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The Simultaneous Saccharification and Fermentation of Pretreated Woody Crops to Ethanol

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ABSTRACT

Four promising woody crops (Populus *maximowiczii* x *nigra* (NE388), P. *trichocarpa* x *deltoides* (N11), P. *tremuloides*, and Sweetgum *Liquidambar styraciflua*) were pretreated by dilute sulfuric acid and evaluated in the simultaneous saccharification and fermentation (SSF) process for ethanol production. The yeast *Saccharomyces cerevisiae* was used in the fermentations alone, and in mixed cultures with β-glucosidase producing *Brettanomyces clausenii*. Commercial Genencor 150L cellulase enyme was either employed alone or supplemented with β-glucosidase. All SSFs were run at 37°C for 8 d and compared to saccharifications at 45°C under the same enzyme loadings. *S. cerevisiae* alone achieved the highest ethanol yields and rates of hydrolysis at the higher enzyme loadings, whereas the mixed culture performed better at the lower enzyme loadings without β-glucosidase supplementation. The best overall rates of fermentation (3 d) and final theoretical ethanol yields (86–90%) were achieved with P. *maximowiczii* x *nigra* (NE388) and Sweetgum *Liquidambar styraciflua*, followed by P. *tremuloides* and P. *trichocarpa* x *deltoides* (N11) with slightly slower rates and lower yields. Although there were some differences in SSF performance, all these pretreated woody crops show promise as substrates for ethanol production.

Index Entries: Simultaneous saccharification and fermentation (SSF); dilute acid pretreatment; woody crops; cellulase; β-glucosidase.

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INTRODUCTION

The simultaneous saccharification and fermentation (SSF) process for conversion of cellulose into ethanol was first studied by Takagi et al. (*1,2*) more than 10 years ago, and the process still shows great potential for economic production of ethanol. Ethanol is a clean-burning, high octane fuel, and in light of our current concerns (i.e., urban air pollution, global warming, strategic vulnerability, and the trade deficit), the conversion of biomass to ethanol becomes an attactive alternative to fossil fuels. The SSF process employs a fermentative microorganism, in combination with cellulase enzyme, to minimize accumulation of sugars in the fermenter. As a result, inhibition of the enzyme by its product sugars is reduced, and higher hydrolysis rates and yields are possible than for straight saccharification (*2*). However, to produce ethanol from the SSF process that is competitive in price with petroleum-derived fuels, hydrolysis yields must be further increased, enzyme costs must be reduced, and ethanol production rates must be improved. SSF modeling, integration, and process engineering studies are presently underway to address some of these challenges. A recent economic analysis (*3*) of the SSF process with xylose fermentation estimates the selling price at $1.35/gallon. As continued research is conducted in the area of biomass-to-ethanol, further reduction of cost will be realized, with the goal to replacing petroleum fuels.

Yeast selection for SSF has been described in several publications (*4–9*). Some of this work involved the selection of thermotolerant yeast (*7–9*), with the goal of selecting a yeast that can ferment at a temperature close to the optimal hydrolysis temperature for the cellulase enzyme, 45°C (*7*). However, although an increase in temperature can speed up the hydrolysis, loss of cell viability counters these gains, and 37 to 40°C still appears to be the best temperature for the SSF process (*7,9*). Saddler et al. (*10*) also found that a temperature of 37°C maintained fermentation of released sugars by *Zymomonas* and *S. cerevisiae* strains.

Cellobiose-fermenting yeast have also been studied because additional β-glucosidase activity can speed up the SSF reaction (*5,11–13*). The importance of end product inhibition of the cellulase enzymes during cellulose hydrolysis has been modeled by Howell (*14*). Some publications discuss the advantage of the cellobiose-fermenting yeasts in decreasing end product inhibition of cellobiose to the cellulase enzyme (*15,16*). In general, *S. cerevisiae*, a strong glucose fermenter with a fast rate of fermentation, has been found to perform well if the enzyme preparation is high in β-glucosidase, whereas a mixed culture of *B. clausenii* and *S. cerevisiae* provides better yields, rates, and concentrations if the enzyme is lower in β-glucosidase.

Another important element in the SSF process is the choice of substrate. Several cellulosic substrates have been evaluated in the SSF process, including sugar cane bagasse, rice straw, wheat straw, wood fractions, and paper mill byproducts (*16–22*). Although these substrates are all poten-

tially important, fast growing trees may prove economically attractive as substrates for ethanol production, and an important consideration is the acceptability of these fast growing woody crops for biological conversion to ethanol. Therefore, this project was undertaken to evaluate the most promising of these woody crops as substrates for the SSF process. Because high β-glucosidase activity has been shown for high yields by Spindler et al. (*18*), the cellulase enzyme was used alone and with β-glucosidase supplementation to establish the highest possible cellulose conversions.

MATERIALS AND METHODS

Materials

Four woody crops were employed in this study, Populus *maximowiczii* x *nigra* (Hybrid NE388), from Pennsylvania State University, *trichocarpa* x *deltoides* (Hybrid N11), from the University of Washington, Washington State, *tremuloides* (Aspen), from Colorado, and a native strain of Sweetgum *Liquidambar styraciflua*, from North Carolina State University. P. *maximowiczii*, P. *trichocarpa*, and *S. liquidambar* were supplied to us through the coordination of the biomass production laboratory of Oak Ridge National Laboratory (ORNL), Oak Ridge, TN. The fermentation yeasts used were *S. cerevisiae* (D_5A), a SERI strain genetically derived from Red Star Brewers Yeast, and *B. clausenii* Y-1414, obtained from the U.S. Department of Agriculture (USDA) Northern Regional Research Laboratory (NRRL), Peoria, IL. Chemicals were purchased from the Sigma Chemical Company, St. Louis, MO, and yeast extract and peptone growth media were ordered from Difco, Detroit, MI. Cellulase enzyme came from Genencor Inc., San Francisco, CA, and β-glucosidase (Novozyme-188) from NOVO Laboratories, Inc., Wilton, CT. Shaker flasks, 250-mL Pyrex graduated vessels, and Braun Biostat V fermentation vessels were used for the fermentations.

Methods

Shaker flask SSFs were carried out in 250-mL flasks outfitted with stoppers constructed to vent CO_2 through a water trap. These flasks contained 100 mL of fermentation broth, and were agitated at 150 rpm in a shaker incubator at 37°C. A 1% yeast extract and 2% peptone (w/v) media was used with a substrate loading of 7.5% (w/v) cellulose. A lipid mixture of ergosterol (5 mg/L) and oleic acid (30 mg/L) was added to the media for improved ethanol yield (23). Also, penicillin and streptomycin at 10 mg/L were used to minimize bacterial contamination. The inocula were grown in a shaker flask with (YP) media and 2% (w/v) glucose at 37°C, and a 1/10 (v/v) yeast culture to total volume of media was added to the fermentation. The substrate was autoclaved in fermentation flasks, and sterile media, lipids, antibiotics, and enzyme were added before the inoculum.

Ethanol concentrations in the supernatant were measured by gas chromatography, using a Porapak Q80/100 column. The internal standard was 4% isopropanol. For the larger scale 3 L SSFs, residual sugars (glucose and cellobiose) were determined as glucose by incubation of the sample with 2 mg/mL almond extract β-glucosidase from Sigma for 1 h at 37°C, and total sugars were measured on the model 27 glucose analyzer from Yellow Springs Instruments, Yellow Springs, OH. Viable cell densities were measured as colony forming units (CFU) by plating serial dilutions on YPD or YPC plates.

Cellulase enzyme loadings of 7, 13, 19, and 26 IU/g cellulose substrate were used in the shake flask screening experiments to span the range of activity previously shown to be important for SSFs. In this paper, IU stands for international units of filter paper activity in micromoles of glucose/minute (*24*). β-glucosidase enzyme was employed in this study at ratios of 1, 2, and 8 parts to 1 part of cellulase, as measured by IU of β-glucosidase/IU of cellulase. The β-glucosidase activity was determined by Nitrophenyl-β-glucoside (PnPGU) assay at a temperature of 37°C, because this is the temperature for the SSFs. The activity of cellulase increases with increasing temperatures to an optimum at 45°C (*7,9*), the temperature selected for saccharification without fermentation studies. The IUPAC revisions of measured cellulase activities indicate that the level of β-glucosidase in an enzyme preparation may affect the results of the cellulase assay in filter paper units (*24*).

Wood crops were completely debarked, and two 500 g batches of Wiley milled (2 mm screen) wood were pretreated with dilute sulfuric acid (0.45% v/v) in a 2 g Par Reactor. All woods were pretreated at 140°C for 1 h, with stirring at 185 rpm. After reaction, the wood slurries were washed several times with hot water in a large Buchner funnel lined with a linen sheet to bring the pH of 1.3 up to 4.5. These batches were combined, immediately placed in freezer storage bags, and stored at −20°C. Approx 70% of the pretreated woods dry wt was found to be cellulose, 29% lignin and acid insoluble ash, and 1% xylan.

Before pretreatment, approx 22% of the wood consists of lignin, 50% cellulose, and 14% xylan. Exact values are listed in Table 1 and will be addressed in the discussion.

Shaker flask results are reported as percent of maximum theoretical ethanol yields, and do not account for substrate used in cell growth. Thus, the maximum expected ethanol yield is about 95%, assuming about 5% of the substrate is needed for cell growth. These calculations are based on the measured ethanol concentrations and a 56.7% theoretical ethanol yield conversion of cellulose to ethanol only. However, the saccharification with cellulose is reported on the basis of percent of the maximum amount of sugars possible. Thus, comparison of the SSF and straight saccharification results must consider that subsequent fermentation of the sugars produced in the latter will also result in about a 5% loss to cell growth and maintenance.

Table 1
Chemical Composition of Woody Crops* Before[b] and After[a] Dilute Acid Pretreatment

Woody Crop*	1[b]	1[a]	2[b]	2[a]	3[b]	3[a]	4[b]	4[a]
Klason Lignin	21.8	29.0	22.5	29.0	20.0	29.0	21.9	29.0
Cellulose	48.6	69.5	51.8	72.0	49.8	63.3	49.5	68.0
Xylan	14.6	1.5	11.5	0.5	18.0	4.0	17.5	0.9
% Total**	86.0	99.5	86.5	101.5	88.0	96.0	89.5	98.0

*Woody Crops
1 = Populus maximowiczii x nigra (hybrid NE388)
2 = Populus trichocarpa x deltoides (hybrid N11)
3 = Populus tremuloides (aspen)
4 = Sweetgum Liquidambar styraciflua (native)

**The remaining mass balance for wood before pretreatment consists of 4% hemicellulosic sugars and other extractives.

Although in previous work with Sigmacell-50 (*4,5,9*), we were able to measure the residual cellulose via a wash filtration method, this method for measuring residual cellulose in real substrates, such as pretreated woody crops, was not effective because of lignin interference. Therefore, for our detailed analysis, an alternate method was developed that could estimate the residual cellulose by poisoning the yeast cells with NaF and allowing the excess enzyme to complete the saccharification from a given point and time of SSF. Background measurements of glucose were subtracted, which stem from the β-glucosidase enzyme itself, and from some residual glucose not taken up by the cells at the time of sample. At lower enzyme levels, additional cellulase and β-glucosidase were added to complete the saccharification.

The straight saccharification yields are calculated as the amount of glucose produced compared to the potential glucose in the cellulose feed. The substrate level was limited to 7.5% cellulose for the small- and large-scale SSFs because mixing problems were encountered at higher cellulose levels during previous SSF evaluations performed with *S. cerevisiae* and pretreated wheat straw at higher substrate levels (*18*).

RESULTS

Supplementation of Genencor 150L enzyme (batch II) with β-glucosidase was used to increase ethanol yields and saccharification rates to values equal to previous work, because our original supply of cellulase (batch I) was exhausted on preliminary small-scale screenings with Sigmacell-50. Previous work indicates an approx 35% reduction in PnPGU activity/g of protein for (batch II) when compared with (batch I) (*9,18*). Without the β-glucosidase supplementation, approx 12% decrease in ethanol production was observed. Thus, although batch II enzyme was still valuable for comparing the yeast characteristics on a consistent basis, supplementation with β-glucosidase increases the specific cellulase activity significantly (*7*).

Table 2 illustrates the final saccharification yields at 45°C for all four acid pretreated woody crops at the selected cellulase loadings and supplementation with β-glucosidase. The best overall rates of hydrolysis and final conversions to simple sugars were observed for P. *maximowiczii* and S. *liquidambar*. The highest enzyme loading of 26IU cellulase with a 8:1 ratio of β-glucosidase was necessary to achieve 75% cellulose conversion, although the 2:1 ratio of β-glucosidase with 26IU cellulase was very close behind. The saccharifications took 5 d to achieve the highest yields, 8 d for the lower loadings.

It is interesting to compare the straight saccharification results in Table 2 with the SSF results of Table 3. Here, we see near completion of fermentations with the same two woody crops in 3 d with *S. cerevisiae* and the higher enzyme loadings. It is apparent that the SSF process performs with better efficiency than straight saccharification if we compare the final yields of both Tables 2 and 4. This is because of the low residual sugars

Table 2
Summary of Final (8-day) Saccharifications Yields
For Acid Pretreated Woody Crops With Selected Cellulase and Beta-Glucosidase Loadings at 45°C*

IU beta-glucosidase:IU Cellulase	0:1				2:1				8:1			
A IU Cellulase/g Cellulose	7	13	19	26	7	13	19	26	7	13	19	26
Woody Crops** 1	24	48	66	72	35	53	69	75	23	50	82	82
2	14	28	40	47	23	38	50	51	14	31	35	65
3	17	30	48	65	20	36	52	51	16	30	43	64
4	32	46	53	66	36	52	71	77	34	48	68	74

* Saccharification yields are expressed in percents of theoretical conversion
**Woody Crops
1 = Populus maximowiczii x nigra (hybrid NE388)
2 = Populus trichocarpa x deltoides (hybrid N11)
3 = Populus tremuloides (aspen)
4 = Sweetgum Liquidambar styraciflua (native)

Table 3
Summary of Ethanol Yields in SSFs After 3 Days
For Pretreated Woody Crops At 37°C For Selected Cellulase and Beta-Glucosidase Loadings*

IU beta-glucosidase:IU Cellulase	0:1				2:1				8:1			
IU Cellulase/g Cellulose	7	13	19	26	7	13	19	26	7	13	19	26
Yeast	S. cerevisiae											
Woody Crops**												
1	20	30	41	50	52	65	72	71	56	65	83	86
2	21	24	35	39	40	45	49	56	39	47	59	59
3	16	24	32	47	47	58	63	69	46	62	70	72
4	18	29	42	53	60	70	74	81	65	76	86	86
Yeast	Mixed Culture***											
Woody Crops**												
1	32	42	46	50	42	52	60	64	43	55	63	71
2	20	23	28	32	28	33	36	41	33	40	54	58
3	32	45	50	55	47	58	64	65	54	60	65	65
4	41	46	48	50	43	52	52	57	53	54	59	59

* Ethanol yields are calculated as percents of theoretical yield
** Woody Crops
1 = Populus maximowiczii x nigra (hybrid NE388)
2 = Populus trichocarpa x deltoides (hybrid N11)
3 = Populus tremuloides (aspen)
4 = Sweetgum Liquidambar styraciflua (native)

***Mixed Culture = Saccharomyces cerevisiae and Brettanomyces clausenii

Table 4
Summary of Final Ethanol Yields (8-day) in SSFs
For Pretreated Woody Crops Run at 37°C For Selected Cellulase and Beta-Glucosidase Loadings*

IU beta-glucosidase:IU cellulase	0:1				2:1				8:1			
IU Cellulase/g Cellulose	7	13	19	26	7	13	19	26	7	13	19	26
Yeast	S. cerevisiae											
Woody Crops**												
1	42	61	75	80	70	84	87	89	79	86	88	90
2	39	47	55	62	57	62	68	72	56	61	77	82
3	34	51	66	76	70	79	83	84	69	81	83	84
4	41	61	70	77	73	81	82	86	77	83	86	86
Yeast	Mixed Culture***											
Woody Crops**												
1	53	67	74	77	56	75	83	83	65	84	86	86
2	39	43	47	52	44	52	56	60	52	66	73	80
3	63	66	74	76	72	76	78	78	73	78	79	79
4	68	72	77	78	68	76	76	79	67	79	82	84

* Ethanol yields are calculated as percents of theoretical yield
** Woody Crops
1 = Populus maximowiczii x nigra (hybrid NE388)
2 = Populus trichocarpa x deltoides (hybrid N11)
3 = Populus tremuloides (aspen)
4 = Sweetgum Liquidambar styraciflua (native)

*** Mixed Culture = Saccharomyces cerevisiae and Brettanomyces clausenii

present in the SSF relieves inhibition to the cellulase enzyme experienced in straight saccharification.

Table 4 compares the final (8-d) ethanol yields for SSF with different substrates at selected enzyme loadings and yeast combinations run in 100 mL shaker flasks. The rates and yields are consistently higher for P. *maximowiczii*, compared to the other two populus and *S. liquidambar* legume with *S. cerevisiae* as the fermenting yeast. However, the mixed culture achieves the best overall rates and highest yields for S. *liquidambar* at the lower enzyme loadings without β-glucosidase supplementation. In all cases, lower rates and yields resulted with P. *trichocarpa*, compared to the other substrates.

The ethanol yields for S. *liquidambar* are slightly higher than those for P. *maximowiczii* at lower enzyme loadings with *S. cerevisiae*, but then level out to values about equal to those for the other substrates at high enzyme loadings. It is interesting to note that the increase in ethanol yields between the 2:1 and 8:1 ratios of β-glucosidase differ for the two cultures. For *S. cerevisiae*, the 8:1 and 2:1 ratios of β-glucosidase to cellulase (IU/IU) give about the same ethanol yields, whereas there is a greater difference for the mixed culture. Apparently, *S. cerevisiae* is more efficient as a single culture with the lower addition of β-glucosidase than it is in coculture.

All four of the woody crops reached high conversions of cellulose (86–100%) to ethanol with both yeast cultures at higher cellulase enzyme loadings. Although the mixed culture of *S. cerevisiae* and *B. clausenii* was slightly less sensitive to β-glucosidase supplementation, we find that without β-glucosidase supplementation, the average final yield was 63.7% of theoretical ethanol, compared to 58.5% for *S. cerevisiae* alone. This observation can be explained by production of β-glucosidase during SSFs by the cellobiose-fermenting yeast, *B. clausenii*, while *S. cerevisiae* can achieve similar results with β-glucosidase supplmentation.

Table 1 lists the composition of the substrates before and after pretreatment with dilute acid. If we review this table, it is apparent that there are no large differences in substrate composition that might explain the subtle differences in the fermentability of sugars from these substrates. It might be postulated that the enzyme accessibility to the substrate is somehow affected by the physical response of lignin to pretreatment. All these substrates were pretreated the same, and show similar composition within experimental error. Approx 4% of the composition not listed here was measured as hemicellulosic sugars and the rest of the mass balance for the pretreated wood is classified as other extractives.

Two larger-scale (3 L) stirred reactor SSFs were conducted with the woody crop P. *maximowiczii* for a more detailed analysis. These were run with *S. cerevisiae* and the mixed culture of *S. cerevisiae* and *B. clausenii* as before, except yeast cell and residual sugar were monitored in addition to the ethanol concentration. The enzyme loading selected was 19 IU cellulase/g cellulose, with an 8:1 ratio of β-glucosidase to cellulase. Because of

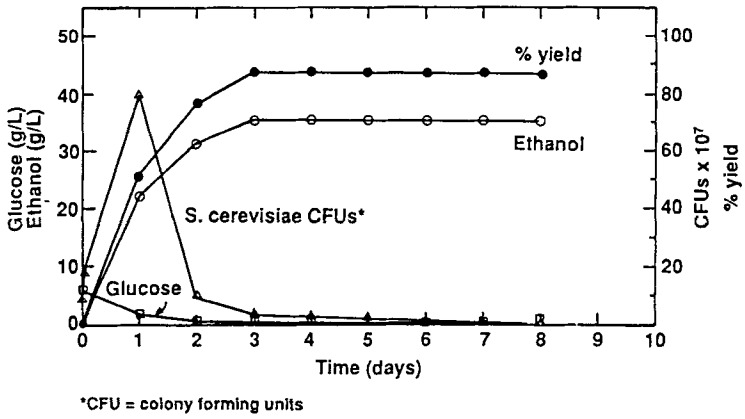

Fig. 1. Stirred fermenter SSF with *S. cerevisiae* at 37°C, a cellulase loading of 19 IU/g cellulose, and 7.5% cellulose for Populus NE388 and a β-glucosidase/cellulase activity ratio of 8:1.

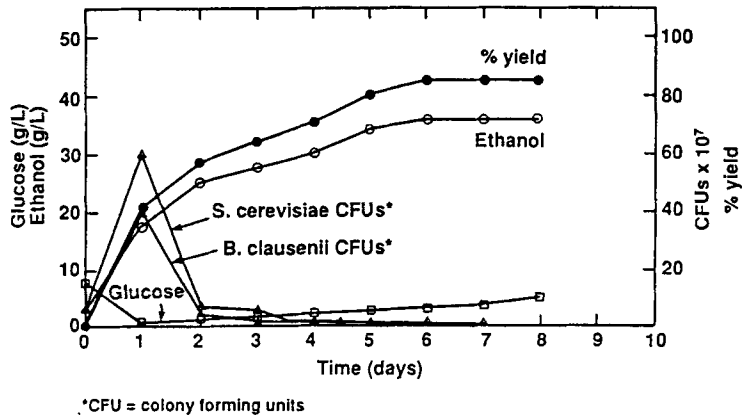

Fig. 2. Stirred fermenter SSFs with a mixed culture of *S. cerevisiae* and *B. clausenii* at 37°C with a cellulase loading of 19 IU/g cellulose and 7.5% cellulose for Populus NE388 and β-glucosidase/cellulase activity ratio of 8:1.

the high supplementation with β-glucosidase, no cellobiose was detected, and all residual sugar was measured as glucose. Cellulose disappearance measured as glucose correlated closely with the values achieved for ethanol, and although filtration of cellulose was not possible for actual residual cellulose readings, we feel confident in our product yield from these substrates.

Figure 1 presents the concentrations measured for the stirred SSF fermentations conducted with *S. cerevisiae*. The ethanol concentration continually increases whereas the cell viability first incrases and then declines from 8×10^8 to 10^6 CFUs. Glucose initially increased to about 7 g/L, and then quickly dropped to less than 1 g/L within 2 d. Figure 2 illustrates these same parameters for the mixed culture SSF with the cell

count for *B. clausenii*, starting at 10^8, rising to 4×10^8 in 1 d, and then declining to 10^5 while *S. cerevisiae* again levels off at 10^6. Although residual sugar is low in this SSF, a steady increase in glucose eventually occurs as the cell viability for *B. clausenii* declines. *B. clausenii* has a lower ethanol tolerance than does *S. cerevisiae*, and therefore, a lower survival rate in the SSF process.

The percent ethanol yields for the stirred fermentations correspond closely to the results shown in the shaker flask SSFs of Tables 3 and 4. Once again, *S. cerevisiae* exhibits a faster rate of conversion than the mixed culture at 19 IU cellulase/g cellulose, supplemented with a 8:1 β-glucosidase-to-cellulase ratio.

CONCLUSIONS

Although all the pretreated woody crops reached high final conversions of cellulose to ethanol, the final yields and rates of hydrolysis were the highest for P. *maximowiczii* with *S. cerevisiae* and β-glucosidase supplementation. However, the mixed culture of *S. cerevisiae* and *B. clausenii* achieved slightly higher final yields and 3 d rates of hydrolysis for S. *liquidambar* at the lower enzyme loadings without β-glucosidase supplementation.

A 79% or better ethanol yield was achieved in SSF at the highest enzyme loading of 26 IUs cellulase with 8:1 supplementation of β-glucosidase. *S. cerevisiae* excelled with P. *maximowiczii* as a substrate, reaching 84% or better of theoretical ethanol yields at 13, 19, and 26 IUs cellulase/g cellulose for both β-glucosidase ratios of 2:1 and 8:1.

The overall rates and yields with the mixed culture without β-glucosidase were better than for *S. cerevisiae* because of the additional β-glucosidase activity associated with *B. clausenii*, whereas *S. cerevisiae* did better with large β-glucosidase addition. These results point out the necessity to provide high β-glucosidase levels to prevent accumulation of the powerful inhibitor cellobiose. Thus, the use of mixed cultures of a cellobiose-fermenting organism with an ethanol-tolerant strain or supplementation of the enzyme broth with β-glucosidase will continue to be desirable to obtain high yields until an enzyme is developed that has higher levels of β-glucosidase. From these data, we can conclude that these short rotation woody crops respond well to dilute acid pretreatment, show fast enzyme hydrolysis rates, and give high ethanol yields through fermentation by selected yeast.

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