Functional Assembly and Characterization of a Modular Xylanosome for Hemicellulose Hydrolysis in Yeast

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ABSTRACT: Five trimeric xylanosomes were successfully assembled on the cell surface of Saccharomyces cerevisiae. Three dockerin-tagged fungal enzymes, an endoxylanase (XynAc) from Thermomyces lanuginosus, a β-xylosidase (XlnDt) from Aspergillus niger and an acetyl xylan esterase (AwAXEf) from Aspergillus awamori, were displayed for the synergistic saccharification of birchwood xylan. The surface-expression scaffoldins were modular constructs with or without carbohydrate binding modules from Thermotoga maritima (family 22) or Clostridium thermocellum (family 3). The synergy due to enzyme–enzyme and enzyme–substrate proximity, and the effects of binding domain choice and position on xylan hydrolysis were determined. The scaffoldin-based enzymes (with no binding domain) showed a 1.6-fold increase in hydrolytic activity over free enzymes; this can be attributed to enzyme–enzyme proximity within the scaffoldin. The addition of a xylan binding domain from T. maritima improved hydrolysis by 2.1-fold relative to the scaffoldin without a binding domain (signifying enzyme–substrate synergy), and 3.3-fold over free enzymes, with a xylose productivity of 105 mg g⁻¹ substrate after 72 h hydrolysis. This system was also superior to the xylanosome carrying the cellulose binding module from C. thermocellum by 1.4-fold. Furthermore, swapping the xylan binding module position within the scaffoldin resulted in 1.5-fold more hydrolysis when the binding domain was adjacent to the endoxylanase. These results demonstrate the applicability of designer xylanosomes toward hemicellulose saccharification in yeast, and the importance of the choice and position of the carbohydrate binding module for enhanced synergy.


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catalytic domains toward the biomass substrate, and several cohesin domains for dockerin-associated enzyme attachment (Lamed and Bayer, 1988; Lamed et al., 1983). Based on the modular nature of the cellulosomal subunits and the availability of distinct cohesin–dockerin pairs, designer cellulomes (Bayer et al., 1994; Fierobe et al., 2002, 2005), carrying cellulases and/or xylanases from various microorganisms have been studied in cell-free systems for synergistic action toward the saccharification of complex biomass. Tsai et al. (2009) extended this concept to \textit{Saccharomyces cerevisiae} with the functional surface-display of a trimeric mini-cellulosome based on the specificity of interactions between distinct cohesin–dockerin pairs (Pages et al., 1997) from \textit{C. thermocellum}, \textit{C. cellulolyticum}, and \textit{Ruminococcus flavefaciens}. This system was used for the ordered display of three cellulases and achieved a 2.4-fold greater hydrolysis of phosphoric acid swollen cellulose (PASC) with a 2.6-fold improvement in ethanol concentration over free enzymes. The scaffoldin and enzyme ratios were further optimized for a synthetic yeast consortium with a final ethanol yield of approximately 0.48 g ethanol per g cellulose consumed (Tsai et al., 2010). A similar study by Wen et al. (2010) reported the consolidated bioprocessing of PASC in yeast to a final ethanol titer of approximately 1.8 g L\(^{-1}\).

Similar to cellulomes, naturally occurring "xylanomes" have been found both in anaerobic fungi and bacteria (Coughlan and Hazlewood, 1993; Jiang et al., 2006; Woodward, 1984). Earlier studies have shown synergistic activities of secreted xylanolytic enzymes (La Grange et al., 2001; Sunna and Antranikian, 1997; Wong et al., 1988), independent non-scaffoldin associated surface-expressed enzymes (Fujita et al., 2002; Katahira et al., 2004; Sakamoto et al., 2012) in yeast, or enzymes displayed on a cell-free modular scaffoldin in combination with cellulases (Moraís et al., 2010, 2011).

In this study, we have combined the concepts of hemicellulase enzyme synergy and yeast surface-display modular scaffolds with the aim of enhanced saccharification of hemicellulosic substrates in yeast. Applying the system of highly specific cohesin–dockerin pairs used by Tsai et al. (2009), the functional modular "designer-xylanosome" developed in this study consists of an engineered trimeric scaffoldin from the Scaf6 system (Fierobe et al., 2005) for surface-display in \textit{S. cerevisiae}. The set of enzymes selected for display on the modular scaffoldin included two fungal hemicellulases: an endo-\(\beta\)-1,4 xylanase XynA (\textit{Thermomyces lanuginosus}) (Damaso et al., 2003) and a \(\beta\)-xylanosidase XlnD (\textit{Aspergillus niger}) (La Grange et al., 2001), and an acetyl xylan esterase AwAXE from \textit{Aspergillus awamori} (Koseki et al., 2005). Since these enzymes naturally occur without internal binding domains, we compared the effectiveness of two carbohydrate binding modules on xylan hydrolysis in the engineered xylanomes: a \textit{Thermotoga maritima} xylan-binding domain (A2) from family 22 that has been previously characterized to bind soluble xylan (Kleine and Liebl, 2006), and a C. thermocellum cellulose binding domain from family 3 which has been known to bind crystalline cellulose (Fierobe et al., 2005; Shoham et al., 1999). In addition to comparing the hydrolytic activity between systems with different binding modules, we also investigated the influence of position of the xylan binding domain relative to the hemicellulases on xylan hydrolysis.

To our knowledge, this is the first report of a system of xylanolytic enzymes surface-displayed on a modular scaffoldin in yeast for hemicellulose hydrolysis. The designer xylanosome system reported here enables control of the number of enzymes displayed per scaffoldin and the order of enzymes with respect to the CBD, allowing synergy due to enzyme–enzyme and enzyme–substrate proximity.

**Materials and Methods**

**Strains, Plasmids, and Media**

\textit{Escherichia coli} DH5\(\alpha\) (Invitrogen, Carlsbad, CA) was used for plasmid maintenance and amplification. \textit{S. cerevisiae} BY4741 \textit{d}TRP1 (\textit{MATa his3\(\Delta\)1 leu2\(\Delta\)0 met15\(\Delta\)0 ura3\(\Delta\)0 trp1\(\Delta\)0; Open Biosystems, Lafayette, CO) was used for surface expression of the scaffolds and enzymes. \textit{E. coli} cultures were grown in Luria-Bertani (LB) medium supplemented with 100 \(\mu\)g mL\(^{-1}\) ampicillin for selection. Yeast cultures were grown in selective SDC(A,T) or SGC(A,T) medium (2% glucose or 2% galactose, respectively, 0.67% yeast nitrogen base without amino acids, 0.5% casamino acids, adenine-sulfate (100 \(\mu\)g mL\(^{-1}\)), tryptophan (100 \(\mu\)g mL\(^{-1}\)), and supplemented with uracil (100 \(\mu\)g mL\(^{-1}\) when needed), or in selective SD medium (2% dextrose, 0.67% yeast nitrogen base without amino acids, adenine-sulfate (100 \(\mu\)g mL\(^{-1}\)), histidine-HCl (100 \(\mu\)g mL\(^{-1}\)), leucine (150 \(\mu\)g mL\(^{-1}\)), uracil (100 \(\mu\)g mL\(^{-1}\)), and tryptophan (100 \(\mu\)g mL\(^{-1}\)) for \textit{MET15}-marked vectors.

To construct the plasmid for the constitutively expressed surface display scaffoldin tfc (Fig. 1), the gene fragments encoding the three cohesin domains from \textit{C. cellulolyticum} (c), \textit{C. thermocellum} (t), and \textit{R. flavefaciens} (f), were amplified from plasmid pAGo2Scaf6 (Goyal et al., 2011) derived from the plasmid pETscaf6 (Fierobe et al., 2002). The amplified fragments, which include an N-terminal MFA1 secretion signal, were ligated downstream of the \textit{PGK1} promoter on a CEN/ARS expression vector Ycplac33-3\-Ago-scaf3 (Goyal et al., 2011) carrying the sequence for the C-terminal Ag\(\alpha\) surface-expression anchor (Murai et al., 1997). The final tfc scaffoldin construct comprises the cohesin domains in the order t-f-c from the N- to C-terminus of the protein, with a myc tag on the N-terminus for fluorescence detection. The construction of other plasmids (Fig. 1) carrying the scaffoldins (pAGo2-tfcCBD, -tfcXBD, -XBDtfc, and -tfXBDc) followed similar procedures with the xylan binding module XBD (excised from pUC57A2 with the A2 gene sequence (Winterhalter et al., 1995) synthesized by GenScript) or CBD (amplified
from pAGα2Scaf6) inserted into the desired location on pAGα2tfc. Details on the construction of the surface-expression vectors and the primers used in this study are in Supporting Information SI-1 and SI-2, respectively.

The gene coding for the XynA enzyme was fused to the dockerin from _C. cellulolyticum_ and inserted into the 2µ-based pCEl15 expression vector (Moses et al., 2005) to form pCEL15XynAc. Similarly, the gene coding for AwAXE was fused to the dockerin from _R. flavefaciens_ and inserted into the 2µ-based YEplac112 expression vector (Geitz and Sugino, 1988) to form pYEplac112AwAXEf. The gene coding for β-xylosidase XlnD was reverse transcribed from the mRNA of _A. niger_ strain 90196 (La Grange et al., 2001). The XlnD cDNA was fused to the dockerin from _C. thermocellum_ and inserted into the CEN/ARS based plasmid pXP114 (Fang et al., 2011), to form pXP114XlnDt. All gene sequences coding for the enzymes included an N-terminal MFα1 secretion signal sequence. Details on the construction of the enzyme expression vectors are provided in Supporting Information SI-3. The scaffoldins and dockerin-tagged enzymes used in this study are summarized in Table I.

**Surface Display of Scaffoldins on Yeast Cell Surface**

Yeast strains carrying the surface-expression plasmids were precultured in selective medium SDC (A, T) for 18 h at 30°C.
and inoculated to an OD_{600} of 0.5 in 75 mL SDC (A, T) medium supplemented with 10 mM CaCl2. Cells were grown at 20°C for 36 h, harvested by centrifugation (3,000g, 4 mins) at 4°C and resuspended in binding Buffer A (50 mM Na-Citrate pH 7.0, 20 mM CaCl2).

**Enzyme Expression, Concentration, and Xylanosome Assembly on Cell Surface**

Yeast strains carrying enzyme expression plasmids were precultured at 30°C for 18 h in selective medium, and inoculated to an OD of 0.5 into 75 mL medium supplemented with 10 mM CaCl2. Cells were then grown for 36 h at 20°C for enzyme expression and secretion. The enzymes in the culture medium (supernatant) were collected by centrifugation (3,000g, 10 min) at 4°C and concentrated 30-fold with Amicon (PLGC06210, Millipore, Billerica, MA) membrane filter discs. The filtered enzymes were incubated with yeast cells displaying the scaffoldin for 2 h in Buffer A at 4°C. Following incubation, the cells were washed three times, and stored at 4°C in Buffer A for further application.

**Immunofluorescence Microscopy and Whole Cell Fluorescence**

Yeast cells displaying the surface expression scaffoldin were washed with phosphate-buffered saline (PBS; 0.8% NaCl, 0.02% KCl, 0.144% Na2HPO4, and 0.024% KH2PO4), and resuspended in 250 μL PBS containing 1 mg mL⁻¹ bovine serum albumin (BSA) and 0.5 μg anti-c-Myc for 4 h with occasional mixing. Cells were pelleted and washed with PBS, and incubated for 2 h in 250 μL PBS with 1 mg mL⁻¹ BSA and 0.5 μg anti-mouse IgG conjugated with Alexa488 (Molecular Probes, Life Technologies, Grand Island, NY). The cells were then washed three times and resuspended to OD_{600} 1.0. The percentage fluorescence was measured using fluorescence microscopy (Olympus BX51), and the images captured using Wasabi software. To determine whole cell fluorescence, the relative fluorescence units was measured with an AMINCO Bowman Series 2 luminescence spectrometer at excitation and emission wavelengths of 485 and 535 nm, respectively. Cells displaying no scaffoldin were used as control. All fluorescence measurements were conducted in triplicate.

**Resting Cells Assay**

Yeast cells expressing scaffoldins were incubated with three times the saturating level of enzymes to allow cohesin–dockerin interactions for surface display. The cells were centrifuged at 3,000g, 10 min at 4°C and the supernatant carrying residual enzymes was tested for activity (enzyme wash-off). The cell pellets were washed at least three times with Buffer A to remove any non-specifically bound enzyme. The cells with surface displayed scaffoldins were then resuspended in Buffer A supplemented with 1% birchwood xylan (Sigma, St. Louis, MO) to allow hydrolysis for 48 h at 50°C. The temperature for hydrolysis was chosen to test for maximum activity from the thermostable xylanolytic enzymes displayed on the cell surface. The hydrolysate was collected across 48 h and the hydrolytic activity was measured using DNS (Bailey et al., 1992; Miller, 1959), PNPA, or PNPX assays. Three controls were used: cells used in the analysis. To determine the amount of enzymes displayed on the cell surface, a volumetric single enzyme loading–activity correlation was established for free enzymes. The activity measured from cells displaying scaffoldin-bound enzymes could then be correlated to a volumetric enzyme loading. The accuracy of the method was validated for single enzyme displays on tfc by subtracting the activity measured in the enzyme wash-off from the total enzyme loaded, and comparing this value to the activity measured from the scaffoldin-bound enzymes.

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**Table I.** Scaffolds and dockerin-tagged enzymes used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Description from N- to C-terminus</th>
<th>Expression vector</th>
<th>Selection</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scaf-ctf</td>
<td>Original template scaffoldin w/cohesin “c” (C. cellulolytica), CBD (C. thermocellum, family 3 CBM), cohesin “t” (C. thermocellum), cohesin “f” (R. flavefaciens), and c-Myc tag</td>
<td>pAG25Scaf6</td>
<td>Amp’</td>
<td>Goyal et al. (2011)</td>
</tr>
<tr>
<td>tfc</td>
<td>Modular scaffoldin w/Myc-tag “t” “f” and “c”</td>
<td>pAG2tfc</td>
<td>URA3</td>
<td>This study</td>
</tr>
<tr>
<td>tfcCBD</td>
<td>Modular scaffoldin w/Myc-tag “t” “f” “c” and CBD</td>
<td>pAG2tfcCBD</td>
<td>URA3</td>
<td>This study</td>
</tr>
<tr>
<td>tfCBSXbd</td>
<td>Modular scaffoldin w/Myc-tag “t” “f” “c” and XBD</td>
<td>pAG2tfCBSXbd</td>
<td>URA3</td>
<td>This study</td>
</tr>
<tr>
<td>XBDtc</td>
<td>Modular scaffoldin w/Myc-tag “t” “f” “c” XBD and “c”</td>
<td>pAG2XBDtc</td>
<td>URA3</td>
<td>This study</td>
</tr>
<tr>
<td>tfCBSXbdC</td>
<td>Modular scaffoldin w/Myc-tag “t” “f” “c” XBD and “c”</td>
<td>pAG2tCBSXbdC</td>
<td>URA3</td>
<td>This study</td>
</tr>
<tr>
<td>XlnDt</td>
<td>β-xylanosidase XlnD from A. niger fused w/dockerin from C. thermocellum</td>
<td>pXP14lxlnDt</td>
<td>MET15</td>
<td>This study</td>
</tr>
<tr>
<td>AwaAXEf</td>
<td>Acetyl xylan esterase AwaAXE from A. awamori w/dockerin from R. flavefaciens</td>
<td>YEplac112AwaAXEf</td>
<td>TRP1</td>
<td>This study</td>
</tr>
<tr>
<td>XynAc</td>
<td>Endo-β-1,4 xylanase XynA from T. lanuginosus w/dockerin from C. cellulolyticum</td>
<td>pCEL15XynAc</td>
<td>URA3</td>
<td>This study</td>
</tr>
<tr>
<td>CBD</td>
<td>Family 3 cellulose binding domain from C. thermocellum</td>
<td>pAG25Scaf6</td>
<td>Amp’</td>
<td>This study</td>
</tr>
<tr>
<td>XBD</td>
<td>Family 22 xylan binding domain (A2) from T. maritima</td>
<td>pUC57A2</td>
<td>Amp’</td>
<td>This study</td>
</tr>
</tbody>
</table>
Enzyme Activity Assays

Reducing sugars released by free or surface-displayed enzymes were assayed by the method of DNS (Bailey et al., 1992) in the presence of 1% (w/v) birchwood xylan (Sigma) at 50°C in 50 mM Na-Citrate buffer (pH 5.0) supplemented with 10 mM CaCl₂. The samples were collected periodically during hydrolysis, the reaction stopped with 3 mL DNS reagent, the color developed at 95°C for 15 min, and absorbance read at 540 nm. One unit is defined as the amount of enzyme that releases 1 μmol of reducing sugars per minute. The β-xylanosidase and acetylxylan esterase enzyme activities were determined by measuring the amount of p-nitrophenol released from p-nitrophenol-β-D-xioside (PNPX; Sigma) and p-nitrophenol-acetate (PNPA; Sigma), respectively. For each reaction, 50 μL of enzyme was incubated with 500 μL of 10 mM PNPX or PNPA solution and 500 μL of 100 mM citrate buffer, pH 5.0 at 37°C for 10 min. The reaction was stopped with 3 mL of 200 mM borate buffer, pH 9.8, and the absorbance read at 405 nm. In both PNPX and PNPA assays, one unit is defined as the amount of enzyme that hydrolyzes 1 μmol of p-nitrophenol at 37°C, pH 5.0 per minute.

HPLC Analysis of Xylan Hydrolysate

Samples from the resting cell assay were centrifuged at 3,000g for 10 min at 4°C and filtered with a 0.45 μm pore-size Acrodisc filter. The oligosaccharide products of enzymatic hydrolysis of birchwood xylan were analyzed on a Shimadzu HPLC (Prominence UFLC) system with an Aminex HPX-42A column (BioRad, Hercules, CA) and a Shimadzu RID-10A detector. Fifty systems with an Aminex HPX-87H ion exclusion column maintained at 60°C with 5 mM sulfuric acid as eluent at a flow rate of 0.4 mL min⁻¹. The acetic acid released by the acetylxylan esterase was measured using an Aminex HPX-87H ion exclusion column maintained at 60°C with 5 mM sulfuric acid as eluent at a flow rate of 0.6 mL min⁻¹. Internal standards were used to prepare standard curves for quantitative analysis from the HPLC chromatograms. Xylobiose and xylotriose (both HPLC grade) were purchased from Wako chemicals USA, Ltd. (Richmond, VA); D-xylose (molecular biology grade) was purchased from Sigma; and glacial acetic acid was purchased from Fisher Chemical (Fairlawn, NJ).

RESULTS

Construction and Surface Expression of the Modular Scaffoldin

A mini-xylanosome on the yeast surface allows enzyme and binding domain synergy for the hydrolysis of hemicellulose. We designed a modular trimeric scaffoldin comprising the C. thermocellum (t), C. cellulolyticum (c), and R. flavefaciens (f) cohesin domains and a carbohydrate binding module (the Thermotoga maritima family 22 xylan binding domain “XBD” or the C. thermocellum family 3 cellulose binding domain “CBD”) for the saccharification of birchwood xylan. The basic scaffoldin tfc with no internal carbohydrate binding module was constructed with the Aga1 anchor system (Murai et al., 1997) and an N-terminal MFA1 secretion signal, under the control of the PGK1 promoter. This construct was further modified to include a carbohydrate binding module, XBD or CBD at the C-terminus (Fig. 1) to study the importance of the domain on xylan hydrolysis. These binding modules have been shown to have only binding and no catalytic activity on polymeric sugars (Kleine and Liebl, 2006; Poole et al., 1992). In addition to these constructs, two other XBD-based scaffoldins (tfcXBD and tfcCBD) were constructed (Fig. 1) to evaluate the effect of binding module position within the scaffoldin on hydrolysis. The five scaffoldins allow a detailed analysis of xylan hydrolysis based on (1) enzyme synergy, (2) binding module synergy (for both XBD and CBD), and (3) positional effects of the carbohydrate binding module for this system of enzymes.

The scaffoldin construct also carried a c-Myc tag on the C-terminus for fluorescence detection of cells displaying the scaffoldins. The fluorescence images confirmed display of the modular scaffoldin constructs on the yeast cell surface (Fig. 2). All five systems had similar levels of fluorescence (Supporting Information SI-4), with approximately 80% of the cells displaying the scaffoldin on the surface.

Assembly of Enzymes on the Displayed Scaffoldin

Three fungal hemicellulase enzymes, T. lanuginosus endo-β-1,4 xylanase (XynA), A. niger β-xylanosidase (XlnD), and A. awamori acetyl xylan esterase (AwAXE), were tagged at the C-terminus to distinct dockerins (-c, -t, and -f, respectively) that bind specifically to the corresponding

![Figure 2](image-url)
cohesins (see Fig. 1). The dockerin-tagged enzymes were secreted into culture medium, concentrated and tested for specific activity by enzyme-specific assays. All three dockerin-tagged enzymes were found to be active in the culture medium (Table II).

To determine the functionality of these glycosyl hydrolases on the surface displayed scaffoldin, yeast cells displaying the trimeric scaffoldin tfc was incubated with greater than saturating levels of enzymes at 4°C for 2 h for cohesin–dockerin interactions. Excess enzymes were then washed off and the cells were tested for hydrolytic activity of either single enzymes or enzyme-complexes on birchwood xylan to confirm functionality of the xylanosome. The whole cell hydrolysis assays were normalized to the number of scaffoldins surface-displayed by relative fluorescence (Supporting Information SI-4).

**Comparing the Hydrolytic Activity of the Scaffoldins**

The extent of xylan hydrolysis was determined by measuring the reducing sugars released for the three scaffoldin constructs tfc, tfcXBD, and tfcCBD. The cells were cultured to express the trimeric scaffoldins and incubated with three times the saturating-level of the dockerin-tagged enzymes to account for any fluctuations in growth or surface-expression. The cells were washed and resuspended in Buffer A with 1% xylan, and hydrolysate samples were collected periodically for 48 h.

**Table II.** Activity of dockerin-tagged hemicellulases from culture medium.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Activity of enzyme&lt;sup&gt;a&lt;/sup&gt; (U/mL)</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>XynAc (2 μ-based plasmid)</td>
<td>9.5 ± 0.03</td>
<td>DNS</td>
</tr>
<tr>
<td>XlnDt (2 μ-based plasmid)</td>
<td>0.5 ± 0.02</td>
<td>PNPX</td>
</tr>
<tr>
<td>AwAXEf (CEN/ARS-based plasmid)</td>
<td>0.18 ± 0.02</td>
<td>PNPA</td>
</tr>
</tbody>
</table>

<sup>a</sup>Indicates the standard deviation observed across three independent experiments.

Figure 3. Enzyme saturation for single enzymes (a) XynAc, (b) XlnDt, and (c) AwAXEf; double enzymes (d) XynAc, XlnDt; and triple enzymes (e) XynAc, XlnDt, AwAXEf on the scaffoldin tfc. Cells displaying the tfc scaffoldin were cultured and concentrated to an OD<sub>600</sub> of 40. The enzymes were secreted from *Saccharomyces cerevisiae*, and the culture medium was concentrated 30-fold. Hundred percent activities refer to: 36 mg L<sup>-1</sup> reducing sugars (DNS) for XynAc; 3.2 U mL<sup>-1</sup> (PNPX) for XlnDt; 1.3 U mL<sup>-1</sup> (PNPA) for AwAXEf; 48 mg L<sup>-1</sup> reducing sugars (DNS) for XynAc and XlnDt; 70 mg L<sup>-1</sup> reducing sugars (DNS) for XynAc, XlnDt, and AwAXEf. Three controls were used for these assays to form a baseline for background activity: a yeast strain surface displaying tfc without enzyme incubation, a yeast strain without tfc incubated with enzymes (to account for non-specific binding), and binding Buffer A. For all three controls, the value for reducing sugars released in these experiments was ≤1% of the 100% activity observed in the saturation experiments.
All three strains with scaffoldins tfc, tfcXBD, and tfcCBD displaying single, double, or triple enzymes showed a consistent increase in the amount of reducing sugars released across 48 h of hydrolysis of birchwood xylan (Fig. 4a–c), following which no significant increase was noted in the hydrolysis profile (data not shown). For all three scaffoldins, incorporation of two enzymes (XynAc and XlnDt) increased hydrolysis at least 1.7-fold relative to XynAc alone. Incorporation of all three enzymes (XynAc, XlnDt, and AwAXEf) increased hydrolysis at least 3.0-fold relative to XynAc alone. These results show that the acetyl xylan esterase plays a significant role in the xylanosomal system for the hydrolysis of birchwood xylan.

The importance of the binding modules was also evident (Table III, Fig. 4). The dockerin-tagged endoxylanase XynAc displayed a 1.4- or 1.8-fold higher activity (at 48 h) when displayed on a scaffoldin with a binding module (CBD or XBD, respectively) as compared to tfc with no binding module (Fig. 4a). In addition, the strain with tfcXBD showed approximately 1.4-fold higher activity relative to the strain with the binding domain from C. thermocellum (tfcCBD). The positive contribution of enzyme–substrate proximity on hydrolytic activity was observed in both scaffoldin systems; the rates of hydrolysis using tfcXBD and tfcCBD displaying all three enzymes were 2.1- and 1.5-fold higher, respectively, relative to tfc. These results also clearly indicate the better performance of the xylanosome carrying the XBD as compared to CBD in hydrolyzing xylan.

In addition, a comparison was done to study the effectiveness of the yeast surface display systems with...
two hemicellulases and an acetylxylan esterase over free culture medium enzymes in the hydrolysis of hemicellulose birchwood xylan. The results are summarized in Table III. The strain displaying tfc showed 1.6-fold higher activity relative to comparable amounts of free enzymes, which can be attributed to enzyme–enzyme proximity in the modular xylanosome. The scaffoldin with XBD from _T. maritima_ displaying all three enzymes showed a marked

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Free enzyme</th>
<th>tfc(a)</th>
<th>tfcXBD(b)</th>
<th>tfcCBD(c)</th>
<th>Fold synergy compared to free enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>XynAc</td>
<td>214 ± 18</td>
<td>233 ± 16</td>
<td>389 ± 11</td>
<td>308 ± 18</td>
<td>1.1x 1.8x 1.4x</td>
</tr>
<tr>
<td>XynAc XlnDt</td>
<td>324 ± 23</td>
<td>420 ± 10</td>
<td>699 ± 14</td>
<td>537 ± 13</td>
<td>1.3x 2.2x 1.7x</td>
</tr>
<tr>
<td>XynAc XlnDt AwAXEf</td>
<td>476 ± 17</td>
<td>740 ± 12</td>
<td>1575 ± 17</td>
<td>1127 ± 19</td>
<td>1.6x 3.3x 2.4x</td>
</tr>
<tr>
<td>Fold synergy due to binding module</td>
<td>1x</td>
<td>2.1x</td>
<td>1.5x</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.3-fold improvement in xylan hydrolysis over comparable amounts of free enzymes in culture medium, demonstrating additional improvement due to enzyme-substrate proximity.

Our studies show the distinct contributions of enzyme–enzyme and enzyme–substrate proximity on enhancing the rate of hydrolysis. In addition, the results of this study demonstrate the influence of choice of binding domain for xylan hydrolysis. These results confirm the functionality of the engineered modular scaffoldin with a CBD for hemicellulose hydrolysis.

**Effect of Position of Binding Domain on Xylan Hydrolysis**

The xylan binding module (XBD) was shown to be superior to CBM in the engineered surface-scaffoldin. Our next objective was to evaluate the effect of the position of XBM on xylan hydrolysis. To test the influence of binding domain proximity to specific enzymes within the modular xylanosome, a set of scaffoldin constructs with XBD at three different locations (tfcXBD, tXBDtfc, and XBDtfc) were created (Fig. 1) and the rates of xylan hydrolysis from these constructs were compared (Fig. 5).

Different levels of hydrolysis were observed for the three locations studied (tfcXBD, tXBDtfc, and XBDtfc). An interesting observation was that the binding domain was most influential in improving hydrolysis when placed adjacent to the xylanase XynAc bound to position “c” within the scaffoldin (tfcXBD or tXBDtfc) as compared to a farther location (XBDtfc) within the modular system. The single enzyme XynAc and double enzyme systems showed at least 1.3-fold higher hydrolysis from the scaffoldin with the adjacent XBD as compared to XBDtfc (Fig. 5a,b). The three scaffoldins displaying XynAc, XlnDt, and AvAXEf with the xylan binding module showed a 1.3- to 2.5-fold higher hydrolytic activity at 48 h relative to tfc which lacks a binding domain (Fig. 5c). When tested with all three enzymes docked on the scaffoldin, both tfcXBD and tXBDtfc showed ~1.5-fold higher hydrolytic activity on xylan as compared to XBDtfc. These results indicate that having a xylan binding module closest to the endoxylanase may be beneficial for the quick release of shorter polysaccharide chains that could then be broken down by the β-xylosidase.

**Characterization of Xylan Hydrolysate from Xylanosome tfcXBD**

To determine the composition of the xylan hydrolysate from tfcXBD, samples taken at various incubation times were assayed by HPLC and the amounts of xylose, xylobiose, xylotriose, and acetic acid were quantified relative to internal standards (Fig. 6a,b). Most of the hydrolysis occurred within the first 24 h, with an accumulation of xylotriose (225 mg g⁻¹ substrate), xylobiose (192 mg g⁻¹ substrate) and xylose sugars (65 mg g⁻¹ substrate), as compared to the 0 h sample. The rate of hydrolysis decreased between 24 and 72 h, with an increase in the conversion of xylotetrose and higher oligosaccharides to xylobiose (290 mg g⁻¹ substrate) and xylose (105 mg g⁻¹ substrate). The decrease in hydrolytic rate could be a result of xylose and xylobiose product inhibition of the hydrolyase enzymes (Dobberstein and Emeis, 1991; Poutanen and Puls, 1988; Sunna and Antranikian, 1997). Therefore, a combined bioprocessing approach involving fermentation of the sugars by a yeast strain capable of xylose uptake and metabolism should prove useful in further saccharification of the xylan. Analysis of the hydrolysate for acetic acid via HPLC (Fig. 6) showed that most of the side chain acetate groups were released in the first 24 h of hydrolysis (46 mg g⁻¹ substrate), following which the rate of accumulation was minimal (50 mg g⁻¹ substrate at 72 h).

In conclusion, these results clearly demonstrate the synergistic activity of the hemicellulase and acetyltransferase enzymes toward xylan saccharification, and confirm the applicability of the tfcXBD surface-display system for the production of monomeric pentose sugars. The modular tfc construct allows for easy addition and swapping of domains at different positions within the scaffoldin (including two carbohydrate binding modules) that enabled comparative studies of the different xylanosomal systems. Furthermore, the study highlights the importance of choice of carbohydrate binding modules and position within the scaffoldin on substrate hydrolysis. Current work focuses on combining this xylanosome with the mini-cellulosome developed by Tsai et al. (2010) for the complete saccharification and fermentation of complex crop residues in yeast.
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