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Effect of hemicellulolytic enzymes to improve sugarcane bagasse saccharification and xylooligosaccharides production

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ABSTRACT

Enzymatic hydrolysis of lignocellulosic biomass is limited by economic considerations arising from enzyme production costs and specific activities. The effect of six hemicellulases on raw sugarcane bagasse and two types of pretreated sugarcane bagasse was investigated using experimental designs. Our strategy was successful for developing more efficient and less expensive enzymatic mixture, and also revealed that hemicellulase mixtures with multiple activities could be less effective than expected. In this study, only two hemicelulases, the endo-1,4-xylanases (GH11) from *Penicillium funiculosum* (XynC11/CAC15487) and the feruloyl esterase (CE1) from *Clostridium thermocellum* (CtFAE/ATCC27405), effectively broke-down hemicellulose from pretreated sugarcane bagasse (up to 65%), along with the production of xylooligosaccharides (XOS). Our results also demonstrated that GH11 and CE1 can improve biomass saccharification by cellulases. Treatment with these two enzymes followed by a commercial cellulase cocktail (Accellerase®1500) increased saccharification of pretreated lignocellulose by 24%. Collectively, our data contributes to the rational design of more efficient and less expensive enzyme mixtures, targeting the viable production of bioethanol and other biorefinery products.

1. Introduction

Enzymatic hydrolysis of pretreated lignocellulosic biomass is an ideal alternative for bioethanol production [1]. The pursuit of enzymatic cocktails with improved performance has prompted years of bio-prospecting, optimization of strains through genetic engineering and development of pretreatment strategies to mitigate inhibitory effects. The recent shift to comprehensive studies of purified proteins for developing more efficient and less expensive enzymatic mixture, reflects the evolution of this field of study [2].

Plant lignocellulosic biomass, a sustainable feedstock with minimal effect on agricultural food production, is mainly comprised of polymeric sugars (cellulose, hemicellulose and pectin) and polyphenolics (lignin) found within the plant cell wall [3]. While cellulose consists of linear chains of hundreds or thousands of glucose molecules, hemicellulose is a branched polymer consisting of a mixture of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose) and sugar acids. The composition of hemicellulose can vary significantly among plant species and even within the separate components of a single plant (leaves, stem and roots) [2].

Hemicellulose is typically found in large quantities in the plant cell wall (20–35% of natural lignocellulosic biomass) and the efficiency of its extraction and conversion is an important factor and determinant for economic viability of a biomass processing facility [2]. A diverse combination of enzymes is required for complete hemicellulose hydrolysis: endo and exoxylanases initiate the break down of cross-linked hemicelluloses generating xylooligosaccharides (XOS) of varying lengths, β-xylosidases are able to hydrolyze XOS into xylose; α-arabinofuranosidase hydrolyses arabinose units into both furanose and pyranose forms; α-glucoronisidase hydrolyses methyl glucuronic acid substitutes; while acetylxylan esterase and ferulic acid esterase hydrolyze acetyl groups substitutes and ferulic acid, respectively [4].

The economically viable production of biofuels via the enzymatic hydrolysis process is dependent on reduced production costs and enzymatic mixtures with increased specific activities (i.e. increased sugar production at reduced enzyme loadings). While the discovery and production of new enzymes through bioprospection, genetic engineering and strain improvements have

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been partially accomplished, these studies have led to the identification of thousands of cellulases, hemicellulases, ligninases and pectinases whose function and relative activities are largely unknown. According to Banerjee et al. [5], the development of a standardized approach to efficiently evaluate the relative performance of individual proteins and enzymatic mixtures was deemed crucial to maximize their specific activities for commercial applications. Better understanding of which enzymes, and their proportions, is essential for lignocellulosic degradation, and could lead to rational design of more efficient, and consequently less expensive enzyme mixtures [6].

The effect of hemicellulases on different pretreated sugarcane bagasse was investigated in the present study using experimental designs (Plackett & Burman) to analyze the interaction of different enzymes for degradation of three biomass types, as well as identify the best condition to promote hemicellulose conversion. Three sugarcane bagasse samples were tested: raw sugarcane bagasse (RAW), sugarcane bagasse pretreated with phosphoric acid (PASB) and sugarcane bagasse pretreated with acetic acid and hydrogen peroxide (PACH). Six recombinant hemicellulolytic enzymes were evaluated: two endo-1,4-xylanases (GH10 and GH11), two α-L-arabinofuranosidases (GH51 and GH54), one β-xylosidase (GH43) and one feruloyl esterase (CE1). Enzymes which had significant effects were selected for a central compound rotational design (CCRD) seeking to define the optimal protein concentration. Collectively, our experimental designs succeeded in producing more efficient and less expensive hemicellulolytic enzyme mixtures for sugarcane bagasse hydrolysis.

2. Material and methods

2.1. Hemicellulolytic enzymes

Six recombinant hemicellulolytic enzymes were studied: two endo-1,4-xylanases (GH10 and GH11), two α-L-arabinofuranosidases (GH51 and GH54), one β-xylosidase (GH43) and one feruloyl esterase (CE1). The GH11 endo-1,4-xylanase (XynC11/CAC15487) from *Penicillium funiculosum* and the GH54 α-L-arabinofuranosidases (AbfB54/AAB53944) from *Aspergillus niger* were expressed heterologously and produced in *Aspergillus nidulans* [7]. The GH10 endo-1,4-xylanases (SCXyl10/KC904514) from the sugarcane soil metagenome [8], GH51 α-L-arabinofuranosidases (BsAbf51/BSU28720) from *Bacillus subtilis*, and GH43 β-xylosidase (BsXyn43/BSU17580) from *Bacillus subtilis* were expressed heterologously and produced in *Escherichia coli* [9]. The CE1 feruloyl esterase (CtFAE/ATCC27405) was cloned from the cellulosome xylanase Z feruloyl esterase sequence (XynZ) from *Clostridium thermocellum* that encodes both xylanase and feruloyl esterase domains [10,11]. The specific oligonucleotides forward FAE1 (5′-ACCATGCCGCCTTCGGGATA-3′) and reverse FAE2 (5′-TCATGTGTTAGCCGGCTTTGGA-3′) were synthesized to amplify the region containing the feruloyl esterase domains. All enzymes and clones were stored at −80 °C in the enzyme bank at the Brazilian Bioethanol Science and Technology Laboratory – CTBE/CNPEM, Campinas, Brazil.

2.2. Expression and purification of enzymes

E. coli BL21 (DE3) (Promega, Madison, WI, USA) was transformed with pET28a vector (Novagen, Madison, WI, USA) for heterologous expression of the four enzymes: GH10 (SCXyl10), GH51 (BsAbf51), GH43 (BsXyn43) and CE1 (CtFAE). The enzyme production and purification processes were performed as described previously [11].

The pEXPYR shuttle vector [12] was used for the heterologous expression of GH11 (XynC11) and GH54 (AbfB54) in *A. nidulans* A773 (pyrG89, wA3, pyroA4), purchased from Fungal Genetic Stock Center (FGSC, University of Missouri, Kansas City, MO, USA). Cloning of the selected genes into pEXPYR was confirmed by sequencing, and the plasmid was transformed into *A. nidulans* A773 as previously described [13]. Transformant selection, culture conditions, and enzyme purification methods were selected as described by Gonçalves et al. [7]. Pure fractions of GH11 and GH54 were assayed for enzymatic activity as previously described [14] and analyzed by SDS–PAGE.

2.3. Determination of protein concentration and SDS-PAGE

The protein concentration was determined according to the methodology proposed by Bradford [15] using bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) as the standard. Spectrophometric readings were taken at 595 nm. Electrophoresis (SDS-PAGE) was used to separate the proteins and estimate their molecular weight. This process was performed in 12% (w/v) polyacrylamide gel according the protocol proposed by Laemmli [16]. Gel was stained by coomassie blue. Samples were denatured in sample buffer at 99 °C for 5 min. A mixture of high molecular weight proteins (PageRuler™, Unstained Protein Ladder, Thermo Scientific, Waltham, MA, USA) was used as the molecular weight standard.

2.4. Enzymatic activity assay

The substrates xylan beechwood and arabinan from sugar beet were used to measure xylanases (GH11 and GH10) and GH54 activities, respectively. The enzymatic reaction mixtures consisted of 50 μL of the substrate (0.5% w/v), 40 μL of citrate phosphate buffer (0.1 M) at pH 5.0, and 10 μL of the purified enzyme incubated at 50 °C in a Thermostat® (Eppendorf, Hamburg, Germany) for 10 min. The reaction was stopped by adding 100 μL of 3,5-dinitrosalicylic acid and was immediately boiled for 5 min at 99 °C and cooled [17]. The solution was analyzed at 540 nm, in an Infinite M200® spectrophotometer (Tecan-Switzerland) to measure the release of reducing sugars. One enzyme unit was defined as the quantity of enzyme that released reducing sugar at a rate of 1 μmol/min. To measure the GH51 and GH43 activities, 2 mM of the substrates 4-nitrophenyl-α-L-arabinofuranoside (pNP-ara) and *p*-nitrophenyl-β-xylobioside (pNP-xyl) were used, respectively, according to methods previously described [18]. One enzyme unit was defined as the quantity of enzyme that released *p*-nitrophenyl at a rate of 1 μmol/min. The feruloyl esterase activity was performed using the substrate α–naphthylacetate according to the method previously described by Koseki et al. [18]. One enzyme unit was defined as the amount of enzyme that released 1 μmol α–naphthol per minute under the assay conditions.

2.5. Sugarcane bagasse and compositional analysis

Three different substrates were used in the analyses: raw sugarcane bagasse (RAW), kindly provided by the Usina Vale do Rosário (São Paulo, Brazil); sugarcane bagasse pretreated with phosphoric acid P.A., solid-liquid ratio of 1:5 m/v at 180 °C for 5 min [19] (named PASB); and sugarcane bagasse pretreated with a 1:1 mixture of 8.74 M glacial acetic acid and 21.6 M hydrogen peroxide, solid–liquid ratio of 1:20 m/v at 60 °C for 7 h (named PACH). Both pretreated sugarcane bagasse samples (PASB and PACH) were washed continuously with distilled water until removing the excess acid and reaching a neutral pH followed by drying at 60 °C for 24 h [20].