pH Control in a Miniaturized Bioreactor

by

Aaron A. Griswold

Submitted to the Department of Mechanical Engineering in Partial
Fulfillment of the Requirements for the Degree of
Bachelor of Science in Mechanical Engineering

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ABSTRACT

A miniaturized bioreactor with a volume on the order of 100 μl has been built with the aim of increasing the efficiency of the screening process for various microbial cultures. Unlike larger reactors currently in use, the current miniaturized design lacks a method of pH control. Without pH control, cell growth can be hindered or even stopped altogether when the growing medium becomes too acidic. Using technology already in place to optically measure the pH inside the reactor in conjunction with a valve and a base-filled reservoir, a simple closed-loop (feedback) control system has been developed. The volume of base injected into the reactor must be minimized because the reactor itself is so small. Data is recorded and control signals are outputted by a computer running LabView software. While the control system developed in this thesis shows promise, further development is needed before it can be put to good use.

Thesis Supervisor: Klavs F. Jensen
Title: Professor of Chemical Engineering

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List of Symbols

P_b	–	pressure of liquid base
P_r	–	internal reactor pressure
Φ	–	pH phase shift
ρ_w	–	density of water
g	–	acceleration due to gravity
h	–	water column height
t_v	–	valve operation time
V_b	–	base injection volume
d_b	–	distance traveled by a bubble in the base injection line
r_t	–	inner radius of the tube in which a bubble lies
P_0	–	underpressure applied to pneumatic valve
P_1	–	overpressure applied to pneumatic valve

1.0 Introduction

Fermentations of bacterial cultures are used extensively for both production and research purposes. The variety of uses of the fermentation process is growing rapidly. In production, fermentations are used to generate products such as enzymes, antibiotics, and vaccines. In the laboratory, the uses of bacterial cell culture include metabolic engineering and the generation of biochemical diversity.

Today's fermentation methods are time consuming, labor intensive, and expensive. Starting with the screening phase, experiments are typically carried out using Petri dishes, micro titer plates, and shakes flasks. Next comes the development phase. Here, the physiology of the strain is characterized in greater detail, including optimal growth conditions. These experiments are generally carried out in bioreactors with volumes of 0.5 – 10 L. From there, the process is scaled up until production scale is reached (100,000 – 300,000 L).[i]

Professor Jensen's research group has built a miniaturized bioreactor, and developed a setup and measurement procedure to perform one fermentation run at a time, taking measurements on optical density (to measure cell growth), dissolved oxygen (a nutrient required for cell growth), and pH. This miniaturized bioreactor has the potential to greatly increase efficiency in the fermentation of bacterial cell cultures. Many of these tiny reactors can be run simultaneously, which would yield a much greater quantity of results.[ii] However, a drawback of the previous system was the lack of pH control. *E. coli* growth produces an acid at a non-constant rate and will eventually lower the pH of the growing medium to about 5, which causes cell growth to stop. Operation of the bioreactor at a specified pH near 7 would eliminate this growth limitation and allow cell cultures to grow healthier and to achieve a higher biomass.

2.0 Theoretical Analysis

Control systems are built for four primary reasons: Power amplification, remote control, convenience of input form, and compensation for disturbances.[iii] The control system that is the subject of this thesis is intended to perform the last task. The production of acid and the subsequent lowering of the pH is the disturbance that is to be compensated for. This can be done by introducing a base to the growing medium that will then neutralize the acid.

The internal volume of the reactor is a mere 100 µl, and consequently the volume of base injected into the reactor must be much smaller. This requires a stronger base, but care must be taken because too strong a base can have adversary effects on cell growth. The base must be stored in a reservoir connected to the reactor through a controllable valve. The pressure on the base, P_b, must be greater than the internal pressure of the reactor, P_r, in order to push a volume of base into the reactor when the valve opens. P_r can be held constant by connecting a water column to it as shown in Figure 1. When this is the case,

$$P_r = \rho_w g h. \quad (1)$$

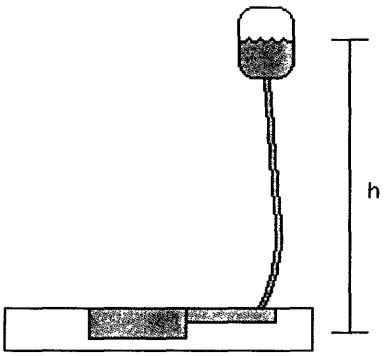


Figure 1: Reactor with attached water column

A typical closed-loop control system includes the following components: a plant, or physical system that is to be controlled; a sensor (or sensors) monitoring the state of the system; a reference value indicating the desired state of the system; and a controller that outputs a signal to the plant depending on the error between the system state and reference input. Figure 2 shows a block diagram representation of a typical closed-loop control system.

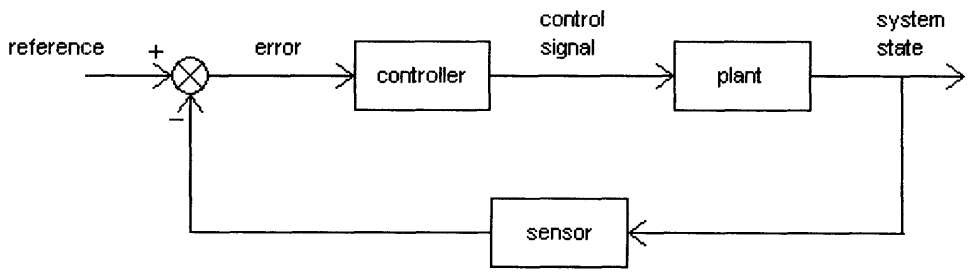


Figure 2: Block diagram of a generic closed-loop control system

In the case of the uncontrolled system in use prior to this thesis, the plant is simply the reactor, and the system state to be monitored is the pH inside the reactor. The sensing method used to obtain values of the pH inside the reactor has already been established by Professor Jensen's group and is as follows. A function generator applies a given frequency-modulated voltage to a blue LED whose light is transmitted via fiber optics to a pH sensor foil located inside the reactor. The sensor foil fluoresces at the same frequency and wavelength, but phase shifted dependant upon the pH inside the reactor. This light is then collected and transmitted by a fiber-optic cable to a lock-in amplifier, where it is then interpreted as a phase shift value, Φ. The pH can then be calculated using the calibration equation,

$$pH = 5.78 + 0.45 \ln\left(\frac{-42.584}{\Phi + 44.978} - 1\right). \quad (2)$$

This calibration equation was obtained by measuring the phase shift when the reactor was at known pH levels. The pH measurement procedure, as well as those for measuring dissolved oxygen and optical density, is controlled and documented by a computer running LabView software. Because only one lock-in amplifier, and one function generator are used to perform all three measurements, none of them can be done simultaneously. Additionally, the pH foil signal degrades over time while exposed to light, so it is best to measure at discrete intervals in order to lessen the frequency at which the foil must be replaced.

LabView can be used not only to take measurements, but also to control various electronic instruments and actuators. The computer running the LabView software will therefore serve as the controller. It must operate an electromechanical valve that opens and closes the connection between the base reservoir and the reactor. While electromechanical valves are getting smaller and smaller as technology progresses, the internal volumes are still too great to simply use one by itself. However, Professor Jensen's group has developed a pneumatic valve with a much smaller internal volume. This valve can be controlled by varying the pressure applied to a gas port that pushes or pulls on an elastomeric membrane. An electromechanical 3-way valve can be controlled through LabView and used to apply two different pressures, P_0 and P_1, to the gas port, as shown in Figure 3. If

$$P_1 > P_b > P_0, \quad (3)$$

then the pneumatic valve will be open when P_0 is applied to the gas port, and it will be closed when P_1 is applied to the gas port (default).

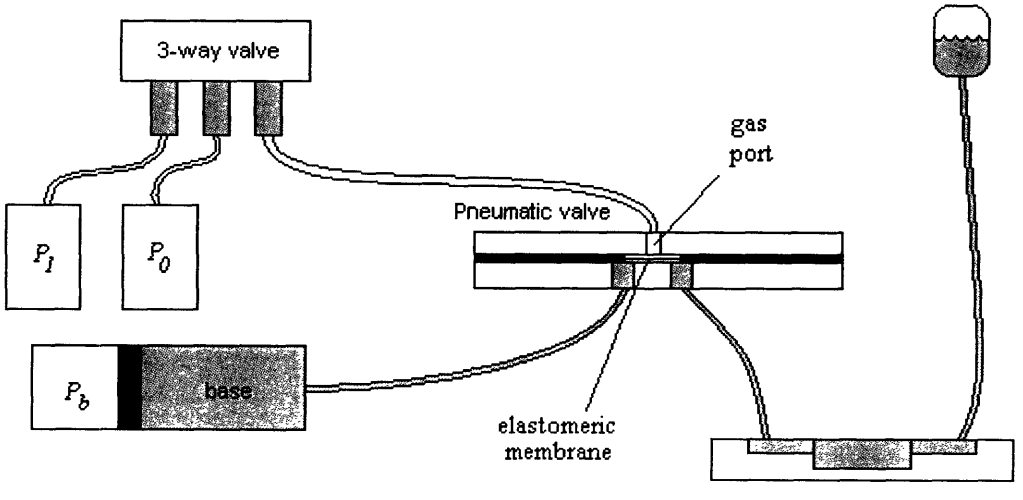


Figure 3: Reactor, water column, pneumatic valve, and 3-way valve configuration

The pneumatic valve, the 3-way valve, the base-filled reservoir, and all their associated pressures become a part of the plant to be controlled by the computer. Figure 4 shows the block diagram for this particular closed-loop control system.

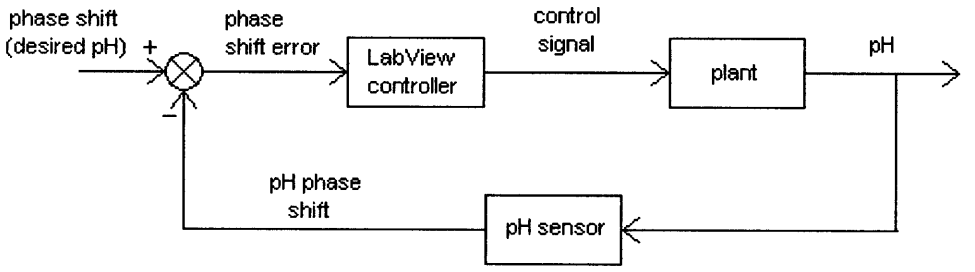


Figure 4: Block diagram of the closed-loop control system

3.0 Apparatus and Procedure

The following sections outline the physical setup and control schematic used to achieve pH control, as well as the methods used to test and operate the system.

3.1 Physical Apparatus

With all of the physical requirements of the control system established, the detail of what actual components are needed to be used becomes more evident. The base reservoir requires 3 ports; one port connected to the pressure source, one port acting as an outlet and connected to the pneumatic valve, and another sealable port used to replenish the supply of base in the reservoir. A disposable filter capsule, seen in Figure 5, has all of the required characteristics. The filter capsule is regularly intended to be used in conjunction with a pressurized gas that pushes a liquid through tiny pores in the filter, and out the port in the bottom.

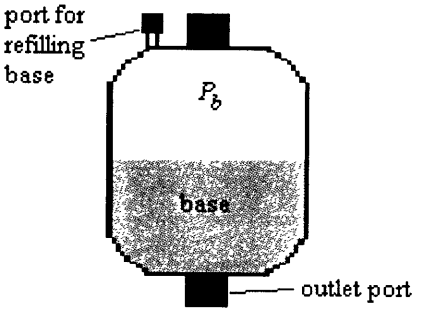


Figure 5: Disposable filter capsule

Nitrogen gas was chosen as the pressure source due to its inertness and availability. The same nitrogen tank can be used to supply the pressure on the base, P_b, and the pressure on the pneumatic valve, P_1, if there is a T-junction in the line and pressure regulators on both branches downstream from the junction, as shown in Figure 6.

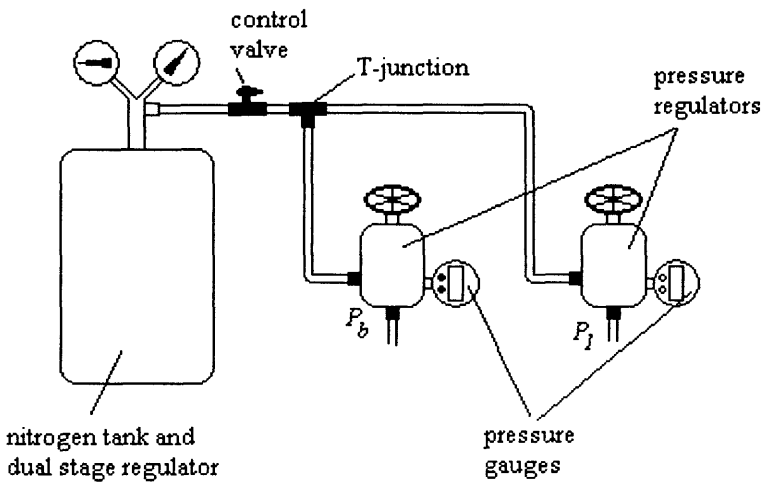


Figure 6: Pressure supply and regulators

The 3-way valve selected is a micro-inert 3-way valve from The Lee Company. After connecting the 3-way valve and the pneumatic valve to their pressure sources, a test was performed to determine if atmospheric pressure is indeed low enough to allow the pneumatic valve to open when applied. If the valve we to not open, a vacuum generator would be needed to provide a great enough underpressure to open the valve. However, the valve did indeed open, and the resulting valve configuration is shown in Figure 7.

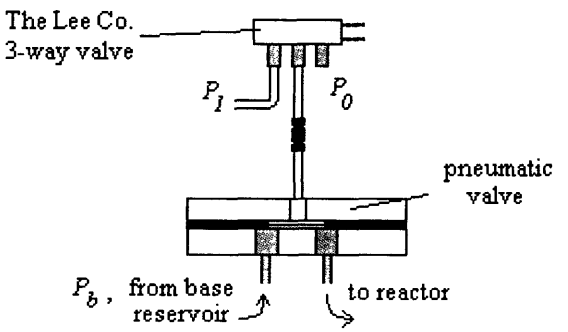


Figure 7: Pressure supply and regulators

To ensure proper mixing of the base and the growing medium, a magnetic spin bar is located inside the reactor, and a permanent magnet motor is placed directly below the reactor. When the motor runs, the spin bar spins, mixing the contents of the reactor.

The entire physical apparatus for this system includes numerous components, many of which were taken from the already existing (non-controlled) system. A complete diagram and list of parts is located in Appendix A.

3.2 Control Schematic

The control schematic for this closed-loop system is contained within the LabView program. Figure 8 shows a flowchart that represents the functionality of the program.

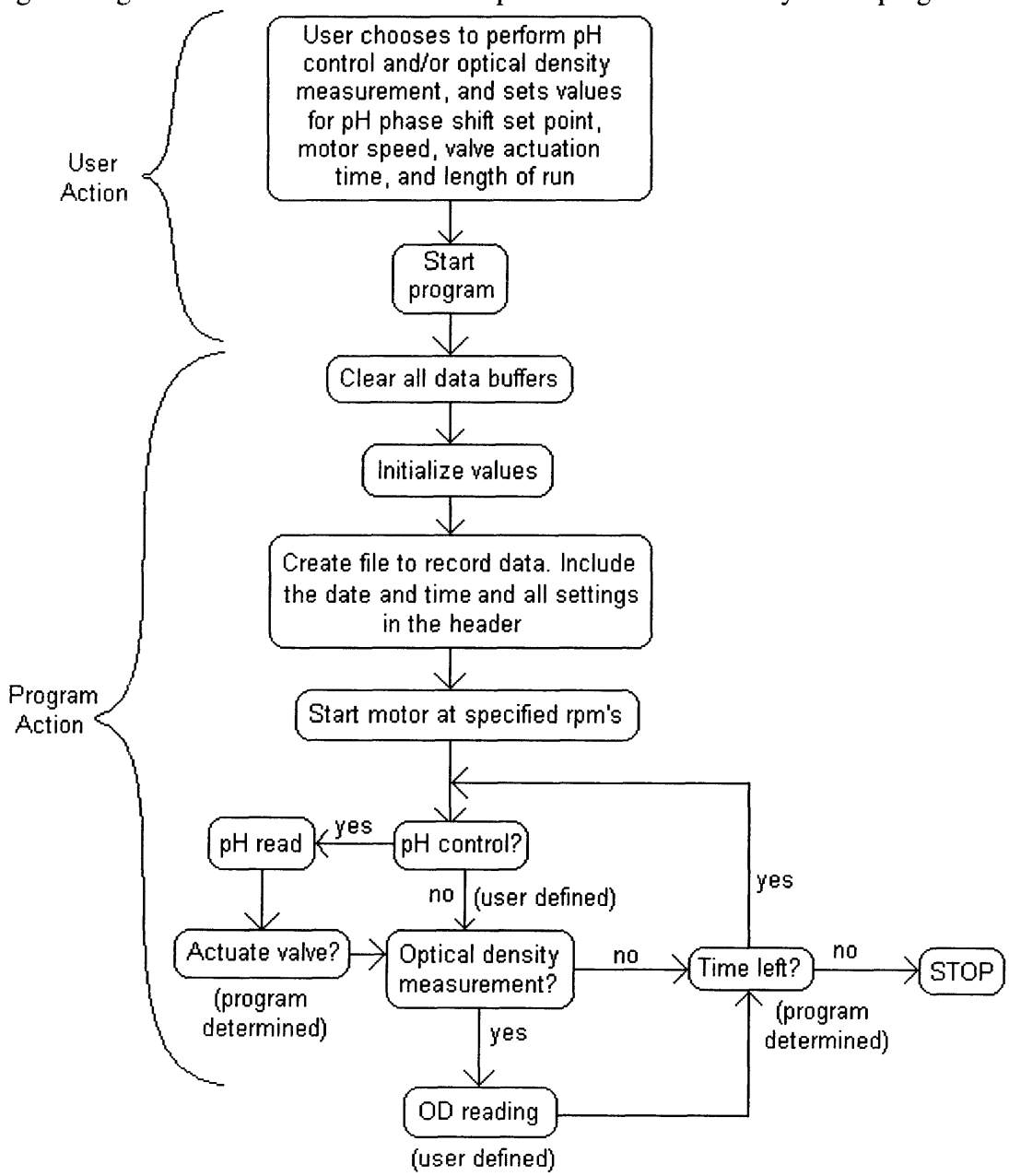


Figure 8: Functionality of the LabView program

The user running the program is prompted for a set point for the pH phase shift, Φ. After initializing the values and creating the file to which data will be saved, the program then proceeds to run a loop that repeats every five minutes in which it obtains the current value of Φ, compares it to the set point, and then either opens the valve for a short period of time, or leaves it closed. Tests were performed to determine what the minimum possible base injection volume is. It is important to minimize this volume in order to prevent overshooting the set point. For more on the subject of determining the minimum injection volume, see section 3.3.2.

3.3 Experimental Procedure

The following sections outline in detail the procedure used to prime the system, test the system against an acid buffer solution, and to perform a fermentation run with pH control.

3.3.1 Priming the System

The system is ready for use when the base injection line is filled up to the edge of the reactor, and the reactor itself has been inoculated with *E. coli* cells. There should be no bubbles present in any of the tubes. The steps to achieve this primed state of the reactor are as follows:

1. With the base under pressure, open the valve and the reactor outlet, and allow the base to flow into the reactor.
2. When the injection line is completely filled with base up to the reactor, close the valve.
3. Next flush the reactor with water to remove any base inside.
4. Connect a syringe filled with cell medium to the input line (normally connected to the water column), and connect an empty syringe to the output line.
5. Gradually push with the filled syringe, and pull with the empty one such that the reactor fills with the cells.
6. Clamp the output line, disconnect both syringes, and connect the water column to the input line, taking care to prevent any bubbles from forming inside the line.

The reactor is now ready for a pH-controlled run.

3.3.2 Testing the System

The base injection volume, V_b, should be as small as possible while still compensating for the acid production. To minimize V_b, the pressure on the base, P_b, and the valve operation time, t_v, can be reduced. The control system was tested against an acid buffer, and P_b and t_v were varied from trial to trial. The system was primed with a base of pH 10 as described in section 3.3.1, except that instead of *E. coli,* an acid buffer was used. The pH

phase shift, Φ, was set to -55 (which corresponds to a pH value of 6.3), the pH was measured every three minutes, and the LabView program operated the valve accordingly and recorded the results. After it was determined which set of parameters yielded the smallest injection volume, a small bubble was introduced to the base feed line. The distance traveled by the bubble over the course of a single injection, d_b, was measured, and the injection volume was calculated by using the equation

$$V_b = d_b \pi r_t^2, \tag{4}$$

where r_t is the radius of the tube in which the bubble lies. The results from these experiments are located in section 4.0.

3.3.3 Fermentation Runs

To perform a fermentation run, the system must first be primed as described in section 3.3.1. The reactor must be positioned with the pH sensor foil directly over the end of fiber optic cable. The LabView program must be given values for the pH phase shift set point, the location of the data file to be saved, the speed of the stirring motor, and how long a duration to perform the fermentation run. After this is complete, the LabView program requires no further input, and it will continue to run until stopped or until the run reaches its ending time.

4.0 Experimental Results

The minimum base injection volume experiments described in section 3.3.2 indicate that the optimal parameters for minimizing the volume are P_b = 5.0kPa and t_v = 30 ms. Figure 7 shows pH vs. time for this experiment. A chart containing results from all trials is located in Appendix B. The result shown in Figure 9 has the smallest overall positive slope, meaning that over time, the pH increases the most slowly. There were three trials with either a non-positive slope or a slope so close to zero that the base injection likely would not keep up with acid production. Using Eq. 4 and the values d_b = 0.25 in and r_t = 0.0225 in, V_b is calculated to be 2 μl. This is $1/50^{th}$ the volume of the reactor itself.

As simple as the fermentation run may seem, there appear to be some major sources of error. The best result obtained from the test runs can be seen in Figure 10. For the first two hours, the system behaved as expected, raising the pH to almost 7 whenever it drops below 6.3. But shortly after the two hour mark, the value of Φ that is returned is out of the domain of possible values, and when plugged into the pH sensor foil calibration equation (Eq. 2), the result is undefined. Results from other fermentation runs do not show any change in the pH due to a base injection. Figure 11 shows one such example. It appears as if no base was ever introduced to the growing medium, and no neutralization occurred.

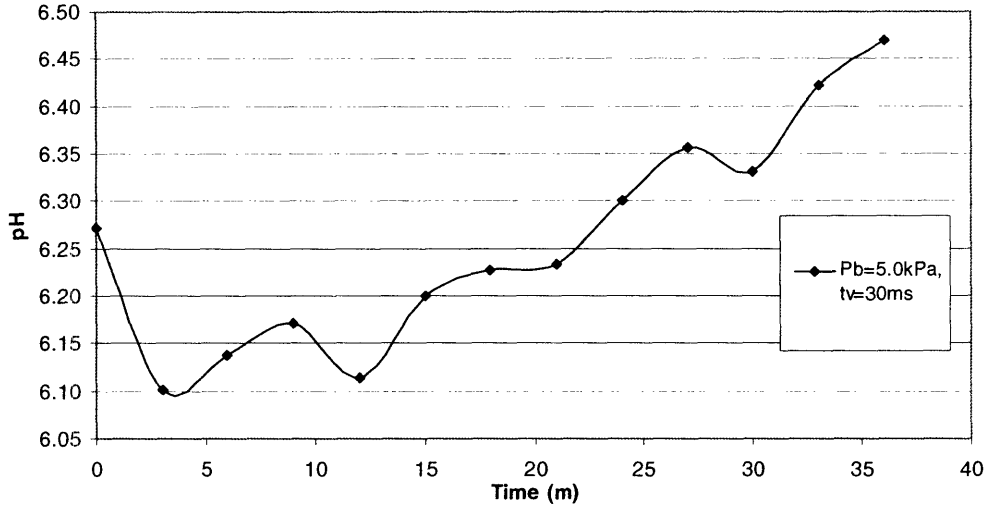


Figure 9: pH vs. time for the trial achieving the minimum base injection volume.

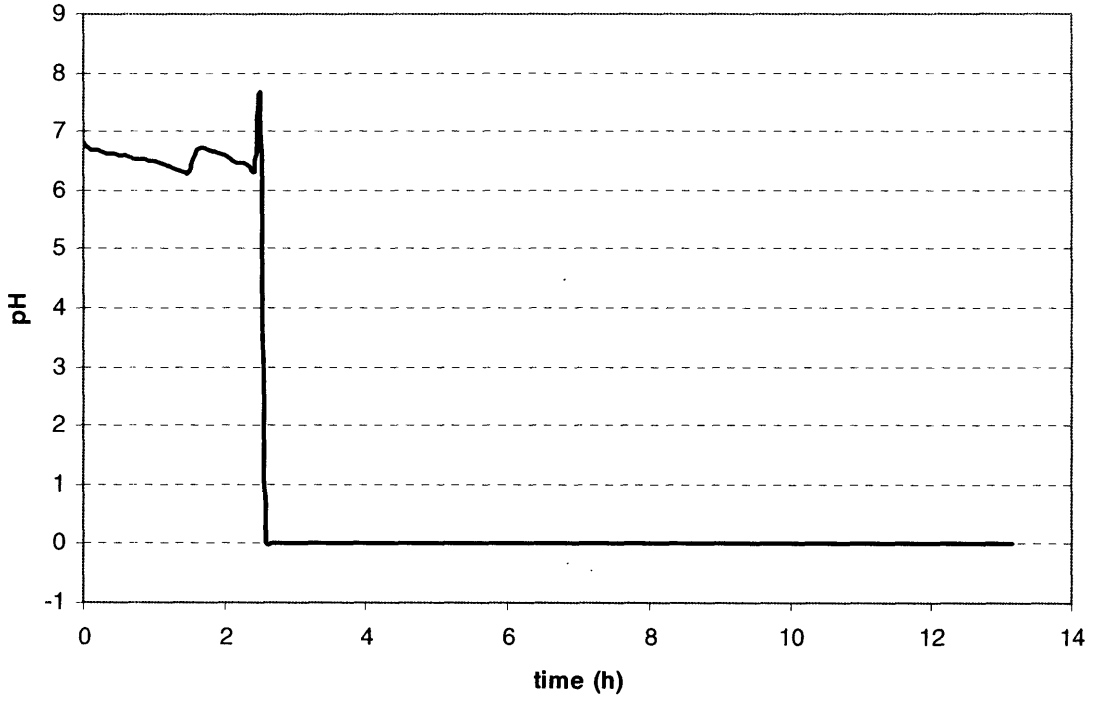


Figure 10: pH vs. time recorded during a fermentation run on 3-9-2004

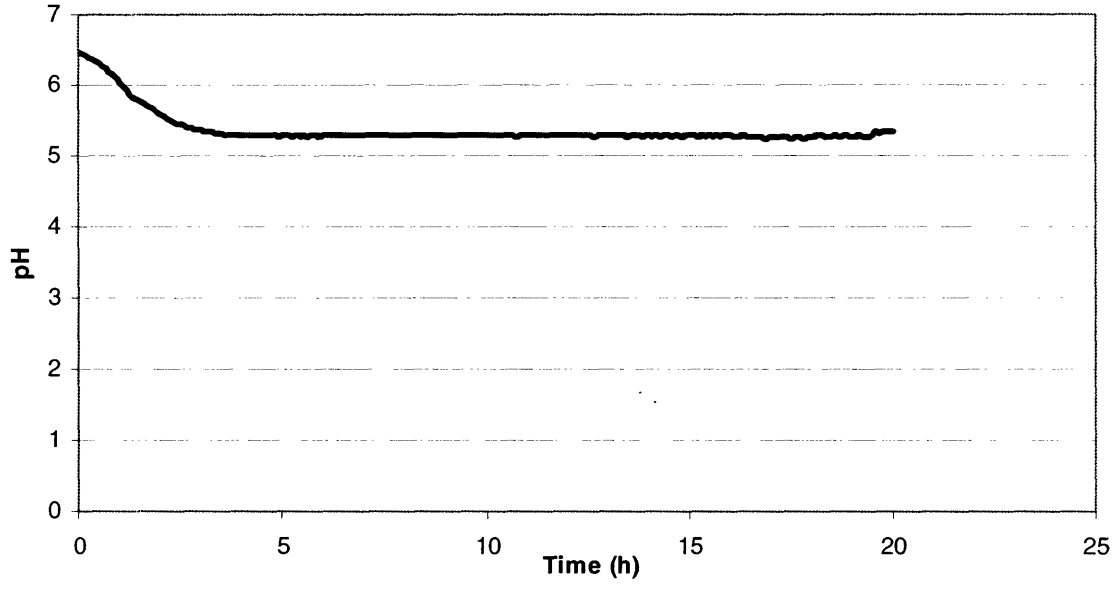


Figure 11: pH vs. time recorded during a fermentation run on 3-12-2004

5.0 Discussion

The minimum base injection volume is 2% of the volume of the reactor, which is very encouraging. The first two hours of the fermentation run shown in Figure 8 indicate that the 2 μl volume base injection will occur on the order of once per hour. Therefore, in the course of a 24 hour fermentation run, the volume inside the reactor could increase up to 50%. The elastomeric PDMS top to the reactor chamber allows evaporation, and can stretch to accommodate any small extra volume.

While the results from the minimum base injection experiments are encouraging, the results from the fermentation runs are rather disturbing. This is especially so because an observer could hear the 3-way valve actuating when it was supposed to, and when disconnected from the reactor, base flowed through the injection line as expected. Liquid could easily be pushed through all inlets and outlets of the reactor when not in use, so there was nothing blocking the injection line. One hypothesis was that the base was not strong enough, or that perhaps it was not actually basic at all, but the problem persisted even when new base was obtained.

The most promising hypothesis as to the cause of the problem was that the injection line was too long and elastic, which resulted in expansion of the tubes to accommodate the added pressure and volume. If this is the case, then the problem can be solved by developing a new reactor with an integrated pneumatic valve, as seen in Figure 12. With the valve located so close to the reactor itself, there is no tubing capable of expanding between the valve and the reactor.

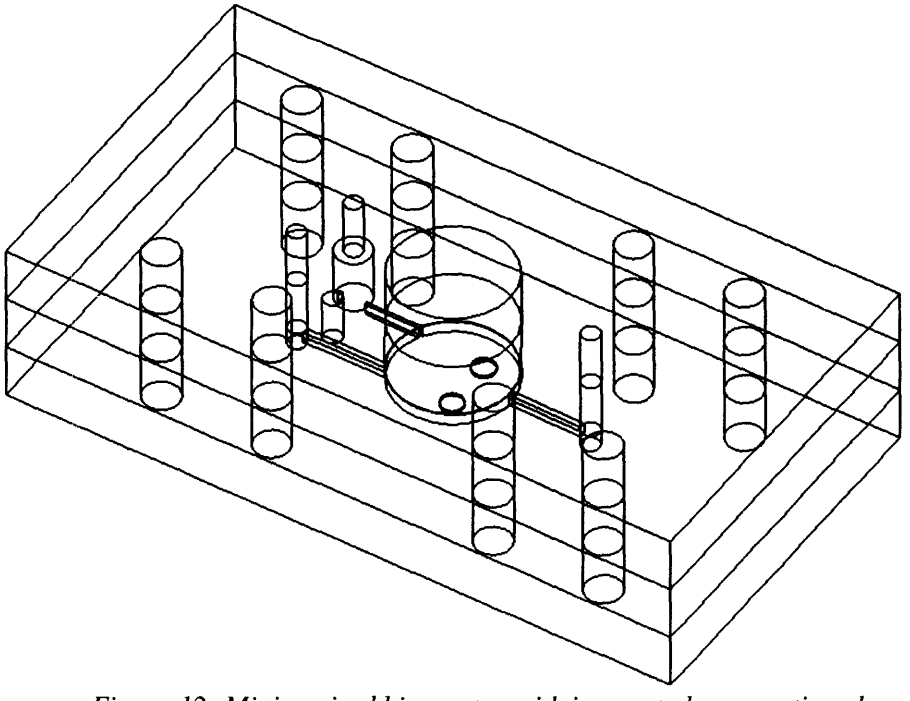
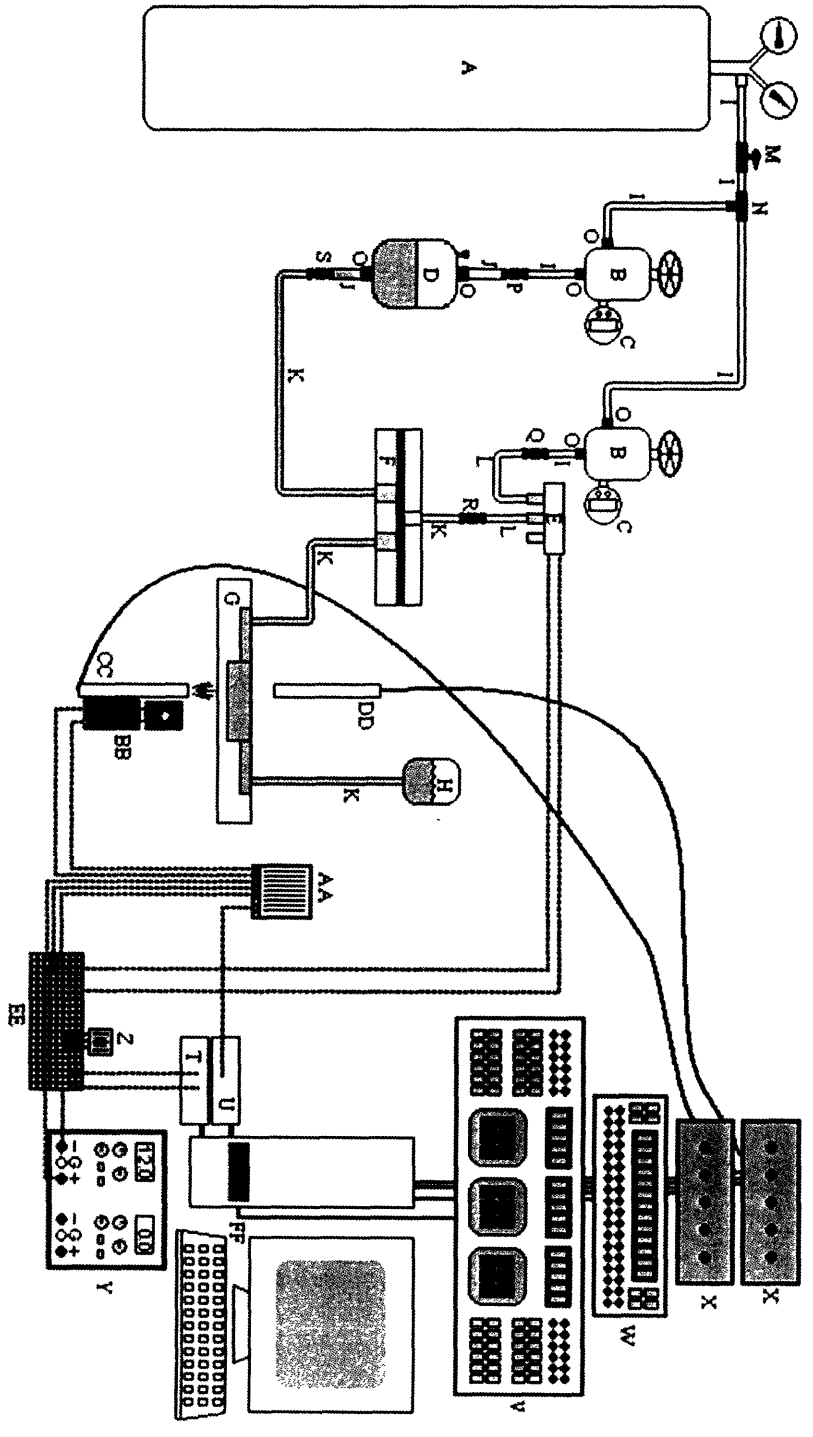


Figure 12: Miniaturized bioreactor with integrated pneumatic valve

6.0 Conclusions

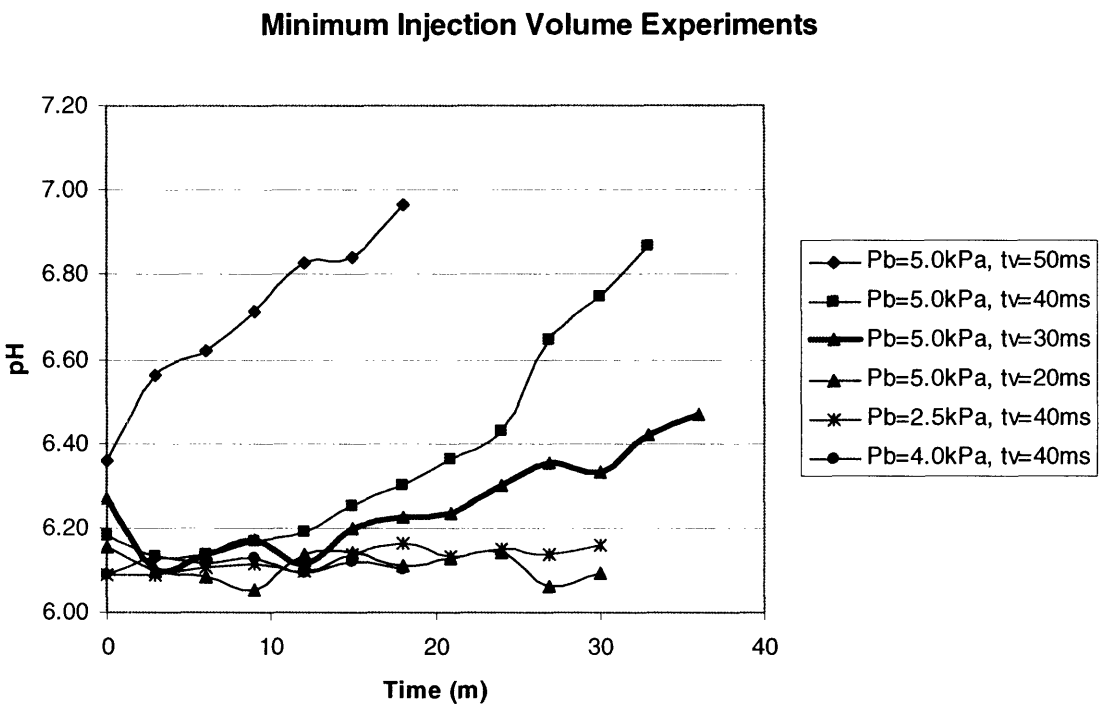
While no conclusive results were achieved with regards to the stable pH control within a miniaturized bioreactor, the concepts developed in this thesis have shown some promise. Further development of the reactor and integrated valve are required. The continued development of a smaller total package of the system will help to reduce the uncertainties that are resulting, and should yield extended results similar to the first two hours of the fermentation run shown in Figure 10. This will help to advance the area of microfermentation research, and could even help revolutionize the biochemical industry.

Appendix A: Apparatus (key on next page)



A. nitrogen tank and dual stage regulator
B. Bellofram air regulator (model #960-130-000)
C. Advanced Custom Sensors pressure gauge (model #1200)
D. Polycap HD disposable filter capsule (VWR catalog # 28137-894)
E. The Lee Co. 3-way valve (model #LHDA1231115H)
F. pneumatic valve
G. miniaturized bioreactor
H. small water bottle
I. ¼" O.D., 3/16" I.D. tubing
J. 5/16" O.D., ¼" I.D. tubing
K. 0.062" O.D., 0.045" I.D tubing
L. 1/8" O.D., 1/16" I.D. tubing
M. Swagelok brass ball valve ¼" O.D. tube fitting (model #B-42S4)
N. Swagelok brass union tee ¼" O.D. tube fitting (model #B-400-3)
O. Swagelok brass connector ¼" O.D. tube fitting to male NPT (model #B-400-11-4)
P. Upchruch flangeless ferrule and nut for 5/16" O.D. tubing, flangeless ferrule and nut for ¼" O.D. tubing, and ½"·20 to ¼"·28 adapter (model #'s U660x, U662x, U655x, U650x, U665)
Q. Upchurch flangeless ferrule and nut for ¼" O.D. tubing, flangeless ferrule and nut for 1/8" O.D. tubing, and ½"·20 to ¼"·28 adapter (model #'s U655x, U650x, P300x, P335x, U665)
R. Upchruch flangeless ferrule and nut for 1/8" O.D. tubing, flangeless ferrule and nut for 1/16" O.D. tubing, ¼"·28 union (model #'s P300x, P335x, P200x, P235x, P620)
S. Upchruch flangeless ferrule and nut for 5/16" O.D. tubing, flangeless ferrule and nut for 1/16" O.D. tubing, and ½"·20 to ¼"·28 adapter (model #'s U660x, U662x, P200x, P235x, U665)
T. National Instruments 48-bit isolated digital I/O card and connector block (model #'s NI6527 and CB-100)
U. National Instruments 12-bit DAQ card and connector block (model #'s NI-PCI-6040E and SCB-68)
V. Stanford Research Systems lock-in amplifier (model #SR830)
W. Agilent function generator (model #33120A)
X. Electro Standards Laboratory, Inc. data switches (model #7204)
Y. Protek dual DC power supply (model # 3015)
Z. The Lee Co. Ov'r Driver
AA. Instech permanent magnet motor controller (model 604KIT)
BB. Instech permanent magnet motor (model 604KIT)
CC. fiber optic cable and LED's
DD. fiber optic cable
EE. Radio Shack breadboard (model #276-174)
FF. Computer with LabView6.1

Appendix B: Base Injection Volume Experiment Data



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[ii] Z. Zhang, N. Szita, P. Boccazzi, A. Sinskey, K. Jensen, "Monitoring and Control of Cell Growth in Fed-Batch Microbioreactors," Proc. Of Micro Total Analysis Systems 2003, Seventh International Conference on Miniaturized Chemical and Biochemical Analysis Systems, Squaw Valley, California, USA, 2003, pp. 765 – 768.

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