IBRI

Final Report

North Carolina State University

Integrated Biomass Refining Institute at North Carolina State University

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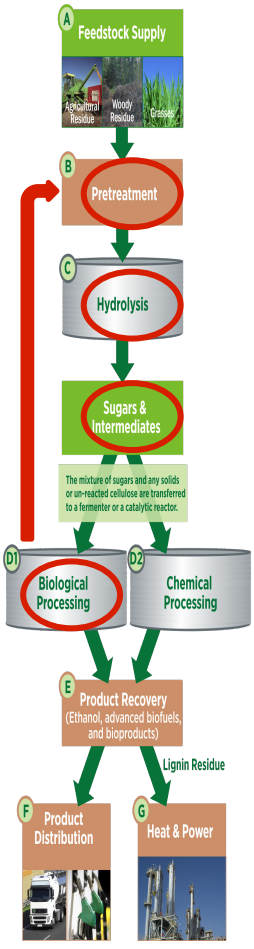
PROJECT OBJECTIVES

The overall objective of the Integrated Biomass Refining Institute (IBRI) is to advance the fundamental understanding of novel biomass conversion technologies leading to the production of biofuels and bioproducts, expanding the range of feedstock that can be utilized and compounds produced from a biomass refinery. The outcomes of this project will be new analytical facilities for biofuels and bioproducts research at North Carolina State University (NCSU), establishment of the capabilities of a cellulosic ethanol screening pilot facility to monitor and control processes, and publications in the open literature and presentations at public conferences regarding novel crops and technologies for cost-effective biomass processing.

PROJECT SCOPE

Significant biochemical pathway barriers to ethanol production from lignocellulosic material include: (1) Biomass Recalcitrance – the restricted access of enzymes or chemicals to cellulose caused by the close association of lignin with cellulose, which limits the extent and rate of hydrolysis; (2) Pretreatment Chemistry – the degree of crystallinity of natural and pretreated cellulose, which also limits the extent and rate of hydrolysis; and (3) Catalyst and Fuels Organism Development – inefficient or slow fermentation into ethanol of mixed sugar oligomers that result from pretreatment. The Integrated Biomass Refining Institute dealt with two primary aspects of biomass conversion, one investigating optimization of fermentation systems and one investigating optimal biomass pretreatment methods for lignocellulosic material, focusing on two important feedstocks, loblolly pine and switchgrass.



Pretreatment informs the ultimate fates of sugars and lignin; it is the central processing step linking biomass composition with biomass utilization. Optimal processing effectiveness suggests the establishment of feedback loops whereby downstream performance and capabilities inform the development of upstream catalysts and processes, whether one is considering sugar or lignin processing. IBRI's emphasis has been on sugars, with research focused on effectively bridging pretreatment technologies with fermentative capabilities.

Initial project research focused on feedstock evaluation, in order to assess the viability of regional biorefineries. The advances from the sustainable production and crop genetics work, along with information from partners and literature, were used to inform techno-economic models for biomass availability and costs. In the new technology arena, three innovative conversion schemes addressed barriers in both the biochemical and thermochemical conversion pathways. In the biochemical pathway, barriers in biomass fractionation and mixed sugar fermentation were addressed. In the thermochemical pathway, barriers in fuels catalysts were addressed.

The majority of effort in IBRI has been focused on study of pretreatment and conversion technologies. These research and development efforts directly support the key 2012 technical targets of achieving higher yields of cleaner sugars and lower fuel conversion costs in the processing steps of pretreatment, enzymatic hydrolysis, and fermentation.

PROJECT RESEARCH AREAS

Sugar and Mixed Oligomer Fermentation Characterization and Enhancement

Hydrolysis of cellulose and starches, whether enzymatic or chemical, performed individually or as part of a simultaneous saccharification fermentation process, will generate pentoses and hexoses, and sugar oligomers of various chain length distributions, concentrations, and compositions. Efficient and effective conversion of those sugars to ethanol and other fermentation products will require organisms with new and/or improved performance.

Temperature dependence of mixed-sugar and oligomer fermentations

Bioethanol production in the USA is primarily based on large-scale industrial ethanol fermentations, typically performed using strains of *Saccharomyces cerevisiae*. However, *S. cerevisiae* has weaknesses that reduce its usefulness in many fermentations. First, due to its optimal fermentation temperature around 25-30°C, it is a poor candidate for higher temperature processes that could greatly reduce production costs (Abdel-Banat et al., 2010). An example of such a process is simultaneous saccharification and fermentation (SSF), where the enzymes involved in the saccharification process have an optimal operating temperature of 45-55°C (Banat et al., 1998). Second, with the exception of a few genetically engineered strains, *S. cerevisiae* also lacks the ability to utilize most non-glucose sugars under normal fermentation conditions (Ho et al., 1998). This is especially troublesome for cellulosic biomass fermentations due to the high xylose and arabinose content of cellulose (Sedlak and Ho, 2004).

Kluyveromyces marxianus has been proposed as an alternative ethanol producer because of several advantages it possesses. First, *K. marxianus* has the ability to utilize sugars not normally used by *S. cerevisiae* such as D-xylose and L-arabinose, the second and third most prevalent sugars after glucose in cellulosic biomass (Saha, 2003) and other sugars found in potential feedstocks (Rodrussamee et al., 2011). *K. marxianus* also ferments more effectively at higher temperatures, with many strains highly productive at 45°C (Wilkins et al., 2008) and some showing growth at temperatures above 50°C (Nonklang et al, 2008; Abdel-Banat et al., 2010). This thermotolerance allows for better performance in SSF operations, decreased operating costs, and more widespread use in warm climates (Suryawati et al., 2008). In addition, *K. marxianus* exhibit higher ethanol and temperature tolerances than another commonly used xylose-fermenting yeast, *Pichia stipitis*, and therefore may be more suited to many industrial ethanol fermentations due to their high concentrations of ethanol (Jeffries et al., 2007). These properties may allow *K. marxianus* to perform more effectively than either *S. cerevisiae* or *P. stipitis* for the production of fuel ethanol.

Currently there is no type strain or genome sequence for *K. marxianus*. Consequently, research has been spread out, with many strains of *K. marxianus* having been examined for growth and ethanol production on a wide variety of both simple (Rodrussamee et al., 2011; Wilkins et al., 2008) and complex substrates. (Oda and Nakamura, 2009; Yuan et al., 2008; Suyawati et al., 2008). Comparatively little is known about either the response of the organism on leaner media or the specifics of *K. marxianus*'s metabolic response to elevated temperatures and ethanol concentrations at these higher temperatures. This research activity is a first step in discovering these properties and elucidating the mechanisms behind them. Three strains of *Kluyveromyces marxianus* were examined in this work, designated CBS 712, UCD-

VEN 744, and CBS 745; each strain was cultured on growth media supplemented with one of three sugars at 30 and 45°C under aerobic and anaerobic conditions.

Table 1. Overall results summary indicating ethanol yields, time to maximum yield, and peak densities and CFU ml^{-1} for each culture.

	CBS 712				UCD-VEN 744				CBS 745			
condition and substrate	ethanol yield	Time (hr)	OD_{600}	CFU ml^{-1}	ethanol yield	Time (hr)	OD_{600}	CFU ml^{-1}	ethanol yield	Time (hr)	OD_{600}	CFU ml^{-1}
Aerobic 30°C												
glucose	76.7%	18	8.16	$7.9x10^7$	68.3%	24	7.15	$8.0x10^7$	70.1%	24	8.95	$1.4x10^8$
xylose	0	N/A	9.15	$1.3x10^8$	0	N/A	6.27	$1.2x10^8$	0	N/A	6.25	$1.2x10^8$
arabinose	0	N/A	5.88	$8.1x10^7$	0	N/A	1.85	$3.5x10^7$	0	N/A	1.75	$4.5x10^7$
Aerobic 45°C												
glucose	66.7%	18	4.38	$3.0x10^8$	0	N/A	0.14	$6.7x10^6$	0	N/A	3.03	$8.4x10^6$
xylose	0	N/A	0.81	$3.6x10^6$	0	N/A	0.11	$8.7x10^5$	0	N/A	0.52	$1.5x10^6$
arabinose	0	N/A	0.21	$2.2x10^6$	0	N/A	0.17	$9.5x10^5$	0	N/A	0.22	$1.8x10^6$
Anaerobic 30°C												
glucose	88.5%	36	1.84	$2.2x10^7$	90.7%	84	1.41	$2.0x10^7$	90.6%	36	1.36	$1.6x10^7$
Anaerobic 45°C												
glucose	34.5%	36	0.31	$2.8x10^6$	7.5%	12	0.24	$1.7x10^6$	15.2%	12	0.27	$2.2x10^6$

Substantial substrate effects were observed in the fermentations of these strains. Ethanol production was only detected from glucose and no ethanol was produced from xylose or arabinose at either 45°C or 30°C. This suggests that the pentose phosphate pathway does not produce ethanol and that ethanol production is entirely triggered by the glycolysis pathway. In addition, no significant quantities of either acetic or lactic acid were detected when xylose or arabinose was the carbohydrate source. This is in contrast to previous work, which detected both xylitol and acetic acid production during the investigation of other strains of *K. marxianus* (Wilkins et al., 2008; Yablochkova et a., 2003).The production of xylitol is indicative of xylose not entering the pentose phosphate pathway, suggesting that there are multiple pathways for xylose metabolism in *K. marxianus*.

The effect of temperature was significant for both growth and ethanol production and again showed variance among the strains. Growth of strain UCD-VEN 744 was eliminated at 45°C in all aerobic cultures. In contrast, CBS 712 and CBS 745 exhibited very slight growth on xylose, but still grew relatively

normally on glucose. CBS 712 was least affected by the higher temperature and was able to produce ethanol with a reduction in yield of 10%, making it the most promising for high temperature fermentations.

The anaerobic fermentations tested here at 30°C exhibited lower cell densities, but increased ethanol yields. On a per cell basis, ethanol production was greatly increased in anaerobic culture at 30°C; CBS 712 exhibited a 41-fold increase in ethanol production per cell. The UCD-VEN 744 and CBS 745 strains exhibited 53-fold and 113-fold increases. These gains are mitigated somewhat by the corresponding increase in time to reach full sugar utilization.

The differences in fermentation performance among the three strains used in this study suggests both potential differences in the metabolic pathways of these strains and more fundamental differences between all of the *K. marxianus* strains and the most widely studied *Kluyveromyces*, *K. lactis*. Further examination of the genetic, regulatory, or other differences between strains of *K. marxianus* could prove very useful for understanding how to improve their potential for use in ethanol production from lignocellulosic substrates.

Development of techniques and technologies for monitoring and modeling of mixed oligomer fermentation

Fermentation Monitoring

In industrial settings involving complex fermentations such as simultaneous saccharification and fermentation (SSF) of high-gravity corn mash mixtures, process-monitoring methods usually involve periodic sampling and off-line analysis. In contrast, interesting preliminary results have shown that Raman spectroscopy has the potential to provide real-time on-line measurements of oligosaccharide (maltose, maltotriose, DP4+) concentrations during fermentation, as well as ethanol, glucose, and glycerol. In this work, the Kaiser Optical Systems Incorporated Raman RXN System™ employed 935-nm (near-infrared) laser light as the excitation source, thereby suppressing significantly the fluorescence background typical of very-high-density corn mash and other fermentation media containing solid particles. Further development of this analytical tool to other oligosaccharides, as well as additional metabolic intermediates (e.g., low molecular-weight acids and sugar-phosphates) would dramatically accelerate and refine the development of accurate metabolic flux models for ethanolic fermentations, and would likely provide additional insight into species-specific metabolic fluxes in mixed cultures.

In situ Raman spectroscopy was employed for real-time monitoring of simultaneous saccharification and fermentation (SSF) of corn mash by an industrial strain of *Saccharomyces cerevisiae*. An accurate univariate calibration model for ethanol was developed based on the very strong C–C stretching band at 883 cm^{-1}. Multivariate partial least squares (PLS) calibration models for total starch, dextrins, maltotriose, maltose, glucose, and ethanol were developed using data from eight batch fermentations and validated using predictions for a separate batch. The starch, ethanol, and dextrins models showed significant prediction improvement when the calibration data were divided into separate high- and low-concentration sets. Collinearity between the ethanol and starch models was avoided by excluding regions containing strong ethanol peaks from the starch model and, conversely, excluding regions containing strong saccharide peaks from the ethanol model. The two-set calibration models for starch (R^2 = 0.998, percent error = 2.5%) and ethanol (R^2 = 0.999, percent error = 2.1%) provide more accurate predictions than any previously published spectroscopic models. Glucose, maltose, and maltotriose are modeled to accuracy comparable to previous work on less complex fermentation processes. Our results demonstrate that Raman spectroscopy is capable of real-time *in situ* monitoring of a complex industrial biomass fermentation. To our knowledge, this is the first PLS-based chemometric modeling of corn mash fermentation under typical industrial conditions, and the first Raman-based monitoring of a

fermentation process with glucose, oligosaccharides, and polysaccharides present.

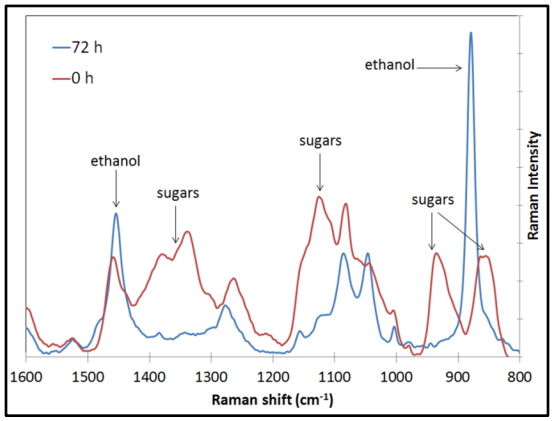


Figure 1. Raman spectra of a fermentation at inoculation (0 h, red) and completion (72 h, blue). Differences in the peaks of the spectra are correlated with compositional changes through chemometrics.

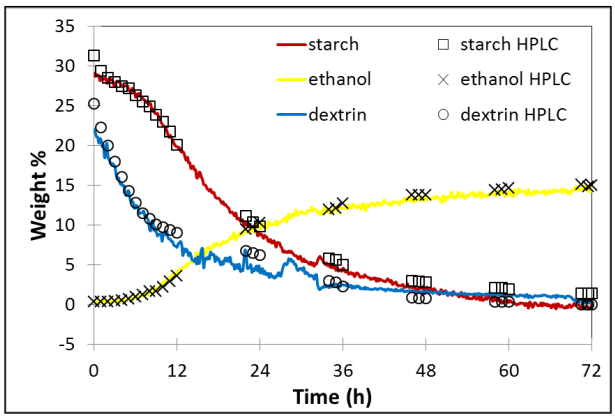


Figure 2. Temporal plots of the validation batch two calibration set models for starch, dextrin, and ethanol. The superior fit can be easily seen.

From: Gray, S.R., S.W. Peretti, and H.H. Lamb. 2013. Real-time monitoring of high-gravity corn mash fermentation using in situ Raman spectroscopy. Biotechnology and Bioengineering, 110(6): 1654-1662.

Fundamental Insights into Biomass Pretreatment and Reactivity

Cellulase enzymes can produce monomeric glucose from an ideal amorphous cellulose in 5-10 hours, while highly crystalline cellulose will be essentially unreacted after 100 hours. Real biomass samples fall someplace in between these two extremes. It is clear there are a number of key macromolecular features such as cellulose crystallinity, surface area, pore size and pore size distribution, and the location and structure of residual lignin and hemicelluloses impact the reactivity of real biomass samples. In addition to the rate of reaction, pretreatment processes must consider a number of very practical issues including the recovery of other biomass sugars, the formation of fermentation inhibitors, and the capital and operating costs.

IBRI research evaluated several key pretreatment barriers of interest to both NCSU and its industry partner. This research task provided a systematic understanding of the features that control the reactivity of biomass cellulose. New analytical tools were developed or adapted as necessary to provide additional insight into the relationships between biomass source, biomass pretreatment, and biomass reactivity.

Two important biomass resources, switchgrass and loblolly pine, were the primary feedstocks for IBRI research. These two feedstocks represent crops that can be cost-effectively grown in North Carolina and the southeastern US, they represent feedstocks with known, near-term deployment potential, and the research team has hands-on experience with these two systems.

The team focused on alkaline pretreatments that have practical, near-term potential. Work by NCSU and its industry partner has highlighted practical aspects of these alkaline pretreatments, particularly when it comes to the formation of fermentation inhibitors. The specific nature of the alkaline pretreatments may vary between the two biomass feedstocks, where the loblolly pine may require a more aggressive pretreatment, such as “green-liquor” pretreatment, which is well-known to the pulp and paper industry.

Effects of residual hemicellulose on enzymatic hydrolysis

Hemicellulose is an important component in hardwood and agricultural residues. Removal of hemicelluloses, which represent about 10-25% of biomass content, makes residual biomass more digestible by enzymes. This research will evaluate the impact of hemicellulose removal on biomass structure at the molecular level using a variety of analytical instruments such as x-ray diffraction (XRD), nuclear magnetic resonance (NMR), and electron microscope. There will be a particular focus on the "hard-to-move" hemicellulose that is strongly associated with biomass, likely through the presence of lignin-carbohydrate complex (chemistry issue) and/or a part of secondary cell wall (geometry and diffusion issue). It is speculated that lignin carbohydrate complex (LCC) may be critical for improved enzymatic hydrolysis. In this research area, work concentrated on the fundamental impact of ozonolysis and ultrasonication pretreatments of lignocellulosic materials on the structure of lignin, hemicelluloses, and cellulose of the biomass.

Specific activities in this research area were focused on experiments to determine the impact of ozone pretreatment on delignification and fermentable sugar recovery from lignocellulosic biomass.

Ozonolysis pretreatment

Ozonolysis pretreatment conditions for obtaining maximum glucose concentration in pretreated biomass from each of the four energy cane varieties being investigated were identified statistically. As in the case of hydrolysis of energy cane samples selected from optimized ozone pretreatments on the basis of acid insoluble lignin (AIL) content after treatment, samples in this set of experiments were also enzymatically hydrolyzed at enzyme loadings of 0, 10, 20, 30% (g enzyme protein/g dry biomass) with Cellic CTec2 (Novozymes NA, Franklinton, NC). Untreated samples were hydrolyzed at equivalent enzyme loadings as control. The 0% enzyme loading looked at the effect of soaking alone on release of sugars. Samples (treated and untreated) were directly used for hydrolysis without a washing step. In order to avoid the effect of any inhibitors on carbohydrate conversion during hydrolysis, additional studies were carried out with pretreated solids washed with water. Hydrolysis was conducted at a solid loading of 8% in a 20 ml solution volume made up (apart from enzyme volume) by sodium citrate buffer as the (pH 4.9) and tetracycline hydrochloride solution equivalent to 40µg/ml to prevent contamination of the hydrolyzate.

Figures 3 and 4 show the concentration of acid insoluble lignin (AIL) and acid soluble lignin (ASL), respectively, in pretreated biomass before and after washing along with the original composition of the untreated biomass (Control). From Figure 3 it can be seen that AIL concentration is highest in the control for all the four varieties. Between unwashed and washed samples, unwashed samples have a higher lignin concentration than washed samples, implying the removal of lignin-degraded compounds during the wash. However these may not be statistically significant. In Figure 4, it can be observed that ASL concentration is highest in unwashed samples potentially due to the accumulation of

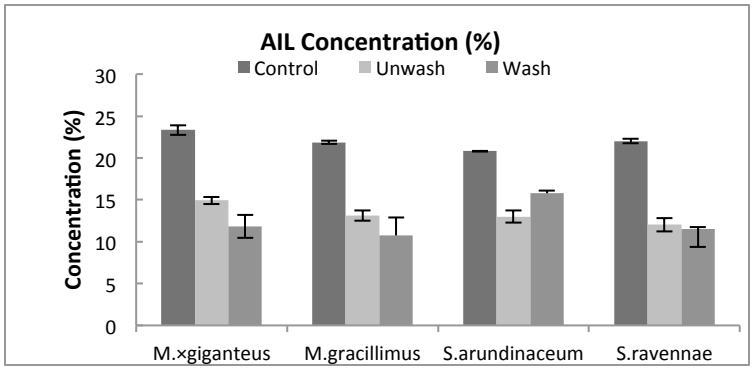


Figure 3. Comparison of Acid Insoluble Lignin (AIL) in untreated (control), pretreated unwashed and washed energy canes.

lignin degradation products during pretreatment. Furthermore, ASL concentration was lower than the concentration observed in the control, indicating the removal of soluble lignin during the wash step.

The concentration of glucose and xylose in unwashed and washed samples pretreated with ozone and from untreated biomass samples are presented in Tables 2 and 3. Although seemingly higher, significant differences were not observed ($P > 0.05$) between untreated and ozonated samples, indicating minimal or no impact of ozone on carbohydrates in the biomass.

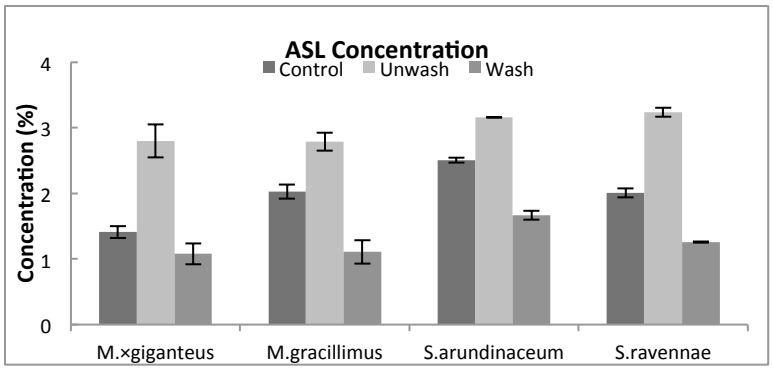


Figure 4. Comparison of Acid Soluble Lignin (ASL) in untreated (control), pretreated unwashed and washed energy canes.

Table 2. Comparison of glucose concentration in control, pretreated unwashed and pretreated washed energy canes.

	Untreated	**Unwashed**	**Washed**
M. ×giganteus	44.51 ± 4.57	46.61 ± 0.05	53.58 ± 4.03
M. gracillimus	37.65 ± 4.75	47.49 ± 4.71	53.29 ± 14.98
S. arundinaceum	37.87 ± 3.46	42.42 ± 7.57	44.23 ± 2.92
S. ravennae	39.00 ± 3.63	42.78 ± 0.44	49.4 ± 4.90

Table 3. Comparison of xylose concentration in control, pretreated unwashed and pretreated washed energy canes.

	Untreated	**Unwashed**	**Washed**
M. ×giganteus	18.59 ± 1.9	19.53 ± 0.02	14.05 ± 0.55
M.gracillimus	18.92 ±2.58	17.66 ± 1.74	16.02 ± 4.42
S. arundinaceum	20.45 ± 1.92	16.23 ± 3.27	16.60 ± 0.59
S. ravennae	20.06 ± 1.85	17.04 ± 0.6	15.53 ± 1.49

Sugar yields (mg/g) after hydrolysis of untreated and ozonated energy cane varieties and overall carbohydrate conversions (%) obtained from the various energy cane varieties were measured. Results indicate that the maximum yields of glucose and xylose for *M. x giganteus* (370.4 mg/g and 96.2 mg/g) and *M. gracillimus* (416.8 mg/g and 92.2 mg/g) were observed in the pretreated samples that were washed and hydrolyzed with 10% enzyme loading. In case of the *Saccharum* varieties, maximum glucose yields of 442.8 and 389.9 mg/ g for *S. arundinaceum* and *S. ravennae*, respectively, were also obtained with the washed samples hydrolyzed with 10% enzyme loading. It was however observed that for *S. ravennae*, glucose yields from hydrolysis of unwashed samples at 10% enzyme loading were not significantly different ($P>0.05$) from washed samples. In all cases unwashed samples resulted in lower sugar production compared to control potentially due to the presence of inhibitory compounds which were removed during the washing step. We have not been able to complete the detection of these inhibitory compounds using GC analytical methods. It appears that their concentration is lower than the detection range and we are currently investigation options to resolve this issue. Overall, it was observed

that the inhibitory effect was more pronounced in *Miscanthus* varieties compared to *Saccharum* varieties as the washed samples had significantly higher sugar yields compared to unwashed samples.

At all enzyme loadings across the varieties, the % conversion of (hemi) cellulose to glucose and xylose followed the general trend of control < unwashed solids < washed solids. Unwashed solids had lower conversion than washed solids due to enzymatic inhibition, caused by the lignin degradation products generated during pretreatment. Twenty % and 30% enzyme loadings were found to produce more than 100% conversion to glucose for all the samples except for unwashed pretreated *M. × giganteus* at 20% loading. The high conversion observed could be due to the interference of gluconic acid (a product of hydrolysis with Cellic Ctec2) and some other compounds with glucose during HPLC analysis (Cannella et al, 2012). Higher conversions were observed for all the varieties when the pretreated biomass was washed with water and hydrolyzed with 10% Cellic Ctec2. The conversions of cellulose to glucose were 76.5%, 92.4%, 105.4% and 100% for *M. x giganteus, M. gracillimus, S. arundinaceum,* and *S. ravennae* respectively. From the conversion (%), it can be said that the *Saccharum* varieties are more susceptible to hydrolysis using Cellic Ctec 2, than the *Miscanthus* varieties. It may be noted that excessively high enzyme loadings (up to 30% g enzyme protein/g biomass) were initially used to eliminate any enzyme limitation effects since a relatively less understood pretreatment method was being investigated. However the results indicate that direct hydrolysis of biomass may be a possibility if the economic feasibility can be established.

Results from the ozonolysis and hydrolysis experiments indicate that a uniflow reactor configuration with ozone flow maintained at 0.25 LPM and 40 mg/L for 2h for biomass at 30% moisture (db) maximizes delignification and sugar recovery for energy canes. Washing is believed to be an integral step to remove inhibitors prior to hydrolysis with CTec® Cellic2 at a loading of 10%. These results highlight the potential of ozone for the pretreatment of lignocellulosic biomass without the need for harsh chemicals and costly reaction vessels (resistant to corrosion). Besides, the gaseous ozonolysis process reduces the waste stream that may be generated with conventional pretreatment methods. A logical next step of this study is to investigate reaction kinetics for scaling up the process and to conduct an in-depth economic analysis for the process.

Since the primary outcome for

Raw Miscanthus					
Total Solid (%)		**95.15 ± 0.26**			
Water Content (%)		**4.85 ± 0.26**			
Ash (%)		**2.23 ± 0.06**			
Extractive 1 (%)		**1.80 ± 0.03 (Acetone)**			
Extractive 2 (%)		**2.42 ± 1.45 (EtOH + Toluene)**			
Lignin (%)		**22.81 ± 0.12**			
AIL (%)		**20.68 ± 0.13**			
ASL (%)		**2.13 ± 0.01**			
ASL (%)		NaOH Concentration			
		0.0%	0.5%	1.0%	1.5%
PT Time	30 min	**1.32**	**0.98**	**0.82**	**0.65**
	60 min	**1.51**	**1.16**	**0.90**	**0.85**
AIL (%)		NaOH Concentration			
		0.0%	0.5%	1.0%	1.5%
PT Time	30 min	**19.22**	**15.03**	**6.58**	**6.51**
	60 min	**21.34**	**16.40**	**6.89**	**5.41**
Lignin (%)		NaOH Concentration			
		0.0%	0.5%	1.0%	1.5%
PT Time	30 min	**20.54**	**16.01**	**7.41**	**7.16**
	60 min	**22.85**	**17.56**	**7.79**	**6.25**
AIL/ASL		NaOH Concentration			
		0.0%	0.5%	1.0%	1.5%
PT Time	30 min	**14.61**	**15.38**	**8.01**	**10.12**
	60 min	**14.14**	**14.15**	**7.67**	**6.44**

ozonolysis of lignocellulosic biomass is delignification, for comparative purposes alkaline pretreatment on *Miscanthus giganteus* was conducted and the results shown in the table above.

Ultrasonication pretreatment

Ultrasonication of switchgrass at 170 micron (100%) amplitude, for 60 min in a stainless steel beaker with temperature control was the most effective pretreatment strategy tested in this research area. SEM images highlighted the treatment's tendency to physically disrupt significant portions of the treated biomass due to mechanical action. The trend of considerably high fermentable sugar generation from untreated switchgrass hydrolyzed at higher enzymatic loadings, however, presents a new investigational direction relative to economic viability of pretreatment and hydrolysis processes, and bears further scrutiny.

It can be inferred from the results, which investigated ultrasonication at 20KHz for 5, 7.5, and 10 min at 50, 75 and 100% amplitude (corresponding with 75, 112.5, 150 µm at the face of the sonotrode), that ultrasonication alone is not an effective pretreatment technique. The reported range of particle size that can be affected by ultrasonication (0.15–100 mm) is large and therefore higher particle sizes need to be explored to determine the true potential of this technique. Considering that ultrasonication does not provide significant change in chemical composition of biomass, it may however be utilized as a preliminary size reduction step to decrease the severity of primary pretreatment methods.

Effects of residual lignin on enzymatic hydrolysis

Residual lignin on biomass will inhibit enzymatic hydrolysis in several ways. Lignin may coat the biomass surface and also link strongly through lignin-carbohydrate complexes (LCC). Non-specific binding with enzyme systems is also observed. Therefore, the structure and distribution of lignin are important, as well as lignin content after pretreatment. Investigation of the effect of residual lignin on enzymatic hydrolysis will use a variety of analytical tools such as nuclear magnetic resonance (NMR), microscopy, and Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS).

Previous work determined the impact of lignin content on enzymatic hydrolysis for different wood species. Progress has been made in isolation of LCC fractions. LCC structures need to be quantified in these fractions using NMR. Activities in this research area included the following:

a) Develop novel methods for characterizing residual lignin and LCC.
b) Confirm and quantify LCC structures using NMR.
c) Improve isolation of LCC fractions.
d) Study the impact of lignin on enzymatic hydrolysis by regenerating wood and pulp samples to analyze the specific impact of the presence of lignin.
e) Study the impact of lignin distribution within the fiber on enzymatic hydrolysis. Preliminary indications are that lignin distribution on the fiber surface and within the secondary wall has a very significant impact on enzymatic hydrolysis.
f) Study the impact of lignin removal on cellulose crystallinity. It is expected that cellulose crystallinity is affected by the method of lignin removal and consequently enzymatic hydrolysis.

Enzymatic hydrolysis of Avicel

In this study, Avicel was used as a cellulose model to investigate the factors affecting the kinetics of enzymatic hydrolysis using the unique experimental design of multi-stage enzymatic hydrolysis. By comparing these processes, i.e. single- and multi-stage enzymatic hydrolysis and a multi-stage process with and without sugar addition, the effect of cellulose crystallinity might be revealed. Since the same enzyme-to-substrate ratio was used in every stage, the changes in substrate can also be monitored. This fundamental study and kinetics information will be useful to determine dominant rate-reducing factors,

which may provide opportunities to improve the current technology by minimizing the limiting factors for enzymatic hydrolysis.

The kinetics of single-stage enzymatic hydrolysis of Avicel went through a rapid hydrolysis rate in the first 12 h, as shown in Figure 5. The carbohydrate conversions in the first 12 h were 25.8% and 38.6% with an enzyme loading of 5 and 20 FPU/g, respectively. After that, the reaction rate decreased significantly. From 12 to 72 h, the carbohydrate conversions were 25.5% and 28.9% for an enzyme loading of 5 and 20 FPU/g, which were less than in the first 12 h, although the reaction time was five times longer. Nearly no increase of carbohydrate conversion was obtained from 72 h to 96 h. The overall 96 h carbohydrate conversions of Avicel were limited to 49.7% and 69.6% with 5 and 20 FPU/g, respectively.

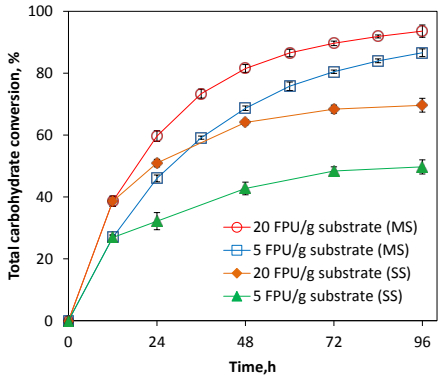


Figure 5. Carbohydrate conversion during single-stage (SS) and multi-stage (MS) enzymatic hydrolysis with an enzyme loading of 5 and 20 FPU/g substrate, respectively.

The reasons for the slower and more limited conversion of single-stage enzymatic hydrolysis can be originated from three aspects: enzyme deactivation, product inhibition, and the changes in substrate reactivity. This study was designed to evaluate these factors using multi-stage enzymatic hydrolysis as a process tool. The same ratio of fresh enzyme to substrate was applied in each stage to minimize the impact of enzyme deactivation and allow for an examination of the changes in Avicel reactivity. By conducting multi-stage enzymatic hydrolysis, a significant improvement in carbohydrate conversion was obtained within the same reaction time (96 h), compared to the single-stage (Figure 3). At the end of the 8th stage, carbohydrate conversion reached 86.6% and 93.6% with 5 and 20 FPU/g, respectively, which were 36.9% and 25.4% higher than single-stage hydrolysis. One of the advantages of multi-stage enzymatic hydrolysis is that it substantially reduces product inhibition because the enzymes and buffer were replaced in every 12 h (Yang et al., 2010). Therefore, the effect of product inhibition on a multi-stage process can be investigated by adding sugars along with fresh enzyme and buffer at the beginning of each stage, as demonstrated in the following section.

Effect of product inhibition on multi-stage enzymatic hydrolysis

Product inhibition is an important reason for the decreased rate of enzymatic hydrolysis (Converse et al., 1988; Qing et al., 2010; Smith et al., 2010). Sugar products combined with enzyme can inhibit the hydrolysis ability of an enzyme. A frequently used method for studying this phenomenon is to add sugars into substrate suspension at the beginning of the reaction (Lee & Fan, 1983; Qing et al., 2010). Another approach is to use ultrafiltration to remove sugars while keeping the enzyme in the reactor. However, it was observed that a substantial amount of enzyme leaked from the reactor (Converse et al., 1988). In order to study the impact of product inhibition on enzymatic hydrolysis without the interference of enzyme deactivation, glucose was added into the hydrolysis system along with fresh

enzyme and buffer in every stage. Using this approach, product inhibition at low and high enzyme loading was investigated.

Figure 6a shows that glucose-supplemented eight-stage enzymatic hydrolysis obtained about 20% lower carbohydrate conversion than regular multi-stage enzymatic hydrolysis at an enzyme loading of 5 FPU/g. However, there was no noticeable difference in the case of 20 FPU/g as shown in Figure 6b. The glucose concentrations at the end of the 8th stage were 40.9 g/l and 54.5 g/l with an enzyme loading of 5 and 20 FPU/g. These sugar concentrations can be considered high enough to cause product inhibition, but the higher glucose concentration had no impact on higher enzyme loading (20 FPU/g) in this study. These results implied that product inhibition had a significant impact at low enzyme loading, while this impact was marginal at high enzyme loading. A similar observation is supported by earlier work (Dekker et al., 1987; Hogan et al., 1990; Ramos et al., 1992). This phenomenon might be explained by the mechanism of competitive inhibition. At low enzyme loading, it is considered that a signification portion of enzymes was inhibited by forming an enzyme-inhibitor complex. Compared to the amount of uninhibited enzymes in multi-state enzymatic hydrolysis without glucose-supplemented process, less uninhibited enzymes can bind to substrate to form an enzyme-substrate complex (ES) when glucose was supplemented. At high enzyme loading, however, it was likely that there was still a substantial amount of enzymes that were not inhibited by sugars to form an ES. When there is an enough amount of ES in the multi-stage process, even with glucose addition in the case of 20 FPU/g, product inhibition can be overcome. Some earlier works have identified that the product inhibition in enzymatic hydrolysis of Avicel is competitive inhibition (Borchert & Buchholz, 1987; Ohmine et al., 1983). Besides, the differences between glucose-supplemented multi-stage enzymatic hydrolysis and single-stage enzymatic hydrolysis were largely due to the effect of enzyme deactivation on the hydrolysis process, which were 20.3% and 25.4% for enzyme loadings of 5 and 20 FPU/g, respectively, as shown in Figure 6.

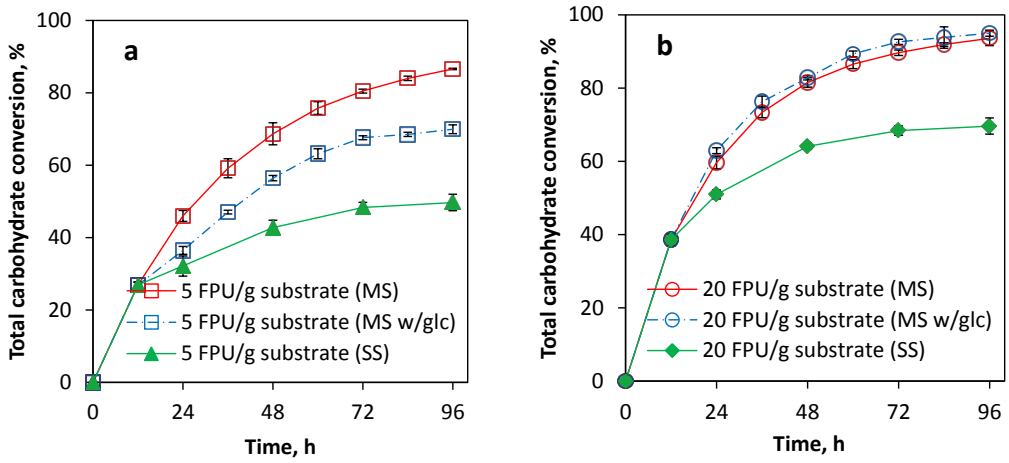


Figure 6. Comparison of glucose-supplemented eight-stage enzymatic hydrolysis, eight-stage, and single-stage enzymatic hydrolysis with an enzyme loading of (a) 5 FPU/g, and (b) 20 FPU/g.

It should be noted that determining a definite mechanism for product inhibition is a difficult task. Enzymatic hydrolysis usually takes place in a system containing multiple enzymes (exoglucanase, endoglucanase, and β-glucosidase) and multiple substrates (cellulose is first converted to oligosaccharides, then to monosaccharides), which requires many assumptions in order to make a model. In addition, the insolubility of the substrate is another concern. Michaelis-Menten equations are

valid for soluble substrate, but the applicability of this concept to insoluble substrates is not well understood. For insoluble substrates, the substrate architecture (e.g. surface area, pores, and smoothness) and crowding effect (e.g. substrate concentration gradient) can be very different for different substrates and the time of enzymatic hydrolysis. Some studies related to product inhibition of cellulose found that the inhibition pattern could be competitive, noncompetitive, or mixed, depending on the cellulase binding constant, enzyme concentration, maximum adsorption of the enzyme, substrate concentration, and β-glucosidase activity (Gusakov & Sinitsyn, 1992). For a more complicated substrate such as lignocellulosic substrate, noncompetitive inhibition has been reported (O'Dwyer et al., 2007).

Changes of substrate reactivity during multi-stage enzymatic hydrolysis

The change in substrate reactivity during enzymatic hydrolysis is described as the substrate transforming into a less digestible (or more recalcitrant) form as a function of time. These changes in substrate are considered to have a negative impact on the hydrolysis rate. In eight-stage enzymatic hydrolysis, it has been shown that the digestibility of Avicel linearly decreased in each stage (Figure 7). With an enzyme loading of 20 FPU/g, the carbohydrate conversion during the 1st stage was 38.6%, but it dropped to 30.6% during the 4th stage and to 21.2% in the 8th stage. An average decrease of 2.6% in carbohydrate conversion was found every 12 h. A similar trend was observed for the enzyme loading of 5 FPU/g with a 1.6% average decrease every stage. Generally, Avicel digestibility with an enzyme loading of 5 FPU/g decreased more slowly than that with 20 FPU/g. The 12 h carbohydrate conversion declined from 26.9% to 16.3% after the eight stages.

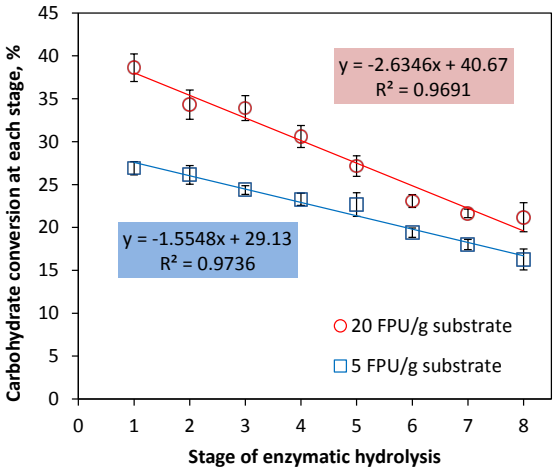


Figure 7. Changes in substrate reactivity during multi-stage enzymatic hydrolysis with an enzyme loading of 5 and 20 FPU/g, respectively.

The changes in substrate reactivity have been explained by the changes in crystallinity index, accessible surface area, chemical composition, and enzyme-substrate interaction (Arantes & Saddler, 2010; Jeoh et al., 2007; Lee & Fan, 1983; Sathitsuksanoh et al., 2011; Smith et al., 2010). Researchers have investigated the reactivity of Avicel during enzymatic hydrolysis. Two-stage enzymatic hydrolysis was conducted on Avicel PH-101 with complete removal of residual enzyme after the first stage, and no decline in the reaction rate was observed in the first hour of the second stage (Yang et al., 2006). However, it was found that the reactivity of Avicel PH-105 was significantly reduced due to the decrease of cellulose's accessibility to cellulase, which was measured by the adsorption of nonhydrolytic fusion protein (Hong et al., 2007). The structure of Avicel PH-101 is simple and chemically uniform because it has no extra cell wall components such as hemicellulose and lignin. The degree of polymerization and particle size of Avicel are not expected to increase, which should not negatively affect enzymatic hydrolysis (Hallac & Ragauskas, 2011; Hong et al., 2007; Yeh et al., 2010). For these reasons, the potential

increase of crystallinity and the presence of remaining adsorbed enzyme due to the limitation of mild washing are considered to be the major factors that affect the reactivity of Avicel in this study.

Changes in crystallinity during enzymatic hydrolysis

The crystallinity of untreated Avicel and hydrolysis residue from multi-stage enzymatic hydrolysis was measured by XRD. Figure 8 shows that the normalized XRD spectrum of untreated Avicel and extensively hydrolyzed Avicel in the 8th stage of hydrolysis with an enzyme loading of 5 and 20 FPU/g mostly overlap, which indicates that the crystalline structure of Avicel had marginal change even after extensive hydrolysis. Small decreases in the crystal face of $1\bar{1}0$ and 110 were observed in the hydrolyzed samples. The significance of this minor change is not clear. It has been reported that no significant change was observed in the degree of crystallinity during enzymatic hydrolysis of Avicel PH-101, up to 90% conversion (Hall et al., 2010). The invariant XRD spectra of untreated and enzymatically treated Avicel also demonstrated that the crystalline structure of Avicel remained nearly unchanged (Figure 8). A possible reason for the unchanged crystallinity could be that the structure of Avicel is highly crystalline and chemically uniform. However, one issue about the crystallinity measured by XRD is that it is a bulk measurement and measured in the dry state. For example, the drying process carried out before crystallinity measurement is known to change the structure of cellulose. This deficiency can be avoided by solid-state 13C nuclear magnetic resonance (NMR) measurement, because the analysis can be conducted in wet state. Although there is some evidence showing that NMR-measured crystallinity changes would be similar, the total crystallinity might differ (Park et al., 2009). An in situ analysis of crystalline structure under reacting conditions at the localized surface is not available (Barnette et al., 2011) and thus, whether the crystallinity has any significant changes in wet state during enzymatic hydrolysis needs to be further explored (Hall et al., 2010).

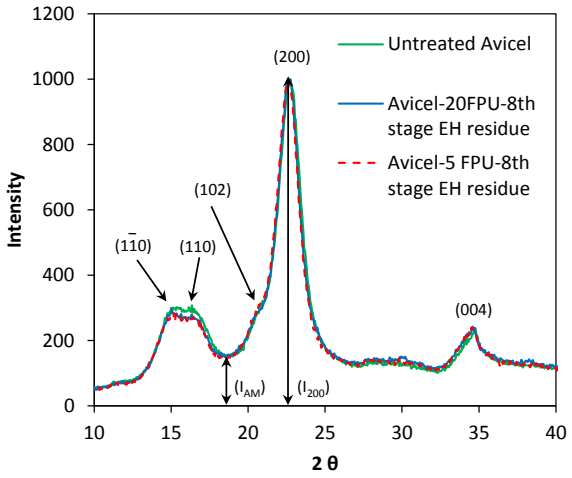


Figure 8. X-ray diffraction spectra of untreated Avicel PH-101 and substantially converted Avicel residues from the 8th stage of multi-stage enzymatic hydrolysis with an enzyme loading of 5 and 20 FPU/g, respectively.

Techniques for measuring lignin distribution

The presence of lignin in a cellulosic matrix reduces the efficiency of hydrolyzing enzymes by acting as a physical barrier to heterogeneous catalysis and also presenting a surface site for nonproductive adsorption. For a given substrate, removing incremental amounts of lignin will invariably increase the amount of monomeric sugar released into solution by a constant dose of enzymes. However, two different substrates, i.e., pretreated softwood and hardwood fibers, at the same bulk lignin content can show drastically different enzymatic hydrolysis efficiencies (Figure 9). The difference in hydrolysis

efficiencies is explained to a limited extent by morphological parameters such fiber dimensions, pore size, and initial surface area accessible to enzymes but in the limit that all lignin is removed from the substrate the enzymatic hydrolysis efficiencies tend to converge. A hypothesis is proposed that differences in hydrolysis efficiencies for substrates with identical bulk lignin contents can be explained by differences in lignin distribution throughout the substrate matrix.

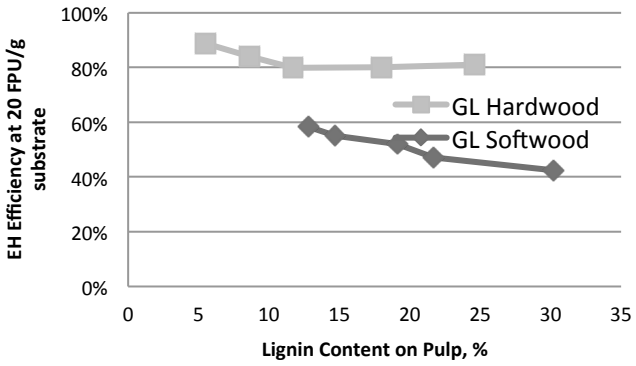


Figure 9. Enzymatic hydrolysis performance of green liquor pretreated hardwood and softwood samples at similar bulk lignin contents.

Three techniques have been investigated for measuring the distribution of lignin in a pretreated wood fiber. X-ray photoelectron spectroscopy (XPS) can be used to semi-quantitatively determine lignin content on the surface of fibers. This technique can only provide information about the top ~10 nm of the substrate and cannot provide information about the lignin distribution across the entire cell wall. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) has the potential to provide detailed spatial and chemical component information across the fiber cell cross section. This technique can potentially differentiate the spatial distribution of S and G lignin in hardwood fibers. However, this technique is still a developing science and is extremely sensitive to sample preparation and any surface contamination induced from preparing cross sections of the wood or fiber. The third analytical tool investigated for measuring lignin distribution is a confocal laser scanning microscope (CLSM). The CLSM can image fibers and produce a high-resolution three-dimensional image of where lignin is distributed in a fiber. Additionally, the lignin distribution across the cell wall can be accessed at multiple z-planes of the same fiber and multiple fibers can be examined relatively quickly compared to the analysis time required for XPS and ToF-SIMS. Figure 10 presents a three dimensional reconstruction of a pretreated softwood fiber analyzed using a CLSM. Figure 11 presents one particular z-plane of the fiber shown in Figure 10 and shows quantitative image analysis data for two separate directional scans across the fiber cell wall.

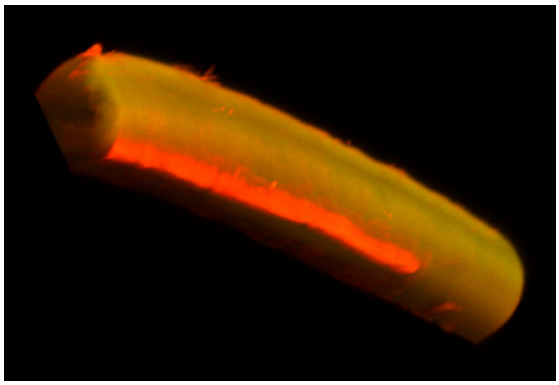


Figure 10. Three-dimensional reconstruction of z-stack CLSM images of a green liquor and oxygen pretreated softwood tracheid.

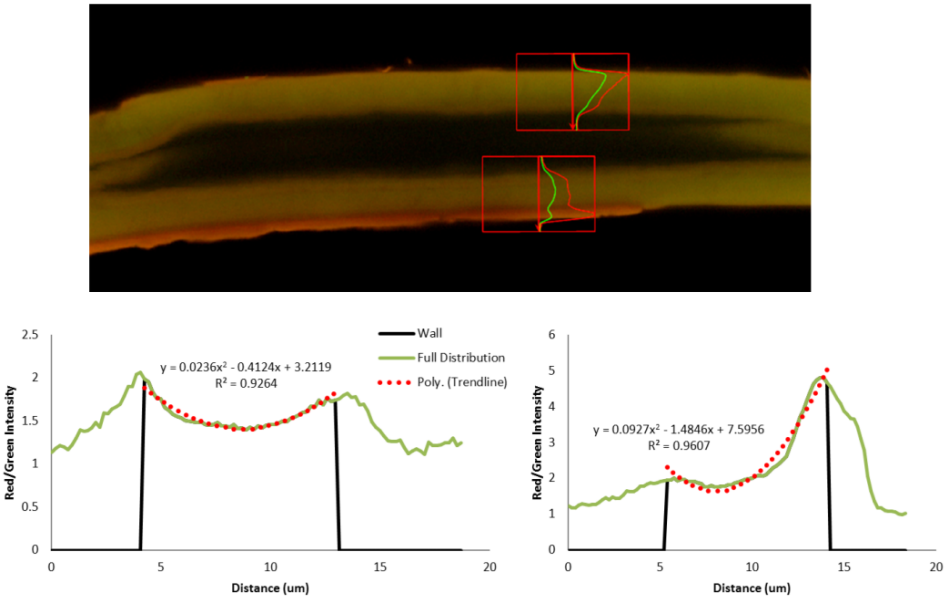


Figure 11. Cell wall lignin distribution analysis of a green liquor and oxygen pretreated softwood tracheid analyzed via CLSM.

The techniques discussed have their advantages and disadvantages when trying to measure the lignin distribution in a lignocellulosic substrate. The most promising technique for quantifying lignin distribution is the CLSM for the following reasons: 1) sample preparation is simpler and much faster than preparation for XPS or ToF-SIMS, 2) a three dimensional image of a fiber can be acquired in a matter of minutes, making it possible to sample multiple fibers and produce statistically sound results in a relatively short period of time; XPS and ToF-SIMS require orders of magnitude more analysis time. The next steps of the project will involve analyzing an array of softwood samples that have been pretreated using different techniques to remove lignin. Using different techniques for lignin removal can produce substrates with the same bulk lignin content but they will likely have different lignin distributions because the enzymatic hydrolysis performance of these substrates is drastically different. The ultimate goal is to gain a better understanding of how lignin distribution impacts the ability of hydrolyzing enzymes to break down carbohydrates into simple sugars.

Quantification of lignin-carbohydrate linkages with high-resolution NMR spectroscopy

A quantitative approach to characterize lignin–carbohydrate complex (LCC) linkages using a combination of quantitative 13 C NMR and HSQC 2D NMR techniques has been developed. Crude milled wood lignin (MWLc), LCC extracted from MWLc with acetic acid (LCC-AcOH) and cellulolytic enzyme lignin (CEL) preparations were isolated from loblolly pine (*Pinus taeda*) and white birch (*Betula pendula*) woods and characterized using this methodology on a routine 300 MHz NMR spectrometer and on a 950 MHz spectrometer equipped with a cryogenic probe. Structural variations in the pine and birch LCC preparations of different types (MWL, CEL and LCCAcOH) were elucidated. The use of the high field NMR spectrometer equipped with the cryogenic probe resulted in a remarkable improvement in the resolution of the LCC signals and, therefore, is of primary importance for an accurate quantification of LCC linkages. The preparations investigated showed the presence of different amounts of benzyl ether, c-ester and phenyl glycoside LCC bonds. Benzyl ester moieties were not detected. Pine LCC-AcOH and birch MWLc preparations were preferable for the analysis of phenyl glycoside and ester LCC linkages

in pine and birch, correspondingly, whereas CEL preparations were the best to study benzyl ether LCC structures. The data obtained indicate that pinewood contains higher amounts of benzyl ether LCC linkages, but lower amounts of phenyl glycoside and c -ester LCC moieties as compared to birch wood.
From: Balakshin, M., E. Capanema, H. Gracz, H. Chang, and H. Jameel. 2011. Quantification of lignin-carbohydrate linkages with high-resolution NMR spectroscopy. Planta 233:1097-1110.

Dilute sulfuric acid pretreatment of transgenic switchgrass

Conventional Alamo switchgrass and its transgenic counterparts with reduced/modified lignin were subjected to dilute sulfuric acid pretreatment for improved sugar production. At 150 °C, the effects of acid concentration (0.75%, 1%, 1.25%) and residence time (5, 10, 20, 30 min) on sugar productions in pretreatment and enzymatic hydrolysis were investigated, with the optimal pretreatment conditions determined for each switchgrass genotype based on total sugar yield and the amounts of sugar degradation products generated during the pretreatment. The results show that genetic engineering, although did not cause an appreciable lignin reduction, resulted in a substantial increase in the ratio of acid soluble lignin:acid insoluble lignin, which led to considerably increased sugar productions in both pretreatment and enzymatic hydrolysis. At an elevated threshold concentration of combined 5-hydroxyfuranmethal and furfural (2.0 g/L), the overall carbohydrate conversions of conventional switchgrass and its transgenic counterparts, 10/9-40 and 11/5-47, reached 75.9%, 82.6%, and 82.2%, respectively.
From: Zhou, X., J. Xu, Z. Wang, J.J. Cheng, R. Li, and R. Qu. 2012. Dilute sulfuric acid pretreatment of transgenic switchgrass for sugar production. Bioresource Technology 104(1): 823-827.

Chemical recovery cycles in cellulosic ethanol processes using alkaline pretreatment

The team at NCSU has developed a great deal of experience and some intellectual property in the area of alkaline pretreatments and associated chemical recovery systems. IBRI research emphasis involved the evaluation of novel alkaline pretreatment technology on the feedstocks of interest to this project, loblolly pine and switchgrass. Research techniques included alkaline pretreatment of transgenic switchgrass in order to improve sugar production from lignocellulosic biomass. Focus was on investigation of the impact of specific alkaline pretreatments on fiber structure, chemical composition, and lignin structure.

IBRI research activity during Phase 2 focused on:

a) Investigation of the fundamental mechanism of alkaline pretreatment of transgenic switchgrass with lower lignin for improved sugar yield using the combination of sodium hydroxide and lime at low temperature.
b) Investigation of the main obstacles to high sugar conversion from ionic liquid-pretreated switchgrass.

Sodium hydroxide pretreatment of genetically modified switchgrass

Overcoming biomass recalcitrance to bioconversion is crucial for cellulosic biofuels commercialization. In this study, Alamo switchgrass (*Panicum virgatum* L.) was genetically transformed to suppress the expression of 4-coumarate-CoA ligase (4CL). The transgenic plants were determined to have lignin content reductions of up to 5.8%. The ratios of acid soluble lignin (ASL) to acid insoluble lignin (AIL) and syringyl/guaiacyl (S/G) in transgenic plants were 21.4–64.3% and 11.8–164.5%, respectively, higher than those of conventional biomass. Both conventional and transgenic plants were pretreated with 0.5%, 1%, and 2% (w/v) NaOH for 15, 30, and 60 min at 121 °C, followed by enzymatic hydrolysis with commercial cellulases and xylanases. At the optimal conditions, the glucan and xylan conversion efficiency in the best transgenic plants were 16% and 18% higher than the conventional

plant, respectively. The results show that down-regulation of 4CL gene promoted enzymatic hydrolysis of plant cell walls following a mild alkali pretreatment.
From: Wang, Z., R. Li, J. Xu, J.M. Marita, R.D. Hatfield, R. Qu, and J.J. Cheng. 2012. Sodium hydroxide pretreatment of genetically modified switchgrass. Bioresource Technology 110(4): 364-370.

Low temperature alkali pretreatment of transgenic switchgrass

Genetically modified switchgrass (cv. Alamo) and its conventional plant were both pretreated using two groups of conditions: lime at 50 °C and the combination of lime and NaOH at ambient temperature. The results show that the transgenic plant (with altered lignin content and composition) was more susceptible to alkali pretreatment than the conventional plant. At the recommended conditions (0.1 g/g of raw biomass and 12 h) for lime pretreatment at 50 °C, the glucan and xylan conversions of transgenic switchgrass were 12 and 10%, respectively, higher than those of the conventional plant. These increases were reduced to 7 and 8% for glucan and xylan conversions, respectively, when the best conditions (0.025 g of lime/g of raw biomass, 0.1 g of NaOH/g of raw biomass, and 6 h) for combined alkali pretreatment at ambient temperature were employed. The advantage of transgenics over a conventional plant in sugar production could be maximized if proper pretreatment conditions were used.
From: Wang, Z., J. Xu, P. Pandey, J.J. Cheng, R. Li, and R. Qu. 2012. Improvement of sugar production from transgenic switchgrass with low temperature alkali pretreatment. Energy & Fuels 26(5): 3054-3061.

Switchgrass-derived black liquor pretreatment of corn stover

To improve the cost-effectiveness of biomass-to-sugar conversion, sodium hydroxide (NaOH) pretreatment of switchgrass was carried out at 21 °C using previously determined optimum conditions (2% NaOH (w/v), 6 h), and the spent alkaline liquid (black liquor) was collected and used for pretreatment of corn stover, a feedstock exhibiting a higher susceptibility to NaOH attack, for improved enzymatic hydrolysis at a reduced cost. The results showed that, because of the high pH and the appreciable amount of carbohydrates in the black liquor, sugar production during enzymatic hydrolysis of corn stover pretreated with black liquor was comparable to that of biomass pretreated with 1% NaOH. After black liquor pretreatment at the best residence time (24 h), the total reducing sugar, glucose, and xylose yields of corn stover reached 478.5, 287.7, and 145.3 mg/g raw biomass, respectively, indicating the viability of this novel pretreatment technology.
From: Xu, J., X. Zhang, and J.J. Cheng. 2012. Pretreatment of corn stover for sugar production with switchgrass-derived black liquor. Bioresource Technology 111(5): 255-260.

Modeling biochemical conversion of lignocellulosic materials

To deeply understand the factors that affect the conversion of lignocellulosic biomass to fermentable sugars, experimental results should be bridged with process simulations. The objective of this paper is to review published research on modeling of the pretreatment process using leading technologies such as dilute acid, alkaline, and steam explosion pretreatment, as well as the enzymatic hydrolysis process for converting lignocellulose to sugars. The most commonly developed models for the pretreatment are kinetic models with assumptions of a first-order dependence of reaction rate on biomass components and an Arrhenius-type correlation between rate constant and temperature. In view of the heterogeneous nature of the reactions involved in the pretreatment, the uses of severity factor, artificial neural network, and fuzzy inference systems present alternative approaches for predicting the behavior of the systems. Kinetics of the enzymatic hydrolysis of cellulosic biomass has been simulated using various modeling approaches, among which the models developed based on Langmuir-type adsorption mechanism and the modified Michaelis-Menten models that incorporate

appropriate rate-limiting factors have the most potential. Factors including substrate reactivity, enzyme activity and accessibility, irreversible binding of enzymes to lignin, and enzyme deactivation at high conversion levels, need to be considered in modeling the hydrolysis process. Future prospects for research should focus on thorough understanding of the interactions between biomass reactants and chemicals/enzymes — the key to developing sophisticated models for the entire conversion process.
From: Wang, Z., J. Xu, and J.J. Cheng. 2011. Modeling biochemical conversion of lignocellulosic materials for sugar production: a review. BioResources 6(4): 5282-5306.

Cellulose conversion with alkaline extraction/ionic liquid dissolution

In previous work, we pretreated corn stover (CS) by combining alkaline extraction (AE) with ionic liquid (IL) dissolution and a high enzymatic hydrolysis rate; total sugar conversion was determined. When the same pretreatment conditions were used for the pretreatment of switchgrass (SG), however, the total sugar conversion was lower than that from corn stover at even longer hydrolysis time. Research is planned to investigate if the AE/IL process can be broadly applied to overcome the property differences of various biomass sources to achieve a comparable hydrolysis rate and sugar conversion.

In this research, 80% of the cellulose in switchgrass was converted to glucose in 24 hours using a new pretreatment methodology. Alkali extraction (AE) of raw biomass, i.e., switchgrass (SG), is followed by a dissolution step using an ionic liquid (IL) as a solvent. The IL is able to readily dissolve the polysaccharides (cellulose and hemicellulose) and, in some cases, significant amounts of lignin. The polysaccharides are recovered by a regeneration step in which a nonsolvent (i.e., water) is added to the IL-switchgrass mixture causing the polysaccharides (and, in some cases, other biomass components) to precipitate out of solution. The IL and other biomass components are left in the residual solution.

To achieve our target of the conversion of 90% of the cellulose in switchgrass to glucose in 24 hrs using this process, we have explored what role the IL dissolution plays in this combination process. To do this, untreated switchgrass was pretreated with the 4 ILs (Fig. 12) and the pretreated switchgrass was then enzymatically hydrolyzed (note: no alkali extraction was performed for this study to examine the influence of the IL alone). The untreated SG was dissolved in the ILs at 100ºC. As shown in Fig. 12, the mixtures with the acetate ILs, [C2mim]Ac and [C4mim]Ac, were darker than those with the chloride ILs, [C2mim]Cl and [C4mim]Cl. This is attributed to the greater solubility of lignin (resulting in a darker discoloration) in the acetate ILs than in the chloride ILs. This improved solubility of lignin also significantly enhanced the rate of the switchgrass dissolution process for the acetate ILs relative to that of the chloride ILs.

To determine the lignin content in the samples, untreated SG (particle size 2 mm) was first extracted with benzene-ethanol to remove soluble extractives. The solid yield after extraction was 95.72% and the extractive-free switchgrass (EF-SG) was analyzed for its chemical composition and used in the following experiments.

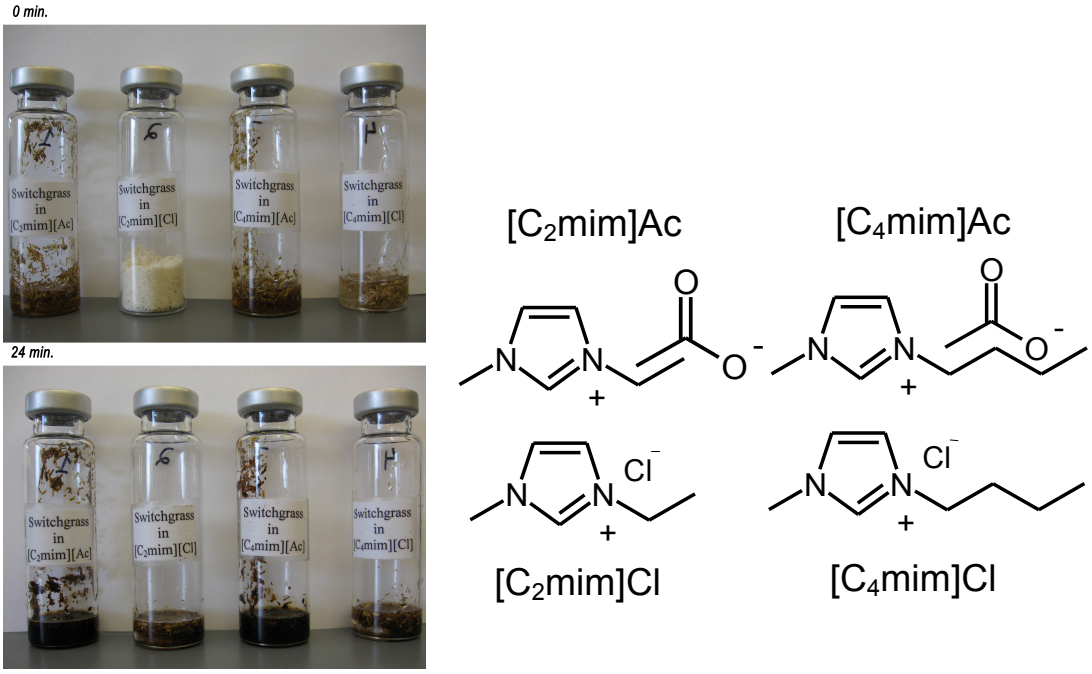


Figure 12. Dissolution at 100°C of SG in the four ILs studied (dissolution time shown) and structures of the ions.

For the EF-SG, the acetate ILs extracted more lignin than the chloride ILs (more lignin remained in the residual liquid rather than in the regenerated solids). The wash solutions for all of the ILs, however, had a similar low carbohydrate content (Table 4). Thus, the acetate ILs are able to fractionate SG to a greater extent than the chloride ILs. This fractionation is confirmed by the compositional analysis of the regenerated solids (Table 5).

Table 4. Analysis of the liquid wash after regeneration of IL-dissolved SG.

	[C_2mim]Ac	[C_4mim]Ac	[C_2mim]Cl	[C_4mim]Cl
Yield (%)	83.72	84.51	91.31	90.93
Lignin (%)	11.52	10.64	4.99	4.47
Total sugar (%)	3.44	3.31	3.02	2.01

Table 5. Main components of the recovered SG after IL dissolution.

	EF-SG	[C_2mim]Ac	[C_4mim]Ac	[C_2mim]Cl	[C_4mim]Cl
Lignin (%)	20.07	15.00	16.93	21.44	22.38
Cellulose (%)	37.93	40.29	42.22	42.63	43.00
Xylan (%)	25.21	25.05	27.08	28.57	28.86

The recovered SG from the IL dissolution was then enzymatically hydrolyzed. The cellulose conversion is displayed in Figure 13. The two acetate ILs resulted in a much higher cellulose conversion than the two chloride ILs. This is attributed to the fact that the recovered SG samples from the acetate ILs dissolution contained a much lower lignin content (15-16%) than those from the chloride ILs (21-22%). This implies that the lignin content is a crucial influencing factor for the enzymatic hydrolysis under these conditions.

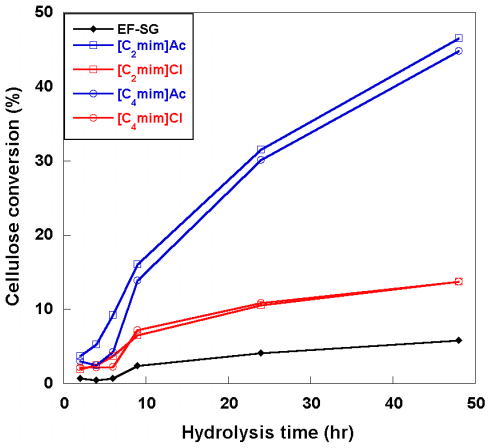


Figure 13. Enzymatic hydrolysis of IL-dissolved extractive-free switchgrass.

Complementary biomass pretreatment and reactivity research by industry

Industrial partner Novozymes North America in Franklinton, NC conducted research in parallel and in cooperation with research tasks at NCSU. Activities included:

a. Complement work by NCSU to determine the effect of residual lignin on enzymatic hydrolysis by preparing pretreated samples of non-woody biomass. Conduct verification tests to determine hydrolysis performance of pretreated samples in order to correlate hydrolysis performance with analytical characterization of lignin by NCSU.

b. Complement work by NCSU to determine the effect of residual hemicellulose on enzymatic hydrolysis by preparing pretreated samples of non-woody biomass. Conduct verification tests to determine hydrolysis performance of pretreated samples in order to correlate hydrolysis performance with analytical characterization of hemicellulose by NCSU.

c. Complement work by NCSU to determine the effect of cellulose crystallinity on enzymatic hydrolysis by conducting verification tests to determine hydrolysis performance of pretreated samples in order to correlate hydrolysis performance with analytical characterization of cellulose crystallinity by NCSU.

d. Continue optimization of pretreatment and hydrolysis processes on representative feedstocks (com stover, switchgrass, and woody biomass) using both alkaline and acid pretreatments.

Summary of the complementary work is provided below in the form of abstracts from two journal articles produced by Novozymes scientists.

In this study, raw corn stover was subjected to dilute acid pretreatments over a range of severities under conditions similar to those identified by the National Renewable Energy Laboratory (NREL) in their technoeconomic analysis of biochemical conversion of corn stover to ethanol. The pretreated corn stover then underwent enzymatic hydrolysis with yields above 70% at moderate enzyme loading conditions. The enzyme exhausted lignin residues were characterized by 31 P NMR spectroscopy and functional moieties quantified and correlated to enzymatic hydrolysis yields. Results from this study indicated that both xylan solubilization and lignin degradation are important for improving the enzyme accessibility and digestibility of dilute acid pretreated corn stover. At lower pretreatment temperatures, there is a good correlation between xylan solubilization and cellulose accessibility. At higher pretreatment temperatures, lignin degradation correlated better with cellulose accessibility, represented by the increase in phenolic groups. During acid pretreatment, the ratio of syringyl/guaiacyl functional groups also gradually changed from less than 1 to greater than 1 with the increase in

pretreatment temperature. This implies that more syringyl units are released from lignin depolymerization of aryl ether linkages than guaiacyl units. The condensed phenolic units are also correlated with the increase in pretreatment temperature up to 180° C, beyond which point condensation reactions may overtake the hydrolysis of aryl ether linkages as the dominant reactions of lignin, thus leading to decreased cellulose accessibility.
From: Moxley, G., A.R. Gaspar, D. Higgins, and H. Xu. 2012. Structural changes of corn stover lignin during acid pretreatment. J Ind Microbiol Biotechnol 39:1289–1299.

Dilute acid pretreatment is a leading pretreatment technology for biomass to ethanol conversion due to the comparatively low chemical cost and effective hemicellulose solubilization. The conventional dilute acid pretreatment processes use relatively large quantities of sulfuric acid and require alkali for pH adjustment afterwards. Significant amounts of sulfate salts are generated as by-products, which have to be properly treated before disposal. Wastewater treatment is an expensive, yet indispensable part of commercial level biomass-to-ethanol plants. Therefore, reducing acid use to the lowest level possible would be of great interest to the emerging biomass-to-ethanol industry. In this study, a dilute acid pretreatment process was developed for the pretreatment of corn stover. The pretreatment was conducted at lower acid levels than the conventional process reported in the literature while using longer residence times. The study indicates that a 50% reduction in acid consumption can be achieved without compromising pretreatment efficiency when the pretreatment time was extended from 1–5 min to 15–20 min. To avoid undesirable sugar degradation and inhibitor generation, temperatures should be controlled below 170° C. When the sulfuric acid-to-lignocellulosic biomass ratio was kept at 0.025 g acid/g dry biomass, a cellulose-to-glucose conversion of 72.7% can be achieved at an enzyme loading of 0.016 g/g corn stover. It was also found that acid loading based on total solids (g acid/g dry biomass) governs the pretreatment efficiency rather than the acid concentration (g acid/g pretreatment liquid). While the acid loading on lignocellulosic biomass may be achieved through various combinations of solids loading and acid concentration in the pretreatment step, this work shows that it is unlikely to reduce acid use without undermining pretreatment efficiency simply by increasing the solid content in pretreatment reactors, therefore acid loading on biomass is indicated to be the key factor in effective dilute acid pretreatment.
From: Chen, Y., M.A. Stevens, Y. Zhu, J. Holmes, G. Moxley, and H. Xu. Reducing acid in dilute acid pretreatment and the impact on enzymatic saccharification. J Ind Microbiol Biotechnol 39:691–700.

Design of superior hydrolytic enzymes for conversion of lignocellulosic materials to ethanol

A novel analytical tool was developed and employed to investigate the relationship between biomass substrate and enzymatic hydrolysis. Modified hydrolytic enzymes (hydrolases), such as cellulase(s), β-glucosidase(s), xylanase(s) were prepared using immobilized proteases, to generate active hydrolases that exhibit a lower molecular weight. Potentially, smaller enzyme fractions will display increased accessibility to biomass substrates for improved catalytic generation of sugars used in the subsequent production of ethanol. These modified hydrolytic enzymes will be compared to traditional commercial products to examine the conversion of lignocellulosic starting materials (switch grass/loblolly pine) to sugars. Control/modified enzyme will be quantified using enzyme linked immune-sorbent (ELISA) assays, a new experimental approach for investigating effects of residual hemicellulose, lignin, and LCC on enzymatic hydrolysis.

Design of protease modified cellulolytic enzymes with high catalytic functionality

Hydrolytic enzymes, such as cellulase and β-glucosidase, are commonly used in the biofuels industry. This work characterizes the residual functionality after creating *modified* cellulase (*m*cellulases)

and β-glucosidase (*m*βGSDs) truncated variants, using three different immobilized proteases, namely trypsin, chymotrypsin, and amino-peptidase. The experimental goal was focused on generating multiple, smaller sized *m*cellulase/*m*βGSD molecular species, such that high catalytic capabilities were retained. After cleavage, (*i*) the degree of hydrolysis was determined using OPA assay methods, (*ii*) SDS-PAGE protein banding patterns were visualized, and (*iii*) residual cellulase and βGSD enzymatic activities were measured using filter paper and cellobiose as substrates, respectively. The method for generating functional *m*cellulase/*m*βGSD prototypes was optimized for these experimental conditions. This study describes a simple model system for creating *m*cellulase/*m*βGSD hydrolase fractions with potential for improved catalytic efficiencies due to increased accessibility to recalcitrant biomass substrates. Ultimately, *modified* enzyme preparations will be tested in practical "real world" applications using switch grass and/or woody biomaterials.

Utilization of protease modified hydrolytic enzymes for sugar production from conventional switchgrass

Hydrolytic enzymes, such as cellulases and β-glucosidase, are commonly used in the biofuels industry. The experimental goal for this study was focused on generating multiple, smaller-sized cellulase/βGSD molecular variants with aminopeptidase, such that high catalytic capabilities were retained. After cleavage, (*i*) the degree of hydrolysis was determined using OPA assay methods, (*ii*) SDS-PAGE protein banding patterns were visualized, and (*iii*) residual cellulase and βGSD enzymatic activities were measured after incubation with peptidase based on filter paper and cellobiase assays, respectively. (*iv*) Truncated enzyme forms were then utilized for hydrolysis of alkaline-pretreated switchgrass. The catalytic efficiency of this reaction, defined as mg sugar produced per unit of enzyme, was increased when peptidase-modified cellulase forms were used to degrade the grass, especially in the absence of sodium azide. Conversely, peptidase treatment of β-glucosidase showed little effect using this bioprocessing scheme. The role of native protease activity in this system was identified as well. Taken together, native/exogenous proteolysis appeared to exhibit synergistic effects, in that equivalent sugar outputs were achieved at lower catalytic levels.

Equipment for Cellulosic Ethanol Screening Facility

Process and analytical equipment were planned for purchase in support of a cellulosic ethanol screening facility being developed at NCSU with third-party support. The equipment as part of the pilot plant will be used to develop new technology, integrate operations, optimize system performance, and demonstrate the feasibility of producing ethanol and other bioproducts from native biomass resources, including those from forestry, agricultural, and industry sources. The facility will facilitate potential scale-up of bench-scale research processes performed by IBRI researchers. The projected timeline for completion of the pilot facility was extended considerably from the original timeline because of constraints on the third-party funding; therefore the emphasis for this project has been primarily on analytical equipment in support of the pilot facility, to be housed primarily in the Biofuels Analytical Laboratory, a shared-use analytical facility. Commissioning of the biomass-to-bioproducts pilot facility is now scheduled for fall 2013.

Development of Biofuels Analytical Laboratory

Installation, operation, and maintenance of equipment, and development of analytical protocols were accomplished as part of the development of a Biofuels Analytical Laboratory. The laboratory has operated as a shared analytical facility in support of research by IBRI investigators. The laboratory will continue to operate as a resource for research and development in biomass, bioenergy, and bioproducts at NCSU (http://www.ibri.ncsu.edu/facilities). Equipment currently installed includes:

- IC chromatograph (Dionex ICS-5000 anion exchange chromatographic dual system coupled with electrochemical detectors)
- HPLC (Dionex U-3000 dual system with refractive index detectors)
- NIR Rapid content analyzer (Foss XDS with capabilities to analyze powders, slurries and coarse materials. WinISI software enables multivariant data analysis)
- BET Analyzer (Gemini VII 2390p physiosorption analyzer from Micromeritics coupled with SmartPrep 065 degassing unit)
- GC X GC – TOFMS (LECO Pegasus 4D system with two GC columns in series connected to time-of-flight mass spectrometer)
- Pyroprobe (CDS Pyroprobe 5200 for pyrolysis at temperatures up to 1400 ^{o}C)
- TGA/DSC analyzer (Q600 from TA instruments provides simultaneous measurement of weight change and true differential heat flow during pyrolysis)
- UV-Vis Analyzer (Shimadzu UV-1800 spectrophotometer)
- Raman Analyzer (Kosi RXN-Raman spectrophotometer)
- Autoclaves and a biosafety hood
- Incubator shakers, centrifuges (Beckmann Allegra 25R), analytical balances, soxhlet extraction units, and other assorted supporting laboratory equipment
- Pyrolysis reactor (Hanwoul Engineering)
- Chemisorption analyzer (Micromeritics)

Novel Energy Crops for Biofuels Production

Supplying biorefineries with adequate biomass to support high levels of production requires the development of (1) local/regional models for energy crop cultivation, harvest and transport, (2) novel uses for existing crops, possibly through redirection of metabolic activity, and (3) conversion of inexpensive "waste" matter to materials more amenable to processing into fuels and products. The proposed research focused on understanding production practices and metabolic activities that lead to sustainable high yielding energy crop rotations and productive strategies for recycling organic material into energy crops.

Southeastern energy crop field trials

Key issues that need to be addressed for canola cultivation include: suitable varieties and nutrient regimes to maximize yields; how canola grows and develops under southeastern conditions in order to determine how best to manage the crop and incorporate it into existing agricultural rotations; as well as pest and disease risks and suitable management methods.

2010 National Winter Canola Variety Trial

Winter canola production is a good fit for small-grains cropping systems because both use the same equipment. Wheat crops following canola have also shown a 10 percent or greater increase in yield compared with continuous wheat. Canola is a broadleaf crop, which allows use of more effective herbicides to control grassy winter annual weeds. Canola and wheat have no major diseases in common. Growing canola in rotation with wheat breaks the hard-to-control weed and disease cycles of wheat monoculture systems. Because canola is an oilseed, its commodity price is not tied to prices of cereal grains, which spreads economic risk over more than one commodity class.

Objectives of the National Winter Canola Variety Trial (NWCVT) are to evaluate the performance of released and experimental varieties, determine where these varieties are best adapted, and increase visibility of winter canola across the nation. Breeders, marketers, and producers use information

collected from the trials. The number of environments and entries tested have increased over the past decade. The NWCVT is planted at locations in the Great Plains, Midwest, northern United States, and Southeast. The wide diversity of environments has improved our knowledge and understanding of winter canola variety performance.

Several varieties of winter canola were grown in field trials and evaluated at two locations in North Carolina; results were contributed to the national variety trial.

Table 6. Results for the 2010 National Winter Canola Variety Trial at Jackson Springs, NC

Name	Yield (lb/a)			Yield (% of test avg.)	Winter Survival (%)			Plant Height	Moisture	Test Weight	Protein	Oil
	2010	2009	2-Yr.	2010	2010	2009	2-Yr.	(in.)	(%)	(lb/bu)	(%)	(%)
Croplan Genetics												
Hyclass154W	2140	---	---	98	---	---	---	47	---	---	25.2	41.8
DL Seeds Inc.												
Baldur	2101	---	---	96	---	---	---	45	---	---	24.6	42.7
Dimension	2371	---	---	109	---	---	---	49	---	---	23.4	44.9
Dynastie	2171	---	---	100	---	---	---	44	---	---	23.4	42.6
Flash	2369	---	---	109	---	---	---	48	---	---	25.3	42.0
Safran	2282	---	---	105	---	---	---	46	---	---	25.9	40.5
Sitro	2457	---	---	113	---	---	---	51	---	---	24.0	43.1
Visby	2566	---	---	118	---	---	---	48	---	---	25.0	41.6
Kansas State University												
Wichita	2203	---	---	101	---	---	---	44	---	---	25.9	41.8
MOMONT												
Hybristar	2254	---	---	103	---	---	---	47	---	---	24.4	43.8
Hybrisurf	2528	---	---	116	---	---	---	46	---	---	24.0	44.2
Kadore	1447	---	---	66	---	---	---	39	---	---	25.4	39.9
University of Arkansas												
ARC00005-2	2063	---	---	95	---	---	---	42	---	---	24.8	42.4
ARC00024-2	2030	---	---	93	---	---	---	43	---	---	25.3	42.0
ARC2189-2	1789	---	---	82	---	---	---	44	---	---	26.1	41.2
ARC99009-1	1930	---	---	89	---	---	---	44	---	---	25.7	41.9
Mean	2180	---	---	---	---	---	---	46	---	---	24.9	42.3
CV	18	---	---	---	---	---	---	9	---	---	4.4	3.0
LSD (0.05)	NS	---	---	---	---	---	---	NS	---	---	NS	NS

Bold - Superior LSD Group - Unless two entries differ by more than the LSD, little confidence can be placed in one being superior to the other.

Table 7. Results for the 2010 National Winter Canola Variety Trial at Williamsdale, NC.

Name	Yield (lb/a)			Yield (% of test avg.)	Winter Survival (%)			Plant Height	Moisture	Test Weight	Protein	Oil
	2010	2009	2-Yr.	2010	2010	2009	2-Yr.	(in.)	(%)	(lb/bu)	(%)	(%)
Croplan Genetics												
HyClass154W	1705	---	---	94	---	---	---	42	---	---	27.8	40.4
DL Seeds Inc.												
Baldur	1663	---	---	91	---	---	---	41	---	---	26.3	**41.9**
Dimension	2295	---	---	126	---	---	---	49	---	---	26.0	**43.2**
Dynastie	1840	---	---	101	---	---	---	41	---	---	25.5	**42.7**
Flash	1330	---	---	73	---	---	---	45	---	---	26.9	**41.8**
Safran	1814	---	---	100	---	---	---	43	---	---	26.2	**41.9**
Sitro	2163	---	---	119	---	---	---	46	---	---	26.5	41.4
Visby	2301	---	---	126	---	---	---	46	---	---	26.9	41.1
Kansas State University												
Wichita	1822	---	---	100	---	---	---	43	---	---	28.0	41.3
MOMONT												
Hybristar	1701	---	---	94	---	---	---	45	---	---	26.9	**42.2**
Hybrisurf	1704	---	---	94	---	---	---	44	---	---	26.3	**42.5**
Kadore	1636	---	---	90	---	---	---	39	---	---	26.3	41.2
University of Arkansas												
ARC00005-2	1780	---	---	98	---	---	---	44	---	---	27.5	41.0
ARC00024-2	1836	---	---	101	---	---	---	51	---	---	27.6	40.4
ARC2189-2	1860	---	---	102	---	---	---	50	---	---	27.4	40.2
ARC99009-1	1784	---	---	98	---	---	---	41	---	---	26.8	**42.0**
Mean	1819	---	---	---	---	---	---	44	---	---	26.8	41.6
CV	19	---	---	---	---	---	---	12	---	---	2.8	1.8
LSD (0.05)	NS	---	---	---	---	---	---	NS	---	---	NS	1.6

Bold - Superior LSD Group - Unless two entries differ by more than the LSD, little confidence can be placed in one being superior to the other.

From: 2010 National Winter Canola Variety Trial, Kansas State University, January 2011. Contribution no. 10-165-T from the Kansas Agricultural Experiment Station.

Small-scale canola storage and processing in North Carolina

The objective of this effort was to assess the methods and feasibility of small-scale canola storage and processing. Data collected from our storage and processing setup at the Lake Wheeler Research Station, Raleigh, NC and from other sources have been compiled to determine the feasibility of operating a small-scale canola processing business for biodiesel and animal feed markets. The steps from seed to oil include: seed cleaning, seed storage, oilseed pressing, and oil filtering and degumming. The economic feasibility component examines two oilseed processing capacities and the associated cost and revenue estimates.

Two different production scales (based on farm acreage) were chosen to demonstrate the costs and returns of storage and processing of canola. Both of the scenarios were based on the assumption that a farmer produces and processes the canola crop on the farm and then sells the crude oil and the meal. The information provided came from oilseed processors and biodiesel producers in the region and from our experience.

The smallest scale system represents a 20-acre farm; the larger scale system represents a 126-acre farm. These are merely points of reference given the range of scenarios. Stakeholders should use this information as a decision-making tool to determine the feasibility of storage and processing based on their situation.

There are several essential points to highlight before proceeding. First, based on the average market value for crude soybean oil (about $.42/lb -$.48/lb) the net cost of crude canola oil should not exceed

$3.20/gallon ($.42/lb) to have competitive pricing. This is essentially the break-even cost. In addition, the net cost includes returns from meal sales, which are crucial to economic viability.

The third point is that canola production costs comprise a high proportion of overall costs. We have included an estimate of $230/acre based on a modified enterprise budget estimate provided in the North Carolina Winter Canola Production Guide (George et al., 2009). Economic viability of small-scale production and processing is largely determined by production costs. Farmers should work out an enterprise budget and have a good idea of yield expectations to properly assess their situation.
From: George N, Tungate K, Hobbs A, Atkinson A. 2008. The North Carolina Canola Production Guide (revised). Golden LEAF Foundation and North Carolina Solar Center, North Carolina State University.

Enzymatically enhanced industrial-type sweetpotatoes for biofuel production

This project involves continued research towards the development of enzymatically enhanced industrial-type sweetpotatoes for use in the production of bio-fuels. Our initial efforts have focused upon the development of storage root specific promoters and plant transformation technologies necessary for the production of transgenic high dry-matter, "industrial-type" sweetpotatoes (i.e. sweetpotatoes not suitable for use as a food crop) optimized for the production of ethanol and butanol. Work to date has revealed that sweetpotato transformation is highly genotype specific and time consuming. Herein, we propose to continue to test the materials developed during the initial phases of this project, and to further develop transformation protocols that will speed up the production of new transgenic sweetpotatoes suitable for biofuel production in North Carolina and the southeast US.

Specific objectives in this research area were:

1. Continue to test the efficacy of amylolytic enzymes from hyperthermophilic microorganisms for their ability to degrade sweetpotato starches, and engineer those genes into sweetpotato.
2. Continue to develop industrial type enzymatically enhanced sweetpotatoes that express optimized levels of starches and fermentation enzymes for biofuel production.
3. Optimize sweetpotato transformation of industrial-type genotypes.

Work was focused primarily on objective #3, "Optimize sweetpotato transformation of industrial-type genotypes," employing our novel sweetpotato transformation technology, although significant progress was made for objectives 1 and 2. We have focused our efforts on four different industrial-type sweetpotato lines that have been developed by traditional breeding here at NC State University: DM01-158, FT4-89, DM0-074 and FTA-94, all of which are high dry matter, white skinned and fleshed genotypes that show promise for biofuel production. Work was also conducted with 'Tanzania,' which is an African landrace that is also a high dry matter type. All of these lines were transformed with a "test" gene (GUS) to determine the efficacy of our protocol. Briefly, cuttings from each genotype were transformed with the test gene, and then grown until they produced storage roots. The protocol for transformation is summarized in Figure 14. These storage roots are then tested to determine whether or not the gene has been transferred. When the gene is present, the storage roots are then sprouted to determine if the gene is also present in the sprouts. When the gene is determined to be present in the sprouts by GUS bioassay (Figure 14, panels h and i), the presence of the gene is independently confirmed in the laboratory by PCR reaction.

Experimental summary and results

There have been a total of 10 independent transformations using wild-type *Agrobacterium rhizogenes* K599 employing 5 clones of sweetpotato (DMO1-074, FTA-89, FTA-94, DMO1-158, and 'Tanzania'). Approximately 18 nodal cuttings were inoculated per clone per experiment. These clones were transformed with GUS/GFP driven by a 35S promoter. The leaves and roots have tested positive for the GUS gene. The transformation efficiencies ranged from 10% to 57% depending on the clone (Table 6). As is often seen with plant transformation, both normal and altered phenotypes were observed once the plants were sprouted. In a second set of transformations, the thermophilic alpha-amylase was transformed into these clones. Transformation efficiencies employing the alpha-amylase construct were similar to those seen with the GUS construct. The leaves tested positive for enzyme activity of the thermophilic alpha-amylase as indicated from the starch gel assay (Figure 15) and a DNS enzyme assay to measure released maltose (Table 8). The starch gel assay depicted in Figure 15 is a standard polyacrylimide protein gel that has been co-polymerized with starch, and is then stained with iodine. Enzyme activity is detected by starch clearing.

Figure 14. The experimental process showing the steps involved in an ex vitro system of transformation of sweetpotato nodal cuttings using *Agrobacterium rhiozogenes* K599. a. Rockwool; b. Perlite; c. Soil; d. Greenhouse; e. Harvest; f. Sprout; g. GUS stained roots; h., i. GUS stained leaf.

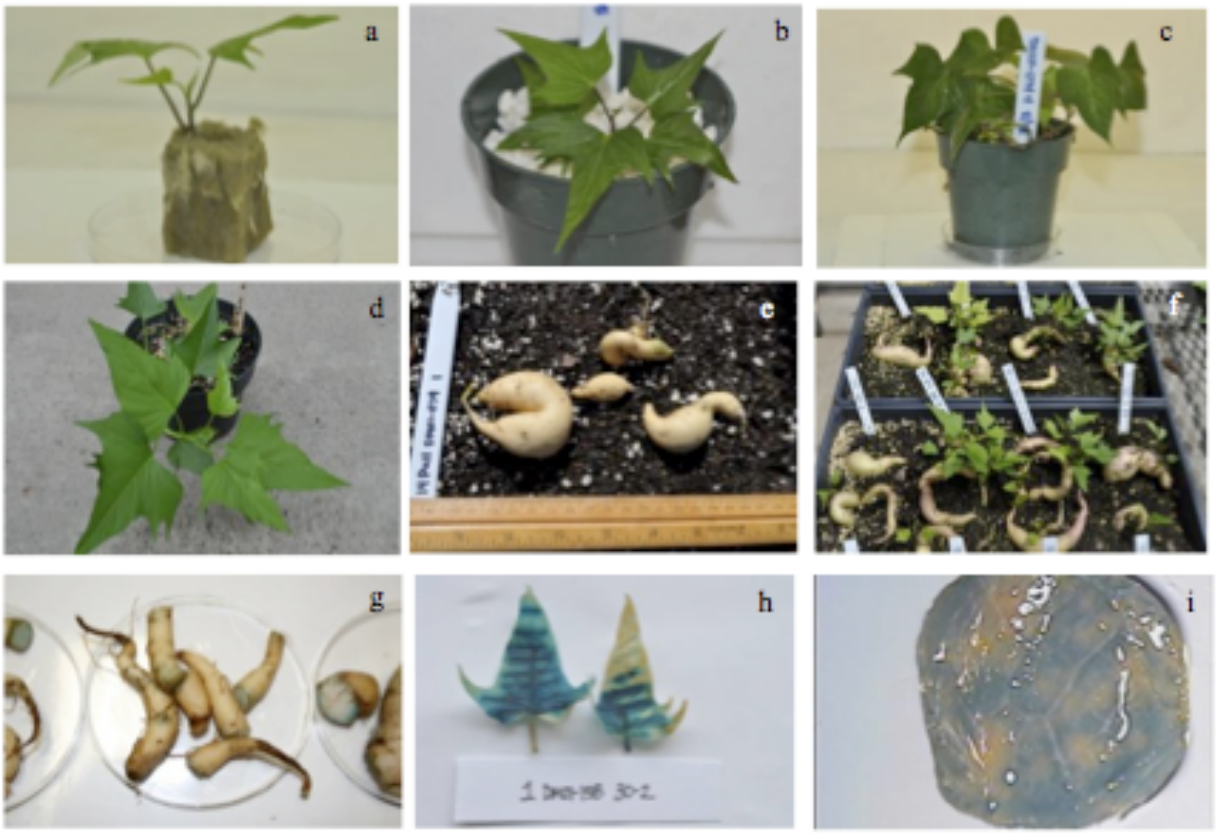


Table 6. Efficiency of GUS transformation. Transformation efficiencies for the varieties of sweetpotato are indicated as frequency (B/A, %) of PCR-positive for the GUS gene. Transformation efficiencies when considering total number of sprouts are indicated as frequency (D/C,%). DMO1-158 showed the highest transformation efficiency of the all the varieties.

Variety	Gene	Experiment no.	Inoculated (A)	Positive plants (B)	Frequency (B/A, %)	Sprouts (C)	Positive sprouts (D)	Frequency (D/C, %)
DMO1-074	GUS	1	5	2	40	12	6	50
		15	2	0	0	0	0	0
DMO1-158	GUS	1	30	17	56.7	144	37	25.7
		2	17	8	47.1	59	16	27.1
		15	12	2	16.6	25	2	8
FT4-89	GUS	1	2	2	100	6	2	33.3
		2	11	1	9.1	42	1	2.4
		15	11	0	0	30	0	0
FTA-94	GUS	1	16	9	56.3	47	13	27.7
		15	13	3	23.1	34	3	8.8
Tanzania	GUS	15	13	5	38.4	28	5	17.9

Table 7. Efficiency of α-amylase transformation. Transformation efficiencies for the varieties of sweetpotato are indicated as frequency (B/A, %) of PCR-positive for the GUS gene. Transformation efficiencies when considering total number of sprouts are indicated as frequency (D/C,%). DMO1-158 showed the highest transformation efficiency of the all the varieties.

Variety	Gene	Experiment no.	Inoculated (A)	Positive plants (B)	Frequency (B/A, %)	Sprouts (C)	Positive sprouts (D)	Frequency (D/C, %)
DMO1-074	Amy	7	8	5	62.5	24	11	45.8
	Amy	16	15	1	6.7	35	2	5.7
DMO1-158	Amy	3	33	23	69.7	82	44	53.7
	Amy	4	29	3	10.3	37	4	10.8
	Amy	6	17	6	35.3	47	12	25.5
	Amy	7	19	18	94.7	54	36	66.7
	Amy	16	0	18	0	0	54	0
FT4-89	Amy	3	14	8	57.1	43	9	20.9
	Amy	6	4	4	100	6	6	100
	Amy	7	6	6	100	18	13	72.2
	Amy	16	18	0	0	18	0	0
FTA-94	Amy	3	15	13	86.7	38	20	52.6
	Amy	4	2	0	0	6	0	0
	Amy	6	10	2	20	29	3	10.3
	Amy	7	16	11	68.8	48	19	39.6
	Amy	16	18	0	0	54	0	0
Tanzania	Amy	6	7	0	0	17	0	0
	Amy	7	21	21	100	53	50	94.3
	Amy	16	14	8	57	42	14	31.1

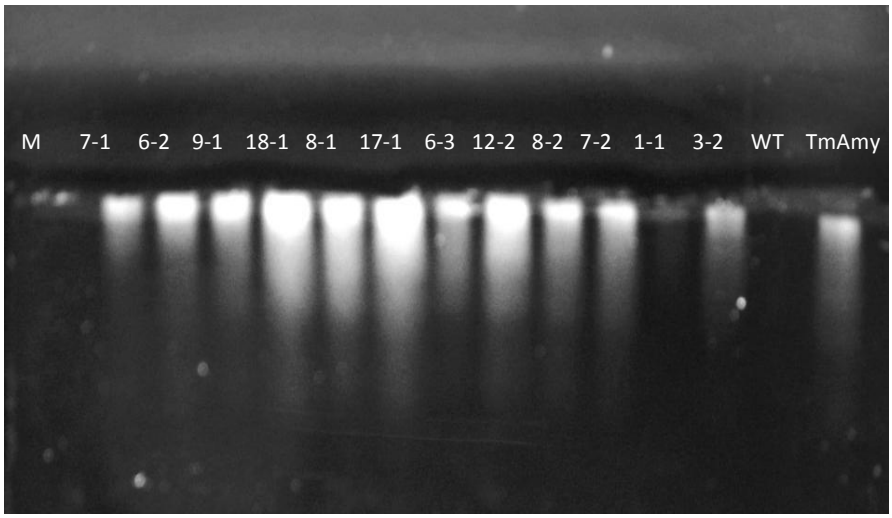


Figure 15. Starch SDS-PAGE Gel showing active starch degradation by transgenic leaves carrying the alpha-amylase gene. This indicates that the transgenic plants are carrying active enzymes.

Table 8. Enzyme activity of α-amylase from *T. martitima* and transformed sweetpotato. DNS assay was used to determine the specific enzyme activity of the transgenic alpha-amylase.

Variety	Event	Experiment no.	Total Activity (U/ml)	Specific activity (U/mg solid)	Specific activity (U/ mg protein)	U/g protein
Tm Amy		3	17.03	n/a	66517.79	6.52
DMO1-158	7-2	3	114.63	39526.03	32026.47	32.03
DMO1-158	12-2	3	94.83	22104.97	44492.02	44.49
DMO1-158	17-1	3	123.6	38746.08	62725.20	62.73
DM01-158	18-1	3	110.48	35754.69	90600.55	90.60
FT4-89	1-1	3	53.63	20391.13	11872.64	11.87
FT4-89	6-2	3	36.83	1055.80	12921.40	12.92
FT4-89	6-3	3	96.21	30066.05	21425.07	21.43
FT4-89	7-1	3	17.03	4355	4665.75	4.67
FTA-94	3-2	3	73.88	33737.08	27697.92	27.70
FTA-94	8-1	3	112.55	24574.24	36073.72	36.07
FTA-94	8-2	3	18.66	5441.63	5729.97	57.30
FTA-94	9-1	3	138.33	34156.30	48452.89	48.45

Growing Duckweed on Swine Wastewater for Ethanol Production

Researchers at NCSU have developed a duckweed production system that efficiently recovers the nutrients from swine wastewater. The system integrates low-cost anaerobic digestion for organics reduction and biogas production with utilization of nutrient-rich digester effluent as the medium for duckweed growth, resulting in nutrient recovery, generation of water for reuse, and production of large quantities of duckweed biomass.

In a preliminary experiment, using a native NC strain of *Spirodela polyrrhiza*, we successfully exploited nutrient limitation to induce starch accumulation. Duckweed was grown on wastewater then transferred it to water where it was grown for another 5 days. Starch analysis of the tissue showed its dry weight to be 45.8% starch (high-starch duckweed). This result indicated that growth on limiting nutrients could induce starch accumulation beyond the 14-16% levels we have observed in duckweed growing on swine wastewater. Our most recent work is to develop a large-scale process for production of high-starch duckweed biomass using nutrient-rich effluent from hog production.

The objectives of this research area are to develop harvesting, dewatering and drying methods, to quantify starch accumulation as a function of limiting nutrients, to obtain yield data for ethanol production from duckweed, and to estimate the cost and energy requirements to produce ethanol from duckweed biomass. We will also investigate seasonal variation in starch accumulation, collecting data on daily temperature, light intensity, day length and weather variation and monitoring duckweed growth rate and starch levels. All of these data will be used to determine the effects of seasonal variation of physical environmental parameters known to affect plant growth and metabolism. These data will allow us to quantify the relationship between biomass yield, growth rate, starch accumulation,

and starch accumulation rate and the seasonal environmental variations of light intensity, day length, and temperature. These results will allow us to develop a duckweed production protocol for pond management and harvesting that will give consistent starch levels throughout the year.

Biomass production, processing costs and scale-up potential are critical factors in commercialization of plant biomass for ethanol production. Previous research at N.C. State University has investigated the use of hog wastewater for production of duckweed biomass that could be used as a feedstock for ethanol production. Researchers demonstrated ethanol production from duckweed biomass using existing corn-based methods. These achievements provided proof-of-concept that duckweed biomass could be used to produce ethanol for fuel.

In this work, a scaled-up, pilot version of the system was built on an operational hog farm in Johnston County to design and test protocols for cropping, harvesting, and drying of duckweed biomass, to produce ethanol from this biomass, and to conduct the first economic analysis of ethanol production from duckweed biomass.

Duckweed Biomass Yield

The duckweed production system consists of two ponds. The first pond, the "culture" pond, produces high-protein duckweed utilizing hog wastewater as a source of nutrients to support growth. That duckweed can be transferred to a second pond of fresh water, the "starch accumulation" pond, which uses nutrient starvation to induce starch accumulation by the duckweed plants. Our results indicate that hog wastewater will serve as an excellent source of nutrients to support fast growth of duckweed biomass at yields of approximately 15 tons dry weight/acre over North Carolina's 30-week growing period. The protein content of the dry biomass harvested from the culture pond ranges from 35 to 45%. On the basis of protein content, this makes duckweed dry biomass roughly equivalent to soybeans. North Carolina Agricultural statistics show that soybean yield is approximately one ton per acre; however, duckweed yield is 15-fold higher. Under the starvation conditions of the starch accumulation pond, duckweed biomass will increase 1.5-fold and peak at a starch content of 25-30%. For comparison, North Carolina Agricultural statistics report average corn yields of 78 bushels/acre in North Carolina. Assuming a 70% starch content, this is equivalent to approximately 1.5 tons of starch per acre. Assuming a 1.5-fold increase in biomass and 25% starch content the duckweed system starch yield would be approximately 5.6 tons/acre.

Harvesting and Drying

The goal of the harvesting and drying studies was to identify low-cost, mechanized methods for harvesting duckweed and for drying the biomass to a moisture content of 10-15%. We devised a simple harvesting method that consist s of a floating funnel attached to a land-based suction pump that pumps the duckweed to a screen-lined cart positioned next to the pond. A slurry of duckweed and water is pumped into the cart, free water drains from the duckweed and returns to the pond. To shorten the harvesting time, we utilize a floating boom that skims the floating duckweed into a small area surrounding the floating harvesting funnel. This skimming concentrates the duckweed and minimizes harvesting time. We believe this simple method can be scaled up and further mechanized. Simple efficient drying of duckweed can be achieved by taking advantage of the plant's high surface area to volume ratio and the absence in duckweed of mechanisms that terrestrial plants have to resist dessication. Biomass is spread as a mat and periodically raked or stirred. This method yields dry duckweed biomass with a 10-15% moisture content in 1-2 days. We have stored this dry biomass for 12 months without any noticeable change in its appearance or components.

Ethanol Production

A pilot-scale enzymatic hydrolysis and fermentation of high-starch duckweed (*Spirodela polyrrhiza*) were performed in a 14-liter bioreactor. The starch content of the dry duckweed is 31%. 998 g of dry duckweed biomass was mixed with buffer to make slurry of about 5 L (4.95 L after fermentation). The enzymatic hydrolysis of duckweed was carried out using α-amylase (Sigma A3404), pullulanase (Sigma P2986), and amyloglucosidase (Sigma 10115). Although the loadings of a-amylase, pullulanase, and amyloglucosidase applied were respectively 50%, 90%, and 50% lower than those proposed in the standard protocol due to the cost consideration, the glucose recovery was still pretty high (96.8%). In the fermentation of hydrolysate (72h), although the yeast loading was cut by 38% due to the insufficient capacity for yeast cultivation in the lab, the ethanol production still reached 97.8%. Therefore, the overall starch conversion rate is 94.7%. Assuming that the 300 m^2 pond in Barham Farm can produce 700 lbs of fresh duckweed per week, and the moisture content of fresh duckweed is 90%, the annual yield (9 months) of the dry biomass can reach 2700 lbs. If the average starch content of duckweed is 31%, and the conversion rate of starch to ethanol is 94.7%. The theoretical annual ethanol production of that pond can reach 475.3 lbs, which is equal to 72.2 gals of pure ethanol (0.20 gal/day or 6.01 gal/month). In other words, we can get an annual ethanol yield of 973.9 gal/acre from duckweed, which is higher than that of corn or even switchgrass (which are respectively around 350 and 500 gal/acre).

Development of Biomass Conversion Systems for Fuel Production

A systems biology approach for the understanding of ethanol production and stress responses in *Dekkera bruxellensis*

The goal of this work was to develop a better understanding of the pathways *Dekkera bruxellensis* uses to produce ethanol and resist inhibitors in both cellulosic and traditional substrates. *Dekkera* is better able to survive both in the harsher environments found in cellulosic fermentations and at higher alcohol concentrations than traditional *Saccharomyces* yeast strains. There is limited genetic information on *Dekkera*, thus the proteome was examined to better understand these pathways. It was determined that the *D. bruxellensis* proteome is not closely related to the partial *Dekkera* genome currently available, so genomic analysis was necessary. NMR-based methods were planned to understand the fluxes that take place in the cell during ethanol production from different carbon sources, with a focus on *Dekkera*'s response to acetic acid, ethanol, furfurals and plant phenolics. Transcriptome sequencing was done by the US Dept of Energy Joint Genome Institute comparing fermentations with and without oxygen; genome sequence results were delayed beyond the end of the project, and effort on this activity was redirected.

In this work, the ability of *Dekkera bruxellensis* to withstand increasing concentrations of both lactic and acetic acids was compared to *Saccharomyces cerevisiae*. Plate reader experiments were designed to establish growth curves of both yeasts under treatments of 25mM, 50mM, 75mM and 100mM concentrations of lactic and acetic acids. These experiments ran at 30°C for one week with an optical density reading taken every two hours. Results reveal that certain strains of *D. bruxellensis* had similar or higher percent growths when treated with 25-50mM concentrations of either lactic or acetic acids when compared to *S. cerevisiae* (Figures 16 and 17). Most notable was *D. bruxellensis* strain 96 with significant growth yields at 25mM acetic acid and 50mM lactic acid. Results also revealed differences in growth among the different *D. bruxellensis* strains. In order to better understand and interpret these results, acid stress gene sequences were to be compared utilizing the known acid stress genes from the sequenced genome of *S. cerevisiae* and primers made to the entire gene (Table 9) and used for PCR of all yeast strains.

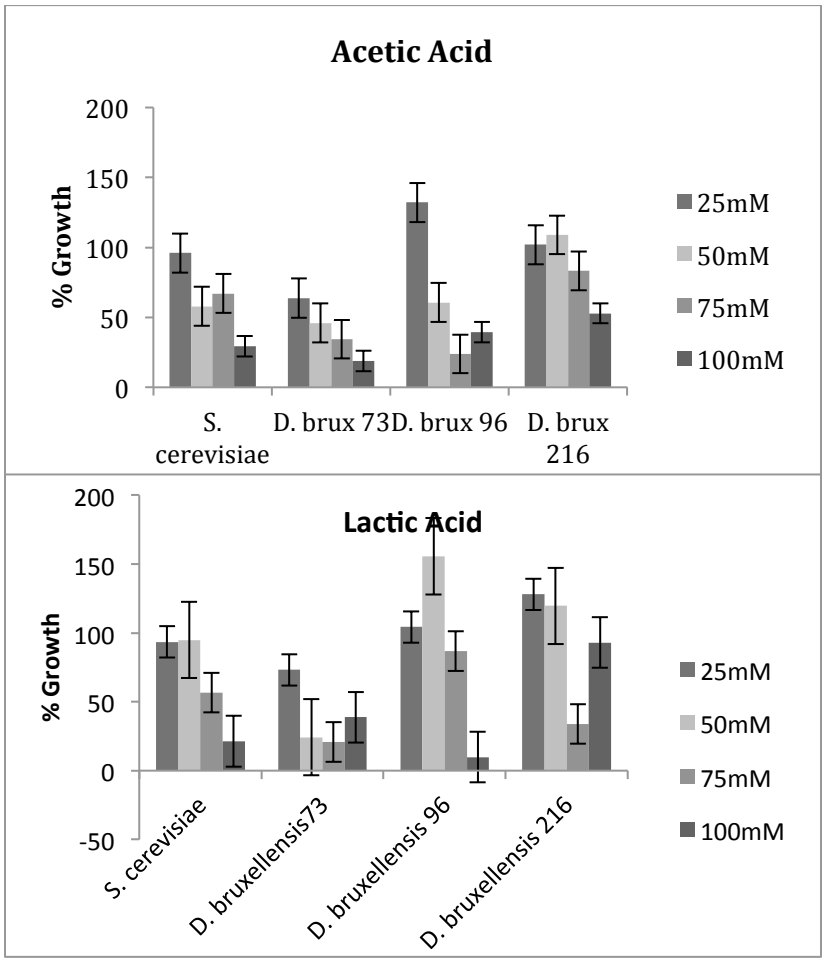


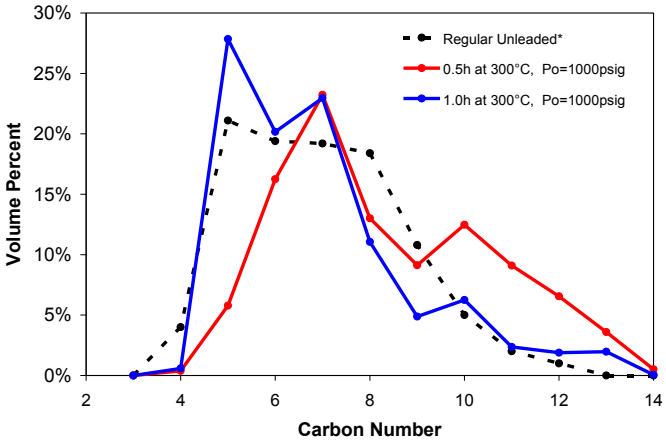
Figure 16 and 17. Growth of *S. cerevesiae* and *D. bruxellensis* yeast strains treated with acetic acid and lactic acid.

Table 9. Genes and gene functions of *Saccharomyces cerevesiae*.

Gene	Function	Gene	Function
ACT1	Actin	JEN1	Lactic acid transport
IPP1	Inorganic phosphatase	PBS2	Osmotic stress
AFT1	Acid shock and adaptation	PDR12	Plasma membrane ABC transporter
ARN1	Metal metabolism	SAP30	Telomere maintenance
ATG22	Vacuolar effluxers	SCW11	Cell wall protein
DSE1	Mating and filamentation pathways	SED1	Cell wall mannoprotein
HAA1	Transcriptional activator of membrane stress proteins	SUN4	Cell wall septation
HOG1	Osmoregulation	UBC13	Ubiquitin-conjugating enzyme involved in the error-free DNA post-replication repair pathway
VAC14	Maintenance of vacuole size and acidity		

Novel thermocatalytic process for converting triglycerides into gasoline and jet fuel

The objective of this project was to move toward commercialization an innovative thermochemical process (trademarked Centia™) for converting bio-based fats and oils into hydrocarbon-based transportation fuels with physical and combustion characteristics virtually identical to petroleum-derived fuels. Most thermocatalytic processes for converting triglycerides into transportation fuels are still too inefficient to compete with petroleum-derived fuels—with the possible exception of diesel. In order to produce surrogate jet fuel and gasoline from triglycerides, Centia™ employs a carbon-efficient catalytic step to convert C15-17 linear alkanes into C5-14 mid-distillate hydrocarbons (branched and linear alkanes). The figure on the left compares carbon number distributions obtained by reforming an n-C17 feed (using an NCSU proprietary catalyst) to that of regular unleaded gasoline. The figure also illustrates the progressive nature of the reforming process; a shorter (0.5 h) batch reaction time yields a higher average molecular weight with more material in the C10-14 (jet fuel) range. This work focused on screening and optimizing catalysts for this reforming step based on our preliminary research and evaluation of catalyst stability.



Development and upgrade of a catalytic micro-reactor (Fig. 18) were completed. These include new mass flow controllers, back-pressure regulator and liquid feed system for either high-pressure gas phase or trickle bed operation. We originally planned to use an HPLC pump for the high-pressure liquid feed system; however, HPLC pump did not provide adequate precision at very low flow rates (<0.1 ml/min). Fortunately, a high-pressure syringe pump was available with a range of microliters to 100 ml/min. A second on-line gas chromatograph for analysis of light gases (including methane, CO, and CO2) was also added and calibrated. Undecane hydrocracking over a Pt-H-Beta zeolite catalyst at 300°C and 250 psig was used to qualify the micro-reactor systems. Stable, steady-state operation for over 24 h was achieved with on-line analysis of products.

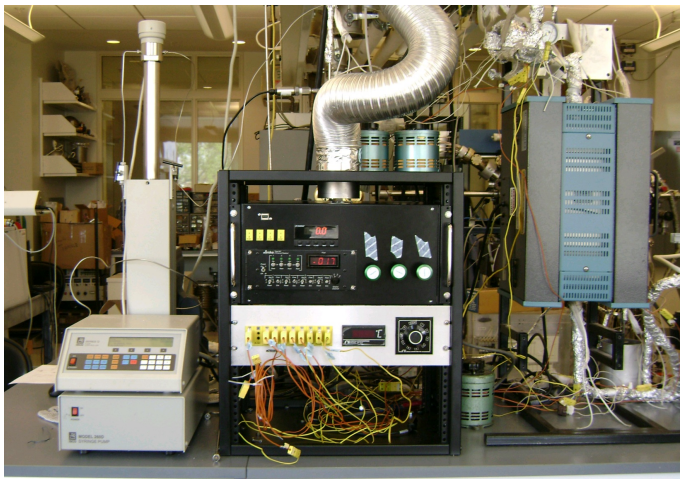


Figure 18. Photo of catalytic micro-flow reactor with liquid feed system.

Novel thermocatalytic process for deoxygenation of surrogate fast pyrolysis oil

Work in this research area was designed to gain a better understanding of the deoxygenation reactions that occur during pyrolysis oil upgrading over carbon-supported noble metal catalysts. The approach was to employ model compounds that are representative of particular classes of oxygenated compounds found in fast pyrolysis oils. These compounds and surrogate bio-oil mixtures were deoxygenated over Pd/C and Ru/C catalysts in a continuous down-flow trickle bed micro-reactor. The objective of this seed project is to elucidate the reaction pathways and kinetics for catalytic deoxygenation of model compounds in the surrogate mixture. Follow-on work will use this information to construct a detailed kinetic model for catalytic upgrading of fast pyrolysis oils.

Research focused on bio-oil model compound hydrodeoxygenation (HDO) over Pd/C, Re/C, PdRe/C and Ru/C catalysts. Experiments with guaiacol HDO were conducted at low conversions to extract kinetic information and identify primary pathways, and initial anisole HDO experiments were performed. Installation of a Micromeritics ASAP 2920 Autochem II instrument was completed, and temperature-programmed reduction (TPR) and temperature-programmed hydride decomposition (TPHD) data were obtained for Pd/C, Re/C and PdRe/C catalysts.

Anisole HDO

For anisole HDO experiments, 0.5 g of each catalyst was loaded on top of a quartz wool plug. A thermocouple was fixed in the bed to monitor its temperature throughout. Pd/C E117 was reduced for an hour in 100 sccm H_2 at 300°C—all others were reduced at 400°C then cooled to a reaction temperature of 260 or 300°C. The H_2:reactant molar ratio was held constant (~60:1) and the typical weight hourly space velocity (WHSV) was ~1 h^{-1}. Tests with an empty tube showed no significant amounts of products; thus thermal decomposition was excluded.

Anisole HDO was studied in order to observe C-O bond selectivity in the removal of the methoxy group from a phenyl ring, which lacks the hydroxyl group of guaiacol. It also demonstrates the series-type reaction pathway for deoxygenation. These experiments largely confirm the reaction networks established from guaiacol HDO. At the same conditions as guaiacol HDO experiments, the degree of deoxygenation performed by all the catalysts has increased (Fig. 19). The trend is largely the same: PdRe/C is superior, Ru/C and Re/C perform comparably and Pd/C giving the lowest benzene and cyclohexane yields. Once again, PdRe/C deoxygenates more of the feed more completely than either Pd- or Re/C Norit or Ru/C. In contrast to guaiacol, however, Pd/C E117 has substantially lower conversion, while Pd/C Norit performs comparable to Re/C. This could be an indication of some influence of the activated carbon support, though it is peculiar that the behavior is as markedly different from Pd/C in guaiacol HDO.

Looking at the selectivity to saturated products distinguishes the behavior of catalysts that perform similarly in terms of yield of fully-deoxygenated products. Again, benzene is a more desirable product because cyclohexane represents the waste of H2 to hydrogenate the ring. Comparison of selectivities shows that Pd/C Norit and Ru/C are primarily selective to cyclohexane. The greater selectivity to hydrogenation instead of HDO shown by Pd is apparent from the production of methoxycyclohexane, only produced over Pd/C catalysts. Re/C, on the other hand, has particularly low hydrogenation activity, converting almost all of the anisole to benzene and acting no further. The bimetallic catalyst is somewhere in the middle, producing around 3 benzene molecules for each cyclohexane.

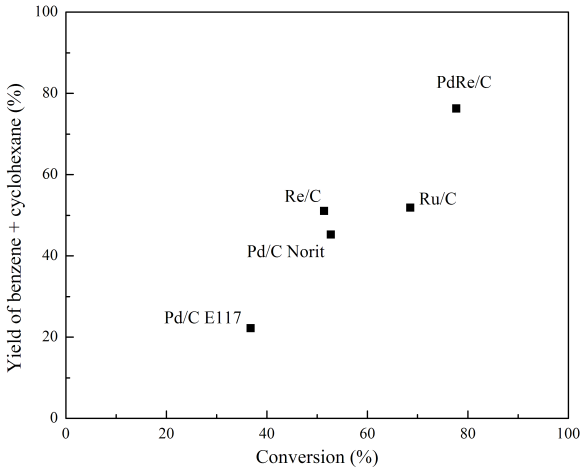
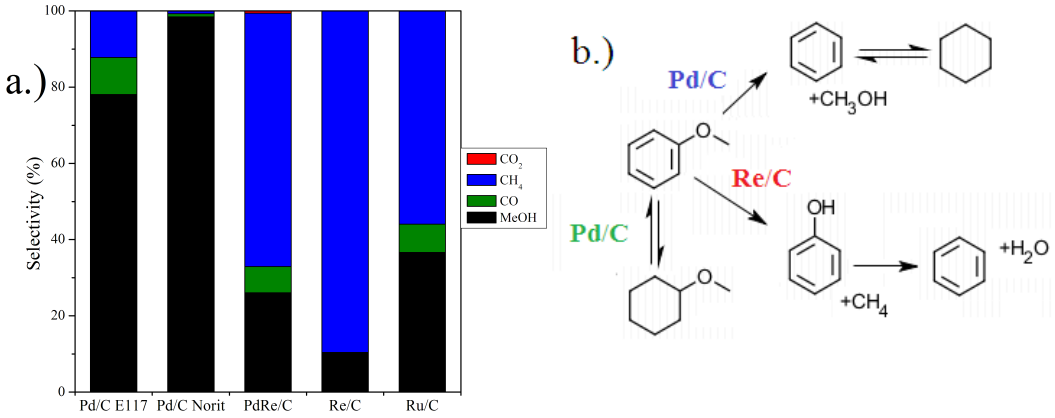


Figure 19. Yields of fully-deoxygenated products versus conversion in anisole HDO at 300°C, ambient pressure, WHSV ~1 h^{-1}, 100 sccm H_2.

As with guaiacol HDO pathways, anisole HDO pathways follow from the combination of C1 and C5 and C6 product selectivities (Fig. 20a). Again, the methoxy group is cleaved from the phenyl ring either by demethoxylation (blue in Fig. 20b), or by demethylation followed by HDO (red in Fig. 20b), in both cases producing benzene that can be subsequently saturated. The preference of Pd/C for demethoxylation is clear from selectivity to methanol; the preference of Re/C for demethylation, from its high selectivity to CH4; the ability of Ru/C to break either C-O bond non-selectively, from the ~50% selectivity to CH4 and ~50% selectivity to CO and methanol (Fig. 20a). PdRe/C is likewise able to break either C-O bond, but the behavior of Re predominates, as indicated by its selectivity to CH4. The demethylation pathway and the CH4 observed during reaction explain the amounts of phenol and cyclohexanol produced by Pd/C E117. The apparent shift in selectivity at lower conversion versus guaiacol HDO is more difficult to explain. The hydroxyl group may play a role in the stability of intermediate species on the Pd surface, directing C-O bond selectivity for the o-methoxy group in guaiacol.



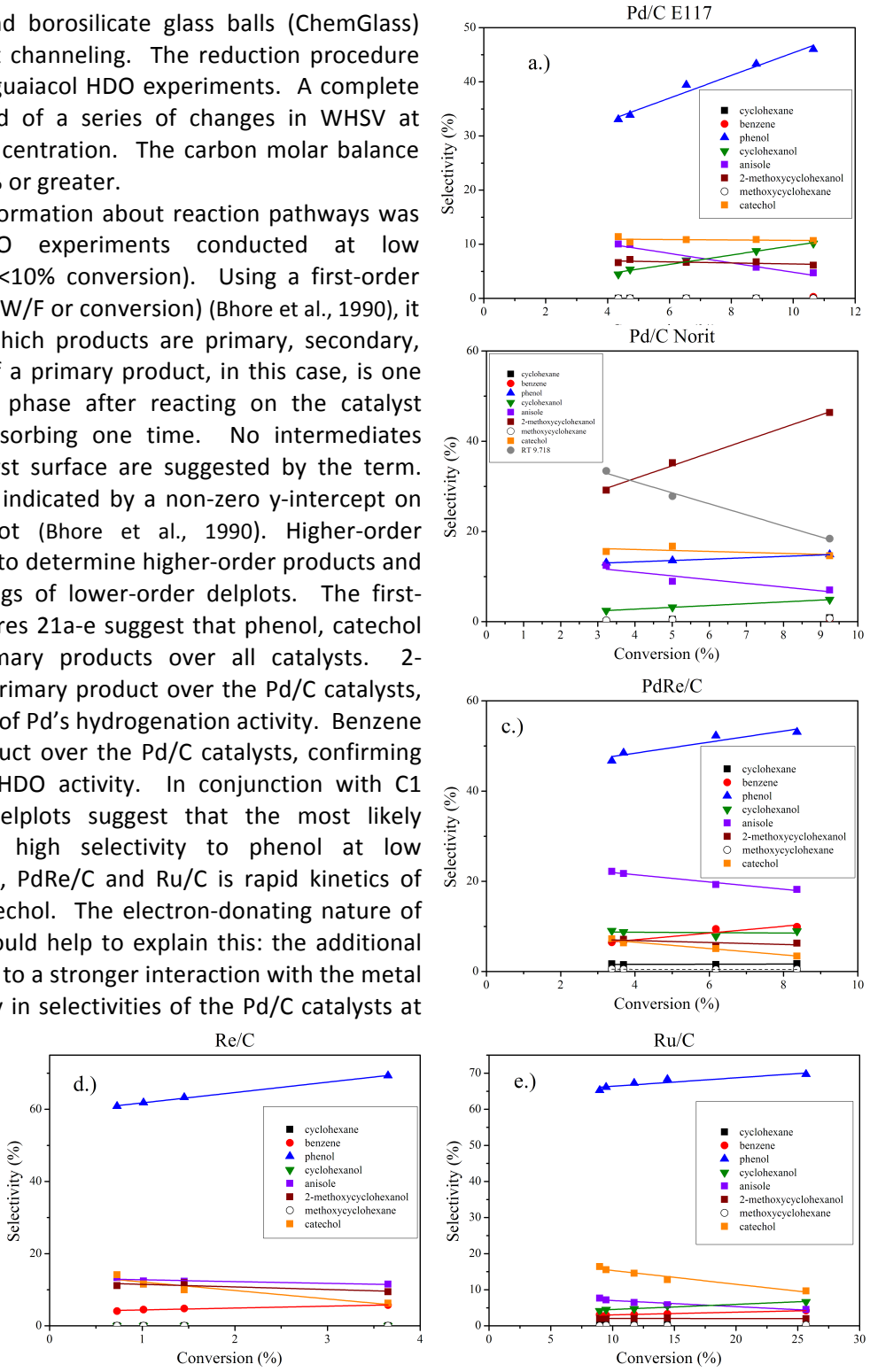
Figures 20a and 20b. Selectivities to C1 products in guaiacol HDO and predominant reactions of anisole over indicated catalysts.

Guaiacol HDO at low conversion

Low conversion experiments were performed to elucidate primary products and reaction networks. In order to get lower contact times (1/WHSV) while keeping the feed vaporized, less catalyst was used

(typically 0.150 g) and borosilicate glass balls (ChemGlass) were used to prevent channeling. The reduction procedure was the same as for guaiacol HDO experiments. A complete reaction set consisted of a series of changes in WHSV at constant reactant concentration. The carbon molar balance was closed within 90% or greater.

More detailed information about reaction pathways was obtained from HDO experiments conducted at low conversion (typically <10% conversion). Using a first-order delplot (selectivity vs. W/F or conversion) (Bhore et al., 1990), it is possible to infer which products are primary, secondary, etc. The definition of a primary product, in this case, is one observed in the gas phase after reacting on the catalyst surface and then desorbing one time. No intermediates formed on the catalyst surface are suggested by the term. Primary products are indicated by a non-zero y-intercept on the first-order delplot (Bhore et al., 1990). Higher-order delplots may be used to determine higher-order products and to confirm the findings of lower-order delplots. The first-order delplots in Figures 21a-e suggest that phenol, catechol and anisole are primary products over all catalysts. 2-Methoxyphenol is a primary product over the Pd/C catalysts, which is an indication of Pd's hydrogenation activity. Benzene is not a primary product over the Pd/C catalysts, confirming these catalysts' low HDO activity. In conjunction with C1 selectivities, these delplots suggest that the most likely explanation for the high selectivity to phenol at low conversion over Re/C, PdRe/C and Ru/C is rapid kinetics of deoxygenation of catechol. The electron-donating nature of the hydroxyl group could help to explain this: the additional electron density leads to a stronger interaction with the metal surface. The disparity in selectivities of the Pd/C catalysts at low conversion is similar to their C1 product selectivities in guaiacol HDO at high conversion. The low activity of Pd/C Norit seems to favor the production of catechol and an unknown product that is suspected to be 1,2-cyclohexanediol at low conversion, and



Figures 21a-e. Delplots for guaiacol HDO: a) Pd/C E117, b) Pd/C Norit, c) PdRe/C, d) Re/C, e) Ru/C.

its slow rate for catechol conversion as compared to Re/C is clear.

Guaiacol HDO predominant pathways

Predominant pathways for reactions of guaiacol at 300°C are thus confirmed in Figure 22. Pd/C has a strong hydrogenation function, leading primarily to saturated products, including a substantial yield of 2-methoxycyclohexanol, which represents the loss of valuable H_2. At low conversion, some anisole and catechol are produced, but the vast majority of deoxygenation that occurs follows hydrogenolysis of the phenyl C-O bond to make methanol and phenol. Saturation proceeds sequentially.

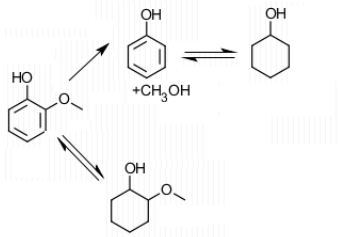


Figure 22. Predominant pathways for guaiacol HDO over Pd/C catalysts.

Deoxygenation of guaiacol over Re/C follows hydrogenolysis of the methyl C-O bond to form catechol and CH4. HDO then takes place through successive dehydroxylation reactions: phenol and water molecules are produced by the first, then benzene and another water molecule (Runnebaum et al., 2012; Lin et al., 2011). Since benzene is a primary product over PdRe/C, Re/C and Ru/C, one or both of the following must be true: 1) HDO of catechol and phenol is achieved very rapidly and/or 2) the energy of adsorption of catechol and phenol to these metal surfaces must be stronger than it is on Pd. Lin et al observed no catechol as a product in batch experiments over NiMoS- and CoMoS/Al2O3, assuming that further deoxygenation was catalyzed rapidly or that it formed coke on the catalyst (Lin et al., 2011). Further information on kinetics for each catalyst can be elucidated from data collected at low conversion. Re is unique among the three metals for its ability to cleave the hydroxyl group first to make anisole, thus that pathway has been included. It lends this activity as well as its preference to demethylation followed by HDO steps to PdRe/C. Though Pd does lend some hydrogenation activity to the bimetallic catalyst, its primary role may be in stabilizing the Re, perhaps by the same H spillover mechanism by which Pd catalyzes Re reduction, to keep Re from deactivating by oxidation. Clearly, the combination of the two metals gives the catalyst water-gas shift activity at these reaction conditions, as neither monometallic catalyst produces CO_2.

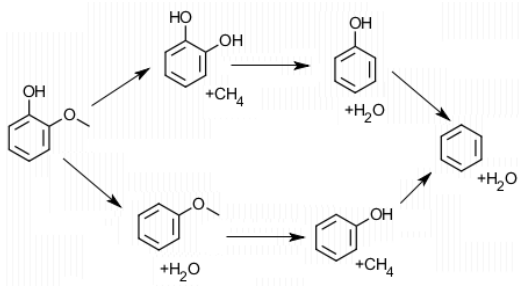


Figure 23. Predominant pathways for guaiacol HDO over Re/C catalysts.

Ru/C is able to perform both of the reactions of Pd/C and Re/C. It is more effective at HDO and tends to have lower hydrogenation activity than Pd/C, thus no 2-methoxycyclohexanol is formed. Instead, HDO proceeds by the demethylation and subsequent deoxygenation pathway or by the demethoxylation. Ru/C does not, however, continue in sequence to achieve yields of fully-deoxygenated products that are as high as Re/C and PdRe/C, nor does it have WGS activity, as the combination of metals in PdRe/C does.

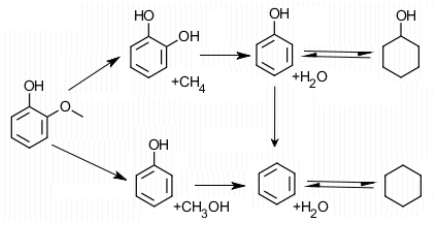


Figure 24. Predominant pathways for guaiacol HDO over Ru/C catalysts.

Practical Models for Biomass Cultivation, Collection, Transportation and Utilization

Abundant, sustainable, and affordable biomass, coupled with robust process technology for biofuel production, are the two keys to success in establishing a transportation biofuels industry.

Bioenergy plantations

The Goal of *North Carolina's Strategic Plan for Biofuels Leadership*, set by the Biofuel Center of North Carolina (BFCNC), is that by 2017, 10% of liquid fuels sold in North Carolina will come from biofuels locally grown and produced.

The study objective was to determine the technical and economic feasibility of achieving financially attractive biofuel investments, considering the existing base of timberland, agricultural residues, and purpose-grown bioenergy plantation crops as feedstocks to developing biomass-to-ethanol conversion technologies that are most likely capable of startup in a timeframe consistent with the BFCNC Goal.

Summary Conclusions

Without the favorable confluence of many factors, the BFCNC Goal is unlikely be met by 2017:

- The current lack of an economically viable technology for converting biomass to biofuel is the most critical obstacle. While such technology is likely to eventually emerge, given a traditional developmental schedule, 2017 is overly optimistic, since none are currently known to be past the Pilot Demonstration Unit stage.
- Ethanol revenue per gallon must achieve a stable level of $3.00 per gallon. Currently, the market price of ethanol fluctuates +/- $1.00 per gallon around a 10-year average of $1.50, based on ethanol from corn grain, which includes a subsidy adding $0.45 per gallon.
- While forest biomass is abundant, and can supply all feedstock needed to meet the Goal, delivered cost is likely in the current pulpwood delivered cost range of $70 per Bone Dry Ton (BDT), representing more than 50% of the cash manufacturing cost in many processes.

Candidate crops for bioenergy plantations have been identified that could reduce the delivered cost to the $50-$60 per BDT, but still not low enough to provide integrated plantation – to – biomass conversion – to market economics on a trajectory to achieve an economic return of 6% Internal Rate of Return for the landowner and 12% Internal Rate of Return for the biofuel producer. Agricultural residues appear to be one of the lowest cost class of feedstock, with attractive carbohydrate contents. One of our most promising options is a multi-feed plant that processes woody biomass during dry harvesting conditions, and captures agricultural residues as soon as possible after harvest.

Options exist for accelerating progress to the Goal. The BFCNC can continue to play a central role in further developing information on both process technology and biomass supply economics. In particular, providing introductions to facilitate partnerships among interested parties, where each partner brings specific knowledgeable resources together, is critical to innovation.

Other Major Conclusions

Financially feasible cellulosic ethanol production potential – with or without purpose-grown bioenergy plantations – involves favorable interaction among an incredible number of moving parts. Foremost,

ethanol revenue (from both market and subsidy components) must be significant and dependable in terms of both amount and time duration to attract investment. Biomass must grow productively without significant landowner investment AND at the same time be readily broken into its cellulosic components in a way that is easily converted by either enzymes (biochemical) or heat (thermochemical) so that the molecules can be converted readily to liquid fuel. Moreover, the conversion process itself must be simple to keep investment costs affordable. More specific comments follow. The process options in this specific technology area lose some of the traditional advantages of "Economy of Scale" by virtue of feedstock cost increasing as a function of cost (largely due to transportation costs).

Forests and Forest Products

1. North Carolina forests can sustainably supply all current demands for forest products by harvesting only 10% of the privately owned timberland on the state. Nine Regions have been identified where greater than 500,000 BDT per year wood can be sustainably harvested and delivered within a 50-mile radius.
2. The historical major timber-consuming industries – solid wood products and pulp and paper – face stagnant to declining demand, but are likely to be supplanted in prominence by biomass–to–power boilers (to meet State-mandated Renewable Portfolio Standards) and wood pellets produced for export to Western Europe, where power generators can afford to pay high prices for biomass, conversion and transportation to offset carbon taxes. These factors will keep the market price of biomass at current levels.
3. North Carolina privately owned forests have significant potential to sustainably produce more than double the current excess growth over removals, and to do so widely across the state; the potential can be achieved by increasing the harvest of up to 10% of the underutilized middle-aged timber stands (<40<100 Years) annually, and replanting with faster growing seedlings at higher densities than currently practiced. The additional wood volumes will be more expensive to harvest than current harvest sites, will require standards for habitat and environmental impacts, but the total cost should be within the $70 to $100 per BDT typical of current pulpwood volumes in North Carolina.
4. Even though the potential exists for abundant harvested biomass, without assurance – such as guaranteed supply – price contracts by the biomass-consuming industry and willingness to harvest by the landowners, the more intensive harvesting scheme is likely to lag the investments in conversion plants.

Agriculture Residues

5. North Carolina also has significant agricultural residues available during harvest season, which can likely be collected and transported to biomass-to-ethanol plants at lower cost than forest biomass. However, the agricultural residues are nor adequate to support significant investment as the only source of raw material.
6. Sweet Sorghum Cane is the most attractive crop for further development, but is not currently a major factor in North Carolina agriculture. Farmers with land suitable for Sweet Sorghum production have the most suitable overall crop for ethanol production; in conjunction with a plant capable of processing wood, sweet sorghum could attract more revenue as fuel feedstock than current markets offer, and in do so, attract more farmers to plant land.

Bioenergy Plantations

7. Eight potential bioenergy crops were considered promising for dedicated plantations to feed processing plants on the basis of capability to achieve delivered biomass costs cheaper than current pulpwood costs.
    a. The most promising woody biomass is loblolly pine seedlings planted at double current planting density, harvested and replanted on an 8-year rotation basis.
    b. Switchgrass is less promising despite superior growth rates due to the difficulties associated with annual harvest and year-round storage.
    c. Sweet Sorghum is the most interesting agricultural candidate if planted for biofuel with two crops per year. The sugar squeezings coupled with high carbohydrate containing Bagasse is an interesting combination.
    d. We view all annual crops as adjuncts to a plant designed for woody biomass.

Technology

8. More than 20 technologies for converting biomass to ethanol are under development today using both private and public funds. The processes can generally be categorized as:
    a. Biochemical – where biomass is first pretreated to open the physical structure to the action of enzymes or bacteria. Carbohydrates in wood are converted to sugars, and the sugars are subsequently fermented to ethanol.
        i. Conventional ethanologenic fermentation organisms achieve a maximum 51% stoichiometric conversion of sugars to ethanol, creating an upper limit of ~120 gallons per BDT for woody biomass.
        ii. Acetogenic fermentation technology is one of the most promising developments, theoretically yielding 50% higher yield (albeit with greater process complexity and investment cost).
    b. Thermochemical – where biomass is first dried and gasified. Steam reforming converts a high percentage of the biomass components to carbon monoxide and hydrogen, which are then subsequently catalytically converted to a mixture of potential fuel compounds, including dimethyl ether, methanol, and ethanol.
        i. Thermochemical systems are under active development because of a potential yield advantage (100 to 120 gallons per BDT), but with a significantly higher investment cost than biochemical systems.
        ii. Thermochemical systems are capable of processing even complex softwood biomass, but are not as advantaged on agricultural residues.
9. To our knowledge, none of the processes have successfully advanced past a Pilot Demonstration Unit development phase. Assuming one or more do so before the end of 2010, a normal scale-up step would be taken and could be in operation by 2013. This development timetable suggests widespread application by 2017 to meet the BFCNC Goal is not likely.

Financial Issues

10. The revenue the producer receives in the market drives the financial return for investment in biomass – to – ethanol. The market price for ethanol has fluctuated between $1.50 and $2.50 per gallon for the past 10 years, including a subsidy that is currently $0.45 per gallon. Ethanol from cellulosic biomass could earn as much as $1.01 per gallon total subsidy, though the enabling legislation expires in 2012.

11. If ethanol value is fixed by the market price + subsidy to on the order of $2.50 per gallon, then an investment return greater than 12% can only be achieved in a fermentation plant if:
    a. Biomass cost is less than $70 per BDT
    b. Enzymes cost less than $0.50 per gallon
    c. Ethanol yield is greater than 85 gallons per ton
    d. Capital Investment is less than $3.50 per annual gallon produced.
12. In a higher yield thermochemical plant with >100 gallons per BDT, the same limits apply, but capital cost can rise to $7.00 per annual gallon.
13. Overall, thermochemical processes, in principle, are superior to biochemical, but the lack of commercialization suggests the learning curve is steeper than thought by investors. At least one process – the Choren development in Germany – has been "one year away" for more than four years.
14. Points 8 and 9 apply to plants where greater than 35 million gallons of biochemical or 50 million gallons of thermochemical fuel is produced; this implies a minimum sized plant requires 500,000 BDT feedstock that contains a minimum of 60% carbohydrate content.

What would you have to believe to make biofuels from cellulosic biomass a financial opportunity?

1. The world is one terrorist action away from $100 - $150 per barrel energy, implying that gasoline prices > $4 per gallon will create greater development of biofuels.
2. The thermochemical platform could announce larger (>30 million gallon per year plant) scale development in 2011, either from pure thermochemical or hybrid technology that combines gasification with fermentation.
3. Repurposing a kraft pulp mill to Green Liquor pretreatment can be quickly scaled up in the event a specific mill opportunity is identified.
4. While most biochemical developments incorporate ethanologenic fermentation, an alternative process using acetogenic fermentation is under construction (ZeaChem, Boardman, OR) with the promise of 50% greater ethanol yield.

North Carolina is well positioned from the standpoint of woody biomass to meet the Biofuel Center goal in the future. Development of agricultural crops specifically for a biofuel industry is more likely to follow, where the farmer sees a specific profitable customer for the crop.

Extracting Value Prior to Combustion

While many research projects target production of ethanol from biomass, today almost all transportation fuel produced from biomass is derived from corn. While woody biomass - abundant in the United States and especially North Carolina - is competitive with corn grain with respect to growth rate of carbohydrate (tons per acre per year) and lower in cost ($ per ton of carbohydrate harvested), the chemical and physical complexity of wood versus corn leads to lower ethanol yields (gallons of ethanol per bone dry ton of biomass) and higher capital costs ($ capital Investment per annual gallon of ethanol produced). North Carolina ranks ninth in the nation in timberlands with forests covering 57% of the total land area. In addition, North Carolina sends $20 billion/yr out of state to purchase fossil fuel.

A simple process scheme that addresses both the complexity and the capital cost issue is to add a relatively small wood-to-ethanol processing plant (5-15 million gallons ethanol per year) adjacent to a wood-to-energy power generating stations or coal-fired power stations. Briefly, the process calls for hot water or steam treatment of wood (chips or forest residues) in order to extract the least recalcitrant

hemicellulose carbohydrates in wood. Hemicelluloses represent ~15 - 25% of softwoods and hardwoods, and are easily dissolved from wood in hot water. Following extraction, the wood residue is dried and sent to a boiler where it is burned using conventional technology. The hemicelluloses contain only about 40% of the BTU value of the residue, so the tradeoff of transportation fuel value versus combustion fuel value does not pose an economic penalty. The extracted sugars need to be cleaned up to remove inhibitors and also concentrated before they can be fermented to produce ethanol. Another option is to make valuable biomaterial products from this stream including super-absorbents and pigments.

In order to develop a process to harvest the hemicellulose prior to combustion the following tasks were undertaken.

1. *Determine preferred conditions for extracting hemicellulosic sugars.* A high-pressure reactor with internal stirring will be used to establish the conditions necessary for hemicellulose removal. The conditions studied will include: time: 10-60 min, temperature: 150-170 °C, pH: 1-4. The sugars will be measured using a Dionex IC. The heat value of the extracted wood will also be determined for the various conditions.
2. *Produce sufficient quantities of the extract.* A 70-liter pressure vessel will be used to produce large quantities of the sugar hydrolysate. The conditions used will be based on the findings from Task 1.

Autohydrolysis pretreatment of mixed hardwoods to extract value prior to combustion

Biomass pretreatment by autohydrolysis uses hot water to extract soluble components from wood prior to converting the woody residuals into paper, wood products, or fuel, etc. Mixed hardwood chips were autohydrolyzed in hot water at 150, 160, 170, and 180 C, for 1 and 2 h. The tradeoff between fermentable sugar yield and caloric value of the residual solids was studied for a process that will be referred to as “value prior to combustion.” The extracted liquid was treated with dilute sulfuric acid to break down sugar oligomers into fermentable monomers. Material balances were performed around autohydrolysis to evaluate the role of temperature and residence time on sugar production and residual solid heating value. The composition (sugars and byproducts) of the extracted liquid was determined. As the autohydrolysis temperature increased, the material balance became less precise, presumably due to more volatile byproducts being formed that were not quantified. More hemicelluloses were extracted from the wood by the hot water extraction process under higher temperature and longer residence time, but a greater degree of sugar degradation was also observed. After hot-water extraction the heating value of the solid residues was higher than the original wood. The total energy content of the residual solid after extraction ranged from 74 to 95% of the original energy content of the feed.
From: Pu, Y., T. Treasure, R. Gonzalez, R. Venditti, and H. Jameel. Autohydrolysis pretreatment of mixed hardwoods to extract value prior to combustion. 2011. BioResources, 6(4):4856-4870.

Process economics of value prior to combustion

A process economic analysis of co-producing bioethanol and electricity (value prior to combustion) from mixed southern hardwood and southern yellow pine is presented. Bioethanol is produced by extracting carbohydrates from wood via autohydrolysis, membrane separation of byproducts, enzymatic hydrolysis of extracted oligomers, and fermentation to ethanol. The residual solids after autohydrolysis are pressed and burned in a power boiler to generate steam and electricity. A base case scenario of biomass combustion to produce electricity is presented as a reference to understand the basics of bio-power generation economics. For the base case, minimum electricity revenue of $70–$96/MWh must be realized to achieve a 6–12% internal rate of return. In the alternative co-production cases, the ethanol facility is treated as a separate business entity that purchases power and steam from the biomass power plant. Minimum ethanol revenue required to achieve a 12% internal rate of return was

estimated to be $0.84–$1.05/l for hardwood and $0.74–$0.85/l for softwood. Based on current market conditions and an assumed future ethanol selling price of $0.65/l, the co-production of cellulosic bioethanol and power does not produce finance-able returns. A risk analysis indicates that there is a probability of 26.6% to achieve an internal rate of return equal or higher than 12%. It is suggested that focus be placed on improving yield and reducing CAPEX before this technology can be applied commercially. This modeling approach is a robust method to evaluate economic feasibility of integrated production of bio-power and other products based on extracted hemicellulose.

From: Treasure, T., R. Gonzalez, R. Venditti, Y. Pu, H. Jameel, S. Kelley, and J. Prestemon. 2012. Co-production of electricity and ethanol, process economics of value prior to combustion. Energy Conversion and Management 62:141-153.

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