Biotechnology Journal
www.biotechnology-journal.com

Biotechnol. J. 2015, 10, 1912–1919

DOI 10.1002/biot.201500081

Research Article

Enhanced hydrolysis of lignocellulosic biomass: Bi-functional enzyme complexes expressed in *Pichia pastoris* improve bioethanol production from *Miscanthus sinensis*

Sang Kyu Shin[1,], Jeong Eun Hyeon[1,2,*], Young In Kim[1], Dea Hee Kang[1], Seung Wook Kim[3], Chulhwan Park[4] and Sung Ok Han[1]*

[1] Department of Biotechnology, Korea University, Seoul, Republic of Korea
[2] Institute of Life Science and Natural Resources, Korea University, Seoul, Republic of Korea
[3] Department of Chemical and Biological Engineering, Korea University, Seoul, Republic of Korea
[4] Department of Chemical Engineering, Kwangwoon University, Seoul, Republic of Korea

Lignocellulosic biomass is the most abundant utilizable natural resource. In the process of bioethanol production from lignocellulosic biomass, an efficient hydrolysis of cellulose and hemicellulose to release hexose and pentose is essential. We have developed a strain of *Pichia pastoris* that can produce ethanol via pentose and hexose using an assembly of enzyme complexes. The use of enzyme complexes is one of the strategies for effective lignocellulosic biomass hydrolysis. Xylanase XynB from *Clostridium cellulovorans* and a chimeric endoglucanase cCelE from *Clostridium thermocellum* were selected as enzyme subunits, and were bound to a recombinant scaffolding protein mini-CbpA from *C. cellulovorans* to assemble the enzyme complexes. These complexes efficiently degraded xylan and carboxymethylcellulose (CMC), producing approximately 1.18 and 1.07 g/L ethanol from each substrate, respectively, which is 2.3-fold and 2.7-fold higher than that of the free-enzyme expressing strain. *Miscanthus sinensis* was investigated as the lignocellulosic biomass for producing bioethanol, and 1.08 g/L ethanol was produced using our recombinant *P. pastoris* strain, which is approximately 1.9-fold higher than that of the wild-type strain. In future research, construction of enzyme complexes containing various hydrolysis enzymes could be used to develop biocatalysts that can completely degrade lignocellulosic biomass into valuable products such as biofuels.

Received	22 FEB 2015
Revised	17 JUL 2015
Accepted	26 OCT 2015
Accepted article online	19 OCT 2015

Keywords: Bioethanol · Biorefinery · Enzyme complex · *Miscanthus sinensis* · *Pichia pastoris*

1 Introduction

Lignocellulosic bioethanol is increasingly becoming the preferred energy source compared to non-renewable fossil resources because of its desirable characteristics. These include being the most abundant natural renewable organic material and, given the concerns over CO_2 emissions, its high octane value and good combustion efficiency compared to fossil fuels [1]. Enhanced efficient ethanol production based on the sugar content of lignocellulosic biomass, e.g. the cellulosic portion, and the sugars released from the hemicelluloses fraction are key for the economic feasibility of this fuel [2]. Plant biomass hydrolysates contain cellodextrin, glucose and xylose. Pentose is produced by the acid treatment of hemicellulose, which releases xylose and arabinose [3]. Lignocellulosic biomass is composed of various components, includ-

Correspondence: Prof. Sung Ok Han, Department of Biotechnology, Korea University, Seongbuk-Gu, Anam-ro 145, Seoul 02841, Republic of Korea
E-mail: samhan@korea.ac.kr

Abbreviations: ADH, alcohol dehydrogenase; **AFEX**, ammonium fiber explosion; **AOX**, alcohol oxygenase; **CBM**, carbohydrate binding module; **CMC**, carboxymethyl cellulose; **DNS**, 3,5-dinitrosalicylic acid

* These authors contributed equally to this work.

ing cellulose, and its hydrolysis requires multiple sets of cellulolytic enzymes [4]. To utilize biomass, conventional production of cellulolytic enzymes by cellulolytic microorganisms is usually used, but the saccharification process is considered laborious and not cost effective. Perennial grasses such as *Miscanthus sinensis* are considered to be promising plants for CO_2 conversion as well as substrates for hemicellulosic ethanol production [5]. *M. sinensis* is a fast-growing plant, and 95% of its hemicellulosic content is xylose. This plant can also be grown on polluted soils to reduce soil contamination and can be harvested afterwards as a fuel source [6].

In nature, some cellulolytic anaerobic bacteria such as *Clostridium cellulovorans* have evolved intricate multienzyme complexes that facilitate the breakdown of complex polymers, such as xylan and cellulose found in plant cell walls[3]. The endoglucanases (EC 3.2.1.4) and exoglucanases (EC 3.2.1.91) target and degrade cellulose to release cellobiose and cello-oligosaccharides, which can be converted to glucose by β-glucosidases (EC 3.2.1.21) [7]. However, the total yield of ethanol is low. Xylan is the major hemicellulose component in woody biomass and can be broken down by xylan-degrading enzymes such as endoxylanase, β-xylosidase, α-glucuronidase, α-arabinofuranosidase and esterase [8]. Xylanases have been found in plants, algae, insects, protozoans and microorganisms. Due to their potential applications in various industries [9], xylanases are the subject of growing research interest. Various types of xylanase have now been cloned and expressed in *Pichia pastoris* [10]. This study uses recombinant yeast for the heterologous expression of endo-1,4-β-xylanase genes from *C. cellulovorans* [11]. Efficient enzymatic degradation is based on the assembly of multiple enzymes that enhance the hydrolysis and interaction between the enzymes and their substrates [12].

Cellulosomes are multi-enzyme complexes that are assembled by high-affinity protein-protein interactions. They have been identified and characterized in cellulolytic *Clostridia* and ruminal bacteria. Cellulolytic complexes contain various enzymes that are tightly bound to a large protein called scaffoldin, which itself is devoid of enzymatic activity but aids in the effective breakdown of crystalline cellulose and related plant cell wall polysaccharides such as hemicelluloses and pectin [1]. Scaffoldin binds to the complementary dockerin domain of the catalytic substrate, which is made up of a modular protein with a carbohydrate-binding module (CBM), two X2 domains of unknown function and eight cohesin modules [13]. Cellulolytic *Clostridia* species including *C. cellulovorans* produce and secrete large cellulolytic complexes that contain dockerin and hydrophobic cohesin domains [14]. Cohesins are repeated nine times in the recombinant scaffolding proteins CbpA and CBM, which in turn can be used as highly versatile tags for affinity applications because of their high and specific affinity for cellulose [15]. Previous studies demonstrated that minicellulosomes showed greater and enhanced activity against crystalline cellulose compared to the cellulosomal free enzyme [16]. The use of direct CBM-based purification could greatly enhance the cost effectiveness because cellulose is inexpensive, has excellent physical properties and has a low nonspecific affinity for most proteins, making it convenient for large-scale affinity purification [1].

The methylotrophic yeast *P. pastoris* is widely studied for its potential in industrial ethanol production because it can utilize and ferment xylose with a high ethanol yield. It is also able to ferment glucose and has been proven to be a good host for the heterologous expression of soluble proteins as well as several different classes of membrane proteins, including peptides and glucose transporters [17]. *M. sinensis* is one of the leading cellulosic biofuel crops and is gaining in importance. These crops are quite widespread, which makes the manufacture of this fuel generally available around the world. The biofuel industry is still being developed and potential feedstocks have also been introduced and adapted as bioenergy species based on their high biomass potential [18]. In a previous study [1], a synergic effect for cellulose hydrolysis was demonstrated through the in vivo assembly of minicellulosomes in recombinant *Saccharomyces cerevisiae* strains, resulting in an improved ethanol production from lignocellulosic biomass. In this study, we constructed functional minicellulosomes in *P. pastoris* by the heterologous expression of a recombinant scaffolding protein mini-CbpA, xylanase XynB from *C. cellulovorans* and a chimeric endoglucanase E from *C. thermocellum*.

2 Materials and methods

2.1 Strains and media

The composition of medium in this study was that described in the vector instructions of Invitrogen (MA, USA). *Escherichia coli* strain DH5α was maintained on Luria-Bertani agar plates, and 12.5 μg/mL zeocin was used for determining of integration in genomic DNA. The inoculum medium was prepared as follows: 5 g/L yeast extract, 10 g/L tryptone, 5 g/L sodium chloride and 15 g/L agar with an adjusted pH of 7.5 was sterilized at 120°C for 20 min by autoclaving, followed by the addition of 25 μg/mL zeocin. *P. pastoris* X-33 was purchased from Invitrogen. The yeast was maintained on YPD agar plates containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose (D-glucose) and 20 g/L agar. The inoculum medium was prepared as follows: 10 g/L yeast extract and 20 g/L peptone were sterilized at 120°C for 20 min in an autoclave. Subsequently, 50 mL 40% w/v D-glucose was added after being sterilized using 0.20-μm sterile filters (Sartorius AG, Göttingen, Germany). *P. pastoris* was supplemented from YPD agar, YPD-xylan and YPD-CMC

plates, which included a total concentration of 2% w/v xylan and carboxymethyl cellulose (CMC), respectively. The inoculum was grown aerobically on a rotatory shaker at 200 rpm and 30°C for 24h. Pretreated *M. sinensis* comprising 68% w/v glucose, 19% w/v xylose and 14% w/v lignin was used as the biomass substrate. This composition information was given by the provider (Changhae Ethanol Co., Ltd, Jeonju Republic of Korea). An ammonia fiber explosion (AFEX) process, which treats lignocellulose with 15% liquid ammonia v/v under high pressure at 150°C for 90min, was performed to decrystallize the biomass.

2.2 Construction of the recombinant plasmid

The polymerase chain reaction (PCR) products, such as XynB, cCelE and mini-CbpA, were cut by restriction enzymes following the instructions of the manufacturer (TaKaRa, Otsu, Japan). The cut genes were inserted into the pPsADHα vector. The chimeric CelE was fused with dockerin and endoglucanase E, and overlap PCR for cohesin-dockerin interaction was performed. In the pPsADHα1 vector, the alcohol oxygenase (AOX) promoter was replaced by the alcohol dehydrogenase 2 (ADH2) promoter, which is activated by O_2 limitation. These transformants were selected on YPD plates containing 25 μg/mL zeocin and 2% w/v sorbitol. Single colonies were selected, and the sequence of the isolated plasmids was analyzed by sequencing to verify the presence of the correct insert. The procedures for the small-scale preparation of the plasmids, digestion with the restriction enzymes, and ligation and transformation all followed standard methods.

2.3 Transformation and selection of *P. pastoris*

P. pastoris X-33 was cultured in YPD medium for12 h according to the manufacturer's instruction (Invitrogen). The plasmids containing the target genes were linearized by *Bst*XI. Electrocompetent *P. pastoris* cells were prepared according to instructions (Bio-Rad, CA, USA). This competent cell was used for transformation of each gene. Plasmid residues and competent cells were pulsed five times for each transformation. Pulses of 2000 V for an average of 5.90 ms electrophoresis time in a 0.2-cm gap cuvette were used. The protocol in this section followed the instructions and operation manual for *P. pastoris* electrophoresis transformation (Bio-Rad and Invitrogen). Transformation was confirmed by colony PCR to ensure that the genes were of correct size. Colony PCR conditions were as described previously [19]. Electrophoresis results were analyzed on a 1.0% agarosegel.

2.4 Cultivation conditions of *P. pastoris*

Before main culture, *P. pastoris* was pre-cultured at 30°C with shaking at 200 rpm for 48 h in 5 mL YPD. The main cultures of transformants for protein expression were grown to an OD value of 5 measured at 600 nm. *P. pastoris* was cultured 300 mL YPD medium in 500-mL baffled flask at 30°C with 200 rpm shaking. Full-grown *P. pastoris* was harvested by ultracentrifugation and washed twice with distilled water to remove residues. This *P. pastoris* was cultured into 20 mL medium with substrates, such as xylan, CMC and *M. sinensis* as carbon source, in closed 50-mL bottles. Inoculum volume was adjusted to an OD value of 20 at 600 nm. Transformed *P. pastoris* were cultured at 30°C with 100 rpm shaking.

2.5 Expression and harvest of recombinant protein

The *P. pastoris* strains cultured for 20 h in baffled flask containing 50 mL YPD medium were collected by centrifugation and resolved in 50 mL YPD medium. Yeast transformants containing xylanase XynB and the chimeric endoglucanase CelE were added. We used YPD plates containing 1 g/L xylan and CMC for analysis of enzyme activity. One drop of medium of recombinant *P. pastoris* was dropped on plates and washed after 1 h. Congo red (1 g/L) was used for staining CMC and Xylan and destained using1 g/L NaCl. A halo proving substrate degrading activity was detected on YPD-Xylan and YPD-CMC.

2.6 Minicellulosome assembly by zymogram analysis

Native PAGE and zymogram, as described previously [1], were performed to identify the cohesin-dockerin interaction. Native PAGE for the secreted and high-expression XynB and cCelE was performed. The culture filtrates of the transformants were run on a native gel and stained with Coomassie Blue R250. The zymogram analysis was performed on a native PAGE gel that contained 0.2% xylan and CMC. The compositions of solutions A, B and C were as described [1]. Gels were washed consecutively in solutions A and B at room temperature for 1 h. The native PAGE gel was renatured in solution C at 4°C overnight, and then dyed with 0.25% Congo red for 5 min and decolored in 1 M NaCl for 1 h.

2.7 Enzyme activity assay

The analysis conditions for enzyme complexes and the measurement of released sugars were as described previously [20]. Following incubation of the enzyme solution at 60°C for 2 h with the same volume of substrate mixture containing 1% w/v xylan and 1% w/v CMC in 50 mM sodium acetate buffer (pH 5.0), the hydrolytic activity of the enzyme was measured. The 3,5-dinitrosalicylic acid (DNS) assay method was used to determine content of released sugars from xylan and CMC. The DNS reagent was added in the same ratio to the enzyme reaction mix-

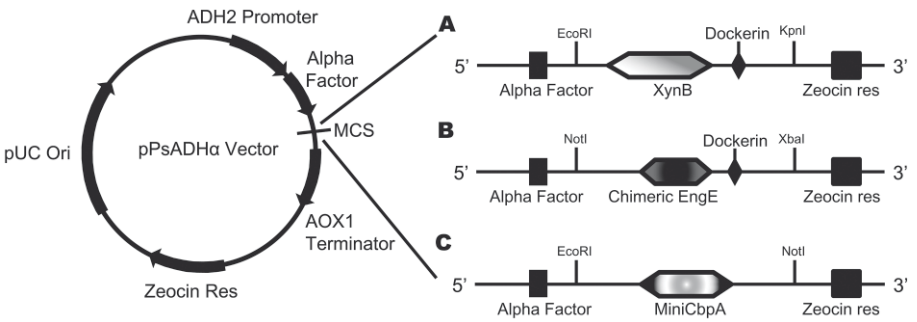


Figure 1. Diagram illustrates the construction of the plasmids used in this study. Construction of the *Pichia pastoris* expression vector containing *Clostridium cellulovorans* xylanase XynB, recombinant scaffolding protein mini-CbpA and *Clostridium thermocellum* chimeric endoglucanase CelE. The plasmids used for constructing the strains are depicted: (**A**) pPsADHα-XynB, xylanase B; (**B**) pPsADHα-cCelE, chimeric CelE; and (**C**) pPsADHα-M, mini-CbpA.

ture. The DNS and enzyme mixture was heated at 95°C for 5 min to develop the DNS reagent. Absorbance at 540 nm was measured to calculate the amount of released sugars in the mixture.

2.8 Direct CBM-based purification

CBM-based purification using Sigmacell Type 50 (Sigma Aldrich, MO, USA) was performed as previously described [1]. Crystalline cellulose (10 µg/mL) was bound to the resulting enzymes at room temperature for 1 h with continuous shaking. The samples were centrifuged at 1600 ×g to separate the cellulose-bound proteins. Nonspecific proteins that did not bind to cellulose were removed by washing the samples three times. Proteins specifically bound by CBM were analyzed by SDS-PAGE after elution with 50 mM Tris, pH 12.5.

2.9 Yeast fermentation and analysis of ethanol concentration

The fermentation method for ethanol production was performed as described previously [1]. The *P. pastoris* cells were grown to exponential phase at 30°C and 200 rpm in 500 mL YPD medium in 1-L Erlenmeyer flasks for 48 h. *P. pastoris* cells were collected by centrifugation and washed twice with sterile distilled water to remove any residues. The washed cells were inoculated into 20 mL YPD medium in closed 50-mL bottles. Fermentation was performed at 30°C with shaking at 100 rpm. Ethanol was analyzed as described [1]. The culture medium was centrifuged, and the supernatant was filtered before measuring the ethanol composition. Gas chromatography (model GC7890; Agilent, CA, USA) with aDB-WAXetr column (50.0 m × 0.32 mm) was performed to determine ethanol concentrations as described [1], using commercial-grade ethanol for standards.

2.10 Monosaccharide assay using HPLC

An HPLC system (Waters, MA, USA) with a refractive index detector and an HPX-87H column (300 mm × 7.8 mm) was used for the monosaccharide assay following the protocol of the manufacturer. The temperatures of the detector and column were 40°C and 50°C, respectively; flow rate was set to 0.6 mL, with an injection volume of 10 µL. The mobile phase was 0.005 M H_2SO_4. All analytical samples and solutions were filtered through a 0.2-µm cellulose acetate filter before injection.

3 Results

3.1 Heterologous expression of xylanase XynB for the hydrolysis and fermentation of xylan

To assemble the minicellulosome via the mini-CbpA scaffolding protein from *C. cellulovorans*, we constructed a xylanase XynB expressing strain. The gene encoding XynB was fused to the gene coding for the secretion signal sequence factor from *P.pastoris* (Fig. 1). To confirm the secretion of xylanase, a plate assay was performed using 1g/L xylan as a substrate, followed by Congo red staining. A clear yellow halo was observed when recombinant

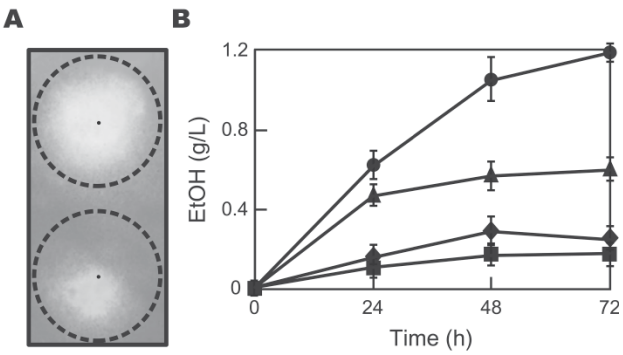


Figure 2. Heterologous expression of xylanase and the production of ethanol from the xylan substrate. (**A**) Congo red-stained YPD agar plates containing 1g/L xylan. Each plate was incubated for 48h at 30°C. The different colonies on the plate were incubated with the following cells: upper, *P. pastoris*-pPsADHα-XynB; and lower, *P. pastoris*-pPsADHα (control). (**B**) The preculture of the strains was inoculated into 20 mL YPD-xylan medium in a closed 50-mL bottle. These cultures were anaerobically cultivated at 30°C with shaking at 100 rpm. Error bars represent standard deviation of triplicate experiments. Closed squares, *P. pastoris* (control); closed diamonds, *P. pastoris*-pPsADHα-M; closed triangles, *P. pastoris*-pPsADHα-XynB; closed circles, *P. pastoris*-pPsADHα-M and pPsADHα-XynB.

yeast with the plasmid for XynB grew on the xylan plates (Fig. 2A). No halo was observed for the control plasmid pPsADHα-containing colony. A positive result indicated that XynB was expressed in an active form and that the fusion of the dockerin domain at the Cterminus of the catalytic domain was successfully enhanced [1]. To produce ethanol from xylan efficiently, *P.pastoris* was transformed with a plasmid containing the mini-CbpA and xylanase XynB genes. In the time course of xylan fermentation, values were consistently higher for strains expressing mini-CbpA and XynB in xylan medium at 30°C. The experiments were performed in triplicate (Fig. 2B). The results demonstrated that scaffolding proteins function and the resultant dockerin-fused enzymes acted on lignocellulosic substrates via the assembly of the minicellulosome. The highest ethanol concentration was approximately 1.18 g/L from xylan after 72 h of fermentation. The cell growth and ethanol concentration were consistent over the time course of the fermentation.

3.2 Heterologous expression of chimeric endoglucanase for the hydrolysis and fermentation of CMC

To confirm the endoglucanase production potential, a plate assay was performed using 1g/L CMC as a substrate, followed by Congo red staining. Yeast cells harboring the plasmid pPsADHα-cCelE, which encodes the chimeric cCelE, hydrolyzed the substrate and a clear halo was observed (Fig. 3A). However, with the control plasmid pPsADHα, no halo appeared around the colony. The chimeric cCelE did not interfere with the dockerin domain and was expressed in an active form [1]. Wild-type *P. pastoris* was unable to hydrolyze cellulose to cellobiose. We previously demonstrated direct ethanol fermentation using yeast cells expressing a plasmid containing mini-CbpA and chimeric endoglucanase cCelE. This suggested that CMC was hydrolyzed to glucose by the sequential reactions of cCelE. Figure3B shows that the time course of CMC fermentation was consistently longer for *P. pastoris* expressing mini-CbpA and cCelE in the CMC medium at 30°C. The highest ethanol concentration was approximately 1.07 g/L from CMC after 72 h of fermentation (Fig. 3B). These results indicate that the *P.pastoris* strain transformed with the pPsADHα plasmid as a control did not produce ethanol, and that ethanol production was higher for cells expressing mini-CbpA and chimeric cCelE. The results demonstrate the practicability of using a cellulosic material medium for the synergistic effect of minicellulosomes and its use in fermentation.

3.3 Confirmation of enzyme complexes and monosaccharide analysis

The assembly of the recombinant cellulosome of mini-CbpA and cellulosomal enzymes XynB and cCelE was

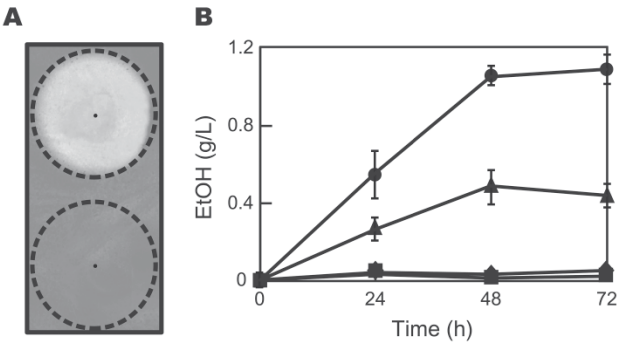


Figure 3. Expression of chimeric endoglucanase cCelE and fermentation from carboxymethyl cellulose(CMC) as a substrate. (**A**) Congo red-stained YPD agar plates containing 1g/L CMC; 5μL concentrated supernatant harvested from the following cells was applied to the different wells: upper, *P. pastoris*-pPsADHα-cCelE; and lower, *P. pastoris*-pPsADHα (control). Clear halos are indicative of CMC hydrolysis. (**B**) The preculture of strains was inoculated into 20 mL YPD-CMC medium in a closed 50-mL bottle. These cultures were anaerobically cultivated at 30°C with shaking at 100 rpm. Error bars represent standard deviation of triplicate experiments. Closed squares, *P. pastoris* (control); closed diamonds, *P.pastoris*-pPsADHα-M; closed triangles, *P. pastoris*-pPsADHα-cCelE; closed circles, *P. pastoris*-pPsADHα-M and *P. pastoris*-pPsADHα-cCelE.

confirmed by native PAGE and zymogram analysis. The purified XynB and cCelE were shown to be bound to the mini-CbpA in vitro (Fig. 4A). The purified mini-CbpA and cellulosomal enzymes units were mixed at a molar ratio of 1:2. As the mini-CbpA was designed to contain a CBM at its Nterminus, the purification of intact mini-CbpA using the cellulose purification method was achieved ina single step and was analyzed with nativePAGE. The calculated molecular mass of the mini-CbpA was 58.2 kDa (57 208 Da mini-CbpA plus 1012Da His-tag residues), which is in good agreement with the calculated molecular mass (Fig. 4B). The composition of degraded polysaccharides in the culture medium was analyzed by HPLC to determine the feeding ratio of *P. pastoris* and the ethanol production. No glucose or xylose was detected from CMC, and xylose hydrolysis occurred during the 72-h anaerobic fermentation in the culture broth. In addition, no xylobiose or cellobiose was detected in some of the fermentation samples. Therefore, the process of utilization of monosaccharides by *P. pastoris* uptake requires clarification.

3.4 Ethanol fermentation of *M. sinensis* as lignocellulosic biomass by recombinant strains

Direct fermentation of *M. sinensis* to ethanol was performed using the biomass-utilizing *P. pastoris* strains at 30°C. The recombinant *P. pastoris* strains containing pPsADHα-XynB and pPsADHα-cCelE produced higher levels of ethanol than the control *P. pastoris* strain. The final recombinant strain with minicellulosome-producing ability exhibited direct fermentation of lignocellulosic *M.sinensis*. The lignocellulosic biomass of *M. sinensis*

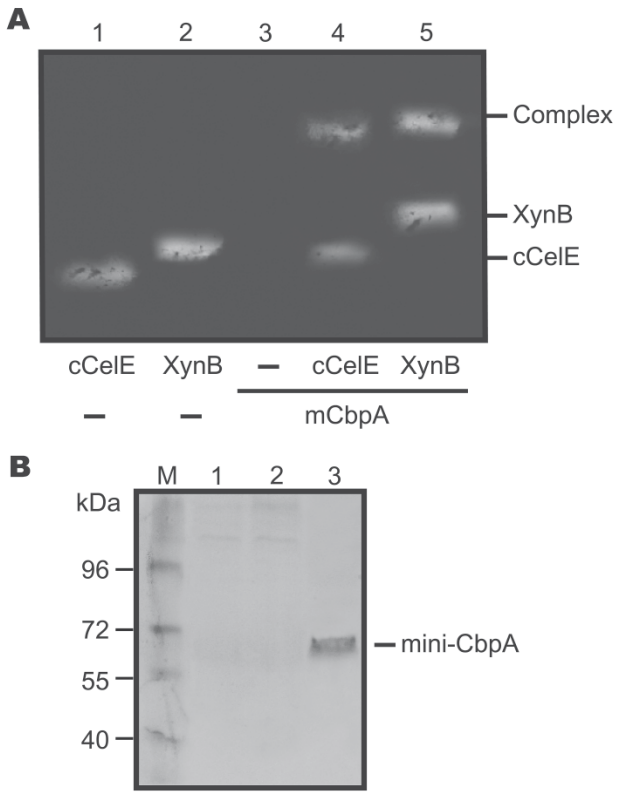


Figure 4. The assembly of minicellulosomes containing each subunit. (**A**) Electrophoretic analysis of expression in *P. pastoris* by nativePAGE visualized by zymogram and Congo red staining. Lane 1: pPsADHα-cCelE; lane 2: pPsADHα-XynB; lane 3: pPsADHα-M; lane 4: pPsADHα-cCelE and pPsADHα-M; and lane 5: pPsADHα-XynB and pPsADHα-M. (**B**) Direct CBM-based purification and electrophoretic analysis. The expression of mini-CbpA in *P.* based on SDS-PAGE visualized by Coomassie blue staining. Lanes: M, molecular weight standards; 1, wash 1 with 1 M NaCl in 20 mM Tris, pH 8.0; 2, wash 2 with 20 mM Tris, pH 7.5; and 3, purified mini-CbpA after cellulose purification.

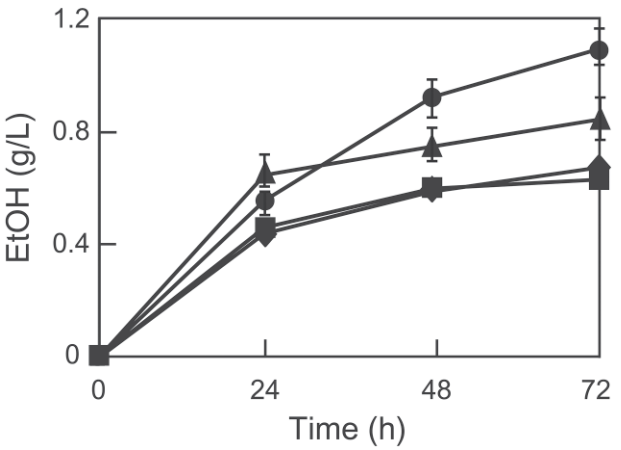


Figure 5. Production of ethanol from biomass (*Miscanthus sinensis*). The preculture of strains was inoculated into YPD medium and further cultured at 30°C anaerobically. Error bars represent standard deviation of triplicate experiments. Closed diamonds, *P. pastoris* (control); closed squares, *P pastoris*-pPsADHα-M; closed triangles, *P. pastoris*-pPsADHα-XynB and *P. pastoris*-pPsADHα-cCelE; closed circles, *P. pastoris*-pPsADHα-XynB, *P. pastoris*-pPsADHα-M and *P. pastoris*-pPsADHα-cCelE.

was hydrolyzed to fermentable sugars such as xylose and glucose by cooperative reactions of XynB and cCelE, yielding 1.08 g/L ethanol after 72 h of fermentation, which is approximately 1.9-fold higher than that of free-enzyme expression strain. The experiments were performed in triplicate (Fig. 5). *P. pastoris* (control) and *P. pastoris*-pPsADHα-M (control) showed an ethanol production of around 0.4 g/L without any hydrolysis enzymes. This result was due to the small amount of glucose released from the biomass by the AFEX pretreatment process [21]. The small amount of glucose released from the biomass was converted to bioethanol by *P. pastoris* (control) and *P. pastoris*-pPsADHα-M (control).

4 Discussion

In nature, cellulolytic anaerobic bacteria such as *C. cellulovorans* have evolved intricate multi-enzyme complexes that facilitate the breakdown of complex polymers, such as xylan and cellulose found in plant cell walls [22]. We have previously reported the expression of endoglucanase cCelE and BglA of *C. thermocellum* in *S. cerevisiae* [23]. In another report, cellulosomal xylanase XynB and the mini-CbpA scaffolding gene from *C. cellulovorans* were expressed in *Bacillus subtilis*, forming minicellulosomes based on in vivo interactions between cohesin and dockerin [14]. In the present study, we successfully constructed xylanase XynB and chimeric endoglucanase cCelE and assembled a minicellulosome for the enhanced production of ethanol from cellulosic biomass in *P. pastoris*. This is the first report on the use of minicellulosomes in vivo using mini-CbpA from *C. cellulovorans* in recombinant *P. pastoris* with the potential for the assembly of a variety of designer cellulosomes. The use of eukaryotic *P. pastoris* as the expression host offers advantages such as correct protein processing and folding and easy foreign protein isolation combined with the some advantages of *E.coli*, including cost effectiveness and easy scale-up potential [1, 24]. Accordingly, this study demonstrated the expression of XynB and cCelE in engineered *P. pastoris* as a microbial platform for the efficient conversion of biomass to valuable products, and the potential for the assembly of functional designer cellulosomes. As the microorganism utilizes glucose preferentially, the xylose fermentation rate is considerably lower when both glucose and xylose were used as substrates. This phenomenon, which is called glucose repression, is regarded as a significant barrier to the successful utilization of mixed sugars in cellulosic hydrolysates [25]. Previous reports demonstrated the use of *P. pastoris* as the engineered host for the co-fermentation of xylose and cellobiose. The

hydrolysis of cellobiose was catalyzed by intracellular β-glucosidase transported by a high-affinity cellodextrin transporter [3]. In previous research [26], the Q_s^{Max}, which is volumetric rate of sugar consumption, was 0.5 g/L/h in the *P. pastoris* strain. The engineered *P. pastoris* in this study also showed same substrate consumption rates.

To improve its hydrolytic ability against insoluble substrates, xylanase XynB and chimeric endoglucanase cCelE were assembled with mini-CbpA from *C. cellulovorans*. The mini-CbpA was derived from scaffolding protein CbpA containing a CBM, which provides the proximity of substrates to the cellulosomal enzymes [14]. Some previous reports on ethanol-producing ability have described the engineering of thermophilic anaerobes for utilizing biomass-derived monosaccharides, and the optimization of natural bacterial consortia (HPP consortium) for utilizing lignocellulose [27, 28]. In this study, we applied not the ethanol-producing ability of *P. pastoris*, but its ability to efficiently express heterologous enzymes and use both pentose and hexose as carbon sources. Also, for more efficient degradation of lignocellulose, we constructed complexes of hydrolytic enzymes. Construction of enzyme complexes containing of XynB and cCelE aids the effective breakdown of polysaccharides because the CBM in the scaffolding protein from *C. cellulovorans* could provide an efficient approach to substrates. The recombinant yeast strain with the minicellulosome-assembling activity exhibits several advantages, including a high expression of the mini-CbpA scaffolding protein and the XynB and chimeric cCelE fused to the dockerin domain. The minicellulosomes could be purified using direct CBM-based purification due to the high specificity and affinity of the CBM on the scaffolding protein for crystalline cellulose. To analyze the synergistic actions between XynB and cCelE on lignocellulose degradation, recombinant cellulosomes were assembled using mini-CbpA, and cCelE was shown to contribute to the breakdown of soluble cellulose rather than insoluble cellulose from the introduction of mini-CbpA [29]. To confirm the synergic effect of enzyme complexes, specific enzyme activities are measured using of mixed substrate containing of 1% xylan w/v and 1% CMC w/v. Specific enzyme activity of CelE and XynB were 1.4 U/mg and 1.8 U/mg, respectively. The enzyme activity of minicellulosome, which contains CelE and XynB, is 6.2 U/mg, demonstrating a 1.9-fold increased synergic effect.

In this study, we successfully established a minicellulosome-harboring *P. pastoris* strain for ethanol fermentation using lignocellulose as the substrate [29]. A laboratory and commercially available yeast strain was used as the host for the expression and assembly of the minicellulosome. Ethanol production of the engineered *P. pastoris* expressing the enzyme complexes increased 1.4-fold over that of the wildtype using pretreated *M. sinensis* as cellulosic substrate. The results obtained in this study demonstrate the potential for the development of engineered *P. pastoris* because this strain could efficiently express heterologous enzymes and use both of pentose and hexose as carbon sources. It is the key for future bioethanol production from lignocellulose.

This work was supported by the Industrial Strategic Technology Development Program (10051513) funded by the Ministry of Trade, Industry & Energy (MI, Korea) and a grant from the Institute of Life Science and Natural Resources, Korea University.

The authors declare no financial or commercial conflict of interest.

5 References

[1] Hyeon, J. E., Yu, K. O., Suh, D. J., Suh, Y. W. et al., Production of minicellulosomes from *Clostridium cellulovorans* for the fermentation of cellulosic ethanol using engineered recombinant *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* 2010, *310*, 39–47.

[2] Bellido, C., Bolado, S., Coca, M., Lucas, S. et al., Effect of inhibitors formed during wheat straw pretreatment on ethanol fermentation by *Pichia stipitis*. *Bioresour. Technol.* 2011, *102*, 10868–10874.

[3] Ha, S. J., Galazka, J. M., Kim, S. R., Choi, J. H. et al., Engineered *Saccharomyces cerevisiae* capable of simultaneous cellobiose and xylose fermentation. *Proc. Natl. Acad. Sci. U.S.A.* 2011, *108*, 504–509.

[4] Shill, K., Padmanabhan, S., Xin, Q., Prausnitz, J. M. et al., Ionic liquid pretreatment of cellulosic biomass: Enzymatic hydrolysis and ionic liquid recycle. *Biotechnol. Bioeng.* 2011, *108*, 511–520.

[5] Graham-Rowe, D., Agriculture: Beyond food versus fuel. *Nature* 2011, *474*, S6–S8.

[6] Heo, H. S., Park, H. J., Yim, J. H., Sohn, J. M. et al., Influence of operation variables on fast pyrolysis of *Miscanthus sinensis* var. *purpurascens*. *Bioresour. Technol.* 2010, *101*, 3672–3677.

[7] Skovgaard, P. A., Thygesen, L. G., Jørgensen, H., Cardona, M. et al., The role of endoglucanase and endoxylanase in liquefaction of hydrothermally pretreated wheat straw. *Biotechnol. Prog.* 2014, *30*, 923–931.

[8] Decelle, B., Tsang, A., Storms, R. K., Cloning, functional expression and characterization of three *Phanerochaete chrysosporium* endo-1,4-beta-xylanases. *Curr. Genet.* 2004, *46*, 166–175.

[9] Patel, S. J., Savanth, V. D., Review on fungal xylanases and their applications. *Int. J. Adv. Res.* 2015, *3*, 311–315.

[10] Han, H., You, S., Zhu, B., Fu, X., et al., Characterization and high expression of recombinant *Ustilago maydis* xylanase in *Pichia pastoris*. *Biotechnol. Lett.* 2015, *37*, 697–703.

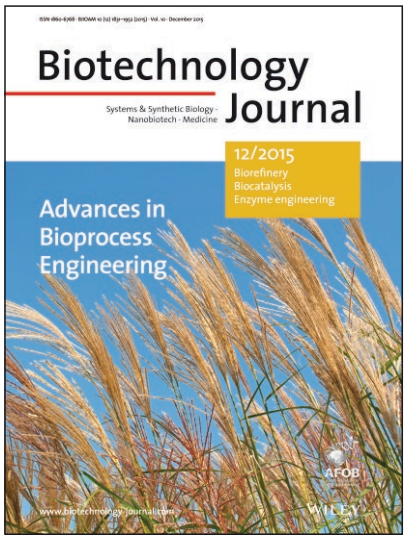
[11] Thomas, L., Joseph, A., Gottumukkala, L. D., Xylanase and cellulase systems of *Clostridium* sp.: An insight on molecular approaches for strain improvement. *Bioresour. Technol.* 2014, *158*, 343–350.

[12] Oliveira, C., Carvalho, V., Domingues, L., Gama, F. M., Recombinant CBM-fusion technology – applications overview. *Biotechnol. Adv.* 2015, *33*, 358–369.

[13] Kim, S. J., Hyeon, J. E., Jeon, S. D., Choi, G.-w., Han, S. O., Bifunctional cellulases complexes displayed on the cell surface of *Corynebacterium glutamicum* increase hydrolysis of lignocelluloses at elevated temperature. *Enzyme Microb. Technol.* 2014, *66*, 67–73.

[14] Cho, H. Y., Yukawa, H., Inui, M., Doi, R. H., Wong, S. L., Production of minicellulosomes from *Clostridium cellulovorans* in *Bacillus subtilis* WB800. *Appl. Environ. Microbiol.* 2004, *70*, 5704–5707.

[15] Murashima, K., Kosugi, A., Doi, R. H., Synergistic effects of cellulosomal xylanase and cellulases from *Clostridium cellulovorans* on plant cell wall degradation. *J. Bacteriol.* 2003, *185*, 1518-1524.
[16] Liang, Y., Si, T., Ang, E. L., Zhao, H., Engineered pentafunctional minicellulosome for simultaneous saccharification and ethanol fermentation in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 2014, *80*, 6677–6684.
[17] Cereghino, J. L., Cregg, J. M., Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiol. Rev.* 2000, *24*, 45–66.
[18] Youngs, H., Somerville, C., Development of feedstocks for cellulosic biofuels. *F1000 Biol. Rep.* 2012, *4*, 10.
[19] Lane, C. E., Benton, M. G., Detection of the enzymatically-active polyhydroxyalkanoate synthase subunit gene, *phaC*, in cyanobacteria via colony PCR. *Mol. Cell Probes* 2015.
[20] Dashtban, M., Qin, W., Overexpression of an exotic thermotolerant beta-glucosidase in *Trichoderma reesei* and its significant increase in cellulolytic activity and saccharification of barley straw. *Microb. Cell Fact.* 2012, *11*, 63.
[21] Harun, S., Balan, V., Takriff, M. S., Hassan, O. et al., Performance of AFEX™ pretreated rice straw as source of fermentable sugars: The influence of particle size. *Biotechnol. Biofuels* 2013, *6*, 40.
[22] Han, S. O., Yukawa, H., Inui, M., Doi, R. H., Regulation of expression of cellulosomal cellulase and hemicellulase genes in *Clostridium cellulovorans*. *J. Bacteriol.* 2003, *185*, 6067–6075.
[23] Han, S. O., Yukawa, H., Inui, M., Doi, R. H., Isolation and expression of the xynB gene and its product, XynB, a consistent component of the *Clostridium cellulovorans cellulosome*. *J. Bacteriol.* 2004, *186*, 8347–8355.
[24] Ebrahimi, S. M., Tebianian, M., Toghyani, H., Memarnejadian, A., Attaran, H. R., Cloning, expression and purification of the influenza A (H9N2) virus *M2e* antigen and truncated *Mycobacterium tuberculosis* HSP70 as a fusion protein in *Pichia pastoris*. *Protein Expression Purif.* 2010, *70*, 7–12.
[25] Kim, J. H., Block, D. E., Mills, D. A., Simultaneous consumption of pentose and hexose sugars: An optimal microbial phenotype for efficient fermentation of lignocellulosic biomass. *Appl. Microbiol. Biotechnol.* 2010, *88*, 1077–1085.
[26] Li, T., Cao, R., Liu, J. J., Production of ethanol from cellobiose by recombinant β-glucosidase-expressing *Pichia pastoris*: Submerged shake flask fermentation. *Afr. J. Biotechnol.* 2012, *11*, 9108–9117.
[27] Shaw, A. J., Podkaminer, K. K., Desai, S. G., Bardsley, J. S. et al., Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield. *Proc. Natl. Acad. Sci. U.S.A.* 2008, *105*, 13769–13774.
[28] Du, R., Yan, J., Li, S., Zhang, L. et al., Cellulosic ethanol production by natural bacterial consortia is enhanced by *Pseudoxanthomonas taiwanensis*. *Biotechnol. Biofuels* 2015, *8*, 10.
[29] Hyeon, J. E., Jeon, W. J., Whang, S. Y., Han, S. O., Production of minicellulosomes for the enhanced hydrolysis of cellulosic substrates by recombinant *Corynebacterium glutamicum*. *Enzyme Microb. Technol.* 2011, *48*, 371–377.



Cover illustration

This special issue, in collaboration with the Asian Federation of Biotechnology and edited by Professors Yoon-Mo Koo and Penjit Srinophakun , covers advances in bioprocess engineering. Several articles in this issue discuss different aspects of lignocellulose-based biorefining. Miscanthus (shown on the cover) is one example of a plant whose lignocellulosic biomass can be used for bioethanol production. © M. Schuppich, Fotolia.com

Biotechnology Journal – list of articles published in the December 2015 issue.

Research Article

Phenolic compounds: Strong inhibitors derived from lignocellulosic hydrolysate for 2,3-butanediol production by *Enterobacter aerogenes*

Sang Jun Lee, Ju Hun Lee, Xiaoguang Yang, Sung Bong Kim, Ja Hyun Lee, Hah Young Yoo, Chulhwan Park and Seung Wook Kim

http://dx.doi.org/10.1002/biot.201500090

Research Article

Salt tolerant chromatography provides salt tolerance and a better selectivity for protein monomer separations

Noriko Yoshimoto, Daisuke Itoh, Yu Isakari, Ales Podgornik and Shuichi Yamamoto

http://dx.doi.org/10.1002/biot.201400550

Research Article

Dual utilization of NADPH and NADH cofactors enhances xylitol production in engineered *Saccharomyces cerevisiae*

Jung-Hyun Jo, Sun-Young Oh, Hyeun-Soo Lee, Yong-Cheol Park and Jin-Ho Seo

http://dx.doi.org/10.1002/biot.201500068

Biotech Method

Phosphonium alkyl PEG sulfate ionic liquids as coating materials for activation of *Burkholderia cepacia* lipase

Yui Matsubara, Shiho Kadotani, Takashi Nishihara, Yoshichika Hikino, Yukinobu Fukaya, Toshiki Nokami and Toshiyuki Itoh

http://dx.doi.org/10.1002/biot.201500413