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Functional Assembly of Minicellulosomes on the *Saccharomyces cerevisiae* Cell Surface for Cellulose Hydrolysis and Ethanol Production▽

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We demonstrated the functional display of a miniscaffoldin on the *Saccharomyces cerevisiae* cell surface consisting of three divergent cohesin domains from *Clostridium thermocellum* (t), *Clostridium cellulolyticum* (c), and *Ruminococcus flavefaciens* (f). Incubation with *Escherichia coli* lysates containing an endoglucanase (CelA) fused with a dockerin domain from *C. thermocellum* (At), an exoglucanase (CelE) from *C. cellulolyticum* fused with a dockerin domain from the same species (Ec), and an endoglucanase (CelG) from *C. cellulolyticum* fused with a dockerin domain from *R. flavefaciens* (Gf) resulted in the assembly of a functional minicellulosome on the yeast cell surface. The displayed minicellulosome retained the synergistic effect for cellulose hydrolysis. When a β-glucosidase (BglA) from *C. thermocellum* tagged with the dockerin from *R. flavefaciens* was used in place of Gf, cells displaying the new minicellulosome exhibited significantly enhanced glucose liberation and produced ethanol directly from phosphoric acid-swollen cellulose. The final ethanol concentration of 3.5 g/liter was 2.6-fold higher than that obtained by using the same amounts of added purified cellulases. The overall yield was 0.49 g of ethanol produced per g of carbohydrate consumed, which corresponds to 95% of the theoretical value. This result confirms that simultaneous and synergistic saccharification and fermentation of cellulose to ethanol can be efficiently accomplished with a yeast strain displaying a functional minicellulosome containing all three required cellulolytic enzymes.

Production of bioethanol from biomass has recently attracted attention due to the mandate for a billion gallons of renewable fuel by the new Energy Policy Act (22). Current production processes using sugar cane and cornstarch are well established (19, 23). However, utilization of a cheaper substrate would render bioethanol more competitive with fossil fuel (29). Cellulosic biomass found in many low-value agricultural or wood pulping wastes is particularly well suited because of its large-scale availability, low cost, and environmentally benign production (23). The primary obstacle impeding the more widespread production of ethanol from cellulose is the absence of a low-cost technology for overcoming its recalcitrant nature (21).

Recently, a new method known as consolidated bioprocessing (CBP) has been proposed that combines enzyme production, cellulose saccharification, and fermentation into a single process to dramatically reduce the cost of ethanol production (22). An ideal microorganism for CBP should possess the capability of simultaneous cellulose saccharification and ethanol fermentation. One attractive candidate is *Saccharomyces cerevisiae*, which is widely used for industrial ethanol production due to its high ethanol productivity and high inherent ethanol tolerance (24). Attempts have been made to engineer *S. cerevisiae* to hydrolyze cellulose (6, 7, 16). However, due to energetic limitations under anaerobic conditions, only a small amount of cellulases can often be secreted. An alternative is to display the cellulolytic enzymes on the yeast cell surface (13, 14). Up to three different cellulases have been displayed, permitting the hydrolysis of cellulose with concomitant ethanol production. While these results point to a potential strategy of combining ethanol-producing capability with cellulose hydrolysis, the efficiency of hydrolysis must be significantly improved before it can be employed for practical applications.

Many anaerobic bacteria have developed an elaborately structured enzyme complex on the cell surface, called the cellulosome, to maximize the catalytic efficiency of cellulose hydrolysis with only a limited amount of enzymes (1, 8, 9). The major component of these cellulosome complexes is a structural scaffoldin consisting of at least one cellulose-binding domain (CBD) and repeating cohesin domains, which are docked individually with a different cellulase tagged with the corresponding dockerin domain (26). Since the interaction between dockerin and cohesin is species specific (17, 25), designer minicellulosomes composed of three different dockerin-cohesin pairs with a cellulose hydrolysis efficiency up to sixfold higher than that of similar free enzymes have been generated (11, 12). Recently, it has been shown that the specific cellulose hydrolysis rates of metabolically active cultures of *C. thermocellum* displaying cellulosomes are more than fourfold higher than those of purified cellulosomes (20). This significant improvement appears to be a surface phenomenon involving adhesion to cellulose for enhanced substrate capture.

In the present report, we demonstrate the functional assembly of a minicellulosome composed of three different cellulases on the *S. cerevisiae* cell surface and the feasibility of using the engineered yeast strains for cellulosic ethanol production. The success of displaying a functional cellulosome on the surface of an organism that already produces high titers of ethanol could

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TABLE 1. Scaffoldins and dockerin-tagged cellulases used in this study

Protein name	Description (from N terminus to C terminus)	Host cell	Tag
Scaf-c	Scaffoldin containing a cohesin from *C. cellulolytica* followed by a CBD	*S. cerevisiae*	c-Myc
Scaf-ct	Scaffoldin containing a cohesin from *C. cellulolytica* followed by a CBD followed by a second cohesin from *C. thermocellum*	*S. cerevisiae*	c-Myc
Sacf-ctf	Scaffoldin containing a cohesin from *C. cellulolytica* followed by a CBD followed by a second cohesin from *C. thermocellum* and a third cohesin from *R. flavefaciens*	*S. cerevisiae*	c-Myc
At	Endoglucanase CelA from *C. thermocellum* fused with its native dockerin	*E. coli*	c-His_6
Ec	Exoglucanase CelE from *C. cellulolytica* fused with its native dockerin	*E. coli*	c-His_6
Gf	Endoglucanase CelG from *C. cellulolytica* fused with a dockerin from *R. flavefaciens*	*E. coli*	c-His_6
BglA	β-Glucosidase BglA from *C. thermocellum* fused with a dockerin from *R. flavefaciens*	*E. coli*	c-His_6

lay a foundation for the achievement of an industrially relevant CBP-enabling microorganism.

MATERIALS AND METHODS

Strains, plasmids, and media. *Escherichia coli* strain JM109 [*endA1 recA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *relA1 supE44* Δ(*lac-proAB*)] was used as the host for genetic manipulations. *E. coli* BL21(DE3) [F^- *ompT gal* $hsdS_B$ (r_B^- m_B^-) *dcm lon* λDE3] was used as the production host for cellulase expression. *S. cerevisiae* strain EBY100 [*MAT***a** *AGA1*::*GAL1-AGA1*::*URA3 ura3-52 trp1 le*u*2-1 his3-200 pep4*::*HIS3 prb1-1.6R can1 GAL*] was used for surface display of scaffoldins. All *E. coli* cultures were grown in Luria-Bertani (LB) medium (10.0 g/liter tryptone, 5.0 g/liter yeast extract, 10.0 g/liter NaCl) supplemented with either 100 μg/ml ampicillin or 50 μg/ml kanamycin. All yeast cultures were grown in SDC medium (20.0 g/liter dextrose, 6.7 g/liter yeast nitrogen base without amino acids, 5.0 g/liter Casamino Acids).

To display scaffoldins, a gene fragment coding for a scaffoldin containing three cohesins from *C. cellulolyticum*, *C. thermocellum*, and *R. flavefaciens* and one CBD was amplified with plasmid pETscaf6 (10) as the template with forward primer F1NdeI (5′-TATA**GCTAGC**GGCGATTCTCTTAAAGTTACAGT-3′ [the boldface portion is a restriction endonuclease site]) and reverse primer R1SalI (5′-ATAT**GTCGAC**GTGGTGGTGGTGGTG-3′). The PCR product was then digested and ligated into the surface display vector pCTCON2 (5) to form pScaf-ctf. Similar procedures, except that the reverse primers were changed to RASalI (5′-ATAT**GTCGAC**ATCTGACGGCGGTATTGTTGTTG-3′) and RBSalI (5′-ATAT**GTCGAC**TATATCTCCAACATTTACTCCAC-3′), were used for the construction of pSacf-c and pSacf-ct.

Plasmids pETEc (15) and pETGf (12), encoding exoglucanase CelE (Ec) and endoglucanase CelG (Gf) of *C. cellulolyticum* fused to the dockerins from *C. cellulolyticum* and *R. flavefaciens*, respectively, were kindly given by H.-P. Fierobe (CNRS, France). Plasmid pETAt, encoding a His_6-tagged endoglucanase (CelA) and a dockerin from *C. thermocellum* (At), was obtained by PCR from pCelA with forward primer F2NdeI (5′-ATAT**CATATG**GCAGGTGTGCCTTTTAACACAAA-3′) and reverse primer R2XhoI (5′-ATAT**CTCGAG**CTAATAAGGTAGGTGGGG-3′). The amplified fragment was cloned into NdeI-XhoI-linearized plasmid pET24a to form pETAt. Plasmid pBglAf, encoding a His_6-tagged dockerin from *R. flavefaciens* fused to a β-glucosidase (BglA) from *C. thermocellum*, was obtained by two-step cloning. First, a gene fragment coding for the His_6-tagged dockerin of *R. flavefaciens* was obtained from pETGf by digestion with BamHI and XhoI and ligated into pET24a to form pETDf. The gene fragment of BglA was amplified by PCR from pBglA with forward primer F3NdeI (5′-ATAT**CATATG**TCAAAGATAACTTTCCCAAAA-3′) and reverse primer R3BglII (5′-ATAT**AGATCT** TTAAAAACCGTTGTTTTTGATTACT-3′) and inserted into NdeI-BamHI-linearized pETDf to form pBglAf. A summary of all of the scaffoldins and dockerin-tagged cellulases used in this study is listed in Table 1.

Display of scaffoldins on the yeast cell surface. For the display of scaffoldins on the yeast cell surface, yeast cells harboring pScaf-c, pScaf-ct, or pScaf-ctf were precultured in SDC medium for 18 h at 30°C. These precultures were subinoculated into 200 ml SGC medium (20.0 g/liter galactose, 6.7 g/liter yeast nitrogen base without amino acids, 5.0 g/liter Casamino Acids) at an optical density (OD) at 600 nm of 0.1 and grown for 48 h at 20°C.

Expression and purification of dockerin-tagged cellulases. *E. coli* strains expressing At, Ec, and Gf were precultured overnight at 37°C in LB medium supplemented with appropriate antibiotics. The precultures were subinoculated into 200 ml LB medium supplemented with 1.5% glycerol and appropriate antibiotics at an initial OD of 0.01 and incubated at 37°C until the OD reached 1.5. The cultures were then cooled to 20°C, and isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 200 μM. After 16 h, cells were harvested by centrifugation (3,000 × *g*, 10 min) at 4°C, resuspended in buffer A (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 10 mM $CaCl_2$), and lysed with a sonicator. The different cellulases were purified with a His-binding resin (Novagen) at 4°C.

Minicellulosome assembly on the yeast cell surface. To assemble the minicellulosomes, either cell lysates containing dockerin-tagged cellulases or purified cellulases were incubated with yeast cells displaying the scaffoldin for 1 h at 4°C in buffer A. After incubation, cells were washed and harvested by centrifugation (3,000 × *g*, 10 min) at 4°C and resuspended in the same buffer for further use.

Immunofluorescence microscopy. Yeast cells displaying scaffoldins or the minicellulosomes on the surface were harvested by centrifugation, washed with phosphate-buffered saline (PBS; 8 g/liter NaCl, 0.2 g/liter KCl, 1.44 g/liter Na_2HPO_4, 0.24 g/liter KH_2PO_4), and resuspended in 250 μl of PBS containing 1 mg/ml bovine serum albumin and 0.5 μg of anti-c-Myc or anti-c-His immunoglobulin G (IgG; Invitrogen) for 4 h with occasional mixing. Cells were then pelleted and washed with PBS before resuspension in PBS plus 1 mg/ml bovine serum albumin and 0.5 μg anti-mouse IgG conjugated with Alexa 488 (Molecular Probes). After incubation for 2 h, cells were pelleted, washed twice with PBS, and resuspended in PBS to an OD at 600 nm of 1. For fluorescence microscopy (Olympus BX51), 5- to 10-μl volumes of cell suspensions were spotted onto slides and a coverslip was added. Images from Alexa 488 were captured with the QCapture Pro6 software. Whole-cell fluorescence was measured with a fluorescence microplate reader (Synergy4; BioTek, VT) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Enzyme assays. Carboxymethyl cellulose (CMC) was obtained from Sigma and used as a substrate. Phosphoric acid-swollen cellulose (PASC) was prepared from Avicel PH101 (Sigma) according to the method of Walseth (27). Enzyme activity was assayed in the presence of a 0.3% (wt/vol) concentration of cellulose at 30°C in 20 mM Tris-HCl buffer (pH 6.0). Samples were collected periodically and immediately mixed with 3 ml of DNS reagents (10 g/liter dinitrosalicylic acid, 10 g/liter sodium hydroxide, 2 g/liter phenol, 0.5 g/liter sodium sulfite). After incubation at 95°C for 10 min, 1 ml of 40% Rochelle salts was added to fix the color before measurement of the absorbance of the supernatants at 575 nm. Glucose concentration was determined with a glucose HK assay kit from Sigma.

Fermentation. Fermentation was conducted anaerobically at 30°C. Briefly, yeast cells were washed once with buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 10 mM $CaCl_2$ and resuspended in SDC medium containing 6.7 g/liter yeast nitrogen base without amino acids, 20 g/liter Casamino Acids, and 10 g/liter PASC as the carbon source. Reducing sugar production and glucose concentration were measured by the methods described above. The amount of residual cellulose was measured by the phenol-sulfuric acid method as described by Dubois et al. (10). Ethanol concentration was measured with a gas chromatograph (model 6890; Hewlett Packard) with a flame ionization detector and an HP-FFTP column.

RESULTS

Functional display of miniscaffoldins on the yeast cell surface. Previously, several highly synergistic designer minicellulosomes that are able to hydrolyze cellulose up to sixfold faster than free enzymes were created (12). We reasoned that significant improvements in both cellulose hydrolysis and ethanol production can be achieved if a similar minicellulosome can be

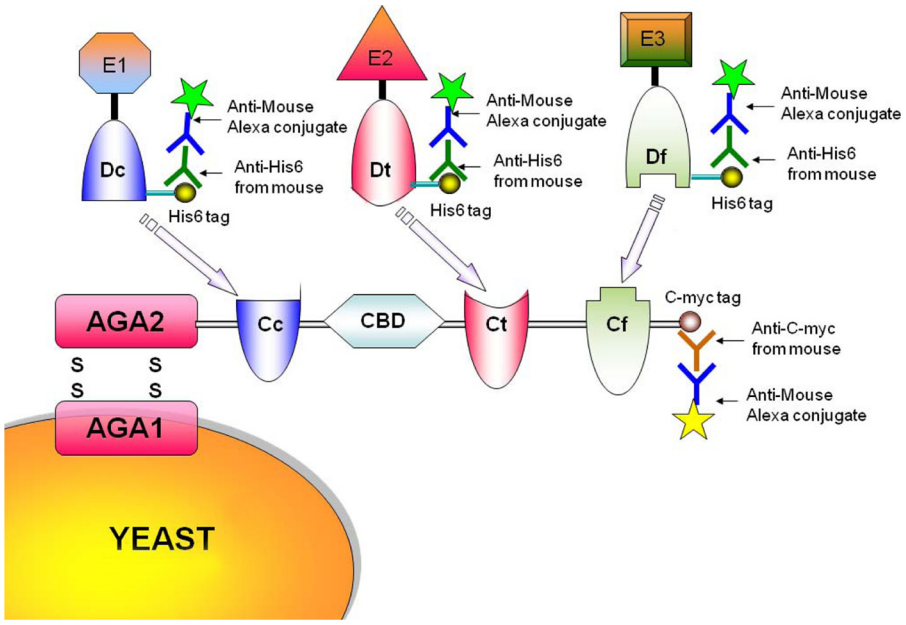

FIG. 1. Functional assembly of minicellulosomes on the yeast cell surface. A trifunctional scaffoldin (Scaf-ctf) consisting of an internal CBD flanked by three divergent cohesin (C) domains from *C. thermocellum* (t), *C. cellulolyticum* (c), and *R. flavefaciens* (f) was displayed on the yeast cell surface. Three different cellulases (E1, E2, and E3) fused with the corresponding dockerin domain (either Dt, Dc, or Df) were expressed in *E. coli*. Cell lysates containing these cellulases were mixed with yeast cells displaying Scaf-ctf for the functional assembly of the minicellulosome.

assembled on the yeast cell surface. A plasmid coding for a trifunctional scaffoldin (Scaf-ctf) consisting of an internal CBD flanked by three divergent cohesin domains from *C. thermocellum* (t), *C. cellulolyticum* (c), and *R. flavefaciens* (f) (Fig. 1) was created for surface display. To further demonstrate the specificity of the different dockerin-cohesin pairs, two smaller scaffoldins, (i) Scaf-c containing a cohesin domain from *C. cellulolyticum* followed by a CBD and (ii) Scaf-ct containing an additional cohesin domain from *C. thermocellum* at the C terminus of the CBD, were generated (Fig. 1). The different scaffoldins were displayed on the yeast cell surface by using the glycosylphosphatidylinositol (GPI) anchor (3) linked at the N-terminal side of the scaffoldins. A c-Myc tag was added to the C terminus of each scaffoldin to allow detection with anti-c-Myc serum.

To probe the surface localization of the scaffoldin, immunofluorescent labeling of cells was carried out with anti-c-Myc serum and Alexa 488-conjugated goat anti-mouse IgG. Cells displaying the scaffoldin domains were brightly fluorescent (Fig. 2A), while no fluorescence was observed in the control yeast cells (EBY100). Since monoclonal antibodies are not able to penetrate the cell wall, the fluorescence images confirmed that the scaffoldins are displayed on the cell surface.

Functionality of the displayed scaffoldins. To investigate the functionality of the displayed scaffoldins, an exoglucanase (CelE) from *C. cellulolyticum* fused to a dockerin domain from the same species (Ec), an endoglucanase (CelG) from *C. cellulolyticum* fused to a dockerin domain from *R. flavefaciens* (Gf), and an endoglucanase (CelA) fused to a dockerin domain from *C. thermocellum* (At) were expressed in *E. coli*. A His_6 tag was added to the C terminus of each of the dockerin domains for detection of the assembly. Cells displaying scaffoldins on the surface were incubated directly with *E. coli* cell lysates containing At, Ec, or Gf for 1 h to form the cellulosome complex. The presence of each cellulase-dockerin pair on cells displaying Scaf-ctf was confirmed by immunofluorescence microscopy with the anti-His_6 antibody (Fig. 2B).

To demonstrate the specificity of different cohesin-dockerin pairs, similar experiments were performed with cells displaying either Scaf-ct or Scaf-c. In Scaf-ct-displaying cells, fluorescence was detected only in the presence of Ec or At, whereas incubation with Gf did not result in any detectable fluorescence (Fig. 2C). Similarly, in Scaf-c-displaying cells, fluorescence was only observed in the presence of Ec (Fig. 2D). These results confirm that the specificity of the cohesins is preserved even when they are displayed on the cell surface, as only the corresponding dockerin-tagged enzymes are assembled correctly.

Functionality of the displayed minicellulosomes. To demonstrate the functionality of the assembled minicellulosomes, cells expressing Scaf-ctf were first saturated with different combinations of Ec, At, and/or Gf. As depicted in Fig. 3, a similar level of fluorescence was detected from the c-Myc or c-His_6 tag when only one dockerin-tagged enzyme was added, indicating the correct 1:1 binding between the cohesin-dockerin pairs. More importantly, a corresponding increase in fluorescent intensity was observed when an increasing number of enzymes were docked on Scaf-ctf. This result confirms that the correct 1:1 binding ratio of each dockerin-cohesin pair was preserved even when it was assembled into a three-enzyme minicellulosome on the cell surface (Fig. 3).

Engineered yeast cells docked with different combinations of cellulases were further examined for functionality in cellulose hydrolysis. Cells were resuspended in Tris buffer containing CMC, and the rate of reducing sugar production was determined. As shown in Fig. 4, cells with any one of the three cellulases docked on the surface showed visible differences in

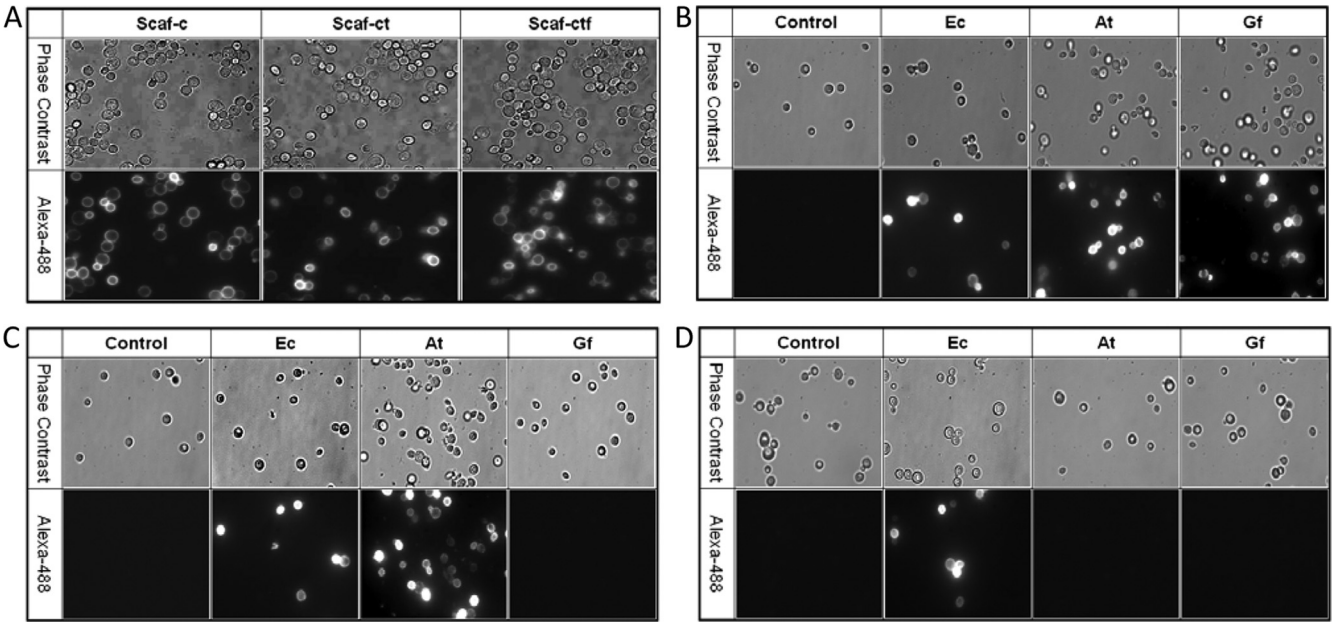

FIG. 2. Phase-contrast and immunofluorescence micrographs of yeast cells displaying minicellulosomes. (A) Cells displaying either scaffoldin Scaf-c, Sacf-ct, or Sacf-ctf. Functional assembly of three dockerin-tagged cellulases (CelE-Dc [Ec], CelA-Dt [At], or CelG-Df [Gf]) on cells displaying (B) Sacf-ctf, (C) Sacf-ct, or (D) Scaf-c. Cells were probed with either anti-c-Myc or anti-c-His_6 serum and fluorescently stained with a goat anti-mouse IgG conjugated with Alexa Fluor 488. Cells displaying only the scaffoldins were used as controls.

cellulose hydrolysis from the control. The endoglucanase At had the highest rate of hydrolysis, followed by Gf and Ec, a trend consistent with the relatively low activity of the exoglucanase CelE on CMC (15). The rate of CMC hydrolysis increased in an additive fashion when two of the cellulases were docked on the cell surface, and the highest rate of hydrolysis was observed when all three cellulases were assembled. The additive effect on CMC hydrolysis confirms that the recruitment of cellulases to the displayed scafoldin has a very minimum effect on their individual functionality.

Synergistic effect of displayed minicellulosomes. The synergistic effect on cellulose hydrolysis is the most intriguing property of naturally occurring cellulosomes. To test whether the synergistic effect of the minicellulosome structure was preserved when displayed on the yeast cell surface, Avicel hydrolysis was compared with that of purified cellulases. In this case, the amount of each cellulase docked on Scaf-ctf was first determined from the binding experiments. These predetermined amounts of cellulases were then mixed together, and the hydrolysis of Avicel with the cellulase mixture was compared with that of whole cells displaying the functional cellulosome containing the same amount of each cellulase. As shown in Table 2, the level of reducing sugar production was consistently higher for cells displaying the cellulosome, confirming that synergy was indeed maintained (12). More importantly, the level of synergy increased from 1.62 to 2.44 when the number of cellulases recruited in the minicellulosome system increased from one to three. This result suggests the potential to further

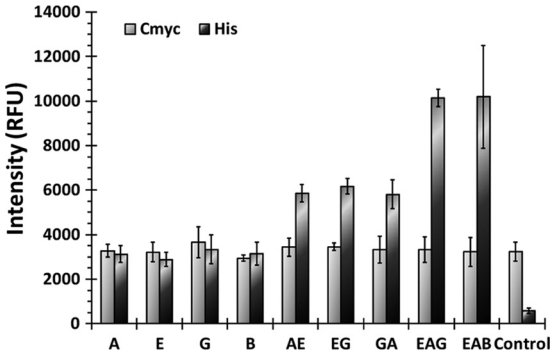

FIG. 3. Fluorescence intensity of cells either displaying scaffoldin Sacf-ctf or with different combinations of dockerin-tagged cellulases (At [A], Ec [E], and Gf [G]) docked on the displayed Sacf-ctf. Cells were probed with either anti-c-Myc or anti-c-His_6 serum and fluorescently stained with goat anti-mouse IgG conjugated with Alexa Fluor 488. Whole-cell fluorescence was determined with a fluorescence microplate reader. Cells displaying only Scaf-ctf were used as controls. RFU, relative fluorescence units.

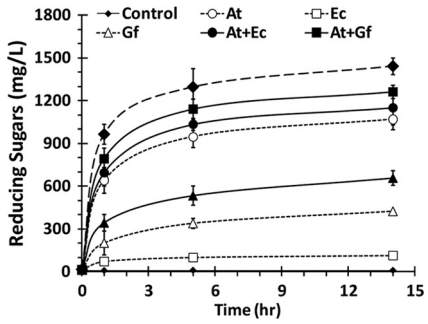

FIG. 4. Whole-cell hydrolysis of CMC by different cellulase pairs (CelE-Dc [Ec], CelA-Dt [At], or CelG-Df [Gf]) docked on the displayed Scaf-ctf protein. Cells displaying only Scaf-ctf were used as controls.

TABLE 2. Amounts of reducing sugars released from Avicel after 24 h of incubation at 30°C either by cells displaying cellulosomes or by the same amount of free enzymes[a]

Cellulase pair(s)	Amt of reducing sugars (mg/liter) released from:		Degree of synergy
	Cellulosome	Free enzymes	
At	46.1	28.3	1.62
At + Ec	80.1	37.6	2.13
At + Ec + Gf	132.3	54.2	2.44

[a] Reactions were conducted either with different cellulase pairs (CelE-Dc [Ec], CelA-Dt [At], or CelG-Df [Gf]) docked on the displayed Scaf-ctf or with the corresponding purified cellulases. The degree of synergy is defined as the amount of sugar released from the cellulosome over the amount of sugar released from free enzymes.

enhance cellulose hydrolysis by increasing the number of displayed cellulases.

Incorporation of β-glucosidase into the minicellulosome. Since *S. cerevisiae* is unable to transport and utilize oligosaccharides, directing the complete hydrolysis of cellulose to glucose is essential. To achieve this goal, a β-glucosidase (BglA) from *C. thermocellum* tagged with the dockerin from *R. flavefaciens* was constructed. The resulting dockerin-tagged BglA retained the same specificity and docking efficiency as Gf (Fig. 3). Figure 5 shows the time course of reducing sugar and glucose released from PASC with different enzyme combinations docked on the cell surface. Although >40% of the PASC was hydrolyzed in the presence of the endoglucanase At, <25% of the reducing sugar was further hydrolyzed to glucose. In comparison, the presence of the exoglucanase Ec not only enhanced reducing sugar production but also increased glucose production threefold. The addition of BglA further improved the rate of glucose liberation, although no difference in reducing sugar formation was observed. This result is very significant, as we demonstrated that a functional minicellulosome containing all three exoglucanase, endoglucanase, and β-glucosidase activities can be successfully assembled on the surface of a heterologous host cell. Our result also confirms the important role of β-glucosidase in achieving a higher conversion of cellulose to glucose. More importantly, the displayed minicellulosome exhibited significant synergy in both reducing sugar and glucose liberation compared to that of free enzymes. It should be noted that the modest improvement in glucose formation by BglA is the result of glucose inhibition, a known behavior of many β-glucosidases, including BglA (8, 28).

Direct fermentation of amorphous cellulose to ethanol. The ability of ethanol fermentation from PASC was examined by using the scaffoldin-displaying strains docked with different cellulases. As shown in Fig. 6, the increase in ethanol production was accompanied by a concomitant decrease in the total sugar concentration. The levels of ethanol production and PASC hydrolysis were directly correlated with the number of cellulases docked on the cell surface. The maximum ethanol production of cells displaying At, Ec, and BglA was 3.5 g/liter after 48 h; this corresponds to 95% of the theoretical ethanol yield, at 0.49 g ethanol/g sugar consumed. Moreover, the glucose concentrations during the fermentation were below the detection limit. This indicates that all of the glucose produced was quickly consumed, resulting in no detectable glucose accumulation in the medium. The level of ethanol production by cells displaying all three cellulases was higher than that of cells displaying only At and Ec, again confirming the importance of β-glucosidase in the overall cellulose-to-ethanol conversion process. More importantly, the synergistic effect of the minicellulosome was also observed, as the ethanol production by a culture with the same amounts of purified At, Ec, and BglA added to the medium was more than threefold lower.

DISCUSSION

Biomass represents an inexpensive feedstock for sustainable bioethanol production. Among the three biological events that occur during the conversion of cellulose to ethanol, i.e., enzyme production, polysaccharide hydrolysis, and sugar fermentation, cellulose hydrolysis is widely recognized as the key step in making bioconversion economically competitive (21). In addition, it is believed that a significant cost reduction can be achieved when two or more steps are combined, such as in CBP (22). To achieve this goal, we demonstrated the functional assembly of a minicellulosome on the yeast cell surface to render the ethanologenic microbe cellulolytic.

First, a chimeric minicellulosome containing three dockerin-cohesin pairs from different species was assembled on the yeast cell surface. Although similar minicellulosomes have been shown to hydrolyze cellulose with high synergy in vitro (12),

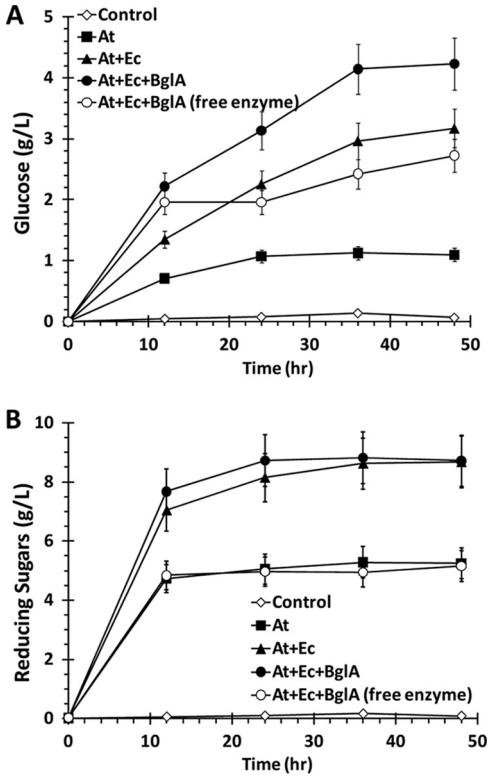

FIG. 5. Production of glucose (A) and reducing sugars (B) from the hydrolysis of PASC by free enzymes and by surface-displayed cellulosomes. Reactions were conducted either with different cellulase pairs (CelE-Dc [Ec], CelA-Dt [At], or β-glucosidase-Df [BglA]) docked on the displayed Scaf-ctf protein or with the corresponding purified cellulases. Cells displaying only Scaf-ctf were used as controls.

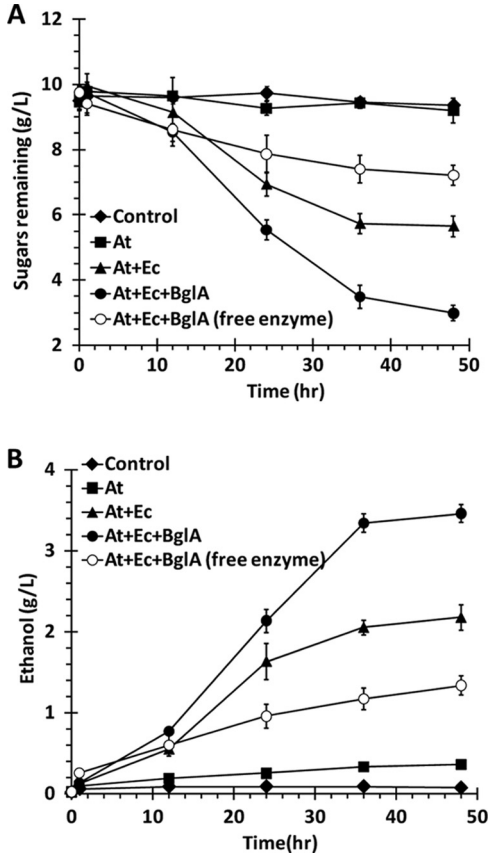

FIG. 6. Time profiles of ethanol production (A) and cellulose hydrolysis (B) from PASC by control strain EBY100 plus free enzymes and yeast cells displaying functional cellulosomes. Fermentations were conducted either with different cellulase pairs (CelE-Dc [Ec], CelA-Dt [At], or β-glucosidase-Df [BglA]) docked on cells displaying Scaf-ctf or with control strain EBY100 plus the corresponding purified cellulases. Cells displaying only Scaf-ctf were used as controls. The individual enzyme amounts were the same in all cases.

successful demonstration for in vivo systems has never been achieved. Immunofluorescence microscopy showed the successful translocation of the miniscaffoldin on the yeast cell surface, and the functionality of the cohesin domains was retained by observing the successful assembly of the corresponding dockerin-tagged cellulases. Since the specificity of the dockerin-cohesin pairs is preserved, it may be possible to direct any enzymatic subunit to a specified position within a modular scaffoldin by tagging with the designated dockerin.

Another interesting property of cellulosome use is its synergistic effect on cellulose hydrolysis compared with that of free enzymes. Recent studies (2, 11) suggest that the close proximity and ordering of the enzyme components appear to be the key. In the present study, the displayed minicellulosome retained this key characteristic. Interestingly, the level of synergy increased with an increasing number of cellulases docked on the cell surface. Cha and colleagues (4) tested the effect of multiple copies of cohesin on the cellulase activities of different minicellulosomes and reported an increase in synergy with increasing numbers of cohesins. This synergistic effect was preserved even when a new minicellulosome composed of a β-glucosidase (BglA), an endoglucanase (At), and an exoglucanase (Ec) was assembled on the yeast cell surface.

To further demonstrate the superiority of using the displayed cellulosome for ethanol production, cellulose hydrolysis and ethanol production were tested with both free enzymes and a displayed minicellulosome. Independent of the number of cellulases incorporated in the minicellulosome, similar levels of enhancement of cellulose hydrolysis, as well as ethanol production, were detected. The ethanol production achieved, in particular, was more than 2.6-fold higher than that of the culture in which all three cellulases were added as free enzymes. This, when combined with an ethanol yield close to 95% of the theoretical maximum, makes this an efficient process for direct fermentation of cellulose to ethanol.

In conclusion, a minicellulosome composed of exoglucanase, endoglucanase, and β-glucosidase activities was successfully assembled on the yeast cell surface. The possibility of displaying enzymes based on the interaction between a displayed anchoring domain and secreted enzymes has recently been reported (18). We are currently working on secreting all three cellulases into the medium to enable their direct assembly into a functional minicellulosome on the yeast cell surface.

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