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Pure enzyme cocktails tailored for the saccharification of sugarcane bagasse pretreated by using different methods

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ABSTRACT

The compositions and physical properties of pretreated lignocellulose vary depending on pretreatment methods; therefore, enzyme cocktails specific to pretreatments are desired for efficient saccharification of lignocellulose. Here, enzyme cocktails consisting of three pure lignocellulolytic enzymes endoglucanase (EG), cellobiohydrolase (CBH) and endoxylanase (XN) with a fixed amount of β-glucosidase were tailored for acid- and alkali-pretreated sugarcane bagasse (ACID and ALKALI, respectively). Based on a mixture design, the optimal mass ratios of EG, CBH, and XN were determined to be 61.25:38.73:0.02 and 53.99:34.60:11.41 for ACID and ALKALI, respectively. The optimized enzyme cocktail yielded a higher or comparable amount of reducing sugars from the hydrolysis of ACID and ALKALI when compared to that obtained using commercial cellulase mixtures. Using the commercial and easily available pure enzymes, this simple method for the in-house preparation of an enzyme cocktail specific to pretreated lignocellulose consisting of only four enzymes with a high level of hydrolysis will be helpful for achieving enzymatic saccharification in the lignocellulose-based biorefinery.

1. Introduction

As the most abundant carbohydrate source on earth, lignocellulose is considered as the most promising biomass that could dominate future biorefineries. In a lignocellulose-derived biorefinery, enzymatic saccharification is a prerequisite for the production of sugar-based fuels and value-added chemicals [1]. Cellulose, hemicellulose, and lignin are the three main constituents of lignocellulose, in which cellulose is closely associated with hemicellulose and lignin, and cellulose itself exists in crystalline and less-crystalline (amorphous) structures. Owing to their heterogeneous features, the concerted activities of different enzymes specific to different substrates are involved in the breakdown of lignocellulose in nature [2].

The major enzymes involved in the hydrolysis of lignocellulose include endoglucanase (EG), cellobiohydrolase (CBH), and β-glucosidase (BG) which act on cellulose, and endoxylanase (XN) which acts on xylan [3,4]. In cellulose hydrolysis, EGs randomly attack the internal bonds of cellulose polymer chains in amorphous regions, producing oligosaccharides with varying degrees of polymerization. CBHs, also referred to as exocellulases, cleave the cellulose chains either from the reducing end (CBH 1) or non-reducing end (CBH 2) in a progressive manner to yield cellobiose as the major product. Then, cellobiose is finally converted by BGs into glucose [2,3]. For the hydrolysis of xylan, which is the main component of hemicellulose, XNs are involved in the cleavage of the β-1,4-glycosidic linkages of the xylan backbone to generate various xylooligosaccharides [4].

In the production of bio-based products and fuels, lignocellulosic biomass is subjected to chemical pretreatment prior to enzymatic saccharification. Pretreatment of lignocelluloses enhances the substrate accessibility to enzymes by disrupting the recalcitrant structure of lignocellulose [5,6]. During the pretreatment process, reduction in the cellulose crystallinity could occur, and cellulose-surrounding physical barriers such as hemicellulose and lignin are removed depending on the pretreatment method [7]. Acidic and alkaline pretreatments are the two representative methods of chemical pretreatment. The composition and physical properties of biomass vary substantially depending on the type and process conditions of the pretreatment applied [8,9]. Specifically, during an acidic pretreatment using dilute sulfuric acid, a substantial amount of hemicellulose, mainly xylan, is solubilized and removed from lignocellulose [7,10]. In contrast, alkaline pretreatment methods such as with diluted ammonia, ammonia fiber expansion, and soaking in aqueous ammonia result in the removal of much of the lignin [11,12]. Thus, depending on the pretreatment, different combinations of enzymes in terms of type, amount, and ratio are required, and customization of the cellulase mixture (or cocktail) specific to the pretreatment is essential for maximization of the sugar yield from the

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pretreated biomass [9,13].

In general, commercial cellulase mixtures such as Celluclast 1.5 L and Cellic CTec2, both from Novozymes, and Accellerase 1000 or 1500 from DuPont, are widely utilized for the hydrolysis of pretreated lignocellulose. As crude extracts derived from the powerful cellulolytic fungal strains of *Trichoderma reesei*, these commercial cellulase preparations provide broad and superior enzymatic activities. However, as the purification and functional identification of all enzymes from the crude extracts is not an easy task, only limited information is currently available on the composition of these commercial cellulase mixtures. For example, Celluclast 1.5 L contains a substantial amount of components identified as miscellaneous (10%) and of unknown function (15%) when analyzed by liquid chromatography-tandem mass spectrometry in addition to the functionally defined enzymes [14]. This makes it difficult to rationally design the cellulase mixtures that are optimized for a pretreated lignocellulosic biomass using commercial cellulase mixtures. Therefore, development of an enzyme cocktail consisting of a defined composition using known enzymes whose activity is comparable to or higher than commercial cellulases is required.

In this study, we established in-house preparations of enzyme cocktails using three pure enzymes that were purchased from commercial sources: an endoglucanase, E-CELTR™; a cellobiohydrolase, E-CBHI™ from *Trichoderma longibrachiatum*; an endoxylanase, E-XYTR1™ from *Trichoderma viride*; and the addition of a β-glucosidase of Novozyme 188 ™ from *Aspergillus niger*, for the hydrolysis of two sugarcane bagasses that were subjected to two different pretreatment methods: acid and alkali pretreatment. As the rational statistical approach, the mixture design was employed for the pretreatment-specific customization of the cellulase cocktails. Furthermore, the hydrolytic performance of the customized enzyme cocktails was compared with those of commercial cellulase cocktails. Despite the abundant reports on the optimization of lignocellulose- or chitin-degrading enzymes [9,13–16], this is the first study to optimize enzyme formulations using commercially available pure enzymes. Using these commercial and easily accessible pure enzymes, our study provides a simple method for the in-house preparation of cellulase cocktails with defined compositions composed of a minimal number of enzymes. This is expected to be helpful for the future development of pretreatment-specific enzyme cocktails.

2. Materials and methods

2.1. Substrates and enzymes

Substrates such as carboxymethyl cellulose (CMC), Avicel PH-101, and beechwood xylan that were used for analyzing the specific activities of enzymes were purchased from Sigma-Aldrich (St. Louis, MO, USA). For the pretreatment-specific customization, purified enzymes, E-CELTR, E-CBHI, and E-XYTR1, were purchased from Megazyme (Bray, Wicklow, Ireland). The β-glucosidase Novozyme 188 was purchased from Sigma-Aldrich. Celluclast 1.5 L and Cellic CTec2 were purchased from Novozymes (Bagsværd, Denmark). In this study, for 1 filter paper unit (FPU) of cellulase activity at pH 6.0 and 50 °C, 0.488 mg and 1.850 mg of Celluclast 1.5 L and Cellic CTec2 were needed, respectively.

2.2. Pretreatment of sugarcane bagasse

Sugarcane bagasse from Thailand was kindly donated by GS Caltex (Daejeon, Korea). After washing and air-drying at room temperature for 72 h, the sugarcane bagasse was milled by passing through a 0.5-μm screen using a high-speed rotary cutting mill (MF 10; IKA, Staufen, Germany). For the preparation of acid-pretreated biomass (ACID), the sugarcane bagasse was suspended in 1% (w/v) sulfuric acid at a solid-to-liquid (S/L) ratio of 1:10, and then the slurry was digested at 190 °C for 3 min holding time with 3 min ramping time using Ethos EZ Microwave Digestion Labstation (Milestone, Shelton, CT, USA) [7]. For the preparation of alkali-pretreated biomass (ALKALI), the sugarcane bagasse was soaked in a 21% (w/w) aqueous ammonia solution at an S/L ratio of 1:6, and incubated at 70 °C for 10 h [12]. The two pretreated sugarcane bagasse samples were filtered to separate solids from liquids through a filtration cloth (pore size: 22–25 μm; Calbiochem, La Jolla, CA), followed by thoroughly washing with distilled water until reaching a pH of 6–7, and the solid fraction was dried at 45 °C. Compositional analysis was performed according to the Laboratory Analytical Procedure (LAP) of the National Renewable Energy Laboratory (NREL; Golden, CO, USA) [17–19]. The carbohydrate contents of ACID and ALKALI, including the contents of cellulose and hemicellulose (xylan, galactan, mannan, and arabinan) were evaluated as follows. After the two-stage sulfuric acid digestion of carbohydrates in ACID and ALKALI, which was performed to completely monomerize the cellulose and hemicellulose, their monomeric sugars (glucose, xylose, galactose, mannose, and arabinose) were measured by high performance liquid chromatography (HPLC; Agilent 1100, Agilent Technologies, Waldbronn, Germany) equipped with an SP0810 column (Shodex, Showa Denko, Kawasaki, Japan) set at 80 °C and a refractive index detector (RID; G1362A, Agilent Technologies). Distilled water was used as the mobile phase at a flow rate of 0.5 mL/min.

2.3. Enzymatic saccharification of pretreated sugarcane bagasse

The specific activities of EG, CBH, XN, and BG were analyzed to examine their CMCase, Avicelase, xylanase, and β-glucosidase activities. A reaction mixture containing each substrate, CMC (1%, w/v), Avicel (5%, w/v), beechwood xylan (1%, w/v), or cellobiose (1%, w/v), was incubated with EG, CBH, XN, or BG. For the soluble substrates, namely, CMC, xylan, and cellobiose, the enzymatic reaction time was 20 min for each enzyme. However, for the insoluble substrate, Avicel, the enzymatic reaction time was 12 h since the hydrolysis of Avicel is much slower than those of the soluble substrates. The enzymatic reaction was performed in 50 mM sodium citrate buffer (pH 6.0) at 50 °C with a total volume of 500 μL. The amount of sugars released from the substrates was determined by the dinitrosalicylic acid-based measurement of reducing sugar using D-cellobiose (for CMC and Avicel), D-glucose (for cellobiose), and D-xylose (for xylan) as the standards. The specific activity of an enzyme is presented in units of U/mg of enzyme, where one unit (U) is defined as the amount of enzyme that releases 1 μmol of reducing sugar per minute.

To obtain the optimal pH conditions, two buffers, 0.1 M sodium citrate for pH 3.0–6.0 and 0.1 M Tris-Cl for pH 7.0–8.0, were used at 50 °C. To determine the optimal temperature condition, the reactions were performed at various temperatures ranging from 20 °C to 80 °C at pH 6.0. For the enzymatic hydrolysis of pretreated sugarcane bagasse, 2% (w/v) ACID or ALKALI was incubated in 0.1 M sodium citrate buffer (pH 6.0) containing 50, 100, and 200 mg of EG or CBH per gram of glucan at 50 °C. After the mixture design-based customization, 2% (w/v) ACID or ALKALI was incubated with the enzyme mixture containing EG, CBH, and XN for a total amount of 100 mg/g glucan with BG at 10 mg/g glucan in 0.1 M sodium citrate buffer (pH 6.0) at 50 °C. For the comparison with the customized enzyme mixtures, two commercial enzymes were used: Celluclast 1.5 L at 5 FPU/g glucan supplemented with 10 mg BG/g glucan, and Cellic CTec2 at 5 FPU/g glucan with total protein loadings of 2.44 and 9.25 mg/g glucan, respectively. All experiments were performed in triplicate.

2.4. Mixture design for the optimal formulation of enzymes

To obtain the optimal compositions of EG, CBH, and XN for the hydrolysis of two types of pretreated sugarcane bagasse, a [3,3]-augmented simplex-centroid model was applied with Minitab 17 software (Minitab, State College, PA, USA). In the simplex-centroid model-based mixture design, 10 experimental composites were designed, and