# DE-SC0024998: STTR Topic 13.b
# Alternative Use of Commercial Equipment
# *Low-Cost Production of Sustainable Aviation Fuels (SAF) from Perennial Feedstocks using Simultaneous Ball Milling and Enzyme Hydrolysis*
# *STTR Final Report Phase I*


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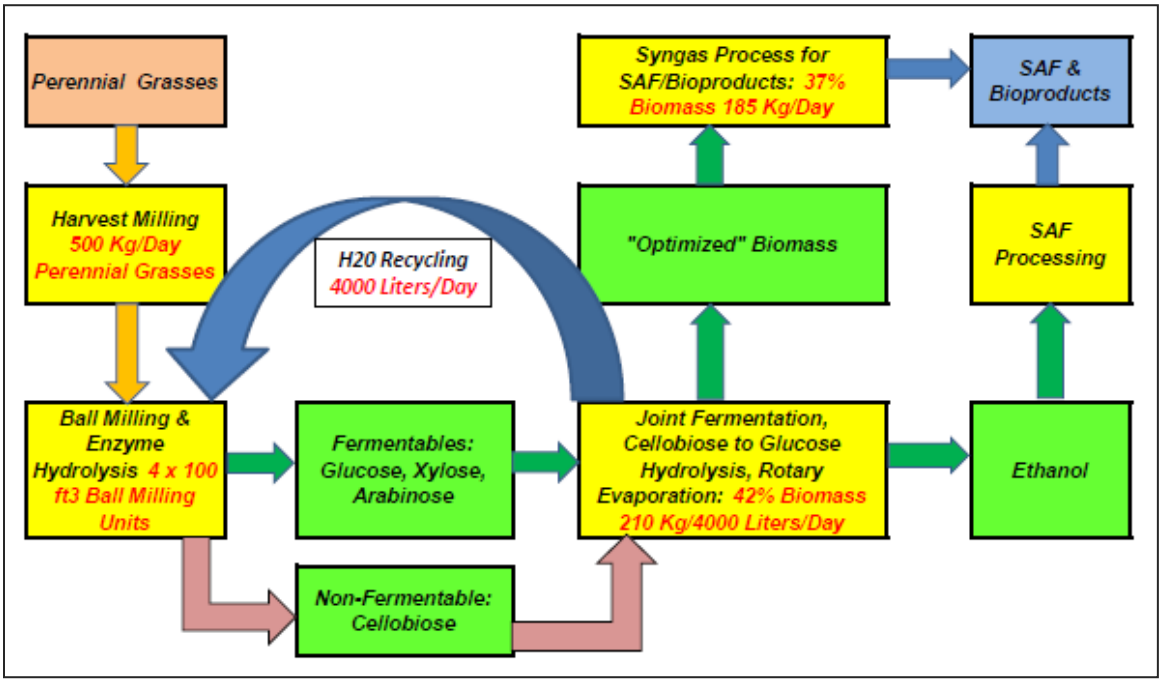

DOE STTR Phase I Project: DE-SC0024998
*Low-Cost Production of Sustainable Aviation Fuels (SAF) from Perennial Feedstocks using Simultaneous Ball Milling and Enzyme Hydrolysis*

Table of Contents:

**DE-SC0024998: Topic 13.b Alternative Use of Commercial Equipment**
***Low-Cost Production of Sustainable Aviation Fuels (SAF) from Perennial Feedstocks using Simultaneous Ball Milling and Enzyme Hydrolysis***
***Final Report Phase I***

## Introduction

The objective of this STTR Phase I project was to demonstrate that existing commercially available dry ball milling equipment could be used as components of a simultaneous wet ball milling/enzyme hydrolysis process that could provide the processing breakthroughs necessary for making the use of low-value perennial grasses as feedstocks for biofuel and bioproducts economically viable.

This project builds on our success in preliminary work funded by the Maryland Energy Innovation Institute (MEII). That work demonstrated that our combined wet ball milling/enzyme hydrolysis process eliminates the need for pretreatment and can produce ethanol at sufficient conversion rates that perennial grasses could serve as feedstocks to produce ethanol in conjunctions with existing ethanol to SAF (Sustainable Aviation Fuels) commercial processes.

Among the immediate benefits of combining simultaneous ball milling and enzyme hydrolysis was to eliminate the current paradigm of using thermal and/or chemical pretreatments to improve the enzymatic conversion of biomass to fermentable sugars. While these pretreatments are effective in raising fermentable sugar yields from biomass, they also introduce significant processing complexity and costs. These include the capital costs of pretreatment equipment, energy and chemicals, as well as the often-overlooked costs of removing pretreatment chemicals that inhibit downstream fermentation and other chemical processes.

## Phase I Accomplishments

At the start of this Phase I project, the focus was to improve the performance of simultaneous ball milling and enzyme hydrolysis by improving individual aspects of the ball milling hardware and the enzyme conversion process (Chapter 1). The work was conducted in commercial 5 liter MSE 304 grade stainless steel Ball Mill Jars. Two jars were used simultaneously on a Morse # 1-5154-3 stationary drum roller. This equipment was previously acquired by Atlantic Biomass, LLC for the MEII work listed above. This system was a competent simulation of a larger scale commercial system. Later verification of commercial ball milling operational parameters such as daily biomass slurry output, loading/offloading procedures, and hydrolysis temperature control were conducted in a commercial Orbis 2ft$^3$ (56.6 liter) dry ball milling unit (Figure 1-1).

As Phase I work continued, a system oriented approach toward system design was used. This allowed us to move beyond developing the process of changing grasses to biofuels and bioproducts from individual “boxes” that fed products in one direction, toward a highly integrated process that had downstream feedback loops that could benefit both upstream and downstream functions. This approach led to the following three discoveries:

1. Enzyme hydrolysis to fermentable sugars occurred in both ball milling and during downstream processes such as fermentation. This removed inhibitions that enabled the conversion of a key biomass intermediate, cellobiose, to glucose without increasing processing costs.

2. The combined ball milling, hydrolysis, fermentation, and distillation processes which are required for ethanol feedstock production, results in a low impurity byproduct usable as a pyrolysis/syngas feedstock. This overcomes purity and heterogeneous compositions issues that currently limit the economics of using intact biomass for syngas production.

3. Reducing the ratio of the length of input perennial grass biomass to the interior diameter of a ball milling vessel to less than 15 percent resulted in over 90 percent of input biomass to be converted to a slurry containing fermentable sugars on a periodic cycle of about 24 hours.

The combination of these discoveries, along with the other process improvements developed in Phase, I would lead to integrated commercial systems that would have very high rates of total biomass conversion and income from low value perennial grasses. Implementing and improving these discoveries in a Phase II prototype system is the next step toward commercialization. Plans for a prototype unit are included in this report.

**Report Organization**

This Phase I report consists of three Chapters.

- Chapter I: Operation Parameter Testing
- Chapter II: Optimizing Product Outputs
- Chapter III: Prototype System Development

Chapter I presents results of experiments to develop and optimize the conversion process that were listed in the Phase I grant proposal. These include:

- Effects of Carbohydrate Composition of Perennial Grasses

- Processing Mixed Grasses
- Synergy between Ball Milling and Enzyme Hydrolysis
- Optimizing Ball Milling Speed
- Maximizing Removal of Slurry After Hydrolysis
- Reducing Weight of Milling Containers
- Establishing Enzyme Performance Guidelines

Chapter II focuses on the system development of the entire perennial grass to bioproduct process. It reports on how two of discoveries listed above maximize product outputs.

Chapter III focuses on developing a continuous process for simultaneous ball milling/enzyme hydrolysis unit that would serve as the basis of a Phase II 500 kg/day input prototype unit. Phase I test runs were conducted at the 5 liter scale as well as in a commercial Orbis 2 $ft^3$ (56.6 liter) ball milling unit (Figure 1-1). Testing focused on addressing operational procedures that minimize equipment requirements and costs. In addition, a crop-to-fuel economic model is presented based on Phase I results.

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**Figure 1-1 Commercial Scale Ball Mill used for process development.**

## Chapter I: Operation Parameter Testing

Individual production parameters were tested and quantified with a minimum of variables to isolate causes and effects whenever possible. These tests usually took one ball milling/enzyme hydrolysis cycle which was either 24 or 48 hours. Individual parameters that were tested included:

- Carbohydrate Composition of Perennial Grasses
- Processing Mixed Grasses
- Synergy between Ball Milling and Enzyme Hydrolysis
- Ball Milling Speed
- Maximizing Removal of Slurry After Hydrolysis
- Reducing Weight of Milling Containers
- Establishing Baseline Enzyme Performance

### Section 1.1 Carbohydrate Composition of Perennial Grasses

Three perennial grasses were used in this Phase I project: switchgrass, phragmites, and miscanthus. The first two have been identified as the best high yield candidates for cellulosic ethanol production, and the third is a widespread high yielding invasive species. Carbohydrates in these grasses can be hydrolyzed into sugars that can be fermented into ethanol and other chemicals. These include the six-carbon (C-6) sugar glucose hydrolyzed from cellulose that is generally used in yeast fermentation, and also the five-carbon (C-5) hemicellulose sugars, xylose and arabinose. We performed an acid hydrolysis compositional analysis (based on NREL procedures) to determine the amount of both sugar types in the feedstocks for fuel and chemical production. As shown in Table 1-1, by including the C-5 hemicellulose sugars, from 52 to 65% of the total biomass could potentially be converted into fermentable sugars available for conversion to fuels and chemicals.

**Table 1-1**
**Acid Hydrolysis Analysis of Perennial Grass Carbohydrates in this study**
(Acid hydrolysis procedures were based on, "Determination of Structural Carbohydrates and Lignin in Biomass Laboratory Analytical Procedure (LAP)" NREL, April 2008)

| Sample Type | Sample ID | Cellulose (% total biomass) | Hemicellulose (% total biomass) | Cellulose and Hemicellulose (% total biomass) | % Increase over Glucose |
|---|---|---|---|---|---|
| Switchgrass PA 2023 Fall Harvest (50°C) | 4A | 34% | 28% | 62% | |
| | 4B | 32% | 26% | 58% | |
| | **Avg.** | **33%** | **27%** | **60%** | 82% |
| | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| Switchgrass PA 2023 Fall Harvest | 11A | 32% | 29% | 61% | |
| | 11B | 31% | 25% | 56% | |
| | 11C | 31% | 25% | 56% | |
| | **Avg.** | **31%** | **26%** | **57%** | 84% |
| | | | | | |
| Phragmites Ohio Summer 2024 Harvest | 12A | 30% | 20% | 50% | |
| | 12B | 30% | 24% | 55% | |
| | 12C | 30% | 24% | 54% | |
| | **Avg.** | **30%** | **22%** | **52%** | 75% |
| | | | | | |
| Miscanthus Ohio Winter 2025 Harvest | 21A | 48% | 21% | 69% | |
| | 21B | 48% | 21% | 69% | |
| | 21C | 41% | 19% | 60% | |
| | **Avg.** | **45%** | **20%** | **65%** | 45% |

The miscanthus values were within range of previously reported values (Table 1-2).

**Table 1-2: Miscanthus Cell Wall Composition** [1]

| | Cellulose (%) | Hemicellulose (%) | Lignin (%) |
|---|---|---|---|
| *Miscanthus giganteus Illinois* | 42.8 | 22.02 | 19.67 |
| Average of 80 genotypes | 40.7 | 21.0 | 24.0 |
| Range of 80 genotypes | 27.7-48.6 | 19.6-27.1 | 15.5-27.8 |

Switchgrass cellulose values were somewhat lower and their hemicellulose values were somewhat higher than previously reported (Tables 1-2 and 1-3).

**Table 1-3**
**Comparison of Biomass Compositions**
(Kim, et al 2021)[2]

| (A) | Composition (%) | | | |
|---|---|---|---|---|
| Biomass | Cellulose | Hemicellulose | Lignin | Ref. |
| Industrial hemp | 32.6–44.5 | 16.6–15.5 | 17.0–21.5 | 20 |
| Corn stover | 37.0 | 22.7 | 18.6 | 12 |
| Sugarcane bagasse | 37.9 | 21.5 | 27.2 | 26 |
| Hardwood | 39.8 | 16.6 | 31.0 | 27 |
| Corn pericarp | 22.5 | 23.7 | 4.7 | 28 |
| Switchgrass | 39.5 | 10.3 | 17.8 | 29 |
| Poplar | 43.8 | 14.8 | 29.1 | 30 |

These variations may be due to time of harvest. In a European comparison of four M. *giganteus* genotypes, one M. *sacchariflorus* genotype, and ten M. *sinensis* genotypes, cell wall composition was found to vary as a function of harvest time and growing conditions. As shown in Table 1-4, winter harvest of miscanthus resulted in reduced hemicellulose content and increased cellulose and lignin content.

**Table 1-4**[3]
**Miscanthus Cell Wall Content at Winter Harvest**

| Winter Harvest (%) [a] | Denmark | Sweden | England | Germany | Portugal |
|---|---|---|---|---|---|
| Hemicellulose | -0.7% | -3.5% | -2.3% | -1.0% | -4.0% |
| Cellulose | +15.5% | +14.5% | +9.2% | +7.4% | +27.1% |
| Lignin | +22.5% | +23.7% | +13.8% | +16.0% | +21.5% |

[a] - Change from Autumn Harvest (Mean Values per Country)

## Section 1.2 Processing Mixed Grasses

As listed above, three perennial grasses were tested in Phase I. The first was switchgrass (Figure 1-2) harvested at the end of 2023 and stored until summer of 2024. It was milled by the grower to approximately 1”-1.5” by using a commercial bedding milling unit and stored dry.

**Figure 1-2**
**Switchgrass Arriving at OSU/Wooster Lab in July 2024**

In order to match the phragmites and miscanthus (Figure 1-3) to this “as received from grower” milled condition,

**Figure 1-3**
**Miscanthus in northeastern Ohio prior to harvest**

a hammer mill (Figure 1-4) equipped with a 3/8” output screen was used (Figure 1-5) for milling.

**Figure 1-4 Milling of phragmites and miscanthus feedstocks**

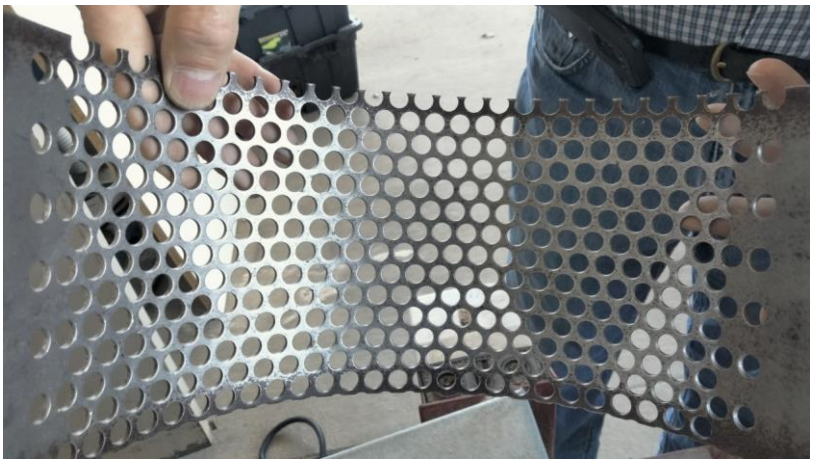


**Figure 1-5 3/8" Output Screen**

**Figure 1-6**
**Miscanthus Before and After Hammer Milling**

The hammer mill turned the grass stems into pieces about 1-1.5" in length relatively quickly (Figure 1-6). A larger automated hammer mill would be used in the commercial application of this system.

Ball Milling and Enzymatic Hydrolysis Results

Simultaneous wet ball milling and enzyme hydrolysis was conducted in equipment previously acquired by Atlantic Biomass, LLC for work funded by the Maryland Energy Innovation Institute (MEII).

The ball milling vessels were 5 liter MSE 304 grade stainless steel Ball Mill Jars (Figure 1-9). Wall thickness was 3 mm and empty weight was about 5 kg. Two jars were used simultaneously on a Morse # 1-5154-3 stationary drum roller (Figure 1-10).

All three grasses were ball milled and hydrolyzed individually with a variety of enzyme mixtures. To assess the effects of using mixed feedstocks, and if the composition of the grasses might inhibit one another, they were also mixed together and tested in ball milling/enzyme hydrolysis runs.

All sugar concentrations were measured using Agilent 1200 HPLC units with a Bio-Rad HPX-87H column and RID (refractive index detection). Analysis runs were performed at both OSU/Wooster and Hood College. Both units were calibrated and tested with the same reference values to enable data comparison. The NREL document, "Determination of Structural Carbohydrates and Lignin in Biomass Laboratory Analytical Procedure (LAP)" April 2008 was used for procedure information.

Table 1-5 details the sugars produced from these mixed grasses runs.

**Table 1-5**
**Monomeric Sugars Produced from Mixed Perennial Grasses**
**(HPLC values)**

| Amounts in g/L | Miscanthus & Switchgrass | Miscanthus & Phragmites | Miscanthus, Switchgrass & Phragmites (A) | Miscanthus, Switchgrass & Phragmites (B) |
|---|---|---|---|---|
| Cellobiose | 7.35 | 10.34 | 8.41 | 9.56 |
| Glucose | 29.98 | 24.78 | 24.09 | 25.81 |
| Xylose | 8.67 | 7.23 | 7.45 | 8.35 |
| Arabinose | 1.02 | 1.02 | 0.95 | 1.15 |
| Acetic Acid | 2.35 | 2.4 | 1.88 | 2.23 |

The values are relatively consistent for all sugars except glucose. The higher glucose values for miscanthus and switchgrass are probably due to the slightly higher values for cellulose in switchgrass and miscanthus as compared to phragmites (Table 1-1).

Since these results remained consistent and hydrolysis inhibition did not seem to be occurring in the mixed grasses runs, mixtures of the grasses were used in the multi-day steady-state runs (Chapter 3). The January-February 2025 run used a switchgrass and phragmites mixture while the later runs used a switchgrass and miscanthus mixture.

## Section 1.3 Positive Synergism of Simultaneous Ball Milling and Enzyme Hydrolysis

Current research tends to show that biomass saccharification (sugar production) is initiated by enzyme activity at amorphous zones of cellulosic fibrils and then continues to crystalline cellulose zones[4]. The actual process is complex because of the presence of cellulose, hemicellulose, pectin, and lignin molecules that are often cross-linked (Figure 1-7).

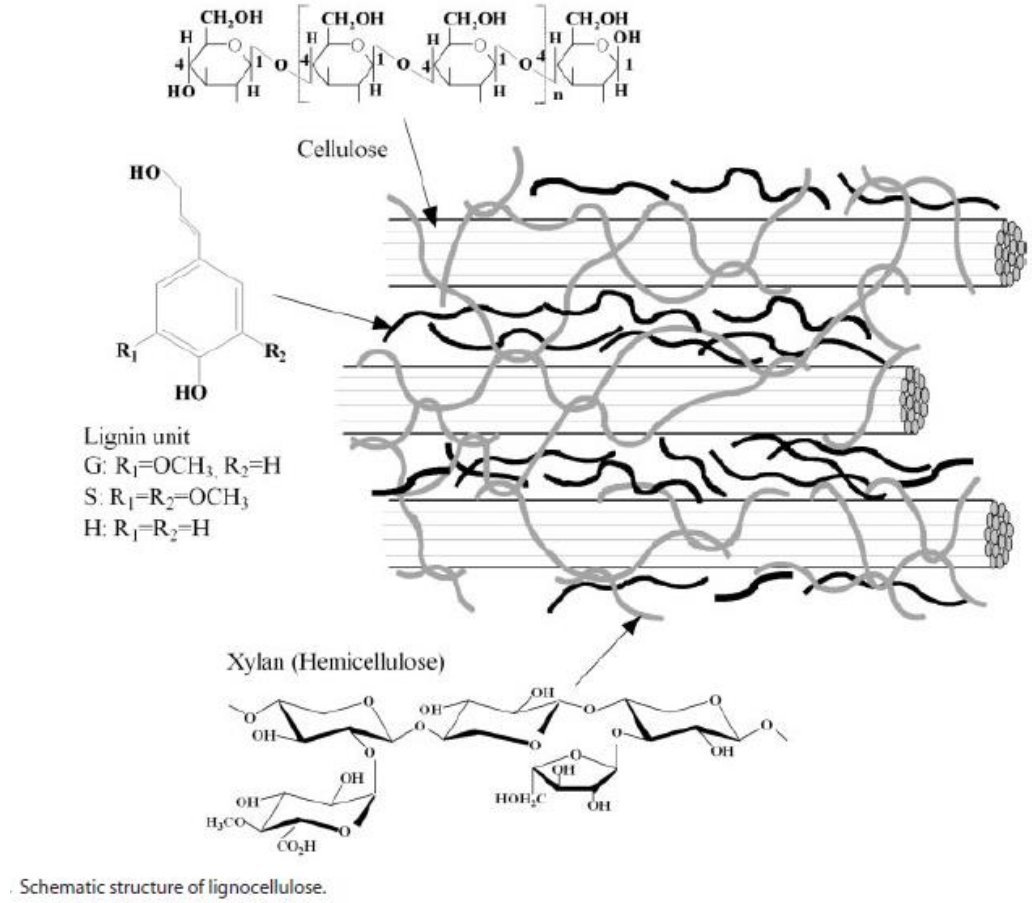



**Figure 1-7: Simplified Plant Wall Structure** [5]

The general process of enzymatic biomass deconstruction and saccharification involves enzymes from three different families.

- Endo-Glucanases: Breaks bonds between glucose residues in the cellulose backbone to allow enzyme processing from ends of smaller chains.
- Exo-Glucanases: Processes the smaller cellulose chains from the “broken” end into cellobiose.
- Glucosidases: Converts 2 glucose unit cellobiose into glucose monomers.

However, without pretreatment, mixtures of these enzymes produce low quantities of saccharified biomass. This can be explained by the limited number of amorphous access points available. Chemical and heat pretreatment generally increases conversion rates by increasing these access points.

Our earlier work has shown on a qualitative level that ball milling can also increase saccharification by mechanically reducing the size of biomass. Results with perennial grasses showed that ball milling decreased from the input size from 1”- 1.5” to sub-micron sizes for more than 90 percent of the biomass (Figure 1-8).

**Figure 1-8**
**Before and After Switchgrass Ball Milling & Enzyme Hydrolysis**

To measure the effectiveness of simultaneous ball milling with and enzyme hydrolysis, one of the first steps of the Phase I work program was to quantify saccharification with and without ball milling. This was done by processing the same quantities of perennial grass biomass, liquid and enzymes with different quantities of ball milling media for the same periods of time. The range of these runs was between 0% and 100% of ball milling weight as recommended by the milling equipment provider MSE Supplies. Specifics of these runs are shown in Table 1-7

1. Milling Ball Weight Distribution

A mixture of three stainless steel milling ball sizes was used; 10 mm, 20 mm, and 25 mm. The weight percent distribution is shown in Table 1-7. This distribution was maintained for all Phase I runs unless it was changed for specific testing conditions.

**Table 1-7**
**Milling Ball Weight Distribution**

| MSE 304SS Media Set | **Avg. per-ball wt. (g)** | **100% Avg.** |
|---|---|---|
| **Small: 10 mm** | | 785 |
| Small wt. (g) | 4.12 | 3,230 |
| % of total wt. | | 26% |
| **Medium: 20 mm** | | 195 |
| Medium wt. (g) | 33.12 | 6,459 |
| % of total wt. | | 53% |
| **Large: 25 mm** | | 40 |
| Large wt. (g) | 64.77 | 2,591 |
| % of total wt. | | 21% |
| | | |
| **Total # balls** | | 1,020 |
| Total Ball wt. (g) | | **12,280** |

2. Milling Containers

The milling runs for this task were performed in 5 liter MSE 304 grade stainless steel Roller Mill Jars (Figure 1-9). Two jars were run simultaneously on a Morse # 1-5154-3 stationary drum roller (Figure 1-10).

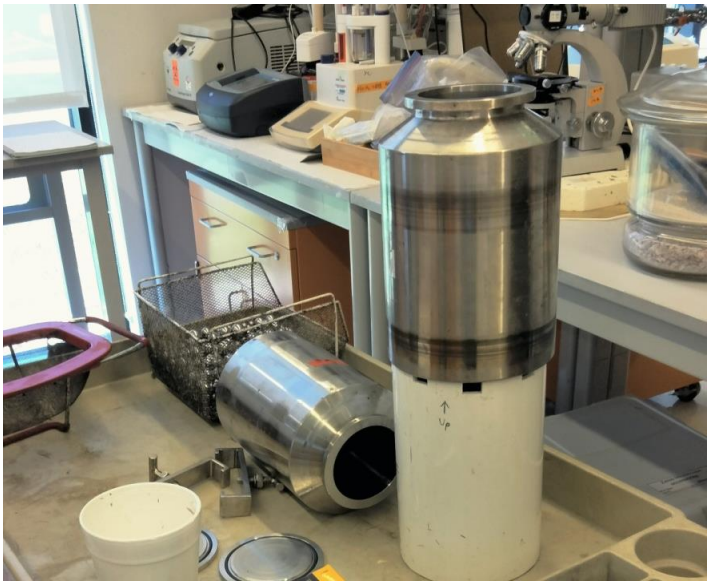


**Figure 1-9**
**5 liter MSE Roller Mill Jars Prepared for Loading**

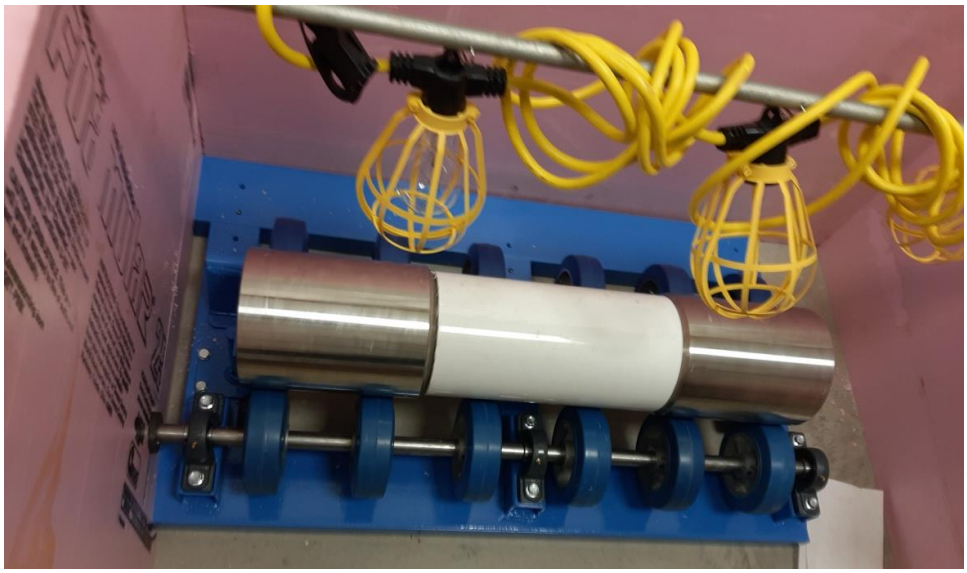


**Figure 1-10**
**MSE 5 liter Jars on Morse # 1-5154-3 stationary drum roller**
**Equipment provided by Atlantic Biomass, LLC**

3. Biomass Source/Loading Information

Switchgrass grown in Wapwallopen, Pennsylvania by Will Brandau, Chairman of the Association of Warm Season Grass Producers (AWSGP), was used for these differential ball milling runs. This biomass was harvested at the end of 2023 and was milled to approximately 1”-1.5” by a commercial bedding milling unit. The grasses were loaded at a ratio of 15% biomass: 85% water. This is the maximum biomass ratio that had been tested and maintained in earlier runs.

4. Enzyme Mixture

Novozyme CTec2, a mixture of cellulase enzymes and Novozyme Xyanase X2753, a hemicellulase enzyme mixture, were used. These enzymes have been cited in many published papers and are generally considered industry standards for comparing yields. Unfortunately, because of supply-chain issues, these enzymes were not available beginning in January 2025. We therefore had to search out alternatives and verify their performance for the remainder of Phase I (details in Section 1.7).

5. Hydrolysis Time Period

Milling times of 24 and 48 hours were tested to determine optimal time period and measure total potential sugar production.

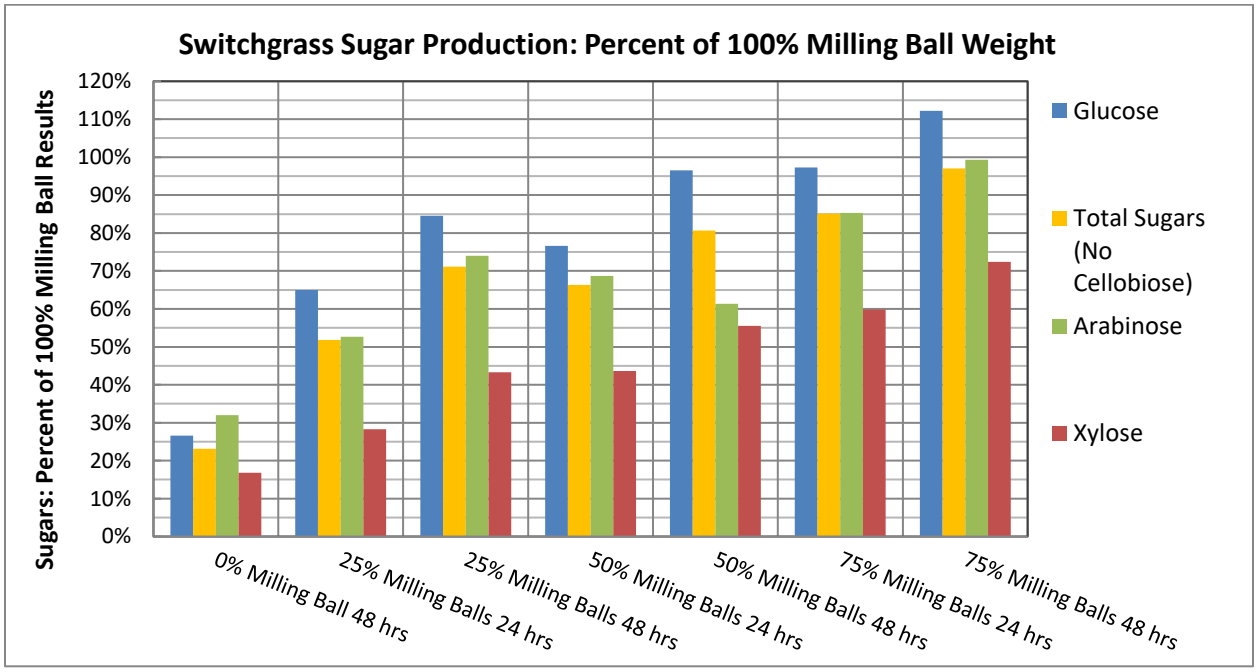
Results of these runs are compiled in Figure 1-11. Three primary findings were produced.

1. Zero Percent Milling Balls Produced Low Saccharification Results: Percent conversion of carbohydrates to sugars without ball milling after 48 hours were about 15 to 30 percent of the values obtained with enzyme hydrolysis and a 100 percent ball mill load for 24 hours.

2. 24 and 48 Hour Milling Results Had Significant Differences: For all different milling ball quantities, substantial increases for all sugars were recorded at 48 hours as compared to 24 hours.

3. Results Equal to 100 Percent Milling Balls were Only Obtained at 48 Hours: The only readings that matched the 100 percent milling ball results were the 75 percent 48 hour results for glucose, arabinose, and total sugars. The 24 hour results at 75 percent for all sugars were about 10-15 percent lower than the 48 hour results.

**Figure 1-11**




## Scanning Electron Microscopy (SEM) Images of Processed Switchgrass Biomass

The surface structure and morphology of switchgrass samples, both prior to and with different types of processing, were examined using a JCM-6000 benchtop scanning electron microscope (SEM; Model JCM 6000-OG-2, JEOL, Peabody, MA, USA). The purpose of this analysis was to see if changes in cell wall structures were different for the different processes. Small sections of each sample were mounted onto aluminum stubs using double-sided carbon adhesive tape and subsequently coated with a gold-palladium

(Au/Pd) alloy using a Cressington sputter coater (Ted Pella Inc., Redding, CA, USA). SEM images were acquired at an accelerating voltage of 15 kV, with magnifications ranging from 100× to 500×.

Switchgrass images included; (1-12a) unprocessed, (1-12b) processed with 0 percent milling balls and biomass enzymes, (1-12c) processed with 100 percent milling balls only, and (1-12d) processed with 100 percent milling balls and biomass enzymes.

**Figure 1-12a**
**Unprocessed Switchgrass**
Intact architecture with a
rigid, fibrillary surface morphology

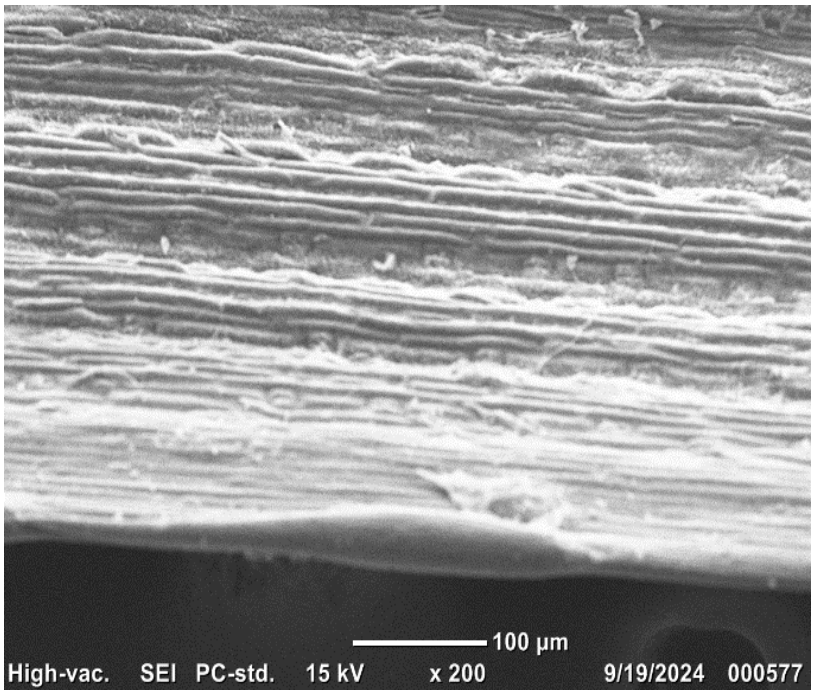


**Figure 1-12b**
**Enzyme Treatment Only**
Overall morphology remained largely similar to that of unprocessed switchgrass

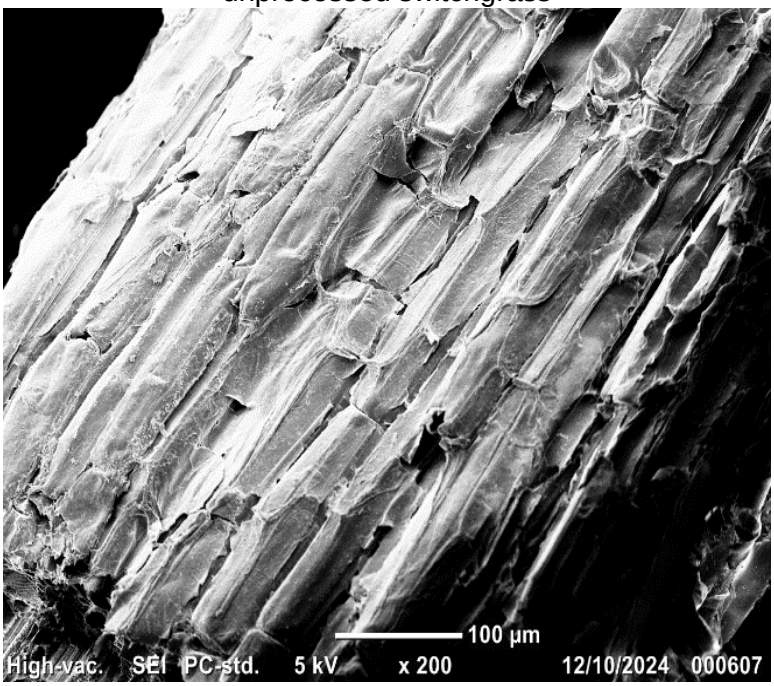



**Figure 1-12c**
**Ball Milling Only**
Mechanical pretreatment produced porous structures and disintegrated vascular tissues, facilitating increased enzymatic accessibility

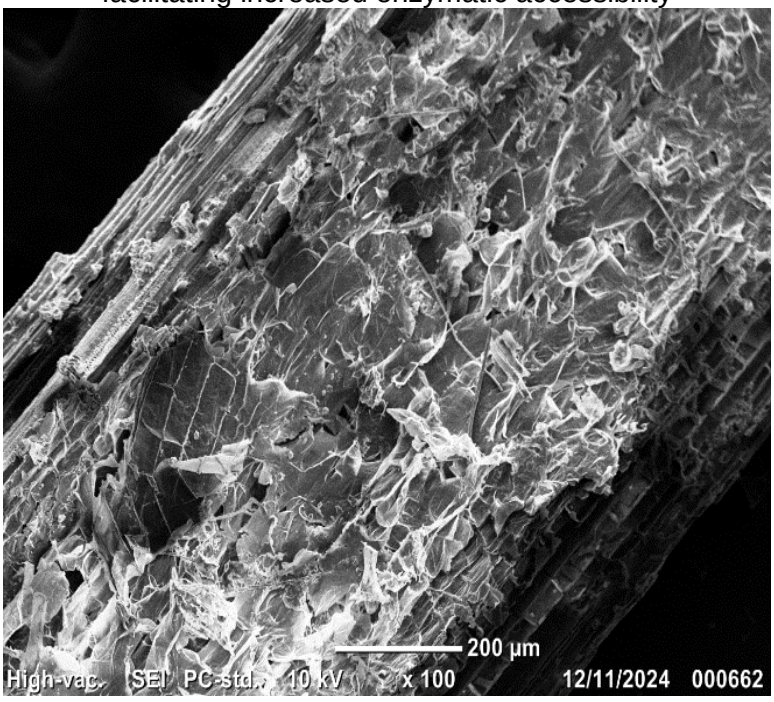
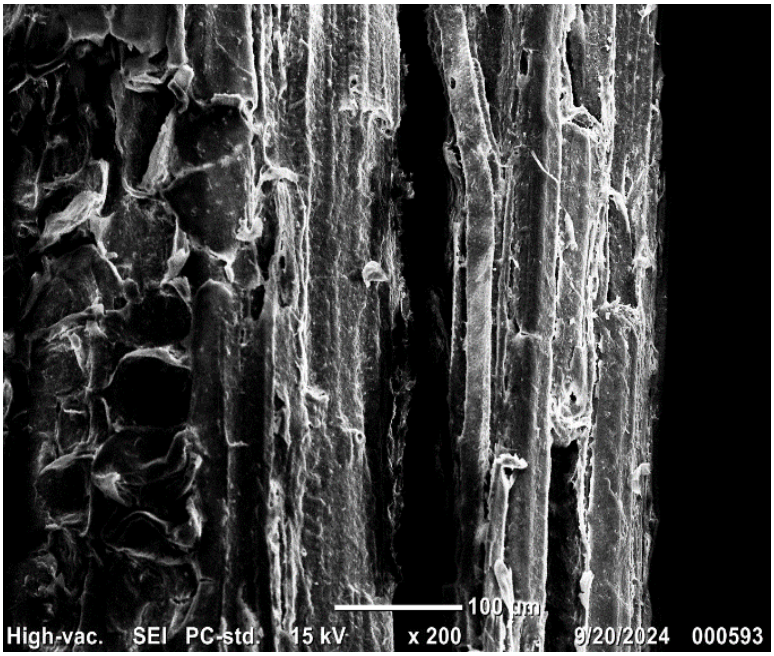


**Figure 1-12d**
**Ball Milling & Enzyme Hydrolysis**

SEM analysis corroborated enhancing substrate-enzyme interactions promoting efficient depolymerization into fermentable monomeric sugars. These visual results showed globular cell structures and reduced particle sizes—features indicative of increased enzymatic accessibility




The changes in the structures track with the differences in sugar production seen with 0 percent and 100 percent milling balls. Also, they show the increased efficiency (synergy) of simultaneous ball milling and enzyme hydrolysis (1-13c compared to 1-13d). From these results we reached two conclusions:

1. Ball milling added significant increases to carbohydrate hydrolysis sugar quantities, especially xylose: This probably indicates that ball milling mechanical deconstruction was sufficient enough to open up cross-linkages between cellulose and hemicellulose for enzyme access in a manner better than many heat or chemical pretreatments.

2. 100 percent of Milling Ball quantity was retained for the remainder of Phase I: This quantity of ball milling media provided significantly better results at 24 hours. Simply put, it reduces cost-effective hydrolysis processing time from 48 to 24 hours. Therefore it is also recommended as a starting point for commercial processing.

## Section 1.4 Ball Milling Speed

An important variable in ball milling operation is rotation speed. An ideal speed maintains a cascading motion of all the milling balls which uniformly reduces the biomass to the target size (Figure 1-13).

**Figure 1-13**
**Jar, Ball and Pebble Milling Theory and Practice**
**Operating Division of ER Advanced Ceramics, Inc.**

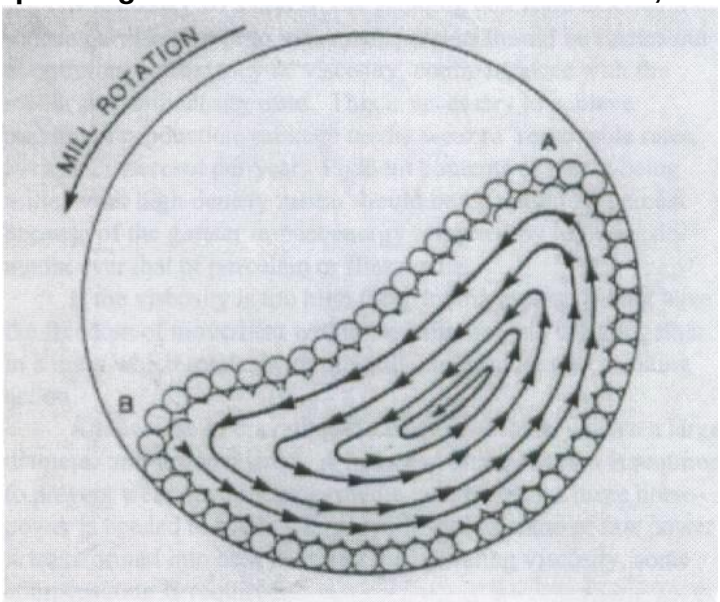



*FIG. 3- Showing grinding action of mill under cascading conditions with an angle of break of 50 degrees.*

When mills are rotated at too fast a rate centifuging will occur. Individual media are thrown clear of the media mass and move independantly until they rejoin the charge at the bottom of the mill. Unground material is held with the centrifuging balls and resulting in uneven disintegration or dispersion.

If too slow, not enough of the wet biomass is exposed to the milling balls to be reduced in size. If too fast, individual milling balls cling to the wall and do not break away from the mass due to centrifugal effects. This reduces overall milling effectiveness and also produces biomass with different sizes, some which are not conducive to enzyme access.

The basic equation for calculating rotational speed is NC (critical speed) = 54.2 / (square root of R) = 76.6 / (square root of D). R = inside radius and D = inside diameters. All measurements are in feet.

Different rotational speeds (percentage of NC) are required for wet and dry milling and for different materials to be milled. Generally for wet milling, speeds equal to 60% NC ± 5% produce optimal results.

To determine the effects of rotational speed for perennial grass hydrolysis, runs were conducted at NC = 46% and NC = 60%. To assess the effects of different grass compositions, one run used switchgrass as the feedstock and the other miscanthus. While 46 percent is considerably lower than the generally accepted values of about 60%, it was tested to see if lower energy use could produce close to optimal results.

Milling efficiency was measured by glucose production from cellulose enzyme hydrolysis during ball milling. As shown in Table 1-8, there were significant increases in glucose production from cellulose in both grass samples run at NC= 60% as compared to those run at NC=46%. The differences between miscanthus and switchgrass results were caused by different cellulose concentrations in their biomass and differences in total biomass loadings.

**Table 1-8**
**Miscanthus and Switchgrass Milling at NC=46% and NC=60%**

| | Net Glucose | **Increase (g/L)** | **Increase %** |
|---|---|---|---|
| Miscanthus: 45% Cellulose: 12% Biomass/88% Fluid Loading | | | |
| Miscanthus NC=46% rpm 24h Wooster OSU | 7.70 | | |
| **Miscanthus NC=60% rpm 24h Wooster OSU** | **34.25** | **26.55** | **345%** |
| | | | |
| Switchgrass: 31% Cellulose: 15% Biomass/85% Fluid Loading | | | |
| Switchgrass NC=46% rpm 24h Wooster OSU | 16.18 | | |
| **Switchgrass NC=60% rpm 24h Wooster OSU** | **42.90** | **26.72** | **165%** |

Based on these results, RPMs in the NC=60% range were selected for use in both the MSE 5 liter vessels and later in the 2 ft.$^3$ Orbis commercial milling unit.

## 1.5 Maximizing Removal of Slurry after Hydrolysis

Because of the high viscosity of the sugar containing post-hydrolysis slurry, some of it sticks to the milling balls and is difficult to recover when the slurry is poured out. Use of water is not a solution since this would dilute the sugar solution and increase fermentation volume. This needed to be improved to reach commercially acceptable sugar recovery

rates and concentrations. Initial, pre-Phase I, attempts to improve slurry removal involved blowing compressed air into the 5 liter milling containers (Figure 1-14).

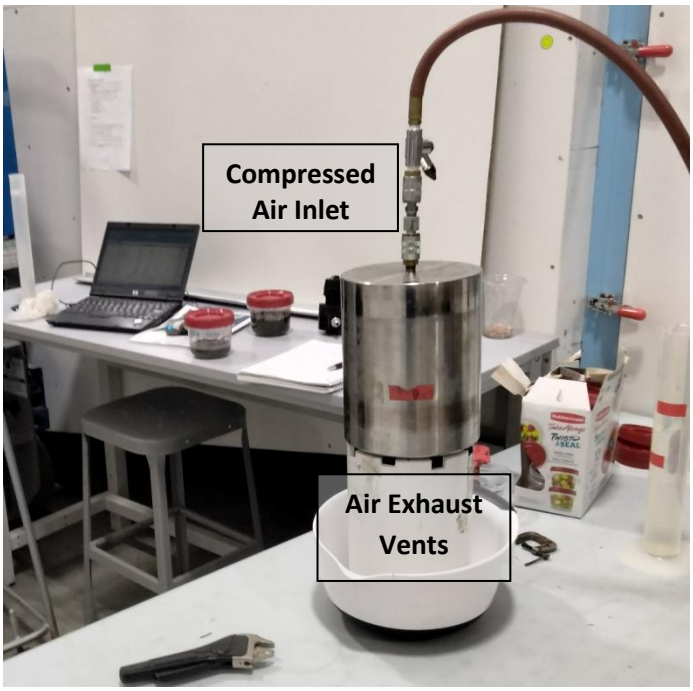



**Figure 1-14**

However, this made slurry recovery worse. The room temperature compressed air evaporated liquid inside the containers leaving the milling balls covered in dry slurry (Figure 1-15).

**Figure 1-15**

Our solution was to heat the air to the same temperature as the experiment and saturate it at that temperature with water. This prevented evaporation of the slurry. Preliminary runs were promising (Figure 1-16) but much work was needed to make the process operational.


**Figure 1-16**

Phase I STTR Work

Both air pressure and water temperature had to be controlled to make this process work. In Phase I the purchase of a 1-gallon capacity high pressure vessel (maximum pressure 100 psi) from Sigma-Aldrich (Figure 1-17) provided us with a controlled method to add varying amounts of heated water to compressed air to further the development of our slurry removal process.

**Figure 1-17**

Since the lab provided by OSU/Wooster did not have a compressed air system, air pressure up to 150 psi was provided by a portable air compressor (Figure 1-18) while water heating was provided by partially submerging the pressure vessel in a water bath (Figure 1-19). Two air compressors (the first one did not provide enough pressurized capacity) and the pressure vessel were purchased with DOE STTR Phase I funds.

**Figure 1-18**
**Large Capacity Air Compressor**

Test runs were hampered by water back flow out of the pressure vessel siphon inlet tube into the air compressor storage tank. This was corrected by mounting a control valve in the input line at the pressure vessel (Figure 1-19). Modulating this valve and the air pressure valve on the air compressor controlled input water/air pressure while also providing back flow control.

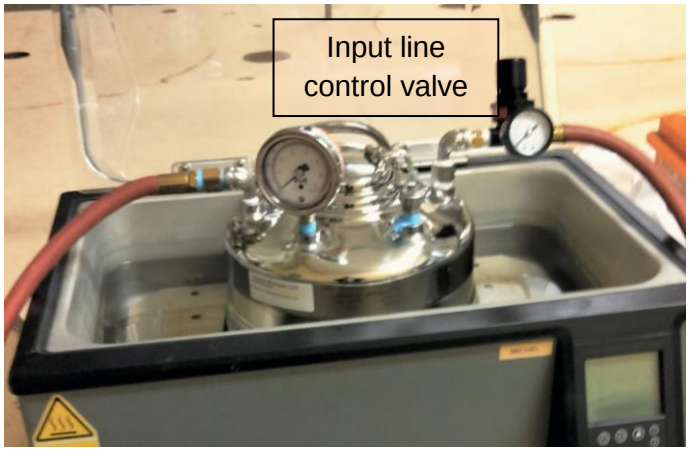



**Figure 1-19**
**In-Line Air Pressure Valve and Pressure Vessel in Water Bath**

Effectiveness of the pressurized water vapor/air system to remove sugar containing slurry was measured in two ways.

1. Comparing the slurry sugar concentrations before and after the compressed air/water system was turned on. Lower sugar concentrations in the slurry that had been treated with the compressed air/water showed dilution of the slurry.
2. Comparing the slurry quantity recovered with that put in the milling vessel produced a percent recovery value.

For commercial applications, final sugar concentrations should be very near to pre-removal levels (low dilution) while slurry recovery values should be over 90 percent.

Slurry Sugar Concentration Results

The runs used to measure ball milling effectiveness (Section 1.2) were also used as test runs for this system. Initially compressed air/water was run for up to 30 minutes. The higher volume compressor allowed higher pressure for longer period which reduced compressed mixture runs to about 15 minutes.

Table 1-9 summarizes the before and after compressed air/water slurry results.

**Table 1-9**
**Slurry Dilution due to Compressed Air/Water recovery from Ball Milling**

| 10/23-25/24 | **0% Milling Balls with Enzymes** | | |
|---|---|---|---|
| Sample A | T48 Before Slurry Removal | T48 After Air/Water Slurry Removal | % Lost/Gained |
| Total Sugars | 12.24 | 11.90 | -2.8% |
| Glucose | 8.96 | 8.66 | -3.3% |
| Arabinose | 0.48 | 0.46 | -4.2% |
| Xylose | 2.80 | 2.78 | -0.7% |

| 10/2-3/2024 | **25% Milling Balls by Wgt.** | | |
|---|---|---|---|
| Sample A T48 | T48 Before Slurry Removal | T48 After Air/Water Slurry Removal | % Lost/Gained |
| Total Sugars | 37.68 | 35.96 | -4.6% |
| Glucose | 28.52 | 27.62 | -3.2% |
| Arabinose | 1.11 | 0.86 | -22.5% |
| Xylose | 7.22 | 6.86 | -5.0% |

| 10/2-3/2024 | **50% Milling Balls by Wgt.** | | |
|---|---|---|---|
| Sample B | T48 Before Slurry Removal | T48 After Air/Water Slurry Removal | % Lost/Gained |
| Total Sugars | 42.73 | 42.10 | -1.5% |
| Glucose | 32.55 | 32.03 | -1.6% |
| Arabinose | 0.92 | 0.91 | -1.1% |
| Xylose | 9.26 | 9.16 | -1.1% |

| 10/22/2024 | **65% Milling Balls by Wgt.** | | |
|---|---|---|---|
| Sample B | T48 Before Slurry Removal | T48 After Air/Water Slurry Removal | % Lost/Gained |
| Total Sugars | 54.25 | 54.99 | 1.4% |
| Glucose | 42.22 | 42.83 | 1.4% |
| Arabinose | 1.06 | 1.07 | 0.9% |
| Xylose | 10.97 | 11.09 | 1.1% |

Except for the arabinose values in the 25 percent milling ball sample, where the high dilution percent was probably caused by the relatively low concentrations of arabinose, dilution values were 5 percent or less. This indicates that the process works with commercially acceptable values and that the major water and air temperature and flow issues have been resolved.

Slurry Recovery Results

A representative slurry recovery run was completed on 16 May 2025. Two samples of 75 dry grams of switchgrass was ball milled and hydrolyzed with 500 ml of total fluid. As shown in Figure 1-20, slurry recovered was over 400 ml in both samples. Percentage slurry recovery is shown in Table 1-10.


**Figure 1-20**

**Sample A** **Sample B**

**Table 1-10**
**Estimated Slurry Recovery**

| Sample | Input Liquids Biomass & Fluids (ml) | | Recovered Slurry (ml) | % Recovered |
|---|---|---|---|---|
| A | 500 | | 450 | 90% |
| B | 500 | | 410 | 82% |

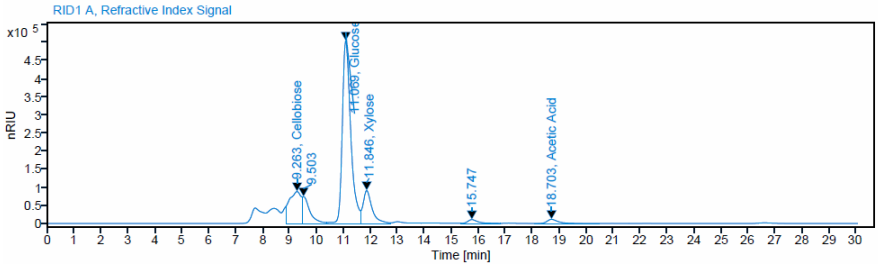
While the switchgrass input was the same in both samples, different enzyme mixtures were used. As a result, sugar production was higher in Sample A (Figure 21). This higher monomeric sugar production lowered the viscosity of the slurry which probably led to the higher slurry recovery rate. This sugar production seen in Sample A is in the commercially acceptable range which shows that a 90 percent slurry recovery rate and low sugar dilution is possible with this system.

**Figure 1-21**
**HPLC 16 May 2025 Reports**
**Sample A**

**Sequence Summary Report**
T48 Original ALI Run 5.16.25 2025-05-16 15-32-23

Agilent

| | | | |
|---|---|---|---|
| **Sample name:** | Sample A SWG T48 ALI 5.16.25 | | |
| **Injection date:** | 2025-05-16 15:33:32-04:00 | **Sample type:** | Sample |
| **Acq. method:** | Hydrolysis Fixed Eth6 5.2.25<br>10 ul 30 min.M | **Location:** | 2 |
| **Analysis method:** | Hydrolysis Fixed Eth6 5.2.25<br>10 ul 30 min.M | **Injection:** | 1 of 1 |
| **Acq. operator:** | SYSTEM | **Injection volume:** | 10.000 |
| **Last changed:** | 2025-05-16 11:44:32-04:00 | | |

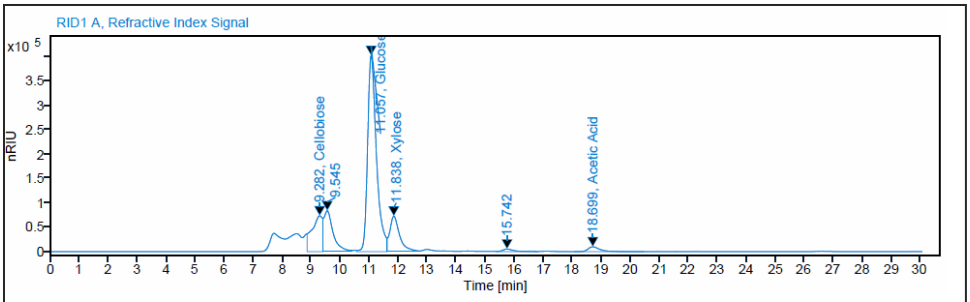



**ESTD report based on Area**

| RT [min] | Signal | Type | Area | Height | Amount [g/l] | Name |
|---|---|---|---|---|---|---|
| 9.263 | RID1A | BV | 2694814.25000 | 88455.5313 | 15.02673 | Cellobiose |
| 11.069 | RID1A | VV | 10938994.00000 | 505535.0625 | 60.39446 | Glucose |
| 11.846 | RID1A | VV | 2165314.00000 | 91198.2031 | 12.26515 | Xylose |
| 18.703 | RID1A | VV | 345628.46875 | 11540.4922 | 4.19999 | Acetic Acid |

**Sample B**

| | | | |
|---|---|---|---|
| **Sample name:** | Sample B SWG T48 ALI 5.16.25 | | |
| **Injection date:** | 2025-05-16 16:04:45-04:00 | **Sample type:** | Sample |
| **Acq. method:** | Hydrolysis Fixed Eth6 5.2.25<br>10 ul 30 min.M | **Location:** | 3 |
| **Analysis method:** | Hydrolysis Fixed Eth6 5.2.25<br>10 ul 30 min.M | **Injection:** | 1 of 1 |
| **Acq. operator:** | SYSTEM | **Injection volume:** | 10.000 |
| **Last changed:** | 2025-05-16 11:44:32-04:00 | | |




**ESTD report based on Area**

| RT [min] | Signal | Type | Area | Height | Amount [g/l] | Name |
|---|---|---|---|---|---|---|
| 9.282 | RID1A | BV | 1827216.75000 | 73442.5703 | 10.18887 | Cellobiose |
| 11.057 | RID1A | VV | 8505039.00000 | 402759.5625 | 46.95653 | Glucose |
| 11.838 | RID1A | VV | 1720221.50000 | 72894.5625 | 9.74398 | Xylose |
| 18.699 | RID1A | VV | 286873.12500 | 10001.0313 | 3.48601 | Acetic Acid |

The following three photos in Figures 1-22 show the progress we have made in removing the slurry with monomeric sugars from the milling balls. Patent applicability of this process is being pursued.

**Figure 1-22a**
**Post Hydrolysis Milling Balls: No Slurry Removal**

**Figure 1-22b**
**After Compressed Air Treatment Only**

**Figure 1-22c**
**After Pressurized Air/Water Treatment**

## Section 1.6 Reducing the Weight of Ball Milling Vessels

Industrial ball milling units are built to heavy-duty standards. As seen in this 2 ft.$^3$ ball milling vessel built by Orbis Machinery (Figure 1-23), walls of the loading port continue with the 12+mm steel wall construction of the ball milling cylinder.

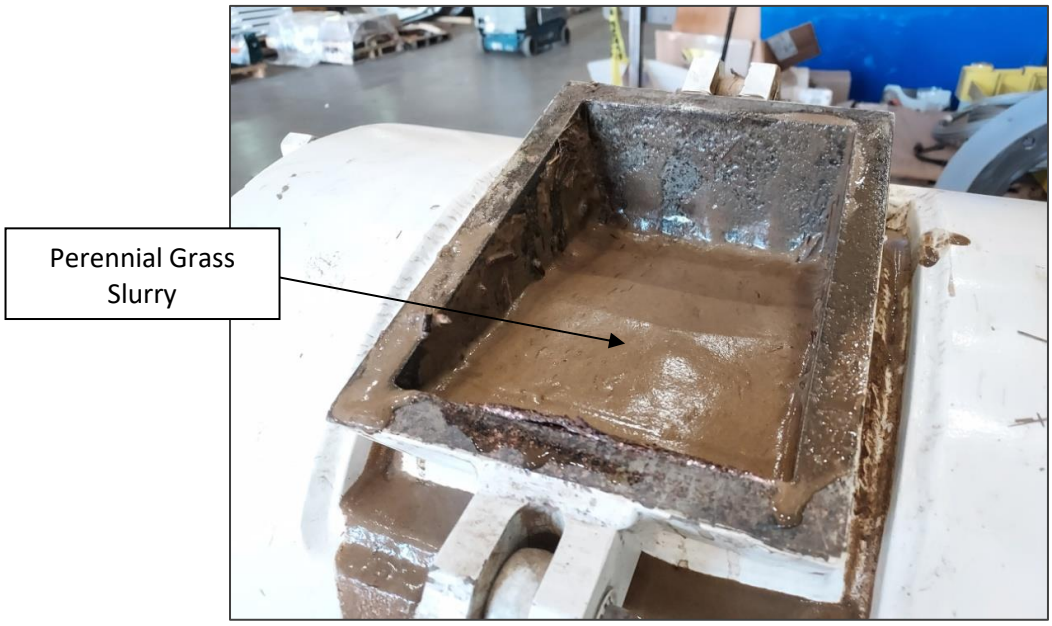



**Figure 1-23**
**Orbis Ball Mill Access Port**

The 5 liter MSE ball milling vessels used in Phase I also use this design approach. The 304 stainless steel walls are 3 mm in thickness (Figure1-24) and the empty weight of the vessel is about 5 kg. We have been using the MSE 5 liter cylinders since 2021 and have not seen any dings in the interior cylinders walls.

**Figure 1-24**

The reason for this heavy duty design and construction is that these mills are designed to absorb pressures from milling high density ores or industrial gravels for a number of years. In our use, the biomass provides much lower tumbling object density which leads to the hypothesis that thinner walls could be used for biomass while maintaining effective milling and minimal cylinder wall damage.

While the weight of these cylinders is not an issue in stationary applications, it could become an issue for the transport of portable units to forward locations and for portable operations in areas with roads not suited to heavy-weight vehicles. For example the small 2 $ft.^3$ Orbis cylinder weighs about 347 lbs (Orbis provided information) and a $50ft.^3$ commercial cylinder for Phase II scale operations would be much higher. We have been working with several partners who are interested in portable and/or forward deployed units and as a result did some testing with thin-walled ball milling cylinders.

The testing was done in 304 stainless steel 5 liter milk cans. These cans had walls .7mm thick and weighed approximately 1.2 kg. They were modified with nut and bolt cap attachments and quick-release air pressure valves for slurry removal (Figure 1-25).


**Figure 1-25**

They were tested side-by-side with the MSE milling cylinders because the milk cans had similar dimensions as the MSE milling cylinders (Figure 1-26). One such test run was the 75% ball milling weight run. The slower speed, NC=46%, run results for the 5 liter milk can and the 5 liter MSE cylinder are in Table 1-11. The sugar concentration values are within $\pm$ 10% for the two cylinders which indicates that the simultaneous ball milling and

enzyme hydrolysis processes were proceeding in a similar manner in the similar sized and shaped cylinders.

**Figure 1-26**
**MSE 5 Liter Cylinder (L) 5 Liter Milk Can (R)**

**Table 1-11**
**Comparison of Thin Walled Milk Can and Thick Walled MSE Cylinder Sugar Production**
(Sugars are Measured by HPLC Analysis)

| | **5 Liter 304 Milk Can** Sample B 75% Milling Media T22 **NC =46%** | **5 Liter 304 MSE** Sample B 75% Milling Media T48 10/16-17/24 **NC=46%** | Milk Can % of MSE |
|---|---|---|---|
| | g/L | g/L | |
| Total Fermentable Sugars | 40.53 | 43.98 | 92.2% |
| Glucose | 31.60 | 34.58 | 91.4% |
| Arabinose | 1.42 | 1.37 | 103.6% |
| Xylose | 7.51 | 8.03 | 93.5% |

Some durability runs up to 72 hours were completed. Figure 1-27 shows the milk cans after two sets of 48 hour test runs and one, 72 hour durability run that had 100% milling balls to simulate the effects of a longer runs.

While not enough runs were conducted to access long-term durability, the condition of these cans show that these and probably larger versions of the same design can at least

be used for prototype testing and short term installations. It also shows that additional testing should be pursued to determine if the Phase II prototypes 50 $ft^3$ and 100 $ft^3$ ball milling units could use thinner, lighter weight and lower cost milling cylinder walls.


**Figure 1-27**
**5 Liter Milk Cans after Durability Runs**

For a cost comparison, the 5 liter milk can lists for $49.99 and a 304 stainless steel 40 liter (1.4 $ft.^3$) milk can lists for $249. The 5 liter MSE ball milling cylinder currently lists for $1,267.95. The 40 liter milk container could be run on our Morse 1-5154-3 stationary drum roller for scale-up testing.

## Section 1.7 Establishing Baseline Enzyme Performance

### Current State of Biofuel/Bioproduct Enzyme Availability

The key enzyme accessibility assumption made in July 2024 at the beginning of this Phase I project was that Novozyme enzymes CTec2 and HTech2 would be commercially available during the Phase I research period.

In the early part of this century Novozyme, a division of Novo Nordisk (a large Norwegian based pharmaceutical company) was awarded two contracts by US DOE for the development of new biomass hydrolysis enzymes - $13.8 million in 2001-2005, and $12.3 million in 2008-2012. With the US DOE grants Novozyme developed CTec and HTec enzymes that showed better activity than wild type enzyme mixtures. CTec2 became the

standard for cellulose to glucose enzyme activity measurements and is used in most published reports. Hence, they provide baseline results that enable researchers to compare results without having to account for enzyme performance difference.

However, significant changes happened to their enzyme availability in 2024.

On the business side, Novozyme was spun off by Novo Nordisk (which is now focused on Ozempic, Wegovy, and related drugs) in 2024 to a new company called Novonesis. On their website (https://www.novonesis.com/en), the company does not show a focus on biofuel/bioproduct enzymes and does not offer CTec2 for sale. Only the more expensive CTec3 is offered under special conditions.

As for the CTec2 and HTec2 enzymes, they are offered for sale by biologic supply companies such as Sigma-Aldrich. However, because of supply-chain issues, delivery times in 2024 were first extended to over three months. Then, beginning in October 2024, the enzymes were not available. This continued until the end of the Phase I research program in July 2025. We were forced to find and then test alternative commercial enzymes.

We eventually settled on cellulases and hemicellulases from American Laboratories, LLC a provider of animal feed grade enzymes. This decision was made on the basis of availability rather than quality. Three issues had to be resolved before using these enzymes in test runs:

- Background Sugars in Enzyme Mixtures
- Enzyme Activity Compared to CTec2
- Contamination in Enzyme Packing

Background Sugars in Enzyme Mixtures

All commercial enzymes are delivered in liquids or solid packing materials. The first step in our test runs was to extract a sample of enzymes and fluids before the test biomass is loaded to determine the background sugar content that would have to be subtracted from final HPLC readings. These were called T0 (time zero) samples. There was considerable difference between the CTEc2 and ALI enzymes. The following three TO HPLC reports are of; CTec2 cellulase, CTec2 cellulase and 150 ALI hemicellulase, and ALI 400 cellulase and ALI 150 hemicellulase. The background glucose values range from 4.99 g/L to 18.45. Similar increases are seen in xylose and cellobiose values (Figures 1-28a, 1-28b, and 1-28c).

**Figure 1-28a**
**CTec2 T0 Background Sugar Values**

## Sequence Summary Report (Extended)

8.5.25 T0 Cellulases 2025-08-05 14-46-00

Agilent

| | | | |
|---|---|---|---|
| **Data file:** | C:\USERS\PUBLIC\DOCUMENTS\CHEMSTATION\1\DATA\8.5.25 T0 CELLULASES 2025-08-05 14-46-00\T0 12 ml CTec2 Cellulase 8.5.25 2025-08-.D | | |
| **Sample name:** | T0 12 ml CTec2 Cellulase 8.5.25 | | |
| **Injection date:** | 2025-08-05 15:20:27-04:00 | **Sample type:** | Sample |
| **Acq. method:** | Hydrolysis Fixed Eth7 6.30.25 10 ul 30 min.M | **Location:** | 3 |
| **Analysis method:** | Hydrolysis Fixed Eth7 6.30.25 10 ul 30 min.M | **Injection:** | 1 of 1 |
| **Acq. operator:** | SYSTEM | **Injection volume:** | 10.000 |
| **Last changed:** | 2025-08-05 13:13:39-04:00 | | |

**ESTD report based on Area**

| RT [min] | Signal | Type | Area | Height | Amount [g/l] | Name |
|---|---|---|---|---|---|---|
| 9.184 | RID1A | MM | 261064.21875 | 11577.8701 | 1.45574 | Cellobiose |
| 11.175 | RID1A | MM | 905105.31250 | 49770.9219 | 4.99711 | Glucose |

**Figure 1-28b**
**CTec2 & ALI 150 Hemicellulase T0 Background Sugar Values**

## Calibration Report

CTec2/ALI 150 1:83 3.13.25

Agilent

**Percent report based on Area**

| Name | RT [min] | Area | Height | RF | Amount [g/l] |
|---|---|---|---|---|---|
| Glucose | 10.575 | 2808670.5000 | 160511.4531 | 0.00001 | 15.071 |
| Xylose | 11.508 | 89457.0391 | 2371.6475 | 0.00000 | 0.261 |

**Figure 1-28c**
**ALI 400 Cellulase & ALI 150 Hemicellulase T0 Background Sugar Values**

## Calibration Report

Day-1 Background 9.8.25

**Percent report based on Area**

| Name | RT [min] | Area | Height | RF | Amount [g/l] |
|---|---|---|---|---|---|
| Cellobiose | 8.994 | 2337802.7500 | 118085.3047 | 0.00001 | 12.604 |
| Glucose | 10.746 | 3309272.7500 | 176727.5313 | 0.00001 | 18.435 |
| Xylose | 11.451 | 68346.5313 | 3337.7031 | 0.00000 | 0.133 |
| Ethanol | 25.756 | 276789.0938 | 1515.0922 | 0.00001 | 2.714 |

The significantly higher background sugar values in the American Laboratories enzymes are to be expected given the relative low density of enzymes in the delivered ALI mixtures. For example, the CTec2 cellulase mixture is listed by Sigma-Aldrich as being essentially saturated, 1,000 mg/ml while the ALI 400 cellulase mixture was calculated to have 245.1 mg/g of enzyme (24.5%) and the ALI hemicellulase mixture has 75.1 mg/g (7.5%) of the enzyme mixture.

## ALI Cellulase Activity Compared to CTec2

We had conserved enough CTec2 enzyme to be able to use it as a control in some key test runs including multi-day steady-state runs (Details in Chapter 3). This allowed us to determine the ALI dosages needed achieve cellulose to glucose results comparable to CTec2 results. As shown in Table 1-12 we determined an ALI cellulase dosage that produced both similar enzyme loading (mg/g) for both cellulose (row 7) and total biomass (row 8) as well as similar cellulose to glucose conversion percentages (row 9).

**Table 1-12**
**Cellulase Activity Comparison**

| Ball Milling/Enzyme Run | Orbis 2ft$^3$ with ALI Cellulase | Steady-State 2: 5 Liter with CTec2 |
|---|---|---|
| 1. ALI Cellulase (g) Added | 348 | |
| 2. CTec2 Cellulase (ml) added | | 12 |
| 3. Switchgrass/Miscanthus Biomass (g) | 2691 | 400 |
| | | |
| 4. CTec2 Enzyme mg/ml enzyme liquid | | 1,000 |
| 5. ALI cellulase mg/g enzyme powder | 245.1 | |
| 6. Total enzyme (mg) | 85,295 | 12,000 |
| 7. Enzyme mg/g biomass | **32** | **30** |
| 8. Enzyme mg/g cellulose | **79.2** | **75.0** |
| | | |
| 9. % Cellulose to Glucose Conversion | **48.6%** | **46.7%** |

With these calculations we will be able to equate our ALI results to published CTec2 results.

### Contamination in Enzyme Packing

As shown in Figure 1-28C above and in Table 1-13, the enzyme preparations used were contaminated by ethanol producing microorganisms. The addition of ALI enzymes packed in calcium carbonate without the addition of any ethanol producing organisms contained ethanol. Note also that ethanol production occurred in both switchgrass and miscanthus. While in

terms of the overall objective of producing ethanol this might not be a negative development, it is a negative development in that microbial contamination might lead to other production issues such as enzyme interference during storage. And so this effect, in addition to performance issues and costs, calls for the development of microbial and glucose contaminant free cellulase and hemicellulase enzyme preparations by Atlantic Biomass (see below).

**Table 1-13**
**Comparison of Ethanol Production from ALI Enzymes**

| ALI Enzymes in Maltodextrin (HPLC Results in g/L) | | | | ALI Enzymes in Calcium Carbonate (HPLC Results g/L) | | |
|---|---|---|---|---|---|---|
| Date | Sample | Ethanol (g/L) | | Date | Sample | Ethanol (g/L) |
| 5.15.25 | Sample A Switchgrass T24 | 0 | | 5.13.25 | Sample A Miscanthus New ALI T24 hrs. | **0** |
| 5.16.25 | Sample A Switchgrass T48 hrs. | 0 | | 5.14.25 | Sample A Miscanthus New ALI T48 hrs. | **4.717** |
| 5.15.25 | Sample B Switchgrass T24 hrs. | 0 | | 5.13.25 | Sample B Switchgrass New ALI T24 hrs. | **0** |
| 5.16.25 | Sample B Switchgrass T48 hrs. | 0 | | 5.14.25 | Sample B Switchgrass New ALI T48 hrs. | **5.346** |
| 5.20.25 | Sample A Switchgrass T24 hrs. | 0 | | 5.20.25 | Sample B Switchgrass New ALI T24 hrs. | **2.935** |
| 5.21.25 | Sample A Switchgrass T48 hrs. | 0 | | 5.21.25 | Sample B Switchgrass New ALI T48 hrs. | **7.671** |

### Use of Pectinase Enzymes

Pectin is another cell wall carbohydrate. It is hydrolyzed into galacturonic acid, a 6-carbon fermentable sugar. We have produced a thermophillic pectin methylesterase enzyme (PME)[6], and saw good conversion rates to galacturonic acid and significant increases in overall fermentable sugar production when it was used with hemp and other biomass feedstocks. It was hoped that similar results would happen with perennial grasses.

In the absence of published data on the percentage of pectin in perennial grasses, we ran a series of runs with and without pectinases to determine potential galacturonic production. As shown in rows 4-7 of Table 1-14, galacturonic acid concentrations only added about 3-5 percent to total fermentable sugar production. Simply put, perennial

grass stems and leaves have low pectin content. Currently it is not cost-effective to try to produce these minimal fermentable sugar increases.

**Table 1-14**
**Galacturonic Acid Production with Different Enzyme Mixtures**

| **All Samples 75 g Switchgrass** | **All HPLC Values in g/L** | | | | | |
|---|---|---|---|---|---|---|
| **Sample** | **Gal Acid** | **Glucose** | **Xylose** | **Arabinose** | Total Sugars | Gal-Acid % Total Sugars |
| 1) T24 Cellulase only 12/12/24 | | 27.01 | 11.39 | 0.79 | 39.19 | |
| 2) T24 Hemicellulase only 12/12/24 | | 17.06 | 3.82 | 0.68 | 21.55 | |
| 3) T24 Pectinase only 12/17/24 | 1.15 | 4.07 | 1.35 | 0.37 | 6.95 | 16.6% |
| 4) T24 Cellulase + Hemicellulase 12/17/24 | | 34.39 | 11.37 | 1.16 | **46.92** | |
| 5) T24 Cellulase + Pectinase 12/19/24 | 1.26 | 28.90 | 9.60 | 1.37 | 41.13 | **3.1%** |
| 6) T24 Hemicellulase + Pectinase 1 2/19/24 | 1.19 | 17.12 | 3.47 | 0.59 | 22.36 | **5.3%** |
| 7) T24 Cellulase+ Hemicellulase +Pectinase 1/3/25 | **1.36** | 29.017 | 10.85 | 1.7 | **42.93** | **3.2%** |
| 8) T24 Cellulase+ Hemicellulase 1/3/25 | 0 | 27.797 | 10.889 | 1.556 | **40.242** | |

## Baseline Carbohydrate to Fermentable Sugar Conversion Ratios

Sugars suitable for fermentation are referred to as simple sugars or monomeric sugars. This means they are one unit of a complex carbohydrate that provides the structure of plant cell walls.

In grasses, with the low concentration of pectin, the two primary structural carbohydrates are cellulose and hemicellulose. Table 1-15 shows the monomeric forms and the number of carbon atoms in the monomeric forms.

**Table 1-15**

| Carbohydrate Type | Number of Carbon Atoms in Monomeric Ring | Monomeric Forms |
|---|---|---|
| Cellulose | 6 | Glucose |
| Hemicellulose | 5 | Xylose, Arabinose |

Conversion rates of over 70 percent to fermentable sugars have been reported without pretreatment for crop carbohydrate residues such as soybean hulls and sugar beets (Kim 2025). However conversion rates for crops such as perennial grasses that have not been pretreated are low (~20%) due the complexity of the non-processed biomass[7]. In order to be commercially successful, perennial grass conversion rates need to exceed 70 percent and ideally reach greater than 90%.

As detailed in section 1.3 above, grass to monomeric sugar enzyme hydrolysis involves enzymes from three different families each with different tasks.

- Endo-Glucanases: Breaks bonds between glucose residues in the cellulose backbone to allow enzyme processing from ends of smaller chains.
- Exo-Glucanases: Processes oligomeric glucose chains from the “broken” end into cellobiose.
- Glucosidases: Converts cellobiose (composed of two glucose molecules) into glucose monomer.

If the process “stalls out” at any of the steps, monomeric fermentable sugar production does not reach the maximum possible glucose concentrations.

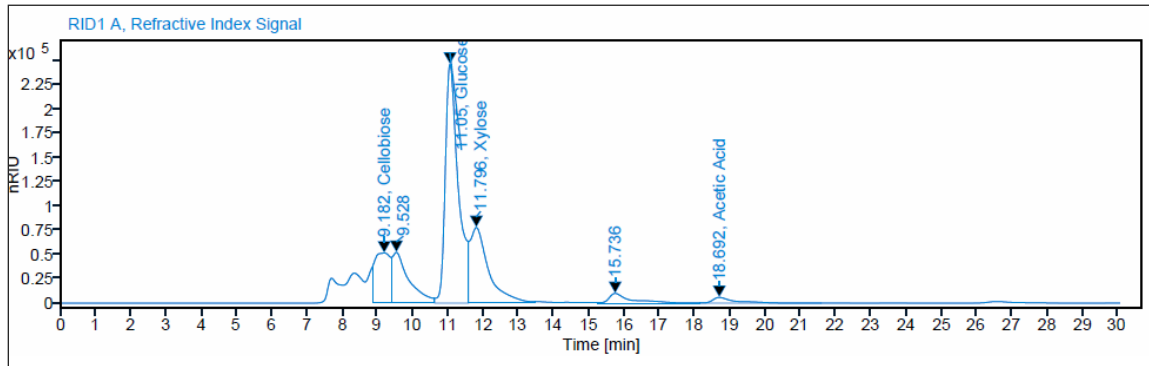
In Phase I enzyme performance tests, post hydrolysis HPLC results showed both glucose and cellobiose concentrations. As shown in Table 1-16 and Figure 1-29, the cellobiose values were a considerable percentage of the glucose value. This indicates that a substantial portion of the cellulose was not getting through the entire pathway and was instead stuck at cellobiose formation.

**Table 1-16**
**August 7, 2025 72 Hour Hydrolysis Results**

| | ALI T72 | | | CTec2 T72 | |
|---|---|---|---|---|---|
| | g/L | % of Glucose | | g/L | % of Glucose |
| Cellobiose | 12.91 | **31.1%** | | 9.538 | **30.8%** |
| Glucose | 41.51 | | | 30.96 | |
| Xylose | 13.83 | | | 14.31 | |
| Arabinose | 0 | | | 0 | |

**Figure 1-29**
**Ball Milled/Enzyme Hydrolysis Phragmites HPLC Result**

| | | | |
|---|---|---|---|
| **Sample name:** | Sample B T24 Phagmites | | |
| **Injection date:** | 2025-05-23 15:23:47-04:00 | **Sample type:** | Sample |
| **Acq. method:** | Hydrolysis Fixed Eth6 5.2.25 10 ul 30 min.M | **Location:** | 3 |
| **Analysis method:** | Hydrolysis Fixed Eth6 5.2.25 10 ul 30 min.M | **Injection:** | 1 of 1 |
| **Acq. operator:** | SYSTEM | **Injection volume:** | 10.000 |
| **Last changed:** | 2025-05-23 14:51:21-04:00 | | |




**ESTD report based on Area**

| RT [min] | Signal | Type | Area | Height | Amount [g/l] | Name |
|---|---|---|---|---|---|---|
| 9.182 | RID1A | BV | 1543748.50000 | 51520.4766 | 8.60820 | Cellobiose |
| 11.050 | RID1A | VV | 6296819.00000 | 245970.7656 | 34.76489 | Glucose |
| 11.796 | RID1A | VV | 2877278.25000 | 77845.3594 | 16.29799 | Xylose |
| 18.692 | RID1A | VV | 261226.23438 | 5908.7266 | 3.17435 | Acetic Acid |

To overcome this cellobiose buildup, research by Fernandes, et al, 2022[8], proposes that increased glucose production could be achieved by converting cellulose to glucose through the addition of extra Beta-Glucosidase (B-G) enzyme. We conducted some experiments with and without B-G. Some runs showed increases in glucose production. For instance, the runs conducted on 3-4 September 2024 showed that with the addition of B-G enzymes, the cellulose to **glucose conversion ratio reached 70 percent** which was the first time we had seen this rate in Phase I runs (Table 1-17).

**Table 1-17**
**Effects of Beta-Glucosidase on Cellulose Hydrolysis**
(HPLC was not calibrated for Cellobiose on this run)

| 9/3-4/24 | Sample B: No B-Glucosiadase | Sample A: .3 g B-Glucosiadase | % Increase |
|---|---|---|---|
| Glucose in Enzyme Solution | 5.75 | 5.75 | |
| Glucose | 37.86 | 42.13 | |
| Net Glucose | 32.11 | 36.38 | 13.3% |
| Arabinose | 1.25 | 1.73 | 38% |
| Xylose | 15.55 | 15.41 | -1% |
| Total Sugars | 48.91 | 54.61 | **12%** |
| % Total Carbohydrate Conversion | 59.3% | 66.2% | 12% |
| % Cellulose to Glucose @34% | 63.0% | **71.3%** | 13% |
| % Xylan to Xylose @21 | 49.4% | 48.9% | -1% |

Achieving this 70 percent conversion value was important because it shows simultaneous ball milling/enzyme hydrolysis can achieve this milestone value without pretreatment and using commercially available enzymes.

Beta-glucosidase and cellobiose work was not completed because of the high cost of purified B-G enzymes and because this problem was somewhat beyond the Phase I scope of work. We did revisit the issue and found a potential solution to the "cellobiose problem" when we ran ethanol production experiments (Chapter II).

## Hemicellulose Conversion Rates

Due to a shortage of the Novozyme Htec X2753 xylanase enzyme, we were unable to compare ALI hemicellulase to the Novozyme Htec as we did with cellulases. However, we were able to calculate xylanase loading (mg of enzyme/g of hemicellulose) for the ALI hemicellulase we were using (Table 1-18). For comparison with cellulose to glucose conversion, data from Table 1-12 is included in Table 1-18.

**Table 1-18**
**ALI Hemicellulase Activity**

| Ball Milling/Enzyme Run | Orbis 2ft3 | Steady-State 2 |
|---|---|---|
| ALI Hemicellulase g | 170 | 12 |
| Biomass g | 2691 | 400 |
| | | |
| Enzyme mg/g enzyme powder | 75.1 | 75.1 |
| Total enzyme mg | 12,767 | 901 |
| Enzyme mg/g hemicellulose | **21.6** | **10.2** |

| **Hemicellulose to Xylose & Arabinose % Conversion** | **27%** | **42%** |
|---|---|---|
| | | |
| CTec2 Enzyme mg/ml enzyme liquid | | 1,000 |
| ALI cellulase mg/g enzyme powder | 245.1 | |
| Total enzyme (mg) | 85,295 | 12,000 |
| Enzyme mg/g cellulose | **79.2** | **75.0** |
| **% Cellulose to Glucose** | **48.6%** | **46.7%** |
| Ratio of Hemicellulase to Cellulase Concentration | 27.3% | 13.6% |

The data in this table led to the following three conclusions:

- While cellulose conversion to glucose was 60-70%, with hemicellulase it was less than 50% in both trials (Table 1-18). Additional work on carbohydrate conversion and condition optimization is needed.

- The ALI 800,000 HCU/g hemicellulase was able to deliver about the same carbohydrate to sugar conversion result (42%) as CTec2 (46.7%) in the multiday Steady-State 2 run even though the enzyme concentration for the hemicellulase was only 14 percent of the cellulase. This is consistent with hemicellulose being a shorter and less recalcitrant polymer within the cell wall.

- Because of the relatively efficient hemicellulose conversion rate at low enzyme concentrations, there is a good probability of conversion rates in the 70+ percent range with higher enzyme concentrations. Given the relatively low commercial price of the ALI enzymes, they will be retained for initial Phase II use.

- The lower hemicellulose conversion rate observed for the Orbis 2ft$^3$ run (Table 1-20) was probably due to a lower pH of the slurry during that experiment. See Chapter III for details.

**Enzyme Development for the Biofuel/Bioproduct Industry**

Because of the combination of poor commercial as-delivered enzyme quality, high cellulase enzyme prices, and supply-line availability problems, the development of new cellulase and hemicellulase mixtures for perennial grass biomass is a high priority for the economical production of biomass based biofuels and bioproducts. Ideally, a second round of DOE enzyme development projects would spearhead this work. However, since

that does not appear to be on the current schedule of Federal funding, companies, including small businesses like Atlantic Biomass, LLC will be pursuing it on their own limited budgets.

From Phase I results, the following cellulase and hemicellulase performance objectives have been developed.

1. Utilize low cost enzymes rather than expensive high efficiency or custom made enzymes. The goal is to maximize Total Biomass to Sugar Conversion instead of enzyme efficiency: Spending the money to optimize enzyme genetics would not be cost-effective and high efficiency enzymes developed for cellulosic ethanol are not available. Instead higher concentrations of less effective enzymes will be used to achieve biomass to monomeric sugar conversion rates of 75-85%.

2. Enzyme Mixtures will Contain All Three Biomass Hydrolysis Enzyme Groups: Since all three groups; endo, exo, and B-glucosidase are required for biomass to monomeric sugar conversion, hydrolysis efforts will be focused on end-to-end hydrolysis. Enzyme mixtures may include enzymes from different organisms or from organisms with multiple enzyme activities.

3. Enzyme Recycling is A Priority: Retaining enzyme activity over more than one 24 hour cycle could significantly reduce enzyme costs. Conditions will also be developed that conserve enzyme activity over maximizing rate. See Chapter 3 for progress report on retaining enzyme activity.

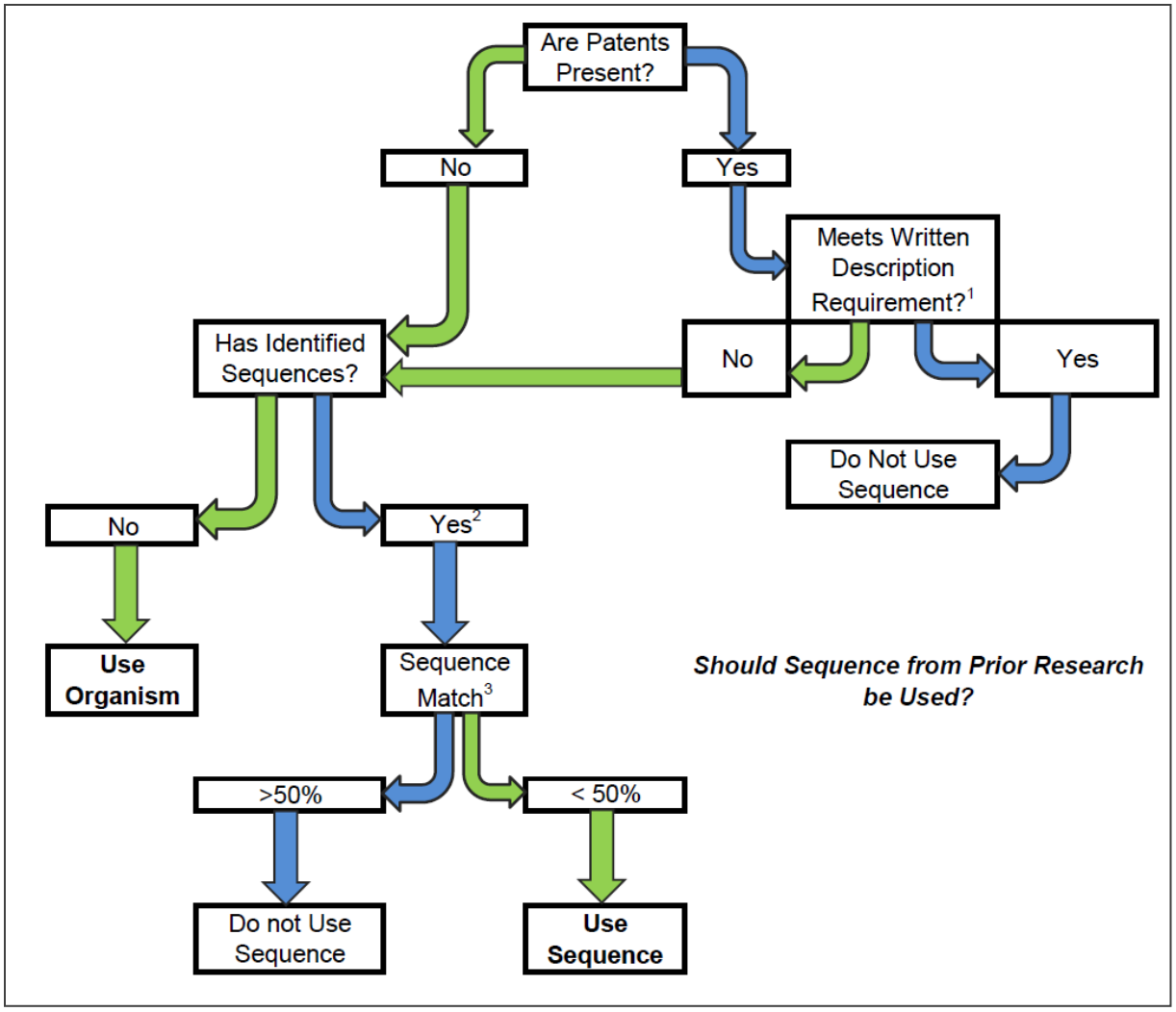
4. Enzyme production costs will be optimized. Enzyme development will operate within profitable enzyme cost analysis. For cost effective production of SAF, enzyme costs should not exceed about $.80/gallon SAF. Enzyme costs will be minimized to be sure operational costs are economical. For example, while an organism may produce highly efficient enzymes, if the costs of extracting the enzyme brings operational costs above the limit, a less efficient enzyme with low extraction costs may be selected instead.

**IP and Business Barriers to Cellulase and Hemicellulase Development**

Besides the technical challenges of enzyme development, in the biofuel/bioproduct sphere overcoming IP barriers are also a formidable challenge. Novozyme, while they were developing the CTec and HTec enzymes also quickly built an IP defense of their work through multiple gene sequence patents. This approach has also been used by other biomass enzyme developers. While the US Court system ruled against the

patenting of entire organisms as a result of a 2013 lawsuit, **NOVOZYMES A/S, AND NOVOZYMES NORTH AMERICA, INC.** *Plaintiffs-Appellants,* **v. DUPONT NUTRITION BIOSCIENCES APS (formerly Danisco A/S), GENENCOR INTERNATIONAL WISCONSIN, INC., DANISCO US INC., AND DANISCO USA INC.,** overlapping sequence patents within the same organism (in the 2013 case it was *Bacillus bacterium)* make it nearly impossible to use those organisms as sources of "wild-type" enzyme DNA sequences on which to build. One other important result of this case was that the courts accepted Novozyme's argument that a competing homologous DNA sequence was restricted from use if it had 90 percent homology with the sequence that had been (or was becoming) patented. Based on these results we developed the following decision tree (Figure 1-31) that we use whenever we have found preliminary positive results. Our use thus far has shown that a relatively small number of organisms have been "mined" for biomass deconstruction/hydrolysis enzyme sequences which means we have to find, identify, and test new organisms for enzyme activity.

**Figure 1-30**
**Atlantic Biomass, LLC Patent Avoidance Decision Tree**
(Footnotes Apply to detailed Decision Tree Use Guidance)



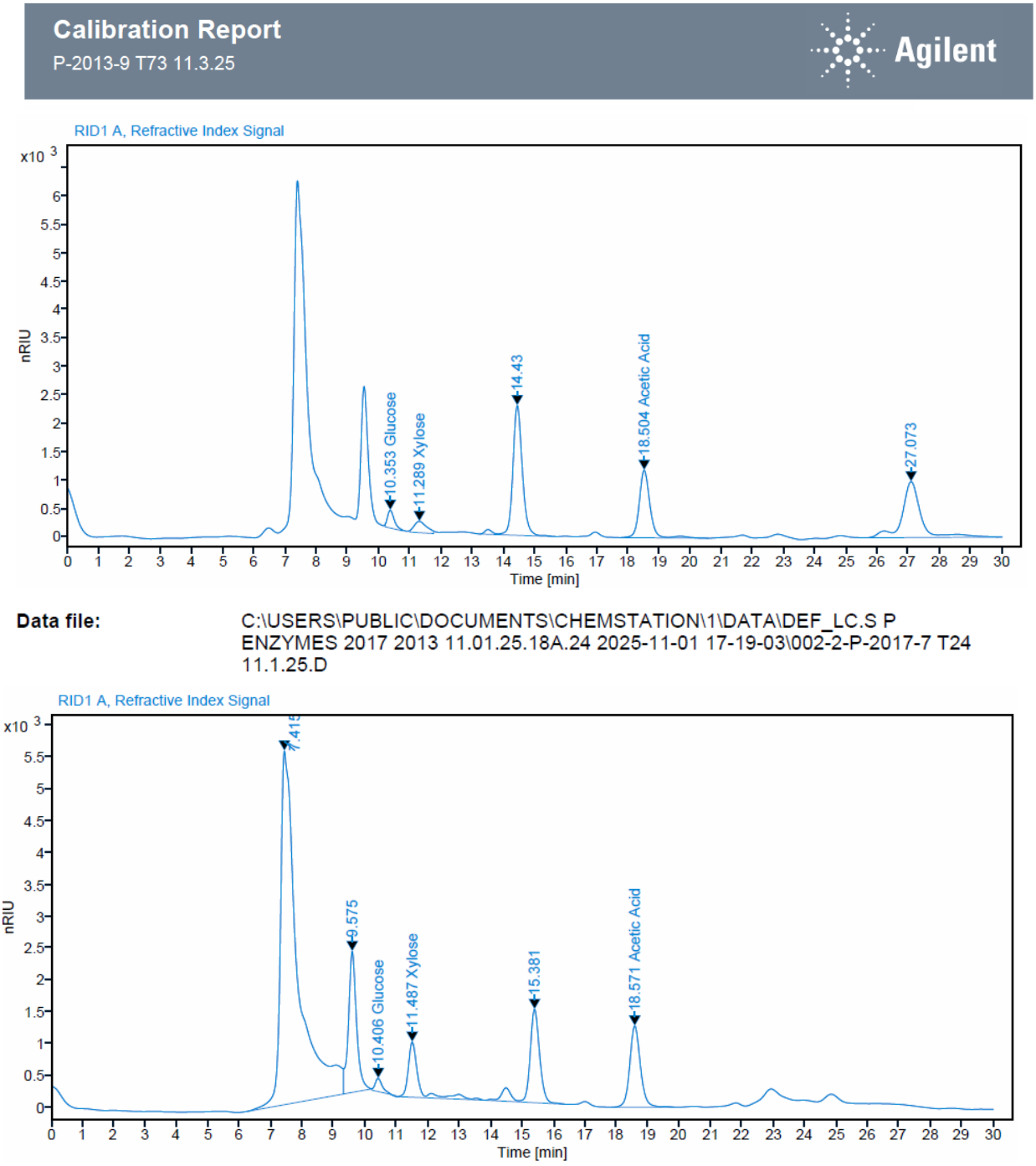

Thus far we have tested enzymes from three organisms that met the patent avoidance criteria. The samples were taken from low enzyme concentration culture supernatant and not higher concentration cell extracts or cell surface extracts. To simulate our Phase I testing all three samples were tested in 10 percent switchgrass biomass ground to 40 mesh (.4 mm) to simulate ball milling. All three samples were not buffered and had substantial pH drops which probably interfered with enzyme activity.

**Table 1-19**
**Before/After Enzyme Hydrolysis pH Readings**

| Enzyme | 0 hours pH | 73 hours pH |
|---|---|---|
| 2017-1 | 7.54 | 5.91 |
| 2017-7 | 7.67 | 5.69 |
| 2013-9 | 7.99 | 4.88 |

Despite these conditions some glucose and xylose production, was detected which indicates cellulase and hemicellulase activity. We will be continuing research on these two organisms.

**Figure 1-31**
**Organism P-2013-9 & P-2011-17 Monomeric Sugar Production**



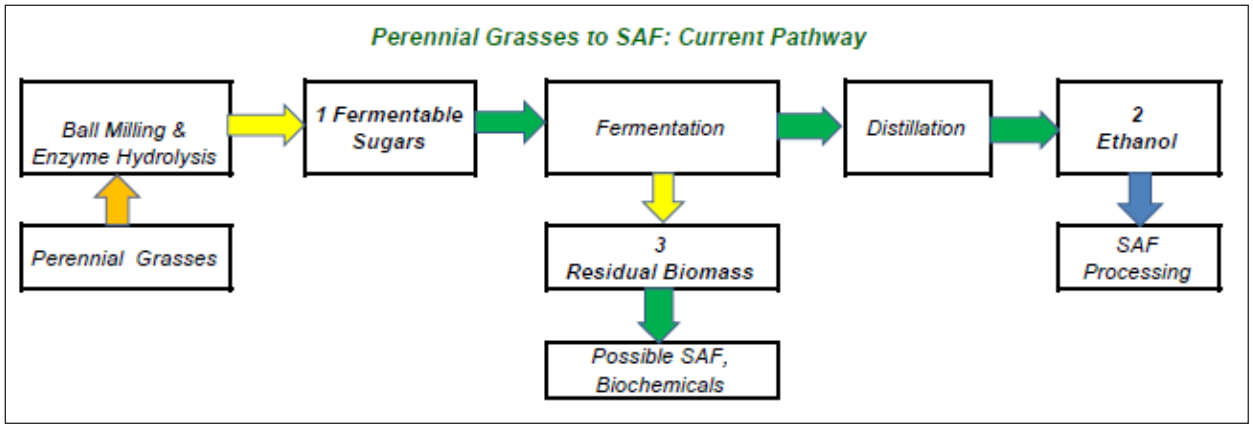
## Chapter II Maximizing Product Outputs

There are three outputs within the perennial grass to biofuel/bioproducts system:

1. Fermentable Sugars
2. Ethanol
3. Residual Biomass Products

Each of these (Figure 2-1) must be maximized in order to produce an economically feasible commercial system.

**Figure 2-1**




In this Phase I project, significant improvements were made in the production of fermentable sugars and the production of biofuels/bioproducts from residual perennial grass biomass. Together they point to a system with good economic potential.

## Section 2.1. Increasing Fermentable Sugar Production

As discussed above in Section 1.7, restarting the Beta-Glucosidase (B-G) cellobiose to glucose hydrolysis pathway is a major innovation needed to make perennial grasses and other biomass economically successful feedstocks.

As shown in Table 1-17, B-G was shown to increase glucose production. However, these increases did not occur in all runs. For example, 6 September 2024 runs of identical switchgrass samples run with and without B-G showed only a minimal glucose increase with added B-G (Table 2-1). Unfortunately, the HPLC analyzers were not calibrated for cellobiose so we were not able to see any relationships between cellobiose and glucose concentrations.

**Table 2-1**
**Effects of B-G on Glucose Production**

| 9.6.24 | Slurry A &B Switchgrass | | |
|---|---|---|---|
| | Without B-G | With B-G | % Increase with B-G |
| | | | |
| Glucose | 37.86 | 38.98 | 3.0% |
| Xylose | 15.549 | 16.76 | 7.8% |
| Arabinose | 1.251 | 1.25 | -0.2% |

As stated above, we stopped beta-glucosidase and cellobiose work at that point due to the high cost of purified B-G enzymes and that the problem was somewhat beyond the Phase I scope of work. We revisited it when we ran ethanol production experiments.

## Ethanol Production Effects on Cellobiose Conversion

As shown in Section 1.1 the C-5 hemicellulose monomeric sugars, xylose and arabinose, are a substantial portion of the fermentable sugars available from perennial grasses. Depending on the efficiency of hemicellulase enzymes that produce xylose and arabinose, they could increase ethanol yield up to 30 percent over just using glucose for fermentation (Table 2-3). However, yeasts (Saccharomyces) commonly used in fermentation of corn kernels to ethanol cannot utilize the C-5 sugars.

To overcome this fermentation deficiency, we have been using a modified E.coli strain, FBR5, that was developed by the USDA/ARS Midwestern Laboratory[9]. This strain is able to convert the C-5 xylose and arabinose sugars as well as glucose to ethanol (Table 2-2).

**Table 2-2**
**April 2025 FBR5 Fermentation Runs**
Note: the xylose and arabinose values in Table 2-2 are lower than expected due to testing a new hemicellulase mixture.

| **Miscanthus** | T0 hrs (g/L) | T24 hrs (g/L) | Sugars Converted (g/L) | % Converted |
|---|---|---|---|---|
| | | | | |
| Cellobiose | 6.31 | 1.27 | 5.04 | 80% |
| Glucose | 22.10 | 0.00 | 22.10 | 100% |
| Xylose | 4.57 | 0.70 | 3.88 | 85% |
| Arabinose | 0.65 | 0.00 | 0.65 | 100% |
| Total Sugars | 33.63 | 0.00 | 31.66 | 94% |
| Acetic Acid | 2.04 | 2.27 | | |
| Ethanol | 0 | 6.30 | | |

| **Switchgrass** | T0 hrs (g/L) | T24 hrs (g/L) | Sugars Converted (g/L) | % Converted |
|---|---|---|---|---|
| | | | | |
| Cellobiose | 5.98 | 1.27 | 4.71 | 79% |
| Glucose | 19.73 | 0.00 | 19.73 | 100% |
| Xylose | 2.81 | 0.74 | 2.08 | 74% |
| Arabinose | 0.00 | 0.00 | 0.00 | |
| Total Sugars | 28.52 | 2.01 | 26.52 | 93% |
| Acetic Acid | 1.73 | 2.36 | | |
| Ethanol | | 6.39 | | |

While the cellobiose concentrations dropped significantly during fermentation, we were focused on ethanol production and did not immediately pursue the results thinking it might be something like a HPLC plot integration error. However, when we did look at the HPLC plots more closely, both the T0 and T48 HPLC reports had similar cellobiose split peaks. What was different was that the post-fermentation T48 peak was smaller (Figures 2-2a and 2-2b).

**Figure 2-2a**
**Miscanthus: T0 Before Fermentation HPLC Report**

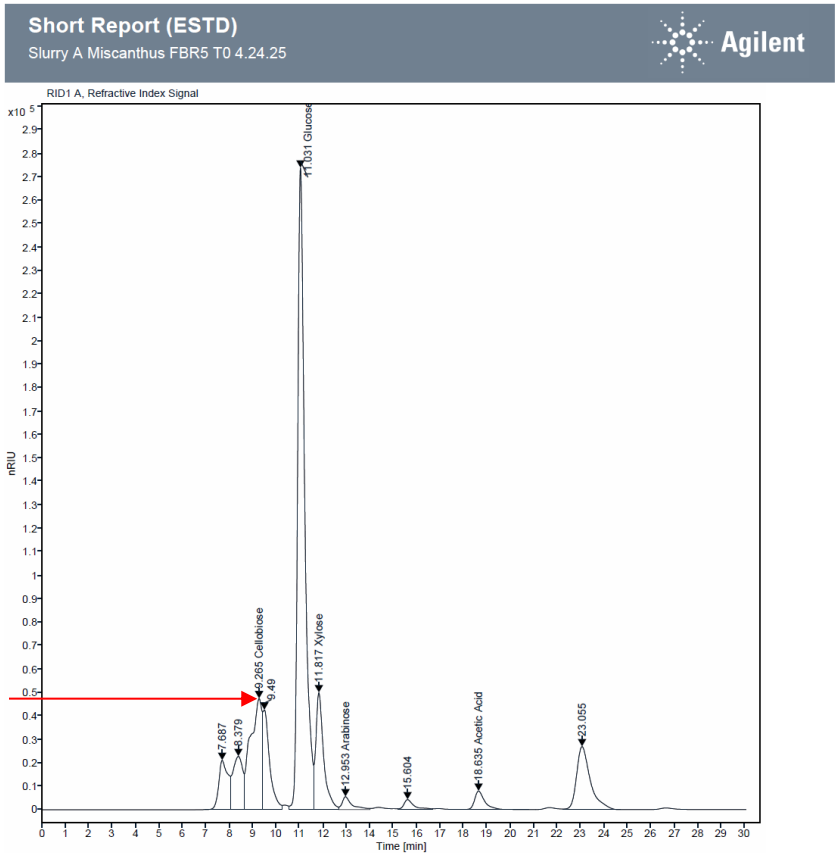
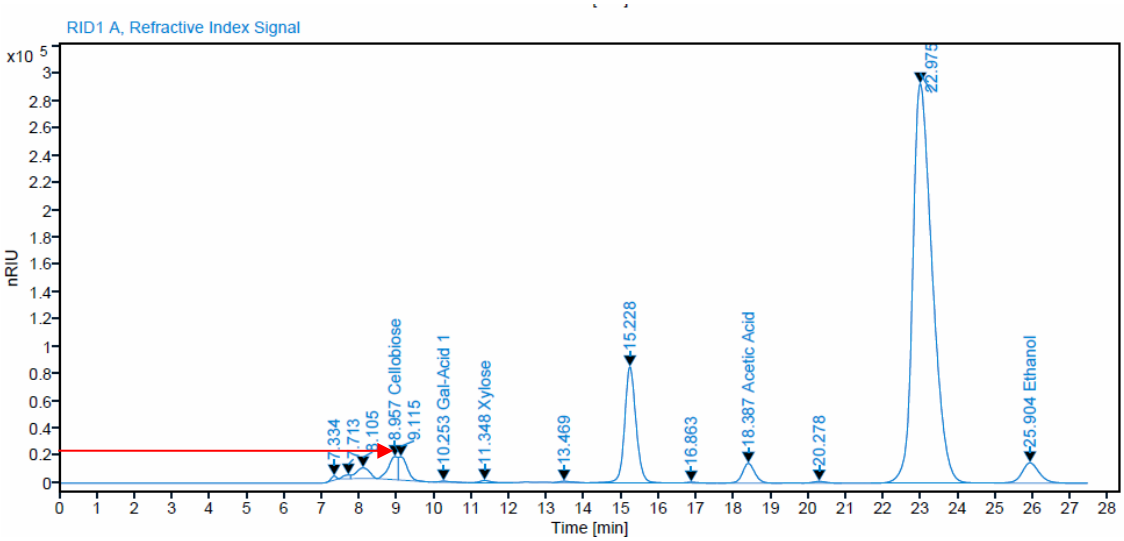



**Figure 2-2b**

**Miscanthus: T48 After Fermentation HPLC Report**

| | | | |
|---|---|---|---|
| **Sample name:** | A Miscanthus FBR5 T48 4.26.25 | | |
| **Description:** | | | |
| **Sample amount:** | 0.000 | **Sample type:** | Sample |
| **Instrument:** | Agilent HPLC | **Location:** | 2 |
| **Injection date:** | 2025-05-07 16:15:44-04:00 | **Injection:** | 1 of 1 |
| **Acq. method:** | def_LC.M 5.5 5.6.25 EtoH Cal 10 ul Injection Cal .M | **Injection volume:** | 10.000 |
| **Analysis method:** | def_LC.M 5.5 5.6.25 EtoH Cal 10 ul Injection Cal .M | **Acq. operator:** | SYSTEM |
| **Last changed:** | 2025-05-07 16:53:51-04:00 | | |




## Glucose Inhibition Effects: A Possible Solution

In an example of how a beneficial product can also inhibit a process, Bei Ouyang, et al[10], cite research on cellulose to glucose conversion and state that, “In the tandem cellulolysis process, the hydrolytic end product, glucose, inhibits BGL, leading to the accumulation of cellobiose, which in turn inhibits endo-1,4-_-D-glucanase (EG) and cellobiohydrolase (CBH).“ They propose, “*To broaden the applications of BGLs [Beta Glucosidase] in industry, it would be beneficial to enhance the capability of BGLs to tolerate non-mild conditions such as high temperatures, high concentrations of glucose, extreme pH, and high concentrations of organic solvents, to name a few.”*

In light of this, the April FBR5 results led to a possible alternative solution – instead of improving B-G performance, the glucose concentration could be reduced to reduce inhibition of B-G. The lowering of glucose concentration was something that already occurs during the downstream ethanol fermentation step.

To test this hypothesis, FBR5 was inoculated into a slurry composed of ball mill/enzyme hydrolyzed switchgrass and miscanthus. It differed from the April 2025 run in that glucose conversion to ethanol was designed to be incomplete (less than 100 percent conversion). This was done to see if lowering, rather than eliminating the glucose concentration, would lead to a lower cellobiose concentration. As shown in Table 2-3, a 35.1 percent decrease in glucose concentration occurred that was accompanied by a 24.5 percent decrease in cellobiose concentration.

**Table 2-3**
**Partial Glucose Fermentation to Ethanol Effects on Cellobiose**

| | HPLC Readings (g/L) | HPLC Readings (g/L) | Sugars Converted (g/L) | % Sugars Converted |
|---|---|---|---|---|
| | T0 hrs | T49 hrs | T0 to T49 | T0 to T49 |
| **Cellobiose** | **12.41** | **8.75** | **3.66** | **24.5%** |
| **Glucose** | **47.03** | **27.16** | **19.87** | **35.1%** |
| Xylose | 14.06 | 9.91 | 4.15 | 24.5% |
| Arabinose | 1.47 | 1.27 | 0.20 | 11.2% |
| Acetic Acid | 2.40 | 2.26 | | |
| Ethanol | 0.56 | 5.05 | | |

**Figure 2-3a**
**Partial Fermentation: Before Fermentation T0 HPLC Report**

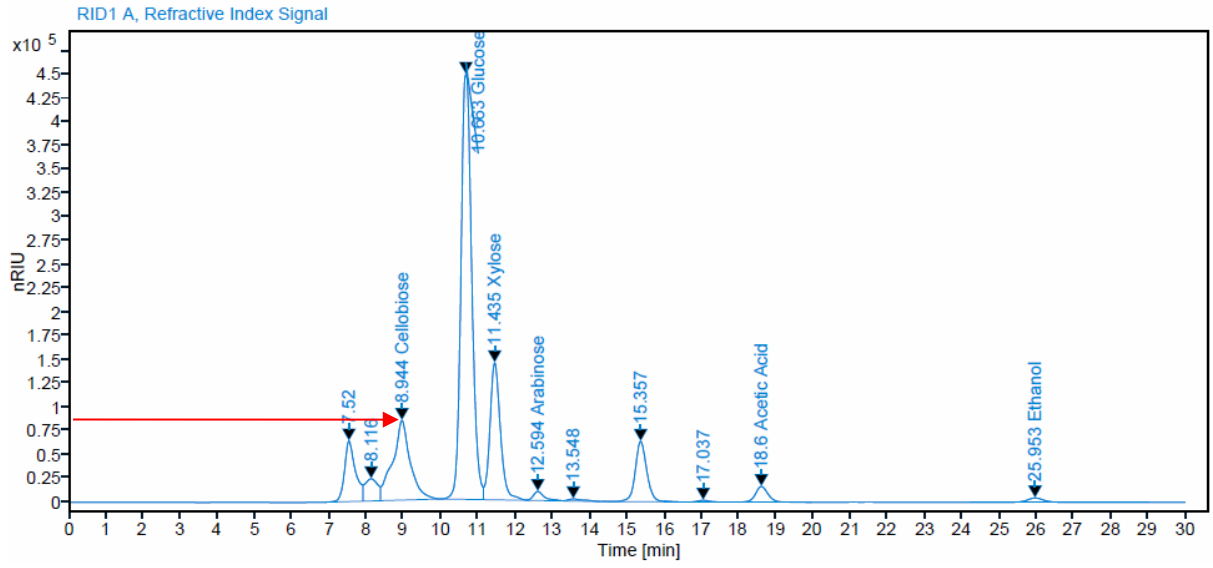
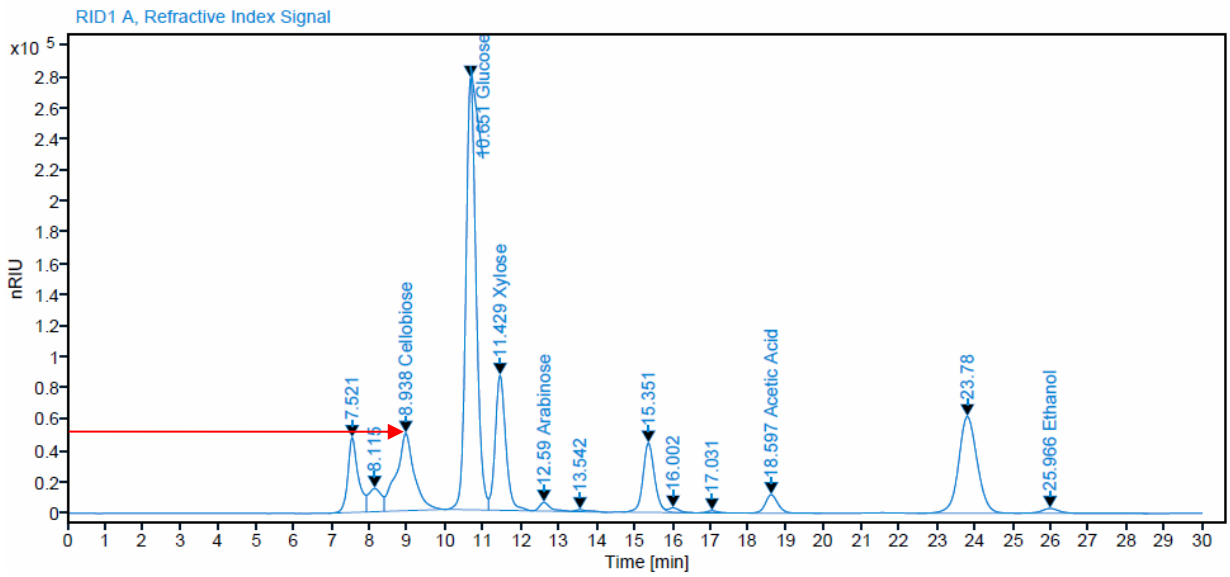


**Figure 2-3b**
**Partial Fermentation: After Fermentation T49 HPLC Report**




The question remains, how was the cellobiose converted? The most probable answer for both sets of runs is by Beta-glucosidase remaining in the slurry. The majority of the combined ball milling/enzyme hydrolysis runs in Phase I had high enzyme concentrations. This was because we were adapting the principles of Mes-Hartree enzyme recycling activity in Phase I[(11)]. The basis of this approach is to begin with sufficient enzymes to achieve very high biomass to sugar conversion rates. Therefore, residual B-G enzymes that were in the slurry but had been inhibited by high glucose concentrations would be available to bind with and convert cellobiose.

While these results are encouraging, more testing will be needed to verify the effects and conversion mechanism. Varying quantities of B-G enzymes will be added at different times during FBR5 fermentation. Time series HPLC analysis will be done to track cellobiose, glucose and ethanol concentrations. While cellobiose is reported as having very low solubility in ethanol, the effects of ethanol, which in FBR5 fermentation must be limited to less than 4 percent to maintain FBR5 viability, on cellobiose will also be tracked. Atlantic Biomass, LLC will be conducting this research without waiting for DOE Phase II or other funding sources because of its importance to the overall project.

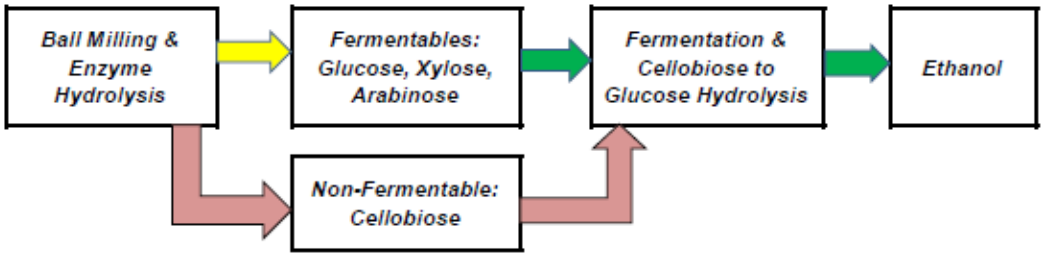
**Effects of Cellobiose to Glucose Conversion on Commercial Ethanol Production**

By continuing enzymatic hydrolysis in the fermentation step, this grass to bioproducts process has taken on the characteristics of Simultaneous Saccharification and Fermentation (SSF). It uses fermentation as a feedback loop to improve saccharification. In turn the increase in cellobiose to glucose saccharification increases the amount of

fermentable sugars which leads to increased ethanol fermentation without increasing production costs. Because these processes are occurring simultaneously, the glucose concentration will not excessively build up since it is being used for additional ethanol production.

**Figure 2-4**

*Combined Saccharification & Fermentation*




In Table 2-5, the effects of using the 80 percent conversion of cellobiose to glucose to increase potential glucose for fermentation are presented.

*Cellobiose is composed of two glucose molecules, so each conversion step produces two glucose molecules for fermentation.*

**Table 2-5**
**Effects of Cellobiose Conversion to Glucose on Glucose Availability**

| | | 8/7/2025 | | |
|---|---|---|---|---|
| | | ALI T72 | | CTec2 T72 |
| Total Liquid (ml) | | 2000 | | 2000 |
| Total Biomass (g) | | 300 | | 300 |
| Pre-Fermentation Concentrations | | Concentrations in g/L | | |
| Cellobiose (g/L) | | 12.91 | | 9.538 |
| Net Glucose (g/L) | | **35.12** | | **22.62** |
| **Potential Increases from Fermentation Hydrolysis** | | | | |
| **Cellobiose to Glucose at 80% from Fermentation Hydrolysis (g/L)** | | **20.66** | | **15.26** |
| **% Increase in Glucose Available for Fermentation** | | **58.8%** | | **67.5%** |

## Section 2.2 Use of Residual Biomass: Conversion of Post-Fermentation Slurry to Higher-Value Products

### Introduction

The production of SAF and biochemicals from perennial grass carbohydrates (cellulose and hemicellulose) uses only carbohydrate fraction of the biomass. In the three perennial grasses that were tested; switchgrass, phragmites, and miscanthus, the carbohydrate percentages ranged from about 53 to 66 percent as shown in Table 2.6.

**Table 2-6**
**Perennial Grass Carbohydrate & Ash Composition**

| Sample Type | Sample ID | Cellulose (% biomass) | Hemi-cellulose (% biomass) | Cellulose and Hemicellulose (% biomass) | % Ash |
|---|---|---|---|---|---|
| | | | | | |
| PA Switchgrass (50°C) | 4A | 34% | 28% | 62% | |
| | 4B | 32% | 26% | 58% | |
| | **Avg.** | **33%** | **27%** | **60%** | |
| | | | | | |
| PA Switchgrass | 11A | 32% | 29% | 61% | 0.7% |
| | 11B | 31% | 25% | 56% | 1.3% |
| | 11C | 31% | 25% | 56% | 2.1% |
| | **Avg.** | **31%** | **26%** | **58%** | |
| | | | | | |
| Ohio Miscanthus | 21A | 48% | 21% | 69% | 0.6% |
| | 21B | 48% | 21% | 69% | 0.5% |
| | 21C | 41% | 19% | 60% | 0.4% |
| | **Avg.** | **45%** | **20%** | **66%** | |

Use of the remaining biomass for biofuels or biochemicals could significantly increase the overall efficiency and profitability of the biomass to biofuel or biochemical process. This could be accomplished by converting the remaining biomass into syngas feedstocks for fuels and chemicals via a thermochemical process involving pyrolysis.

### Biomass Characteristics Necessary for Efficient Thermochemical Conversion

However, for syngas to be produced at commercial quantity and purity levels, the input biomass has to have a relatively high lignin concentration while the carbohydrate and ash concentrations should be low.

For example, pyrolysis, an oxygen free decomposition process occurring at approximately 400-500$^0$C, has been proposed as a viable option for the production of a variety of bioproducts from biomass. There are three groups of products from a biomass pyrolysis reaction. In terms of value they are; 1) liquids (bio-oils), 2) gases (syn-gas feedstocks), and 3) biochar (land nutrients).

While pyrolysis is a relatively straight forward and efficient process when the feedstock is highly uniform, high in lignin content, and low in water content it becomes complex and less efficient when plant biomass is used.

As shown in Butler, et al, 2012[(12)], high ash, hemicellulose and moisture correlated with negative pyrolysis results.

**Table 2-7**

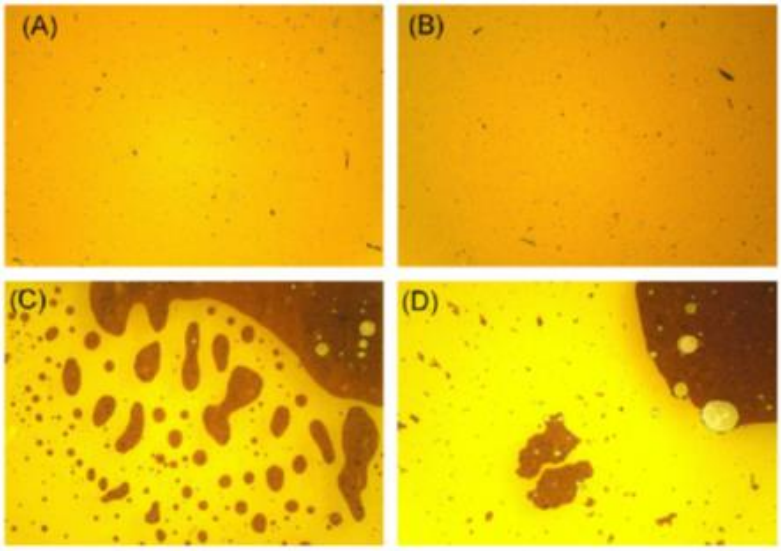
| Percentage Content | Miscanthus | Wood (Spruce & Willow) Average | % Miscanthus Increase |
|---|---|---|---|
| Moisture (%) | 7.6 | 3.2 | +139% |
| Ash (%) | 3.4 | .7 | +383% |
| Hemicellulose | 22.7 | 10.8 | +111% |

Liquid organic and bio-oil yields were lower for miscanthus while char production and residual water were higher (Table 2-8).

**Table 2-8**

| | Miscanthus (% dry biomass) | Wood Average (% dry biomass) | % Miscanthus Difference |
|---|---|---|---|
| Liquid Organic Yield | 37.3 | 49.6 | -25% |
| Bio-Oil Fraction | 48.95% | 43.4% | -11.34% |
| Char Yield | 21.8 | 13.8 | +158% |
| Water Content | 34.7 | 20.6 | +169% |

This study also found that the oils produced from wood to be homogenous while the plant produced oils were inhomogeneous.




Microscopy images (×50) for bio-oil fractions from spruce (A), Salix (B), Miscanthus (C) and wheat straw (D).

**Figure 2-4**

## Syngas Results with Post-Hydrolysis Perennial Grass Biomass

Instead of the “raw” non-processed miscanthus used in the pyrolysis tests, we used a post-hydrolysis slurry mixture of miscanthus and switchgrass (Figure 2-5). It is important to point out that this was biomass remaining after income producing fermentable sugars and ethanol had been removed. So, the only additional cost in processing the residual perennial grass biomass into a potentially high quality feedstock was drying.

**Figure 2-5**
**Dried Post-Hydrolysis Perennial Grass Biomass**

As shown in Table 2-9 the perennial grass remaining after hydrolysis was found to contain relatively low carbohydrate (31%) and higher lignin (41%) concentrations as compared to pre-hydrolysis composition.

**Table 2-9**
**Pre and Post Hydrolysis Perennial Grass Composition**

| | % Before Hydrolysis | % After Hydrolysis |
|---|---|---|
| | | |
| Cellulose | 38% | 4% |
| Hemicellulose | 23% | 27% |
| Lignin | 23% | 41% |
| Other | 16% | 28% |
| Total | 100% | 100% |

This biomass was washed, centrifuged, and dried after ball milling/enzyme hydrolysis to remove liquefied monomeric sugars. This procedure was used to simulate fermentation which would have converted the monomeric sugars into ethanol since the fermentation process was not ready at this stage of Phase I.

Two uses for this biomass, which also had a low ash value, were tested.

- Fluidized Bed Combustion of Slurry for Process Heat Energy Production
- Slurry to Syngas Production via Chemical Looping Partial Oxidation

The fluidized bed combustion test was run first since it was simpler and would provide data that would indicate if efficient slurry to syngas production might be possible.

*The following sections were primarily written by Shekhar G Shinde of the William G. Lowrie Department of Chemical & Biomolecular Engineering Department of the Ohio State University who led the research.*

**Fluidized Bed Combustion of Slurry for Process Heat Energy Production**

A fluidized bed combustor is an efficient reactor system that can convert any type of carbonaceous feed, such as agricultural waste, into heat or syngas. Complete combustion of the fuel results in the formation of heat and $CO_2$, whereas partial combustion results in syngas ($H_2$+ CO).

A bubbling fluidized bed (BFB) is a type of gas-solid reactor where solid particles, such as oxygen carriers, catalysts, or inert materials, are suspended in an upward-moving gas stream to create a reactor bed that resembles a fluidized bed was used in these tests.

When gas is constantly introduced into the vertical reactor at a certain velocity, the sand particles inside the reactor begin to exhibit liquid-like behavior and become suspended. The gas velocity at which this phenomenon occurs is referred to as the minimum fluidization velocity. The fluidized condition occurs when the upward drag force exerted by the gas precisely counteracts the effective weight of the solid particles. Here, the upward force by air balances the weight of the sand particles.

This fluidized bed setup produces syngas from the partial combustion of fuel, which is further converted to combustion products to yield maximum heat. During partial combustion of biomass, the air sent into the reactor is controlled to limit the oxygen supply. The reactions taking place in the reactor are represented by equations 1 to 6. Reactions 1 to 4 represent the combustion reactions, whereas reactions 5 and 6 represent the partial combustion reactions

$$C + O_2 = CO_2 \quad (1)$$

$$2C + O_2 = 2CO \quad (2)$$

$$2H_2 + O_2 = 2H_2O \quad (3)$$

$$CH_4 + 2O_2 = CO_2 + 2H_2O \quad (4)$$

$$C + CO_2 = 2CO \quad (5)$$

$$C + H_2O = CO + H_2 \quad (6)$$

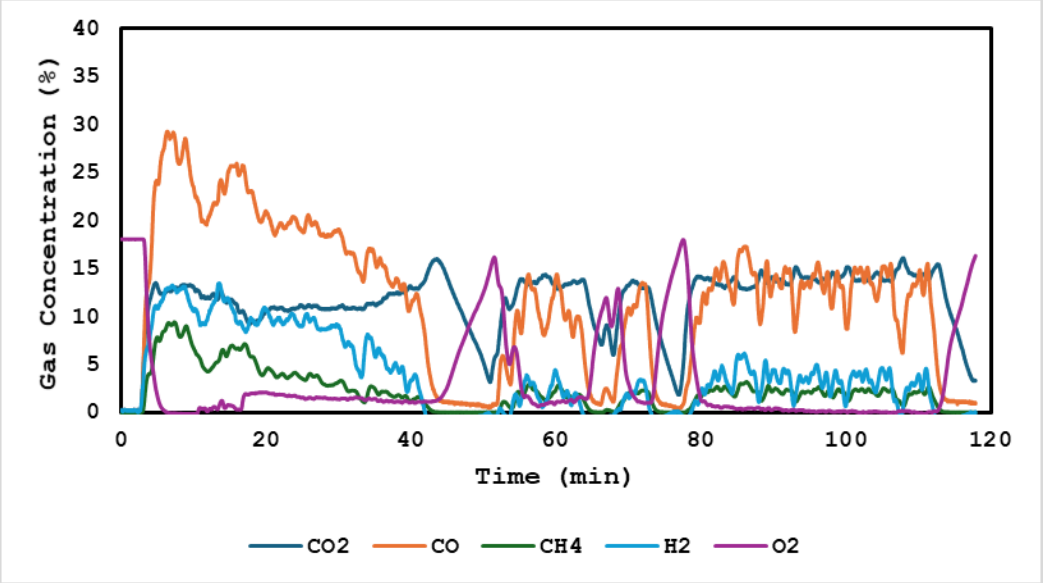
The procedures for this fluidized bed experiment are as follows:

- 300–500 microns of sand particles are sieved, and 300 grams of those particles are weighed.
- These sand particles are loaded into the reactor.
- Gas (air) is injected from the bottom via a gas distribution plate.
- The temperature of the reactor is then increased to the desired temperature of operation. (900 °C).
- As gas velocity rises, it approaches the minimum fluidization velocity.
- At this stage, the solid particles are lifted and start acting like a boiling liquid.
- The biomass is then dropped onto the vertical reactor using a calibrated screw loader.
  Biomass is combusted to carbon dioxide ($CO_2$), carbon monoxide (CO), methane ($CH_4$), hydrogen($H_2$), oxygen($O_2$) and other gases at $900^o$ C
- The gases exit from the top of the reactor.

## Results

During the initial period, a high $O_2$ content is seen in the reactor. However, as time progresses and biomass is introduced into the reactor, $O_2$ content decreases, and there is a steady rise in the CO, $CO_2$, and $H_2$ profiles. Initially, a high concentration of CO is seen in the reactor outlet due to the lower air supplied at the reactor inlet. As time progresses, the air inlet into the reactor increases, which increases the $CO_2$ composition in the gas profile, indicating that the reaction is moving towards complete combustion of the biomass feedstock due to an adequate supply of oxygen from the air being let in. (Figure 2-6).

**Figure 2-6**
**Complete gas profile for biomass combustion in the fluidized bed reactor**




As shown in Figure 2-7, the reactions maintained steady-state from 80 to 120 minutes. During this period, there is no transition in the gas profiles. The air supplied is sufficient to convert the biomass into stable gases at outlet.

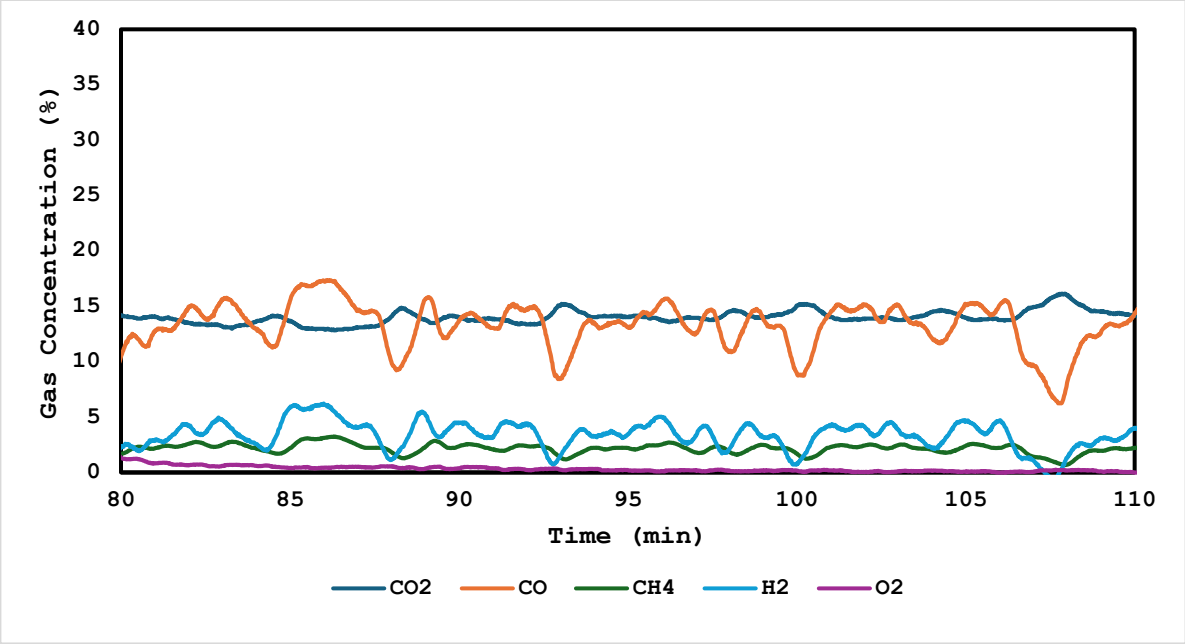



**Figure 2-7**
**Steady state gas profiles for biomass combustion in the fluidized bed from minutes 80 to 110**

Figure 2-8 represents the carbon conversion percentage in the reactor. Carbon conversion gives an idea about the efficiency of the reaction. It tells how much of the inlet carbon from the biomass is converted into gases. A higher carbon conversion indicates that almost all the carbon from the biomass is converted into gases. A lower carbon conversion, on the other hand, indicates that the carbon from the biomass is accumulating in the reactor.

The carbon conversion observed for this experiment was close to 100%, affirming the applicability of the process to this perennial grass biomass mixture.

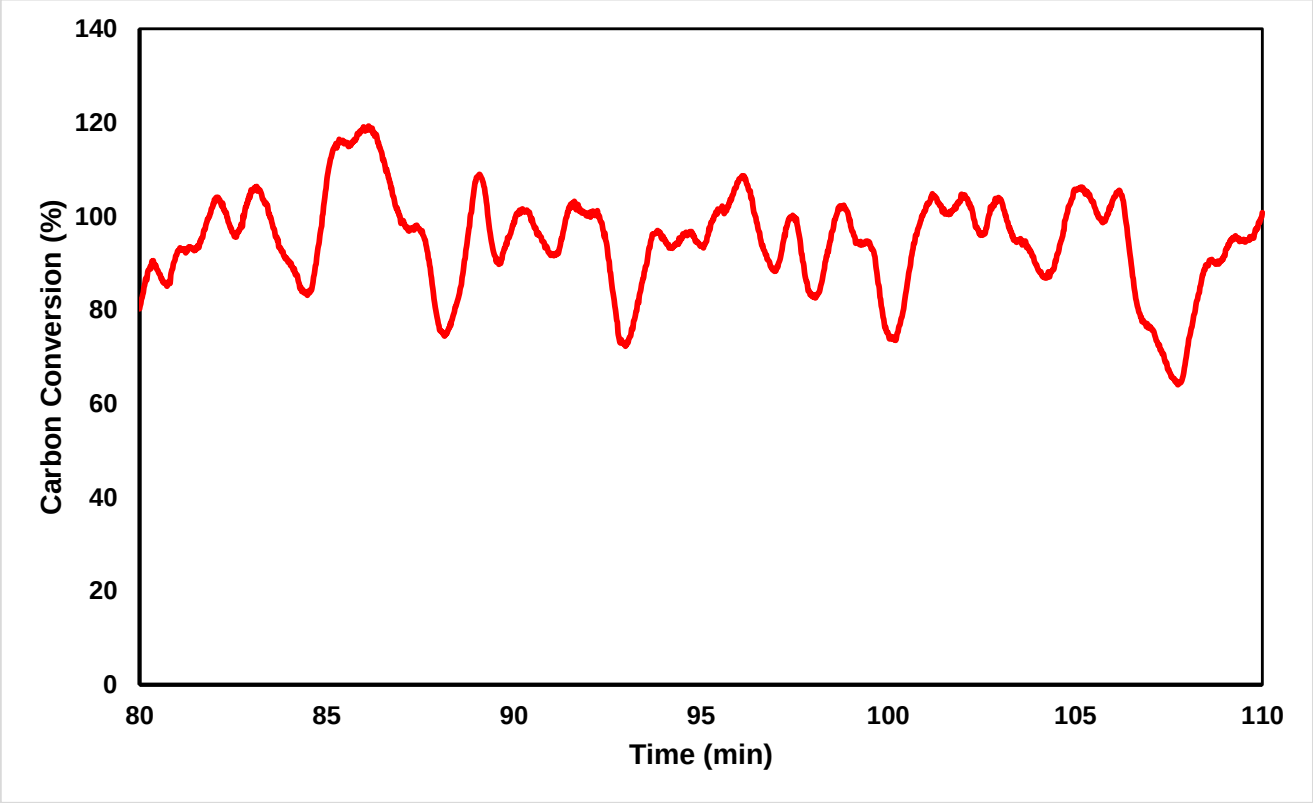



**Figure 2-8**
**Carbon conversion for the complete combustion of biomass using a fluidized bed**

## Conclusion

Complete combustion of the fuel results in the formation of heat and $CO_2$, whereas partial combustion results in syngas ($H_2$+ CO). Stable gas profiles were established during the run, indicating that the fluidized bed can be used to convert agricultural residue into useful gases, commodity chemicals, and heat. An increase in air supply into the reactor enables a higher oxygen supply to the biomass, thereby enabling the transition from partial combustion to complete combustion of the biomass fuel. ***The carbon conversion obtained in the reactor is close to 100 % which proves the efficiency of the system.***

## Slurry to Syngas via Chemical Looping Partial Oxidation

The Biomass to Syngas (BTS) chemical looping process is an advanced thermochemical process that eliminates the need for using an air separation unit, a tar reformer, a steam reformer, or a water-gas-shift unit for the production of sustainable fuels and chemicals[13].

In the BTS process, syngas is produced by partially oxidizing the biomass with the help of oxygen carriers in a co-current gas-solid moving bed reducer reactor. This converts the biomass feedstock to high-purity syngas, with an adjustable $H_2$:CO molar ratio. The co-current moving bed reducer eliminates back-mixing, channeling, or bypassing of solid and gas reactants, resulting in a syngas composition that is close to the thermodynamic equilibrium. The reduced oxygen carriers are regenerated in a fluidized bed combustor reactor via the oxidation reaction with air. The BTS process uses the iron-titanium composite metal oxide (ITCMO) material as the oxygen carrier, which is capable of cracking the volatiles produced in biomass pyrolysis as well as regulating the syngas composition.

As illustrated in Figure 2-9, chemical looping partial oxidation for direct biomass gasification consists of cyclic reduction-oxidation reactions with recycling metal oxides to produce syngas from biomass and air.

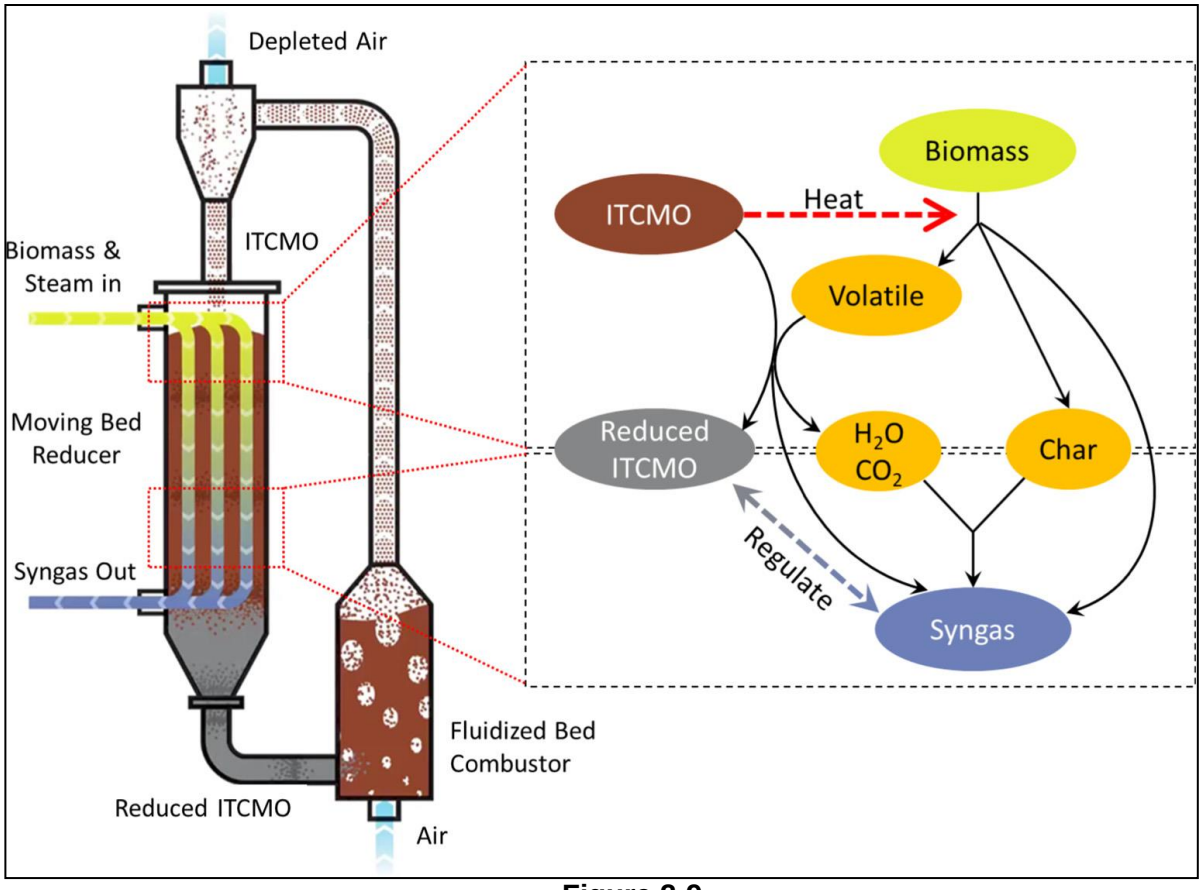



**Figure 2-9**
**Biomass to Syngas (BTS) chemical looping process**

As the biomass enters the top of the reducer, the carbonaceous fuel (biomass) comes into direct contact with the high-temperature oxygen carriers, and rapid biomass pyrolysis

occurs. The gaseous volatiles travel co-currently downward with respect to the oxygen carrier flow. The organic compounds in the pyrolyzed biomass volatiles are cracked and oxidized to form CO, $H_2$, $CO_2$, and $H_2O$ as they flow through the bed of oxygen carriers rich with available lattice oxygen. As the oxygen carriers travel downward through the reducer and oxidize the biomass feedstock, oxygen vacancies form in the oxygen carriers. With the oxygen vacancy formation in the oxygen carrier, the partial oxidation of fuels to generate CO and $H_2$ is favored over the full oxidation of fuels to generate $CO_2$ and $H_2O$. At the bottom of the reducer, the oxygen carriers are separated from the syngas. The reduced oxygen carriers are transported to the combustor, where they are subsequently regenerated by air and then recycled back to the reducer, while the syngas is sent downstream for conditioning and processing. Thus, chemical looping gasification processes can produce high-purity syngas.

**Experimental Setup and Procedures**

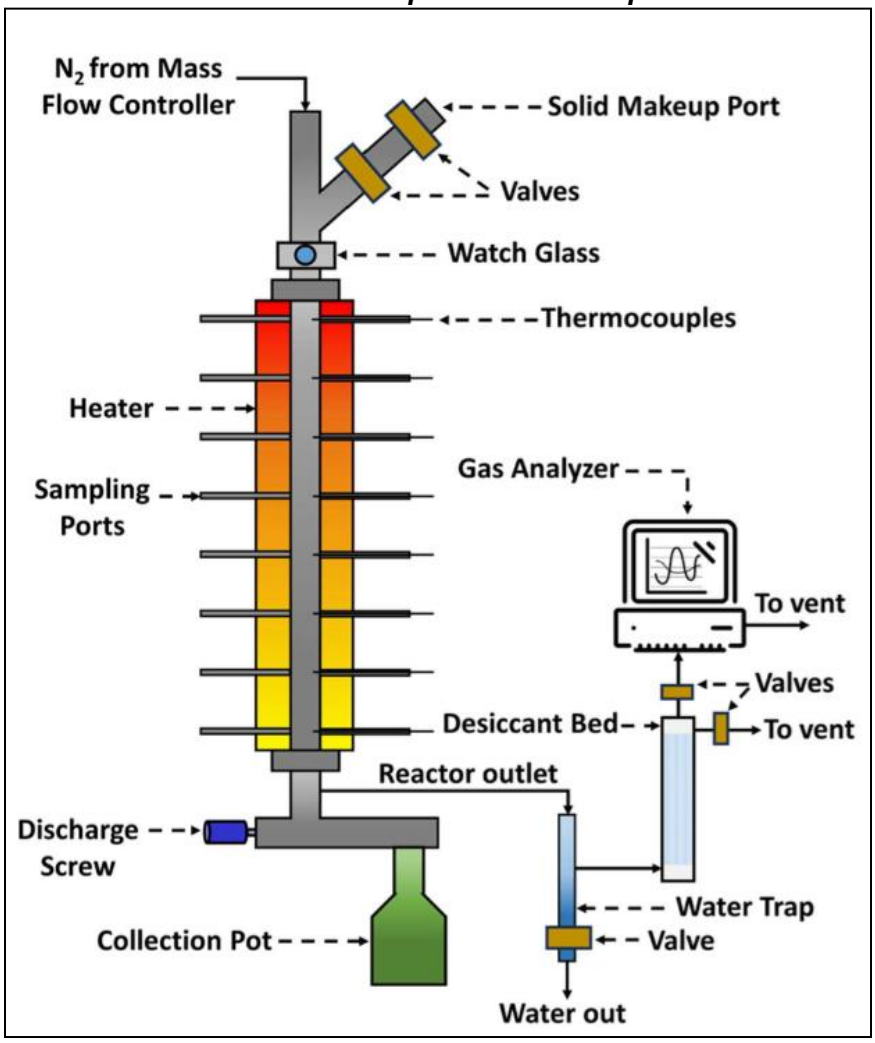
The experiments were carried out in a 1.5-inch internal diameter and 32-inch long moving bed reactor (Figure 2-10). The reactor has nine thermocouple ports equipped with type-K thermocouples and an equal number of sampling ports located 180° opposite the thermocouple ports. These ports are spaced 4 inches apart, with the first port located 2.5 cm within the heating zone. To simulate the operating conditions of a full-scale system, the reactor's external surface is covered with a set of clamshell heaters. These heaters provide an operating temperature range between $700^0$ C and $1050^0$C, with a +/- $5^0$C setpoint tolerance. The upper section of the unit has a lock-hopper, through which solids (Biomass + particles) are introduced into the reactor. A quartz window is installed in the reactor above the heated zone to ensure that the reaction zone is entirely full during operation.

The experimental procedure involved heating the biomass samples from room temperature to the target temperature (either 850°C or 1000°C) at a ramp rate of 55°C/min under a flow of $N_2$. Once the desired temperature was reached, the gasifying gas (80% $CO_2$) was introduced and maintained for 2 hours to allow for complete gasification. This approach provided a comprehensive understanding of the biomass's thermal behavior and reactivity under different conditions relevant to the moving bed reactor operation.

A controlled flow of nitrogen gas ($N_2$) was introduced as a tracer into both the lock hopper section and the reactor. The mass flow controller used for this purpose is sourced from Alicat Scientific (MC-2 SLPM). The solids velocity is regulated by a screw feeder located at the bottom of the moving bed, which is connected to a DC motor that ensures a linear relationship between voltage and flow rate. Then, the gases were directed through a water trap and a drierite desiccant before being analyzed for $H_2$ using SEIMENS

CALOMAT 6 with a thermal conductivity detector (TCD) and for CO, $CO_2$, and $CH_4$ using SEIMENS ULTRAMAT 23 with an infrared (IR) analyzer.

***Figure 2-10***
***Bench Scale Experimental Setup***




Prior to bench-scale testing, the biomass was characterized using thermogravimetric analysis (TGA) to determine its composition, including the amounts of volatiles, moisture, char, and ash. The biomass was then subjected to gasification under a CO2 atmosphere to understand its gasification characteristics. A SETSYS Evolution TG instrument was used for these tests. To investigate the lower and upper bounds of the gasification kinetics, two different target temperatures, 850°C and 1000°C, were selected, with a CO2 concentration of 80%. The TGA experimental setup can be seen in Figure 2-11.

***Figure 2-11***
***TGA Experimental Setup***

## Results

Thermogravimetric analysis (TGA) was conducted to evaluate the thermal decomposition and gasification potential of the biomass. The results, as presented in Table 2-10, indicate that the biomass possesses a suitable composition of volatiles, char, and ash for gasification. This composition is crucial to produce syngas.

**Table 2-10**
**Biomass Composition from TGA Analysis**

| **Parameter** | **850 C** | **1000 C** | **Unit** |
|---|---|---|---|
| Initial Weight | 21.34 | 22.55 | g |
| Post devolatilization | 7.05 | 6.88 | g |
| **%Volatiles** | **66.96** | **69.49** | **%** |
| Post gasification | 2 | 2.28 | g |
| **%Char** | **23.62** | **20.40** | **%** |
| Final wt | 2.01 | 2.28 | g |
| **%Ash** | **9.42** | **10.11** | **%** |
| **Time for char conversion** | **80** | **10** | **min** |

The TGA experiments at 850°C and 1000°C revealed significant differences in gasification kinetics. At the lower temperature of 850°C, as shown in Figure 2-12, complete gasification of the biomass was achieved in approximately 80 minutes. In contrast, the experiment at 1000°C, shown in Figure 2-13, demonstrated a much faster reaction rate, with complete gasification occurring in just 10 minutes. This significant reduction in gasification time at 1000°C is attributed to the accelerated reaction kinetics at the higher temperature.

Crucially, the proximate analysis values for volatiles, char, and ash were found to be consistent across both temperature profiles, which validates the experimental results and confirms that the observed differences in gasification time are solely a function of temperature-dependent reaction kinetics rather than variations in the biomass composition.

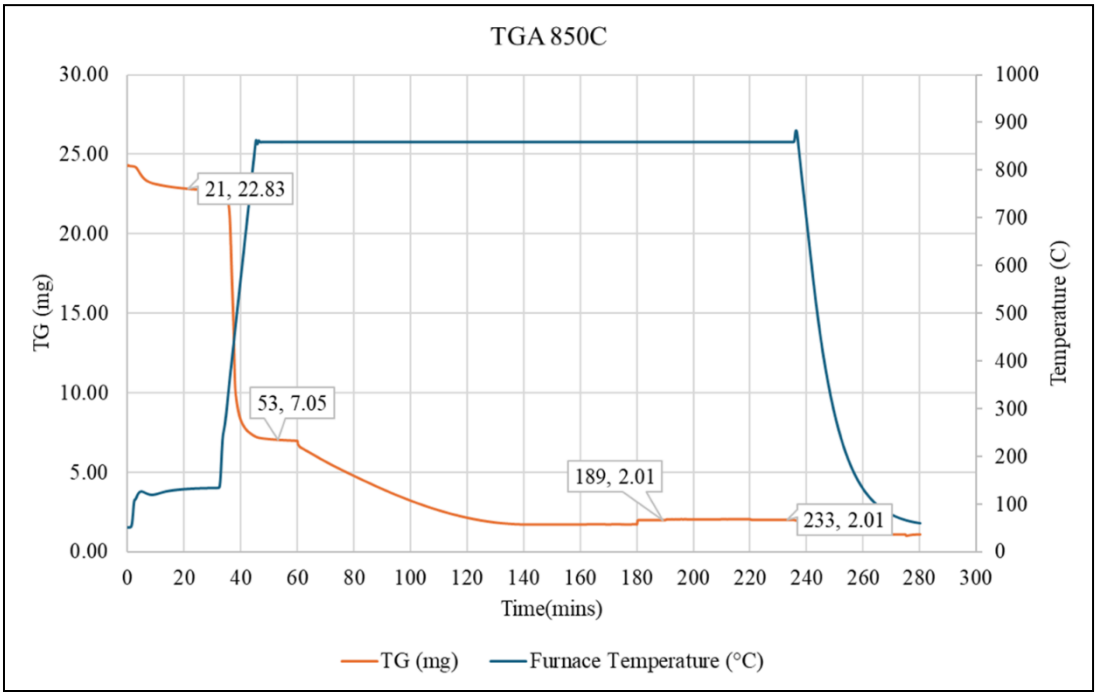



**Figure 2-12**
**Results from TGA Experiment performed at 850 ºC**

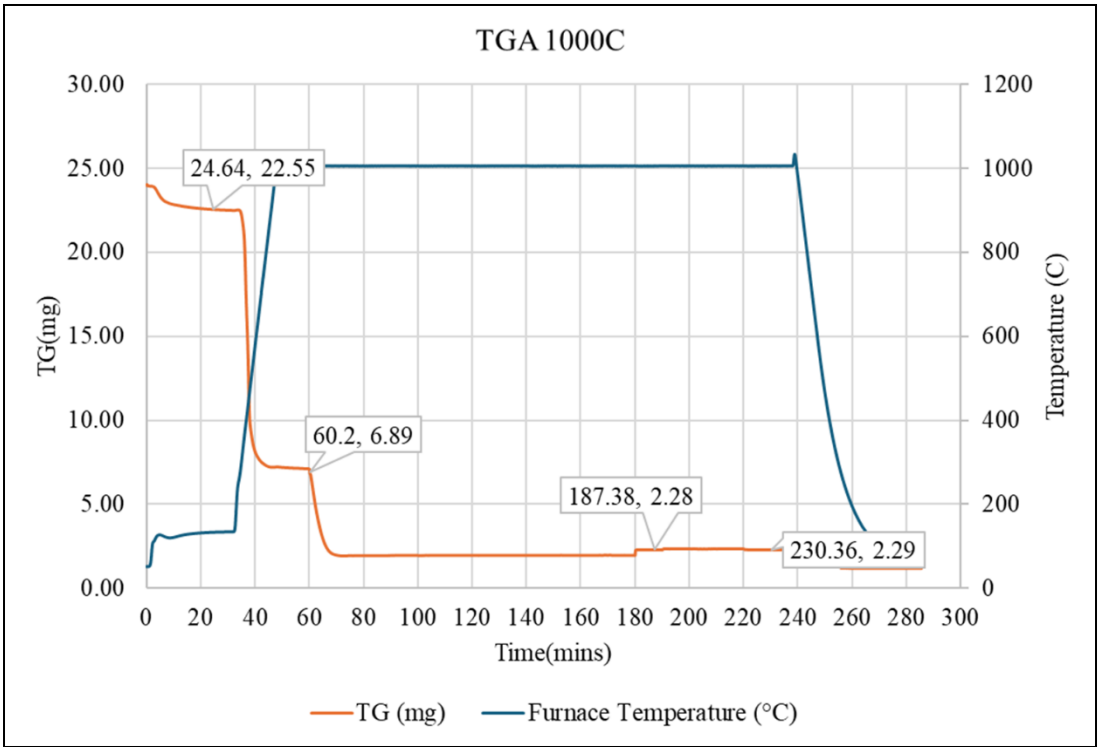



**Figure 2-13**
**Results from TGA Experiment performed at 1000 °C**

The experimental results of the moving bed reactor, as shown in Figure 2-14, demonstrate a successful and stable steady-state operation for 60 minutes. During this period, the syngas purity was consistently maintained between 70% and 80%. This finding serves as a key validation of the moving bed reactor technology's effectiveness in converting biomass into a valuable product.

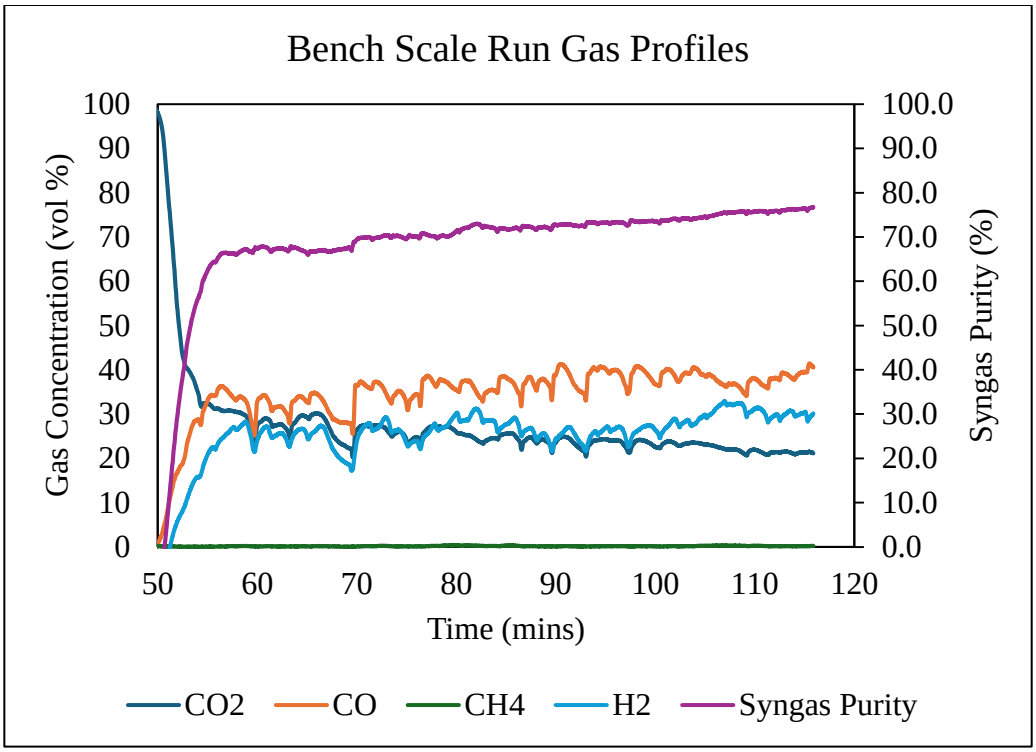



**Figure 2-14**
**Experimental result of the moving bed reactor for a steady state operation of 60 minutes**

## Conclusion

The study's findings, based on both thermogravimetric analysis (TGA) and bench-scale reactor experiments, provide strong evidence of this perennial grass slurry's suitability and the process's efficiency. ***The TGA results showed that the slurry's composition of volatiles, char, and ash is well-suited for gasification***. Most importantly, the TGA proved that the biomass is capable of rapid conversion, with ***complete char gasification occurring in just 10 minutes at a temperature of 1000°C.***

Further, the bench-scale reactor validated the process's practical application, achieving stable steady-state operation for an extended period. During this time, the biomass was consistently and effectively transformed into a valuable product, with syngas purity maintained between 70-80%. This confirms the biomass's capability to be converted into a valuable, high-purity syngas stream.

In summary, the results from this study confirm that the BTS process can efficiently convert perennial grass slurry biomass into syngas, ***highlighting post-hydrolysis perennial grass slurry biomass as an effective and potentially profitable feedstock for producing valuable fuels and chemicals.***

## Synergism with Simultaneous Ball Milling/Enzyme Hydrolysis Process

These results have three important impacts on using simultaneous ball milling and enzyme hydrolysis with perennial grasses to produce cost-effective fuels and chemicals.

1. Simultaneous ball milling and enzyme hydrolysis of perennial grasses produces a high purity feedstock for syngas production without any additional pretreatment costs.

2. Producing fuels and biochemicals with perennial grass slurry through the Biomass to Syngas (BTS) chemical looping process eliminates the inefficiencies of oil product pyrolysis of harvested perennial grasses.

3. Combined perennial grasses-to-ethanol-to-fuels & chemicals and BTS syngas production utilizes up to 80 percent of total perennial grass biomass.

## Chapter 3: Prototype Development

### Section 3.1 Economic Goals and Design Objectives

Before going into the technical aspects of prototype design, the economics of liquid fuel production from petroleum and biomass need to be discussed.

Annual average Crude oil prices have ranged from $39 to $97 per barrel over the past 10 years. At crude oil prices below $80/barrel, which they have been for most of 2024 and 2025, liquid fuel production is a low margin business. The petroleum industry, which controls the majority of liquid fuel production, is operating at a price point that is precluding significant capital investment. With a crude oil input price of about $60/barrel, and a 2/3 gasoline and 1/3 SAF or similar high value fuel production mix, about $0.57/gallon of gasoline of income is available for refining as well as all additional expenses (Table 3-1).

**Table 3-1**
**Current (2025) Crude Oil Economics**

| | |
|---|---|
| Crude Oil Price/Barrel | **$60.00** |
| Gasoline Wholesale Income (20 gallons @ $1.85/gal) | $37.00 |
| SAF Wholesale Income (10 gallons @ $4.00/gal) | $40.00 |
| Total SAF and Gasoline Income/Barrel | **$77.00** |
| Net Profit for Processing Costs/Barrel | $17.00 |
| Money Available for New Investments, etc./gallon | **$0.57** |

Hence, petroleum business organizations and strategies aim at different ways to maximize profits by manipulating source and supply and by minimizing costs for production and sales.

Biofuels that compete with, and also complement petroleum derived fuels, rely on the production of multiple valuable products in order to survive in the current economic conditions. The primary biofuel production industry is corn ethanol. As shown in Table 3-2, the production of ethanol alone from corn kernel starch would currently be a money loser (line 9). However, the production of ethanol results in the production of two valuable byproducts, distillers grains and CO2. Distillers grains, (sold Dry distllers grains, and locally as wet distillers grains) is a high protein animal feed. When the value of this byproduct is added to the value of ethanol the integrated production of both produces a decent profit even when ethanol sells for around $1.76/gallon wholesale (Table 3-2).

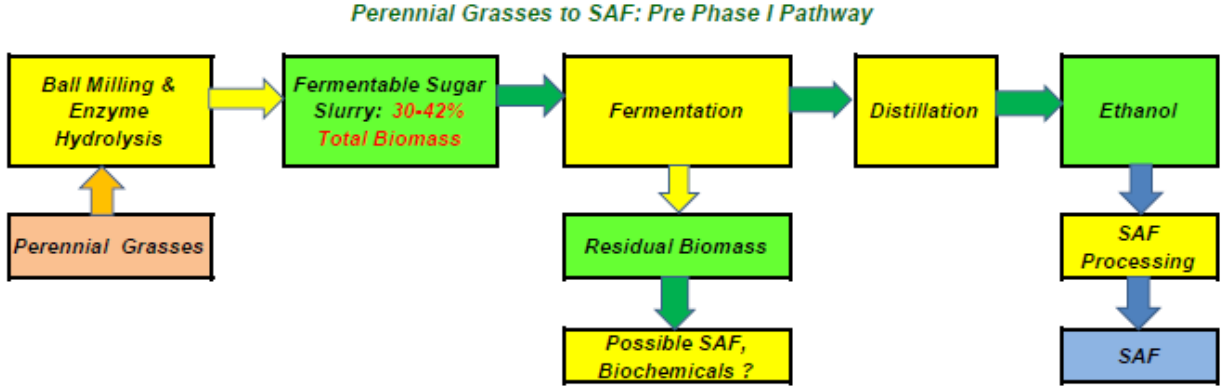
**Table 3-2**
**Corn Kernel Ethanol Profit/Loss With/Without DDG Production**

| | | Corn Ethanol alone | Corn Ethanol With Dried Distillers Grains |
|---|---|---|---|
| 1 | Ethanol Gallons/Bushel Corn | 2.8 | 2.8 |
| 2 | Ethanol Gallons/Acre | 500 | 500 |
| 3 | Ethanol Wholesale Price/Gallon | $1.76 | $1.76 |
| **4** | **Ethanol Income/Acre** | **$880.00** | **$880.00** |
| | | | |
| 5 | Corn Price/Bushel | $4.50 | $4.50 |
| 6 | Corn Price/Acre @179 Bushels | $805.50 | $805.50 |
| 7-a | Ethanol Refining Costs/gallon | $0.95 | $0.95 |
| 7-b | Ethanol Refining Costs/Acre | $475.00 | $475.00 |
| **8** | **Total Ethanol Production Costs/Acre** | **$1,280.50** | **$1,280.50** |
| | | | |
| **9** | **Net Ethanol Income/Acre** | **($400.50)** | **($400.50)** |
| | | | |
| 10 | Dried Distillers Grain: $1.30/ bushel = to $3.64/gal ethanol | | $3.64 |
| **11** | **DDG Income/Acre** | | **$1,820.00** |
| **12** | **Net Income: DDG & Ethanol/Acre** | **($400.50)** | **$1,064.70** |

## Implications for Perennial Grass to SAF/Bioproduct System Design

To produce SAF or similar valued bioproducts from biomass economically, similar strategies will have to be used since the pathway proposed before the beginning of this Phase I project (Figure 3-1) does not provide enough potential per/ton or per/acre income to compete with petroleum derived SAF. To maximize the value of biomass conversion to SAF, the amount of carbohydrates utilized must be maximized. Specifically, only about 42 percent of total biomass consists of carbohydrates and half of these are non glucose compounds strategies must be implemented to convert the non glucose sugars (Xylose and arabinose) and to utilize the residual biomass consisting of non-fermentable components like lignin and proteins. Furthermore, carbohydrate conversion must ensure that unfermentable oligomers and cellobiose do not accumulate and that they are converted to monomeric fermentable sugars. Cellobiose values of up to 30 percent of glucose values were observed in Phase I (Table 1-18).

**Figure 3-1**
**Pre-Phase I Proposal**



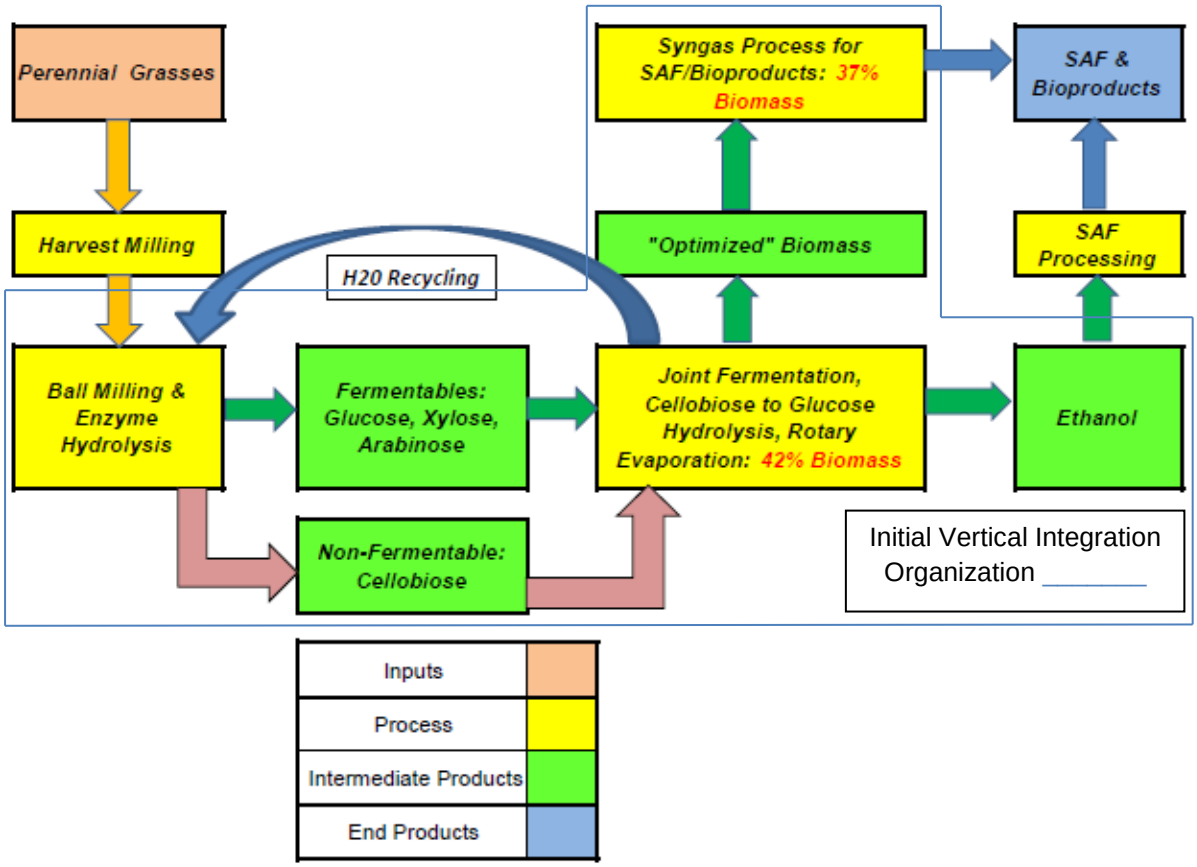

Fortunately, the results and discoveries made in Phase I allowed us to develop a new system that could increase biomass use to about 79 percent without adding biomass cleaning or gas scrubbing procedures. This is accomplished by:

- Integrating cellobiose to glucose enzyme conversion with slurry fermentation (Section 2.1)
- Using ball milling/hydrolysis, fermentation, and ethanol distillation processes developed in Phase I to produce a monomeric sugar free biomass that can produce low contamination syngases for bioproduct/SAF production (Section 2.2).
- Vertically integrating key processes into one organization.

Figure 3-2 summarizes the organization and operational flow of this “Twin Pathway” Bioproducts/SAF system.

**Figure 3-2**

*Twin Pathway Bioproducts/SAF Production*




The functional integration of ball milling/hydrolysis with fermentation/distillation and syngas production argues for a vertical business organization of these three operations (blue line in Figure 3-2).

## Estimated "Twin-Pathway' Economics

In the "Twin-Pathway" system, the production of syngas is analogous to the production of DDGs in the corn kernel to ethanol system in that it could increase income. As shown in Table 3-3, the combination of ethanol-to-SAF and biomass syngas to SAF and/or bioproducts could produce enough net income for the system to be economically viable without subsidies.

**Table 3-3**
**P/L Estimates of Ethanol to SAF and Twin-Pathway Systems**

| | | Pre-Phase I Model | Twin Pathway with Syngas |
|---|---|---|---|
| 1 | SAF from Ethanol: Gallons/Acre | 280 | 280 |
| 2 | SAF Wholesale Price | $4.25 | $4.25 |
| **3** | **SAF Income/Acre**[2] | **$1,190.00** | **$1,190.00** |
| | | | |

| | | | |
|---|---|---|---|
| 4 | Grass Price/Acre[1] | **$500.00** | **$500.00** |
| 5 | Ethanol & SAF Refining Costs/Gallon SAF | $1.80 | $1.80 |
| 6 | Ethanol & SAF Refining Costs/Acre | $504.00 | $504.00 |
| **7** | **Total Production Costs/Acre SAF** | **$1,004.00** | **$1,004.00** |
| | | | |
| **8** | **Net SAF Income/Acre** | **$186.00** | **$186.00** |
| | | | |
| 9 | Syngas Production Tons/Acre | | **3.7** |
| 10 | Syngas (gallons equivalent)/Acre | | 607 |
| 11 | Syngas Wholesale/Gallon | | **$4.00** |
| **12** | **Syngas Income/Acre** | | **$2,429** |
| 13 | Syngas Production Costs/Acre | | 516.12 |
| 14 | Net Syngas SAF Income | | **$1,913** |
| 15 | Net Income Ethanol and Syngas SAF/Acre | **$186.00** | **$2,098.68** |

[1] *Current low-scale switchgrass for animal bedding prices run $250-300/acre*.

[2] *SAF prices are used to represent the value of the fuel and related biochemical products. All attempts have been made to present estimates based on published prices and Phase I production estimates. Calculations for some production estimates used proprietary information that can be shared under proper agreements.*

The Twin Pathway approach has an additional benefit for enzymes costs. While their cost/gallon for ethanol to SAF may be high, these same enzymes would significantly improve syngas quality and reduce production costs because carbohydrate hydrolysis and removal is also the first step in biomass preparation for syngas productions. This is a task that would have to be paid for in a biomass syngas system operating separately from a Twin-Pathway entity.

## Section 3.2 Testing of Prototype Continuous Flow Parameters

### Continuous Milling/Enzyme Hydrolysis Runs

To meet the economic cost estimates listed above, commercial simultaneous ball milling/enzyme hydrolysis needs to minimize down time to unload sugar rich slurries and add new biomass, buffers, and enzymes. This is primarily achieved by establishing a continuous process based on maintaining consistent input/output quantities. Such a process would maintain standardized unloading/loading cycles that could be operated with minimal cost inputs.

Three Phase I runs were dedicated to testing continuous operational concepts. These runs included monitoring hydrolysis inhibition effects and loading capacity that limit conversion during continuous simultaneous ball milling/enzyme hydrolysis. These three runs were:

- Daily Loading and Unloading 20 Percent of the biomass and slurry capacity for Ten Days,
- Daily Loading and Unloading of 50 Percent of the biomass and slurry capacity for Six Days, and
- Loading and Unloading more than 50 Percent of the biomass and slurry capacity in a commercial 2ft.$^3$ Orbis Machinery Ball Milling unit.

**Overcoming Ball Milling Operating Conditions That Negatively Affect Continuous Operation**

To achieve continuous operation, we adapted a fed batch fed system into something that performs like a continuous counter-flow feed/removal system. This is a hybrid batch fed system with periodic loading and unloading.

From our initial Phase I work we discovered that two processing objectives had to be determined before testing a hybrid batch-feed system could begin. These were:

1. Determine the maximum amount of biomass that could be processed as a function of ball milling vessel volume, and

2. Determine the maximum amount of processed biomass slurry that could be periodically (ideally every 24 hours) removed and replaced with a similar amount of unprocessed biomass and water without interfering with ball milling and enzyme hydrolysis.

The equation for this is:

- Maximum volume of Biomass Processed: **A**

- Maximum volume of processed biomass (slurry) removed or biomass added: **B** = x % A

- Maximum efficiency is where B > .8A

**5 Liter Maximum Biomass Loading and Maximum Loading/Unloading Volumes**

Phase I experiments showed that the maximum volume of a ball milling vessel available for loading of biomass and liquids was about 50 percent of total volume. This is called the working volume. The other 50 percent volume was filled with milling balls.

Thus in a 5 liter ball milling vessel, approximately 2.5 liters would be available. This would be **A**. From earlier runs, the maximum biomass loading (biomass to liquid ratio) was about 15 percent. This translates into 400 g biomass.

**Table 3-4**
**Maximum Calculated Biomass Loading**

| Milling Container | Capacity of Milling Container (Liters) | Maximum Liquid and Biomass (Liters) **A** | Biomass (grams) | % Biomass Loading |
|---|---|---|---|---|
| MSE 5 liter milling jars | 5 liters | 2.5 | 400 | 14.8% |

What was also discovered was that in attempting to measure **B** the total of biomass and liquid that could be added or removed could NOT be loaded all at once into the 5 liter vessel. In fact loading just 100 grams of biomass with 650 ml of $H_2O$ (15.4 percent), instead of the standard 75 grams of grasses and 500 ml $H_2O$, caused clogging in the 5 liter vessel (Figure 3-3).

**Figure 3-3**
**100 g Mixed Grasses with 650 ml $H_2O$ after 24 Hours**
**9 January 2025**

As a result, the loading of the 5 liter vessels for the first steady-state run in February 2025 was done in a series of 75 g grasses/500 ml $H_2O$ additions. This meant it took five days to load the 5 liter vessel to its maximum working volume of 2500 ml liquid and 375 g mixed perennial grasses.

Because of these clogging issues, the initial value for **B** was selected as 75 g biomass/500 ml liquid. So B = 0.2 (500/2500) x A.

**10 Day 20 Percent Load/Unload Results**

The daily input loads for two (Sample A and Sample B) 5 liter MSE milling vessels consisted of:

- 37.5 grams dry switchgrass and 37.5 grams dry phragmites
- 500 ml DDI $H_2O$
- Novozyme CTec cellulase
- ALI 150 hemicellulase
- Different enzyme dosages were used in A and B to measure dosages effects. No buffers were added

Three variables were measured:

- Daily sugar production with HPLC
- Daily pH with a pH meters
- Daily cellulase activity with a Megazyme Enzyme Cellulase G5 assay kit.

Removal of the 500 ml of slurry was relatively easy because of its low viscosity. It could be removed with automotive hand-pumps (Figure 3-4) without resorting to the water pressure misting approach.

**Figure 3-4**
**Automotive Oil Hand-Pump**

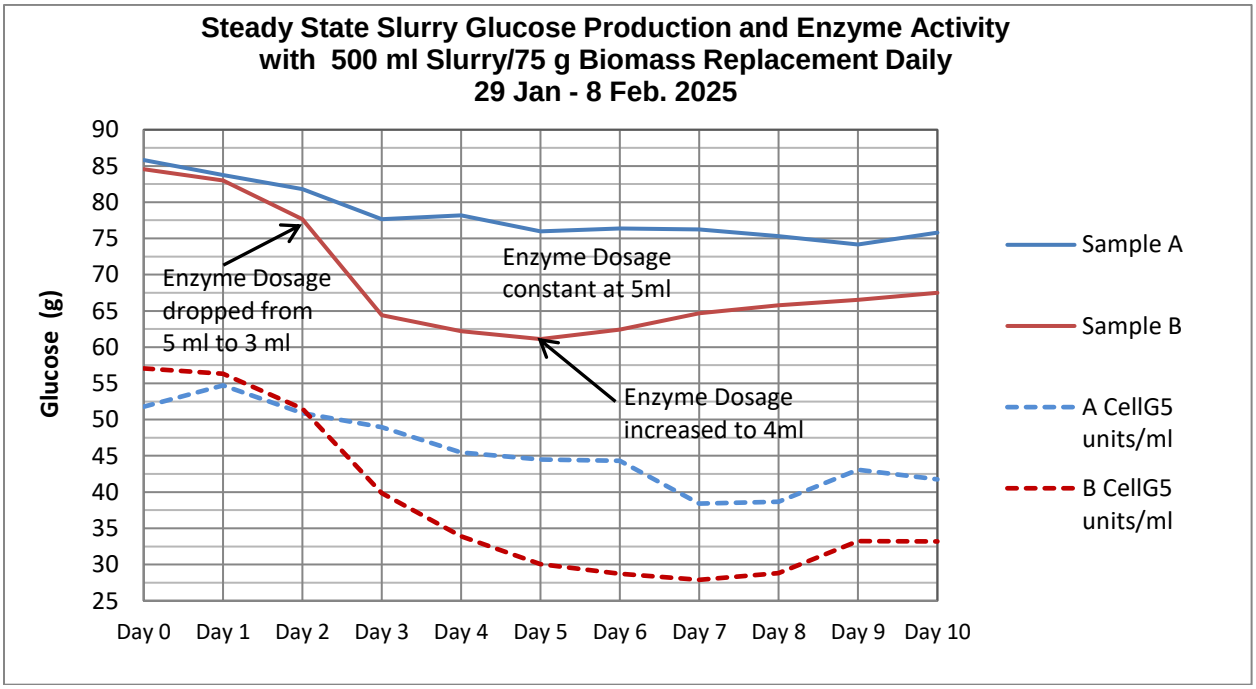
Biomass that measured over 10 mm (meaning it wasn't completely ball milled), was about 1-2 percent of the 75 grams each day.

Enzyme loading was investigated by changing the CTec enzyme dose each day between 3 and 5 ml. As shown in Figure 3-5a, glucose production in Sample B fell off when the daily cellulase dosage dropped from 5ml CTec2 to 3 ml CTec2 (from 48.5 % cellulose to glucose to 38.8%) and partially came back when it was increased to 4 ml/day. In Sample A, it slowly fell while the 5 ml CTec2 dosage remained constant.

These results, however, were not consistent with the CellG5 results. Note that for both Samples A and B CellG5 concentrations fell off after Day 2 when the cellulase dosage was only lowered in Sample A. However, while the activity dropped in Sample A, glucose production did not. This may indicate that the maximum enzyme activity recorded was in excess of what was a sufficient dosage to continuously produce glucose.

As for glucose production in Sample B, it did come back on Days 6 when cellulase dosage was increased. The CellG5 value started to increase after Day 8.Could there be another inhibitor that delayed CellG5 activity?

**Figure 3-5a**
**Continuous Run 20% Unload/Load**

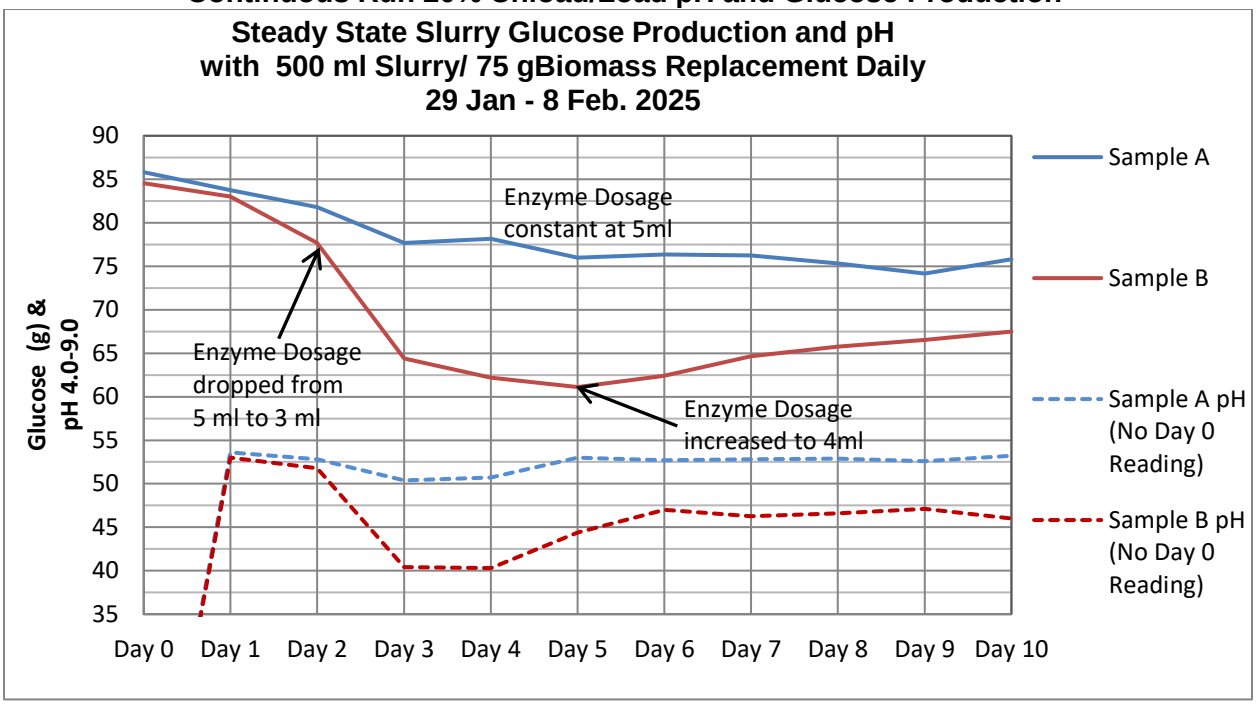



In Table 3-5b the sugar production values are compared to the pH of the slurry. The key pH value is 5.0. Generally, when biomass deconstruction enzymes fall into this range they lose much of their enzyme activity. Note that after Day 2, the pH fell for Sample B from about 5.2 to 4.0. This also tracks with the loss of CellG5 activity. Then on Day 4 it started to increase and tracked with the increased dosage, glucose production, and CellG5 activity.

In Sample A the pH stayed above 5.0 and tracked with glucose production so there does not appear to be much loss of activity due to the lack of pH control.

The source of the free hydrogen lowering pH is probably the production of acetic acid and other organic acids from hemicellulose and pectin hydrolysis.

**Figure 3-5b**
**Continuous Run 20% Unload/Load pH and Glucose Production**




## Lessons Learned from 20 Percent Working Volume Unload/Run Continuous Run

Lessons learned from this 20 percent total working volume continuous ball milling/enzyme hydrolysis run were:

- Slurry at 20 percent of the total working volume (500 ml of 2500 ml) can be removed without the air/water pressure process.

- The 24 hour cycles were sufficient to convert +95% of biomass to particles in the sub millimeter range.

- Slurry pH values of < 5.0 appear to be significant enzyme activity inhibitors. In Sample B the conversion rate of cellulose to glucose fell from 51.1% to 38.8% when pH fell from about 5.2 to 4.0.

- The input biomass solution should be buffered to about 6.5-7.0 pH before the run begins and maintained throughout the run.

- HPLC sugar, organic acid, and ethanol monitoring should be conducted a minimum of every 24 hours.

- Continuous pH monitoring, or at least every 6 hours, is required.

- Automated buffering during the run with a low-cost buffer such as calcium carbonate or acetate is necessary for multi-day continuous processing runs.

**6 Day 50 Percent Load/Unload Test Conditions**

There were two primary objectives of this 2nd continuous processing run:

- Determine maximum unload/load quantities **B**, and
- Compare CTec2 and ALI enzyme activity at similar enzyme activity concentrations.

Three variables were measured:

- Daily sugar production with HPLC,
- Daily pH with pH meters, and
- Daily cellulase activity with a Megazyme Enzyme Cellulase G5 kit.

Maximum Unload/Load Quantities

Going back to Table 3-4, the maximum working volume, **A,** of the 5 liter MSE vessels was thought to be about 2500 ml. However, in testing the total volume not occupied by milling balls (A) was about 2,700 ml. This meant that total biomass could be 400g (15% of 2,700). To determine the maximum amount of slurry that could be removed and replaced (maximum-B), a starting B value of 0.5 x A was selected. If this worked, the amount of daily processed biomass could be raised to 200g of perennial grasses.

As in the earlier 20 percent continuous run, the total of 400 grams of biomass could not be loaded all at once because of clogging issues. Four days of loading was required to reach the 400 grams of perennial grasses.

Once loaded and continuous operation started, the daily input loads that replaced the removed slurry for each of the two (Sample A and Sample B) 5 liter MSE milling vessels consisted of:

- 100 grams dry switchgrass and 100 grams dry miscanthus,

- 1300 ml DDI $H_2O$,
- CTec2 cellulase, and
- ALI 150 hemicellulase (Samples A and B)
- Cellulase dosages from ALI and CTec2 were calculated to deliver similar mg enzyme/g cellulose concentrations in each samples.

**From Table 1-14**

| | ALI Cellulase (ml) | CTec2 Cellulase (ml) |
|---|---|---|
| 7. Enzyme mg/g biomass | **32** | **30** |
| 8. Enzyme mg/g cellulose | **79.2** | **75.0** |

This slurry would be screened at 1 mm (Figure 3-6) prior to removal every 24 hours of processing. This screening was done to retain biomass that was not yet fully processed for additional ball milling size reduction and enzyme hydrolysis.

**Figure 3-6**
**MSE 5L Slurry Removal Screen**

**Results**

To interpret these 50 percent unload/load continuous results, three sets of measurements were used:

- Biomass: > 1mm biomass remaining after unload/load cycles,
- Hydrolysis Outputs: Monomeric sugar, acid, and ethanol from enzyme hydrolysis, and
- Slurry pH: pH was measured in unloaded slurries.

**Ethanol Production:**

As reported in Section 1.7, negative control samples had sugars and acetic acid when analyzed by RID HPLC. These samples were called T0 samples since they were taken after the enzymes were processed for less than two hours. After 24 and 48 hours ethanol was also detected in some of the hydrolysis samples. These values were taken into account when calculating feedstock conversion.

Table 3-5 shows ethanol concentrations measured from slurry samples at T24, T48 and T72, of the continuous run. Note that ethanol was first identified at T24 for the ALI enzymes (Sample A) while it did not show up until T72 when CTec2/ALI hemicellulase was used (Sample B). Also note that the combined ALI cellulase and hemicellulase enzymes produced higher ethanol concentrations. Since ethanol production is directly related to the concentration of ethanol fermenting sugars and the presence of fermentative microorganisms, this probably indicates that both the ALI cellulase and hemicellulase enzymes in Sample A were contaminated with a fermentation microorganism, while in Sample B, the ALI hemicellulase was and the CTec2 cellulase was not.

**Table 3-5**
**Ethanol Concentrations in 50 % Unload/Load Continuous Run**

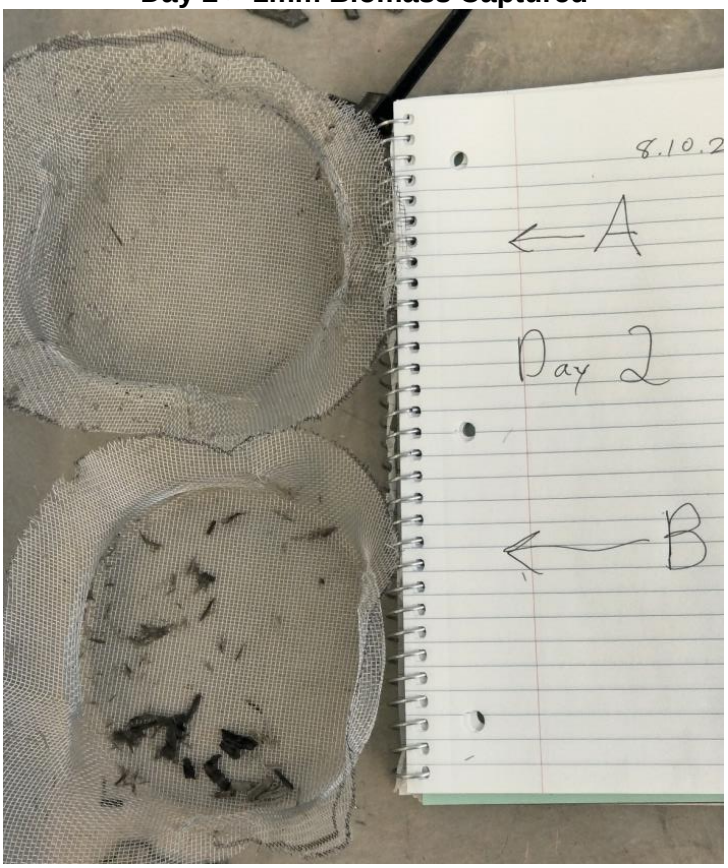
| | ALI T24 | CTec2 T24 | | ALI 48 | CTec2 T48 | | ALI 72 | CTec2 T72 | | ALI Day 1 | CTec2 Day 1 | | ALI Day 2 | | CTec2 Day 2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ethanol | **3.4** | **0** | | **3.4** | **0** | | **4.4** | **3.13** | | **3.08** | **3.03** | | **5.10** | | **2.52** |

Did this ethanol production inhibit the process or effect either biomass deconstruction or monomeric sugar production?

**Biomass Deconstruction**

Figure 3-7 shows the amount of biomass captured by the 1 mm screens between Day 1 and Day 2. Note that Sample A had virtually no biomass greater than 1 mm.

**Figure 3-7**
**Day 2 > 1mm Biomass Captured**




However, by Day 3 the amount of biomass > 1mm increased significantly (Figure 3-8) and by the end of the 6 day run, > 1mm biomass greatly exceeded the 1 percent generally seen in earlier runs (Figure 3.9).

**Figure 3-8**
**Samples A & B Day 3 AND 6 Biomass > 1mm Captured**

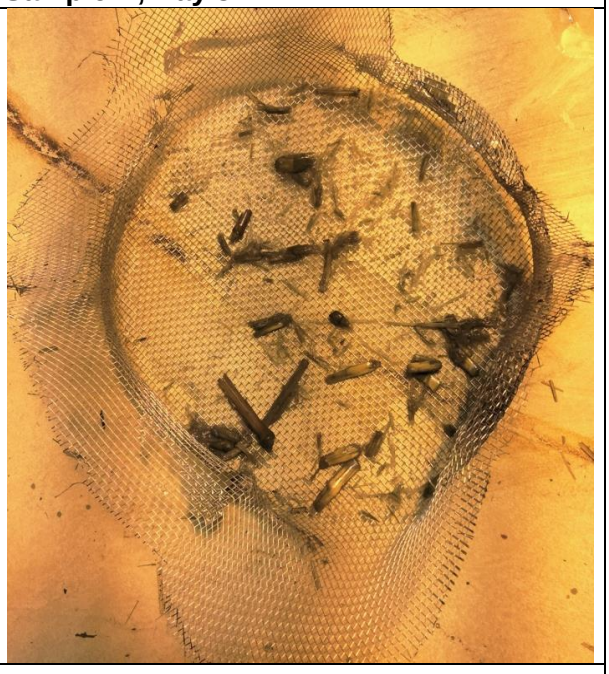
| Sample A, Day 3 | Sample B, Day 3 |
|---|---|
|  |  |

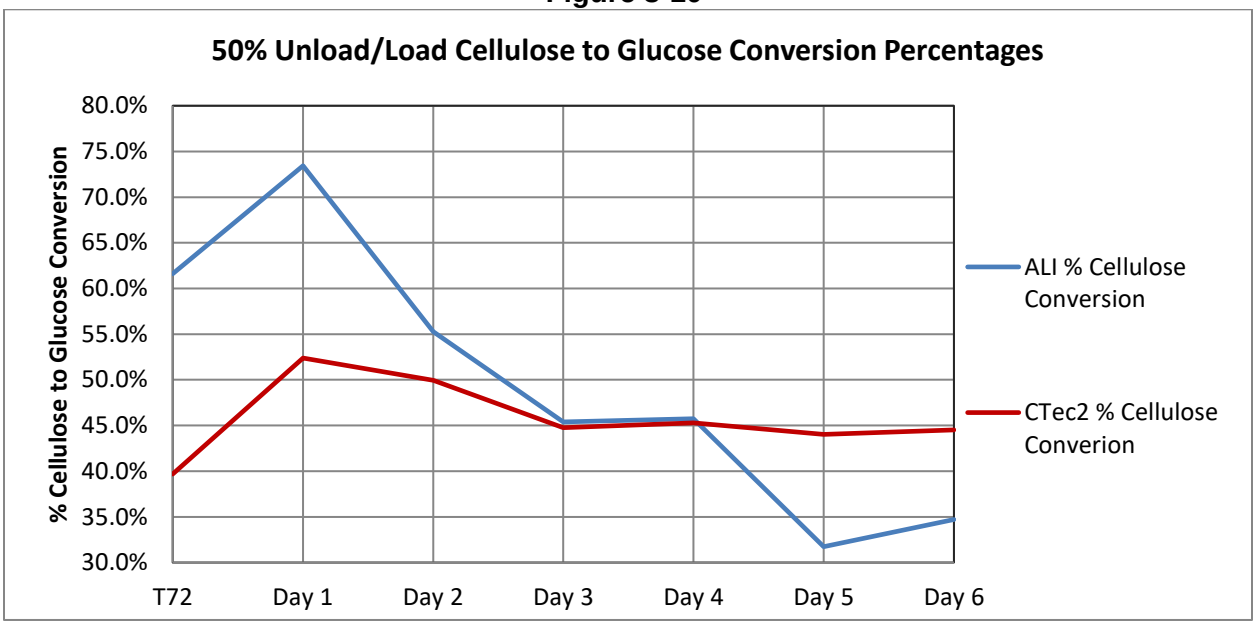
**Figure 3-9**
**Samples A & B After Day 6 Biomass > 1mm Recovered**

| Sample A, Day 6 | Sample B, Day 6 |
|---|---|
|  | |

**Effect of Ethanol Production and pH Concentration on Biomass Deconstruction and Monomeric Sugar Production**

The daily percentages of cellulose to glucose conversion (Figure 3-10) generally track with the increases in unprocessed biomass, shown in Figures 3-7, 3-8, and 3-9.

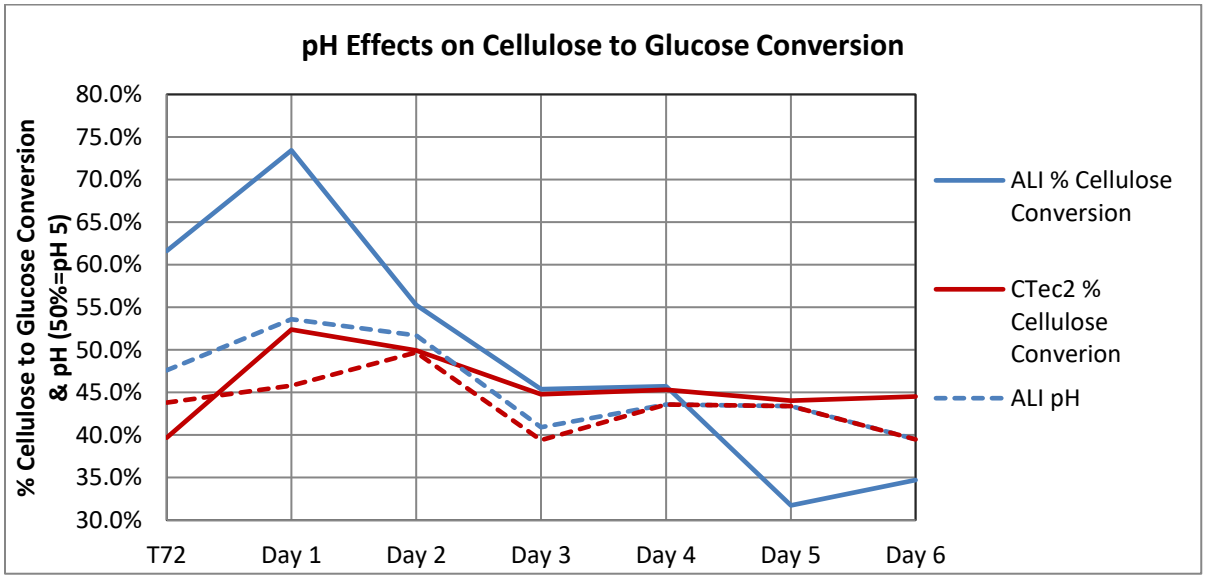
**Figure 3-10**




Was the production of ethanol directly responsible for the decreases in sugar conversion or did ethanol production set off another effect?

Figure 3-11, shows that the decreases in cellulose to monomeric sugar conversion rates coincide with decreases in pH concentrations below 5.5.

**Figure 3-11**



As for the source of the lower pH, in addition to acetic acid produced during hydrolysis, the production of ethanol is the probable cause. This acidification is due to carbonic acid production from $CO_2$ produced during ethanol fermentation.

**Effect of pH on Biomass Hydrolysis Enzyme Activity**

Since decreases in cellulose or hemicellulose hydrolysis are caused by decreases in enzyme activity, measurement of cellulase or hemicellulase activity would verify if pH concentrations have a direct effect. As discussed in the 20 percent unload/load results section, biomass deconstruction and hydrolysis enzymes lose most of their enzyme activity when pH decrease below 5.5 to 5.0. These values are included in enzyme producer fact sheets. During the 20 percent unload/load run enzyme activity values were not continuously monitored.

During the 50 percent unload/load run, the Megazyme CellulaseG5 assay was used to measure for cellulase activity in slurries samples taken before unload/load procedures. Samples were taken every 24 hours. Figures 3-12-a and 3-12-b, show the correlations between decreases in pH concentrations and CellG5 activity.

**Figure 3-12-a**

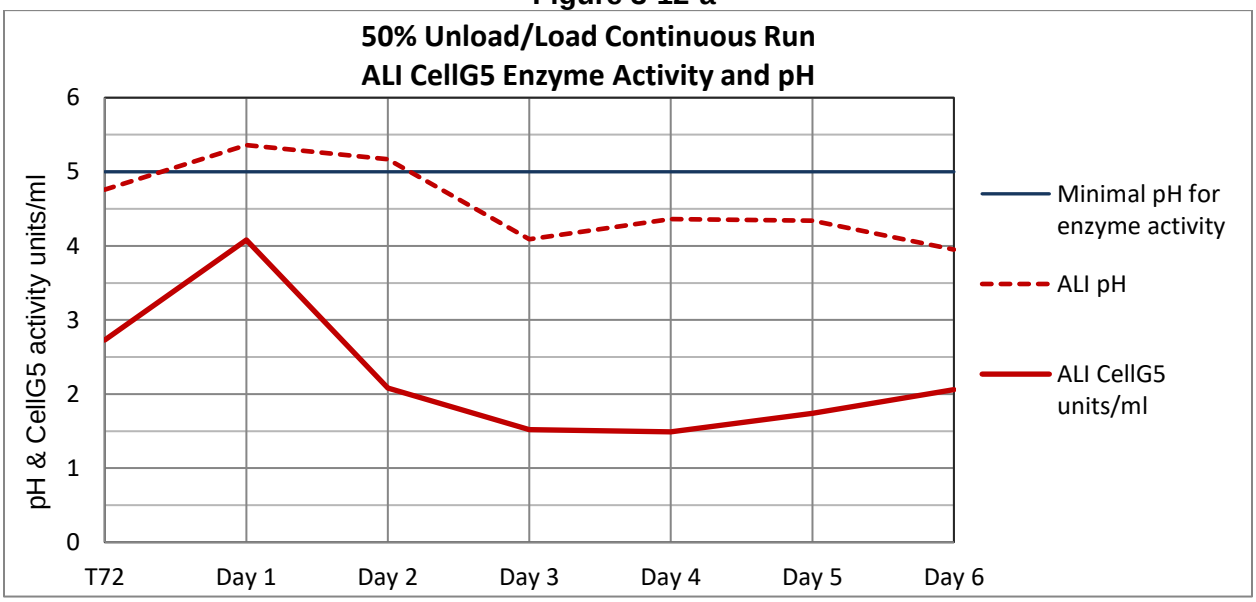
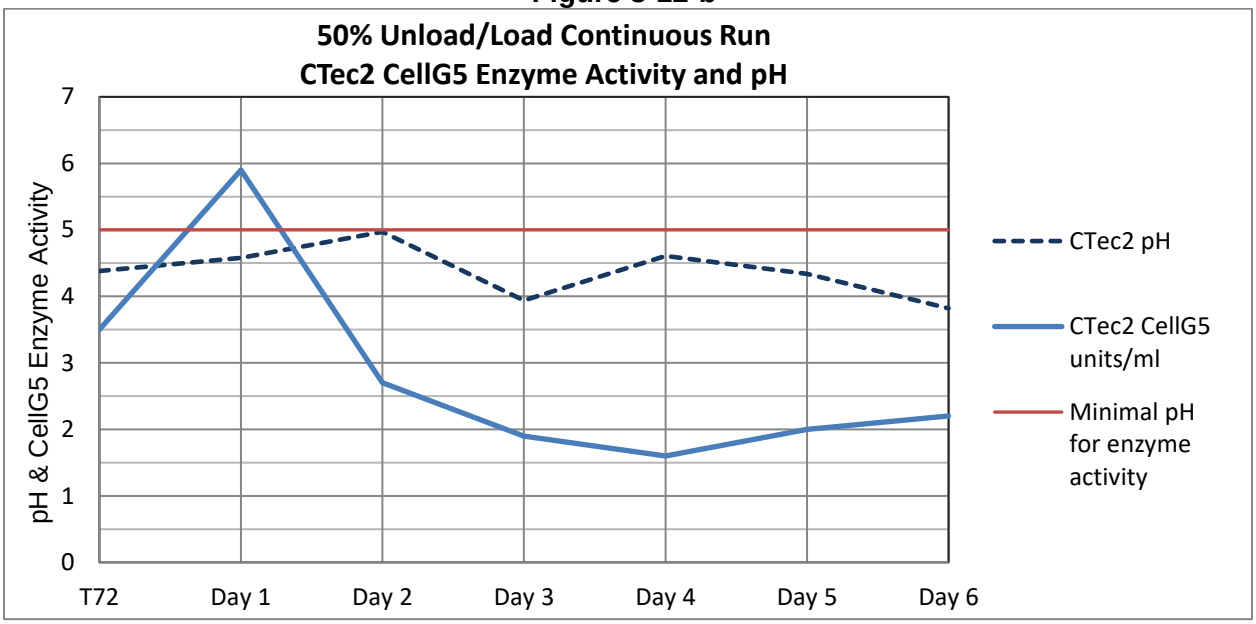


**Figure 3-12-b**




What is especially noteworthy in these graphs is that the enzyme activity (CellG5 units/ml slurry) decreased so significantly even though the enzyme concentrations (enzyme mg/g cellulose) in the slurries remained the same (Table 3-6). This argues for continuous pH monitoring and automated pH control and use of a buffer in the initial mixes.

**Table 3-6**
**Enzyme Activity Loss Due to pH Decline Below Minimum pH Levels**

| | ALI Cellulase | | CTec2 Cellulase | |
|---|---|---|---|---|
| | **79.2** mg/g Cellulose | | **75** mg/g Cellulose | |
| Sample | CellG5 unit/ml | % Maximum (Day 1) | CellG5 unit/ml | % Maximum (Day 1) |
| Day 1 | 408 | | 59 | |
| Day 2 | 208 | 51.0% | 27 | 45.8% |
| Day 3 | 152 | 37.3% | 19 | 32.2% |
| Day 4 | 149 | 36.5% | 16 | 27.1% |
| Day 5 | 174 | 42.6% | 20 | 33.9% |
| Day 6 | 206 | 50.5% | 22 | 37.3% |

In addition, because the pH concentration and enzyme activity curves track fairly well, lower cost continuous pH testing can be used instead of the more expensive enzyme assays for the purpose of signaling the need for pH adjustment during prototype and commercial runs. A low cost pH buffer could also be added along with the enzymes at the beginning of the experiment.

**Lessons Learned from 50 Percent Working Volume Unload/Load Continuous Run**

- About 50 percent of total working volume of the 5 liter ball milling vessel could be daily unloaded and loaded. Values of B = 0.5 x A could be used in later runs.

- A cellulose to glucose conversion rate of about 70 percent (including cellobiose production) can be achieved, but to maintain it over multiple days requires the continuous monitoring and adjusting of operational parameters. Specific lessons that were learned include the following.

- To maintain this level of production, enzyme activity needs to be maintained near the maximum possible for a specific enzyme concentration.

- To maintain enzyme activity, a slurry pH level sufficiently above the minimum enzyme activity pH value of 5.0-5.5 needs to be maintained for the duration of the ball milling/enzyme hydrolysis run. A target pH would be in the 6-.5-7.0 range.

**Continuous Unload/Load Open Issues**

At the completion of the 5 liter continuous unload/load runs there were two unresolved questions;

- What quantity of enzyme activity remains after 24 hour periods that can be recycled in subsequent 24 hour runs?

- Can a working volume (A) greater than 50 percent of total ball milling quantity and an unload/load value of B = 0.5 x A be maintained in a ball milling system designed for commercial use?

**Carry-Over Enzyme Activity**

In the 50 percent unload/load run enzyme activity was to be measured with the Megazyme CellG5 assay. However, with low pH values degrading enzyme activity, those values were not collected. It is planned that early in Phase II, or in the enzyme development work that is scheduled by Atlantic Biomass, a 50 percent load/unload run will be conducted to measure both pH and carry-over enzyme activity. This data will provide detailed enzyme use parameters to determine enzyme development needs and daily enzyme usage and pH data for the selection of buffers and pH control strategies.

**Verifying 5 Liter Data in Commercial Ball Milling Equipment**

A 2ft$^3$ (55.6 liters) commercial dry ball milling unit built by Orbis Machinery was used to scale up and verify 5 liter findings. Results are presented in the following section 3.3.

## Section 3.3 Verifying Phase I Operational Finding in a Commercial Ball Milling Unit

As stated in the title of this Phase I SBIR/STTR award category, **DE-FOA-0003202: Topic 13.b Alternative Use of Commercial Equipment,** Phase I research cannot lose track of the commercial hardware that would be used in a commercial system.

While the important 5 liter findings on enzyme impurities, pH inhibition of enzyme activity, and glucose concentration inhibition of cellobiose to glucose hydrolysis do not need to be verified at a larger scale, key continuous operation parameters need to be developed. Specific operational parameters that would be tested or monitored in the Orbis Machinery 2ft$^3$ (55.6 liters) commercial dry ball milling unit include the following:

- Daily maximum slurry unloading/ biomass and liquid loading quantities,
- Loading/offloading procedures,
- Effects of biomass length to milling vessel diameter ratios,
- Slurry pH and
- Hydrolysis temperature maintenance.

***Suffice to say, this testing in the Orbis 2ft$^3$ unit was very important. Unloading/loading results were unexpected in a positive way. They point to a simplified continuous process with lower operating costs.***

### Characteristics of a Commercial Dry Ball Milling Unit

Orbis Machinery of Waukesha, Wisconsin, is a primary US manufacturer of commercial stand-alone ball milling units. They manufacture units in sizes ranging from 2ft$^3$ to 280 ft$^3$. The design and technology of their units come from the dry milling needed to convert low-grade Minnesota taconite iron into higher iron containing pellets suitable for steel production in Cleveland. Hence their units are very heavy-duty. The 55.6 liter, 2 ft$^3$ unit used for these tests is a stand-alone mill that Orbis and their customers use to test potential feedstocks and processes. While designed for dry milling, it can also handle loading/unloading wet mill biomass. It is built to the same material specifications, i.e. steel selection, wall thickness, as their larger units. While the Orbis ball milling operation is the same as that used in the 5 liter units, there are two major design differences in the Orbis unit that make operations more efficient.

- Biomass Loading/Unloading Access
- Rotation Motor Connection to Milling Container

## Loading/Unloading Access

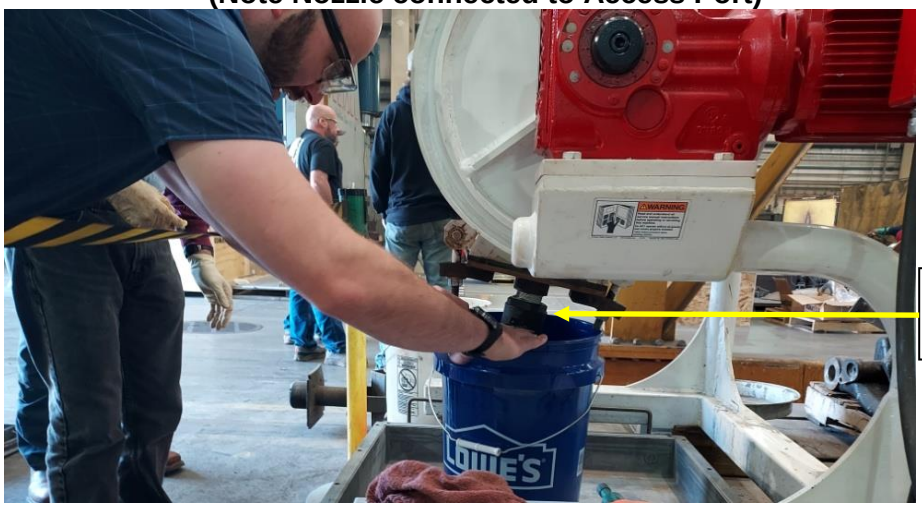
The Orbis units use a side-mounted access port for biomass loading and unloading (Figure 3-13).

**Figure 3-13**
**2ft$^3$ Orbis Stand-Alone Ball Milling Unit**


This simplified arrangement allows for biomass/liquid loading while the milling vessel is spun around with the port pointed up (Figure 3-14 ) and slurry unloading while the port is pointed down (Figure 3-15). Various screening and nozzle configurations can be used for slurry unloading.

**Figure 3-14**
**Loading Biomass in the Orbis Ball Milling Unit**


**Figure 3-15**
**Slurry Unloading**
**(Note Nozzle connected to Access Port)**




For comparison, the 5 liter MSE vessels have to be removed from the roller rig for slurry unloading and biomass loading (Figure 3-16) through end-plate access.

**Figure 3-16**
**Experimental Off-Loading 5 Liter MSE Vessel from Morse Roller Rig**

**Rotation Motor Connection to Milling Container**

While the roller drive used for the 5 liter milling vessels provides the flexibility to test a variety of milling vessel sizes, there is a lack of a continuous friction fit which means energy is lost. Hence, it cannot be used to determine efficient energy use.

The Orbis design incorporates a variable-speed direct drive electric motor that is shaft mounted to the end plate of the milling vessel (Figure 3-17). This design eliminates energy loss due to vessel-to-motor fittings.

**Figure 3-17**
**Checking RPM on Shaft Mounted Direct Drive Electric Motor**

## Operational Parameter Testing

Testing was performed at the Orbis Machinery factory in Waukesha, Wisconsin over a two-day period under a Phase I joint testing contract.

In order to provide a technical context for the results, enzyme loading, milling ball quantities, milling RPM, and the ball milling/enzyme hydrolysis period were based on the August 2025 50 percent unloading/loading run.

Four primary parameters were tested:

- Daily maximum slurry unloading/ biomass and liquid loading quantities,
- Hydrolysis temperature maintenance,
- Loading/offloading procedures, and
- Slurry pH.

## Maximum Slurry Unloading/Biomass Loading: Testing and Results

As discussed above, increasing maximum daily production (B) is the key to reducing commercial system ball milling volume requirements. The main roadblocks to increasing

B > 0.5 x A encountered in the 5 liter continuous runs was the clogging caused by removing/replacing biomass in quantities above B = 0.5 x A.

A potential cause of these limitations could be the ratio between the length of the grass biomass, around 1.5", and the interior diameter of the ball milling vessel. In the MSE 5 liter vessels the ratio of biomass length to interior diameter is about 26 percent. The increased diameter of the Orbis unit reduced the ratio of the biomass length to diameter to 11 percent (Table 3-7). Larger units would further decrease this ratio. This decreased ratio could increase the amount of biomass that could be loaded and processed at one time. Additionally, it could reduce the amount of time needed to mechanically reduce the size of the biomass to provide enzyme access. Together, these changes could increase the value of B to greater than .5 x A.

**Table 3-7**
**Ball Milling Vessel Grass to Diameter Ratio**

| | Vessel Interior Diameter (inches) | Grass Length (inches) | Ratio of Grass Length to Diameter |
|---|---|---|---|
| MSE 5 Liters | 6.7 | 1.75 | 26% |
| | | | |
| Orbis 2 $ft^3$ | 16 | 1.75 | 11% |

## Testing Procedures

Because of time limitations for testing caused by budget limitations, the Orbis testing had to be limited to one 24 hour period. This precluded small quantity sequential loading. However, it did offer the opportunity to try to achieve a value for B above .5A.

The goal was to load about 3,464 dry grams, the projected total based on the August 50 percent unloading/loading run calculations.

While we attempted to load all 3,464 grams of biomass all at once at T0 (Figure 3-14), the working volume in the milling vessel was less than anticipated. This was because the Orbis ball mill quantity specification took up a higher percentage of the milling vessel volume than we had used in the 5 liter MSE vessels (Figure 3-18).

As a result, the total dry biomass that could be initially loaded was 2,241 grams of 50% miscanthus and 50% switchgrass. 14.9 liters of $H_2O$ was loaded which produced a biomass loading value of 12.4 percent. Enzymes concentrations based on the 50 percent

continuous run concentrations were also loaded. In keeping with the operational conditions of that earlier run no buffer was added.

**Figure 3-18**
**Milling Balls in Orbis Unit**

After a four hour run, the Orbis unit was stopped and the access plate removed to view hydrolysis progress. A sample was taken for HPLC analysis. There was no milling ball clogging and much of the biomass had already been converted to slurry (Figure 3-19). Based on these observations additional biomass and water was loaded to determine a possible maximum **A** value.

**Figure 3-19**
**Orbis Ball Milling/Hydrolysis @ T4**

Loading to the 2,691 dry grams/21.65 liters total raised the volume to the total of the milling vessel and did cause some spillage during loading. This quantity was probably right at the maximum possible or slightly over. Biomass and liquid totals are in Table 3-8.

**Table 3-8**
**Orbis Biomass and Liquid Loading**

| Loading Time | Biomass Added (dry grams) | Total Biomass 50% SWG/ 50% Miscanthus (dry grams) | $H_2O$ Added (liters) | Total $H_2O$ (liters) | % Biomass Loading |
|---|---|---|---|---|---|
| T0 | 2241 (83.3%) | | 14.9 | | |
| T5 | 450 (16.7%) | 2,691 | 6.75 | 21.65 | 12.43% |

At T8, the ball milling unit was stopped, the access plate was removed and hydrolysis progress was again observed (Figure 3-20).

**Figure 3-20**
**Orbis Ball Milling/Hydrolysis at T8**

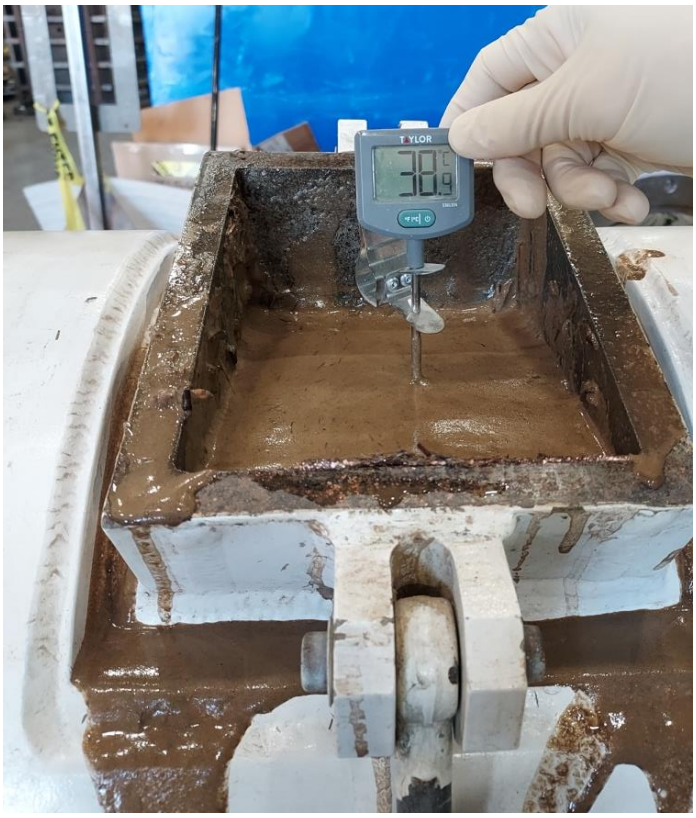
Note that no biomass stems were visible and that the slurry was up to the maximum level of the $2ft^3$ ball milling vessel. Instead of multiple day low volume sequential fillings, the maximum load (A) was being processed in eight hours. The question remained how much of the biomass would be reduced in size for enzyme hydrolysis?

**Processing Temperature Results**

To maximize hydrolysis enzyme activity, the slurry temperature needs to be at a minimum $35^0C$ while $40^0C$ is optimal for most commercial enzymes not modified for thermostability. To minimize operating costs, maintaining these temperatures should be done without the addition of such devices as heating blankets on the milling vessels.

During the Orbis run the facility ambient temperature was $25^0$C. Temperatures of the slurry were measured at T4, T8, and T23. They ranged from 35.6 to $38.9^0$C. These temperatures were achieved by frictional heating of the milling balls against the milling vessel. Therefore, near optimal slurry temperatures were met without any additional energy costs. Also, there was probably enough heat being produced that $38^0$C slurry temperatures could be maintained at lower ambient temperatures of $13^0$C (55F) by wrapping the ball milling vessels with non-electric insulation coatings.

**Figure 3-21**
**Temperature at T23 hours**




## Unloading Slurry

The simultaneous ball milling/enzyme hydrolysis run was ended at T23 hours, one hour less than usual. A sample for HPLC analysis was taken.

Under commercial operating conditions, the slurry would have been removed with a one-step process that would have included screening the slurry with a 1 mm screen to keep partially processed biomass in the ball milling vessel.

However, because this was the first perennial grass run in the Orbis unit, there were uncertainties about how much residual biomass there would be, what would be the range of biomass lengths, and most importantly would the slurry be so viscous that it would not cleanly unload. (The conditions that were encountered that led to the development of the pressurized misting slurry removal system.) Because of these potential conditions, it was decided to remove as much of the unprocessed biomass as possible separate from unloading the slurry. By following these procedures, calculations on the weight of partially processed biomass, the percent of the slurry, and the percent of total biomass they comprised had to be made separately and later combined with slurry and biomass input calculations.

So, the first step was to manually unload as much of the unprocessed biomass as possible through the access port.

**Figure 3-22**
**Manually Offloading Slurry at T23**

Once that was completed, the nozzle attachment shown in Figure 3-14 was then used to remove the majority of the slurry that was collected. However, because there is only one outlet on the Orbis milling vessel, there was no means to equalize pressure. Hence, negative pressure inside the vessel limited slurry outflow. We discussed with Orbis personnel how we could apply the pressurized water misting system (Section 1.5) to alleviate this problem in the prototype and commercial systems. Once the slurry was collected in buckets, partially processed biomass was again manually removed.

Approximately 11.5 liters of slurry (about 53 percent of total) was recovered for later fermentation. This percentage was approximately the same daily percentage removed during the 50 percent unload/load runs. This meant the preliminary Orbis **B** value was around .5 x A.

However, in looking into the ball milling vessel, there was considerable slurry still remaining because of the negative pressure issue (Figure 3-23).

**Figure 3-23**
**Slurry Remaining After T23 Removal**

While removing it would demonstrate that a higher value of **B** was possible, there were two conditions that had to be considered when calculating values for A and B.

First, would the Orbis process produce sufficient fermentable sugars that were recovered in the slurry, and

Second, was the partially processed biomass, which would have been in the slurry in a commercial slurry removal operation, rather than be experimentally separated, be of such quantity that it would/would not interfere with the next cycle of ball milling?

## Fermentable Sugar Results

Sugar hydrolysis results were similar to those recorded in the 50 percent unloading/loading runs. This was probably because of similar low slurry pH values. The pH of the Orbis slurry was 5.25 after T23. Also, 16.7 percent of the biomass was processed for only 18 hours having been added at T5.

**Table 3-9**
**Comparison of Glucose % Conversion Results**

| Fermentable Sugars | (g/L) in Perennial Grasses | T5 Hydrolysis (g/L) | T23 Hydrolysis (g/L) | % Converted | 50% Unload/Load Steady State % Glucose Converted |
|---|---|---|---|---|---|
| Glucose (38% Total Biomass) | 42.4 | 2.84 | 18.19 | 42.9% | 48.1% |
| Xylose & Arabinose (21% Total Biomass) | 26.2 | 2.51 | 7.03 | 27% | |
| Cellobiose | | 5.8 | 5.6 | | |

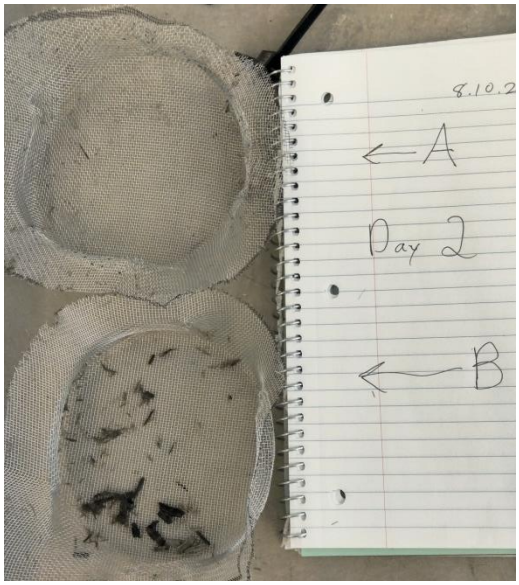
## Residual Biomass

In addition to the residual biomass previously collected, the 11.5 liters of slurry was filtered through a 1mm screen and the captured biomass was added to the biomass previously collected. The combined total was rinsed and dried.

Point of Reference for Comparative Analysis

Early in the 50 percent unload/load run, Day 2, residual biomass quantities were low (Figure 3-24). These showed that a low quantity of partially processed biomass could be maintained with the right pH and enzyme mixtures, even though the perennial grasses had been loaded at a ratio to liquid of 15 percent. This biomass ratio is considered on the high end of current milling and enzyme hydrolysis techniques.

**Figure 3-24**
**Day 2 (Samples A & B)**




These results therefore serve as a reference point for what is possible for perennial grass continuous processing.

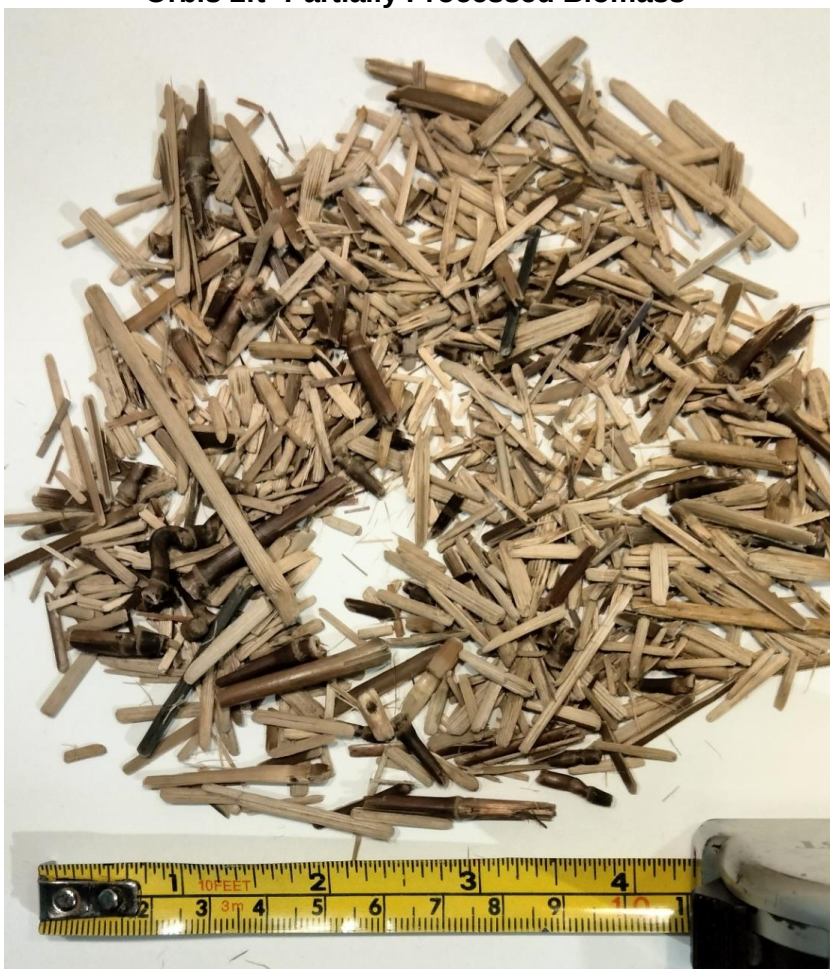
## Orbis Partial Processed Biomass Results

After drying, the residual perennial grasses (Figure 3-25) weighed 290g (10.8% of total loaded biomass).

**Figure 3-25**
**Orbis Residual Biomass after T23 Hours**

Looking at the residual biomass in more detail (Figure 3-26) it seems that much of the biomass had not had enough ball milling to be reduced in size.

**Figure 3-26**
**Orbis 2ft$^3$ Partially Processed Biomass**



The simplest reason for this lack of milling was that the load of 2,691 grams exceeded the working space (total volume – milling ball volume) capacity of the 2ft$^3$ Orbis unit. This meant that within 23 hours some of the biomass had not mixed sufficiently with the milling balls and was probably floating apart from the main milling mixture.

To stop this build-up of partially processed perennial grasses, the probable best course of action is to cut back the daily biomass load (A) to something that would keep partially processed biomass to less than 1 percent of total biomass.

Looking back at the Day 2 results presented above, the starting point for calculating an input biomass total that would produce less than 1 percent of partially processed biomass would be to subtract the biomass overload that produced that high percentage. In this

Orbis run it would be subtracting the 290 g of partially processed biomass from the total of 2,691g producing a load of 2,401g. This resulting biomass would be reduced to about a 11.1 percent loading factor (2.401 grams/21.65 liters), which, in concert with pH being maintained around 6.5-7.0 would most likely keep partially processed biomass low after 24 hours of ball milling and enzyme hydrolysis. (Final biomass loading and liquid content would be refined for optimum conversion.)

Because there would be very little biomass > 1mm in length, this 2,400 g of biomass in about 21 liters of slurry could be unloaded every 24 hours leaving about 1 liter (5%) of slurry behind. The value for B, would be: B = .95 x A. This is about twice the value that was the maximum in the 5 liter milling vessels.

As for enzymes still active in the slurry, which could be an unwanted added cost, these enzymes could be regulated by initial dosages. Furthermore, some Beta-glucosidase and related enzymes will be needed to convert cellobiose to glucose during the fermentation stage so those need to be retained after the ball milling/enzyme hydrolysis process.

With a B value of about .95 x A, the continuous operation of a ball milling/enzyme hydrolysis process becomes less a continuous partial offload slurry/partial on-load biomass process and instead is a continuous batch-load system that can also operate on an on-demand basis. This change offers the potential for lower operating costs since continuous, long term pH, ethanol, and sugar monitoring would be not required. And, instead of scheduling a consistent flow of perennial grasses to keep the continuous running units operating, the continuous batch load units could be started up as needed to respond to uneven harvesting quantities.

Going one step further, with the understanding that this B=.95 x A approach will have to be tested early in the Prototype Phase to verify results, this value for B (Table 3-10) will serve as the baseline for calculating the ball milling volume requirements for the 500 kg/day prototype system (Section 3.4).

**Table 3-10**

| | |
|---|---|
| Total Loaded Orbis 2ft$^3$ Biomass **A** (g) | 2,401 |
| Residual Biomass (g) | 50 |
| Calculated Daily Maximum Ball Milled/Hydrolyzed **B** (g) for 2 ft$^3$ | 2,351 |
| | |
| Biomass Ball Milled/ft$^3$ (g) | 1,175.5 |
| Biomass Ball Milled/ft$^3$ (kg) | 1.2 |
| ft$^3$ Ball Milling/500 kg | 425 |

The values in this table are conservative. Decreases in required milling ball quantities, increases in biomass loading percentages up to 15 percent, minimal extended processing times above 24 hours, adding slurry buffering, and improving enzyme usage could assure these values are met and could provide measureable and economically useful improvements.

## Conclusions

- A commercial dry milling unit such as the Orbis design could serve as the basis for a commercial wet ball milling/enzyme hydrolysis biofuel/bioproduct unit that would use low-value perennial grasses as feedstocks.

- The Orbis test run, while preliminary, showed that limiting factors of the smaller 5 liter vessels such as the ratio of ball milling vessel diameter to input biomass length could be overcome by the design of commercial ball milling units.

- Improvements in ball milling due to the commercial design can lead to daily maximum slurry removal volume being nearly equal to maximum biomass/liquid input. B = 0.95 x A. This significantly lowers ball milling volume requirements in prototype and commercial systems.

- The periodic slurry removal/biomass loading time would be about every 24 hours. This time period is short enough that a properly buffered biomass input would not have to be modified to maintain a 6.0-6.5 pH level for that period. This offers the possibility of lower operating costs. Items such as continuous pH monitoring would be eliminated.

- Near optimum enzyme activity temperatures in the slurry can be maintained by frictional heat produced during ball milling. This eliminates the cost for process heating.

- “Tuning” of ball milling parameters including; milling ball quantities and biomass loading ratios, and enzyme dosages could provide economically beneficial improvements. These will be tested and verified in the prototype stage.

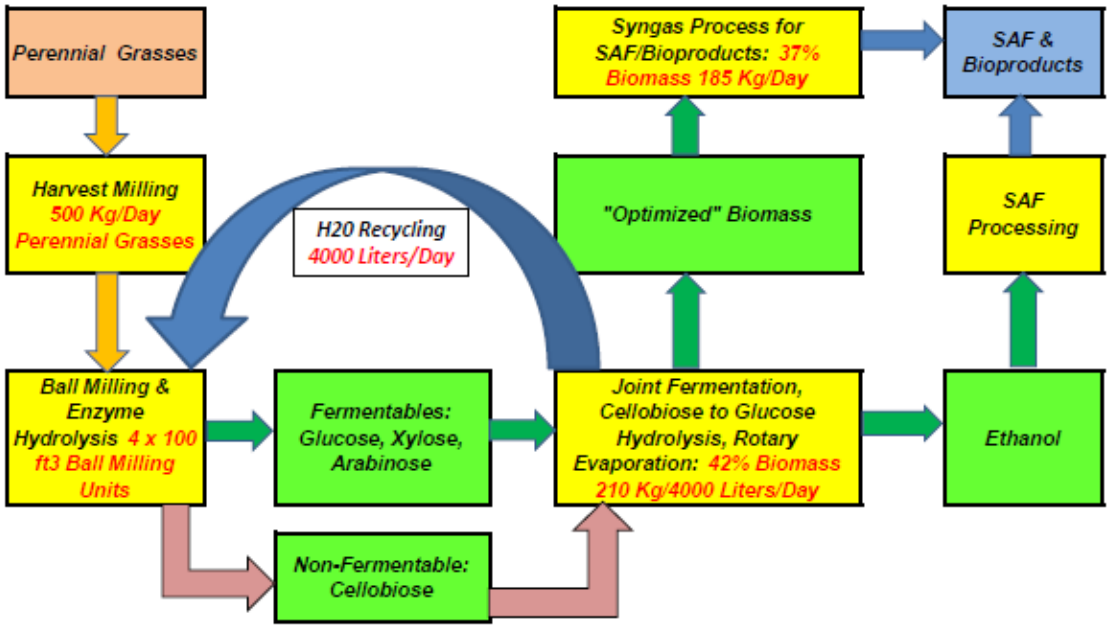
- Efficient removal of all slurry cannot be done with the single port arrangement on the Orbis unit. While convenient to use, the lack of a second air-port caused negative pressure inside the milling vessel that restricted outflow.

- The addition of the pressure misting system (Section 1.5) to the Orbis ball milling vessel could alleviate this condition.

## 3. 4 500 kg/Day Prototype Design

Figure 3-27 presents the overall 500 kg/day prototype design with the quantities of biomass and liquid that would be flowing through it. The production goal is to convert 79 percent of the input perennial grass biomass into SAF and/or bioproducts by the twin pathway ethanol and syngas system.

**Figure 3-27**
**500 Kg/Day Perennial Grass Prototype System**




While ideally the entire system would be included in the Phase II prototype, funding and DOE project objectives will limit what can be included.

At a minimum therefore, the Phase II prototype system should be able to perform the following tasks:

1. Convert 500 kg of perennial biomass to sugar containing slurry daily.

2. Demonstrate cellobiose to glucose conversion simultaneously with C-6 and C-5 fermentation to ethanol on a pilot scale (up to 100 liters/day).

3. Demonstrate production of very low sugar containing “optimized” biomass for syngas production (up to 50 kg/day).

The hardware needed to perform these tasks would consist of:

- One 10 to 25 $ft^3$ Orbis ball milling unit to test/verify thinner wall design, pressurized misting unit system, and B = .95A loading/unloading.

- 3-4 Orbis 100 $ft^3$ combined ball milling/enzyme hydrolysis units with fittings for Section 1.5 pressurized misting slurry removal system. Possible thinner wall design in one unit.

- Automated pH monitoring for all ball milling/enzyme hydrolysis units.

- Enzymes supply for demonstration runs.

- Associated hammer milling, combined biomass weighing/feed system (figure 3-28), and liquid feed hardware.

**Figure 3-28**
**Commercial Biomass Feed Hopper**

- A minimum of one portable Section 1.5 pressurized misting slurry removal system that could be used serially on all 100 $ft^3$ ball milling vessels.

- A pilot scale (up to 100 liters) modular combined fermentation/distillation system using a pressurized vessel component for rotary evaporative type distillation.

Note: Currently, Orbis Machinery is the preferred contractor for the ball milling hardware for the following reasons: 1) the equipment design has been shown to be compatible with our process, 2) we have developed a working arrangement with the company, and 3) the equipment is produced in the USA. If lower priced USA produced units that meet our design and function criteria are available they would be considered as well.

**Estimated Costs**

Because of supply chain uncertainties it is very difficult to accurately calculate costs for a hardware program that would be starting at the earliest in Q2 or Q3 2026. It is especially hard for this project since a great deal of the cost is for high quality steel that is affected by tariff uncertainties. Given this situation, the best current estimate for the system outlined above is between $1.8 and $2.0 million. Details will be provided on request.

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