Bioresource Technology 101 (2010) 2770–2781

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Mixture optimization of six core glycosyl hydrolases for maximizing saccharification of ammonia fiber expansion (AFEX) pretreated corn stover

Dahai Gao [a,b,*], Shishir P.S. Chundawat [a,b], Chandraraj Krishnan [c], Venkatesh Balan [a,b], Bruce E. Dale [a,b]

[a] Biomass Conversion Research Laboratory (BCRL), Department of Chemical Engineering and Materials Science, Michigan State University, MBI Building, 3900 Collins Road, Lansing, MI 48910, USA
[b] Great Lakes Bioenergy Research Center (GLBRC), 164 Food Safety and Toxicology Building, Michigan State University, East Lansing, MI 48824, USA
[c] Department of Biotechnology, Indian Institute of Technology, Chennai 600 036, India

ARTICLE INFO

Article history:
Received 21 August 2009
Received in revised form 12 October 2009
Accepted 19 October 2009
Available online 30 November 2009

Keywords:
Cellulase
Hemicellulase
Enzymatic hydrolysis
AFEX pretreatment
Cellulosic ethanol

ABSTRACT

In this work, six core glycosyl hydrolases (GH) were isolated and purified from various sources to help rationally optimize an enzyme cocktail to digest ammonia fiber expansion (AFEX) treated corn stover. The four core cellulases were endoglucanase I (EG I, GH family 7B), cellobiohydrolase I (CBH I, GH family 7A), cellobiohydrolase II (CBH II, GH family 6A) and β-glucosidase (βG, GH family 3). The two core hemicellulases were an endo-xylanase (EX, GH family 11) and a β-xylosidase (βX, GH family 3). Enzyme family and purity were confirmed by proteomics. Synergistic interactions among the six core enzymes for varying relative and total protein loading (8.25, 16.5 and 33 mg/g glucan) during hydrolysis of AFEX-treated corn stover was studied using a high-throughput microplate based protocol. The optimal composition (based on% protein mass loading) of the cocktail mixture was CBH I (28.4%): CBH II (18.0%): EG I (31.0%): EX (14.1%): βG (4.7%): βX (3.8%). These results demonstrate a rational strategy for the development of a minimal, synergistic enzymes cocktail that could reduce enzyme usage and maximize the fermentable sugar yields from pretreated lignocellulosics.

1. Introduction

Depleting petroleum reserves and potential climate change further fueled by increasing fossil fuel consumption have attracted significant attention towards the use of alternative renewable resources for production of fuels and chemicals. Lignocellulosic biomass provides a plentiful resource for the sustainable production of biofuels and biochemicals and could serve as an important contributor to the world energy portfolio in the near future (Lynd et al., 1991).

Lignocellulosics are comprised of an intertwined, complex matrix of cellulose, hemicellulose and lignin. Successful biological conversion of lignocellulosic biomass requires an efficient and economical pretreatment method, high glucose/xylose yields during enzymatic hydrolysis and fermentation of both C6–C5 sugars to ethanol (Jørgensen et al., 2007). The prohibitively high cost of enzymes is one of the major factors affecting the implementation of economically feasible lignocellulosic biorefineries (Dale et al., 1996). In order to minimize costs pertaining to enzyme production, identification of major enzymes and optimization of their relative ratios could help reduce enzyme usage without sacrificing hydrolysis yield or significantly lowering the rate of hydrolysis. Lignocellulosic biomasses have diverse compositions depending on their source (e.g. softwood, hardwood, grasses) (Pauly and Keegstra, 2008), while thermochemical treatments also modify the physico-chemical nature of the substrate through varying pretreatment chemistries (Mosier et al., 2005). Most acidic and alkaline pretreatments remove a significant fraction of hemicellulose and/or lignin to enhance enzyme accessibility (e.g. dilute acid, steam explosion, ammonia recycle percolation). While, pretreatments like AFEX do not physically extract any of the hemicellulose and lignin as separate fractions, but still modify the cell wall ultra-structure through mechanisms that are currently not well understood (Chundawat et al., 2009). It is reasonable to assume that varying types of pretreated biomass would require a minimal set of enzymes that would have to be tailor-made (e.g. include more hemicellulases for AFEX vs. dilute acid; Berlin et al., 2007; Rosgaard et al., 2007).

The cost of enzymes for efficient hydrolysis of dilute acid pretreated corn stover has decreased dramatically in recent years (Aden et al., 2002). There have also been substantial advances to optimize enzyme mixtures for acid pretreated biomass (Berlin et al., 2007; Rosgaard et al., 2007). However, there have been

* Corresponding author. Address: Biomass Conversion Research Laboratory (BCRL), Department of Chemical Engineering and Materials Science, Michigan State University, MBI Building, 3900 Collins Road, Lansing, MI 48910, USA. Tel.: +1 517 432 0157; fax: +1 517 337 7904.
E-mail address: gaodahai@msu.edu (D. Gao).

0960-8524/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2009.10.056

few, if any, reports on optimization of enzymes for alkali pretreated biomass. AFEX is one of the leading alkaline pretreatment technologies which significantly enhances enzymatic digestibility without physically stripping out hemicellulose and lignin from the biomass (Mosier et al., 2005; Yang and Wyman, 2008), unlike other pretreatments. An optimal enzyme mixture for hydrolysis of AFEX-treated biomass must be different from acid treated biomass due to presence of residual hemicelluloses, and perhaps other factors such as changes in cellulose crystallinity. Hence, hemicellulases must be included in a minimal cellulase mixture to obtain high yields of both glucose and xylose (Berlin et al., 2007).

The most important cellulases and hemicellulases necessary to digest pretreated biomass (based on protein abundances in typical fungal enzyme extracts) include endoglucanases (EG), endo-xylanases (EX), cellobiohydrolases (CBH I and II), β-glucosidases (βG) and β-xylosidases (βX) (Rosgaard et al., 2007; Sorensen et al., 2007). EG randomly hydrolyzes internal glycosidic bonds within cellulosic microfibrils (Wood and McCrae, 1982), while CBH enzymes act processively along cellulosic chains cleaving off cellobiose units from either end (CBH I acts at reducing ends and CBH II acts at non-reducing ends) (Wood and McCrae, 1986) with βG ultimately hydrolyzing cellodextrins to glucose (Schmid and Wandrey, 1987). EX cleaves the xylan backbone at internal β-1,4 xylosidic bonds, while βX hydrolyzes short xylooligomers to xylose (Shallom and Shoham, 2003). All these enzymes are thought to work harmoniously, creating new accessible adsorption sites or active substrates for each other to act upon (Jørgensen et al., 2007).

In this work, six core cellulases and hemicellulases were isolated using various purification and heterologous expression strategies. Various combinations of these enzymes were tested on AFEX-treated corn stover to determine optimal combinations at three total protein loadings (8.25, 16.5 and 33 mg/g glucan) using a suitable design of experiments methodology. Synergistic interactions among different enzymes were then determined through various mixture optimization experiments. Optimum combinations were predicted from suitable statistical models which were able to further increase hydrolysis yields. These results demonstrate the potential to rationally design enzyme mixtures targeted towards a particular feedstock and pretreatment that can help maximize hydrolysis yields and minimize enzyme usage in cellulosic biorefineries.

2. Methods

2.1. AFEX pretreatment

Detailed procedures outlining the methodology for AFEX pretreatment have been described elsewhere (Balan et al., 2009). Pre-milled (passed through a 10 mm sieve) corn stover (Pioneer Hybrid seed variety (33A14) based stover, provided by NREL, was harvested in 2002 from the Kramer farm in Wray, CO) with 60% moisture (kg water/kg dry biomass), was transferred to a high-pressure Parr reactor. Heated liquid ammonia (1 kg of ammonia/kg of dry biomass) was charged to the reactor vessel resulting in immediate rise in temperature to 130 °C. The reactor was maintained at 130 °C for 15 min through an external heating mantle (within ±10 °C). At the end of 15 min, the pressure was reduced to atmospheric level resulting in precipitous drop in temperature of the reactor contents. The instantaneous pressure drop in the vessel caused the ammonia to vaporize, cooling the biomass to below 30 °C. The pretreated material was left under the hood overnight to ensure complete removal of residual ammonia. The AFEX-treated stover was then milled to under 100 μm based on the methodology employed earlier (Chundawat et al., 2008) and kept under refrigeration until further use. The composition of the milled AFEX corn stover was found to be 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.

2.2. Crude enzyme mixtures

Spezyme CP and Multifect Xylanase were a gift from Genencor (Danisco US Inc., Genencor Division, Rochester, NY), while Novo 188 (Sigma–Aldrich Corp., St. Louis, MO, Novozyme 188®, C6105) was procured from Sigma. The protein concentration was determined colorimetrically using the Pierce (Pierce Biotechnology, Rockford, USA) BCA (bicinchoninic acid) assay kit with bovine serum albumin (BSA) as the standard (Smith et al., 1985).

2.3. Heterologous enzyme expression

Recombinant *Pichia pastoris* strain (FGSC #10077) containing the β-xylosidase gene was obtained from the Fungal Genetics Stock Center (FGSC) at the University of Missouri (Kansas City, MO). The gene encoding β-xylosidase (βX) was isolated from *Aspergillus nidulans* and integrated into the genome of *P. pastoris* X-33 by Bauer et al. (2006). The recombinant strain FGSC #10077 was used to express β-xylosidase (βX). The recombinant strain was maintained on YPD plates containing yeast extract (1% w/v), peptone (2% w/v), dextrose (2% w/v) and agar (2% w/v). Plate cultures were stored at 4 °C for routine use. Culture stocks were stored in 40% glycerol at −80 °C for long term use. A lab scale 1 L BIOSTAT B plus fermentor (Sartorius AG, Goettingen, Germany) was used for fed-batch fermentation. High cell-density was achieved by cultivation of *P. pastoris* in batch phase for 24 h in glycerol medium. Fed-batch fermentation was then performed by adding methanol to induce expression of the recombinant protein. The batch fermentation phase was carried out using a buffered methanol-complex medium (BMMY). The BMMY medium was composed of (in 1 L) 10 g yeast extract, 20 g peptone, 100 ml yeast nitrogen base (20.4 g of yeast nitrogen base without ammonium sulfate and amino acids and 60 g of ammonium sulfate in 600 ml water), 100 ml of 1 M potassium phosphate buffer at pH 6.0, 2 ml of 0.02% (w/v) biotin and 5 ml of methanol. The medium was inoculated with seed culture to an initial OD_{600} of about 1.0. The dissolved oxygen (DO) concentration was maintained over 20% throughout the fermentation with an in-house control algorithm manipulating air/O_2 inflow and agitation speed. Methanol was added to the culture as an inducer for expression of recombinant enzyme based on substrate depletion, identified by spike in DO levels. The fed-batch fermentation was carried out for 120 h at pH 6 and 30 °C.

2.4. Protein purification

Details of enzyme purification steps are shown in Table 1. Enzyme purification was performed using a FPLC system (GE Healthcare, Buckinghamshire, United Kingdom). The following FPLC columns were used: 51 ml HisPrep 26/10 desalting column (GE Healthcare, Lot #17-5087-01), 6 ml Resource Q anion exchange column (GE Healthcare, Lot #17-1179-01), 1.7 ml Mono S cation exchange column (GE Healthcare, Lot #17-5180-01), 1.7 ml Mono Q anion exchange column (GE Healthcare, Lot #17-5179-01) and 1 ml PHE hydrophobic interaction column (GE Healthcare, Lot #17-1186-01). The crude enzyme samples were filtered (using 0.2 μm filter) and buffer exchanged to initial buffer (buffer A) using HisPrep 26/10 desalting column before injecting onto respective columns. CBH I and CBH II isolated from Spezyme CP (after steps 4.3 and 2.3 as shown in Table 1) were further polished using APTC (*p*-aminophenyl-1-thio-β-D-cellobioside) based affinity chromatography to remove minor endoglucanase contaminants (Sangseethong and Penner, 1998). Protein samples were concentrated using a tangential flow-filtration system (10 kDa Vivaflow membrane,