Enhanced microbial cellulose utilization of recalcitrant cellulose 
by an ex vivo cellulosome-microbe complex

Running title: Cellulosome-microbe synergy

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ABSTRACT

A cellulosome-microbe complex was assembled *ex vivo* on the surface of *Bacillus subtilis* displaying a mini-scaffoldin that can bind with three dockerin-containing cellulase components -- an endoglucanase Cel5, a processive endoglucanase Cel9, and a cellobiohydrolase Cel48. The hydrolysis performance of the synthetic cellulosome bound to living cells, the synthetic cellulosome, a non-complexed cellulase mixture with the same catalytic components, and a commercial fungal enzyme mixture was investigated on low-accessibility recalcitrant Avicel and high-accessibility regenerated amorphous cellulose. The cell-bound cellulosome exhibited 4.5- and 2.3-fold higher hydrolysis ability than cell-free cellulosome on Avicel and RAC, respectively. The cellulose-microbe synergy was not completely explained by the removal of hydrolysis products from the bulk fermentation broth by free living cells, and appeared due to substrate channeling of long chain hydrolysis products assimilated by the adjacent cells located in the boundary layer. Our results implied that long-chain hydrolytic products in the boundary layer may inhibit cellulosome activity greater than the short-chain products in bulk phase. The findings that cell-bound cellulosome expedited microbial cellulose utilization rate by 2.3-4.5 fold would help develop better consolidated bioprocessing microorganisms (e.g., *B. subtilis*) that can hydrolyze recalcitrant cellulose rapidly at low secretory cellulase levels.

**Keywords:** *Bacillus subtilis*, cellulase, consolidated bioprocessing, cellulosome-microbe synergy, cellulose-cellulosome-microbe complex, synthetic cellulosome
INTRODUCTION

Biofuels and commodity chemicals produced from cellulosic biomass are of interest as sustainable substitutes for functionally-similar molecules based on petroleum. The primary obstacle to biological production of such products is cost-effective technology to overcome the recalcitrance of cellulosic biomass (19, 22, 37).

Consolidated bioprocessing (CBP) - in which saccharolytic enzyme production, plant cell wall solubilization, and fermentation occur in a single step – is widely seen as a promising low cost processing route (18, 22, 24, 37). CBP microorganisms can be developed according to three strategies: (i) engineering naturally-occurring cellulolytic microorganisms for improved product formation-related properties, such as Clostridium thermocellum (6), Clostridium cellulovorans (29), and Clostridium phytofermentans ISDg (30), (ii) engineering natural high-yield product-forming microorganisms by expressing recombinant cellulases, such as Saccharomyces cerevisiae (16, 31, 34), and (iii) engineering one host with both recombinant product-forming and cellulose-utilizing abilities, such as Escherichia coli (15) and Bacillus subtilis (2, 26, 37).

Nature has evolved two distinctive cellulase systems for degrading cellulosic material: non-complexed cellulase mixtures and complexed cellulases, called cellulosomes. Aerobic fungi (e.g., Trichoderma reesei) usually secrete high levels (e.g. >1-10 g protein/L) of several different functionally-distinct cellulase components. By contrast, some anaerobic bacteria, such as C. thermocellum and C. cellulovorans, produce low levels of cellulosomes (i.e., ~0.1 g/L), in which many glycoside hydrolases are linked.
together by non-hydrolytic scaffoldins through the high-affinity interaction between cohesins in scaffoldins and enzyme-borne dockerins (3, 7-10). *C. thermocellum* exhibits among the highest growth rates on cellulose among described microbes (24), although it produces less cellulase per cell mass than aerobic microorganisms. This observation raises an interesting question – how anaerobic cellulolytic microorganisms can hydrolyze cellulose rapidly and effectively without the production of ample secretory cellulase, where the biosynthesis of cellulase means a large bioenergetic burden for anaerobic cellulolytic bacteria. Recently, *in vitro* evidence pertaining to designer cellulosomes suggests that designer cellulosomes exhibit faster hydrolysis rate than their non-complexed counterparts due to an enzyme proximity synergy (25, 32, 41). Zverlov et al. (43) reported that a *C. thermocellum* mutant featuring a completely defective scaffoldin protein exhibited a 15-fold reduction in specific cellulase activity on crystalline cellulose. Furthermore, Lynd et al. (21) found out that *C. thermocellum* along with cell-bound cellulosome exhibited ca. 2.8-4.7-fold enhanced cellulose hydrolysis rates on Avicel compared to purified cellulosome in the presence of another soluble sugar-utilizing microorganism (21). Several recent studies have expressed mini-cellulosomes on the surface of microorganisms, such as *B. subtilis* (1, 5) and *S. cerevisiae* (31, 34) but did not quantitatively evaluate the enzyme-microbe synergy.

In this study, mini-CipA was displayed on the cell surface of *B. subtilis* through a cell wall-binding module of a *B. subtilis* cell wall hydrolase, LytE. A tri-functional mini-cellulosome was assembled *ex vivo* on the cell surface of *B. subtilis*. The hydrolysis performance of a three-enzyme mixture, cell-free mini-cellulosome, cell-bound mini-
cellulosome as well as a commercial fungal cellulase mixture was compared on low-accessibility Avicel and high-accessibility regenerated amorphous cellulose (RAC).

MATERIALS AND METHODS

Chemicals. All chemicals were reagent grade or higher, purchased from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA), unless otherwise noted. Microcrystalline cellulose – Avicel PH105 (20 µm) – was purchased from FMC (Philadelphia, PA). RAC was prepared from Avicel as previously described (19, 38). The oligonucleotides were synthesized by Integrated DNA Technologies (Coraville, IA). The PCR enzyme was high-fidelity Phusion DNA polymerase from New England Biolabs (Ipswich, MA). A commercial Trichoderma cellulase mixture (50013) was gifted from Novozymes North America (Franklinton, NC). The purified fungal enzymes of cellobiohydrolase I (CBH I, Cel7A) and endoglucanase II (EG II, Cel5) from Trichoderma spp. were purchased from Megazyme (Wicklow, Ireland).

Strains and media. The strains and plasmids used in this study are listed in Table 1. E. coli JM109 was used as a host cell for DNA manipulation. E. coli BL21 Star (DE3) (Invitrogen, Carlsbad, CA) and B. subtilis WB600 (35) were used as the hosts for recombinant protein expression. B. subtilis was transformed through a new simple and fast transformation technology as described elsewhere (38). The Luria-Bertani (LB) medium was used for E. coli cell culture and recombinant protein expression. 2 X Mal medium was used for B. subtilis recombinant protein expression (38). The final concentrations of antibiotics for E. coli were 100 mg/L ampicillin and 25 mg/L chloramphenicol. The chloramphenicol concentration for B. subtilis was 5 mg/L.
Construction of plasmids. The primers used in this study are listed in Table 2. For constructing pNWP43N-LysM, the DNA sequence encoding the *B. subtilis* cell wall hydrolase (LysM, GenBank Accession number: U38819, 25-230 amino acids) was amplified from the genomic DNA of *B. subtilis* 168 by a primer pair of LysM_For and LysM_Rev_Flag; the DNA sequence encoding a vector pNWP43N was amplified from pNWP43N-BsCel5 (38) by a primer pair of pNWP43N_For and pNWP43N_Rev. The two PCR products were both digested with NheI/XhoI and then ligated, yielding pNWP43N-LysM. For constructing pNWP43N-LysM-mini-cipA (pNWP43N-LMC), the DNA sequence encoding LysM was amplified by using a primer pair of LysM_For and LysM_Rev based on the *B. subtilis* genomic DNA by PCR, followed by double digestion by XhoI/EcoRV. The DNA sequence encoding truncated mini-CipA (GenBank Accession number: L08665, 26-723 amino acids) was amplified from the genomic DNA of *C. thermocellum* by a primer pair of MC_For and MC_Rev_Flag, followed by double digestion by EcoRV/NheI. The two resultant fragments were ligated into the XhoI/NheI-digested vector pNWP43N to produce pNWP43N-LMC. The DNA sequence encoding truncated mini-CipA was amplified from the genomic DNA of *C. thermocellum* ATCC27405 by a primer pair of mini-CipA_For and mini-CipA_Rev. The PCR product was digested with NdeI/XhoI and then ligated into the NdeI/XhoI-digested vector pET20b (Novagen, Madison, WI), yielding pET20b-mini-CipA. pET20b-Bscel5’ was obtained by using the overlap extension PCR. The DNA sequence encoding mature BsCel5 (GenBank Accession number: CAA82317) was amplified from genomic DNA of *B. subtilis* 168 by a primer pair of BsCel5_For / BsCel5’_Rev. The DNA fragment encoding a dockerin module (DocK, 821-895 amino
acids) of *C. thermocellum* CelK (NCBI Reference Sequence: YP_001036843) was amplified from the genomic DNA of *C. thermocellum* by a primer pair of DocK_For/DocK_Rev. The two resultant fragments were assembled by using a primer pair of BsCel5_For/DocS_Rev through overlap extension PCR. These resultant fragments were cloned into NdeI/Xhol-digested pET20b, generating pET20b-Bscel5’. pET20b-Ctcel9 was obtained by using PCR amplification and overlap extension PCR. The DNA encoding the mature *C. thermocellum* Cel9 (GenBank Accession number: CAA43035) was amplified from the genomic DNA of *C. thermocellum* by a primer pair of CtCelF_For/CtCelF_Rev. The PCR product was digested with NdeI/XhoI and ligated into the corresponding sites of the vector pET20b, yielding pET20b-Ctcel9. The DNA sequence encoding a mature *C. phytofermentans* Cel48 (GenBank Accession number: ABX43721) were amplified from pP43N-Cpcel48 (39) by a primer pair of CpCel48_For/CpCel48_Rev. Plasmid pET20b-Cpcel48 was constructed in the same way as that of pET20b-Bscel5’. The dockerin of Cpcel48 was DocS of the *C. thermocellum* Cel48S (GenBank Accession number: L06942, 673-741 amino acids). All plasmid sequences were verified by DNA sequencing. The resulting plasmids are listed in Table 1.

**Production of dockerin-containing cellulases in E. coli.** The strain *E. coli* BL21 Star (DE3) containing the protein expression plasmid was cultivated in the LB medium supplemented with 1.2% glycerol at 37 °C. Protein expression and purification was conducted routinely as published elsewhere (19, 37, 38).

**Removal of the *B. subtilis* surface proteins by LiCl.** *B. subtilis* cells harboring pNWP43N-LMC were pre-cultured in the LB medium at 37°C till the A₆₀₀ reached about 1.2, which remained at a logarithmic growth phase. Two hundred microliter of the cell
culture was inoculated into 50 mL of 2X Mal medium and then grown at 30°C till $A_{600}$ reached three. Two mL culture of the *B. subtilis* cells were washed two times in Buffer A (50 mM HEPES buffer, pH 7.5, containing 50 mM NaCl and 10 mM CaCl$_2$). The cell pellets were resuspended in 80 µL of Buffer B (50 mM HEPES buffer, pH 7.5, containing 5 M LiCl, 50 mM NaCl, and 10 mM CaCl$_2$). After incubation for 20 min on ice followed by centrifugation at 8,000 g at 4°C for 10 min, a fraction of the supernatant after 10% trichloroacetic acid precipitation was loaded in SDS-PAGE. The other fraction of the supernatant was diluted in Buffer A by five folds, and then mixed with 50 µg RAC. After centrifugation, the adsorbed LMC by RAC was examined by SDS-PAGE, as described elsewhere (37, 38). For validation of the formation of *ex vivo* mini-cellulosome, the resuspended cells with bound LMC were mixed with 0.05 mg purified dockerin-tagged cellulases (BsCel5′ or cellulase mixture with equimolar of BsCel5′, CtCel9 and CpCel48) at 4°C for 1 h. The cells were washed in Buffer A two times. The cell-bound mini-cellulosome was eluted by LiCl, adsorbed by RAC, and examined by SDS-PAGE, as described above.

**Confocal immunofluorescence microscopy.** *B. subtilis* cells (200 µL of cell culture at $A_{600} = 3.0$) having surface-displayed LMC or cell-bound mini-cellulosome were washed in ice-cold phosphate-buffered saline (PBS, 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na$_2$HPO$_4$, and 0.24 g/L KH$_2$PO$_4$) two times, and then mixed with 4% para-formaldehyde at 4°C for 30 min. After wash in 1 mL of PBS two times, the cells were resuspended in 250 µL of PBS containing 1 mg/mL of bovine serum albumin (BSA) and 0.5 µg of monoclonal anti-Flag M2 (Sigma F1804) or monoclonal anti-His (Sigma H1029) antibody with occasional mixing for 2 h. The cells were washed in 1 mL of PBS for two times, followed by
resuspension in 250 µL of PBS containing 1 mg/mL BSA and 0.5 µg anti-mouse IgG conjugated with FITC (Sigma F9137). After incubation for 2 h, cells were washed with 1 mL of PBS two times, and then resuspended in PBS to obtain the cell solution with $A_{600} = 1$. The cells were examined by the ZEISS LSM 510 confocal LASER microscope (Thornwood, NY).

**RAC and Avicel hydrolysis.** All cellulose hydrolysis experiments were conducted in 50-mL serum bottles with a rotary shaking rate of 250 rpm at 37 °C. Equimolar of BsCel5’, CtCel9 and CpCel48 were pre-mixed together, where molecular weights of BsCel5’, CtCel9 and CpCel48 were 44,918, 80,108 and 107,184, respectively. Equimolar of mini-CipA was mixed with the three-cellulase mixture for the formation of tri-functional mini-cellulosome. Similarly, the number of LMC on the surface of *B. subtilis* was determined as described elsewhere (4). The cell culture containing equimolar of LMC was mixed with the three-cellulase mixture for the formation of cell-bound cellulosome. For RAC hydrolysis, the LMC-displayed *B. subtilis* cells collected from 33.3 mL of the cell culture with $A_{600} = 3.0$ were mixed with 0.1 mg (total) three-cellulase mixture in 10 mL of the ice-cooled Buffer A containing 0.4% RAC followed by hydrolysis at 37°C. The LysM-displayed *B. subtilis* cells collected from 33.3 mL of the cell culture with $A_{600} = 3.0$ were mixed with tri-functional mini-cellulosome containing 0.1 mg (total) three-cellulase mixture in 10 mL of the ice-cooled Buffer A containing 0.4% RAC followed by hydrolysis at 37°C. For Avicel hydrolysis, the LMC-displayed *B. subtilis* cells collected from 133.3 mL of the *B. subtilis* cell culture with $A_{600} = 3.0$ were mixed with 0.4 mg of the three-cellulase mixture in 10 mL of the ice-cooled Buffer A containing 0.4% Avicel followed by hydrolysis at 37°C. The LysM-displayed *B. subtilis*
cells collected from 133.3 mL of the cell culture with A$_{600}$ = 3.0 were mixed with tri-functional mini-cellulosome containing 0.4 mg (total) three-cellulases in 10 mL of the ice-cooled Buffer A containing 0.4% Avicel followed by hydrolysis at 37°C. The cellulose hydrolysis by the same amount of the LMC-displayed *B. subtilis* cells or LysM-displayed *B. subtilis* cells without heterologously-added cellulase was performed as a negative control. The same cellulase (mass) concentrations of mini-cellulosome and the three-cellulase mixture were also used to hydrolyze RAC and Avicel in 10 mL Buffer A in the presence of 60 units of β-glucosidase (Bgl, Novozymes 188 from Sigma) per gram of cellulose, respectively. A commercial Novozymes cellulase and a two-enzyme *Trichoderma* fungal cocktail containing EG II and CBH I at the same mass concentration as the bacterial three-cellulase mixture were used to hydrolyze RAC and Avicel at 37°C in 50 mM citrate buffer (pH 5.0) containing 50 mM NaCl and 10 mM CaCl$_2$ in the presence of 60 units of β-glucosidase per gram of cellulose, respectively. In the two-enzyme cocktail, the mass amount of EG II was the same as the sum of BsCel5’ and CtCel9 and the mass amount of CBH I was the same as CpCel48. The cellulose hydrolysis by the same amount of β-glucosidase was performed as a negative control. One mL of the reaction sample was withdrawn at indicated time intervals. The concentration of soluble sugars in the supernatant was measured by using the phenol–sulfuric acid method with glucose as the standard; while the residual cellulose was determined by quantitative saccharification with glucose as the standard (42). All hydrolysis experiments were performed in triplicate.

**Other assays.** Protein mass concentration was measured by the Bio-Rad Bradford protein dye reagent method as a reference of bovine serum albumin. Their protein

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masses based on the Bradford method were calibrated by their UV absorbance at 280 nm in 6 M guanidine hydrochloride (38). The purity of protein samples was examined by SDS-PAGE followed by Coomassie Blue staining. The activity of individual cellulase was measured as described elsewhere (19).

RESULTS

Functional display of mini-CipA on the *B. subtilis* cell surface

Mini-CipA, a fragment of *C. thermocellum* CipA containing three cohesins and one CBM3b (10, 13), was expressed in *B. subtilis* using the *B. subtilis* - *E. coli* shuttle vector pNWP43N-LMC. This vector had an expression cassette containing a NprB signal peptide-encoding sequence, a *B. subtilis* cell wall-binding module (LysM) from a *Bacillus subtilis* cell wall hydrolase LytE (4, 36), a mini-CipA, and a C-terminal Flag-tag, called LMC, under the control of a strong constitutive P43 promoter. Because cell wall hydrolase LytE is located at cell separation sites and poles of *B. subtilis* through its cell wall-binding module (LysM) (4, 36), LMC can be displayed on the cell wall of *B. subtilis*. Controls included plasmid pNWP43N-LysM that expressed a surface displayed LysM with a C-terminal Flag tag and plasmid pNWP43N that did not produce any related surface displayed protein.

After cell cultivation, *B. subtilis* cells harboring pNWP43N-LMC and pNWP43N-LysM produced cell surface-bound LMC and LysM, respectively. Through LiCl elution, the cell wall protein solutions containing cell surface-displayed LMC and LysM were examined in SDS-PAGE (Fig. 2A, Lane 1 and Lane 2), respectively. By the addition of
RAC that binds with high specificity to CBM3b-containing LMC, the LMC (Fig. 2A, Lane 4) was easily separated from other cell wall proteins. The apparent molecular masses for LMC (~105,000) and LysM (~30,000) determined by SDS-PAGE were a little higher than their calculated values (96,711 and 23,215) based on their deduced amino acid sequences, perhaps due to the serine-rich linker sequence in LysM (20). The LMC concentration was estimated to be 1.2 mg/L of the cell culture ($A_{600} = 3.0$) based on the band intensity in the SDS-PAGE, as described elsewhere (4). Approximately 20,000 molecules of LMC were estimated to be displayed on the surface of each $B. subtilis$ cell.

**Expression and purification of cellulases and mini-CipA in E. coli**

Cellulases used for the assembly of tri-functional mini-cellulosome were (i) a non-cellulosomal $B. subtilis$ family 5 endoglucanase (BsCel5), (ii) a cellulosomal $C. thermocellum$ family 9 processive endoglucanase (CtCel9), and (iii) a non-cellulosomal $C. phytofermentans$ ISDg family 48 cellobiohydrolase (CpCel48) (Fig. 1). BsCel5 contains a catalytic module, a dockerin module from $C. thermocellum$, and a C-terminal His tag; CtCel9 contains a catalytic module, CBM3c, a dockerin module, and a C-terminal His tag; and CpCel48 contains a catalytic module, CBM3b, a dockerin module, and a C-terminal His tag. Mini-CipA, a truncated mini-scaffoldin from CipA of $C. thermocellum$, contains three cohesins and one CBM3b. Mini-CipA and three cellulase components expressed in $E. coli$ BL21 were purified to a homogeneous protein (Fig. 2B).

The cellulases used in this study were the same as those in our previous work except CpCel9 (19). Since the activities of CBM-free BsCel5 and CBM-containing CpCel48
were higher than those of CBM-containing BsCel5 and CBM-free CpCel48, respectively (data not shown), CBM-free BsCel5 and CBM-containing CpCel48 were used. In addition, it was found that CBM-containing CpCel48 was expressed at a much higher level than its CBM-free counterpart in E. coli and B. subtilis (data not shown). In this study, family 9 cellulase (CtCel9F) from C. thermocellum was used instead of CpCel9 due to: (i) CtCel9F was expressed at higher levels than CpCel9, (ii) these two enzymes exhibited comparable activities at the temperatures tested, and (iii) CtCel9F contained its own dockerin. Two other dockerin modules from two dockerin-containing cellulases of C. thermocellum were added to the C-terminal of BsCel5 and CpCel48, respectively, because these three cellulases used in this study will be co-expressed in developing consolidated bioprocessing B. subtilis strains (19).

Ex vivo assembly of mini-cellulosomes on the B. subtilis cell surface

The LMC-displayed B. subtilis cells were mixed with excess Cel5’ or a three-enzyme cellulase mixture containing equimolar Cel5’, Cel48, and Cel9. After LiCl elution followed by RAC specific adsorption, LMC + Cel5 exhibited only two bands responsible for LMC and Cel5 at an approximately molar ratio of 1:3, examined by SDS-PAGE (Fig. 3, Lane 2), indicating that one LMC can bind with about three Cel5 molecules. When the cells were mixed with the three-cellulase mixture, LMC bound with the three cellulase components nearly equally (Fig. 3, Lane 3), indicating that each dockerin-containing cellulase component was non-selectively bound with three cohesins of LMC. A negative control LysM-displayed B. subtilis cells did not bind any dockerin-containing cellulase (data not shown).
The ex vivo assembly of mini-cellulosome on B. subtilis cell surface was also examined by confocal immunofluorescence microscopy. When the primary anti-Flag antibody aiming at the C-terminal Flag tag in LMC- or LysM-displayed cells was used, the green fluorescence signals were observed on the surface of the cells displaying LMC and LysM but not on a negative control (B. subtilis WB600/pNWP43N) (Fig. 4A). These results indicated that LMC and LysM were displayed on the B. subtilis cell surface. LMC- and LysM-displayed B. subtilis cells were mixed with excess CtCel9, followed by the primary anti-His antibody that can bind with the His-tag of CtCel9. LMC-displayed B. subtilis cells with CtCel9 exhibited a strong green fluorescence signal (Fig. 4B), suggesting the ex vivo formation of a LMC-CtCel9 complex. By contrast, LysM-displayed B. subtilis cells, a negative control, did not present a detectable fluorescence signal (Fig. 4B). It was noted that the fluorescence signal for LMC-CtCel9 in Fig. 4B was much stronger than that in Fig. 4A because three anti-His antibodies can bind with three CtCel9 linked by one LMC while one anti-Flag antibody can bind with one LMC.

Comparative hydrolysis experiments

Cellulose hydrolysis activity in the presence of the same mass concentrations of cellulase were compared for: living cell-bound mini-cellulosome, cell-free mini-cellulosome, and a (bacterial) three-cellulase mixture with a BsCel5’: CtCel9: CpCel48 molar ratio of 1:1:1 on two model cellulosic materials, RAC and Avicel. Since mini-cellulosome can tightly bind on cellulose, the cellulose-enzyme-microbe (CEM) complex was formed. Cell-bound mini-cellulosome hydrolyzed RAC more rapidly than cell-free mini-cellulosome.
and the three-enzyme mixture (Fig. 5A). At 72 h, a digestibility of 28.4% was achieved
by the mini-cellulosome, about 1.57-fold higher than that of the three-cellulase mixture.
This phenomenon was attributed to the enzyme proximity effect (25, 32, 41). More
notable, the cell-bound mini-cellulosome hydrolyzed RAC 2.25-fold higher in
digestibility than the mini-cellulosome (Fig. 5A). The similar hydrolysis trend in an
increasing order of the cellulase mixture, cellulosome, and cell-bound mini-cellulosome
was observed on Avicel (Fig. 5B). The cell-bound mini-cellulosome exhibited 4.54-fold
higher in Avicel digestibility than did the mini-cellulosome (Fig. 5B). The comparison of
the CEM synergy (Fig. 6) indicated that the cellulose-microbe complex expedited
cellulose hydrolysis rate more significantly on recalcitrant Avicel than on RAC.

To understand the causes that the CEM complex hydrolyzed cellulose more rapidly than
the mini-cellulosome, two control experiments were conducted: (1) non-active mini-
cellulosome-displayed \textit{B. subtilis} cells by the addition of 1 g/L NaN₃ inhibiting cells’
sugar uptake ability; and (2) the mini-cellulosome plus active LysM-displayed \textit{B. subtilis}
cells, which were able to assimilate all soluble sugars in the bulk phase. The non-active
cells associated with cell-bound cellulosome did not hydrolyze cellulose as rapidly as
active cellulosome-bound cells (Fig. 5) possibly due to accumulated sugars in the
supernatant, which inhibited mini-cellulosome activity. The mini-cellulosome plus active
LysM-displayed \textit{B. subtilis} cells where no significant soluble sugars were accumulated in
the supernatant (data not shown) exhibited less hydrolysis ability than the active
cellulosome-bound cells (Fig. 5).
The hydrolysis performance of bacterial cellulase systems was compared to those of a commercial fungal cellulase mixture and a two enzyme cocktail made of purified *Trichoderma* CBH I and EG II at the same protein mass concentration. The two-fungal-enzyme cocktail hydrolyzed cellulosic materials more efficiently than the three-bacterial-cellulase cocktail and the tri-functional mini-cellulosome at 72 h, although each bacterial cellulase component exhibited much higher specific activities at short reaction timeframes (e.g., 10 min to 1 h) (data not shown). The commercial fungal mixture worked better than the two fungal-cellulase mixture, possibly due to its optimized enzyme ratio. Although the non-complexed three-bacterial-cellulase mixture or bacterial mini-cellulosome exhibited less ability in hydrolyzing solid cellulosic materials than the commercial fungal cellulase, the cell-bound cellulosome showed equal hydrolytic ability on RAC and approximately 30% higher hydrolytic ability on Avicel (Fig. 6).

DISCUSSION

We assembled the *ex vivo* tri-functional mini-cellulosome on the surface of the Gram-positive *B. subtilis* strain through the high-affinity interaction between the dockerin modules of cellulase components and the three cohesin modules of mini-CipA. This enabled the comparison of cellulose hydrolysis rate by the cellulose-enzyme-microbe (CEM) complex relative to the non-complexed cellulase mixture or cellulosome (Fig. 5). The CEM synergy was not primarily due to removal of hydrolysis products from the bulk fermentation broth, as suggested by control experiments (Fig. 5). For enzymatic hydrolysis occurring on the surface of solid cellulosic substrate, the concentration of hydrolysis products in the boundary layer was thought to be much higher than that in
bulk phase according to the boundary layer theory (11). Such high concentration hydrolysis products, especially for long chain cellodextrins, in the boundary layer was expected to inhibit cellulase activity more strongly than glucose and cellobiose in the bulk phase because beta-glucosidase that does not have a CBM usually works in the bulk phase. Because the distance between the cell and mini-cellulosome through a LMC (i.e., 20-50 nm) is much shorter than the thickness of boundary layer on solid substrate cellulose for cellulolytic microorganisms (e.g., 10-100 µm) (33), the adjacent cells located in the boundary layer can assimilate long chain hydrolysis products before their diffusion to the bulk phase so to effectively eliminate product inhibition to cellulases and cellulosomes (41). This explanation was partially supported by the observance of some polycellulosomal protuberance between cellulose and C. thermocellum cell under transmission electron microscope (27) and the fast assimilation of long chain cellodextrins by adjacent cellulolytic cells rather than further hydrolysis to cellobiose and glucose by cellulases in the bulk phase (41, 42).

The CEM synergy was more significant on recalcitrant Avicel than on highly-reactive amorphous cellulose (Fig. 6). This difference may be explained by stronger boundary-layer product inhibition on crystalline cellulose than amorphous cellulose. Because cellbiohydrolase is more sensitive to product inhibition than endoglucanase (i.e., $K_{ICBH} \ll K_{IEG}$) and endoglucanase exhibits more hydrolysis ability on amorphous cellulose than on Avicel (19), the aggregated cellulosome exhibited less product inhibition on amorphous cellulose than on recalcitrant Avicel (17). Displaying cellulosome on the
surface of a microorganism would be effective to enhance the cellulolytic host’s ability to
effectively hydrolyze recalcitrant cellulosic fragment of pretreated heterologous biomass.

Both *B. subtilis* and *S. cerevisiae* are important industrial microorganisms. As a potential
CBP host, *B. subtilis* could be better than *S. cerevisiae* due to (i) a natural ability to take
up long-chain cellodextrins, (ii) a natural ability to co-utilize C5 and C6 sugars, (iii) an
inherent ability to secrete a large amount of proteins, and (iv) a small size cell (0.7 × 2
µm) vs. a large size yeast (2.5 × 10 × 4.5 × 21 µm) (i.e., a better mass transfer for a
smaller cell). The first two features have been introduced into recombinant yeasts (12,
28). In spite of intensive efforts, real recombinant cellulose-utilizing yeasts that can
produce ample cellulase and hydrolyze cellulose to support cell growth and cellulase
synthesis without the help of other soluble organic nutrient are not yet available (18). By
contrast, a recombinant cellulose-utilizing *B. subtilis* has been created to produce lactate
from cellulose without the addition of exogenous cellulase and any water-soluble organic
nutrients (37). Since anaerobic cellulolytic microorganisms must produce more secretory
cellulase than do aerobic counterparts based on weight ratio of cellulase to cellular
protein for supporting their growth on cellulose (23), cellulase synthesis always
represents a significant bioenergetic burden for anaerobic microorganisms (42). The
bacterium *C. thermocellum*, for example, produces ~10-20% (wt/wt) cellulase relative to
cellular proteins for fast cellulose hydrolysis with nearly all of cellulosome displayed on
its cell surface (40). It appears that cellulolytic, anaerobic bacteria evolved cell-bound
cellulosomes so to increase specific cellulase activity and to decrease their bioenergetic
burden (42). However, this cellulase evolution mechanism is speculated not to occur to
fungi and yeasts because a) ATP supply is much more plentiful and b) relatively large
cellulolytic fungi and yeasts may not have enough cell surface to display 10%-20% wt.
cellulase relative to cellular protein due to low ratios of surface/volume, where
surface/volume is inversely proportion to a radius of a cell. Therefore, in nature
cellulolytic fungi is hypothesized to evolve to secrete a large amount of cellulases.

For high-yield biofuels production from cellulosic material, it is vital to increase
carbohydrate allocation of desired biofuels by decreasing carbohydrate allocation to
synthesis of cellulase and cell mass under anaerobic conditions (14, 22). This study
showed that displaying cellulosome on the surface of a microbe can enhance microbial
cellulose hydrolysis rate by several-fold without increasing cellulase synthesis burden.
Since fungal cellulases exhibited higher hydrolysis ability in a long time range (19), the
coe-expression of dockerin-containing fungal cellulases by recombinant cellulolytic B.
subtilis strains may be another worthy direction. Another potential direction would be in
vitro assembly of dockerin-containing fungal cellulases produced by Trichoderma spp.
and a recombinant yeast or bacterium that can produce a cell-surface displayed scaffoldin.

ACKNOWLEDGEMENT

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work was also partially supported by the CALS Biodesign and Bioprocessing Research
Center and the Integrated Internal Competitive Grants Program at Virginia Tech.
REFERENCES


FIGURE LEGENDS

Fig. 1. Schematic representation of the recombinant proteins used in this study.

Fig. 2. SDS-PAGE of cell wall proteins eluted from the cell-surface of *B. subtilis* strains (A) and the purified recombinant cellulases and mini-CipA produced by *E. coli* (B). A: Lane 1, the LiCl-eluted supernatant from the *B. subtilis* (pNWP43N-LMC) cells; Lane 2, the LiCl-eluted supernatant from the *B. subtilis* (pNWP43N-LysM) cells; Lane 3, the LiCl-eluted supernatant from the *B. subtilis* (pNWP43N) cells; Lane 4, adsorbed LMC eluted supernatant from the *B. subtilis* (pNWP43N-LMC) cells by using RAC. B: Lane 1, mini-CipA; Lane 2, CpCel48; Lane 3, CtCel9; and Lane 4, BsCel5’.

Fig. 3. SDS-PAGE of RAC affinity pull-down for cell-wall proteins eluted from *B. subtilis* strains. Lane 1, cell-bound LMC from the *B. subtilis* (pNWP43N-LMC) cells; Lane 2, cell-bound unifunctional mini-cellulosome from the *B. subtilis* (pNWP43N-LMC) cells premixed with BsCel5’; and Lane 3, cell-bound tri-functional mini-cellulosome from the *B. subtilis* (pNWP43N-LMC) cells premixed with BsCel5’, CtCel9 and CpCel48.

Fig. 4. Confocal fluorescence microscopy images of LMC, a negative control (blank plasmid), and LysM on the surface of *B. subtilis* cells (A) as well as the cell-bound mini-cellulosome on the surface of *B. subtilis* cells relative to a negative control (B). The cells displaying Flag-tag LMC and LysM were probed with the anti-Flag antibody followed by the rabbit anti-mouse IgG conjugated with FITC (A). The mini-cellulosome containing
LMC + CtCel9 was probed with the anti-His6 antibody followed by the rabbit anti-mouse IgG conjugated with FITC, where CtCel9 contains a His6 tag (B).

Fig. 5. Hydrolysis of RAC (A) and Avicel (B) by the enzyme mixtures supplemented with excess β-glucosidase: the bacterial cellulase mixture (▲), the mini-cellulosome (■), the Novozymes fungal cellulase mixture (●), the two-enzyme *Trichoderma* fungal mixture (EG II and CBH I) (▲), as well as the cell-bound mini-cellulosome (■), the mini-cellulosome in the presence of LysM-displayed *B. subtilis* cells (□), and the cell-bound mini-cellulosome in the presence of 1 g/L NaN₃ (○).

Fig. 6. Comparison of cellulose digestibility by the bacterial cellulase mixture, the cell-free mini-cellulosome, the cell-bound mini-cellulosome, the commercial fungal cellulase mixture, and the two-fungal-enzyme cocktail on RAC (A) and Avicel (B) at hour 72. The error bars represent the standard deviation of triplicate samples.
Table 1. Strains and plasmids in this study.

<table>
<thead>
<tr>
<th>Stains or plasmids</th>
<th>Characteristics</th>
<th>References</th>
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<tr>
<td><strong>E. coli</strong></td>
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<td></td>
</tr>
<tr>
<td>JM109</td>
<td>recA1, supE44 endA1 hsdR17 (&quot;k,m&quot;&quot;) gyr96 relA1 thi (lac-proAB) F'[traD36 proAB' lacFlacZ ΔM15]</td>
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<td>BL21 Star (DE3)</td>
<td>F ompT hsdSB (rB mB') gal dcm rne131 (DE3)</td>
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<td><strong>B. subtilis</strong></td>
<td></td>
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<tr>
<td>WB600</td>
<td>nprE aprA epr bpf mpr nprB</td>
<td>(35)</td>
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<tr>
<td>pNWP43N</td>
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<tr>
<td>pNWP43N-LysM</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;, with LysM expression cassette cloned</td>
<td>This work</td>
</tr>
<tr>
<td>pNWP43N-LysM-mini-cipA</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;, with LysM-mini-cipA expression cassette cloned</td>
<td>This work</td>
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<tr>
<td>pET20b</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;, overexpression vector containing T7-dependent promoter</td>
<td>Novagen, Madison, WI</td>
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Table 2. The primers used to amplify gene fragments.

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<th>RE*</th>
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*RE, restriction enzyme site. Restriction enzyme sites included in primer sequences for cloning purposes are indicated in bold, and the Flag tag sequences are indicated by underlined text.

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<td><em>thermocellum</em></td>
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2 *RE, restriction enzyme site. Restriction enzyme sites included in primer sequences for cloning purposes are indicated in bold, and the Flag tag sequences are indicated by underlined text.
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<th>Notation</th>
<th>M.W.</th>
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**Key to symbols**
- Catalytic domain
- Linker
- CBLMx
- Cohesin
- Dockeins
- 6 x His tag
- 8 Xynhi
- Cell wall binding domain
- Flap tag