Cellulosic ethanol production from AFEX-treated corn stover using *Saccharomyces cerevisiae* 424A(LNH-ST)

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Current technology using corn stover (CS) as feedstock, Ammonia Fiber Expansion (AFEX) as the pretreatment technology, and *Saccharomyces cerevisiae* 424A(LNH-ST) as the ethanologenic strain in Separate Hydrolysis and Fermentation was able to achieve 191.5 g EtOH/kg untreated CS, at an ethanol concentration of 40.0 g/L (5.1 vol/vol%) without washing of pretreated biomass, detoxification, or nutrient supplementation. Enzymatic hydrolysis at high solids loading was identified as the primary bottleneck affecting overall ethanol yield and titer. Degradation compounds in AFEX-pretreated biomass were shown to increase metabolic yield and specific ethanol production while decreasing the cell biomass generation. Nutrients inherently present in CS and those resulting from biomass processing are sufficient to support microbial growth during fermentation. This platform offers the potential to improve the economics of cellulosic ethanol production by reducing the costs associated with raw materials, process water, and capital equipment.

AFEX | fermentation | Lignocellulose | biofuel

Cellulosic ethanol has been widely regarded as a promising alternative liquid fuel due to its projected positive attributes in terms of economic, environmental, and social sustainability (1, 2). The ability to generate and convert fermentable sugars from lignocellulosic materials to ethanol in a cost-effective fashion is the central technological challenge to fully unlock its commercial potential (3). Unfortunately, fermentation of hydrolysates derived from pretreated lignocellulosic biomass is often preceded by washing (4), nutrient supplementation (5, 6), and detoxification (7, 8), which are very costly processes (9, 10). The fermentability of a hydrolysate is strongly dependent on the feedstock pretreatment and strain selection. Improvements in these areas can significantly change the economic performance of fermentation using lignocellulosic biomass.

Ammonia Fiber Expansion (AFEX) has been shown to be an effective pretreatment method for generating a highly fermentable hydrolysate (11). AFEX produces inhibitory degradation compounds at a reduced level (12, 13) and preserves nutrients in biomass (14) for fermentation. Residual ammonia further enriches the nutrient content of the AFEX-pretreated biomass. *Saccharomyces cerevisiae* is a well-established fermenting strain in existing commercial-scale ethanol industries (15). For cellulosic ethanol production, recombinant strain 424A(LNH-ST) from Purdue University reportedly exhibited excellent cofermentation of glucose and xylose (6). Metabolic engineering of the yeast was achieved through integrating multiple copies of three xylose-metabolizing genes i.e., xylose reductase, xylitol dehydrogenase, and xylulokinase into the yeast's chromosome (16, 17). However, markedly slow xylose utilization in lignocellulosic hydrolysate by 424A(LNH-ST), which reduced overall fermentation performance, has been reported (6). Nevertheless, the reasons for this observation remain unclear.

We seek to take advantage of the promising features of AFEX pretreatment and *S. cerevisiae* 424A(LNH-ST) to formulate and demonstrate an industrially-relevant strategy for fermentation using lignocellulosic biomass. We evaluate the requirements for: *a*) high starting cell density, *b*) hydrolysate conditioning, and *c*) nutrient supplementation to conduct lignocellulosic fermentation. We also construct a comprehensive mass balance based on conversion of carbohydrates in untreated biomass to ethanol to elucidate the current status and the bottlenecks of the technology. To better understand the phenomenon of slow xylose utilization in hydrolysate, the interactions between degradation products, xylose metabolism, cell growth, and media nutrient content are described.

Results

Optimization of Fermentation Conditions. Optimal conditions for fermentation of AFEX-corn stover (CS)-hydrolysate are pH 5.5 and 30 °C (among the conditions tested) with respect to overall fermentation yield and rate. While fermentation at pH 6.5 exhibited the highest xylose consumption rate, it had the lowest metabolic yield (79.5%) among tested pH values with the highest level of glycerol (5.0 g/L) formation. Judging from the overall ethanol yield, pH 5.5 was determined to be the optimal pH (Fig. 1 *A* and *B*). Regarding temperature optimization, xylose utilization in fermentations at 35 °C and 37 °C essentially ceased after 72 h; 10.9 g/L and 16.1 g/L of xylose were left unconsumed. Xylose fermentation at 25 °C was slower than at 30 °C (Fig. 1*C*).

The initial rate of xylose consumption is directly correlated to the initial cell density (Fig. 1*D*). However, the difference between the extent of xylose consumption (after 144 h) was rather small (<3 g/L). Final ethanol yield was almost independent of initial cell density. To be more industrially-relevant, relatively low cell densities (≤1.1 g dry-cell-wt./L) were used. With the exception of hydrolysate fermentation at pH 3.5, glucose fermentations were completed within 18 h (glucose consumption profiles are not shown in Fig. 1).

Laboratory Media vs. AFEX-CS Hydrolysate. Fermentations using 424A(LNH-ST) in complex media (YEP) and AFEX-CS-hydrolysates derived from saccharification at 6% glucan loading were compared side-by-side under identical conditions (Fig. 2). These hydrolysates were fermented without conditioning (no washing, nutrient supplementation, or detoxification) at 1.1 g dry-cell-wt./L starting cell density. The difference between Hydrolysate I and II is that an additional 10 g/L glucose and 12 g/L xylose were supplemented into Hydrolysate II to examine the scenario in which soluble oligomeric glucose and xylose in the hydrolysate were also used.

Strain 424A(LNH-ST) was able to grow well in both complex media and AFEX-CS-hydrolysates and achieved cell densities greater than 6.0 g/L within 12 h of fermentation (Table 1; Table S1). In complex media, 70 g/L glucose and 40 g/L xylose were completely consumed within 48 h (Fig. 2*A*). The volumetric

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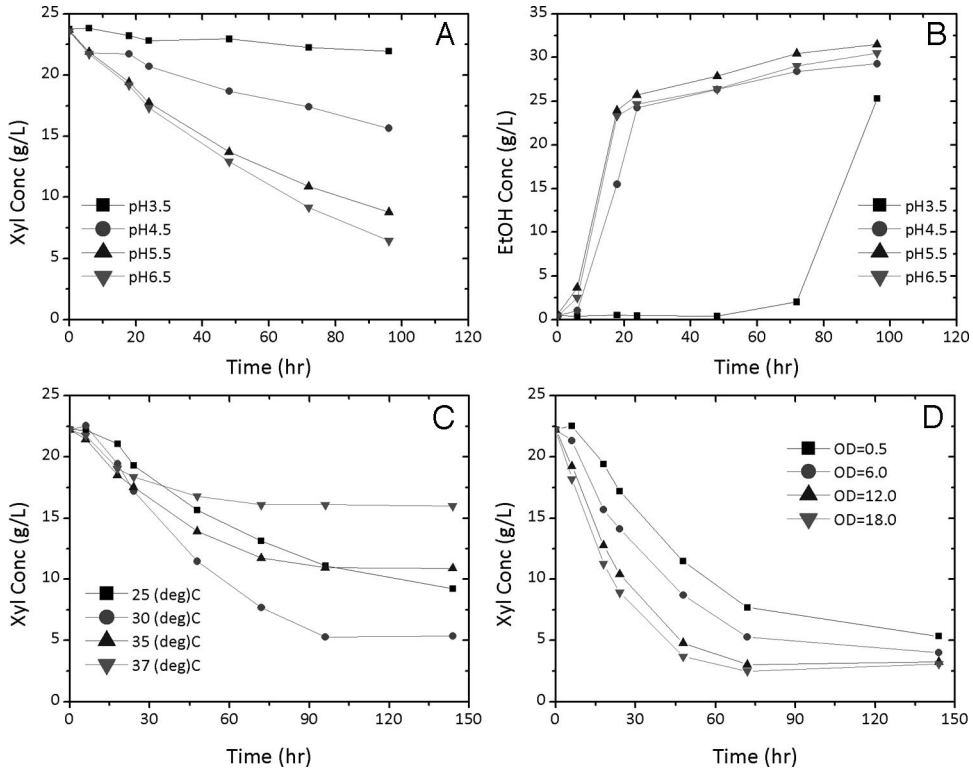

Fig. 1. Effect of (*A* and *B*) pH, (*C*) temperature and (*D*) initial OD on fermentation using hydrolysate from enzymatically-digested AFEX-treated CS using *S. cerevisiae* 424A(LNH-ST). (Glucose consumption profiles are not shown in this graph). Note: Ethanol production from 0 to 25 g/L shown in (*B*) is largely due to glucose fermentation

glucose consumption rate was 7.3 g/L/hr; 0–6 h, which was roughly 7-fold higher than the xylose consumption rate. For fermentations in AFEX-CS-hydrolysate, volumetric xylose consumption rates were an order of magnitude lower than for glucose. This indicates that xylose utilization was more susceptible to inhibition. Surprisingly, specific glucose consumption rates in hydrolysate achieved 10.6 g/L/hr/g cell; 0–6 h, substantially higher than that in complex media (Table 1). Fermentations in the hydrolysates achieved higher metabolic yields with lower xylitol formation (0.46 g ethanol/g consumed sugars; 0.3 g/L xylitol) than complex media (0.43 g ethanol/g consumed sugars; 3.2 g/L xylitol).

Washing Removes Both Degradation Products and Biomass Nutrients. The impacts of washing AFEX-pretreated CS (before enzymatic hydrolysis) on xylose utilization and the requirements for nutrient supplementation were investigated. Washing of AFEX-CS does not improve xylose utilization unless additional nutrients are provided. Xylose fermentation for unsupplemented washed-CS hydrolysate had the lowest rate (0.12 g/L/hr, 0–96 h) followed by unsupplemented unwashed-CS hydrolysate (Fig. 3). This observation is attributed to the loss of nutrients through washing. Besides removing degradation products (18), the wash stream of AFEX-CS contains residual ammonia from pretreated materials and other biomass components that are important

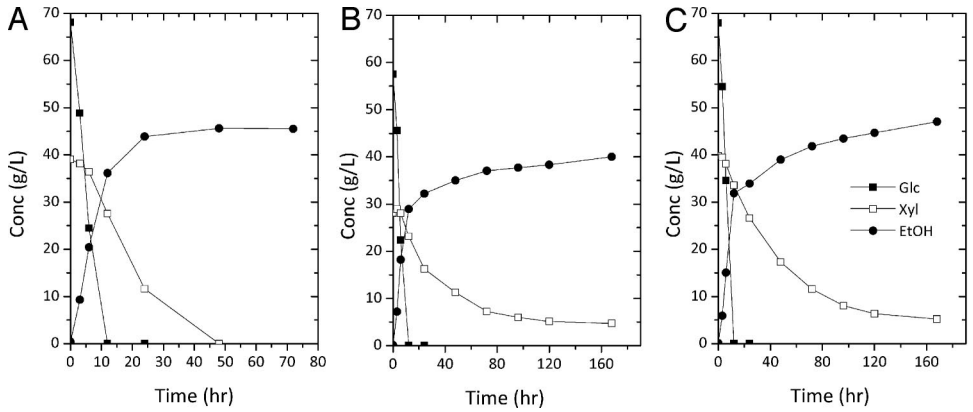

Fig. 2. Fermentation of (*A*) complex media; (*B*) CS-Hydrolysate I; (*C*) CS-Hydrolysate II using *S. cerevisiae* 424A(LNH-ST). Fermentations were initiated with 1.1 g (dry-wt.)/L of 424A(LNH-ST) inoculum, carried out at 30 °C, pH 5.5, and 150 rpm under largely anaerobic condition. The hydrolysates were neither detoxified nor externally nutrient-supplemented.

Table 1. Summary of fermentation parameters for complex media and hydrolysates

	Media*	Monomeric Sugar Conc, g/L		Final EtOH	Volumetric Productivity, g/hr/L			Specific Productivity, g/hr/L/g cells			Metab.	Process	Cell density**,
		Glc	Xyl	Conc, g/L	Glc¶	Xyl¶	EtOH¶	Glc¶	Xyl¶	EtOH¶	Yield‖, %	Yield, %	g dry wt/L
A	YEP†	68.0	39.0	45.6 ± 0.2	−7.3 ± 0.2	−1.1 ± 0.1	0.9 ± 0.0	−6.5 ± 0.3	−2.9 ± 0.3	4.9 ± 0.1	83.6 ± 0.9	83.6 ± 0.9	9.5 ± 0.2
B	CS-Hydrolysate I‡	57.5	28.1	40.0	−5.9	−0.5	0.8	−7.9	−1.8	4.9	92.9	88.5	6.7
C	CS-Hydrolysate II§	68.0	39.8	47.0 ± 0.3	−5.6 ± 0.4	−0.6 ± 0.0	0.8 ± 0.0	−10.6 ± 0.8	−2.0 ± 0.0	5.7 ± 0.1	90.0 ± 0.8	85.6 ± 0.6	6.5 ± 0.1

*Fermentations were conducted at 30 °C, pH5.5 and 150rpm agitation under largely anaerobic conditions, initial cell density was at 1.1 g dry-cell-wt./L.
†5 g/L Bacto Yeast Extract + 10 g/L Bacto Peptone.
‡Hydrolysate from AFEX-treated CS (enzymatically-digested at 6% glucan loading for 96 hr at pH 4.8, 50 °C).
§Hydrolysate from AFEX-treated CS (enzymatically-digested at 6% glucan loading for 96 hr at pH4.8, 50°C) with supplemental sugars of 10 g/L glucose and 12 g/L xylose, which is the monomeric sugar equivalent of the oligomeric sugar content in the hydrolysate.
¶Productivity over the first 6,12 and 24 h, respectively.
‖Metabolic yield calculated based on total consumed glucose and xylose, theoretical EtOH yield was assumed as 0.51 g/g sugar.
**Cell density after 24 hr of fermentation, one unit of absorbance at 600 nm is approximately equal to 0.55 g dry-cell-wt./L

nutrient sources for fermentation (Table S2). With YEP supplementation, washed-CS hydrolysate had a better xylose fermentation (0.20 g/L/hr, 0–96 h) than unwashed-CS. Evidently, even under nutrient-rich conditions, the presence of degradation products affected xylose utilization.

The Effects of Soluble Substances from AFEX-CS on Xylose Fermentation. The relationship between xylose fermentation with the levels of degradation products and nutrient content in fermentation media was further investigated using the wash stream from AFEX-CS. In nutrient-rich conditions, cell growth decreased as the concentration of AFEX-CS wash stream increased. However, the level of specific xylose consumption remained virtually constant at 0.25 g/L/hr/g cell; 0–24 h (Fig. 4*A*). This suggested that xylose utilization was lowered due to the reduced cell mass production in the presence of degradation products, while xylose metabolism in cells was apparently not affected. In nutrient-limiting conditions, where the AFEX-CS wash stream was the sole source of nutrient, cell growth is proportional to the concentration of the wash stream. The relationship between the specific xylose consumption rate and the wash stream concentration exhibited a left-skewed curve with the highest rate (0.15 g/L/hr/g cell, 0–24 h) at 4% solids loading wash stream (Fig. 4*B*). These results imply that, in nutrient-limiting conditions, (*a*) a low level of degradation products result from AFEX pretreatment stimulated xylose metabolism within cells, but the rate of xylose utilization was limited by cell growth (nutrient

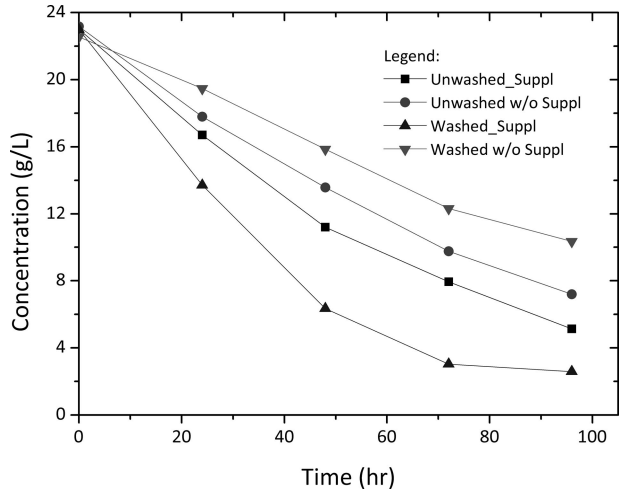

Fig. 3. Effect of washing and nutrient supplementation on xylose consumption in the fermentation of hydrolysates from enzymatically-digested AFEX-treated CS. (Glucose consumption profiles are not shown in this graph.)

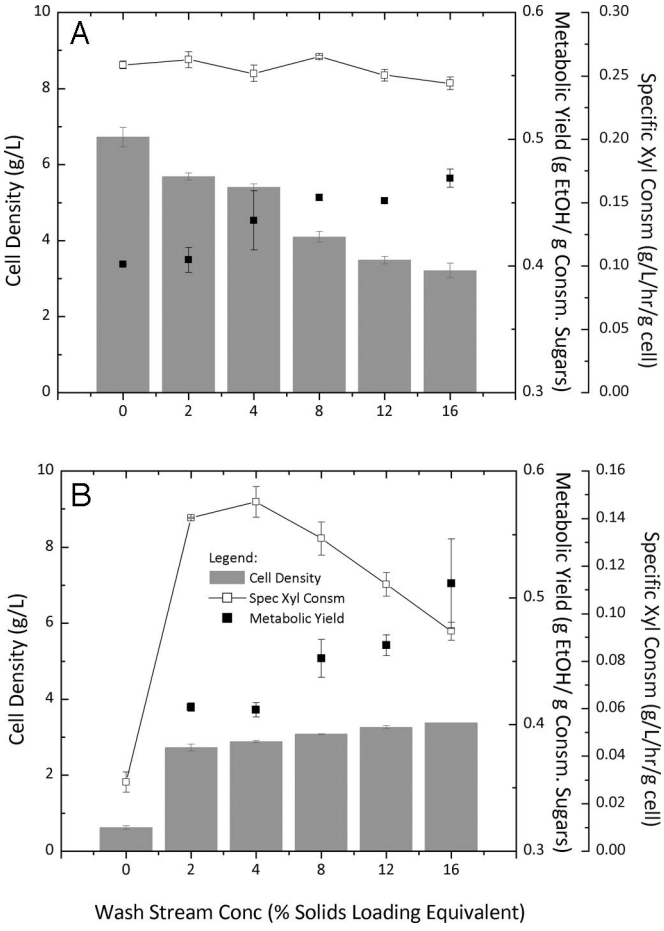

Fig. 4. Effect of AFEX-CS wash stream concentration on cell growth, metabolic yield, and specific xylose consumption rate under (*A*) nutrient-rich and (*B*) nutrient-limiting conditions. Fermentations were initiated with 0.3 g (dry-wt.)/L of 424A(LNH-ST) inoculum, carried out at 30 °C and 150 rpm. Data points presented were at 24 h.

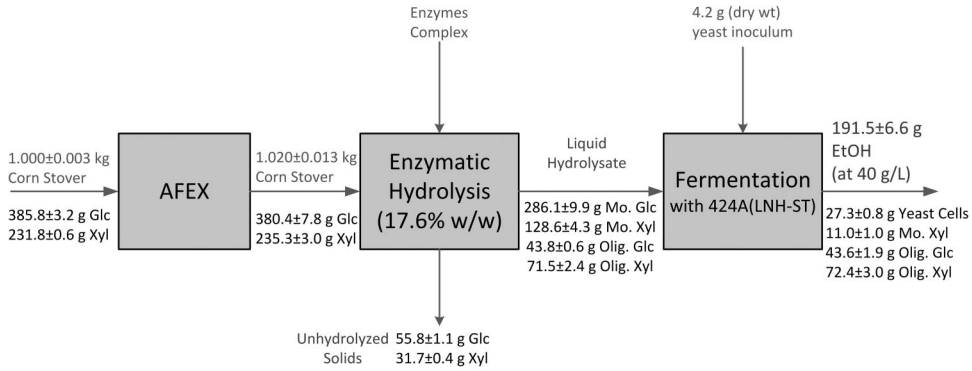

Fig. 5. CS to ethanol mass balance analysis. The analysis was based on AFEX as feedstock pretreatment technology and *S. cerevisiae* 424A(LNH-ST) as the ethanologenic strain; enzymatic hydrolysis was conducted at 6.0% glucan loading (equivalent to 17.6% wt/wt solids loading). Carbohydrate contents in CS are expressed as the hydrated monomers. Glc: Glucose; Xyl: Xylose; Mo: Monomeric; Olig: Oligomeric.

availability) and (*b*) at high wash stream concentrations (≥8% solids loading), inhibition of xylose metabolism was the primary factor affecting xylose utilization. Under both conditions, biomass degradation products resulting from the pretreatment (soluble products in the wash stream) were shown to increase metabolic ethanol yield (Fig. 4 *A* and *B*). Fermentation using a wash stream at 16% solids-loading-equivalent as the sole nutrient source (Fig. 4*B*) achieved comparable cell growth to the YEP-supplementation fermentation (Fig. 4*A*).

Current Status and Bottlenecks of the Technology. Current technology using CS as feedstock, AFEX as the pretreatment technology, and *S. cerevisiae* 424A(LNH-ST) as the ethanologenic strain in Separate Hydrolysis and Fermentation (SHF) with a starting cell density at 1.1 g dry-cell-wt./L was able to achieve 191.5 g EtOH/kg untreated CS (Fig. 5), 60.8% of the theoretical maximum yield, at an ethanol concentration of 40.0 g/L (5.1 vol/vol%) without the need for washing, detoxification, and nutrient supplementation.

During AFEX pretreatment, there was no sugar loss and total material mass was increased by 1–2%, probably due to the ammonia binding onto the biomass (19). The mass balance around the enzymatic hydrolysis step achieved 98.9% and 107.1% closure for glucose and xylose, respectively. In enzymatic hydrolysis at 17.6% solids loading, 85.8% of the total input sugars (glucose and xylose) were hydrolyzed and solubilized, of which, 78.2% was hydrolyzed to their monomers (Fig. 5, Table S3). About two-thirds (62.0%) of the total oligomeric sugars were xylose. Fermentation using 424A(LNH-ST) effectively converted monomeric glucose and xylose with 88.5% ethanol yield. As expected, oligomeric sugars were not used by 424A(LNH-ST).

The three process parameters having the strongest influence on ethanol production economics are yield, titer, and rate. Enzymatic hydrolysis at high solids loading has been identified as the primary bottleneck affecting overall yield and titer for the cellulose-to-ethanol bioconversion. One-third of the total output sugars were oligomers or polymers, which could not be used by 424A(LNH-ST). To improve overall ethanol productivity, efforts should focus on increasing the xylose consumption rate during fermentation.

Discussion

Significance of the Process Integration. Despite abundant published research on ethanol production from lignocellulosic materials, comprehensive system-wide studies with industrially-relevant performance metrics are still lacking. Washing and detoxification steps, although often incorporated to improve the fermentability of the hydrolysate, may well be prohibitively expensive (9). The straightforward cellulosic ethanol technology reported here, which converts sugars from lignocellulosic materials to ethanol without washing, detoxification and nutrient supplementation, is significant in the search for a highly competitive cellulosic ethanol production strategy. A final ethanol titer of 40 g/L, a benchmark concentration for commercial cellulosic ethanol production, was achieved.

Using AFEX as the pretreatment and *S. cerevisiae* 424A(LNH-ST) as the fermenting strain were the key innovations responsible for achieving this progress. Hydrolysate from lignocellulosic biomass is generally regarded to be nutrient-deficient (5). However, our results strongly contradict this perception. The perceived nutrient deficiency is likely due to pretreatment at high temperature and acidic pH followed by washing which degrade and/or remove nutrients. In contrast, nutrients provided through AFEX-pretreated CS were sufficient to support robust yeast growth. The saccharolytic enzyme preparation provides relatively little nutrient value (Fig. S1, II). Cellulosic ethanol technology therefore need not depend on commercial nutrient supplements such as yeast extract or corn steep liquor. Yeast cells can also be produced as a valuable co-product using this platform. A recent publication using *E. coli* KO11 to ferment enzymatic hydrolysate from AFEX-treated CS has also confirmed the general fermentability of CS (11).

Important Steps in Improving Yield and Titer. However, further improvements in overall yield and titer are required to make this technology more commercially-attractive. Results presented herein suggest that efforts to increase the availability of fermentable sugars at high concentrations deserve the highest priority. This can be done through (1) optimization of the enzyme formulation for effective saccharification of both cellulose and hemicellulose; (2) development of ethanologenic strains which consume a wider range of substrates (both mono- and oligosaccharides) and (3) integration of enzymatic hydrolysis and fermentation in the configurations of Simultaneous Saccharification and Co-Fermentation (SSCF) or Consolidated Bioprocessing (CBP) to alleviate sugar inhibition at high solids loading and reduce enzyme dosage. In this report, enzymatic hydrolysis and fermentation have been conducted separately to facilitate understanding of the individual processes. Nevertheless, Simultaneous Saccharification and Fermentation (SSF) was shown to be beneficial compared to Separate Hydrolysis and Fermentation (SHF) (20), despite a lower reaction temperature that was adopted to allow fermentation at the expense of greater efficiency during enzymatic hydrolysis. The full potential of these configurations will be better realized if both hydrolysis and fermentation share common optimal conditions. Developing an

Table 2. Summary of parameters for four experiments examining the effects of pH; temperature; initial cell density; and washing and nutrient supplementation on xylose fermentation of corn stover hydrolysate

Experiment Parameter	Effect of pH	Effect of Temperature	Effect of Initial Cell Density	Effect of Washing and Nutrient Suppl.
Initial pH	3.5, 4.5, 5.5, 6.5	5.5	5.5	5.5
Temperature, °C	30	25,30,35,37	30	30
Initial Cell Density, OD600 nm	0.5	0.5	0.5, 6.0, 12.0, 18.0	0.5
Washing on CS	No	No	No	Yes (Minimal Washing)
Nutrient Supplementation	No	No	No	Yes (10 g/L Yeast Extract, 20 g/L Peptone)
Relevant Figure	Fig. 1*A* and *B*	Fig. 1*C*	Fig. 1*D*	Fig. 3

oligosaccharide-utilizing strain will be particularly advantageous for AFEX-centered cellulose technology as a sizeable portion of sugars in the hydrolysate are oligomers. As a near-future projection, the overall yield could be increased to 251.4 g EtOH/kg untreated CS with final titer of 52.5 g/L or 6.7%(vol/vol) (Fig. 1*B*) if complete utilization of soluble sugars is achieved (Table S4).

Interaction between Degradation Products and Xylose Fermentation. Xylose fermentation was more susceptible than glucose fermentation to inhibition by the degradation products from AFEX pretreatment, resulting in prolonged fermentation. However, as our results indicate, the effects of AFEX degradation products on fermentation are complex. Generally, AFEX degradation products increase metabolic yield by reducing the formation of fermentation by-products. Certain degradation compounds have been postulated to act as electron acceptors to provide redox balance in xylose metabolism (10, 21). An equally important benefit of the degradation products from AFEX-pretreated biomass is that they increase metabolism of sugars which translates into higher specific ethanol production rates. Degradation compounds such as organic acids and 4-hydroxybenzaldehye have been shown to stimulate fermentation when present at moderate levels (22, 23). Nonetheless, they inhibit the cells from propagating to a density warranted by available nutrients. In a commercial setting, a two-fold increase from the existing xylose consumption rate is likely required. Instead of supplementing with excess nutrients or detoxifying hydrolysates, improving xylose fermentation through cell recycle to increase cell density might be a more cost-effective solution.

Materials and Methods

Corn Stover (CS). CS was supplied by the National Renewable Energy Laboratory (Golden, Colorado). It was milled and passed through a 4 mm screen. The moisture content was approximately 7% (total weight basis). The milled CS was kept at 4 °C for long term storage. This CS contains 34.1% cellulose, 20.4% xylan, 3.3% arabinan, and 2.3% protein on a dry weight basis.

AFEX Pretreatment. The AFEX pretreatment was conducted in a 2.0 L pressure vessel (Parr) equipped with thermocouples and a pressure sensor. The vessel was heated to 100–110 °C before 240 g of prewetted CS at 60% moisture (dry weight basis) was loaded. The lid was bolted shut. Concurrently, 150 g anhydrous ammonia was added to a separate 500 ml stainless steel cylinder (Parker Instrumentation) and heated until the gas pressure reached 4.48 MPa (650 psi). Heated ammonia was then transferred into the reactor to initiate the reaction. After 15 min, the pressure was released through an exhaust valve. The initial and final temperatures of the pretreatment were 130 ± 5 °C and 110 ± 5 °C, respectively. After AFEX treatment, pretreated CS was air-dried overnight under a fume hood. The basic schematic for the AFEX-apparatus was as described (13).

Minimal Washing. Washing of CS was conducted by spraying distilled water on AFEX-treated CS at a ratio of 1 g dry CS to 5 ml of water (20% solids loading). The water content of the wetted CS was reduced to 77 ± 2% (total weight basis) by using an in-house manufactured press. Washed CS solids were then enzymatically-hydrolyzed. This wash stream was used for fermentation studies.

Enzymatic Hydrolysis. The AFEX-treated CS was enzymatically-hydrolyzed by commercial enzymes mixtures. The cellulase mixture consisted of Spezyme CP [86.7 ml/kg CS; 15 FPU/g cellulose] and Novozyme™ 188 [43.7 ml/kg CS; 32 *p*NPGU/g cellulose]. The hemicellulase mixture was Multifect Xylanase [12.7 ml/kg CS] and Multifect Pectinase [8.9 ml/kg CS]. The spectrum of activities for the commercial enzymes were as reported (24). The Spezyme™ and Multifect enzymes were obtained from Genencor Inc. and Novozyme™ 188 was purchased from Sigma-Aldrich Co. These enzyme mixtures, unless otherwise stated, were used in all hydrolysis experiments. The cellulose loading for the hydrolysis was kept at 6.0% by weight (60 g of cellulose per 1 kg of hydrolysis mixture) which corresponds to 17.6% and 16.2% solids loading, in unwashed and washed AFEX-CS respectively. The reaction was carried out for 96 h at pH 4.8 (0.05 M phosphate buffer), 50 °C, and 250 rpm agitation. Each hydrolysis was conducted in a 1.0 L baffled flask with 500 g total saccharification mixture. Chloramphenicol (Cm) was added to a final concentration of 50 mg/L to minimize the risk of contamination. After 96 h, the hydrolysis mixture was centrifuged twice at 5,000 g for 30 min to separate the liquid solution from unhydrolyzed solids. The supernatant (not sterilized) was used for fermentation.

Microorganism and Seed Culture Preparation. Xylose-fermenting *S. cerevisiae* 424A(LNH-ST) was obtained from Dr Nancy W. Y. Ho from Purdue University (West Lafayette, IN) through Material Transfer Agreement (MTA06–119). Details of the metabolic engineering of this yeast have been reported (16, 17). To prepare seed culture, the strain was grown on YEP (5 g/L yeast extract, 10 g/L peptone, 20 g/L glucose) plate for 1–2 days at 30 °C. The cells were transferred to liquid YEP media supplemented with 50 g/L glucose in an unbaffled flask. The 424A(LNH-ST) seed was grown overnight at 30 °C, 150 rpm agitation, under a largely anaerobic conditions. The cell density of a typical 424A(LNH-ST) overnight seed culture reached 15 units absorbance at 600 nm.

Fermentation. Fermentations were conducted at a working volume of 70 ml in a 250 ml unbaffled flask at 30 °C, initial pH 5.5, 150 rpm agitation. A designated volume of seed culture was centrifuged (15,000 × *g*) for 5 min and the yeast cell pellet was resuspended into the media to initiate fermentation. The flasks were capped with rubber stoppers pierced with a needle to vent carbon dioxide formed during fermentation. Samples were taken during the course of fermentation. Cell density was measured using a spectrophotometer at 600 nm (DU Series 700 UV/Vis, Beckman Coulter). One unit of absorbance is approximately equal to 0.55 g (dry-wt-cell)/L. Glucose, xylose, glycerol, and ethanol profiles were measured using HPLC as previously described (11).

Complex Media and Hydrolysates. Three different fermentation media were prepared: (*a*) Complex media (YEP + 70 g/L glucose + 40 g/L xylose); (*b*) Hydrolysate I: AFEX-CS hydrolysate at 6.0% glucan loading; (*c*) Hydrolysate II: AFEX-CS hydrolysate at 6.0% glucan loading with supplemental sugars (10 g/L glucose + 12 g/L xylose), which is the monomeric sugar equivalent of the oligomeric sugar content in the hydrolysate. The initial pH of the hydrolysates was adjusted to 5.5 using reagent grade KOH. The starting cell density of fermentation was at 2.0 (OD 600 nm) which is approximately 1.1 g dry-cell-wt./L. Hydrolysates were fermented without prior detoxification or nutrient supplementation. Volumetric productivities of glucose, xylose, and ethanol were calculated from their concentration gradients over the first 6, 12, and 24 h of fermentation, respectively. Specific productivities (g/L/hr/g cell) were calculated by dividing volumetric productivities by the respective values for dry cell mass. Metabolic ethanol yield was estimated based on total consumed glucose and xylose. The theoretical maximum yield was 0.51 g EtOH/g sugar.

Effect of pH, Temperature, Initial Cell Density, Nutrient Supplementation and Washing on Xylose Fermentation in Hydrolysate. Several fermentations were carried out to investigate the effect of (*a*) pH, (*b*) temperature, (*c*) initial cell density, (*d*) nutrient supplementation, and (*e*) minimal washing on xylose

utilization in the cofermentation of hydrolysate. Enzymatic hydrolysis was carried out with the cellulase mixture only. Initial glucose and xylose concentrations in the hydrolysate were 55 ± 2 g/L and 23 ± 1 g/L, respectively. Other experimental details were as listed in Table 2.

Wash Stream (With or Without YEP Supplementation). The wash stream (See *Minimal Washing*) was used to investigate the effects of soluble compounds on AFEX-CS on fermentation. Each fermentation medium contains the wash stream, 10 g/L glucose, 40 g/L xylose, and 50 mg/L Cm. YEP (5 g/L Yeast Extract and 10 g/L Peptone), was added to create nutrient-rich conditions. The final concentrations of the wash stream in the fermentation media ranged from 0 to 16% solids-loading-equivalent. The reaction was carried out at 15 ml working volume in 20 ml screw-capped vial. Three glass beads (1 cm in diameter) were added into each vial to aid stirring. Cell density and specific xylose consumption over the first 24 h of fermentation were estimated.

CS to Ethanol Analysis. To construct a CS to ethanol mass balance analysis, sugars (glucose and xylose in monomeric, oligomeric, and polymeric forms) and ethanol content were measured before and after each process, i.e., pretreatment, enzymatic hydrolysis, and fermentation.

Detailed experiment procedure on mass balance construction was as described in *SI Text*.

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1. Greene N, *et al.* (2004) Growing energy: How biofuels can help end America's oil dependence. (Natural Resources Defense Council).
2. Farrell AE, *et al.* (2006) Ethanol can contribute to energy and environmental goals. *Science* 311:506–508.
3. Lynd LR, *et al.* (2008) How biotech can transform biofuels. *Nat Biotechnol* 26:169–172.
4. Wyman CE, *et al.* (2005) Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover. *Bioresour Technol* 96:2026–2032.
5. Kadam KL, Newman MM (1997) Development of a low-cost fermentation medium for ethanol production from biomass. *Appl Microbiol Biotechnol* 47:625–629.
6. Sedlak M, Ho NWY (2004) Production of ethanol from cellulosic biomass hydrolysates using genetically engineered *Saccharomyces* yeast capable of cofermenting glucose and xylose. *Appl Biochem Biotechnol 114*:403–416.
7. Palmqvist E, Hahn-Hagerdal B (2000) Fermentation of lignocellulosic hydrolysates. I: Inhibition and detoxification. *Bioresour Technol* 74:17–24.
8. Houghton J, Weathermax S, Ferrell J (2006) *Breaking the Biological Barriers to Cellulosic Ethanol: A Joint Research Agenda* (Department of Energy)
9. von Sivers M, Zacchi G, Olsson L, Hahn-Hagerdal B (1994) Cost analysis of ethanol production from willow using recombinant *Escherichia coli. Biotechnol Progr* 10:555–560.
10. Karhumaa K, Sanchez RG, Hahn-Hagerdal B, Gorwa-Grauslund MF (2007) Comparison of the xylose reductase-xylitol dehydrogenase and the xylose isomerase pathways for xylose fermentation by recombinant Saccharomyces cerevisiae. *Microb Cell Fact* 6:.
11. Lau MW, Dale BE, Balan V (2008) Ethanolic fermentation of hydrolysates from ammonia fiber expansion (AFEX) treated corn stover and distillers grain without detoxification and external nutrient supplementation. *Biotechnol Bioeng* 99:529–539.
12. Wyman CE, *et al.* (2005) Coordinated development of leading biomass pretreatment technologies. *Bioresour Technol* 96:1959–1966.
13. Teymouri F, Laureano-Perez L, Alizadeh H, Dale BE (2004) Ammonia fiber explosion treatment of corn stover. *Appl Biochem Biotechnol* 115:951–963.
14. Xu F, *et al.* (2008) Partition of enzymes between the solvent and insoluble substrate during the hydrolysis of lignocellulose by cellulases. *J Mol Catal B: Enzym* 51:42–48.
15. Jeffries TW, Jin YS (2004) Metabolic engineering for improved fermentation of pentoses by yeasts. *Appl Microbiol Biotechnol* 63:495–509.
16. Ho NW, Chen Z, Brainard AP, Sedlak M (1999) Successful design and development of genetically engineered Saccharomyces yeasts for effective cofermentation of glucose and xylose from cellulosic biomass to fuel ethanol. *Adv Biochem Eng Biotechnol* 65:163–192.
17. Chen JH, Ho CT (1997) Antioxidant activities of caffeic acid and its related hydroxycinnamic acid compounds. *J Agric Food Chem* 45:2374–2378.
18. Chundawat SPS, Venkatesh B, Dale BE (2007) Effect of particle size based separation of milled corn stover on AFEX pretreatment and enzymatic digestibility. *Biotechnol Bioeng* 96:219–231.
19. Solaiman SG, Horn GW, Owens FN (1979) Ammonium Hydroxide Treatment on Wheat Straw. *J Anim Sci* 49:802–808.
20. Ohgren K, Bura R, Lesnicki G, Saddler J, Zacchi G (2007) A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. *Process Biochem* 42:834–839.
21. Sonderegger M, *et al.* (2004) Fermentation performance of engineered and evolved xylose-fermenting Saccharomyces cerevisiae strains. *Biotechnol Bioeng* 87:90–98.
22. Pampulha ME, Loureirodias MC (1989) Combined Effect of Acetic-Acid, Ph and Ethanol on Intracellular Ph of Fermenting Yeast. *Appl Microbiol Biotechnol* 31:547–550.
23. Zaldivar J, Martinez A, Ingram LO (1999) Effect of selected aldehydes on the growth and fermentation of ethanologenic Escherichia coli. *Biotechnol Bioeng* 65:24–33.
24. Dien BS, *et al.* (2008) Enzyme characterization for hydrolysis of AFEX and liquid hot-water pretreated distillers' grains and their conversion to ethanol. *Bioresour Technol* 99:5216–5225.