Modeling Butanol Production by *Clostridium beijerinckii*

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Abstract

Although fossil fuels are currently the most economically sensible source of energy, many other alternative energy sources are being explored as replacements for fossil fuels. Millions of dollars are being spent on ethanol research. However, ethanol's energy content is only two-thirds that of gasoline, while butanol, another promising alternative biofuel, has similar energy content when compared to gasoline. The genus *Clostridium* is known for its ability to produce butanol well into the stationary phase of growth and to grow in easily facilitated, inexpensive media. *Clostridium* carries out butanol fermentation from a variety of 5 and 6 carbon sugars, one of which is xylose. A mathematical model was developed using the known metabolic pathway of xylose to butanol, as well as basic Michaelis-Menten kinematics. Experiments were conducted using the bacterium *Clostridium beijerinckii* in bench-top fermentors to calibrate the parameter of the model so other products could be determined analytically and protein expression could be studied. The goal of this research was to develop methods to obtain optimum yields of butanol production. To achieve this goal, two separate but complementary approaches were undertaken. First, the development and verification of a model to guide the selection of parameters that optimize butanol production. Second, to identify proteins and associated enzymes that are activated at various stages of the fermentation process, which can be targeted for enhanced protein expression in future research.

Materials and Methods

- *Clostridium beijerinckii* ATCC 35702 were grown in 500 mL 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 1L with deionized water.
- Fermentors were sparged with N_2 to create anaerobic conditions.
- Samples were taken at 24, 48, 72, 96, and 144 hours, placed in 15 mL centrifuge tubes and measured for turbidity (OD 600).
- Samples were centrifuged at 8,000 rpm for 15 minutes.
- Supernatant was removed and placed in another 15 mL centrifuge tube for future data measurements and calculations.
- 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 Pressure 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.

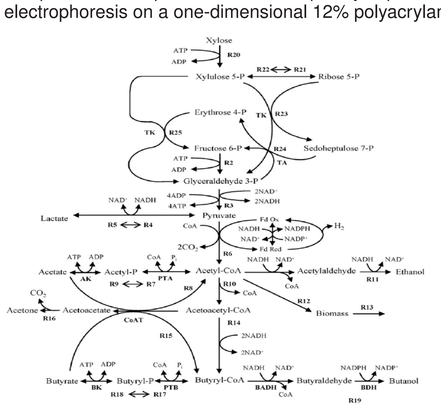
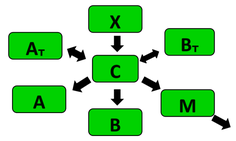


Figure 1. Acetone, Butanol, Ethanol (ABE) fermentation pathway of *Clostridium* using xylose as a substrate (Shinto et al. [5]).

Mathematical Model

From the metabolic pathway (Figure 1), Shinto [4,5] developed a complex mathematical model using a system of 20 differential equations. It contains all of the individual reaction rates for each enzyme complexes indicated by arrows 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 examines only 3 products and 3 intermediates as seen in the schematic (Figure 2) and model equations below.



Variable	Substance	I.C.
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

Figure 2. Simplified xylose pathway, table of variables and initial conditions (I.C.).

Important sequences of enzyme reactions were modeled using combined Michaelis-Menten reactions. 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.

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

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

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

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

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

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

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

Mathematical Results

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.

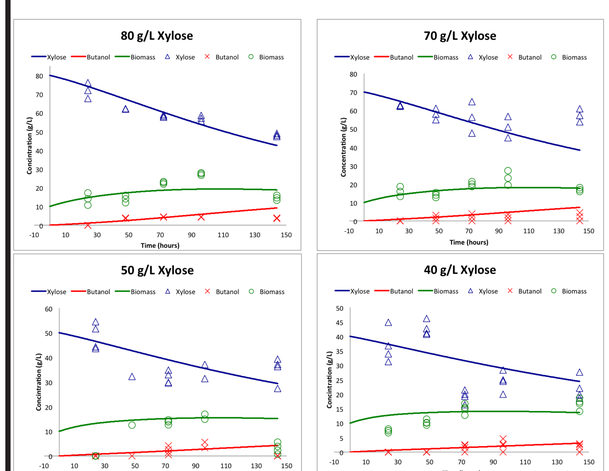


Figure 3. Comparison of numerical (solid lines) and experimental (symbols) results.

Results and Discussion

Our hypothesis was that an increase in the initial xylose concentration would result in an increase in the butanol production of the bacteria. However, it was found that the initial concentration of xylose had little effect on the amount of butanol formed (Figure 4).

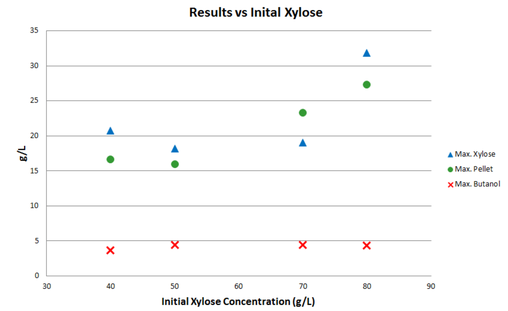


Figure 4. Dependence of maximum xylose metabolized, biomass produced, and butanol produced with respect to initial concentrations of xylose.

Each initial xylose concentration resulted in roughly the same amount of butanol formed. Increasing the amount of xylose in the growth media seemed to increase the xylose consumption and the amount of biomass formed as a product. The use of xylose as a substrate resulted in the incomplete consumption of the xylose. There are known to be certain rate-limiting intermediate reactions in the Pentose Phosphate Pathway that cause this result, and this pathway may be improved upon with future genetic manipulations (Gu et al. [3]).

Figure 5. Fermentor culture in 7-liter BIOFLO-110 bioreactor.

Figure 6. Flask cultures on incubated shaker table.

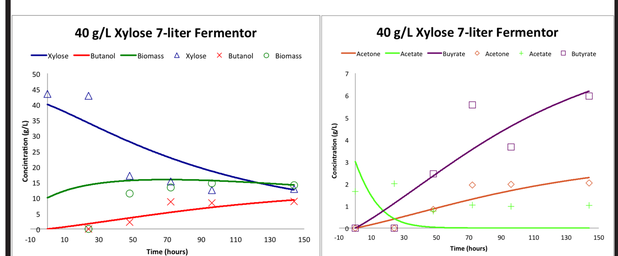


Figure 7. 7-Liter fermentor comparison of numerical (solid lines) and experimental (symbols) results. All parameter values were the same as those used in Figure 3, except V_1 increased by a factor of two and K_5 by a factor of 3.

When scaled up to a 7-liter bioreactor, the 40 g/L culture consumed about 30 g/L xylose and produced roughly 9 g/L butanol (Figure 7). Here, the xylose consumption is 50% greater than in the small flasks, and the butanol production is 100% greater. It is interesting to note, however, that the biomass concentration remained about the same in both the flasks and the 7-liter fermentor.

Protein Analysis

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 length of the fermentation. These differences may be related to the production of different end products over the course of the reaction. Additionally we can predict that additional proteins expressed at later time points may be related to solvent or acid resistance.

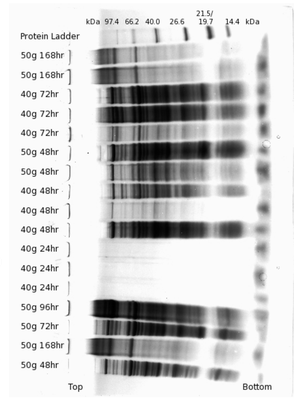


Figure 8. (Right) 1-D gel protein profiles. The first number represents the concentration of xylose in g/L. The second number indicates the time each sample was taken.

Future Work

- Extension of the mathematical model to include additional sugar sources, alternate substrates, pH, and more complex enzyme kinetics.
- Further analysis of substrates and products by high pressure liquid chromatography.
- Detailed proteomic analysis of protein assays.
- Manipulation of pH and intermediate reactants (e.g., butyric and acetic acid) during fermentation.
- Identify causes for differences observed between flasks and 7-liter bioreactor.
- Complete a sensitivity analysis on the math model.

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Acknowledgments

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