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Conversion for Avicel and AFEX pretreated corn stover by *Clostridium thermocellum* and simultaneous saccharification and fermentation: Insights into microbial conversion of pretreated cellulosic biomass

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ABSTRACT

In this study, efforts were taken to compare solubilization of Avicel and AFEX pretreated corn stover (AFEX CS) by SSF and *Clostridium thermocellum* fermentation, with an aim to gain insights into microbial conversion of pretreated cellulosic biomass. Solubilization rates for AFEX CS are comparable for the two systems while solubilization of Avicel is much faster by *C. thermocellum*. Initial catalyst loading impacts final cellulose conversion for SSF but not for *C. thermocellum*. Hydrolysis of the two substrates using cell-free *C. thermocellum* fermentation broth revealed much smaller difference in cellulose conversion than the difference observed for growing cultures. Tests on hemicellulose removal and particle size reduction for AFEX CS indicated that substrate accessibility is very important for enhanced solubilization by *C. thermocellum*.

1. Introduction

Cellulosic biomass is of interest as a sustainable source of organic fuels, chemicals, and materials because of its large scale potential availability, low purchase cost, and more desirable environmental attributes as compared to row crops (Lynd, 2008). Various strategies, depending on the extent of consolidation of steps in the conversion process, have been proposed for cellulosic biomass processing featuring enzymatic hydrolysis (Lynd et al., 2002). Of these, simultaneous saccharification and fermentation (SSF) serves as a good basis for evaluating substrates or cellulase systems and consolidated bioprocessing (CBP) offers potential for low processing costs if limitations (e.g. yield and titer) of currently available microbes can be overcome. *Trichoderma reesei* cellulase is commonly used in SSF studies. *Clostridium thermocellum* is a widely studied candidate microorganism for CBP due to its ability to rapidly hydrolyze cellulosic material and ferment the hydrolysis products to ethanol accompanied by organic acids.

Various pretreatment technologies have been studied to make cellulosic biomass more amenable to enzyme and microbial conversion (Hsu, 1996; Mosier et al., 2005a; Wyman et al., 2005). Ammonia fiber expansion (AFEX) alters lignocellulosic ultra and macro structures due to the catalytic effect of ammonia (Balan et al., 2009). Following AFEX pretreatment, accessible surface area is increased and hemicellulose is partially depolymerized. Ammonia-soluble lignin and hemicelluloses are extracted and displaced to the surface of the plant cell wall, which helps create pores in the biomass and disrupt biomass structure (Balan et al., 2009). AFEX can achieve greater than 90% conversion of cellulose and hemicellulose to fermentable sugars for a variety of lignocellulosic materials (Yang and Wyman, 2008). Degradation products from AFEX pretreatment showed very little inhibitory effect on yeast fermentation. Moreover, AFEX preserves sufficient nutrients for yeast fermentation (Lau and Dale, 2009).

Avicel, a product of pure cellulose in the form of fine powder derived from wood pulp by partial acid hydrolysis and spray drying of the washed pulp slurry (FMC BioPolymer, Philadelphia, PA), has

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been used as a model substrate for evaluating the performance of cellulase and microbial systems. However, pretreated cellulosic substrates are quite different from Avicel. Particle size is typically larger for pretreated substrates, particularly under conditions expected to be practical in industry. Depending on the pretreatment technology and conditions, pretreated cellulosic substrates may contain lignin, hemicelluloses, and other components in addition to cellulose (Kim and Holtzapple, 2005; Kim and Lee, 2005; Liu and Wyman, 2005; Lloyd and Wyman, 2005; Mosier et al., 2005b; Teymouri et al., 2005). These components could block or hinder the access of enzymes or microbe to cellulose. Pretreatment can generate inhibitory compounds which can be inhibitory to cellulase as well as microorganisms (Palmqvist and Hahn-Hägerdal, 2000; Palmqvist et al., 1996). These observations suggest that solubilization of Avicel and pretreated substrates could be quite different for different conversion systems such as SSF and *C. thermocellum*. While extensive data are available for SSF of pretreated lignocellulosic substrates, however, for *C. thermocellum*, Avicel has been widely used in microbial studies and in particular for enzymatic studies and only a few reports have utilized pretreated substrates (Lynd and Grethlein, 1987; Raman et al., 2009; Saddler and Chan, 1982; Weimer and Chou, 1986).

Efforts to improve industrial properties of *C. thermocellum* such as ethanol tolerance and yield are underway but have not yet progressed to the point that comparison to SSF under industrially relevant conditions is relevant. It is of interest, however, to compare the intrinsic cellulose solubilization ability of the non-complexed, mesophilic fungal cellulase systems operative in SSF to the complexed, thermophilic, bacterial cellulase system produced by *C. thermocellum*. This study reports comparison of Avicel and AFEX pretreated corn stover solubilization by SSF and *C. thermocellum*.

2. Methods

2.1. Strains and culturing conditions

AFEX CS used in this study was prepared in the lab of Bruce Dale at Michigan State University. The composition of AFEX CS was reported previously with 34.4% glucan, 22.4% xylan, 4.2% arabinan, 0.6% mannan, 1.4% galactan, 3.8% uronyl, 11% lignin and 5.6% acetyl content (Gao et al., 2010). The particles, ranging between 0 and 6000 μm (maximum length), have a volume weighted average particle size around 1400 μm. Avicel PH 105 was obtained from FMC Corporation (Philadelphia, PA). Spezyme CP cellulase, Multifect pectinase, and Multifect xylanase were kindly provided by Genencor International, Inc. (Rochester, NY). Novozyme 188 β-glucosidase was obtained from Sigma–Aldrich (St. Louis, MO). The activities of these commercial enzymes were reported by Dien et al. (2008).

Saccharomyces cerevisiae, strain D5A (NREL), prepared in YPD media (Sigma Y1375) was used for SSF inoculation. The KN medium, developed by Kadam and Newman (1997) and consisting of 0.3% (v/v) corn steep liquor supplemented by 5 mM MgSO4, was used in all SSF experiments. *C. thermocellum* ATCC 27405 was obtained from the American Type Culture Collection (Manassas, VA). A single colony was isolated and maintained as a stock culture. Chemically-defined Media for thermophilic clostridia (MTC), with components in solutions B, C, D, E, and F, was prepared according to Zhang and Lynd (2003) with the exception that no thiamine was added in solution E and solution F was 100 g/L MOPs sodium salt at 10-fold reaction concentration (no supplemental solution of minerals). All chemicals were reagent grade and were obtained from Sigma (St. Louis, MO), unless indicated otherwise. Solution A contained either Avicel PH105 or AFEX CS supplemented with an appropriate amount of Milli-Q DI water (Millipore, Billerica, MA). Solution B, C, D, E, and F were injected into Solution A using a syringe. Prior to combining all the solutions, they were purged with N_2 (Airgas Northeast, White River Junction, VT) in 250-ml serum bottles and sterilized by autoclaving at 121 °C for 45 min.

2.2. Particle size reduction and hemicellulose removal

For particle size reduction, the original AFEX CS was milled to pass through a 500-μm sieve using a knife mill (Thomas scientific, mill model 174931.00, Swedesboro, NJ). To test the influence of hemicellulose on glucan solubilization by SSF and *C. thermocellum*, hemicellulose was removed by enzymatic hydrolysis conducted in 125-ml serum bottles (Wheaton, Millville, NJ). 0.75 g AFEX CS was added into the bottles and supplemented with 30.5 ml DI water and 2.5 ml 1 M citrate buffer at pH 4.5. The bottles were crimp-sealed and sterilized by autoclaving at 121 °C for 45 min. Thereafter, a 2 ml enzyme preparation consisting of 0.0262 ml Multifect pectinase, 0.0372 ml Multifect xylanase, and 1.9366 ml DI water was added to the AFEX CS slurry. The bottles were then transferred into a shaking incubator (New Brunswick Scientific, innova 4080, Edison, NJ) with temperature controlled at 37 °C and rotation speed set at 200 rpm. After 72 h, the reaction volume was transferred into 50-ml conical tubes (Becton Dickinson Labware, Franklin Lakes, NJ) and centrifuged at 5000*g* in a Biofuge 15R (Heraeus Instruments, Germany). Supernatant samples were analyzed for glucose and xylose following dilute acid hydrolysis according the quantitative saccharification procedure (Ruiz and Ehrman, 1996). The pellet was re-suspended with DI water to a total volume of 40 ml and centrifuged again. The supernatant was then discarded. For *C. thermocellum* fermentation, the pellet was re-suspended with DI water to a total volume of 35 ml and transferred into a 125-ml serum bottle. For SSF, the pellet was re-suspended with DI water to a total volume of 41 ml, transferred into a 125-ml serum bottle, and added with 2.5 ml 1 M citrate buffer with pH at 4.5. The bottles were crimp-sealed, purged with N_2, and autoclaved at 121 °C for 25 min.

2.3. SSF

0.75 g AFEX CS or 0.22 g Avicel PH105 was added into 125-ml serum bottles and supplemented with 41 ml DI water and 2.5 ml 1 M citrate buffer with pH at 4.5. The bottles were crimp-sealed, purged with N_2, and sterilized by autoclaving at 121 °C for 45 min. After cooling, a 2 ml filter-sterilized solution consisting of 0.15 ml CSL, 0.03 g MgSO4, and 1.85 ml DI water was added by syringe. For a cellulase loading of 10 FPU/g glucan, the bottles were then injected with 2 ml filter-sterilized enzyme solution consisting 0.03667 ml Spezyme cp, 0.03667 ml β-glucosidase, and 1.926 ml DI water. For other cellulase loadings, the amount of these components was changed accordingly, keeping the overall volume at 2 ml and an activity ratio of 3 (IU–FPU) for β-glucosidase over Spezyme CP. Finally, 2.5 ml yeast inocula prepared in YPD (Sigma Y1375) media was injected. The bottles were placed in a shaking incubator (New Brunswick Scientific, innova 4080) with temperature controlled at 37 °C and rotation speed set at 200 rpm. For Avicel and AFEX CS treated with both particle size reduction and hemicellulose removal, 5 ml homogeneous samples were taken at 24, 48, and 96 h after inoculation. For AFEX CS without additional treatments, the contents of an entire bottle was collected at the indicated times. For AFEX CS treated only by size reduction or hemicellulose removal, the content of an entire bottle was taken at 96 h. The collected samples were centrifuged. The supernatant was discarded after sampling for HPLC measurement. The pellets were re-suspended to sampling volume with DI water and centrifuged

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again. The resulting pellets were analyzed for residual glucan and xylan using quantitative saccharification (Ruiz and Ehrman, 1996).

2.4. "C. thermocellum" fermentation

0.75 g AFEX CS or 0.22 g Avicel PH105 was added into 125-ml serum bottles and supplemented with 35 ml DI water (or less for >10% v/v inoculation). The bottles were crimp-sealed, purged with N_2, and sterilized by autoclaving at 121 °C for 45 min. After that, 2 ml B, 1 ml C, 1 ml D, 1 ml E, and 5 ml F stock solutions were added by syringe. For 10% v/v inoculation, the bottles were then injected with 5 ml inocula from an exponential phase culture grown on 5 g/L Avicel PH 105 in MTC media. For other inoculum sizes, the volumes of DI water and inoculation were changed accordingly, keeping the post inoculation liquid volume at 50 ml. The bottles were placed in a shaking incubator (New Brunswick Scientific, innova 4080) with temperature controlled at 55 °C and rotation speed set at 200 rpm. Sample collection and processing were the same as described for SSF.

2.5. Cellulose hydrolysis by cell-free cellulase preparations from "C. thermocellum"

C. thermocellum culture at stationary phase (about 24 h) prepared on 5 g/L Avicel PH105 in MTC media was transferred into 50-ml centrifuge bottles (cat 3138-0050, Nalgene, Rochester, NY) in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) and centrifuged for 15 min at 27,000g in an Avanti J-25 centrifuge (Beckman Coulter, Brea, CA). The supernatant was filtered into a sterile and anaerobic serum bottle using a 0.2 μm PES syringe filter (cat 431229, Corning Incorporated, Corning, NY) in the anaerobic chamber. 20 ml of this filtered supernatant was injected into a 125-ml serum bottle containing 5 ml DI water and 0.22 g Avicel PH 105 or 0.75 g AFEX CS. Before adding the cellulase broth, the contents of the serum bottles were purged with N_2 and sterilized by autoclaving at 121 °C for 45 min. After adding the cellulase-containing broth, the bottles were placed in a shaking incubator (New Brunswick Scientific, innova 4080) with temperature controlled at 55 °C and rotation speed set at 200 rpm. Samples were collected at 24, 48, and 96 h. After centrifuging, 1 ml supernatant was added with 27 ml DI water. The mixture was subjected to dilute acid hydrolysis for glucose production which was used to calculate cellulose conversion.

2.6. Analytical methods

The carbohydrate contents of the AFEX CS and residual pellets collected and dried after SSF and *C. thermocellum* fermentation were determined by quantitative saccharification (Ruiz and Ehrman, 1996). The quantities were scaled-down to one-third. 0.1 g biomass was used for AFEX CS and all the residual solids was taken for Avicel. For total hydrolysis products released after hemicellulose removal, dilute acid hydrolysis was performed by adding 1 ml 72% (wt) H_2SO_4 to 28 ml supernatant and autoclaving at 121 °C for one hour. Product concentrations were obtained using a Waters HPLC system (#2695, Milford, MA) with an Aminex HPX-87H column (Bio-rad, Hercules, CA) operated at 60 °C. Conversions were calculated as a percentage of originally-present glucan or xylan solubilized, based on analysis of residual solids. Due to the insoluble nature of the substrates, cell protein was used to estimate cell growth. Cell protein was extracted according to a protocol reported previously (Zhang and Lynd, 2003) and was measured using a 2-D Quant Kit from GE Healthcare (Piscataway, NJ).

3. Results and discussion

3.1. Solubilization of Avicel and AFEX CS by SSF and "C. thermocellum"

This study compared the solubilization of Avicel and AFEX CS by SSF using a commercial fungal cellulase preparation and by *C. thermocellum* fermentation. The comparison was conducted with a substrate concentration of 4.4 g/L glucan. Fig. 1 shows the time course of cellulose and xylan conversion over a 4 day reaction period. For Avicel, *C. thermocellum* achieved over 95% conversion after 1 day while SSF resulted in about 65% conversion after 4 days. For AFEX CS, *C. thermocellum* and SSF obtained similar cellulose (60–70%) and xylan conversion (~70%) after 4 days. For *C. thermocellum*, solubilization of Avicel was much faster than for AFEX CS. For SSF, cellulose conversions were similar after 4 days for the two substrates. Cellulose conversion for SSF with a cellulase loading of 10 FPU/g glucan and *C. thermocellum* fermentation was quite comparable for the AFEX CS. However, the rate of utilization of Avicel was much faster for *C. thermocellum* than that for SSF. This suggests that there may be potential for improving the rate solubilizing pretreated substrates by microbial conversion, and also invites the question of why solubilization of the pretreated substrate is much slower relative to Avicel for *C. thermocellum* but not for SSF.

3.2. Solubilization of AFEX CS by SSF with various cellulase loadings and by "C. thermocellum" with various inoculum sizes

To investigate the effect of initial catalyst loading on the cellulose solubilization by SSF and *C. thermocellum* fermentation, SSF was conducted on AFEX CS with various cellulase loadings and *C. thermocellum* was grown on Avicel and AFEX CS with various inoculum sizes. For the convenience of sampling using syringes and needles, AFEX CS was milled to pass through a 500-μm sieve. Results are given in Fig. 2 for SSF and in Fig. 3a for *C. thermocellum* fermentation. For SSF, lower cellulase loading resulted in lower final cellulose conversion (up to 15% for the conditions tested). *C. thermocellum*, however, produced the same final cellulose conversion regardless of inoculation size for both substrates. Under the conditions tested, SSF needed at least a cellulase loading of 10 FPU/g glucan to reach final conversions similar to those obtained with *C. thermocellum*.

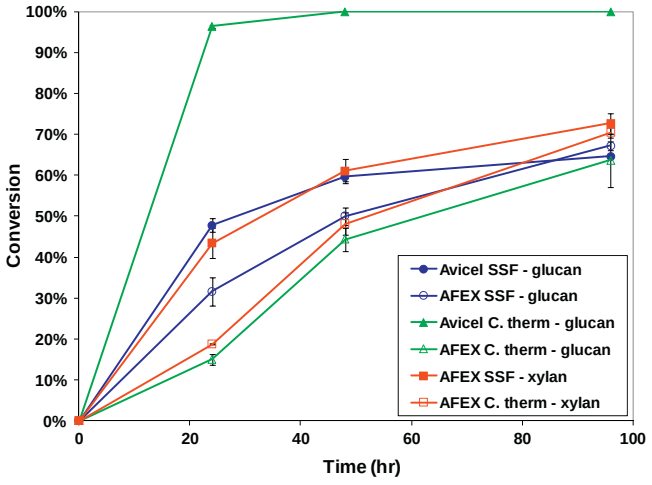

Fig. 1. Conversion for SSF and *Clostridium thermocellum* (C. therm) fermentation using AFEX CS and Avicel: substrate is 4.4 g/L glucan; cellulase loading for SSF is 10 FPU/g glucan with an activity ratio of 3 for β-glucosidase over Spezyme CP (IU to FPU); inoculation for *C. thermocellum* is 10% v/v grown on Avicel; AFEX CS has a volume weighted average particle size of 1.4 mm (size range 0–6 mm).

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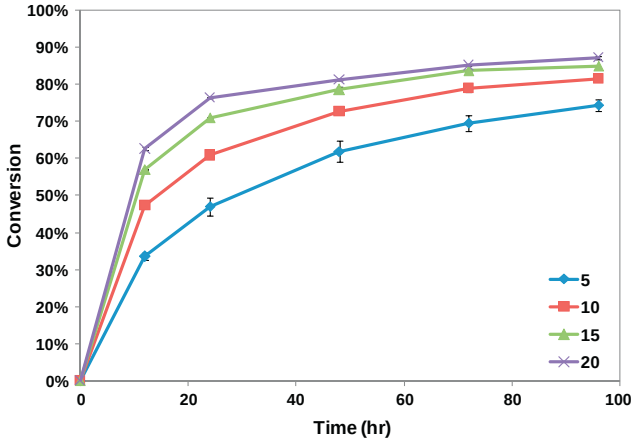

Fig. 2. Glucan conversion for SSF using AFEX CS with various cellulase loadings 5, 10, 15, 20 FPU/g glucan: substrate is 4.4 g/L glucan; AFEX CS was milled to pass a 0.5 mm sieve.

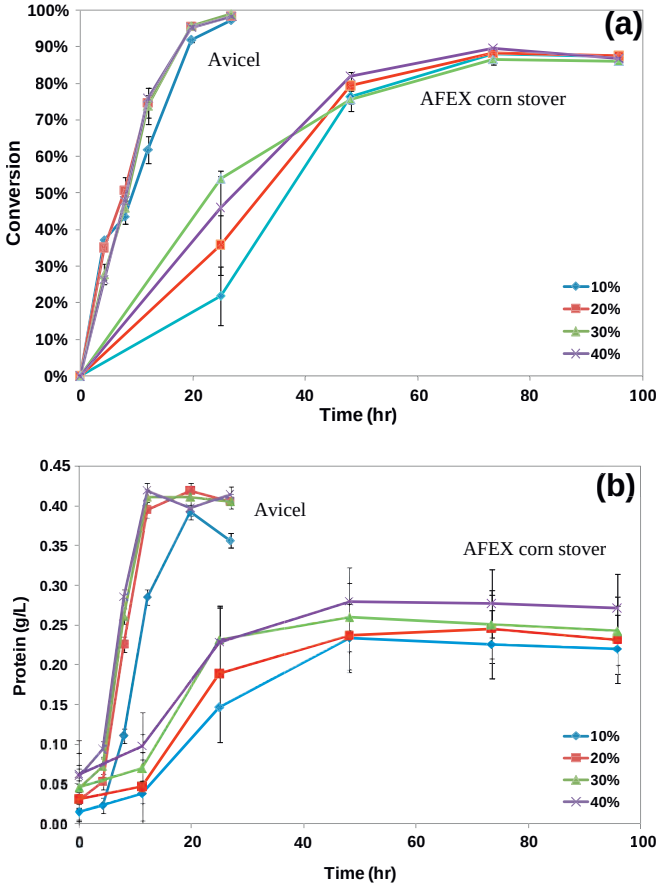

Fig. 3. *C. thermocellum* fermentation using Avicel and AFEX CS with various sizes of inoculation 10%, 20%, 30%, 40% v/v. (a) glucan conversion, (b) pellet protein (cell growth): substrate is 4.4 g/L glucan; AFEX CS was milled to pass a 0.5 mm sieve.

Varying the initial catalyst loading changed the final conversion of AFEX CS after 4 days for SSF but not for *C. thermocellum* fermentation. The trend in Fig. 2 suggests that SSF with lower cellulase loadings needs a much longer period to obtain the same final conversion with the possibility that it can never reach the same conversion as has been observed by others (Spindler et al., 1988; Vu and Kim, 2009). The same final conversion was obtained by *C. thermocellum* fermentation with various sizes of inoculation for both Avicel and AFEX CS. This indicates that growing catalyst during the solubilization process is quite advantageous compared to fixed catalyst loading in SSF. However, solubilization of the pretreated substrate remained much slower than that of Avicel even when the inoculum was large. Thus, inoculum size does not explain the difference between the rate of solubilization of Avicel and AFEX CS for *C. thermocellum*.

3.3. Cell growth and cellulase hydrolysis for "C. thermocellum" on Avicel and AFEX CS

To investigate whether the slower solubilization of AFEX CS by *C. thermocellum* was caused by limited cell growth on the pretreated material, cellular growth as pellet protein was measured during the course of fermentation. Fig. 3b shows the cellular growth for both Avicel and AFEX CS with various sizes of inoculation. Significant growth was observed on both substrates, with higher maximum cell concentration seen on Avicel. The cell concentration peaked much faster on Avicel than it did on AFEX CS, consistent with the faster rate of substrate hydrolysis observed on Avicel.

Significant cell growth was observed on AFEX CS for *C. thermocellum* fermentation (Fig. 3b). This suggests that cell growth is probably not inhibited by the pretreated substrates, particularly given the low substrate concentration (1.5% w/w total solids) used in this study. Enzymatic hydrolyzate from AFEX CS has been reported to show little inhibitory effect on a genetically engineered *S. cerevisiae* strain with 4% solids concentrations (Lau and Dale, 2009) and on *Escherichia coli* KO11, *Zymomonas mobilis* AX101, and *Clostridium phytofermentans* (unpublished results from the Dale lab). No inhibitory effect was observed for Avicel solubilization by *C. thermocellum* with 0.33% w/w lignin residue obtained by enzymatic hydrolysis (data not shown). It was also reported that *C. thermocellum* cellulosome activity can actually increase in presence of organic acid anion (e.g. up to 12 g/L acetate) (Xu et al., 2010).

Cellulose hydrolysis using the supernatant from stationary phase Avicel-grown *C. thermocellum* cultures was performed on Avicel and AFEX CS. The difference in cellulose conversion was much smaller than the conversion difference observed with growing cultures, 15% vs. 55% after 2 days. To reach 50% of maximum attainable cellulose conversion (50% for Avicel and 45% for AFEX CS), it took about 6 h for Avicel and 30 h for AFEX CS for the case with cultures (a ratio of 6), while it took about 37 h for Avicel and 47 h for AFEX CS for cellulose hydrolysis by cell-free *C. thermocellum* fermentation broth (a ratio of 1.3). The greater solubilization performance observed with growing culture is consistent with *C. thermocellum* deriving more benefit from cell-associated cellulosome while growing on Avicel as compared to AFEX CS. Cell-associated cellulosome could have been blocked by unhydrolyzable components such as lignin in the AFEX CS. Thus, solubilization performance for *C. thermocellum* utilizing AFEX CS should improve when substrate is more readily available for cell adherence. Aside from not benefiting from enzyme-microbe synergy, the reduced solubilization performance for cell-free hydrolysis can also be partially attributed to end product inhibition. Cellobiose was reported to have significant inhibition on *C. thermocellum* cellulase activity (Johnson et al., 1982).

3.4. Solubilization of AFEX CS by SSF and "C. thermocellum" with substrate particle size reduction and hemicellulose removal

Supplementing Multifect xylanase and Multifect pectinase has been shown to significantly increase cellulose hydrolysis by Spezyme CP for AFEX CS (Dale et al., 2007), *Miscanthus* (Murnen et al., 2007), and switchgrass (Bals et al., 2010). To study the effect of hemicellulose on final cellulose conversion, the two enzymes

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cannot be supplemented directly to *C. thermocellum* fermentation because *C. thermocellum* grows at a pH (~7) and temperature (55 °C) higher than tolerated by these two enzymes. Thus it was chosen to remove hemicellulose in AFEX CS by enzymatic hydrolysis using multifect xylanase and multifect pectinase prior to conducting SSF and *C. thermocellum* fermentation on the remaining solids. Particle size has also been reported to have a significant impact on the performance of enzymatic cellulose hydrolysis (Dasari and Berson, 2007; Jin et al., 2011; Yeh et al., 2010). To investigate the effect of particle size reduction, the original AFEX CS was milled to pass through a 500-μm sieve. The effect of hemicellulose removal and particle size reduction on four-day cellulose and xylan conversion is given in Fig. 4 for AFEX CS. Final conversions of cellulose and xylan for both SSF and *C. thermocellum* were observed to increase compared to the control. For SSF, xylan conversion was almost exclusively from the prior step of hemicellulose removal by xylanase and pectinase. For *C. thermocellum*, a few percent additional xylan hydroysis occurred during fermentation. For both SSF and *C. thermocellum* fermentation, cellulose and xylan conversions were increased compared to control after the particle size of AFEX CS was reduced by knife-milling to pass through a 500-μm sieve. After particle size reduction, a slightly greater increase in cellulose and xylan conversions was observed for *C. thermocellum* compared to SSF. Cellulose conversion increased significantly compared to controls for both systems when combining the treatments of hemicellulose removal and particle size reduction (particle size was reduced first and then hemicellulose was removed), as shown in Fig. 5.

Substrate accessibility is important for cellulose solubilization (Arantes and Saddler, 2011; Chandra et al., 2007, 2009; Lee et al., 1994). Avicel PH105 used in this study is a pure substrate in the form of fine particles with an average diameter of 20 μm, whereas AFEX CS contains lignin and hemicellulose and particle size is much larger (milled to pass a 500-μm sieve). Cellulose conversion increased significantly after removing the hemicellulose components or reducing the average particle size of the AFEX CS. Increase in accessible surface area was reported for hemicellulose removal (Grethlein, 1985), while no direct correlation was made between accessible surface area and particle size reduction. This can be explained because most accessible surface area of cellulosic materials (e.g. Avicel) is internal area rather than external area (Hong et al., 2007). Studies have observed increased cellulose conversion with smaller particle size (Dasari and Berson, 2007; Jin et al., 2011; Yeh et al., 2010). Particle size reduction was more effective than hemicellulose removal for *C. thermocellum* fermentation while they were quite similar for SSF. Further testing could be done comparing the solubilization of Avicel to that of pretreated substrates with particle size reduced to around 20 μm or lignin removed.

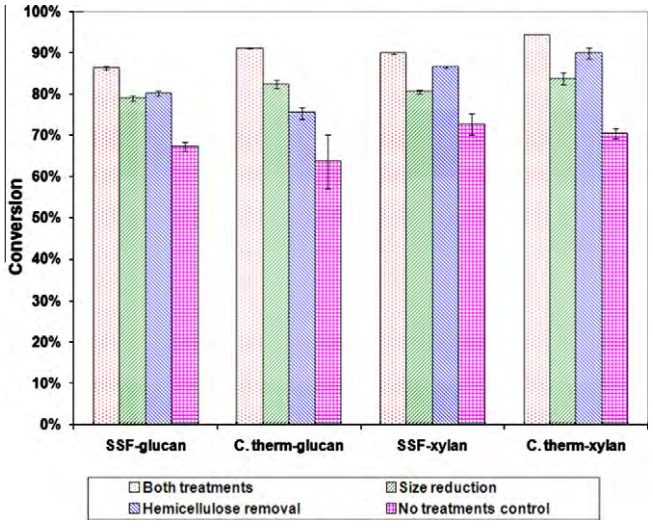

Fig. 4. Four-day glucan and xylan conversions for SSF and *C. thermocellum* (C. therm) fermentation using AFEX CS with various conditions: no treatment control, hemicellulose removal (HR), size reduction (SR) (<0.5 mm), and both treatments (HR and SR); substrate is 4.4 g/L glucan originally; cellulase loading for SSF is 10 FPU/g glucan originally (there will be some glucan loss during hemicellulose removal, so the cellulase loading is a little higher than 10); inoculation for *C. thermocellum* is 10% v/v grown on Avicel.

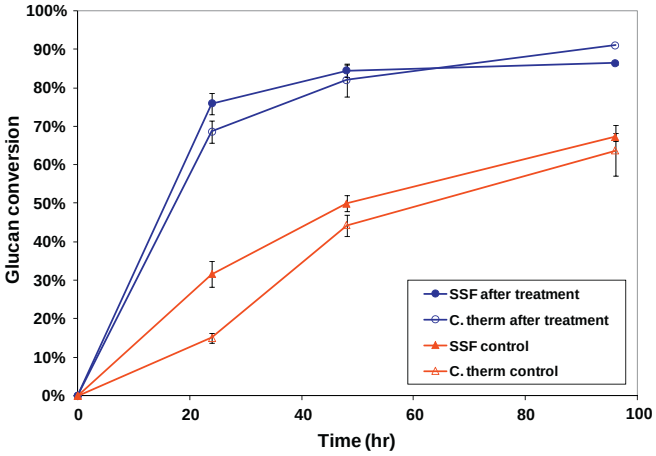

Fig. 5. Conversion for SSF and *C. thermocellum* (C. therm) fermentation using AFEX CS treated by both hemicellulose removal and particle size reduction (<0.5 mm): substrate is 4.4 g/L glucan; cellulase loading for SSF is 10 FPU/g glucan; inoculation for *C. thermocellum* is 10% v/v grown on Avicel; AFEX CS control has a volume weighted average particle size of 1.4 mm (size range 0–6 mm).

4. Conclusion

Rates and extents of solubilization of AFEX CS are comparable for *C. thermocellum* fermentation and SSF at 10 FPU/g glucan. However, rates of Avicel solubilization are much faster by *C. thermocellum* than SSF. Initial catalyst loading affects final conversion for SSF but not for *C. thermocellum*. The difference between solubilization of Avicel and AFEX CS for *C. thermocellum* cannot be explained by increasing the initial catalyst or inhibition of cell growth as a result of pretreatment. Particle size reduction is more effective than hemicellulose removal on increasing the extent of cellulose solubilization for *C. thermocellum* while they are similar for SSF.

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