IMPROVED BIOREFINERY FOR THE PRODUCTION OF ETHANOL, CHEMICALS, ANIMAL FEED AND BIOMATERIALS FROM SUGAR CANE

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Submitted by
Professor Donal Day
Audubon Sugar Institute
LSU Agricultural Center
3845 Highway 75
St. Gabriel, LA 70776

Contact:
Donal F.Day, PhD.
Telephone: 225-642-0135
Fax: 225-642-8790
E-mail: dday@agctr.lsu.edu

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EXECUTIVE SUMMARY

The Audubon Sugar Institute (ASI) of Louisiana State University's Agricultural Center (LSU AgCenter) and MBI International (MBI) sought to develop technologies that will lead to the development of a sugar-cane biorefinery, capable of supplying fuel ethanol from bagasse. Technology development focused on the conversion of bagasse, cane-leaf matter (CLM) and molasses into high value-added products that included ethanol, specialty chemicals, biomaterials and animal feed; i.e. a sugar cane-based biorefinery. The key to lignocellulosic biomass utilization is an economically feasible method (pretreatment) for separating the cellulose and the hemicellulose from the physical protection provided by lignin. An effective pretreatment disrupts physical barriers, cellulose crystallinity, and the association of lignin and hemicellulose with cellulose so that hydrolytic enzymes can access the biomass macrostructure (Teymouri et al. 2004, Laureano-Perez, 2005). We chose to focus on alkaline pretreatment methods for, and in particular, the Ammonia Fiber Expansion (AFEX) process owned by MBI. During the first two years of this program a laboratory process was established for the pretreatment of bagasse and CLM using the AFEX process. There was significant improvement of both rate and yield of glucose and xylose upon enzymatic hydrolysis of AFEX-treated bagasse and CLM compared with untreated material. Because of reactor size limitation, several other alkaline pretreatment methods were also co-investigated. They included, dilute ammonia, lime and hydroxy-hypochlorite treatments. Scale-up focused on using a dilute ammonia process as a substitute for AFEX, allowing development at a larger scale. The pretreatment of bagasse by an ammonia process, followed by saccharification and fermentation produced ethanol from bagasse. Simultaneous saccharification and fermentation (SSF) allowed two operations in the same vessel. The addition of sugarcane molasses to the hydrolysate/fermentation process yielded improvements beyond what was expected solely from the addition of sugar. In order to expand the economic potential for building a biorefinery, the conversion of enzyme hydrolysates of AFEX-treated bagasse to succinic acid was also investigated. This program established a solid basis for pre-treatment of bagasse in a manner that is feasible for producing ethanol at raw sugar mills.

Project objectives and tasks

The rationale for this project was to develop and evaluate scalable, integrated technologies utilizing bagasse, molasses and CLM for the production of ethanol, chemicals, biomaterials and animal feeds that can be used in a sugar mill based biorefinery. All efforts in this project were directed towards investigating those processes that could be integrated into a raw sugar mill.

Objectives

1) Develop a scalable AFEX biomass pretreatment process for bagasse (**Tasks 1 and 2**).

2) Develop and demonstrate processes that utilize components from sugar cane; bagasse, CLM and molasses for ethanol production (**Tasks 4, 5, 6**). This required scaling up AN AFEX reactor from 1-gallon capacity to 5-gallon capacity both to obtain processing data for future modeling and economic analysis of pilot plant scale AFEX pretreatment and to produce larger amounts of AFEX-treated material for fermentations. Continue investigation of those processes thought to offer opportunities to contribute to biorefinery economics (**Tasks 3, 5**). Scale up the fermentation experiments to collect data for process analysis and to produce representative stillage. This includes an investigation of gasification of bagasse for the production of Syngas in sub- and supercritical conditions. This objective was added later to the original proposal.

Task 1. AFEX Pretreatment, Enzymatic Hydrolysis, and Solid/liquid Separation

The primary objective of this task was to develop, optimize and demonstrate an AFEX pretreatment process to produce fermentable sugars and other products from bagasse and CLM.

Sub Task 1. AFEX pretreatment of bagasse and CLM.

Summary

The primary objective of this study was to evaluate the efficiency of the AFEX process in the pretreatment of bagasse pith and cane leaf matter (CLM). Enzyme hydrolysis of AFEX-treated bagasse pith and CLM generates streams of fermentable sugars containing both C5 (xylose and arabinose) and C6 sugars (glucose). Maximal utilization of all the biomass polymeric sugar is essential to make the economics of biomass processing feasible. Alkaline treated biomass, unlike acid treated material, has a significant amount of hemicellulose that needs to be hydrolyzed to monomeric sugars. AFEX-treated bagasse, pith and CLM were hydrolyzed with combinations of cellulase and xylanase to maximize both glucose and xylose yields. The generated C5 and C6 sugar streams are useful in the production of fuels and chemicals such as ethanol and succinic acid.

The AFEX process conditions, which include reaction time, reaction temperature, ammonia loading, and moisture content of the biomass, were varied to determine those conditions that provide the highest glucose and xylose yield. The efficiency of the AFEX process was evaluated using enzyme hydrolysis. The most effective AFEX treatment conditions for bagasse pith and CLM were found to be 100^{o}C, a biomass moisture content of 40%, a reaction time of 30 minutes, and an ammonia loading of 2 g ammonia per gram dry biomass. Combinations of cellulase and xylanase produced higher glucose and xylose yields from AFEX-treated material compared to hydrolysis with only cellulase. A process was developed for the separation of hemicellulose and cellulose from AFEX-treated CLM and bagasse pith using selective hydrolysis of hemicellulose by using cellulase-free xylanases for selective hydrolysis of the hemicellulose. For AFEX-treated CLM, about 52% of the xylan was converted to xylose while about 96% of the cellulose was left in the biomass. For AFEX-treated bagasse pith, about 56% of the xylan, but only 5% of the cellulose, was hydrolyzed.

The enzyme hydrolysate of AFEX-treated bagasse can be converted to ethanol via Separate Hydrolysis and Fermentation (SHF) process. *Zymomonas mobilis* pZB5, an organism capable of converting both glucose and xylose to ethanol, was used for this fermentation. The fermentations were run at two different solids loadings, 4% and 8%. These fermentations were successful and all the generated sugars were converted. However, the ethanol concentration was low, 15 g/l at an 8% solids loading. The concentration of the individuals products strongly influences product recovery cost and is critical to the success of recovery and the downstream processing systems. The efficient recovery of ethanol requires an ethanol concentration of at least 4% (w/v), which in turn requires a starting concentration of fermenting sugar higher than 8% (w/v) (Grohmann, 1993). Due to the extreme sensitivity of current cellulolytic enzymes to end-product inhibition, high concentrations of sugar (glucose) in the enzyme hydrolysis process could not be achieved. This shortcoming of the SHF process can be addressed by a process called Simultaneous Saccharification and Fermentation (SSF). In SSF, a cellulase enzyme and microorganisms are added to the substrate at the same time in a fermentation vessel. The microorganism will metabolize the sugars as soon as they are released. The SSF process typically results in a higher concentration of ethanol as compared to the SHF process. Furthermore, performing SSF in a fed batch manner (solid biomass is incrementally fed to the fermentation vessel) may yield even higher concentrations of ethanol.

In an effort to increase ethanol concentrations, fed batch simultaneous saccharification and fermentation (SSF) was investigated for ethanol production from materials. In these fermentations, the solids loading were incrementally increased by batch feeding the biomass to the fermentor. Two microorganisms, *Zymomonas mobilis* and *Saccharomyces cerevisiae* were tested. Up to 25% solids loading and a concentration of ethanol of up to 28 g/l was achieved in these fermentations. The fermentation results from *Saccharomyces cerevisiae* were promising, but the fermentations with *Z. mobilis* gave pooryields.

Sub-Task 2. Conversion of AFEX treated materials to succinic acid

Summary

Succinic acid is a four-carbon dicarboxylic acid used to manufacture polymers and resins for lacquers, dyes and perfumes, industrial solvents, and newly developed products such as de-icing chemicals. Succinic acid is produced predominantly from petroleum-based feedstocks. It can be produced via bacterial fermentation using renewable biomass-derived sugars. MBI has identified and developed a bacterium, *Actinobacillus succinogenes*, capable of utilizing both hexose and pentose sugars simultaneously for the production of succinic acid. This organism produces 75-80 g/l of succinic acid in 48 hours with greater than 90% yields. Fermentability of the enzyme hydrolysate from AFEX-treated CLM was assessed for succinic acid production. Using SHF processes, with two different solid loadings for succinic acid fermentation with *Actinobacillus succinogenes FZ45* generated sugars (C5 and C6) were converted to succinic acid.

MBI's existing ASPEN Plus-based process model for production of succinic acid from pure sugars was modified to evaluate the technical and economic performance of succinic acid production from AFEX-treated biomass. The existing ASPEN Plus-based process model for production of ethanol from AFEX-treated switchgrass was also modified to evaluate the technical and economic performance of ethanol production from AFEX-treated bagasse. Both models showed that the capital and manufacturing costs would be decreased if the cost associated with hydrolysis was decreased. Lowering the purchase price of cellulases directly lowers the manufacturing cost. Improving cellulase activity in the presence of increased solids loading would provide a lower AFEX cost, a higher succinic acid and ethanol titer, and lower downstream processing costs.

Sub-task 3. Produce large quantities of AFEX-treated bagasse and CLM

In order to scale the process to something larger than laboratory experiments, sufficient quantity of pretreated feedstocks are required. This was addressed two ways; by scaling up the AFEX reactor and by using an AFEX stimulant (not part of this project).

Task 1. Results

Optimization of AFEX treatment conditions for bagasse, pith and bulk production of AFEX-treated fractionated bagasse and CLM solids

Table 1. Composition of Bagasse, Bagasse Pith, and CLM (based on dry weight)

	Glucan %	Xylan %	Galactan %	Arabinan %	Ash %	Extractive %
CLM	27.09	20.01	1.44	2.34	5.7	3.2
Bagasse	34.6	18.49	00.45	0.95	6.5	2.4
Bagasse pith(first batch)	19.7	12.32	0.57	0.95	25	1.9
Bagasse pith (second batch)	26.28	14.37	1.13	1.3	16	2.36

*See Methods for Analytical Processes

A factorial design for the experiments was performed for bagasse pith and CLM to optimize the AFEX process conditions which included reaction time, reaction temperature, ammonia loading, and moisture content of the biomass. This factorial design had proven successful for determining AFEX conditions for other crop residue materials such as corn stover. The efficiency of the AFEX pretreatment was evaluated by maximum enzymatic hydrolysis achieved. Table 2 summarizes the glucose and xylose yields obtained from 72 hr enzyme hydrolysis of these samples.

Table 2. Enzymatic Hydrolysis of AFEX-treated feedstocks (100°C, 40% Loading, 2:1, and 30 min) Ground and Unground Bagasse and CLM with 15 FPU of Spezyme Cp plus 42 units of Novo 188 per gram of Cellulose (the unground AFEX-treated samples were not ground prior to the hydrolysis)

AFEX-treated Sample	**72 hr Glucose Yield%**	**72 hr Xylose Yield %**
Ground bagasse	69±2	59±1
Unground bagasse	66±1	57±1
Ground CLM	84±2	58±2
Unground CLM	81±2	61±2

As part of this task, we evaluated the effect of the particle size of the biomass on the efficiency of the AFEX treatment. Several batches of unground bagasse and CLM (particle size was about 2.5-3 cm) were AFEX-treated (100°C, 40%, 2:1, and 30 min). Hydrolysis results (Table 2) of these samples were similar to those of bagasse and CLM samples that were ground (1mm pass through Mesh #9) prior to AFEX treatment. These

results indicate that prior grinding of biomass is not necessary for AFEX treatment of bagasse and CLM.

AFEX treatment significantly increased the digestibility of biomass. Maximum glucose and xylose yields were obtained from bagasse pith that was AFEX-treated at 100°C, 40 or 60% moisture content, 2:1 ratio of ammonia loading to dry biomass for 30 min. 72 hour hydrolysis of this sample with 15 FPU of cellulase and 42 units of Novo 188 per g of cellulose resulted in 90% glucose and 70% xylose yield. Enzyme hydrolysis of the untreated sample gave only 9% glucose yield and 3% xylose yield. Hydrolysis data showed that digestibility of AFEX-treated bagasse pith is higher than bagasse treated under similar AFEX conditions. However, glucan content of the tested bagasse pith is lower than for bagasse. Therefore, the amount of glucose released from bagasse is higher than for bagasse pith alone.

Table 3 gives all the conditions for AFEX tested on bagasse pith. **Table 3.** AFEX Conditions for Pretreatment of Bagasse Pith and 72 hr Hydrolysis Results

AFEX CONDITIONS FOR TREATMENT OF BAGASSE PITH			**72 hr hydrolysis with 15FPU of Spezyme Cp+42CBU of Novo 188/g cellulose**	
TEMPERATURE	**AMMONIA LOADING: BIOMASS**	**MOISTURE CONTENT**	**GLUCOSE YIELD (%)**	**Xylose yield (%)**
100°C	2:1	40%	92	66
100°C	2:1	40%	93	68
100°C	1:1	40%	79	63
100°C	1:1	40%	75	61
90°C	1:1	40%	62	54
90°C	1:1	40%	66	56
90°C	2:1	40%	83	65
100°C	1:1	60%	75	58
100°C	1:1	60%	72	54
90°C	2:1	60%	80	60
90°C	2:1	60%	81	51
90°C	1:1	60%	57	48
90°C	1:1	60%	66	55
100°C	2:1	60%	98	75
100°C	2:1	60%	90	70
Untreated			9	3

Optimally AFEX-treated bagasse, pith and CLM solids were hydrolyzed using the enzyme mixtures including cellulase, β-glucosidase, and xylanase to produce C5 and C6 sugar stream.

The enzyme cocktail for this task contained cellulase (Spezyme Cp), xylanase (NS50030), and β-glucosidase (Novo 188). AFEX-treated (100°C, 40%, 2:1, and 30 min) bagasse, CLM and bagasse pith were hydrolyzed with several different combinations of these enzymes. Table 4 summarizes the hydrolysis results.

Table 4. Hydrolysis of AFEX-treated Bagasse, CLM and Bagasse Pith (100°C, 40% moisture content, 2:1 ammonia loading, 30 min) with Different Combinations of Cellulase and Xylanase (Yields are calculated based on the theoretically calculated available sugar content.)

AFEX treated biomass	Spezyme Cp (FPU), Novo 188 (CBU)/g of cellulose	Xylanase(NS50030) ml/g dry biomass	72 hr Glucose Yield (%)	72 hr Xylose Yield (%)
Bagasse	**15,42**	**0**	**72±2**	**58±2**
Bagasse	15, 42	0.2	82±0.2	69±1
Bagasse	15,42	0.1	80±2	68±1
Bagasse	15,42	0.05	83±0.5	67±0.5
Bagasse	10,42	0.2	81±1	74±1
Bagasse	10,42	0.1	81±0.5	79±1
Bagasse	5,42	0.2	68±2	74±2
Bagasse	**5,42**	**0.1**	**70±1**	**69±2**
CLM	**15,42**	**0**	**85±1**	**58±0.5**
CLM	15, 42	0.2	85±2	67±2
CLM	15,42	0.1	88±1	63±1
CLM	15,42	0.05	86±2	64±1
CLM	10,42	0.2	89±2	62±0.5
CLM	10,42	0.1	88±1	63±2
CLM	5,42	0.2	85±1	62±1
CLM	**5,42**	**0.1**	**81±1**	**62±0.6**
Bagasse pith	**15,42**	**0**	**80±2**	**59±2**
Bagasse pith	15, 42	0.2	91±2	74±1
Bagasse pith	15,42	0.1	87±0.2	68±0.5
Bagasse pith	15,42	0.05	83±2	67±1
Bagasse pith	10,42	0.2	82±1	75±2
Bagasse pith	**10,42**	**0.1**	**81±3**	**62±3**
Bagasse pith	5,42	0.2	71±1	65±1
Bagasse pith	5,42	0.1	70±2	65±2

Presumably, xylanases improve cellulose hydrolysis by removing/hydrolyzing hemicellulose on cellulose surface, thereby increasing the accessibility of cellulose to cellulase. Hydrolysis of bagasse and bagasse pith support this concept and showed that addition of xylanase increases both glucose and xylose yields compared to hydrolysis with only cellulase. Our previous work had shown similar results for the hydrolysis of AFEX treated corn stover. However, in CLM hydrolysis addition of xylanase didn't show any significant change.

As the data show addition of xylanase allowed us to trade off cellulase and xylanase activity without significant reduction in total released sugars. These data suggest that it is reasonable to expect incremental improvement in the hydrolysis of biomass by systematically fine tuning the cellulolytic and xylanolytic compositions of enzyme complexes for particular substrates. Having an enzyme cocktail with adequate cellulase and hemicellulase activity might help to reduce the overall enzyme loading and subsequently costs for the biomass hydrolysis.

Fed batch SSF fermentation of AFEX treated CLM:

Each of these fermentations was initiated with 10% solid loading of AFEX-treated CLM (100°C, 40% moisture content, 2:1 ammonia loading, and 30 minutes treatment time). Enzyme loading was 20 FPU Spezyme Cp and 42 CBU of Novo 188/g cellulose. During the first 24 hrs of the fermentation process, two more batches of biomass equal to the initial loading plus required amount of enzyme were added to the fermentors. Both fermentations were run for 120 hrs at pH 5.0. The temperature was set at the optimal temperature for the microorganisms, 35° C for *S. cerevisiae* and 32°C for *Z. mobilis.* The overall biomass loading was about 21% in both fermentations. Since the temperature in the *Z. mobilis* fermentation was low, it took longer for the biomass to become solubilized compared to the *S. cerevisiae* fermentation.

The time course of these fermentations is presented in Figures 1 and 2 and the results are summarized in Table 5.

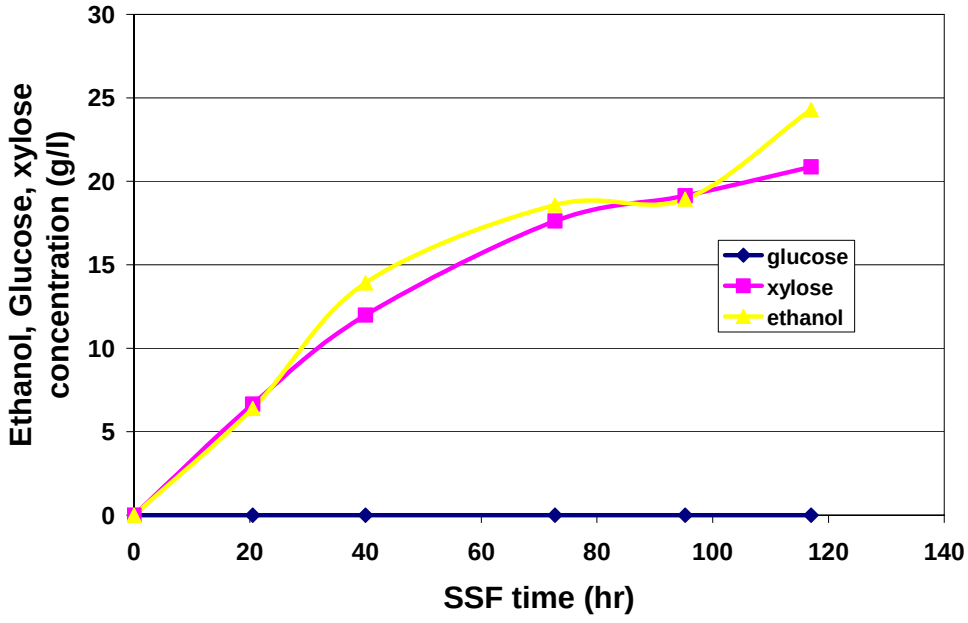


Figure 1. Time course of the fed batch SSF fermentation of AFEX treated CLM with *S. cerevisiae.*

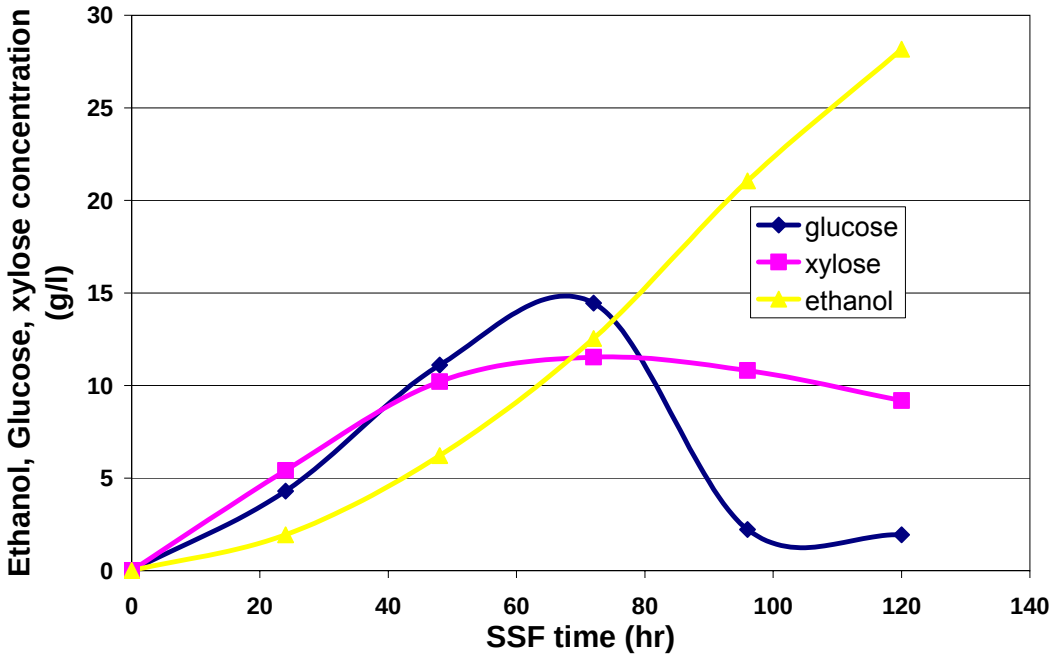


Figure 2. Time course of fed batch SSF fermentation of AFEX treated CLM with *Z. mobilis.*

Table 5. Summary of the Ethanol Fed Batch SSF of AFEX-treated CLM

Biomass: AFEX treated CLM (100°C, 40% moisture, 2:1 ammonia loading, 30 min.)		
Microorganism	*S. cerevisiae*	*Z. mobilis*
Initial solid loading	10%	10%
Glucan content	31.92%	31.92%
Xylan content	20.38%	20.38%
Initial dry solid loading (g)	120	120
Dry solid loading in two equal separate additions (g)	120 (in each addition)	120 (in each addition)
Total solid loading	360	360
Overall solid loading	21%	21%
Available Glucose (g)	127.66	127.66
Available Xylose (g)	83.39	83.39
Theoretical Ethanol (g)*	65.1(from C6)	107.64 (from C6+C5)
Initial enzyme loading	20 FPU Spezyme cp+ 42 CBU Novo 188/g cellulose	20 FPU Spezyme Cp+ 42 CBU Novo 188/g cellulose
Enzyme loading in each addition	20 FPU Spezyme Cp+ 42 CBU Novo 188/g cellulose	20 FPU Spezyme Cp+ 42 CBU Novo 188/g cellulose
120 hr glucose concentration (g/l)	0	1.9
120 hr xylose concentration (g/l)	20.8	8.38
120 hr ethanol titer (g/l)	24.8	28.15
Total ethanol (g)	49.85	57.8
Ethanol Yield** %	76.57	53.72

* Based on 0.51 g ethanol per gram of sugar.

** Based on obtained ethanol compared with theoretical values for ethanol.

In the *S. cerevisiae* fermentation, all the produced glucose was consumed and about 25 g/l of ethanol was produced. Based on the available glucose in the biomass, the ethanol yield for *S. cerevisiae* fermentation was about 77%. Considering that the enzyme hydrolysis of the AFEX-treated CLM at low solid loading and at 50°C results in about 80-85% glucose yield, the 77% ethanol yield for high solid loading was promising.

Fermentation with *Z. mobilis* was slower than the fermentation with *S. cerevisiae.* At the end of this fermentation, there was some unconsumed glucose and xylose. The final ethanol concentration reached a maximum of 28 g/l. Based on the available glucose and xylose in the biomass, the overall ethanol yield was only 54%. The lower ethanol yield in this fermentation is presumably due to the lower temperature and also the lower efficiency of the *Z. mobilis* in converting both glucose and xylose to ethanol compared to the efficiency of *S. cerevisiae* for converting glucose to ethanol. However, even though the ethanol yield in the *Z. mobilis* fermentation was low, it is noteworthy that the total amount of generated ethanol was higher than from a similar fermentation with *S. cerevisiae*, 57.8 g ethanol vs. 49.87 g (Table 5). As our economic analysis showed,

ethanol titer has a significant impact on the production cost and increasing the titer would improve the process economics.

Fed batch SSF fermentation of AFEX treated bagasse. Based on the CLM fed batch SSF results (mentioned above), *S .cerevisiae* was chosen for this fermentation. The fermentation was performed in a 5L New Brunswick fermentor. The fermentation was run at pH 5.0 and at 35°C for 120 hr. The initial solid loading was 10%. Enzyme loading was 20 FPU Spezyme Cp and 42 CBU of Novo 188/g cellulose. In the first 24 hr of the fermentation, two more batches of biomass plus required amount of enzyme were added to the fermentor. Agitation was provided with two impellers, one pitched-blade impeller at the bottom and one disk impeller at the top (submerged in the fermentation broth). The biomass was well solubilized; the mixing was adequate and the temperature was stable.

Figure 3 presents the time course of this fermentation and Table 6 summarizes the results.

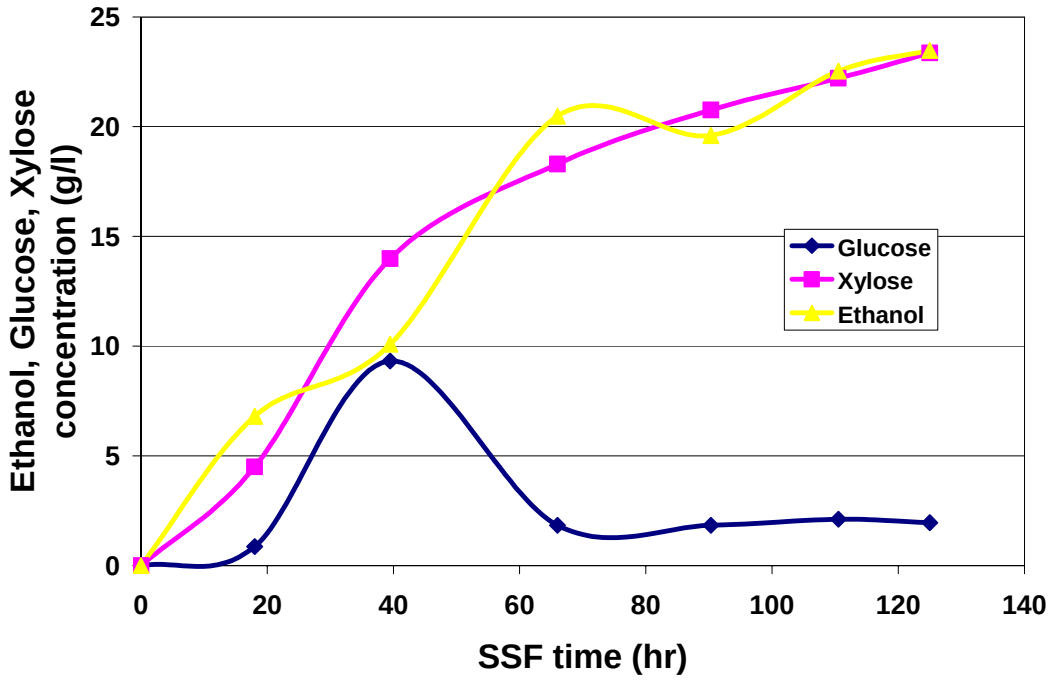


Figure 3. Time course of fed batch SSF of AFEX treated bagasse with *S. cerevisiae*.

Table 6. Summary of the Fed Batch SSF Fermentation of AFEX-treated Bagasse with *S. cerevisiae*

Biomass: AFEX treated bagasse (100°C, 40% moisture, 2:1 ammonia loading, and 30 min.)	
Microorganism	*S. cerevisiae*
Initial solid loading	10%
Glucan content	34.57%
Xylan content	18.49%
Initial dry solid loading (g)	300
Dry solid loading in two equal separate additions (g)	300 (in each addition)
Total solid loading (g)	900
Overall solid loading	25%
Available Glucose (g)	345.7
Available Xylose (g)	189.1
Theoretical Ethanol (g)*	176.3 (from C6)
Initial enzyme loading	20 FPU Spezyme Cp+ 42 CBU Novo 188/g cellulose
Enzyme loading in each addition	20 FPU Spezyme Cp+ 42 CBU Novo 188/g cellulose
120 hr glucose concentration (g/l)	1.9
120 hr xylose concentration (g/l)	23.5
120 hr ethanol titer (g/l)	23.6
Total ethanol (g)	108
Ethanol Yield %**	61.25%

* Based on 0.51 g ethanol per gram of sugar.

** Based on obtained ethanol compared with theoretical ethanol.

Almost all the generated glucose was consumed and about 24 g/l ethanol was produced. Based on the available glucose in the biomass, the ethanol yield was about 61%. The enzyme hydrolysis of the AFEX-treated bagasse at low solid loading and at 50°C resulted in about 70% glucose yield, the 61% ethanol yield for high solid loading process was encouraging. Almost complete consumption of glucose reconfirmed the absence of inhibitory compounds to the fermentation process. In this process, about 58% of the xylan was converted to xylose and produced about 109 g of xylose. Using a robust and efficient microorganism capable of converting both glucose and xylose to ethanol could significantly impact the overall economy of ethanol production from cellulosic material such as bagasse.

Fed batch SSF fermentation of AFEX treated bagasse pith

The fermentation was run at pH 5.0 and at 35°C for 80 hr. The initial solid loading was 10%. Enzyme loading was 20 FPU Spezyme Cp and 42 CBU of Novo 188/g cellulose.

In the first 24 hr of the fermentation, two more batches of biomass plus required amounts of enzyme were added to the fermentor. Agitation was provided with two impellers, one pitched-blade impeller at the bottom and one disk impeller at the top (submerged in the fermentation broth). The biomass was well solubilized; the mixing was adequate and the temperature was stable.

Figure 4 presents the time course of this fermentation and Table 7 summarizes the results.

Table 7. Summary of the Ethanol Fed Batch SSF of AFEX-treated Bagasse Pith

Biomass: AFEX treated bagasse pith (100°C, 40% moisture, 2:1 ammonia loading, 30 min.)	
Microorganism	*S. cerevisiae*
Initial solid loading	10%
Glucan content	26.29%
Xylan content	14.29%
Initial dry solid loading (g)	300
Dry solid loading in two equal separate additions (g)	300 (in each addition)
Total solid loading (g)	900
Overall solid loading	26%
Available Glucose (g)	262.87
Available Xylose (g)	146.1
Theoretical Ethanol (g)*	134.1 (from C6)
Initial enzyme loading	20 FPU Spezyme Cp+ 42 CBU Novo 188/g cellulose
Enzyme loading in each addition	20 FPU Spezyme Cp+ 42 CBU Novo 188/g cellulose
120 hr glucose concentration (g/l)	0
120 hr xylose concentration (g/l)	21.6
120 hr ethanol titer (g/l)	24
Total ethanol (g)	85.5
Ethanol Yield %**	64%

* Based on 0.51 g ethanol per gram of sugar.

** Based on obtained ethanol compared to theoretical ethanol.

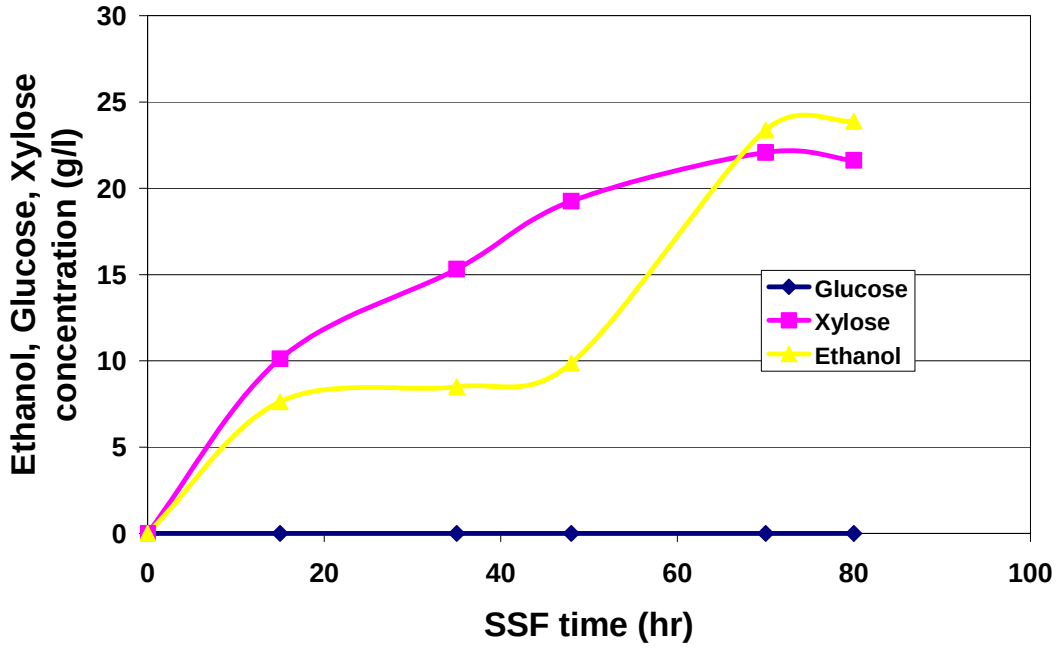


Figure 4. Time course of fed batch SSF fermentation of AFEX treated bagasse pith with *S. cerevisiae.*

In conclusion, these fermentations showed that the fed batch SSF fermentation of AFEX-treated biomass was feasible and resulted in higher ethanol titer compared to regular SHF fermentation. These fermentations showed that for future work a more thermo stable and more efficient microorganism that converts both C6 and C5 sugars to ethanol is needed to make the cellulosic ethanol production more efficient.

Evaluating recombinant E. coli KO11 for production of ethanol: Recombinant *E. coli* KO11, like *Zymomonas mobilis* 37821 (pZB5), utilizes both C6 and C5 sugars for ethanol production. The optimal temperature for *E.coli* KO11 (37°C) is closer to the optimal temperature of cellulase enzyme (50°C) compared to that for *Z. mobilis* (32°C). However, temperature is not the only key factor; another critical factor in SSF process is the pH. Therefore, to find the most suitable pH for our SSF experiment with KO11, this microorganism was grown in the media with a mixture of sugars (glucose+xylose+arabinose) at three different pH's (5,6, and 7). Table 8 summarizes the optical density (OD) measurements and the sugar consumption of this microorganism at different pH for the course of 48 hr of growth.

Table 8. Growth of KO11 on a Media Containing a Mixture of Sugars at Different pH

			g/l remain in the media			
pH	Time, hr	OD	Glucose	Xylose	Arabinose	Total Sugar
5.00	0.00	0.00	4.17	2.66	2.02	8.86
	2.50	0.28	4.06	2.67	2.04	8.77
	4.50	0.70	3.67	2.67	1.98	8.33
	7.50	0.99	3.00	2.50	1.87	7.37
	24.00	0.73	2.05	2.68	1.95	6.68
	48.00	0.82	1.53	2.70	1.99	6.22
6.00	0.00	0.00	4.44	2.75	2.52	9.71
	2.50	0.32	4.31	2.75	2.50	9.57
	4.50	1.37	3.68	2.74	2.33	8.74
	7.50	2.37	2.50	2.60	2.09	7.19
	24.00	3.11	0.00	2.59	0.00	2.59
	48.00	3.68	0.00	2.29	0.00	2.29
7.00	0.00	0.00	4.21	2.72	2.91	9.84
	2.50	0.37	4.06	2.71	2.87	9.65
	4.50	1.66	3.32	2.69	2.66	8.66
	7.50	2.72	2.21	2.67	2.35	7.22
	24.00	3.72	0.00	2.10	0.00	2.10
	48.00	3.92	0.00	0.00	0.00	0.00

The highest growth rate was observed at pH 7 followed by pH 6. As the above data suggest, xylose utilization for this microorganism is not as efficient and as fast as glucose and arabinose utilization. At pH 7, both glucose and arabinose were totally used up in 24 hr of growth while xylose was consumed after 48 hr. The growth rate was slightly slower at pH 6 and after 48 hr, and some xylose still remained. pH 5 was not a favorable environment for this microorganism. Based on our previous work and the available literature, the most favorable pH for the cellulase enzyme is 5. Therefore, to keep both enzyme and microorganism productive, we chose pH 6 for our SSF experiments.

An SSF experiment with AFEX-treated bagasse pith (100°C, 40% moisture content, 2:1 ammonia loading, and 30 minutes) and KO11 was set up in a shake flask. Fermentation was carried out with 10% solid loading, 15 FPU of cellulase and 42 units of β-glucosidase per gram of glucan, at 37°C and pH 6 for 96 hr. At the end of the fermentation there was some unconsumed xylose while there was no glucose or arabinose left in the media. Based on the available sugars (glucose+xylose+arabinose), the ethanol yield was about 57%. The ethanol yield was considerably lower than expected, based on our enzyme hydrolysis. Earlier enzyme hydrolysis of the AFEX-treated bagasse pith with the same enzyme loading showed 83% yield based on the available sugars (glucose+xylose+arabinose). SSF experiments are typically run at lower temperatures compared to enzyme hydrolysis and yet result in relatively higher yield. This suggests that the pH could be the major contributing factor for the low ethanol yield observed in this experiment. The results indicated that the *E. coli* was not suitable for this application. All further experimentation was conducted using *Zymomonas mobilis* 37821 (pZB5) and *Saccharomyces cerevisiae.*

The fermentability of the generated mixed C5 and C6 sugars was evaluated via fermentation of succinic acid using *Actinobacillus succinogenes* (MBI strain FZ45).

The fermentability of the generated mixed sugar stream from AFEX-treated CLM was evaluated via SHF fermentation of succinic acid using *Actinobacillus succinogenes* (MBI strain FZ45) at two different solids loadings. Table 9 summarizes the fermentations results.

Table 9. Succinic Acid Fermentation from AFEX-treated CLM with *Actinobacillus succinogenes.*

Biomass: AFEX treated CLM (100°C, 40% Moisture, 2:1 ammonia loading, 30 min.)		
Solid loading	8%	12%
Glucan content	27.09%	27.09%
Xylan content	20.01%	20.01%
Dry solid loading(g)	220	165 (less volume compared to 8%)
Available Glucose (g)	66.21	49.66
Available Xylose (g)	50.00	37.51
Hydrolysis		
Enzyme loading	20 FPU Spezyme Cp + 42 CBU Novo 188/g cellulose	20 FPU Spezyme Cp + 42 CBU Novo 188/g cellulose
72 hr hydrolysis Glucose yield	83%	72%
72 hr hydrolysis Xylose yield	60%	49%
Total G+X at 72 hr (g)	83.16	54.38
Fermentation		
Microorganism	*A. succinogenes*	*A. succinogenes*
Succinic acid at 72 hr (g)	102.3	63.2
Succinic acid titer [g/l]	37.2	45.9
Theor. Max., succinic acid[g]/[g] sugar in fermentation	98.7	60.1
Yield: [g] succinic acid/[g] biomass	0.47	0.38
Yield: [g] succinic acid/[g] consumed sugars	1.09	1.05

Total consumption of all the generated sugars at the hydrolysis stage confirmed the absence of inhibitory compounds in the hydrolysate of AFEX-treated CLM. Lower levels of hydrolysis in the higher solid loading fermentation is most probably due to the sensitivity of the cellulase enzyme to the end product (glucose) concentration, and/or to cellobiose or to changes in the local environment as solids concentration is increased, limiting full enzymatic access to the material or adsorbing the enzymes to the substrate through pysico-chemical means. In order to lower the fermentation cost, yeast extract was replaced by LFS as the nutrient source for the fermentation. Yields of over 100% for production of succinic acid from the generated sugar from CLM are due to the fact that

LFS contains glycerol and *Actinobacillus succinogenes* is also capable of converting glycerol to succinic acid.

Scale –up of AFEX

Sub-Task 3. Scale up AFEX reactor from 1 gallon to 5 gallons

MBI purchased a 5-gallon pressure reactor from Parr Instrument Company to replace the 1-gallon reactor currently being used for the AFEX process. The 5-gallon reactor is made out of T316 stainless steel with following specifications:

1. With 120 in/lb footless magnetic stirrer
2. With PTFE gasket and split ring closure
3. Head to include 0-1000 psi gage, 1000 psi rupture disk, double valve gas release valve and thermowell with thermocouple, back to back anchor stirrer with PTFE wiper blades
4. Variable speed motor 0-180rpm
5. Flexible mantle heater
6. Fixed head style vessel mounted in custom support stand for either vertical or horizontal operation with pneumatic controls
7. Programmable temperature control

The 1-gallon reactor, the 5-gallon reactor and the pump set-ups were rearranged so that both reactors could be connected to the pump to allow simultaneous use of both reactors. The 1-gallon reactor operates only in a vertical position and is equipped with an anchor-shaped stirrer that reaches the bottom of the reactor. With the 5-gallon reactor, we are limited to being able to stir and AFEX-treat approximately 150 g of bagasse or CLM. However, the 5-gallon reactor can be operated in either the vertical or horizontal positions. The reactor is loaded and unloaded in a vertical position and rotates to horizontal for the stirring operation. The stirrer has a special design (back-to-back anchor) which provides better mixing. Initially, 750 g of bagasse was treated at the optimal conditions identified for the AFEX treatment of bagasse (100°C, 40% moisture content, 2:1 ammonia:biomass, and 30 min) in the 1-gallon reactor. The temperature was well controlled and the biomass was stirred in the horizontal position. The final pressure was approximately 450-500 psi, similar to that seen in the 1-gallon reactor. After 30 minutes of treatment, the reactor was rotated back to the vertical position and the pressure was released (releasing pressure took longer than the 1-gallon reactor.). The AFEX-treated biomass was unloaded and left under a fume hood to volatize the remaining ammonia. The treated sample did not appear well mixed; some patches of the biomass were lighter in color compared with the rest of the biomass. The treated sample was enzymatically hydrolyzed to evaluate the AFEX efficiency. The hydrolysis results are summarized in Table 10. The glucose and xylose yields were slightly lower compared to samples treated in the 1-gallon reactor under the same conditions.

To provide better mixing and ammonia distribution, the Teflon blades on the mixer were extended and a dip tube was added to the ammonia feeding port. After implementing these modifications, the reactor was run with 600 g biomass instead of 750 g. The sample generated from this run was more homogenous than those of the previous runs. The hydrolysis data showed slightly higher glucose and xylose yields (Table 10), similar to yields from the 1-gallon reactor; however, the mixing still was not satisfactory. To insure a better mixing pattern, the lower anchor was modified with two extension rods extending downward on the opposite sides of the main shaft. The rods have tines perpendicularly welded on them to provide a pitchfork-like action for mixing the biomass. Then 600 g of bagasse was pretreated with the modified mixer. The treated sample was more thoroughly mixed than the earlier samples. The hydrolysis results were similar to the hydrolysis result from the 1-gallon reactor. The AFEX run was performed in duplicate, and the hydrolysis in quadruples. The results are reported as the average.

Table 10. Hydrolysis Results of Bagasse Treated in the 5-gallon Reactor

AFEX ID#	Biomass g	Stirrer	Temperature	% Moisture	Time Min.	Ammonia loading	Glucose yield %	Xylose yield %
831-99A	750	original	100°C	40	30	2:1	64±1	49±2
831-100A	750	original	100°C	40	30	2:1	65±2	48±2
853-16A	600	modified	100°C	40	30	2:1	70±2	56±1
853-17A	600	modified	100°C	40	30	2:1	69±2	54±2
1 gallon reactor	150	original	100°C	40	30	2:1	70±2	58±2

To optimizethe AFEX conditions and the reactor setting, more runs needed to be performed. However at this point of the project the heater on the 5-gallon reactor failed. After the repair (which took approximately a month), a few more AFEX runs were conducted to verified the results obtained above. Data in Table 11 shows the reproducibility of these results. We have also treated bagasse under similar conditions with the exception that the run time was increased from 30 minutes to 45 minutes (853-22A). Hydrolysis results for this run exhibited higher glucose and xylose yields compared to the sample treated for 30 minutes. These results were verified through repeat runs and, as Table 2 shows, the improvement is not significant. AFEX runs of bagasse with 60% moisture content have also been attempted and the hydrolysis data has not shown any improvement. Bagasse sample treated at 60% moisture content at 100°C and 2:1 ammonia loading for 45 minutes gave a similar glucose yield (70% ± 1) compared to a sample treated with 40% moisture content. Lower hydrolysis yields were demonstrated when the ammonia loading was lowered.

Further fine tuning of the stirrer and the overall operation, has enabled us to treat 750 grams of biomass per run, versus the 600 grams of biomass previously treated. The

treated biomass were well mixed and showed similar hydrolysis results to the AFEX run performed with 600 grams of biomass.

Table 11. 72-hr Hydrolysis Results of Bagasse Treated in the 5-gallon Reactor.)Yields are calculated based on the available sugars. Hydrolyses were conducted with 15 FPU of cellulase and 42 units of Novo-188 per gram of cellulose.)

AFEX ID#	Biomass g	Temperature	% Moisture	Time Min.	Ammonia loading	Glucose yield %	Xylose yield %
853-16A	600	100°C	40	30	2:1	70±2	56±1
853-21A	600	100°C	40	30	2:1	69±1	55±1
853-26A	600	100°C	40	30	2:1	67± 1	50±1
853-22A	600	100°C	40	45	2:1	78± 2	56±2
853-29A	600	100°C	40	45	2:1	68±1	50±2
853-25A	600	100°C	40	45	2:1	72± 1	54±1
853-25B	600	100°C	40	45	2:1	70± 1	53±1
853-30A	750	100°C	40	45	2:1	70± 3	46±2
853-26B	600	100°C	40	30	1.5:1	61± 1	49±1
853-27A	600	100°C	40	30	1.5:1	56±2	45±2
1 gallon reactor	150	100°C	40	30	2:1	70± 2	58±2

Previous years research showed that in the 1-gallon reactor, the most effective AFEX conditions for the treatment of CLM were 100ºC, 40% moisture content, 2:1 ammonia to biomass loading for 30 minutes. These conditions were evaluated in the 5-gallon reactor and gave similar hydrolysis results to the sample treated in the 1-gallon reactor. Lower hydrolysis yields were demonstrated when the ammonia loading was lowered. When a longer treatment time (45 minutes) was evaluated, the hydrolysis yields were similar to those from runs conducted at 30 minutes. The hydrolysis data and the applied AFEX conditions are summarized in Table 12.

Table 12. 72-hr Hydrolysis Results of CLM Treated in the 5-gallon Reactor. (Yields are calculated based on the available sugars. Hydrolyses were conducted with 15 FPU of cellulase and 42 units of Novo-188 per gram of cellulose.)

AFEX ID#	Biomass g	Temperature	% Moisture	Time Min.	Ammonia loading	Glucose yield %	Xylose yield %
853-23A	**600**	**100°C**	**40**	**30**	**2:1**	**85±1**	**51±1**
853-24A	**600**	**100°C**	**40**	**45**	**2:1**	**83±1**	**50±1**
853-28A	**600**	**100°C**	**40**	**30**	**2:1**	**83±1**	**49±1**
853-33A	**750**	**100°C**	**40**	**30**	**2:1**	**79±1**	**51±1**
853-28B	**600**	**100°C**	**40**	**30**	**1.5:1**	**77±1**	**46±1**
1 gallon reactor	**150g**	**100°C**	**40**	**30**	**2:1**	**84 ±2**	**58±2**

The AFEX treatment of bagasse and CLM at 100°C, 40% moisture content, 2:1 ammonia loading for 30 minutes was repeated. The hydrolysis results verified that the most effective conditions for AFEX treatment of bagasse and CLM in the 5-gallon reactor are the same as conditions identified for the 1-gallon reactor.

Materials and Methods (Task 1)

Materials:

Sugarcane bagasse, bagasse pith and CLM were provided by Audubon Sugar Institute (ASI). They were milled by knife mill (Fitzmill Model JT) to pass through a 1mm screen (Mesh #9). The composition of the bagasse, bagasse pith and CLM are summarized in Table 1.

Cellulase: Spezyme Cp (Genencor, Rochester, NY).

β-glucosidase: Novozyme 188 (Sigma, St. Louis, MO)

Xylanase: NS50014 and NS50030 (Novozyme, Franklinton; NC), NS50030 activity: 500

FXU/g (FXU: Fungal Xylanase Unit); activity of NS50014: 750 FXU/g; density of both enzymes is 1.08 g/ml.

Anhydrous ammonia: Linde Gas LLC, (Lansing, MI). All other chemicals were purchased from Sigma (St. Louis, MO).

Analytical methods:

Composition (glucan, xylan, galactan, arabinan, lignin and ash) of the biomass was determined by following the National Renewable Energy Laboratory (NREL) procedure, (NREL LAP-002). NREL procedures are available at:
http://www.ott.doe.gov/biofuels/analytical_methods.html.

Extractive content was measured according to NREL procedure LAP-010.

Enzymatic hydrolysis was carried out by following the NREL LAP-009 procedure. Hydrolysate samples were analyzed for glucose, xylose, cellobiose, arabinose, mannose and galactose content by HPLC, using an Aminex-HPX-87P column (Bio-Rad). All hydrolyses were performed in duplicate and the data are presented as the average of the duplicates.

Selective hydrolysis of hemicellulose by cellulase-free xylanase: Hydrolyses were performed according to NREL LAP-009 with the exception of replacing the cellulase with xylanase. The detail of loading is presented in the Results section. All experiments were performed in duplicate and the data are presented as the average of the duplicates.

AFEX treatment:
The biomass (bagasse, bagasse pith, and CLM) was treated by the AFEX process in a one-gallon pressure reactor (PARR). Biomass with the desired moisture content level was added to the reactor. To ensure uniform distribution of heat and ammonia, the reactor was equipped with an agitator and mixed the biomass at a speed of 100rpm during the entire process. The reactor was heated by means of an electrical heating mantel. While the reactor was heating, the desired amount of ammonia was pumped into the reactor. The reaction timer was started when the reactor was within 5°C of the set-point. When the desired reaction time elapsed, ammonia was quickly evacuated via a manual ball valve. The treated biomass was removed from the reactor and left in a fume hood to evaporate the residual ammonia. The treated biomass was stored at 4°C until used. All AFEX runs were performed in duplicate and data are presented as the average of the duplicates.

Succinic acid production from AFEX treated CLM and bagasse via separate hydrolysis and fermentation (SHF):
AFEX-treated biomass for two different solids loadings (8 and 12% for CLM, 8 and 12% for bagasse) was combined with 125 g/l of liquid feed syrup [LFS] (GPC, Muscatine IA) and the volume adjusted to nearly final fermentation volume with water. The fermentation vessels (5L BioFloIII fermentor) containing the biomass and LFS were sterilized for 60 min at 121°C, 19psi pressure, cooled to and maintained at 50°C. The pH of the biomass/ LFS slurries was determined and adjusted to a pH 5.1 through the addition of 8 N H_2SO_4. Only minor pH adjustments were necessary. Enzymes (20 FPU Spezyme Cp + 42 CBU Novo 188/g cellulose)were filter sterilized and added to the fermentor , but no further pH adjustments were necessary over the course of the 72 hour hydrolysis. Samples were taken every 24 hours for monitoring the hydrolysis of AFEX-treated CLM. Samples were analyzed for sugars by HPLC using refractive index detection and an Aminex HPX-87P column with a de-ashing guard column, held at room temperature. After 72 hours of the hydrolysis, the pH was brought to 6.9 via the addition of $Mg(OH)_2$/ Na_2CO_3 and bringing the temperature to 38°C. Fomenters were inoculated with a 5% seed inoculums. Seed was raised in a medium containing: 80 g/l glucose, 85 g/l LFS, 3 g/l yeast extract, 0.2 mg/l d-biotin and 5 mM phosphate. The pH was maintained at 7.0 with $Mg(OH)_2$. Agitation was set at 250 rpm, temperature at 38^{o}C and carbon dioxide was spurge at a rate of 0.025 v.v.m. The seed was grown for about 13 hours and until the OD_{660} was 2.84. After inoculating the fermenters, the fermentations

were carried out for 72 hours. The fermentations were monitored by analyzing the 24 hr samples for succinic acid, glucose, lactic acid, pyruvate, ethanol, and formic acid concentrations. These components were determined by reverse phase high pressure liquid chromatography (HPLC) using a Waters 1515 isocratic pump with a Waters 717 Auto sampler and a Waters 2414 refractive index detector set at 35^{o}C. The HPLC system was controlled, data collected and processed using Waters Breeze software (version 3.3). A Bio-Rad Aminex HPX-87H (300mm x 7.8mm) column was used with a cation H guard column held at 55^{o}C. The mobile phase was 0.021N sulfuric acid running at 0.5 ml/min. Samples were filtered through a 0.45 μm filter, and 5.0 μl were injected onto the column. Run time was thirty minutes.

A mass flow controller (Brooks model 5850I) was used to monitor and supply CO_2 to the fermenter sparging system at 100 ml/min. A mass flow meter (Brooks model 5860I) was used to measure CO_2 exiting the fermentation vessel by way of the exhaust condenser system. The two CO_2 flow meters were connected to a computer via a 4-20ma Bio-Command Interface. The BioCommand Plus Bioprocessing software logs the inlet and outlet CO_2 flow every 60 seconds. The rate of CO_2 consumption (ml/min) was expressed as the difference between the inlet and outlet rates during any given minute (CO_{2use} = CO_{2in} - CO_{2out}). The moles of CO_2 consumed were calculated using the Ideal Gas Law, (0.88694 liters ÷22.4 liters/mole = 0.0396 moles). The mass flow meters were calibrated by the manufacturer for CO_2 and their precision is 1% of full scale or 2 ml/m. The fermentation setup was monitored for gas leaks, by mixing 5% hydrogen into the CO_2. Hydrogen leaks are detected using a Tif8800 CO/Combustible Gas analyzer.

Ethanol production from AFEX bagasse via separate hydrolysis and fermentation (SHF):
Enzyme cocktail: 20 FPU of Spezyme Cp and 42 CBU of Novozyme 188 /g cellulose.

Inoculum preparation: *Zymomonas mobilis* pZB5 was used for SHF fermentations. The medium for the SHF fermentations contained 5 g/l Bacto yeast extract, 2 g/l KH2PO4, 30 mg/l tetracycline-HCL, 50-100 g/l biomass weight. The inoculum was grown in the same medium with 2% w/v glucose and 1% xylose as substrate. The growth in the inoculum flasks was monitored by measuring the optical density. They were used to inoculate the fermenters when the OD_{600} reached 2.5-3.0.

Bioreactor preparations: The fermentors were autoclaved for 30 min prior loading. The appropriate amounts of biomass for 4% (in 5L BioFloIII fermentor) and 8% (in 2.5L BioFloIII) solids loading along with the required amount of water and medium were added to the fermentors. The fermentors were operated at 50°C, 500 rpm and pH 5.0 and the separate hydrolysis stage was started by adding the enzyme cocktail. The hydrolyses were carried out for 72hrs and samples were taken every 24 hours. After the hydrolyses, the fermentors were operated at 28°C, 500 rpm and pH 5.0 and the SHF fermentations were started by adding 5.8% inoculum of *Z. mobilis* pZB5. The fermentations were carried out for 72 hrs and samples were taken every 24 hours. Sugars concentrations were determined by HPLC using standard methods for the BioRad Aminex HPX-87P column. Ethanol and organic acid byproduct were determined by HPLC using standard methods for the BioRad Aminex HPX-87H column.

Fed batch SSF ethanol fermentation with Saccharomyces cerevisiae:
Enzyme preparations: An enzyme cocktail was made for each fermentor and Spezyme Cp (20 FPU/g cellulose) and Novo 188 (42 CBU/g cellulose) were added. The enzyme cocktail was filter sterilized with a 50mm pre-sterilized 0.2μm filtration unit (www.nalgenelabware.com).

Medium and inoculum preparations: A *Saccharomyces cerevisiae* strain obtained from NREL was used. The medium used in the SSF fermentations contained corn steep liquor (CSL) 5% v/v, 0.62 g/l $MgSO_4.7H_2O$, 2 g/l NH_4SO_4. The inoculum was grown in the same medium as used in the SSF fermentations, but with 2% w/v glucose as substrate. The inoculum was grown in 1000 ml baffled Erlenmeyer flasks containing 150 ml of medium each. The flasks were incubated at 30° C and 150 rpm in a Model G25 incubator shaker (NBS, Edison, NJ). The growth in the inoculum flasks was monitored by measuring the optical density. They were used to inoculate the fermentors when the OD_{600} reached 2.5-3.5, after about 14-15 hours of incubation.

Bioreactor preparations: The amount of biomass necessary to give a 10% solids loading was placed into 3 or 5L fermentor (NBS, Edison, NJ), with the required amount of water containing the CSL and the medium salts. The fermentors were autoclaved for 1 hour. After autoclaving, the fermentors were operated at 35° C, 700 rpm, and pH 5.0. The pH was controlled through the automatic addition of 1M NaOH, and 4N H_2SO_4. The pH of the fermentation was monitored by checking the sample pH using an externally calibrated electrode and adjusting the fermentor automatic pH controller as necessary. The SSF fermentation was started by adding 150 ml of enzyme cocktail and 150 ml of *S. cerevisiae* inoculum. The target amount of biomass was three times of the starting loading; therefore, in the first 24 hrs of the process, two more batches of biomass (each equal to the initial loading) plus the required amount of enzyme cocktail were separately added to the fermentor. Fermentation was carried out for 120 hr and samples were taken for sugar and ethanol analysis every 24 hours.

Fed batch SSF ethanol fermentation with Zymomonas mobilis:
This fermentation was similar to the fermentation mentioned above with the following exception:

Inoculum preparation: The medium for the SSF fermentations contained 5 g/l Bacto yeast extract, 2 g/l KH2PO4, 30 mg/l tetracycline-HCL. The inoculum was grown in the same medium with 2% w/v glucose and 1% xylose as substrate. Growth in the inoculum flasks was monitored by measuring the optical density at 600 nm. Inoculum flasks were used to inoculate the fermentors when the OD_{600} reached 2.5-3.0.

The fermentor was operated at 32° C, 700 rpm, and pH 5.0 with 10% solids loading. The pH was controlled through the automatic addition of 1M NaOH, and 4N H_2SO_4. The SSF fermentation was initiated by adding 150 ml of enzyme cocktail and 150 ml of *Z. mobilis* inoculum. The target amount of biomass was three times of the starting loading; therefore, in the first 24 hr of the process, two more batches of biomass (each equal to the

initial loading) plus the required amount of enzyme cocktail were separately added to the fermentor. Fermentation was carried out for 120 hr and every 24 hours samples were taken for sugar and ethanol analysis.

Fed batch SSF fermentation of AFEX treated CLM: Two different microorganisms, *S. cerevisiae* and *Z. mobilis* 37821 (pZB5) were used for the fed batch SSF fermentation of AFEX-treated CLM. These fermentations were performed in 3L New Brunswick Microferm fermentorsAgitation in both fermentors was provided by three impellers with the same set up (one pitched-blade impeller at the bottom and two disk impellers on the top). In general, the mixing appeared adequate to maintain solids in a suspended slurry.

Task 2. Alkaline treatment, solid/liquid separation and Lignin recovery

The goal of this task was to investigate the effects of post- AFEX extractions on biomass. The concentration was on a second extraction with caustic (or water). Monitoring was by enzyme digestion. Several other methods were tried, on both AFEX and non-AFEX treated materials, but these results are subject of a different contract.

Results

Biomass Analysis

The key to this task is biomass analysis. Analytical methods developed or utilized by ASI, MBI and NREL were compared to establish the composition of the various test materials. Table 13 compares the compositions of pith, CLM, and bagasse determined at ASI and MBI. The glucan content of pith agreed within 2% for ASI and MBI. For CLM, ASI measured a slightly lower hemicellulose and cellulose content than, however lignin content agreed within 1% and extractives agreed with those of published literature within 2%.

Table 13: Compositional analysis of sugar cane biomass. Weight percent (g/100 g dry matter).

Substrate	Hemicellulose	Cellulose	Lignin	Ash	Extractives	Total
Pith (ASI)	16.7%	17.3%	44.1%	20.9%	4.6%	103.5%
	13.9%	17.0%	49.2%	27.6%	3.8%	111.4%
Pith (MBI)	14.0%	19.7%	20.4%			
CLM (ASI)	19.7%	23.7%	21.2%	6.0%	5.0%	75.7%
	20.9%	22.4%	23.8%	5.8%	4.9%	77.8%
CLM (ASI)*	23.3%	28.5%	21.5%	13.4%	5.3%	92.0%
CLM (MBI)	24.5%	31.9%	20.8%			77.2%
Bagasse (ASI)*	22.8%	34.5%	20.1%	8.8%	3.2%	89.4%
Bagasse (MBI)	19.9%	34.6%				

*These results are from work conducted at Audubon Sugar Institute and were published in The Sugar Bulletin, vol. 82, July 2004.

This table shows that for the pith samples, the mass balances exceeded 100% and both the ash and lignin contents were rather high. In bagasse, acid insoluble ash can be high and this is counted twice, both in the lignin and ash fractions. Ashing the lignin fraction is

required to correct this. The mass balances for CLM are lower than the literature values, but consistent with MBI. Comparisons with the literature can only be approximate, however, because biomass composition is known to vary with harvesting time and geographic location.

Fractionation of AFEX-treated bagasse pith and CLM solids

Pith and Cane Leaf Matter (CLM) pretreated with the AFEX process (pith and CLM) were received from MBI for further pretreatment with water and sodium hydroxide.

Aqueous extracts were found to contain primarily lignin and small quantities of hemicellulose. There was a higher weight percent loss of the biomass when it was treated with alkali (25 %) as compared to pretreatment with water (48 %). These extractions were done at 100 °C.

Water extractions were performed at 120 and 140 ^{o}C resulted in an enhanced glucan conversion with saccharification in the range of 67-88 % (72 hour glucan yield, 120 ^{o}C). The range of saccharification yield for AFEX-only treated material was 52-70 %. To consolidate results, the water and sodium hydroxide extractions were repeated at 120 and 140 °C. The goal of the second extraction was to remove lignin and hemicellulose, producing a biomass with higher cellulose content. Removal of saccharification and fermentation inhibitors occurs during the second pretreatment. Table 14 shows the quantity of lignin solubilized by the hydrothermal treatment. There was greater removal of both lignin and ash at 120 °C.

The composition of the hydrolysate from water treatment is Table 15. Most of the sugars extracted are oligomers with xylose oligomers being dominant. All of the arabinose is recovered..

Lignin removal after one hour treatment with 1 % NaOH for AFEX treated samples is shown in Table 16. As was found in the previous year, more Klason lignin and acid soluble lignin was removed compared to the water extraction. The composition of the base treated hydrolysate is given in Table 16. Most of the sugars extracted are xylose oligomers (Table 17).

Table 14: Quantity of solubilized lignin after post-AFEX water extraction in the hydrolysates expressed as g/100 g dry substrate.

	Klason Lignin	**Acid Soluble Lignin**	**Acid Insoluble Ash**
Pith, 120°C	7.9	4.3	1.8
Pith, 140°C	3.9	3.6	1.9

CLM 120°C	6.8	4.6	1.5
CLM 140°C	5.9	4.4	1.1

Table 15: Quantity of solubilized oligomers after post-AFEX water extraction in the hydrolysates expressed as g/100 g dry substrate.

	Oligomer		
	Glucose	**Xylose**	**Arabinose**
Pith, 120°C	2.7	6.0	1.3
Pith, 140°C	2.2	5.6	1.3
CLM 120°C	1.9	8.7	2.1
CLM 140°C	0.6	7.0	2.3

Table 16: Quantity of solubilized lignin in the hydrolysates from post-AFEX NaOH extraction expressed as g/100 g dry substrate.

	Klason Lignin	**Acid Soluble Lignin**	**Acid Insoluble Ash**
Pith, 120°C	10.8	6.3	2.4
Pith, 140°C	8.5	7.1	1.7
CLM 120°C	8.9	7.7	1.5
CLM 140°C	8.4	8.6	1.1

Table 17: Quantity of solubilized oligomers after post-AFEX NaOH extraction in the hydrolysates expressed as g/100 g dry substrate.

Oligomer			
	Glucose	**Xylose**	**Arabinose**
Pith, 120°C	1.2	6.0	2.6
Pith, 140°C	5.7	11.1	2.0
CLM 120°C	1.0	8.9	1.8
CLM 140°C	1.6	13.2	3.3

The % glucan in the extracted samples was higher than the % glucan in the untreated samples after a second extraction. The extraction processes removed some of the hemicellulose and the extractives from the biomass which results in higher glucan content and also makes the biomass more digestible. As expected, the extracted samples gave high glucose yields and for NaOH extracted samples, all the available glucan was converted to glucose.

Conclusion: An alkali pretreatment increased the saccharification of AFEX-treated biomass.

Post-AFEX Extractions

The weight loss depended on the solvent used; water gave least weight loss followed by acid, followed by alkaline extraction. An analysis was performed of the AFEX extracts. These extracts were concentrated and spray-dried to produce a powder. The carbohydrate and ash contents of the powders are given in Figure 5.

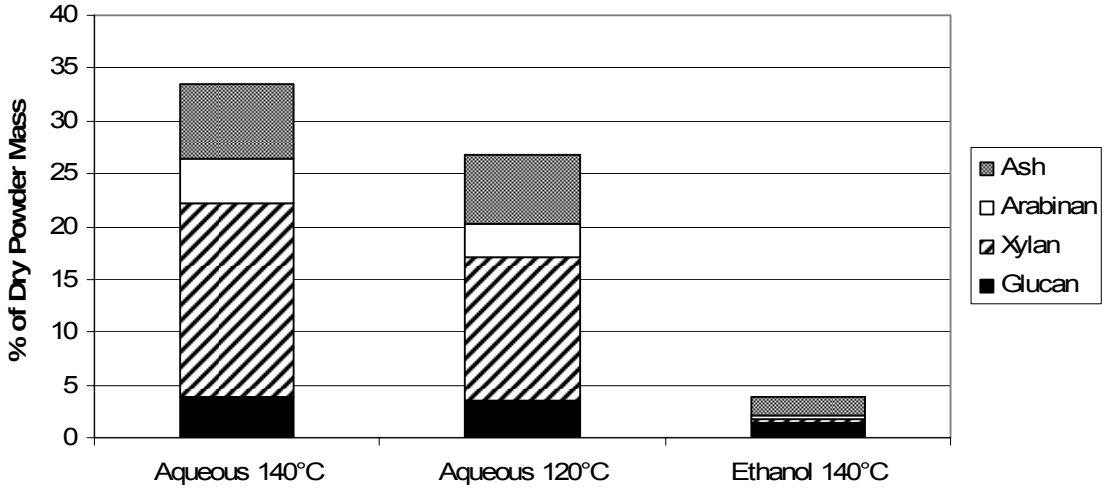


Figure 5: Carbohydrate and ash content of powders produced by spray drying from the liquid obtained from post AFEX extractions of CLM and bagasse.

This graph shows that the extract is composed mostly of a non-carbohydrate and we expect it to be mostly lignin. Based on this consideration, the estimate of lignin purity is about 95% in the ethanol extract to close to 75 % in the aqueous extract produced at 120°C. Aqueous extraction removes significantly more sugars than ethanol. Also, as expected, most of the sugars removed are pentoses from the hemicellulose and only a small amount of glucan is removed. This glucan is likely to be amorphous which is extractable whereas crystalline cellulose requires more severe conditions to dissolve. For the ethanol extracted sample, none of the hemicellulose was removed. This is because ethanol does not act as a catalyst for hydrolysis the way that water does. Furthermore, sugar solubility is very low in ethanol. The aqueous samples also contained more ash than the ethanol extracts.

Ethanol

The compositions of the fibers from the ethanol extractions are shown in Table 18. The weight loss is the quotient of the dry fiber mass after pretreatment divided by the original dry fiber mass. Most of the glucan in biomass is in the form of crystalline cellulose, and it is assumed that none of it is removed by pretreatment. Thus during pretreatment, other components like hemicellulose, lignin, ash, and extractives are partially removed, creating a substrate with a slightly higher cellulose content on a dry weight basis.

Table 2 shows the analysis for the fibers from post AFEX ethanol extraction at 120°C and 140°C. Samples that were treated by AFEX at different conditions were combined in order to have a large enough substrate for extraction in the Parr apparatus. The Sample ID's are the ID's from AFEX. In order to evaluate this procedure, Table 3 also includes the weight losses and the glucan contents based on those weight losses.

Table 18: Composition of fibers after AFEX pretreatment and ethanol extraction. Weight per cent (g/100 g dry matter).

Treatment	Sample ID	Wt. loss	Hemi-cellulose	Cellulose	Cellulose (based on wt. loss)	Lignin	Ash	Total
Untreated CLM			23.3%	28.5%		22%	13 %	87%
Untreated Bagasse			22.8%	34.5%		20%	9%	86%
AFEX, Bagasse, 120°C	99A, 99B, 100A, 100B	12.70 %	36%	49%	40%	25%	13 %	123%
			27%	48%		24%	13 %	112%
AFEX, CLM, 120°C	83 / 85 / 86	8.50%	29%	29%	31%	32%	11 %	101%
			33%	36%		27%	11 %	107%
AFEX, CLM, 120°C*		- 4.60%	19%	10%	27%	46%	7%	82%
			30%	15%		45%	7%	96%
AFEX, Bagasse, 140°C	91A, 91B, 94B, 98B	10.70 %	40%	39%	39%	22%	14 %	114%
			52%	50%		22%	22 %	114%
AFEX CLM, 140°C	64A, 65B, 67A, 68A	7.90%	22%	28%	31%	27%	13 %	89%
			29%	29%		25%	13 %	96%
AFEX, CLM, 140°C	91A, 91B, 94B, 98B	9.90%	46%	33%	32%	26%	14 %	119%
			42%	32%		26%	14 %	113%
AFEX, CLM, 140°C	83B, 86B, 87A, 88A	14.70 %	43%	33%	33%	25%	13 %	114%
			33%	25%		25%	13 %	97%

*Because of the low glucan content measured in this analysis, this sample was repeated, this time with proper grinding, screening, and removal of extractives. The composition was then 31-36% hemicellulose, 29-35% glucan, and 24-25% lignin.

The main carbohydrate in the extracts from ethanol was glucose whereas in the aqueous extract, it is xylose. At higher temperatures, amorphous glucose is removed faster but the xylan removal may stay the same.

Water

For the water extracts, there is an even greater variability, however, the lignin content is increased compared to untreated bagasse and CLM, and the hemicellulose content is overall reduced (Table 19).

Table 19: Composition of fibers after AFEX pretreatment and water extraction. Weight per cent (g / 100 g dry matter).

Treatment	Sample ID	Wt. loss	Hemi-cellulose	Cellulose	Cellulose (based on wt. loss)	Lignin	Ash	Total
Untreated CLM			23.3%	28.5%		22%	13%	87%
Untreated Bagasse			22.8%	34.5%		20%	9%	86%
No AFEX, CLM, 120°C		6.20%	25%	31%	30%	21%	5%	83%
			25%	30%		25%	30%	109%
AFEX, CLM, 120°C	64A, 65B, 66A, 66C	37.20 %	20%	32%	45%	24%	9%	85%
			21%	32%		26%	9%	88%
AFEX, CLM, 120°C	86A, 86B, 83B, 85B	30.00 %	16%	25%	41%	43%	12%	96%
			16%	26%		40%	12%	94%
AFEX, Baggasse, 120°C	99A, 99B, 100A, 100B	22.20 %	22%	38%	44%	28%	11%	98%
			23%	32%		32%	11%	98%
AFEX CLM, 140°C	67A, 68A	23.70 %	26%	8%	37%	41%	13%	88%
			29%	29%		38%	10%	106%
AFEX, Bagasse, 140°C	90A, 90B	Not known	23%	35%	Not known	37%	15%	110%
			23%	34%		38%	15%	109%
AFEX, Bagasse, 140°C	91B, 93B	25.70 %	34%	47%	46%	47%	12%	140%
			21%	34%		41%	12%	108%
AFEX, Bagasse, 140°C	91A, 98A, 98B	27.40 %	20%	39%	48%	30%	6%	95%
			19%	31%		40%	13%	103%

Enzymatic Hydrolysis

Table 20 shows our results for the enzymatic digestibility at 72 hours for 15 FPU/g glucan of Spezyme® and 42 CBU/g glucan of Novozyme®. Many more tests were performed, however a large number of samples showed contamination in that a higher digestibility was measured at 24 or 48 hours than 72 hours. In those instances, microbial infection occurred in the sample flask and consumed some of the glucose. We are taking steps to avoid this problem by autoclaving flasks before experiments and limiting exposure to air during sampling. The samples given in Table 3 did not suffer from this problem.

Table 20: Enzyme Hydrolysis of fibers after AFEX pretreatment and extraction.

Treatment	Sample ID	72 hr. glucan yield	Range of Glucan conversion (72 hrs) Prior to Extraction	Glucan content based on compositional analysis	Glucan content based on weight loss
AFEX, CLM, 100% EtOH, 140°C, 1 hr.	83B, 86B, 87A, and 88A	9%	75% - 84%	10%	27%
		16%		15%	
AFEX, CLM, 100% EtOH, 140°C, 1 hr.	83B, 85B, 86A, 86B	15%	77% - 84%	29%	31%
		14%		36%	
AFEX, CLM, 100% EtOH, 140°C, 1 hr.	91A, 91B, 94B, 98B	10%	69% - 75%	50%	39%
		9%		39%	
AFEX, CLM, EtOH, 140°C, 1 hr.	64A, 65A, 67A, 68A	31%	44% - 49%	28%	31%
		26%		29%	
No AFEX, CLM, Aqueous, 120°C, 1 hr.*		38%	13%	31%	30%
		42%		30%	
AFEX, CLM, Aqueous, 120°C, 1 hr.	64A, 65B, 66A, 66C	68%	48% - 62%	32%	45%
		68%		32%	
AFEX CLM, Aqueous, 120°C, 1 hr.	86A, 86B, 83B, 85B	88%	75% - 84%	25%	41%
		-		26%	
AFEX, Bagasse, Aqueous, 120°C, 1 hr.	99A, 99B, 100A, 100B	84%	52% - 70%	38%	44%
		67%		32%	

*This sample was not AFEX treated before extraction and the yield of raw CLM is 13%.

Table 20 presents the glucose yields of post AFEX extracted samples and compares it to the yields for just AFEX pretreated samples. However, because the material used for the

extractions was a composite of materials treated at different AFEX conditions, a range is presented of the lowest and highest digestibility of the samples collected in that lot. The ethanol extracted samples consistently exhibited a lower yield than the non-extracted samples. The reason for this is unknown, but we believe that ethanol may have collapsed the pores of the biomass structure, making it less accessible to cellulose enzymes. It is believed that such pore structures are maintained, in part, through the action of bound water. Ethanol has a drying effect. For the samples that were extracted with water at 120°C, the first was not AFEX treated at all, and it had a digestibility of 38-42%, which was less than all the AFEX digestibility but significantly higher than the untreated CLM (13%). The 64A - 66C and 86A-85B samples both clearly had a higher yield after aqueous extraction than with just AFEX, and the improvements were between 4-20% and 4-13%. These higher digestibilities would give an ethanol yield increase of 0.4 to 1.75 gallons per dry ton CLM. For the last sample, a digestibility of 67-88% was measured. This is a large range, but the range from only AFEX treatment was 52%-70%. These results indicate that an aqueous post AFEX extraction is promising. Furthermore, enzyme hydrolysis requires an aqueous slurry with solid concentration of about 10% or less. The substrate after AFEX is relatively dry after ammonia recovery, and will have to be contacted with water before saccharification and fermentation. The extract is composed mostly of lignin and some of the hemicellulose sugars. If market can be found for the lignin or pentose sugars, aqueous extraction provides a ready source.

Washing

A recent publication from Michigan State University (MSU) (Shishir et al., 2007) indicated that washing AFEX-treated corn stover improved the glucan conversion of the biomass in high solid loading hydrolysis. Therefore, in an effort to improve the enzymatic hydrolysis and ultimately the fermentation yield, the AFEX-treated bagasse was washed with water. The solid and liquid portions were separated by filtration. The glucan and xylan content of both washed and unwashed AFEX-treated biomass were analyzed. Glucan and xylan contents of the washed sample were 39.4% and 18.8% respectively, and the unwashed sample contained 35.8% glucan and 16.3% xylan. To demonstrate the effect of washing, enzyme hydrolyses at different solid loadings were conducted with both washed and unwashed samples. The results are presented in Table 21. The hydrolysis yields from washed samples were unexpectedly lower or equal to the hydrolysis results of unwashed samples

Table 21. 72-hr Hydrolysis Results of Washed and Unwashed AFEX-treated Bagasse Samples with 15 FPU of Spezyme Cp and 42 CBU of Novo 188 per Gram of Cellulose. (Yields are based on sugar content of the biomass.)

	Glucose yield
1% glucan loading	
Unwashed sample	76% ±2
Washed sample	72% ± 0
2% glucan loading	
Unwashed sample	75% ± 2
Washed sample	74% ± 0

3% glucan loading	
Unwashed sample	70% ± 1
Washed sample	70% ± 2

Materials and methods (Task 2)

Alkaline and water extraction. For each test, 33.6 g of AFEX treated pith at 74.5 % solids (*i. e.* 25 g of bone dry pith) was placed in a 5-gal Parr reactor containing 500 ml of deionized water. The same test is done using 31.7 g of AFEX-treated CLM, at 78.8 % solids (*i. e.* 25 g of bone dry CLM). The vessel was stirred by a spiral agitator and constantly heated. The approximate heat-up time to 120 °C was 19 min and 21 min to reach 140 °C. Once the target temperature was reached, it was maintained for an hour. After one hour, the heating jacket was shut off and the vessel cooled to 65 °C within 3-4 hr. The Parr reactor was fitted with a frit to filter the solution. An adapter was added to the bottom port and attached with tygon tubing to the side arm of a filter flask. The top of the flask was attached to vacuum. The liquid was collected in the vacuum flask. The fiber was recovered from the reactor. Both liquid and solid were refrigerated.

Following AFEX treatment, bagasse pith and CLM solids were treated with a base or other solvent to separate cellulose and hemicellulose. The residual solid, which is rich in cellulose, was recovered by filtration. Ethanol was added to the supernatant to precipitate the extracted hemicellulose, which can be recovered by filtration. Other ways such as membrane filtration or crystallization were considered for recovering solubilized non-cellulose components.

The AFEX-treated bagasse pith and CLM samples were extracted with water and NaOH at two different temperatures (120 and 140°C) by ASI. The generated solids were sent to us for composition analysis and enzyme hydrolysis. The samples received were very wet. A portion of each sample was used for composition analysis and the rest for enzyme hydrolysis. The samples for the composition analysis should be dry; therefore, for the water extracted samples, the liquid was squeezed out using a Buchner funnel and vacuum and the samples were dried at 45°C. The rest of the samples were used for enzyme hydrolysis as received. The moisture content of the samples was taken into account for the hydrolysis.

For NaOH extracted samples, the liquid was squeezed out from the whole sample and the solid portion was washed several times with water to bring the pH of the wash close to 7. A portion of the samples were dried for composition analysis and the rest were used for enzyme hydrolysis.

Bagasse samples pretreated with different concentrations of NaOH and NH_4OH (1%,2%,4%,6%) prior to treatment were AFEX-treated at 100°C, 40% moisture content and 1:1 ammonia loading for 30 min. In some cases, the duplicate results of this series of

experiments showed relatively large deviations; therefore, it is not possible to draw firm conclusions.

Several bagasse samples treated with different combinations of peroxide/hypochlorite solution (referred to as Ox-B) were received from LSU. These samples were AFEX-treated under two different sets of AFEX conditions (100°C, 40% MC and two ammonia loadings: 2:1 and 1:1 for 30 minutes). All of the Ox-B-AFEX-treated bagasse samples were hydrolyzed and the obtained data showed that no significant improvements were observed on the glucose yield.

Unground CLM was AFEX-treated at 100°C, 40% moisture content and 2:1 ammonia loading for 30 min. Enzyme hydrolysis of this sample gave glucose and xylose yields that were not significantly different from those obtained from ground CLM AFEX-treated under the same conditions. These data indicate that grinding of CLM offers no direct benefit to the efficacy of AFEX pretreatment.

Several batches of AFEX-treated bagasse (100°C, 40% MC, 2:1, and 30min) were produced and combined for ethanol fermentation. The fermentation was performed with two solid loadings, 4% and 8%, with recombinant *Zymomonas mobilis* 37821(pZB5) using separate hydrolysis and fermentation (SHF). Even though these hydrolyses were performed with much higher solid loading (4 and 8%) compared to regular hydrolysis loading (in a small shake flask ~2%), the hydrolysis yields were higher and we were able to ferment all the produced glucose and xylose to ethanol.

Enzyme hydrolyses with 15, 20 and 30 FPU of Spezyme Cp/g glucan were performed on both AFEX-treated bagasse and CLM. AFEX-treated CLM and bagasse were hydrolyzed with a combination of cellulase (Spezyme Cp) and several xylanases. Using the combined enzyme mixture resulted in an increase in both glucose and xylose yields.

Two cellulase-free xylanases were examined for selective hydrolysis of hemicellulose of AFEX-treated CLM and bagasse. The best case for AFEX-treated CLM gave a 52% yield of xylose after 72 hours of hydrolysis and 60% after 168 hours, while leaving more than 93% cellulose in the biomass. For AFEX-treated bagasse, the best case gave a 53% xylose yield and 5% glucose yield after 72 hours of hydrolysis and 60% and 6% yields for xylose and glucose respectively, after 168 hours of hydrolysis.

Composition (glucan, xylan, galactan, arabinan, lignin and ash) of the biomass was determined by following the National Renewable Energy Laboratory (NREL) procedure, (NREL LAP-002). NREL procedures are available at:
http://www.ott.doe.gov/biofuels/analytical_methods.html.

Extractive content was measured according to NREL procedure LAP-010.

Enzymatic hydrolysis was carried out by following the NREL LAP-009 procedure. Hydrolysate samples were analyzed for glucose, xylose, cellobiose, arabinose, mannose and galactose content by HPLC, using an Aminex-HPX-87P column (Bio-Rad). All hydrolyses were performed in duplicate and the data are presented as the average of the duplicates.

Task 3

Sugar cane bagasse is a lignocellulose biomass produced as a byproduct from sugar extraction in vast amounts that will continue to increase in light of increased worldwide sugar production. Considerable amounts of bagasse are currently used as a combustible energy source, for paper pulp production or animal feed. However the relatively high carbohydrate and low lignin content make bagasse an attractive substrate for ethanol production. Approximately 24 and 38 % (dry weight) of bagasse consists of hemicellulose and cellulose respectively, and bioconversion of both fractions to ethanol must be considered for a viable process. Pretreatment of bagasse is necessary to reduce the recalcitrance to enzymatic hydrolysis. Previous studies have reported the pretreatment of sugar cane bagasse with either physical or chemical methods such as acid (Martin et al., 2002), steam (Kaar et al., 1998) or alkali (Fox et al., 1987; Holtzapple et al., 1991). Chemical pretreatments generally remove the hemicellulose or lignin fraction thereby promoting cellulose hydrolysis (Mosier et al., 2005). Dilute sulphuric acid has been widely used to pretreat sugar cane bagasse (Van Zyl et al., 1989; Martin et al., 2002) and results in the hemicellulose fraction being released directly as pentoses such as xylose and arabinose. Without detoxification, the subsequent fermentation can be seriously inhibited. Alkali pretreatment generally leaves the hemicellulose fraction relatively intact. The ammonia freeze explosion (AFEX) process is a particularly attractive pretreatment of sugar cane bagasse (Holtzapple et al., 1991) as ammonia can be potentially recycled while only some hemicellulose is removed and the formation of sugar degradation products isminimized (Mosier et al., 2005). Furthermore this process enables both the cellulose and hemicellulose fractions to be hydrolyzed enzymatically.
The objective of this task is to develop and optimize a process using enzymatic hydrolysis to produce individual C5 sugars.

We evaluated the degree of hydrolysis of sugar cane bagasse pretreated by the AFEX process or ammonium hydroxide with combinations of cellulase, β-glucosidase and hemicellulase enzymes. Significant xylanase activity in enzyme cocktails appears to be required for greater hydrolysis of both glucan and xylan fractions of ammonia pretreated sugar cane bagasse.

Sugar cane bagasse is an attractive waste substrate for ethanol bioconversion. Approximately 24 and 38 % (dry weight) of bagasse consists of hemicellulose and cellulose, respectively, and bioconversion of both fractions to ethanol must be considered for n economically viable process. We have evaluated the degree of hydrolysis of pretreated bagasse with combinations of cellulase, β-glucosidase and hemicellulase. Ground bagasse was pretreated either by the AFEX process (2 NH_3: 1 biomass, 100°C, 30 min; kindly provided by MBI) or with ammonium hydroxide (0.5 g NH_4OH of a 28 % (v/v) per g dry biomass; 160°C, 60 min) and in both samples, the amounts of glucan and xylan fractions remained largely intact. The enzyme activities of 14 commercial enzyme preparations and supernatants of six laboratory grown fungi were determined. Four commercial preparations and four laboratory preparation with significant xylanase activity were evaluated for their ability to boost xylan hydrolysis when used in combination with cellulase and β-glucosidase (10 FPU: 20 CBU/g glucan). When evaluated at a 1 % glucan loading, one commercial enzyme preparation (added at 10 % level of total enzyme protein) boosted xylan hydrolysis by at least 40 % of both pretreated bagasse samples. The glucan hydrolysis was also boosted by the xylanase. Xylanase addition at 10 % protein level also improved hydrolysis of xylan and glucan fractions up to 10 % glucan loading (28 % solids loading). Significant xylanase activity in enzyme cocktails appears to be required for greater hydrolysis of both glucan and xylan fractions of ammonia pretreated sugar cane bagasse.

Results

Table 22 shows the composition of sugar cane bagasse and the bagasse after treatment with the AFEX process or with ammonium hydroxide. The pretreatment process resulted in an increase in the glucan concentration especially in the ammonium hydroxide pretreated bagasse. Both pretreatment processes left the hemicellulose fraction of bagasse largely intact.

Table 22. Composition analysis (g/100 g dry biomass) of ammonium hydroxide- and AFEX-treated sugar cane bagasse

Component	Raw sugar cane bagasse	Pretreated sugar cane bagasse	
		0.02 g NH_3/ 100 g H_2O, 160° C, 1 h	AFEX (2 NH_3: 1 biomass, 100° C, 30 min)
Ash	4.2	2.8	10.0
Ethanol extractives	1.1	3.9	5.8
Acid soluble lignin	5.3	5.0	8.2
Acid insoluble lignin	19.7	16.1	14.4
Glucan	38.4	56.6	41.7
Xylan	24.1	24.0	20.4

Arabinan	1.9	1.2	1.2
Mannan	0	1.7	1.5

The cellulolytic activities of 14 commercial preparations are shown in Table 23. Filter paper activity could only be detected in some samples and the highest activities were obtained in BioCat xylanase (sample 1), Spezyme CP (sample 8) and Fibrezyme LBL (sample 12). Samples showed a considerable range in CMCase (an indicator of endo-β-1,4-glucanase), avicelase (an indicator of exoglucanase) and β-glucanase activities. BioCat products showed high activities of the various cellulolytic enzymes (samples 1, 4). Novozym 188 (sample 5) as expected had the highest β-glucosidase activity.

Table 23. Specific activities (mean of triplicate determinations ± standard deviation) of cellulolytic enzymes in commercial preparations

Enzyme	Protein Conc	Filter paper	β-glucanase	Endo-glucanase	Exo-glucanase	Cellobiohydralase	β-glucosidase
		activity		(CMCase)	(Avicelase)		
	mg/ml	Units/mg	µmol/min/mg	µmol/min/mg	µmol/min/mg	µmol/min/mg	µmol/min/mg
BioCat xylanase 10 mg/ml	0.51±0.01	1.43	69.4±9.7	78.6±10.5	9.50±0.79	1.27±0.05	4.65±0.26
BioCat hemicellulase 10 mg/ml	0.52±0.01	0.67	63.9±10.1	41.7±10.2	11.43±0.61	2.12±0.24	1.77±0.36
T. lanuginosus xylanase, 10 mg/ml	0.08±0.03	ND	35.4±1.1	164.8±60.5	33.32±3.88	16.76±8.36	1.33±1.03
BioCat cellulase, 10 mg/ml	0.55±0.05	ND	106.7±17.6	79.5±6.1	8.64±0.61	2.05±0.31	8.11±0.79
Novozym 188	38.01±1.2	0	1.9±0.1	1.0±0.2	0.35±0.05	0.25±0.11	14.75±0.45
Multifect xylanase	7.63±0.39	0.04	3.1±0.8	6.3±0.6	0.46±0.07	0.28±0.14	3.30±0.10
T. reesei ATCC 26921 cellulase	26.10±1.78	ND	50.1±5.9	32.2±3.8	0.21±0.02	0.88±0.11	1.10±0.28

Spezyme CP	51.81±5.66	1.4	57.9±11.0	21.8±2.1	0.09±0.02	1.79±0.05	1.82±0.08
Cellulase from *Aspergillus*	4.90±0.58	ND	15.7±4.6	16.5±3.7	0.06±0.05	0.05±0.02	0.02±0
GC 220	56.63±8.68	ND	85.0±18.0	25.2±6.2	0.13±0.01	2.59±0.04	3.84±0.28
PowerPulp TX200A	3.34±0.07	0.42	3.4±1.2	5.6±2.0	0.31±0.09	0.06±0.03	1.07±0.13
FibreZyme LBL	28.81±1.00	1.27	66.5±8.5	97.1±7.6	0.38±0.05	1.61±0.12	3.09±0.21
FibreZyme LWT	33.61±1.86	ND	91.5±19.8	123.1±7.4	0.41±0.02	2.87±0.18	2.66±0.08
BioAce	37.94±4.29	ND	88.1±21.2	114.6±3.5	1.25±0	2.82±0.07	1.82±0.05

Table 24 shows the hemicellulolytic specific activities of the 14 commercial samples. β-xylanase activities ranged between 10 and 2967 U/mg. Especially notable was the low β-xylanase activities of Novozym 188 (sample 5) and Spezyme CP (sample 8), two enzyme preparations commonly used in the hydrolysis of lignocellulose (ref?). The purpose of the analysis was to establish the hemicellulolytic enzyme profile that might be appropriate for addition to a cellulase/β-glucosidase enzyme mixture. The most suitable enzyme preparations were identified as BioCat xylanase, BioCat hemicellulase. *T. lanuginous* xylanase, Multifect xylanase, PowerPulp TX200A and FibreZyme LBL. These six preparations showed the highest xylanase specific activity. Furthermore BioCat xylanase, Multifect xylanase, and PowerPulp TX200A revealed specific β-xylosidase activity greater than one and BioCat xylanase and Multifect xylanase were found to have the highest α-arabinofuranosidase activity. These results suggest that BioCat xylanase, Multifect xylanase, PowerPulp TX200A and FibreZyme LBL might be the best hemicellulolytic enzyme preparations that should be added to a cellulase/β-glucosidase enzyme mixture in order to achieve the most effective hydrolysis of sugar cane bagasse to a mixture of hexoses and pentoses.

Table 24. Specific activities (mean of triplicate determinations ± standard deviation) of hemicellulolytic enzymes in commercial preparations

Sample	Enzyme preparation	β-xylanase	β-xylosidase	α-arabinofuranosidase
		μmol/min/mg	μmol/min/mg	μmol/min/mg
1	BioCat xylanase 10 mg/ml	1235±57	5.87±0.31	5.07±0.45
2	BioCat hemicellulase 10 mg/ml	609±20	0.82±0.09	0.39±0.10
3	*T. lanuginosus* xylanase, 10 mg/ml	2967±18	0.11±0.09	1.18±0.07
4	BioCat cellulase, 10 mg/ml	36±1	0.80±0.10	0.55±0.06
5	Novozym 188	10±1	0.22±0	0.09±0.1
6	Multifect xylanase	209±10	4.90±0.58	3.21±0.40
7	*T. reesei* ATCC 26921 cellulase	20±1	2.11±0.23	1.29±0.05
8	Spezyme CP	15±2	0.56±0.02	0.38±0.04
9	Cellulase from *Aspergillus*	49±10	0.01±0.01	0.01±0
10	GC 220	20±2	0.06±0.01	0.02±0
11	PowerPulp TX200A	369±10	1.27±0.01	0.98±0.04
12	FibreZyme LBL	69±1	0.94±0.03	0.68±0.03
13	FibreZyme LWT	56±1	1.48±0.10	0.93±0.04
14	BioAce	22±1	1.33±0.03	0.88±0.01

The cellulolytic and hemicellulolytic enzyme profiles of number of selected fungi were evaluated The selection of the fungi was based on their properties determined in previous research. *A. carneus* ABO374 was isolated from soil in the Southern Cape region of South Africa and found to efficiently release reducing sugars from wheat straw. The supernatant of this strain cultivated on wheat straw also improved digestibility of wheat straw in in vitro and field sheep feeding trials (WH van Zyl, A. Botha, CW Cruywagen & B. A. Prior. 2005. Unpublished data). The *T. lanuginosus* strains SSBP and ATCC 34626 were shown to be very efficient producers of xylanases and the β-xylanase activity maintained up to 60° C (Singh et al., 2000a; Singh et al., 2000b). *A. carneus* cultivated on AFEX pretreated sugar cane bagasse produced the highest specific activities of cellulolytic enzymes and β-glucosidase. *T. lanuginosus* strain SSBP produced lower cellulolytic specific activities when compared with the ATCC 34626 when cultivated under similar conditions. Most activities of cellulolytic enzymes were higher when strain ATCC 34626 was grown on beechwood xylan rather than for AFEX-pretreated bagasse suggesting that the pure xylan is a better substrate for this enzymatic prep. The highest specific β-xylanase activity was produced by strain ATCC 34626 when cultivated on beechwood xylan

whereas lower activity was found when the *T. lanuginosus* strains were grown on AFEX-pretreated bagasse and very little β-xylanase was produced by *A. carneus*. These results also revealed that the *T. lanuginosus* strains SSBP and ATCC 34626 yielded the highest specific xylanase specific activities and these values are higher thanthose found in most commercially available samples with the exception of the commercial *T. lanuginosus* xylanase which is apparently a recombinant enzyme. *A. carneus* produced the highest activity of β-xylosidase and α-arabinofuranosidase whereas the *T. lanuginosus* strains produced much lower activities of these auxiliary enzymes as has been previously reported (Singh et al., 2000a).

In a simultaneous saccharification and fermentation (SSF) to ethanol process, the temperature is restricted to 30° C if a yeast strain such as *S. cerevisiae* is used. Therefore the activities of the β-xylanase were evaluated at 50° C (which is closer to optimum for many of the enzymes selected) and 30° C (which would appropriate for a SSF process). The activity of most β-xylanases was lower at 30° C than at 50° C. However, the activities of Multifect, PowerPulp and FibreZymeLBL β-xylanases were only slightly lower or not significantly diminished at the fermentation temperature. The other activities of the commercial and laboratory produced β-xylanases were much lower at 30° C than at 50° C.

Spezyme CP is a commercially marketed cellulolytic enzyme preparation produced by *Trichoderma reesei* that has been widely used in the hydrolysis of cellulose-rich biomass due to the dominance of endoglucanases and exoglucanases in the preparation. However this preparation lacks adequate β-glucosidase activity (Lynd et al., 2002) to achieve complete hydrolysis to glucose and ,as a result ,there is an accumulation of cellobiose. Therefore Spezyme CP is usually supplemented with β-glucosidase in order to promote complete hydrolysis. In the literature the ratios of cellulase to β-glucosidase have ranged widely and have depended upon the nature of the lignocellulose material to be hydrolysed. For example Berlin et al. (2005) used a ratio of 1 FPU: 2CBU to hydrolyse various softwood substrates, Martin et al. (2002) used a ratio of 1 FPU: 5.4 CBU while the NREL procedure (LAP-009) recommended a ratio of approximately 1 FPU: 1 pNPGU to hydrolyze stream pretreated sugar cane bagasse. A ratio of 1 FPU: 2 CBU was selected for hydrolysis experiments here and the ratio was based on the reported commercial enzyme activities of 60 FPU/ml for Spezyme CP and 282 CBU/ml for Novozym 188. Based on the data in Table 23, my calculation was 1 FPU Spezyme CP: 3.3 pNPGU Novozym 188.

Table 25 shows the effect of three levels of Spezyme CP/Novozym 188 (in an activity ratio of 1:2) on the hydrolysis of ammonium hydroxide-pretreated and AFEX-pretreated bagasse and Avicel. As the enzyme level increased, greater amounts of glucose and xylose were released from the bagasse samples and glucose from the Avicel. No cellobiose was detected in any of the samples suggesting that the β-glucosidase activity was not limiting in any of the enzyme mixtures. Arabinose was only released from the AFEX-pretreated bagasse. At a 10 FPU: 20 CBU/ g glucan ratio, the greatest amount of glucose was released from Avicel followed by the AFEX-pretreated bagasse. At the lowest enzyme level only 28 % and 33 % of the glucan in respective NH_4OH- and AFEX-pretreated bagasse samples were hydrolyzed to glucose whereas 66 % of the Avicel glucan was hydrolyzed suggesting that some glucan was inaccessible in the bagasse samples to enzyme hydrolysis. At the highest enzyme level (60 FPU: 120 CBU), 68 % and 78 % of the glucan was hydrolyzed in the respective bagasse samples and Avicel samples.

Increasing the level of enzyme appeared to have a much lower impact on the hydrolysis of the Avicel sample that the bagasse samples. This might be due to a synergistic effect of the other enzymes present in the Spezyme CP/Novozym 188 acting on the cellulose and hemicellulose components in bagasse.

Table 25. Sugar release from ammonium hydroxide- and AFEX pretreated sugar cane bagasse (at 1 % glucan level) by Spezyme CP and Novozym 188 (1:2 ratio activity)

Enzyme activity Spezyme/Novozyme	Sugar concentration (g/l)					% hydrolysis	
FPU:CBU/g glucan	cellobiose	glucose	xylose	arabinose	total*	glucan	xylan
	Ammonium-hydroxide pretreated						
10:20	0	3.11	2.09	0	5.2±0.27	28	41
30:60	0	5.73	3.16	0	8.89±0.76	52	62
60:120	0	7.64	3.8	0	11.44±0.43	68	75
	AFEX-pretreated						
10:20	0	3.68	2.87	0.98	7.53±0.54	33	65
30:60	0	6.14	3.78	1.19	11.11±1.13	55	84
60:120	0	7.51	4.36	1.17	13.04±0.37	68	94
	Avicel						
10:20	0	7.31	0	0	7.31±0.65	66	
30:60	0	8.27	0	0	8.27±0.40	74	
60:120	0	8.68	0	0	8.68±0.20	78	

Four xylanase preparations with suitable activity profiles were evaluated for their ability to boost the hydrolysis by Spezyme CP and Novozym 188 (10 FPU: 20 CBU/g glucan) of NH_4OH-(Table 26) and AFEX-pretreated bagasse (Table 27) and Avicel (Table 28). These enzyme preparations contained multiple cellulolytic and hemicellulolytic activities, therefore the total protein concentration was used as the basis to supplement the Spezyme CP/Novozym mixture instead of using the activity of a single enzyme such as xylanase. Table 26 shows that at a 10 % protein level, Multifect and BioCat xylanase had a significant increase in total sugar release compared withthe control whereas with PowerPulp xylanase and FibreZyme LBL xylanase no increase was found. When the enzymatic preparations were added at 50 % protein level, significant increases were observed in all instances. No cellobiose was detected. In spite of the hydrolysis increasing from 31 % to respectively 43, 44 and 55 % when Multifect, FibreZyme LBL and BioCat xylanases were added at the 50 % protein level, the degree of hydrolysis remained low. The hydrolysis of AFEX-pretreated bagasse was boosted significantly by the addition of a 10 % level of Multifect and BioCat xylanase to Spezyme CP and Novozym 188 (10 FPU: 20 CBU/glucan) and at a 50 % level, all the xylanase preparation increased significantly (Table 27). Interestingly the release of glucose also increased markedly and the degree of the

glucan hydrolysis increased from 39 % to respectively 61 % and 73 % by the addition of Multifect and BioCat xylanase at the 50 % protein level.

Table 26.

Effect of addition of commercial xylanase preparation to hydrolysis of ammonium hydroxide pretreated
sugar cane bagasse by Spezyme CP (10FPU/g glucan)/Novozym 188 (20CBU/g glucan)

Enzyme preparation	% protein loading	Sugar (g/l)					% hydrolysis
		Cellobiose	Glucose	Xylose	Arabinose	Total*	Glucan
None	0	ND	3.49	1.94	0.26	5.64±0.34	31
Multifect	10	ND	4.3	2.72	ND	7.02±0.84	39
	50	ND	4.76	2.9	ND	7.66±1.43	43
Power pulp	10	ND	2.57	1.67	0.18	4.42±0.45	23
	50	ND	3.88	2.19	0.18	6.24±0.64	35
FibreZyme LBL	10	ND	3.31	1.97	0.17	5.44±0.27	30
	50	ND	4.88	2.55	0.55	7.98±1.20	44
BioCat xylanase	10	ND	3.7	2.22	0.13	6.05±0.45	33
	50	ND	6.1	2.83	0.28	9.21±0.19	55

ND: not detected
*mean of triplicate determinations ± standard deviation

Table 27. Effect of addition of commercial xylanase preparation to hydrolysis of AFEX-pretreated sugar cane
bagasse by Spezyme CP (10FPU/g glucan)/Novozyme 188 (20CBU/g glucan) (triplicate determinations)

Enzyme preparation	% protein loading	Sugar (g/l)					% hydrolysis	
		Cellobiose	Glucose	Xylose	Arabinose	Total	Glucan	Xylan
None	0	ND	4.32	2.86	0.38	7.57±0.30	39	55
Multifect	10	ND	4.94	3.99	0.66	9.59±0.51	44	62
	50	ND	6.77	4.65	0.73	12.15±1.28	61	91
Power pulp	10	ND	4.79	3.29	0.29	8.37±0.82	43	61
	50	0.05	5.98	3.78	0.24	10.05±2.31	54	68
FibreZyme LBL	10	0.01	4.49	3.28	0.24	8.02±0.10	41	60
	50	0.01	6.16	3.79	0.46	10.43±1.20	55	72
BioCat xylanase	10	ND	5.91	3.96	0.29	10.16±0.58	53	72
	50	0.09	7.97	4.3	0.34	12.69±1.56	73	79

ND: not detected
*mean of triplicate determinations ± standard deviation

Table 28.

Effect of addition of commercial xylanase preparation to hydrolysis of Avicel by Spezyme CP (10FPU/g glucan)/Novozym 188 (20CBU/g glucan) (triplicate determinations)

Enzyme preparation	% protein loading	Sugar (g/l)			% hydrolysis
		Cellobiose	Glucose	Total*	
None	0	0.02	7.27	7.29±0.31	65
Multifect	10	ND	6.58	6.58±0.51	59
	50	ND	8.49	8.49±0.92	76
Power pulp	10	0.04	7.96	7.96±0.01	72
	50	0.05	8.81	8.86±0.36	80

ND: not detected
*mean of triplicate determinations ± standard deviation

An analysis of the relationship between xylanase activity added and sugar release found that increasing units of enzyme activity greatest greater impact on the release of glucose than on xylose (to be evaluated statistically). Obviously this preparation contains a mixture of activities, making it difficult to remove C-5 and preserve C-6 sugars separately.

The interaction between xylanase and the release of glucose from glucan was further evaluated by adding Multifect and PowerPulp xylanases to Spezyme CP and Novozym 188 in the hydrolysis of Avicel. Table 28 shows that both Multifect and PowerPulp xylanases boosted the amount of hydrolysis significantly when added as protein at 50% the protein concentration of the Spezyme (a 50 % protein level) whereas at 10 % protein level only the Power Pulp xylanase had a significant impact. PowerPulp and Multifect xylanase added at a50 % protein level increased the hydrolysis from 65 % to 80 % and 76 % respectively. Table 3 shows that both xylanase preparations had relatively low cellulolytic enzyme activities and therefore these results suggest that the xylanase might act synergistically in the hydrolysis of cellulose by cellulase. Perhaps it permits easier access to the cellulose through the removal of C-5 sugars. This suggestion needs to be confirmed by further investigation.

The impact of laboratory-produced enzymes added at a 10 % protein level on the hydrolysis by Spezyme CP and Novozym 188 is shown in Table 29. Enzymes produced by *A. carneus* failed to boost the hydrolysis of NH_4OH- and AFEX-pretreated bagasse. The enzyme preparation produced by *T. lanuginosus* ATCC 34626 grown on beechwood xylan was the most effective of the various laboratory produced enzyme preparations in increasing the amount of glucose, xylose and total sugar released. The percent hydrolysis of the glucan fraction of NH_4OH- and AFEX-pretreated bagasse increased respectively from 32 % to 40 % and from 49 % to 54 %. As shown in the studies with the commercial xylanase preparations, greater amounts of sugar were released from the AFEX-pretreated bagasse than the NH_4OH-pretreated bagasse when the laboratory-produced enzymes were added to Spezyme CP and Novozym 188 suggesting that the AFEX-pretreated bagasse is more easily hydrolyzed.

Table 29.

Effect of addition of concentrated lab xylanase preparations (10 % protein) to hydrolysis of pretreated
sugar cane bagasse (1 % glucan) by Spezyme CP (10FPU/g glucan)/Novozyme 188 (20CBU/g glucan)

Organism	Growth conditions	Sugar (g/l)					% hydrolysis	
		Cellobiose	Glucose	Xylose	Arabinose	Total*	Glucan	Xylan
Ammonium hydroxide pretreated bagasse								
A. carneus ABO372	AFEX	0.06	3.23	2.32	0.18	5.80±0.43	30	49
T. lanuginosus SSBP	AFEX	0.07	4.17	3.16	0.22	7.62±0.14	39	69
T. lanuginosus ATCC 34626	AFEX	ND	3.74	2.82	0.3	6.87±0.78	34	62
T. lanuginosus ATCC 34626	Beechwood	ND	4.41	3.41	0.25	8.07±0.91	40	72
None		0.03	3.53	2.2	0.17	5.93±0.30	32	47
AFEX pretreated bagasse								
A. carneus ABO372	AFEX	0.14	5	4.02	0.3	9.46±0.48	48	73
T. lanuginosus SSBP	AFEX	0.12	5.51	4.1	0.27	9.99±0.96	52	74
T. lanuginosus ATCC 34626	AFEX	0.01	5.87	4	0.28	10.16±0.93	53	73
T. lanuginosus ATCC 34626	Beechwood	ND	6.01	4.55	0.25	10.82±0.71	54	82
None		ND	5.48	3.94	0.27	9.69±0.66	49	72
Avicel								
A. carneus ABO372	AFEX	0.08	9.53	ND	ND	9.61±0.44	88	

T. lanuginosus SSBP	AFEX	0.11	10.75	ND	ND	10.86±0.97	100
T. lanuginosus ATCC 34626	AFEX	0.03	7.68	ND	ND	7.71±0.41	70
T. lanuginosus ATCC 34626	Beechwood	0.08	8.59	ND	ND	8.67±0.18	79
None		0.08	8.83	ND	ND	8.91±0.33	82

Growth conditions: Fungus grown either on AFEX pretreated bagasse or beechwood xylan

ND: Not detected

*mean of triplicate determinations ± standard deviation

As the Multifect xylanase was shown to be one of the most effective xylanase preparations in enhancing the hydrolysis of bagasse, the effect on substrate loading on the hydrolysis of NH_4OH- and AFEX-pretreated bagasse by Spezyme CP and Novozym 188 with and without Multifect xylanase was evaluated (Table 30). At all substrate loading doses, there were greater amounts of xylose, arabinose and glucose released when xylanase was present. Due to the nature of the NH_4OH-pretreated bagasse, a maximum substrate loading of only 4 % could be tested whereas the AFEX-pretreated bagasse could be tested up to a10 % substrate loading dose. However, it was apparent that greater concentrations of both glucose and xylose were released from the AFEX-pretreated bagasse compared with NH_4OH-pretreated bagasse. Surprisingly the % hydrolysis of the glucan fraction of bagasse appeared to be unaffected by the load increase but the xylan hydrolysis declined with an increase in loading in the AFEX-pretreated bagasse. It is notable that there was a greater accumulation of cellobiose with an increase in the loading of the AFEX-pretreated bagasse. Furthermore the accumulation was greater in the absence of Multifect xylanase. This suggests that additional β-glucosidase activity might be necessary at higher loading doses.

Table 30. Effect of substrate loading on hydrolysis of AFEX and NH3-pretreated sugar cane bagasse by Spezyme (10 FPU/g glucan)
and Novozyme 188 (20 CBU/g glucan) with or without Multifect xylanase added at 10 % protein (mean of triplicate determination)

% glucan loading	Sugar (g/l)						
	Cellobiose	Glucose	Xylose	Arabinose	Total*	% hydrolysis	
						glucan	xylan
Ammonium hydroxide pretreatment							
With xylanase							
1	0.05	4.27	2.77	0.19	7.29±0.58	39	58
2	ND	9.21	6.05	0.49	15.75±0.75	41	65
4	0.07	22.93	13.21	0.99	37.21±1.30	52	70
AFEX-pretreatment							
1	0.06	5.73	4.51	0.42	10.71±1.21	53	84
2	0.32	14.23	8.74	1.05	24.34±0.55	67	83
5	0.91	28.47	19.45	2.57	51.41±2.83	55	75
10	5.43	46.39	34.56	4.5	90.89±0.75	52	66

ND: not detected
*mean of triplicate determinations ± standard deviation

	Sugar (g/l)						
	Cellobiose	Glucose	Xylose	Arabinose	Total*	% hydrolysis	
						glucan	xylan
Without xylanase							
	0.06	3.76	2.32	0.18	6.32±0.37	35	49
	ND	6.61	4.26	0.37	11.24±1.40	30	46
	ND	17.79	9.93	0.85	28.57±2.34	40	53

Conclusions

Enzyme preparations were assayed for their activities and certain preparations have been identified as being more suitable for use in hydrolysis experiments of pre-treated bagasse.

Methods and Materials (Task 3)

Pretreatment of sugar cane bagasse

Bagasse from sugar cane (*Saccharum officinarum*) was obtained from the Raceland Raw Sugar Corp. sugar mill, Raceland, Louisiana and ground to a particle size of less than a 12 mm in length. Ground sugar cane bagasse has submitted to MBI International, Lansing, MI for

pretreatment using the AFEX process (Holtzapple et al., 1991). Briefly, approximately 1 kg of the bagasse was treated in a one gallon reactor at 100 °C for 30 min with a 2:1 ammonia loading to biomass and 40 % moisture level. After pretreatment, bagasse was removed from the reactor, dried to remove ammonia and stored in sealed plastic bags at 4°C. Ground sugar cane bagasse (2.5 kg) was also pretreated by adding a 28 % stock solution of ammonium hydroxide to achieve a final concentration of 0.02 g NH_3 /g water in a final water mass of 20 kg. The slurry was placed in a pressure reactor and heated at 160 °C for 60 min. The solid mass was removed from the slurry by filtration through muslin cloth and washed with 40 kg of water. Less than 1 % of mono sugars in the bagasse were removed by this pretreatment.

Analysis of the composition of sugar cane bagasse and pretreated bagasse

The carbohydrate composition and the content of ethanol extractives, ash and lignin were determined using the analytical procedures published by DOE's NREL laboratory (www1.energy.gov/biomass/analytical_procedures.html). The moisture content of the bagasse samples was determined using a moisture analyzer (Computrac MAX 1000, Arizona Instrument Corporation, Tempe, Arizona).

Enzyme preparations

Commercial preparations

A commercial preparation produced by *Trichoderma reesei* (Spezyme CP) and supplied by Genencor (Rochester, NY) was used as the main cellulase enzyme in this study. This preparation was supplemented with a β-glucosidase preparation (Novozym 188) produced *Aspergillus niger* and supplied by Sigma (C6105). Various commercial enzymes with cellulose and xylanase activity were kindly provided by various North American suppliers and their cellulolytic and hemicellulolytic activities were evaluated. Some enzyme samples were in powder form and were suspended in 100 mM sodium acetate buffer (pH 5) at a concentration of 10 mg/ml.

Laboratory-produced enzyme preparations

Thermomyces lanuginosus strains SSBP and ATCC 34626 and *Aspergillus carneus* Abo 372 were obtained from the culture collection of the Department of Microbiology at the University of Stellenbosch, South Africa. The fungi were maintained on malt extract agar plates. With a sterilized needle, colonies were inoculated into 1 ml YPD (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose), and cultivated for 2 days at the optimum temperature for each of the fungal species (*T. lanuginosus*, 50°C; *A. carneus,* 30°C) with shaking. The contents of each inoculant were decanted into 200 ml growth medium (0.67% Yeast Nitrogen Base, with amino acids, 0.2% L-Asparagine monohydrate, 0.5% KH_2PO_4), containing 4 g of AFEX pretreated sugar cane bagase or 4 g beechwood xylan (Sigma X 4252) as carbon source and cultivated for 5 days at the optimum temperature. Fungal growth was clearly visible in the flasks after 4 days. The culture fluid was filtered through 4 layers of muslin cloth and then, centrifuged at 5 000 X g for 10 min to remove fungal debris. After centrifugation, 0.1 % sodium azide was added to the supernatant

and concentrated by filtration through an Amicon concentration apparatus using a 10 kDa membrane filter.

Enzyme and protein assays

The filter paper activity of enzyme samples was determined at 50 °C according to standardized NREL filter paper assay (Adney & Baker, 1996). Carboxymethylcellulase (CMCase; endoglucanase), avicelase (exoglucanase) and β-D-(1,3;1,4)-glucanase activity was determined by measuring the release of reducing sugars from respectively 3 % carboxymethyl cellulose (Sigma), 3 % Avicel (FMC Biopolymer pH-102) and 0.5 % barley β-glucan (Sigma) at 50°C and pH 5.0 (100 mM acetate buffer) for 10 min (Wood and Bhat, 1988). The reaction was terminated by addition of dinitrosalicyclic acid reagent and subsequently boiled for 5 min. The reducing sugar concentration was determined from the absorbance at 540 nm using a glucose standard curve as reference. One unit (IU) of activity was defined as the amount of enzyme that released 1 μmol of glucose as reducing sugar equivalents per minute.

β-Xylanase activity was determined by following the release of reducing sugars from a 1.0% birchwood xylan (Sigma) solution at 50°C for 5 min (Bailey *et al*., 1992). The reaction was terminated by the addition of dinitrosalicyclic acid reagent and subsequently boiled for 5 min. The reducing sugar concentration was determined spectrophotometrically at 540 nm from a xylose standard curve. One unit of activity was defined as the amount of enzyme that released 1 μmol of xylose as reducing sugar equivalents per minute.

Cellobiohydrolase (CBHI), β-glucosidase, β-xylosidase and α-arabinofuranosidase activities were determined by following the release of 4-nitrophenol from their respective 4-nitrophenol conjugates:4-nitrophenyl-β-D-lactopyranoside, 4-nitrophenyl-β-D-glucopyranoside, 4-nitrophenyl-β-D-xylopyranoside and 4-nitrophenyl-α-L-arabinopyranoside (Sigma) for 15 min at 50° C and pH 4.0 (50 mM Na acetate buffer). The reaction was stopped by the addition of 1 M Na_2CO_3 and absorbance read at 410 nm from a 4-nitophenol standard curve. One unit of activity was defined as the amount of enzyme that released 1 μmol of 4-nitrophenol per minute.

The protein concentration was determined using the Coomassie Brilliant Blue dye-binding method (Bio-Rad; Bradford, 1976).

Batch hydrolysis of pretreated sugar cane bagasse and Avicel

Enzymatic saccharification experiments of pretreated samples were performed in triplicate in 20 ml glass scintillation vials at 50 °C and 100 rpm? for 72 h as described in the NREL (LAP-009) procedure. Briefly, the reaction mixture contained 0.1 g cellulose (dry weight) (except with the substrate loading experiment), 0.5 ml 1 M sodium citrate buffer (pH 4.8), 40 μl tetracycline (10 mg/ml), 30 μl cycloheximide (10 mg/ml), Spezyme CP and Novozym 188 in a ratio of 1:2 and distilled water to give a final volume of 10 ml. Adjustments were also made for the addition of various activities of commercial and laboratory-produced xylanases. The moisture content in the AFEX-pretreated bagasse (24.53 %) and NH_4OH-pretreated bagasse (78.42 %) was included in the calculation of the total volume. Avicel (FMC Biopolymer pH-102; 4.54 % moisture) was also included as a control. Substrate blanks excluded the enzyme activities whereas enzyme blanks

excluded the substrates and the degree of spontaneous hydrolysis was used to correct data. Samples were withdrawn initially and after 72 h and centrifuge at 10,000 rpm in Eppendorf tubes to remove the biomass. Subsequently the liquid portion was filtered through 0.45 µm (pore size) filters (25mm (diameter) Whatman GD/X filter (PTFE filter media with polypropylene housing, Cat. No. 6874-2504, Whatman Inc. Florham Park, NJ) and the liquid subjected to HPLC for reducing sugar analysis. HPLC analysis was conducted using an isocratic HPLC system equipped with a refractive index detector (Spectra System. RI 150, Thermo Electron Corp., Milan, Italy). Sugars were separated on a BioRad Aminex-HPX-87P column (BioRad Lab. Inc. Hercules, CA) at a flow rate for the mobile phase (DI water) of 0.6 ml/min at 85°C for 30 min. Reducing sugar concentration was determined as described above. Generally, the reducing sugar concentrations agreed within 10 % of the total sugar determined by HPLC. In instances where the values did not agree, analyses were repeated.

Calculations

The percent hydrolysis of the glucan fraction of sugar cane bagasse was calculated adding the glucose and double the cellobiose concentrations, correcting for the hydration (each glucose molecule had one molecule of water added during hydrolysis and therefore the glucose concentration was multiplied by 0.9) and dividing by the grams of glucan. Similarly, the percent hydrolysis of the xylan fraction was calculated by adding the xylose and arabinose concentrations, correcting for hydration (total xylose and arabinose was multiplied by 0.88) and dividing by the grams of total xylan and arabinan [should that be arabinose?].

Task 4. Produce ethanol through fermentation of bagasse and molasses

This task was transferred to DE-FG36-05GO85007 and will be reported in full there.

In order to scale-up biomass processing an alternative pretreatment was required as the AFEX technology was not able to handle the biomass at the required scale .

An AFEX simulant, which involves ammonium hydroxide pretreatment, was tested at 10% loading of raw sugarcane bagasse to quantify selected inhibitors (i.e. lignin, total phenolic compounds, and pH) formed during pretreatment and to investigate their combined effect on enzymatic hydrolysis. Also investigated was the release of sugars (i.e. glucose, cellobiose, xylose, arabinose) after treatment with increasing levels of ammonium hydroxide.

Increasing concentrations of ammonium hydroxide (0-0.040 g NH_3 per g of water) resulted in an increased release of TPC (0.0052 - 0.0490 g vanillin per g dry biomass), lignin (0.0116-0.070 g beechwood per ml solution), and an increase in pH (5.0- 10.8) (Table 31). A change in the treatment process from 10 min to 60 min favored the release of TPC, lignin and the formation of organic acids which caused a drop in pH.

Table 31. pH, Lignin and TPC removal Post-Ammonium Hydroxide Pretreatment of Sugarcane Bagasse.

Treatment			Total TPC* (g vanillin per g dry biomass)	Lignin (g beechwood per ml solution)	pH Solution
Ammonium Hydroxide (g NH_3 in solution per g of water)	Temperature (°C)	Time (min)			
0.000	120	10	0.0052	0.0120	5.6
0.000	120	60	0.0060	0.0116	5.0
0.002	120	10	0.0130	0.0229	9.5
0.002	120	60	0.0210	0.0239	9.0
0.020	120	10	0.0280	0.0419	10.3
0.020	120	60	0.0430	0.0426	10.2
0.040	120	10	0.0350	0.0464	10.8
0.040	120	60	0.0490	0.0710	10.6

No significant differences from the control were observed in the release of cellobiose, glucose, or xylose fractions following pretreatment with ammonium hydroxide. However, the release of arabinose increased with increasing concentrations of ammonium hydroxide (Table 32).

Rinsing the sugarcane bagasse with water post-ammonium hydroxide treatment removed some of the inhibitors mentioned above thus increasing cellulose and hemicellulose conversion to fermentable sugars (Table 33). A 55% or 41% cellulose conversion and 18% or 13% hemicellulose conversion were obtained for washed or unwashed treated biomass, respectively. This approach was successfully followed under the auspices of DE-FG36-05GO850 and will be fully reported elsewhere. Using this approach we can routinely achieve 90% cellulose conversion, with an SHF-SSF fermentation using a batch fed process, achieve a 30% solid loading and, with molasses supplementation, routinely obtain greater than 5% ethanol in the beer.

Table 32. Selected Sugars Released Post-Ammonium Hydroxide Treatment of Sugarcane Bagasse.

Treatment			Concentration (g/L solution)			
Ammonium Hydroxide (g NH_3 in solution per g of water)	**Temperature (ºC)**	**Time (min)**	**Cellobiose**	**Glucose**	**Xylose**	**Arabinose**
0	120	10	ND	0.03	ND	ND
0	120	60	ND	0.04	0.07	ND
0.002	120	10	0.09	0.02	0.03	ND
0.002	120	60	0.02	0.05	0.06	0.06
0.020	120	10	ND	0.09	0.03	ND
0.020	120	60	0.04	0.05	0.02	0.35
0.040	120	10	0.04	0.01	0.03	0.77
0.040	120	60	0.03	0.05	0.04	0.53

ND= None Detected.

Table 33. Enzyme Hydrolysis Post-Ammonium Hydroxide Treatment of Sugarcane Bagasse

Treatment			Percent of Maximum Theoretical Yield			
Ammonium Hydroxide (g NH_3 in solution per g of water)	**Temperature (ºC)**	**Time (min)**	**Cellulose Hydrolyzation (Washed)**	**Hemicellulose Hydrolyzation (Washed)**	**Cellulose Hydrolyzation (Not Washed)**	**Hemicellulo Hydrolyzati (Not Washe**
Control (AVICEL)	NA	NA	92.0	0	92.0	0
0.000	120	10	7.4	0.9	7.3	0.9
0.000	120	60	6.9	1.0	8.1	1.1
0.002	120	10	13.3	1.3	12.4	1.6
0.002	120	60	21.1	4.1	14.2	2.1
0.020	120	10	27.7	8.7	24.2	7.6
0.020	120	60	45.2	15.5	37.0	12.5
0.040	120	10	28.8	9.4	24.0	6.2
0.040	120	60	55.2	18.4	40.7	12.5

Task 5
Thermochemical Conversion of Bagasse

Because of the importance of Syngas production and Fischer –Tropsch conversion to ethanol as a potential biofuel process a small investigative component on pyrolysis of sugarcane biomass was undertaken in order to gain sufficient expertise in this field to evaluate its potential for a sugar-mill based biorefinery. This involved two tasks.

> *Sub-Task 5.1.* Design and execute an experimental matrix of subcritical aqueous alkali-catalyzed conversion tests with sugarcane CML solids. Characterize liquid, gas and solid products of the conversions.

The liquid products of pyrolysis were characterized. Each pyrolysis liquid sample was extracted into acidic and basic fractions, and into each of three solvents; dichloromethane, ethyl acetate, or toluene. These solvents were selected for their ability to solubilize a wide variety of species presumed to be present in pyrolysis products. Samples were run on a GC-MS. Components were selected for identification by the magnitude of their peak intensity. The product distribution was normalized based on the peak with the highest count intensity.

It was found that the acidic toluene extraction yielded the highest proportion of components, despite a wide variation in test conditions. The compound tentatively identified (NIST GSMS library) as tricyclo[5.2.1.0(1,5)]dec-5-en-8-ol ($C_{10}H_{14}O$) yielded the highest intensity peak for these extractions. Some of the other likely candidates are listed in Table 34.

Table 34: Preliminary Characterization of Liquid Pyrolysis Products.

Compound	Probability	Molecular Wt.
tricyclo[5.2.1.0(1,5)]dec-5-en-8-ol	36.9	150
1,3-dimethylbenzene	28.5	106
paraxylene	21.3	106
α,β-dimethylbenzene ethanol	20.1	150

It should be noted that the NIST library searches produced several low probability matches with substances commonly found in the pharmaceutical industry (e.g., creatinine and dimethoxyphenylethylamine). A comparison with recent literature (Ind. Eng. Chem. Res., Vol. 42, No. 14, 2003) suggests, for example, that the compound identified by the NIST library as creatinine (a protein produced by muscle tissue), which had a retention time of 9.571 minutes and a probability of 44, would more likely be propionic acid methyl ester with a retention time of 9.567 minutes.

The first four pyrolysis runs were conducted at 600ºC and the fifth run was carried out at 850ºC. The first and last runs had no catalyst added, while the second, third, and fourth runs had Na_2CO_3, MgO, and CaO added, respectively (shown in Table 35) in hopes of improving the methane and CO levels in the gas.

Table 25: The Various Conditions used in Pyrolysis Tests on Sugarcane Biomass.

Run	Catalyst	Soak Temperature	Time at Soak
RTA1	None	600ºC	16.10 min
RTA2	4.23g Na2CO3	600ºC	15.00 min
RTA3	4.40g MgO	600ºC	15.50 min
RTA4	4.19g CaO	600ºC	16.10 min
RTA5	None	850ºC	17.20 min

Gas chromatography revealed the largest constituent in the product gas stream to be CO2, followed by CO. The last two tests, however, had markedly reduced concentrations of CO2, yet they were the only runs that showed evidence of methane formation. On average, the gas from the first three runs contained 84 wt% CO2 and 16 wt% CO with a standard deviation of 4.6 wt%. The weight percent level of the various product gases is provided in Table 36.

Table 36: Composition of Gaseous Products of Pyrolysis Tests.

Run	Wt% CO_2	Wt% CO	Wt% CH_4
RTA1	83	17	0
RTA2	80	20	0
RTA3	89	11	0
RTA4	63	24	13
RTA5	52	41	7

Ashing of the Na_2CO_3, MgO, and CaO catalysts resulted in a dry weight losses of 10%, 1%, and 23%, respectively. The low weight loss for sodium carbonate indicated that carbonate was not breaking down and contributing to the formation of carbon dioxide. The approximate calculated weight loss of 42% is based on the stoichiometry of the following chemical equation:

$$Na_2CO_3\ (s) \rightarrow NaO\ (s) + CO_2\ (g)$$

The weight loss, after accounting for the calcium oxide catalyst was surprisingly high.

An important observation was made that may have an impact on the chemistry that took place in the Rotational Thermal Apparatus (RTA) pyrolysis runs (Figure 6). A plot of the oxygen concentration profiles revealed inconsistencies in the oxygen profiles during tests. The profiles

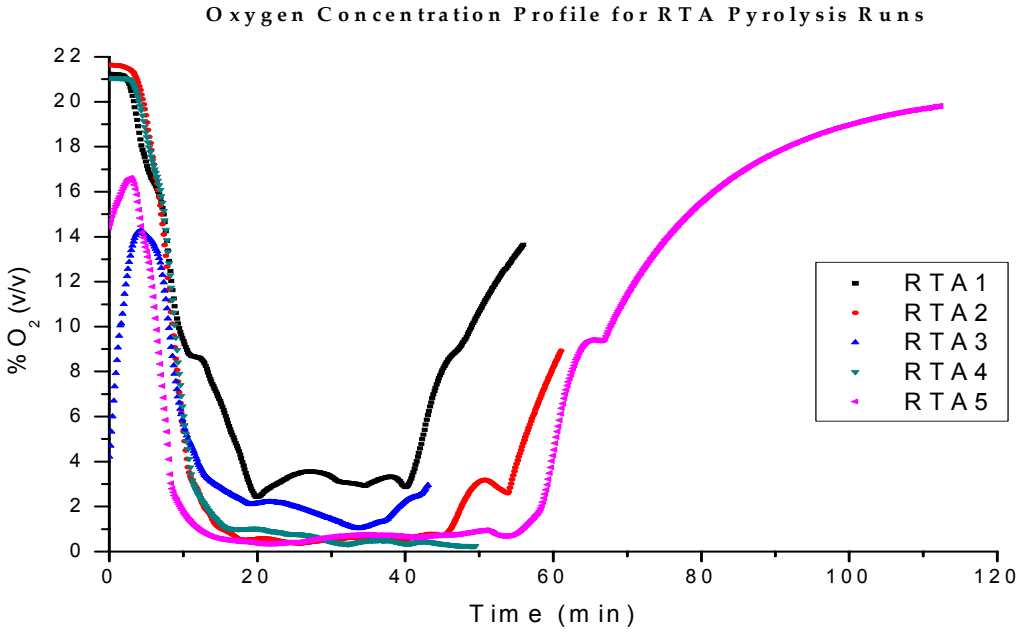


Figure 6: Oxygen Concentration Profiles from Rotational Thermal Apparatus Pyrolysis Studies.

of the third and fifth runs showed increases during the first five minutes of the reactions prior to decreasing, whereas the remaining RTA runs saw decreases in oxygen concentrations from time t=0. Furthermore, the slope of the initial declines in oxygen concentration was fairly consistent for each of the runs, except for RTA run 1, which exhibited an unusual plateau effect while the oxygen concentrations were decreasing.

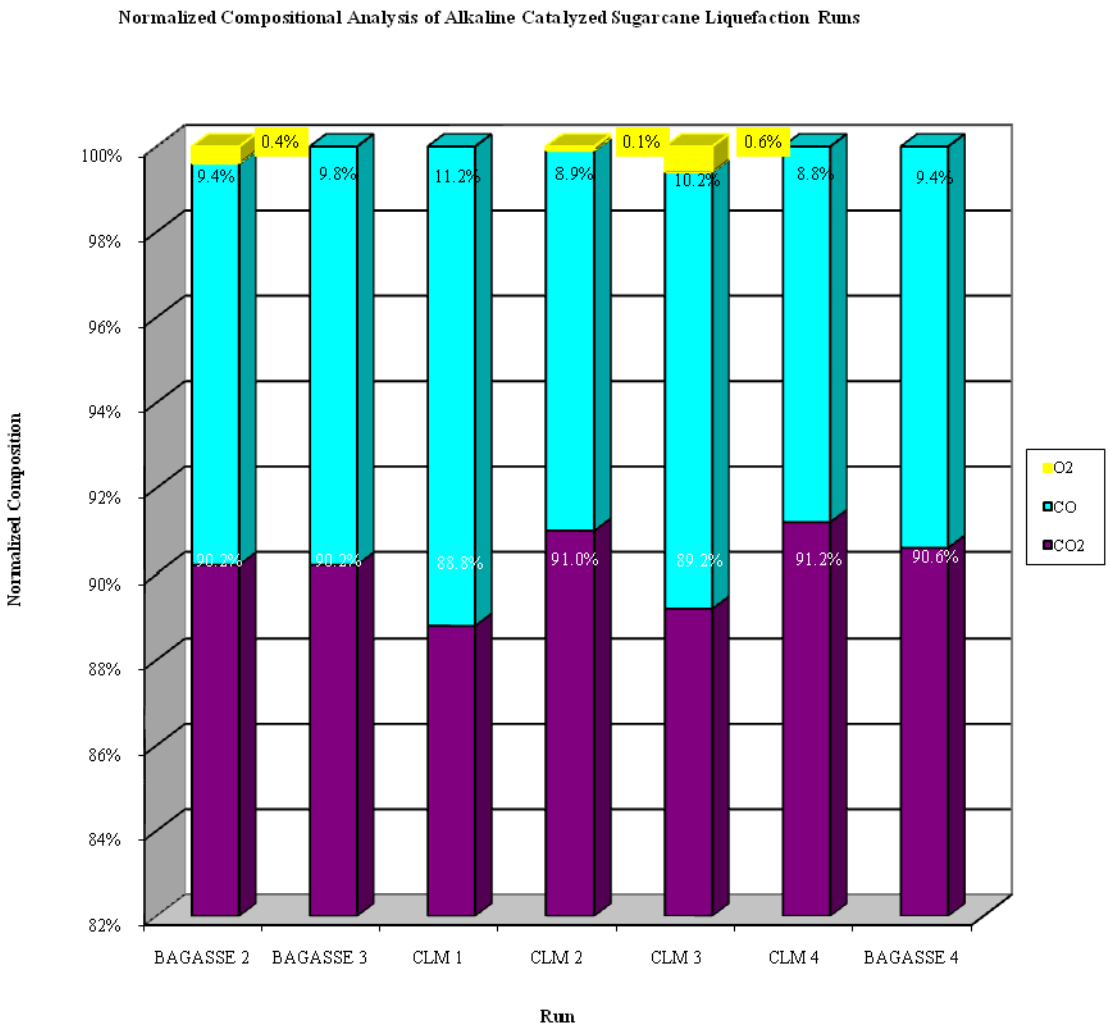
Table 37 below depicts the breakout of the mass balance for the starting material mass in terms of the liquid, solid and gaseous product portions. The gases were measured via a water displacement method and the solids were measured by weight. The organic liquid product was determined by difference. The first four runs depict a consistent gas mass of about 13 g. The final four runs have lower mass yields for the gases but demonstrate an average liquids product mass of about 53 g, while the first four runs average about 46 g of liquid product. The particularly low gas product masses in runs 5 and 6 are attributable to a possible leak in the tubing during gas collection. The presence of the leak makes it difficult to accurately assess the impact of the nitrogen purge on the overall product distribution.

Table 37. Mass balance for alkaline-catalyzed liquefaction runs

Mass Balances on Liquefaction Runs 1-8				
Run	**Starting Material Mass (g)**	**Liquid Product Mass (g)**	**Solid Product Mass (g)**	**Gas Product Mass (g)**
1	90.0	NA	10.4	NA
2	90.0	48.0	28.7	13.3
3	86.0	42.0	30.8	13.2
4	86.0	46.9	25.6	13.5
5	86.0	55.7	23.0	7.3
6	86.0	53.4	26.3	6.3
7	90.0	53.0	27.7	9.3
8	90.0	49.8	28.0	12.2

Figure 7 depicts the normalized compositional gas analyses from the alkaline-catalyzed liquefaction runs. Oxygen was detected in the gas product on the order of 2 – 4 % w/w. Does this indicate incomplete pyrolysis?? It is unlikely that oxygen could be present in the final product gas in such a quantity, especially in the runs that were purged for twenty minutes with nitrogen. Upon running the GC with a 25 μL helium blank injection, it was verified that air was indeed entering the chromatographic system upon injection in a fairly reproducible fashion. Since it is improbable that free nitrogen or oxygen would be formed under the conditions for pyrolysis, they have been effectively omitted from the final analysis of the permanent gas products by deducting the total amount of nitrogen in the system and the stoichiometric equivalent of oxygen. A negligible amount of oxygen remained from CLM runs 2 and 3 and bagasse run 2. Carbon dioxide was present in the highest abundance, constituting a normalized average of approximately 90.2 % w/w of the permanent gas mass, while carbon monoxide was the second most prolific permanent gas contributing a normalized average of 9.7 % w/w of the gas mass.

Figure 7. Normalized compositional analysis of base catalyzed liquefaction runs



GC-MS was utilized to provide a qualitative assessment of the major constituents present in the liquid product from the alkaline-catalyzed liquefaction runs. The two major classes of compounds contained within the liquid product as revealed by the GC-MS characterization work (Table 38) are phenols and ketones. Typically, match qualities at 60 or above are considered to be reliable library matches for the unknown peak. A few match qualities are posted that are below this threshold. These were included to illustrate a possibly important distinction between compounds present in the bagasse-derived liquids and the CLM-derived liquids. Compounds containing the chemical formula C_8H_{14} arise in the GC-MS results for both CLM runs. In other circumstances, the lower quality matches were included to demonstrate that both the CLM and bagasse liquid products contain similar types of compounds, such as the dimethylated ketones (2,3-dimethyl-2-cyclopenten-1-one). The loss in match quality in compounds such as the former ketone is unavoidable since the determination of the actual configuration of identical functional groups on cyclic or ring compounds is often ambiguous when using a mass spectrometer, which classifies compounds according to the mass to charge ratios of their respective fragment patterns.

Table 38. Qualitative characterization of liquid products from subcritical aqueous alkali-catalyzed sugarcane conversion

Bagasse Pyrolysis Run 1 - DCM Extraction GC-MS Results

Residence Time	Compound	Formula	% Quality
6.552	3 - Methyl - 2 - cyclopenten - 1 - one	C6H8O	59
7.441	Phenol	C6H6O	87
7.993	2 - Hydroxy - 3 - methyl - 2 - cyclopenten - 1- one	C6H8O2	52
8.632	2 - Methoxy - phenol	C7H8O2	87
	Mequinol (4 - Methoxy - phenol)	C7H8O2	87
9.358	4 - Ethyl - phenol	C8H10O	81
	2 - Ethyl - phenol	C8H10O	81
10.311	4 - Ethyl - 2 - methoxy - phenol	C9H12O2	70
10.312	2 - Methoxy - 4 - methylbicyclo[3.2.1]oct - 2 - ene	C10H6O	70
10.322	5 - Isopropyl - 3,3 - dimethyl - 2 - methylene - 2,3 - dihydrofuran	C10H16O	62
10.864	2,6 - Dimethoxy - phenol	C8H10O3	90

Bagasse Pyrolysis Run 2 - DCM Extraction GC-MS Results

Residence Time	Compound	Formula	% Quality
6.533	2 - Methyl - cyclopenten - 1 - one	C6H8O	72
7.443	Phenol	C6H6O	83
8.114	2,3 - Dimethyl - 2 - cyclopenten - 1 - one	C7H10O	52
8.634	2 - Methoxy - phenol	C7H8O2	94
9.338	4 - Ethyl - phenol	C8H10O	81
10.302	7,7 - Dimethylbicyclo[3.3.0]octan - 2 - one	C10H16O	83
10.866	2,6 - Dimethoxy - phenol	C8H10O3	95

CLM Pyrolysis Run 1 - DCM Extraction GC-MS Results

Residence Time	Compound	Formula	% Quality
4.856	3,4 - Dihydro - 2H - Pyran	C5H8O	64
6.546	2 - Methyl - 2 - cyclopenten - 1 - one	C6H8O	86
7.326	3 - Methyl - 2 - cyclopenten - 1 - one	C6H8O	64
	2 - Methyl - 3 - hexyne	C7H12	64
7.456	Phenol	C6H6O	87
7.651	1,2 - Dimethyl - cyclohexene	C8H14	58
7.673	6 - Methyl - 3 - heptyne	C8H14	50
8.106	2,3 - Dimethyl - 2 - cyclopenten - 1 - one	C7H10O	59
8.626	2 - Methoxy - phenol	C7H8O2	93
9.341	3 - Ethyl - phenol	C8H10O	68
9.352	4 - Ethyl - phenol	C8H10O	83
10.316	4 - Ethyl - 2 - methoxy - phenol	C9H12O2	90
10.880	2,6 - Dimethoxy - phenol	C8H10O3	81

CLM Pyrolysis Run 2 - DCM Extraction GC-MS Results

Residence Time	Compound	Formula	% Quality
4.790	3,4 - Dihydro - 2H - pyran	C5H8O	72
5.473	2 - Cyclopenten - 1 - one	C5H6O	64
6.491	2 - Methyl - 2 - cyclopenten - 1 - one	C6H8O	90
7.249	3 - Methyl - 2 - cyclopenten - 1 - one	C6H8O	91
7.390	Phenol	C6H6O	90
7.585	3,4 - Dimethyl - 2 - cyclopenten - 1 - one	C7H10O	90
7.628	2,3 - Dimethyl - 2 - cyclopenten - 1 - one	C7H10O	86
7.910	Cyclohexane	C8H14	64
8.051	2,3 - Dimethyl - 2 - cyclopenten - 1 - one	C7H10O	83
8.571	2 - Methoxy - phenol	C7H8O2	96
9.286	3 - Ethyl - phenol	C8H10O	81
9.297	4 - Ethyl - phenol	C8H10O	87
9.535	2- Methoxy - 4 - methyl - phenol	C8H10O2	96
10.250	4 - Ethyl - 2 - methoxy - phenol	C9H12O2	95
10.813	2,6 - Dimethoxy - phenol	C8H10O3	93

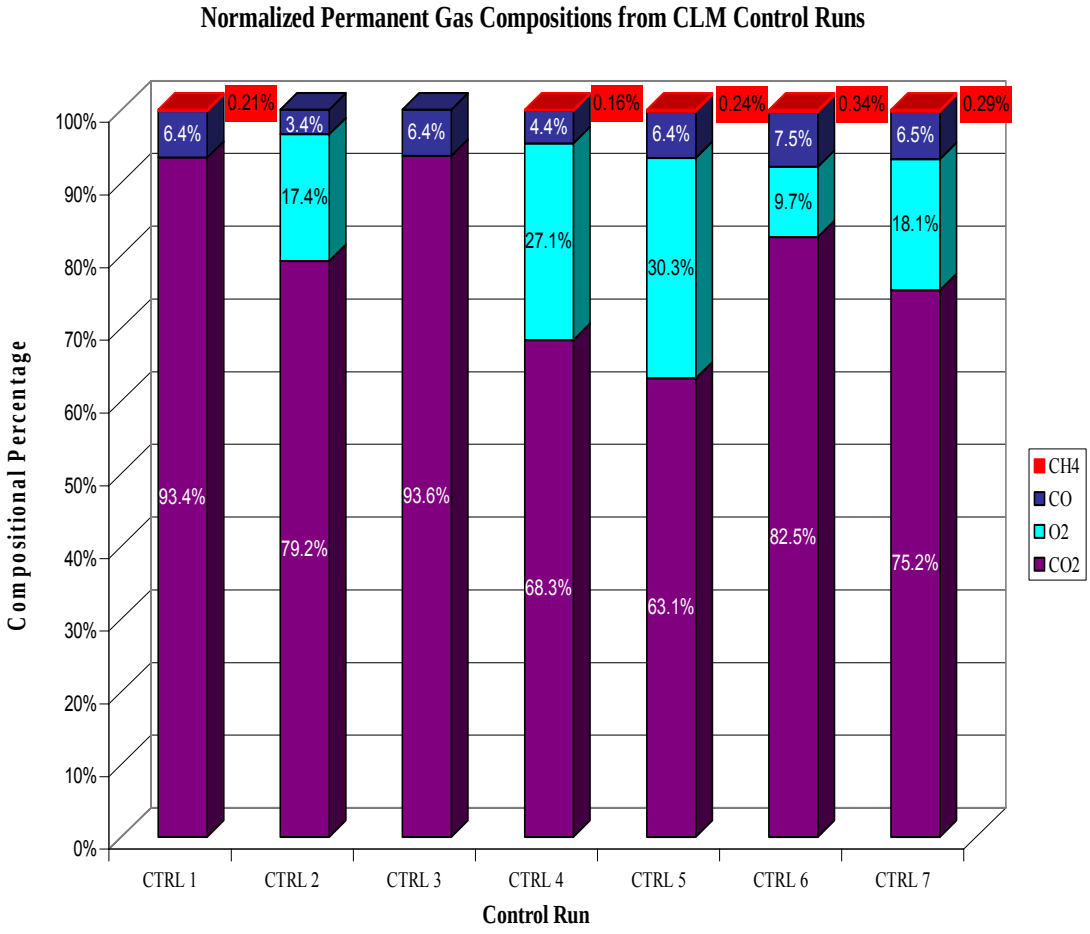
Due to the potential contribution of the sodium carbonate catalyst to the product yield and composition of the sugarcane residue liquefaction runs, it was deemed necessary to run a set of control tests having no catalyst. All of these tests were run with CLM at an identical 20:1 mass ratio of water to moist biomass. The CLM had an average as-received moisture level of 14 %. The CLM and water were continuously mixed with a helical impeller and heated to 300 °C at a rate of 4 °C/min. The CLM slurry was soaked at 300 °C for one hour. The control (#1) was run under an air environment, while controls (#2 through 7) were either purged or sparged with an inert gas. Control #2 was purged with nitrogen for 5 min using an in-line pressure of 30 psia. Control # 3 was purged with nitrogen for 50 minutes using an in-line pressure of 40 psia. Argon was used to purge the reactor system in control run 4 for 13 min at a flowrate of 2 l/min. In control # 5, argon was used to sparge the system at 105 °C for 5 min. Control run 6 was sparged with argon at 105 °C for 7 min. Control # 7 was sparged with argon at 105 °C for 5 min. Table 39 indicates the mass balance for the liquefaction control runs.

Table 39. Mass balance for non-catalyzed liquefaction control runs

Mass Balance on CLM Liquefaction Control Runs 1-7				
Run	**Starting Material Mass (g)**	**Liquid Product Mass (g)**	**Solid Product Mass (g)**	**Gas Product Mass (g)**
1	86.0	52.7	22.4	10.9
2	86.0	40.0	36.6	9.4
3	86.0	45.7	29.3	11.0
4	86.0	34.9	34.3	16.8
5	86.0	45.2	35.4	5.4
6	86.0	54.7	28.6	2.7
7	86.0	40.4	41.4	4.2

Figure 8 depicts the normalized compositional gas analyses for the liquefaction control runs.

Figure 8. Normalized gas composition for liquefaction control runs with CLM



GC-MS analysis was also conducted on the liquid products from the CLM liquefaction control runs. The same five phenolic compounds predominate in the mass spectral analyses of three different control runs and the column retention times are almost identical as shown in Table 40. The identical phenolic compounds are also found in the previous alkaline-catalyzed liquefaction runs. total recoverable phenolics was determined for a randomly selected control run to obtain estimate of the concentration of phenolics present in the aqueous organic product. A phenolic concentration of 1 mg/l was obtained using the EPA total recoverable phenolics spectrophotometric determination method. A spectrophotometric method (Folin-Ciocalteau), yielded roughly 20 times the concentration of phenolics, or 20 mg/l. A comparison of these two methods using a known concentration of a standard phenolic, such as vanillin, is required to more accurately quantify the amount of deviation between these two procedures.

Table 3. GC-MS analysis of the most dominant compounds in the CLM control run liquids

Run	Compound	Area %	Retention Time	Match Quality
Control 3	2-Methoxy-phenol	13.46	7.49	94
	Phenol	12.51	5.15	91
	4-Ethyl-phenol	8.22	8.87	94
	2,6-Dimethoxy-phenol	7.85	11.82	93
	4-Ethyl-2-methoxy-phenol	5.15	10.76	91
	2,3-Dimethylcyclopent-2-en-1-one	3.34	6.54	93
	1,4-Dimethoxy-phenol	2.86	9.38	91
Control 5	2-Methoxy-phenol	14.53	7.50	94
	Phenol	11.78	5.17	91
	2,6-Dimethoxy-phenol	11.31	11.82	90
	4-Ethyl-phenol	11.09	8.88	94
	4-Ethyl-2-methoxy-phenol	5.83	10.76	87
	2,3-Dimethylcyclopent-2-en-1-one	2.86	6.53	94
	2-Methoxy-4-methyl-phenol	2.65	9.38	95
	4-Methyl-phenol	2.43	7.17	97
Control 7	2-Methoxy-phenol	14.39	7.49	95
	Phenol	12.06	5.14	91
	4-Ethyl-phenol	10.59	8.87	94
	2,6-Dimethoxy-phenol	6.79	11.82	90
	4-Ethyl-phenol	5.26	10.76	94
	Spiropentane	3.89	4.97	53
	4-Methyl-phenol	3.85	7.15	87
	2,3-Dimethylcyclopent-2-en-1-one	3.19	6.54	90

Conclusion

1. The composition of pyrolysis liquids is highly aromatic consequently it may be susceptible to naphtha reforming, leading to gasoline fractions as fuel.

2. Gas phases are predominantly carbon dioxide thus, a rich Syngas for chemical synthesis would be the next step for gasification.

Bagasse is approximately 50 % water. Both pyrolysis and gasification must make used of water-mediated reactions to achieve a competitive advantage over drier substrates. Simple hot water and near supercritical water may offer sufficient chemical reaction power to produce both syngas and pyrolysis liquids with specific characteristics without the co-formation of toxic chemicals.

Subtask 5.2 Determine experimentally the yields of syngas per unit mass of bagasse.

Results from five samples collected at temperatures ranging from 673 °C to 765 °C were analyzed by gas chromatography are given in Table 41. A ShinCarbon ST micropacked GC column was employed. A thermal conductivity detector (TCD) was used to detect differences in thermal conductivity between the column effluent flow (carrier gas plus sample components) and the reference flow of the carrier gas. A voltage proportional to this difference is generated which becomes the output signal. The analytical method employed the following method for each gas analysis, in triplicate. The initial oven temperature was set at 40 °C and held there for 3 min. The column was then heated to 120 °C at a rate of 12 °C/min. Subsequently, the temperature was raised to 150 °C at a rate of 15 °C/min. This ramp was followed by a final elevation to 200 °C at a rate of 40 °C/min. The inlet temperature for the GC was 200 °C and the detector temperature was 280 °C. The column pressure was regulated at 60 psi and the carrier gas was helium. The duration of each test was approximately 15 min, including equilibration time at the initial temperature. A 2 m by 1 mm ID micropacked column was able to resolve six permanent gases: hydrogen, oxygen, nitrogen, carbon monoxide, methane, and carbon dioxide. Injections with a gas-tight syringe were made at three different volumes, including 100 μL, 250 μL, and 500 μL. Results indicate appreciable levels of hydrogen.

Table 4: Hydrogen composition in flue gas at various combustion temperature conditions.

Injection Volume	705 °C	728 °C	721 °C	673 °C	765 °C
100 μL	0.29 wt%	0.34 wt%	0.11 wt%	0.34 wt%	0.10 wt%
500 μL	0.58 wt%	0.91 wt%	0.33 wt%	0.81 wt%	0.76 wt%

Saturation of the detector likely occurred with the 500 μL injection, which helps explain why the amounts detected were not five times higher than those obtained for the 100 μL injection. Signs of the overloading included peaks with significant tailing for the major species such as nitrogen and carbon dioxide. Alternatively, argon and nitrogen can be used instead of helium to eliminate problems with hydrogen detection when using helium, although detector sensitivity is considerably diminished for the detection of gases other than hydrogen.

A goal of subtask 5.2 was to develop a theoretical model that effectively simulates the combustion processes occurring within the gasification unit. COMSOL Multiphysics software, which employs finite element analysis, has been used in the construction of the gasification model. Results provided a schematic representation of the sugarcane bagasse downdraft gasifier. Considerable attention was devoted to refining the mesh of the gasifier, which partitions the geometry model into a network of small units of simple shapes. The large geometric scale variations presented a significant challenge to mesh because of the inclusion of some very thin layers. The thin domains were meshed with a swept mesher, which is less sensitive to thin regions than the free mesher. The interactive meshing tool was used to separately mesh the rest of the domains using a free mesher.

Initially, the fluid transport through the downdraft gasifier was simply modeled using a laminar - flow with the Navier-Stokes equations. In the fully developed laminar flow model there were only velocity components in the main direction of the flow. In reality, however, the gasifier is likely to have a mixture of turbulent flow and laminar flow. Unlike the flow in the laminar regime, flows in the turbulent flow regimes exhibit a velocity profile will vary perpendicularly to flow as the flow proceeds downstream.

Inclusion of a turbulent flow regime domain requires a simplification of the momentum transport equation to avoid the large number of finite elements that would be necessary to account for all of the dynamics of the fluid flow. Moreover, turbulence also presents a difficulty when trying to resolve fluid flow near walls. These zones contain small chaotic eddies as well as boundary layer formation, both of which must be resolved from the main body fluid flow. The first requirement for turbulent flow analysis involves the evaluation of the mean flow characteristics through the use of a Reynolds averaged representation of the turbulent flows that is inserted into the Navier-Stokes equation assuming a constant viscosity. Closure of the turbulence model is then achieved by incorporating the k-ε assumption which broadly implies that momentum transport attributable to small eddies is translated to transport by shear, or turbulent viscosity. The turbulent viscosity term has been replaced by field variables for which the transport equations are defined, namely the turbulence kinetic energy, k, and the dissipation rate of turbulence energy, ε.

Convergence of the solution for the turbulent flow model is of utmost importance and the model is currently being tested in a stepwise fashion for increasing velocities. Currently, the model solution has converged for a velocity of 0.0005 m/s. Velocity values above this level have resulted in non-convergence because the k-ε closure application mode utilizes an empirical relation between the value of the flow velocity and the wall friction substitutes the thin boundary layer near the wall. Although this method is relatively accurate for high Reynolds numbers, it begins to break down for moderate to low Reynolds number turbulent models as in the sugarcane bagasse gasifier model. In this case, we have had to refine the mesh near the wall so that the "viscous" sublayer can be resolved. Due to modeling restrictions with the current version of COMSOL, it may be necessary to develop both a purely laminar model and a wholly turbulent flow model. Experimentation on agasifier at transitional flows will then be required to obtain data points that can be used to plot a curve between the laminar and turbulent regimes.

Figure 9 depicts the turbulent flow model for the gasifier at 0.0005 m/s. Air passing through the uppermost region of the gasifier almost immediately encounters a region of turbulence, which is caused by the presence of a thin circular arc about the inner perimeter of the gasifier. After this zone, the gas flow proceeds down through the gasifier until it reaches the packed bed containing the sugarcane bagasse and cane trash. Flow through the packed bed becomes strangled and is then forced through another porous bed containing charcoal and bagasse in the cylindrical combustion core. Gas flow through the combustion chamber approaches the highest velocity in the entire gasifier. The tightly banded streamlines are indicative of higher velocity rates. The gas that leaves the combustion cylinder is subjected to a turbulent flow field, which can be seen from the sizeable eddies that form both at the exit of this cylinder and also at entrance to the

exhaust tube of the gasification reactor. In both cases, the turbulence arises from gas flow that experiences a significant expansion or constriction to movement, respectively.

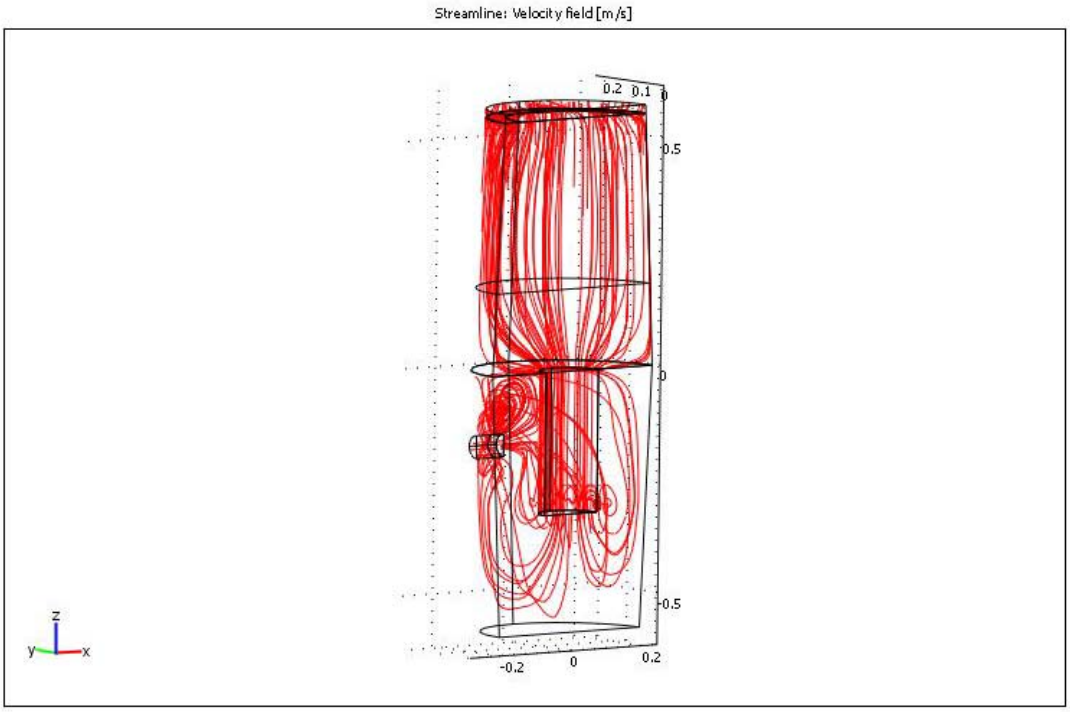


Figure 9. Velocity profile for the downdraft sugarcane bagasse gasifier

During the second quarter advances were also made on the temperature profile modeling of the gasifier. COMSOL Multiphysics was again used to facilitate the theoretical modeling of the heat transfer within the gasification process. A general heat transfer application mode was applied that includes both conduction and convective heat transport terms. Conduction through the galvanized steel walls of the gasifier is included in this model by accounting for the thermal conductivity of the steel. Convection, however, plays a much larger role in the heat transport than do heat losses via conduction in the gasifier. Presently, the source of heat, q, is designated to be approximately 25 W/m^3. The heat emanates from the combustion core, where the gasification reactions occur.

Figure 10 indicates the steady state temperature profile of the gasification reactor after approximately one hour of operation. The temperature at the core center has reached 1042 °C. The illustration also depicts the loss of heat through the galvanized steel walls to the outside, which is held constant at an ambient temperature of 25 °C. The arrows represent the temperature gradients within the gasifier.

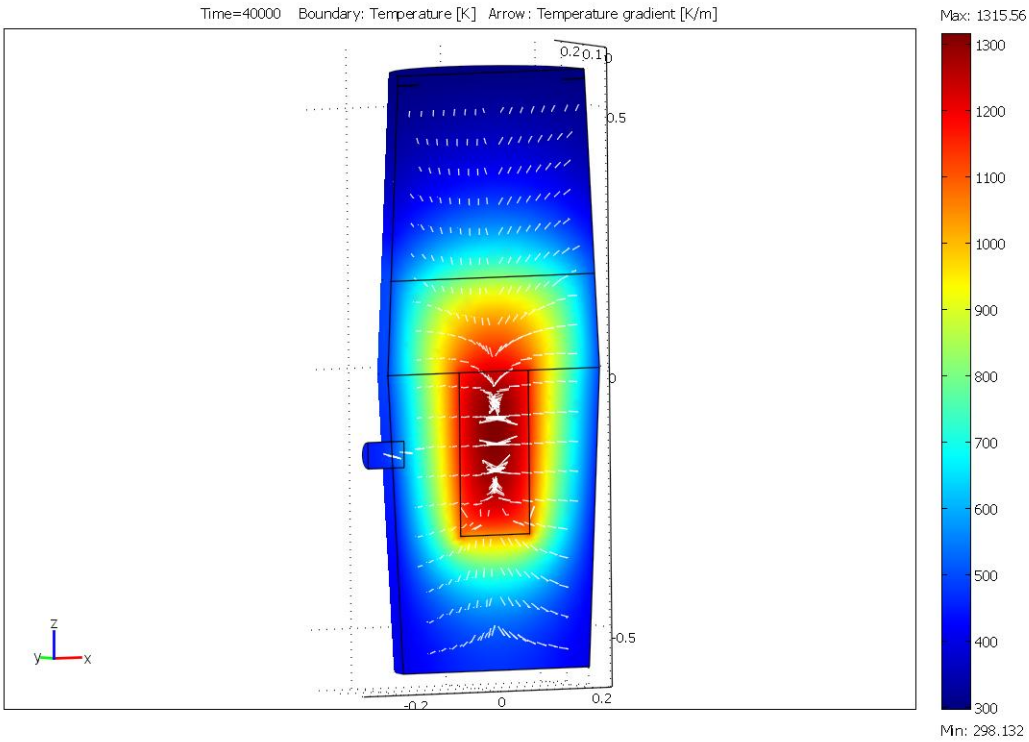


Figure 10. Temperature profile of a gasification reactor (after 1hr operation)

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A laboratory scale gasifier prototype was designed (see Figure 11 below) but not constructed because of safety concerns at the proposed site. A model for the heat transfer through the reaction cells was created. The model predicted that the shortest time to temperature would be approximately 30 minutes. This did not correlate well with experimental results using a reaction cell filled with sand, which indicated heat transfer rates that were on the order of 6 times faster.

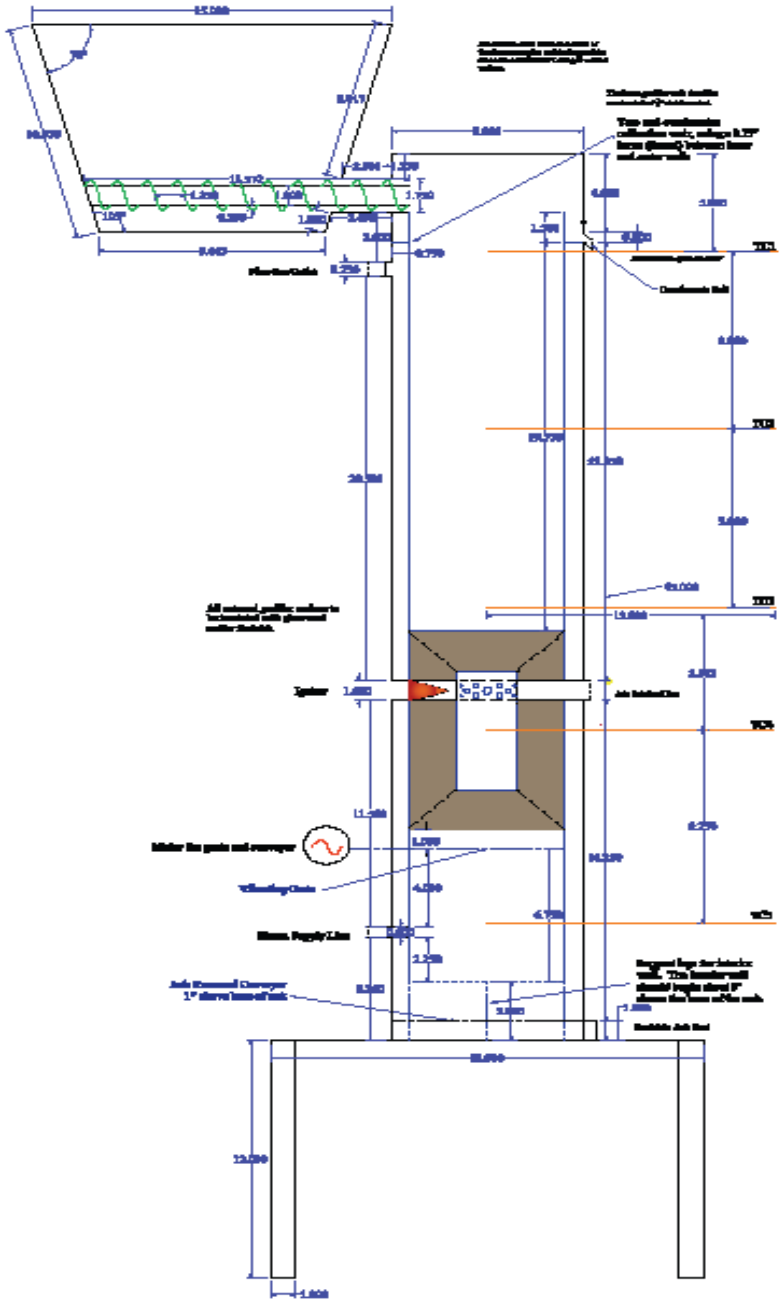

Figure 11.Proposed laboratory prototype gasification reactor for bagasse.

Temperature control studies in a molten metal furnace were conducted. An accurate portrayal of the hysteresis resulting from the single-loop feedback controller was rendered. The initial overshoot in temperature was typically between 6-9 % of the set-point temperature. Successive temperature overshoots are no more than 3 % higher than the set-point temperature. The resulting dip in temperature between overshoots is always less than 1 % absolute deviation from set-point temperature. The time to reach the desired set-point temperature from ambient outside air temperature is on the order of 5 to 6 minutes using a reaction cell filled with sand. The use of water within the cell might translate to a maximum time savings of 45 seconds to 1 minute. The bulk of the heating time occurs while the heat is being conducted through the Hastelloy walls of the reaction cell. These walls tend to behave as highly unfavorable thermal conductors. It was discovered that the greatest hindrance to the heating occurs during the ramp from ambient temperature to 100 °C. It is believed that this is the combined effect of thermal inertia and the time required to drive off any residual moisture within the cell.

Conclusions

Gasification research was terminated at the end of the project. Although the gasification approach is promising for producing ethanol from biomass it was felt that the expense of a gasifier of sufficient scale to prove the result was beyond the capability of a University based research group. Gasification also minimized the chances of finding a range of by-products from the process that would improve the economic viability of a sugar mill based borefinery.

Materials and Methods (task 5)

The compositional gas analysis was conducted on an HP 5890 GC unit using a Shincarbon ST® micropacked column (Restek Corporation; Bellefonte, PA) fitted with a thermal conductivity detector (TCD). Helium was selected as the carrier gas and set at a pressure of 22 psi and a flowrate of approximately 10 ml/min. An internal standard (SCOTTY Gases; Pittsburgh, PA) was used to calibrate the measurements and had the following permanent gases with the corresponding molar compositions: 5.00 % CO_2, 5.03 % CO_2, 4.00 % H_2, 3.96 % CH_4, 5.05 % N_2, with the balance consisting of helium. The gas standard injection volume ranged between 50 and 100 µl, while the sample gas injection volume varied between 50 and 200 µl. The temperature program profile used to perform the gas chromatography is provided here: Begin at 28 or 30 °C, hold for 3 min and then ramp to 120 °C at 45 °C/min with no hold time; then ramp to 150 °C at 40 °C/min with no hold time; continue to ramp to 200 °C/min with a 2.75 min hold before terminating the program. The total analysis run time was either 9.97 min or 9.93 min depending on the initial starting temperature, respectively. A slightly abridged version of this program was used for control runs 4 and 5. In this program the beginning temperature was 35 °C and after being held constant for 3 min the oven temperature was increased to 200 °C at a rate of 40 °C/min, followed by a 2.50 min hold time before program termination. The total run time for this abridged profile was 9.63 min. Both the injector and detector temperatures in this program were set to 110 °C, while the helium carrier gas was set to a pressure of 40 psi at a flowrate of 20 ml/min.

TASKS 5.1 and 5.2

Eight subcritical aqueous alkali-catalyzed sugarcane liquefaction experiments were conducted using a 20:1 mass ratio of water to moist sugarcane biomass. As-received bagasse had 10 %

moisture content and cane leaf matter (CLM) had 14 % moisture content. Alkaline catalyst was added in the form of sodium carbonate in the amount of 5 % w/w per run. The biomass slurry was continuously stirred and heated at a ramp rate of approximately 2.5 – 4.0 °C/min to 300 °C and soaked at this temperature for approximately one hour. After an hour-long soak, the heating coil was switched off and the reactor was allowed to return to ambient room temperature (20 °C) over the course of about 12 hours.

Gases were collected at ambient temperature while the system was under slightly positive pressure and measured via volume displacement of water in a glass bell jar. The liquid products from these eight runs were initially filtered on No. 42 Whatman filter paper to remove the entrained particulate matter. The coloration of the liquid products after filtration closely resembled that of apple juice. Upon storage at 4 °C for about 72 hours, the appearance of the liquid products darkened considerably to a dark olive-green color with a yellowish cast. Storage at durations beyond one year has not resulted in any additional color intensification. The liquid products have a distinctive smoky, hickory aroma that has not changed throughout the storage period.

The first four tests were carried out under an atmospheric environment, whereas the final four tests were run after first purging with nitrogen gas for 20 min to reduce the likelihood of oxygen presence in the system. No gas was collected from the Parr reactor from the first run. The second run used a much slower ramp rate corresponding to about 1 °C/min. The biomass slurry in the fourth run was left to liquefy at the soak temperature for about 90 min. Runs 5 - 8 were purged under nitrogen for approximately 20 – 30 min and on run 6 the reactor drain was accidentally left partially open for the first few minutes of the run, potentially resulting in the loss of some liquid product. Loss of some gas product is highly probable in run 5 due to a suspected minor leak in the tubing fittings.

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Project summary

Project Summary

The rationale for this project was to develop and evaluate scalable, integrated technologies utilizing bagasse, molasses and CLM for the production of ethanol, chemicals, biomaterials and animal feeds that can be used in a sugar mill based biorefinery. All efforts in this project were directed towards investigating those processes that could be integrated into a raw sugar mill.

Specific Objective: Develop a scalable AFEX biomass pretreatment process for bagasse (**Tasks 1 and 2**).

The pretreatment of bagasse via the AFEX process, followed by saccharification and fermentation demonstrated the potential of AFEX as a pretreatment for bagasse. The AFEX process conditions, which include reaction time, reaction temperature, ammonia loading, and moisture content of the biomass, were varied to determine those conditions that provide the highest glucose and xylose yield. The maximum yield from bagasse was obtained with AFEX treatment times twice as long as required for corn stover. The most effective AFEX treatment conditions for bagasse, pith and CLM were found to be 100°C, a biomass moisture content of 40%, a reaction time of 30 minutes, and an ammonia loading of 2 g ammonia per gram dry biomass.

The efficiency of the AFEX process was evaluated using enzyme hydrolysis. Combinations of cellulase and xylanase produced higher glucose and xylose yields from AFEX-treated material compared to hydrolysis with only cellulase. A process was developed for the separation of hemicellulose and cellulose from AFEX-treated CLM and bagasse pith using selective hydrolysis of hemicellulose by using cellulase-free xylanases for selective hydrolysis of the hemicellulose. For AFEX-treated CLM, about 52% of the xylan was converted to xylose while about 96% of the cellulose was left in the biomass. For AFEX-treated bagasse pith, about 56% of the xylan, but only 5% of the cellulose, was hydrolyzed.

Specific Objective: Develop and demonstrate processes that utilize components from sugar cane; bagasse, CLM and molasses for ethanol production (**Tasks 4, 5, 6**).

This research broke down into several areas, technologies to produce ethanol from AFEX treated bagasse and technologies to produce succinic acid from the xylose stream. Enzyme hydrolysis of AFEX-treated bagasse generates streams of fermentable sugars containing both C5 (xylose and arabinose) and C6 sugars (glucose). The feasibility of integrating simultaneous saccharification and fermentation (SSF) allowing two operations in the same vessel was demonstrated. Additionally, the addition of sugarcane molasses to the hydrolysate/fermentation process yielded improvements beyond what was expected solely from the addition of sugar.

Alkaline treated biomass, unlike acid treated material, contains a significant amount of hemicellulose that needs to be hydrolyzed to monomeric sugars. The generated C5 and C6 sugar streams are useful in the production of fuels and chemicals such as ethanol and succinic acid. Succinic acid is a four-carbon dicarboxylic acid used to manufacture polymers and resins for lacquers, dyes and perfumes, industrial solvents, and newly developed products such as de-icing chemicals. It can be produced via bacterial fermentation using renewable biomass-derived sugars. MBI identified a bacterium, *Actinobacillus succinogenes*, capable of utilizing both hexose and pentose sugars simultaneously for the production of succinic acid. Fermentability of the enzyme hydrolysate from AFEX-treated CLM was assessed for succinic acid production. Using SHF processes, with different solid loadings for succinic acid fermentation with *Actinobacillus succinogenes FZ45,* generated sugars (C5 and C6) were converted to succinic acid. MBI's existing ASPEN Plus-based process model for production of succinic acid from pure sugars was modified to evaluate the technical and economic performance of succinic acid production from AFEX-treated biomass. The model showed that the capital and manufacturing costs would be decreased if the cost associated with hydrolysis was decreased. Improving cellulase activity in the presence of increased solids loading would provide a lower AFEX cost, a higher succinic acid and ethanol titer, and lower downstream processing costs.

Specific Objective: Investigate the feasibility of gasification of bagasse for ethanol production

Gasification research was terminated at the end of the project. Although the gasification approach was promising for producing ethanol from biomass it was felt that the expense of a gasifier of sufficient scale to prove the result was beyond the capability of a University based research group. Gasification also minimized the chances of finding a range of by-products from the process that would improve the economic viability of a sugar mill based borefinery.

Products and Technology Transfer Activities

Publications, conference papers and other public releases:

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