Deconstruction of Lignocellulose into Soluble Sugars by Native and Designer Cellulosomes

Sarah Moraïs,[a,b] Ely Morag,[c] Yoav Barak,[a,d] Dan Goldman,[e] Yitzhak Hadar,[b] Raphael Lamed,[f] Yuval Shoham,[e] David B. Wilson,[g] and Edward A. Bayer[a]

Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot, Israel[a]; Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, Rehovot, Israel[b]; Designer Energy, Rehovot, Israel[c]; Chemical Research Support, The Weizmann Institute of Science, Rehovot, Israel[d]; Department of Biotechnology and Food Engineering, Technion-Israel Institute of Technology, Haifa, Israel[e]; Department of Molecular Microbiology and Biotechnology, Tel Aviv University, Ramat Aviv, Israel[f]; and Department of Molecular Biology and Genetics, Cornell University, Ithaca, New York, USA[g]

ABSTRACT **Lignocellulosic biomass, the most abundant polymer on Earth, is typically composed of three major constituents: cellulose, hemicellulose, and lignin. The crystallinity of cellulose, hydrophobicity of lignin, and encapsulation of cellulose by the lignin-hemicellulose matrix are three major factors that contribute to the observed recalcitrance of lignocellulose. By means of designer cellulosome technology, we can overcome the recalcitrant properties of lignocellulosic substrates and thus increase the level of native enzymatic degradation. In this context, we have integrated six dockerin-bearing cellulases and xylanases from the highly cellulolytic bacterium, *Thermobifida fusca*, into a chimeric scaffoldin engineered to bear a cellulose-binding module and the appropriate matching cohesin modules. The resultant hexavalent designer cellulosome represents the most elaborate artificial enzyme composite yet constructed, and the fully functional complex achieved enhanced levels (up to 1.6-fold) of degradation of untreated wheat straw compared to those of the wild-type free enzymes. The action of these designer cellulosomes on wheat straw was 33 to 42% as efficient as the natural cellulosomes of *Clostridium thermocellum*. In contrast, the reduction of substrate complexity by chemical or biological pretreatment of the substrate removed the advantage of the designer cellulosomes, as the free enzymes displayed higher levels of activity, indicating that enzyme proximity between these selected enzymes was less significant on pretreated substrates. Pretreatment of the substrate caused an increase in activity for all the systems, and the native cellulosome completely converted the substrate into soluble saccharides.**

IMPORTANCE **Cellulosic biomass is a potential alternative resource which could satisfy future demands of transportation fuel. However, overcoming the natural lignocellulose recalcitrance remains challenging. Current research and development efforts have concentrated on the efficient cellulose-degrading strategies of cellulosome-producing anaerobic bacteria. Cellulosomes are multienzyme complexes capable of converting the plant cell wall polysaccharides into soluble sugar products en route to biofuels as an alternative to fossil fuels. Using a designer cellulosome approach, we have constructed the largest form of homogeneous artificial cellulosomes reported to date, which bear a total of six different cellulases and xylanases from the highly cellulolytic bacterium *Thermobifida fusca*. These designer cellulosomes were comparable in size to natural cellulosomes and displayed enhanced synergistic activities compared to their free wild-type enzyme counterparts. Future efforts should be invested to improve these processes to approach or surpass the efficiency of natural cellulosomes for cost-effective production of biofuels.**

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Address correspondence to Edward A. Bayer, ed.bayer@weizmann.ac.il.

The cellulosome is a multienzyme complex produced by certain anaerobic cellulolytic microorganisms that is specifically designed to overcome the natural recalcitrance of plant cell wall polysaccharides. Cellulose and hemicellulose (mostly xylan) contained in the plant cell wall are both particularly abundant resources and represent attractive renewable substrates for biofuel production from plant biomass and agricultural waste (1, 2). During recent years, major progress has been accomplished in the production of "designer cellulosomes," i.e., artificial enzymatic complexes that have been demonstrated to degrade efficiently crystalline cellulose (3–8). This progress is consistent with global initiatives to promote alternative solutions to the problems of biomass waste and dependence on fossil fuels. In designer cellulosomes, artificial scaffoldins serve as docking backbones that contain both a cellulose-specific carbohydrate-binding module (CBM) and multiple cohesin modules of divergent specificities. The CBM directs the enzymatic complex to the cellulosic substrate, while the cohesins allow the specific incorporation of matching dockerin-bearing enzymes.

In past efforts, we have investigated the conversion of an entire noncellulosomal, free-enzyme system to function in the cellulosome mode (9–14). The well-characterized enzymatic system of the aerobic thermophilic bacterium *Thermobifida fusca* was selected, as it comprises a definite and manageable number of en-

zymes (six well-defined cellulase enzymes and four xylanases). Two cellulases of *T. fusca* (endoglucanase Cel5A and exoglucanase Cel48A) and all four xylanases (Xyn10A, Xyn10B, Xyn11A, and β-xylosidase Xyl43A) have been successfully incorporated into designer cellulosomes, which exhibited enhanced enzymatic activities for degradation of either purified cellulosic substrates or native wheat straw (10, 11, 13, 14). One exoglucanase (Cel6B), however, failed to adapt to the cellulosome mode of action, as it displayed a distinct preference for the free mode of action (9). Interestingly, an endoglucanase (Cel6A) from the same family readily acclimated to the cellulosome mode (9). It thus appears that some enzymes can be freely interchanged between the cellulosomal and free (noncellulosomal) modes, whereas others appear to be more restricted.

In the current communication, we designed a hexavalent scaffoldin bearing six cohesins of divergent specificities, thus allowing precise incorporation of the six cellulosome-competent *T. fusca* enzymes into hexavalent designer cellulosomes. Their combined action in the degradation of the natural complex wheat straw substrate was analyzed compared to that of their wild-type counterparts. Wild-type *T. fusca* β-glucosidase (Bgl1C) was also included in the free state together with the former enzymes for enhanced synergy (19). Finally, in an attempt to increase enzyme absorption and hydrolysis rates, we examined the effect of reduced recalcitrance on wheat straw deconstruction. Thus, two pretreatment strategies that selectively reduced the amount of lignin were conducted prior to substrate degradation by designer cellulosomes.

RESULTS

***Thermobifida fusca* enzymes.** The schematic modular structures of the wild-type enzymes used in this study are shown in Fig. 1. Four different wild-type *T. fusca* xylanases were used, Xyn10B, Xyn11A, Xyn10A, and Xyl43A, as well as three cellulases, Cel5A, Cel48A, and Bgl1C. The family-1 *T. fusca* β-glucosidase Bgl1C was prepared in the present study for synergy with designer cellulosomes and free enzymes. All of the other enzymes have been used as designer cellulosome components in previous studies (13, 14, 20).

Wild-type Xyn10B and Xyn11A xylanases were characterized previously (16, 17). In the native state, wild-type Xyn10B enzyme lacks a CBM (17), and Xyn11A contains a family-2 CBM (CBM2), which shows binding specificity for both cellulose and xylan (16). For the purposes of this study, this CBM is designated XBM, owing to its capacity to bind to xylan and its previously determined effects on enzyme activity (14). Xyl43A carries an X module at its C terminus with no apparent binding function (46). Wild-type Xyn10A contains a CBM2, which exhibited binding ability to cellulose but not to xylan.

The native *T. fusca* cellulases, Cel5A and Cel48A, are typical free (noncellulosomal) enzymes, each of which contains a family-2 cellulose-specific CBM2. Bgl1C consists of a single catalytic module (19).

Construction and expression of designer cellulosome components. In order to convert *T. fusca* enzymes into the cellulosomal mode, each enzyme was joined to a dockerin of divergent specificity (13, 14, 20). The recombinant dockerin-appended proteins designed for use in this study are shown schematically in Fig. 1.

Thermobifida fusca enzymes have been the topic of several previous studies (13, 14, 20), in which dockerins of different specificities were added at the C termini of the native xylanases and N termini of native cellulases, thereby generating the chimeric dockerin-containing enzymes.

In order to integrate Xyn10B into an enzymatic complex, a *Clostridium thermocellum* dockerin (21) was fused at its C terminus, resulting in chimaera 10B-*t*. Interestingly, the incorporation of the resultant 10B-*t* into a chimeric CBM-containing scaffoldin imparts a cellulose-binding component to this enzyme that is inherently lacking in the wild-type enzyme. In chimaera 11A-XBM-*a*, a dockerin from *Acetivibrio cellulolyticus* was appended at the C terminus of the original Xyn11A, whereby the original catalytic module and the essential xylan-binding CBM (termed herein XBM) were both retained.

The Xyn10A enzyme was converted to the cellulosomal mode by replacing its native CBM using a third type of divergent dockerin (chimaera 10A-*f*). Chimaera 10A-*f* is thus a recombinant xylanolytic enzyme consisting of two fused modules: a catalytic module of the family-10 xylanase A from *T. fusca* and a dockerin from *Ruminococcus flavefaciens* (20, 22). In this case, the cellulose-binding function was deemed unnecessary to the action of this enzyme, since the binding ability is recovered upon its incorporation into the chimeric CBM-containing scaffoldin.

The chimeric form of Xyl43A, 43A-*c*, was designed to contain a dockerin from *Clostridium cellulolyticum* at its C terminus. The X module is essential to β-xylosidase activity and was thus retained in the chimera. The wild-type enzyme exhibits binding to xylan but not to cellulose, the latter of which will be acquired by the chimeric enzyme through incorporation into a CBM-containing scaffoldin (20).

The endoglucanase chimaera *g*-5A is a recombinant cellulose-hydrolyzing enzyme consisting of two fused modules: a catalytic module of the family-5 endoglucanase Cel5A from *T. fusca* and the dockerin 2375 from *Archaeoglobus fulgidus*. The exoglucanase chimaera *b*-48A was designed to contain the catalytic module of *T. fusca* exoglucanase Cel48A ligated with a type II dockerin from *Bacteroides cellulosolvens* at its C terminus.

In order to incorporate the complement of dockerin-bearing xylanases into a defined functional designer cellulosome, an elaborate scaffoldin, containing a cellulose-binding CBM and appropriate matching cohesins, is required. The hexavalent chimeric scaffoldin Scaf·CATGBF includes six different cohesin types together with the cellulose-binding CBM3a, as shown schematically in Fig. 1. From the N to C terminus, the cohesin modules of the chimeric scaffoldin include C from *C. cellulolyticum*, A from *A. cellulolyticus*, T from *C. thermocellum*, G from *A. fulgidus*, B from *B. cellulosolvens*, and F from *R. flavefaciens*, with a potent cellulose-binding *C. thermocellum* family-3a carbohydrate-binding module (CBM3a) positioned between A and T. Scaf·CATGBF allows the specific incorporation of the above-described dockerin-containing enzymes and will direct the complex to the complex cellulosic substrate via the CBM.

All purified recombinant proteins showed a single major band on SDS-PAGE (not shown), and in each case their mobility was consistent with their molecular mass.

Enzymatic properties of the produced enzymes. The affinity constant of Bgl1C for pNPG was examined using the Michaelis-Menten method (data not shown). At 50°C and at pH 6, the release of *p*-nitrophenol was linear with time and proportional to enzyme concentration. The apparent K_m value was 0.6 mM, while the k_{cat} was 2.08 s^{-1}.

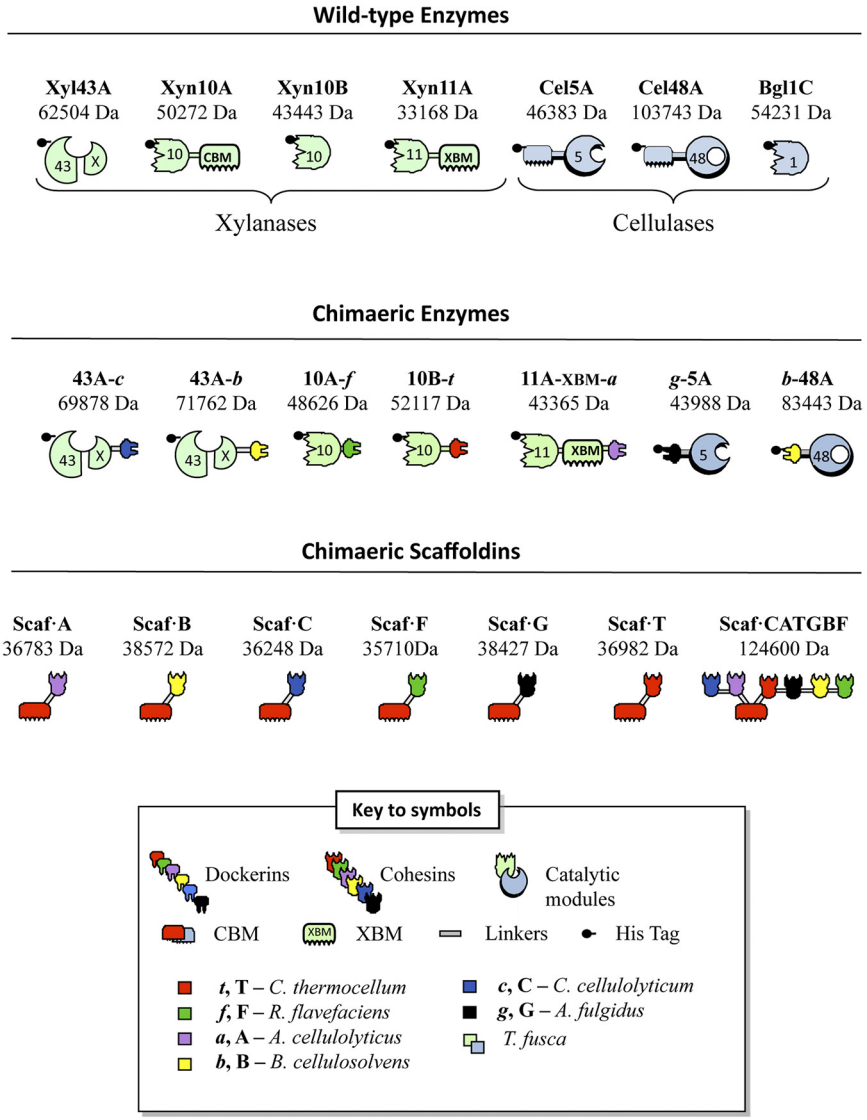

FIG 1 Schematic representation of the recombinant proteins used in this study. The source of the representative module (see key) is indicated as follows: mint green or powder blue, *T. fusca* xylanase or cellulase, respectively; yellow, *B. cellulosolvens*; green, *R. flavefaciens*; red, *C. thermocellum*; lavender, *A. cellulolyticus*; blue, *C. cellulolyticum*; and black, *A. fulgidus*. In the shorthand notation for the engineered enzymes, the numbers 5, 10, 11, 43, and 48 refer to the corresponding GH family (GH5, GH10, GH11, GH43, and GH48) of the catalytic module; uppercase characters (A, B, C, F, G, and T) indicate the source of the cohesin module, and lowercase (*a*, *b*, *c*, *f*, *g*, and *t*) indicate the source of the dockerin module: *A. cellulolyticus*, *B. cellulosolvens*, *C. cellulolyticum*, *R. flavefaciens*, *A. fulgidus*, and *C. thermocellum*, respectively.

The dockerin-bearing β-xylosidase, 43A-*c*, was checked for its activity on pNPX, and the apparent K_m value was 0.6 mM, while the k_{cat} was 12.33 s^{-1} (close to the values obtained for the wild-type enzyme (46). The results of temperature and pH activity studies were comparable to those obtained with the wild-type enzyme (data not shown).

The chimeric cellulase *g*-5A was tested for its activity as a single enzyme on PASC and demonstrated activity levels similar to those of the wild-type Cel5A (data not shown), suggesting that the replacement of the native family-2 CBM by the dockerin module had no significant impact on the catalytic module for this insoluble amorphous cellulosic substrate.

The effect of dockerin replacement or addition to all other enzymes was examined previously (11, 13, 14, 20).

Cohesin-dockerin specificity. The scaffoldin chimaera Scaf·CATGBF was examined by affinity-based enzyme-linked immunosorbent assay (ELISA) (23) to ensure specific affinity to the converted, dockerin-bearing *T. fusca* enzymes. The six cohesins specifically bound their respective dockerin in an exclusive manner (data not shown).

Analysis of complex formation. To examine designer cellulosome complexes, we employed the ability of each complex to bind microcrystalline cellulose by virtue of its resident CBM. For this purpose, the designer cellulosome preparations were added to cellulose, and the bound and unbound fractions were examined by SDS-PAGE. Dockerin-bearing enzymes that remained in the unbound fractions indicated that they failed to interact properly with the matching cohesins of the scaffoldin protein. As seen in Fig. 2,

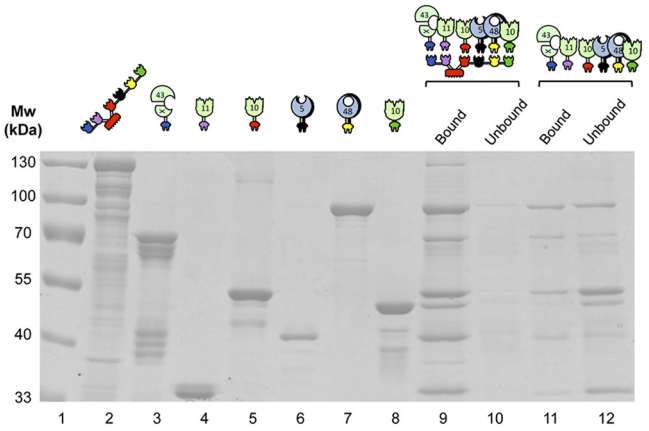

FIG 2 Assessment of complex formation. Dockerin-bearing enzyme components were mixed with and without the hexavalent scaffoldin in equimolar quantities and then combined with cellulose. The cellulose-binding ability of the resultant designer cellulosome complex was then determined by SDS-PAGE. Samples include lane 1, molecular weight marker; lane 2, Scaf·CATGFB; lane 3, 43A-*c*; lane 4, 11A-*a*; lane 5, 10B-*t*; lane 6, *g*-5A; lane 7, *b*-48A; lane 8, 10A-*f*; lanes 9 and 10, hexavalent designer cellulosome, unbound and bound fractions; lanes 11 and 12, mixtures of the six enzymes without scaffoldin, unbound and bound fractions. The designer cellulosome components were associated with the cellulose-bound fraction, whereas the free dockerin-bearing enzymes remained in the unbound fraction.

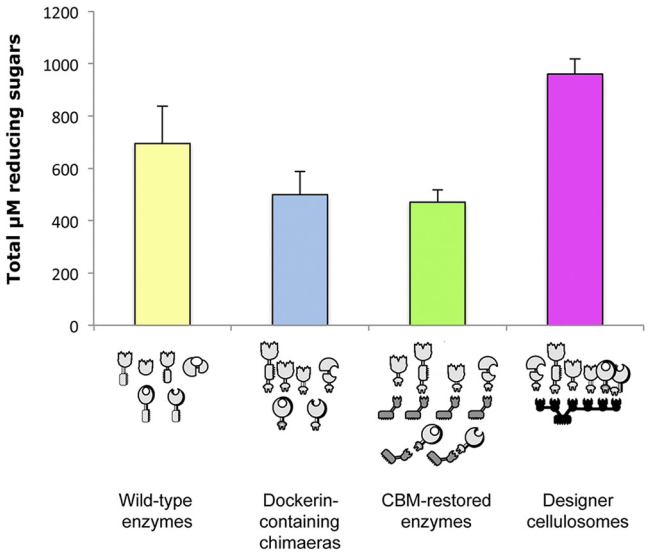

FIG 3 Comparative degradation of wheat straw by hexavalent designer cellulosomes and free-enzyme systems. The composition of the complexes and free-enzyme systems is indicated by the pictograms shown in Fig. 1. Enzymatic activity is defined as μM of total reducing sugars following a 24-h reaction period. Each reaction was performed in triplicate, and standard deviations for wheat-straw hydrolysis are indicated.

it appears that complex formation was nearly complete, as all of the designer cellulosome components were present in the bound fractions, and only negligible amounts of the protein bands were found in the unbound fraction. In the absence of scaffoldin, the dockerin-bearing enzymes mostly failed to bind to the cellulose matrix and remained in the unbound fraction. Residual binding activity was observed for the CBM-lacking chimaeras 43A-*c*, 10A-*f*, *g*-5A, and *b*-48A, suggesting diminished but residual active binding properties of the catalytic module to the cellulosic substrate. The experiment was repeated after incubating the complex for 72 h at 50°C, and the results demonstrated the general stability of the designer cellulosome complexes under those conditions (data not shown).

Pretreatment strategies. Prior to all the experiments of this study, the wheat straw substrate was subjected to mechanical treatment (blending; see Materials and Methods). The action of enzymes and enzymatic complexes was examined either directly following the mechanical treatment or following an additional pretreatment for reduction of lignin content preserving the cellulose/hemicellulose fractions in order to promote enzymatic degradation. In this context, chemical pretreatment with sodium hypochlorite served to increase the cellulose content and reduced significantly the lignin content in the wheat straw while largely maintaining the hemicellulose content (Table 1). Conversely, the biological pretreatment of the wheat straw substrate had no significant effect on lignin content in the present study, in contrast to previously reported biological pretreatments, whereby enzymes secreted by microorganisms were shown to solubilize lignin and increase access to cellulose by cellulase enzymes (24, 25). In addition, the biological pretreatment strategy employed here slightly reduced cellulose content and promoted decomposition of hemicelluloses (Table 1).

TABLE 1 Chemical composition of untreated and treated wheat straw as determined by the improved TAPPI method (45)

Wheat straw	% of dry matter		
	Cellulose	Hemicellulose	Lignin
Untreated	32 ± 1	30 ± 1	21 ± 1
Chemically treated	63 ± 2	31 ± 1	3 ± 1
Biologically treated	27 ± 2	18 ± 1	22 ± 2

Untreated wheat straw degradation. Complexation of the dockerin-bearing enzymes onto the hexavalent Scaf·CATGBF scaffoldin generated a dramatic increase in the amount of reducing sugar production over that of the wild-type enzyme system, following a 24-h incubation with untreated wheat straw (Fig. 3). As in previous publications (13, 14, 20), the control for the targeting effect comprised the combination of a monovalent designer scaffoldin together with a dockerin-bearing enzyme in order to emulate the CBM2-containing free wild-type enzymes. The chimeric dockerin-bearing enzymes, either with or without the monovalent chimeric scaffoldin, exhibited similar activity, somewhat lower than that of the combined wild-type enzyme system (Fig. 3). These results imply that neither the CBM-targeting effect nor the addition of dockerin modules on *T. fusca* enzymes is responsible for the activity enhancement observed with designer cellulosomes.

The assembly of the enzymes onto the hexavalent scaffoldin resulted in a significant proximity effect of the enzymes with a 1.4-fold increase in enzymatic activity after 72 h of wheat straw degradation (Fig. 4). The addition of the Bgl1C to the enzymatic complex served to further increase the observed enhancement of activity (1.6-fold) compared to the mixtures of the free wild-type enzymes. Similar amounts of native *C. thermocellum* cellulosomes exhibited higher levels of wheat straw degradation than the hexavalent designer cellulosome (2.4- and 2.9-fold in the absence and presence of Bgl1C, respectively).

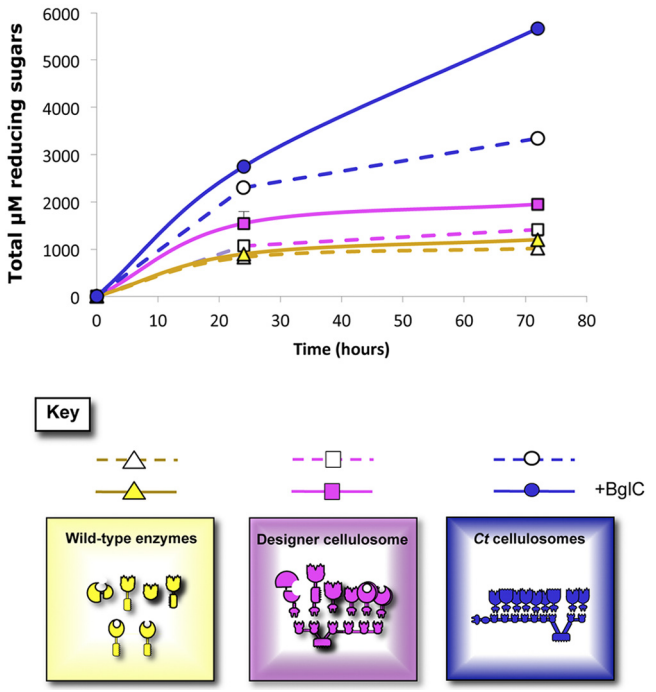

FIG 4 Kinetics studies of wheat straw hydrolysis by native cellulosomes, hexavalent designer cellulosomes, and the free wild-type enzyme system. Curves are labeled as follows: the degradation by the *C. thermocellum* cellulosomes (blue circle), the degradation by the hexavalent designer cellulosome (pink square), and the degradation by the six wild-type enzymes (yellow triangle). Dashed lines represent enzymes without Bgl1C, and continuous lines represent enzymes with Bgl1C added. Enzymatic activity is defined as μM of total reducing sugars. Triplicates of each reaction were carried out. Standard deviations are indicated.

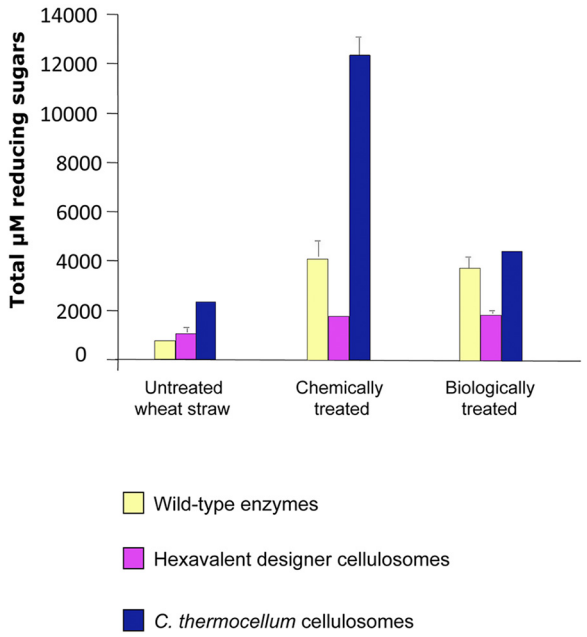

FIG 5 Comparative degradation of untreated and treated wheat straw by native cellulosomes, hexavalent designer cellulosomes, and the wild-type free-enzyme system. Blue bars represent the degradation by the *C. thermocellum* cellulosomes; pink bars, the degradation by the hexavalent designer cellulosome; and yellow bars, the degradation by the six wild-type enzymes. Free Bgl1C was added to all the samples, and free Xyl43A was added to the *C. thermocellum* cellulosomes. Chemical pretreatment was sodium hypochlorite at 12% for 1 h, and biological pretreatment was *P. ostreatus* degradation for 28 days. Enzymatic activity is defined as μM total reducing sugars following a 24-h reaction period. Each reaction was performed in triplicate, and standard deviations for wheat straw hydrolysis are indicated.

Overall reaction yields (based on cellulose/hemicellulose content) comprised about 14.1% for the hexavalent designer cellulosome, compared to 8.7% for the corresponding complement of wild-type enzymes and 28.5% for native *C. thermocellum* cellulosomes (in the presence of Bgl1C, the yields comprised about 16.5%, 10.4%, and 49%, respectively).

Pretreated wheat straw degradation. The effect of reduced recalcitrance on wheat straw deconstruction was also examined, since disrupting the physical barrier of lignin/hemicellulose has been shown to increase subsequent enzyme adsorption and hydrolysis rates (26). For this purpose, we evaluated a biologically based (*Pleurotus ostreatus* growth on the wheat straw substrate) pretreatment and a chemically based (sodium hypochlorite) pretreatment of wheat straw prior to degradation by designer cellulosomes versus that of the free enzymes and native cellulosomes (in each case, identical amounts of Bgl1C and Xyl43A were added).

After 24 h of incubation, the wild-type enzymes exhibited markedly higher levels of degradation over hexavalent designer cellulosome complexes on pretreated wheat straw, reaching sugar yields of 37% and 15%, respectively (Fig. 5; Table 2). Natural *C. thermocellum* cellulosomes exhibited maximum (near-complete) levels of degradation of the chemically treated wheat straw substrate.

Similarly, for the biologically pretreated substrate, the advantage of enzyme proximity in the designer cellulosomes was absent and the free wild-type enzyme mixture exhibited higher enzymatic activities. Sugar yields comprised about 28% for designer cellulosomes and 50.6% for the wild-type enzymes, whereas natural *C. thermocellum* cellulosomes demonstrated a higher capacity of degradation and achieved 61.3% of sugar yield (Table 2).

Soluble sugar production. The fact that the enzymatic composition of the native and designer cellulosome is markedly different should be considered when analyzing the data in Table 3. Both the designer cellulosomes and the equivalent free-enzyme mixture are composed of the same six enzymes (four xylanases and two cellulases) in precisely the same stoichiometric amounts. Native cellulosomes, however, are inherently heterogeneous and vary greatly in composition. The nine enzymes borne by an individual scaffoldin subunit may thus comprise various mixtures of the ~70 different dockerin-containing proteins of *C. thermocellum* (e.g., cellulases, xylanases, and other carbohydrate-active enzymes).

As observed in the present study, compared to the free wild-type enzymes, hexavalent designer cellulosomes produced larger quantities of all sugars (xylose, xylobiose, glucose, and cellobiose) from untreated wheat straw (Table 3). Native cellulosomes produced more xylobiose (presumably due to the absence of an appropriate β-xylosidase to convert xylobiose into xylose units) and cellobiose than the hexavalent designer cellulosome. The high levels of cellobiose can be explained by the higher and more diversified cellulase content in native cellulosomes. In addition, cellotriose and xylotriose were detected only in samples degraded by native cellulosomes, suggesting the presence of additional types of enzymes in the composition of the native cellulosomes (and ab-

TABLE 2 Soluble sugar yields obtained after enzymatic degradation on untreated and pretreated wheat straw samples

Enzyme	% of dry matter		
	Untreated wheat straw	Chemically treated wheat straw	Biologically treated wheat straw
Wild-type enzymes	8 ± 1	37 ± 5	50 ± 6
Hexavalent designer cellulosomes	11 ± 2	15 ± 0.5	28 ± 3
C. thermocellum cellulosomes	22 ± 4	93 ± 7	61 ± 0.5

sent in the six selected enzymes of the free and designer cellulosome mixture). Generally, adding the *T. fusca* β-glucosidase (Bgl1C) to the enzymatic mixtures increased glucose levels at the expense of cellobiose and slightly contributed to xylose and xylobiose production.

In both pretreated samples, the levels of cellobiose measured upon degradation by native cellulosomes were significantly enhanced compared to those measured after treatment with designer cellulosomes or wild-type enzymes (Table 3). High xylobiose concentrations were found in all the samples in descending order, from the wild-type free enzymes, native cellulosomes, and hexavalent designer cellulosomes. The high levels of cellobiose observed in samples subjected to treatment by native cellulosome and the high xylobiose concentration following treatment by wild-type enzymes are consistent with the differences in their enzyme content, i.e., cellulases and xylanases, with higher ratios and more

TABLE 3 Soluble sugar production following digestion of hatched wheat straw and pretreated wheat straw over a 24-h incubation period by various enzyme combinations[a]

Substrate	Enzyme combination	mg/g substrate ± SD (μM/g substrate)						
		Arabinose	Xylose	Xylobiose	Xylotriose	Glucose	Cellobiose	Cellotriose
Untreated wheat straw	WT free enzymes (six enzymes)	ND	17 ± 1 (110)	28 ± 0.8 (98)	ND	3 ± 1 (17)	41 ± 3 (119)	ND
	Hexavalent designer cellulosomes	5 ± 3 (30)	22 ± 2 (145)	35 ± 4 (125)	ND	5 ± 0.6 (30)	44 ± 5 (129)	ND
	Native cellulosomes	8 ± 2 (55)	1 ± 0.5 (10)	80 ± 8 (282)	8 ± 2 (19)	4 ± 0.6 (22)	105 ± 11 (306)	9 ± 3 (18)
	WT free enzymes + Bgl1C	ND	15 ± 4 (101)	37 ± 8 (132)	ND	36 ± 7 (198)	ND	ND
	Hexavalent designer cellulosomes + Bgl1C	3 ± 1 (23)	33 ± 4 (217)	47 ± 5 (167)	ND	37 ± 3 (208)	ND	ND
	Native cellulosomes + Bgl1C	7 ± 3 (48)	11 ± 1 (70)	106 ± 12 (375)	ND	58 ± 7 (322)	34 ± 3 (100)	ND
Chemically pretreated wheat straw	WT free enzymes (six enzymes) + Bgl1C	11 ± 4 (70)	70 ± 5 (466)	300 ± 13 (1,062)	9 ± 2 (22)	13 ± 2 (71)	19 ± 3 (56)	ND
	Hexavalent designer cellulosomes + Bgl1C	17 ± 6 (112)	32 ± 4 (211)	113 ± 9 (400)	ND	9 ± 1 (49)	11 ± 1 (31)	ND
	Native cellulosomes + Bgl1C + Xyl43A	13 ± 2 (89)	29 ± 2 (194)	212 ± 11 (750)	12 ± 1 (28)	11 ± 1 (60)	375 ± 15 (1,094)	ND
Biologically pretreated wheat straw	WT free enzymes (six enzymes) + Bgl1C	0.3 ± 3 (2)	34 ± 2 (225)	256 ± 14 (906)	ND	2 ± 0.7 (11)	21 ± 2 (60)	ND
	Hexavalent designer cellulosomes + Bgl1C	9 ± 2 (57)	29 ± 2 (192)	107 ± 6 (378)	ND	1 ± 0.4 (6)	34 ± 4 (98)	ND
	Native cellulosomes + Bgl1C + Xyl43A	11 ± 3 (74)	40 ± 6 (268)	201 ± 10 (712)	ND	5 ± 2 (29)	89 ± 8 (260)	ND

[a] Data were obtained by high-performance liquid chromatography analysis. Values for xylose and glucose were corroborated using a sugar assay kit. An unidentified peak, present only after enzymatic treatments, eluted at ~4 min (between the xylose and xylobiose peaks), is possibly a modified monosaccharide, as previously described (13, 14, 20). ND, not detected.

diversified types of cellulases in the *C. thermocellum* cellulosomes than in the six *T. fusca* enzymes selected as components of the free-enzyme system.

As a general observation, minute quantities of arabinose were found in some samples, presumably as a residual by-product independent of enzyme content.

DISCUSSION

Designer cellulosome technology has been proposed to increase the native microbial enzymatic degradation of particularly recalcitrant lignocellulosic substrates. By integrating high numbers of enzymes in designer cellulosome complexes, we hoped to approach the complexity of the arrangement of enzymes in natural cellulosomes. In the course of our studies, we developed an incremental approach in which we initially established enhanced synergy between two types of enzymes in a divalent designer cellulosome and systematically increased the number of enzymes by adding them sequentially into trivalent, tetravalent, and now hexavalent designer cellulosomes. The type of cellulosic substrate influences enzyme synergy and thus represents a fundamental parameter of the experimental system. In the present work, wheat straw was chosen as the substrate, since, among the agricultural residues, it is the largest biomass feedstock in Europe and the second largest in the world after rice straw (1). In the experiments described in this article, this natural complex cellulosic substrate was subjected to either physical pretreatment only, in which the experimental conditions are closer to those in nature, or physical pretreatment together with chemical or biological pretreatment, which would presumably facilitate industrial biomass conversion. Moreover, the wheat straw substrate is certainly a more appropriate model feedstock than synthetic cellulosic substrates such as Avicel. In any case, encouraging sugar yields were obtained, using the natural complex cellulosic substrate.

In a previous publication, we demonstrated the strong proximity effect among *T. fusca* cellulases and xylanases on the degradation of the wheat straw substrate (13). While increasing the number of enzymes in our designer cellulosome complexes in the present study, we continue to observe a significant proximity effect between the two types of glycoside hydrolases. In a recent series of articles, Qing and colleagues revealed strong concentration-dependent inhibition of cellulase activity by xylose, xylooligomers, and xylan (27). Moreover, xylooligomers were more inhibitory to cellulase than either xylan or xylose, and, surprisingly, more than equimolar concentrations of glucose or cellobiose. These findings may account for the importance of cellulase-xylanase proximity while degrading a complex substrate such as wheat straw. These authors demonstrated the advantage of adding hemicellulase to the substrate several hours prior to adding cellulase, possibly as a result of a higher observed adsorption affinity of cellulase and xylanase to xylan than to glucan (27, 28). In another publication (29), a strong relationship between xylan removal and the extent of cellulose digestion was established, which indicated that enhanced xylanase-mediated xylan solubilization increased the accessibility of cellulases to cellulose by exposure of new cellulose chains, thereby amplifying cellulose hydrolysis.

Similarly, another study concluded that a xylan structure barrier was one of the major mechanisms that limited the accessibility of the cellulase enzymes to the cellulose and that synergistic cooperation between xylanases and cellulases improves cellulose accessibility by increasing fiber swelling and fiber porosity and plays a major role in enhancing enzyme accessibility (18).

However, our results on pretreated substrates imply that the advantage of the proximity effect between the enzymes within a designer cellulosome is relevant only for complex substrates. Indeed, both chemical and biological pretreatment served to reduce the complexity of the substrate, and their preferred enzymatic degradation was in the free mode of action, in which the enzymes are not concentrated on a specific part of the substrate but can relocate freely after the attack and degradation of a specific part of the substrate. This conclusion was also reported by Fierobe and colleagues in 2005 (5), while studying the action of designer cellulosomes versus free enzymes on both homogeneous and complex cellulosic substrates. Substrate pretreatment also favors inhibitor formation that might affect wheat straw degradation by designer cellulosomes (30).

The addition of *T. fusca* β-glucosidase Bgl1C in the free mode to all enzyme systems served to increase the level of reducing sugars on untreated and pretreated wheat straw produced by both the free-enzyme system and the native and designer cellulosomes. This increase in reducing sugars reflects both the production of additional reducing ends by degrading cellobiose into two molecules of glucose and the release of cellobiose feedback inhibition on the cellulase components of the enzyme cocktails. Similarly, the effect of β-glucosidase supplementation was recently observed only at the early phase of hydrolysis of pretreated switchgrass, where accumulation of cellobiose and oligomers is significant (31). Moreover, it has been demonstrated recently that increased β-glucosidase expression or addition in *C. thermocellum* served to enhance significantly its cellulase activity (32, 33).

Natural *C. thermocellum* cellulosomes exhibited highly enhanced sugar yields on pretreated substrates with near-complete sugar conversion on sodium hypochlorite-treated wheat straw after only 24 h of degradation and more than 60% sugar yield on *P. ostreatus* pretreated samples. The greater sugar yields obtained with native cellulosomes reflects the diversity of complementary enzymes present in native cellulosomes compared to our controlled composition of only six defined enzymes in designer cellulosomes. The data also suggest that designer cellulosome technology still lags behind the hydrolytic competence of natural cellulosomes for degradation of lignocellulosic biomass. In addition, despite the attractive advantages of biological pretreatments (e.g., low cost and environmental considerations), *P. ostreatus* pretreatment is a particularly slow process and, as such, apparently inappropriate for wheat straw biomass conversion processes, since the fungus appeared to prefer using hemicellulose (and cellulose) rather than lignin for growth. In this context, it has been reported that *P. ostreatus* has a preference for cotton straw for growth (34). The screening of a faster and improved strain of *P. ostreatus* on the wheat straw substrate could be an attractive alternative (or other white-rot fungi). Thus, further development will be required before this biological pretreatment strategy can be considered in lieu of chemical processes.

Future studies should address whether we can incorporate more cellulases in the designer cellulosome complexes, since cellulases comprise a major portion of natural cellulosomes relative to other glycoside hydrolases. In recent research (35), an optimal ratio of xylanases to cellulases of 1:3 was recommended using free-enzyme systems, suggesting the use of hemicellulases as accessory enzymes within a cocktail of cellulases for enhancement of

both glucose and xylose yields for consequent reduction in the total enzyme loading. Future studies using designer cellulosomes should therefore address this issue in order to further increase conversion of cellulosic biomass to soluble sugars. In this context, the incorporation of the remaining four *T. fusca* cellulases (Cel6A, Ce6B, Cel9A, and Cel9B) into, or together with, the designer cellulosome complexes is required to obtain a more complete picture of the total xylanase/cellulase-converted system compared to the wild-type free mode of action. Priority should be given to the processive endoglucanase Cel9A, as it is considered to be the most important enzyme for synergistic activity of the complete *T. fusca* system (36) and exhibits synergism with Cel48A on cellulosic substrates (M. Kostylev and D. B. Wilson, unpublished results). At an optimal ratio of 9:1 (Cel48A/Cel9A), it was observed that Cel9A effectively degrades clean crystalline surface, and as the surface is eroded, functional binding of Cel9A is reduced, allowing Cel48A to degrade efficiently loosely bound cellulose chains (as opposed to crystalline surfaces). This action has been proposed to cleanse the surface eroded by Cel9A, thereby enabling the processive endoglucanase to continue the degradation process (M. Kostylev, M.E. Himmel, and D. B. Wilson, unpublished results). In addition, the family-9 enzymes are very common components in all naturally produced cellulosomal systems, and the *T. fusca* family-9 endoglucanase (Cel9B) is the most active endoglucanase as a single enzyme on cellulosic substrates in this bacteria. The question is whether these enzymes will act together with the other cellulosome-converted enzymes to produce designer cellulosomes of enhanced activity on crystalline cellulosic substrates, notably wheat straw.

Fierobe and colleagues (4, 5) have previously shown that when engaging the *C. cellulolyticum* family-9 processive endoglucanase into designer cellulosomes, the overall synergistic activity of the complexes increased. In fact, they have demonstrated that the cellulosomal family-9 endoglucanase is the most important factor for synergistic cellulose degradation observed for these designer cellulosomes.

The *T. fusca* exoglucanase Cel6B should also be reexamined carefully for its possible antagonism within the designer cellulosome complex. As reported earlier (9), the family-6 enzymes (both endo- and exoglucanases) are not part of any known bacterial cellulosomal complex and are known only as free, CBM-bearing cellulases. In contrast to Cel48A (the family-48 cellulases are key enzymes in many natural secreted cellulosomes and exhibited enhanced activities in the cellulosome mode), the chimeric form of the Cel6B exoglucanase (*t*-6B) failed to adapt well to the cellulosomal mode of action and therefore appears only in free cellulase systems. In this context, this enzyme could contribute to the *T. fusca* designer cellulosomes, preferably as a free enzyme. Interestingly, as opposed to the *t*-6B exoglucanase, the other *T. fusca* family-6 enzyme—an endoglucanase—was shown to adapt well to the cellulosome mode of action (9). An alternative for overcoming the reduced activity of the *T. fusca* family-6 exoglucanase-containing designer cellulosomes would be to construct a chimera with the wild-type enzyme's intact CBM, as Fierobe and colleagues reported successful integration into designer cellulosomes of a fungal, noncellulosomal family-6 endoglucanase bearing its original CBM together with cellulosomal bacterial enzymes (37).

Cellulosic biomass is a resource with enough potential to satisfy significant transportation fuel demands. However, the very low rates of lignocellulose decomposition are a barrier to commercialization of biological conversion processes. Sugar production rates must therefore be increased by at least an order of magnitude over those found in nature (47). The assembly of six distinct enzymes into a hexavalent scaffoldin subunit to form a homogeneous multienzyme complex represents a significant advancement in our endeavors to achieve designer cellulosomes that efficiently deconstruct cellulosic substrates. Research and development efforts using biological conversion strategies are accelerating with significant advances in enzyme technologies, and these efforts must be maintained so that these processes can become cost-competitive with fossil fuels.

MATERIALS AND METHODS

Cloning. Wild-type enzymes (Cel5A, Cel48A, Xyn10B, Xyn11A, Xyn10A, Xyl43A), chimeras (*b*-48A, 10B-*t*, 11A-XBM-*a*, 10A-*f*, 43A-*b*), and recombinant scaffoldins (Scaf·A, Scaf·B, Scaf·F, Scaf·G, Scaf·C, Scaf·T) were cloned as described previously (13, 14, 16, 17, 20, 38, 39).

Scaf·CATGBF was assembled from cohesin modules and CBM, which were cloned from the appropriate genomic DNAs. Cohesin C (cohesin1 from scaffoldin C from *Clostridium cellulolyticum*) was amplified using the forward primer 5′ CTTAG**CCATGG**GCGATTCTCTTAAAGTTACA 3′ and reverse primer 5′ CTAGA**GGTACC**GATGGCCTGGTGGGCGT AGC 3′ (NcoI and KpnI sites are in boldface type), cohesin A (cohesin 3 from *Acetovibrio cellulolyticus* scaffoldin C) was cloned using 5′ TATCG **GGTACC**ATTTACAGGTTGACATTGGAAGT 3′ and 5′ TACGT**GAAT TC**AACCTTCAAATTGCCTGATA 3′ primers (KpnI and EcoRI sites are in boldface type), CBM-T (CBM3a and cohesin 3 from the cellulosomal scaffoldin subunit *Clostridium thermocellum* YS) was amplified using 5′ A TCGT**GAATTC**GACAAACACACCGACAAACACA 3′ and 5′ CATGCA **GGATCC**CTATATCTCCAACATTTACTCCAC 3′ primers (EcoRI and BamHI sites are in boldface type), cohesin G (cohesin 2375 from *Archaeoglobus fulgidus*) was cloned using 5′ CATGC**GGATCC**GTTCCTCCGAA AACTACCAT 3′ and 5′ ATCGA**GAATTC**GCTTCTTCCTGAGAGACA AT 3′ primers (BamHI and EcoRI sites are in boldface type), cohesin B (cohesin 3 from *Bacteroides cellulosolvens* scaffoldin B) was amplified using 5′ GTCAT**GAATTC**CGGGGAAAAGTTCACCAG 3′ and 5′ ATCGT **GAGCTC**TTAGTTACAGTAATGCTTCC 3′ primers (EcoRI and SacI sites are in boldface type), and cohesin F (cohesin 1 from *Ruminococcus flavefaciens* strain 17 scaffoldin B) was amplified using 5′ TGACT**GAGC TC**CGCCGGTGGTTTATCCGCTGTG 3′ and 5′ CATGA**CTCGAG**TTA ATGGTGATGGTGATGGTG 3′ primers (SacI and XhoI sites are in boldface type). The different modules were assembled in the linearized pET28a plasmid to form pScaf·CATGBF.

The *g*-5A chimera, i.e., the catalytic module of Cel5A attached directly to a dockerin from *A. fulgidus*, was constructed from pCel5A using primers 5′ CTAGA**GGTACC**CGGCACGCAGCCCGGCACCG 3′ (KpnI site is in boldface) and 5′ GTCA**CTCGAG**TCAGGACTGGAGCTTGCTCC 3′ (XhoI site is in boldface) for amplifying the DNA encoding the catalytic module and primers 5′ GTATT**CCATGG**CACATCACCATCACCATCA CGCAGAAGAAGCAAACAAGGG 3′ (NcoI site is in boldface) and 5′ G TCA**GGTACC**GCCTTACCCAGTAAGCCATT 3′ (KpnI site is in boldface) for amplification of the *A. fulgidus* dockerin 2375. The two modules were ligated (T4 DNA ligase; Fermentas UAB, Vilnius, Lithuania) into NcoI-XhoI (New England BioLabs Inc., Beverly, MA)-linearized pET21a (Novagen Inc., Madison, WI) to form p*g*-5A.

Primer 5′ TTATGT**CATATG**CACCATCACCATCACCATACCTCG CAATCGACGACTCCTC 3′ (NdeI site is in boldface) and 5′ TGCTAA**G AATTC**CTATTCCTGTCCGAAGATTCCCCCGTTG (EcoRI site is in boldface) were used to clone the full-length Bgl1C DNA, and the PCR product was then ligated into NdeI-EcoRI-linearized pET21a to form pBgl1C.

The *C. cellulolyticum* dockerin from scaffoldin A was amplified from the bacterial genomic DNA using the primers 5′ TATTCG**ACTAGT**AAA TTTATATATGGTGATGTTG 3′ and 5′ TTATGC**GAGCTC**TTATTCTT CTTTCTCTTCAACAG 3′ (SpeI and SacI sites in boldface) and was added

to the Xyl43A catalytic module C-terminal cloned using forward primer 5′ TCATGA**CATATG**CACCATCACCATCACCATACTTCTCCCCAAG TCACGTCCT 3′ (NdeI site is in boldface) and 5′ CTATGA**ACTAGT**GG AGGGGGACTGAGGCCGGTA 3′ (SpeI site is in boldface) as a reverse primer to form p43A-*c*.

All enzyme constructs were designed to contain a His tag for the subsequent purification. PCRs were performed using ABgene Reddymix x2 (Advanced Biotechnologies Ltd., United Kingdom), and DNA samples were purified using a HiYield gel/PCR fragment extraction kit (Real Biotech Corporation, RBC, Taiwan).

Protein expression and purification. Wild-type enzymes (Cel5A, Cel48A, Xyn10B, Xyn11A, Xyn10A, Xyl43A), chimeras (*b*-48A, 10B-*t*, 11A-XBM-*a*, 10A-*f*, 43A-*b*), and recombinant scaffoldins (Scaf·A, Scaf·B, Scaf·F, Scaf·G, Scaf·C, Scaf·T) were prepared as described previously (13, 14, 16, 20, 38, 39). The plasmids pBgl1C, pg-5A, and p43A-*c* were expressed in *E. coli* BL21 (lDE3) pLysS cells, and the proteins were purified on an Ni-nitrilotriacetic acid (NTA) column (Qiagen), as reported earlier (12). The plasmid pScaf·CATGBF was expressed, and the protein was purified on phosphoric acid-swollen cellulose, 7.5 mg ml^{-1} (pH 7) (PASC), according to the previously described methodology (38), followed by an additional gel filtration purification step using an AKTA-prime system and Hiload 16/60 Superdex 200 column (GE Healthcare). Purity of the recombinant proteins was tested by SDS-PAGE on 12% acrylamide gels, and the fractions containing the pure recombinant protein were pooled and concentrated using Vivaspin concentrators. Protein concentration was estimated from the absorbance at 280 nm based on the known amino acid composition of the protein using the Protparam tool (http://www.expasy.org/tools/protparam.html). Proteins were stored in 50% (vol/vol) glycerol at –20°C.

Kinetic studies. Temperature-equilibrated reactions of a 180-μl final volume (100 mM buffer citrate [pH 6], 1 g/liter bovine serum albumin [BSA], 0 to 15 mM *p*-NP-β-D-glucopyranoside [pNPG], or *p*-NP-β-D-xylopyranoside [pNPX] at 50°C) were initiated by adding 10 μl of the enzyme (0.05 μM). The absorbance was read at 420 nm at 14-s intervals for 10 min using an ELISA plate reader (Bio-Tek Synergy HT, Winooski, VT). Absorbencies were then converted to molarities using an extinction coefficient of 3,365 $M^{-1} \cdot cm^{-1}$ for the absorbance of 4-paranitrophenol in buffer citrate (pH 6) at 50°C.

Affinity-based ELISA. The matching fusion-protein procedure of Barak et al. (12, 23) was followed to determine cohesin-dockerin specificity of interaction.

Cellulose-binding assay. Equimolar mixtures of pure proteins (70 picomole each in 50 mM citrate buffer, pH 6.0, 12 mM $CaCl_2$, 2 mM EDTA) were incubated for 2 h at 37°C and mixed with 20 mg of microcrystalline cellulose (Avicel, FMC Biopolymer Philadelphia, PA) in a final volume of 200 μl. The tubes were incubated at 4°C for 1 h with gentle mixing before being centrifuged at 16,000 × *g* for 2 min, and the supernatants (containing unbound protein) were carefully removed. The proteins in the unbound fractions were precipitated by trichloroacetic acid (TCA; at a final concentration of 10%) and incubated on ice for 10 min before being centrifuged for 5 min at 16,000 × *g*. The pellet was then washed three times in 200 μl cold-acetone and centrifuged for 5 min at 16,000 × *g*. The protein pellet was dried for 5 min at 95°C and finally resuspended in 25 μl of SDS-containing buffer.

The polysaccharide pellet (bound fraction) was washed twice by resuspension in 100 μl of the Tris buffer saline (TBS; supplemented with 0.05% Tween 20 to eliminate nonspecific binding), centrifuged at 16,000 × *g* for 2 min, resuspended in 40 μl of SDS-containing buffer, and boiled for 10 min to dissociate any bound protein.

Equimolar mixture of enzymes without the scaffoldin was used as a negative control to ensure specificity of binding. The chimeric enzyme 11A-*a* [lacking XBM (14)] was used to avoid direct binding of the 11A-*a* enzyme to cellulose. Bound and unbound fractions and single proteins were analyzed by SDS-PAGE using a 10% polyacrylamide gel. A similar assay was realized after an incubation period of 72 h at 50°C.

Activity assay. All assays were performed in triplicate. PASC degradation was assayed with Cel5A and *g*-5A, varying from 0 to 0.32 μM in a final volume of 200 μl (50 mM acetate buffer [pH 5.0], 12 mM $CaCl_2$, 2 mM EDTA). The suspensions were incubated at 50°C for 1 h, reactions were terminated by immersing the sample tubes in ice water, and the samples were centrifuged at max speed to remove the substrate.

Hatched wheat straw (blended at 0.2 to 0.8 mm) provided by Valagro (Poitiers, France) was treated as described previously (5, 40). A typical assay mixture consisted of A 200-μl reaction (50 mM citrate buffer [pH 6.0], 12 mM $CaCl_2$, 2 mM EDTA, 3.5 g/liter wheat straw) containing the enzymes (that interacted formerly at 37°C for 2 h with the scaffoldin; in case of the adaptor scaffoldin, it was initially subjected to interaction with the *b*-48A enzyme, and then other enzymes and primary scaffoldin were added) at about 0.3 μM (optimized equimolar ratio). Bgl1C or Xyl43A enzymes were added at 0.3 μM where specified. Reaction mixtures were incubated for up to 72 h at 50°C.

Gel-filtrated *Clostridium thermocellum* cellulosomes were kindly provided by Designer Energy (Rehovot, Israel) and assayed on wheat straw as described above except for acetate buffer (pH 5) and incubation at 60°C. The amount of cellulosomes equivalent to 0.3 μM hexavalent designer cellulosome in 200 μl was calculated at 20.3 μg.

To evaluate reducing sugar concentration, dinitrosalicylic acid (DNS) solution (150 μl) was added to 100 μl of supernatant fluids (41), and the reaction mixture was boiled for 10 min. Optical density was measured at 540 nm, and activity was determined from a glucose standard curve.

Sugar analysis. Sugar content was analyzed using a high-performance anion-exchange chromatography (HPAEC) system equipped with a PA1 column (Dionex, Sunnyvale, CA). Samples that consisted of the supernatants of the reaction mixtures obtained after centrifugation were loaded onto the PA1 column, and NaOH (200 mM) served as the elution solution (using 1 ml/min as the elution flow rate). Arabinose, xylose, xylobiose, xylotriose, glucose, cellobiose, and cellotriose standards were loaded separately to determine elution time and peak areas as a function of the sugar concentration in order to identify sugars present in each sample as well as their concentrations. Arabinose and xylose were observed in blanks at low levels (samples consisted of double-distilled water instead of the enzymatic mixture); these values were deducted in all the samples.

Xylose concentrations were analyzed by a D-xylose assay kit purchased from Megazyme (Wicklow, Ireland), and glucose concentration was determined using a glucose assay kit (product code GAGO20; Sigma-Aldrich); both kits were used according to the manufacturer's instructions.

Chemical wheat straw pretreatment. Wheat straw was subjected to pretreatment with sodium hypochlorite at a concentration of 12% at room temperature for 1 h.

Biological wheat straw pretreatment (solid-state fermentation). *Pleurotus ostreatus* Florida strain F6 (ATCC 58053) was grown on 2% agar basidiomycete synthetic medium (BSM [42]) plates at 28°C for 7 days and served as the inoculum for growth in plastic cups containing sorghum seeds at 28°C for 8 days (sorghum seed preparation: 900 ml of double-distilled water was added to 1 kg of sorghum seeds and autoclaved for 1 h; after 24 h, the seeds were mixed with 100 g $CaSO_4$ and 10 g $CaCO_3$ for acidification and to avoid adhesion of the seeds; finally, 42 g of the mixture was placed in 100-ml plastic cups and autoclaved for 1 h). Wheat straw was supplemented with 150 mg manganese sulfate (Sigma) per gram of substrate (43), and 10 g of substrate was placed in 100-ml plastic cups, moistened at 130%, and autoclaved twice for 20 min at 120°C (water could evaporate through the unclosed plastic cups). Five sorghum grains were added to 10 g (dry weight) of prepared wheat straw substrate and incubated at 28°C for 28 days (44). Fungal growth was monitored by measuring the dry weight of the samples.

Determination of wheat straw substrate chemical composition. Chemical composition of the samples was determined according to the following improved TAPPI methods (45). For hemicellulose content, samples were boiled in 2% HCl for 2 h, washed with double-distilled water and ethanol, and dried at 105°C to a constant weight (about 2 to 3 h). For

cellulose content, samples were boiled in Kürschner reagent (30% HNO_3 solution in ethanol) for 1 h; washed successively with ethanol, double-distilled water, and ethanol; and dried at 105°C to a constant weight (about 2 to 3 h). For lignin content, samples were swollen in 72% H_2SO_4 at room temperature for 2 h, diluted with double-distilled water to 8 to 10% acid, hydrolyzed in the boiling diluted acid solution (8 to 10%) for 2 h, washed with double-distilled water and ethanol, and dried at 105°C to a constant weight (about 2 to 3 h). Total solid content was determined by drying the samples at 105°C for 2 h.

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