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The addition of accessory enzymes enhances the hydrolytic performance of cellulase enzymes at high solid loadings

Jinguang Hu, Richard Chandra, Valdeir Arantes, Keith Gourlay, J. Susan van Dyk, Jack N. Saddler *

Forest Products Biotechnology and Bioenergy Group, University of British Columbia, Vancouver, BC, Canada

HIGHLIGHTS

- Synergism between cellulases and accessory enzymes improved high solid hydrolysis.
- Required amount of accessory enzyme xylanase and AA9 were highly biomass dependent.
- More xylanase was needed when hydrolysis performed at higher biomass concentration.
- Relatively low amount of AA9 was satisfied for all hydrolysis conditions tested.
- Enzyme dosage was considerably reduced by optimizing enzymes for particular biomass.

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ABSTRACT

The pretreatment process used and the nature of the biomass feedstock will influence the role that accessory enzymes can play in synergistically interacting with cellulases to effectively deconstruct the substrate. The work reported here assessed the possible boosting effects of the xylanase and lytic polysaccharide monooxygenase (AA9, formerly known as GH61) on the hydrolytic potential of cellulase enzyme mixtures during hydrolysis of steam pretreated poplar and corn stover at high (10–20% w/v) substrate concentrations. A higher proportion of xylanase was required when the substrate had a relatively high xylan content and at high substrate concentrations. In contrast, a relatively small amount of AA9 (about 2 mg/g cellulose) was enough, regardless of the nature or concentration of the substrate. The overall protein loading required to achieve effective hydrolysis of high concentrations of pretreated biomass substrates could be substantially reduced by optimizing the ratio of enzymes in the "cellulase" mixture.

1. Introduction

The transition from a traditional "oil-refinery" to a "bio-refinery" based on renewable lignocellulosic biomass is very desirable if we are to move to a more environmentally friendly economy (Ragauskas et al., 2006). The enzyme based biorefinery process typically involves the key process steps of, pretreatment, hydrolysis and fermentation with one of the challenges being to economically produce sugars at the high concentrations that can be achieved by the sugarcane and starch (corn, wheat) based processes (Jorgensen et al., 2007; Zhang et al., 2009). It has been estimated that ethanol must be produced at a concentration of at least 4–5% to economically convert lignocellulosic biomass to ethanol (Jorgensen et al., 2007; Varga et al., 2004; Szijarto et al., 2011). To achieve this final ethanol concentration requires a high initial sugar concentration. A combination of incomplete enzyme hydrolysis, below theoretical ethanol yields and lignin constituting at least 25% of the substrate, implies that a pretreated biomass substrate loading of close to 20% (w/v) will be required. However, unlike starch hydrolysis, which can readily be carried out at a 20–30% substrate concentration (Bayrock and Ingledew, 2001), lignocellulosic hydrolysis at high biomass loadings is much more challenging due to problems such as the inherent recalcitrance of the substrate, inefficient mass transfer (e.g. rheological problems) and the increased levels of enzyme inhibition due to both sugars and various degradation products (Zhang et al., 2009; Cara et al., 2007).

Several strategies have attempted to overcome the challenges associated with the high solid hydrolysis including, the use of customized reactors (Jorgensen et al., 2007; Zhang et al., 2009; Cara et al., 2007), fed-batch approaches to improving biomass liquefaction (Zhang et al., 2009; Rudolf et al., 2005) and the use of simultaneous saccharification and fermentation (SSF) to decrease end products inhibition (Varga et al., 2004; Rudolf et al., 2005). Substrate concentrations as high as 40% dry weight of pretreated wheat straw could be liquefied using a horizontally placed drum

* Corresponding author. Tel.: +1 604 822 9741.
E-mail address: jack.saddler@ubc.ca (J.N. Saddler).

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with a horizontal rotating shaft mounted with paddles (Jorgensen et al., 2007) while a 30% dry weight concentration of pretreated poplar could be hydrolyzed using a PEG mixer that is typically used in pulp bleaching (Zhang et al., 2009). Several studies have shown that a combined fed-batch substrate loading and SSF approach can increase final ethanol concentrations to approximately 50 g/L (Jorgensen et al., 2007; Varga et al., 2004; Rudolf et al., 2005). However, all of this previous work required the addition of high enzyme/protein loadings while at the same time the cellulose hydrolysis yields were often around 60% after 72 h hydrolysis.

In a typical bioconversion process, a pretreatment step is usually required to both recover the hemicellulose and lignin in a useable form and to increase the accessibility of the cellulose to the enzymes. Although a more "severe" pretreatment that uses higher temperatures, pressures or chemical loadings typically results in a more easily digestible cellulose-rich substrate, these severe pretreatment conditions may destroy the more labile hemicellulose component (Chandra et al., 2007; Yang and Wyman, 2008). Thus, for most pretreatments, such as steam pretreatment, mild severity conditions are often used to provide a compromise between increasing the ease of cellulose hydrolysis while recovering more than 70% of hemicellulose (Bura et al., 2003; Ewanick et al., 2007). However, under these milder pretreatment conditions, a higher proportion of the hemicellulose remains associated with the cellulose-rich water insoluble fraction, restricting access to the cellulose component. Recent work has shown that accessory enzymes such as xylanases and lytic polysaccharide monooxygenase (LPMO) Auxiliary Activities Family 9 (AA9, formerly known as GH 61) can greatly enhance cellulose accessibility by removing the xylan "coat" and/or disrupting the crystalline cellulose structure (Hu et al., 2011, 2014; Harris et al., 2010), resulting in significant improvements to the hydrolytic performance of cellulase enzymes. However, this earlier work used low substrate concentrations which are unlikely to be used at a commercial scale.

Earlier work has shown that, at high substrate concentrations, the removal of the hydrophilic xylan by xylanases facilitates liquefaction by either increasing the free water in the hydrolysis system and/or reducing the viscosity/particle size of the pretreated biomass (Viamajala et al., 2009; Di Risio et al., 2011). It is also likely that lytic polysaccharide monooxygenase enzymes such as AA9 might act in a complementary manner by improving cellulose accessibility to the enzymes (Vaaje-Kolstad et al., 2010; Quinlan et al., 2011) and by releasing unproductively bound processive enzymes. Thus a combination of xylanases and AA9 could potentially make a greater contribution when hydrolysis is performed at high substrate concentrations as cellulases would have greater difficulty accessing the cellulose and would likely have an increased tendency to bind non-productively at high solids loadings. In the work reported below the potential beneficial effects of supplementing commercial cellulase mixtures with xylanases and lytic polysaccharide monooxygenase (AA9) during the hydrolysis of high concentrations of steam pretreated poplar (SPP) and steam pretreated corn stover (SPCS) were assessed. We hoped to assess the synergistic interaction between cellulases and these accessory enzymes by partially replacing a portion of the cellulases with purified xylanases and AA9 enzymes, without increasing the overall enzyme (protein) loading. Since the accessory enzymes themselves cannot hydrolyze cellulose on their own (Hu et al., 2013, 2014), the observed increase in hydrolysis of the cellulose, using the same level of protein/enzyme loading, is due to the synergistic cooperation between the cellulases and the added accessory enzymes. It was apparent that the hydrolytic potential of some cellulase mixtures could be improved by optimizing the components of the cocktail when performing hydrolysis at high substrate concentrations.

2. Methods

2.1. Lignocellulosic biomass preparation and composition

The agricultural residue corn stover and the hardwood substrate poplar were selected and the steam pretreatment was conducted in a 2 L StakeTech III steam gun (Stake Technologies, Norvall, ON, Canada) in the Forest Products Biotechnology/Bioenergy Laboratory at the University of British Columbia as described elsewhere (Bura et al., 2009). The pretreatment condition for poplar was selected, based on previous work in our laboratory (Bura et al., 2009), to maximize overall sugar recovery (hemicellulose and cellulose) while providing a cellulosic component that could be readily hydrolyzed with relatively low enzyme loadings. The corn stover sample was pretreated at low severity in order to maintain a relatively high hemicellulose/xylan content in the water insoluble cellulosic fraction. After steam pretreatment, the solid fractions of the pretreated biomass were collected and washed with water and vacuum filtered to a final moisture content above 60%. A small fraction of this sample was kept at 4 °C for chemical composition analysis and daily experiments; and the rest was stored at −20 °C to prevent contamination.

The chemical composition of the water insoluble fraction after steam pretreatment was determined using the modified Klason lignin method derived from the TAPPI standard method T222 om-88, as previously described (Nakagame et al., 2010). The pretreatment conditions and chemical composition of the pretreated substrates are shown in Table 1.

2.2. Enzyme preparations

Commercial cellulase enzyme mixtures Celluclast 1.5 L, CTec 2, and CTec 3 were generous gifts from Novozymes, Franklinton, NC, USA. Novozymes 188 (protein content 233 mg/mL, Novozymes A/S, Bagsvaerd, Denmark) was the β-glucosidase, HTec was the xylanase and *Thermoascus aurantiacus* Auxiliary Activity Family 9 (AA9) was the lytic polysaccharide monooxygenase that were also generously provided by Novozymes. The glycoside hydrolase family 10 xylanase was purified from a commercial HTec xylanase preparation, as described previously (Hu et al., 2013). The term, xylanase enzyme, used throughout the paper refers to the purified xylanase.

2.3. Enzymatic hydrolysis

The hydrolysis experiments were carried out at 2%, 10%, and 20% (w/v) solids loading, respectively, in sodium acetate buffer (50 mM, pH 4.8), 50 °C, 150 rpm in a rotary shaker. Celluclast 1.5 L was used over a range of protein loadings (per gram of cellulose) with enough β-glucosidase supplementation (2 CBU (cellobiose unit) of β-glucosidase per 1 FPU (filter paper unit) of cellulase) to prevent inhibition from cellobiose accumulation. CTec 2 and CTec 3 were used directly (per gram of cellulose) without β-glucosidase supplementation. The accessory enzymes were added using the cellulase replacement strategy as described previously (Hu et al., 2011) which involved replacing varying amounts of the cellulase with an amount (protein) of the indicated accessory enzymes to maintain the total protein loading at the same level.

At the end of hydrolysis (48 h), samples were heated at 100 °C for 10 min to inactivate the enzymes. Supernatants were separated and collected after centrifugation at 16,000*g* for 10 min. The samples were stored at −20 °C for further analyses. All hydrolysis experiments were performed in duplicate and mean values and standard deviations are presented.