Mode of action of GH30-7 reducing-end xylose-releasing exo-xylanase A (Xyn30A) from the filamentous fungus *Talaromyces cellulolyticus*

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Supplementary Information

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  1 MRHPIPILAV LGRASAWSYS QTLSANIQVN ALQRYQEMIG
 41 GGCSGAFGWA CQQFPTTGLT PENQEEVTKI LFDENIGGLS
 81 IVRNDIGSSP GSTILPTCPA TPAGPFNYQW DGSDSCQFNL
121 TKTALKYNPE LYVYANAWSA PGCMKTVGTE NDGGQICGVR
161 GTNCTYDWRQ AYADYLVQYV KFYQAEGIDI SLLGAWNEPD
201 FNPVTYESME SDGFQAKDFL EILYPTVKKA FPNLDVSCCD
241 ATGARQERNI LYEVQQAGGE HFFDVATWHN YQSSPERPFN
281 VVGKPNIMTE WADGSGPWNT TWDVSGQLAE GLQWALYMHN
321 AFTNSDTSGY NHWWCAGGGA DNVLISITGN SYEVSSRLWA
361 FASYFRFARP GSVRIGATSS VENVYVSAYE NKNGTVSIPV
401 INAAHFPYEV TIDLQGLKAR KRVSTFLTDN SHNVTLMDQS
441 ELHGSVLKAT VPPRAVQVFW LE
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FIG S1. Sequence of tryptic peptides from Xyn30A. The fragment masses were searched the MASCOT server (Matrix Science, London, United Kingdom) against the NCBI-nr database. Matched peptides in the amino acid sequence of *T. cellulolyticus* Xyn30A are shown in bold (sequence coverage: 18%).

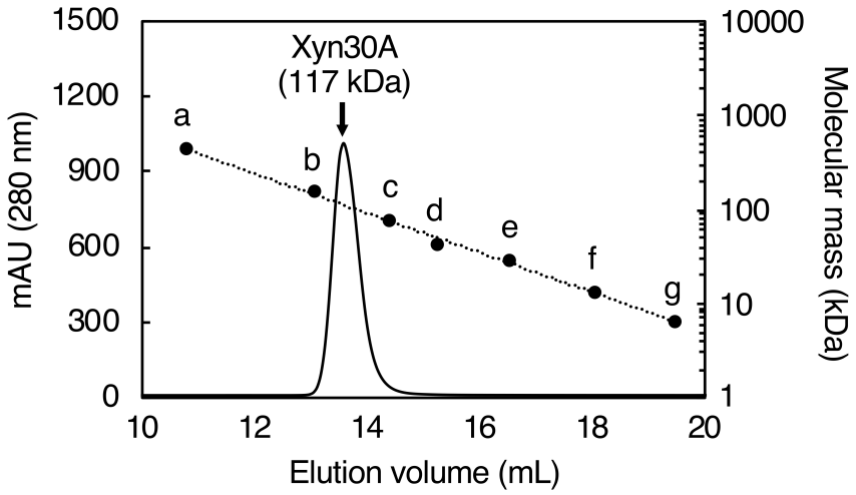

FIG S2. Gel filtration chromatography elution profile of Xyn30A. Xyn30A was subjected to gel filtration chromatography (Superdex 200 increase 10/300 GL, GE Healthcare) equilibrated with 20 mM sodium acetate (pH 4.0) containing 150 mM NaCl. The calibration curve used to estimate the molecular mass of Xyn30A is indicated by a dashed line. Dots (a–g) indicate the molecular mass of the marker proteins (Gel Filtration Calibration Kit HMW and LMW, GE Healthcare). Marker proteins are as follows: a, ferritin (440 kDa); b, aldolase (158 kDa); c, conalbumin (75 kDa); d, ovalbumin (43 kDa); e, carbonic anhydrase (29 kDa); f, ribonuclease A (13.7 kDa); g, apoprotein (6.5 kDa).

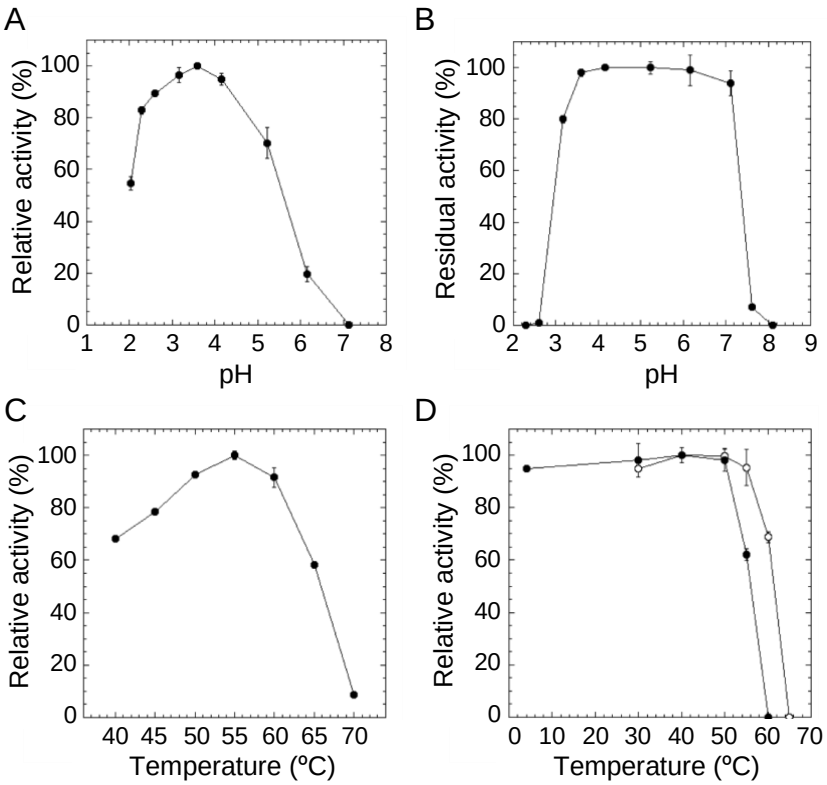

FIG S3. Thermal and pH optima and stability of Xyn30A. Enzyme activity was measured in a reaction mixture containing purified Xyn30A and 2 mM Xyl_3 under standard assay conditions. (A) Effect of pH on activity. (B) pH stability of Xyn30A. Xyn30A was preincubated at a pH range of 2.0–8.0, at 45ºC, for 30 min. (C) Effect of temperature on enzyme activity. (D) Thermal stability of Xyn30A. Xyn30A was preincubated in 50 mM sodium acetate (pH 4.0), at 30–65ºC, for 30 min (open circle) or at 4–65ºC for 24 hours (filled circle).

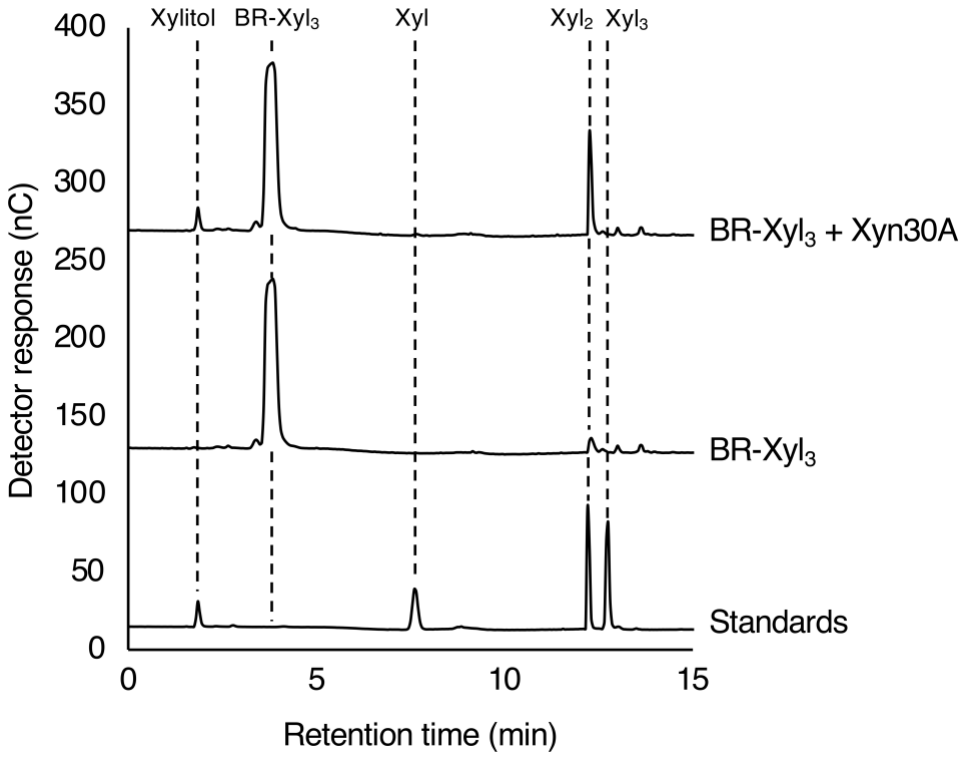

FIG S4. Degradation of BR-Xyl_3 by Xyn30A. Hydrolysate of BR-Xyl_3 (upper), BR-Xyl_3 (middle), and standards (lower) were analyzed by HPAEC-PAD. Hydrolysis reaction was performed at 45°C for 30 min in a mixture consisting of 2 mM BR-Xyl_3 and 100 μg mL^{-1} Xyn30A in 50 mM sodium acetate (pH 4.0).

^{1}H NMR analysis of xylotriose hydrolysate

The anomeric ratios of xylose (X1), xylobiose (X2), and xylotriose (X3) were determined from ^{1}H NMR spectra of X3 hydrolysate as follows.

1. Protons in oligoxyloses

Protons in oligoxyloses contained in the X3 hydrolysate were named according to their position and anomer as shown in **Fig. S5**. For example, (X2α, 5'ax) indicates the proton at 5' axial position of α-xylobiose.

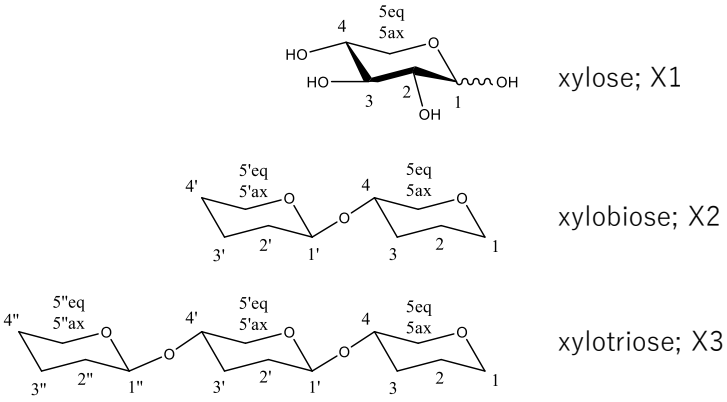

FIG S5. Position and number of protons of oligoxyloses.

2. ^{1}H NMR spectra

^{1}H NMR spectra of the reaction mixture were measured at (a) 0, (b) 5, and (c) 33 min after the start of the reaction at 23˚C, as shown in **Fig. S6**. **Fig. S7** shows an enlarged view of the spectra in the range of 3.7–4.1 and 3.3–3.5 ppm. Overlapped peaks in the spectra are described in **Table S1** according to the study of Balazs et al. (1).

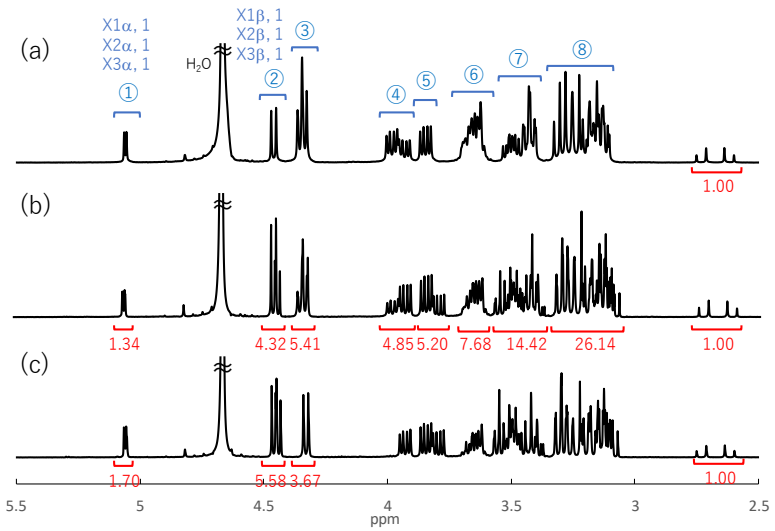

FIG S6. ^{1}H NMR spectra of xylotriose hydrolysate (a) 0, (b) 5, and (c) 33 min after the start of the reaction. The numbers in red font indicate integrals of the peaks normalized to that of the peak of citric acid at 2.70 ppm. The text in blue font indicates the assignment of the peaks (see **Table S1**).

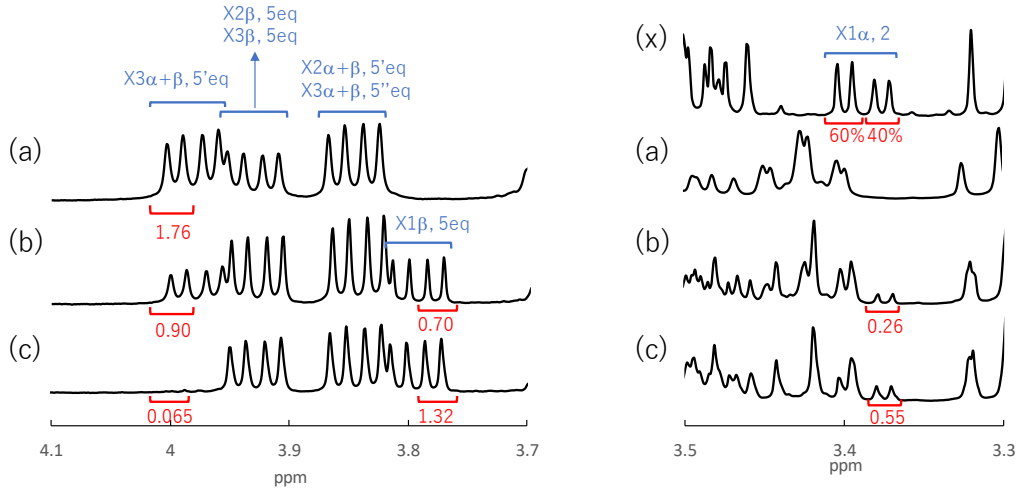

FIG S7. Enlarged view of ^{1}H NMR spectra (**Fig. S6**) in the range of 3.7–4.1 and 3.3–3.5 ppm at (a) 0, (b) 5, and (c) 33 min. The numbers in red font indicate integrals of the peaks normalized to that of the peak of citric acid at 2.70 ppm (see **Fig. S6**). The text in blue font indicates the assignment of the peaks (see **Table S1**). The spectrum (x) for X1 was separately measured and used for comparison with spectra for the reaction mixtures (a–c).

Table S1. Chemical shifts and corresponding peak assignments of XOSs

Number	Peak area (ppm)	Assignment
1	5.05	(X1α,1) (X2α,1) (X3α,1)
2	4.45	(X1β,1) (X2β,1) (X3β,1)
3	4.35	(X2α+β,1') (X3α+β,1') (X3α+β,1'')
4	4.05–3.9	(X2β,5eq) (X3β,5eq) (X3α+β,5'eq)
5	3.9–3.8	(X1β,5eq) (X2α+β,5'eq) (X3α+β,5''eq)
6	3.7–3.6	(X2α,3) (X2α+β,4) (X2α,5eq) (X2α,5ax) (X3α,3) (X3α,4) (X3α,5eq) (X3α,5ax) (X3β,4) (X3α+β,4')
7	3.55–3.35	(X1α,2) (X1α,3) (X1α,4) (X1α,5eq) (X1α,5ax) (X1β,4) (X2α,2) (X2β,3) (X2α+β,4') (X3α,2) (X3β,3) (X3α+β,3') (X3α+β,4'')
8	3.35–3.2	(X1β,2) (X1β,3) (X1β,5ax) (X2β,2) (X2β,5ax) (X2α+β,2') (X2α+β,3') (X2α+β,5'ax) (X3β,3) (X3β,5ax) (X3α+β,2') (X3α+β,5'ax) (X3α+β,2'') (X3α+β,3'') (X3α+β,5''ax)

3. Analysis of the hydrolysate at 33 min

Conversion of X3 was calculated using the peak of the proton of (X3α+β, 5'eq) at 4.0 ppm; half of this peak was not overlapped with other peaks (**Fig. S7**). Conversion at 33 min was 96% based on the peak areas at 0 min (1.76) and at 33 min (0.065). Thus, X3 was nearly entirely consumed at 33 min, and the mixture contained only X1 and X2; the small amount of X3 remaining was negligible.

The amount of X1α and β was calculated from the peaks at 3.78 ppm and 3.37 ppm (**Fig. S7**); peak area of X1β = 2.64 (66%), X1α = 1.38 (34%). Using these values, the amounts of X2α and X2β were calculated as X2β = 2.94 (83%) and X2α = 0.60 (17%) based on the peaks at 4.45 and 5.05 ppm, respectively (**Fig. S6,** (c) X3α = X3β = 0). The anomeric ratio in equilibrium state was separately measured using ^{1}H NMR in D_2O; the anomeric ratio was α:β = 35:65 mol% for X1, X2, and X3. The observed anomeric

ratio for X1 in the reaction mixture was close to equilibrium state, whereas the ratio of X2β was greater than that under conditions of equilibrium. Therefore, the enzyme was identified as a retaining glycoside hydrolase. The anomerization during the reaction was slow under these conditions.

4. Analysis of hydrolysate at 5 min

Conversion of X3 at 5 min, and the anomeric ratio of X1, were calculated as described in the section "3. *Analysis of the hydrolysate at 33 min*"; conversion occurred at 49%, X1α/β = 32/68. To simplify the calculation, these values were rounded to 50% and 30/70, respectively. Based on 50% conversion and the integrals of (X1α,2) and (X1β,5eq), which were equal to 0.65 and 1.40 (total 2.05), respectively (**Fig. S7**), the total of the anomers of X2α and X2β, and X3α and X3β, was also 2.05, respectively.

We were unable to determine the amount of X2α, β and X3α, β because their signals overlapped, and because experimental errors were present in the integrals of the peaks in ^{1}H NMR spectra. The following equations were based on peak assignment and integrals, as shown in **Fig. S6** (b) and **Table S1**, where a, b, c, d, e, and f are equal to the integral of one proton in X1α, X1β, X2α, X2β, X3α, and X3β, respectively.

$$a+c+e = 1.34 \quad (1)$$

$$b+d+f = 4.32 \quad (2)$$

$$c+d+2e+2f = 5.41 \quad (3)$$

$$d+e+2f = 4.85 \quad (4)$$

$$b+c+d+e+f = 5.20 \quad (5)$$

$$4c+d+5e+2f = 7.68 \quad (6)$$

$$5a+b+2c+2d+3e+3f = 14.42 \quad (7)$$

$$3b+3c+5d+5e+7f = 26.14 \quad (8)$$

The values (a–f) are valid if the sum of the error for each Equation (1–8) reaches minimum. Thus, Formula (9) was constructed on conditions that: a = 0.65, b = 1.40, c = variable in the range of 0 to 2.05, d = 2.05−c, e = variable in the range of 0 to 2.05, and f = 2.05−e.

$$(a+c+e-1.34)^2 + (b+c+f-4.32)^2 + (c+d+2e+2f-5.41)^2 + (d+e+2f-4.85)^2 + (b+c+d+e+f-5.20)^2 + (4c+d+5e+2f-7.68)^2 + (5a+b+2c+2d+3e+3f-14.42)^2 + (3b+3c+5d+5e+7f-26.14)^2 \quad (9)$$

Indeed, c and e were independently changed by 5% of 2.05 (21 points between 0 and 2.05), and the values became minimal (the value is 3.15) when the values c−f are those listed in **Table S2**. These values satisfy the Formula (10) as depicted in **Fig. S8**.

$$e = 0.82 - c \quad (10)$$

Overall, the values of a−f, expressed by equations (11) satisfied all the equations; these values were: a = 0.65, b = 1.40, c = variable in the range of 0 to 0.82, d = 2.05−c, e = 0.82−c, and f = 1.23+c. (11)

Table S2. The values of c-f when the value of formula (9) becomes minimal

c	d	e	f
0	2.05	0.82	1.23
0.1025	1.9475	0.7175	1.3325
0.205	1.845	0.615	1.435
0.3075	1.7425	0.5125	1.5375
0.41	1.64	0.41	1.64
0.5125	1.5375	0.3075	1.7425
0.615	1.435	0.205	1.845
0.7175	1.3325	0.1025	1.9475
0.82	1.23	0	2.05

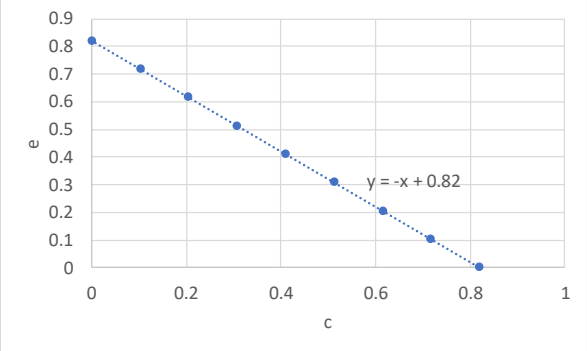

FIG S8. Plots of the values c and e listed in Table S2 and linear approximation of the plots.

5. *Determination of values a-f*

Unique determination of the values a-f was conducted for three conditions: 1) the enzyme randomly cleaves α and β anomers of X3; 2) the enzyme selectively cleaves the α anomer of X3; and 3) the enzyme electively cleaves the β anomer of X3.

Condition 1. Random cleavage of X3α and β anomers

When the enzyme randomly cleaves X3, the anomeric ratio of X3 remains constant. Thus, e = 0.65 and f = 1.40, and accordingly c = 0.17 and d = 1.88, as determined using Equations in (11). These values satisfy all the requirements mentioned above.

Because 92% of X2 is composed of the β anomer, the enzyme is considered a retaining glycoside hydrolase. The higher ratio of X2β suggests the occurrence of a slow anomerization rather than that of an enzymatic reaction, which is consistent with results obtained at 33 min.

	X3	**X2**	**X1**
α	e = 0.65	c = 0.17 (8.3 %)	a = 0.65
β	f = 1.40	d = 1.88 (92 %)	b = 1.40

Condition 2. Selective cleavage of the X3α anomer

Under conditions of selective cleavage of X3α, X3β must be anomerized to X3α during the reaction to achieve 50% conversion; this is because the initial amount of X3α is less than 50% of X3. Because X3α is preferentially consumed under this assumption, e and f should be 0 and 2.05, respectively; accordingly, c = 0.82 and d = 1.23 as determined using Equations in (11). Although these values suggest selective production of X2α followed by anomerization, these results were not consistent with those obtained at 33 min. Thus, this assumption was deemed erroneous.

	X3	**X2**	**X1**
α	0	0.82 (40 %)	0.65
β	2.05	1.23 (60 %)	1.40

Condition 3. Selective cleavage of the X3β anomer

If selective cleavage of X3β is assumed, residual e = 1.435 and f = 0.615. These values were not generated by Equations in (11). Thus, this assumption was deemed erroneous.

	X3	**X2**	**X1**
α	1.435	–	0.65
β	0.615	–	1.40

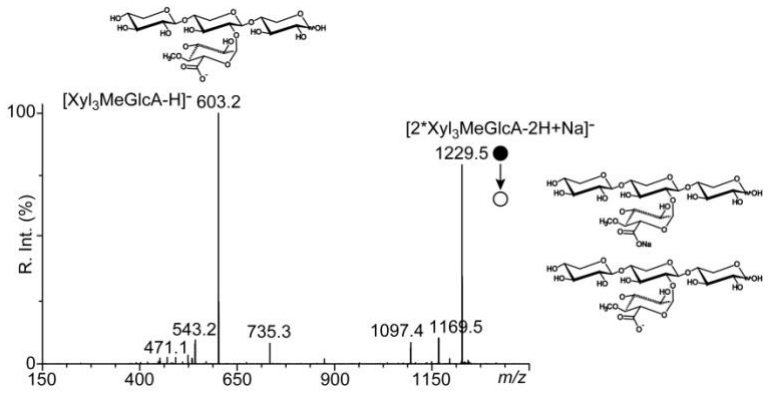

FIG S9. ESI(-)-MS^2 spectrum of the non-covalent dimer $[2*Xyl_3MeGlcA - 2H + Na]^+$. The non-covalent dimer $[2*Xyl_3MeGlcA - 2H + Na]^+$ was formed in-source from the major XOS, $Xyl_3MeGlcA$, released by Xyn30A (**Fig. 6A** in the main text).

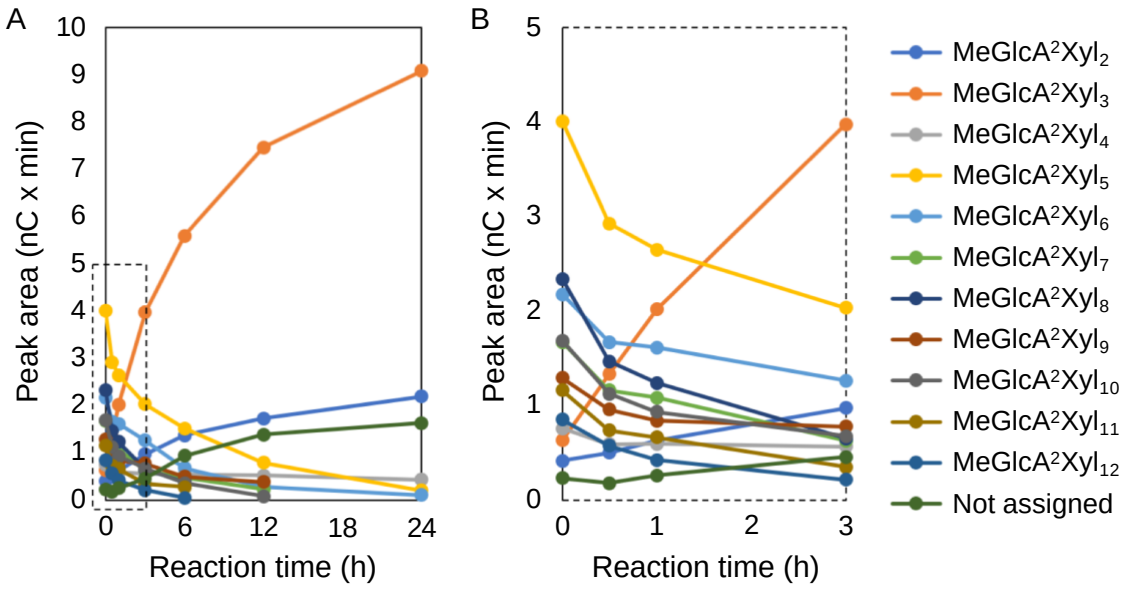

FIG S10. HPAEC-PAD analysis of products generated by Xyn30A. Peak areas of acidic XOSs on a HPAEC-PAD chromatogram (**Fig. 5B** in the main text) are plotted. (A) Time-course analysis of second hydrolysis of Xyn30B hydrolysate by Xyn30A. (B) Enlarged view of the dotted-box area in (A). The line "not assigned" represents the peak indicated by a dashed line in **Fig. 5B**.

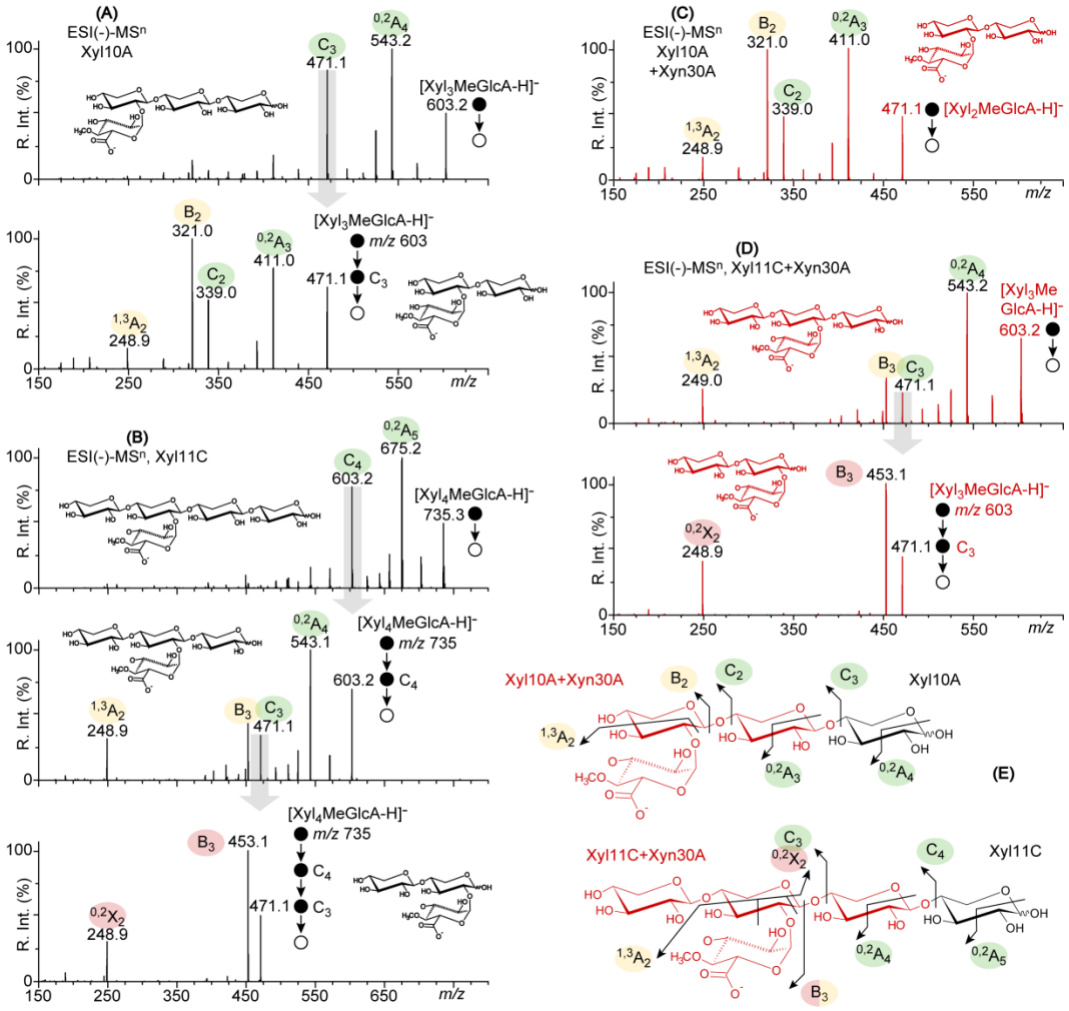

FIG S11. ESI(-)-MS^n spectra of major acidic XOSs. (A, B) ESI(-)-MS^n spectra of Xyl$_3$MeGlcA from Xyl10A and Xyl$_4$MeGlcA from Xyl11C (2). (C, D) ESI(-)-MS^n spectra of the counterparts of Xyl$_2$MeGlcA and Xyl$_3$MeGlcA generated by second digestion by Xyn30A. (E) Structural elucidation of XOSs conducted by assigning pairs of diagnostic product ions. Black line: XOSs from Xyl10A/Xyl11C. Red line: XOSs from second digestion by Xyn30A.

References

1. Balazs YS, Lisitsin E, Carmiel O, Shoham G, Shoham Y, Schmidt A. 2013. Identifying critical unrecognized sugar-protein interactions in GH10 xylanases from *Geobacillus stearothermophilus* using STD NMR. FEBS J 280:4652–4665.
2. Fouquet T, Sato H, Nakamichi Y, Matsushika A, Inoue H. 2018. Electrospray multistage mass spectrometry in the negative ion mode for the unambiguous molecular and structural characterization of acidic hydrolysates from 4-*O*-methylglucuronoxylan generated by endoxylanases. J Mass Spectrom 54:213–221.