



Development of tailor-made synergistic cellulolytic enzyme system for saccharification of steam exploded sugarcane bagasse

Benjarat Bunterngrsook,¹ Thanaporn Laothanachareon,¹ Chayanon Chotirotsukon,¹ Hiroyuki Inoue,²
 Tatsuya Fujii,² Tamotsu Hoshino,² Niran Roongsawang,¹ Sanchai Kuboon,³ Wasawat Kraithong,³
 Wikanda Techanan,⁴ Natthakorn Kraikul,⁴ and Verawat Champreda^{1,*}

Enzyme Technology Laboratory, Microbial Biotechnology and Biochemicals Research Unit, National Center for Genetic Engineering and Biotechnology, 113 Thailand Science Park, Khlong Luang, Pathumthani 12120, Thailand,¹ Research Institute for Sustainable Chemistry, National Institute of Advanced Industrial Science and Technology, 3-11-32 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-0046, Japan,² National Center for Nanotechnology, Innovation Cluster 2 Building, Thailand Science Park, Khlong Luang, Pathumthani 12120, Thailand,³ and PTT Global Chemicals PCL, Energy Complex Tower A, Chatuchak, Bangkok 10900, Thailand⁴

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Designing a tailor-made synergistic system is a promising strategy for developing an effective enzyme for saccharification of lignocellulosic materials. In this study, a cellulolytic enzyme mixture comprising selected core recombinant enzymes for hydrolysis of sugarcane bagasse pretreated by alkaline-catalyzed steam explosion was optimized using a mixture design approach. The optimized enzyme system comprised a cellobiohydrolase (Cel7A) from *Talaromyces cellulolyticus*, an endo-glucanase (Cel7B) from *Thielavia terrestris*, a β -glucosidase (BGL) and an endo- β 1,4-xylanase (XYN) from *Aspergillus aculeatus* at the ratio of 0.34:0.27:0.14:0.25. The maximum reducing sugar yield of 797 mg/g biomass, comprising 543 and 96.8 mg/g glucose and xylose, respectively were achieved, equivalent to 92.44% and 47.50% recoveries, respectively from the pretreated substrate at the enzyme dosage of 20 mg/g biomass. The sugar yield from the quaternary enzyme mixture was 17.37% higher than that obtained with Accellerase 1500.

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Lignocellulosic agricultural biomass is a renewable carbon source for production of commodity biofuels and chemicals as well as a wide spectrum of specialty bio-products in biorefinery industry with the key advantage on its carbon-neutral nature (1). Lignocellulose, the major structural component in plant cell wall, consists of cellulose, a linear homopolymer of β -1,4-linked glucose organized into a highly ordered fibrous structure with high crystallinity which is embedded in the network of the amorphous hemicellulose, a branched heteropolymer of pentoses, hexoses, and sugar acids. The polysaccharides are shielded by lignin, a three-dimensional heteropolymer of phenolic alcohols, making it highly recalcitrant to external physical, chemical, and biological attacks (2,3). An efficient pretreatment step is thus required in order to increase enzymatic digestibility of lignocellulosic materials by different means, e.g., solubilization of hindered hemicellulose, destruction of the lignin shield, and reducing crystallinity of cellulose, which in overall result in higher enzyme's accessibility and efficiency towards hydrolysis of the cellulose fraction (4).

Complete saccharification of lignocellulosic biomass requires a repertoire of core and accessory hydrolytic enzymes in different glycosyl hydrolase families which act in a synergistic manner (5). Core cellulolytic enzymes include three modes of actions: endo-glucanase, exoglucanase or cellobiohydrolase and β -glucosidase

(3,6,7). Endoglucanases randomly hydrolyze intramolecular β -1,4-glucosidic bonds of cellulose while cellobiohydrolases cleave the cellulose polymer from either the reducing and non-reducing ends depending on their types, producing cellobiose as the products (7). The released di- or oligo-saccharides are then hydrolyzed to glucose by β -glucosidases. The cellulases play synergistic and cooperative roles with a variety of hemicellulases attacking the heterogeneous hemicellulose structure. Among them, endo- β -1,4-xylanases and β -xylosidases are the major hemicellulolytic enzymes responsible for hydrolysis of xylan that is the main component of hemicellulose in herbaceous biomass. Synergism of these enzymes to accessory hydrolytic and non-hydrolytic enzymes, e.g., esterases, lyases, and oxidases, attacking the hemicellulose and lignin-carbohydrate complex as well as various auxiliary enzymes and proteins (8) further enhances hydrolysis efficiency on lignocelluloses.

Due to variations in physical and chemical natures of different agricultural residues pretreated by different methods (9,10), dissecting and identifying enzyme activities with major and minor roles and their cooperativity on hydrolysis of lignocelluloses provides a way toward customization of a more active enzyme system with maximal efficiency against the target substrate with specified characteristics (11). Most studies have dealt with combinations of crude commercial cellulases, particularly those from *Trichoderma reesei*, which is naturally equipped with active complex lignocellulose degrading enzyme system synergized with additional crude

* Corresponding author. Tel.: +66 2564 6700 (x 3446); fax: +66 2564 6707.
 E-mail address: verawat@biotec.or.th (V. Champreda).

enzyme preparations (12,13) or isolated enzymes, e.g., xylanases, feruloyl esterase and auxiliary components from various microbial origins (8,14,15). These synergistic enzyme mixtures based on “crude” cellulolytic enzyme preparations as the core component have been customized on hydrolysis of various agricultural wastes, e.g., rice straw, barley straw, alfalfa hay and sugarcane bagasse (16–22). So far, a limited number of studies have focused on developing synergistic enzyme mixtures based on key isolated enzyme components solely, either using purified or recombinant enzymes with varying efficiency on hydrolysis of some specific cellulosic substrates, including corn stover (14,23) and wheat straw (24), but not on sugarcane bagasse. This strategy is considered advantageous compared to the crude enzyme-based mixtures on potentially lower enzyme dosage (23) and allow possibility for production of the enzymes in well-established heterologous host, e.g., yeasts for consolidate bioprocessing (25,26).

In this study, a synergistic multi-species enzyme system optimized for hydrolysis of hydrothermally pretreated sugarcane bagasse has been customized from recombinant enzymes derived from potential cellulolytic fungi previously reported as promising candidates for biomass saccharification. *Aspergillus aculeatus* has been previously shown as a source for complex cell wall degrading enzymes with potentials on hydrolysis of non-starch polysaccharides in cassava substrates (27–29). *Talaromyces cellulolyticus* was shown as a potent producer of a highly efficient cellulase for biomass hydrolysis (23) while *Thielavia terrestris* was reported for production of thermostable complex cellulase system (30). The enzyme components used in this study were chosen based on a pre-screening experiment for glycosyl hydrolase components with the highest respective activities from our enzyme collection. The work demonstrates the first tailor-made isolated enzyme system with high performance for saccharification of sugarcane bagasse, one of the world's most abundant agricultural wastes for bio-refinery. The work paves the way towards the development of an efficient customized enzyme system with higher performance compared to widely used commercial cellulases.

MATERIALS AND METHODS

Biomass preparation Sugarcane bagasse was collected from the central area of Thailand. The biomass was physically processed using a SM2000 cutting mill (Retsch, Haan, Germany) and sieved through a 0.85 mm mesh. The bagasse was pretreated by alkaline-catalyzed steam explosion (kindly supplied from PTT Global Chemicals, PCL, Thailand). The bagasse was pre-impregnated in NaOH solution before pretreatment by steam explosion in the temperature range of 160–210°C under the maximum pressure of 20 bars for less than 10 min. The solid fraction was separated by filtration on filter paper using a Büchner funnel and washed with distilled water and dried at 60°C before subjecting to enzymatic hydrolysis. The pretreated bagasse contained 58.82% cellulose, 20.39% hemicellulose, 5.81% lignin and 14.97% ash as determined by the standard NREL analysis method (31). The biomass has a crystallinity index of 40.19% and the crystalline size of 1.14 nm according to X-ray diffraction analysis (32).

Strains, enzyme, and plasmid The recombinant GH7 cellobiohydrolase I (Cel7A) from *T. cellulolyticus* was produced using the homologous expression system and purified as described previously (23,33). The recombinant GH10 endo-β-1,4-xylanase from *A. aculeatus* BCC17849 was prepared by expression in *Pichia pastoris* according to Laothanachareon et al. (20). Expression vector pPICZαa (Invitrogen, Carlsbad, CA, USA) was used for expression of the GH7 endoglucanase from *T. terrestris* and GH1 β-glucosidase from *A. aculeatus*. *Escherichia coli* DH5α was used as the host strain for DNA cloning. *P. pastoris* (Invitrogen) was used for recombinant protein expression. The commercial *T. reesei* cellulase Accellerase 1500 was purchased from Dupont (Rochester, NY, USA).

Recombinant plasmid construction The mature genes encoding a GH7 endoglucanase from *T. terrestris* (Genbank accession no. G2R8K6) and a GH1 β-glucosidase from *A. aculeatus* (GenBank accession no. P48825) were synthesized with codon optimization for expression in *P. pastoris* (Genscript, Piscataway NJ, US). The gene fragments were digested with *EcoRI* and *XbaI*, and cloned into pPICZαa expression vector digested with the same restriction enzymes. The recombinant plasmids were transformed into *E. coli* DH5α using heat shock method and selected on LB agar containing 25 µg/ml of Zeocin. The gene was verified by colony PCR for

subsequent by DNA sequencing (Macrogen, Seoul, South Korea). The recombinant plasmids pPICZα-Cel7B and pPICZα-BGL were linearized by *PmeI* and transformed into *P. pastoris* KM71 by electroporation at 1.5 kV/cm, 200 Ω and 25 µF. The yeast transformants were selected on YPD agar containing 100 µg/mL of Zeocin and then Zeocin-resistant clones were screened for gene-integrated clones by colony PCR screening.

Enzyme expression For protein production, the recombinant strains containing plasmids pPICZα-Cel7B or pPICZα-BGL were cultured in 200 mL BMGY [2% peptone, 1% yeast extract, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogenous base (w/o amino acid), 0.4 µg/mL biotin, and 1% glycerol] until the OD₆₀₀ of the culture reached 8–10, and then the cells were pelleted by centrifugation. The cell pellets were resuspended in 20 mL BMMY (BMMY containing 3% methanol instead of glycerol) for induction of the target gene. The cells were then cultivated under the same conditions for 3 d, and then the supernatant was collected by centrifugation at 5000×g for 5 min at 4°C. The enzyme was concentrated and desalted using a Macrosep column molecular weight cut-off 10 kDa (Pall, Port Washington, NY, USA). The enzyme was exchanged to 100 mM sodium acetate buffer (pH 5.0) prior to subsequent experimental studies. Concentration of the purified enzyme was determined using the Bradford assay kit (Bio-Rad, Hercules, CA, USA).

Enzyme activity assay Polysaccharide degrading activities were analyzed based on the amount of liberated reducing sugars using the 3,5-dinitrosalicylic acid (DNS) method (34). A one milliliter reaction mixture contained the appropriate dilution of enzyme in 100 mM sodium acetate buffer, pH 5.0 and 1% (w/v) carboxymethylcellulose for endo-glucanase activity, beechwood xylan for xylanase activity and microcrystalline cellulose Avicel PH101 for cellobiohydrolase activity. The reaction was incubated at 50°C for 10 min for endo-glucanase and xylanase activities and 60 min for cellobiohydrolase activity. The β-glucosidase activity was analyzed based on the amount of *p*-nitrophenol liberated from *p*-nitrophenyl-β-D-glucopyranoside. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µmole of product per min. Analysis of thermostability and pH stability were performed according to Bunterngsook et al. (35).

Experimental mixture design The interactions among four recombinant enzymes were studied using an experimental mixture design approach (36). A [4,2]-augmented simplex lattice design implemented in the Minitab 17.0 software (Minitab Inc., State College, PA, USA) was used to define an optimal enzyme mixture for the maximal sugar yield. The design contained 15 experimental points, which were examined in triplicate with four components and a lattice degree of 2. In the mixture design, the sum of all components was always 5 mg/g biomass. Four independent variables in the mixture design consisted of Cel7B (X₁), Cel7A (X₂), BGL (X₃) and XYN (X₄) for hydrolysis of 5% (w/v) of pretreated bagasse in 100 mM sodium acetate buffer (pH 5.0). The reaction was incubated at 50°C with shaking at 200 rpm for 72 h. The reducing sugar released was measured using the DNS method (34). The sugar profile in the liquid fractions of the hydrolysis reaction was determined by HPLC as described in Laothanachareon et al. (20). The reducing sugar yield (Y₁) was applied as dependent variables for the analysis and simulation of the respondent model. After the regression analysis, the full cubic model was used to predict the optimized ratio of the mixture components. The canonical form of the full cubic model is shown in Eq. 1:

$$Y = \sum_{i=1}^3 \beta_i X_i + \sum_{i < j}^3 \beta_{ij} X_i X_j + \sum_{i < j}^3 \delta_{ij} X_i X_j (X_i - X_j) + \sum \sum \sum_{i < j < k}^3 \beta_{ijk} X_i X_j X_k \quad (1)$$

where Y is the predicted response, β_i is a linear coefficient, β_{ij} is a quadratic coefficient and β_{ijk} is a cubic coefficient. δ_{ij} is a parameter of the model. β_iX_i represents the linear blending portion and the parameter β_{ij} represents either synergistic or antagonistic blending.

Physical analysis of the biomass The microstructure of bagasse samples was analyzed by SU5000 scanning electron microscope (SEM) (Hitachi, Tokyo, Japan) with electron beam energy of 10 kV. The samples were dried and coated with gold for SEM analysis.

RESULTS

Protein expression and characterization of recombinant enzymes Induction of heterologous gene expression led to production of the target enzymes in the culture supernatants (Fig. 1). Cel7B showed a major protein band with the molecular weight of 66 kDa with minor species possibly due to differential glycosylation. BGL was expressed as two separated bands with the major band at 116 kDa and the minor one at the expected Mw. The sizes of Cel7B and BGL were larger than the deduced molecular of 51 and 86 kDa, respectively, possibly due to hyperglycosylation in *P. pastoris*. In contrast, XYN was expressed as a

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