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Synthetic multi-component enzyme mixtures for deconstruction of lignocellulosic biomass

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ABSTRACT

A high throughput enzyme assay platform, called GENPLAT, was used to guide the development of an optimized mixture of individual purified enzymes from ten "accessory" and six "core" enzymes. Enzyme mixtures were optimized for release of Glu, Xyl, or a combination of the two from corn stover pretreated by ammonia-fiber expansion (AFEX). Assay conditions were a fixed enzyme loading of 15 mg/g glucan, 48 h digestion, and 50 °C. Five of the ten tested accessory proteins enhanced Glu or Xyl yield compared to the core set alone, and five did not. An 11-component mixture containing the core set and five accessory enzymes optimized for Glu released 52.1% of the available Glu, compared to 38.5% with the core set alone. A mixture optimized for Xyl released 39.9% of the Xyl, compared to 26.4% with the core set alone. We predict that there is still considerable opportunity for further improvement of synthetic mixtures. Furthermore, the strategy described here is applicable to the development of more efficient enzyme cocktails for any pretreatment/biomass combination and for detecting enzymes that make a heretofore unrecognized contribution to lignocellulose deconstruction.

1. Introduction

An essential step in the conversion of lignocellulosic biomass to ethanol or other liquid transportation fuels is the enzyme-catalyzed depolymerization of polysaccharides. However, the high cost of enzymes hinders the development of a viable lignocellulosic ethanol industry (Lynd et al., 2008). One approach to reduce the cost of enzymes is improving their specific activities, i.e., increasing the amount of Glu, Xyl, and other fermentable sugars released per unit of protein.

Current commercial preparations, mainly derived from fermentation of the filamentous fungus *Trichoderma reesei*, contain more than 80 proteins (Nagendran et al., 2009; Banerjee et al., 2010a). Beyond the major cellulases and xylanases, little is known about the involvement of most of these proteins in biomass conversion. Reducing the levels of nonessential enzymes and enhancing the relative proportions of the critical enzymes are therefore potential strategies to increase the specific activities, and thereby lower the cost, of biomass-converting enzymes.

One strategy to the rational improvement of enzyme cocktails is to construct enzyme mixtures *de novo*. This would allow complete control over the individual components and their relative proportions. In a previous paper, we described an experimental platform, named GENPLAT (for Great Lakes Bioenergy Research Center [GLBRC] Enzyme Platform), that can be used to construct and test synthetic enzyme mixtures (Banerjee et al., 2010b). Critical attributes of GENPLAT include the production of individual enzymes free of contaminating activities in a heterologous host such as *Pichia pastoris*, a liquid handling robot that can perform thousands of pipeting steps in a 96-well format, appropriate experimental design, and automated Glu and Xyl assays. We demonstrated the capability of GENPLAT to develop an optimized mixture of six "core" enzymes, defined as cellobiohydrolase 1 (CBH1, GenBank CAA49596), cellobiohydrolase 2 (CBH2, P09787), endo-β1,4-glucanase 1 (EG1, AAA34212), β-glucosidase (BG, AAA18473), endo-β1,4-xylanase 3 (EX3, BAA89465), and β-xylosidase (BX, CAA93248) (Banerjee et al., 2010b). In this paper we show that the hydrolytic efficiency of the core set can be significantly improved by the addition of other "accessory" enzymes. Of the ten tested accessory enzymes, five caused a significant increase in Glu and/or Xyl yield from AFEX-pretreated corn stover. The specific activity of an optimized 11-component mixture equalled commercial enzymes (Accellerase 1000 or Spezyme CP) for Glu yield and

Abbreviations: Glu, glucose; Xyl, xylose; GH, glycosyl hydrolase; CBH, cellobiohydrolase; BG, β-glucosidase; EG, endo-β-1,4-glucanase; BX, β-xylosidase; EX, endo-β-1,4-xylanase; α-Glr, α-glucuronidase; Abf, arabinosidase; MW, molecular weight; AFEX, ammonia-fiber-expansion pretreatment; pNP, para-nitrophenyl; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; GC, gas chromatography.

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surpassed them for Xyl yield, indicating that 11 enzymes alone can perform as well as more complex, unbalanced mixtures.

2. Methods

2.1. Biological materials

All experiments in this study were carried out using the same batch of AFEX-treated "GLBRC corn stover" (*Zea mays* L.) ground to 0.5 mm particle size, as described by Banerjee et al. (2010b). GLBRC stover has a Glu content of 34.4%, Xyl content of 22.4%, and lignin content of approximately 11.0% (Banerjee et al., 2010b). The production and sources of the core set of enzymes are described by Banerjee et al. (2010b). All enzymes are from *T. reesei* or *T. longibrachiatum*. Most of the accessory enzymes used in the current paper were produced in *P. pastoris* from native *T. reesei* genes, with the exception of endo-β-1,4-xylanase 2 (EX2, GenBank AAB29346) and Cel5A (also known as EG2; GenBank AAA34213), which were expressed in *T. reesei* as His-tagged versions. The gene for arabinosidase B (AbfB; Trire2|55139) with an *Eco*RI site at the 5′ end and an *Xba*I site at the 3′ end was synthesized by GenScript (Piscataway, NJ) using codon usage optimized for *P. pastoris*. The other enzymes in this paper, their alternate names and/or abbreviations, and their GenBank accession numbers are Cel12A (EG3, BAA20140); Cel61A (EG4, CAA71999); Cel61B (EG7, AAP57753); α-glucuronidase (α-Glr, CAA92949); arabinosidase 2 (Abf2, AAP57750); Cip1 (AAP57751); and Cip2 (AAP57749). Gene-specific primers were designed with restriction fragment cleavage sites to facilitate cloning into the *P. pastoris* expression vector pPICZ (Supplementary Table S1).

The cloning and expression of individual genes in *P. pastoris* are described by Banerjee et al. (2010b), with the following changes. After concentration of the supernatants (300 ml/batch) to 50 ml, $(NH_4)_2SO_4$ was added to 80% saturation (51.6 g/100 ml), incubated with gentle rocking at 4 °C for 45 min, and centrifuged at 8000×*g* for 30 min. The pellets were redissolved in 3 ml of 25 mM sodium acetate, pH 5, and desalted twice using 10-DG columns (Bio-Rad, Richmond, CA). Glycerol was added to a final concentration of 20% (v/v) glycerol, and the enzymes were stored at −80 °C in 250 μl aliquots.

2.2. Transformation and enzyme production in T. reesei

For construction of the *T. reesei* expression vector pCB1004-EV, a fragment of the *CBH1* promoter (−876 to −12 relative to the ATG translational start) was amplified using PCR primers (Supplementary Table S1) with 5′ *Kpn*I and 3′ *Xho*I restriction sites added, and cloned into the multiple cloning site of pCB1004 (obtained from the Fungal Genetics Stock Center, University of Missouri, Kansas City, MO; http://www.fgsc.net/). An oligonucleotide encoding an *Xba*I site plus a 6x His-tag plus a stop codon (TGA) was used as a 5′ PCR primer. Together with a 3′ PCR primer corresponding to a sequence in the *CBH2* terminator region (downstream of the polyA$^+$ site) and containing a *Sac*I site (Table S1), an 898-bp fragment was amplified and cloned into the multiple cloning site of pCB1004. cDNAs were made as described (Banerjee et al., 2010b). The genes, excluding the stop codons, were amplified with gene-specific primers with the addition of restriction sites compatible with the multiple cloning site between the promoter and His-tagged terminator region of pCB1004-EV. The resulting plasmids were transformed into *T. reesei* strain QM9414 (US Department of Agriculture National Center for Agricultural Utilization Research, Peoria, IL). Fungal transformation was done according to the method of Cho et al. (2006). Transformants were selected on potato dextrose agar (PDA) containing 150 μg/ml hygromycin. Stable transformants were subcultured onto PDA plates without hygromycin to facilitate sporulation. Spores from transformants (~10^7/flask) were inoculated into ten 250-ml baffled flasks each containing 100 ml of lactose-based inducing medium (Karlsson et al., 2001) and incubated for 5 d at 30 °C with shaking at 200 rpm. On the sixth day, the culture filtrates were separated from the mycelium by filtering through Whatman #1 paper. The supernatants were then concentrated to 8 ml by addition of ammonium sulfate to 80% saturation (51.6 g/100 ml). After desalting with a Zeba Desalt Spin Column (Thermo Scientific, Rockford, IL), the 6x-His tagged proteins were purified using a Ni–NTA superflow column (Qiagen, Valencia, CA) following the manufacturer's protocol. The purity of the tagged proteins in the eluted fractions was assessed by SDS–PAGE and mass spectrometry-based proteomics analysis. The enzymes were stored in aliquots of 250 μl in 20% (v/v) glycerol at −80 °C. Protein concentration was determined by the bicinchoninic acid (BCA) method (Pierce Biotechnology, Rockford, IL).

2.3. Enzymatic hydrolysis

Automated enzyme assays were performed on GENPLAT as described (Banerjee et al., 2010b). Briefly, AFEX-treated corn stover was ground to 0.5 mm and dispensed as a slurry into 96-well plates at a final loading of 0.2% glucan. Typical enzyme loading was 15 mg protein/g glucan. Typical incubations were for 48 h at 50 °C. Design-Expert® software (Stat-Ease, Inc., Minneapolis, MN; http://www.statease.com) was used for the experimental design and analysis. In the current work, an augmented quadratic design of experiment was used instead of an augmented special cubic design (Banerjee et al., 2010b). Mixtures containing 7, 8, 9, 10, 11, or 12 components required 36, 45, 55, 66, 78, or 91 individual reactions, respectively. The lowest proportion of any single enzyme in the core set was set to 4%, and the lowest proportion of any accessory enzyme was set to 0%. All assays were replicated once, sampled twice, and assayed for Glu and Xyl twice, for a total of eight replicates of each mixture.

2.4. Sugar analysis

Glu and Xyl were assayed colorimetrically as described (Banerjee et al., 2010b; Santoro et al., 2010). Data were collected for 24 and 48 h digestion. Only the 48 h data are presented here; 24-h data are available upon request. Substrate specificities of the individual enzymes were assayed as described (Banerjee et al., 2010b). α-Glucuronidase (α-Glr) was assayed by the method of de Vries et al. (1998).

Monosaccharides (Glu, Xyl, Ara, Man, Gal, Rha, and Fuc) were analyzed by gas chromatography (GC) of the sugar alditol acetates (Blakeney et al., 1983). Enzyme hydrolyzates were centrifuged (15,000×*g*, 10 min) to remove insoluble residue. Ethanol (100%) was added to 50 μl of the supernatant to a final concentration of 70% (v/v). After 5 h at −20 °C, the samples were centrifuged for 10 min at 10,000×*g*. After transfer of the supernatants to new tubes, 10 μl of inositol (5 mg/ml) was added to each tube as internal standard, the samples were derivatized to their corresponding alditol acetates, and the sugars separated and quantitated by GC.

3. Results and discussion

3.1. Expression of accessory enzymes

All of the accessory proteins tested in this paper, with the exception of Cel5A and EX2, were produced by expression in *P. pastoris* followed by concentration and desalting. Cel5A and EX2 were expressed in *T. reesei* and purified by metal-affinity

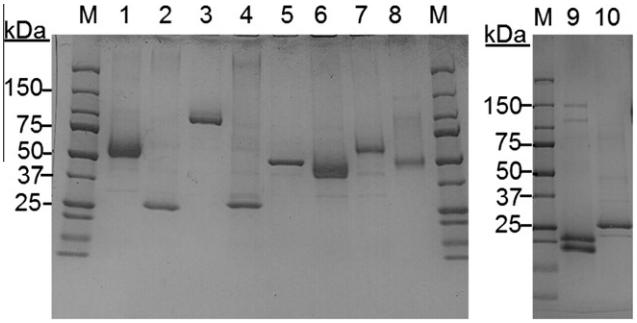

Fig. 1. SDS–PAGE of *T. reesei* accessory proteins expressed in *P. pastoris* (lanes 2–7, 10) or *T. reesei* (lanes 8 and 9). Lane 1, Cel61A; lane 2, Cel61B; lane 3, α-Glr; lane 4, Abf2; lane 5, AbfB; lane 6, Cip1; lane 7, Cip2; lane 8, Cel5A; lane 9, EX2; lane 10, Cel12A. The identical batches of protein were used in all of the experiments in this paper. Three micrograms of protein were loaded per lane.

chromatography (Fig. 1). The molecular weights (MWs) of the accessory proteins were within 10% of the values deduced from their gene sequences, with the exception of Cel61A. This protein has a MW of ~55 kDa when expressed in *P. pastoris* (Fig. 1), whereas the MW predicted from the gene sequence is 35 kDa. However, native Cel61A as well as Cel61A overexpressed in *T. reesei* were reported to have MW's of ~55 kDa (Karlsson et al., 2001; Saloheimo et al., 1997). EX2 expressed in *T. reesei* runs as a doublet on SDS–PAGE (Fig. 1). Mass spectrometry (MS)-based proteomic analysis identified both bands as EX2, and ion exchange chromatography resolved the doublet into two peaks, both having xylanase activity (data not shown). It is unknown why EX2 behaves as a doublet on SDS–PAGE, but the same behavior was observed for endoxylanase 3 (Banerjee et al., 2010b).

The specific activities of the ten accessory enzymes on a variety of substrates were comparable to the activities reported in the literature. They did not show any evidence of contaminating activities that could invalidate their utility (Table 1). EX2 and Cel5A, the two enzymes expressed in *T. reesei*, did not have any detectable activity against CMC-cellulose or pNP-cellobioside and are therefore not contaminated with either EG or CBH1 (Table 1). The minor proteins visible in the preparations of EX2 and Cel5A (Fig. 1) were analyzed by MS-based proteomics (Supplementary Table S2). None of these proteins are predicted to be able to interfere with interpretation of the results.

Cel61A, Cel61B, and α-Glr had no activity against any of the tested substrates, as expected. Cip1 has no reported enzymatic activity whereas Cip2 has been reported to be a glucuronoyl esterase (Li et al., 2007). Cip1 and Cip2 expressed in *P. pastoris* had low but consistent activity againsts pNP-glucoside. This is probably not due to contamination by endogenous *P. pastoris* enzymes because the other enzymes made in *P. pastoris* did not show this activity (Table 2). Cip1 has been reported to have activity against pNP-cellobioside (Foreman et al., 2010).

3.2. Sequential construction of an 11-component enzyme mixture

Ten accessory enzymes were sequentially added to a core set of six enzymes. The core set is composed of CBH1, CBH2, BG, EG1, EX3, and BX (Banerjee et al., 2010b) (Fig. 2). The accessory enzymes were chosen based on abundance in the secretome of *T. reesei* and their known or predicted involvement in deconstruction of corn cell walls (Nagendran et al., 2009). For all experiments, the predicted and experimental optimized proportions are shown in Tables 2 and 3. The data on which the models are based are shown in the Supplementary Tables S3-S14. Statistical analyses of all models are shown in Supplementary Tables S15 and S16.

Cel61A and Cel61B were tested in parallel as a seventh component. For Glu release, the optimized mixture contained 25% Cel61A, which resulted in a 7.2% increase in Glu yield compared to the core set alone (Table 2). Addition of Cel61A caused a shift in the optimal proportion of EG1 from 25% to 15% and the proportion of BG decreased from 14% to 5% (Table 2). The effect on EG1 proportion suggests that Cel61A and EG1 share similar activities, such that Cel61A can substitute for EG1, at least in part. The effect on the optimal BG proportion is less clear, because BG serves two functions: to convert cellobiose to Glu, and to relieve feedback product inhibition of CBH1 and CBH2 by cellobiose. In contrast to Cel61A, Cel61B was ineffective at increasing Glu yields; the optimized mixture contained 0% Cel61B and it did not enhance Glu yield (Table 2). Harris et al. (2010) also reported that Cel61A but not Cel61B enhanced cellulose hydrolysis. Neither Cel61A nor Cel61B contributed to Xyl release (Table 3).

Because some proteins might influence Glu and Xyl release only in the presence of other proteins, Cel61A and Cel61B were tested together (Tables 2 and 3). When combined, the Glu yield was the same as with Cel61A alone, confirming that Cel61B makes no contribution (Tables 2 and 3). Since Cel61B did not contribute to Glu or Xyl release, it was excluded from further experiments.

Glucuronylarabinoxylan (GAX) is the major hemicellulose in the cell walls of corn (Pauly and Keegstra, 2008). Deconstruction of GAX is predicted to require α-glucuronidase (α-Glr), arabinosidase (Abf), xylanase (EX), and β-xylosidase (BX). EX and BX are already a part of the core set. One α-Glr and two Abf's were tested as components eight and nine (Fig. 2). α-Glr as the eighth component did not affect the optimal proportions of components 1–7, nor did it affect Glu release, but was required at 18% for optimal Xyl release (Tables 2 and 3). Despite this, α-Glr caused an increase in Xyl release of only 2% (Table 3).

T. reesei secretes at least three arabinosidases: Abf1, Abf2, and AbfB (Nagendran et al., 2009). Abf1 and AbfB are in family GH54 and Abf2 is in GH62. Abf2 and AbfB were used to supplement the eight-component mixture (core set + Cel61A + α-Glr). Neither Abf2 nor AbfB increased Glu yield compared to the 7-component mixture (Table 2). However, Abf2 enhanced Xyl yields by ~5% (Table 3). Although an optimized mixture was predicted to contain 5% AbfB, this enzyme did not increase Xyl yields when included as the only Abf, either at 24 h (data not shown) or 48 h (Table 3). Further experiments were performed with only Abf2.

Table 1
Enzyme activities of proteins used in this paper. The identical batches of proteins were used in all of the experiments in this paper. Units for pNP derivatives (*) are μmol pNP released/mg enzyme/min; units for polysaccharide hydrolases (**) are μmol Glu equivalents released/mg enzyme/min.

Substrate	Cel61A	Cel61B	α-Glr	Abf2	AbfB	Cel5A	Cip1	Cip2	EX2	Cel12A
pNP-cellobioside*	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	2.44
pNP-glucoside*	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	1.03	0.22	<0.01	<0.01
pNP-xyloside*	<0.01	<0.01	<0.01	<0.01	<0.01	0.20	0.11	<0.01	10.5	<0.01
pNP-arabinoside*	<0.01	<0.01	<0.01	39.5	20.7	0.03	<0.01	0.07	1.1	0.02
1% birchwood xylan**	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	1.8	<0.01
1% CM-cellulose**	<0.01	<0.01	<0.01	<0.01	<0.01	0.03	<0.01	<0.01	<0.01	<0.01
0.5% barley β-glucan**	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Table 2
Optimization of Glu release. "Model expectation" is the Glu yield (as a % of total available Glu) predicted for the given enzyme proportions derived from the data shown in Supplementary Tables S3-S14. "Experimental average" is the actual Glu yield for the given enzyme proportions, ±1 SD of the mean ($n = 8$). Data for the core set alone are from Banerjee et al. (2010b). Dashes indicate that the enzyme was not tested in that particular mixture. Enzyme loading was fixed at 15 mg/g glucan throughout.

Optimized for Glu release (48 h)	Number of components	Optimized enzyme proportions (%)														Model expectation	Experimental average
		CBH1	BG	EG1	BX	EX3	CBH2	Cel61A	Cel61B	α- Glr	Abf2	AbfB	Cip1	Cip2	Cel5A	EX2	Cel12A		
Core set (CS) alone	6	29	14	25	5	22	5	–	–	–	–	–	–	–	–	–	–	40.0	38.5 ± 0.5
CS + Cel61A	7	28	5	15	4	19	4	25	–	–	–	–	–	–	–	–	–	47.2	45.7 ± 0.0
CS + Cel61B	7	30	10	27	6	23	4	–	0	–	–	–	–	–	–	–	–	37.0	37.0 ± 0.1
CS + Cel61A + Cel61B	8	31	6	19	4	11	4	25	0	–	–	–	–	–	–	–	–	47.0	46.1 ± 0.0
CS + Cel61A + α-Glr	8	26	7	22	4	13	4	24	–	0	–	–	–	–	–	–	–	46.5	47.6 ± 0.2
CS + Cel61A + α-Glr + Abf2	9	26	7	20	4	17	5	21	–	0	0	–	–	–	–	–	–	47.0	47.5 ± 0.1
CS + Cel61A + α-Glr + AbfB	9	31	4	18	4	13	8	22	–	0	–	0	–	–	–	–	–	46.1	42.6 ± 0.0
CS + Cel61A + α-Glr + Abf2 + Cip1	10	23	7	20	4	20	4	22	–	0	0	–	0	–	–	–	–	46.5	44.4 ± 0.2
CS + Cel61A + α-Glr + Abf2 + Cip2	10	25	5	19	4	15	9	23	–	0	0	–	–	0	–	–	–	46.5	44.4 ± 0.5
CS + Cel61A + α-Glr + Abf2 + Cip1 + Cip2	11	23	8	19	4	17	7	22	–	0	0	–	0	0	–	–	–	47.0	44.9 ± 0.7
CS + Cel61A + α-Glr + Abf2 + Cel5A	10	25	4	13	4	18	4	20	–	0	0	–	–	–	12	–	–	47.0	45.0 ± 0.7
CS + Cel61A + α-Glr + Abf2 + Cel5A + EX2	11	25	4	8	4	13	4	18	–	0	0	–	–	–	11	13	–	50.5	52.1 ± 0.1
CS + Cel61A + α-Glr + Abf2 + Cel5A + EX2 + Cel12A	12	24	4	7	4	15	4	20	–	0	0	–	–	–	10	12	0	51.0	52.3 ± 0.1

The genes for Cip1 and Cip2 are strongly co-induced with cellulase genes in *T. reesei* and the proteins are among the most abundant in the commercial preparation Spezyme CP and in the culture filtrates of *T. reesei* grown on corn stover (Foreman et al., 2003; Nagendran et al., 2009). Cip1 has been reported to enhance Glu yield from acid-treated corn stover in the presence of cellulases (Scott et al., 2009). Cip2 is a glucoronyl esterase acting on linkages between glucuronic acid and lignin (Duranová et al., 2009; Li et al., 2007). Cip1 and Cip2 were tested individually and together in combination with the eight-component mixture (core set + Cel61A + α-Glr + Abf2) (Fig. 2). Neither Cip1 nor Cip2 increased Glu or Xyl (Tables 2 and 3). The optimum predicted proportion of Cip1 and Cip2 was 0%, indicating that these proteins are not needed in the deconstruction of AFEX-treated corn stover. One possible reason why at least Cip2 is unnecessary is that its target ester bonds are already cleaved by the basic AFEX treatment.

Most cellulolytic microorganisms produce multiple β-1,4-endoglucanases (EG) of different CAZy families (Zhang and Lynd, 2004). *T. reesei* is known to make multiple EG's, including Cel7B (EG1), Cel12A, and Cel5A, all of which are major proteins in the secretome of *T. reesei* (Nagendran et al., 2009). When Cel5A was added as a tenth component, the predicted optimal loading was 12%. This was mainly at the expense of EG1, indicative of overlapping activities, as might be predicted because both of them are endo-β-1,4-glucanases. At 48 h, Cel5A caused no increase in Glu yield (Table 2). However, at 24 h, Cel5A caused a 5% increase in Glu yield at an optimal loading of 15% (data not shown). Thus, Cel5A does make a difference and was therefore retained as part of the enzyme mixture. For Xyl, Cel5A was optimal at 0% and had no effect on yield (Table 3).

The two major families of endo-β-1,4-xylanase are GH10 and GH11, and *T. reesei* secretes one of each (Nagendran et al., 2009). EX3, which is part of our core set, is in family GH10, whereas EX2 is in GH11. GH10 and GH11 xylanases differ in several ways, notably that GH10 enzymes have broader substrate specificity and those in GH11 are among the smallest known glycan hydrolases (Collins et al., 2005). Addition of EX2 to a 10-component set increased Glu yields by 7.1% and Xyl yields by 5.7%. The optimum proportion of EX2 for Glu release was 13% and for Xyl release it was 23% (Tables 2 and 3). For Glu release, the EX2 was partially replacing both EG1 and EX3, and for Xyl release it was replacing EG1, EX3, and α-Glr. Clearly, EX2 is performing a function not performed by the other enzymes, and a combination of EX2 and EX3 is superior to EX3 alone for both Glu and Xyl release.

Finally, another alternative endo-β-1,4-glucanase, Cel12A, was added as the twelfth component. Cel12A was predicted to be optimal at 0% for both Glu and Xyl and had no effect on yield of either sugar. The enzyme proportions for the final 11-component mixtures are shown graphically in Fig. 3.

Under some conditions, e.g., with different feedstocks and fermenting organisms, it may be desirable to optimize enzyme mixtures for release of both Glu and Xyl at some intermediate relative proportions. The experimental design software used in GENPLAT (Design-Expert®) can be used to model optimized release of mixtures of Glu and Xyl in addition to one or the other, as was done in Tables 2 and 3. To illustrate this, the models were re-optimized for a Glu to Xyl ratio of 2:1 (Table 4). As expected, the levels of each enzyme tended to fall between the optima for Glu or Xyl alone (Table 4). Experimental yields using the proportions optimized for 2:1 Glu:Xyl for the final mixture yielded 50.1% of the total Glu (a 2% penalty yield compared to an enzyme cocktail optimized for Glu alone) and 33.0% of the available Xyl (a 6.9% penalty yield) (Table 4).

In conclusion, the best mixture obtained in these experiments had 11 components and released 52.1% of the available Glu, compared to 50.5% for Accellerase 1000, 48.8% for Spezyme CP, and

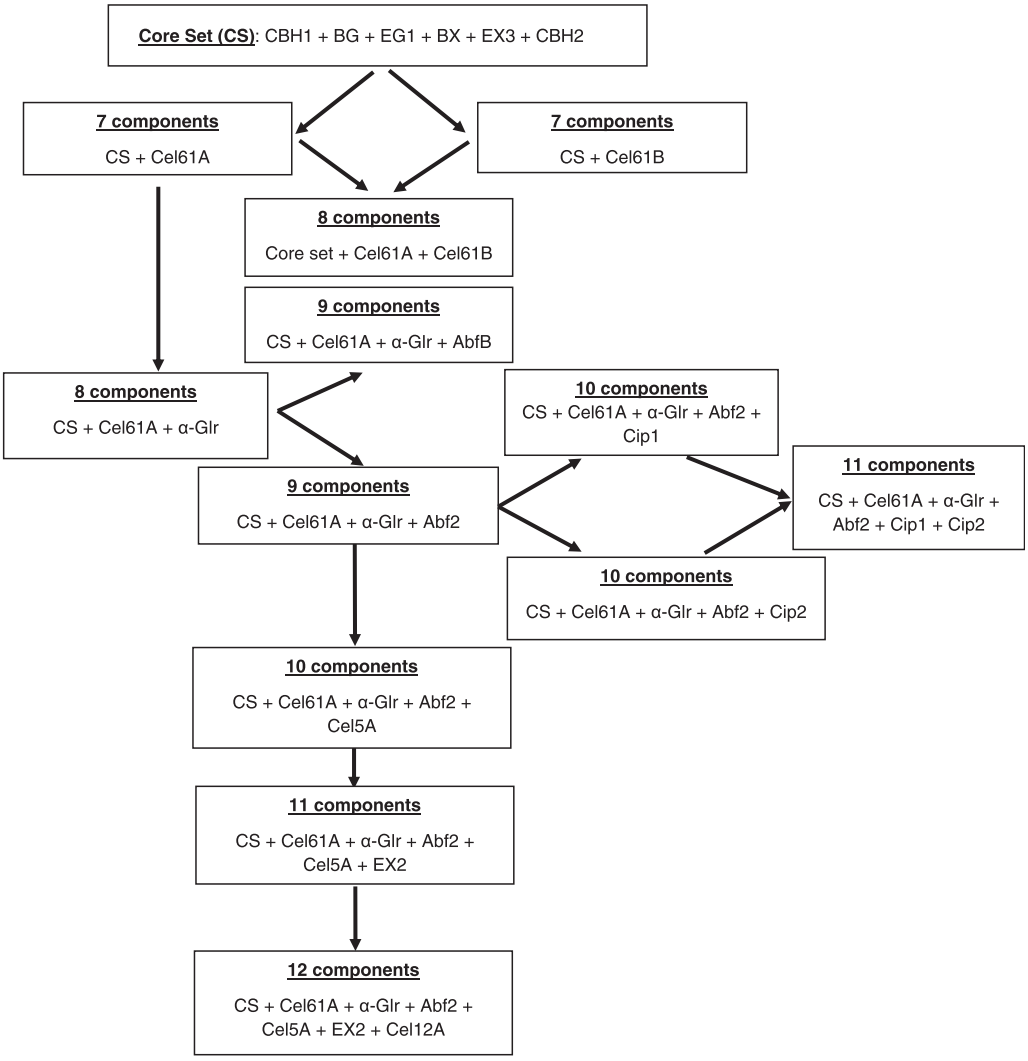

Fig. 2. Flowsheet of the analysis of accessory enzymes.

38.5% for the core six alone (Table 2; Banerjee et al., 2010b). Optimized for Xyl, the best 11-component mixture released 39.9% of the available Xyl, compared to 28.8% for Accellerase 1000, 28.8% for Spezyme CP, and 26.4% for the core six alone (Table 3; Banerjee et al., 2010b). That is, the 11-component mixture performed as well as either of the commercial enzymes, which contain more than 80 proteins, for Glu and significantly better for Xyl.

3.3. Comparison of Glu and Xyl assays by automated colorimetry and gas chromatography

GENPLAT utilizes automated colorimetric assays of Glu and Xyl (Banerjee et al., 2010b; Santoro et al., 2010). This is a relatively untested method compared to chromatographic separation, reducing sugar assays, or GC of alditol acetate derivatives. Since it is possible that multi-component enzyme assays release compounds that inhibit or otherwise interfere with the colorimetric assays, we also analyzed representative samples from GENPLAT by the standard GC alditol acetate method (Blakeney et al., 1983). As shown in Table 5, the two analytical methods are within 10% of each other, with no particular bias toward either method, and both showed good agreement with statistically predicted model expectations. This was true for both sugars released by the 6-component or the 11-component mixtures (Table 5).

The GC method quantitates Ara as well as Glu and Xyl. In the 11-component mixture, Abf2 made no difference to Glu yields and increased Xyl yields by 5.7% (Table 3). From the GC results, the 11-component mixture released 33.3% of the theoretical Ara content of corn stover (experimentally determined to be ~4.2% of the total sugar composition). Neither the 6-component mixture optimized for Glu or Xyl, nor the 11-component mixture optimized for Glu (which contains 0% Abf2), released any detectable Ara (data not shown). Thus, addition of Abf2 results in enhanced release of Ara as well as Xyl. This released Ara could be important in the overall energy capture from corn stover if the downstream fermenting organism has the capacity to ferment Ara.

3.4. Kinetic and dose response analyses

All of the previous optimization experiments were done at fixed protein loading (15 mg protein/g glucan) and fixed time (48 h). In order to determine if synthetic mixtures differed from commercial enzyme preparations in their behavior at different times of digestion or different enzyme loadings, additional comparative studies were performed.

For examining the effect of protein loading, the 6-component core set and 11-component mixture (both at proportions optimized for 15 mg/g glucan loading and 48-h incubation) were

Table 3
Optimization of Xyl release. "Model expectation" is the Xyl yield (as a % of total available Xyl) predicted for the given enzyme proportions derived from the data shown in Supplementary Tables S3-S14. "Experimental average" is the actual Xyl yield for the given enzyme proportions, ±1 SD of the mean (n = 8). Data for the core set alone are from Banerjee et al. (2010b). Dashes indicate that the enzyme was not tested in that particular mixture. Enzyme loading was fixed at 15 mg/g glucan throughout.

Optimized for Xyl release (48 h)	Number of components	Optimized enzyme proportions (%)														Model expectation	Experimental average
		CBH1	BG	EG1	BX	EX3	CBH2	Cel61A	Cel61B	α- Glr	Abf2	AbfB	Cip1	Cip2	Cel5A	EX2	Cel12A		
Core set (CS) alone	6	5	5	25	17	34	14	–	–	–	–	–	–	–	–	–	–	27.8	26. 4 ± 0.5
CS + Cel61A	7	5	4	18	29	39	4	0	–	–	–	–	–	–	–	–	–	28.5	28.4 ± 0.6
CS + Cel61B	7	12	4	27	18	35	4	–	0	–	–	–	–	–	–	–	–	26.4	26.2 ± 0.3
CS + Cel61A + Cel61B	8	4	5	19	28	40	4	0	–	–	–	–	–	–	–	–	–	28.6	27.5 ± 0.6
CS + Cel61A + α-Glr	8	4	4	16	21	33	4	0	–	18	–	–	–	–	–	–	–	30.3	29.8 ± 0.4
CS + Cel61A + α-Glr + Abf2	9	4	4	10	15	30	4	0	–	13	21	–	–	–	–	–	–	35.9	35.5 ± 0.1
CS + Cel61A + α-Glr + AbfB	9	4	4	22	19	30	4	0	–	12	–	5	–	–	–	–	–	29.0	28.8 ± 0.3
CS + Cel61A + α-Glr + Abf2 + Cip1	10	4	4	14	17	28	4	0	–	9	20	–	0	–	–	–	–	35.0	35.1 ± 1.0
CS + Cel61A + α-Glr + Abf2 + Cip2	10	4	4	11	19	28	4	0	–	12	18	–	–	0	–	–	–	35.0	33.3 ± 1.3
CS + Cel61A + α-Glr + Abf2 + Cip1 + Cip2	11	4	4	14	16	28	4	0	–	13	16	–	0	0	–	–	–	34.0	34.1 ± 1.4
CS + Cel61A + α-Glr + Abf2 + Cel5A	10	4	4	11	16	29	4	0	–	15	18	–	–	–	0	–	–	35.0	34.2 ± 0.1
CS + Cel61A + α-Glr + Abf2 + Cel5A + EX2	11	4	4	5	16	19	4	1	–	8	15	–	–	–	2	23	–	39.5	39.9 ± 0.9
CS + Cel61A + α-Glr + Abf2 + Cel5A + EX2 + Cel12A	12	4	4	4	15	17	4	2	–	11	17	–	–	–	0	22	0	38.9	40.1 ± 0.1

Fig. 3. Graphical representation of the final 11-component mixture optimized for **(A)** Glu or **(B)** Xyl, taken from Tables 2 and 3. Numbers in parantheses are the predicted optimal proportional percentages.

compared against Accellerase 1000 and Spezyme CP at seven loading concentrations (0–30 mg protein/g glucan) at three time points (6, 12, and 48 h). At 6 h, Glu release was nearly linear with protein loading for all four (Fig. 4). Spezyme CP, the 6-component core set, and the 11-component mixture were comparable, and Accellerase 1000 was superior to all three. At 12 h, Accellerase 1000 was still superior to the other three, but began to plateau above 20 mg/g glucan. Spezyme CP remained more linear above ~20 mg/g glucan and therefore approached Accellerase 1000 in effectiveness at the highest loading (30 mg/g glucan). At 48 h, all four showed a tendency to plateau above ~15 mg/g glucan. Glu yields with the two commercial enzymes and the 11-component mixture were very similar at 48 h, especially above 15 mg/g glucan. The Glu yields (~40% for the core set and ~50% at 15 mg/g glucan for the other three) are consistent with the results in Banerjee et al. (2010b) and elsewhere in this paper (Table 2).

The core set and the 11-component set performed equally well at 6 h for Glu, whereas at 48 h the 11-component mixture was superior (Fig. 4). Thus, it appears that the core enzymes are relatively more important at earlier time points whereas the accessory enzymes are relatively more important at later time points.

For Xyl release as a function of protein loading, the 11-component mixture was superior to the others at low loadings, but at high loadings Spezyme CP was as effective (Fig. 5A). Both the 11-component mixture and Spezyme CP were superior to the other two at high loadings. At 12 h and loadings above 15 mg/g glucan, the 11-component mixture and Spezyme CP were also equivalent (Fig. 5B), but at 48 h the 11-component mixture was superior to Spezyme CP as well as the other two (Fig. 5C). One interesting trend was that, at 6 and 12 h, the synthetic mixtures tended to plateau at ~5 mg/g glucan, whereas the two commercial preparations continued to release more Xyl up to 30 mg/g glucan (Fig. 5A,B). This effect was less pronounced at 48 h (Fig. 5C). In constrast, at

Table 4
Optimization of Glu + Xyl release in a ratio of 2:1. "Model expectation" is the Glu or Xyl yield as a percent of total available Glu or Xyl. "Experimental average" is the actual Glu or Xyl yield for the given enzyme proportions, ±1 SD of the mean ($n = 8$). Dashes indicate that the enzyme was not tested in that particular mixture. Enzyme loading was fixed at 15 mg/g glucan throughout.

Optimized for Glu + Xyl release (2:1) (48 h)	Number of components	Optimized enzyme proportions (%)														% Glu release		% Xyl release	
		CBHI	BG	EG1	BX	EX3	CBH2	Cel61A	Cel61B	α-Glr	Abf2	AbfB	Cip1	Cip2	Cel5A	EX2	Cel12A	Model expectation	Experimental average	Model expectation	Experimental average
CS + Cel61A	7	26	4	13	4	26	4	23	–	–	–	–	–	–	–	–	–	46.9	45.1 ± 1.0	25.7	26.7 ± 0.2
CS + Cel61B	7	23	4	21	4	45	4	–	0	–	–	–	–	–	–	–	–	35.6	35.3 ± 0.7	24.8	23.0 ± 1.0
CS + Cel61A + Cel61B	8	26	4	14	6	26	4	20	0	–	–	–	–	–	–	–	–	44.0	44.8 ± 1.2	27.0	26.0 ± 0.3
CS + Cel61A + α-Glr	8	22	4	21	4	18	4	18	–	10	–	–	–	–	–	–	–	45.0	44.5 ± 1.0	27.7	27.6 ± 0.7
CS + Cel61A + α-Glr + Abf2	9	23	4	17	4	20	4	17	–	2	9	–	–	–	–	–	–	46.7	46.7 ± 0.3	31.5	31.1 ± 0.4
CS + Cel61A + α-Glr + AbfB	9	28	4	19	4	21	5	19	–	0	–	0	–	–	–	–	–	45.3	46.2 ± 0.1	25.1	26.0 ± 0.3
CS + Cel61A + α-Glr + Abf2 + Cip1	10	20	4	19	5	23	4	19	–	0	6	–	0	–	–	–	–	45.3	46.5 ± 0.9	28.6	30.5 ± 0.3
CS + Cel61A + α-Glr + Abf2 + Cip2	10	23	4	19	7	19	4	21	–	0	4	–	–	0	–	–	–	46.7	47.2 ± 0.1	31.0	30.4 ± 0.5
CS + Cel61A + α-Glr + Abf2 + Cip1 + Cip2	11	22	7	18	6	20	4	18	–	2	3	–	0	0	–	–	–	47.0	50.0 ± 1.7	29.0	30.8 ± 1.3
CS + Cel61A + α-Glr + Abf2 + Cel5A	10	22	4	14	4	21	4	18	–	0	4	–	–	–	10	–	–	47.3	47.1 ± 0.4	29.0	27.9 ± 0.2
CS + Cel61A + α-Glr + Abf2 + Cel5A + EX2	11	19	4	5	8	16	4	15	–	0	2	–	–	–	8	18	–	49.2	50.1 ± 0.7	34.0	33.0 ± 0.1
Cel61A, α-Glr, Abf2, Cel5A, EX2, Cel12A	12	21	4	11	4	12	4	17	–	1	1	–	–	–	5	20	0	50.8	51.5 ± 1.4	33.0	33.5 ± 0.6

Table 5
Comparison of Glu and Xyl quantitation by gas chromatography (GC) and by automated colorimetric assays (GENPLAT).

Number of components	Model optimized for release of:	Model expectation (% release)	Experimental (% release)	
			GC	GENPLAT
6	Glucose	40.0	41.7 ± 1.5	38.7 ± 0.5
	Xylose	27.8	29.0 ± 2.6	26.4 ± 0.5
11	Glucose	51.0	48.1 ± 1.0	52.3 ± 0.1
	Xylose	38.9	43.0 ± 1.9	40.1 ± 0.1

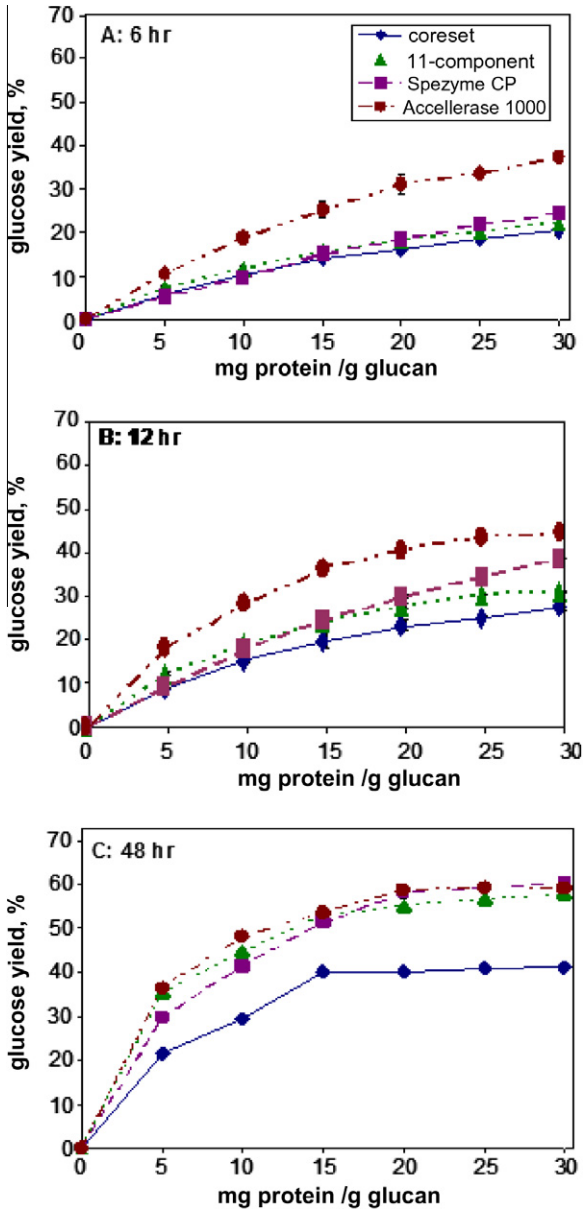

Fig. 4. Glu yield, expressed as percentage of available Glu, from AFEX-treated corn stover as a function of protein loading. (**A**), 6 h digestion; (**B**), 12 h digestion; (**C**), 48 h digestion. Six- and 11-component mixture proportions were those optimized for 48 h (Table 2).

48 h the two commercial preparations tended to plateau above ~20 mg/g glucan, whereas Glu release with the 11-component mixture continued to rise, albeit gradually.

A more detailed time course was performed with the same four enzyme preparations at 15 mg/g glucan (Fig. 6). Consistent with

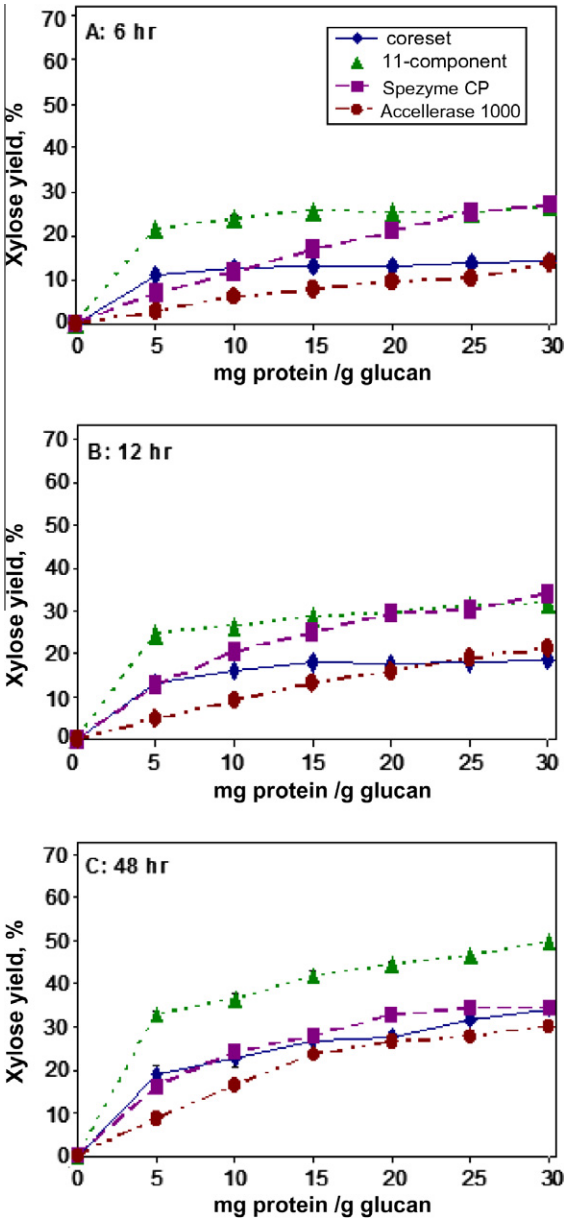

Fig. 5. Xyl yield (expressed as percentage of available Xyl) from AFEX-treated corn stover as a function of protein loading at three different incubation times. (**A**), 6 h digestion; (**B**), 12 h digestion; (**C**), 48 h digestion. Six- and 11-component mixture proportions were those optimized for 48 h (Table 3).

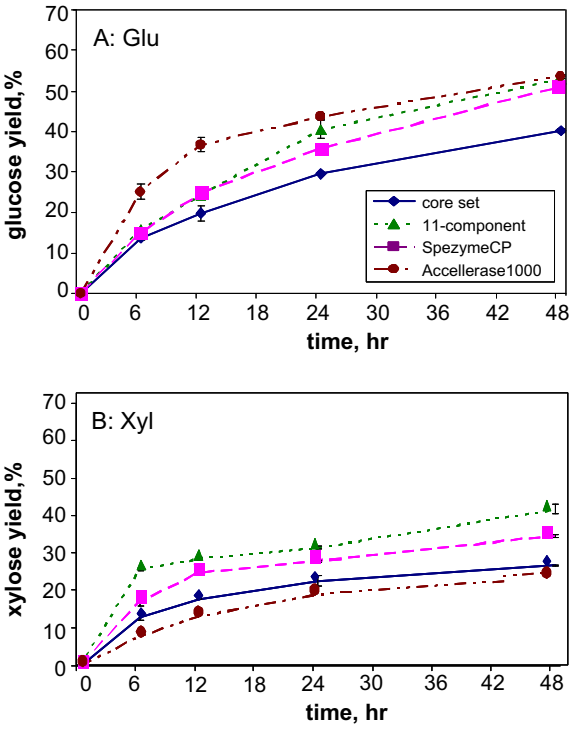

Fig. 6. Time course of Glu (**A**) and Xyl (**B**) release from AFEX-treated corn stover. Error bars (often not visible) represent ±1 SD of the mean. Enzyme loading in all cases was kept constant at 15 mg/g glucan.

the previous results, Accellerase 1000 performed best at early time points for Glu release, whereas all but the core set were approximately equal at 48 h (Fig. 6A). This suggests that the 11-component mixture lacks one or more enzymes needed for Glu release at earlier time points compared to Accellerase 1000. For Xyl release, the 11-component set performed best at all time points (Fig. 6B).

Glu release showed a pronounced tendency to plateau with increased protein loading at 48 h (Fig. 4C). This is probably not due to enzyme inactivation because the time course indicates that Glu is still being released even at 48 h (Fig. 6A). A hypothesis consistent with these observations is that the concentration of enzyme substrate sites becomes a limiting factor in digestions longer than ~12 h. That is, at short times there are sufficient numbers of substrate sites to accommodate protein concentrations up to 30 mg protein/g glucan, whereas after ~12 h the concentration of sites decreases to a point at which enzyme concentrations above ~15 mg/g glucan can no longer find sites to act on. The enzymes that can find sites continue to work, so Glu yields continue to rise (Fig. 6). Such a "site-limiting" model is consistent with our earlier results showing that Glu yields increased substantially (by as much as 20–30%) when the stover was ground more finely (i.e., <500 μm particle size compared to <100 μm) (Banerjee et al., 2010b). Finer grinding is predicted to expose a higher concentration of chemical bonds susceptible to enzyme cleavage, and therefore more of the enzyme molecules would be productively engaged for a longer time, leading to higher Glu yield (Banerjee et al., 2010b; Chundawat et al., 2007). If true, a site-limiting model emphasizes the importance of pretreatments, whether mechanical or chemical, that expose the maximum percentage of enzymatically susceptible chemical linkages.

3.5. Advantages of GENPLAT

With the long-term goal of rationalizing the construction and testing of enzyme preparations for biomass deconstruction, we have implemented a platform, called GENPLAT, to optimize multi-component mixtures of enzymes. Previous efforts to make synthetic mixtures have not gone beyond six components (Banerjee et al., 2010b; de Vries et al., 2000; Gao et al., 2010). Here we show the construction of an 11-component cocktail whose specific activity equals (for Glu) or exceeds (for Xyl) two commercial preparations that contain over 80 proteins. Working with defined mixtures offers several important advantages relevant to the long-term goal of improving the efficiency, and hence lowering the cost, of enzymes for biomass conversion. It allows unambiguous identification of which enzymes do and do not contribute to sugar release from any particular biomass subjected to any particular pretreatment. In the specific experimental conditions of the present study, the core set (comprising CBH1, CBH2, EG1, BG, EX3, and BX), plus Cel61A, EX2, and Cel5A were found to affect Glu release, whereas Cel61B, Cip1, Cip2, α-Glr, Abf2, AbfB, and Cel12A

did not. For Xyl release, the core set, α-Glr, EX2, and Abf2 were important, whereas Cel61A, Cel61B, Cip1, Cip2, Cel5A, Cel12A, and AbfB were not. Another advantage of defined-mixtures is that one can thereby determine the optimal proportions of the critical enzymes. The relative proportions of enzymes in natural mixtures are a function of complex, partially understood, and only partially controllable endogenous regulatory mechanisms in the production host, e.g., *T. reesei*. Our results indicate that CBH1, Cel61A, and EX3 are the three enzymes needed in the highest amounts for optimal Glu release from AFEX-treated corn stover, whereas EX2, Abf2, EX3, and BX are the most important for Xyl release.

GENPLAT should be a useful tool for the discovery of superior versions of known enzymes by replacement experiments. The property of "superiority" will be context-dependent, and could relate to higher specific activity, enhanced thermal stability, altered pH optimum, insensitivity to chemical inhibitors released by pretreatments, resistance to proteases, or cooperativity with other enzymes. Superior versions of enzymes could be ones found in natural sources or derived by protein engineering (Heinzelman et al., 2009). It should be more informative to screen alternative enzymes in a defined multi-component mixture on a realistic substrate than to test them individually on model substrates.

GENPLAT can also be used to identify proteins previously notsuspected to contribute to lignocellulose digestion. For example, in our experiments we found that the protein known as Cel61A makes a significant contribution to Glu release when combined with the core set. The importance of Cel61A had been discovered earlier by screening culture filtrates of novel fungi for factors that enhance commercial *T. reesei* enzyme mixtures (Merino and Cherry, 2007; Harris et al., 2010). GENPLAT provides a different and arguably more efficient way to find these types of novel accessory enzymes because it uses a defined rather than undefined enzyme system as the testing platform. In synthetic mixtures any novel protein, such as Cel61A, can be manipulated to any desired level and proportion relative to the other enzymes. Even well-studied biomass degrading microorganisms such as *T. reesei* secrete proteins of unknown biochemical function, whose roles in lignocellulose degradation have never been tested (Nagendran et al., 2009).

4. Conclusion

Mixtures of defined enzymes provide several advantages for understanding lignocellulose deconstruction. As shown here, multi-component mixtures can be prepared and optimized using robotic liquid handling and statistical experimental design. GENPLAT is adaptable to the analysis of mixtures with more than 11 components. Although it is not known with certainty, mixtures with fewer than 20 components will probably be sufficient to attain maximum release of Glu and Xyl from any pretreatment/biomass material (the specific enzymes and proportions will be different in each case). Using a quadratic design-of-experiment model, mixtures of 20 components can be optimized with fewer than 200 individual reactions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.07.028.

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