Production of Fermentable Sugars from Agricultural Waste

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Cellulosic Ethanol Group
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The environmental and economic strains associated with oil have created the need to explore alternate sources for fuel production and use. One such alternative is ethanol derived from cellulosic material. This report specifies the design of a process used for the conversion of pecan husks into fermentable sugars. The design consists of physical grinding of the raw material, acid pretreatment of the cellulosic powder resulting from grinding, and enzymatic hydrolysis of the remaining polymeric sugars. Enzymes were produced through the culturing of a fungus called T. reesei. Results of testing indicate that optimal conditions for fungus growth are a pH = 5.0, a temperature = 26°C, and glucose concentration = 30 g/L. The optimal conditions determined for acid pretreatment are 1% HCL [w/w], a temperature of 200°C, and a residence time of 5 minutes. For enzymatic hydrolysis, a temperature of 60°C, Enzyme/Pecan Ratio of 5 ml/g, and a pH of 6.0 were chosen as the optimal operating conditions. Finally, an economic analysis of the large-scale ethanol production process was conducted. This analysis was based on the performance of the final design, and results indicate that a plant producing ethanol derived from pecan husks could produce ethanol at a cost of $1.81 per gallon.
1 Executive Summary

Ethanol has been proposed as an alternative fuel that can alleviate stresses on oil supplies and reduce carbon emissions. Utilizing the 10 steps of the design process, a final design has been determined to convert organic waste into fermentable sugars which can then be converted to ethanol. However, only the processes creating the sugars are within the scope of the project. That is, the conversion of sugars to ethanol was not investigated.

The final design consists of a series of operations beginning with mechanical pretreatment and followed by chemical pretreatment with acid. These two preliminary operations serve to make the cellulosic material more react-able by reducing crystallinity and exposing greater surface area to enzymes and water. Acid pretreatment also begins the breakdown of hemicellulose.

After acid treating, enzymatic hydrolysis was performed with enzymes obtained from a fungus called *Trichoderma reesei*. The chemical reactions aided by these enzymes broke down the cellulose and hemicellulose to yield fermentable sugars. The fungus was grown in the lab and the desired enzymes helped “attack” the bonds found in cellulosic chains.

For each of these operations, experiments were performed and analyzed to optimize sugar yields. Variables such as pH, temperature, and acid strength were evaluated in attempts to obtain optimal results and sugar yields.

Determination of sugar yields were obtained with a gas chromatograph and spectrophotometer. The data allowed for a hypothetical, industrial-scale version of the process to be analyzed economically. The economic analysis was based on lab data as well as similar analyses found in literature. Such an analysis was used to compare this process to current fuel-production methods.
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5 Introduction

In the interest of determining whether cellulosic ethanol is a viable alternative to oil, a bench-scale cellulosic ethanol production process was developed. The scope of the project extends only to the formation of fermentable sugars; not the fermentation of sugars to ethanol. An economic analysis was performed on the resulting product (assuming industry-best fermentation techniques) and compared to prices for oil and corn ethanol. Project success was judged by certain working criteria: The feedstock must be a locally-produced, sustainable, waste product (such as pecan husks, rice straw, or cotton gin waste). Also, the production process’s environmental impact must be minimal. Process cost was estimated and compared to other fuel options.

The process for ethanol conversion includes the following unit operations: pretreatment by physical grinding and acid hydrolysis; fermentation of a fungus to create necessary enzymes; and enzymatic hydrolysis. Each of these unit operations has been tested and analyzed.

6 Final Design

The process for ethanol conversion includes the following unit operations: pretreatment by physical grinding and acid hydrolysis; fermentation of a fungus to create necessary enzymes; and finally enzymatic hydrolysis. A flow chart of the process can be seen in Fig. 1. The feedstocks were chosen based on environmental and economic considerations. These included using only agricultural byproducts to ensure maximum economic benefit, as well as including only locally-produced byproducts to reduce transportation costs.
The pretreatment of the feedstock includes a preliminary grinding by blender and a further processing with a coffee grinder. This helps ensure the optimum surface area for reaction exposure in later steps. The second step of pretreatment includes an acid hydrolysis process that has been refined by testing and analysis using two subsequent experimental designs. As the optimum acid strength, time, and temperature were calculated for each operation, a final design was decided upon and implemented in the final process of this project.

After pretreatment, enzymatic hydrolysis was chosen to enhance conversion of sugars. This step was also analyzed based on experiments to ensure optimum yield and efficient use of resources. To supply the enzymes for this reaction, a fungus was grown in the lab, creating a regenerative production process that alleviates both cost and environmental concerns. Experiments were also utilized to optimize the growth of fungus.

### 6.1 Design Constraints

The constraints of this project include economic, environmental, health, and safety concerns. The economic constraints specific to ethanol production require that the process be
relatively inexpensive. Although it was a desired goal to be cheaper than the production of other fuels, this was not considered as the basis for project success. Economic factors considered include the cost of materials, labor, and theoretical cost of energy used. A more in depth analysis of the cost of this project can be seen in 7.6.

The environmental constraints of the project include low emissions of harmful chemicals. This constraint was considered during selection of a final design process. In these processes, the first step’s only concern is the use of acid, which can either be recovered or neutralized. The second step, enzymatic hydrolysis, is a sustainable process with little waste. The fungus is optimally grown to react with the working chemicals. The fungus should also produce the greatest amount of effective enzymes. This result can be seen in 7.5. Another environmental concern for this project was the use of a sustainable feedstock in the form of an agricultural waste product. The decision to use locally-produced feedstocks was dictated by the need to reduce transport costs while maximizing material availability.

Health and safety constraints influenced the temperatures and acid strengths used in the laboratory. While considering the optimal performance of all processes, the lowest possible temperatures were utilized in order to reduce health risks. The weakest acids possible were also used to prevent serious health concerns. Special attention was given to these steps to ensure that the health and safety of the group were not compromised throughout the production process.

7 Methods

Each unit operation was investigated independently. The approach to each is presented below. Section 7.2 describes the analysis of the composition of the three feedstocks considered:
pecan husks, rice straw, and cotton gin waste. This analysis was conducted through the use of a gas chromatograph. A similar test method, which also used gas chromatography, was used to analyze the amount of fermentable sugars in the acid and enzymatic hydrolyzates. This test method is outlined in Appendix B.

7.1 Physical Pretreatment

The energy required to break down the pecan husks for acid treatment takes the form of electrical power that drives a grinder. The grinder used in the lab experiments is a blender and a coffee bean grinder. The physical degradation of the pecan husks allows for greater chemical reactivity in later steps.

7.2 Compositional Analysis

The composition of three different feedstocks – pecan husks, cotton gin waste, and rice straw – was determined using the HP 5890 Gas Chromatograph. The procedure described in the ASTM 1821-01 standard document was used to measure the amount of glucose, xylose, mannose, galactose, and arabinose (in each of their polymeric forms) present in each feedstock.

ASTM 1821-01 outlines the procedure for first hydrolyzing a sample of a biomass using 12M H₂SO₄, and then converting each monosaccharide into its corresponding alditol acetate to be measured using gas chromatography. An internal standard of inositol – which is also a sugar – was utilized to quantify the amount of each monosaccharide per sample. The advantage of using an internal standard during GC analysis is that it helps account for variability in the analyte (alditol acetate) signals. An analyte signal is prone to variability during analysis, but the degree of this variability should be consistent with that of the internal standard since the two are similar in chemical structure and behavior. Thus, analyzing each analyte signal relative to the internal
standard is a more accurate method of analyzing GC data. Calibration solutions were prepared using known concentrations of each monosaccharide being analyzed, and loss factor solutions were prepared to account for losses that occur during the experimental procedure.

A new column – a Supleco-225, 15m x 250µm ID, 0.25µm film thickness polar capillary column – was installed to obtain better separation between analytes. Preliminary tests indicated that better separation is indeed obtained with this new column compared to the previous non-polar column. An electronic HP 3396 Series III Integrator will be used to produce peak data, including the critical area percent data that is directly related to the concentration of each sugar. Microsoft Excel was utilized to perform the simple algebraic steps for converting the chromatogram peak data into amounts of each polymeric sugar present in the feedstock.

7.3 Acid Hydrolysis

Acid hydrolysis serves as a pretreatment to hydrolyze sugars from hemicellulose, a relatively reactable substance. Experiments were used to optimize this process by varying temperature, residence time, and acid concentration. The experimental conditions are shown in Table 1. These experiments were completed and analyzed using the gas chromatograph.
Table 1. Acid Hydrolysis DOE

<table>
<thead>
<tr>
<th>Run #</th>
<th>Temp. [deg C]</th>
<th>Time [min]</th>
<th>HCl % [w/w]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200</td>
<td>120</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>120</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>200</td>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>120</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>8</td>
<td>200</td>
<td>120</td>
<td>0.5</td>
</tr>
</tbody>
</table>

To perform an individual experiment, the steel capsule is loaded with ground pecan husk, acid, and water. The capsule is submerged in the fluidized bath reactor for the residence time, then filtered, neutralized, and stored at 4\(^\circ\) C until gas chromatograph analysis. A detailed experimental procedure can be found in Appendix C.

### 7.4 Enzyme Production

Enzymes, which served to degrade cellulose, were grown with the fungus *Trichoderma reesei*. The fungus was grown in Erlenmeyer flasks incubated on a shake table and later grown in the BioFlow III reactor vessel. Each batch consisted of 100 mL Mandel’s Medium, necessary buffer solution, and was inoculated from a starter culture (Petri dish cultures grown from an ATCC dehydrated sample) after autoclaving at 126\(^\circ\)C for 15 minutes. Once cell growth ceased (after 6 days), the sample was centrifuged for 5 minutes to collect the supernatants and obtain the
enzymes in solution. The effectiveness of these enzymes was tested by performing an enzymatic hydrolysis of raw pecan husks for one hour at 50°C and pH = 4.76. A detailed account of the procedure can be found in Appendix D.

The test variables are shown in Table 2. The results of these tests were then used to select optimal conditions for use in the BioFlow reactor. However, due to instrument calibration problems, the enzymes from the BioFlow had low activities. To obtain improved enzymes, a large shake-flask with 400 mL of Mandel’s Medium was inoculated and later harvested.

<table>
<thead>
<tr>
<th>Batch #</th>
<th>Carbon Source</th>
<th>Carbon Conc. [g/L]</th>
<th>pH</th>
<th>Temperature [deg C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glucose</td>
<td>10</td>
<td>4.0</td>
<td>26*</td>
</tr>
<tr>
<td>2</td>
<td>Glucose</td>
<td>10</td>
<td>5.0</td>
<td>26*</td>
</tr>
<tr>
<td>3</td>
<td>Glucose</td>
<td>10</td>
<td>6.0</td>
<td>26*</td>
</tr>
<tr>
<td>4</td>
<td>Pecan</td>
<td>10</td>
<td>4.0</td>
<td>26*</td>
</tr>
<tr>
<td>5</td>
<td>Pecan</td>
<td>10</td>
<td>5.0</td>
<td>26*</td>
</tr>
<tr>
<td>6</td>
<td>Pecan</td>
<td>10</td>
<td>6.0</td>
<td>26*</td>
</tr>
<tr>
<td>7</td>
<td>Glucose</td>
<td>30</td>
<td>5.0</td>
<td>26*</td>
</tr>
<tr>
<td>8</td>
<td>Pecan</td>
<td>30</td>
<td>5.0</td>
<td>26*</td>
</tr>
<tr>
<td>9</td>
<td>Glucose</td>
<td>10</td>
<td>4.0</td>
<td>30</td>
</tr>
<tr>
<td>10</td>
<td>Glucose</td>
<td>10</td>
<td>5.0</td>
<td>30</td>
</tr>
<tr>
<td>11</td>
<td>Glucose</td>
<td>10</td>
<td>6.0</td>
<td>30</td>
</tr>
</tbody>
</table>

* The shake table had difficulty controlling temperature at 26°C. The actual temperature varied between 26°C and 28°C
7.5  Enzymatic Hydrolysis

The enzymatic hydrolysis was optimized by experimentally varying pH, temperature, and enzyme-substrate ratio. Initially, the enzymes for the tests were obtained from the BioFlow Reactor III, however due to problems discussed in the previous section, the enzymes did not perform well. Still, the analysis of these results (as discussed in 8.4) facilitated the development of a new testing and analysis plan and provided some insight regarding the tested variables’ influence. The second batch of enzymes (using the optimum culture conditions discussed in 8.4) was obtained by performing large shake-flask fermentation.

Hydrolysis experiments were performed in 125 ml Erlenmeyer flasks on raw, dry, and ground pecan husks. pH was controlled with buffer solutions (succinic acid and maleic acid), temperature was controlled with the shake table, and the enzyme-pecan ratio was controlled by varying the amount of pecan added to the flasks. The test matrix is shown in Table 3. A detailed test procedure can be found in Appendix E.

<table>
<thead>
<tr>
<th>Run #</th>
<th>Temp. [deg C]</th>
<th>mL Enzyme/50 mg Pecan</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>1.5</td>
<td>6.0</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td>1.5</td>
<td>6.0</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>0.25</td>
<td>6.0</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>0.25</td>
<td>4.0</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td>1.5</td>
<td>4.0</td>
</tr>
<tr>
<td>6</td>
<td>28</td>
<td>0.25</td>
<td>4.0</td>
</tr>
<tr>
<td>7</td>
<td>28</td>
<td>0.25</td>
<td>6.0</td>
</tr>
<tr>
<td>8</td>
<td>60</td>
<td>1.5</td>
<td>4.0</td>
</tr>
</tbody>
</table>
7.6 **Economic Analysis**

One of this project’s design goals was to minimize the cost of ethanol production for favorable comparison to oil and corn ethanol. Many economic determinants must be examined in order to properly assess this cost. These determinants are summarized in Fig. 2. While the figure refers to corn, the same economic factors apply to cellulosic ethanol. Note that the economic inputs to the farm will be reflected in the price of the agricultural waste product itself. All other economic factors will be analyzed on a per ethanol volume basis. The group will estimate the cost of each of the unit operations demonstrated on the bench-scale, however, the cost will be analyzed for a commercial plant. Since the group is not transporting, fermenting, or separating the alcohol, the costs of these operations must be found in literature.

Data were obtained from a report published by the National Renewable Energy Laboratory, or NREL (Wooley, 1999). In this report, the economics of a cellulosic ethanol plant were evaluated. While woodchips were used as the feedstock in this report’s analysis, the methodology and economic model presented may be applied using any feedstock. However, different feedstocks contain different amounts of fermentable sugars, and also respond differently to the various treatments and processing steps utilized in a plant. Thus, different amounts of feed and energy would be required to produce an equivalent volume of ethanol, which affects the costs associated with producing ethanol. These differences in feed performance created a need to properly address these differences for each process unit in order to calculate an appropriate total process cost.
Weighted performance factors were utilized to account for differences in feed performance. These performance factors varied depending on the process unit being described. In general, these factors could be described by the ratio of polymeric sugar conversion into fermentable sugars. Equation 1 shows the general methodology for calculating the adjusted cost for a process unit. In Eq. 1, $P_{NREL}$ is the performance reported by NREL, $P_M$ is the performance measured for operation using pecan husk as feed, $CC$ represents the capital cost of associated with the process unit, and $VC$ represents the variable cost. All costs were considered on a per volume ethanol basis.

$$Cost = CC + \frac{P_{NREL}}{P_M} \times VC \quad (1)$$

The scope of this project involved the design and testing of three main process units: acid pretreatment, enzyme production, and enzymatic hydrolysis. Therefore, costs for each of these units were evaluated by calculating performance factors based on the measured performance of each. Costs for all other units and processes were obtained from the NREL report.
processes include physical pretreatment, sugar fermentation, ethanol separation, wastewater treatment, storage, and transportation. Assuming these costs are equivalent to those described in the NREL report is a reasonable assumption since these processes are affected less by using a different feedstock. The most dominant differences occur within the three processes tested and evaluated in this project.

The total cost of ethanol can then be determined by summing the cost of each unit operation. The cost of energy, feedstocks, and reagents must be less than the value of the fuel produced for there to be any marginal benefit to this process. Furthermore, the marginal profit must be large enough to make the return on capital greater than the interest rate. Currently, tax incentives make the operation cost of these processes artificially low, which contribute to the spreading interest in all types of ethanol production. When those tax incentives are removed, it is likely that fuel costs would escalate significantly, or production would decrease. Economic analysis for this project assumes no tax incentive.

8 Results

The project required measurements from a variety of tests related to the production of sugar and composition of feedstock. The results from these measurements are presented below. The economic analysis results are also presented in this section, as they derive from the models shown below in addition to materials and equipment costs that were taken from current energy and materials prices.
8.1 Compositional Analysis Results

The amounts of five fermentable sugars – glucose, xylose, galactose, arabinose, and mannose – were measured using gas chromatography. Each of these sugars is fermentable, so they have the potential to be converted into ethanol for use as fuel. The higher the sugar content in a cellulosic material, the more “potential” it has as use as a feedstock for ethanol production. Samples were dried in a convective air oven at 80-90°C, and sugar content was reported on a percent dry mass basis. Additionally, the data reported represents the amount of sugar in each its polymeric form – as a long chain and matrix of simple sugars – present in each feedstock. The results of testing are shown in Fig. 3.

![Figure 3. Fermentable sugar content in three different cellulosic materials](image)

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>Xylose</th>
<th>Galactose</th>
<th>Arabinose</th>
<th>Mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pecan Husk</td>
<td>11.69</td>
<td>4.00</td>
<td>3.16</td>
<td>1.68</td>
<td>0.00</td>
</tr>
<tr>
<td>Rice Straw</td>
<td>16.25</td>
<td>9.31</td>
<td>1.22</td>
<td>1.66</td>
<td>0.00</td>
</tr>
<tr>
<td>Cotton Gin Waste</td>
<td>18.16</td>
<td>7.57</td>
<td>0.51</td>
<td>0.98</td>
<td>0.71</td>
</tr>
</tbody>
</table>
The amount of sugar present in each of the three agricultural waste products tested fell within approximately the same range. According to these results, rice straw and cotton gin waste both contain more fermentable sugar than pecan husks, making them more favorable for ethanol production in this regard. However, it should be noted that the amount of fermentable sugar present in both rice straw and cotton gin waste was low compared to literature values (pecan husk is not reported in literature). For instance, the total amount of fermentable sugar present in cotton gin waste according to Jeoh et. al was 52.3%, which is much higher than the 27.93% measured in this project (Jeoh, 1998). Similarly, a fermentable sugar content of 56% for rice straw was reported by Chandel et. al., a number that is significantly higher than the 28.44% measured in this report (Chandel, 2007).

The low values obtained from testing could possibly be attributed to non-uniform cellulosic material compositions. Different methods for preparing samples for analysis could have contributed to discrepancies as well. Another factor that may have influenced these low composition measurements were the methods used for storing the materials. Approximately four months passed between the cellulosic materials being gathered, washed, dried, and grinded, and the time when compositional testing was conducted. To explore the prospect of moisture being absorbed by tested samples during this period, small amounts of each material were re-dried at 80-90°C for approximately 24 hours. The result revealed that the pecan husk contained 8.7% moisture, the rice straw contained 8.4%, and the cotton gin waste contained 9.9% when each was tested. Re-calculating the composition of each sugar on a dry-weight basis resulted in the sugar content in pecan husk to increase from 20.53% to 22.24%, in rice straw to increase from 28.44% to 30.71%, and in cotton gin waste to increase from 27.93% to 31.00%. Therefore, moisture appeared to have only partially accounted for the low sugar contents. However, with water
presumably present within each material during a period of four months, it is possible that some
degree of degradation took place within each cellulosic material, which may have resulted in the
destruction of fermentable sugars prior to analysis.

Another possible explanation may lie in the test procedure. For analysis, the 12M sulfuric
acid used to break the polymeric sugars into their constituent simple sugars was meant to
accomplish this hydrolysis to completion. It is possible that the concentration of this sulfuric
acid was not exactly 12M, which may have resulted in a less effective or less complete breaking
of polymeric sugars than expected.

Despite the low sugar contents measured, enough confidence was gained in the gas
chromatograph during the course of this project to compare the relative compositions of the
materials tested. Additionally, the values obtained provide a basis for calculating the conversion
of polymeric sugars into their corresponding fermentable sugars.

8.2 Acid Hydrolysis Results

Two acid hydrolysis designs of experiments (DOEs) were completed. The first relied on a
broad test range to get a first approximation of the variable’s influences. The second used the
knowledge from the original DOE to narrow the range of interest, obtain a predictive equation
and use that equation to optimize the process. Each individual run was analyzed for a total of
five different sugars. The sugars were then summed and divided by the original amount of pecan
material to obtain a yield. The raw data and plots associated with the first DOE are shown in
Appendix F.

The first set of experiments revealed interesting trends. While the positive association
with temperature was expected, the negative trends for hydrochloric acid concentration and time
were not. Assuming simple and traditional reaction kinetics, it would be expected that higher
acid concentration and longer times would lead to improved sugar yields. The most likely driving force behind this trend is the degradation of the sugars into furfural and other undesirable gaseous products. Such degradation is consistent with work done at the National Renewable Energy Labs (Wooley, 1999).

Since high temperatures and long residence times appear to have ruinous consequences on sugar production the second DOE reduced the range of these variables. The tabulated results of the second hydrolysis can be seen in Appendix F. Again, it is more valuable to look at the main effects plot, Fig. 4., to understand the trends.

![Main Effects Plot for Total Sugar](image)

**Figure 4. Main Effects Plot of Acid Hydrolysis DOE #2**

The trends from the second DOE are more in line with the original predictions (positive relationship with time and acid concentration), but it still appears that the sugar degrades with extended residence times. Analysis of the second DOE resulted in a reasonably good predictive
equation ($R^2 = 0.96$). The coefficients of the equation can be seen in Appendix F. This equation was then used to simulate 125 different combinations of acid concentration, temperature, and time with the purpose of finding an optimum solution. The simulations demonstrated the optimum acid hydrolysis condition is an acid concentration of 1% HCL [w/w], a temperature of 200°C, and a residence time of 5 minutes. While these conditions perform quite well and are used in the final design solution (see Section 6), it is unfortunate that each variable is at a testing extreme, indicating that a future round of tests with a variable range inclusive of the optimum conditions should be completed. While time did not permit such a test, it would verify that optimum conditions are not, for instance, at an acid concentration of 2% HCL w/w, a temperature of 210°C, and a residence time of 3 minutes.

8.3 Enzyme Production Results

Four variables were investigated to optimize enzymatic production culture conditions. One, the food type, always resulted in negligible results for the non-glucose feed stock. Therefore only data for glucose-grown enzymes are presented. Raw data of the experiments can be seen in Appendix G, and a main effects plot of the data is presented in Fig. 5 (sugar yield vs. culture conditions).
As exhibited by Fig 5, enzymes were much more active at the mid-range pH, the high glucose concentration and the lower temperature. While the effect of pH was hard to predict before the experiment was run, the trends for temperature and glucose concentration were as expected. *T. reesei* typically produces highly active fungus at lower temperatures. At higher temperatures it grows faster, only with less active enzymes. The association between high glucose concentration and superior enzyme performance is intuitive; the more food there is to eat, the more fungus will grow, and more enzymes will be produced. Based on this data, the top performing culture condition was selected for the design solution (pH = 5.0, Temperature = 26°C, and glucose concentration = 30 g/L). This culture condition performed so much better than the others that it was repeated to verify its validity. Unfortunately, the first two attempts at verification did not support the original data. It should be noted that both of these attempts were performed during a time in which the pH probe had lost calibration. It is believed that the actual culture conditions were much too acidic and de-natured the enzymes. A third attempt at

![Main Effects Plot](image_url)

*Figure 5. Main Effects Plot of Sugar Yields for Fungus Growth Culture Conditions*
verification was made in conjunction with the final attempt to produce enzymes for the enzymatic hydrolysis experiments. This final attempt proved more successful, and was used to compare the enzymes to those discussed in the literature. A Filter Paper Test was performed to measure the enzymes Filter Paper Units (FPU), a measure of enzyme activity. The detailed test procedure can be seen in Appendix I. The results of the test indicate the enzymes have a FPU of 5.75, these are highly active for enzymes produced on a bench scale, but significantly below the necessary activity for industrial production processes.

8.4 Enzymatic Hydrolysis Results

Three variables - temperature, pH, and enzyme to pecan mass ratio - were investigated to optimize the enzymatic hydrolysis operation. The trends associated with these variables can be seen in Fig. 6, where the y-axis represents glucose yield from total pecan mass. Raw data from the experiments can be found in Appendix H.
The trends revealed by these experiments are all logical. Higher temperatures provide more energy to speed reactions and higher enzyme concentrations allow for more, quicker sugar conversion. A pH of 6.0 appeared a good, fairly neutral environment for the enzymes to operate in. The results of these experiments led to the formation of a predictive equation, which was then used to optimize the process. A temperature of 60°C, Enzyme/Pecan Ratio of 5 ml/g, and a pH of 6.0 were chosen as the operating conditions.

8.5 Modeling Results

Once the optimum conditions for enzymatic hydrolysis had been decided, kinetic data was taken to determine a rate form (see Fig. 7). The kinetics can be fit to a first-order reaction or a Michaelis-Menten (M-M) model. Both forms are shown in Appendix J. Due to the extremely long time necessary for the reaction to approach equilibrium, the first-order model is more likely and indicates mass-transfer limitations (i.e. pecan particles are too large). The M-M model
would be more likely if the reaction had proceeded quickly, as is typical of enzymatic chemistry. Either of the rate models can be used to investigate the hydrolysis operation in a variety of circumstances (i.e. different reactor types, configuration, etc.), but due to the unexpected slowness, such analysis is not of great value to the project.

![Figure 7. Kinetic Data for Enzymatic Hydrolysis](image)

### 8.6 Series Process Results

After all unit operations had been optimized (see Fig. 8 for final design process with operating conditions), the experiments were run in series to prove their viability and obtain some sense of interaction between the processes. Note that the series experiment was only analyzed for glucose, but the data associated with it can be reasonably extrapolated to overall sugar yield. After all, glucose is by far the dominant sugar and previous testing indicated a linear relation between glucose released and other-sugar released.
The overall cellulose to sugar conversion for the final design is 96%. However, this yield is based on the compositional analysis done by the group, which as discussed in 8.1, may be artificially low. If in fact, the compositional analysis was biased, it is expected that the overall conversion drop to no lower than 56% (based on a scaling factor for compositions of known cellulosic material). While this is obviously a large difference, both yields reflect significant sugar conversion on par or superior to other bench-scale processes.

It should also be noted that there were some synergistic effects observed by running the processes in series. Namely, the enzymatic operation was able to achieve greater conversion as a result of the acid pretreatment. Such a result was the desire and intention for aligning the unit operations in series. It also may reflect a decrease in the mass-transfer limitations discussed in 8.5, as the cellulose is not only chemically degraded during acid treatment, but physically degraded as well.

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**Figure 8. Final Design with Operating Conditions**
8.7 **Economic Analysis Results**

Performance factors were calculated for each of the three process units studied in this project. The conversion of substrate (cellulose) into product (glucose) was considered for both acid pretreatment and enzymatic hydrolysis to calculate the performance factors for these two units. Finally, for enzyme production, performance was based on results from filter paper testing, which provides a means for measuring total enzyme activity.

8.7.1 **Physical Pretreatment Costs**

The operation for physical size reduction of feed in a scaled up cellulosic ethanol plant incurs capital and energy costs. The cost of the mechanical pretreatment is dominated by electricity, but due to significant differences between bench-scale grinding and industrial grinding, tests that measure the power consumption required to grind a given amount of feed for this project’s bench-scale process simply would not have been useful. For instance, the quality and grade of blades used in a coffee grinder differ greatly compared to that of an industrial-type blade. Thus, comparing these two scales on a mass-to-mass basis simply would not have been a legitimate assumption.

Instead, data reported in the NREL report were used. Using this data was reasonable since the physical properties of wood chips and pecan husks are very similar. The figure reported in the NREL report was $0.06 per gallon ethanol produced (Wooley, 1999).

8.7.2 **Acid Hydrolysis Costs**

The costs required for acid hydrolysis are incurred primarily through acid purchase and consumption of the energy required to maintain the high temperatures needed for this process.
Various ratios of feed, liquid, and acid must be considered for this process since the heat required could vary drastically depending on these amounts. For instance, the process tends to be most economical if the ratio of liquid to biomass is kept low.

The conversion of cellulose into glucose during the acid hydrolysis stage reported in literature was 7.9% (Wooley, 1999), while the conversion measured in this project using pecan husk as a feed was 5.3%. This equates to a performance factor of 1.49. The capital cost incurred for acid hydrolysis operation is $0.16/gallon, while the variable cost is $0.10/gallon. Thus, for this process, a cost of $0.31 per gallon of ethanol produced is required.

**8.7.3 Enzyme Production Costs**

The main cost incurred for enzyme production is associated with the nutrients needed to grow the fungus which produces the enzymes. Operating costs are relatively low since low reaction temperatures are utilized for this process.

The performance factor calculated for enzyme production was based on the effectiveness of enzymes that were produced at this unit. Hydrolysis testing was conducted on filter paper to determine the effectiveness of the enzymes produced in terms of FPU. The units FPU stand for “Filter Paper Units,” and are a measure of enzyme activity. The total cost of enzyme production reported in literature was $0.27/gallon, while the enzyme activity was 200 FPU/L (Wooley, 1999). The measured enzyme activity in this project was 143 FPU/L. This equates to a performance factor of 1.40, and a total process cost of $0.38 per gallon ethanol produced.

**8.7.4 Enzymatic Hydrolysis Costs**

An alternate form of breaking polymeric sugars into their constituent simple sugars is simultaneous saccharification and fermentation, or SSF. Wooley et. al used SSF in their
economic model rather than enzymatic hydrolysis, so another source had to be utilized. Fortunately, NREL published another paper about converting corn stover into ethanol a few years after the previous report was published (Aden, 2002). This paper reported that it was possible to create ethanol at $1.07/gallon +$0.12 / -$0.05. The report also stated that 8% of this cost was due to enzymatic hydrolysis. This equates to approximately $0.09/gallon ethanol produced. The upper limit value was used in order to account for variations. Another performance factor was developed based on the conversion of cellulose converted into glucose during the enzymatic hydrolysis step. This weighting was calculated using the 20% conversion reported in literature, and 30.8% measured in this project. Thus, the calculated cost of enzymatic hydrolysis for pecan husks was $0.06 per gallon ethanol produced.

### 8.7.5 Ethanol Fermentation Costs

Since fermentation of sugars into ethanol is beyond the scope of this project, in order to account for the economics of this process, data were obtained from literature (Wooley, 1999). Costs incurred for this process include the energy required to run a reactor, and the production costs of fermenting agents. The fermentation of xylose and glucose into ethanol currently occurs at an industry-best conversion of 95%. This conversion was used by Wooley et al. to calculate the cost per unit volume ethanol produced. The value this group reported was a total cost of $0.15/gallon ethanol produced.

### 8.7.6 Additional Costs

Additional costs that were considered include labor costs, transportation, feed handling, and storage. Again, these figures were obtained from Wooley et. al. A table of these additional costs is shown in Table 4.
8.7.7 Cumulative Process Costs

The cumulative cost for ethanol production using pecan husks as feed is simply the sum of the costs for all process steps as well as all additional costs considered. The result of summing these costs together is a total production cost of $1.81 per gallon ethanol produced. The breakdown for each process unit’s relative cost is shown in Fig. 9. As shown in Fig. 9, acid pretreatment and fungus growth are the two of the most economically taxing processes in cellulosic ethanol production. In order for cellulosic ethanol production to become a viable replacement for oil as a fuel, the cost of these two process units must be reduced.
It should be noted here that the NREL report economic data was obtained from was published almost ten years ago. Accounting for inflationary factors as well as the rising cost of oil would undoubtedly cause the $1.81/gallon figure to rise substantially.

9 Conclusions and Recommendations

The design solution, centered on acid pretreatment and enzymatic hydrolysis, effectively converted the cellulosic material into fermentable sugars. Final operating conditions were optimized for each unit operation; exact values can be seen in Fig. 8. Working criteria were met. The feedstock (pecan husk) is a sustainable, agricultural waste-product. The conversion process itself has minimal environmental impact beyond that associated with any large facility. An economic analysis was completed, demonstrating a $1.81/gallon cost of production. While this
cannot currently compete with corn ethanol or petroleum fuels, it does demonstrate the potential
for cellulosic ethanol to eventually become a viable and significant source of fuel.

Further exploration of several aspects of the project is recommended. All of the operating
conditions for the unit operations in the current process are at extremes of their respective testing
ranges. This indicates further experimentation may indicate that even more extreme values are
optimal. However, it must be noted that while more extreme conditions may lead to higher sugar
production, diminishing marginal returns may make such processes cost-prohibitive.

Investigation of variables this project did not consider is strongly recommended; most
notably, the effect of the pecan husks particle size, but also possible effects of differing medium
compositions for fungus growth, alternating feedstocks, agitation rates, oxygen levels, etc.

There would also be great value in investigating the interaction effects of the unit operations.
The series process experiment indicates that there are favorable interactions between acid
pretreatment and enzymatic hydrolysis. Further understanding of these and other possible
interactions may allow for more efficient sugar production.
10 Bibliography


West Virginia University. “Production Design for Cellulosic Ethanol Facility Capable of Producing 50MMgal/yr.” <www.che.cemr.wvu.edu/publications/projects>
A Budget and Hours

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Total 584.05
Figure A.1 Hours Worked by Individuals

![Bar chart showing hours worked by individuals over the months of September to April. The chart includes data for Team Plan, Ryan Actual, Reid Actual, Jason Actual, Nina Actual, and Will Actual.]

Figure A.2 Hours Worked Cumulative

![Line chart showing cumulative student-hours over the months of September to April. The chart includes data for Team Plan and Team Actuals.]

Page A-3
B  Procedure for Converting Monosaccharides into their Corresponding Alditol Acetates

As Outlined in ASTM E-1821-01: Determination of Carbohydrates in Biomass by Gas Chromatography

1) Prepare appropriate calibration solution. Use an analytical balance to weigh out the appropriate amount of each carbohydrate. An appropriate volume should be determined based on the desired number of calibration samples to be analyzed. A volume of 25 mL should suffice in most cases. The solution should be discarded after 4 weeks. The concentrations in de-ionized water of each monosaccharide should be approximately as follows:

- Arabinose(C₅H₁₀O₅)  1.00 mg/mL
- Xylose(C₅H₁₀O₅)     7.00 mg/mL
- Mannose(C₆H₁₂O₆)     1.00 mg/mL
- Galactose(C₆H₁₂O₆)     1.00 mg/mL
- Glucose(C₆H₁₂O₆)     20.0 mg/mL

2) Prepare inositol (internal standard) solution. Use an analytical balance to weigh out the appropriate amount of inositol. An appropriate volume should be determined based on the desired number of samples to be analyzed. In most cases 50 mL should suffice. It is suggested that the solution be discarded after one week. An appropriate amount of inositol solution should be added to hydrolyzates immediately after filtration and neutralization. The concentration in de-ionized water should be as follows:

- Inositol(C₆H₁₂O₆)     20.0 mg/mL

3) All subsequent additions should be done using gloves, protective eyewear, and under a ventilated hood. Prepare potassium borohydride solution. A potassium borohydride calculator and procedure outline has been created. The only input required in the calculator are the total specimens to be analyzed (this should include all calibration samples). Handle the potassium borohydride with caution, and pour slowly when adding to water. A 40°C water bath will be needed to dissolve the KBO₄ in water. The solution should be prepared fresh before each use, and discarded after 6 hours.
4) Begin conversion procedure by pipetting 900 µL of the calibration solution to two small (10 mL) test tubes. Pipet 100 µL of the inositol solution into the same two test tubes and mix (in all cases where mixing is required, vortex mix if possible) for 5 s. For all steps following step 5, these two samples should be processed in conjunction with each hydrolyzate sample.

5) Pipet 1000 µL of each hydrolyzate specimen (which should already contain the internal standard at this point) into a small test tube (10 mL).

6) Pipet 150 µL of concentrated ammonium hydroxide to each specimen (including the standards) and mix for 5 s. If the sample was acidic prior to this step, a higher volume may be required. The pH after the addition of the NH₄OH should be at least 10.3. Solutions with a pH of less than 10.3 will show irreproducible reduction reactions during the next step.

7) Pipet 500 µL of potassium borohydride solution and mix for 5 s. Place test tubes in a water bath at 40ºC for 90 min to allow for the reduction of monosaccharides into their respective alditols (sugar alcohols).

8) Remove the test tubes from the water bath and stop the reduction reaction by adding 500 µL of glacial acetic acid, dropwise. Allow the fizzing to subside before adding each subsequent drop. Mix for 5 s once the addition is complete. Allow cooling for 10 approximately 10 minutes.

9) Transfer 400 µL of each specimen into a new test tube using a pipet (the test tubes should be larger than the 10 mL tubes that have been used to this point). An alternative to transferring each specimen into a new test tube is to simply pipet off the appropriate volume of each specimen, leaving 400 µL in each original test tube. (In this case, the test tubes used originally should be larger than 10 mL.)

10) Pipet 500 µL of 1-methylimidazole to each test tube and mix for 5 s.

11) Convert the alditols into their corresponding alditol acetates by pipetting 2.0 mL of acetic anhydride to each test tube dropwise. Add slowly since this reaction can be quite rigorous. Mix for 5s, and allow the reaction to proceed for 30 min without special cooling.
12) Decompose the excess acetic anhydride by adding 5.0 mL of water (using a syringe) and mix for 5 s. Allow to cool for approximately 10 min.

13) Pipet 2.0 mL of dichloromethane (methylene chloride) into each test tube and mix for 15 s. Allow the phases to completely separate so that little or no haziness is present in either phase (allow at least 15 min).

14) Using a syringe and long tip, remove the top 5.0 mL of the aqueous phase from each test tube and discard.

15) While cooling the test tube in an ice bath, using a syringe add 5.0 mL of 3.5 M potassium hydroxide, dropwise, while cooling. Allow the phases to completely separate so that little or no haziness is present in either phase (allow at least 15 min).

16) Using a syringe and long tip, transfer 1.0-1.5 mL of the lower phase (dichloromethane solution of alditol acetates) into a 5 mL glass vial.

17) Analyze each specimen using gas chromatography. Conditions used are currently as follows:

- **Detector Type**: Flame Ionization
- **Column Type**: Supelco–225, 15 m x 250 μm ID, 0.25 μm film thickness (polar)
- **Oven Temperature**: 210°C (constant)
- **Injection Port Temperature**: 200°C
- **Detector Temperature**: 250°C
C Acid Hydrolysis Test Procedure

1) Turn on Air Supply to Fluidized Bath (F.B.)
2) Adjust as needed with valve at the F.B. inlet
3) Turn on the Heat
   a. If you’re shooting for 200°C then crank it all the way up until you get there. When you get to 200°C turn the knob to about 5
4) Prepare your capsule
   a. Add 1.00 g of Pecan Husk to the Capsule
   b. Add the water you need (use spreadsheet to determine)
   c. Add the acid you need (use spreadsheet to determine)
5) Torque capsule about 105° in Manuel’s vice. This is about 1.75 “hexagons”
6) Place Capsule in F.B. for the time you want and make sure the temperature is stable
7) Wait….
8) Remove Capsule and place in Water Bath
9) Put the Capsule under running water
10) Dry and place back on scale to make sure the seal held
11) Get Ready to go unscrew the capsule. WEAR GLOVES AND BRING A PYREX BOTTLE to hold under the capsule as you unscrew. Liquid will come out or the capsule once you start to untighten it and you need to be able to capture it.
12) Filter Sample
13) Wash with Distilled water – KEEP TRACK of how much water you add (if you plan ahead you can accomplish this very easily with the mass balance).
14) Take several milliliters of the sample and place it in a vial.
15) Neutralize the liquid in the vial until pH is about 7.0
16) Label vial with your run order# (i.e “AH - #4) and put it in the refrigerator
17) Dump the leftover solution into the waste container (near the plugged up sink – you’ll know if by the large amount of disgusting liquid in it).
18) Turn off F.B. BUT LEAVE ON THE AIR – the air must continue to run through the aluminum until it is below 50°C. If you’re leaving before it cools then just leave the air on overnight.
19) WASH the steel capsule
D Enzyme Production Test Procedure

1) Measure out 50 mg of cellulosic substrate onto filter paper
2) Dump cellulosic substrate into test tube (BUT NOT THE FILTER PAPER)
3) Wash it down with 2.100 mL of Sodium Acetate Buffer
4) Add 0.250 mL of Enzyme Solution
5) Add 0.250 mL of distilled water
6) Set Temperature in Incubator
7) Place Test Tube(s) in a test tube rack
8) Tape Rack to Incubator Table
9) Set Incubator to 200 rpm and correct temperature
10) Let sit for 1 hour
11) Remove Test tubes from Incubator
12) Neutralize to pH~7.0
13) Add 0.200 mL of Inosital Solution
14) Add 2.5 mL of distilled water
15) Draw solution into unused syringe
16) Place unused filter over syringe and push the solution into a vial
17) Label Vial
18) Store in refrigerator at 4°C
E Enzymatic Hydrolysis Test Procedure

1) Measure out the necessary mass of cellulosic substrate onto filter paper
2) Fill Erlenmeyer flask with approx. 45 mL of necessary buffer
3) Add 5 mL of Enzyme Solution
4) Dump cellulosic substrate into test tube (BUT NOT THE FILTER PAPER)
5) Set Temperature in Incubator
6) Place flasks in the incubator
7) Set Incubator to 200 rpm
8) Let sit for 1 hour
9) Remove flasks from incubator
10) Neutralize to pH~7.0
11) Add 0.200 mL of Inosital Solution
12) Add 2.5 mL of distilled water
13) Draw solution into unused syringe
14) Place unused filter over syringe and push the solution into a vial
15) Label Vial
16) Store in refrigerator at 4°C
F Acid Hydrolysis Test Data

Table F.1 First Acid Hydrolysis DOE

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</table>

Figure F.1 Main Effects Plot for Acid Hydrolysis DOE #1
Figure F.2. Scatterplot of Acid Hydrolysis DOE #1

Table F.2 Second Acid Hydrolysis DOE

<table>
<thead>
<tr>
<th>RunOrder</th>
<th>Temp</th>
<th>Time</th>
<th>HCL Conc</th>
<th>Total Sugar</th>
<th>5-Carbon</th>
<th>6-Carbon</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200</td>
<td>30</td>
<td>1</td>
<td>0.0070</td>
<td>0.0032</td>
<td>0.0038</td>
</tr>
<tr>
<td>2</td>
<td>200</td>
<td>5</td>
<td>1</td>
<td>0.1599</td>
<td>0.0847</td>
<td>0.0752</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>5</td>
<td>0.25</td>
<td>0.1013</td>
<td>0.0442</td>
<td>0.0571</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>5</td>
<td>0.25</td>
<td>0.0231</td>
<td>0.0031</td>
<td>0.0201</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>5</td>
<td>1</td>
<td>0.0516</td>
<td>0.0074</td>
<td>0.0443</td>
</tr>
<tr>
<td>6</td>
<td>200</td>
<td>30</td>
<td>0.25</td>
<td>0.0229</td>
<td>0.0078</td>
<td>0.0152</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>30</td>
<td>1</td>
<td>0.0251</td>
<td>0.0020</td>
<td>0.0231</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
<td>30</td>
<td>0.25</td>
<td>0.0004</td>
<td>0.0004</td>
<td>0.0000</td>
</tr>
</tbody>
</table>
Table F.3 DOE Analysis for Second Acid Hydrolysis

Estimated Effects and Coefficients for 5-Carbon (coded units)

<table>
<thead>
<tr>
<th>Term</th>
<th>Effect</th>
<th>Coef</th>
<th>SE Coef</th>
<th>T</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>0.01907</td>
<td>0.005287</td>
<td>0.005287</td>
<td>3.61</td>
<td>0.172</td>
</tr>
<tr>
<td>Temp</td>
<td>0.03176</td>
<td>0.01588</td>
<td>0.005287</td>
<td>3.00</td>
<td>0.205</td>
</tr>
<tr>
<td>Time</td>
<td>-0.03150</td>
<td>-0.01575</td>
<td>0.005287</td>
<td>-2.98</td>
<td>0.206</td>
</tr>
<tr>
<td>HCL Conc</td>
<td>0.01043</td>
<td>0.00521</td>
<td>0.005287</td>
<td>0.99</td>
<td>0.504</td>
</tr>
<tr>
<td>Temp*Time</td>
<td>-0.02746</td>
<td>-0.01373</td>
<td>0.005287</td>
<td>-2.60</td>
<td>0.234</td>
</tr>
<tr>
<td>Temp*HCL Conc</td>
<td>0.00749</td>
<td>0.00375</td>
<td>0.005287</td>
<td>0.71</td>
<td>0.608</td>
</tr>
<tr>
<td>Time*HCL Conc</td>
<td>-0.01194</td>
<td>-0.00597</td>
<td>0.005287</td>
<td>-1.13</td>
<td>0.461</td>
</tr>
</tbody>
</table>

S = 0.0149538   PRESS = 0.0143114
R-Sq = 96.48%   R-Sq(pred) = 0.00%    R-Sq(adj) = 75.34%
# G Enzyme Production Test Data

## Table G.1 Enzyme Production Raw Data

<table>
<thead>
<tr>
<th>pH</th>
<th>Conc [g/l]</th>
<th>Temp [deg C]</th>
<th>5-carbon</th>
<th>6-carbon</th>
<th>Total Sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>30</td>
<td>26</td>
<td>0.161</td>
<td>0.429</td>
<td>0.590</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>30</td>
<td>0.029</td>
<td>0.074</td>
<td>0.103</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>26</td>
<td>0.009</td>
<td>0.098</td>
<td>0.107</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>30</td>
<td>0.006</td>
<td>0.038</td>
<td>0.044</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>26</td>
<td>0.004</td>
<td>0.047</td>
<td>0.050</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>30</td>
<td>0.003</td>
<td>0.039</td>
<td>0.042</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>26</td>
<td>0.005</td>
<td>0.046</td>
<td>0.051</td>
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<tr>
<td>5</td>
<td>10</td>
<td>30</td>
<td>0.006</td>
<td>0.030</td>
<td>0.036</td>
</tr>
</tbody>
</table>


## H Enzymatic Hydrolysis Test Data

### Table H.1 Enzyme Production Results (Glucose Feed Only)

<table>
<thead>
<tr>
<th>Run</th>
<th>pH</th>
<th>Conc [g/l]</th>
<th>Temp [deg C]</th>
<th>5-carbon</th>
<th>6-carbon</th>
<th>Total Sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>30</td>
<td>26</td>
<td>0.1609</td>
<td>0.4290</td>
<td>0.5899</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>30</td>
<td>30</td>
<td>0.0291</td>
<td>0.0738</td>
<td>0.1028</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>10</td>
<td>26</td>
<td>0.0091</td>
<td>0.0978</td>
<td>0.1069</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>10</td>
<td>30</td>
<td>0.0064</td>
<td>0.0377</td>
<td>0.0440</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>10</td>
<td>26</td>
<td>0.0037</td>
<td>0.0466</td>
<td>0.0503</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>10</td>
<td>30</td>
<td>0.0033</td>
<td>0.0387</td>
<td>0.0420</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>10</td>
<td>26</td>
<td>0.0054</td>
<td>0.0461</td>
<td>0.0515</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>10</td>
<td>30</td>
<td>0.0065</td>
<td>0.0299</td>
<td>0.0364</td>
</tr>
</tbody>
</table>

### Table H.2 Enzyme Production Results DOE Analysis (Glucose Feed Only)

The regression equation is

\[ \text{Total Sugar} = 0.841 + 0.0144 \text{ pH} + 0.0146 \text{ Conc [g/l]} - 0.0358 \text{ Temp [deg C]} \]

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coef</th>
<th>SE Coef</th>
<th>T</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>0.8410</td>
<td>0.7909</td>
<td>1.06</td>
<td>0.348</td>
</tr>
<tr>
<td>pH</td>
<td>0.01435</td>
<td>0.07074</td>
<td>0.20</td>
<td>0.849</td>
</tr>
<tr>
<td>Conc [g/l]</td>
<td>0.014558</td>
<td>0.005776</td>
<td>2.52</td>
<td>0.065</td>
</tr>
<tr>
<td>Temp [deg C]</td>
<td>-0.03583</td>
<td>0.02501</td>
<td>-1.43</td>
<td>0.225</td>
</tr>
</tbody>
</table>

\[ S = 0.141473 \quad \text{R-Sq} = 67.9\% \quad \text{R-Sq(adj)} = 43.8\% \]
I Filter Paper Test Procedure

1. Reagents

   Whatman No. 1 filter paper cut into 1- by 6-cm strips (50 mg)

   Buffer = 0.05 M citrate pH 4.8

   Glucose standards in buffer

   Gas Chromatograph agents for sugar analysis

2. Procedure

   0.5 mL enzyme

   1.0 mL buffer

   One strip filter paper

   Mix in Vortex to coil paper in solution. Incubate 1 hour at 50deg. Analyze sugar concentration with Gas Chromatograph

3. The milligram of glucose produced in this test is the filter paper (FP) activity. FP activity is roughly quantitative up to about 3 mg of glucose and is adequate for monitoring fermenters, screening mutants, etc. It should be expressed in relation to concentration as "at 1 mg/mL the powder has a FP activity of 1.0" or "at full strength the culture filtrate has a FP activity of 3.5". For quantitative work, if FP activity is greater than 2.0, the assay is repeated using diluted enzyme, and the dilution to give 2.0 mg of glucose is estimated.
4. Units per mL = __mg glucose x 0.0925__

mL enzyme

or for 0.5 mL assay as described units per mL = mg glucose x 0.185 x dilution factor

or units per mL = __0.37__ to give 2.0-mg glucose.

dilution

5. This assay measures total cellulase, Cx, plus C1, but C1 is the limiting component for most cellulase preparations
J Kinetic Models

**Figure J.1 Log-Log Plot For Traditional Rate Form**

\[ y = 0.9977x - 1.4661 \]
\[ R^2 = 0.9976 \]

**Figure J.2 Lineweaver-Burk Plot For M-M Rate Form**

\[ y = 29.482x - 0.0337 \]
\[ R^2 = 0.9987 \]