Modeling Butanol Production by *Clostridium beijerinckii*

Youngstown State University, Department of Mathematics[1], Department of Biology[2]

Robert DeVita[2], Aaron Esbenshade[2], Estee George[1], Sarah Ritchey[1], George Yates[1], Jozsi Jalics[1] Gary Walker[2]

Abstract

Although fossil fuels are currently the most economical source of energy, alternative energy sources are being explored as replacements for fossil fuels. Millions of dollars are spent on ethanol research. However, the energy content of ethanol is only two-thirds that of gasoline. Butanol, another alternative biofuel, has similar energy content compared to gasoline. Bacteria in the genus *Clostridium* are known for their ability to produce butanol well into the stationary phase of growth and grow in readily available and inexpensive media. *Clostridium* produce butanol from a variety of five and six carbon sugars, one of which is xylose. The goal of this research was to develop methods to optimize butanol production. To achieve this, two separate but complementary approaches were taken. First, a model was developed and verified to guide the selection of optimal parameters. Second, proteins that are activated at various stages of the fermentation process were identified. Associated genes can be targeted for enhanced protein expression in future research. The mathematical model is based on the known xylose-butanol fermentation pathway and Michaelis-Menten enzyme kinetics. Experiments were conducted using the bacterium *Clostridium beijerinckii* in bench-top shakers to verify the model and calibrate parameters of the model. Once the model was tested against data collected from experimental runs, it was used to simulate butanol production over a range of conditions to predict optimal parameter values. Since the simulation and empirical results give parameters for maximum butanol production, this interdisciplinary approach shows great promise for the future economical production of biofuels.

Introduction

Clostridium beijerinckii is an anaerobic, gram positive, endospore-forming bacterium. The bacterium is a mesophile; it grows at moderate temperatures, usually between the temperatures of 25 °C and 40 °C. Like all microorganisms, it has a lag phase in which no growth occurs, a log phase in which the bacteria are growing exponentially, a stationary phase in which no noticeable growth is observed, and a death phase in which the growth is seen to decline.

Clostridium beijerinckii, like most other bacteria in the class *Clostridia*, has the ability to ferment the solvents acetone, butanol, and ethanol. *C. beijerinckii* is known for its ability to grow in relatively inexpensive media and for its ability to produce solvents, mainly butanol, well into their log phase of growth. For these reasons, *C. beijerinckii* was chosen for this research. Since endospores have the ability to survive for several years, the bacterium is easy to research once acquired. The bacteria can ferment a large variety of carbon sources such as glucose, cellobiose, galactose, and mannose to produce carboxylic acids, and eventually solvents such as acetone, butanol, and ethanol. Of these solvents, butanol, is viewed as a very promising source of reusable fuel. Due to the rising costs of gasoline, scientists have begun looking at butanol-fermenting bacteria as an alternative to gasoline[1, 2]. Butanol was chosen as a possible replacement or addition to gasoline for several reasons. Butanol has many advantages over other fuels derived from fermentation such as ethanol. The energy content of ethanol is 30% less than that of butanol and butanol's energy content is very similar to that of gasoline. Butanol also has a low vapor pressure, which makes its shipment possible by use of existing gasoline supply channels. It is also less flammable, less hazardous to handle, and easily mixed with gasoline [7]. Unfortunately, the production of butanol above a certain concentration has proven to be toxic to the bacteria. This results in lower than optimal butanol concentrations, or decreased productivity, and increased fermentation times when compared to ethanol fermentation [26]. Eventually, the bacterium ceases to produce butanol, and the butanol causes the bacterium's cell membrane to lyse, killing the cell. This is caused by an alteration in the fatty acids in the bacterium's cell membrane, which causes the bacterium's cytoplasm to leak out of the cell. The toxicity of butanol is the major factor limiting the profitability of butanol-fermenting bacteria [1, 2, 30].

The *Clostridium* fermentation process normally has two distinct phases: an acidic phase and a solventogenic phase. The acidic phase is growth associated and

includes the lag and exponential phases of the bacterial growth curve. In this first phase, bacteria are converting carbon sources to acetic and butyric acid. The solventogenic phase consists of the uptake of these acids produced in the acidic phase and converts the acids into butanol, acetone, and ethanol. This phase is also relatively non-growth associated. Each phase has a distinct set of enzymes involved, and many of the enzyme interactions are unknown. It can also be noted that it is not possible to skip the first phase of the fermentation process.

There are several ways to increase butanol production. One way is the separation and extraction of butanol itself. One example of this method is liquid-liquid extraction. In this procedure, the fermentation broth contains a layer of organic solvent, usually an alcohol containing a long carbon chain. Since butanol is more similar to the organic layer than to the aqueous layer (fermentation broth), the butanol separates into the organic layer, allowing the bacteria (which remain in the aqueous layer) to ferment larger amounts of butanol. The organic layer containing the desired product, butanol, can be easily separated by means of distillation. Unfortunately, most organic solvents are toxic to the bacteria, and those that come in contact with the organic layer during fermentation are often killed [1, 2, 6, 8, 9, 18]. Therefore, nontoxic organic solvents are used, but they are often expensive to produce.

Ideally, the carbon source for *C. beijerinckii* is completely composed of biomass. Previous studies have used a variety of vegetation types for sources of carbon [27, 31-38]. Due to its fast growth rate and its ability to grow in tightly packed conditions, wood is an ideal carbon source. Wood is composed of four main components: cellulose, hemicellulose, lignin, and ash. Using a process called autohydrolysis, wood chips are hydrolysed under high pressure using water. Another similar process called acid hydrolysis uses acid to break apart the cellulose and hemicellulosic bonds. In both processes, hemicellulose, acid soluble lignin, and ash are removed from the wood, while a very large portion of lignin and cellulose remain in the wood. During autohydrolysis and acid hydrolysis, hemicellulosic bonds are broken apart, resulting in a variety of simple sugars, such as xylose. This liquid hydrolysate can be used as liquid media to ferment butanol. However, the acid soluble lignin in the hydrolysate often contains phenolic compounds that inhibit acetone, butanol, and ethanol production by *Clostridia*[28, 29, 12]. However, several procedures which may remove the majority of these compounds have been studied [10-17]. The remaining portion left in the wood chips (mostly cellulose and lignin) can be used as a heat source in the process. Several recent studies suggest

separating the phenolic compounds in the acid-soluble lignin from the hydrolysate and selling them as antioxidants can be profitable [19-25]. Currently, pharmaceutical companies synthetically produce antioxidants. This procedure may lead to a cheap, natural way to produce antioxidants. Overall, this entire process results in a new, energy-efficient method that will decrease carbon emissions significantly.

Materials and Methods

Clostridium beijerinckii ATCC 35702 was grown in 500mL screw top Erlenmeyer flasks with 250 mL of growth media and placed in a bench-top shaker at 37 °C. In a separate case, it was grown in a 7-liter fermentor at 37 °C. Growth media used during fermentations consisted of: various concentrations of xylose (40g/L - 80g/L), 5.0g yeast extract, 0.68g anhydrous K_2HPO_4, 1g KH_2PO_4, 1g $MgSO_4 * 7H_2O$, 0.5g $FeSO_4$, 0.1g aminobenzoic acid, 3.0g ammonium acetate. The flask was filled to the 1-Liter mark with deionized water. Fermentors were sparged with nitrogen for 30 seconds to create anaerobic conditions. Samples were taken at 24, 48, 72, 96, and 144 hours. 15 mL from each flask were placed in 15 mL centrifuge tubes at each time point and measured for turbidity (OD 600). Samples were then centrifuged at 8,000 rpm for 15 minutes. The supernatant was removed and placed in another 15 mL centrifuge tube for future data measurements and calculations. The samples were stored in a freezer at -20 °C. Bacterial cell pellets were resuspended in 1x TBS and centrifuged at 8,000 rpm for 15 minutes. The composition of the supernatant was analyzed using High Performance Liquid Chromatography (HPLC). The pelleted cells were resuspended and lysed using sonication, and proteins were purified and subsequently separated using electrophoresis on a one-dimensional 12% polyacrylamide gel.

Model Development

Based on the metabolic pathway shown in Figure 1, Shinto [4,5] developed a mathematical model using a system of 20 differential equations. The model includes all the reactions for each enzyme complexes indicated by arrows labeled R1-R25 in Figure 1. We developed a simplified system of 7 differential equations to model the conversion of xylose to butanol in *Clostridium beijerinckii*. Our model includes only 3 products and 3 intermediates as seen in the schematic (Figure 2).

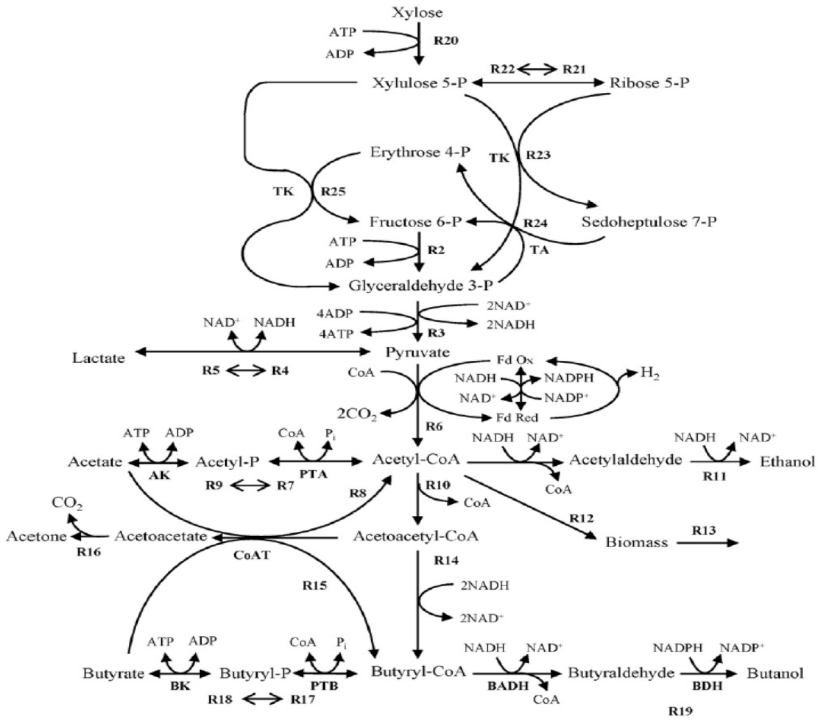

Figure 1: This figure shows the metabolic pathway of xylose into many intermediates and products. This diagram was used by Shinto[4,5] to show the pathways their mathematical model goes along.

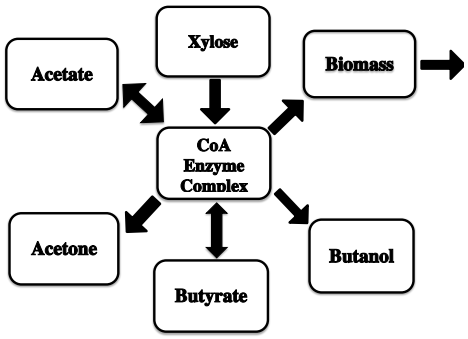

Figure 2: The figure above shows the pathway that converts xylose to acetate, acetone, biomass, butyrate and butanol. It is based on figure 1. The main difference between the two models is that we grouped all the intermediates that we can not get experimental data into one intermediate that we called CoA enzyme complex.

Table 1: Variables and Initial Conditions

Variable	Substance	Initial Conditions
X	Xylose	X_0
A_T	Acetate	3
A	Acetone	0
B_T	Butyrate	0
B	Butanol	0
M	Biomass	10
C	CoA Enzyme Complex	0

These products and two of the intermediates were chosen because they could be measured in the supernatant using High Density Liquid Chromatography. Also, because lactate and ethanol are not produced in significant quantities, we neglected them in our simplified model. The remaining intermediate, labeled CaA enzyme complex in figure 2, is a hybrid of all of the intermediate steps between xylose and the 3 products and 3 intermediates that were grouped together. This simplification is justified under the assumption that there is a limiting reaction in the reactions among the intermediates that makes all of these reactions proceed at this slower rate.

Important sequences of enzyme reactions were modeled using combined Michaelis-Menten reactions, and the system of equations (1)-(8) are given. Competition between substrates and products were incorporated as forms of inhibition in the xylose consumption and the biomass production. The parameters in the model were selected, so that the model fit the data for all initial conditions for which we collected data. Basically the enzyme reactions denoted R_2, R_3, R_6, and $R_{20} - R_{25}$ from Figure 1 are combined into a single chemical reaction, namely

$$\underline{\mathbf{X}} \xrightarrow{Q_1} \underline{\mathbf{C}}$$

where

$$Q_1 = \frac{-V_1 X M}{K_1 + X(1 + \frac{B}{K_7})}$$

In most reactions the initial substance does not form into the product on its own; it uses enzymes to facilitate the process. So the reaction $\underline{\mathbf{X}} \xrightarrow{Q_1} \underline{\mathbf{C}}$, may actually be $\underline{\mathbf{E}} + \underline{\mathbf{X}} \xrightarrow{Q_1} \underline{\mathbf{E_{XC}}} \xrightarrow{Q_2} \underline{\mathbf{C}} + \underline{\mathbf{E}}$.

This equation was formed using the basic Michaelis-Menten reaction equation,

$$\frac{dP}{dt} = \frac{SV_{max}}{S + K_m}$$

where S is the concentration of the substance substance and P is the concentration of the product. This simplified reaction is justified by letting V_1 and K_1 be the effective maximum reaction rate and Michaelis-Menten constant respectively for the limiting values for the 9 reactions that convert xylose to Acetyle-CoA. This equation can be formed under the assumptions that the total concentration of enzyme is constant and there is no net rate of change of the enzyme complexes.

Butanol inhibits the growth of cellular cultures and thus reduces the amount of Xylose metabolized. So we added the $(1 + \frac{B}{K_7})$ term to represent this inhibition. When there is more butanol, the $(1 + \frac{B}{K_7})$ term will increase, which causes $\frac{dX}{dt}$ to decrease, slowing down the rate xylose is metabolized. This is a type of competitive inhibition.

Notice in Figure 2 that the reactions for both Acetate and Butyrate are denoted by double sided arrows. That means that they both are formed from the CoA Enzyme Complex, and then they also make CoA Enzyme Complex. Since they involve different enzymes, they are modeled using two Michaelis-Menten reaction equations, one for each direction of the reaction shown in equation (2). The basic form of the Michalelis-Menten reaction equation was used to model the butanol, biomass, and acetone produced since there was no significant inhibition detected. Then to account for the death phase of the cells, a death coefficient K_8 times the concentration of biomass, M was subtracted from the biomass equation.

This system of equations is balanced. So for example, when xylose is metabolized to CoA, the rate that CoA is changing will be the negative of the rate of change of xylose. Thus, the rate of change of CoA will be the sum of the negative rates of change of xylose, butanol, butyrate, acetone, and acetate plus the negative rate of change of the biomass not including the death coefficient.

We also multiplied each equation by the biomass, M. This takes into account the effect that a culture that has more cells will produce more of each product and consume more of each initial substance. So the biomass M represents the number of cells in the culture at any given time.

$$\frac{dX}{dt} = -\frac{V_1 X M}{K_1 + X(1 + \frac{B}{K_7})} \tag{1}$$

$$\frac{dA_T}{dt} = \frac{V_3 C M}{K_3 + C} - \frac{V_{11} A_T M}{K_{11} + A_T} \tag{2}$$

$$\frac{dM}{dt} = \frac{V_6 C M}{(K_6 + C)(1 + \frac{B}{K_9})} - K_8 M \tag{3}$$

$$\frac{dC}{dt} = -\frac{d(B + A + A_T + B_T + M + X)}{dt} - K_8 M \tag{4}$$

$$\frac{dB_T}{dt} = \frac{V_4 C M}{K_4 + C} - \frac{V_{10} B_T M}{K_{10} + B_T} \tag{5}$$

$$\frac{dB}{dt} = \frac{V_5 C M}{K_5 + C} \tag{6}$$

$$\frac{dA}{dt} = \frac{V_2 C M}{K_2 + C} \tag{7}$$

$$\tag{8}$$

The system of differential equations were solved numerically using XPPAUT. Simulations of the model are shown in Figure 3 along with the results of our experiment. The lines represent the numerical results, and the points represent data taken from cultures and analyzed using HPLC. The parameters (V_j and K_j) used in the numerical computations are identical for all cases in Figure 3. The parameters used are as follows:

V_1	= 0.8	K_1	= 3100	K_9	= 1
V_2	= 0.1	K_2	= 0.135	K_{10}	= 55500
V_3	= 0.1	K_3	= 100	K_{11}	= 1550
V_4	= 1	K_4	= 0.5		
V_5	= 1	K_5	= 0.5		
V_6	= 6.5	K_6	= 0.4		
V_{10}	= 0.2	K_7	= 50		
V_{11}	= 10.2	K_8	= 0.004		

Results

We hypothesized that an increase in the xylose concentration initially fed to the bacteria in the small-scale shaker flasks would result in an increase in butanol production. However, it was found that the initial concentration of xylose had little effect on the amount of butanol formed. These results are shown in Figure 5. In fact, it appears that using a 60g/L xylose solution results in almost twice as much butanol production than any other experimental group, including those with a higher concentration of initial xylose. Each trial which was fed a different initial concentration of xylose resulted in roughly the same amount of butanol produced. Rather than an increase in butanol production, an increase in the amount of xylose present in the growth media resulted in an increase in xylose consumption and, therefore, an increase in the biomass formed. The use of xylose as a substrate accounted for the incomplete consumption of the xylose. There are known to be certain rate-limiting intermediate reactions in the Pentose Phosphate Pathway that cause this occurrence. It is hypothesized that this pathway can be improved upon, though, with future genetic manipulations (Gu et al. [3]).

When scaled up to a 7-liter bioreactor, the culture, which was fed an initial concentration of 40 g/L xylose, consumed about 30 g/L xylose and produced roughly 9 g/L butanol. These results are illustrated in Figure 7. In this trial, the xylose consumption is 50% greater than in the small flasks, and the butanol production is 100% greater. One possibility for the greater success rate in the 7-liter fermenter may have to do with the differing amounts of oxygen in the flasks and the fermenter. The shaker flasks were sparged with nitrogen every 24 hours in order to rid the cultures of oxygen. However, in order to do this, the lids of the flasks are taken off and then put back on. In this process, oxygen could well have entered the flasks. In the fermenter, however, a stream of nitrogen was constantly flowing

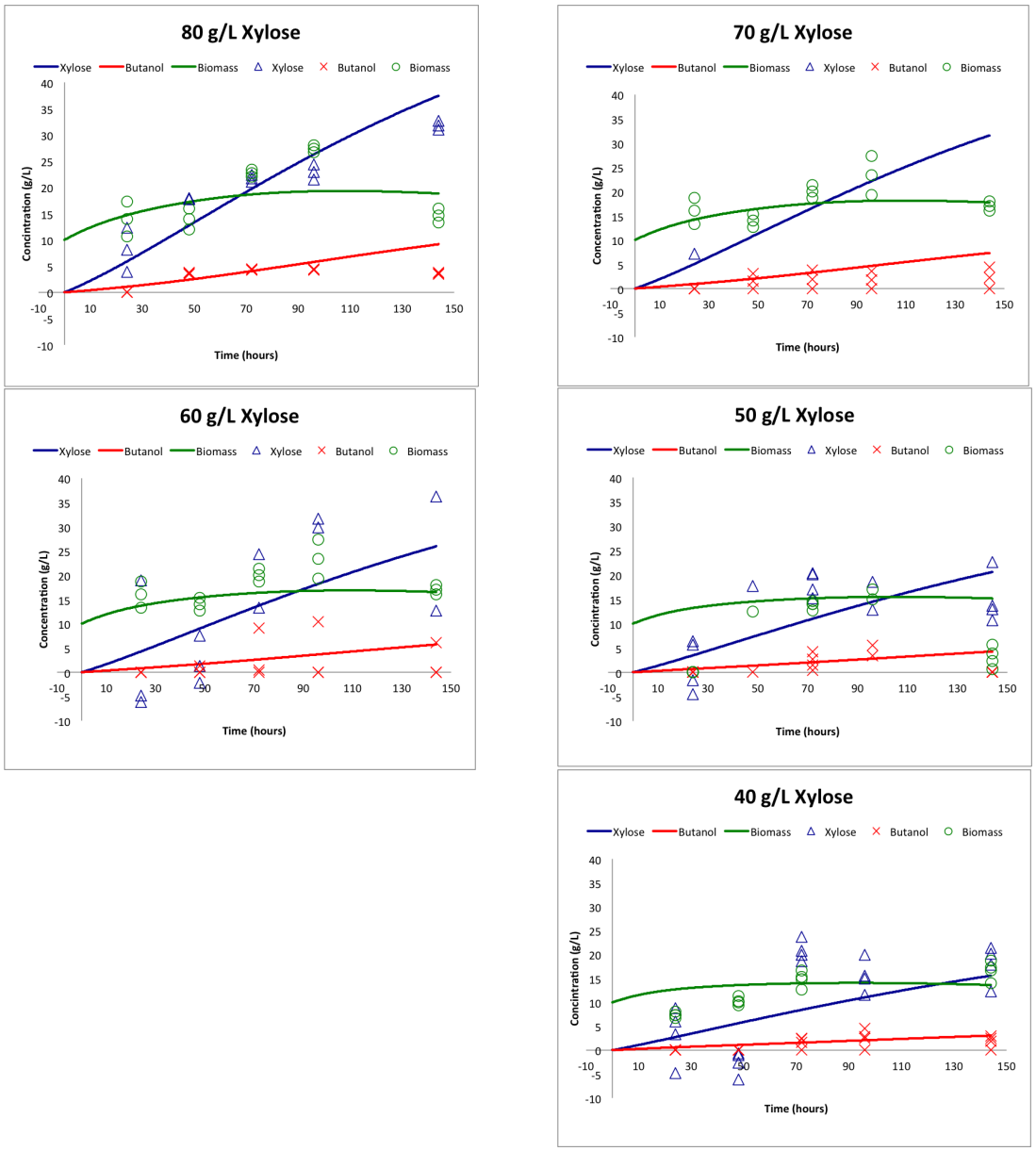

Figure 3: These graphs depict the concentrations of starting materials and products at various times throughout each experiment. The title of each graph gives the initial concentration of xylose fed to the bacteria. The solid curves represent the numerical projections for xylose consumed and product formed. The symbols express the uptake of xylose and formation of product in each experiment. The numerical results for each data set were determined using the same parameters. The initial concentrations for each data set were set as the initial concentrations given by the experiments. All of the trials were run in triplicate, and the data, which consisted of measurements of concentrations, turbidity, and the weight of biomass formed, were collected every 24 hours for 144 hours. The blue curves and blue triangles represent the concentrations of xylose consumed throughout the trials. These concentrations were calculated by subtracting the concentrations of xylose calculated or measured at each point in time from the initial concentrations of xylose. The curves and symbols representing butanol and biomass concentrations give the concentrations of each produced.

Figure 4: The graphs above show the experimental results for the amounts of acetic acid, butyric acid, and acetone produced for each initial concentration of xylose fed to the bacteria. The titles of the graphs indicate the initial concentrations of xylose, and all concentrations are given in g/L. All of these experiments were run in triplicate, and data were collected every 24 hours for 144 hours.

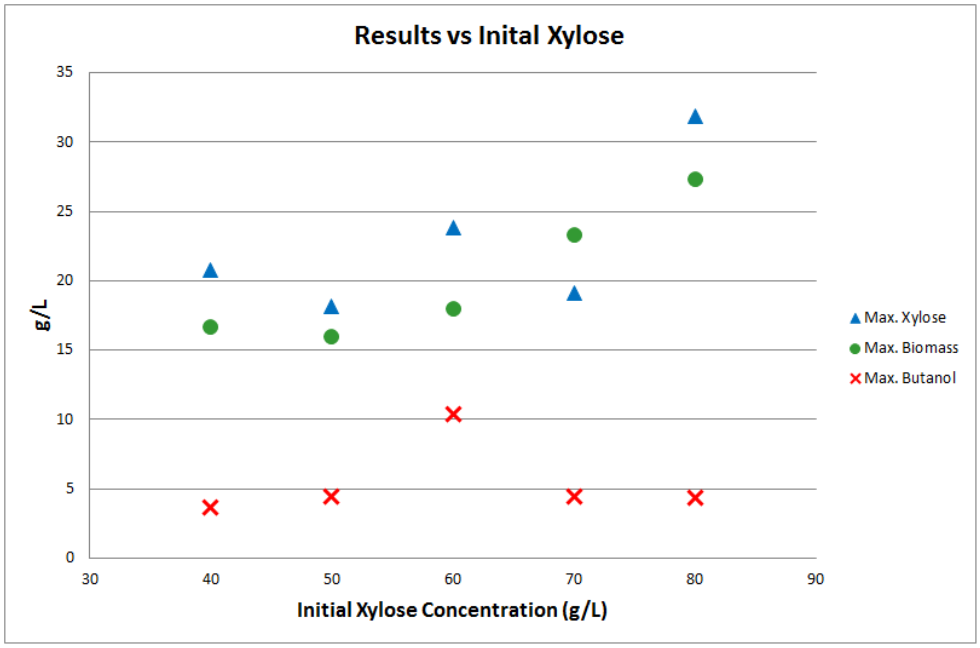

Figure 5: This figure shows the maximum amounts of xylose metabolized and biomass and butanol produced at any time during each experiment with respect to initial concentrations of xylose in the growth media. The xylose metabolized is calculated by subtracting the concentration of xylose at the time point from the initial concentration of xylose.

Figure 6: Fermentor culture in 7-liter BIOFLO-110 bioreactor.

Figure 7: Flask cultures on incubated shaker table.

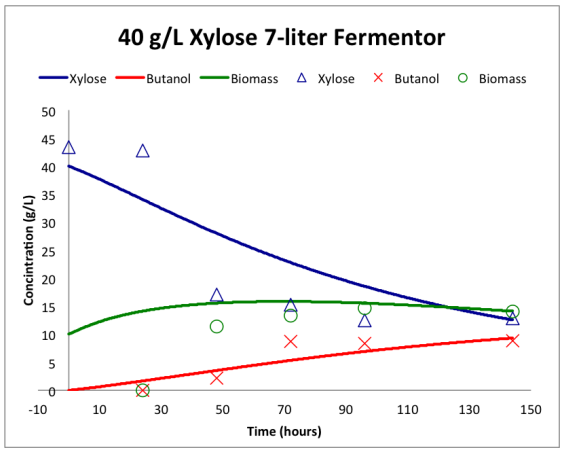

Figure 8: This figure shows the concentrations of xylose, butanol, and biomass in the 7-Liter bioreactor as they change with time. The graph also compares experimental results with numerical, or mathematically predicted, results. The symbols represent experimental results, while the solid curves represent numerical results. All parameter values are the same as those used in Figure 3 with the exception of V_1 and K_5. Here, V_1 is increased by a factor of two, and K_5 is increased by a factor of three. Note that the amount of butanol produced in the bioreactor is significantly greater than the amount of butanol produced during any trial in the shaker flasks.

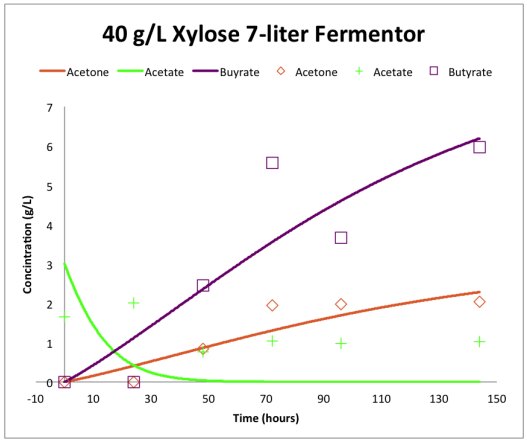

Figure 9: This figure shows the concentrations of butyrate, acetate, and acetone in the 7-Liter fermentor at various times during the experiment. The figure compares experimental results, denoted by symbols, with numerical projections, shown by curves. All parameter values are the same as those used in Figure 4, except, here, V1 is increased by a factor of two and K5 is increased by a factor of three.

through the bioreactor, and the bioreactor was never exposed to the atmosphere. Thus, the oxygen levels in the bioreactor were seemingly much lower in the fermenter than in the flasks. This may have resulted in greater butanol production in the fermenter. It is interesting to note, however, that the biomass concentration remained about the same in both the shaker flasks and the 7-liter fermenter.

The proteins at each time interval for each experiment were analyzed using 1-D gels. Our preliminary gels show distinct differences in protein expression over the duration of the fermentation. These differences may be related to the formation of different end products over the course of the reaction. We can also predict that additional proteins expressed at later time points may be related to solvent or acid resistance.

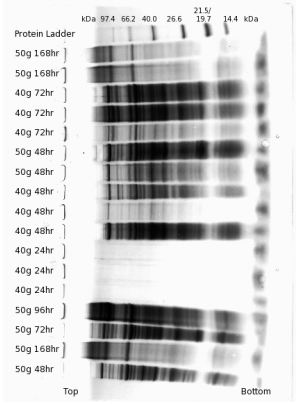

Figure 10: 1-D gel protein profiles. The first number represents the concentration of xylose in g/L. The second number indicates the time each sample was taken.

Discussion

In future research, we would like to extend the mathematical model to include additional sugar sources, alternate substrates, pH, and more complex enzyme kinetics. We would also like to run a more detailed proteomic analysis of protein assays. It would also be interesting to manipulate the pH of intermediate reactants (e.g., butyric and acetic acid) during fermentation. We are also trying to identify causes for differences observed between flasks and 7-liter bioreactor. We are also going to complete a sensitivity analysis on the math model.

Acknowledgments

This work was supported by the National Science Foundation grant DBI-0827205. U.S. Department of Energy, National Energy Technology Laboratory (NETL-DOE Advanced Automotive Fuels Research, Development, and Commercialization Cluster (OH) -Task 3C butanol fermentation).

A special thanks to Dr. Jozsi Jalics (Youngstown State University) for assistance with the mathematical modeling and computational analysis.

Bibliography

[1] Ezeji, T., Milne, C., Price, N. D., Blaschek, H. (2010). Achievements and Perspectives to Overcome the Poor Solvent Resistance in Acetone and Butanol-Producing Microorganisms. *Applied Microbiology and Biotechnology, 85(6), 1697-1712.*

[2] Garcia, V., Pakkila, J., Ojamo, H., Muurinen, E., Keiski, R. L. (2011). Challenges in Biobutanol Production: How to Improve the Efficiency? *Renewable and Sustainable Energy Reviews, 15(2), 964-980.*

[3] Gu, Y., Li, J., Zhang, L., Chen, J., Niu, L., Yang, Y., Yang, S., Jiang, W. (2009). Improvement of Xylose Utilization in Clostridium Acetobutylicum Via Expression of the talA Gene Encoding Transaldolase from Escherichia coli. *Journal of Biotechnology, 143, 284-287.*

[4] Shinto, H., Tashiro, Y., Yamashita, M., Kobayashi, G., Sekiguchi, T., Hanai, T., Kuriya, Y., Okamoto, M., Sonomoto, K. (2007). Kinetic Modeling and Sensitivity Analysis of Acetone-Butanol-Ethanol Production. *Journal of Biotechnology, 131, 45-56.*

[5] Shinto, H., Tashiro, Y., Yamashita, M., Kobayashi, G., Sekiguchi, T., Hanai, T., Kuriya, Y., Okamoto, M., Sonomoto, K. (2008). Kinetic Study of Substrate Dependency for Higher Butanol Production in Acetone-Butanol-Ethanol Fermentation. *Process Biochemistry, 43, 1452-1461.*

[6] Groot, W. J., H. S. Soedjak, P. B. Donck, R. G. J. M. Lans, K. Ch. A. M. Luyben, and J. M. K. Timmer. "Butanol Recovery from Fermentations by Liquid-liquid Extraction and Membrane Solvent Extraction." *Bioprocess Engineering* 5.5 (1990): 203-16. Print.

[7] Ha, Sung H., Ngoc L. Mai, and Yoon-Moo Koo. "Butanol Recovery from Aqueous Solution into Ionic Liquids by Liquidliquid Extraction." *Process Biochemistry* 45.12 (2010): 1899-903. Print.

[8] Kraemer, K., A. Harwardt, R. Bronneberg, and W. Marquardt. "Separation of Butanol from Acetone-butanol-ethanol Fermentation by a Hybrid

Extraction-distillation Process." *Computers and Chemical Engineering* 35.5 (2011): 949-63. Print.

[9] Roffler, S. R., H. W. Blanch, and C. R. Wilke. "In-situ Recovery of Butanol during Fermentation." *Bioprocess Engineering* 2.4 (1987): 181-190. Print.

[10] Persson, Per, Jessica Andersson, Lo Gorton, Simona Larsson, Nils-Olof Nilvebrant, and Leif J. Jnsson. "Effect of Different Forms of Alkali Treatment on Specific Fermentation Inhibitors and on the Fermentability of Lignocellulose Hydrolysates for Production of Fuel Ethanol." *Journal of Agricultural and Food Chemistry* 50.19 (2002): 5318-325. Print.

[11] Martinez, A., M.E. Rodriguez, M.L. Wells, S.W. York, J.F. Preston, and L.O. Ingram. "Detoxification of Dilute Acid Hydrolysates of Lignocellulose with Lime." *Biotechnology Progress* 17.2 (2001): 287-93. Print.

[12] Cho, Dae Haeng, Yun Jie Lee, Youngsoon Um, Byoung-In Sang, and Yong Hwan Kim. "Detoxification of Model Phenolic Compounds in Lignocellulosic Hydrolysates with Peroxidase for Butanol Production from Clostridium Beijerinckii." *Applied Microbiology and Biotechnology* 83.6 (2009): 1035-043. Print.

[13] Jennings, Edward W., and Daniel J. Schell. "Conditioning of Dilute-acid Pretreated Corn Stover Hydrolysate Liquors by Treatment with Lime or Ammonium Hydroxide to Improve Conversion of Sugars to Ethanol." *Bioresource Technology* (2010). Print.

[14] Ezeji, Thaddeus, Nasib Qureshi, and Hans P. Blaschek. "Butanol Production from Agricultural Residues: Impact of Degradation Products OnClostridium Beijerinckii Growth and Butanol Fermentation." *Biotechnology and Bioengineering* 97.6 (2007): 1460-469. Print.

[15] Horvth, Ilona Srvri, Anders Sjde, Bjrn Alriksson, Leif J. Jnsson, and Nils-Olof Nilvebrant. "Critical Conditions for Improved Fermentability During Overliming of Acid Hydrolysates from Spruce." *Applied Biochemistry and Biotechnology* 124.1-3 (2005): 1031-044. Print.

[16] Larsson, Simona, Anders Reimann, Nils-Olof Nilvebrant, and Leif J. Jnsson. "Comparison of Different Methods for the Detoxification of Lignocellulose Hydrolyzates of Spruce." *Applied Biochemistry and Biotechnology* 77.1-3 (1999): 91-104. Print.

[17] Martinez, Alfredo, Maria E. Rodriguez, Sean W. York, James F. Preston, and Lonnie O. Ingram. "Effects of Ca(OH)2 Treatments (overliming) on the Composition and Toxicity of Bagasse Hemicellulose Hydrolysates." *Biotechnology and Bioengineering* 69.5 (2000): 526-36. Print.

[18] Evans, PJ. "Enhancement of butanol formation by Clostridium acetobutylicum in the presence of decanol-oleyl alcohol mixed extractants." *Applied and Environmental Microbiology* 54.7 (1988): 1662. Print.

[19] Gulln, Patricia, Enma Conde, Andrs Moure, Herminia Domnguez, and Juan Carlos Paraj. "Selected Process Alternatives for Biomass Refining: A Review." The Open Agriculture Journal 4 (2010): 135-44. Print.

[20] Garrote, G., J.M. Cruz, H. Domnguez, and J.C. Paraj. "Valorisation of Waste Fractions from Autohydrolysis of Selected Lignocellulosic Materials." Journal of Chemical Technology & Biotechnology 78.4 (2003): 392-98. Print.

[21] Conde, Enma, Andrs Moure, Herminia Domnguez, and Juan Carlos Paraj. "Production of Antioxidants by Non-isothermal Autohydrolysis of Lignocellulosic Wastes." *LWT - Food Science and Technology* 44.2 (2011): 436-42. Print.

[22] Cruz, J., E. Conde, H. Dominguez, and J. Parajo. "Thermal Stability of Antioxidants Obtained from Wood and Industrial Wastes." *Food Chemistry* 100.3 (2007): 1059-064. Print.

[23] Laroze, Liza E., Beatriz Daz-Reinoso, Andrs Moure, Mara Elvira Ziga, Herminia Domnguez. "Extraction of antioxidants from several berries pressing wastes using conventional and supercritical solvents." *European Food Research and Technology* 231.5 (2010): 669.

[24] Gonzalez, J., J. M. Cruz, H. Dominguez, and J. C. Parajo. "Production of Antioxidants from Eucalyptus Globulus Wood by Solvent Extraction of Hemicellulose Hydrolysates." *Food Chemistry* 84.2 (2004): 243-51. Print.

[25] Paraj, J. C., Herminia Domnguez, Andrs Moure, Beatriz Daz-Reinoso, Enma Conde, M. Luisa Soto, M. J. Conde, and Noelia Gonzlez-Lpez. "Recovery of Phenolic Antioxidants Released During Hydrolytic Treatments of Agricultural and Forest Residues." *Electronic Journal of Environmental, Agricultural, and Food Chemistry* 7.8 (2008): 3243-249. Print.

[26] Durre, P. "New Insights and Novel Developments in Clostridial Acetone/butanol/isopropanol Fermentation." *Applied Microbiology and Biotechnology* 49.6 (1998): 639-48. Print.

[27] Sun, Z., and S. Liu. "Production of N-butanol from Concentrated Sugar Maple Hemicellulosic Hydrolysate by Clostridia Acetobutylicum ATCC824." *Biomass and Bioenergy* (2010). Print.

[28] Palmqvist, E., and Barbel Hahn-Hagerdal. "Fermentation of Lignocellulosic Hydrolysates. I: Inhibition and Detoxification." *Bioresource Technology* 74.1 (2000): 17-24. Print.

[29] Palmqvist, E., and Barbel Hahn-Hagerdal. "Fermentation of Lignocellulosic Hydrolysates. II: Inhibitors and Mechanisms of Inhibition." *Bioresource Technology* 74.1 (2000): 25-33. Print.

[30] Lee, Sang Yup, Jin Hwan Park, Seh Hee Jang, Lars K. Nielsen, Jaehyun Kim, and Kwang S. Jung. "Fermentative Butanol Production by Clostridia." *Biotechnology and Bioengineering* 101.2 (2008): 209-28. Print.

[31] Xu, Jian, and Shijie Liu. "Optimization of Ethanol Production from Hot-water Extracts of Sugar Maple Chips." *Renewable Energy* 34.11 (2009): 2353-356. Print.

[32] Mittal, Ashutosh, Siddharth G. Chatterjee, Gary M. Scott, and Thomas E. Amidon. "Modeling Xylan Solubilization during Autohydrolysis of Sugar Maple

and Aspen Wood Chips: Reaction Kinetics and Mass Transfer." *Chemical Engineering Science* 64.13 (2009): 3031-041. Print.

[33] Mendes, CVT. "Extraction of Hemicellulose Prior to Kraft Cooking: a Step for an Integrated Biorefinery in the Pulp Mill." *O papel* 72.9 (2011): 79.

[34] Sattler, Clinton, Nicole Labb, David Harper, Thomas Elder, and Timothy Rials. "Effects of Hot Water Extraction on Physical and Chemical Characteristics of Oriented Strand Board (OSB) Wood Flakes." *CLEAN - Soil, Air, Water* 36.8 (2008): 674-81. Print.

[35] Akpinar, Ozlem, Okan Levent, ?eyda Bostanci, Ufuk Bakir, Levent Yilmaz. "The Optimization of Dilute Acid Hydrolysis of Cotton Stalk in Xylose Production." *Applied Biochemistry and Biotechnology* 163.2 (2011): 313.

[36] Roberto, I. "Dilute-acid Hydrolysis for Optimization of Xylose Recovery from Rice Straw in a Semi-pilot Reactor." *Industrial Crops and Products* 17.3 (2003): 171-76. Print.

[37] Qureshi, N., T. Ezeji, J. Ebener, B. Dien, M. Cotta, and H. Blaschek. "Butanol Production by Clostridium Beijerinckii. Part I: Use of Acid and Enzyme Hydrolyzed Corn Fiber."*Bioresource Technology* 99.13 (2008): 5915-922. Print.

[38] Jeong, Tae-Su, Byung-Hwan Um, Jun-Seok Kim, and Kyeong-Keun Oh. "Optimizing Dilute-Acid Pretreatment of Rapeseed Straw for Extraction of Hemicellulose."*Applied Biochemistry and Biotechnology* 161.1-8 (2010): 22-33. Print.