor and sterilized via autoclave. The bioreactor was brought to initial environmental conditions, and the starter solution was added to the bioreactor vessel via sterilized tubing using a peristaltic pump.

Growth via optical density at 600 nm (OD_{600}) was measured at the beginning of the vessel's inoculation and every 30 minutes afterward. The optical density assays started by drawing culture from the bioreactor's sample port into a sample jar. 1 mL of sample was aseptically transferred from the sample jar to a 1 mL cuvette for a 1:1 dilution. Then, a 1:10 dilution was made by aseptically transferring 0.1 mL of the sample into a 1 mL cuvette and adding 0.9 mL of sterile deionized (DI) water. The spectrophotometer was then calibrated with DI water at a wavelength of 600 nm, and the samples were tested for absorbance.

Students determined the membrane permeability using a crystal violet (CV) uptake assay. Bacterial samples were first washed via centrifugation using phosphate buffered saline (PBS) and brought to an OD_{600} of 1.0 in PBS. A uniform amount of CV staining dye was added to each sample and the samples were incubated in a shaker for several minutes to allow the bacteria to absorb the CV dye. The samples were then centrifuged, separating the bacteria from the PBS/CV solution, and the PBS/CV solution was decanted from the samples. The absorbance of the decanted PBS/CV solution at 590 nm was then measured via the spectrophotometer to determine the percent of CV absorbed compared to a blank of CV in PBS only. Samples were assayed in triplicate for statistical determination of reliability.

Motility assays were performed by students by first washing the bacterial samples via centrifugation with PBS, and bringing them to an OD_{600} of 1.0 in PBS. The samples were then injected via pipette into a motility agar plate that mimicked the bioreactor conditions (e.g. temperature, salinity, fatty acid concentration). The plates were incubated at constant temperature for 12 hours to allow the bacteria to move across the motility media. After the 12 hours, the diameters of the colonies were measured.

Results

The students used the previously presented experimental procedures and tested the bioreactor's capabilities by subjecting *V. cholerae* to temperature changes during growth. First, they decreased the temperature slowly (1^{o}C every hour) from 37^{o}C to 30°C, referred to as a temperature ramp, and measured OD_{600} and CV uptake as the temperature fell (Figure 4). Next, the temperature drop was increased to 3^{o}C every hour, and OD_{600} and CV uptake were again recorded during growth as the temperature fell (Figure 5).

The next set of experiments had the student test the Bioflo's pH controller. *V. cholerae* was grown at 37°C in buffered minimal salts (M9) media instead of LB to allow for pH control. It was then subjected to a pH ramp of +0.5 pH per hour (Figure 6). The pH was controlled using 1.0 M each of sulfuric acid and sodium hydroxide as the acid and base, respectively. Lastly, a set of salinity shock experiments were performed by growing *V. cholerae* at 37°C in M9 (43 mM NaCl) and ramping the salt concentration up to 150 mM, then 300 mM NaCl (Figure 7).

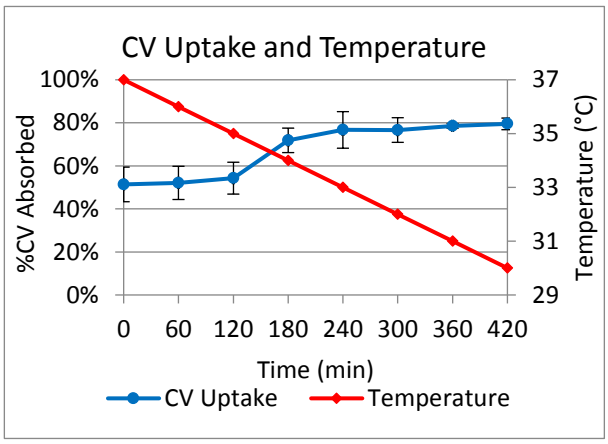

Figure 4: Controlled Temperature Ramp (-1°C per hour)

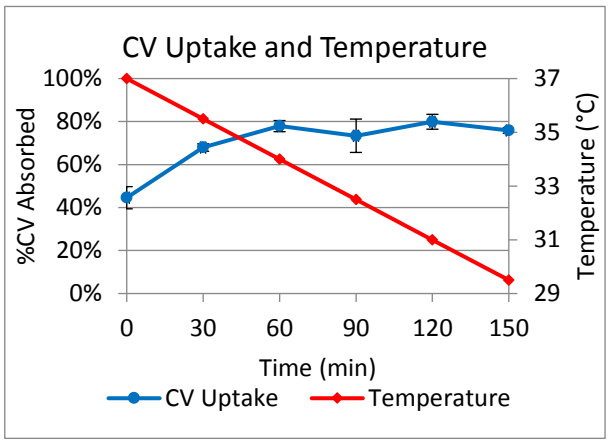

Figure 5: Controlled Temperature Shock (-3°C per hour)

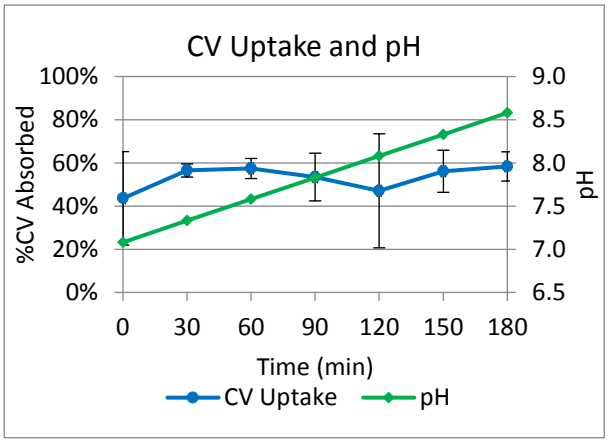

Figure 6: Controlled pH Ramp (+0.5 pH per hour)

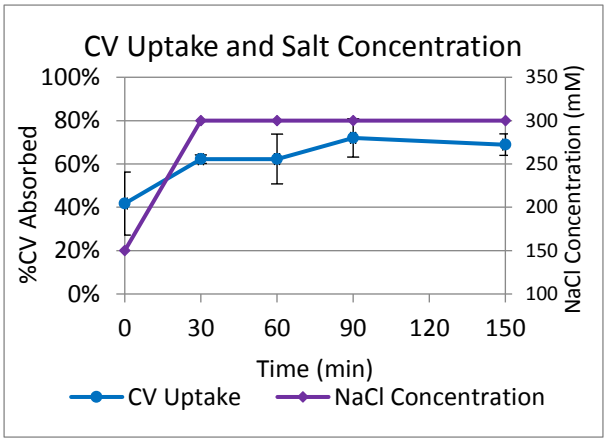

Figure 7: Salinity Ramp (150-300 mM NaCl)

In order to further evaluate the bioreactor's capabilities, the students simulated the transition from a brackish environment to a host's digestive tract. *V. cholerae* was inoculated into the bioreactor in M9 with 300 mM sodium choloride (NaCl) at 30°C and a pH of 8.0. The culture was grown and OD_{600} and CV uptake were measured every 30 minutes. When the culture reached an OD_{600} of 0.8, a motility sample was taken, and the bioreactor environment was transitioned to mimic the upper digestive tract by bringing the growth media to 150 mM NaCl, raising the temperature to 37°C, and lowering the pH to 6.0. After an hour at the new conditions, another motility sample was obtained. Finally, to simulate the small intestines, 150 mM linoleic acid (18:2) was added to the culture. The culture was grown for an additional hour prior to securing the final motility sample. The results of the motility assays are shown in Table 1.

Conditions			Average Colony Diameter (mm)	Error (mm)
Temperature (°C)	NaCl Concentration	Fatty Acid / Concentration		
30	300 mM	n/a	3.0	± 1.30
			2.5	± 0.92
37	150 mM	n/a	11	± 1.30
			13	± 1.84
37	150 mM	18:2 / 150 mM	10.3	± 0.80
			8.25	± 2.00

Table 1: Motility Data

The data shown in Figures 4-7 demonstrate the student's accuracy and consistency using the Bioflo when controlling temperature and pH. These initial trials were also successful in demonstrating that relevant biological data such as growth, membrane permeability, and motility could be analyzed from the samples the students drew from the bioreactor. Because the Bioflo 3000 system controlled variable response performance has worked flawlessly during initial testing parameters, it invites the possibility for even more rapid changes in environmental conditions and a wider array of experimentation possibilities.

Conclusions and Recommendations

This project has provided a means to teach students how to apply engineering principles to biological problems and has served to expose them to cutting edge research in biotechnology. It was initially used for graduate and undergraduate research projects for bioreactor station set-up and feasibility trials. It was then deployed in a junior level unit operations laboratory course where students learned proper aseptic technique and how to develop a standard operating procedure. It was also deployed in a senior chemical processes laboratory course where students designed and conducted experiments to achieve a desired objective (e.g., control pH during growth), collected and analyzed data, and reported their results in both written and oral form.

This apparatus will continue to be a valuable asset for the UTC chemical engineering program. Future work involving this apparatus will be focused on facilitating remote operation. This will enable its use in additional engineering curricula, such as controls systems and computer science, as well as K-12 outreach programs. Also, the apparatus will be used to cultivate other microorganisms of interest for additional laboratory experiments, graduate/undergraduate research projects, and industry partnerships.

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