Additional file 1_ supplementary table and figures

Metabolic engineering enables *Bacillus licheniformis* to grow on the marine polysaccharide ulvan

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Additional tables

- Table S1: Proteins and accession numbers
- Table S2: Bacterial strains and plasmids
- Table S3: M9-mineral media D-glucose composition
- Table S4: M9-mineral media additives
- Table S5: Primer list
- The Supplementary Tables S6 and S7 are available as separate Excel files.

Additional figures

- Figure S1: Sugar composition of the cultivation media
- Figure S2: SDS-PAGE of *F. agariphila* KMM3901^T enzymes expressed recombinantly in *E. coli*
- Figure S3: Consumption of 5-dehydro-4-deoxy-D-glucuronate from *B. licheniformis* DSM13 during cultivation
- Figure S4: Growth of *B. licheniformis* DSM13 on different ulvan hydrolysates
- Figure S5: *B. licheniformis* DSM13 CAZyme repertoire and their expression
- Figure S6: Alignment *B. licheniformis* DSM13 sulfatases with lipoteichoic acid synthases
- Figure S7: Comparison of different *Bacillus* sp. to digest ulvan hydrolysate and ulvan derived monosaccharides
- Figure S8: Growth of the *Bacillus* sp. PL28 and GH105 expression strains
- Figure S9: Activity assays of *Bacillus* sp. PL28 and GH105 expression strains
- Figure S10: C-PAGE results from *Bacillus* sp. PL28 and GH105 expression strains.
- Figure S11: Growth of the different *B. licheniformis* expression strains.
- Figure S12: Activity assay results from *B. licheniformis* SH006, PL28 and GH105 single- and co-expression strain.
- Figure S13: C-PAGE results from *B. licheniformis* SH006 PL28, GH105 and co-expression strains.
- Figure S14: C-PAGE from the cultivation supernatant of *B. licheniformis* strains in M9-mineral media.

Table S1: List of *F. agariphila* proteins used to produce ulvan hydrolysates. (UHA) P30_PL28 and P33_GH105 and (UHB) all listed enzymes. All proteins were recombinantly expressed in *E. coli* as described previously [1].

Name	Locus tag	Uniprot ID	Functional annotation	size [kDa]
P1_GH88	*21900	T2KLZ3	Unsaturated glucuronylhydrolase (GH88)	44.1
P10_PLnc	*21990	T2KNA3	Ulvan lyase (PLnc)	92.6
P17_GH2	*22060	T2KN75	β-Galactosidase (GH2)	112.9
P18_S1_7	*22070	T2KPK5	Arylsulfatase (S1_7)	53.1
P20_GH78	*22090	T2KNB2	α-L-rhamnosidase (GH78)	100.4
P24_GH3	*22130	T2KMH0	β-Glucosidase (GH3)	79.3
P27_GH43	*22160	T2KN85	β-Xylosidase (GH43)	67.3
P30_PL28	*22190	T2KNC2	Ulvan lyase (PL28)	44.1
P31_GH39	*22200	T2KM23	Glycoside hydrolase (GH39)	54.6
P33_GH105	*22220	T2KPL9	Glycoside hydrolase (GH105)	40.7
P36_S1_25	*22250	T2KM26	α-L-rhamnosidase/-sulfatase (GH78/S1_25)	134.3
	*=BN863			

Table S2: Bacterial strains and plasmids

Strain	Description or genotype	Reference or source
Escherichia coli TOP10	*F- mcrA* Δ(mrr-hsdRMS-mrcB) φ80lacZΔM15 Δ*lacX74 nupG* recA1 araD139 Δ(ara-leu)7697 galK16 rpsL(StR) endA1 fhuA2 λ-	Originally purchased from Invitrogen by Thermo Fisher Scientific (Waltham, MS, USA)
Escherichia coli BL21(DE3)	B F-- ompT gal dcm lon hsdSB(rB—mB-) λ(DE3 [lacI lacUV -T7 gene 1 ind1 sam7 nin5])	Originally purchased from New England Biolabs (Ipswich, MS, USA)
Bacillus subtilis B1	Wildtype	Department of Biotechnology & Enzyme Catalysis (University of Greifswald)
Bacillus licheniformis DSM13	Wildtype	Department of Pharmaceutical Biotechnology (University of Greifswald) originating from Veith et al [2]
Staphylococcus carnosus 80-285	Wildtype	Kindly provided by the Enzymicals AG (Greifswald, Germany)
Staphylococcus carnosus 20-282	Wildtype	Kindly provided by the Enzymicals AG (Greifswald, Germany)
Saccharomyces cerevisiae GRF18	Wildtype	Department of Biotechnology & Enzyme Catalysis (University of Greifswald)
Vibrio natriegens ATCC 14048	Wildtype	Originating from the ATCC (Manassas, V, USA)
Pseudomonas putida DSMZ 50198	Wildtype	Originating from the DSMZ (Braunschweig, German)
Pichia pastoris X33	Wildtype	Originally from Invitrogen by Thermo Fisher Scientific (Waltham, MS, USA)
Cutaneotrichosporan curvatus DSM 101032	Wildtype	Department of Computational Synthetic Biology (TU Darmstadt) as described by Hofmeyer *et al.* [3]
Cupriavidus necator H16	Wildtype	Department of Microbial proteomics (University of Greifswald) as described by Pohlmann *et al.* [4]
Bacillus licheniformis MW3	ΔhsdR1, ΔhsdR2	Waschkau *et al.* 2008 [5]
Bacillus subtilis JK138	*sfp*+, Δ*sacA::SpecR*, Δ*lytC::lox72*, Δ*bpr-spo::lox72*, Δ*nprB::lox72*, Δ*mpr::lox72*, Δ*aprE::lox72*, Δ*nprE::lox72*, Δ*vpr::lox72*, Δ*epr::lox72*, Δ*wprA::lox72,* Δ*srfA::(comS,lox72*), Δ*pksX::lox72*, Δ*pps::lox72*, Δ*amyE::lox72*	Krüger *et al.* 2022
Bacillus licheniformis SH006	Δ*hsdR1*, Δ*hsdR2*, Δ*pga*, Δ*apr*	this study
pE194SV	pE194 derivative; *E. coli* / *Bacillus*-shuttle vector; Em*R*; ori pE194; AmpR, ori pUC18	This work
pDhsdR1	pE194SV with Δ*hsdR1* homology flanking region	This work
pDhsdR2	pE194SV with Δ*hsdR2* homology flanking region	This work
pDpga	pE194SV with Δ*pga* homology flanking region	This work
pDapr	pE194SV with Δ*apr* homology flanking region	This work

Table S3: D-glucose-supplemented M9-mineral medium

M9 - mineral media stock solution	**Final concentration per 1 L**
M9 salt solution	1x
20 % glucose	0.4 %
1 M $MgSO_4$	1 mM
1 M $CaCl_2$	0.3 mM
Biotin (1 mg/ml)	4.09 nM
Thiamine (1mg/ml)	3.77 nM
Trace element solution (100x)	1 x

Table S4: M9-mineral medium additives

Additive	Component	Concentration in stock solution
M9 salts (10x)	Na_2HPO_4 *2 H_2O	422.6 mM
	KH_2PO_4	141.4 mM
	NaCl	93.6 mM
	NH_4Cl	93.5 mM
	ddH_2O	1 L
Ulvan solution	Ulvan	2 %
	Sodium phosphate buffer	25 mM
	Sodium chloride	50 mM
	pH 7.5	
Ulvan hydrolysate	Ulvan	2 %
	Sodium phosphate buffer	25 mM
	Sodium chloride	50 mM
	pH 7.5	
	Enzyme mix	5 µL/mL per enzyme
Enzyme mix	Sodium phosphate buffer	25 mM
	Sodium chloride	50 mM
	pH 7.5	
	Enzyme mix	5 µL/mL per crude extract enzyme from table S1
Trace element solution 100 x	$FeCl_3$	13.1 mM
	$ZnCl_2$	0.62 mM
	0.1 M $CaCl_2 \cdot 2H_20$	76 µM
	0.2 M $CoCl_2 \cdot 6 H_2O$	42 µM
	0.1 M HBO_3	162 µM
	1 M $MnCl_2 \cdot 4 H_20$	8.1 µM
	KI	0.5 mM
	Na_2MoO_4	1 mM
	EDTA	13.4 mM
Yeast extract		2 % (w/v)
Sodium chloride		15 % (w/v)

Table S5: List and sequences of primers used in this study.

Primer	Sequence 5´→ 3´
MaZu7	ACGTCATATGAAAATCAGTATGCAAAAAGCAGATTTTTGGAAAAAAGCAGCGATCTCATTACT TGTTTTCACCATGTTTTTTACCCTGATGATGAGCG
MaZu8	CACCATGTTTTTTACCCTGATGATGAGCGAAACGGTTTTTGCGCAAACGGCACCGGATGAA GACACAAGCGCCATTACGAG
MaZu9	ACGTGGTACCTTACAGGCTTTCGACTGCGATGGCTTTCCAGACGC
MaZu10	CACCATGTTTTTTACCCTGATGATGAGCGAAACGGTTTTTGCGCAAAAAGGCCTTAACCATA GCGAAATCGAAGC
MaZu11	ACGTGGTACCTTATTCTTCCAGTTTCAAGACTTCGCTTCCTGCCATCAG
MaZu12	ACGTCATATGTTGATCAACAAAAGCAAAAAGTTTTTCGTTTTTTCTTTCATTTTTGTTATGATG CTGAGCCTCTCATTTGTGAATGGGG
MaZu13	GATGCTGAGCCTCTCATTTGTGAATGGGGAAGTTGCAAAAGCCCAAACGGCACCGGATGAA GACACAAGCGCC
MaZu14	GATGCTGAGCCTCTCATTTGTGAATGGGGAAGTTGCAAAAGCCCAAAAAGGCCTTAACCAT AGCGAAATCGAAGCG
MaZu19	ACGTACCATATGCAAACGGCACCGGATGAAGACACAAGCGCC
MaZu20	ACGTACTCTAGACAGGCTTTCGACTGCGATGGCTTTCCAGAC
MaZu21	ACGTACCATATGCAAAAAGGCCTTAACCATAGCGAAATCGAAGCG
MaZu22	ACGTACTCTAGATTCTTCCAGTTTCAAGACTTCGCTTCCTGCCATCAG
MaZu28	TGATAAGCGTTGGTTTGGCAATCTTATCGGGCTATGCATTTATAAAATG
MaZu29	GCATAGCCCGATAAGATTGCCAAACCAACGCTTATCAATAGAAAAAGAGCATTTTTTGAAAC AAAACTTC
MaZu35	TGCGTTAGCAATTTAACTGTGATAAACTACCGCATTAATAGAAAAAGAGCATTTTTTGAAACA AAACTTC
MaZu36	TAATGCGGTAGTTTATCACAGTTAAATTGCTAACGCAGTCAGGCACCGT
MaZu37	GTTTTTAAAGGCTTTTAAGCCGTCTGTACGTTCCTAACATCTGATGTCTTTGCTTGGCGAATG TTCATCT
MaZu38	TTAGGAACGTACAGACGGCTTAAAAGCCTTTAAAAACGTTTTTAAGGGGTTTGTAGACAAGG TAAAGGATAAAACAG
P1-hsdR1	CTGCAGGGTCTCAACCCGAACAGCGTAAGGCTGATG
P2-hsdR1	CCGTAATTTGAATCTATTAGACAAACATCTTTTGTAGGAATG
P3-hsdR1	GATGTTTGTCTAATAGATTCAAATTACGGGCCTTG
P4-hsdR1	TCTAGAGGTCTCATGAGATCGGTTTTATGAAAGCGTC
P1-hsdR2	CTGCAGCGTCTCAACCCGATAAAAGGATTACTGTGCG
P2-hsdR2	TCCATGTTGTCACAACCTATTGTTGAGAATAAAGGAAAAGGAG
P3-hsdR2	CCTTTATTCTCAACAATAGGTTGTGACAACATGGAAAG
P4-hsdR2	TCTAGACGTCTCATGAGGTGCTTTCATCAATCGTAAATC

Table S6: Summary of the proteomic results (available as separate Excel file)

Table S7: Results of statistical analyses (Welch's T-test, FDR 0.05) (available as separate Excel file)

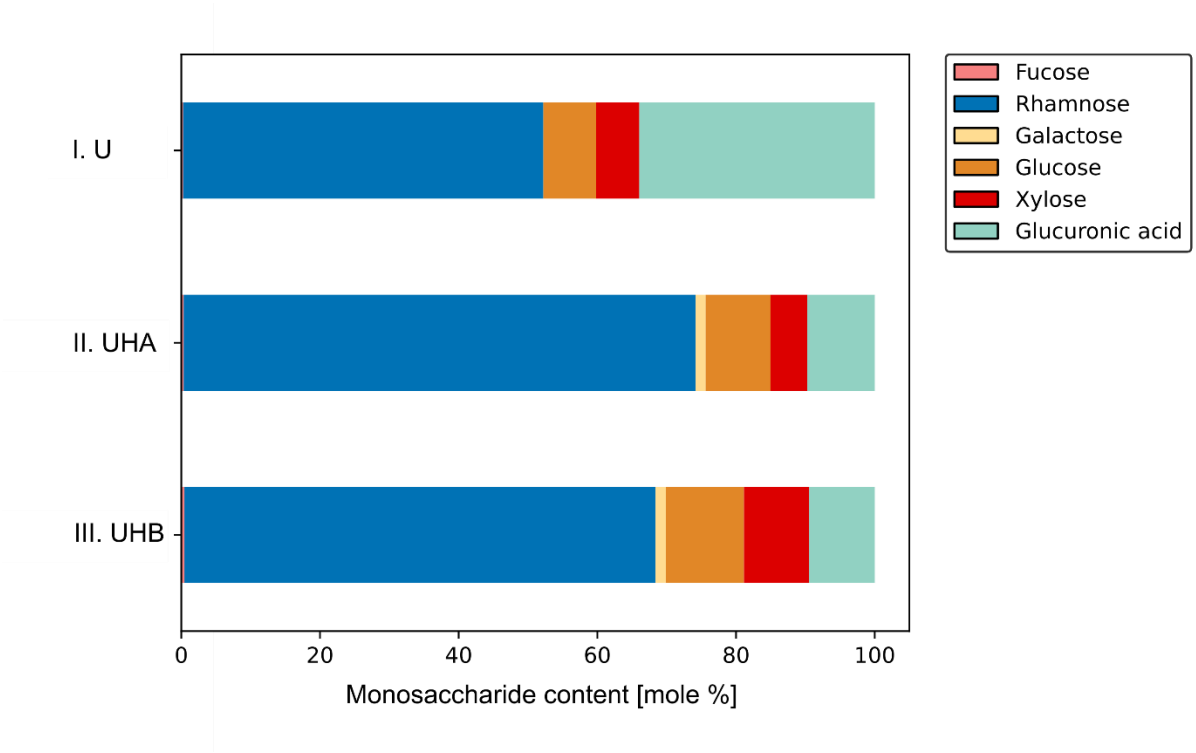

Fig. S1 Sugar composition of the cultivation media. The complex carbon sources were analysed for their detailed monosaccharide composition via acid hydrolysis and HPAEC-PAD. Ulvan (U), the partially P30_PL28 and P33_GH105-hydrolyzed ulvan (UHA) and the completely digested ulvan (UHB) using the whole cascade of ulvan degrading enzymes [1].

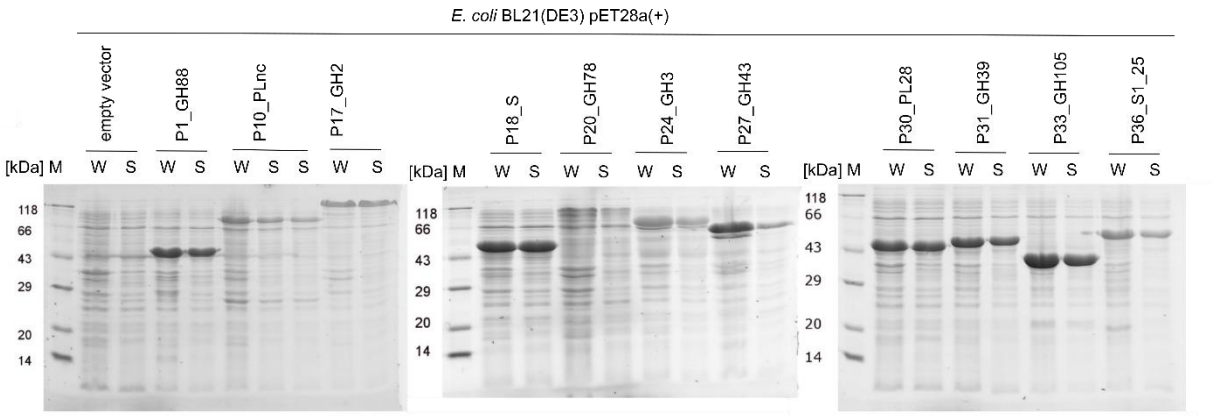

Fig. S2 SDS-PAGE of *F. agariphila* KMM3901^T enzymes expressed recombinantly in *E. coli* BL21(DE3) as described by Reisky *et al.* [1]. The cells were normed (7/OD) and the whole cell (W) extract and soluble protein (S) fraction were analyzed by SDS-PAGE containing 1% (V/V) trichloroethanol (TCE). The protein bands were visualised under UV and the pictures were colour-inverted and decolorized. The protein marker Roti®-Mark from Carl Roth (Karlsruhe, Germany) was used.

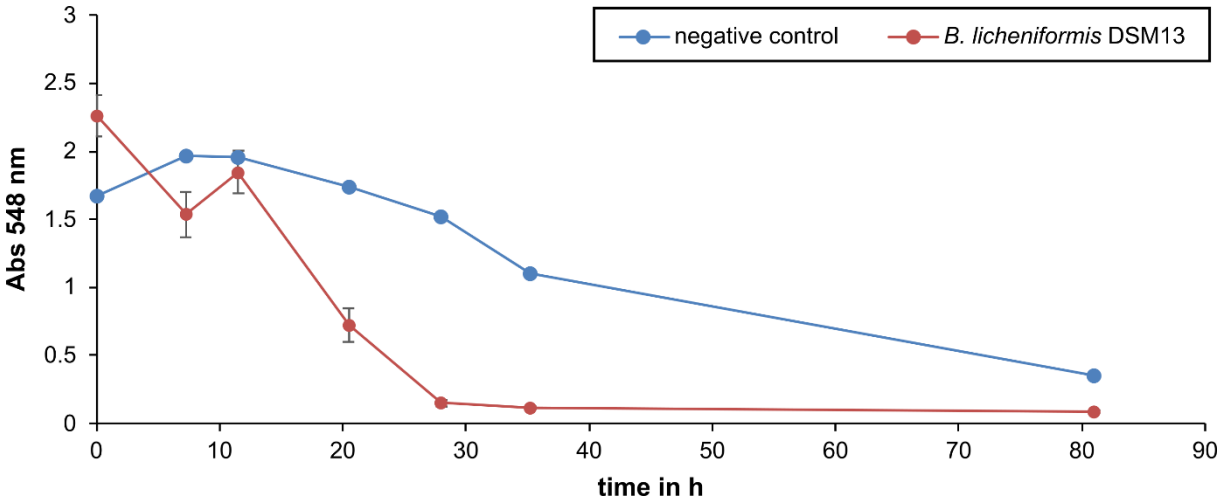

Fig. S3 Consumption of 5-dehydro-4-deoxy-D-glucuronate by *B. licheniformis* DSM13 during cultivation. Thiobarbituric acid assay determined 5-dehydro-4-deoxy-D-glucuronate in cultivation supernatants (M9-ulvan hydrolysate UHB, see Fig. 3a). Cell-free medium served as negative control.

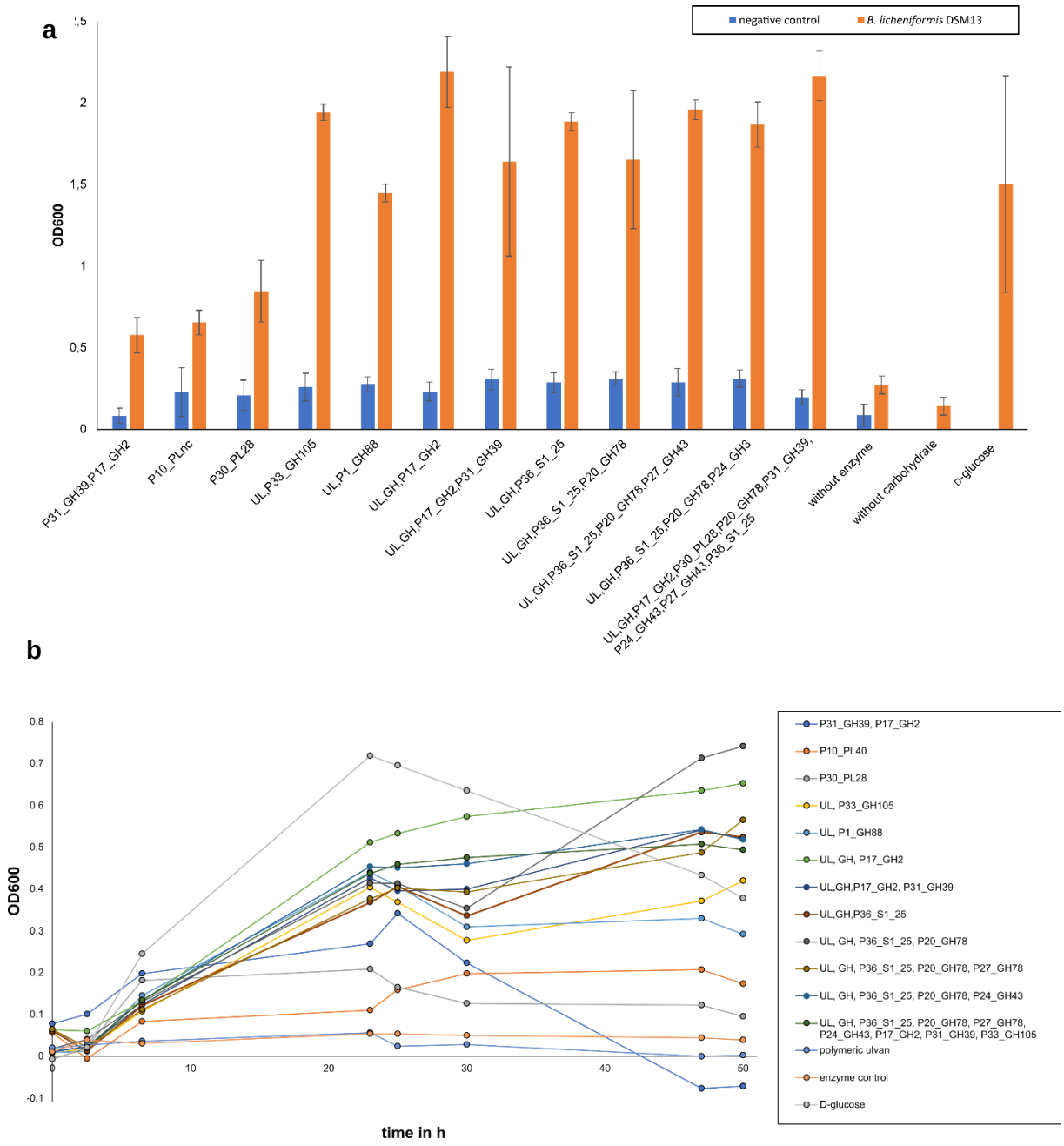

Fig. S4 Growth of *B. licheniformis* DSM13 on ulvan (without enzyme) and ulvan hydrolysates representing different levels of degradation. For hydrolysis, different *F. agariphila* enzymes, enzyme combinations or all enzymes (recombinantly expressed in *E. coli*) were used (Table S1). *B. licheniformis* DSM13 was cultivated in 1 mL in 96 deep-well plates and OD600 was measured after 48 h (**a**). This graph represents the full dataset of the Figure 2 in the main text. For investigation of the growth behaviour the culture was cultivated in 200 µL scale in a low-well plate and measured for 48 h (**b**).

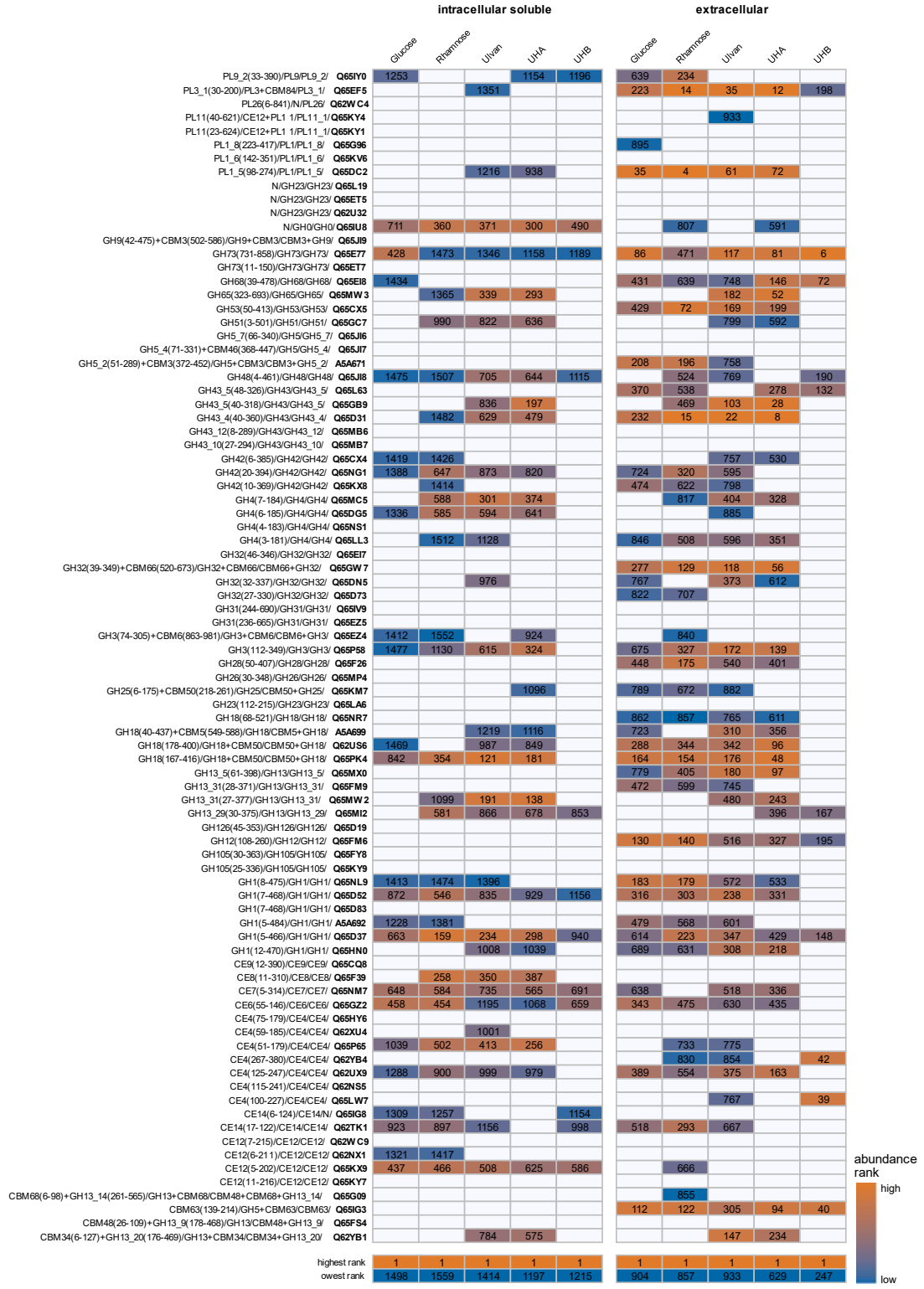

Fig. S5 List of PLs, GHs and CEs identified by dbCAN2 [6] and their abundance in the intracellular soluble and extracellular proteomes. The graph indicates the relative abundance of proteins within the respective sample given as abundance ranks. Abundance ranks were derived from %riBAQ values (Table S6). The lowest rank corresponds to the total number of quantified proteins per sample. Blank tiles represent proteins that were not quantified. UHA/B: Ulvan hydrolysate A/B (see Fig. 3a). Protein IDs are highlighted in bold together with the full output of dbCAN2 analyses (HMMER/Hotpep/DIAMOND/Protein ID).

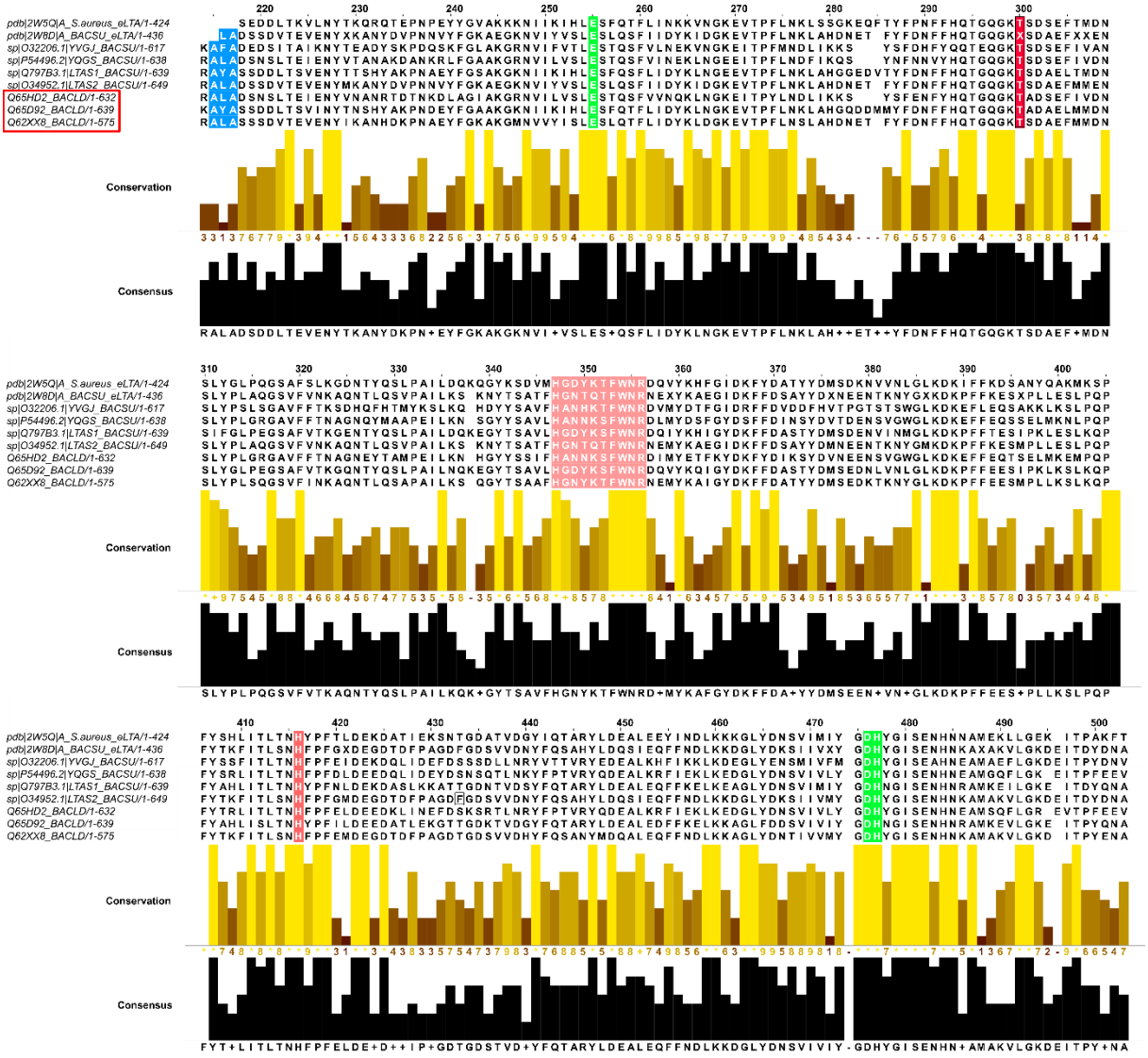

Fig. S6 Alignment of sulfatases from *B. licheniformis* DSM13 with lipoteichoic acid synthases of other Gram-positive bacteria. The lipoteichoic acid synthases (LTA) were the highest hit in the BlastP search. LTAs consist of a transmembrane and an extracellular domain. Amino acid residues in the region of the extracellular LTA (eLTA) domain (210 to 585 As) are displayed. LTAs are synthesized as membrane proteins and are cut at a cleavage sequence AXA (blue). T300 (marked in red) is the catalytic residue in the eLTA of *S. aureus*, while H416 is involved in the reaction mechanism (protonation of the leaving group). Residues marked in green are important for the binding of Mn2+. Residues 347-356 (HxD/NxxFW/YNR) are important for substrate binding [7].

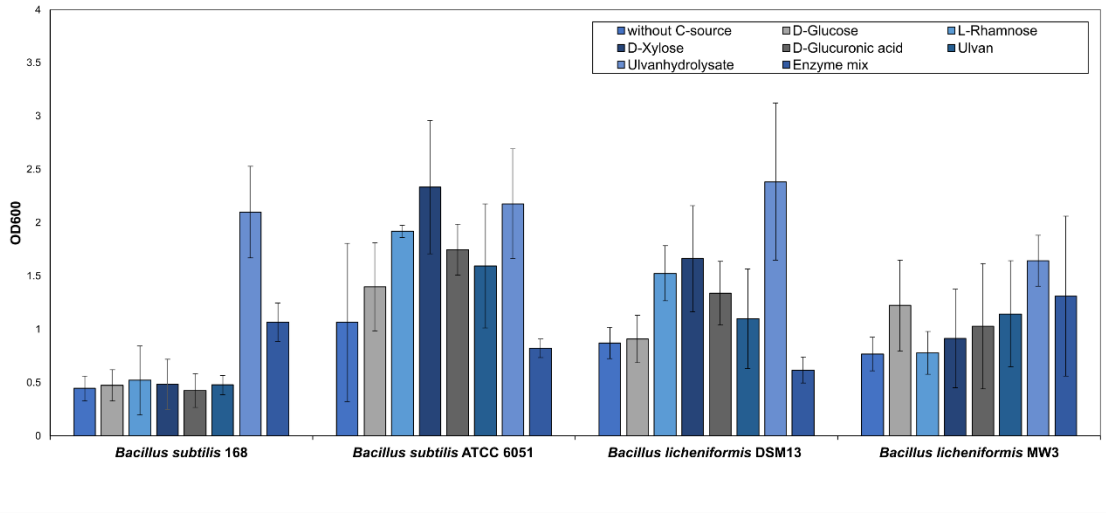

Fig. S7 Comparison of different *Bacillus* sp. for the conversion of ulvan hydrolysate and ulvan derived monosaccharides. The strains were cultivated in 1 mL Belitzky-Minimal media [8] with variation of the carbon source in a 96-deep well-plate. General growth in OD600nm was measured after 24 h, showing a general acceptance of the ulvan hydrolysate for all four *Bacillus* sp. in the chosen Belitzky-Minimal medium conditions.

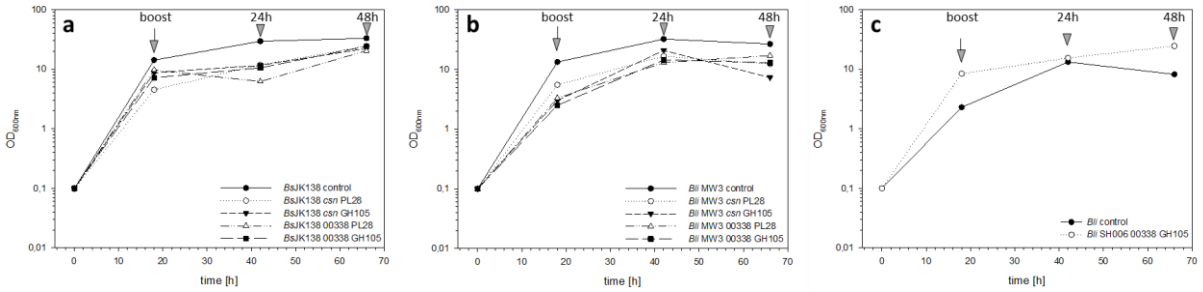

Fig. S8 Growth of the PL28 and GH105 *Bacillus* expression strains. *B. subtilis* JK138. (**a**), B. licheniformis MW3 (**b**) and *B. licheniformis* SH006 (Δapr) (**c**) were grown under simulated fed-batch conditions in Expresso-B medium at 30°C and 250 rpm. Sampling points for activity measurements, 24 h and 48 h after the boost are indicated.

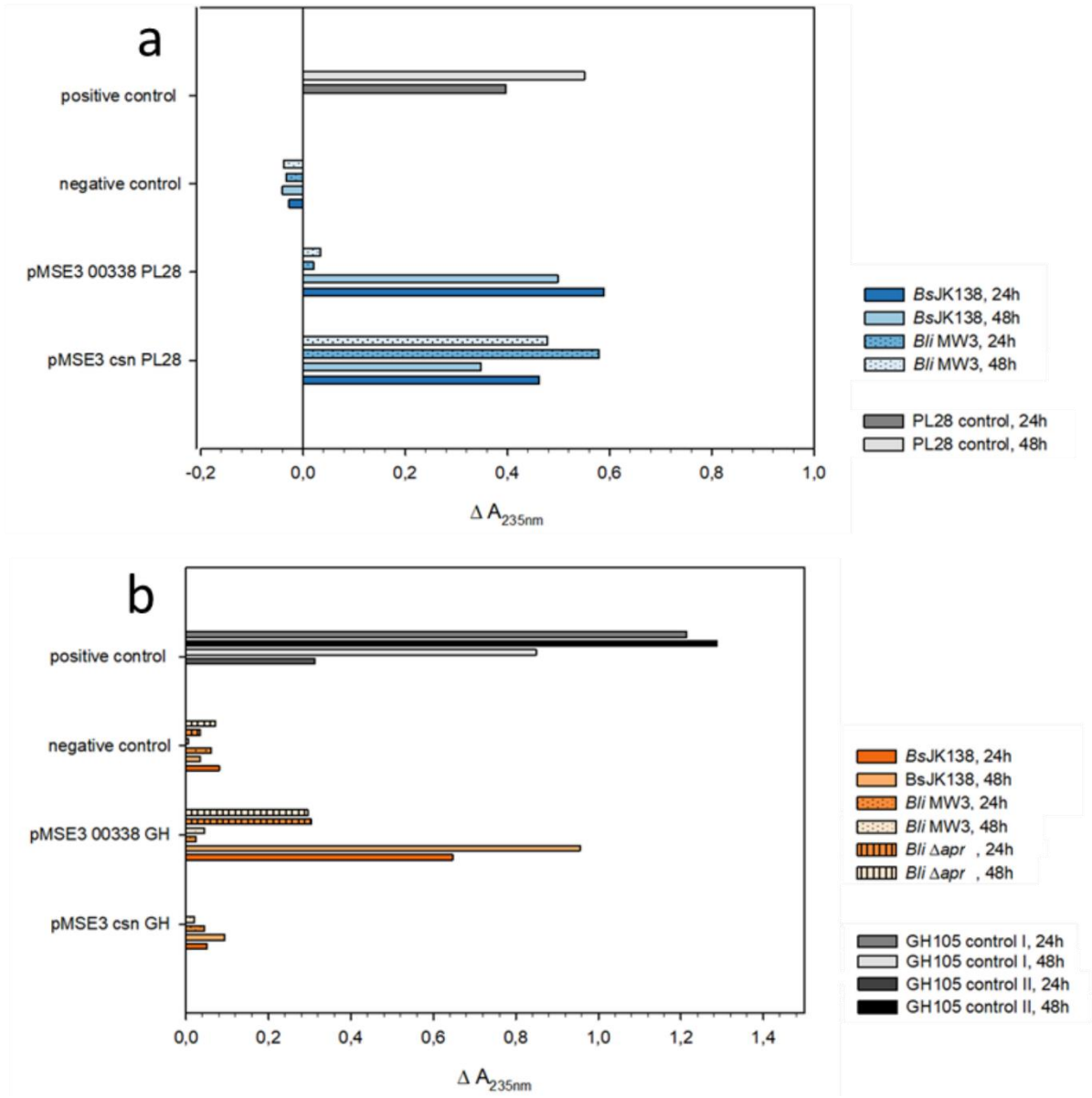

Fig. S9 Activity assay results from *Bacillus* sp. PL28 and GH105 expression strains. (**a**) Results of the ulvan lyase (UL) assay measurement of the lyase product formation at 235 nm over 60 min. The deviation of the absorption of the end-start reveals the lyase product formation (lyase activity), while the deviation of the absorption start-end shows the reversed reaction of the GH105 activity (**b**) cleaving of the lyase moiety using prehydrolyzed ulvan from the recombinantly expressed ulvan lyase PL28.

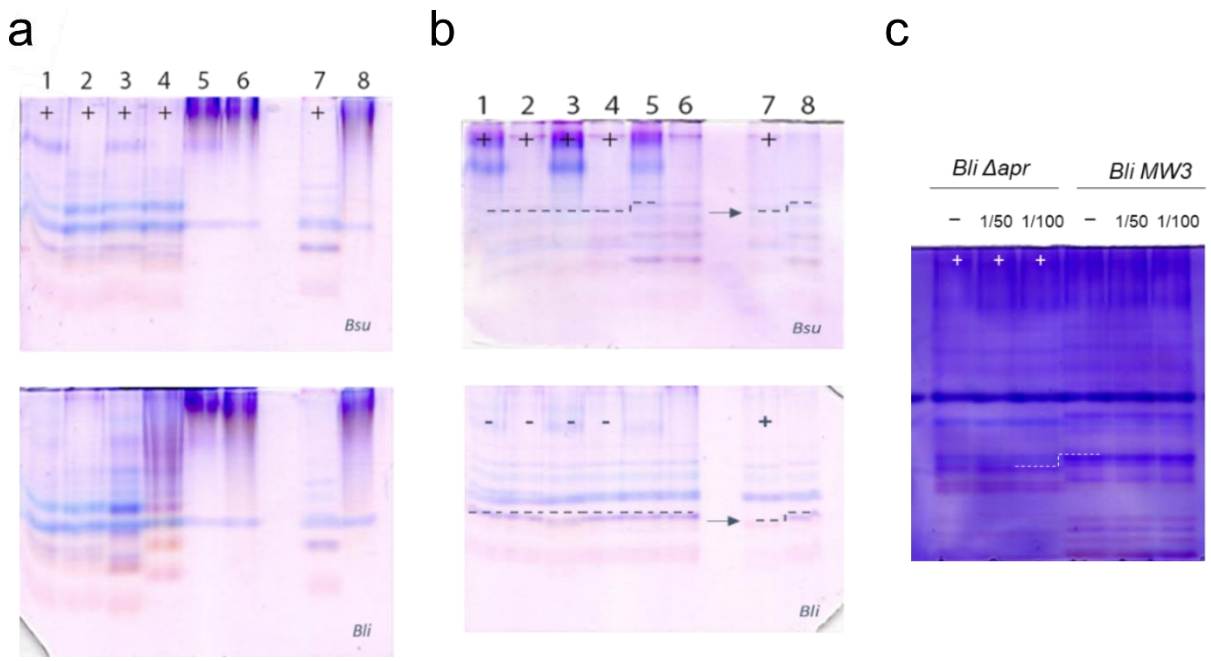

Fig. S10 C-PAGE results from *Bacillus* sp. PL28 and GH105 expression strains. (**a**) The C-PAGE (carbohydrate polyacrylamide gel electrophoresis) corresponds to the ulvan lyase assay shown in Fig. S10 to the results of the ulvan lyase (UL) expression and (**b**) reaction of the GH105 activity cleaving of the lyase moiety using prehydrolyzed ulvan from the recombinantly expressed ulvan lyase PL28. Intracellular (in) and extracellular (ex) fractions of *B. subtilis* JK138 (Bsu) and *B. licheniformis* DSM13 (Bli) were analyzed. Lane 1: TB1 csn-UL ex, Lane 2: TB1 csn-UL in, Lane 3: TB1 00338-UL ex, Lane 4: TB1 00338-UL in, Lane 5: Ko (empty vector) ex, Lane 6: Ko (empty vector), Lane 7: positive control, Lane 8: negative control. (**c**) C-PAGE after 24 h of GH105 expression in *B. licheniformis* SH006 (Bli Δapr) and *B. licheniformis* MW3 (Bli MW3) with the addition of the protease inhibitor in 1/100, 1/50 and no protease (−) addition.

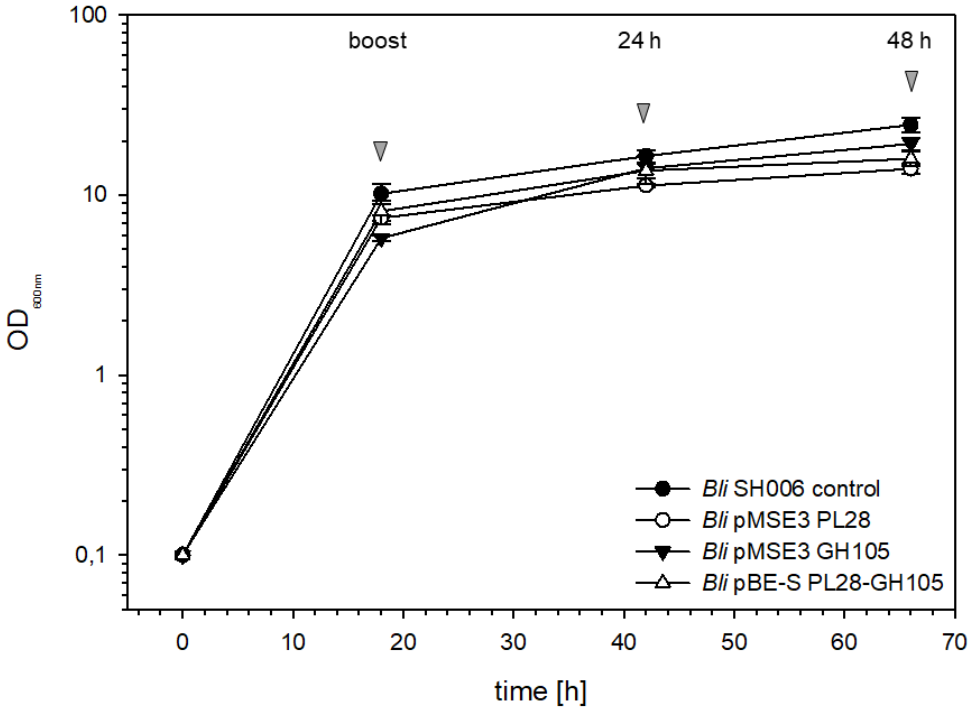

Fig. S11 Growth of the different *B. licheniformis* expression strains. *B. licheniformis* SH006, *B. licheniformis* pMSE3 PL28, *B. licheniformis* pMSE3 GH105 and *B. licheniformis* pBE-S PL28-GH105 were grown under simulated fed-batch conditions in Expresso-B medium at 30°C and 250 rpm. Sampling points for activity measurements, 24 h and 48 h after the boost are indicated.

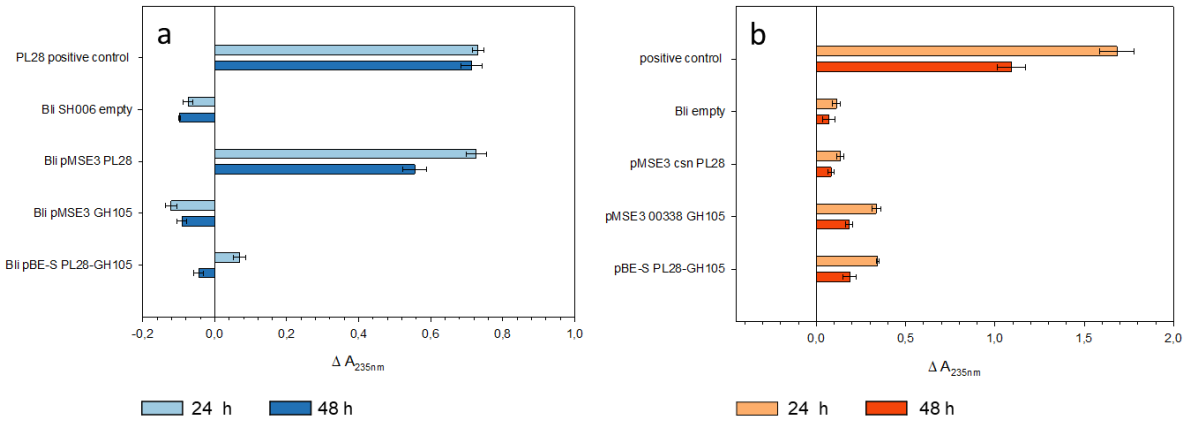

Fig. S12 Activity assay results from *B. licheniformis* SH006, PL28 and GH105 single- and co-expression strain. (**a**) Results of the ulvan lyase (PL28) assay measurement of the lyase product formation at 235 nm over 60 min. The deviation of the absorption of the end-start shows the lyase product formation (lyase activity). (**b**) The deviation of the absorption start-end shows the reversed reaction of the GH105 activity by cleaving of the lyase moiety using prehydrolyzed ulvan from the recombinantly expressed ulvan lyase PL28. Corresponding C-PAGE gels are shown in Fig. S13.

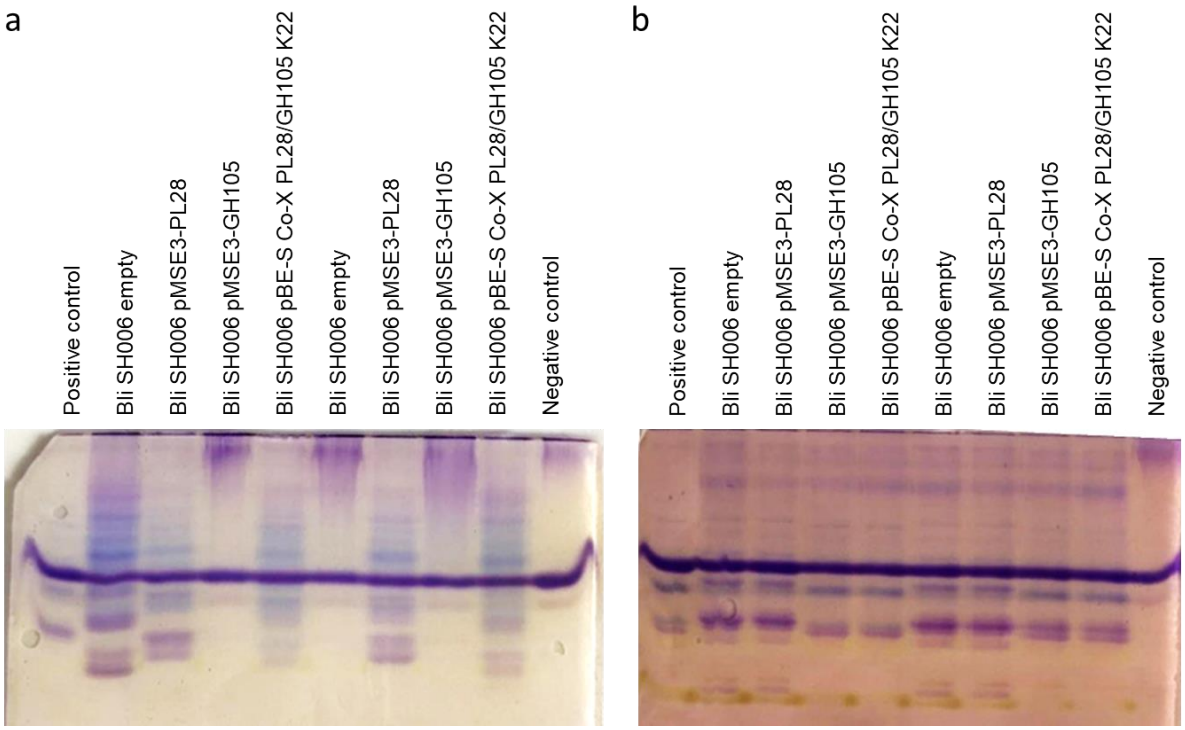

Fig. S13 C-PAGE results from *B. licheniformis* SH006 PL28, GH105 and co-expression strains. The results correspond to the ulvan lyase assay shown in Fig. S12. (**a**) Shows the C-PAGE result of the PL28 ulvan lyase activity (extracellular fraction) of the strain constructs *B. licheniformis* SH006 (Bli SH006) empty, pMSE3_PL28, pMSE3- GH105 or pBE-S Co-X PL28/GH105 K22 (**b**) shows the reaction of the GH105 cleaving of the lyase moiety using prehydrolyzed ulvan.

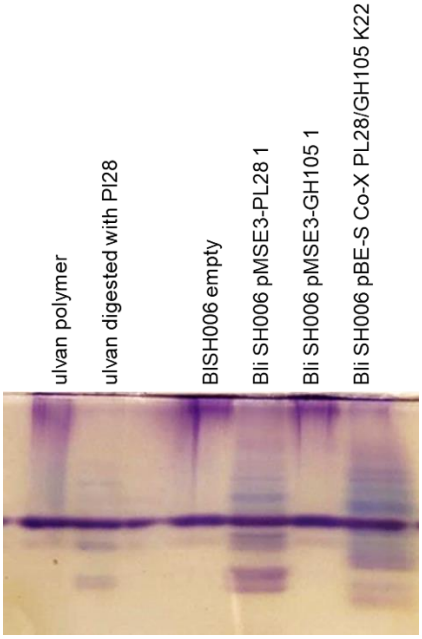

Fig. S14 C-PAGE from the cultivation supernatant of *B. licheniformis* strains in M9-mineral media. *B. licheniformis* (Bli) SH006 PL28, GH105 and coexpression strains were cultivated in M9-mineral media (see Fig. 6) for seven days. A supernatant sample was loaded, as negative control the ulvan polymer and as a positive control the predigested ulvan with PL28 was loaded on the gel.

References

1. Reisky L, Précoux A, Zühlke MK, Bäumgen M, Robb CS, Gerlach N, et al. A complex enzyme cascade degrades the polysaccharide ulvan from green algae. Nat Chem Biol. 2019;803–12.

2. Veith B, Herzberg C, Steckel S, Feesche J, Maurer KH, Ehrenreich P, et al. The complete genome sequence of *Bacillus licheniformis* DSM13, an organism with great industrial potential. J Mol Microbiol Biotechnol. 2004;7:204–11.

3. Hofmeyer T, Hackenschmidt S, Nadler F, Thürmer A, Daniel R, Kabisch J. Draft genome sequence of *Cutaneotrichosporon curvatus* DSM 101032 (Formerly *Cryptococcus curvatus*), an oleaginous yeast producing polyunsaturated fatty acids. Genome Announc. 2016;4:e00362-16.

4. Pohlmann A, Fricke WF, Reinecke F, Kusian B, Liesegang H, Cramm R, et al. Genome sequence of the bioplastic-producing “Knallgas” bacterium *Ralstonia eutropha* H16. Nat Biotechnol. 2006;24:1257–62.

5. Waschkau B, Waldeck J, Wieland S, Eichstädt R, Meinhardt F. Generation of readily transformable *Bacillus licheniformis* mutants. Appl Microbiol Biotechnol. 2008;78:181–8.

6. Yin Y, Mao X, Yang J, Chen X, Mao F, Xu Y. dbCAN: a web resource for automated carbohydrate-active enzyme annotation. Nucleic Acids Res. 2012;40:W445–51.

7. Lu D, Wörmann ME, Zhang X, Schneewind O, Gründling A, Freemont PS. Structure-based mechanism of lipoteichoic acid synthesis by *Staphylococcus aureus* LtaS. Proc Natl Acad Sci. Proceedings of the National Academy of Sciences; 2009;106:1584–9.

8. Stülke J, Hillen W. Regulation of Carbon Catabolism in *Bacillus* Species. Annu Rev Microbiol. 2000;54:849–80.