Secretion of chimeric cellulase from *Clostridium thermocellum* and *Clostridium cellulolyticum* by *Bacillus subtilis*

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Abstract
Lignocellulose recalcitrance poses a significant challenge in utilizing abundant agricultural residues as renewable resources for high-value cellulose-based applications. Some anaerobic bacteria can efficiently degrade lignocellulose using secreted protein complexes known as cellulosomes. Chimeric cellulosomes are created by combining genetic material from various sources to degrade different biomass substrates and function well in extreme conditions. A variety of functional cellulases can be assembled using scaffoldin which is a primary protein structure in a cellulosome through species-specific interaction between a type-1 cohesin module from scaffoldin and a type-1 dockerin module from cellulosomal cellulase.

This study focused on designing a chimeric cellulosomal cellulase which is a fusion of the catalytic module of endoglucanase Cel5CCA from C. cellulolyticum (Ruminiclostridium cellulolyticum) and a type-1 dockerin of endoglucanase Cel8A from C. thermocellum (Acetivibrio thermocellus). The cellulase genes were cloned in Escherichia coli and expressed extracellularly in Bacillus subtilis WB800N. They demonstrated carboxymethyl cellulose hydrolysis (CMCase) in the Congo Red assay. Furthermore, they exhibited protein bands around 50 kDa consistent with the theoretical molecular masses in SDS-PAGE and Western blot analyses. Elisa analysis confirmed a species-specific interaction between the type-1 dockerin of the cellulases and the type-1 cohesin of the scaffoldin mini-CipA. Extracellular mixtures of the cellulases and mini-CipA increased their synergistic CMCase activity by approximately 30% to 50% compared to mixtures of the free cellulases without mini-CipA.

Keywords: Chimeras, *Clostridia*, endoglucanase, mini-cellulosome, protein engineering.

Introduction

Agricultural residues release an estimated five billion metric tons annually[28]. In addition to their conventional applications in composting, feed stock, mushroom cultivation, building materials and biochar, they are also used for high-value products in food-packaging, water treatment, cosmetics, biomedicine and bioenergy[17]. The utilization of crop waste requires the pre-processing which faces challenges due to the lignocellulose recalcitrance caused by the high crystallinity of cellulose, hydrophobic characteristic of lignin and stable covering of lignin-hemicellulose matrix on the cellulose fibers[9].

Compared to chemical and physical methods such as acid hydrolysis and steam explosion, using cellulases and their families requires less energy, yet they are more economical and environmentally friendly. Cellulases are mostly produced by microorganisms and play an ecological role in recycling carbon. They also have significant applications in the textile, paper, detergent, food and beverage industries. The estimated global market value of cellulases is projected to be above 1,685.8 million USD in 2023[8].

Cellulase can be divided into three types including exo-glucanase, endo-glucanase and β-glucosidase, based on the attacked position of cellulose substrates[15]. Additionally, they are categorized into glycoside hydrolase families (GHs) based on their three-dimensional structure and reaction mechanism on the substrate[3]. Cellulases are also divided into two groups: non-cellulosomal (free) and cellulosomal cellulases. Non-cellulosomal cellulases usually contain carbohydrate binding modules (CBM) that guide the catalytic modules to the substrate. The targeting effect caused by CBM has been reported to be the major factor responsible for the enhanced activity of designer chimeric cellulases, while individual chimeric cellulases showed equivalent activity to the corresponding wild-type analogues[30].

On the other hand, the cellulosomal cellulases typically have type-1 dockerin modules that bind to type-1 cohesin of scaffoldins in cellulosomes secreted by microorganisms[7]. The assembly of cellulosomal cellulases by scaffoldins through cohesin-dockerin interaction creates a spatial proximity effect that enhanced the hydrolytic activity of cellulosomes[13].

Chimeric cellulosomes have been created using a variety of genetic sources to enhance their ability to utilize different substrates. When compared to a mixture of free cellulases, the chimeras demonstrated improved synergistic activity on

crystalline cellulose[6] or xylan[16]. Numerous experiments have been conducted on designer chimeric cellulosomes using intracellular expression to investigate their biochemical and physicochemical characteristics[2,6,19,25,30]. Additionally, cell-surface expression is carried out to mimic natural cellulosomes[31]. However, when increasing the size of designer cellulosomes on surface expression, their density on the cell surface also decreased.

Chimeric cellulosomes from *C. thermocellum* and *C. cellulolyticum* have been intracellularly expressed in *E. coli*[6]. Their type-1 cohesin-dockerin interactions have been analyzed in purified forms before[23]. In this study, we designed a chimeric endoglucanase containing the catalytic module Cel5CCA from *C. cellulolyticum* fused with the type-1 dockerin Cel8A from *C. thermocellum* using extracellular expression in *B. subtilis* WB800N. Subsequently, we evaluated the activity of chimeric cellulase with previously designed scaffolldin mini-CipA from extracellular cultures.

Material and Methods

Strains, plasmids and culture media: *Clostridium thermocellum* (*Acetivibrio thermocellus*, DSM1237, ATCC27405) and *Clostridium cellulolyticum* (*Ruminiclostridium cellulolyticum*, DSM5812, ATCC35319) (The Leibniz Institute DSMZ, Germany) were cultured using medium DSMZ #122 to amplify mini-cellulosomal genes[12]. *E. coli - B. subtilis* shuttle plasmids pHT contain an expression cassette controlled by an Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible P_{grac} promoter along with two antibiotic resistant genes, *bla* and *cat*[20]. The *bla* gene codes for the ampicillin resistant enzyme β-lactamase was used for plasmid cloning in *E. coli* OmniMAX™ (Invitrogen). The *cat* gene codes for the chloramphenicol resistant enzyme chloramphenicol acetyltransferase was used for recombinant protein expression in *B. subtilis* WB800N (MoBiTec, Germany).

Ampicillin (Amp) at 100 µg/ml and chloramphenicol (Cm) at 10 µg/ml (final concentration) were used as selection markers in *E. coli* and *B. subtilis* cultivation, respectively. Both strains were cultured in Luria Broth (LB) media at 30°C with shaking at 250 rpm.

Chimeric cCel5CCA cloning: Chimeric cCel5CCA was created by replacing the type-1 dockerin of Cel5CCA from *C. cellulolyticum* with the type-1 dockerin of Cel8A from *C. thermocellum* (Figure 1) through four steps. Step 1: cel8A (Genbank:K03088.1) was amplified from the *C. thermocellum* genome (Genbank:CP000568.1) and inserted into the pHT backbone plasmid to produce pHT1454. Step 2: The type-1 dockerin of cel8A (position:1531-1731, Genbank:K03088.1) was amplified from pHT1454 and inserted into the pHT backbone plasmid to create pHT1756B. Step 3: cel5CCA (Genbank:M93096.1) was amplified from the *C. cellulolyticum* genome (Genbank:CP001348.1) and inserted into the pHT backbone plasmid to create pHT1729. Step 4: The catalytic module of cel5CCA (position:79-1224, Genbank:M93096.1) was amplified from pHT1729 and inserted into pHT1756B to generate pHT1757 encoding the chimeric cCel5CCA.

The plasmid characteristics are provided in table 1. The primers used for DNA amplification, colony PCR and plasmid sequencing are shown in table 2. A DNA ladder (200 to 2000 bp ZipRuler Express DNA Ladder, Fermentas) was used as weight markers in gel electrophoresis. Primer synthesis and DNA sequencing were conducted by Macrogen, Korea.

Congo Red assay: *B. subtilis* WB800N secreting cellulases were cultured on LB-Agar containing Carboxymethyl cellulose (CMC) 0.5%, Cm 10 µg/mL and Isopropyl-β-D-thio-galactoside (IPTG) 0.5 mM at final concentration. The agar plate was incubated at 30°C for 24 hours for colony growth. Congo red dye 1% of 2 mL was added all over the agar surface for 5 minutes. Then, Congo red dye was rinsed by NaCl 1 M.

Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE): *B. subtilis* carrying each plasmid was cultured in 5 mL of LB-Cm media with 1 mM IPTG final concentration at 30°C for 18 hours.

The supernatants were collected by centrifuging the culture media at 13000 rcf for 10 minutes. The supernatant of 1600 µL was added with trichloroacetic acid (TCA) 50% of 400 µL, then kept on ice for 30 minutes for protein precipitation.

Table 1
***E. coli - B. subtilis* shuttle plasmids were used in this research.**

S.N.	Plasmids	Coding genes	Characteristics
1	pHT1454	*cel8A*	Amp^R, Cm^R, P_{grac}, SS_{AmyQ}, cel8A, His-tag
2	pHT1756B	*dock*	Amp^R, Cm^R, P_{grac}, type-1 dockerin
3	pHT1729	*cel5CCA*	Amp^R, Cm^R, P_{grac}, SS_{AmyQ}, Cel5CCA
4	pHT1757	*cel5CCA-dock*	Amp^R, Cm^R, P_{grac}, SS_{AmyQ}, chimeric cCel5CCA, His-tag
5	pHT1701[21]	*coh1-coh2-cbm-coh9-dock2*	Amp^R, Cm^R, 5'-lacA, 3'-lacA, P_{grac}, SS_{AmyQ}, coh1-coh2-cbm-coh9-dock2, StrepII-tag

pHT: *E. coli - B. subtilis* shuttle vectors, Amp^R: Ampicilin resistant gene as known as *bla* gene coding for enzyme β-lactamase, Cm^R: Chloramphenicol resistant gene as known as *cat* gene coding for enzyme Chloramphenicol acetyltransferase, P_{grac}: promoter P_{grac}, SS_{AmyQ}: signal sequence of enzyme amylase AmyQ from *Bacillus amyloliquefasciens*, dock: domain coding for type-1 dockerin from *C. thermocellum* Cel8A, His-tag: 8X Histidine tag. StrepII-tag: Streptavidin tag.

Table 2
Primers used in this research.

Purpose	Primers	Templates	Products (bp)	Nucleic acid sequences (5'-3')
Obtaining *cel8A*	ON915	*C. thermocellum* genome	1420	GGCCATAGATCTGCAAACACTGTGTCAGCGGCA
	ON916B			TGTCCATGTGATCCGCTATAAGGTAGGTGGGGTATGCTCTTTATC
Obtaining type-1 dockerin	ON1873	*E. coli* /pHT1454	272	CCAAGACAGATCCTGACGTCCCTCAGGTTGTTTACGGTGATG
	ON1874			TAGGCGGGCTGCCCCGGGTTAG
Obtaining *cel5CCA*	ON1093	*C. cellulolyticum genome*	1501	GGCGGATCCATGAAAAAAACAACAGCTTTTTTATTATG
	ON1094			GGCTCTAGACTGCTCGAGGGTTATTATCCCCTACTGAATGATACTT
Obtaining catalytic module of *cel5CCA*	ON1871	*E. coli* /pHT1729	1179	AGCATCAGCAGGATCCTATGATGCTTCACTTATTCCGAATC
	ON1872			TGTCCATGTGATCCGACGTCAGGATCTGTCTTGGCTTC
PCR colony for pHT1454	ON707	*E. coli* /pHT1454	1620	AAAGGAGGAAGGATCAATGAGAGGAAGCA
	ON916			TGTCCATGTGATCCGCTATAAGGTAGGTGGGGTATGCTCTTTATC
PCR colony for pHT1756B	ON1207	*E. coli* /pHT1756B	432	GGGAGATTCTTTATTATAAGAATTG
	ON1874			TAGGCGGGCTGCCCCGGGTTAG
PCR colony for pHT1729	ON1093	*E. coli* /pHT1729	1614	GGCGGATCCATGAAAAAAACAACAGCTTTTTTATTATG
	ON872			TCCTTTTTTTGAATTCAAGATCTCCATGGACGCGTGACG
PCR colony for pHT1757	ON1871	*E. coli* /pHT1757	1417	AGCATCAGCAGGATCCTATGATGCTTCACTTATTCCGAATC
	ON1874			TAGGCGGGCTGCCCCGGGTTAG
Sequencing pHT1454	ON314	pHT1454		TGTTTCAACCATTTGTTCCAGGT
	ON1207			GGGAGATTCTTTATTATAAGAATTG
Sequencing pHT1756B	ON1375	pHT1756B		GTTTCAACCATTTGTTCCAGGTAAG
Sequencing pHT1729	ON1207	pHT1729		GGGAGATTCTTTATTATAAGAATTG
	ON872			TCCTTTTTTTGAATTCAAGATCTCCATGGACGCGTGACG
Sequencing pHT1757	ON1207	pHT1757		GGGAGATTCTTTATTATAAGAATTG
	ON1375			GTTTCAACCATTTGTTCCAGGTAAG

Next, the pellet was collected and cleaned from TCA gently twice using chilled acetone of 400 µL and centrifuged at 13000 rcf for 10 minutes. The pellet was added with Laemmli buffer of 40 µL, vortexed and heated at 80°C for 10 minutes for protein denaturation. The protein sample of 10 µL was added per well of agarose 1% gel to run SDS-PAGE electrophoresis at 25 mA and 400 mV for 70 mins with a protein weight marker (10 to 200 kDa PageRuler Unstained Protein Ladder - Thermo Fisher Scientific).

Western blot: Samples for Western blot were prepared following the SDS-PAGE analysis procedure, using a pre-stained protein weight marker (10 to 250 kDa PageRuler Pre-stained Protein Ladder, Thermo Fisher Scientific).

After completing SDS-PAGE electrophoresis, the agarose gel and nitrocellulose membrane were soaked in Towbin buffer for 10 minutes. The membrane, towels and gel were then assembled into the Western blotting system (Bio-Rad Mini Protean II) and filled with a transfer buffer. The cartridge was run at 30 mV - 200 mA for 1 hour and the blotted membrane was rinsed in 20 mL of transfer buffer for 2 minutes.

Subsequently, it was gently shaken in 20 mL of blocking buffer PBS-T (0.1% Tween-20 in Phosphate Buffer Saline) containing 1% BSA (Bovine Serum Albumin) for 1 hour at room temperature. After removing the blocking buffer, the membrane was gently shaken for 1 hour in 20 mL of PBS-T containing an anti-His mouse antibody IgG linked with horseradish peroxidase (Anti-His mAb-HRP, Genescript, A00612) to detect the cellulases tagged with 8X Histidine at

C-terminal region. A chromogenic substrate of HRP, 3,3′,5,5′-Tetramethylbenzidine (TMB, Thermo Fisher Scientific), was added to the membrane to detect cellulase-antibody interaction.

Cohesin-dockerin analysis using modified enzyme-linked immunosorbent assay (Elisa): *B. subtilis* carrying each plasmid was cultured in LB-Cm at 30ºC with 1 mM IPTG for 18 hours. 50 mL culture was centrifuged at 10,000 rcf for 10 minutes at 4ºC to collect the supernatant. The supernatant fractions containing Cel5CCA and Cel8A were collected using Ultra Centrifugal Filters MWCO 30 kDa and 100 kDa (Merck Millipore), while the supernatant fractions containing mini-CipA were collected using Ultra Centrifugal Filters MWCO 50 kDa and 100 kDa. The protein concentration was measured using Bradford assay. The proteins were then precipitated with the anhydrous $(NH_4)_2SO_4$ and centrifuged at 10,000 rcf for 10 minutes at 4ºC to collect the protein pellets, which were quickly washed with PBS twice. Protein pellets of approximately 100 µg were diluted with a coating buffer (PBS-T) at dilution ratios of 1/10, 1/100, 1/1000, 1/10000.

A diluted sample of 100 µL was coated per well of an ELISA 96-well microplate (Nunc-Immuno MicroWell 96-Well Plates, Thermo Scientific) and gently shaken for 2 hours. After removing the coating buffer, 100 µL of the blocking buffer (PBS-T + 1% BSA) was added to each well and gently shaken for 2 hours. Subsequently, the blocking buffer was removed and 100 µL of mini-CipA 1 µg/ml fused with Strep-tag in PBS-T was added. The microplate was gently shaken for 1 hour and washed with 100 µL of PBS-T per well three times. The wells were then incubated with 100 µL of a diluted anti-Strep mouse antibody IgG linked with horseradish peroxidase (anti-StrepII mAb-HRP, GeneScript, 5A9F9) in PBS-T for 1 hour. After rinsing with 100 µL of PBS-T per well three times, 50 µL of TMB was added to each well and the reaction was stopped with 50 µL of HCl 1%. The absorbance of the reaction was measured at the wavelength of 450 nm using a 96-well microplate reader (CLARIOstar, BMG Labtech).

CMCase activity was measured using a 96-well microplate Dinitro salicylic acid (DNS) assay: Samples included *B. subtilis* WB800N carrying pHT1454/Cel8A, pHT1757/Cel5CCA, pHT1701/mini-CipA. A control was prepared with *B. subtilis* WB800N carrying a non-coding gene plasmid. Each sample was cultured in 50 mL of LB-Cm medium for 18 hours at 30ºC with shaking at 250 rpm. When the OD 600 nm reached approximately 1, IPTG was added at the concentrations of 0.00, 0.01, 0.10 and 1.00 mM. Samples were collected at 6, 12, 18 and 24 hours after adding the inducer.

After each culture period, 2 mL of the culture was centrifuged at 10,000 rcf for 10 minutes at 4ºC to collect the supernatant. 30 µL of the supernatant was added to each well of a PCR 96-well microplate. Next, 30 µL of buffer containing 0.5% CMC in 50 mM sodium succinate pH 5.4 and 10 mM $CaCl_2$ was mixed with the supernatant. The microplate was incubated at 55ºC for 1 hour using a PCR thermocycler (Eppendorf 5333 MasterCycler Thermal Cycler). Then, 60 µL of 1% DNS was added to each well and the microplate was incubated at 95ºC for 5 minutes.

The mixtures were transferred to an Elisa 96-well microplate and the absorbance of the reaction was measured at a wavelength of 540 nm using a 96-well microplate reader. A standard curve was prepared at the various concentrations of glucose (0, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml). Three replicates were performed.

CMCase activity of the endoglucanases with scaffoldin: The supernatants of culture media from *B. subtilis* WB800N carrying pHT1454/Cel8A, pHT1757/cCel5CCA, pHT1701/mini-CipA at 18 hours with 0.1 mM IPTG as the inducer were collected to prepare 6 mixtures of proteins. The labels of the mixtures from 1 to 6 respectively contain: (1) Cel8A, (2) Cel8A + mini-CipA, (3) cCel5CCA, (4) cCel5CCA + mini-CipA, (5) Cel8A + cCel5CCA, (6) Cel8A + cCel5CCA + mini-CipA. The ratios of the mixtures include: 1:0:0, 1:1:0 or 1:1:1 where '1' represents the addition of the cellulase or scaffoldin and '0' represents the addition of the control. The mixtures were then measured for CMCase activity using a 96-well microplate dinitro salicylic acid (DNS) assay as described above.

Results

Cloning Cel5CCA: The products of gene amplification from *C. cellulolyticum* genome included cel5CCA (1501 bp) and the catalytic module of cel5CCA (1179 bp) while the products from *C. thermocellum* genome included type-1 dockerin of cel8A (272 bp) and cel8A (1420 bp) (Fig. 1C). The catalytic module of cel5CCA, which was fused with type-1 dockerin of cel8A, was cloned in *E. coli* to create pHT1757. DNA sequencing confirmed that pHT1757 coded for the designer chimeric cCel5CCA (Fig. 1E).

Congo red assay, SDS-PAGE, Western blot: The halo assay on CMC agar plate using Congo Red dye showed large halo rings around the colonies secreting Cel5CCA, (chimeric) cCel5CCA and Cel8A (Fig. 2A). SDS-PAGE (Fig. 2B) and Western blot (Fig. 2C) of the extracellular proteins from *B. subtilis* WB800N carrying the plasmids pHT1757 and pHT1454 detected protein bands of approximately 50 kDa which were consistent with theoretical molecular masses of cCel5CCA (52 kDa, 460 aas) and Cel8A (51 kDa, 461 aas) respectively. The corresponding bands were absent in the control samples of culture media without IPTG. The results of SDS-PAGE, Western blot and Congo red assay indicated that Cel8A and cCel5CCA were secreted in the active forms of full length.

Cohesin-dockerin interaction: The wells coated with Cel8A and cCel5CCA whose type-1 dockerin from *C.*

thermocellum presented signals of cohesin-dockerin interaction, were the same as the wells coated with mini-CipA (positive control) carrying type-1 cohesins (Figures 3B and 3C). In contrast, the wells coated with wild-type Cel5CCA from *C. cellulolyticum* showed no signal of the cohesin-dockerin interaction. When the concentration of coated proteins was reduced, the signals of cohesin-dockerin interaction also decreased. The results confirmed the species-specificity of cohesin-dockerin.

CMCase activity of the endoglucanases: Under the same conditions of the culture process and inducer concentration (Fig. 4A), CMCase activity of Cel5CCA was higher than that of Cel8A. In the early phases after adding the inducer, CMCase activity of secreted Cel5CCA was significantly higher than that of Cel8A at 6 hours and 12 hours, but it decreased gradually and became approximately equal to that of Cel8A at 18 hours and 24 hours. When increasing the concentration of the inducer IPTG, the CMCase activity of both cellulases increased gradually from 0 to 0.1 mM, then changed insignificantly from 0.1 mM to 1 M.

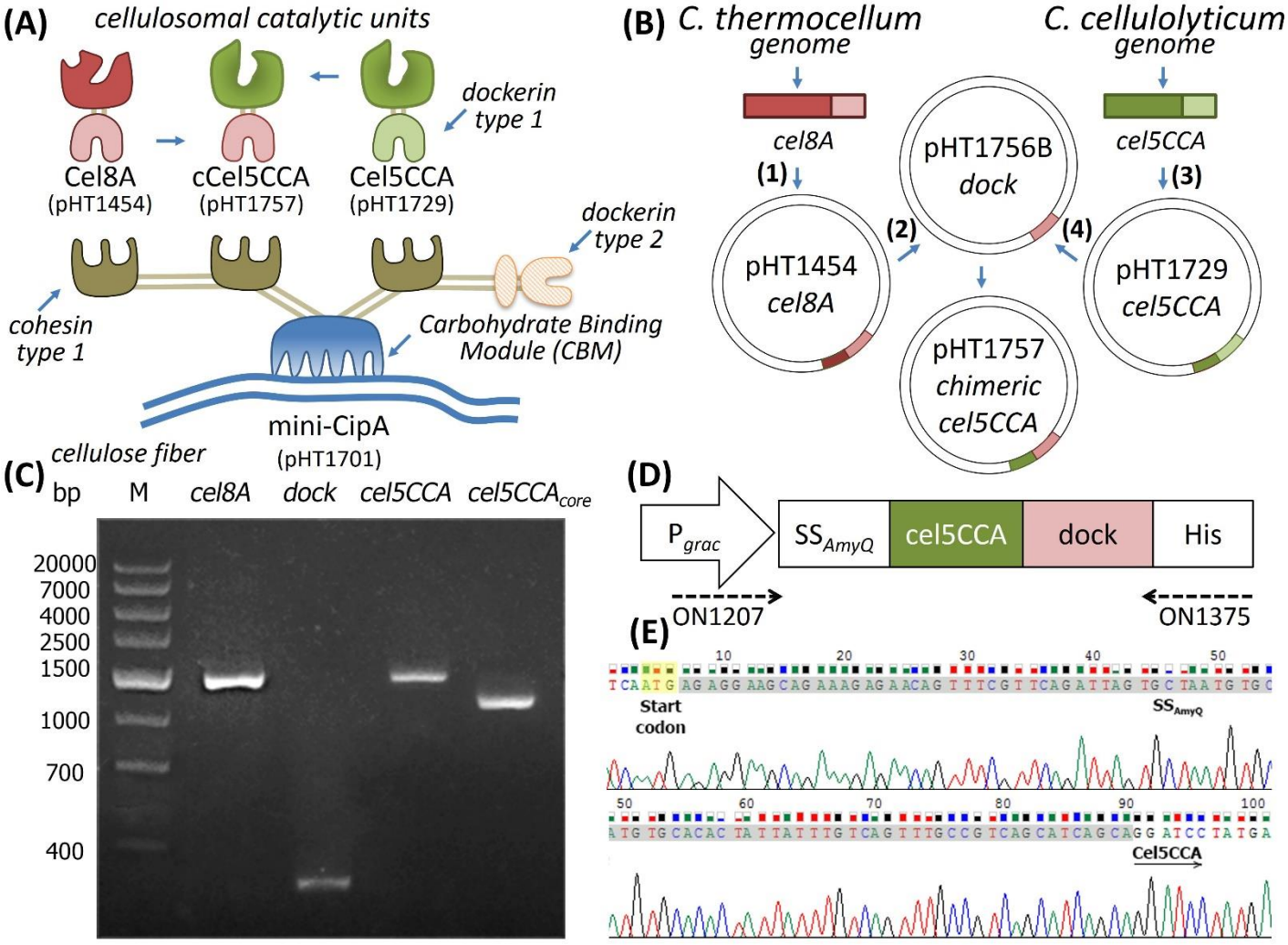

Figure 1: Plasmids encoding chimeric Cel5CCA were designed in this study.
(**A**) A cellulosome consists of numerous cellulosomal catalytic units or cellulases and a scaffoldin mini-CipA. The chimeric cCel5CCA was created by combining the catalytic module of Cel5CCA from *C. cellulolyticum* with the type-1 dockerin of Cel8A from *C. thermocellum*. (**B**) Plasmids encoding for the chimeric cCel5CCA were designed in four steps. Step 1: cel8A was amplified from the *C. thermocellum* genome and inserted into the pHT backbone plasmid to create pHT1454. Step 2: The type-1 dockerin of cel8A was amplified from pHT1454 and inserted into the pHT backbone plasmid to create pHT1756B. Step 3: cel5CCA was amplified from the *C. cellulolyticum* genome and inserted into the pHT backbone plasmid to create pHT1729. Step 4: The catalytic module region of cel5CCA was amplified from pHT1729 and inserted to pHT1756B to create pHT1757 encoding the chimeric cCel5CCA. (**C**) PCR products of *cel8A* (1st well, 1420 bp), type-1 dockerin of *cel8A* (2nd well, 272 bp), *cel5CCA* (3rd well, 1501 bp) and the catalytic module of endoglucanase *cel5CCA* (4th well, 1179 bp). (**D**) The expression cassette of pHT1757 contains the promoter P$_{grac}$, signal sequence SS$_{AmyQ}$, the catalytic module of cel5CCA fused with the type-1 dockerin and an 8X-Histidine tag. The position of the sequencing primers were marked with dashed arrows. (**E**) DNA sequencing of pHT1757 was conducted with the primer ON1207 for the regions of SS$_{AmyQ}$ and *cel5CCA*

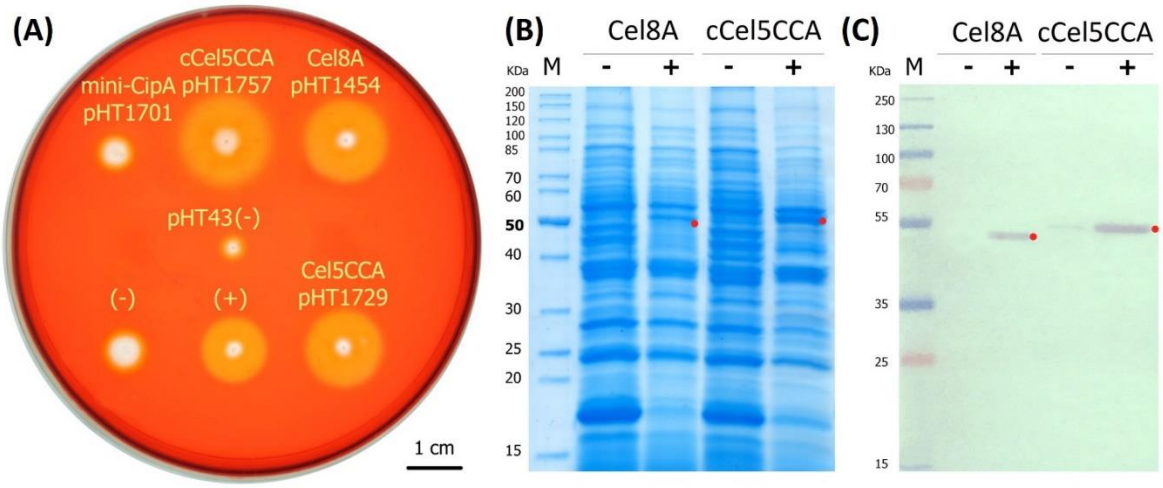

Figure 2: Analyses of extracellular proteins of *B. subtilis* WB800N.
(**A**) CMCase activity of *B. subtilis* WB800N carrying plasmids using Congo Red assay. (-/+): control strains; pHT43: *B. subtilis* WB800N carrying non-coding plasmid. The halo circular regions exhibited around the colonies secreting endoglucanases. (**B**) SDS-PAGE of culture media from *B. subtilis* WB800N carrying plasmids. (**C**) Western blot with anti-8xHis tag antibody. The bands of chimeric cCel5CCA (52 kDa) and Cel8A (51 kDa) were marked with red dots. (-/+) Culture media without/with the addtion of IPTG

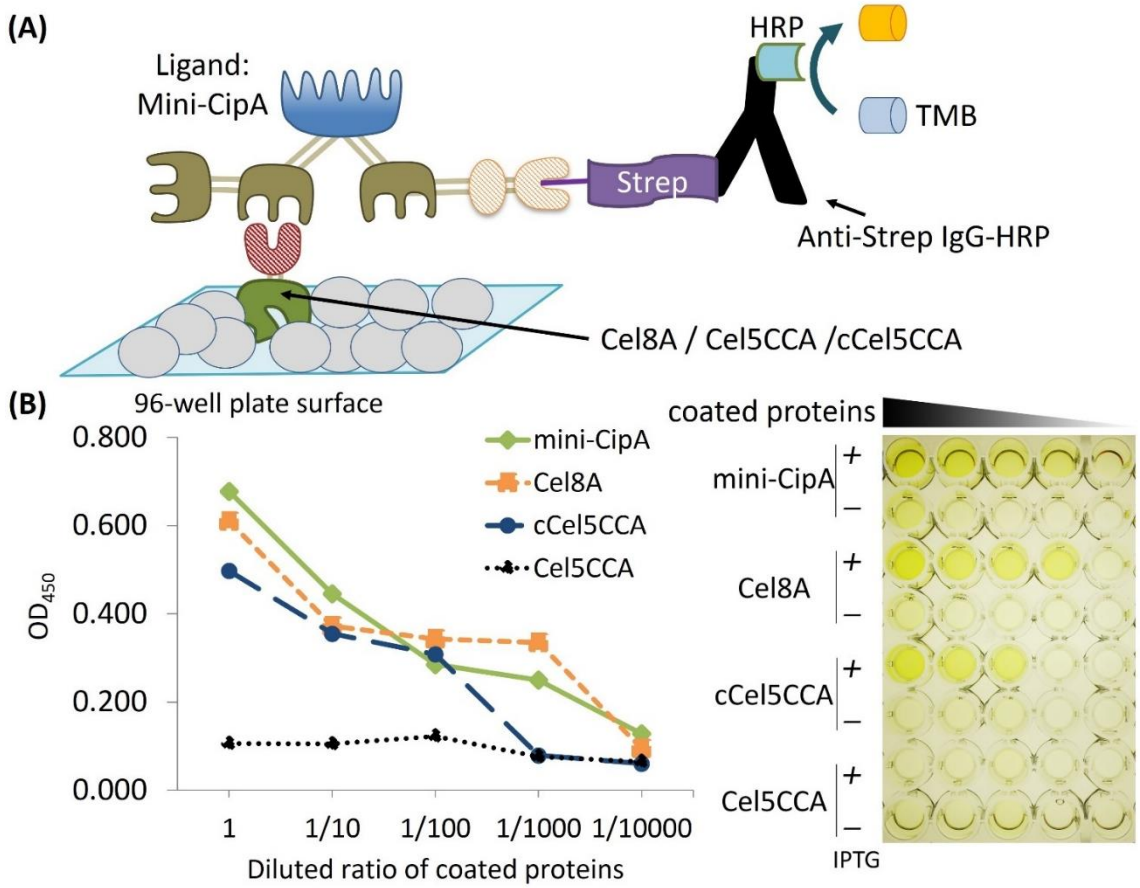

Figure 3: Elisa analysis of type-1 cohesin and dockerin interactions.
(**A**) Each cellulase was coated on microplate well surface, then mini-CipA fused with Strep tag at C- terminus was also added to the well. The interaction between type-1 dockerin of the cellulase and type-1 cohesin of the mini-CipA was dectected by the anti-Strep antibody conjugated with horseradish peroxidase (HRP). TMB, a chromogenic substrate of HRP, would turn yellow after adding HCl. The absorbance at a wavelength of 450 nm (OD_{450}) of the yellow TMB-HRP reaction indicated the amount of cohesin-dockerin interaction in the well. (**B**) A reduced concentration of coated proteins led to a decrease in OD_{450}. (**C**) The concentrations of coated proteins decreased in the wells from left to right, corresponding with the diluted ratio from 1 to 10^{-4}. (+/-): Coated proteins were concentrated from the culture media with/without the inducer IPTG

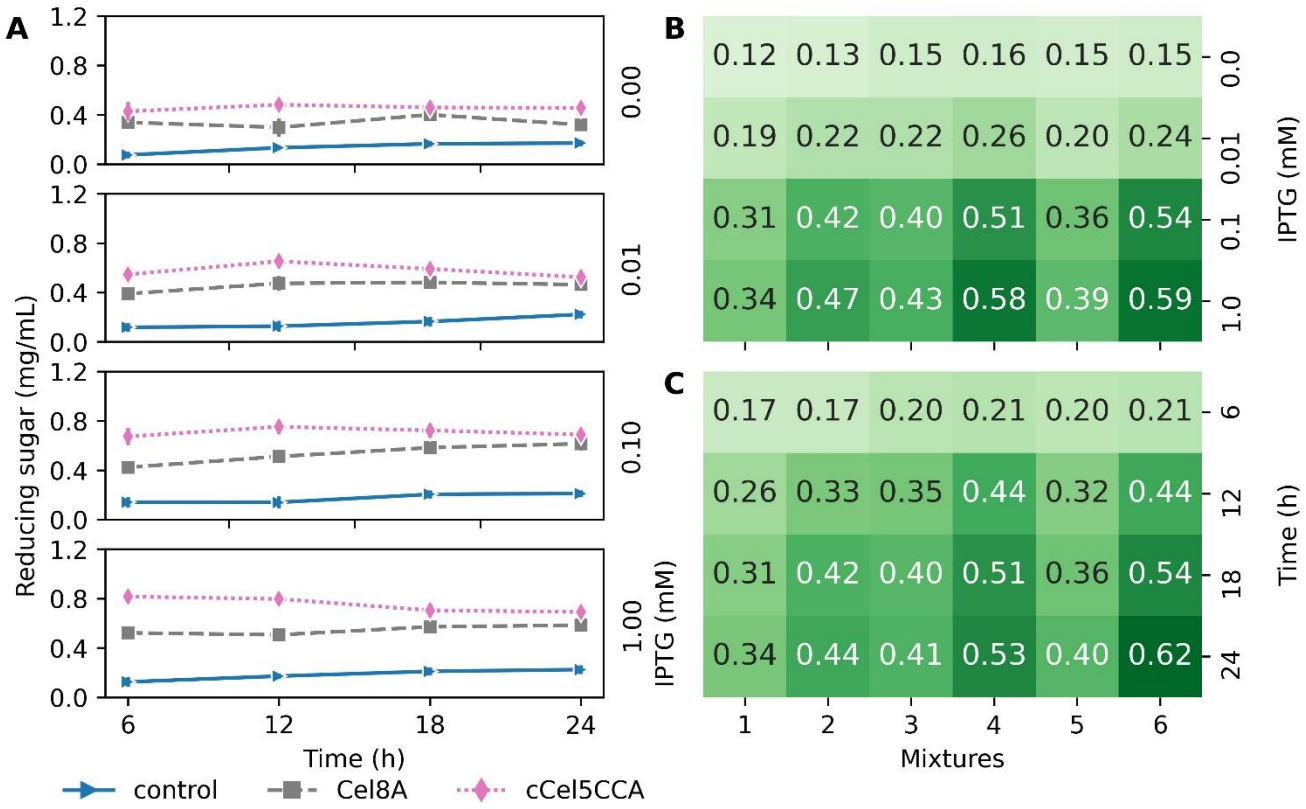

Figure 4: Analysis of CMCase activity of extracellular culture media from *B. subtilis* WB800N.
(**A**) CMCase activity was measured by DNS assay, quantified via equivalent reducing sugar (mg/mL) as the reaction product of Cel8A and cCel5CCA expressed by *B. subtilis* WB800N in culture media at various IPTG concentrations and culture periods with CMC as subtrate. (**B**) The reducing sugar (mg/mL) released by CMCase activity from mixtures of cellulases and scaffoldin at 18 hours. (**C**) Reducing sugar (mg/mL) released by CMCase activity from the mixtures of cellulases and scaffoldin at 0.1 mM IPTG. The labels of mixtures from 1 to 6 respectively contain: (1) Cel8A, (2) Cel8A + mini-CipA, (3) cCel5CCA, (4) cCel5CCA + mini-CipA, (5) Cel8A + cCel5CCA, (6) Cel8A + cCel5CCA + mini-CipA. The ratios of the mixtures include: 1:0:0, 1:1:0 or 1:1:1, where '1' represents the addition of the cellulase or scaffoldin and '0' represents the addition of the control

CMCase activity of the endoglucanases with scaffoldin: When mixing the secreted proteins collected at 18 hours (Fig. 4B) or induced with 0.1 mM IPTG (Fig. 4C), the CMCase activity of the mixtures contaning mini-CipA in the mixtures 2, 4 and 6 increased respectively by 34%, 31% and 52% compared to those of the mixtures 1, 3 and 5 without mini-CipA. CMCase activity of the mixtures containing only cCel5CCA (the mixtures 3) and cCel5CCA+Cel8A (the mixtures 5) were higher than those of the mixtures containing only Cel8A (the mixtures 1).

Discussion

Cel8A is one of the first cellulosomal endoglucanases isolated in *C. thermocellum*[24]. It has a molecular mass of 52.6 kDa and a length of 477 amino acids, including a 32 amino acid signal sequence at the N-terminal region and a 67 amino acid type 1 dockerin at the C-terminal region (Uniprot: A3DC29). The catalytic module belongs to the GH8 family, with a protein structure that is a symmetrical circular $(\alpha/\alpha)_6$ barrel made up of six inner six outer α helices[1].

On the other hand, Cel5CCA is a cellulosomal endoglucanase isolated in *C. cellulolyticum*[5]. It has a molecular mass of 53.6 kDa and a length of 475 amino acids including a 26 amino acid signal sequence at the N-terminal region and a 66 amino acid type 1 dockerin at the C-terminal region (Uniprot: P17901). The catalytic module of Cel5CCA belongs to the GH5 family, with a protein structure that is a $(\beta/\alpha)_8$ barrel consisting of eight β sheets and eight α helices[4].

Schwarz et al[27] reported two bands of 49 kDa and 52 kDa which were suggested to be cleaved and native forms of Cel8A were expressed intracellularly in *E. coli*. Soutschek-Bauer et al[29] also observed a bold band of 48 kDa and a light band of 52 kDa in the extracellular protein electrophoresis when *B. subtilis* 168, a wild strain of *B. subtilis* WB800N, was used to express secreted Cel8A. Joliff et al[11] reported a dominant 46 kDa band when expressing Cel8A extracellularly by *B. subtilis* 168. However, Liu et al[14] reported the only 52 kDa protein band of Cel8A when using *B. subtilis* WB600 and WB800, earlier versions of *B. subtilis* WB800N.

On the other hand, Fierobe et al[5] reported that recombinant Cel5CCA intracellularly expressed in *E. coli* exhibited two bands and suggested a 51 kDa band as the full form, while a 44 kDa band was the cleaved form at dockerin in SDS-PAGE electrophoresis. In this study, only the uncleaved forms of Cel8A and Cel5CCA were detected when using *B. subtilis* WB800N, a deficient protease strain, hence could reduce protein degradation in extracellular expression.

While the affinity constants K_a of IgG antibodies with antigens generally ranged approximately from 10^5 M^{-1} to 10^9 M^{-1},[22] the $K_a > 10^9$ M^{-1} of the cohesin-dockerin interaction was rather high[18,26]. The species-specificity of cohesin-dockerin was tested in purified forms of chimeric cellulosomes intracellularly expressed in *E. coli* of *C. thermocellum* and *C. cellulolyticum*[23]. The cohesin-dockerin is also stable in extracellular forms in this study.

The CMCase activity of chimeric Cel5CCA is higher than that of Cel8A under the same conditions. Several hypotheses have been proposed to explain this phenomenon. First, the mesophilic *C. cellulolyticum* Cel5CCA has a temperature optimum at 50°C while the thermophilic *C. thermocellum* Cel8A has an optimal temperature at 75°C[5,27]. Therefore, Cel5CCA showed higher activity than Cel8A when measuring CMCase activity using the DNS assay at 50°C[32].

Secondly, although both proteins are expressed under the same culture conditions, differences in the composition and the structure of amino acids may result in variations in the speed of translation, folding and secretion. The rate of secretion can impact the concentration of enzymes present in extracellular culture samples at the time of harvest. High-throughput absolute quantification of secreted proteins in realtime without using indirect measurement of catalytic products has not been investigated.

Compared to non-cellulosomal cellulases which typically carry their own CBMs, cellulosomal cellulases rely on the CBMs of scaffoldins to enhance their attachment to substrate fibers. Currently, there is no evidence supporting the hypothesis that utilizing the CBMs of scaffoldins would result in greater energy and material savings when adapting to anaerobic living, compared to using their own CBMs. Non-cellulosomal cellulases from mesophilic aerobic fungi have shown high rates of substrate degradation, but using cellulosomal cellulases could be an effective approach for dealing with complex substrates in harsh conditions[10].

Conclusion

Cellulosomes are secreted protein complexes containing a variety of catalytic modules that can efficiently degrade lignocellulose. Chimeric cellulosomes have been created from different genetic sources to enhance their hydrolytic ability on a wide range of substrates and perform well under extreme conditions. In this study, a chimeric endoglucanase called cCel5CCA was expressed extracellularly in *B. subtilis* WB800N using the catalytic module of *C. cellulolyticum* and the type-1 dockerin of *C. thermocellum*.

Qualitative analyses using Congo red assay, SDS-PAGE, Western blot and Elisa indicated that *B. subtilis* WB800N secreted active chimeric Cel5CCA that can bind to mini-CipA using species-specific cohesin-dockerin interaction. The CMCase activity of Cel5CCA can be increased by 34% when assembled with mini-CipA. When synergistically collaborated with Cel8A and mini-CipA, the mixture of Cel5CCA can enhance CMCase activity by 50%.

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