Journal of Biotechnology 246 (2017) 16–23

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Development of minimal enzyme cocktails for hydrolysis of sulfite-pulped lignocellulosic biomass

Piotr Chylenski[a], Zarah Forsberg[a], Jerry Ståhlberg[b], Anikó Várnai[a], Martin Lersch[c], Oskar Bengtsson[c], Solve Sæbø[a], Svein Jarle Horn[a], Vincent G.H. Eijsink[a,*]

[a] Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences (NMBU), Ås, Norway
[b] Department of Chemistry and Biotechnology, Swedish University of Agricultural Sciences, Uppsala, Sweden
[c] Borregaard AS, Sarpsborg, Norway

ARTICLE INFO

Article history:
Received 24 October 2016
Received in revised form 26 January 2017
Accepted 13 February 2017
Available online 17 February 2017

Keywords:
Lignocellulose
Cellulase
LPMO
Trichoderma reesei
Saccharification

ABSTRACT

Despite recent progress, saccharification of lignocellulosic biomass is still a major cost driver in biorefining. In this study, we present the development of minimal enzyme cocktails for hydrolysis of Norway spruce and sugarcane bagasse, which were pretreated using the so-called BALI™ process, which is based on sulfite pulping technology. Minimal enzyme cocktails were composed using several glycoside hydrolases purified from the industrially relevant filamentous fungus *Trichoderma reesei* and a purified commercial β-glucosidase from *Aspergillus niger*. The contribution of in-house expressed lytic polysaccharide monooxygenases (LPMOs) was also tested, since oxidative cleavage of cellulose by such LPMOs is known to be beneficial for conversion efficiency. We show that the optimized cocktails permit efficient saccharification at reasonable enzyme loadings and that the effect of the LPMOs is substrate-dependent. Using a cocktail comprising only four enzymes, glucan conversion for Norway spruce reached >80% at enzyme loadings of 8 mg/g glucan, whereas almost 100% conversion was achieved at 16 mg/g.

1. Introduction

Lignocellulosic biomass is the most abundant source of renewable carbon and offers great potential for development of bio-based fuels and chemicals. Despite great progress in the past decades, enzymatic hydrolysis of recalcitrant lignocellulose is still not sufficiently efficient, and the high cost of enzymes remains a major hindrance for economic viability of the process (Himmel et al., 2007; Klein-Marcuschamer et al., 2012). The main polysaccharide component of lignocellulose is cellulose, a linear polymer of glucose units linked by β-1,4-glycosidic bonds. Degradation of cellulose requires at least three different groups of enzymes: cellobiohydrolases (CBHs), processively cleaving cellobiose units from the reducing (CBHI) or the non-reducing (CBHII) end of the substrate, endo-β-1,4-glucanases (EG), randomly cleaving internal glycosidic bonds, and β-glucosidases, hydrolyzing cellobiose and other soluble cellodextrins to glucose. These enzymes act synergistically to degrade cellulose (Jørgensen et al., 2007). High efficiency on complex co-polymeric biomass requires the action of several other enzymes, in particular various hemicellulose degrading enzymes (Várnai et al., 2011; Hu et al., 2011, 2015). The discovery of the lytic polysaccharide monooxygenases, active on chitin (Vaaje-Kolstad et al., 2010), cellulose (Forsberg et al., 2011; Phillips et al., 2011; Quinlan et al., 2011) and other substrates (Johansen, 2016), has altered the understanding of lignocellulose degradation and opened new perspectives for development of optimized enzyme cocktails for degradation of biomass (Harris et al., 2010; Hu et al., 2014; Müller et al., 2015). Oxidative cleavage of polysaccharides and oligosaccharides by LPMOs requires molecular oxygen and either an enzymatic (*e.g.* cellobiose dehydrogenase, CDH) or a non-enzymatic (*e.g.* ascorbic acid) external electron donor (Kracher et al., 2016; Vaaje-Kolstad et al., 2010). LPMO action results in formation of one native and one oxidised new chain end, with oxidation being either at the C1 or C4 carbon of the scissile glycosidic bond (Horn et al., 2012). LPMOs are classified in the Carbohydrate Active Enzymes database (CAZy) as auxiliary activity (AA) families 9, 10, 11 and 13 (Levasseur et al., 2013).

Since enzyme related costs play a considerable role in a biomass-based economy, major efforts in improving enzyme cocktails have been made (Harris et al., 2014). Besides searching for new enzymes or engineering of existing ones, optimization of enzyme mixtures for specific lignocellulosic substrates is of interest. There is

* Corresponding author at: Faculty of Chemistry, Biotechnology and Food Science Norwegian University of Life Sciences (NMBU) NO-1432 Aas Norway.
E-mail address: vincent.eijsink@nmbu.no (V.G.H. Eijsink).

http://dx.doi.org/10.1016/j.jbiotec.2017.02.009
0168-1656/

increasing recognition of the fact that a "one-size-fits-all" strategy may not be optimal in the design of more efficient biomass processing. Furthermore, different pretreatment strategies, such as steam explosion or chemical pulping, may lead to varying enzyme requirements in subsequent steps. Early studies on enzyme cocktail optimization focused on optimizing enzyme cocktails for commercial model cellulosic substrates such as Sigmacell (Baker et al., 1998) or filter paper (Kim et al., 1998). Subsequently, several studies were published on customizing enzyme mixtures for industrially relevant pretreated lignocellulosic substrates, such as pretreated corn stover (Banerjee et al., 2010a; Banerjee et al., 2010b; Gao et al., 2010; Zhou et al., 2009), wheat and barley straw (Billard et al., 2012; Kallioinen et al., 2014; Rosgaard et al., 2007), sugarcane bagasse (Kallioinen et al., 2014), perennial energy crops (Banerjee et al., 2010b; Garlock et al., 2012), hardwoods (Banerjee et al., 2010b) and softwoods (Gusakov et al., 2007). In most of these studies, the pretreatment technologies used were based on steam pretreatment or ammonia fiber expansion (AFEX) pretreatment. The compositions of designed enzyme mixtures resulting from these studies varied depending on utilized enzymes, type of biomass and type of pretreatment, highlighting the importance of customizing enzyme mixtures for individual processes. Although saccharification of sulfite-pulped woody biomasses has been described previously (Wang et al., 2009; Zhu et al., 2009), it has not been studied to the same extent as other pretreatments and little is known about the composition of optimal enzyme mixtures for such biomass.

The aim of this study was to optimize enzyme mixtures for degradation of two different lignocellulosic biomasses, Norway spruce and sugarcane bagasse, that had been subjected to a proprietary pretreatment technology based on sulfite pulping that has been developed by Borregaard AS (Sarpsborg, Norway) (Sjöde et al., 2013). This process yields water soluble lignin, which can be utilized in various applications, as well as a cellulose fraction that is almost free of hemicellulose. The applied enzyme cocktails were composed of purified cellulases from *Trichoderma reesei* (Cel7A/CBHI, Cel6A/CBHII and Cel7B/EGI), commercially available β-glucosidase from *Aspergillus niger* and an LPMO from *Streptomyces coelicolor* A3(2), *Sc*LPMO10C (also known as CelS2). The performance of the optimized cocktails was benchmarked using the commercial cellulase cocktails Cellic® CTec2 and CTec3.

2. Materials and methods

2.1. Biomass, pretreatment and compositional analysis

Norway spruce (*Picea abies*) and sugarcane bagasse (*Saccharum* spp.) were pretreated at Borregaard AS (Sarpsborg, Norway), as described in Rødsrud et al. (2012) and Sjöde et al. (2013). The pretreatment consisted of acid sulfite cooking with calcium and sodium, respectively, as counter ions, where the majority of the lignin was made water soluble through sulfonation and the majority of the hemicellulose was hydrolyzed into soluble monosaccharides. The soluble fractions were then washed out of the solid fraction that mainly consisted of cellulose. Compositional analysis of pretreated biomasses was carried out following standardized procedures developed by the National Renewable Energy Laboratory (NREL/TP-510-42618) and data are presented in Table 1. Pretreated biomass was oven dried at 40 °C overnight to a water content in the order of 1–3%. To ensure homogenous distribution of dried biomass to reaction tubes, the Norway spruce-derived material was milled using a Cutting Mill SM 2000 (Retsch, Germany) equipped with a 0.5 mm screen; the sugarcane bagasse-derived material was milled using a Planetary Ball Mill PM 100 (Retsch, Germany) and sieved through a 0.85 mm screen.

2.2. Enzymes

Major wild-type *Trichoderma reesei* monocomponent cellulases were purified from culture filtrate of *Trichoderma reesei* QM 9414 (VTT Culture Collection, D-74075, Finland). Enzymes were purified essentially as described in Bhikhabhai et al. (1984) for *Tr*Cel7B, Ståhlberg et al. (1996) for *Tr*Cel7A and Bergfors et al. (1989) for *Tr*Cel6A. Commercially available, purified *Aspergillus niger* β-glucosidase was from Megazyme (product code E-BGLUC, Bray, Ireland). *Streptomyces coelicolor* LPMO10C (formerly known as CelS2) was expressed and purified as described in Forsberg et al. (2014).

The purified LPMO was saturated with Cu (II), essentially as described in Loose et al. (2014) with the exception that samples were incubated with $CuSO_4$ for 30 min on ice, and desalted utilizing PD midiTrap G-25 desalting columns (GE Healthcare, UK), equilibrated with 20 mM BisTris buffer pH 6.0. Proteins were eluted with 1 mL of equilibration buffer, collected and stored at 4 °C until further use.

All protein concentrations were determined with the Bio-Rad Protein Assay (Bio-Rad, USA) based on the Bradford method (Bradford, 1976), using Bovine Serum Albumin (BSA) as a standard.

2.3. Enzymatic hydrolysis reactions

Enzymatic hydrolysis reactions were carried out in 2 mL screw cap micro tubes (Sarstedt, Nümbrecht, Germany), with 5% total solids loading and 8 mg/g glucan total protein loading in 50 mM sodium acetate buffer pH 5.0, with a total reaction volume of 500 μL. To each micro tube three glass beads, 3 mm in diameter, were added (Assistent, Sondheim, Germany). The tubes were incubated at 50 °C, for 48 h in a ThermoMixer (Eppendorf, Hamburg, Germany) at 1000 rpm. Hydrolysis was terminated by placing the tubes in a boiling water bath for 15 min. Subsequently, 1 mL of ultrapure water (Merck Millipore, Billerica, MA, USA) was added to each tube and after subsequent centrifugation for 10 min at 11 000*g*, supernatants were stored at −20 °C for further analysis.

Benchmark enzymatic hydrolysis reactions were carried out utilizing commercially available cellulase preparations Cellic® CTec2 and Cellic® CTec3, kindly provided by Novozymes A/S, Bagsværd, Denmark.

2.4. Minimal cocktail optimization

MODDE Design of Experiments software, version 10.0 (MKS Data Analytics Solutions, Umeå, Sweden) was used for experimental design and data analysis. A quadratic design with five mixture components and an additional quantitative multilevel factor (reducing agent) was used in all experiments. The best subset of experiments from a candidate set was generated with MODDE by D-optimal design. The optimization process was divided into two stages. In the first stage, three expected major mixture components (the three *Trichoderma reesei* cellulases) were varied from 0 to 100%, whereas the other two enzymes were added at lower levels (0–20%). In the second stage the fractions of all five enzymes were set around the optimum obtained from the first round. The reducing agent was set at five and six concentrations (in mM) for the first and second round, respectively (a full list of factors is presented in Table S1). The center points, located in the middle of the range for each factor, were run in triplicate to control reproducibility of the experiments. Altogether, the first stage consisted of 30 and second stage of 36 individual experiments. Enzymatic hydrolysis data was analyzed, and models were fitted and evaluated using the multiple linear regression (MLR) function of MODDE.