An integrated paradigm for cellulosic biorefineries: Utilization of lignocellulosic biomass as self-sufficient feedstocks for fuel, food precursors and saccharolytic enzyme production

Title:

AN INTEGRATED PARADIGM FOR CELLULOSIC BIOREFINERIES: UTILIZATION OF LIGNOCELLULOSIC BIOMASS AS SELF-SUFFICIENT FEEDSTOCKS FOR FUEL, FOOD PRECURSORS AND SACCHAROLYTIC ENZYME PRODUCTION

Authors:

Ming W Lau[a], Bryan D Bals[a], Shishir P S Chundawat[a], Mingjie Jin[a], Christa Gunawan[a], Venkatesh Balan[a], A Daniel Jones[b] and Bruce E Dale[a]

Affiliations:

[a]Department of Chemical Engineering and Materials Science, DOE Great Lakes Bioenergy Research Center, Michigan State University, 3900 Collins Rd, Lansing, MI 48910.
[b]Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI-48824

Corresponding Author:

Bruce E Dale
Department of Chemical Engineering and Materials Science, Michigan State University, 3900 Collins Rd, Lansing, MI 48910.
Email: bdale@egr.msu.edu
Tel: 517-353-6777
Fax: 517-432-1105

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Abbreviations :

AFEX: Ammonia Fiber Expansion, CS: Corn Stover, CSL: Corn Steep Liqour

ABSTRACT

Simultaneously achieving economic, environmental and social sustainability is a major challenge for the emerging renewable fuel industry. We approach this problem by demonstrating a cellulosic biorefinery paradigm which produces ethanol and food precursors using lignocellulosic biomass as the exclusive source for carbohydrate and minerals. Enzymatic hydrolysate from Ammonia Fiber Expansion (AFEX)-pretreated corn stover at 18%w/w solids loading was found to be nutrient-rich. This hydrolysate was fermented completely within 48hr in two stages to produce ethanol and native yeast cells. An In-house saccharolytic enzyme production using AFEX-pretreated corn stover as carbohydrate source greatly reduces the dependence on commercial enzymes. The inducer mixture is 2.5-7 times more potent than lactose, a common enzyme inducer. Economic analysis indicates that the proposed paradigm is substantially more cost-effective relative to the 2005 NREL model. This improvement is largely attributed to the native yeast cells co-production and the reduction of enzyme cost through the in-house production.

INTRODUCTION

Lignocellulosic biomass, the most abundant organic matter on earth, offers an immense potential for renewable fuels and chemicals production in a sustainable fashion[1]. This biomass can be used as the carbon source for various fermentation products such as ethanol [2], succinic acid [3], lactic acid [4] and long chain hydrocarbons [5]. The key challenge of the commercialization of this nascent cellulosic technology is the cost-competitiveness of the process relative to its petrochemical routes; hence the effort to reduce the overall production cost is the center of extensive research and development. Reducing the costs associated with saccharolytic enzymes (cellulase), detoxification of hydrolysate from pretreated biomass and nutrient supplementation during ethanol fermentation deserves close attention as they are projected to be 45% of the total processing cost of cellulosic ethanol production [6].

Besides carbohydrate and lignin, plant materials contain nutrients such as protein and minerals that might be utilized during fermentation [7]. However, the potential of these biomass constituents to support fermentation is poorly characterized and understood. Therefore, we investigate the nutrient content of the hydrolysate from corn stover, a representative substrate for cellulosic biofuels production, from a system-wide perspective.

Utilizing the nutrient content inherently present in corn stover, we develop an integrated biorefinery approach which features (i) efficient cellulosic ethanol production, (ii) native yeast cells as a co-product and (iii) in-house enzyme production using corn stover as the exclusive carbohydrate source. A techno-economic analysis is conducted to better understand the cost-effectiveness of the proposed scheme relative to the conventional approach [8]. Ultimately, we seek to demonstrate how a lignocellulosic biomass can be used to support fuel and food production in a potentially cost-attractive fashion.

RESULTS

Enzymatic hydrolysis and nutrient content of AFEX-treated corn stover (AFEX-CS) hydrolysate

The amino acid, trace elements and vitamin contents of the enzymatic hydrolysate were analyzed and quantified. The enzymatic hydrolysate contained 800±50 mg/L ammonia and 1231±44 mg total amino acids, of which 16% was in the form of free amino acids **(Figure 1a)**. Glutamic acid, glycine and alanine are three most abundant amino acids found in the hydrolysate. The amino acid concentration is higher than that of a typical malt wort for brewery applications (800-900 mg/L total amino acids) [9].

Ten trace elements known to be important for microbial growth were in excess compared to values suggested by Walker for yeast fermentation [10]. Magnesium (269±6mg/L), a limiting trace element in most industrial fermentations [10], was found to be at a sufficient level **(Figure 1b)**. Respecting vitamin content, the concentrations of panthothenic acid, pyridoxine, nicotinic acid, and biotin exceeded their levels in a typical malt wort [9]. The level of thiamine (0.4 µM) is slightly lower than the reference concentration range (0.57-2.83 µM). AFEX-CS is the predominant source of protein, trace elements and vitamins in the enzymatic hydrolysate. The contribution of commercial enzymes to the nutrient levels is very low **(Figure 1b)**.

AFEX-CS at 18% solids loading (6% glucan loading) was enzymatically-hydrolyzed using commercially-available enzymes. About 85% of the total carbohydrate was hydrolyzed and solublized in the liquid stream (Figure 1C), achieving a total soluble sugars concentration of 110 g/L **(Figure 1c)**.

The mass balance with respect to total nitrogen (N), phosphorus (P) and potassium (K) confirmed that 80-82% of the total nitrogen and potassium from the AFEX-CS was solubilized into the liquid stream (hydrolysate). However, most of the phosphorus content (64%) was left on the residual solids following enzymatic hydrolysis **(Figure 1d)**.

Empirical fermentability test using water extract from AFEX-treated corn stover (CS) and rice straw (RS)

A water extract at 9.0% solids loading equivalent, which partially solubilized the biomass constituents, was generated from both AFEX-CS and AFEX-RS. Glucose was added as carbon source to a final concentration of 100 g/L. After 48 hr of fermentation using *S. cerevisiae*

424A(LNH-ST), glucose in both water extracts was completely consumed. The yeast final yeast cell density (at 48 hr) was 4.5-5.5 g dry.wt/L **(Figure 2 a,b)**. These values were greater than fermentation in yeast nitrogen base (YNB; 13.7 g/L), but not as high as in yeast extract + peptone (YEP; 5 g/L yeast extract + 10 g/L peptone). These results suggested that nutrient levels greater than 9% solids loading equivalent from the pretreated CS and RS are sufficiently high to support yeast fermentation. Furthermore, this suggests that nutrients are in excess in a higher solid loading hydrolysate, and thus could support multiple fermentations.

Two-stage ethanol fermentation using native and recombinant Saccharomyces cerevisiae

To further investigate the potential of using corn stover at high solids loading to support multiple fermentations, AFEX-CS hydrolysate from an 18% w/w loading saccharification process was fermented first by native *S. cerevisiae* (ATCC 4124) for 15 hr, after which the yeast cells were separated from the hydrolysate by sedimentation for 3 hr **(Figure 2c)**. In the second stage, high cell density (11.25 g dry wt./L) of recombinant *S. cerevisiae* 424A(LNH-ST) was inoculated to ferment the remaining sugars **(Figure 2c)**. The glucose fermentation proceeded at a rate of 3.2 g/L/hr (0-15hr) and was completed at 18hr. After sedimentation, 4.1 g dry wt./L yeast cells were collected. In the second stage fermentation at high cell density, 87% of the xylose was consumed within the first 24hr of the recombinant *S. cerevisiae* inoculation. A final ethanol concentration of 39 g/L was achieved with metabolic ethanol yield at 92.1% of the theoretical maximum **(Figure 2e)**.

The recombinant *S. cerevisiae* 424A(LNH-ST) cells were recycled and used in three subsequent cycles of fermentation **(Figure 2d)**. In essence, xylose fermentation using the recycled yeast cells achieved a similar efficiency as that of fresh cells **(Figure 2e)**. This effectively reduced the need for fresh cells in the successive batches of fermentation without significantly decreasing the xylose fermentation rate, a potentially significant cost savings.

In-house enzyme production

Trichoderma reesei RUT-C30 was first cultured in media consisting of 2% w/w corn steep liquor and 20 g/L glucose for 36hr. The saccharolytic enzymes were induced for 96 hr by a mixture of solids and liquid extract from AFEX-CS **(Figure 3 a&b)**. The concentration of saccharolytic enzyme produced was 2.7 g/L which can support an 18% w/w enzymatic hydrolysis at 15 mg enzyme protein per gram of corn stover. About 13% of the protein provided through CSL was converted to saccharolytic enzymes. The *T. reesei* broth was diluted by a factor of 1:6; the

diluted broth was then used to hydrolyze AFEX-CS at 1.0% cellulose loading for 24 h with or without additional enzymes (Accellerase 1000). Enzymatic hydrolysis using the 1:6 diluted in-house *T. reesei* broth achieved a total soluble sugar yield of 15.0 g/L within 24hr (85.6% of theoretical maximum yield) **(Figure 3c)**. This total sugar yield level is comparable to that of the standard enzyme mixture based on commercial enzymes (15.7 g/L; 89.4%) **(Figure 3d)** suggesting that the in-house enzyme production approach can support effective enzymatic hydrolysis at high solids loading. If monomeric sugars are desired, an Accellerase loading of 1.5-2.0 mg protein/g corn stover **(Figure 3d)** is required to achieve similar yields as those of the standard mixture, in which 13.7 g/L of total monomeric glucose and xylose was obtained.

We also found that the AFEX-CS mixture used for the enzyme induction was 2.5-7 times more potent than lactose on a same sugar weight basis **(Figure 3b)**. Enzymatic hydrolysis based on *T. reesei* broth achieved 10.4 g/L (59% of total yield) net increase in sugar yield when AFEX-CS was used for induction instead of lactose on a same sugar-equivalent basis **(Figure 3b)**.

In total, 79 proteins were identified from the secretome of *Trichoderma reesei* (RUT-C30) induced using AFEX treated corn stover, out of which 31 were carbohydrate active enzymes (CAZymes; www.cazy.org). There was no difference in the abundance (within 2-fold) of most cellulases (e.g. Cel7A, Cel6A, Cel74A, Cel7B, Cel5A, and β-mannase) expressed using AFEX treated corn stover or lactose (Table 1). However, AFEX treated corn stover resulted in a significant increase (> 2-fold in protein abundance) in expression of several endocellulases (Cel61A, Cel12A) and hemicellulases (e.g. β-xylosidase, endoxylanases, α-arabinofuranosidases, CIP2, acetyl xylan esterases, α-glucuronidase, polygalaturonase) that were previously missing for lactose-induced *T. reesei*. The relative abundance of the proteins expressed using either AFEX treated corn stover and lactose are shown in **Figure 3e** (with details provided in Supplementary Table 4, 5).

Process flow diagram (PFD)

Based on the results from this study on the two-stage ethanol fermentation and *Trichoderma reesei* RUT-C30 saccharolytic enzyme fermentation, an integrated biorefinery scheme using AFEX-pretreated corn stover as the exclusive source for carbohydrate and minerals was proposed **(Figure 4a)**. This proposed scheme included (i) core bioprocessing and bioconversion units (CBB) for ethanol and native yeast cells production, (ii) in-house enzyme production using *T. reesei* and (iii) auxiliary units that support the CBB and enzyme production units.

The proposed design is as follows. On a basis of 1000 g untreated corn stover, 1020 g of AFEX-pretreated corn stover (CS) was produced. One-third (33.3%) of the AFEX-CS was washed at 18% w/w solids loading, the liquid extract (Stream 5) was used for enzyme induction. The washed corn stover, in which the total carbohydrate was reduced by 7%, was fed to the unwashed AFEX-CS (Stream 2) and the combined stream was enzymatically-hydrolyzed. After 4 days of enzymatic hydrolysis, the liquid hydrolysate was separated from the solids by a Pneuma-press. The sugars in the solids-free liquid were fermented to ethanol using the two stage ethanol fermentation where native yeast cells were produced at a yield of 20.8 g dry yeast cells/1.0 kg untreated CS. The soluble sugars in the pressed residual solids were further diluted to generate a sugar stream for seed culture preparations for *Trichoderma reesei* RUT-C30 and *Saccharomyces cerevisiae*. Taking into account the carbohydrate streams used for preculture preparation and induction, 10% of the carbohydrate in the input untreated corn stover was used for enzyme production.

Techno-economic analysis based on proposed scheme

To determine the potential cost-attractiveness of this approach to biomass refining, a techno-economic analysis of the proposed scheme was created and compared to the conventional approach to ethanol production. This analysis is based on the model developed by NREL [11, 12] and adapted to AFEX pretreatment [8]. Changes in the NREL model were only made in the section devoted to biological conversion; it was assumed that all upstream and downstream processes would be the same. Hydrolysis yields, enzyme, ethanol, and yeast production, and consumption of nutrients were all estimated from experimental data, with results similar to those reported here and elsewhere [2]. The ethanol yield used in the analysis, 276 L/MT, was higher than what is reported both here and in Lau and Dale [2]. This projection was based on the result from the fedbatch fermentation by *Thermoanaerobacterium saccharolyticum* ALK2 on AFEX-CS hydrolysate in which about 60% of the cello-oligosaccharides were consumed during fermentation (Supplementary Fig. 1), increasing the overall yield from 246 L/MT to 276 L/MT.

For the changes in the process design, assumptions on power use and equipment and size were taken from the NREL models wherever possible and estimated from literature values when appropriate. More details on the economic model can be found in the Supplementary Methods.

With the proposed changes in the biological conversion operation, the profit per Mg initial feedstock is $27.64 compared to a loss of $7.31 per Mg, essentially allowing ethanol production to become profitable. The ethanol selling price was assumed to be $0.45/L ($1.70/gal) and the corn stover delivered cost was $65/Mg. Because of the additional costs of cellulase fermentation, the total project investment increases by ~15% over the conventional approach.

Likewise, revenue from ethanol production decreases, as some sugars are used for cellulase fermentation and yeast production, and less electricity can be sold to the grid due to the requirements of additional presses and agitation for the cellulase fermenters. However, the large reduction in enzyme costs (76% of the enzymes are produced in-house in this model) and additional revenue from yeast production is more than enough to offset these additional expenses. Of the two technologies considered, yeast recycling and co-production has a larger impact on profitability than enzyme production. If only enzyme production is considered, then the profit is $7.59 per Mg feedstock compared to $13.88 per Mg if only yeast co-production and recycling is considered.

From this analysis, it appears that the cellulase fermentation is the most expensive to implement of the technologies suggested. A total of 14 fermenters are required for cellulase production, as the maximum allowable size is 1000 m^3, less than that of the hydrolysis and ethanol fermentation tanks [12]. In addition, the air compression package and the agitators necessary for aerobic fermentation approximately double the capital cost of the fermenters, while agitators for the anaerobic ethanol tanks are less expensive and require less electricity. The settling tanks for yeast recycling or co-production do not add a significant expense, particularly as they also allow a decrease in fermentation time, thereby decreasing the capital cost of fermenters. The cost of solid-liquid separation equipment necessary for these two technologies is dominated by the Pneumapress package, which is also present in the conventional approach to ethanol production.

Figure 5 also shows the effect of changing variables on the profitability of the proposed approach. Both yield and selling price of ethanol dominate the economics of this approach, as ethanol accounts for over 80% of the revenue in the proposed approach. Likewise, the selling price of native yeast can have a major impact, as the overall margin in the base case ($28/Mg) is similar to the total revenue of yeast ($22/Mg feedstock). Because the true value of this yeast is unknown, more information is needed to determine if co-production is economically viable. Interestingly, the cost of enzymes does not greatly impact the profit, as a 33% increase in the price translates to a 10% decrease in profit. Thus, despite being a major unknown factor in cellulosic ethanol production, by producing the majority of enzymes on site the influence of enzyme costs decreases. Other major design considerations, including xylose fermentation time, *T. reesei* fermentation residence time, and nutrient requirements for *T. reesei* fermentation did not large impact on the profitability of the refinery, suggesting that the general approach is feasible and robust.

DISCUSSION

Improving overall economics of cellulosic ethanol production is a major challenge for the commercialization of cellulosic biofuels. Based on economic analysis by NREL[8], the costs associated with exogenous enzymes, detoxification and nutrient supplementation accounted for 45% of the total operating costs. Co-product generation, such as electricity production through lignin residues, is shown to be important factor to improve the overall economics.

Nutrients present in corn stover, which were solubilized in the liquid hydrolysate after AFEX pretreatment and enzymatic hydrolysis at high solids loading, are sufficient to support growth for ethanol fermentation without the need for exogenous nutrient supplementation. Furthermore, AFEX-corn stover could support fermentation activities beyond ethanol production because minerals are present in excess. Combining these concepts, an integrated biorefinery scheme was formulated to utilize cellulosic biomass (corn stover) for both ethanol and enzyme productions. The central features of this integrated biorefinery are (i) elimination/reduction in the requirements for nutrient supplementation and detoxification while achieving effective ethanol fermentation, (ii) reduction in the overall cost of saccharolytic enzymes through in-house production and (iii) generation of native yeast cells as a co-product.

Based on the experimental results, AFEX-corn stover can serve as the sole source of carbohydrate and minerals for the integrated biorefinery. Corn steep liquor is required as a protein supplement primarily for enzyme production. The overall fermentation can essentially be completed within two days with effective recycle of xylose-fermenting recombinant *S. cerevisiae* to substantially reduce the requirement for fresh cells inoculum on successive batches of fermentation. This has been achieved without washing or detoxification, as described in a previous report[2].

Recent work has shown that hemicellulases and other accessory enzymes are critical to improving digestibility of pretreated lignocellulosic biomass which facilitates further reduction of the total enzyme loading during hydrolysis [13, 14]. Most commercial enzymes are deficient in hemicellulases and other accessory enzymes necessary to hydrolyze AFEX pretreated lignocellulosic biomass. Sepharose and lactose, which are known to be potent inducers of *Trichoderma reesei* cellulases[5], have been used traditionally for inducing industrial-scale production of biomass degrading enzymes. However, our results show that AFEX-treated lignocellulosic biomass (or water soluble extractives isolated from it) is a more potent inducer for cellulases and hemicellulases that were either absent or were present at low abundances

when expressed using lactose as the sole inducer. Further improvements to industrial fungal strains to optimize utilization of pretreated lignocellulosic biomass as the sole carbon source for production of biomass degrading enzymes, in a cost-effective manner, would greatly reduce the cost of cellulosic biorefineries.

Genencor Inc and Novozymes Inc project that saccharolytic enzymes would cost 0.50 USD per gallon of cellulosic ethanol produced [15] under current technology. This enzyme cost, has been reduced by one or two orders of magnitude in the past decade, but still persists as a major cost hurdle for cellulosic technology. The in-house enzyme production approach demonstrated herein take advantage of the abundant cellulosic biomass as the source of carbohydrate and minerals, eliminating the requirement for expensive substrates such as lactose or sepharose while achieving superior enzyme induction. Cello-oligosaccharides are known to induce cellulase secretion from saccharolytic microorganisms such as *Trichoderma sp* and *Aspergillus sp* [16]. Our laboratory also previously reported that the cell-wall extractives [17, 18] from AFEX-CS at moderate level (3-8% solids loading equivalent) can stimulate microbial growth at least for *S. cerevisiae* [2] and *E. coli* [19]. The combination of the potency of cello-oligosaccharides and the stimulatory effect of degradation products on microbial growth are thought to be the key factors leading to the strong enzyme induction by the AFEX-CS mixture relative to lactose.

The enzyme titer produced by "in-house" fermentation, albeit not relevant for stand-alone commercial enzyme production, is sufficient to support high solids loading (at least 18% w/w solids loading) enzymatic hydrolysis at enzyme loading of 15 mg protein/g corn stover. Therefore, the enzyme mixture requires no further concentration, and no addition of protein stabilizer and/or preservatives to prolong the shelf life of the enzymes. According to our economic modeling, the in-house production approach can substantially reduce the overall cost of enzymes after taking into account the increased capital and operating costs associated with *T. reseei* fermentation.

In this proposed approach, native yeast from the first stage ethanol fermentation is a co-product in addition to the electricity generated from lignin residue. Various commercial applications have been developed to exploit the nutritional and functional properties of yeast. Besides food and animal feed industries, other specialty products from yeast such as invertase, beta-glucans, phospholipids and ergosterol have been pursued to further increase the value of yeast cells [20, 21]. Currently, yeast cell production competes with biofuels production because both share similar raw materials such as molasses. Therefore, the proposed biorefinery approach creates a scenario in which the growth of the biofuels industry can enhance the production of food precursors.

While further issues pertaining to large scale production need to be addressed, the proposed integrated biorefinery approach presents a sustainable solution to produce biofuels in an economically-attractive fashion. This approach is very likely applies to various important cellulosic feedstocks including rice straw, a highly abundant agricultural residue in many countries that contains nutrient levels similar to corn stover **(Figure 2 a,b)**. In this scheme, native yeast serves as an agent to capture and concentrate the nutrients from plant biomass while producing ethanol as fuel. However, a solid-liquid separation must precede fermentation to facilitate yeast cell separation from broth. Therefore, achieving these advantages requires that the bioconversions be completed in the Separate Hydrolysis and Fermentation (SHF) mode, rather than the Simultaneous Saccharification and Co-fermentation (SSCF) mode, which is viewed as a more economical bioconversion approach for cellulosic ethanol production[22].

Biomass hydrolysate from AFEX-corn stover at high solids loading is nutrient-rich and highly fermentable. We found that it contains a nutrient level similar malt wort for beer production[9]. This also contrasts with the general perception that an enzymatic hydrolysate from biomass must be preceded by detoxification and nutrient supplementation to improve its general fermentability. In our opinion, this perception is due to the nature of acidic pretreatment in which detoxification (such as overliming) and/or washing of pretreated materials to remove inhibitory compounds renders the pretreated biomass nutrient-deficient. This is further evidence of the central importance of pretreatment; i.e. the choice of pretreatment approach largely dictates many system-wide advantages or disadvantages of a particular cellulosic biofuel technology[23].

CONCLUSIONS

Commodity products derived from lignocellulosic biomass such as fuels require high processing efficacies, low raw material costs and significant co-product revenue to achieve economic competitiveness. Pursuing this theme, we demonstrate a holistic approach to biomass processing, where the production cost of cellulosic ethanol can be dramatically reduced by integrating fuels, saccharolytic enzymes and food precursors (co-products) production into the cellulosic biorefinery utilizing plant materials (such as corn stover and switchgrass) as the primary carbohydrate, nitrogen and mineral source. This integrated scheme includes (i) core bioprocessing and bioconversion (CBB) unit operations for ethanol and native yeast production, (ii) in-house enzyme production using *Trichoderma reesei*, an industrial workhorse microbe, and (iii) auxiliary units that support the CBB and enzyme production units. The nutrients present in lignocellulose, as well as the additional nitrogen added during certain ammonia based pretreatments (like AFEX), are sufficient to co-produce feed-grade yeast as well as ethanol. This provides both an economic benefit as well as a food supplement that can help offset the “food

vs. fuel" issue of growing dedicated energy crops. In addition, the oligosaccharides produced during AFEX treatment are valuable as enzyme inducers, which are combined with biomass-derived carbohydrates to produce nearly all enzymes in-house. A detailed techno-economic assessment of this integrated approach compared to the traditional paradigm of cellulosic ethanol production showed increased profit margins by $35/Mg dry feedstock (or nearly $0.13 per liter ethanol produced) for the former approach.

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AUTHOR CONTRIBUTIONS

M.W.L. and B.E.D. designed research; M.W.L., B.D.B., S.P.S.C, M.J., C.G. performed research/experiments; M.W.L., B.D.B., S.P.S.C, M.J., C.G., V.B., A.D.J. and B.E.D. analyzed data; and M.W.L., B.D.B., S.P.S.C, M.J., and B.E.D. wrote the paper.

FIGURE LEGENDS

Figure 1

Nutrient contents and balances during the biomass processing of AFEX-Corn Stover.

(a) Amino acid concentration of AFEX corn stover enzymatic hydrolysate (6.0% cellulose loading).
(b) Trace elements and vitamins of AFEX corn stover enzymatic hydrolysate (6.0% cellulose loading).
(c) Carbohydrate balance around enzymatic hydrolysis of 6.0% cellulose loading. **(d)** Nitrogen, Phosphorus and Potassium balances around AFEX and enzymatic hydrolysis.

Figure 2

Fermentation using recombinant *S. cerevisiae* 424A(LNH-ST).

(a) Glucose consumption in the fermentation of 9% SLE water extract from AFEX-CS and AFEX-RS.
(b) Cell density in the fermentation of 9% SLE water extract from AFEX-CS and AFEX-RS. Water extract of AFEX-corn stover and AFEX-rice straw at 9% SLE was prepared as described in [23]. Glucose and phosphate buffer (salts) were added into the water extract to a final concentration of 100 g/L and 0.1M, respectively. The pH of the water extract was adjusted to 5.5. Seed culture was used to inoculate the water extracts at an initial cell density of 2.0 unit OD600nm. Control experiments in YEP and YNB (Yeast Nitrogen Base) was conducted in parallel. These fermentations were conducted in triplicate. Cell density and fermentation products were measured as described [24]
(c) Schematic representation of two-stage ethanol fermentation and native *S. cerevisiae* co-product generation.
(d) Schematic representation of cell recycling of recombinant *S. cerevisiae* for high cell density xylose fermentation.
(e) Sugar and ethanol profiles for the two-stage fermentation.
(f) Xylose consumption of recombinant *S. cerevisiae 424A(LNH-ST)* over three generations of recycling

Figure 3

In-house enzyme production using *Trichoderma reseei* RUT-C30.

(a) Schematic illustration of enzyme production using *Trichoderma reseei* RUT-C30 fermentation.
(b) Enzyme inducers used for *Trichoderma reseei* RUT-C30 fermentation. To compare the induction efficiency of AFEX-CS liquid extract relative to lactose, 18% solids-loading-equivalent (SLE) AFEX-CS liquid extract (loaded at 27.8% v/v) or 4.17 g/L of lactose was added into the RUT-C30 preculture (60% v/v). Other fermentation procedure and parameter were kept identical. The initial carbohydrate concentration of both inducers were at the same level (4.17 g/L)
(c) Induction comparison between AFEX-CS and lactose.
(d) Net sugar yield of enzymatic hydrolysis on 1.0% cellulose loading AFEX-CS using 1:6 diluted RUT-C30 broth induced by AFEX-CS mixture. RUT-C30 fermentation broth was diluted by a factor of 1:6 (1 part of broth + 5 part of distilled water). The diluted broth was used to conduct enzymatic hydrolysis on AFEX-CS at 1.0% glucan loading for 24hr at pH 4.8, 50C. The AFEX-CS was finely ground and passed through a 0.25mm screen (Ultra Centrifugal Mill ZM 200, Retsch, Germany). Accellerase at varying dosages (0.0, 1.0, 2.0 mg protein/g dry AFEX-CS) was added to the diluted broth to investigate the need for exogenous enzyme supplementation. Control experiments using commercial enzymes mixture were conducted for comparison. The enzyme mixture was consisted of Accellerase 1000 (120 mL/kg CS), Multifect Xylanase (6.2 mL/kg CS) and Multifect Pectinase (4.3 mL/kg CS).
(e) Top 11 secreted *Trichoderma reesei* (RUT-C30) cellulases and hemicellulases differentially expressed during induction using AFEX treated corn stover water extract (WE), AFEX treated corn stover water extract and solid biomass (AFCS+WE), and lactose only.
The extracellular proteins from the *T. reesei* fermentation broths were isolated using chloroform/methanol precipitation method to remove interfering agents to subsequent proteomics analysis [25, 26] . Experimental procedure on proteomics and protein homolog identification is included in Supplementary Methods.

Figure 4

Process flow diagram

(a) Proposed scheme for the biorefinery utilizing corn stover as the sole source for carbohydrate and mineral for ethanol, enzyme and native yeast cell production.
(b) Core bioprocessing and bioconversions (CBB) of the proposed scheme.
(c) Stream description of the flow diagram.

Figure 5

Economic analysis of the impact of yeast co-production and in-house enzymes.

(a) Costs and revenue breakdown of the base case model (adapted from Eggeman and Elander [8], and the proposed processing scheme with both technologies (yeast recycle and recovery and in-house enzyme production) included. All values are normalized to 2008 dollars per Mg feedstock.
(b) Costs and revenues of four potential biological conversion processing schemes. The base case and the proposed scheme are the schemes presented in Figure 5.a. The yeast only scheme includes only the new technology required for the yeast co-product and recycling xylose-consuming yeast, while the enzyme only scheme includes only in-house enzyme production as the only new technology.
(c) Sensitivity analysis of the impact on the pre-tax profit in the proposed scheme of several process flow variables. Each variable was allowed to deviate from its standard value, and the percent change in pre-tax profit of the proposed scheme was determined.
(d) Table showing the low, standard, and high values for the economic variables tested in the sensitivity analysis.

MATERIALS AND METHOD

AFEX-Corn Stover and AFEX-Rice Straw

Corn stover was obtained from the National Renewable Energy Laboratory (NREL). This corn stover contains 33.2% cellulose, 22.4% xylan, 3.3% arabinan and 2.3% protein. The rice straw used in this report contains 34.7% cellulose, 15.1% xylan and 2.2% arabinan. The AFEX pretreatment conditions and procedures for the respective feedstocks are as described in [2, 27].

Enzymatic hydrolysis of AFEX-CS at 6.0% glucan loading

The enzymatic hydrolysis (2.0 kg total saccharification) was conducted in a 3L bioreactor (Applikon, Biobundle, Foster City CA) at 50°C, pH 4.8 for 96hr. To achieve proper liquefaction and stirring throughout the enzymatic hydrolysis, both AFEX-CS and commercial enzymes were fed batch wise. A total protein loading of 7.4 mg protein/g biomass was used. The commercial enzymes and their respective dosages used were Accellerase 1000 (120 mL/kg CS), Multifect Xylanase (6.2 mL/kg CS) and Multifect Pectinase (6.2 mL/kg CS). The protein concentration of the commercial enzymes were analyzed and reported in Supplementary Table 3. The mass balance around enzymatic hydrolysis was constructed as described previously [2].

Nutrient content analysis

Free ammonia in AFEX-CS hydrolysate was analyzed through an enzymatic assay from R-biopharm AG (Cat no: 11112732035, Darmstadt, Germany). The analyses for amino acid concentrations on AFEX-CS hydrolysate were conducted in MSU Macromolecular Structure Facility through a High Performance Liquid Chromatography (HPLC) system equipped with a Nova Pak C18 (3.9mm×150mm; Waters). Operational details of the system were as described [28]. The amino acids involved in the analysis are Asp, Glu, Ser, Gly, His, Thr, Arg, Ala, Pro, Tyr, Val, Met, Ile, Lys and Phe. Nitrogen content of the dry untreated CS, AFEX-treated CS, solid residue, enzyme solution and AFEX-CS hydrolysate were determined using a Skalar Primacs SN Total Nitrogen Analyzer (Breda, The Netherlands). The nitrogen analysis is based on the Dumas method using EDTA as the standards. Trace elements were measured by inductively-coupled-plasma mass spectrometry (ICP-MS) in the MSU Department of Geological Sciences. Five vitamins important for industrial fermentations were analyzed using a LC/MS/MS (Quattro Micro, Waters) using a Water Symmetry C-18 column.

Details regarding the nutrient content analysis was provided in Supplementary Methods.

Two-stage ethanol fermentation

Frozen glycerol stock of native *S. cerevisiae* (ATCC 4124) was inoculated into 2% w/w corn steep liquor media (CSL) supplemented with 20 g/L glucose and grown for 18hr. The grown seed culture was used as inoculum for first-stage fermentation on the 6.0% glucan loading AFEX-CS enzymatic hydrolysate at a working volume of 80mL with an initial cell density equivalent to 0.05 unit OD600nm. The fermentation was conducted for 15hr and the broth was transferred to 50 mL conical tubes (Falcon, BD) to allow cell separation by sedimentation at 30°C for 3 hr. The clarified liquid hydrolysate was pipetted from the tubes to the unbaffled Erlenmeyer flask.

Seed culture of the xylose-fermenting *S. cerevisiae* 424A(LNH-ST) was prepared by inoculating the frozen stock into 3:10 diluted 6.0% glucan loading enzymatic hydrolysate supplemented with 2%w/w of CSL and the culture was grown for 18hr. The cells was harvested and inoculated into the enzymatic hydrolysate from first-stage fermentation at a working volume of 70mL with an initial cell density of 25 unit OD600nm. The fermentation was conducted for another 48hr. The *S. cerevisiae* in the seed cultures and the two-stage ethanol fermentation was grown microaerobically in unbaffled Erlenmeyer flasks at 30°C, 150 rpm, pH 5.5 as previously reported in [2]. Samples were taken at designated times and cell density was measured as described in [24].

Trichoderma reseei RUT-C30 Fermentation

Frozen glycerol spore stock of RUT-C30 was inoculated into 2%w/w CSL supplemented with 20 g/L glucose at pH 5.5 (50mM phosphate buffer). The preculture (50mL) was grown in a 250 mL baffled flask at 30°C, 200rpm for 48hr. During the enzyme induction phase, 18% solids-loading-equivalent (SLE) AFEX-CS water extract (loaded at 27.8% v/v; Supplementary Methods) and/or finely-ground (passed through 0.5mm screen) AFEX-CS (1% w/v) were added into the preculture (60% v/v) as the enzyme inducer. Distilled water and phosphate buffer were added to the respective mixtures to achieve final volume of 50 mL. Additional CSL equivalent to a final concentration of 1% w/w CSL was supplemented after 24hr of induction. The fermentation broth was conducted at 30°C 120 hr and pH was adjusted to 5.5 every 24hr by adding HCl. The working volume of the fermentation during induction phase was 50 mL. Upon completion of the induction phase, the fermentation broth was centrifuged at 2,500 x g for 30 min. The cell-free fermentation broth was used to conduct enzymatic hydrolysis at 1.0% glucan loading AFEX-CS.

The saccharolytic enzymes were separated from the other background protein at smaller molecular weight by using a FPLC system (GE Healthcare, Buckinghamshire, United Kingdom) equipped with a 51 ml HisPrep 26/10 desalting column (GE Healthcare, Lot #17-5087-01). The concentration of fractions that contained the saccharolytic enzymes were quantified by BCA assay (Pierce Biotechnology, Rockfort, IL). The original concentration was calculated by taking into the account of the dilution factors involved.

Trichoderma Extracellular Protein Isolation

The extracellular proteins from the fungal broths were isolated using chloroform/methanol precipitation method to remove interfering agents to subsequent proteomics analysis [26, 29]. Four parts volume cold methanol was added to one part volume of the broth and vortexed well. One part volume cold chloroform followed by three part volumes cold water was then added to the mixture and vortexed again. The mixture was centrifuged at 15,000 g at 4 °C for 10 min following which the aqueous (top) layer was discarded and four part volume cold methanol was added. The mixture was centrifuged at 15,000 g at 4 °C for 30 min following which the liquid supernatant was carefully removed without disturbing the precipitated protein pellet. The protein pellet was air-dried overnight and redissolved in SDS-PAGE sample buffer (NuPAGE® LDS Sample Preparation Buffer, Invitrogen, CA) prior to proteomics analysis.

Proteomics analysis

Redissolved proteins (~400 μg) were loaded on to a SDS-PAGE gel (NuPAGE®, Invitrogen, CA) and electrophoresis was carried out at 50 V for 15 min to stack up the proteins within the gel. Gel bands were then cut out and subjected to in-gel tryptic digestion [30]. The extracted peptides were re-suspended into a solution of 2% Acetonitrile and 0.1% Trifluoroacetic Acid to 20 μl volume. From this solution, 10 μl were automatically injected by a Waters nanoAcquity Sample Manager (www.waters.com) and loaded for 5 minutes onto a Waters Symmetry C18 peptide trap (5 μm, 180 μm x 20 mm) at 4 μL/min in 2% Acetonitrile/0.1% Formic Acid. The bound peptides were then eluted using a Waters nanoAcquity UPLC (Buffer A = 99.9% Water/0.1% Formic Acid, Buffer B = 99.9% Acetonitrile/0.1% Formic Acid) onto a Michrom MAGIC C18AQ column (3u, 200 Angstrom, 100 μm x 150 mm, www.michrom.com) and eluted over 60 minutes with a gradient of 2% B to 30% B in 46 min, spiked to 90%B at 47 minutes and equilibrated back to 5% B after 49 min at a flow rate of 1 μl/min. Eluted peptides were sprayed into a ThermoFisher LTQ-FT Ultra mass spectrometer (www.thermo.com) using a Michrom ADVANCE nanospray source. Survey scans were taken in the FT (25000 resolution determined at m/z 400) and the top ten ions in each survey scan are then subjected to automatic low energy collision induced dissociation (CID) in the LTQ. The resulting MS/MS spectra are converted to peak lists using BioWorks Browser v3.3.1 (ThermoFisher) using the default LTQ-FT Ultra parameters and

searched using the Mascot search algorithm v2.3 (www.matrixscience.com) against fungi protein entries from NCBI, downloaded 11-13-2009, and against the *Trichoderma reesei* protein database, v2.0, downloaded from the DOE Joint Genome Institute (JGI). Mascot parameters for all databases allowed for up to 2 missed tryptic sites, fixed modification of carbamidomethyl cysteine, and variable modification due to oxidation of methionine. The peptide and MS/MS fragment tolerance was ± 10 ppm (monoisotopic) and 0.60 Da (monoisotopic), respectively. The Mascot output was then analyzed using Scaffold (www.proteomesoftware.com) to probabilistically validate protein identifications using the ProteinProphet computer algorithm [31]. Minimum criteria for positive protein assignment were at least two peptides and >95% confidence filter as determined by Scaffold. Uncharacterized and/or putative protein sequences were BLAST against the UniprotKB database to identify homology to similar proteins from other microbes.

Enzymatic Hydrolysis of AFEX-CS at 1.0% glucan loading

RUT-C30 fermentation broth was diluted by a factor of 1:6 (1 part of broth + 5 part of distilled water). The diluted broth was used to conduct enzymatic hydrolysis on AFEX-CS at 1.0% glucan loading for 24hr at pH 4.8, 50C. The AFEX-CS was finely ground and passed through a 0.25mm screen (Ultra Centrifugal Mill ZM 200, Retsch, Germany). Accellerase at varying dosages (0.0, 1.0, 2.0 mg protein/g dry AFEX-CS) was added to the diluted broth to investigate the need for exogenous enzyme supplementation. Control experiments using commercial enzymes mixture were conducted for comparison. The enzyme mixture was consisted of Accellerase 1000 (120 mL/kg CS), Multifect Xylanase (6.2 mL/kg CS) and Multifect Pectinase (4.3 mL/kg CS).

Other Analytical Methods

The levels of oligosaccharides were quantified using NREL-LAP-014, a method based on acid hydrolysis. Glucose, xylose, arabinose and ethanol are quantified using a HPLC system equipped with Biorad Aminex HPX-87H column as described in [24].

REFERENCES

1. Chundawat, S.P.S., Beckham, G.T., Himmel, M.E. & Dale, B.E. Deconstruction of Lignocellulosic Biomass to Fuels and Chemicals. *Annual Review of Chemical and Biomolecular Engineering* **2**, 121-145 (2011).
2. Lau, M.W. & Dale, B.E. Cellulosic ethanol production from AFEX-treated corn stover using Saccharomyces cerevisiae 424A(LNH-ST). *Proc Natl Acad Sci U S A* **106**, 1368-1373 (2009).
3. Kim, D.Y. et al. Batch and continuous fermentation of succinic acid from wood hydrolysate by Mannheimia succiniciproducens MBEL55E. *Enzyme and Microbial Technology* **35**, 648-653 (2004).
4. Patel, M., Ou, M., Ingram, L.O. & Shanmugam, K.T. Fermentation of sugar cane bagasse hemicellulose hydrolysate to l(+)-lactic acid by a thermotolerant acidophilic Bacillus sp. *Biotechnology Letters* **26**, 865-868 (2004).
5. Fortman, J.L. et al. Biofuel alternatives to ethanol: pumping the microbial well. *Trends in Biotechnology* **26**, 375-381 (2008).
6. Aden, A. Golden, Colorado; 2008).
7. Ladisch, M.R. & Svarczkopf, J.A. Ethanol production and the cost of fermentable sugars from biomass. *Bioresource Technology* **36**, 83-95 (1991).
8. Eggeman, T. & Elander, R.T. Process and economic analysis of pretreatment technologies. *Bioresource Technology* **96**, 2019-2025 (2005).
9. Boulton, C. & Quain, D. Brewing Yeast and Fermentation. (Iowa State University Press, Ames, Iowa; 2001).
10. Walker, G.M. in Advances in Applied Microbiology, Vol. 54. (eds. A.I. Laskin, J.W. Bennet & G.M. Gadd) (Elsevier Academic Press, New York, NY; 2004).
11. Aden, A. et al., Vol. NREL/TP-510-32438. (ed. D.o. Energy)2002).
12. Wooley, R. et al., Vol. NREL/TP-580-26157. (ed. D.o. Energy)1999).
13. Gao, D., Chundawat, S.P.S., Krishnan, C., Balan, V. & Dale, B.E. Mixture optimization of six core glycosyl hydrolases for maximizing saccharification of ammonia fiber expansion (AFEX) pretreated corn stover. *Bioresource Technology* **101**, 2770-2781 (2010).
14. Banerjee, G. et al. Synthetic multi-component enzyme mixtures for deconstruction of lignocellulosic biomass. *Bioresource Technology* **101**, 9097-9105 (2010).
15. Schill, S.R. in Ethanol producer magazine2009).
16. Suto, M. & Tomita, F. Induction and catabolite repression mechanisms of cellulase in fungi. *Journal of Bioscience and Bioengineering* **92**, 305-311 (2001).
17. Chundawat SPS. et al. Major cell wall decomposition products produced during ammonia fiber expansion pretreatment of corn stover. *[Manuscript In Preparation]* (2009).
18. Chundawat, S.P.S. et al. Multi-scale visualization and characterization of lignocellulosic plant cell wall deconstruction during thermochemical pretreatment. *Energy & Environmental Science* **4**, 973-984 (2011).
19. Lau, M.W. & Dale, B.E. Effect of primary degradation-reaction products from Ammonia Fiber Expansion (AFEX)-treated corn stover on the growth and fermentation of Escherichia coli KO11. *Bioresource Technology* (2010).
20. Kollar, R., Sturdik, E. & Sajbidor, J. Complete fractionation of saccharomyces cerevisiae biomass. *Food Biotechnology* **6**, 225 - 237 (1992).
21. Fleet, G. in Yeasts in Food and Beverages 1-122006).

22. Wingren, A., Galbe, M. & Zacchi, G. Techno-Economic Evaluation of Producing Ethanol from Softwood: Comparison of SSF and SHF and Identification of Bottlenecks. *Biotechnology Progress* **19**, 1109-1117 (2003).
23. Lau, M.W., Gunawan, C. & Dale, B.E. The impacts of pretreatment on the fermentability of pretreated lignocellulosic biomass: a comparative evaluation between ammonia fiber expansion and dilute acid pretreatment. *Biotechnol Biofuels* **2**, 30 (2009).
24. Lau, M.W., Dale, B.E. & Balan, V. Ethanolic fermentation of hydrolysates from ammonia fiber expansion (AFEX) treated corn stover and distillers grain without detoxification and external nutrient supplementation. *Biotechnology and Bioengineering* **99**, 529-539 (2008).
25. Wessel, D. & Fluegge, U.I. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Analytical Biochemistry* **138**, 141-143 (1984).
26. Jiang, L., He, L. & Fountoulakis, M. Comparison of protein precipitation methods for sample preparation prior to proteomic analysis. *Journal of Chromatography A* **1023**, 317-320 (2004).
27. Zhong, C., Lau, M.W., Balan, V., Dale, B.E. & Yuan, Y.J. Optimization of enzymatic hydrolysis and ethanol fermentation from AFEX-treated rice straw. *Applied Microbiology and Biotechnology* **84**, 667-676 (2009).
28. Bergman, T., Carlquist, M. & Jornvall, H. in Advanced Methods in Protein Microsequence Analysis. (ed. B. Wittmann-Liebold) 45-55 (Springer Verlag, Berlin; 1986).
29. Wessel, D. & Flügge, U.I. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Analytical Biochemistry* **138**, 141-143 (1984).
30. Shevchenko, A., Wilm, M., Vorm, O. & Mann, M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* **68**, 850-858 (1996).
31. Nesvizhskii, A.I., Keller, A., Kolker, E. & Aebersold, R. A Statistical Model for Identifying Proteins by Tandem Mass Spectrometry. *Analytical Chemistry* **75**, 4646-4658 (2003).

FIGURE 1: Nutrient contents and balances during the biomass processing of AFEX-Corn Stover. (A) Amino acid concentration of AFEX corn stover enzymatic hydrolysate (6.0% cellulose loading). (B) Trace elements and vitamins of AFEX corn stover enzymatic hydrolysate (6.0% cellulose loading). (C) Carbohydrate balance around enzymatic hydrolysis of 6.0% cellulose loading. (D) Nitrogen, Phosphorus and Potassium balances around AFEX and enzymatic hydrolysis.

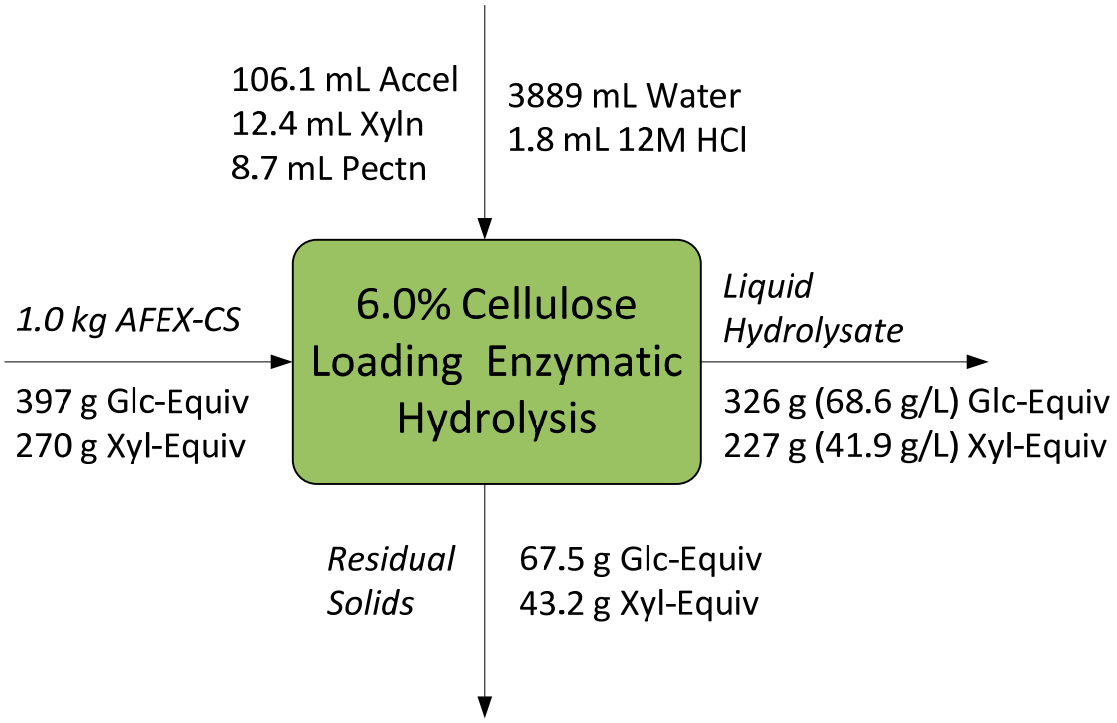
(A)

Components	**AFEX-Hydrolysate (mg/L)**	
	Free	**Total**
NH^{4+}	750±50	
Asp	8.4±1.7	75.9±1.7
Glu	0.0±2.4	133.8±2.4
Ser	16.8±3.8	104.2±3.8
Gly	5.2±5.8	127.2±5.8
His	4.5±2.3	34.3±2.3
Thr	17.6±4.6	98.9±4.6
Arg	17.1±3.2	55.0±3.2
Ala	11.6±2.9	110.2±2.9
Pro	30.4±2.3	108.7±2.3
Tyr	30.0±2.5	28.6±2.5
Val	9.9±2.2	68.8±2.2
Met	2.6±1.9	19.4±1.9
Ile	7.6±2.2	55.4±2.2
Leu	0.0±3.8	93.6±3.8
Lys	18.4±1.3	25.7±1.3
Phe	15.7±3.8	91.6±3.8
Total	195.8±28.3	1231±43.8

(B)

			Unit	AFEX-CS Hydrolysate
Trace Elements	Mg	Magnesium	mg/L	168.42±3.24
	Ca	Calcium		242.87±7.72
	Mn	Manganase		2.32±0.53
	Co	Cobalt	µg/L	11.3±3.8
	Ni	Nickel		13.5±5.3
	Cu	Copper		116.2±9.3
	Zn	Zinc		505.7±51.3
	Mo	Molybdenum		15.9±0.6
	Fe	Iron		296.4±74.5
Vitamins		Panthothenic Acid	µM	1.50±0.12
		Pyridoxine		1.26±0.18
		Nicotinic Acid		10.87±1.38
		Biotin		~0.05
		Thiamine		~0.66

(C)


106.1 mL Accel
12.4 mL Xyln
8.7 mL Pectn
3889 mL Water
1.8 mL 12M HCl
6.0% Cellulose Loading Enzymatic Hydrolysis
1.0 kg AFEX-CS
397 g Glc-Equiv
270 g Xyl-Equiv
Liquid Hydrolysate
326 g (68.6 g/L) Glc-Equiv
227 g (41.9 g/L) Xyl-Equiv
Residual Solids
67.5 g Glc-Equiv
43.2 g Xyl-Equiv

(D)

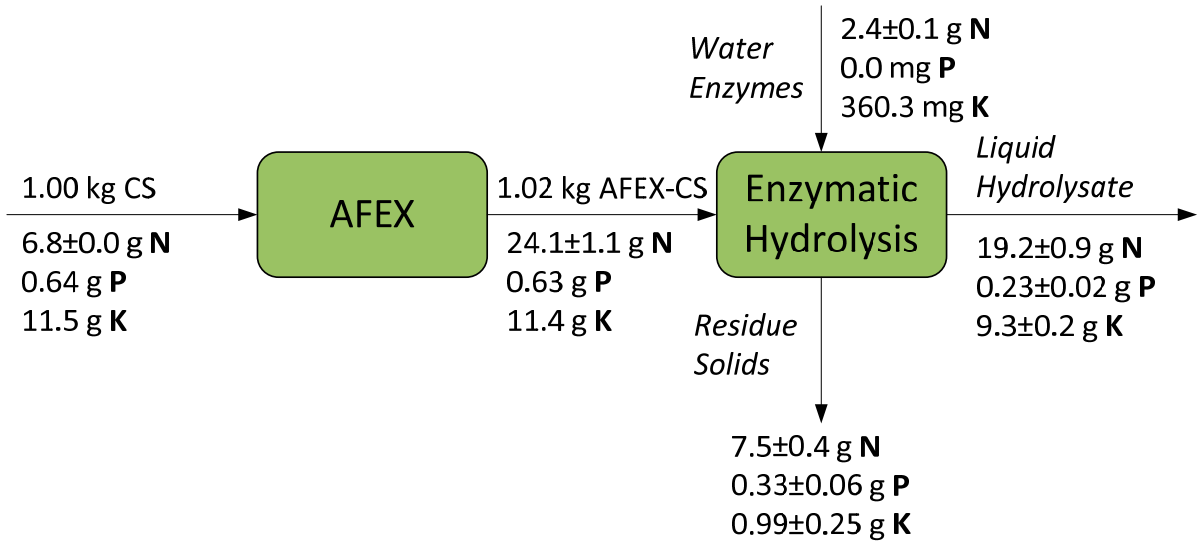
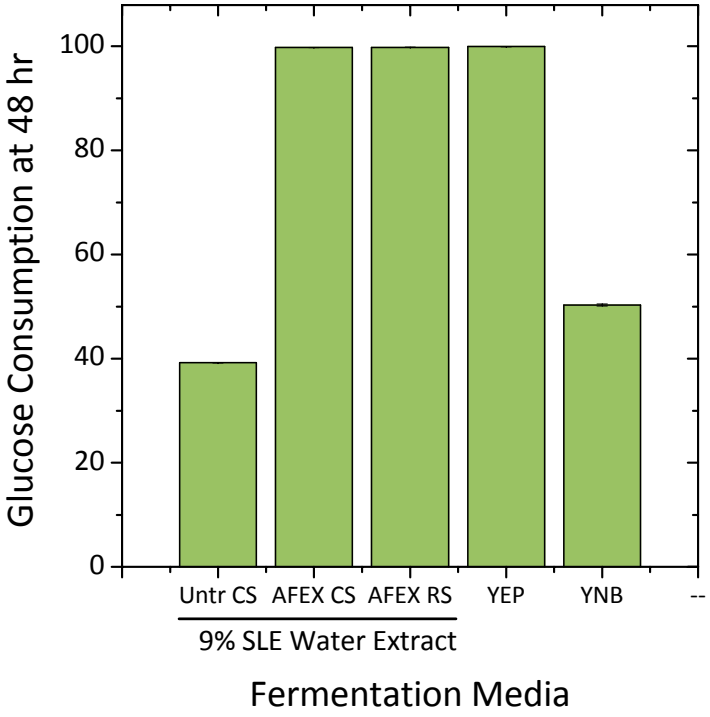

Water
Enzymes
2.4±0.1 g N
0.0 mg P
360.3 mg K
1.00 kg CS
6.8±0.0 g N
0.64 g P
11.5 g K
AFEX
1.02 kg AFEX-CS
24.1±1.1 g N
0.63 g P
11.4 g K
Enzymatic Hydrolysis
Liquid Hydrolysate
19.2±0.9 g N
0.23±0.02 g P
9.3±0.2 g K
Residue Solids
7.5±0.4 g N
0.33±0.06 g P
0.99±0.25 g K

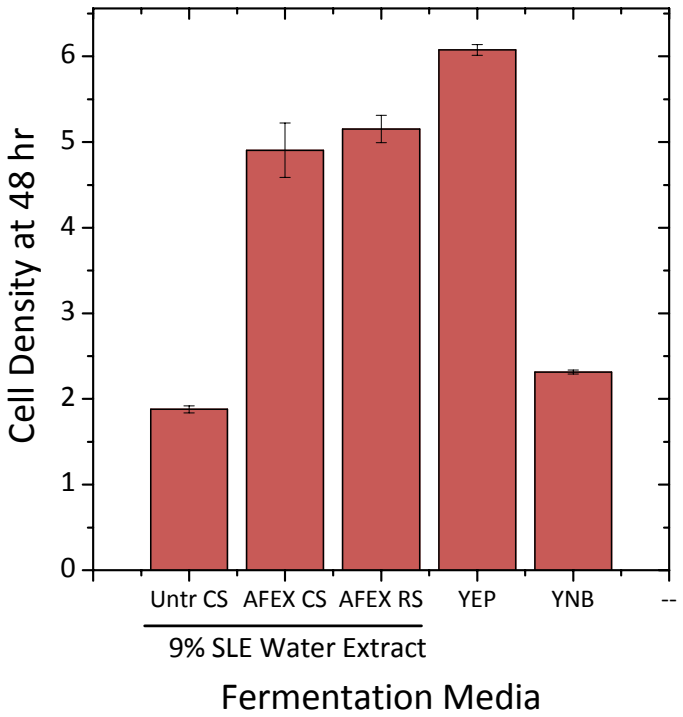
FIGURE 2:

Fermentation using recombinant *S. cerevisiae* 424A(LNH-ST). (A) Glucose consumption in the fermentation of 9% SLE water extract from AFEX-CS and AFEX-RS. (B) Cell density in the fermentation of 9% SLE water extract from AFEX-CS and AFEX-RS. (C) Schematic representation of two-stage ethanol fermentation and native *S. cerevisiae* co-product generation. (D) Schematic representation of cell recycling of recombinant *S. cerevisiae* for high cell density xylose fermentation. (E) Sugar and ethanol profiles for the two-stage fermentation. (F) Xylose consumption of recombinant *S. cerevisiae 424A9LNH-ST)* over three generation of recycling

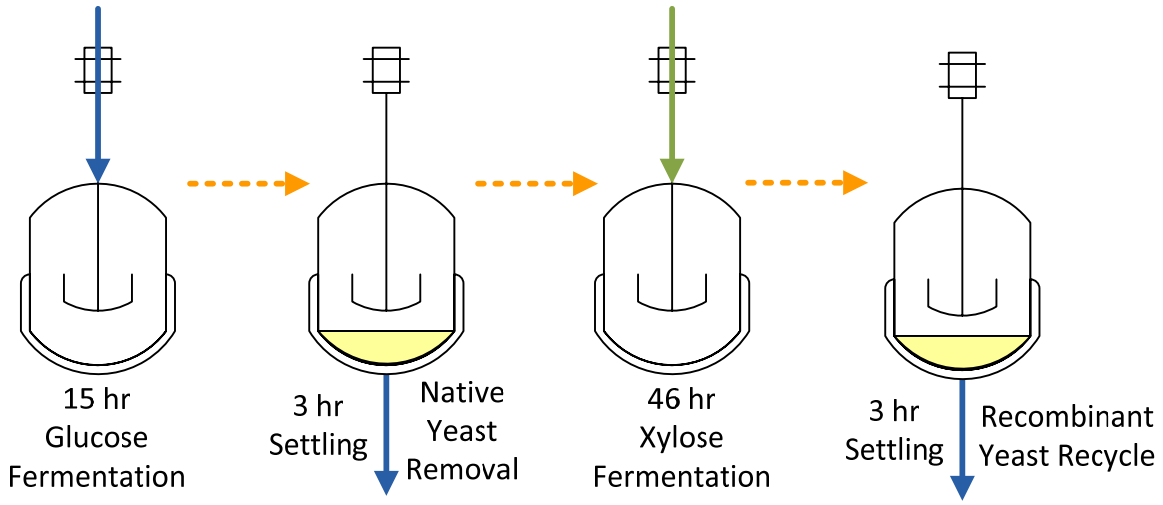
A: Glucose Consumption Density



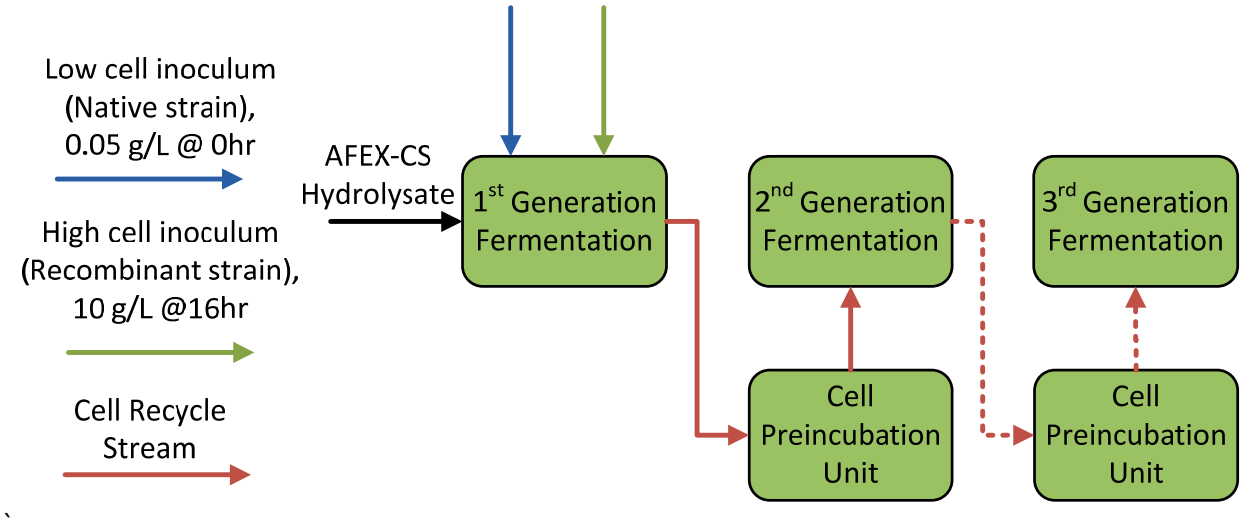
B: Cell



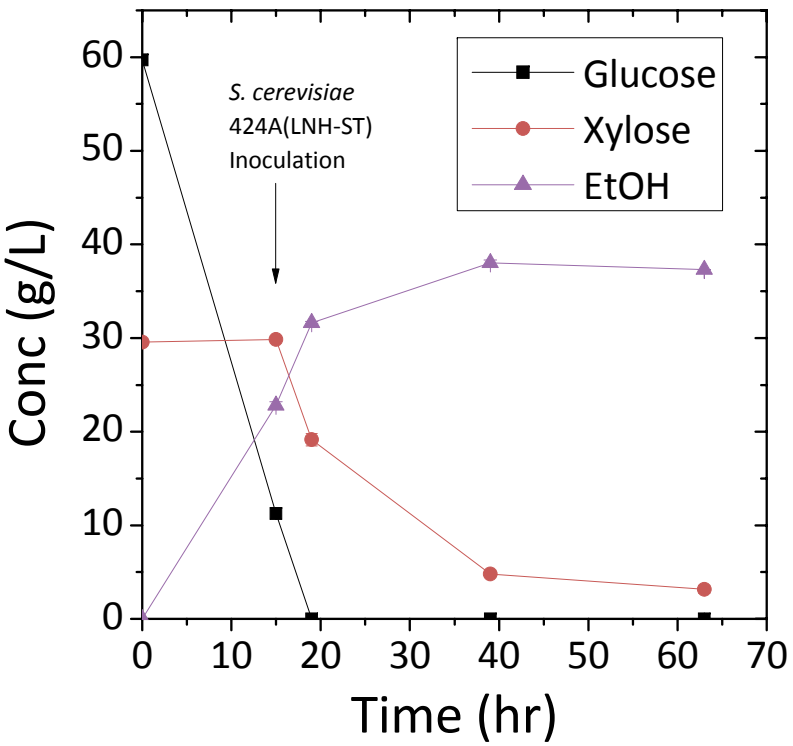
(C) Two-stage ethanol fermentation and native *S. cerevisiae* co-product generation



(D) Cell recycling of recombinant *S. cerevisiae* for high cell density xylose fermentation



(E) Sugar and ethanol profiles for the two-stage fermentation



(F) Xylose consumption of recombinant *S. cerevisiae* 424A (LNH-ST) over three generations of recycling

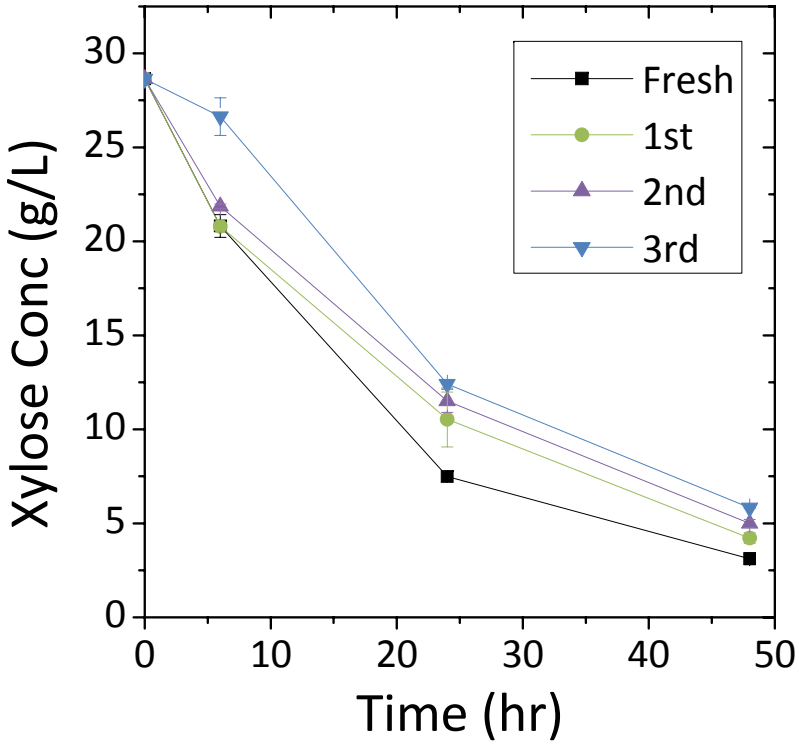
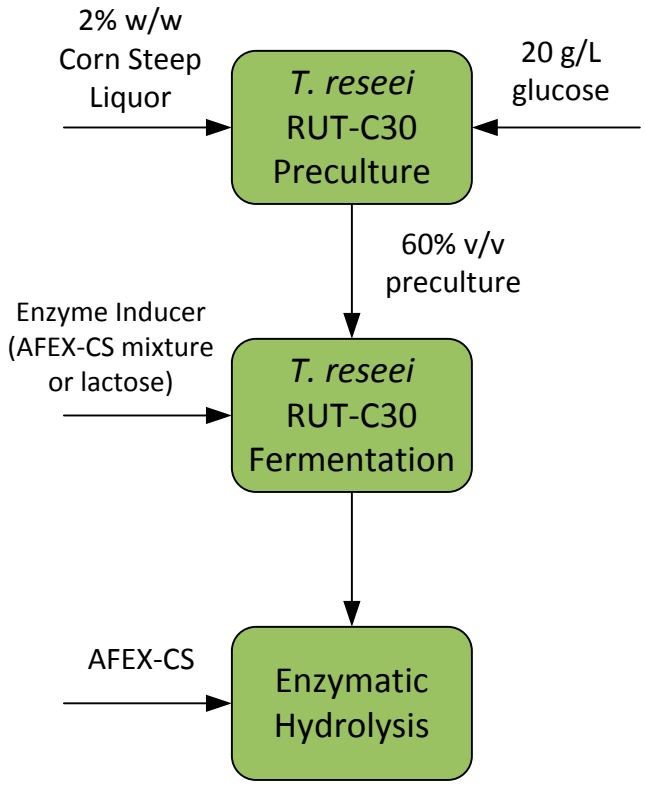


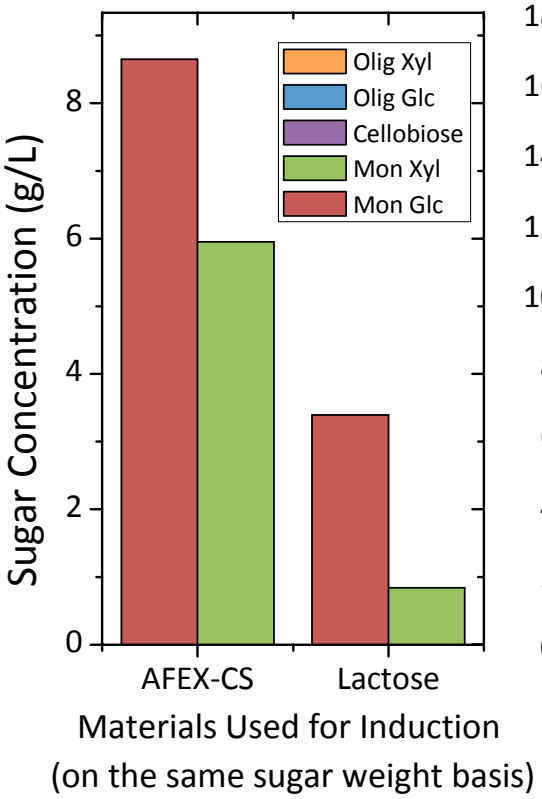
FIGURE 3: In-house enzyme production using *Trichoderma reseei* RUT-C30. (A) Schematic illustration of enzyme production using *Trichoderma reseei* RUT-C30 fermentation. (B) Enzyme inducers used for *Trichoderma reseei* RUT-C30 fermentation (C) Induction comparison between AFEX-CS and lactose. (D) Net sugar yield of enzymatic hydrolysis on 1.0% cellulose loading AFEX-CS using 1:6 diluted RUT-C30 broth induced by AFEX-CS mixture (E) Top 11 secreted Trichoderma reesei (RUT-C30) cellulases and hemicellulases differentially expressed during induction using AFEX treated corn stover water extract (WE), AFEX treated corn stover water extract and solid biomass (AFCS+WE), and lactose only.

(A) **(B)**

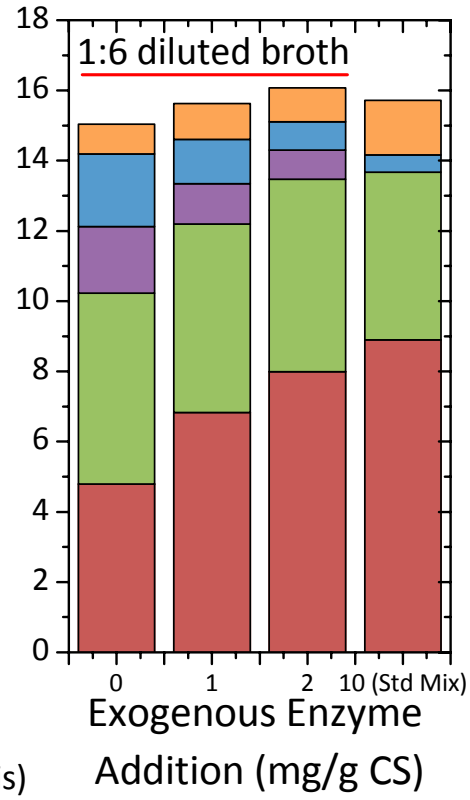


	AFEX-CS mixture	Lactose
Fig 3 C	5.4% SLE water extract (which contains 4.17 g/L of total sugar)	4.17 g/L
Fig 3 D	5.4% SLE water extract + 1% w/v AFEX-CS	N.A.
Fig 3 E	(WE): 5.4% SLE water extract (AFCS + WE): 5.4% SLE water extract + 1% w/v AFEX-CS	4.17 g/L

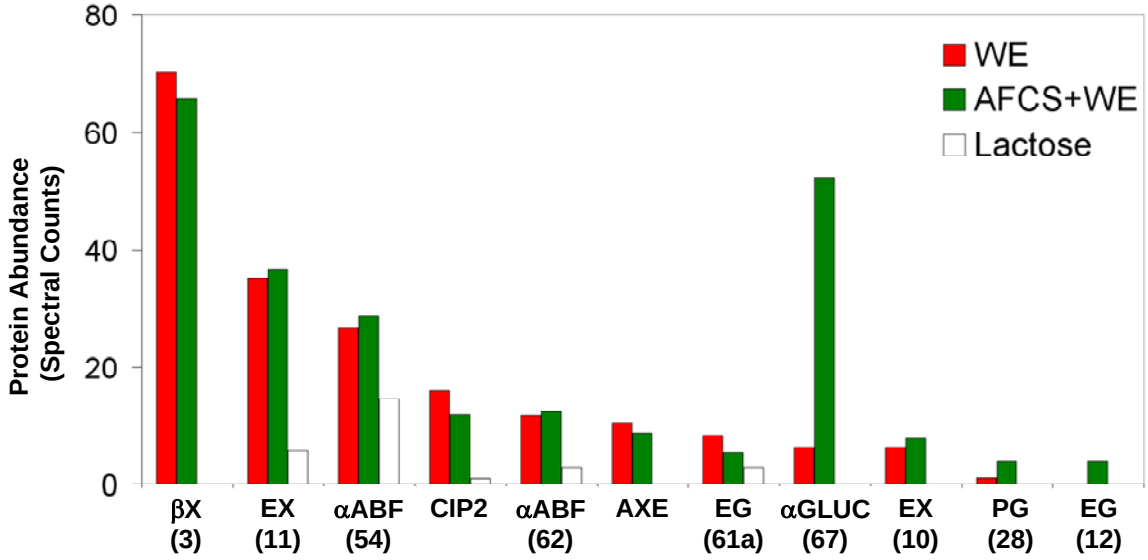
(C)


Olig Xyl
Olig Glc
Cellobiose
Mon Xyl
Mon Glc
Sugar Concentration (g/L)
0
2
4
6
8
AFEX-CS
Lactose
Materials Used for Induction
(on the same sugar weight basis)

(D)


1:6 diluted broth
0
2
4
6
8
10
12
14
16
18
0
1
2
10 (Std Mix)
Exogenous Enzyme
Addition (mg/g CS)

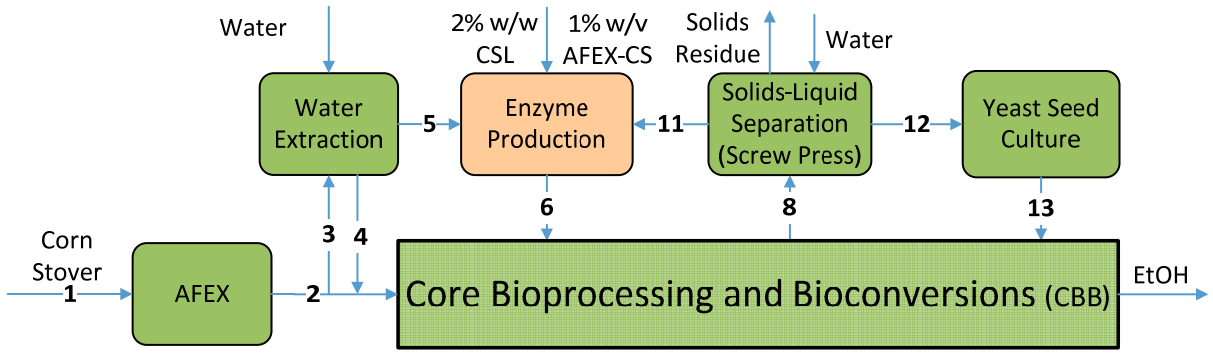
(E)



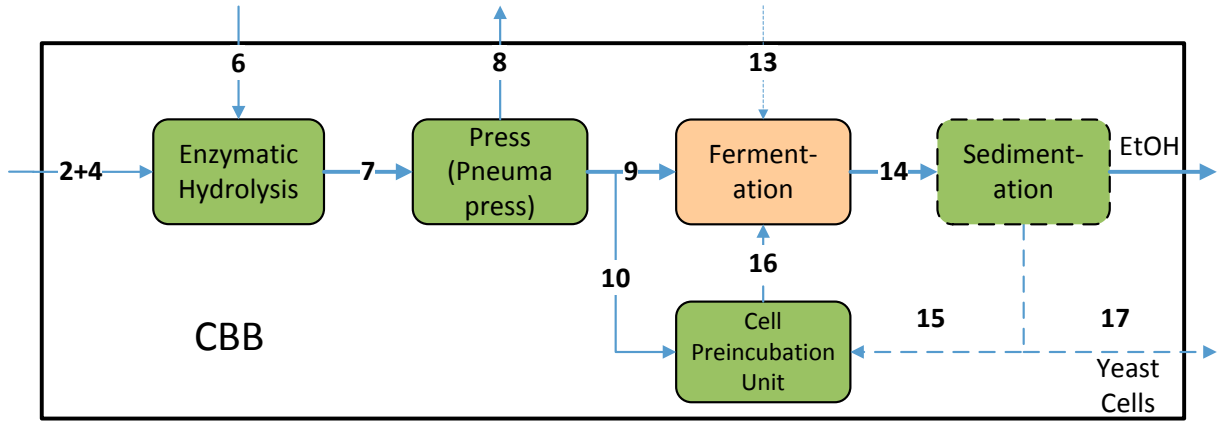
Note: Only proteins with >2-fold difference in abundance with respect to lactose control are shown here. Glycosyl hydrolase family for each protein is depicted in parenthesis along the x-axis. Protein acronyms used are; βX (β-xylosidase), EX (endoxylanase), αABF (α-arabinofuranosidase), CIP (cellulose induced protein), AXE (acetyl xylan esterase), EG (endoglucanase), αGLUC (α-glucuronidase), and PG (polygalacturonase).

Figure 4: Process flow diagram. (A) Proposed scheme for the biorefinery utilizing corn stover as the sole source for carbohydrate and mineral for ethanol, enzyme and native yeast cell production. (B) Core bioprocessing and bioconversions (CBB) of the proposed scheme. (C) Stream description of the flow diagram.

(A)



(B)

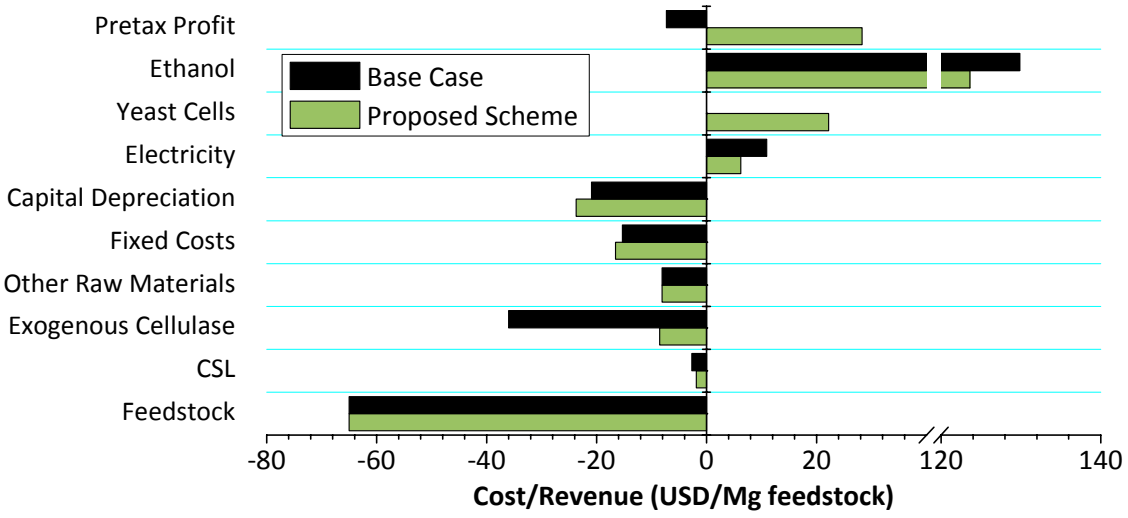


(C)

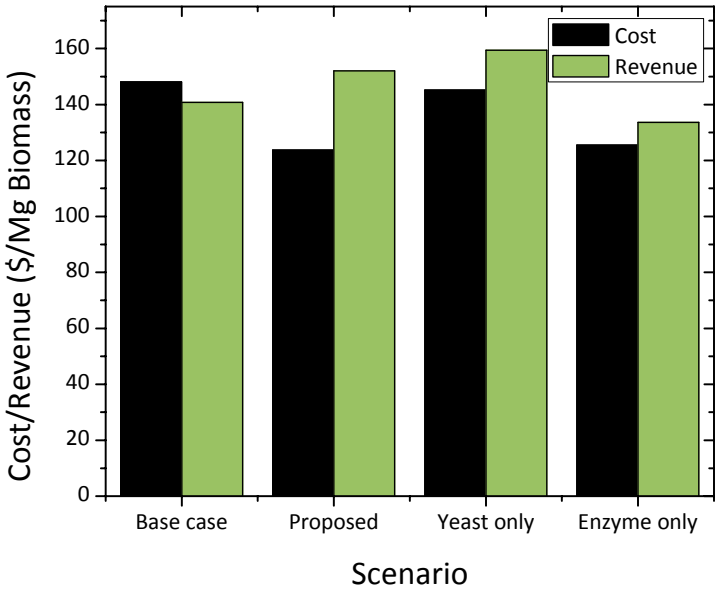
Stream	Description	Explanation[a]/Assumption[b]
1	Untreated Corn Stover	
2	AFEX-pretreated corn stover (AFEX-CS);	
3	AFEX-pretreated corn stover (AFEX-CS); 33% of the total output AFEX-CS by weight	[a] Total carbohydrate content was reduced by 7% during the extraction process as previously reported (1)
4	Washed AFEX-CS; Moisture Content at 75%	
5	Water extract of AFEX-CS at 18% solids loading	
6	Saccharolytic enzyme from *Trichoderma reesei* fermentation	
7	Enzymatic hydrolysate in slurry	
8	Moist residual solids	
9	Liquid enzymatic hydrolysate	
10	Split Stream of liquid enzymatic hydrolysate; 20% of Stream 9 by volume	[b] 18% SLE enzymatic hydrolysate is assumed to achieve similar impact for cell recycling process compared to 5.4% SLE enzymatic hydrolysate in the experiments in this report
11 & 12	Diluted AFEX-CS Hydrolysate; Sugar concentration at 30% that of Stream 9	[a] This diluted hydrolysate is used as the sugar source for *T. reesei* preculture instead of pure sugar in the experiments in this report
13	Fresh native and recombinant *S. cerevisiae* 424A(LNH-ST)	[a] --0.05 g/L of fresh native S. cerevisiae for glucose fermentation [b] –1.0 g dry wt/L of fresh *S. cerevisiae 424A(LNH-ST)* inoculum are needed for xylose fermentation from the fourth recycle event onwards
14	Beer stream at 4% w/v ethanol	
15	Recombinant *S. cerevisiae* recycle	
16	Recombinant *S. cerevisiae* recycle after preincubation in AFEX-CS hydrolysate	
17	Yeast purge stream	[b] 10% of the recombinant *S. cerevisiae* 424A(LNH-ST) is purged after each recycle event

Figure 5: Economic analysis of the impact of yeast co-production and in-house enzymes. (A) Costs and revenue breakdown of the base case model (adapted from Eggeman and Elander (2)), and the proposed processing scheme with both technologies (yeast recycle and recovery and in-house enzyme production) included. All values are normalized to 2008 dollars per Mg feedstock. (B) Costs and revenues of four potential biological conversion processing schemes. The base case and the proposed scheme are the schemes presented in Figure X.A. The yeast only scheme includes only the new technology required for the yeast co-product and recycling xylose-consuming yeast, while the enzyme only scheme includes only in-house enzyme production as the only new technology. (C) Sensitivity analysis of the impact on the pre-tax profit in the proposed scheme of several process flow variables. Each variable was allowed to deviate from its standard value, and the percent change in pre-tax profit of the proposed scheme was determined. (D) Table showing the low, standard, and high values for the economic variables tested in the sensitivity analysis.

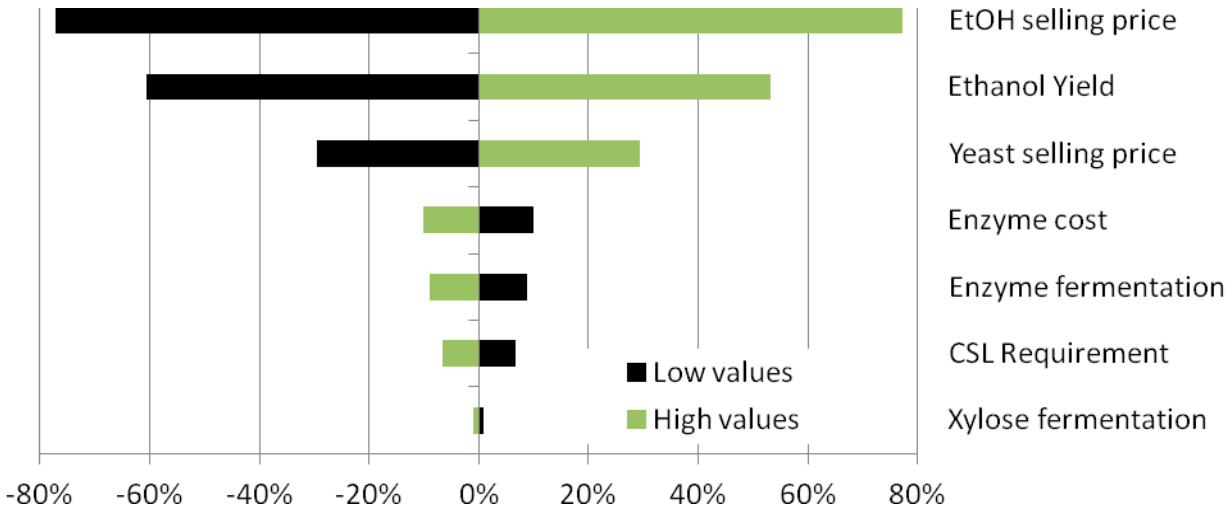
(A)



(B)


Cost
Revenue
Cost/Revenue ($/Mg Biomass)
0
20
40
60
80
100
120
140
160
Base case
Proposed
Yeast only
Enzyme only
Scenario

(C)


EtOH selling price
Ethanol Yield
Yeast selling price
Enzyme cost
Enzyme fermentation
CSL Requirement
Xylose fermentation
Low values
High values
-80%
-60%
-40%
-20%
0%
20%
40%
60%
80%

(D)

	Low	Standard	High	Unit
Xylose fermentation time	12	24	36	hours
CSL Requirement	0	10	20	g/kg BM
T. reesei fermentation time	60	96	132	hours
Enzyme cost	2400	3600	4800	$/Mg enzyme
Yeast selling price	500	800	1100	$/Mg yeast
Ethanol yield	236	275	311	L/Mg BM
Ethanol selling price	1.4	1.7	2	$/gal EtOH

1. Lau MW, Gunawan C, & Dale BE (2009) The impacts of pretreatment on the fermentability of pretreated lignocellulosic biomass: a comparative evaluation between ammonia fiber expansion and dilute acid pretreatment. *Biotechnol Biofuels* 2:30.
2. Eggeman T & Elander R (2005) Process and economic analysis of pretreatment technologies. *Bioresource Technology* 96:2019-2025.

SUPPLEMENTARY INFORMATION: AN INTEGRATED PARADIGM FOR CELLULOSIC BIOREFINERIES: UTILIZATION OF LIGNOCELLULOSIC BIOMASS AS SELF SUFFICIENT FEEDSTOCKS FOR FUEL, FOOD PRECURSORS AND SACCHAROLYTIC ENZYME PRODUCTION.

Ming W Lau[1], Bryan D Bals[1], Shishir P S Chundawat[1], Mingjie Jin[1], Christa Gunawan[1], Venkatesh Balan[1], A Daniel Jones[2] and Bruce E Dale[1]

AUTHOR AFFILIATIONS
[1]Department of Chemical Engineering and Materials Science, DOE Great Lakes Bioenergy Research Center, Michigan State University, 3900 Collins Rd, Lansing, MI 48910, USA
[2]Department of Biochemistry and Molecular Biology, Michigan State University, 212 Biochemistry, East Lansing, MI 48824, USA

CORRESPONDING AUTHOR
Bruce E Dale (b.dale@egr.msu.edu)
Ming W Lau (lauming@egr.msu.edu)

Supplementary Table 1: Full mineral analysis results for untreated CS, AFEX-CS and residual solids after enzymatic hydrolysis

Concentration (mg/kg)	Untreated Kramer Corn Stover	AFEX Kramer Corn Stover	Residual Solid after 6% Glucan Enzymatic Hydrolysis
Cr	3.4E+01	1.2E+01	1.2E+00
Co	5.8E-01	1.2E-01	7.5E-02
Ni	1.6E+01	5.3E+00	1.3E+00
Cu	3.9E+00	3.4E+00	9.5E+00
As	1.0E-01	7.2E-02	Not Detected
Cd	9.1E-02	7.5E-02	2.0E-01
Pb	1.8E+00	1.0E+00	2.4E+00
Mo	4.9E+00	1.9E+00	1.9E+00
U	7.9E-02	6.0E-02	1.8E-01
Mn	1.5E+01	1.2E+01	8.2E+00
Zn	8.5E+00	8.1E+00	1.9E+01
Se	Not Detected	Not Detected	Not Detected
Ba	2.8E+01	2.5E+01	2.9E+01
Fe	1.8E+02	1.2E+02	1.1E+02
Ca	1.9E+03	1.8E+03	1.8E+03
P	**6.3E+02**	**6.5E+02**	**8.5E+02**
Na	Not Detected	Not Detected	2.0E+02
K	**1.1E+04**	**1.2E+04**	**2.5E+03**
Mg	8.7E+02	8.5E+02	2.7E+02
Total	1.5E+04	1.5E+04	5.8E+03

Supplementary Table 2: Full mineral analysis results for 9% SLE water extract of AFEX-CS and AFEX-RS

	9% SLE Water Extract Rice Straw	**9% SLE Water Extract Corn Stover**	**Concentration**
Cr	8.08	Not Detected	µg/L
Co	21.92	82.73	
Ni	61.16	29.79	
Cu	143.47	89.41	
As	64.94	Not Detected	
Cd	Not Detected	Not Detected	
Pb	Not Detected	17.65	
Mo	33.33	23.21	
U	Not Detected	Not Detected	
Mn	12186.09	198.19	
Zn	278.71	126.63	
Se	Not Detected	Not Detected	
Ba	334.01	465.36	
Fe	437.18	152.98	
P	Not Detected	Not Detected	
Na	37.34	Not Detected	mg/L
Mg	96.23	57.33	
Ca	Not Detected	73.40	
K	1.62	1.16	g/L

Supplementary Table 3: Concentration of Nitrogenous Compounds in Commercial Enzymes

	Total Nitrogen	Protein Equivalent
	mg/mL	
Accellerase 1000	8.5±0.1	53.1±0.6
Spezyme CP	13.4±1.1	83.5±6.8
Novozyme 188	10.6±0.1	66.3±0.7
Multifect Xylanase	5.0±0.3	31.0±1.7
Multifect Pectinase	8.3±0.0	51.9±0.2

Supplementary Table 4. Top 37 secreted Trichoderma reesei (RUT-C30) proteins (in descending order of spectral abundance) with known functions expressed using AFEX treated corn stover water extract (WE), AFEX treated corn stover water extract and solid biomass (AFCS+WE), and lactose only. Where; JGI # is the Joint Genome Institute accession number for Trichoderma proteins, GH # is glycosyl hydrolase family number.

						Abundance (Spectral Counts)		
Sr #	Mol Wt	JGI Accession #	Uniprot #	Enzyme Name	GH family #	WE	AFCS+WE	Lactose
1	54 kDa	123989	P62694	Cellobiohydrolase I (Cel7A)	7	76	60	68
2	87 kDa	121127	Q92458	β-Xylosidase	3	70	66	0
3	87 kDa	49081	Q7Z9M8	Xyloglucanase (Cel74A)	74	50	35	28
4	24 kDa	123818	Q02244	Xylanase 2	11	35	37	6
5	51 kDa	123283	Q92455	α-Arabinofuranosidase 1	54	27	29	15
6	50 kDa	72567	P07987	Cellobiohydrolase II (Cel6A)	6	27	21	26
7	52 kDa	123992	Q9P8D0	Swollenin	-	21	17	10
8	48 kDa	123940	Q7Z9N1	Cip2	-	16	12	1
9	33 kDa	73638	Q7Z9M9	Cip1	-	13	12	5
10	35 kDa	76210	Q7Z9N0	α-Arabinofuranosidase 2	62	12	12	3
11	22 kDa	54219	Q99034	Acetyl xylan esterase	-	11	9	0
12	46 kDa	80833	Q65YQ7	Chitinase	18	10	5	20
13	36 kDa	73643	O14405	Endoglucanase 4 (Cel61A)	61	9	5	3
14	93 kDa	51365	A4V8W5	Peptidase	-	7	23	0
15	93 kDa	72526	Q99024	α-Glucuronidase	67	6	52	0
16	38 kDa	120229	Q9P973	Xylanase 3	10	6	8	0
17	48 kDa	122081	P07981	Endoglucanase 1 (Cel7B)	7	4	5	7
18	26 kDa	73897	A4V8W4	Peptidase	-	4	1	6
19	79 kDa	70845	Q8TG99	β-1,3-glucanase	55	4	0	7
20	67 kDa	120873	Q8WZM7	α-1,3-Glucanase	71	4	1	0
21	83 kDa	121746	Q9P491	β-1,3-Glucosidase	55	3	1	19
22	69 kDa	21960	Q157R5	Phosphoesterase	-	3	0	1
23	44 kDa	120312	P07982	Endoglucanase 2 (Cel5A)	5	2	3	5
24	40 kDa	56996	Q99036	β-Mannase	5	2	1	2
25	111 kDa	80240	Q70SY0	β-Galactosidase	35	1	18	0
26	38 kDa	103049	B7ZEN3	Polygalacturonase	28	1	4	0
27	41 kDa	123234	A2TM20	Peptidase	-	1	1	0
28	67 kDa	1885	Q599K8	Glucoamylase	15	1	0	3
29	42 kDa	77579	B3VTV5	Protease	-	0	1	16
30	64 kDa	21725	P78738	Chitinase	20	0	0	16
31	58 kDa	123244	A4V8W2	Peptidase	-	0	0	13
32	56 kDa	45717	Q9P8T8	α-1,2-Mannosidase	47	0	0	10
33	25 kDa	123232	O00095	Endoglucanase 3 (Cel12A)	12	0	4	0
34	49 kDa	72632	Q92456	α-Galactosidase	27	0	3	0
35	39 kDa	121418	A7J2C6	Acetyl esterase	-	0	3	0
36	36 kDa	59791	A2VEC4	Chitinase	18	0	0	5
37	35 kDa	43873	A2VEC1	Chitinase	18	0	0	4

Supplementary Table 5: Top 38-79 secreted Trichoderma reesei (RUT-C30) proteins with uncharacterized or putative functions expressed using AFEX treated corn stover water extract (WE), AFEX treated corn stover water extract and solid biomass (AFCS+WE), and lactose only. Where; JGI # is the Joint Genome Institute accession number for Trichoderma proteins.

			Abundance (Spectral Counts)			Homolog		
Sr #	Mol Wt	JGI #	WE	AFCS+WE	Lactose	Uniprot #	% Identity	Putative Function
38	53 kDa	111849	40	40	2	B6QTH8	76	GH 30 Hydrolase
39	29 kDa	65406	24	17	32	C7Z603	69	GH 16 Hydrolase
40	20 kDa	122127	16	14	20	C7ZJF8	53	Unknown
41	48 kDa	76155	12	5	15	C7ZIV2	71	Phosphoesterase
42	57 kDa	22914	9	12	38	C7Z1Y3	62	GH 72 Hydrolase
43	42 kDa	39942	9	10	12	C7ZPF2	65	GH 17 Hydrolase
44	36 kDa	66792	6	4	10	C7YSI0	68	GH 17 Hydrolase
45	40 kDa	124175	5	2	15	C7YZ45	57	GH 64 Hydrolase
46	49 kDa	69276	5	6	3	A8NHV9	54	GH 30 Hydrolase
47	25 kDa	112018	5	4	6	C7ZBH7	66	Unknown
48	53 kDa	69650	5	2	6	Q5AXA5	65	Oxidoreductase
49	41 kDa	68067	4	8	22	C7YRX5	50	Unknown
50	154 kDa	81517	4	1	9	C7Z8P8	56	DNA deacetylase
51	99 kDa	82235	3	4	4	Q75QW0	65	GH 30 Hydrolase
52	89 kDa	74198	3	3	0	A4RJ44	62	GH 92 Hydrolase
53	21 kDa	102908	2	1	4	C7Z940	45	Unknown
54	44 kDa	71094	2	1	4	C7Z1U0	64	Oxidoreductase
55	37 kDa	104461	1	3	6	C7YGW3	57	Unknown
56	55 kDa	81070	1	1	8	C7Z801	68	Peptidase
57	117 kDa	123456	1	3	0	B6H9U0	68	GH 65 Hydrolase
58	13 kDa	122374	1	0	4	C5FWC7	26	Unknown
59	49 kDa	123538	0	0	12	C7YRY4	72	GH 72 Hydrolase
60	25 kDa	76971	0	0	8	C7E9V9	56	Unknown
61	27 kDa	107704	0	0	8	A1C5D7	53	Unknown
62	33 kDa	31248	0	0	7	A7EEQ5	63	Ribonuclease
63	39 kDa	55887	0	0	7	C7Z7C2	47	Unknown
64	97 kDa	72379	0	0	7	Q9Y7V5	43	Conidiospore Surface Protein
65	77 kDa	81778	0	0	6	A1CJH2	55	Glutaminase
66	20 kDa	65483	0	0	6	C7YH29	60	Super Oxide Dismutase
67	46 kDa	22210	0	0	5	A4R6X2	63	Peptidase
68	14 kDa	108663	0	1	2	B8N8E9	26	Unknown
69	70 kDa	66616	0	1	1	A0A8X4	69	Phosphatase
70	56 kDa	82633	0	0	3	C7Z1Y3	55	GH 72 Hydrolase
71	62 kDa	71170	0	0	4	C7ZAZ1	55	Unknown
72	102 kDa	5836	0	0	4	B8NP78	60	GH 2 Hydrolase
73	66 kDa	44366	0	0	3	Q5AZ98	57	Phosphoesterase
74	24 kDa	103458	0	0	3	B8M1A1	65	GH 25 Hydrolase
75	76 kDa	121475	0	0	3	B6Q6E0	43	Unknown
76	31 kDa	124259	0	0	3	C7Z2C2	38	Phosphatase
77	22 kDa	2537	0	0	2	C7Z6R8	90	RHO-GDI
78	61 kDa	56830	0	1	0	B6GZT9	55	Carboxyl Esterase
79	88 kDa	79921	0	0	2	A1DCD4	69	GH 92 Hydrolase

Supplementary Table 6: List of major assumptions for the proposed process scheme

Feedstock		
Insoluble glucose (IG)	386	g/kg dry biomass (BM)
Insoluble pentose (IX)	262	g/kg BM
Lignin	240	g/kg BM
Oligomeric glucose (IG) conversion during AFEX	0.05	g/g IG
Oligomeric pentose (OX) conversion during AFEX	0.15	g/g IX
Water	0.6	g water/g BM
Screw Press/Washing		
Water requirement	4	g water/g dry biomass
Post-press moisture content	0.75	g water/g total weight
Enzyme Production		
OX requirement	5	g OX/kg BM
Enzyme production rate constant	0.05	h^{-1}
Residence time	96	hours
Sugar consumption	3	g sugar/g enzyme
Scaling size for seed train	0.01	g/g
Corn steep liquor requirement	10	g/kg BM
Cellulose Hydrolysis		
Solid loading	0.18	g initial BM/g total weight
Enzyme loading	10	g enzyme/ kg BM
IG -> MG conversion	0.9	g MG produced/g IG
IX -> OX conversion	0.9	g OX produced/g IX
OX -> MX conversion	0.7	g MX produced/g OX
Residence time	72	hours
Post-hydrolysis press		
Final moisture content	0.5	g water/g total weight
Ethanol Fermentation		
Yeast loading	0.01	g/g BM
Residence time - glucose	15	hours
Residence time - xylose	46	hours
MG fermentation extent	1	g MG consumed/g MG
MG efficiency	0.48	g EtOH/g MG
MX fermentation extent	0.8	g MX consumed/g MX
MX efficiency	0.45	g EtOH/g MX
MX rate	0.05	g MX/g yeast/h
Scaling size for seed train	0.01	g water/g water fermentation
Sugar consumption	1	g MG/yeast produced
Initial yeast density	0.225	g yeast/kg water
Yeast after glucose	6.75	g/kg water
Second washing		
Water requirement	15	g water/g insoluble biomass
Moisture content after press	0.8	g water/g total weight

Settling Tank		
Residence time	3	hours
Recovery efficiency	0.95	g/g yeast
Yeast to recycle for xylose	0.658	g/g yeast
Economics		
Yeast extract selling price	800	$/Mg
Ethanol selling price	1.70	$/gal ethanol
Electricity price (in-house production)	0.05	$/kW*h
Feedstock buying price	65	$/Mg
Biorefinery size	2000	Mg dry biomass/day

Supplementary Table 7: List of major pieces of equipment in the biochemical conversion section of the biorefinery for the proposed process scheme.

Equipment	Size (L)[a]	Number	2008 Cost	Total Cost	Source
Wash table	85000	1	$150,000	$150,000	[1]
Screw press	13750	5	$150,000	$770,000	[2]
Hydrolysis tank	3600000	10	$730,000	$7,290,000	[1]
Holding tank	720000	3	$210,000	$1,290,000	[1]
Pneumapress package	26000	4	$2,070,000	$8,290,000	[1]
Filter press	100[b]	1	$70,000	$70,000	[3]
Glucose Fermentation	2400000	3	$590,000	$1,780,000	[1]
Holding tank	2400000	1	$570,000	$570,000	[1]
Settling tank	1000000	2	$110,000	$220,000	[4]
Xylose Fermentation	3600000	6	$730,000	$4,370,000	[1]
Settling tank	1000000	2	$110,000	$220,000	[4]
Tunnel dryer	0.02[c]	1	$1,120,000	$1,120,000	[3]
T. reesei seed fermenter 1	10000	2	$80,000	$150,000	[1]
T. reesei seed fermenter 2	100	2	$20,000	$50,000	[1]
T. reesei Fermentation	1000000	14	$260,000	$3,600,000	[1]
T. reesei Agitators	1000000	14	$820,000	$11,460,000	[5]
T. reesei Air compressors	5000000	3	$860,000	$2,580,000	[5]
Enzyme holding tank	3600000	1	$730,000	$730,000	[1]
Seed yeast fermenter 1	8500	3	$70,000	$220,000	[1]
Seed yeast fermenter 2	85	3	$20,000	$70,000	[1]
Beer Storage tank		1	$350,000	$350,000	[1]
Total – Major Equipment				$45,450,000	
Minor Equipment				$2,270,000	
Total Project Investment				$336,530,000	

[a] Unless otherwise indicated, the size of the equipment is based on the total mass flow of the process, assuming all material has a specific gravity of 1.

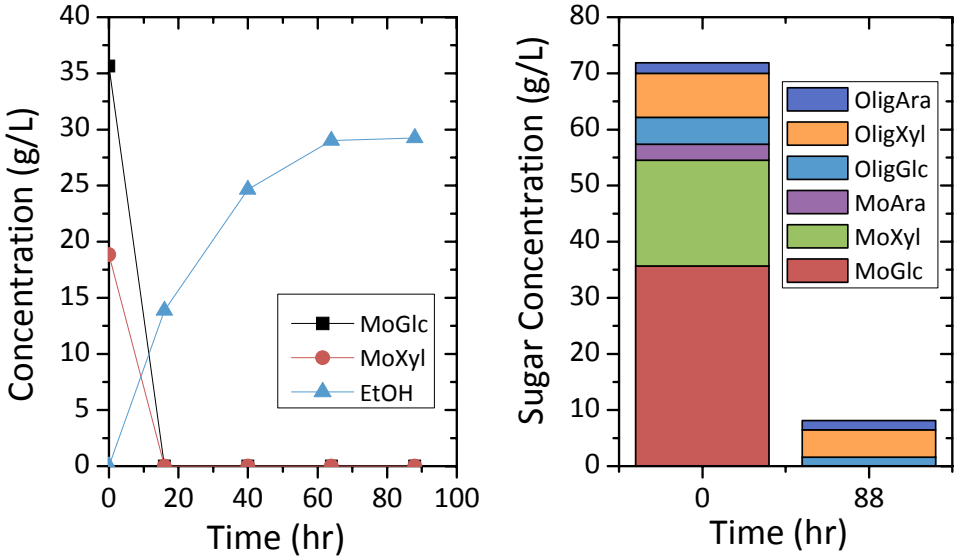
[b] Units for the filter press are the filtration area (m^2) required to effectively filter the insoluble residue.

[c] Units for the tunnel dryer are in thickness of yeast to be dried (m)

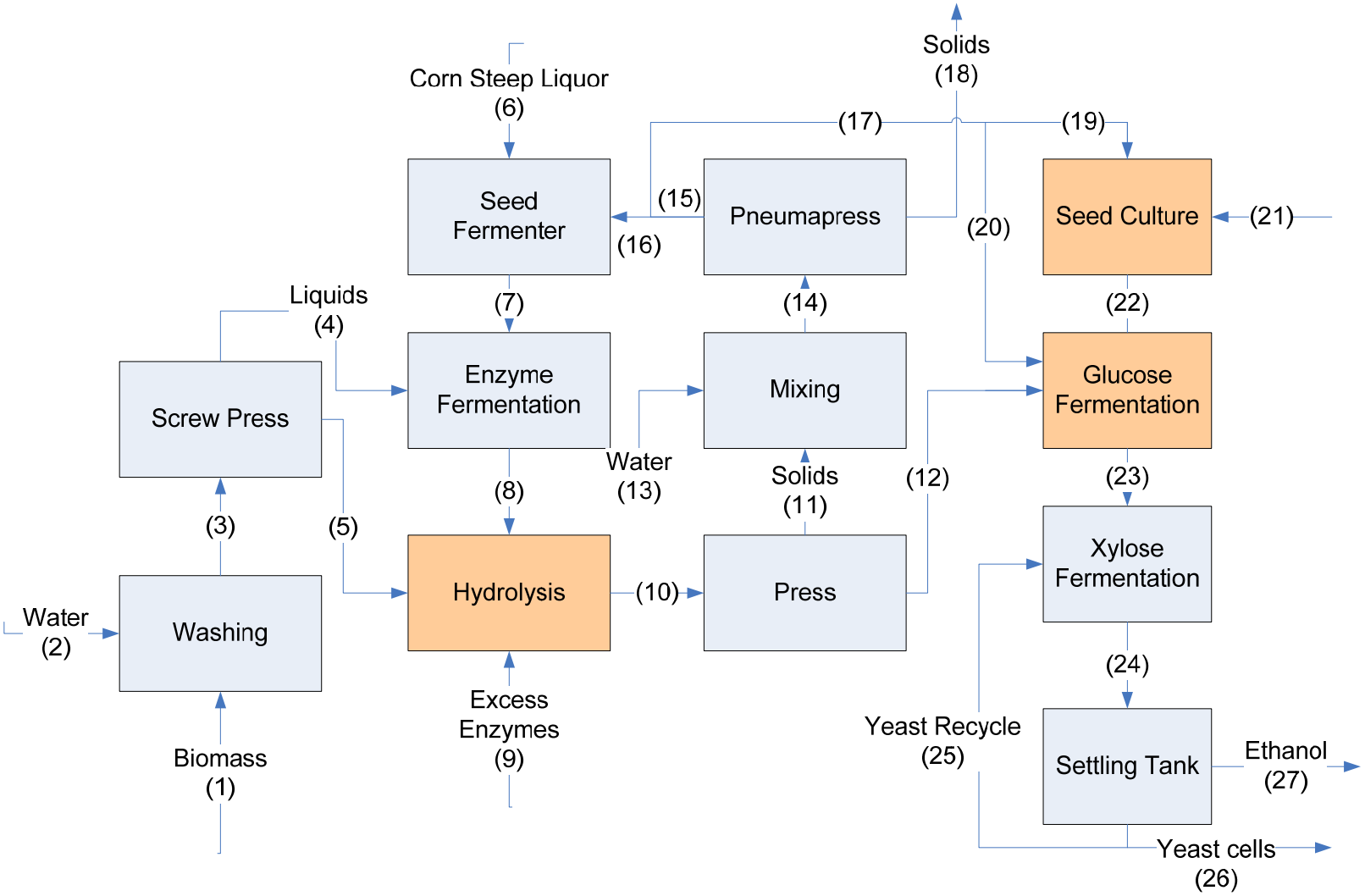
Supplementary Table 8: List of changes in operating cost for the proposed process.

	Amount (kg/Mg biomass)	Cost ($/Mg)	$/Mg biomass
Corn steep liquor	10	185.27	$1.85
Enzyme	2.42	3600	$8.71
Maintenance	2% of capital costs		$5.83
Electricity	Number	kW*h/Mg	kW*h/Mg
Screw Press	5	0.89	4.47
Tunnel dryer	0.08	3.2	21.83
Agitators	14	4.8	67.2
Excess Electricity Costs ($/Mg biomass)			$4.68

Supplemental Fig 1: Fermentation of AFEX-CS hydrolysate using T. saccharolyticum ALK2



Supplemental Fig 2: Process flow diagram of the biological conversion section of the biorefinery with in-house enzyme production and yeast co-production included. Orange squares represent operations also present within the base case scenario



Supplementary Methods

18% SLE water extract preparation

AFEX-pretreated corn stover was washed with distilled water at a ratio of 1 g dry AFEX-CS to 4.6 g of water to produce an aqueous extract (18% solids loading equivalent). In each batch of washing, distilled water was preheated to 60-70°C and added to 100 g (dry weight equivalent) of AFEX-CS. The water content of the wetted AFEX-CS was reduced by pressing. The washing was conducted in three cycles, i.e. water-extract from a previous cycle of washing was used for the next cycle of washing. In the final cycle of washing, the moisture content of the washed AFEX-CS was reduced to 77±3%. The AFEX-CS water extract was used for the fermentation. The preparation steps were as before [6]. The total sugar solubilized was calculated by multiplying total soluble sugar in the water extract with total volume of the water extract from a given mass of dry AFEX-CS.

Nutrient Content Analysis

Ammonia

Free ammonia in AFEX-CS hydrolysate was analyzed through an enzymatic assay from R-biopharm AG (Cat no: 11112732035, Darmstadt, Germany). The solution was diluted to an appropriate level for assay detection. The level of reduction of NADH, which indicates the concentration of ammonia in the solution, was measured as the absorbance 340 nm wavelength using a spectrophotometer. A standard ammonia solution (control experiment) was tested to ensure the accuracy of the results. Other experimental details and enzymatic chemistry explanation can be found in the manufacturer's instruction manuals.

Protein

The analyses for amino acid concentrations on AFEX-CS hydrolysate were conducted in MSU Macromolecular Structure Facility through a High Performance Liquid Chromatography (HPLC) system equipped with a Nova Pak C18 (3.9mm×150mm; Waters). Operational details of the system were as described [7]. The amino acids involved in the analysis are Asp, Glu, Ser, Gly, His, Thr, Arg, Ala, Pro, Tyr, Val, Met, Ile, Lys and Phe.

Free Amino Acids

500 µL of each of the respective solutions were filtered (Millipore Centricon), 20uL of the filtered elute was derivatized with AccQ Tag (Waters), 10% of the total derivatized sample was injected into the HPLC system.

Protein Amino Acids

The three solutions were dried under vacuum (SpeedVac, Savant) and hydrolyzed with 6N HCl at vapor phase at 100°C for 24 hrs. The hydrolyzed dry samples were solubilized in 100 µL of 20mM HCl and 10µL of the mixture was derivatized with AccQTag (Waters). 10% of the derivatized mixture was injected into a Nova Pak C18 (3.9mm×150mm; Waters).

Total Nitrogen Content

Nitrogen content of the dry untreated CS, AFEX-treated CS, solid residue, enzyme solution and AFEX-CS hydrolysate were determined using a Skalar Primacs SN Total Nitrogen Analyzer (Breda, The Netherlands). Liquid samples (1 mL) were dried at 110°C overnight prior to the analysis. The nitrogen analysis is based on the Dumas method using EDTA as the standards. Nitrogen content of the samples was calculated by dividing nitrogen content (g) of the analyzed materials by weight or volume of the samples.

Minerals

Trace elements were measured by inductively-coupled-plasma mass spectrometry (ICP-MS) in the MSU Department of Geological Sciences.

Liquid Samples:

Approximately 1 mL of liquid sample was digested on a hot plate, sub-boiling, in acid cleaned Teflon savillex beakers using 1.9 mL Optima nitric acid and 0.1 mL trace metal clean hydrofluoric acid for 24 hours. After digestion 0.250 mL of trace metal clean 30% hydrogen peroxide was added and the sample evaporated to near dryness on a hotplate. Samples were then brought up to final volume with 5 mL of 2% Optima nitric acid, visual inspection showed a complete digestion of all samples. This solution was run in the ICP-MS for full mass scan analyses.

Solid Samples

Approximately 100 mg of solid samples was added to 5 mL of Optima nitric acid in an acid cleaned Teflon Savillex vial and sonicated for 60 minutes to homogenize the sample. Then the samples were digested, sub-boiling, overnight on a hot-plate. After approximately 24 h, 0.1 mL of trace metal clean hydrofluoric acid and 1 mL of trace metal clean 30% hydrogen peroxide was added and digested for another 24 hours. Finally the samples were allowed to evaporate to near dryness and taken up to a final volume of 5 mL with 2% Optima nitric acid. This solution was run in the ICP-MS for full mass scan analyses.

For major element analysis: potassium (K), magnesium (Mg), calcium (Ca), phosphorus (P), and sodium (Na) samples were diluted 1:300 prior to analysis. For trace element analysis: chromium (Cr), cobalt (Co), nickel (Ni), copper (Cu), arsenic (As), cadmium (Cd), lead (Pb), molybdenum (Mo), uranium (U), manganese (Mn), zinc (Zn), selenium (Se), barium (Ba) and iron (Fe) samples were run without dilution.

Vitamins

Five vitamins important for industrial fermentations were analyzed using a LC/MS/MS (Quattro Micro, Waters) using a Water Symmetry C-18 column. The mobile phase was run at 0.3 mL/min with a gradient of 1 mM perfluoroheptanoic acid and acetonitrile. Mass spectra were acquired for 6 min using electrospray ionization in positive ion mode. The capillary voltage, extractor voltage and RF lens voltage was set at 3.17 kV, 4.00 V and 0.3 V, respectively. The source temperature and desolvation temperature were at 110°C and 350°C. The desolvation gas flow was set at 400L/hr. Collision energies and source cone potentials were optimized for each transition using Waters QuanOptimize software. Data was acquired with MassLynx 4.0 and processed with QuanLynx software.

FermGold™ Corn Steep Liquor (Lot: 154-07) from Cargill, Inc (Minneapolis, MN) was used as the protein supplement for fermentations. To prepare 20%w/w CSL, 200 g of FermGold™ CSL was diluted to total volume of 1.0 liter with distilled water after pH was adjusted to 5.0 with reagent grade KOH. The insoluble solids were separated from the liquid by centrifugation at 5,000 × g for 30 min. The 20% w/w CSL was sterile-filtered (0.22µm) and used for media preparation.

Determination of Protein Concentration in Complex Enzymes

The protein concentrations of commercial enzymes Accelerase 1000, Spezyme CP, Novozyme 188, Multifect Xylanase, and Multifect Pectinase were determined through nitrogen content analyses of the protein precipitate. Each complex enzyme was centrifuged (13,000 x g) for 5 min, and 0.20 mL of clear supernatant of the enzyme was combined with 0.25 mL 100% w/v trichloroacetic acid (TCA) and 0.80 mL distilled water to precipitate the protein in the enzyme solution. After 5 minute of incubation at 4°C, the mixture was centrifuged at 13,000 x g for 5 min and the supernatant was decanted. The precipitate was washed with 1.0 mL cold (4°C) acetone twice, each washing was followed by centrifugation and decanting the residual acetone. The washed protein precipitate was placed in a crucible (a sample holder for nitrogen analyzer) and dried under vacuum.

Nitrogen content within the precipitate was determined using a Skalar Primacs SN Total Nitrogen Analyzer (Breda, The Netherlands). The principle behind the nitrogen analysis is based on the Dumas method using EDTA as the standard. Nitrogen content was converted to protein content by multiplying a factor of 6.25. Errors represented are standard deviation of duplicate experiments. The protein concentrations of the respective commercial enzymes analyzed according to this protocol are presented in **S.T. 3**

Fed-batch Fermentation of Enzymatic Hydrolysate using enzyme secreting ethanologen Thermoanaerobacterium saccharolyticum

Fed-batch fermentation was conducted in a custom-made fermenter (NDS Technologies, NJ) equipped with a pH probe. The fermenter temperature was controlled by an external water bath recirculation system. Feeding and pH were controlled by Sartorius A plus system (Goettingen, Germany). Initial volume of the reactor was 120 mL which consisted of 20 mL enzymatic hydrolysate at 18% solids loading, nutrient supplement and distilled water (for dilution). For nutrient supplementation, 1.0 g yeast extract, 0.5 g peptone, appropriate levels of minerals and vitamins was added. The fermentation media was pH-adjusted to 6.2 with KOH and sparged with nitrogen for about 10 min to create anaerobic condition. The seed culture (10mL) was inoculated to initiate fermentation. Undiluted 18% solids loading enzymatic hydrolysate at pH 6.2 (supplemented with 10 g/L yeast extract and 5 g/L peptone), was used as the feed. Feeding started 4 hr after inoculation at the rate of 4.0 mL/hr until 180 mL of feed volume was added into the fermenter. Samples were taken at the designated periods. Glucose, xylose, arabinose (in monomeric form) and ethanol were analyzed using HPLC. Oligomeric sugars were analyzed through acid hydrolysis based on NREL Protocol LAP-014.

In rich nutrient-supplemented fermentation, nearly to 90% of the total sugars (monomers and oligomers) in the hydrolysate were consumed, and a metabolic yield of 0.45 g EtOH/ g consumed sugars was achieved **S.F. 1**. Fermentation was completed within 64 hr after inoculation; 15 hr after feeding was concluded. Over 60% of the total oligomeric sugars were consumed in this time period. We demonstrated that ALK2 is able to grow and produce ethanol to 30 g/L at 0.45 g/L/hr (0-64hr) from the hydrolysate containing degradation compounds equivalent to 11.7% solids loading of AFEX-Corn Stover **S.F. 1**.

Techno-economic analysis

The techno-economic analysis used in this study is a factor level estimate based on the major pieces of equipment present in the biological conversion area of a refinery. The initial model was developed by the National Renewable Energy Laboratory (henceforth referred to as the NREL model), and includes both traditional saccharification and fermentation [1] as well as enzyme production [8]. The initial model uses dilute acid as a pretreatment method, but was adapted to AFEX pretreatment in a later study developed for the Consortium for Applied Fundamentals and Innovation project (henceforth referred to as the CAFI model) [9]. The economic assumptions of these three models were used whenever possible. In particular, the CAFI model was used as the basis of the biorefinery studied.

The baseline conventional approach was based heavily on the CAFI model, but with minor adjustments in assumptions. In the initial model, total hydrolysis and fermentation time was 168 hours, although current data suggests 72 hours for hydrolysis and 72 hours for fermentation are sufficient. While simultaneous saccharification and fermentation can occur, it was not explicitly modeled as such. Instead, hydrolysis and ethanol yields were estimated based on experimental data [10]. With improved enzyme formulation, yields can improve over those presented in the literature, and monomeric sugars can increase relative to oligomeric sugars. Thus, we project ethanol yields will increase by 20% compared to the older experimental data. In comparison, the CAFI model used 26% higher ethanol yields than that presented in Lau and Dale [9, 10]. Feedstock costs were also increased to $65/Mg, which was estimated to be a viable price for delivered corn stover [5, 11]. Likewise, enzyme costs were changed to $3600/Mg pure enzyme, which at 10 g/kg feedstock is equivalent to approximately $0.50/gal ethanol, a number recently cited by Genencor and Novozyme as the current state of technology. Finally, all costs were updated to 2008 dollars.

The model used in this study uses $/Mg feedstock as the unit of comparison for all costs and revenues. For costs, only equipment, fixed costs, and raw materials are considered. The cost of individual pieces of equipment was estimated based on their size, scaling factor, and the unit price of a base unit. Only major pieces of equipment were considered. Based on the equipment list in the NREL model, these pieces consisted of approximately 95% of cost of the total equipment in the biological conversion area [1]. Thus, the total cost was multiplied by 1.05 to compensate for this difference. The final figure was multiplied by 1.243 to obtain the installed cost, and added to the installed cost of the rest of the refinery ($147 million)[12]. A Lang factor of 1.628 was applied to obtain the total project investment [1], and straight-line depreciation over 20 years was applied to determine the capital cost per Mg feedstock. Fixed costs include salaries, overhead, maintenance, and insurance, and are all determined in the same manner as the NREL model. Raw materials included feedstock, cellulase, corn steep

liquor, and all other necessary chemicals for biorefinery operations (ammonia, wastewater treatment chemicals, etc) which were assumed to be the same as the NREL model.

All heat and power requirements are supplied by burning lignin. No steam is required in the biological conversion step, and all temperature changes are mild changes. Thus, no changes in heat requirements were made relative to the NREL model, as it was assumed that heat integration is possible to supply all changes in energy. For electricity, the added requirements of presses and agitation for the *T. reesei* fermentation were included. Because excess electricity is produced at the biorefinery, changes in electricity use are treated as a change in revenue, decreasing the revenue generated by selling excess electricity. Native yeast co-production is dried and sold as-is. While yeast extract has a very high market value $7000-8000/ metric ton [13], a modest value of $800/Mg of yeast cells is assumed here to account for further processing. Ethanol selling price is assumed to be $1.70/gal, and electricity selling price to the grid is $0.05/kWh.

A process flow diagram of the proposed biological conversion approach is shown in **S.F. 2**. A wash table is used to wet the biomass after AFEX pretreatment, using diluted recycled hydrolysate as the water media. The biomass is then dewatered using a screw press. The cost and performance of the screw press was estimated using a Vincent Corporation twin screw press, which has been successfully used on lignocellulosic biomass [14]. The water effluent is rich in oligomeric sugars produced during AFEX (and recycled from hydrolysis), and is thus used to induce the T. reesei enzyme production. As an initial approximation, the fungus is assumed to consume 3 g sugar for every g enzyme produced. Esterbauer et al. [15] report an average efficiency of 4 g sugar consumed per g enzyme, but also observe efficiencies as high as 2 g sugar per g enzyme. Due to the improvements in sugar release from oligomers relative to lactose as reported in this study, the improved efficiency of 3 g sugar per g enzyme seems reasonable. Enzyme production is modeled as a first order reaction of oligomeric sugar with a rate constant of 0.05 h^{-1}. This is sufficient to produce 76% of the enzymes required for lignocellulosic hydrolysis, consistent with the results presented in this study. Total *T. reesei* fermentation time was assumed to be 96 hours; this was varied in a later sensitivity analysis.

A total of 10 g corn steep liquor (CSL) was consumed per kg biomass to provide the nutrients necessary for *T. reesei* growth and enzyme production. While this is an order of magnitude lower than the experimental data, the experimental value (~100 g/kg biomass) is not reasonable in a commercial scale. For the experiments, the corn steep liquor was filtered to remove insoluble solids, which removes ~35% of the total nitrogen in CSL. Thus, less CSL would be required in a commercial operation. Furthermore, saccharolytic enzyme loading of 15 mg/g was used in our experiments to prove the concept of in-house enzyme production. However, in commercial production, enzyme loading at 3-6 mg/g is shown to be sufficient[16]. Likewise, the

biomass contains approximately 6 g nitrogen per kg biomass in the form of acetamide, nearly four times as much nitrogen as required for enzymes. Acetamide is not consumed by *T. reesei* but is by other organisms. Thus, if the fungus can be modified to consume acetamide and can be adapted more fully to AFEX-treated corn stover, then much lower nutrient supplementation would be required.

After enzymatic hydrolysis, a pneumapress is used to separate the solids and liquids. This press uses compressed air to force more water out of the biomass, reducing the moisture content to 50% of the total weight. This package is used in the NREL model after distillation, and the same economic assumptions are used here. Because no additional solubilization of biomass occurs after hydrolysis, the cost of the pneumapress is no different in this model than in the NREL model. The liquid released from the press is used as the fermentation media. However, the insoluble biomass still retains some water, which includes hydrolyzed sugars. To ensure that all hydrolyzed sugars are used, the biomass is rinsed with fresh water and then dewatered using a filter press. The cost of this press was estimated using design equations from Peters et al. [3] A second pneumapress was deemed too expensive, and thus the final moisture content of the insoluble residue is 80% water. This residue then exits the process and is burnt for heat and power. The rinsed water is separated into multiple streams. Much of the water is used as the *T. reesei* fermentation media and as the rinse water for obtaining *T. reesei* induction. The remaining water is used as a seed culture for yeast fermentation or combined into the fermentation media.

Fermentation is separated between glucose and xylose fermentation. A settling tank is placed in between glucose and xylose fermentation to recover the yeast. As a first approximation, a residence time of 3 hours was used to settle 95% of the yeast, based on the experimental data. Sizing and equipment costs were obtained from van Kasteren et al. [4] The settled yeast were then dried in a tunnel dryer before being sold. Capital cost was estimated using Peters et al. [3], and a value of 3.2 GJ heat per Mg water evaporated was used based off values for drying distiller's grains [17]. This energy was assumed to be in the form of steam, and would reduce electricity production by 30% of the total energy requirement. Glucose fermentation time was 15 hours and xylose was 46 hours. After xylose fermentation, another settling tank is used to recycle the yeast, while the fermentation broth is then sent to distillation.

All major process assumptions are shown in **S.T. 6**. The feedstock is corn stover, and the composition is based on equivalent monomeric sugar content. After AFEX pretreatment, some of the carbohydrates are converted to oligomeric sugars, which are used to induce enzyme production. During cellulose hydrolysis, 18% solid loading is assumed, as it is sufficient to produce 40 g/L ethanol [10]. For simplicity, it is assumed that both in-house enzymes and

exogenous enzymes have the same activity on all carbohydrates, and thus a constant 10 g/kg enzymes is added regardless of the source. In reality, a constant activity would be added, which may mean different amounts of enzymes depending on the scale of in-house production. During hydrolysis, it is expected that most of the enzymes are deactivated by permanently binding to the biomass. In this study, 90% of the enzymes were assumed to be deactivated, and thus any recycled enzymes represent only a small fraction of the total.

For fermentation, it was assumed that monomeric glucose is completely consumed, as demonstrated in experimental data. No oligomeric sugars are consumed, and maximum xylose consumption is only 80% of the total sugar present. In addition, total xylose consumed is based on a linear rate of 0.05 g sugar per g yeast per hour. The experiments presented here suggest that xylose consumption is nearly linear at high cell density, and approximately 80% of the sugar is consumed. When no cell recycle is performed, yeast growth is only present during glucose fermentation. Some cell growth is present during xylose fermentation at high cell density, but it is minor. Glucose fermentation is assumed to be slightly more efficient at producing ethanol, with a metabolic ethanol yield of 0.48 g ethanol per g glucose compared to 0.45 g ethanol per g xylose consumed. Arabinose hydrolysis and fermentation is assumed to be identical to xylose, and thus all model data is in total pentoses.

A list of major pieces of equipment is shown in **S.T. 7**. As stated previously, most costs were obtained from NREL's model. Other sources are also included in the table. As seen from the table, the bulk of the cost is in enzyme fermentation, particularly with the agitation system. Because the fermentation is aerobic, an air compression system is also included. These costs make up over 40% of the total cost in the biological conversion area (consisting of hydrolysis, fermentation, and the new technologies discussed here). However, the biological conversion area only accounts for approximate one fourth of the total capital investment in the refinery. In contrast, the cell recycle regime does not greatly add to the capital costs. Assuming flocculation of yeast can occur, yeast sedimentation can be performed rapidly and thus reducing the need for large tanks. The most expensive piece of equipment involved in the yeast recycle and co-production is the dryer used for native yeast. These costs are actually offset by reducing the total fermentation time, reducing the number of fermenters required. In total, capital costs increase by 13% compared to the base case scenario.

A list of major operating costs in the biological conversion area is shown in **S.T. 8**. The changes made to the biological conversion area decrease the amount of excess electricity produced from 218 kW*h/Mg biomass to 125 kW*h/Mg biomass. Most of the additional electricity demand is due to the agitators for *T. reesei* fermentation, which require 400 kW of power per tank. By using additional steam for heating purposes, the dryer also reduces electricity demand. Interestingly, the screw presses are not a major electricity cost, as not many are needed due to

their high throughput. In terms of raw materials, the dominant cost in the biological conversion area remains the cellulase despite the reduction in use. However, costs are reduced to only 13% of the cost of biomass compared to 55% in the base case scenario. In contrast, the amount of corn steep liquor required by this process under the assumptions present is minor; and thus only accounts for less than $2 per Mg feedstock. Thus, the operating costs of fermentation are also mitigated with this approach.

References

1. Aden, A. et al., Vol. NREL/TP-510-32438. (ed. D.o. Energy)2002).
2. Fedler, C.B., Hanson, J.L., Beruvides, M., Simonton, J. & Lyford, C. (ed. T.S.E.C.O.C.N. CM707)2008).
3. Peters, M.S., Timmerhaus, K.D. & West, R.E. Plant Design and Economics for Chemical Engineers, 5th ed. (McGraw-Hill, Boston; 2003).
4. van Kasteren, J.M.N. & Nisworo, A.P. A process model to estimate the cost of industrial scale biodiesel production from waste cooking oil by supercritical transesterification. *Resources, Conservation and Recycling* **50**, 442-458 (2007).
5. Humbird, D. & Aden, A., Vol. NREL/TP-510-48214. (ed. D.o. Energy)2008).
6. Lau, M.W., Gunawan, C. & Dale, B.E. The impacts of pretreatment on the fermentability of pretreated lignocellulosic biomass: a comparative evaluation between ammonia fiber expansion and dilute acid pretreatment. *Biotechnol Biofuels* **2**, 30 (2009).
7. Bergman, T., Carlquist, M. & Jornvall, H. in Advanced Methods in Protein Microsequence Analysis. (ed. B. Wittmann-Liebold) 45-55 (Springer Verlag, Berlin; 1986).
8. Wooley, R. et al., Vol. NREL/TP-580-26157. (ed. D.o. Energy)1999).
9. Eggeman, T. & Elander, R. Process and economic analysis of pretreatment technologies. *Bioresource Technology* **96**, 2019-2025 (2005).
10. Lau, M.W. & Dale, B.E. Cellulosic ethanol production from AFEX-treated corn stover using Saccharomyces cerevisiae 424A(LNH-ST). *Proceedings of the National Academy of Sciences* **106**, 1368-1373 (2009).
11. Perlack, R.D. & Turhollow, A.F. Feedstock cost analysis of corn stover residues for further processing. *Energy* **28**, 1395-1403 (2003).
12. Eggeman, T. & Elander, R.T. Process and economic analysis of pretreatment technologies. *Bioresour. Technol.* **96**, 2019-2025 (2005).
13. Lawford, H.G. & Rousseau, J.D. Corn steep liquor as a cost-effective nutrition adjunct in high-performance Zymomonas ethanol fermentations. *Appl. Biochem. Biotechnol.* **63-5**, 287-304 (1997).
14. Enochian, R.V., Kohler, G.O., Edwards, R.H., Kuzmicky, D.D. & Vosloh, C.J. (ed. U.S.D.o. Agriculture)1980).
15. Esterbauer, H., Steiner, W., Labudova, I., Hermann, A. & Hayn, M. Production of Trichoderma cellulase in laboratory and pilot scale. *Bioresour. Technol.* **36**, 51-65 (1991).
16. Gao, D., Chundawat, S.P.S., Krishnan, C., Balan, V. & Dale, B.E. Mixture optimization of six core glycosyl hydrolases for maximizing saccharification of ammonia fiber expansion (AFEX) pretreated corn stover. *Bioresour. Technol.* **101**, 2770-2781 (2010).
17. Kwiatkowski, J.R., McAloon, A.J., Taylor, F. & Johnston, D.B. Modeling the process and costs of fuel ethanol production by the corn dry-grind process. *Ind. Crops Prod.* **23**, 288-296 (2006).