Electronic Supplementary Material (ESI) for Chemical Communications

Surface-based Mass Spectrometry Method for Screening Glycosidase Specificity in Environmental Samples

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Supporting Information.

General Methods

Chemicals were purchased from Sigma-Aldrich or Acros Organics and were used without further purification. Matrixes were purchased from Sigma-Aldrich (purity >99.5%). Solvents employed were HPLC quality. Microwave irradiation was performed on Biotage Initiator monomode oven, Biotage AB, Uppsala, Sweden. HRMS spectra were recorded on *Time-of-Flight* mass spectrometer LCT Premier XE (Waters, Milford USA), with electrospray source operating in negative mode (infusion rate 10μL/min; capillary voltage 2000 V; sample cone voltage 200 V), using sodium formate (Sigma) clusters as internal standards. MALDI-MS spectra were recorded on an Applied Biosystems Voyager DE Pro with a nitrogen laser (337 nm) in negative reflector ion mode (20 kV plate voltage, 79% grid extraction, 0.01% guide wire voltage, 200 ns delay and 100-150 shots, averaged per spectra). Laser intensity was set marginally above the threshold of ionization to avoid fragmentation. The *m/z* range was chosen according to the mass of the sample. Resulting spectra were processed by Data Explorer 4.0 software. Functionalized sample plates were stored in glass petri dishes or under high vacuum in the sample holder of the spectrometer. Contact angle measurements on the surface were performed at room temperature using DSA 100 contact angle meter (Krüss). The soil sample was collected from switchgrass-containing garden, San Sebastian, Spain, the compost sample was kindly provided by GHK, S. A. composting plant (San Sebastián, Spain) and the saliva sample was obtained from a male healthy volunteer.

Preparation and characterization of glycoconjugates

Cellopentaose, arabinopentaose, xylotetraose, xyloglucan heptasaccharide and maltopentaose were tagged with DHPE (1,2-Dihexadecyl-*sn*-glycero-3-phosphoethanolamine) by reductive amination as described by Chai et al[1]: DHPE (3.1 mg, 0.004 mmol, 1.0 eq.) were mixed with 2.0 equivalents of the corresponding carbohydrate in a microwave vial and the mixture was suspended in a solution of $NaBH_3CN$ (1 mM in DMSO:AcOH 7:1) and irradiated at 70ºC for two cycles of 10 minutes. The employed glycans were diluted in MeOH to a final concentration of 1 mM.

Identity of all compounds (**1** to **5**) was confirmed by high resolution mass spectrometry (HRMS, figure S1 to S5)

a) Cellopentaose conjugate 1

Chemical formula: $C_{67}H_{129}NO_{31}P$

Theoretical *m/z* for $[M-H]^-$: 1474.8286

Found *m/z* for $[M-H]^-$ by HRMS: 1474.8262

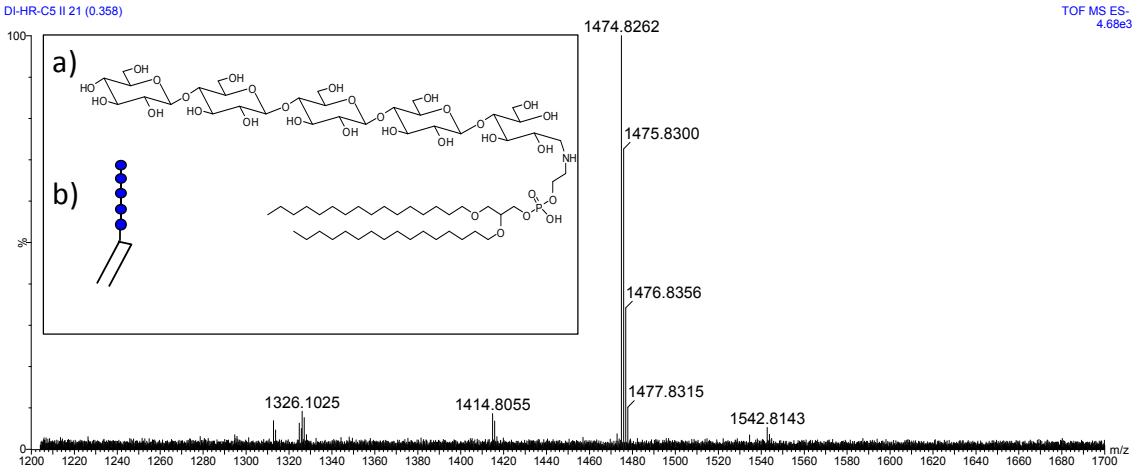


Figure S1. HRMS spectra of compound **1**. Inset picture shows the (a) chemical structure and (b) used pictogram representation according to Consortium of Functional Glycomics (*http://www.functionalglycomics.org*) guidelines.

b) Arabinopentaose conjugate 2

Chemical formula: $C_{62}H_{119}NO_{26}P$

Theoretical *m/z* for $[M-H]^-$: 1324.7758

Found *m/z* for $[M-H]^-$ by HRMS: 1324.7847

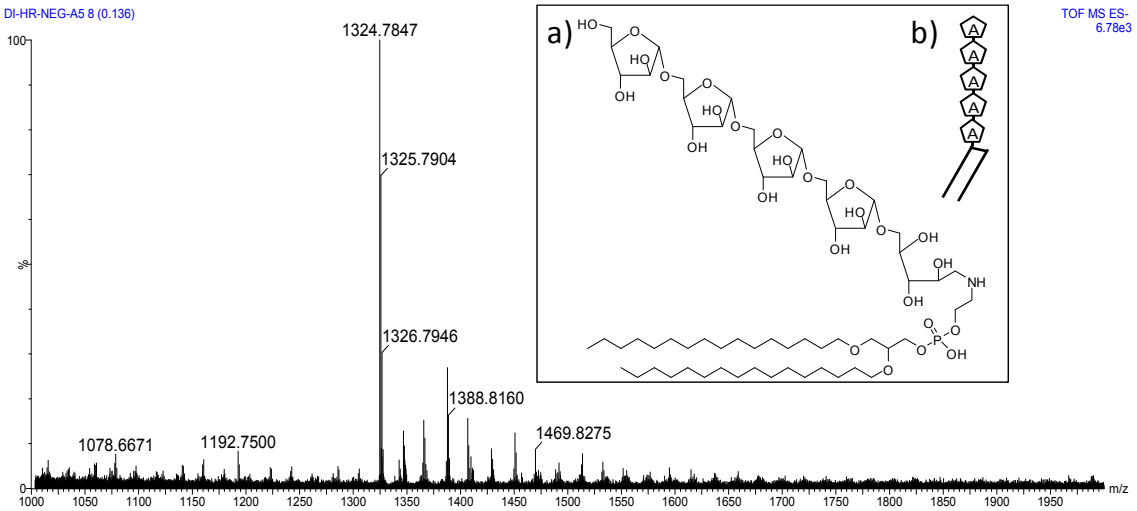


Figure S2. HRMS spectra of compound **2**. Inset picture shows the (a) chemical structure and (b) used pictogram representation.

c) Xylotetraose conjugate 3

Chemical formula: $C_{57}H_{111}NO_{22}P$

Theoretical *m/z* for $[M-H]^-$: 1192.7335

Found *m/z* for $[M-H]^-$ by HRMS: 1192.7375

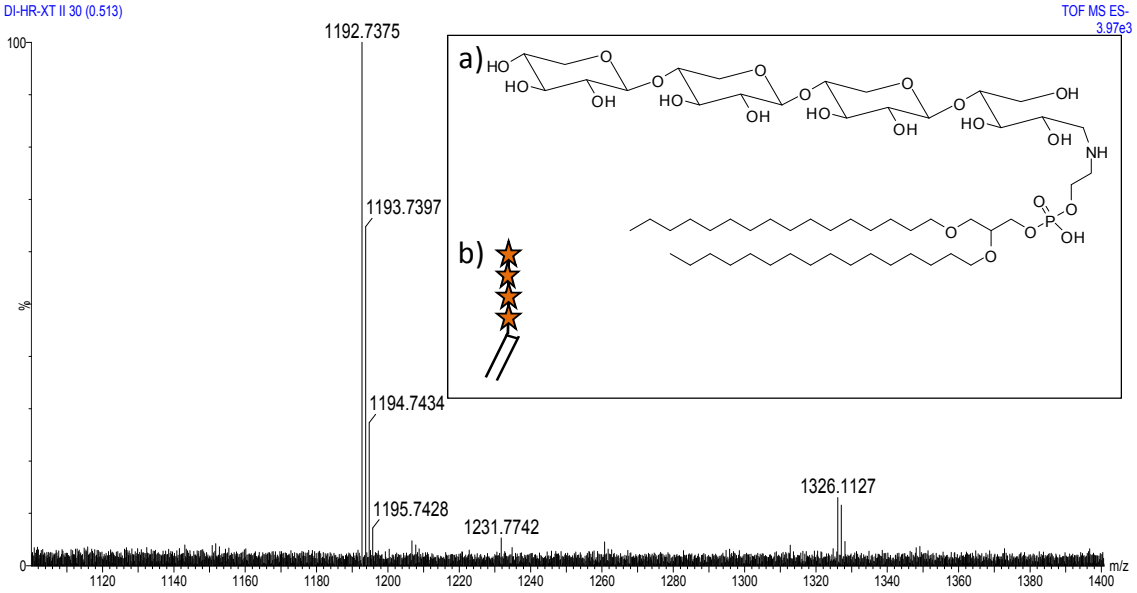


Figure S3. HRMS spectra of compound **3**. Inset picture shows the (a) chemical structure and (b) used pictogram representation.

d) Xyloglucan heptasaccharide conjugate 4

Chemical formula: $C_{76}H_{143}NO_{38}P$

Theoretical *m/z* for $[M-H]^-$: 1708.9026

Found *m/z* for $[M-H]^-$ by HRMS: 1708.9000

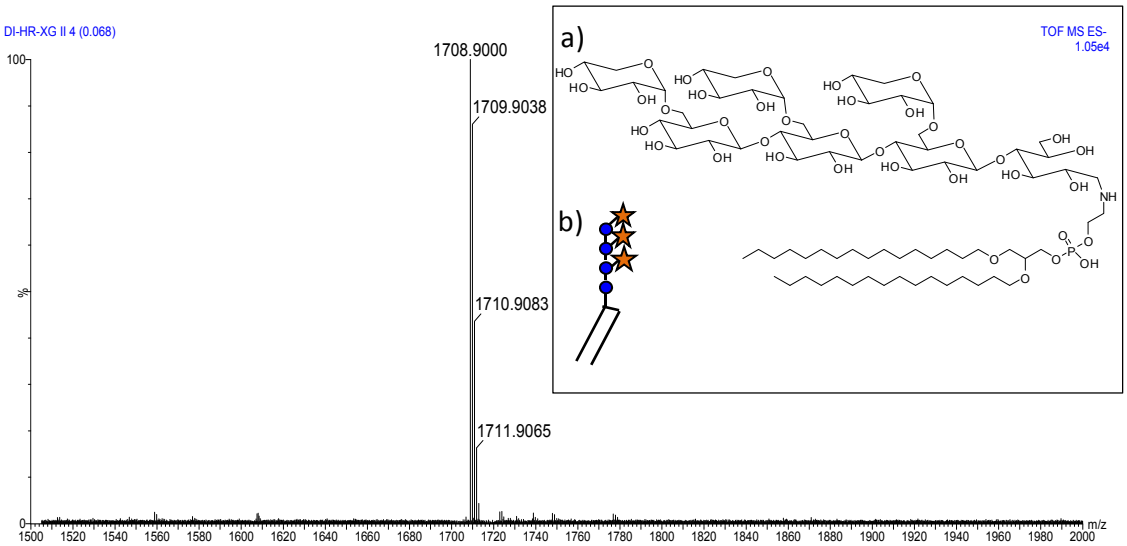


Figure S4. HRMS spectra of compound **4**. Inset picture shows the (a) chemical structure and (b) pictogram representation

e) Maltopentaose conjugate 5

Chemical formula: $C_{76}H_{143}NO_{38}P$

Theoretical *m/z* for $[M-H]^-$: 1708.9026

Found *m/z* for $[M-H]^-$ by HRMS: 1708.9000

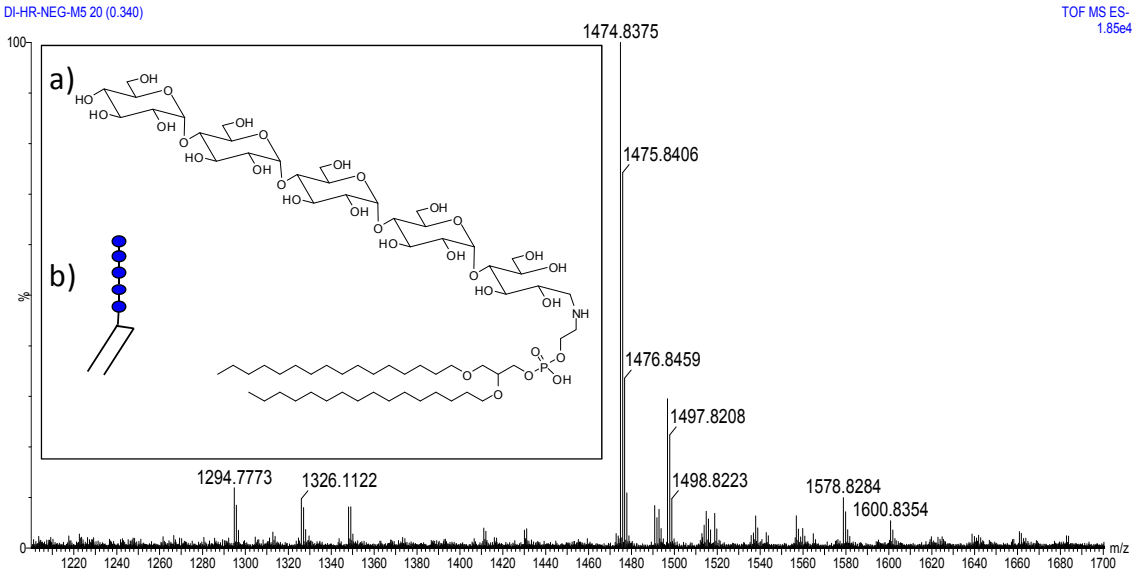


Figure S5. HRMS spectra of compound **5**. Inset picture shows the (a) chemical structure and (b) pictogram representation

Functionalization of sample plate with hydrophobic SAM

An Applied Biosystems disposable MALDI gold plate (part number V503476) was functionalized with a SAM of undecanethiol following a procedure described by Bethencourt et al.[2] Uniformity of the SAM was controlled by contact angle measurements (native gold plate angle: 79.5º; undecanethiol covered plate angle: 100.83º).

Immobilization of tagged glycans on SAM

0.5 µL of tagged glycans **1-5** in MeOH (1 mM) were spotted onto selected wells on the sample plate and air-dried. Unbound material was removed by immersion of the sample plate in milliQ water. Blank spectra of each immobilized glycan were recorder prior to hydrolysis experiments (figure S7).

On-chip hydrolase assays with pure hydrolases

Bacillus amyloliquefaciens endo-cellulase assay. A 1.4 U/ml solution of an endo-cellulase enzyme from *Bacillus Amyloliquefaciens* (Megazyme, ref. E-CELBA) was prepared by dilution in miliQ water of the stock solution (1400 U/ml). 2.4 µL of the working solution (a total of 3.4 µU of enzyme) was spotted onto wells containing conjugate **1** and incubated for the specified time (10, 20 and 30 minutes) at 37ºC. The plate was then immersed in milliQ water, sonicated for 10 seconds, dried under a stream of Ar and analyzed (figure S8) by MALDI-ToF MS (THAP matrix, 7 mg/ml in acetone).

Aspergillus niger endo-arabinanase assay.. A 1.0 U/ml solution of an endo-cellulase enzyme from *Aspergillus niger* (Megazyme, ref. E-EARAB) was prepared by dilution in miliQ water of the stock solution (100 U/ml). 2.4 µL of the working solution was spotted (2.4 µU of enzyme) onto wells containing conjugate **2** and incubated for the specified time (10, 20 and 30 minutes) at 37ºC. The plate was then immersed in milliQ water, sonicated for 10 seconds, dried under a stream of Ar and analyzed (figure S9) by MALDI-ToF MS (THAP matrix, 7 mg/ml in acetone).

Thermotoga maritima endo-xylanase assay. A 100 U/ml solution of an endo-xylanase enzyme from *Thermotoga Maritima* (Megazyme, ref. E-XYLATM) was prepared by dilution in acetate buffer (50 mM, pH 5.0, 1 mg/ml BSA) of the stock solution (3750 U/ml). 2.4 µL of the solution was spotted (a total of 0.24 mU of enzyme) onto wells containing conjugate **3** and incubated for the specified time (10, 20 and 30 minutes) at 37ºC. The plate was then immersed in milliQ water, sonicated for 10 seconds, dried under a stream of Ar and analyzed (figure S10) by MALDI-ToF MS (THAP matrix, 7 mg/ml in acetone).

Paenibacillus sp. endo-xyloglucanase assay. A 550 U/ml solution of an endo-xyloglucanase enzyme from *Paenibacillus sp.* (Megazyme, ref. E-XEGP) was prepared by dilution in MES buffer (100 mM, pH 6.5) of the stock solution (1000 U/ml). 2.4 µL of the working solution was spotted (a total of 1.3 mU of enzyme) onto wells containing conjugate **4** and incubated for the specified number of 30 minutes cycles (1, 2, and 3 cycles) at 37ºC. The plate was then

immersed in milliQ water, sonicated for 10 seconds, dried under a stream of Ar and analyzed (figure S11) by MALDI-ToF MS (THAP matrix, 7 mg/ml in acetone).

General procedure for duplex assays with pure hydrolases

In order to check if this methodology could be used for screening different activities at the same time, two different compounds were immobilized on the same well and were assayed against different pure commercial hydrolases. For this, mixture of conjugates **1**-**2** and **1**-**3** was prepared by spotting sequentially 0.5 µl of each glycan on the same hydrophobically coated well. Hydrolase specificity was demonstrated against compound **1** and **2** when 3.4 µU of *B. amyloliquefaciens* cellulase and 2.4 µU of *A. niger* arabinanase, respectively, were used (figure S12).

General procedure for in-solution enzymatic assay

In order to compare the hydrolysis activity on surface and in solution against described glycolipids from **1** to **4**, same quantities of substrate and enzymes than on-chip conditions were added into eppendorf tubes for each reaction. First, glycan solutions were added into the eppendorff and the methanol was evaporated to dryness. Then, the corresponding enzyme units in their appropriate buffer were added into the glycan containing tube and these mixtures were incubated for 20 min at 37ºC. The enzymatic reactions were stopped by boiling the solution and, afterwards, 2.5 µl of each reaction mixture were deposited onto a stainless steel MALDI plate, evaporated and analyzed (figure S13) by MALDI-ToF MS (THAP matrix, 7 mg/ml in acetone).

Hydrolysis assays using natural glycans.

Natural oligosaccharides (cellopentaose and arabinopentaose) were also assayed against the appropriate enzymes in solution. For that, 10 µl of cellopentaose and arabinopentaose stock solutions (1 mg/ml in water) were mixed with 24 µl of *B. amyloliquefaciens* endo-cellulase (1.4 U/ml in water) and 24 µl of *A. niger* arabinanase (1.0 U/ml in water), respectively. The mixtures were incubated for 20 min at 37ºC and the reactions stopped after boiling the sample. 1 µl of these solutions were spotted onto a stainless MALDI plate with 1 µl of DHB matrix (containing 0.1% TFA) and the spectra were recorded (figure S14)

Table S1 compares the hydrolysis rate (in terms of %) after 20 min of incubation when performing the reaction on surface or in solution with tagged or natural glycans.

	On-Surface (%)	In-Solution with tagged glycans (%)	In-Solution with natural glycans (%)
Cellulase (C2:C3:C4:C5)	8:59:15:18	0:0:0:100	29:37:9:25
Arabinanase (A2:A3:A4:A5)	6:17:5:72	0:2:9:89	13:37:13:37
Xylanase (X2:X3:X4)	26:18:56	10:0:90	n.d.
Xyloglucanase (XG3:XG5:XG7)	15:5:80	19:3:78	n.d.

Table S1. Comparison of enzymatic activity on-surface and in-solution on glycans **1**, **2, 3** and **4** and using natural oligosaccharides after 20 minutes of incubation. Conversions are calculated from the intensity of the peaks from the degradation products.

Analysis of glycosyl hydrolase activity in complex matrices

Environmental sample processing

Cellulase-like protein enrichment. 200 mg of sample (both compost and soil) were suspended into in 50 ml of M9TE media (M9 minimal media with $MgCl_2$ 2 mM, 100 µM of $MgCl_2$ and trace elements - 730 µM $MgSO_4 \cdot 7H_2O$, 180 µM $MnCl_2$, 1 mM NaCl, 33 µM $FeCl_2$, 39 µM $CoSO_4$, 41 µM $CaCl_2 \cdot 2H_2O$, 21 µM $ZnSO_4 \cdot 7H_2O$, 2.4 µM $CuCl_2$, 97 µM H_3BO_3) with filter paper (Whatman 3 MM) disks of 1 cm in diameter dipped into the medium. The flasks were sealed and incubated at 45ºC with orbital shaking. After 3 weeks of incubation, the cultures were centrifuged at 5000 x *g*, 5 min at 4ºC and the supernatant was concentrated 600-fold using Vivaspin 20 filter devices (10000 MWCO PES) through centrifugation at 5000 x *g* at 4ºC. The final total protein concentration was approximately 2 mg/ml.

Hydrolase screening in agar plates. Plates were incubated for 1-2 h at 50 ºC in agar (15 g/L) containing citrate buffer 50 mM pH 5.0 and 1 % (w/v) of carboxymethyl cellulose (CMC) as substrate. The plates were subsequently stained for 30 min with 0.5 % Congo red (w/v) and then washed with NaCl 1 M. Yellowish halos appears only if the CMC is largerly hydrolyzed to oligomers with fewer than five residues (figure S6).

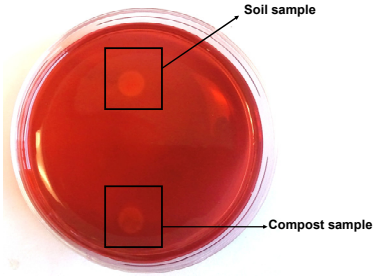


Figure S6. Qualitative test on hydrolytic activity of environmental samples.

Saliva sample processing

1 ml of saliva from a healthy individual was collected through unstimulated passive drool and diluted with 6 ml of miliQ deionized water. The sample was directly spotted onto wells containing mixture of conjugates **1, 2** and **3** and incubated for the 30 minutes at 37ºC. The plate was then immersed in milliQ water, sonicated for 10 seconds, dried under a stream of Ar and analyzed by MALDI-ToF MS (THAP matrix, 7 mg/ml in acetone).

Hydrolase screening on hydrophobic MALDI plate

Onto hydrophobically coated wells, a mixture of conjugates **1-2** and **1-3** was prepared through sequential addition of 0.5 µl of each compound. 2.4 µl of environmental sample extract was deposited and incubated at 45ºC for 20 minutes. When the wells run dry, 2.4 µl more of extract were added and was incubated for 20 minutes more. This process was repeated three times, after which, the plate was sonicated for 20 seconds and analyzed by MALDI-ToF MS (THAP, 7 mg/ml in acetone). Different conversion ratios were calculated (table S2) for each sample and substrate.

	Complex samples		
Substrate (products, %)	**Compost**	**Soil**	**Saliva**
Cellopentaose derivative **1** (C3:C4:C5:C6)	72:0:18:10	71:11:10:8	n.d.
Arabinopentaose derivative **2** (A2:A3:A4:A5)	0:0:26:74	23:39:23:15	0:0:100
Xylotetraose derivative **3** (X2:X3:X4)	0:0:100	0:0:100	0:0:100
Maltopentaose derivative **5** (M2:M3:M4:M5)	n.d.	n.d.	47:0:53

Table S2. Comparison of enzymatic activity of complex samples with compounds **1**, **2**, **3** and **5**. The ratios were calculated as percentage of conversion.

MALDI MS Spectra

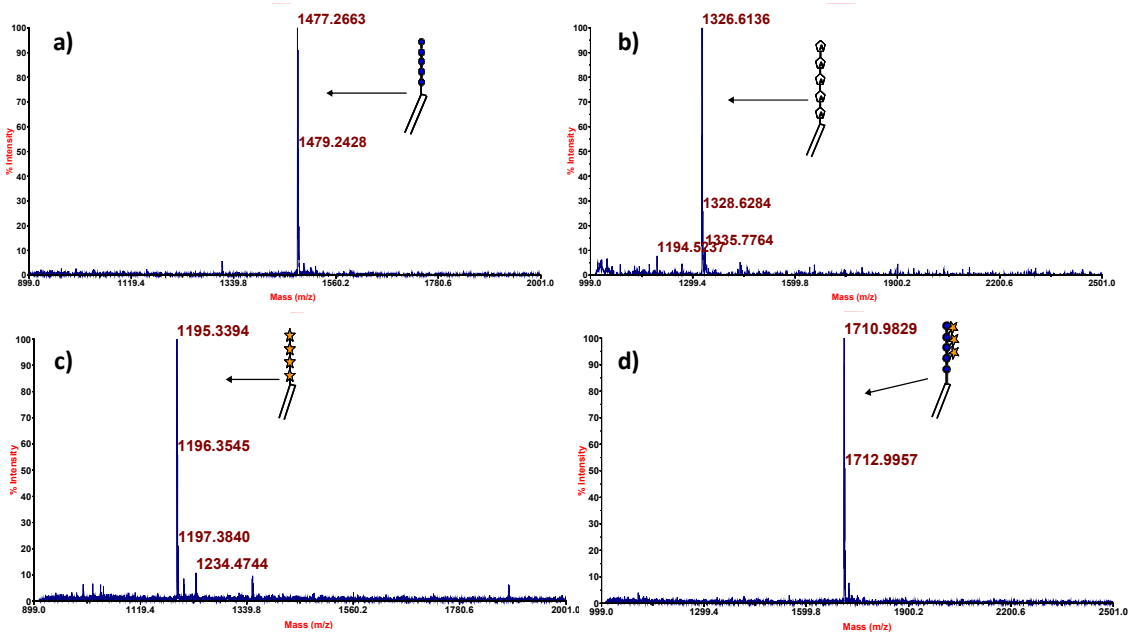
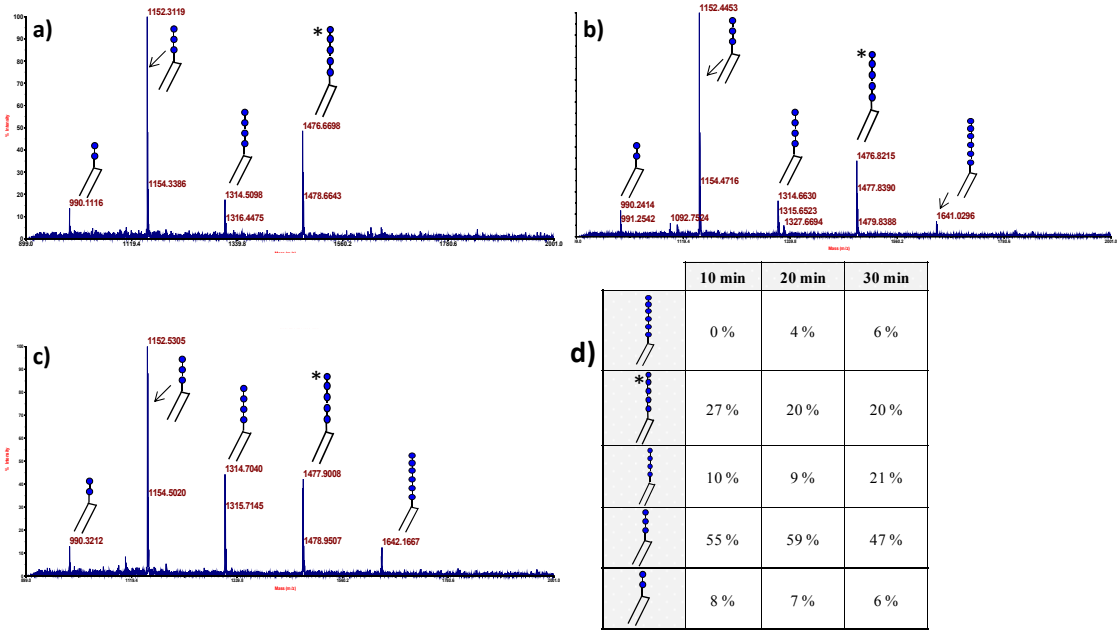
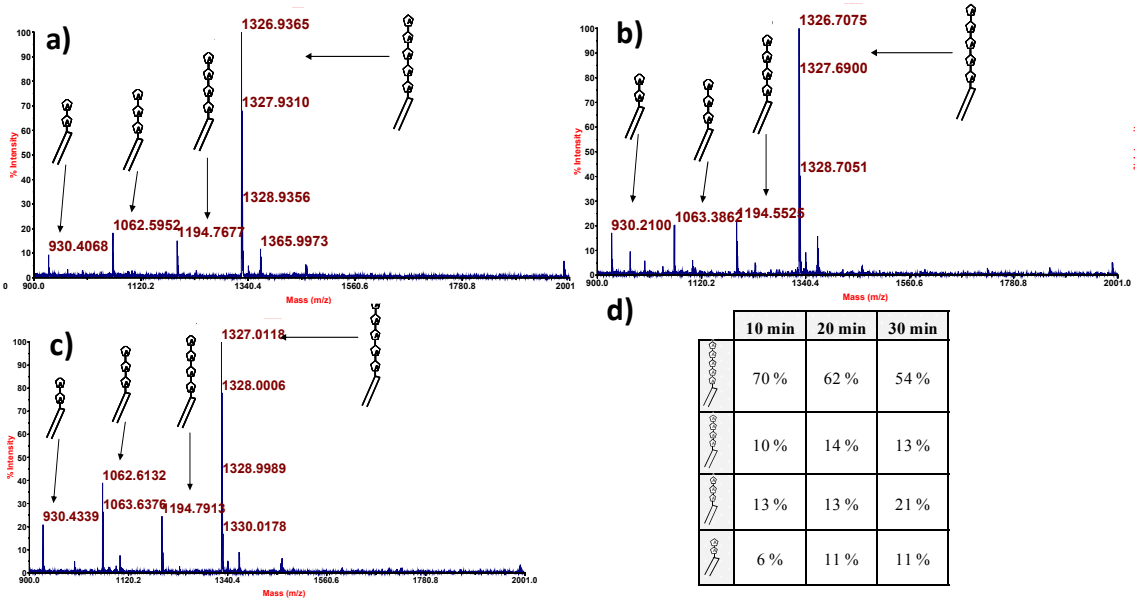


Figure S7. MALDI-Tof MS of used conjugates **1** (*a*), **2** (*b*), **3** (*c*) and **4** (*d*)**.**



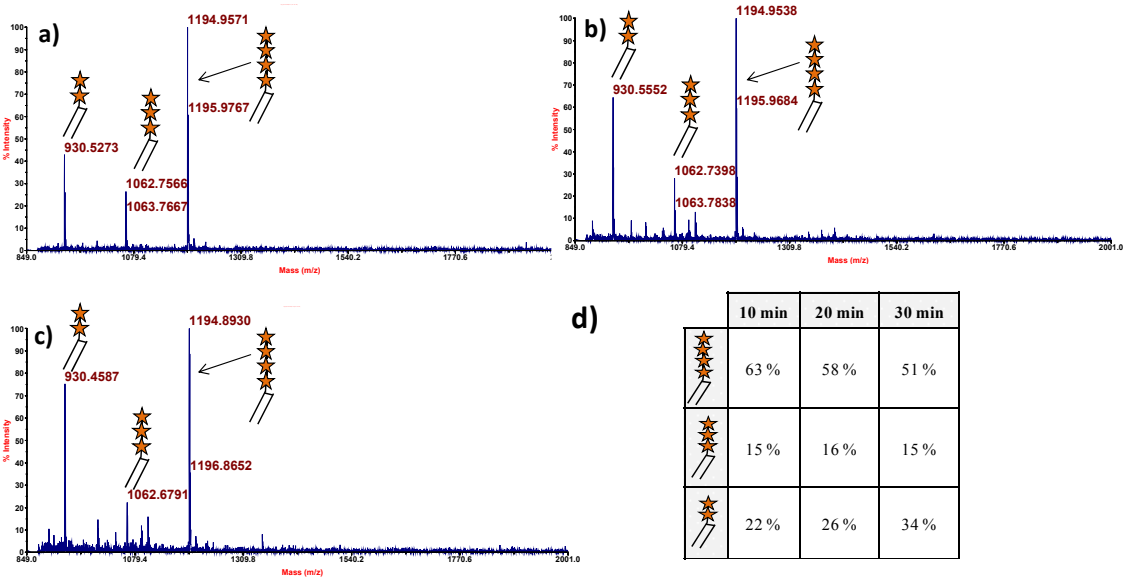
	10 min	20 min	30 min
	0 %	4 %	6 %
*	27 %	20 %	20 %
	10 %	9 %	21 %
	55 %	59 %	47 %
	8 %	7 %	6 %

Figure S8. Activity of diluted (1.4 U/ml, total of 3.4 µU) *B. amyloliquefaciens* cellulase at 10 (*a*), 20 (*b*) and 30 (*c*) minutes of on-chip incubation at 37ºC. The relative hydrolysis rate (*d*) was calculated using DataExplorer software where 100% corresponds to all combined degradation products between 900 Da and the precursor.



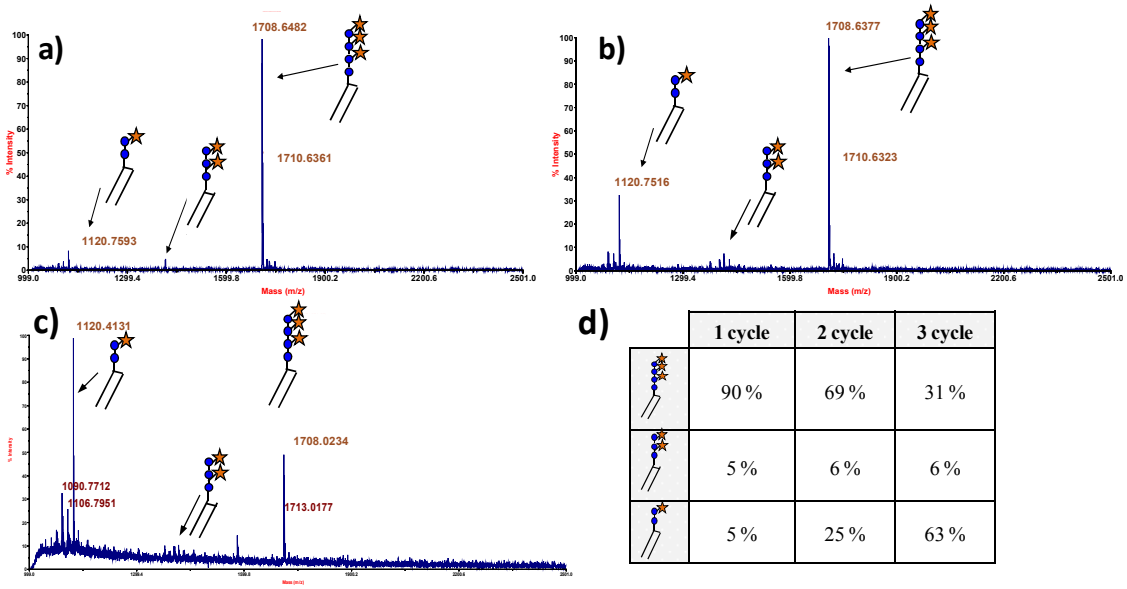
	10 min	20 min	30 min
	70 %	62 %	54 %
	10 %	14 %	13 %
	13 %	13 %	21 %
	6 %	11 %	11 %

Figure S9. Activity of *Aspergillus niger* endo-arabinanase (1 U/ml) at 10 (*a*), 20 (*b*) and 30 (*c*) minutes of on-chip incubation at 37ºC. d) Relative hydrolysis rate of detected hydrolysis products and the precursor.



	10 min	20 min	30 min
	63 %	58 %	51 %
	15 %	16 %	15 %
	22 %	26 %	34 %

Figure S10. Activity of *Thermotoga maritima* endo-xylanase (100 U/ml, total of 0.24 mU of enzyme) at 10 (*a*), 20 (*b*) and 30 (*c*) minutes of on-chip incubation at 37ºC. d) Relative hydrolysis rate of detected hydrolysis products and the precursor.



d)

	1 cycle	2 cycle	3 cycle
	90 %	69 %	31 %
	5 %	6 %	6 %
	5 %	25 %	63 %

Figure S11. Activity of xyloglucanase enzyme after 1 (*a*), 2 (*b*) and 3(*c*) cycles of 30 minutes of on-chip incubation at 37ºC. d) Relative hydrolysis rate of detected hydrolysis products and the precursor.

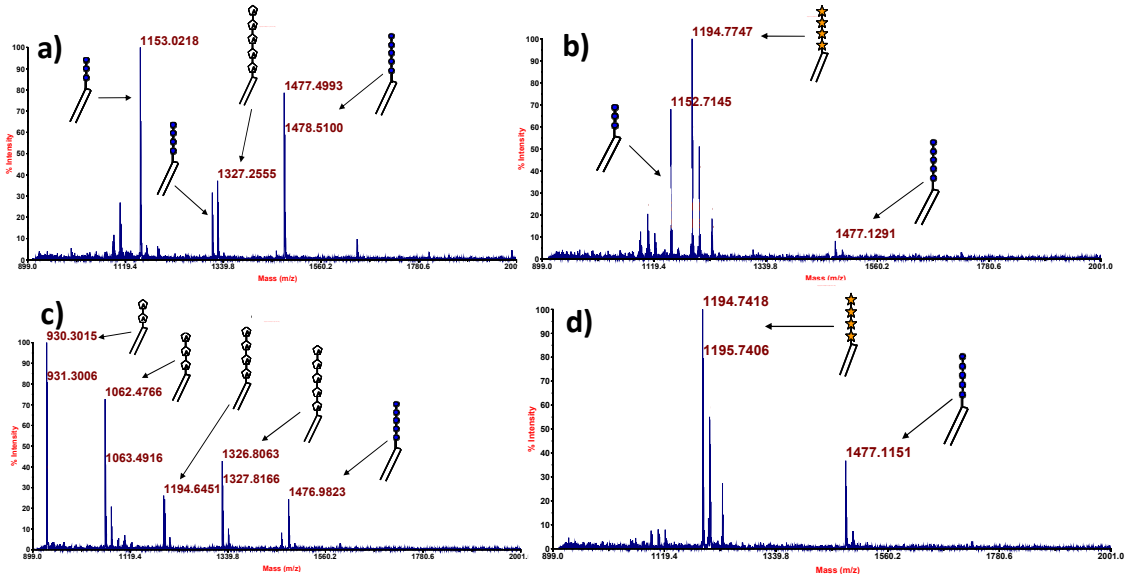


Figure S12. Analysis of specificity of *Bacillus amyloliquefaciens* cellulase (*a* and *b*) and *Aspergillus niger* arabinanase (*c* and *d*) over mixtures of conjugates **1**-**2** and **1**-**3**.

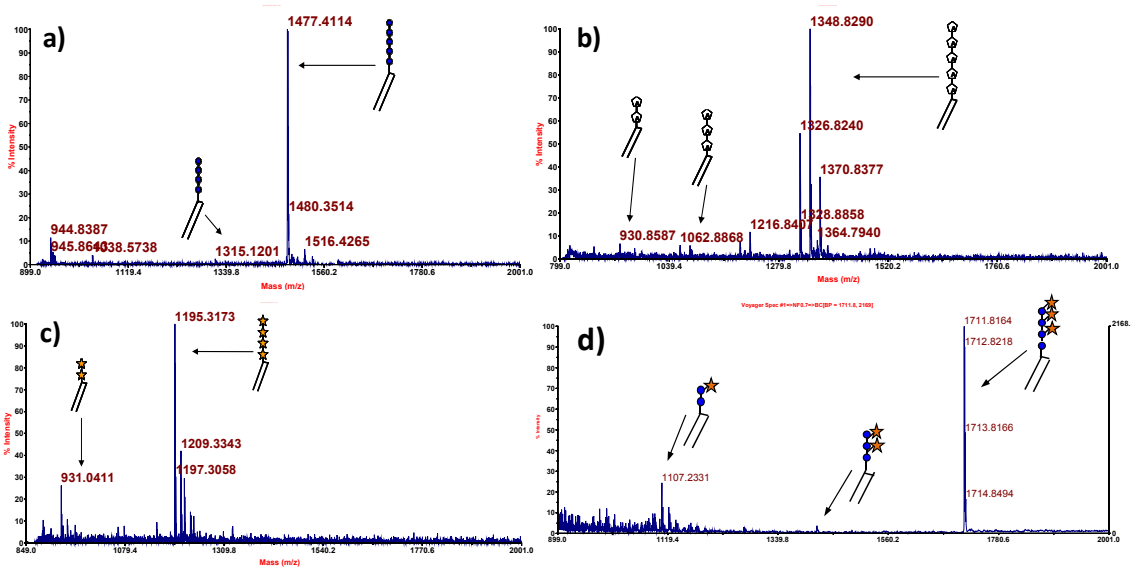


Figure S13. Hydrolysis of conjugates in solution. *a*) Conjugate **1** with *B. amyloliquefaciens* cellulase. *b*) Conjugate **2** with *A. niger* arabinanase. *c*) Conjugate **3** with *T. maritima* xylanase. *d*) Conjugate 4 with *Paenibacillus sp.* endo-xyloglucanase.

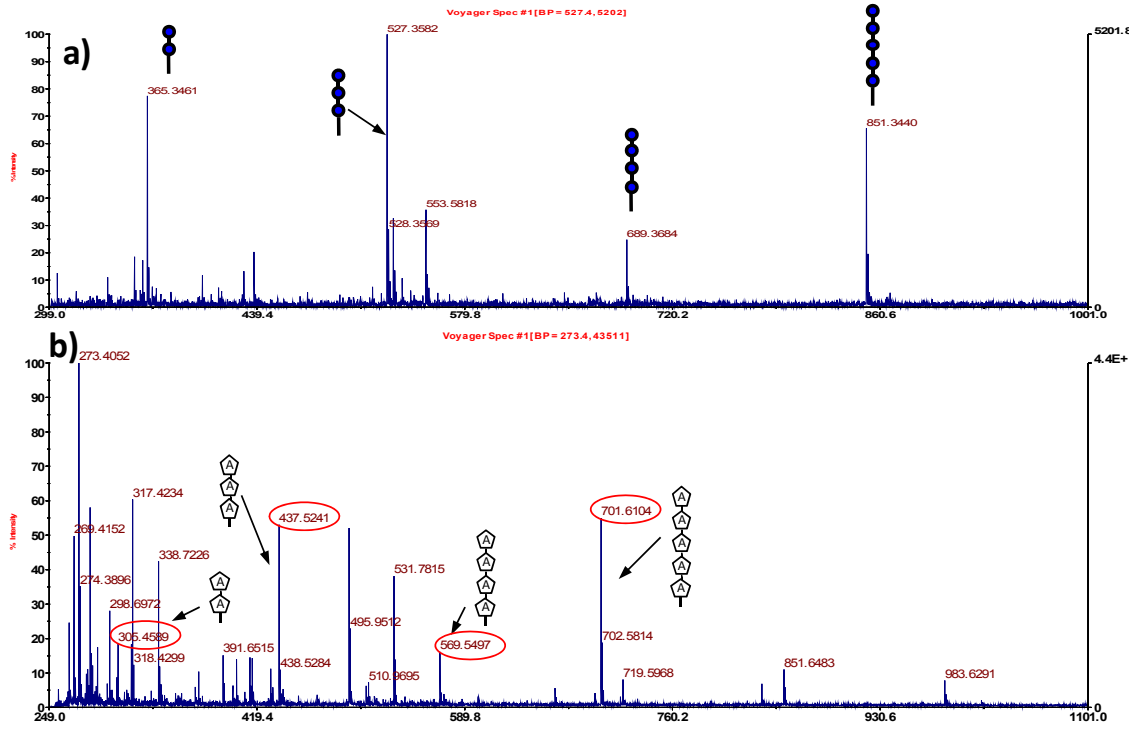


Figure S14. MALDI-Tof MS after 20 min of incubation of (a) *B. Amyloliquefaciens* cellulase with cellopentaose and (b) *A. niger* arabinanase with arabinopentaose. The identified subproducts are remarked with their corresponding pictogram.

References:

1. W. G. Chai, M. S. Stoll, C. Galustian, A. M. Lawson and T. Feizi, *Recognition of Carbohydrates in Biological Systems Pt A: General Procedures*, **2003**, *362*, 160-195.
2. Bethencourt, M. I.; Srisombat, L. O.; Chinwangso, P.; T. R. Lee, T. R. *Langmuir*, **2009**, *25*, 1265-1271.
3. Beloqui, A., Guazzaroni, M.E., Ferrer, M. *Procedures for protein isolation in pure culture and microbial communities.* Handbook of Microbiology of Hydrocarbons, Oils, Lipids and Derived Compounds, Springer **2009**, *63,* 4184-4192.