

**Southeastern Sun Grant Center Quarterly Progress Report
(Quarter 4)**

Project Title: Bacterial adaptations for enhanced cellulose utilization: a systems approach.

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Project Location: Lexington, Kentucky

Reporting Period: June 1, 2008 to Dec. 31, 2008

Date of Report: March 9, 2009

Written by: Sue E. Nokes

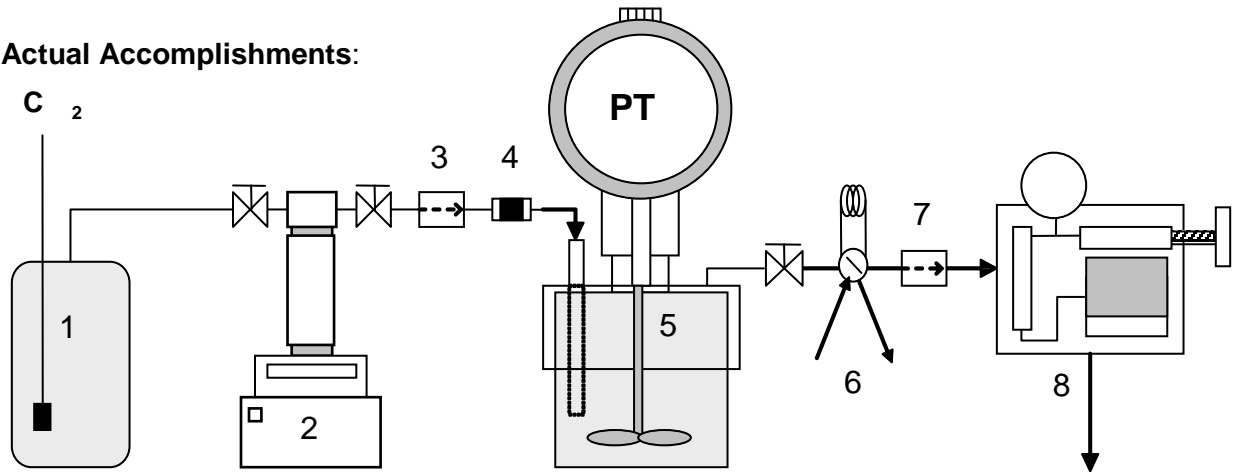
Planned Activities:

1. Examine the buffering of media to determine a robust media to use with the dissolved gas treatments.
2. Collection of the CSTR data collected and compared to previous results for benchmarking.

Dissolved Gas Treatment	$[H_2]_{aq}^{\dagger}$	Rationale for Treatment Selection
0.1 MPa (no added H_2)	< 0.7 mM	Provides baseline for end products at atmospheric pressure (0.1 MPa)

3. Formulate a metabolic control analysis model and relate back to experimental data.

Actual Accomplishments:



- | | |
|---|---|
| 1. Media reservoir (feed, CO ₂ Sparge) | 5. Continuous culture apparatus (0.1 L) |
| 2. Syringe pump | 6. Sample port (2 ml,effluent) |
| 3. Unidirectional flow valve | 7. Unidirection flow |
| 4. Filter frit (0.5 | 8. Effluent Flask (500ml) |

Figure 1: The schematic diagram of the general assembly of the continuous culture system

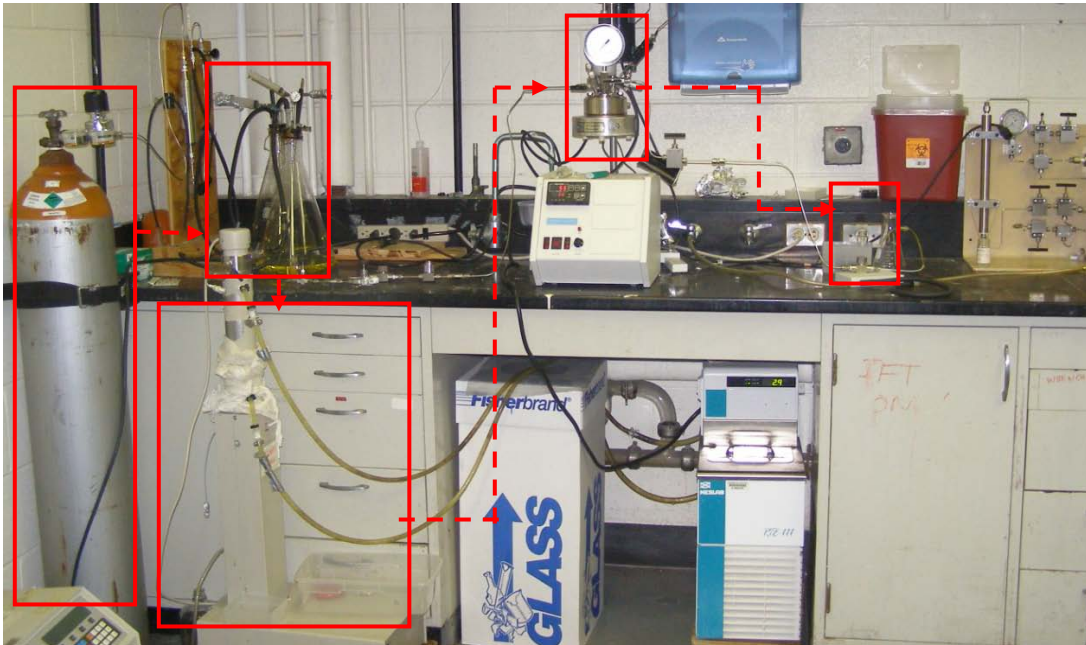


Figure 2: Experimental Setup of Continuous Chemostat System

- A. CO₂ Gas Tank
- B. Media Reservoir
- C. ISCO 500D High Pressure Pump
- D. 100mL Stainless Steel High Pressure Bioreactor
- E. Chiller

Effect of exogenous hydrogen and carbon dioxide on the growth of ATCC 27405 in batch cultures

The purpose of this experiment is to observe the effect of exogenous hydrogen and carbon dioxide on the growth of *C. thermocellum* (ATCC 27405) in batch cultures. Two sterilized test tubes containing 10 ml of sodium carbonate buffer medium under anaerobic condition were prepared with following treatments:

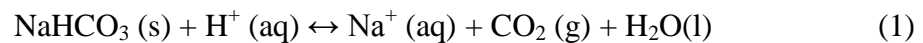
Test Tube A: Purge with CO₂ and inoculate with 1 ml of *C. thermocellum* from freezer stock

Test Tube B: Purge with H₂ and inoculate with 1 ml of *C. thermocellum* from freezer stock

0.2 ml of 10% (w/v) cellobiose was added to each of the test tubes to make 2 g cellobiose/liter solution. Cell density was measured from optical density (OD) values at 600 nm using Biowave

II diode array spectrophotometer. The graphical result of optical density over time is shown in Figure 4. There was a significant difference in cell growth observed in Test Tube A and B within 24 hours after inoculation. At 24 hours, enormous cell growth was observed in Test Tube B (gassed with H₂) but much less growth was observed in Test Tube A (gassed with CO₂). Also, microbial growth in Test Tube A posed a much longer lag period than Test Tube B. The maximum optical density was observed at approximately 72 and 24 hours in Test Tube A and B, respectively. However, the optical density in Test Tube B at the end of 94.1 hours was 60% higher than that in Test Tube A. This indicates a faster and greater cell growth with test tubes gassed with H₂.

The observation of less microbial growth in CO₂ gasses test tube might be a result of lowered pH in the presence of CO₂ gas in sodium carbonate buffer media. For instance, the addition of CO₂ pushes the dissociative reaction of sodium carbonate (Equation 1) in reverse direction resulting in more acidic pH in the solution (McMurry and Fay, 1995).



Several studies have reported detrimental effect of acidic pH on microbial growth (Islam et al., 2006; Weimer and Zeikus, 1977; Russell et al., 2008). However, the possible mechanism(s) that caused the inhibition in ATCC 27405 were unknown. An experiment was conducted later in this study to test the effect of pH on ATCC 27405 growth on 4 g/L cellobiose in batch fermentation.

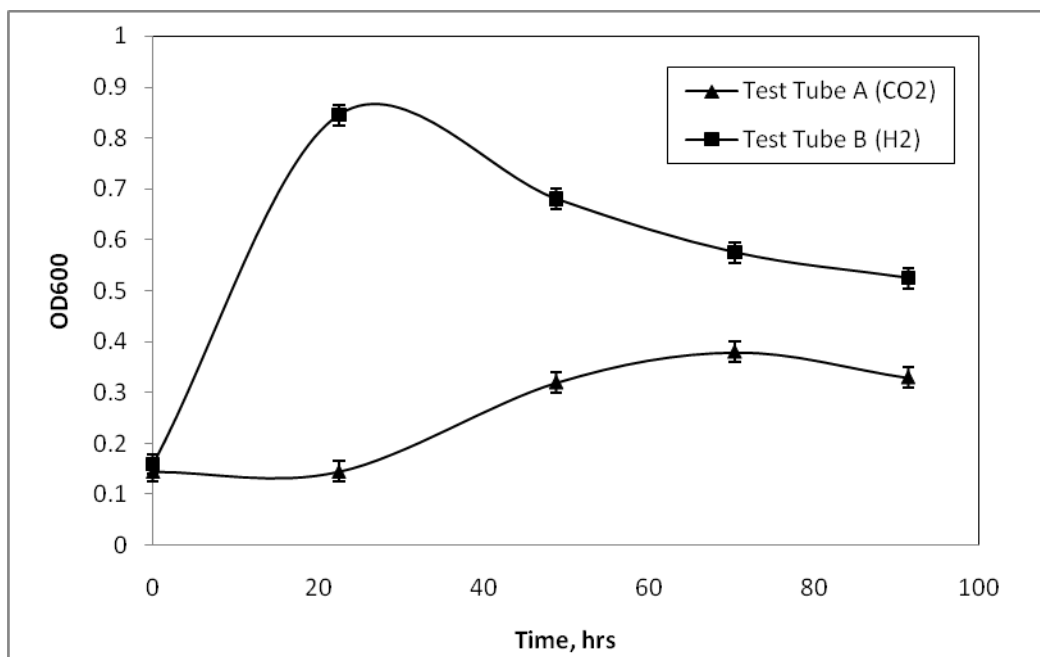


Figure 3: OD₆₀₀ of *C. thermocellum* in test tubes A and B over time

In addition to the OD₆₀₀ measurement, pH of each culture broth was also measured at the end of the batch experiment. Result showed that the test tube purged with H₂ had overall higher final pH values (approximately 0.08 to 0.16 higher) than the test tubes purged with CO₂. A further test was conducted to measure initial pH without inoculation. The result showed that by changing the headspace gas of the test tube to CO₂, the pH of the carbonate buffer medium immediately decreased from 6.7 to 6.55, whereas the pH of the test tube gassed with N₂ and H₂ increased from 6.7 to 6.86 and 6.96, respectively (Table 1).

Same test was performed using potassium phosphate monobasic buffer medium. Monopotassium phosphate is a commonly used buffering agent in most basal medium with pKa (negative logarithm of the acid dissociation constant, Ka) equals to 6.82 (Sigma-Aldrich). It is known that the buffering capacity of a weak acid reaches maximum when its pH value matches pKa. Monopotassium phosphate, therefore, acts as a better buffering agent than sodium carbonate which only has pKa value around 6.37 (Sigma-Aldrich). Initial pH of medium in CO₂

gassed tubes was measured to be 5.99 which was significantly lower than that of the test tubes gassing with N₂ or H₂ (Table 1). This decrease in pH value coincided with a decline in cell growth rate (Figure 4). This indicated that *C. thermocellum* could delay initiate growth in an acidic pH medium. Russell (1992) suggested that anion accumulation as a result of organic acids production is responsible for the toxic effect of fermentation acids at low pH. Essentially, the transport of protonated species across the cell membrane in response to the pH gradient ceased in the presence of large concentration of end-product acids exceed the buffering capacity of the media (Dharmagadda, not published).

Table 1: Initial pH measurement with different gas headspace in sodium carbonate buffer medium and potassium phosphate buffer medium

Headspace Gas	Buffer Medium Type	
	Sodium Carbonate	Potassium Phosphate
	pH	pH
Carbon dioxide	6.55 (0.09)	5.99 (0.05)
Nitrogen	6.86 (0.04)	6.56 (0.06)
Hydrogen	6.96 (0.05)	6.54 (0.04)

Effect of pH and dissolved gas on cell growth and product formation

In the previous experiment, medium was prepared at the initial pH of 6.7 before autoclaving. Known that the source of metabolic inhibition might be due to the reduced pH, a series of sealed test tubes containing 10 ml of phosphate buffer media was prepared with initial pH adjusted from 6.5 to 7.5 (0.2 increment in between). Each set of the pre-adjusted pH medium was gassed with three different gasses: CO₂, N₂, and H₂. When 1 ml of ATCC 27405 was inoculated into each set of the test tubes containing 10 ml of prepared medium and kept in 55° C water bath, growth was observed based on the optical density measured by the spectrophotometer. The final optical density (24 hours after inoculation) of ATCC 27405 in phosphate buffer medium at different initial pH values with different headspace gases was shown in Figure 4.

Growth took place when the initial pH was between 6.7 and 7.5. When ATCC 27405 was grown in basal medium that contained the headspace gas of CO₂, the final optical density was greatest at initial pH of 7.3. If the pH of the medium decreased, the optical density significantly reduced approximately 30% with every 0.2 decrease in pH until pH reached 6.5. Similar trend was observed in strain ATCC 27405 grown in N₂-gassed medium except that the final optical density reached greatest if the initial pH was 7.3. Comparatively, the extent of cell growth based on optical density was significantly greater (approximately 1.2 to 1.8-fold) in the medium gassing with N₂ than that with CO₂. Similarly, strain ATCC 27405 grown in H₂-gassed medium had significant higher optical density (approximately 1.1 to 1.8-fold) than that with CO₂. However, the change in medium pH did not cause a significant change in optical density for H₂-gassed tubes except at initial pH of 6.5 where the optical density measurement was minimal. Lowe et al (1993) also reported similar growth condition of *C. thermocellum* at pH of 7.0.

The effect of dissolved H₂ on the metabolism of thermophilic bacteria is believed to have mass action effect that acts upon the regulation of reduced and oxidized electron carriers in the metabolic pathway (Lamad, 1988) causing the changes in biochemical pathway and the function of plasma membrane (Jones and Greenfield,1982). Within the cellular membrane, the membrane fluidity, permeability as well as the cellular transport may be altered by the presence of dissolved gas at atmospheric pressures (Jones and Greenfield,1982; Chin et al, 1976).

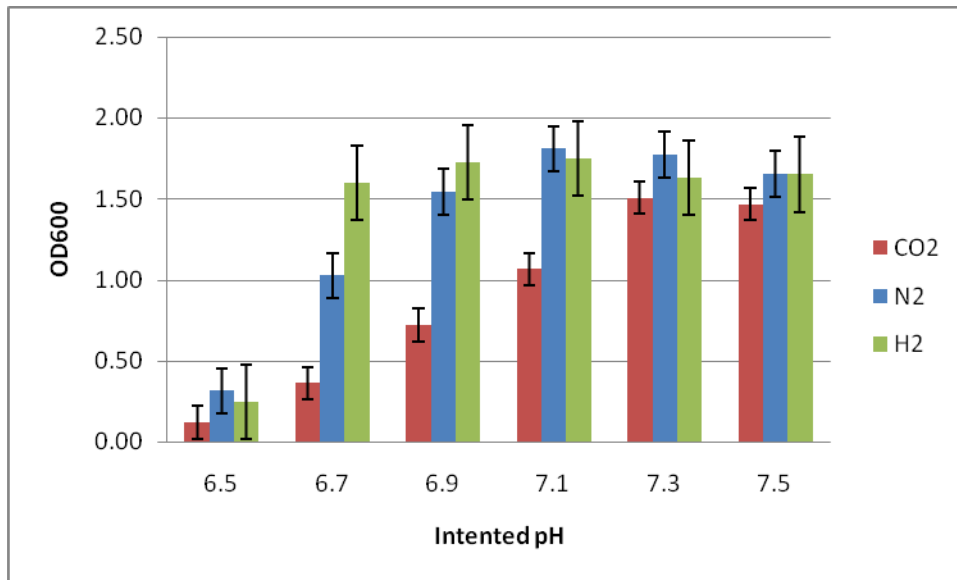


Figure 4: The effect of initial pH on the growth of *C. thermocellum* ATCC 27405 in phosphate buffer medium with different headspace gases

In addition to the OD₆₀₀ measurement, ethanol yield of each basal broth at the end of the batch experiment was also analyzed using HPLC. The amount of ethanol produced by strain ACTT 27405 followed closely with the corresponding final optimal density (Figure 6). The maximum ethanol yield from the culture was observed when the medium was gassed with N₂ or H₂ at initial pH of 7.1 or 7.3, whereas the maximum ethanol yield from the culture broth with CO₂ as headspace was observed when the initial pH was 7.3. No literature reports have studied the effect of pH on the ethanol production of *C. thermocellum*; however, the positive relationship between the microbial growth and product formation was well recognized.

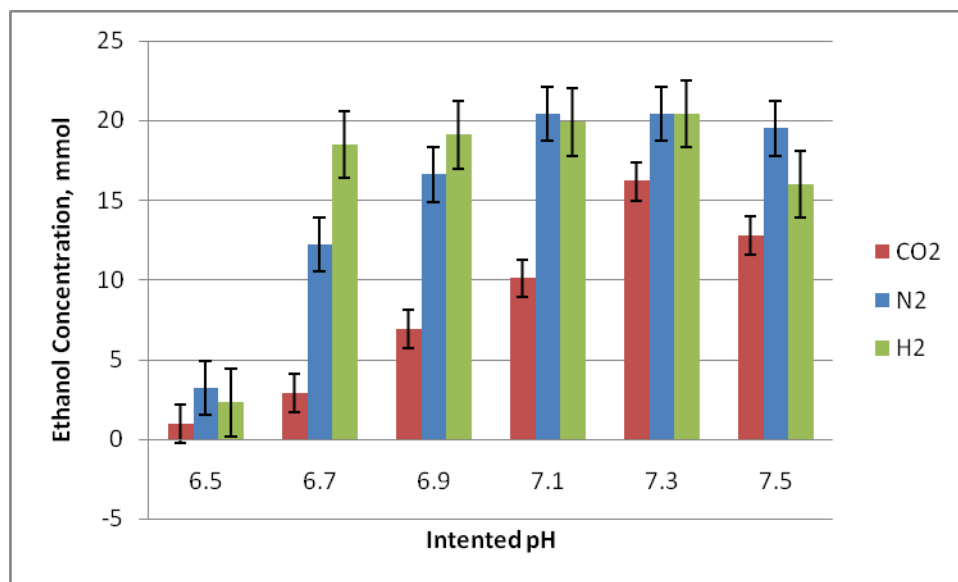


Figure 5: The effect of initial pH on the ethanol yield of *C. thermocellum* ATCC 27405 in phosphate buffer medium with different headspace gases

Cell growth curve and data

As a result of the previous studies, a new medium (TP medium) was developed. The original 4 g/L carbonate buffer was replaced by 0.1M phosphate buffer due to the better buffering capacity phosphate buffer has. Also, initial pH was adjusted to 7.1 before autoclaving with nitrogen as headspace gas. One continuous culture experiment was conducted using the new medium. The fermentation run began by inoculating the 350 ml glass chemostat, establishing batch fermentation conditions within the bioreactor, and initiating flow within the stirred and N₂-aerated reactor to establish chemostat operating conditions. The first inoculation of the *C. thermocellum* was achieved at 328 K and atmospheric pressure. The system was kept in near-batch mode (relatively small dilution rate) for 48 hours. Sample was drawn at the end of 48 hours and the cell density was measured from OD values at 600 nm using an UV spectrometer. After that, the system was switched to continuous mode with the dilution rate targeting at 0.05 h⁻¹ of a medium containing 4 g cellobiose/liter solution. Samples were then taken after the system reached steady state (98% turnover, after 78.2 hours).

The OD_{600} reading of sample at batch condition was 1.12 which corresponds to 0.52 gDCW/L. After the continuous mode was initiated, the cell concentration was able to maintain at 0.46 gDCW/L for three consecutive days. The cell growth curve (Figure 6) expressed a typical substrate limiting growth where the medium controlled the growth rate. When the N_2 was turned off 48 hours after the system reached steady state, the optical density decreased near 30 % in 24 hours and near 50 % in 48 hours. When N_2 was turned on again, the optical density gradually increased to 1.443 in three days. When the concentration of the phosphate buffer increased to 0.1 M, the optical density increased to as high as 2.029. The system was able to maintain steady state at optical density close to 1.75. However, when H_2 was introduced to replace N_2 , the optical density quickly dropped to 1.420 in 24 hours and was only able to maintain steady state at 1.16.

This preliminary experiment showed several characteristics of ATCC 27405. First, the presence of oxygen in the growing environment of culture broth could significantly delay or inhibit the growth of ATCC 27405. Second, the increase of monobasic potassium phosphate concentration increased the media buffer capacity and therefore, better maintained the strain ATCC 27405 at high optical density. Finally, the introduction of H_2 in replace of N_2 reduced the growth yield of ATCC 27405 possibly due to highly unstable hydrogen ions unable to dissolve in liquid broth. It can be seen that the fermentation broth was well buffered with pH maintained at around 6.5.

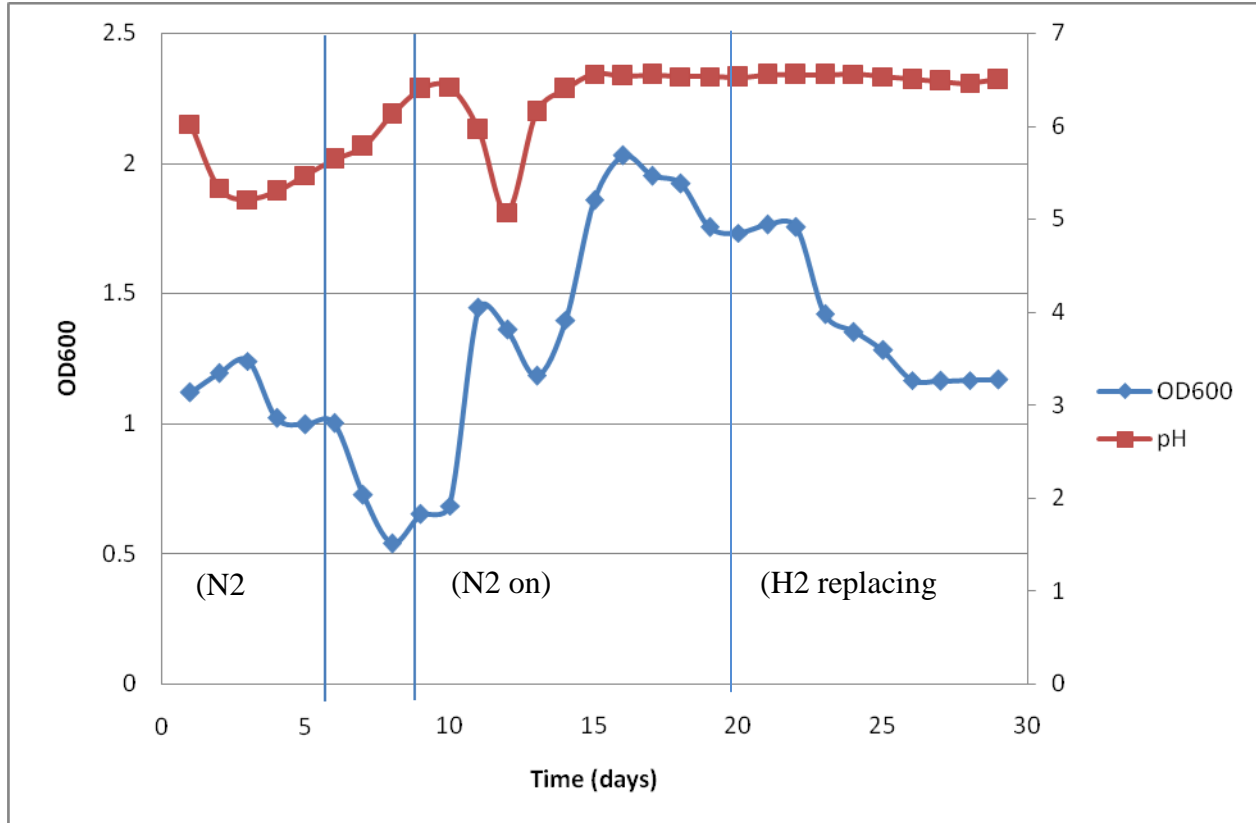


Figure 6: Growth curve and the corresponding pH of *C. thermocellum* in continuous chemostat experiment

Metabolic Control Analyses Method: MCA model was developed from observed data, and tested. The next step is to merge this model with the membrane permeability functions that predict dissolved gas effects on cellobiose transport into and out of the cell. Experiments were planned for the laboratory and data will be collected this week to further refine the model.

Explanation of Variance: The only variance from planned activities was that our long-term chemostat became contaminated, so the data collected is suspect and the experiment must be repeated.

Plans for Next Quarter:

1. Rerun chemostat for conditions above; prevent contamination
2. Run chemostat for other treatment conditions.
3. Conduct whole-cell membrane permeability tests to begin developing membrane model to predict cellobiose transfer into the cell as a function of dissolved gas content.

Patents: None

Publications / Presentations: None

Financial:

1. *Cummulative Expenditures to Date:* \$92,725
2. *Remaining Balance:* \$137,275