Supplementary materials

Xylan Deconstruction by Thermophilic *Thermoanaerobacterium bryantii* Hemicellulases is Stimulated by Two Oxidoreductases

Zhuolin Yi [1,2,†], Xiaoyun Su [1,2,†], Abigail E. Asangba [1,2,3,†], Ahmed M. Abdel-Hamid [2,4], Siddhartha Chakraborty [2,5], Dylan Dodd [1,2,5], Peter G. Stroot [3], Roderick I. Mackie [1,2,3] and Isaac Cann [1,2,3,5,*]

[1] Energy Biosciences Institute, 1206 West Gregory Drive, University of Illinois, Urbana, IL 61801, USA; yizl1982@hotmail.com (Z.Y.); suxiaoyun@caas.cn (X.S.); Asangba.Abigail@mayo.edu (A.E.A.); ddodd2@stanford.edu (D.D.); r-mackie@illinois.edu (R.I.M.)
[2] Carl R. Woese Institute for Genomic Biology, 1206 West Gregory Drive, University of Illinois, Urbana, IL 61801, USA; ahetta@illinois.edu (A.M.A.-H.); sc99@illinois.edu (S.C.)
[3] Department of Animal Sciences, 1207 West Gregory Drive, University of Illinois, Urbana, IL 61801, USA; pgstroot@gmail.com
[4] Department of Botany and Microbiology, Faculty of Science, Minia University, El-Mina 61519, Egypt
[5] Department of Microbiology, University of Illinois, Urbana, IL 61801, USA
* Correspondence: icann@illinois.edu; Tel.: +1-217-333-2090
† These authors contributed equally to this work.

Supplemental Table S1. Characteristics that differentiate strain mel9^T from other species of *Thermoanaerobacterium*.

Characteristics	Strain mel9^T	*T. thermosaccharolyticum* #	*T. aotearoense* #	*T. saccharolyticum* #	*T. thermosulfurigenes* #	*T. xylanolyticum* #
G + C content (mol%)	37.7	29– 32	34.5–35	36	32.6	36.1
pH range	5.0–8.0	6.5–8.5	3.8–6.8	5.0–7.5	4.0–7.6	5.0–7.5
Optimum pH	6.8–7.0	7.8	5.2	6.0	5.5–6.5	6.0
Optimum temp (°C)	62–65	55–60	60.1–62.6	60	60	60
Maximum temp (°C)	69	62	65.7	68–70	75	70
Spore formation	−	+	+	+	+	+
Fermentation End Products*						
Lactate	−	+	+	+	+	-
Acetate	+	+	+	+	+	+
Butyrate	+	+	-	−	−	−
Ethanol	+	+	+	+	+	+
Butanol	+	−	−	−	−	−
CO_2	+	+	+	+	+	+
H_2	+	+	+	+	+	+

From liu et al. [63], *From Lee et al. [44], except data on *T. thermosaccharolyticum* from Jones and Woods [64].

Supplemental Table S2. Amino acid sequence identities among the enzymes in the hemi-cellulase gene clusters from *T. bryantii* (*T.b*), *T. xylanolyticum* (*T.x*) and *T. thermosaccharolyticum* (*T.t*).

Sequence Identity	Agu67	HEOA	HEOB	Xyl52	Xyl39	Axe1
T.b–T.x	86.5%	91.5%	95.8%	89.3%	86.6%	92.5%
T.b–T.t	91.3%	95.1%	91.9%	92.0%	95.0%	99.4%
T.b–T.s	84.8%	90.2%	91.6%	86.0%	89.0%	92.5%

Supplemental Table S3. End products of synergistic hydrolysis of different xylan-containing plant biomass by the seven enzymes encoded by the *T. bryantii* hemicellulase gene cluster[a,b,c]

Enzymes Combination	Substrates	End-Products (mM)			Substrates	End-Products (mM)		
		X1	X2	X3		X1	X2	X3
1	BeeWX	0.1 ± 0.0	3.5 ± 0.0	2.0 ± 0.2	BWX	ND	0.5 ± 0.2	0.4 ± 0.1
2		0.8 ± 0.0	3.9 ± 0.1	1.6 ± 0.1		1.7 ± 0.2	1.2 ± 0.2	0.5 ± 0.1
3		10.6 ± 0.4	0.8 ± 0.0	<0.1		2.1 ± 0.6	0.7 ± 0.5	0.3 ± 0.2
4		11.5 ± 0.6	0.6 ± 0.1	<0.1		4.3 ± 0.2	0.5 ± 0.0	0.2 ± 0.0
5		12.9 ± 1.5	0.4 ± 0.0	ND		7.6 ± 0.6	1.1 ± 0.2	0.3 ± 0.2
6		12.5 ± 1.6	0.3 ± 0.0	ND		9.7 ± 0.1	1.3 ± 0.0	0.3 ± 0.0
7		13.8 ± 0.9	0.3 ± 0.0	ND		12.5 ± 0.5	1.3 ± 0.1	0.2 ± 0.0
8		18.8 ± 1.1	0.4 ± 0.1	ND		16.1 ± 0.3	1.0 ± 0.2	0.2 ± 0.1
9		14.4 ± 0.8	0.2 ± 0.1	ND		15.2 ± 0.7	0.6 ± 0.0	<0.1
10		17.8 ± 1.2	0.7 ± 0.1	ND		17.0 ± 0.4	1.1 ± 0.2	0.2 ± 0.0
1	OSX	ND	0.8 ± 0.1	0.5 ± 0.0	LAX	<0.1	1.7 ± 0.2	1.0 ± 0.1
2		1.0 ± 0.1	0.7 ± 0.3	0.2 ± 0.0		0.9 ± 0.1	1.7 ± 0.2	0.7 ± 0.0
3		2.3 ± 0.3	0.3 ± 0.0	<0.1		2.9 ± 0.1	0.7 ± 0.0	0.3 ± 0.0
4		3.0 ± 0.5	0.3 ± 0.0	<0.1		3.3 ± 0.0	0.7 ± 0.0	0.2 ± 0.0
5		5.7 ± 0.1	0.3 ± 0.0	ND		5.5 ± 0.2	0.7 ± 0.1	0.2 ± 0.0
6		5.6 ± 0.1	0.3 ± 0.0	ND		5.7 ± 0.6	0.9 ± 0.1	0.2 ± 0.0
7		6.4 ± 0.8	0.2 ± 0.0	ND		5.9 ± 0.2	0.7 ± 0.0	0.1 ± 0.0
8		6.6 ± 0.3	0.2 ± 0.0	ND		7.6 ± 0.7	0.5 ± 0.1	0.1 ± 0.1
9		8.3 ± 0.0	0.2 ± 0.0	ND		7.3 ± 0.1	0.6 ± 0.0	0.2 ± 0.0
10		7.4 ± 1.1	0.2 ± 0.0	ND		6.3 ± 0.2	0.6 ± 0.1	0.1 ± 0.0
1	WAX	0.1 ± 0.1	1.1 ± 0.1	0.5 ± 0.0	Miscanthus	0.2 ± 0.0	0.7 ± 0.0	ND
2		0.4 ± 0.2	1.1 ± 0.3	0.3 ± 0.0		1.2 ± 0.2	<0.1	ND
3		1.2 ± 0.2	1.0 ± 0.2	0.2 ± 0.1		1.4 ± 0.2	ND	ND
4		1.3 ± 0.0	0.8 ± 0.0	0.2 ± 0.0		2.0 ± 0.0	ND	ND
5		1.3 ± 0.3	0.9 ± 0.1	0.2 ± 0.0		2.2 ± 0.2	ND	ND
6		1.6 ± 0.1	1.0 ± 0.0	0.2 ± 0.0		2.0 ± 0.2	ND	ND
7		1.9 ± 0.2	1.1 ± 0.1	0.3 ± 0.0		2.1 ± 0.1	ND	ND
8		1.9 ± 0.1	0.9 ± 0.1	0.1 ± 0.0		3.0 ± 0.3	ND	ND
9		1.8 ± 0.1	1.0 ± 0.0	0.1 ± 0.0		2.5 ± 0.2	ND	ND
10		2.0 ± 0.3	1.1 ± 0.0	0.2 ± 0.0		2.9 ± 0.1	ND	ND

[a] Enzymes combination 1: TbXyn10A; 2: TbXyn10A and TbXyl39A; 3: TbXyn10A and TbXyl52A; 4: TbXyn10A, TbXyl39A and TbXyl52A; 5: TbXyn10A, TbXyl39A, TbXyl52A and TbHEOA; 6: TbXyn10A, TbXyl39A, TbXyl52A and TbHEOB; 7: TbXyn10A, TbXyl39A, TbXyl52A, TbHEOA and TbHEOB; 8: TbXyn10A, TbXyl39A, TbXyl52A, TbHEOA, TbHEOB and TbAgu67A; 9: TbXyn10A, TbXyl39A, TbXyl52A, TbHEOA, TbHEOB and TbAxe1A; 10: TbXyn10A, TbXyl39A, TbXyl52A, TbHEOA, TbHEOB, TbAgu67A and TbAxe1A; [b] ND: not detected. [c] Abbreviations were X1: xylose; X2: xylobiose; X3: xylotriose.

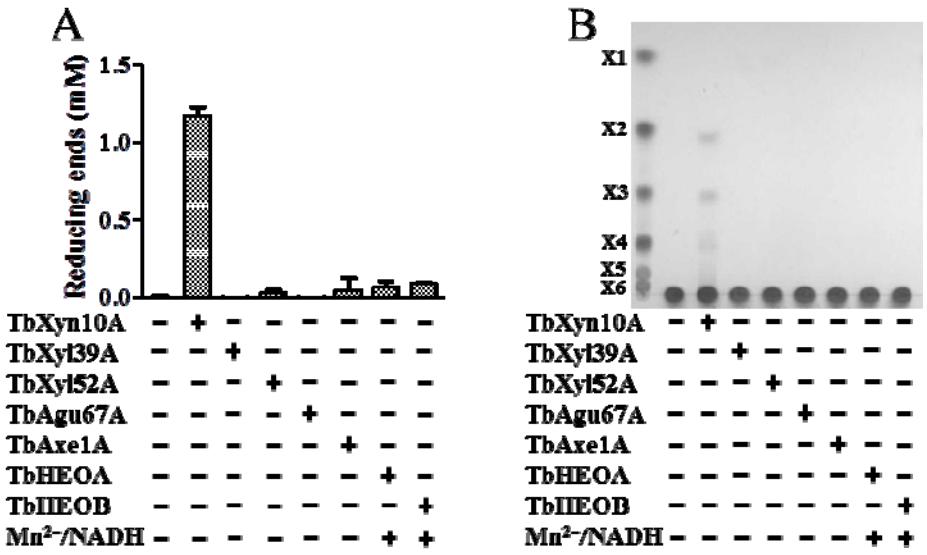
Supplemental Table S4. Conservations of two adjacent oxidoreductases (TbHEOA and TbHEOB) from *T. bryantii* in other bacteria.

Source		Predicted Function	Amino Acid Identities	
Strains	**GenBank no.**		**TbHEOA**	**TbHEOB**
Thermoanaerobacterium thermosaccharolyticum DSM 571	ADL68510.1	oxidoreductase domain protein	95%	/
	ADL68511.1		/	96%
Thermoanaerobacterium xylanolyticum LX-11	AEF17767.1	oxidoreductase domain protein	91%	/
	AEF17766.1		/	92%
Thermoanaerobacterium saccharolyticum JW/SL-YS485	AFK86456.1	oxidoreductase domain protein	90%	/
	AFK86457.1		/	92%
Thermoanaerobacter italicus Ab9	ADD01506.1	oxidoreductase domain protein	75%	/
	ADD01507.1		/	81%
Thermoanaerobacter mathranii subsp.	ADH60019.1	oxidoreductase domain protein	74%	/
	ADH60020.1		/	81%
Paenibacillus sp. oral taxon 786 str. D14	EES74063.1	oxidoreductase	63%	/
	EES74062.1		/	64%
Paenibacillus sp. JDR-2	ACT03815.1	oxidoreductase domain protein	63%	/
	ACT03814.1		/	63%
Rahnella sp. Y9602	ADW72798.1	oxidoreductase domain protein	60%	/
	ADW72799.1		/	64%
Hafnia alvei ATCC 51873	EHM43730.1	putative dehydrogenase	60%	/
	EHM43731.1		/	62%
Enterobacteriaceae bacterium 9_2_54FAA	EFV40373.1	oxidoreductase	60%	/
	EFV40372.1		/	62%
Enterobacter cancerogenus ATCC 35316	EFC53861.1	oxidoreductase	60%	/
	EFC53860.1		/	64%
Enterobacter mori LMG 25706	ZP_09036120.1	hypothetical protein EmorL2_03392	59%	/
	ZP_09036121.1		/	65%
Enterobacter hormaechei ATCC 49162	EGK61318.1	oxidoreductase	58%	/
	EGK61319.1		/	65%
Enterobacter cloacae subsp. cloacae ATCC 13047	ADF60186.1	hypothetical protein ECL_00620	58%	/
	ADF60185.1		/	65%
Enterobacter cloacae EcWSU1	AEW71857.1	glucose--fructose oxidoreductase	58%	/
	AEW71856.1		/	64%
Pantoea sp. Sc1	EIB96373.1	oxidoreductase	57%	
	EIB96374.1		/	63%
Pantoea stewartii subsp. stewartii DC283	EHU00915.1	oxidoreductase domain protein	56%	/
	EHU00916.1		/	62%
Lachnospiraceae bacterium 3_1_57FAA_CT1	EGN35098.1	hypothetical protein HMPREF0994_04650	53%	/
	EGN35097.1		/	63%
Clostridiales bacterium 1_7_47FAA	EEQ58458.1	oxidoreductase domain-containing protein	48%	
	EEQ58457.1		/	54%
Clostridium citroniae WAL-17108	EHE96106.1	hypothetical protein HMPREF9469_04981	48%	/
	EHE96107.1		/	56%
Kineococcus radiotolerans SRS30216	ABS02075.1	Oxidoreductase domain	49%	/
	ABS02074.1		/	57%
Xylanimonas cellulosilytica DSM 15894	ACZ30672.1	oxidoreductase domain protein	49%	/
	ACZ30671.1		/	60%
Clostridium phytofermentans ISDg	ABX40986.1	oxidoreductase domain protein	46%	/
	ABX40987.1		/	57%
Sphaerochaeta globus str. Buddy	ADY11936.1	oxidoreductase domain protein	45%	/
	ADY11935.1		/	52%

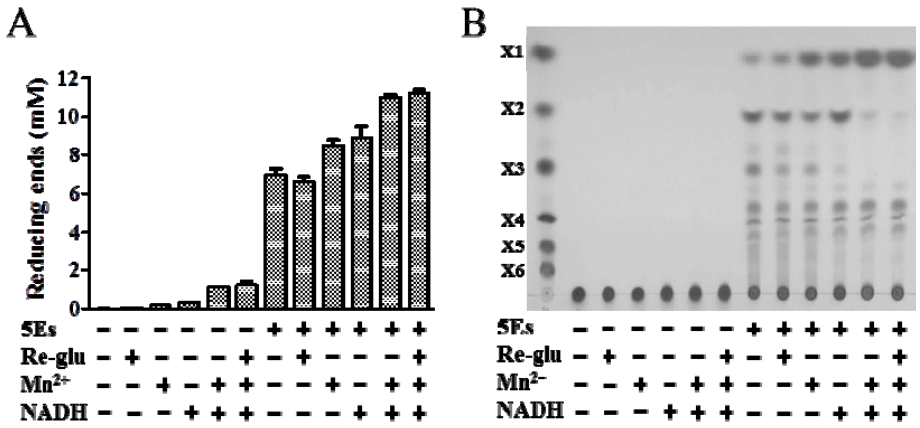
Supplemental Table S5. Nucleotide sequences of primers used for cloning of the genes in the hemicellulase gene cluster.

Gene	Primer direction	Primer sequences [a]
TbXyn10A	Forward	5′-GACGACGACAAGATGCCTTCAAAGGGAAAAATG-3′
	Reverse	5′-GAGGAGAAGCCCGGTTAAAAATCAACTATGCTGTAAAAG-3′
TbAgu67A	Forward	5′-GACGACGACAAGATGTACGACTGCTGGCTTAGGTATAAG-3′
	Reverse	5′-GAGGAGAAGCCCGGTTATTCATAAATTTTCCTTCCATG-3′
TbHEOA	Forward	5′-GACGACGACAAGATGATTAATATAGCCATTATTGGCGC-3′
	Reverse	5′-GAGGAGAAGCCCGGTTACTTAATATCTCTTCCAAATG-3′
TbHEOB	Forward	5′-GACGACGACAAGATGAGCAAAGAAAACGGCATGTAC-3′
	Reverse	5′-GAGGAGAAGCCCGGTCATTCTACCTTTATTGCATTTG-3′
TbXyl52A	Forward	5′-GACGACGACAAGATGATAAGTAAATCTTTTTATGCGC-3′
	Reverse	5′-GAGGAGAAGCCCGGTTATTTCATCCACAGTATGCTGG-3′
TbXyl39A	Forward	5′-GACGACGACAAGATGATAAAAATAAAGATACCAAAAAATTC-3′
	Reverse	5′-GAGGAGAAGCCCGGTCAATATCCGTTTATCTTGCTATC-3′
TbAxe1A	Forward	5′-GACGACGACAAGATGGGACTTTTTGATATGCCATTAC-3′
	Reverse	5′-GAGGAGAAGCCCGGTTAAAGCTCCAGTAAAAATTGC-3′

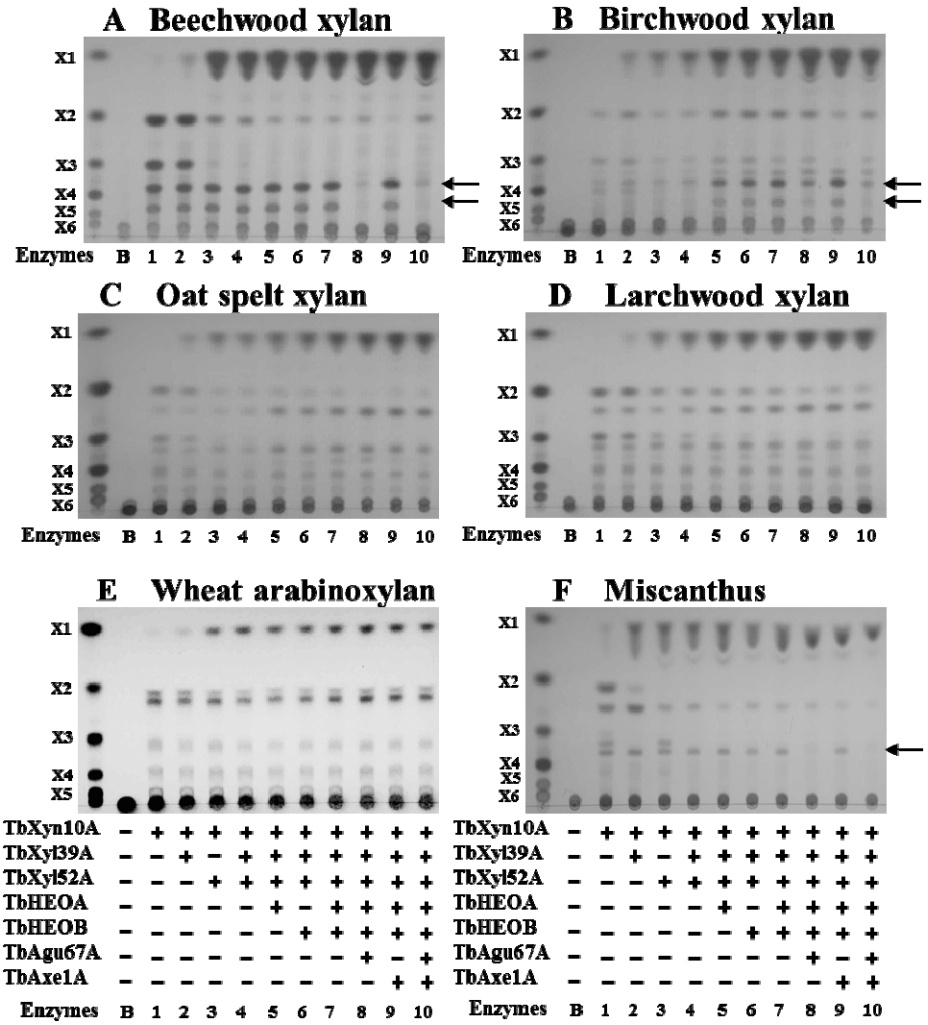
[a]: Underlined nucleotides are added to facilitate ligation-independent cloning into a pET46-Ek/LIC vector.



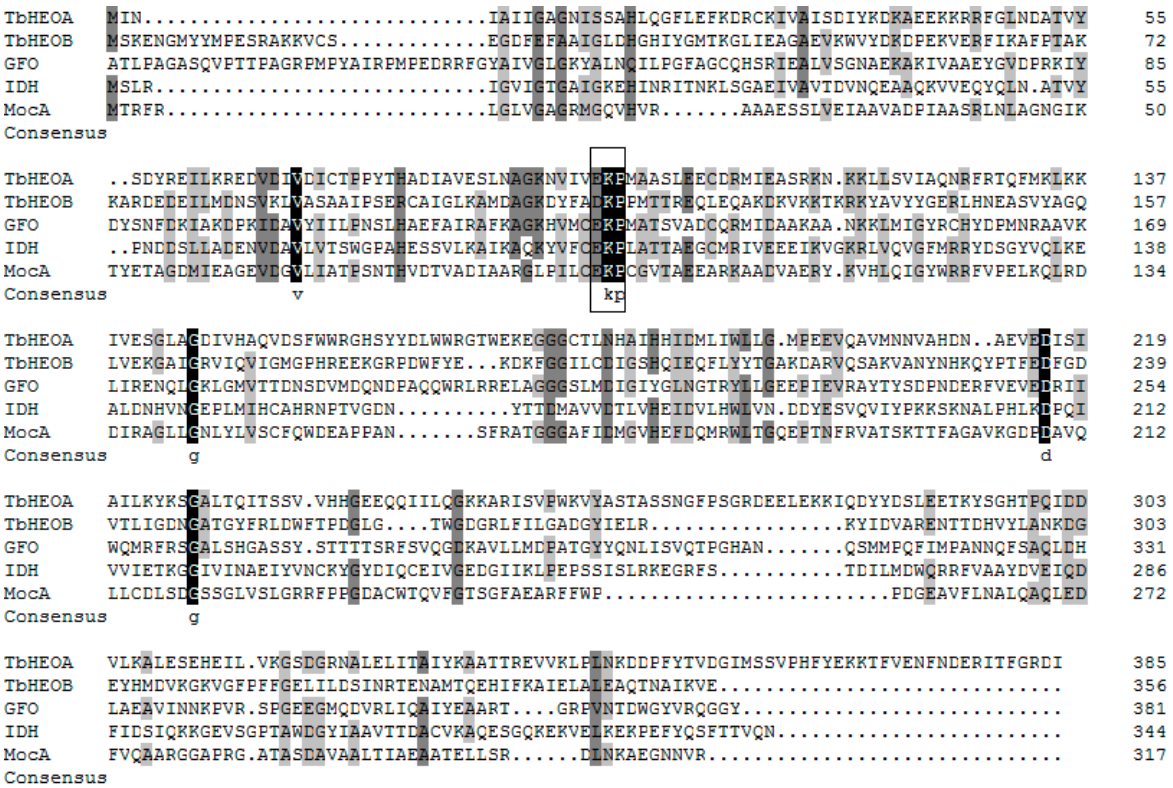
Supplemental Figure S1. Hydrolysis of birchwood xylan (BWX) by a single enzyme (TbXyn10A, TbXyl39A, TbXyl52A, TbHEOA, TbHEOB, TbAgu67A or TbAxe1A) in the gene cluster as analyzed by reducing sugar assay (**A**) and TLC (**B**). Five mg/mL of BWX was incubated with each enzyme (10 nM TbXyn10A, 10 nM TbXyl39A, 5 nM TbXyl52A, 100 nM TbAgu67A, 100 nM TbAxe1A, 100 nM TbHEOA or 100 nM TbHEOB) at 60 °C in a citrate buffer (pH 5.5) for 16 h, in the presence or absence of cofactors (1 mM NADH and 1 mM $MnSO_4$).



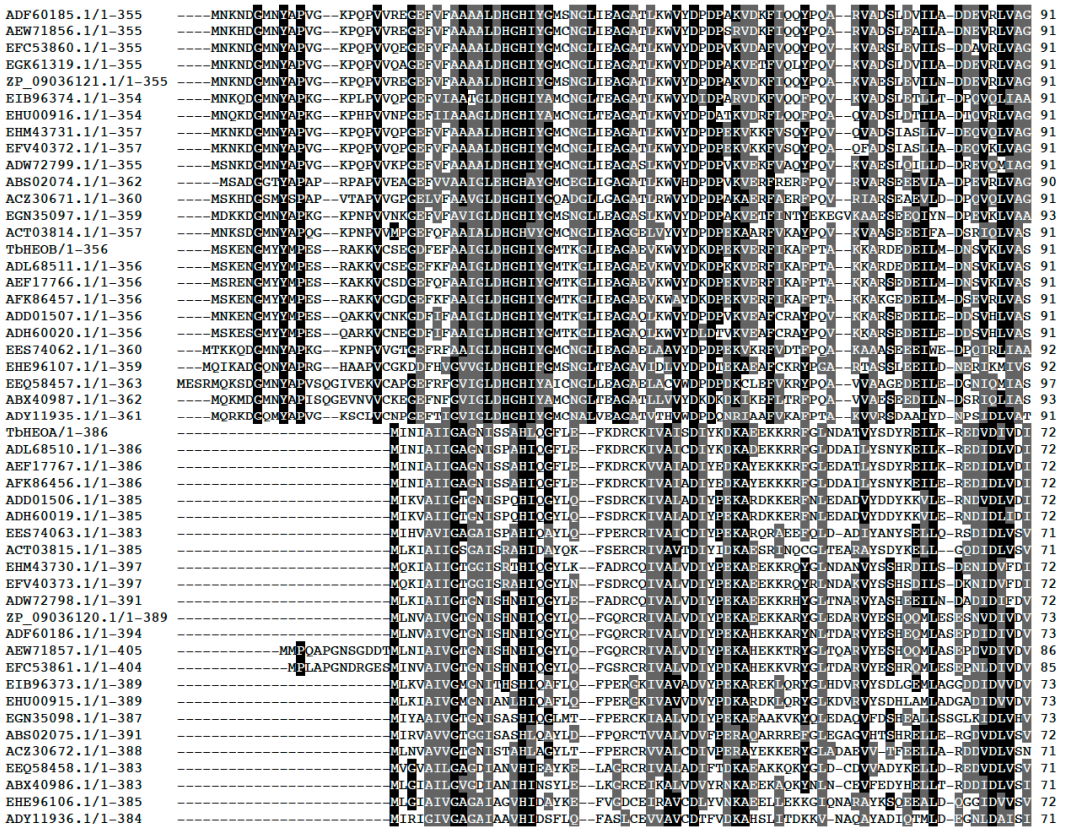
Supplemental Figure S2. Effects of redox-active cofactors and Mn^{2+} ion on the capacity of two new oxidoreductase-like proteins (TbHEOA and TbHEOB) to enhance hydrolysis of BWX by three enzymes mixture composed of TbXyn10A, TbXyl39A, and TbXyl52A. The end products were analyzed by reducing sugar assay (**A**) and TLC (**B**). Five mg/mL BWX was incubated with and without five enzymes mixture (10 nM TbXyn10A, 10 nM TbXyl39A, 5 nM TbXyl52A, 50 nM TbHEOA, and 50 nM TbHEOB) at 60 °C in a citrate buffer (pH 5.5) in the presence or absence of cofactors (1 mM NADH, 1 mM Mn^{2+} or 0.5 mM Re-glu) for 20 h. 5Es: 5 enzymes mixtures (TbXyn10A, TbXyl39A, TbXyl52A, TbHEOA, and TbHEOB). Other abbreviations were as follows: X1: xylose; X2: xylobiose; X3: xylotriose; X4: xylotetraose; X5: xylopentaose; X6: xylohexaose.

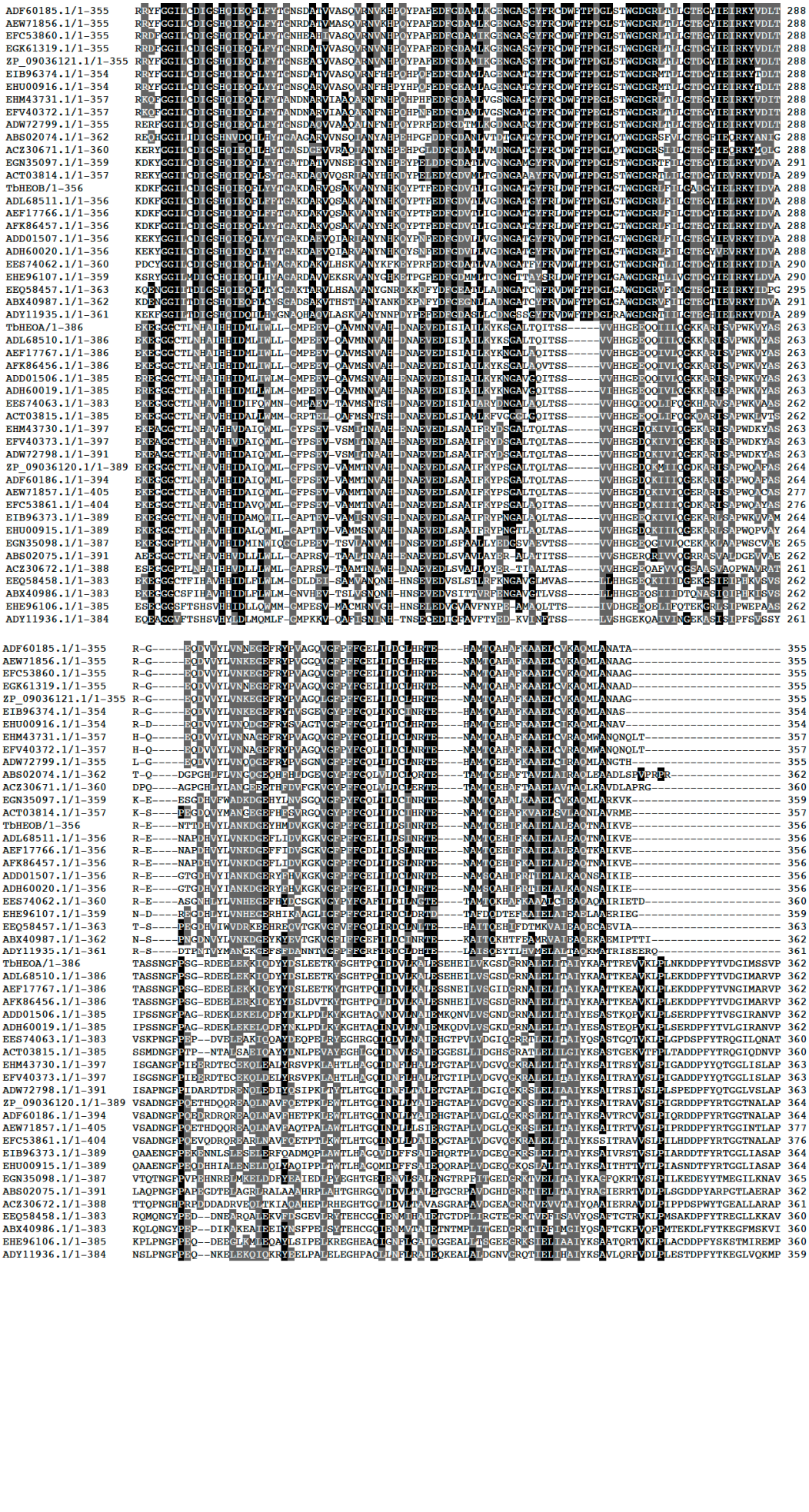


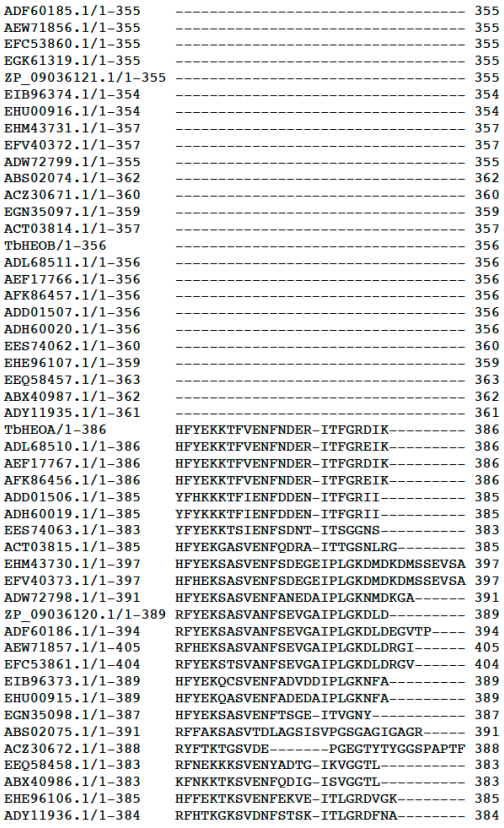
Supplemental Figure S3. The synergistic effect of the seven enzymes encoded in the *T. bryantii* hemicellulase gene cluster during hydrolysis of heterogeneous xylans (**A**–**E**) and Miscanthus (**F**). The enzymatic reactions were carried out at 60 °C in a pH 5.5 citrate buffer supplemented with 1.0 mM NADH and 1.0 mM Mn^{2+}. The substrates (BeeWX, BWX, LWX, and OSX) at 5 mg/mL were incubated with different combinations of the seven enzymes for 16 h. In the case of WAX, a lower concentration of substrate (2.5 mg/mL) was incubated with a lower concentration of enzymes (5 nM TbXyn10A, 5 nM TbXyl39A, 2.5 nM TbXyl52A, 50 nM TbAgu67A, 50 nM TbAxe1A, 25 nM TbHEOA, and 25 nM TbHEOB) for 16 h. Miscanthus was incubated at a higher concentration of substrate loading (10 mg/mL) with higher concentrations of enzymes (1 μM TbXyn10A, 0.5 μM TbXyl39A, 0.5 μM TbXyl52A, 2 μM TbHEOA, 2 μM TbHEOB, 2 μM TbAgu67A, and 2 μM TbAxe1A) for a longer period of 21 h. The end products were analyzed by TLC. (**B**): is a control of substrate without enzyme.



Supplemental Figure S4. Multiple sequence alignment of TbHEOA and TbHEOB with three characterized Oxidoreductases. GFO (PDB accession number: 1OFG): a glucose-fructose oxidoreductase from *Zymonomas mobilis*; IDH: an inositol dehydrogenase from *Bacillus subtilis*; MocA: a rhizopine catabolism protein from *Rhizobium meliloti* L5-30. The conserved motif EKP is highlighted by rectangle.







Supplemental Figure S5. Multiple sequence alignment using Clustal Omega of the two HEOs from *T. bryantii* with putative functional homologs occurring as adjacent genes in other bacteria. GenBank accession numbers of the oxidoreductase-like proteins are the same as listed in Supplemental Table 5. The amino acids are shaded based on conservation. Residues with 50% or more identity in the aligned sequences are shaded in black. Amino acids with similar properties (grouped as GAVLI, FYW, CM, ST, KRH, DENQ, P) in 50% or more of the aligned sequences are shaded in grey.