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

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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

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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

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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

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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

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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

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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

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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

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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)

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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

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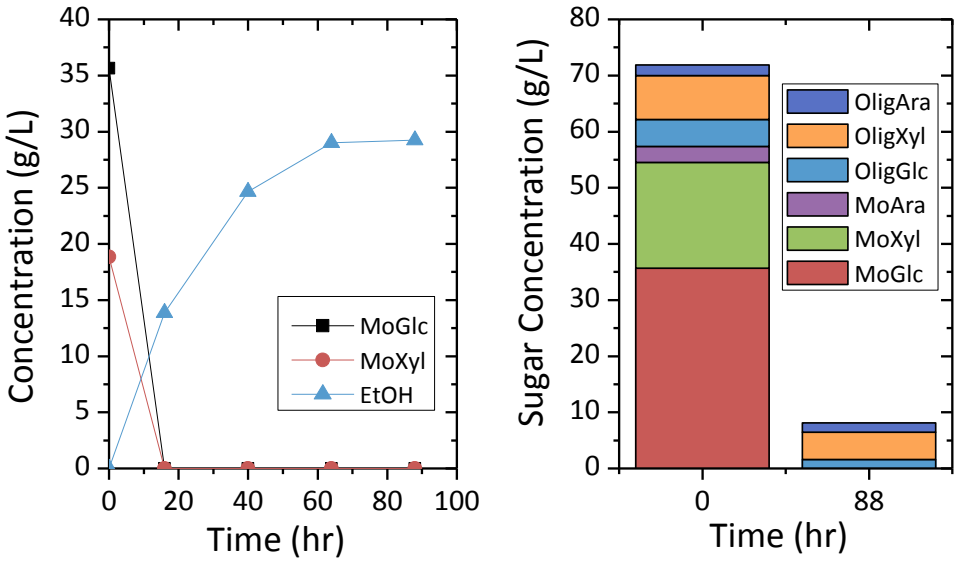
Supplemental Fig 1: Fermentation of AFEX™-CS hydrolysate using T. saccharolyticum ALK2



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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

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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.

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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.

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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**.

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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]. We project that improved utilization of oligomers would occur during fermentation, similar to that shown in **S.F. 1**. Thus, we project ethanol yields will increase by 20% compared to current experimental data with *S. cerevisae* . 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

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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 **Figure 4 A, B.**. 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

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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

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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

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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.

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