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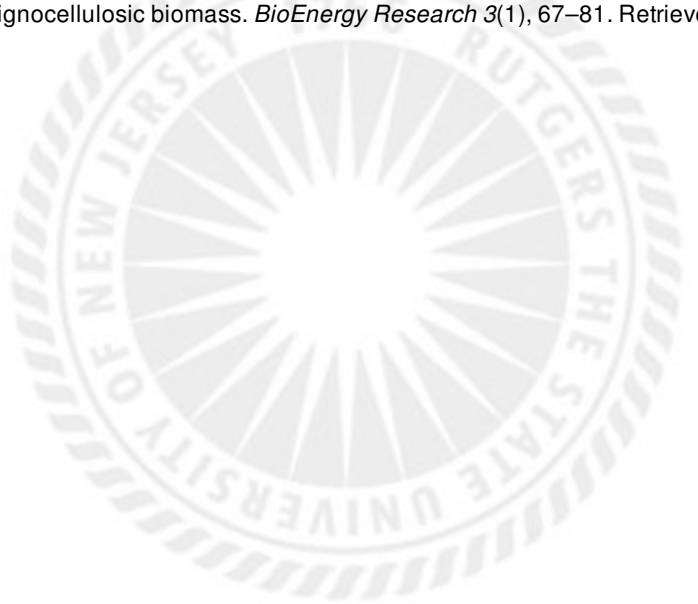

Strategy for identification of novel fungal and bacterial glycosyl hydrolase hybrid mixtures that can efficiently saccharify pretreated lignocellulosic biomass

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Abstract

We have applied a rational four-step strategy to identify novel bacterial glycosyl hydrolases (GH), in combination with various fungal enzymes, in order to define an efficient enzyme cocktail to hydrolyze pretreated lignocellulosic biomass. The fungal cellulases include cellobiohydrolase I (CBH I; GH family 7A), cellobiohydrolase II (CBH II; GH family 6A), endoglucanase I (EG I; GH family 7B) and β-glucosidase (βG; GH family 3). Bacterial endocellulases (LC1 and LC2; GH family 5), β-glucosidase (LβG; GH family 1), endoxylanases (LX1 and LX2; GH family 10) and β-xylosidase (LβX; GH family 52) from multiple sources were cloned, expressed and purified as well. Enzymatic hydrolysis for various enzyme combinations was performed on Ammonia Fiber Expansion (AFEX) treated corn stover at various total protein loadings (30, 15 and 10 mg enzyme/g glucan). The optimal mass ratio of enzymes necessary for obtaining

high glucan and xylan yields was determined using a suitable mixture design of experiments. The optimal hybrid enzyme mixtures contain fungal cellulases (78% of total protein loading), which include CBH I (loading ranging between 9-51% of total enzyme load), CBH II (9-51%), EG I (10-50%), and bacterial hemicellulases (22% of total protein loading) comprising of LX1 (13%) and LβX (9%). The hybrid mixture works effectively at 50 °C and pH 4.5 to efficiently saccharify AFEX treated corn stover giving as high as 95% glucan and 65% xylan conversion, respectively. This strategy of screening enzyme mixtures on pretreated lignocellulose will ultimately help develop enzyme cocktails that can hydrolyze plant cell walls efficiently and economically to produce cellulosic ethanol.

Key words: Glycosyl hydrolases, AFEX, Lignocellulose, Enzymatic Hydrolysis, Ethanol

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Introduction

Using renewable resources for production of fuels and chemicals has attracted significant attention in recent years. Lignocellulosic biomass provides a unique, plentiful resource for the sustainable production of biofuels as well as creating new jobs and providing energy security to the nation [1]. It has been estimated that the amount of carbon fixed by plants is over 100 billion tons per year [2]. Among this fixed carbon, there is approximately 252 million tons of corn stover residue available in the US each year, making it one of the most abundant agricultural feedstocks that could be used to produce cellulosic ethanol [3].

Lignocellulosics are comprised of a complex intermeshed matrix of cellulose, hemicellulose and lignin. Successful conversion of lignocellulosic biomass to ethanol requires an efficient and economical pretreatment method (to reduce recalcitrance to biological conversion), high glucose/xylose yields during enzymatic hydrolysis (to convert cellulose and hemicellulose polymers to monomeric sugars) and efficient microbial fermentation (to convert both C6-C5 sugars to ethanol) [4]. Currently, the high cost of pretreatment and enzymes are the two major factors affecting the economics of lignocellulosic biorefineries[5].

In nature, both fungi and bacteria have their own unique machinery to deconstruct plant cell walls. For fungi, a battery of cellulases, hemicellulases and other accessory enzymes are extracellularly secreted to synergistically hydrolyze cell walls, while releasing monomeric and oligomeric sugars for their metabolism [6]. On the other hand, bacterial enzymes are typically aggregated and assembled on a complex scaffold structure through various integrating modules known as cohesins and dockerins [7]. These enzyme complexes, known as cellulosomes, are attached to the surface of the bacterial cell walls [8-10]. There have been few studies that have looked at studying the synergistic interactions between different catalytic domains of bacterial enzymes that can be cloned, expressed and studied individually along with various fungal enzymes on pretreated lignocellulosic biomass. Some reports have shown exo/exo, exo/endo synergism operate between fungal and bacterial enzymes on crystalline cellulose [11, 12]. But, very few reports are available on the nature of synergistic interactions between bacterial and fungal enzymes, especially bacterial hemicellulases, hydrolyzing pretreated lignocellulosic biomass.

In this study, we have evaluated the hydrolysis of Ammonia Fiber Expansion (AFEX) treated corn stover by different combinations of enzymes from both fungal and bacterial sources. Fungal enzymes (CBH I, CBH II and EG I) were purified from a commercial source (Spezyme CP); while βG was purified from Novozyme 188. Two cellulases (LC1 and LC2), two xylanases (LX1 and LX2), one β-glucosidase (LβG) and one β-xylosidase (LβX) were obtained from various bacterial sources (e.g. Clostridium, Geobacillus, Dictyoglomus). These results indicate the possibility of designing an optimal enzyme cocktail based on enzymes from multiple sources to efficiently hydrolyze pretreated plant biomass, ultimately decreasing the cost of cellulosic ethanol.

Materials and Methods

AFEX Pretreatment

AFEX pretreatment of corn stover was carried out as described in our previous work [13]. Milled corn stover (particle size <100 μm) with 60% moisture (kg water/kg dry biomass), was transferred to a high-pressure Parr reactor. Liquid ammonia (1 kg of ammonia/kg of dry biomass) was slowly charged to the vessel. The reactor was maintained at 90^{o}C for 5 min and then the pressure was released explosively (total residence time ~ 30 min). The instantaneous pressure drop in the vessel caused the ammonia to vaporize, with an explosive decompression and resulted in considerable biomass fiber disruption. The pretreated material was left under the fume hood overnight to remove residual ammonia and then kept at 4°C until further use. The composition of the milled AFEX corn stover, based on NREL LAP protocol [14], was found to be 34.4% glucan and 22.4% xylan content.

Isolation of LX1, LX2, LβX and LβG

Samples from Yellowstone National Park hot springs were obtained during sampling trips in September 2003, and September 2004. Enrichment cultures were performed in YTP-2 medium containing (per liter) 2.0 g yeast extract, 2.0 g tryptone, 2.0 g sodium pyruvate, 1.0 g KCl, 2.0 g KNO_3, 2.0 g $Na_2HPO_4.7H_2O$, 0.1 g $MgSO_4$, 0.03 g $CaCl_2$, and 2.0 ml clarified tomato juice. Enrichments were performed at 70°C in flasks agitated at 200 rpm. A number of aerobic cultures were purified by selection of individual colonies on plates containing the above medium and 16.0 g/liter agar. For preparation of genomic DNA, liter cultures were grown from a single colony in YTP-2 medium and collected by centrifugation. The cell concentrate was lysed using a combination of SDS and proteinase K, and genomic DNA was isolated using a phenol/chloroform extraction. The genomic DNA was precipitated, treated with RNase to remove residual contaminating RNA, and fragmented by hydrodynamic shearing (HydroShear apparatus, Genomic Solutions, Ann Arbor, MI) to generate fragments of either 3-5 kb or 10 kb. The fragments were purified on an agarose gel, end-repaired, and ligated into a high-stability, low copy vector (pSMART-LCKan, Lucigen, Middleton, WI). The recombinant plasmids were then used to transform electrocompetent cells and screened on plates containing 4-methylumbelliferyl-β-D-xylopyranoside (MUX) or 4-methylumbelliferyl-β-D-xcelloobioside (MUC). DNA inserts of the positive clones were sequenced, and the enzymes of interest were subcloned into pET28a and the resulting vector used to transform BL21(DE3) chemically competent cells.

Isolation of LC1 and LC2

Dictyoglomus turgidum strain 6724T (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; German Collection of Microorganisms and Cell Cultures) bacterial cell concentrate was a kind gift of Dr. Frank T. Robb, Center of Marine Biotechnology, University of Maryland Biotechnology Institute. *Clostridium thermocellum* bacterial cell concentrate was a kind gift of Dr. Paul Weimer, USDA-ARS-US Dairy Forage Research Center, Madison, WI. The cell concentrate from each strain was lysed using a combination of SDS and proteinase K, and genomic DNA was purified using phenol/chloroform extraction. The genomic DNA was precipitated, treated with RNase to remove residual contaminating RNA, and fragmented by hydrodynamic shearing (HydroShear apparatus, GeneMachines, San Carlos, CA) to generate fragments of 2-4 kb. The fragments were purified on an agarose gel, end-repaired, and ligated into pEZSeq, a lac promoter vector (Lucigen, Middleton, WI). The recombinant plasmids were then used to transform electrocompetent cells and screened on plates containing 4-methylumbelliferyl-β-D-cellobioside (MUC). LC1 was obtained from screening of the *D. turgidum* library, and LC2 from screening of the *Clostridium thermocellum* library. DNA inserts of the positive clones were sequenced, and the enzymes of interest were subcloned into pET28a and the resulting vector used to transform BL21(DE3) chemically competent cells.

Enzyme expression and purification

Plasmids containing the specific protein genes (for LC 1, LC 2, LX1, LX 2, LβG, LβX) with a 6X N-terminal His tag were transformed into E.coli BL21 (DE3) cells for protein

expression. A starter culture, inoculated from frozen stocks, was grown overnight by incubating at 37°C in kanamycin (30 μg/ml) + 0.4% glycerol. Flasks of LB soy + kanamycin + 0.4% glycerol were inoculated with the overnight culture and grown to an OD600 of 0.7-0.8, induced with 1 mM IPTG (isopropylthio-β-D-galactoside) and then incubated overnight (16-18 hrs) at 37°C. Cells were harvested by centrifugation (4000 rpm, 30 min) and the supernatant removed. Cell pellets were resuspended in Ni-NTA lysis buffer (25 mM Tris-HCl, pH 8.0, 300 mM NaCl) and sonicated using sonics vibra cell large tip. The mixture was centrifuged at 12,000 rpm for 30 min to remove the cell debris. The supernatant was heated to 60°C for 20 min and centrifuged again at 12,000 rpm to further remove precipitated debris. The supernatant was filtered through 0.5 μm filter and applied on 40 ml bed volume HIS-select Nickel affinity gel (Sigma, St. Louis, MO) equilibrated in Ni-NTA lysis buffer. The column was washed with 10 column volumes of Ni-NTA lysis buffer. The bound protein was eluted using 6 column volumes of Ni-NTA elution buffer (25 mM Tris-HCl, pH 8.0, 300 mM NaCl, 300 mM Imidazole) and verified on Pierce 4-20% SDS-PAGE (Thermo Fisher Scientific, Rockford, IL). The Ni-pool was concentrated using amicon ultra-15 membrane, dialyzed against storage buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl and 20% Glycerol) and quantified using the Pierce Bradford assay kit (Thermo Fisher Scientific, Rockford, IL) with bovine serum albumin (BSA) as the standard.

Fungal enzyme purification was performed using an FPLC system (GE Healthcare, Buckinghamshire, United Kingdom) at room temperature, while the fraction collector was refrigerated. Cellulases (CBH I, CBH II and EG I) and βG were purified from

Spezyme CP (Genencor, Rochester, NY) and Novo 188 (Novozyme, Davis, CA) respectively. The purification methodology has been described in our previous work [15].

Enzyme activity assays

The enzyme activity assays were based on a high-throughput microplate based method as described in previous work [13]. A 2.2 ml deep-well microplate (Greiner, Monroe, NC) was used to add 250 µl of 1% (w/v) stock substrate (CMC, Avicel, oat spelt xylan, cellobiose, xylobiose), 50 µl of 1M citrate buffer (pH 5.0) and 200 µl of appropriately diluted enzyme samples (20 ng to 100 µg/well). The microplates were incubated at 50 ^{o}C with shaking at 200 rpm for 10 min (cellobiose), 60 min (CMC, xylan) or 300 min (Avicel). The amount of glucose released was estimated using an enzyme based glucose assay kit (R-Biopharm, Marshall, MI). One unit of cellobiase was defined as one micromole of glucose released per milligram enzyme per minute under the assay conditions. For CMC, Avicel and xylan based substrates the reducing sugars released were estimated using 3,5-dinitrosalicylic acid (DNS) reagent [16]. One unit of CMCase, Avicelase and xylanase activity was defined as one micromole of reducing sugars (as glucose equivalents) released per milligram enzyme per minute under the respective assay conditions.

The *para*-nitrophenyl (*p*NP) based chromogenic substrates used were 4-nitrophenyl-β-D-cellobioside (*p*NPC), 4-nitrophenyl-β-D-glucopyranoside (*p*NPG), 4-nitrophenyl-β-D-xylopyranoside (*p*NPX) and 4-nitrophenyl-β-D-arabinofuranoside (*p*NPAf) (Sigma-Aldrich, St. Louis, MO). The assay mixtures containing 80 µl of 1 mM *p*NP substrate, 10 µl of 0.5 M citrate buffer (pH 5.0) and 10 µl of diluted enzymes (20 ng to 16 ug/well) in

350 µl micro plates were incubated at 50 ^{o}C with shaking at 200 rpm. After 15 min reaction time, 200 µl of 1M Na_2CO_3 was added to assay mixtures to arrest the hydrolytic reaction. The amount of *p*NP released was measured at OD420. One unit of enzyme activity was defined as one nanomole of *p*-nitrophenol released per milligram enzyme per minute under the assay conditions.

Enzymatic hydrolysis of pretreated biomass

The hydrolysis experiments were performed in 2.2 ml deep well microplates (Greiner, Monroe, NC) at 0.2% (w/w) total glucan loading in a total volume of 500 µl per well [13]. The enzyme and buffer mixtures were prepared separately and added simultaneously using a 96-channel automated pipette (JANUS, Perkin Elmer, Waltham). The hydrolysis experiments were performed using both Avicel and AFEX treated corn stover as the substrates. The microplates were incubated at 50 ^{o}C with shaking at 200 rpm for 24 h. The concentration of glucose and xylose in the hydrolyzates were measured using suitable enzyme based assays. All experiments were carried out in duplicates.

Glucose and xylose assays

Glucose and xylose concentrations were measured using enzymatic kits purchased from R-Biopharm (Marshall, MI) and Megazyme (Bray, Ireland), respectively. The glucose assay was based on a two step reaction method. D-Glucose is first phosphorylated to D-glucose-6-phosphate using ATP and hexokinase. The D-glucose-6-phosphate is then reacted with NADP+ by glucose-6-phosphate dehydrogenase to form D-gluconate-6-phosphate and NADPH. The reactions are stoichiometric to the amount of D-glucose. The corresponding increase in NADPH is measured at 340 nm to estimate glucose

concentration. The xylose assay is based on analogous two-step reactions. α-D-xylose is converted to isomeric β-D-xylose by xylose mutarotase. β-D-xylose is then reacted with NAD+ to form D-xylonic acid and NADH. The corresponding increase of NADH is measured at 340 nm to determine the xylose concentration.

Results and discussion

Strategy for enzyme screening on realistic lignocellulosic substrates

A simple four-step strategy was applied for screening and comparing activities of novel enzymes to develop enzyme mixtures that can efficiently saccharify pretreated lignocellulosic biomass (Fig. 1). A typical benchmark enzyme mixture could include fungal based cellulases (CBH I + CBH II + EG I) along with a suitable β-glucosidase (βG). The goal is to compare the activity of novel enzymes with respect to a defined benchmark on realistic substrates like pretreated cellulosic biomass. The first step is to characterize the type of enzyme in order to classify the enzyme to certain GH family (e.g. pNP-glycoside based activity assays and glycosyl hydrolase family determination based on amino acid sequence similarity). The next step is to dope the new enzyme/s along with a benchmark mixture to determine the effect on the digestibility of pretreated biomass. It may be necessary to swap the corresponding type of enzyme from the benchmark mixture before adding the new enzyme to compare relative improvements. This iterative method would allow one to determine the most efficient enzyme/enzyme mixtures that have high activity of pretreated lignocellulosic biomass, and avoid the pitfall of screening individual enzymes on unrealistic substrates (e.g. CMC, pNP-glycosides) [17]. Once a minimal enzyme mixture has been defined it should be possible to further reduce enzyme dosage

by optimizing the relative ratios of the enzymes in the mixture to maximize glucan and xylan digestibility.

Source of bacterial enzymes

The bacterial enzymes were isolated from thermophilic microbial enrichment isolates (See Methods Section and Table 1). The two cellulases (LC1 and LC2) are family 5 glycosyl hydrolases based on amino acid sequence similarity to *Dictyoglomus turgidum* and *Clostridium thermocellum* based endoglucanases, respectively. The two hemicellulases (LX1 and LX2) are family 10 glycosyl hydrolases having sequence similarity to *Geobacillus* based endoxylanases. The β-glucosidase (LβG) and β-xylosidase (LβX) belong to family 1 and 52 based glycosyl hydrolases with sequence similarity to *Clostridium* and *Geobacillus* based enzymes, respectively.

Enzyme activity

The enzymes were tested for their activity on different substrates at pH 5 and 6.5. Only activity data for pH 5 (50 ^{o}C) is shown in Table 2, since all further experiments were conducted under similar assay conditions. LC1 and LC2 have significant pNP-cellobioside and CMC activity. Though, both the endocellulases were found to have significant activity on pNPC, their CMC activity was lower compared to EG I. LX1 and LX2 were found to have much higher xylanase activity than EG I. LβX was found to have high xylosidase activity but poor α-arabinofuranosidase activity. The bacterial β-glucosidase (LβG) had significantly lower cellobiose activity than its fungal counterpart (βG). No noticeable Avicel activity was detected for any of the bacterial enzymes compared to the fungal cellulases.

Figure 2 shows the hydrolysis yields on AFEX corn stover for all 6 bacterial enzymes added together as a mixture at varying pH and temperatures. The enzyme loading was 4 mg/g glucan each for LC1, LC2, LX1 and LX2; 2 mg/g glucan each for LβX and LβG. At pH 6.5 and 50 °C, the xylose yield was approximately 50%. However, the glucan conversion was significantly lower (< 5%). The activity assays showed that none of the bacterial enzymes had any significant activity on avicel. Although, the bacterial enzymes cloned belonged to thermophilic microbes, hydrolysis yields at 70 ^{o}C were much lower compared to 50 ^{o}C. It is possible that the enzymes lost activity at high temperature during the prolonged incubation (24 hours). Since the bacterial and fungal enzymes have a different working pH range, a mixture of both enzymes was tested on pH 6.5 and pH 4.5. For fungal enzymes, the optimal pH was found to be between 4.5-5 (data not shown). When tested under pH 6.5, significant loss in activity was observed. The glucose yield for CBH I, CBH II and EG I equimass mixture glucan hydrolysis yield decreased to 10% compared to 60% at pH 4.5 (24 hrs hydrolysis).

Doping and swapping bacterial/fungal cellulases

The experimental design for the doping and swapping experiments is shown in Table 3, which was conducted under specific assay conditions (pH 4.5-5.0, 50 ^{o}C, 24 hrs) using AFEX treated corn stover. From Figure 3-I, experiments #A-B show that swapping of LβG with βG does not significantly increase the glucose or xylose yield. From the results of experiment #G it can be observed that LβG showed lower glucan conversion even at much higher enzyme loadings (Figure 3-II and Table 3). Experiments #F-J also indicate that the bacterial β-glucosidase has much lower activity compared to its fungal counterpart. The current batch of bacterial enzymes do not possess substantial exo-

cellulase activity to hydrolyze AFEX treated corn stover. Therefore, purified fungal cellulases (CBH I, CBH II, EG I) were doped into the enzyme mix in order to further enhance the glucose yield (Experiments #C-E). Doping either of the three fungal cellulases resulted in increasing the glucan conversion to around 20%. Interestingly, #E has a higher xylose yield possibly due to cross-activity of EG I on xylan. Doping LC1, LC2 or both together into a fungal mixture did not significantly improve either the glucose or xylose hydrolysis yield (Experiments #K-M). The above results demonstrate that bacterial cellulases (LC1, LC2, LβX) do not significantly improve the digestibility of pretreated biomass compared to fungal cellulases (CBH I, CBH II, βG), despite the fact that the bacterial enzymes were found to have significant activity on artificial substrates (like CMC and pNP-glycosides).

Doping of bacterial hemicellulases to fungal benchmark mixture

Although bacterial cellulases (LC1, LC2 and LβG) cannot work effectively on their own or work synergistically with fungal enzymes, the bacterial hemicellulases (LX1, LX2 and LβX) were found to have significant activity on pretreated corn stover (Experiments #O-R). As show in Figure 3-III (experiment design shown in Table 3), doping LX1 and LX2 to the fungal benchmark mixture helps increase the xylose and glucose yield. Hydrolyzing xylan enhances the accessibility of the cellulases to the residual cellulose microfibrils. Hence, resulting in higher glucan conversion in the presence of suitable hemicellulases. When LX1 and LX2 were doped together (Experiment #Q), glucan conversion increased to 70% whereas xylose seems to have no noticeable increase compared with Experiments #O-P. Addition of LβX (Experiment #R), helped increase

the xylan conversion substantially (71% glucan and 76% xylan conversion). In order to enhance both glucan and xylan conversion, LβX is important to hydrolyze soluble xylan based oligosaccharides which are potential inhibitors of endoxylanases and cellulases.

Enzyme mixture optimization

Previous results have demonstrated the synergistic interactions between fungal cellulases (CBH I, CBH II, EG I and βG) and bacterial hemicellulases (LX1, LX2 and LβG). However, the individual enzyme ratio needs to be optimized to further increase glucan and xylan conversions. In order to do this, 73 different enzyme combinations were tested in duplicates (standard deviations were less than 5% of the mean) and the average hydrolysis yields for both glucan and xylan were determined for three different protein loadings (Table 5). βG was loaded at a 10% (mass ratio) of the total remaining enzymes to ensure complete hydrolysis of cellobiose [18]. CBH I and CBH II were added at more than 20% (total protein exluding βG) in all mixtures to ensure sufficient cellulase activity. EG I added was at least 10% of the mixture. Bacterial LX1 and LX2 together were always more than 5% of total enzymes while bacterial LβX was greater than 1%. All of the above constraints were based on the fact that cellobiohydrolase, endoglucanase, beta-glucosidase, endoxylanase and beta-xylosidase are indispensible for an efficient enzyme cocktail. Deconstruction of crystalline cellulose is the major limiting step for hydrolysis, hence requiring significant cellulase protein loading. It is clear that increasing the bacterial hemicellulases loading beyond 10 mg/g of glucan does not significant increase the xylan conversion. Using the current cocktail of enzymes the xylan conversion could not exceed 60-70% conversion even when glucan conversion was over 90%. It is possible that other hemicellulases (e.g. α-arabinofuranosidase, α-glucuronidase) are required to

further increase the xylan conversion. Without suitable complementary hemicellulases, xylan conversion is a bottleneck and hence just increasing enzyme loading alone would not increase the xylan conversion.

Glucan and xylan hydrolysis results for all mixtures are shown in Fig. 4 as a scatter plot. The relatively ratio of the individual enzymes significantly affected the overall sugar yield. At 10 mg/g glucan loading, the highest glucan conversion is 63.1% while the lowest is 20.6%. The highest xylan conversion is 52.3% while the lowest is 20.1% at the same protein loading. Similarly, huge differences in overall conversions can be seen for other protein loadings as well. One of the best mixtures resulting in the highest glucan and xylan conversions contained 19% CBH I, 9% CBH II, 50% EG I, 7.8% LX1, 5.3% LX2 and 9.0% LβX.

Relationship between glucan and xylan conversions

Glucan and xylan conversion for various combinations of enzymes at three different total enzyme loadings is shown in Fig.4. The three clusters for different enzyme loadings demonstrate that at higher enzyme loads, the glucan and xylan conversion is generally high. Another interesting phenomenon observed is that at high enzyme loading, the shape of the data point cluster is narrow while at low enzyme loading, the data points are more scattered. By applying linear regression on xylan conversions vs. corresponding glucan conversion for various enzyme mixtures, a linear relationship between the two is confirmed (Table 4). The P values are close to 0, indicating that the linear relationship has statistical significance. When the total enzyme loading was increased (from 10 to 30

mg/g glucan) the R^2 value increases as well. This validates our visual interpretation of the shape of the data cluster at low vs. high enzyme loading.

CBH I and CBH II are both indispensible for efficient hydrolysis. For all 3 varying enzyme loadings, if the mixture did not contain CBH I, the glucan conversions were quite low. When both CBH I and CBH II are included, higher glucan conversions were possible (>90% glucan conversion at 30 mg/g glucan enzyme loading). Xylan conversion was not obviously affected by the existence of either CBH I or II. Xylan conversion tended to be slightly higher (5~10%) at higher enzyme loadings when both CBH were present. LX1 has slightly higher specific activity compared to LX2 on AFEX treated corn stover.

Figure 5 is helpful in visually summarizing the optimal regions of enzyme ratios for maximizing both glucan and xylan digestibility. Different clusters of data (based on Table 5) were separated based on the overall ratio of cellulases (I), xylanases (II) and βX (III). The three enzyme loadings were plotted as insets (a), (b) and (c) representing 10, 15 and 30 mg/g glucan enzyme loading, respectively. At high enzyme loading, the higher glucan and xylan yielding data points aggregate closely compared to the lower enzyme loading. This suggests that glucan and xylan yields are more sensitive to individual enzyme ratios at lower enzyme loading. At high cellulase loading (94%), the hemicellulase loading is much lower and both glucan and xylan yields are relatively lower. This further confirms our previous assumption that in order to maximize glucan yield, higher xylan hydrolysis yields are desirable. On the other hand, for higher hemicellulase loading (>37%), xylan conversion is slightly lower while glucan conversions drop significantly. Similar trends for βX at around 9% loading of total ratio

are seen as well. To achieve high conversions of glucan and xylan, 78% cellulases (CBH I, CBH II and EG I), 13% xylanase (LX1 and LX2) and 9% βX seemed to be optimal. The optimal cellulase loading (total to 78%) for CBH I and CBH II ranges from 9-51%; while for EG I it ranges from 10-50%.

Conclusion

In this paper, we have examined the activity of both fungal and bacterial enzyme based mixtures on a realistic substrate (i.e. AFEX treated corn stover). The results indicate that certain fungal cellulases and bacterial hemicellulases can work synergistically together to maximize digestibility. Bacterial xylanases (LX1, LX2 and LβX) can increase both the glucan and xylan hydrolysis yield when added along with fungal cellulases. Optimized ratios for individual enzymes were obtained by examining 73 unique enzyme mixture combinations. Close to 90% glucan and 70% xylan conversion was achieved using the best enzyme combination. The current sets of bacterial hemicellulases were not sufficient to hydrolyze AFEX corn stover hemicellulose completely. In order to further increase xylan conversion greater than 70%, other hemicellulases such as α-arabinofuranosidase and α-glucuronidases would be necessary.

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Table 1. Amino acid sequence and glycosyl hydrolase families for the six bacterial enzymes

Name	Amino acid sequence	GH family
LC1	MNNLPIKRGINFGDALEAPYEGAWSGYIIKDEYFKIVKDAGFDHVRIPIKWSVYTQKEAPYSIEKRIFDRVDHLIEEGLKNNLHVIINIHH YEEIMEDPLGEKERFLAIWRQISEHYKDYPNNLYFELLNEPTQNLSSELWNQFLKEAIEVIRRTNPERKIIVGPDNWNSLYNLEKLIIP ENDENIIITFHYYNPFPFTHQGAGWVKIDLPVGVKWLGTEEEKREIERELDMAVSWAEEHGNIPLYMGEFGAYSKADMESRVRWTD FVARSAEKRGIAWSYWEFYSGFGVFDPEKNEWRTPLLRALIPERNI*	5
LC2	MVSFKAGINLGGWISQYQVFSKEHFDTFITEKDIETIAEAGFDHVRLPFDYPIIESDDNVGEYKEDGLSYIDRCLEWCKKYNLGLVLD MHHAPGYRFQDFKTSTLFEDPNQQKRFVDIWRFLAKRYINEREHIAFELLNEVVEPDSTRWNKLMLEYIKAIREIDSTMWLYIGGNN YNSPDELKNLADIDDDYIVYNFHFYNPFFFTHQKAHWSESAMAYNRTVKYPGQYEGIEEFVKNNPKYSFMMELNNLKLNKELLRKD LKPAIEFREKKKCKLYCGEFGVIAIADLESRIKWHEDYISLLEEYDIGGAVWNYKKMDFEIYNEDRKPVSQELVNILARRKT*	5
LX 1	MAKTEQSYAKKPQISALHAPQLDQRYKDSFTIGAAVEPYQLLNEKDAQMLKRHFNSIVAENVMKPINIQPEEGKFNFAEADQIVRFA KKHHMDIRFHTLVWHSQVPQWFFLDKEGQPMVNETDPVKREQNKQLLLKRIETHIKTIVERYKDDIKYWDVVNEVVGDDGELRDS PWYQIAGIDYIKVAFQTARKYGGNKIKLYINDYNTEVEPKRSALYNLVKQLKEEGIPIDGIGHQSHIQIDWPSEEEIEKTIIMFADLGLDN QITELDVSMYGWPPRAYLSYDAIPEQKFLDQADRYDRLFKLYEKLSDKISNVTFWGIADNHTWLDSRADVYYDTDGNVIVDPKAPY TRVEKGNGKDAPFVFDPEYNVKPAYWAIIDHK*	10
LX 2	MCSSIPSLREVFANDFRIGAAVNPVTLEAQQSLLIRHVNSLTAENHMKFEHLQPEEGRFTFDIAIKSSTSPFSSHGVRGHTLVWHNQ TPSWVFQDSQGHFVGRDVLLERMKSHISTVVQRYKGKVYCWDVVNEAVADEGSEWLRSSTWRQIIGDDFIQQAFLYAHEADPEA LLFYNDYNECFPEKREKIYTLVKSLRDKGIPIHGIGMQAHWSLTRPTLDEIRAAIERYASLGVILHITELDISMFEFDDHRKDLAAPTNE MVERQAERYEQIFSLFKEYRDVIQNVTFWGIADDHTWLDHFPVQGRKNWPLLFDEQHNPKPAFWRVVNI*	10
X	MPTNVFFNAHHSPVGAFASFTLGFPGKSGGLDLELARPPRQNVFIGVESSHEPGLYHILPFAETAGEDESKRYDIENPDPNPQKPNI LIPFAKERIEREFRVATDTWKAGDLTLTIYSPVKAVPDPETASEEELKLALVPAVIVEMTIDNTNGTRTRRAFFGFEGTDPYTSMRRID DTCPQLRGVGQGRILGIASKDEGVRSALHFSMEDILTATLEENWTFGLGKVGALIADVPAGEKKTYQFAVCFYRGGYVTAGMDASY FYTRFFHNIEEVGLYALEQAEVLKEQAFCSNELIEKEWLSDDQKFMMAHAIRSYYGNTQLLEHEGKPIWVVNEGEYRMMNTFDLTV DQLFFELKMNPWTVKNVLDFYVERYSYEDRVRFPGDETEYPGGISFTHDMGVANTFSRPHYSSYELYGISGCFSHMTHEQLVNW VLCAAVYIEQTKDWAWRDRRLTILEQCLESMVRRDHPDPEKRNGVMGLDSTRTMGGAEITTYDSLDVSLGQARNNLYLAGKCWA AYVALEKLFRDVGKEELAALAGEQAEKCAATIVSHVTEDGYIPAVMGEGNDSKIIPAIEGLVFPYFTNCHEALKEDGRFGDYIRALRQ HLQYVLREGICLFPDGGWKISSTSNNSWLSKIYLCQFIARHILGWEWDEQAKRADAAHVAWLTHPTLSIWSWSDQIIAGENYRSKY YPRGVTSILWLEEGE*	52
G	MSKITFPKDFIWGSATAAYQIEGAYNEDGKGESIWDRFSHTPGNIADGHTGDVACDHYHRYEEDIKIMKEIGIKSYRFSISWPRIFPE GTGKLNQKGLDFYKRLTNLLLENGIMPAITLYHWDLPQKLQDKGGWKNRDTTDYFTEYSEVIFKNLGDIVPIWFTHNEPGVVSLLGH FLGIHAPGIKDLRTSLEVSHNLLLSHGKAVKLFREMNIDAQIGIALNLSYHYPASEKAEDIEAAELSFSLAGRWYLDPVLKGRYPENAL KLYKKKGIELSFPEDDLKLISQPIDFIAFNNYSSEFIKYDPSSESGFSPANSILEKFEKTDMGWIIYPEGLYDLLMLLDRDYGKPNIVISE NGAAFKDEIGSNGKIEDTKRIQYLKDYLTQAHRAIQDGVNLKAYYLWSLLDNFEWAYGYNKRFGIVHVNFDTLERKIKDSGYWYKEV IKNNGF*	1

Table 2. Activity data for bacterial and fungal enzymes

	Activity Units								
	LC1	LC2	LX1	LX2	LβX	LβG	CBH I	CBH II	EG I
pNPC *	79.6	378.8	11.1	65.6	-	130.1	7.4	-	19.3
pNPL *	207.7	353.1	-	8.3	-	143.8	1.6	-	48.5
pNPG *	-	-	-	-	1.5	320.3	-	-	-
pNPAf *	-	-	-	-	9.4	-	-	-	-
pNPGal *	-	-	-	-		92.9	-	-	-
pNPX *	-	-	0.9	4.2	1545.0	15.1	-	-	-
Avicel **	-	-	-	-	-	-	0.03	0.05	0.01
CMC **	6.6	1.1	-	-	-	-	-	-	2.0
Xylan **	-	-	19.3	24.4	-	-	-	-	34.2
Cellobiose ***	-	-	-	-	7.8	31.1	-	-	-

Where;

pNPC: p-Nitrophenyl β-D-cellobioside

pNPL: p-Nitrophenyl β-D-lactopyranoside

pNPG: p-Nitrophenyl β-D-glucopyranoside

pNPAf: p-Nitrophenyl α-L-arabinofuranoside

pNPGal : p-Nitrophenyl β-D-galactopyranoside

pNPX: p-Nitrophenyl β-D-xylopyranoside

CMC: Carboxymethyl cellulose

* 1U pNP activity=1 nmol pNP release/mg enzyme/min

** 1U Avicel/CMC/Xylan activity =1 μmol glucose equivalent released/mg enzyme/min

*** 1U Cellobiase activity =1 μmol glucose released/mg enzyme/min

- : No detectable activity found

Table 3 Enzyme mixtures of bacterial and fungal enzymes during hydrolysis of AFEX treated corn stover*.

			Individual enzyme loading (mg/g glucan)									
	#	Mix Type	LC1	LC2	LX1	LX2	LβG	LβX	CBH I	CBH II	EG I	βG
I	A	Control	4	4	4	4	2	2				
	B	Swap	4	4	4	4		2				2
	C	Dope	4	4	4	4		2	4			2
	D	Dope	4	4	4	4		2		4		2
	E	Dope	4	4	4	4		2			4	2
II	F	Swap					2		4	4	4	
	G	Swap					10		4	4	4	
	H	**Benchmark**							**4**	**4**	**4**	**2**
	I	Swap/Dope					2	2	4	4	4	
	J	Dope						2	4	4	4	2
	K	Dope	4	4					4	4	4	2
	L	Dope	4						4	4	4	2
	M	Dope		4					4	4	4	2
III	O	Dope			4				4	4	4	2
	P	Dope				4			4	4	4	2
	Q	Dope			4	4			4	4	4	2
	R	Dope			4	4		2	4	4	4	2

* The experimental results for these enzyme combinations are shown in Fig.2

Table 4 Linear regression of xylan vs. glucan conversion at three different total enzyme loadings.

Xylan conversion = Constant + A*glucan conversion					
Enzyme loading (mg/g glucan)	Constant		A		R^2
	Coefficient	P	Coefficient	P	
10	0.24347	0.000	0.3061	0.000	21.80
15	0.29395	0.000	0.23899	0.000	26.20
30	0.34521	0.000	0.24774	0.000	45.10

Table 5. Percent conversion (glucan and xylan) after 24 hours hydrolysis of AFEX treated corn stover by various bacterial and fungal enzyme mixtures at three different total protein loadings. Here, βG was loaded at 10% (of all other enzymes; mass basis) for all mixtures.

	Enzymes ratio (%, protein mass basis)						10 mg/g glucan		15mg/g glucan		30 mg/g glucan	
#	CBH I	CBH II	EG I	LX1	LX2	LβX	Glucan Conversion (%)	Xylan conversion (%)	Glucan Conversion (%)	Xylan conversion (%)	Glucan Conversion (%)	Xylan conversion (%)
1	20	0	10	0	37	33	26.2	43.9	31.8	41.6	47.6	49.3
2	84	0	10	5	0	1	34.8	26.3	44.7	33	70.1	47.3
3	0	52	42	5	0	1	31.9	27.2	37.7	35.9	56.5	49.2
4	0	20	10	34.5	34.5	1	23.1	35.5	28.4	39.3	38.1	46.3
5	84	0	10	2.5	2.5	1	34.7	21.5	44	30.6	60.6	38.3
6	10	10	74	5	0	1	51.4	29.8	60.7	37.5	89	54.1
7	0	84	10	0	5	1	25	20.1	30.3	26	45.4	36.9
8	0	20	10	0	37	33	19.5	40.2	21.8	38.8	31.3	44.5
9	52	0	42	0	5	1	37.5	24.5	49.4	31.5	68	44.6
10	20	0	10	0	69	1	20.1	26.1	31.6	36.3	51.4	50.2
11	0	52	10	37	0	1	28.6	31.2	34.8	38.7	52	48.1
12	0	20	10	0	69	1	20.9	29.5	26.1	34.8	40.4	48.7
13	0	84	10	2.5	2.5	1	25.7	20.6	33	29.1	48.2	41
14	42	42	10	5	0	1	61.3	28.6	66.5	35.2	81.2	46.8
15	51	9	18	5.3	7.8	9	55.9	43	69.5	50.3	83.9	56.1
16	52	0	42	5	0	1	45	30	55.4	37.6	71.3	47.6
17	10	10	10	69	0	1	45.2	34.3	54.9	40.8	80.1	54.4
18	10	10	10	0	69	1	41.9	30.3	51.2	37.8	67.2	48.5
19	0	20	74	5	0	1	23.7	26.1	34.2	35.9	44.8	40.8
20	9	51	18	7.8	5.3	9	53.4	44.1	59.5	46.8	86.2	60.2
21	52	0	10	37	0	1	37.4	34.6	42.6	39.3	63.7	47.9
22	0	20	10	69	0	1	21.7	33.8	27.8	38.3	41.1	49.6
23	20	0	10	34.5	34.5	1	25.6	33.3	33.7	40.7	53.1	50.6
24	52	0	10	5	0	33	30	39.2	38.5	43.2	65.7	55.3
25	9	19	18	5.3	7.8	41	53.3	48.3	62.5	46.8	79.6	56.5
26	52	0	10	0	5	33	30.6	36.3	34.2	36.8	53.3	45.7
27	84	0	10	0	5	1	30	20.9	39.7	25.2	64.6	41
28	0	52	10	0	5	33	20.6	29.9	26.8	35	37.1	39.9
29	42	42	10	0	5	1	52.4	20.3	61.8	28.1	78.3	43.6
30	51	9	18	7.8	5.3	9	58.8	47.9	71.6	51	90.2	60.2
31	20	0	10	69	0	1	25.3	34	32.2	40.3	52.7	52
32	9	19	18	7.8	5.3	41	51.7	50.5	58.4	46.8	81.2	60
33	20	0	42	0	37	1	34.3	30.8	44.5	38.6	65.1	47
34	0	52	10	5	0	33	24.9	39.7	29.5	40	45.3	48.8
35	0	20	74	2.5	2.5	1	24.8	25.7	30.4	33	44.5	40.4
36	20	0	74	2.5	2.5	1	35.4	25.6	45.9	33.4	67.1	47.5
37	0	20	10	37	0	33	23.1	46.5	28	46.4	37.5	48.2
38	0	20	42	0	37	1	24	27.5	30.5	36.8	45.7	49.4
39	9	51	18	5.3	7.8	9	61	43.4	64.7	49.7	81.2	56
40	19	9	50	5.3	7.8	9	63.3	52.2	70.2	42.5	81.9	54.1
41	19	9	18	5.3	7.8	41	46.9	39.2	61.2	48.8	79.5	56.8
42	9	19	50	7.8	5.3	9	56.9	37.3	64.2	47.1	90	59.3
43	20	0	10	0	5	65	18.2	34.2	24.4	36.1	42.6	47.2
44	20	0	10	37	0	33	30.2	45.3	37.9	46.3	53.5	55.1
45	10	10	10	0	5	65	32.9	35.9	43.6	39.3	64	49.7
46	0	52	42	0	5	1	32.8	26.4	35.7	29.5	49.5	42.3
47	0	20	10	0	5	65	16.9	33.8	21.3	35.6	33.1	42.8
48	0	20	42	37	0	1	28.7	35.6	33.6	41.2	48.4	50.3
49	0	20	10	2.5	2.5	65	16.5	33.2	21.9	37.6	33	42.7
50	20	0	10	5	0	65	20.4	35.7	27.2	40.7	46.7	50.3
51	9	19	50	5.3	7.8	9	60.1	48.8	61	46.9	77.7	53.6
52	20	0	74	5	0	1	37.1	31.2	44.2	34.8	64.8	45.8
53	9	19	18	39.8	5.3	9	51.6	48.3	61.8	49.2	83.6	59.7
54	19	9	18	7.8	5.3	41	54.8	44.9	62.4	48.7	85.3	60
55	20	0	42	0	5	33	29.7	42.2	38.6	42.7	57.2	49.5
56	0	20	42	0	5	33	22.6	38.7	29.7	42.1	43.5	50.4
57	20	0	74	0	5	1	31.6	25.7	42.6	32.7	67	48.7
58	20	0	10	2.5	2.5	65	24.5	39.6	30	40	48.8	52.7
59	19	9	50	7.8	5.3	9	63.1	52.3	71.8	54.5	94.7	62.9
60	10	10	10	5	0	65	35.4	43.5	46	45.4	74.7	57.9
61	0	20	10	5	0	65	18.2	38.6	22.4	39	33	44.7
62	52	0	10	0	37	1	34.1	28.9	40	35.2	63.5	49.6
63	20	0	42	5	0	33	29.6	40.1	40.3	47.4	57.3	49.5
64	20	0	42	37	0	1	37.1	36.6	44.8	41.7	58.4	46.3
65	19	9	18	5.3	39.8	9	52.8	47.9	60.8	47.8	76.5	53.8
66	0	84	10	5	0	1	28.4	25.6	33.1	31.1	49.9	44.2
67	0	52	10	0	37	1	24.5	25.8	29.7	33.1	44.5	45.9
68	0	20	74	0	5	1	26	24.1	31.8	30.3	46.8	43.3
69	9	19	18	5.3	39.8	9	47.4	42.6	59.9	49	74.9	53.4
70	18	18	26	10.5	10.5	17	58.6	49.9	65.5	49.4	77.3	53.6
71	19	9	18	39.8	5.3	9	52.2	49.3	58.5	48.2	79	54.8
72	0	20	42	5	0	33	24.9	40.5	30.2	45.4	45.4	53.3
73	10	10	74	0	5	1	43.7	25.1	61.8	36.1	82.4	50.1

Figure 1 Four-step strategy for screening enzymes to develop novel enzyme mixtures to maximize digestibility of pretreated lignocellulose

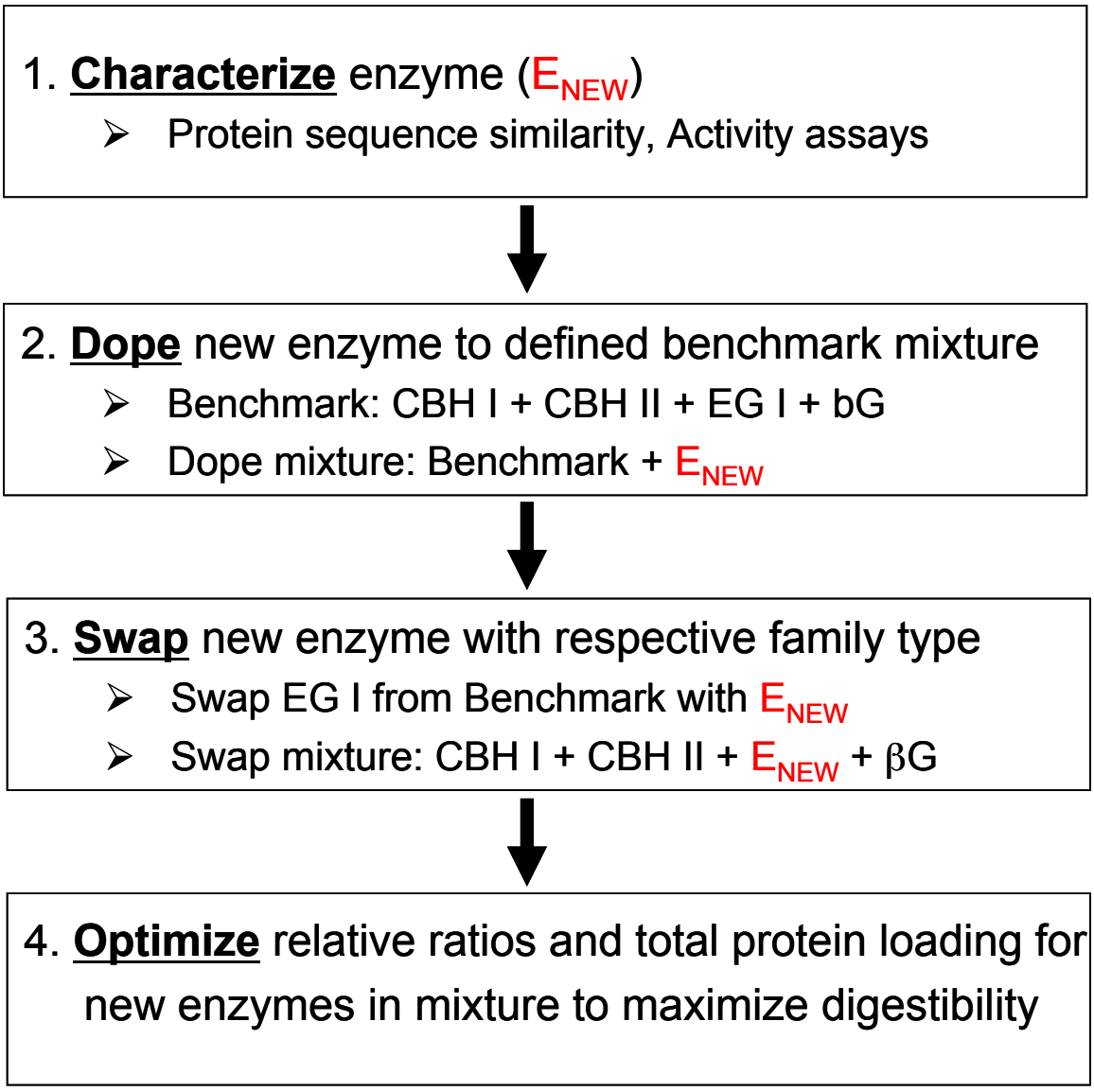

Figure 2 Percent conversion (glucan and xylan) after 24 hours hydrolysis of AFEX treated corn stover. Each enzyme mixture contains both bacterial cellulases and hemicellulases (4 mg/g glucan each for LC1, LC2, LX1 and LX2; 2 mg/g glucan each for LβX and LβG).

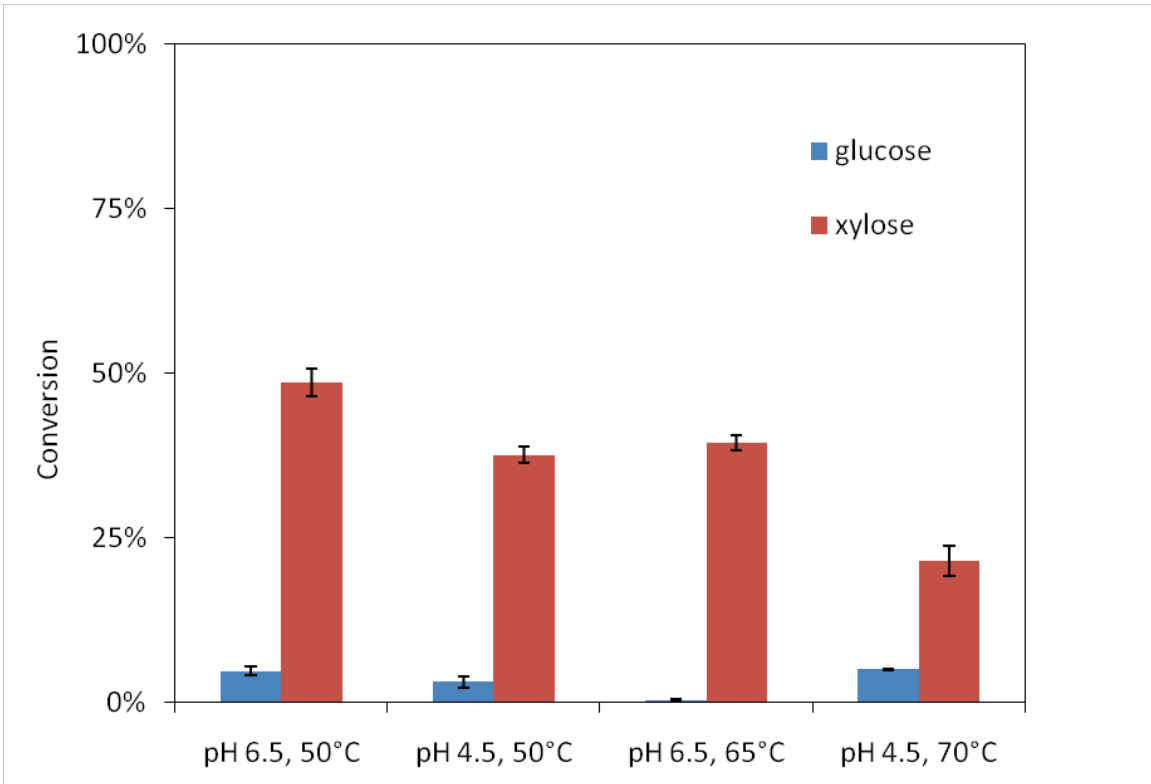

Figure 3 Glucan and xylan conversions after 24 hours hydrolysis of AFEX treated corn stover. Enzyme mixtures used here are I (A to E); II (F to M) and III (H, O to R) based on protein compositions as listed in Table 1.

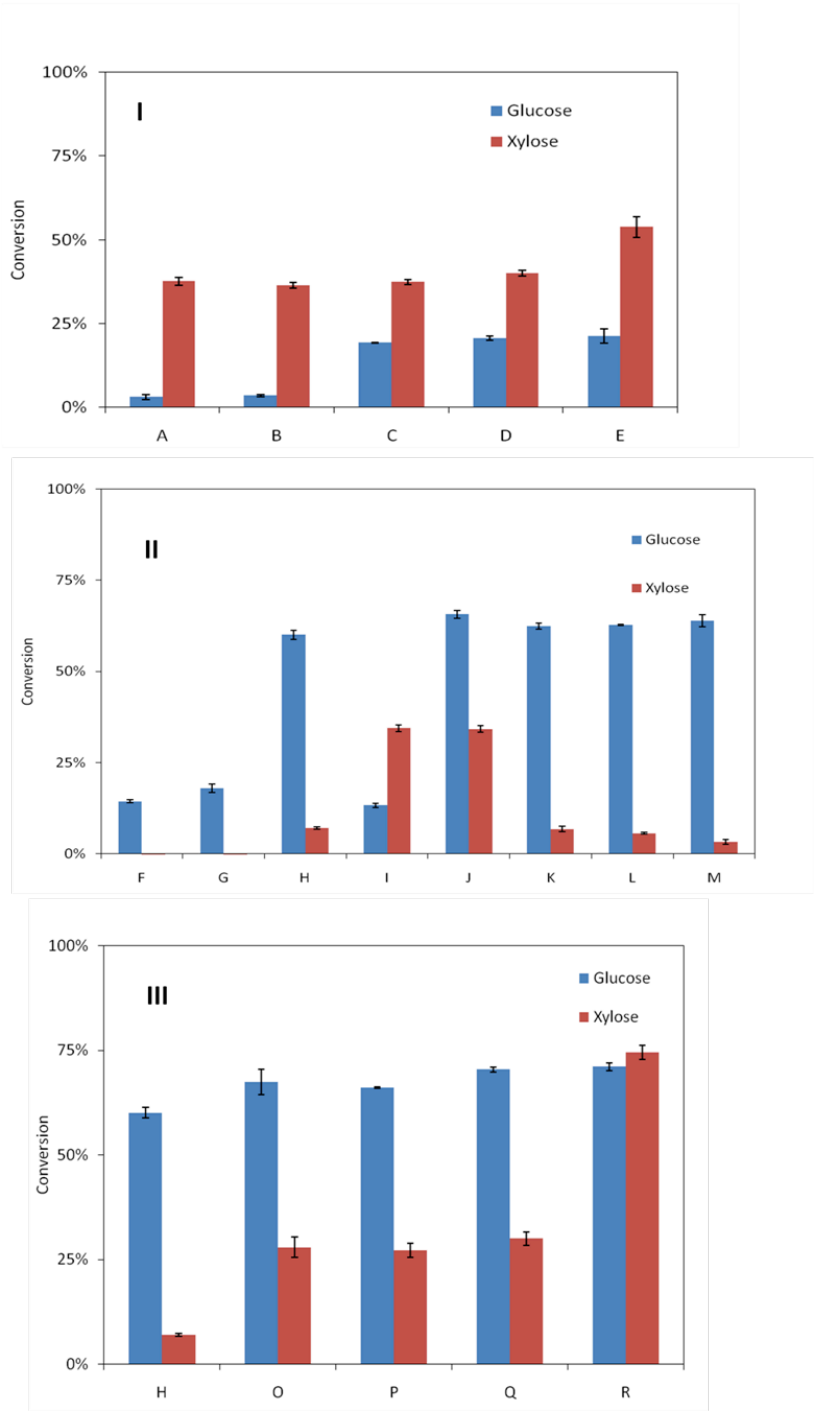

Figure 4 Glucan vs. xylan conversion after 24 hours hydrolysis of AFEX treated corn stover. Three different enzyme loadings (30, 15 and 10 mg/g glucan; not inclusive of 10% βG supplemented) are shown.

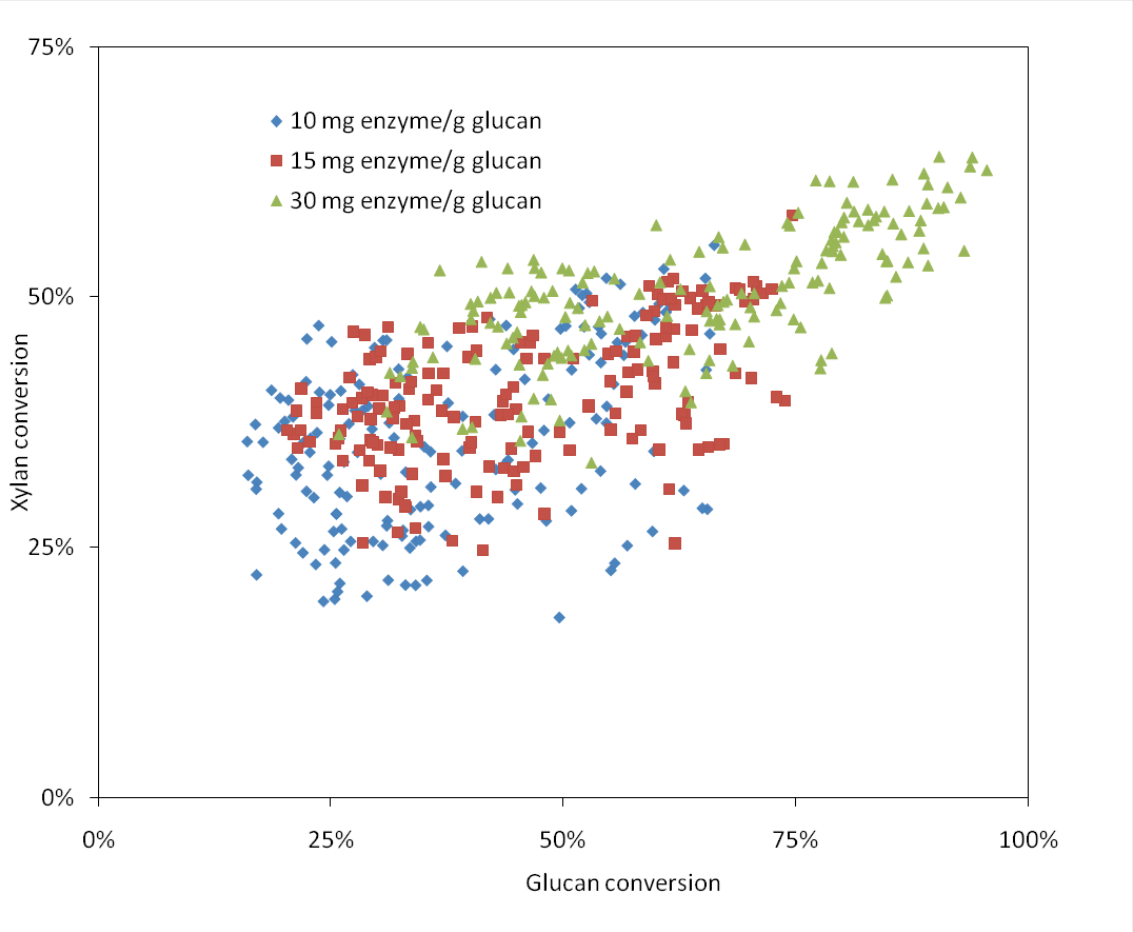

Figure 5 Glucan vs. xylan conversion after 24 hours hydrolysis of AFEX treated corn stover for varying relative ratios of cellulases (I), xylanases (II) and β-xylosidase (III) at three different total enzyme loadings (a, b and c correspond to 10, 15 and 30 mg/g glucan enzyme loading, respectively).