Supplementary Information

Impact of Module-X2 and Carbohydrate Binding Module-3 on the catalytic activity of associated glycoside hydrolases towards plant biomass

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Supplementary information provided with this submission:

Figure S1. Growth and cellulolytic activity of *P. polymyxa* A18.

Figure S2. HPLC (left column) and mass spectrometry (right column) profiles of the enzymatic hydrolysate of tamarind xyloglucan after incubation with full-length and truncated forms of xyloglucanase.

Figure S3. Isothermal titration calorimetry (ITC) data for binding interactions with soluble xyloglucan of (a) CBM3, (b) X2-CBM3, (c) X2, and (d) buffer (control) **.**

Figure S4. Full-length SDS-PAGE gels for binding assay of X2-CBM3 and its independent modules to insoluble polysaccharides.

Figure S5. Alignment of module X2 of different organisms.

Figure S6. Alignment of X2 of PP3 of *P. polymyxa* A18 with (a) X2 of CipC of *Clostridium cellulolyticum* and (b) CBM4-2 X2-L110F of *Rhodothermus marinus*.

Figure S7. (a) Structural model of *P. polymyxa* A18 PP3-X2 as predicted using MODELLER. Representations of already known structures (b) of CipC-X2 of *C. cellulolyticum* (PDB ID 1EHX) and (c) of CBM46 of *Bacillus halodurans* (PDB ID 4UZ8).

Table S1. Annotation of Carbohydrate Binding Modules (CBMs) in *P.polymyxa* A18 genome.

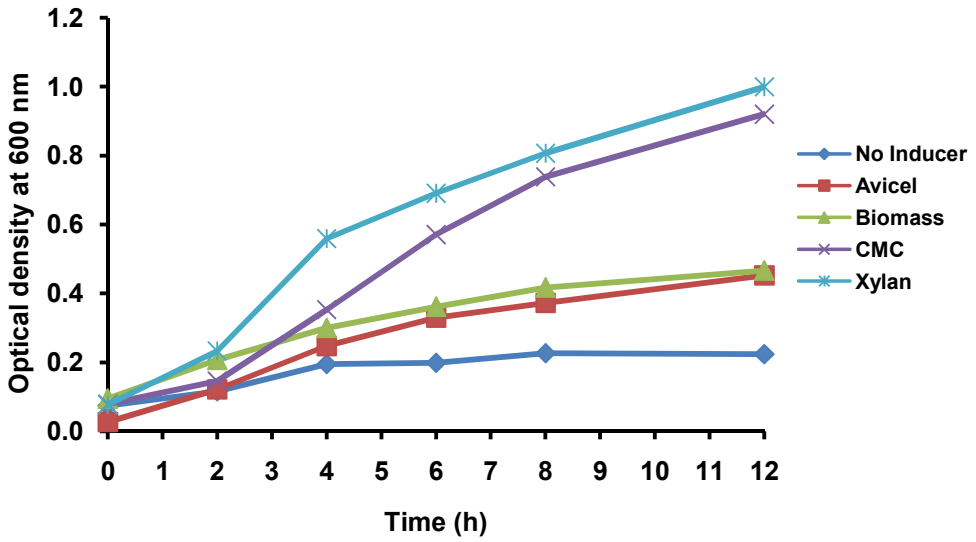
Table S2. Relative transcript levels of genes encoding for CBM containing polypeptides in the presence of different carbon substrates.

Table S3. Comparison of xyloglucanase activity of truncated derivatives towards soluble and insoluble substrates.

Table S4. List of primers used for qRT-PCR.

Table S5. Strains, plasmids, and primers used in the study.

(a)



(b)

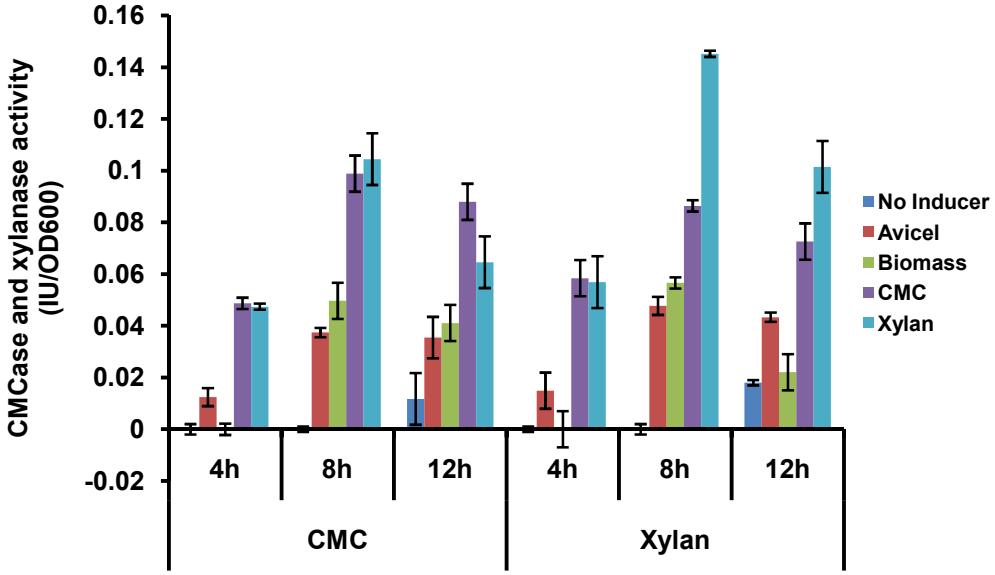


Figure S1. Growth and cellulolytic activity of *P. polymyxa* A18. (a) *P.polymyxa* A18 was grown in different carbon substrates and its growth was monitored by measuring OD_{600} at an interval of 2 hr up to 12 hr. (b) Hydrolytic activity was monitored in the supernatant of *P. polymyxa* A18 grown in different carbon substrates by measuring CMCase and Xylanase activity at an interval of 4 hr.

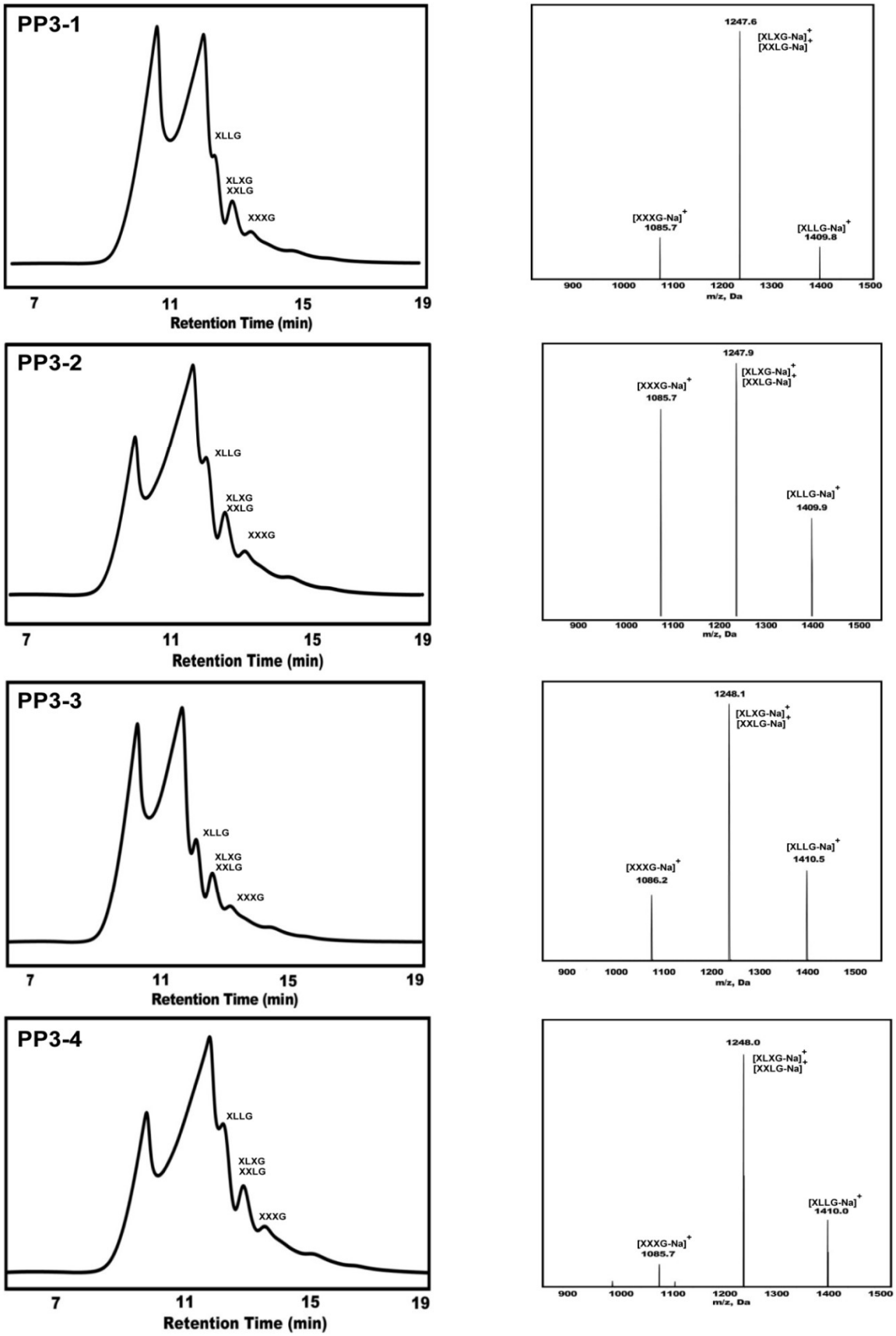


Figure S2. HPLC (left column) and mass spectrometry (right column) profiles of the enzymatic hydrolysate of tamarind xyloglucan after incubation with full-length and truncated forms of xyloglucanase.

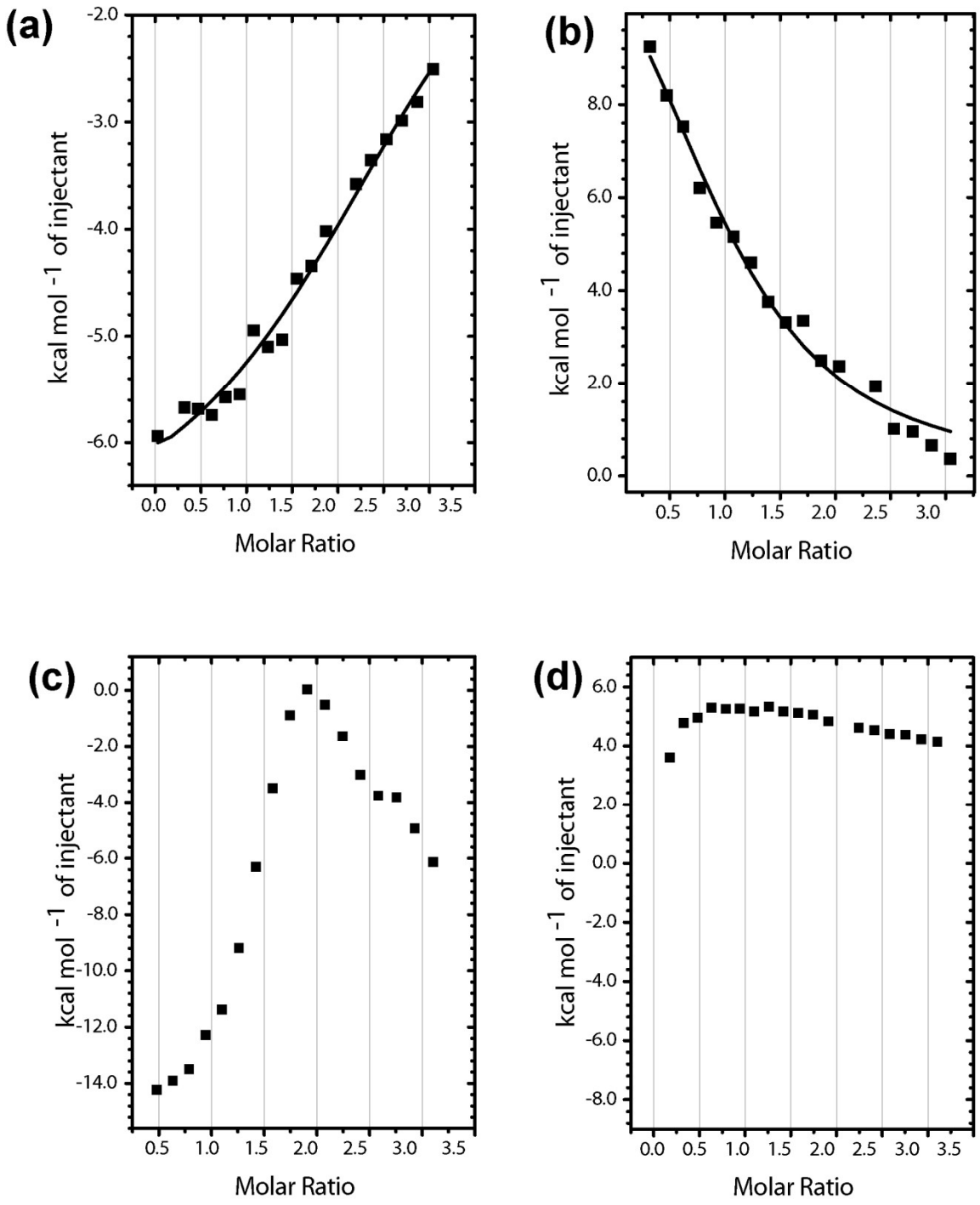


Figure S3. Isothermal titration calorimetry (ITC) data for binding interactions with soluble xyloglucan of (a) CBM3, (b) X2-CBM3,(c) X2, and (d) buffer (control). Binding affinity constants (K_A) and thermodynamic properties are presented in Table 2.

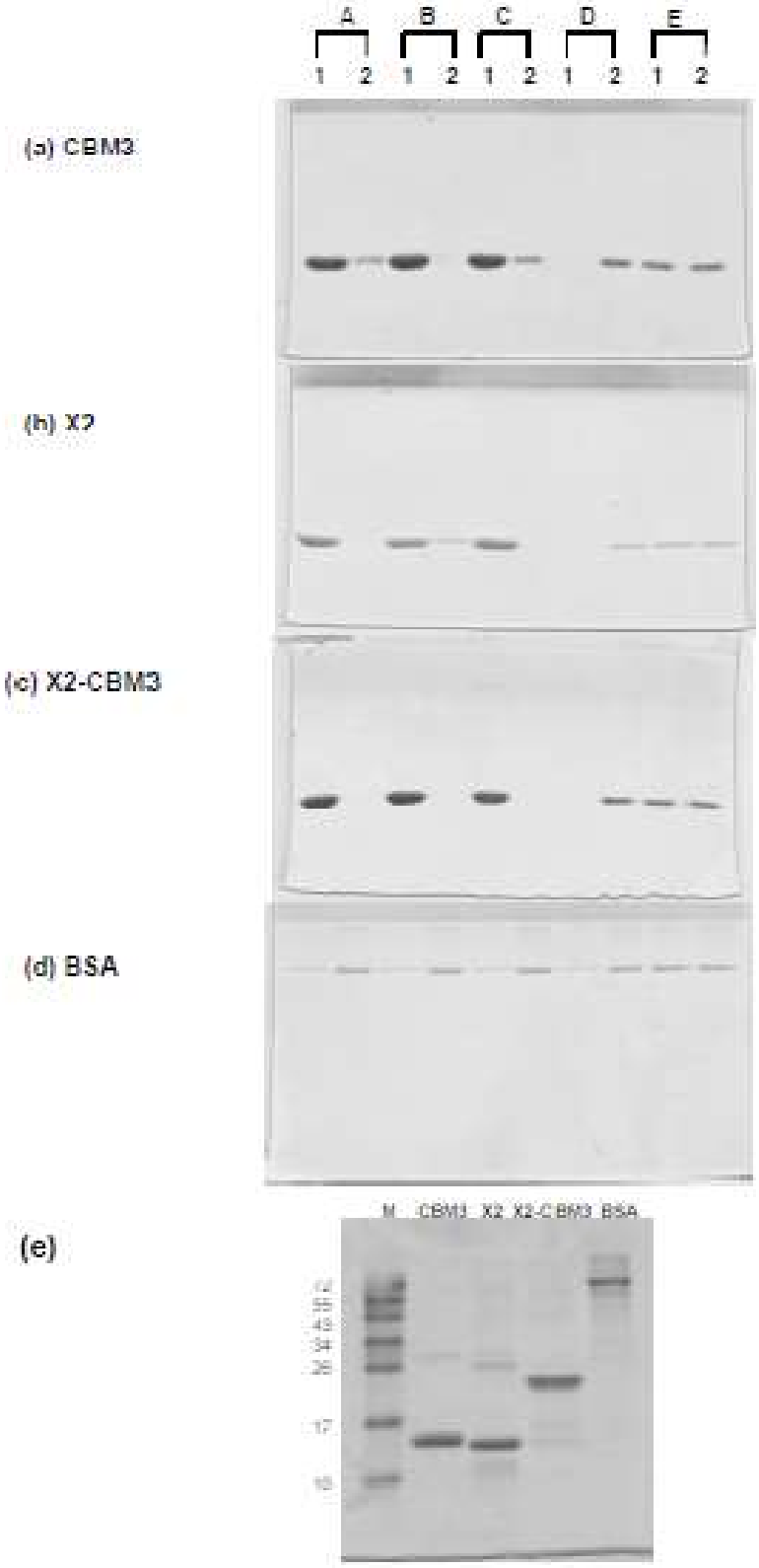


Figure S4. Full-length SDS-PAGE gels for binding assay of X2-CBM3 and its independent modules to insoluble polysaccharides. 10 μg of purified CBM3 (a), X2 (b), X2-CBM3 (c) and BSA (d) was incubated with 4 mg of insoluble substrates, such as (A)

biomass, (B) Avicel, (C) PASC, (D) starch and bound (1) and Unbound*(2) proteins were analysed on SDS-PAGE gel. (E) The same amount of protein used in the binding assay in absence of the polysaccharide was included as a control to observe aggregation over the incubation period. (e) Separation of purified CBM3, X2 and X2-CBM3 on an SDS-PAGE gel along with molecular weight marker (M). *Only 20 µl of 250 µl unbound fraction was loaded on the SDS-PAGE gel.

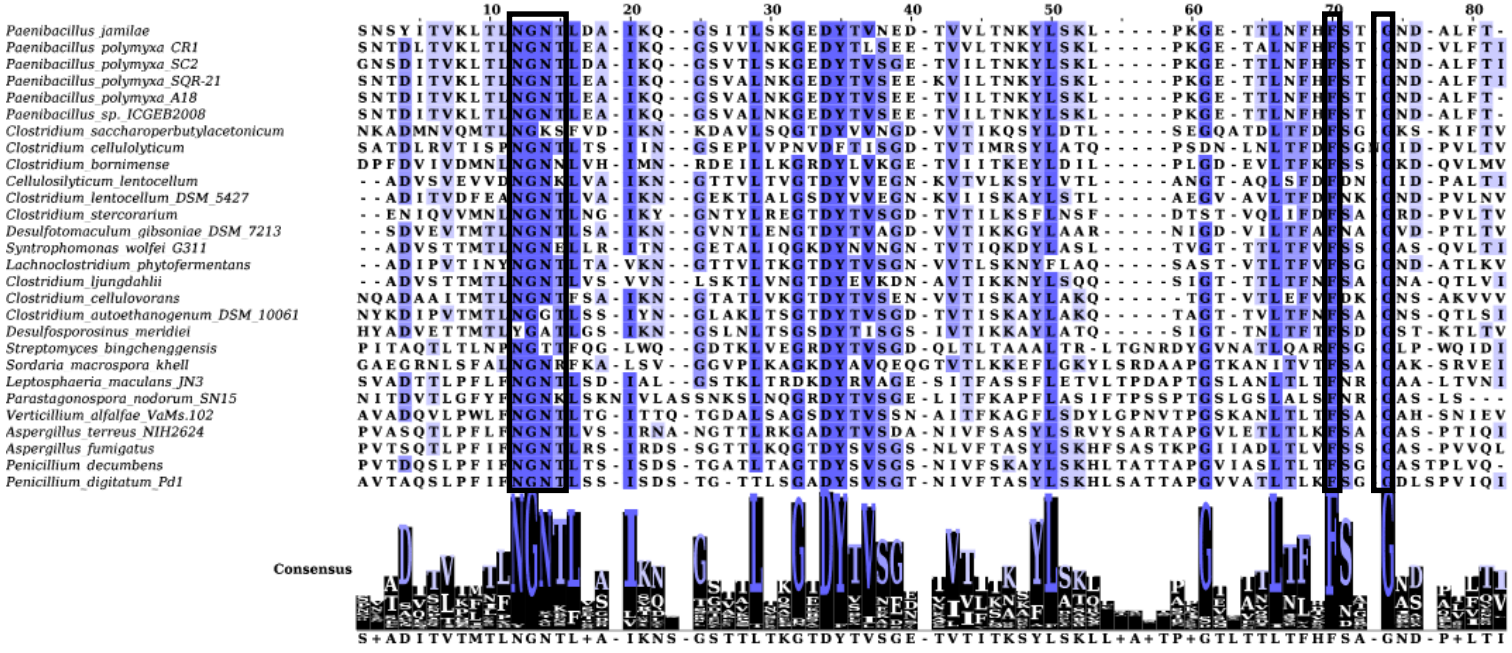
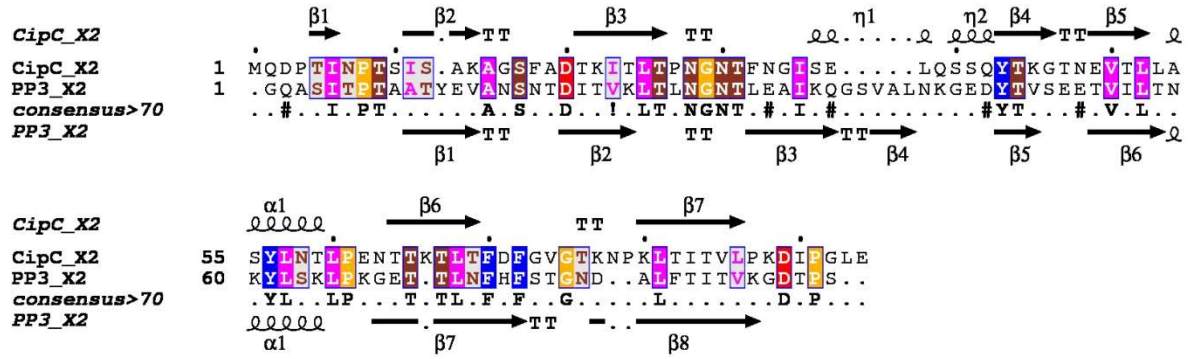


Figure S5. Alignment of module X2 of different organisms. The X2 region was extracted from the PP3 protein of *P. polymyxa* A18 and aligned with X2 of other species using Clustal Omega. Alignment was visualized using Jalview tool. The intensity of blue color defines the degree of conservation. The conserved regions reported earlier[43] have been boxed.

(a)



(b)

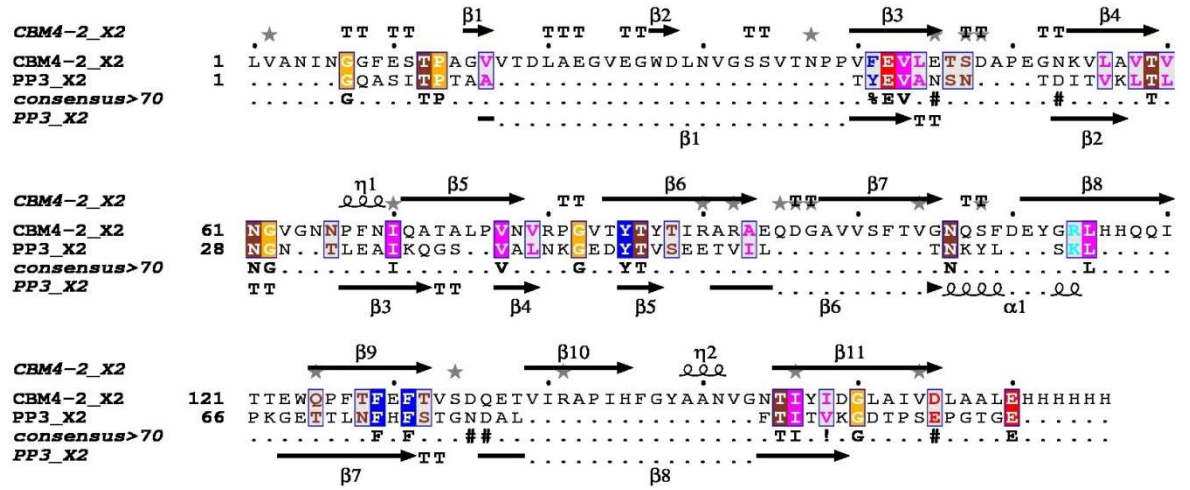


Figure S6. Alignment of X2 of PP3 of *P. polymyxa* A18 with (a) X2 of CipCof *Clostridium cellulolyticum* and (b) CBM4-2 X2-L110F of *Rhodothermus marinus*. The alignment was performed using Clustal Omega. Secondary structural features presented in the alignment are derived from the respective structures of the CipC_X2 module (1EHX), CBM4-2 X2-L110F (4BJ0) and modeled structure of X2 of PP3.

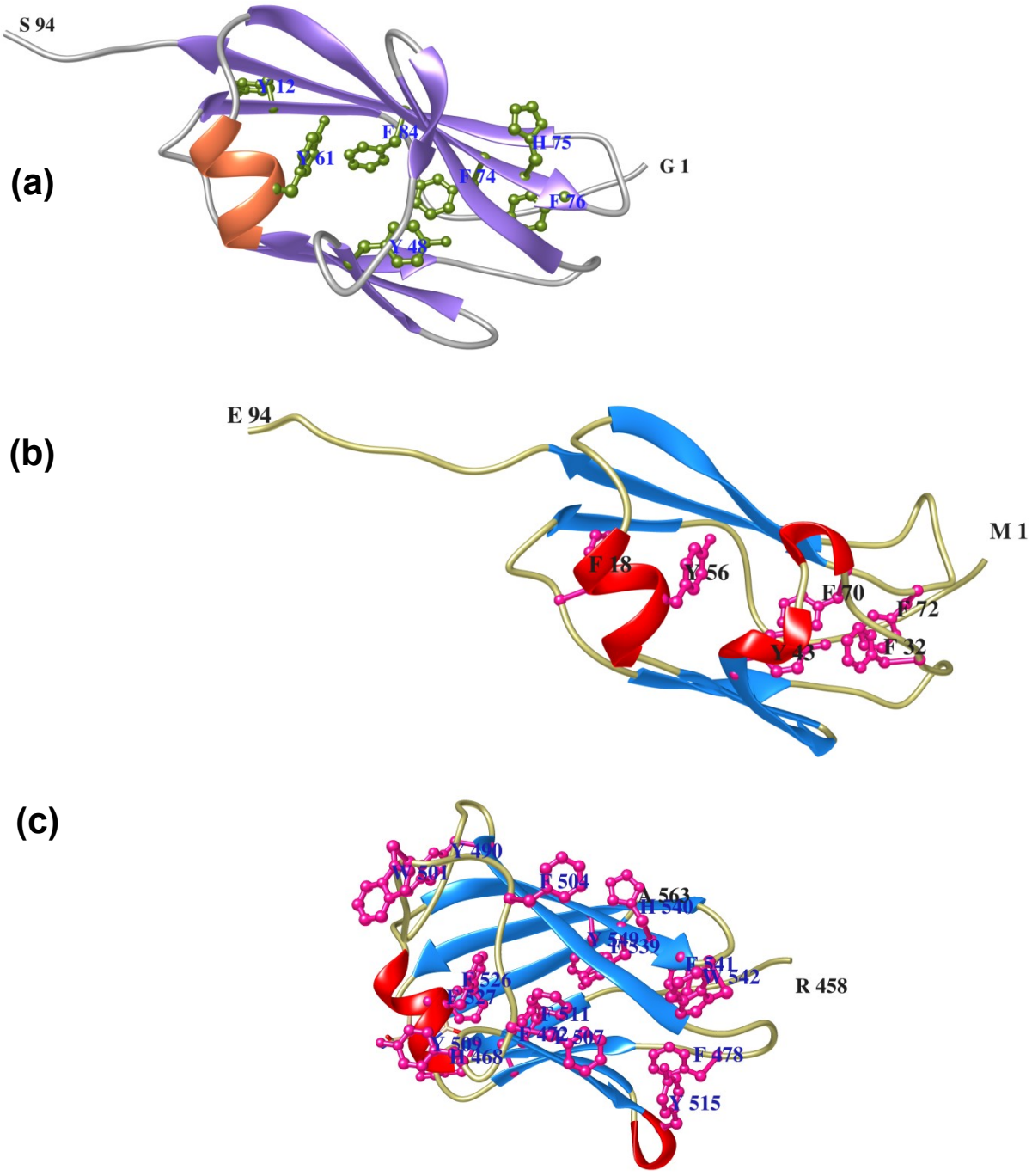


Figure S7. (a) Structural model of *P. polymyxa* A18 PP3-X2 as predicted using MODELLER. Aromatic acid residues and histidine are highlighted in green. **Representations of already known structures (b) of CipC-X2 of *C. cellulolyticum* (PDB ID 1EHX) and (c) of CBM46 of *Bacillus halodurans* (PDB ID 4UZ8).** Aromatic acid residues are highlighted in pink.

Table S1. Annotation of Carbohydrate Binding Modules (CBMs) in *P. polymyxa* A18 genome

NCBI ID	Carbohydrate active enzyme ANnotation dbCAN (46)	CAZyme Analysis Toolkit (CAT)		CBM common to all three (19)
		Sequence similarity (121)	Association links with Pfam (103)	
WP_017427084.1		CBM2 CBM10		
WP_016821067.1		CBM12		
WP_038978019.1		CBM5 CBM12		
WP_013372176.1		CBM13	CBM13	
WP_038978237.1		CBM13		
WP_017426909.1	CBM13	CBM13	CBM13	CBM13
WP_017426818.1	CBM16	CBM16	CBM16	CBM16
WP_016818667.1		CBM2		
WP_017425852.1		CBM2		
WP_016821937.1		CBM2		
WP_017425698.1		CBM2		
WP_016818812.1		CBM2		
WP_017427459.1		CBM2		
WP_016821370.1		CBM2 CBM57	CBM2 CBM57	
WP_017426951.1		CBM2		
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WP_017427423.1		CBM20	CBM20	
WP_038978282.1		CBM20		
WP_017426140.1	CBM22 CBM22	CBM22		
WP_017426122.1	CBM25 CBM25	CBM25 CBM25	CBM25 CBM25	CBM25 CBM25
WP_017427126.1		CBM3		
WP_017427072.1	CBM3 CBM35	CBM3 CBM35	CBM3 CBM35	CBM3 CBM35
		CBM3	CBM3	
		CBM3		
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WP_016821398.1	CBM3	CBM3	CBM3	CBM3
WP_017427741.1	CBM3 CBM46	CBM3 CBM_X2	CBM3 CBM_X2	CBM3 CBM_X2[‡]
WP_017426252.1		CBM32		
WP_017426987.1		CBM32		
WP_017426618.1		CBM32		
WP_017428382.1		CBM32		
WP_017427689.1	CBM32	CBM32		
WP_017427287.1		CBM32		
WP_017428467.1		CBM32		
WP_017425757.1	CBM32	CBM32		
WP_026065438.1		CBM32		
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WP_017428375.1		CBM37		

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		CBM5		
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WP_038978284.1			CBM50	
WP_016821500.1			CBM26	
WP_038978006.1			CBM50	

* CBM35 and CBM36 were predicted to be present as tandem repeats by dbCAN, highlighted in light gray.

Table S2. Relative transcript levels of genes coding for CBM containing polypeptides in the presence of different carbon substrates

CBM containing Polypeptide			Biomass		Avicel		CMC		Xylan	
PP	CBM	GH	Fold change*	P value**	Fold change*	P value**	Fold change*	P value**	Fold change*	P value**
1	3	GH5	1.00	0.0299	-1.32	0.0320	1.53	0.0189	6.89	0.0022
2	3	GH6	7.68	0.0001	9.03	0.0001	6.36	0.0001	1.49	0.0403
3	3, X2	GH74	39.92	0.0001	28.68	0.0003	2.56	0.0127	20.37	0.0001
4	3, 35	GH26, GH44	1.95	0.0079	1.76	0.0171	4.77	0.0033	1.73	0.0408
5	16	PL9	-2.13	0.0235	-7.25	0.0001	-1.41	0.0296	2.51	0.1904
6	46	GH5	4.65	0.0375	6.42	0.0308	1.73	0.0206	11.57	0.0055
7	6	GH3	1.75	0.3595	-1.62	0.0098	1.76	0.0175	6.59	0.0001
8	36, 36	GH43	8.17	0.0006	2.23	0.0485	-1.23	0.4203	-1.15	0.2543
9	13, 32	GH5	-1.01	0.8865	-1.38	0.0018	1.74	0.0001	1.64	0.0293
10	13	PL5	1.44	0.0171	1.10	0.0167	5.04	0.0014	5.23	0.0005
11	36	CE4	-3.15	0.0146	1.24	0.0392	67.51	0.0001	3.00	0.0240
12	35, 35	GH26	1.62	0.0378	-2.63	0.0425	-1.35	0.1783	-1.22	0.3410
13	25, 25	GH14, GH13	1.27	0.8166	-1.26	0.1454	36.12	0.0120	60.60	0.0058
14	48, 41	GH13	1.35	0.0071	-1.67	0.0025	8.44	0.0352	3.57	0.0076

*Fold change was calculated with respect to expression of the same gene in cells grown without any carbon substrate and normalized on 16S rRNA.

**Statistical analysis was performed using an independent Student's *t*-test. P-values <0.05 were considered significant.

Table S3. Comparison of xyloglucanase activity of full-length and truncated derivatives towards soluble and insoluble substrates

Substrate	Encoded domain(s)	Specific activity (IU/mg)	Molar specific activity (IU/µmol)	Fold change
Xyloglucan (soluble)	GH74	2.4 (±0.18)	193.47 (±11)	1
	GH74-X2	3.47 (±0.11)	304.49 (±31.23)	1.57
	GH74-CBM3	3.45 (±0.26)	308.43 (±18.76)	1.6
	GH74-X2-CBM3	4.58 (±0.17)	489.79 (±56)	2.5
Ammonium hydroxide-treated biomass (insoluble)	GH74	0.01 (±0.012)	3.20 (±1.71)	1
	GH74-X2	0.02 (±0.003)	7.06 (±1.29)	2.2
	GH74-CBM3	0.019 (±0.009)	6.91 (±1.12)	2.1
	GH74-X2-CBM3	0.034 (±0.001)	14.71 (±0.62)	4.6

Table S4. List of primers used for RT-qPCR

Polypeptide	Forward oligo sequence	Reverse oligo sequence	Annealing temperature
1	GCCAAGGATAATGCGAT	GAAATTTCTACATATTTATC	43
2	GTAATGTATCGTGCTGG	CCAATTTCAATATAGTTG	41
3	GCAACAAGTAATACGATC	CAATTTCTGCATAAGAATC	45
4	GGTGAACGATAATCACCT	CTGACTTCCAAATAATAATC	47
5	CCCGGAGAAGATGGTAT	AGCATACACTGTTGCCC	47
6	AGGATACCAAGGTCACT	CGACGATTTTAAAATTCC	43
7	GGCGTAGACATGAATACG	TTCTCCACTAAGTGACAG	47
8	CATAATATCTCAGTGCG	GAAAATTCAATAAAATCC	39
9	TAGCCGGAGGCTCCTCA	CTTCCACTTCTGCTGATC	49
10	ACAAATGGCTCTCTTGATC	CCAAGCCTCCATTGTTGA	49
11	CATCATTTTTCACTCCGT	GTTGATCTCCAAATAGTC	45
12	AATGCACCTTCAAGCGGG	CTTGACGTAGTCGATATG	47
13	GTATACTACAAAAAAGGC	CCAGTGCTGAAAAGGTA	45
14	TATGGGGCACCGGACAC	ATATTCCTCTCTGCCACT	47
Reference gene-16S	CCCACCTTCCTCCGGTTT	GCAACGCGAAGAACCTTAC	53
Non coding region1 (NC1)	ATAAGTGACCTGTTCAT	CCGATTTATGAATCTTTA	41
Non coding region2 (NC2)	GCTGCGAAAGCGTAGTT	TAACTTGTTCGGAGTGTT	45

Table S5. Strains, plasmids, and primers used in the study

Name	Description	Reference
Strains		
Paenibacillus polymyxa A18	Native cellulolytic bacterium isolated from termite gut	22
Escherichia coli DH5α	F^- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A*sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-	Life Technologies
Plasmids		
pQE-PP3-1	Xyloglucanasewithout signal peptide expressed under T5 promoter	This study
pQE-PP3-4	GH74 under expressed T5 promoter	This study
pQE-PP3-2	GH74 catalytic domain with X2 domain expressed under T5 promoter	This study
pQE-PP3-3	GH74 catalytic domain with CBM3 expressed under T5 promoter	This study
pQE-X2	X2 domain expressed under T7 promoter	This study
pQE-CBM3	CBM3 domain expressed under T7 promoter	This study
pQE-X2-CBM3	X2-CBM3 domain expressed under T7 promoter	This study
pQE–Endo5A-GS-CBM	Endoglucanase gene fused with X2-CBM3 with linker in between expressed under T7 promoter	This study
pQE–Xyl11D-GS-CBM	Xylanase gene fused with X2-CBM3 with linker in between under T7 promoter	This study
Primers		
Xylg_F	CGTGCATGCCCAAGTGACGATTATACTTG	This study
Xylg_R	CGCGTCGACTTAAGGCTCAATTCCCCATT	This study
GH74_R	CGTGTCGACCTATGCTTGCCCGGTAGATG	This study
GH74_R_kpnI	CGTGGTACCCGACCCACCACCGCCCGAGCCACCG TGCTTGCCCGGTAGATGG	This study
X2_F_sacI	CGCGAGCTCAGTATTACGCCGACGGCTG	This study
X2_F_kpnI	CGCGGTACCAGTATTACGCCGACGGCTG	
X2_R_salI	CAAGTCGACCTA CTGTTCCCGGCTCACTTGG	This study
CBM3_F_sacI	AATGAGCTCGCGAGGGTGCTCTGAAG	
CBM3_F_kpnI	AATGGTACCGCGAGGGTGCTCTGAAG	This study
CBM3_R_salI	CGTGTCGACCTAACCAATTTCTGCATAAGAA	This study

Note: The restriction enzyme sites are underlined.