Enzyme and Microbial Technology 79–80 (2015) 42–48

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

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

Accessory enzymes influence cellulase hydrolysis of the model substrate and the realistic lignocellulosic biomass

Fubao Fuebiol Sun[a,*], Jiapeng Hong[a], Jinguang Hu[b], Jack N Saddler[b], Xu Fang[c], Zhenyu Zhang[a], Song Shen[a]

[a] Key Laboratory of Carbohydrate Chemistry and Biotechnology & Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China
[b] Forestry Products Biotechnology/Bioenergy Group, Wood Science Department, University of British Columbia, 2424 Main Mall, Vancouver BC, V6T 1Z4, Canada
[c] State Key Laboratory of Microbial Technology, Shandong University, Jinan 250100, China

ARTICLE INFO

Article history:
Received 9 January 2015
Received in revised form 24 June 2015
Accepted 25 June 2015
Available online 17 July 2015

Keywords:
Accessory enzyme (xylanase and AA9)
Cellulase preparations (Cellic® CTec1/2/3)
Filter paper activity (FPA)
Enzymatic hydrolysis
Cellulase mixtures/cocktails

ABSTRACT

The potential of cellulase enzymes in the developing and ongoing "biorefinery" industry has provided a great motivation to develop an efficient cellulase mixture. Recent work has shown how important the role that the so-called accessory enzymes can play in an effective enzymatic hydrolysis. In this study, three newest Novozymes Cellic CTec cellulase preparations (CTec 1/2/3) were compared to hydrolyze steam pretreated lignocellulosic substrates and model substances at an identical FPA loading. These cellulase preparations were found to display significantly different hydrolytic performances irrelevant with the FPA. And this difference was even observed on the filter paper itself when the FPA based assay was revisited. The analysis of specific enzyme activity in cellulase preparations demonstrated that different accessory enzymes were mainly responsible for the discrepancy of enzymatic hydrolysis between diversified substrates and various cellulases. Such the active role of accessory enzymes present in cellulase preparations was finally verified by supplementation with β-glucosidase, xylanase and lytic polysaccharide monooxygenases AA9. This paper provides new insights into the role of accessory enzymes, which can further provide a useful reference for the rational customization of cellulase cocktails in order to realize an efficient conversion of natural lignocellulosic substrates.

1. Introduction

One limiting step in an enzyme based biorefinery process is the fast, complete hydrolysis of the cellulose to monomeric sugars, which can be subsequently converted to a wide range of fuels, chemicals and biomaterials [1]. To improve the hydrolytic efficiency of cellulase enzyme preparations, considerable attempts have traditionally focused on their component cellulases because cellulose is the most abundant polysaccharide constituent in lignocellulose. However, it is now recognized that it is necessary to achieve an efficient enzymatic co-hydrolysis of the cellulose and hemicellulose present in pretreated lignocellulosic substrates to fermentable sugars, which requires a supplementation of the cellulases with some accessory enzymes [2–4].

Recent work has shown how important the role that the so-called accessory enzymes can play in the effective enzymatic hydrolysis [5–8]. These accessory enzyme activities can improve the performance of enzyme preparations even when not directly involved in the hydrolysis of cellulose. For example, β-glucosidase activity is of uttermost importance to avoid accumulation of cellobiose and thus severe inhibition of the cellulases [9–11]. Hemicellulases and pectinases can hydrolyze their respective non-cellulosic polysaccharide substrates that coat cellulose fibers, and thus assist in the hydrolysis of cellulose to boost a release of fermentable sugars from lignocellulosic biomass [4,12]. A xylanase-boosting effect has been observed on a range of pretreated lignocellulosic materials, regardless of their xylan content [7]. Another newly found variety, lytic polysaccharide monooxygenases (LPMOs) Auxiliary Activities Family 9 (AA9, formerly known

Abbreviations: AA9, auxiliary activity family 9; BG, β-glucosidase; CTec1, Cellic® CTec1; CTec 2, Cellic® CTec2; CTec 3, Cellic® CTec3; EX, endoxylanase; FPA, filter paper activity; FPU, filter paper unit; Htec, Cellic® Htec; LPMOs, lytic polysaccharide monooxygenases; SPCS, SO_2 steam pretreated corn stalk; SPP, SO_2 steam pretreated poplar; SPSSB, SO_2 steam pretreated sweet sorghum bagass.

* Corresponding author at: School of Biotechnology, Jiangnan University; 1800 Lihu Road, Wuxi 214122, China.
E-mail addresses: fubaosun@jiangnan.edu.cn, sunfubao@hotmail.com (F.F. Sun).

http://dx.doi.org/10.1016/j.enzmictec.2015.06.020
0141-0229/

as GH 61) can greatly enhance cellulose accessibility by disrupting the crystalline cellulose structure [1,13,14], resulting in significant improvements to the hydrolytic performance of cellulase enzymes. These accessory enzymes can contribute significantly to the effectiveness of cellulase preparations, and improve sugar release to varying degrees depending on the type of substrate and pretreatment [2,15]. Therefore, it is of interest to make more efforts to the cellulase supplementation with accessory enzymes.

Very recently, some researchers have also suggested a non-neglectable effect of accessory enzymes made in measuring the enzyme activity with the model substrate [2,16,17]. And the existence of accessory enzymes resulted in a significant deviation at real hydrolytic efficiency of cellulase preparations from the standardized assay. Kabel et al. [16] showed that the activity analyzed in the standard cellulase/xylanase test did not present a high correlation with the corresponding components degradation of wheat bran and grass. Several commercial cellulase preparations exhibited different degradations on wheat bran/grass cellulose as they had different accessory enzyme activities. Likewise, it was concluded by Pryor and Nahar [17] that the accessory enzyme (i.e., inherent xylanase and pectinase) activities were at least one of the main reasons that standardized protocols for measuring enzyme activity are not adequate for assessing activity using pretreated lignocellulosic substrates. Notably, Berlin et al. evidenced the active role of accessory enzymes in cellulase hydrolysis of natural lignocellulosic substrates, arguing that the enzyme preparations with similar cellulase activity would show differences in performance on lignocellulose if they differ in accessory enzyme composition [2,15]. Considering that the commonly used filter paper activity defines a combination of the endoglucanase, the cellubiohydrolase and at most the β-glucosidase activities, some activities of accessory enzymes/proteins cannot be measured by the standardized FPA assay. It is logic to infer that these accessory enzymes should have exerted an active role in the cellulase activity assay, which is neglected by FPA.

To elucidate these, in this study, three leading Novozymes cellulase preparations, Cellic® CTec1/2/3, were compared in the practical hydrolysis of natural lignocellulosic biomass. To ascertain the uttermost effect of accessory enzymes, the hydrolytic performance of these cellulase preparations was carefully assessed by revisit of the FPA assay protocol. Eventually, the role of accessory enzymes was checked out by supplementation of the preparation with some essential accessory enzymes such as xylanase and lytic polysaccharide monooxygenases (LPMOs).

2. Materials and methods

2.1. Lignocellulosic biomass and cellulase enzymes preparation

Poplar, corn stover and sweet sorghum bagasse were steam pretreated at different conditions (200 °C, 5 min; 200 °C, 5 min; 180 °C, 5 min), respectively, as described by Bura et al. [18]. Prior to steam explosion, the dry substrate chips were impregnated with SO_2 in the plastic bags at room temperature overnight. SO_2 was absorbed, in which the absorption of SO_2 was app. 3% weight of dried substrate. Commercial Cellulase preparations, Cellic® CTec1 (CTec1), Cellic® CTec2 (CTec2) and Cellic® CTec3 (CTec3), and endoxylanase preparation, Cellic® Htec (Htec) were supplied by Novozymes (Franklington, NC). Beta-glucosidase preparation, Novozyme 188, and LPMOs preparation, AA9, were kind gifts from Novozymes A/S (Bagsværd, Denmark).

2.2. Enzymatic hydrolysis

Batch enzymatic hydrolysis experiments were conducted in 10 mL total volume in a 50 mL glass bottle with a rubber plug-in and round aluminum cap sealed at 2% substrate consistency [(g dry substrate) × (g dry substrate + g water)$^{-1}$] in 50 mmol L^{-1} acetate buffer at pH4.8. Bottles and substrates were pre-incubated at 50 °C in a shaker (MAX^{Q} 4000, Barnstead Line-lab) for 20 min before the enzyme addition. For the hydrolysis of different lignocellulosic substrates (SO_2 steam pretreated poplar (SPP) and sweet sorghum bagasse (SPSSB)), the cellulases were added at 14.2 FPU and 20 mg protein per gram cellulose. For the complement of cellulase with some accessory enzyme, different amounts of hemicellulase (1.0, 2.0, 4.0 and 8.0 mg protein g^{-1} cellulose), β-glucosidase (1.0, 2.0, 4.0 and 8.0 mg protein g^{-1} cellulose) and LPMOs (0.2, 0.5, 1.0 and 2.0 mg protein g^{-1} cellulose) were added, respectively. The cellulase hydrolysis was performed at 50 °C at a shaking speed of 150 rotations per minute. At an interval time, 500 μL hydrolytic slurry was withdrawn and deactivated on a hot plate at 100 °C for 10 min, followed by centrifugation at 13,000 × *g* for 10 min, and subsequently the supernatant was stored at −20 °C until sugar analysis was made. A prolonged hydrolysis of filter paper was made completely in terms of the standard protocol of NREL, in which each sampling was taken for a set of test tubes alone for the sugar analysis by 3,5-dinitrosalicylic acid method [19]. For a hydrolysis of SO_2 catalyzed steam pretreated corn stalk (SPCS), it was devised only with the SPCS (50.0 mg dried substrate) instead of filter paper based on the standard protocol, in which each cellulase loading (mg protein/g cellulose) was the same to that on the filter paper.

2.3. Analytical method

The substrates were analyzed for acid insoluble lignin and carbohydrates using the Tappi-T-22 om-88 as previously described [20]. The hydrolysate from this analysis was retained and analyzed for soluble lignin by reading the absorbance at 205 nm. Sugars were measured on Dionex (Sunnyvale, CA) HPLC (ICS-3 000) equipped with an AS 50 auto sampler, ED50 electrochemical detector, GP 50 gradient pump and anion exchange column (Dionex-CarboPac PA1). Acetone soluble extractives were estimated using Tappi T 204 om-88 with the following modifications. Briefly, 10 g of air-dried sample was extracted for 8 h with acetone with 6 cycles/h. The acetone in the round-bottomed flask is then evaporated in the fume hood and then dried in the oven at 100 °C overnight to determine the weight of extractives present in the sample flasks. The filter paper activity was determined according to the NREL laboratory analytical procedure [19]. Xylanase and CMCase activities were determined as described elsewhere [21]. Cellobiohydrolase I, β-xylosidase and β-glucosidase activities were determined using *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-β-D-xylopyranoside, and *p*-nitrophenyl-β-D-glucopyranoside as substrates, respectively, as described previously [21]. Protein concentration was measured using the ninhydrin assay using BSA as the protein standard [22]. In addition to the hydrolysis based on FPA assay using the 3,5-dinitrosalicylic acid to detect reducing sugar contents, other enzymatic hydrolysis (%) was calculated based on the glucose content detected with YSI Biochemistry Analyzer (2700 Select). All the FPA based assays were made for 6 to 8 times. All hydrolysis experiments were performed at least in triplicate, and mean values are presented.