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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