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A New Versatile Microarray-based Method for High Throughput Screening of Carbohydrate-active Enzymes*

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Background: There is a growing discrepancy between the putative identification and the empirical characterization of carbohydrate-active enzymes (CAZymes).

Results: We have developed a new versatile and high throughput microarray-based method for screening CAZymes.

Conclusion: The method is a powerful addition to the enzyme screening toolbox.

Significance: The technique enables the rapid screening of CAZymes and facilitates our biological understanding and industrial utilization.

Carbohydrate-active enzymes have multiple biological roles and industrial applications. Advances in genome and transcriptome sequencing together with associated bioinformatics tools have identified vast numbers of putative carbohydrate-degrading and -modifying enzymes including glycoside hydrolases and lytic polysaccharide monooxygenases. However, there is a paucity of methods for rapidly screening the activities of these enzymes. By combining the multiplexing capacity of carbohydrate microarrays with the specificity of molecular probes, we have developed a sensitive, high throughput, and versatile semi-quantitative enzyme screening technique that requires low amounts of enzyme and substrate. The method can be used to assess the activities of single enzymes, enzyme mixtures, and crude culture broths against single substrates, substrate mixtures, and biomass samples. Moreover, we show that the technique can be used to analyze both endo-acting and exo-acting glycoside hydrolases, polysaccharide lyases, carbohydrate esterases, and lytic polysaccharide monooxygenases. We demonstrate the potential of the technique by identifying the substrate specificities of purified uncharacterized enzymes and by screening enzyme activities from fungal culture broths.

Almost all plant cells are encased in a cell wall composed primarily of complex polysaccharides (1). Load-bearing components, principally cellulose and hemicelluloses, are embedded in a matrix of pectic polysaccharides, and in mature secondary walls, further reinforcement is provided by the phenolic polymer lignin (2–4). Cell walls play crucial roles in plant growth and development. They provide structural support, are

physical defensive barriers against biotic and abiotic stress, and are a source of oligosaccharide signaling molecules (2, 5, 6). Collectively, plant cell walls are also the largest source of biomass on earth and have received considerable attention in the context of attempts to produce second generation biofuels and chemicals from lignocellulosic feedstocks as an alternative renewable source to fossil fuels (7–9). A vast repertoire of cell wall-degrading enzymes, notably glycoside hydrolases (GHs),³ are produced by microbes, and these are vital for strategies aimed at the deconstruction of cell wall-based feedstocks used for biorefining (10–12). Carbohydrate-degrading and -modifying enzymes are also extensively used in the production of paper, textiles, detergents, feed, and food (13, 14). Endogenous carbohydrate-degrading and -modifying enzymes are central to many plant development processes. For example, GHs are used to cleave polysaccharides during organ abscission, fruit softening, germination, and plant/microbe interactions. Also, plants typically produce methyl- and acetyl esterases that are used for fine-tuning the *in muro* functionalities of cell wall components to suit local requirements (15, 16).

Techniques for mining genomes and metagenomes have developed rapidly in recent years, and so have medium and high throughput strategies for cloning and expressing recombinant enzymes. Furthermore, bioinformatics resources and associated depositories, such as the carbohydrate-active enzymes database (CAZy) (17) have expanded greatly. However, there are considerable challenges inherent in the exploitation of microbial enzyme diversity for industrial purposes, and the empirical determination of enzyme activities has now become a serious bottleneck. For example, it is estimated that, using cur-

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³ The abbreviations used are: GH, glycoside hydrolase; LPMO, lytic polysaccharide monooxygenase; HG, homogalacturonan; AZCL, azurine cross-linked; CBM, carbohydrate binding module; CDTA, diaminocyclohexanetetraacetic acid; HPAEC, high performance anion-exchange chromatography; DE, degree of methyl esterification; CAZy, carbohydrate-active enzymes database.

rent methods, we can safely predict the activities of no more than 4% of the proteins within the CAZy (18). Although numerous methods are available for monitoring enzyme activities, they generally have some limitations. Well established techniques based on the analysis of the oligomeric fragments produced by enzymatic hydrolysis, such as chromatography combined with mass spectrometry, are available (19). These approaches are powerful; however, they are labor-intensive and generally not suitable for high throughput screening. Methods based on the measurement of reducing sugars such as the 3,5-dinitrosalicylic acid (20) and the Nelson-Somogyi (21) assays are widely used for rapidly screening GH activities. However, these assays lack polysaccharide specificity because reducing ends from different polysaccharides cannot be distinguished. Chromogenic polysaccharide substrates, such as azurine cross-linked (AZCL) and azo-dyed polymers, are extensively used to screen endo-acting GHs, but the current range of available substrates is limited, and the nature of the chemical modification of these polysaccharides renders them unsuitable substrates for other types of enzymes including esterases and exo-acting GHs. Exo-acting enzymes can be analyzed with *para*-nitrophenyl substrates, but these compounds can usually only provide information about the degradation of one substrate per reaction and are not effective substrates for all exo-acting enzymes (22–24).

There is therefore a pressing need for the development of new technology for the rapid screening of GHs and other carbohydrate-active enzymes. We describe the development of a semiquantitative method that combines the multiplexing capacity of robotically produced microarrays with the specificity of monoclonal antibodies (mAbs) and carbohydrate binding modules (CBMs). The method is versatile and can be used to assess enzyme activities against both pure substrates and complex mixtures of cell wall components. In addition to GH activities, the technique can be used for the analysis of other polysaccharide-modifying enzymes including carbohydrate esterases, polysaccharide lyases, and lytic polysaccharide monoxygenases. We demonstrate the applicability of the technique for identifying the activities of novel putative enzymes and for screening crude fungal culture broths.

EXPERIMENTAL PROCEDURES

Biological Materials—Commercial enzymes from Megazyme (Bray, Ireland) used in this work are depicted in Table 1. The enzyme mixture Cellic Ctec2; the purified enzymes eNZ1 (endo-1,4- β -xylanase), eNZ2 (endo-1,4- β -xylanase), eNZ3 (endo-1,4- β -glucanase), and eNZ4 (endo-1,4- β -mannanase) (for details, see Table 1); the 24 crude culture broths obtained from the fungi *Gibberella zeae*, *Poronia punctata*, *Fusarium oxysporum*, *Magnaporthe grisea*, *Ustilago maydis*, and *Deconica inquilina* grown under four different conditions (samples were grown for 5 days at pH 6.5–9.0 in two different growth media (yeast extract-peptone-glucose and FG-4)) (Fig. 6); and the crude recombinant *Aspergillus nidulans* endo-1,4- β -mannanase culture broth (Fig. 9B) were provided by Novozymes (Bagsværd, Denmark). NcLPMO9C was supplied by Vincent G. H. Eijssink.

Pseudacanthotermes militaris proteins were obtained by the amplification of metagenomic DNA fragments by polymerase chain reaction using Phusion high fidelity DNA polymerase (New England Biolabs). The amplicons were cloned as described (25), and recombinant proteins *Pm12*, *Pm23*, and *Pm25* were expressed in fusion with a polyhistidine tag, whereas *Pm83*, *Pm84*, and *Pm85* were produced in fusion with a polyhistidine-thioredoxin tag. Plasmids were used to transform *Escherichia coli* Rosetta DE3 (Merck Millipore, Germany). For protein expression, the autoinducing medium ZYP-5052 (prepared as described in Ref. 26) supplemented with chloramphenicol (34 μ g/ml) was used. Cells were incubated at 37 °C for 5 h and at 17 °C overnight in baffled flasks until an $A_{600\text{ nm}}$ of ~ 11 was reached. Immobilized metal ion affinity chromatography was performed following the protocol described previously (27) for purification of the recombinant proteins. The concentration of purified proteins was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and applying the Beer-Lambert law. The theoretical extinction coefficient and molecular weight values were determined using the ProtParam service. Sequence data can be found in GenBankTM under the following accession numbers: CCO21106.1 (*Pm12*), CCO21108.1 (*Pm23*), CCO21110.1 (*Pm25*), CCO21640.1 (*Pm83*), CCO21632.1 (*Pm84*), and CCO21658.1 (*Pm85*).

Fungal broth supernatants (Fig. 13) were prepared as follows. Baffled Erlenmeyer flasks (500 ml) with 200 ml of Czapek Dox-based medium with apple pomace (35.3 mM NaNO₃, 5.7 mM K₂HPO₄, 6.7 mM KCl, 2 mM MgSO₄, 1 g of Triton-X (0.1%), 1 ml of trace metal solution (36 mM FeSO₄, 11.5 mM ZnSO₄, 17.5 mM CuSO₄) and 20 g of ground apple pomace in 1000 ml of Milli-Q water) were autoclaved and afterward inoculated with 1/4 agar plate of *Trametes versicolor* (DSM-11269, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany), 1×10^4 spores of *Colletotrichum acutatum* (isolate SA 0-1, proprietary), or 1×10^4 spores of *Penicillium expansum* (isolate IK 2020, proprietary) or mock-inoculated as a negative control. The flasks were incubated at 25 °C with shaking at 150 rpm for 7 days in darkness. Samples were collected from day 2 to 7 under sterile conditions and centrifuged twice for 20 min at 4000 rpm at 4 °C to separate spores from supernatants. Supernatants were collected, frozen in liquid nitrogen, and stored at -80 °C until use.

Commercial enzymes were analyzed within their recommended pH range using the following buffers: 0.1 M sodium acetate buffer for pH values 4.0–6.5 and 0.1 M sodium phosphate buffer for pH values 7.0–8.0 except for pH 2–10 analysis where 0.1 M Britton-Robinson buffer was used (Fig. 7). The rest of the samples were analyzed using the following buffers: the eNZ1–4 in 50 mM sodium acetate at pH 5.5; the 24 crude broths (Fig. 6) and the recombinant *A. nidulans* endo-1,4- β -mannanase broth (Fig. 9B) in 0.1 M sodium acetate, pH 5.5 and pH 5.0, respectively; NcLPMO9C in 0.1 M sodium phosphate, pH 8.0; *P. militaris* proteins in 0.1 M sodium acetate, pH 6.5; and fungal broths (Fig. 13) in 0.2 M sodium acetate, pH 6. Enzyme concentrations used are given in the figure legends.

Substrates Used in the Different Experiments—The following defined polysaccharides were used in this work: arabinan (sugar

TABLE 1

Characterized enzymes and enzyme mixture used in this study

N/A, not applicable.

Code	Enzyme name	Source	Microorganism	Catalogue no.
N/A	α -L-Arabinofuranosidase	Megazyme	<i>Bifidobacterium</i> sp.	E-AFAM2
eCEL	Cellulase	Megazyme	<i>Trichoderma longibrachiatum</i>	E-CELTR
N/A	Feruloyl esterase	Megazyme	Rumen microorganism	E-FAERU
eGLC	Endo-1,3- β -glucanase	Megazyme	<i>Trichoderma</i> sp.	E-LAMSE
N/A	Exo-1,3- β -glucanase	Megazyme	N/A	N/A
N/A	Endo-1,3(4)- β -glucanase	Megazyme	<i>Bacillus subtilis</i>	E-LICHN
eMAN	Endo-1,4- β -mannanase	Megazyme	<i>Cellvibrio japonicus</i>	E-BMACJ
N/A	Pectate lyase	Megazyme	<i>Aspergillus</i> sp.	E-PECLY
ePOL	Polygalacturonase M2	Megazyme	<i>Aspergillus aculeatus</i>	E-PGALUSP
eXYL	Endo-1,4- β -xylanase M4	Megazyme	<i>Aspergillus niger</i>	E-XYAN4
N/A	Exo-1,4- β -xylosidase	Megazyme	<i>Bacillus pumilus</i>	E-BXSEBP
eXG	Xyloglucanase (GH5)	Megazyme	<i>Paenibacillus</i> sp.	E-XEGP
eNZ1	Endo-1,4- β -xylanase (GH10)	Novozymes	<i>Aspergillus aculeatus</i>	N/A
eNZ2	Endo-1,4- β -xylanase (GH11)	Novozymes	<i>Thermomyces lanuginosus</i>	N/A
eNZ3	Endo-1,4- β -glucanase (GH5)	Novozymes	<i>Aspergillus aculeatus</i>	N/A
eNZ4	Endo-1,4- β -mannanase (GH5)	Novozymes	Fungal origin (proprietary)	N/A
N/A	Cellic Ctec2	Novozymes	Multienzyme product	N/A
N/A	NcLPMO9C	V. G. H. Eijssink	<i>Neurospora crassa</i>	N/A

beet), arabinoxylan (wheat flour), β -glucan (barley), β -glucan lichenan (icelandic moss), galactomannan (carob), glucomannan (konjac), pachyman (1,3- β -D-glucan), pectic galactan (lupin), and xyloglucan (tamarind) from Megazyme; gum arabic (acacia tree), hydroxyethylcellulose, and xylan (beechwood) from Sigma-Aldrich; pectin with degrees of methyl esterification (DEs) of 11, 16, and 81% (lime) from DuPont Nutrition Biosciences (Brabrand, Denmark); and feruloylated arabinoxylan (wheat flour) from Institut National de la Recherche Agronomique (Nantes, France). Polysaccharide substrates used in Fig. 10 were extracted as described (28). Briefly, cell wall polysaccharides were extracted from the alcohol-insoluble residues of *Arabidopsis thaliana* (rosette), *Salix alba* (willow with bark), *Zea mays* (corn stover, whole), *Hordeum vulgare* (barley, stem, and leaves), and *Equisetum arvense* (horsetail, stem, and leaves). Sequential extractions with 50 mM CDTA and 4 M sodium hydroxide (NaOH) with 1% (v/v) NaBH_4 were performed. Extracts were neutralized to pH 6–7 with glacial acetic acid.

Preparation of the Substrates—Defined polysaccharides were dissolved in deionized water to 4 mg/ml except for pachyman, which was dissolved in 4 M NaOH. Afterward both single polysaccharides and polysaccharide mixtures were prepared by diluting them in printing buffer (55.2% glycerol, 44% water, 0.8% Triton X-100). Substrate concentrations used (generally 0.1 mg/ml) are specified in the figure legends. Substrate mixtures contained each polysaccharide at the same concentration. Additionally, the pH of pachyman solution was neutralized with glacial acetic acid considering final concentration. Regarding the plant extracted polysaccharides (Fig. 10), a 2-fold dilution followed by a 5-fold dilution was performed in printing buffer.

Sample Incubation and Microarray Printing—Substrate solutions (defined polysaccharides or plant extracts) were added into wells of 384-microwell plates (PP microplate, V-shape, catalogue number 781280, Greiner Bio-One), and afterward the enzymatic samples were added (substrate/sample, 1:1, v/v) to a final volume of 20 μ l/well. Controls were prepared for each substrate solution without enzyme, keeping all other conditions identical. Each reaction was performed in

triplicate (except plant extracts that had duplicates at two different concentrations). The filled plates were incubated (Eco-tron, INFORS HT, Switzerland) at 100 rpm for a specific time and temperature (specified in the figure legends) followed by 10 min at 80 °C. After spinning down the plates for 10 min at 4000 rpm, the plate content was printed at 22 °C and 55% humidity onto nitrocellulose membrane with a pore size of 0.45 μ m (Whatman) using a microarray robot (Sprint, Arrayjet, Roslin, UK).

Microarray Probing—The printed arrays were blocked for 1 h in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.7 mM KH_2PO_4 , pH 7.5) with 5% (w/v) low fat milk powder (MPBS). Then arrays were incubated for 2 h with probes (for details, see Table 2) that for the different experiments included anti-rat and anti-mouse mAbs and CBMs (PlantProbes, Leeds, UK; Institut National de la Recherche Agronomique, Nantes, France; BioSupplies, Bundoor, Australia; and NZYTEch, Lisbon, Portugal). Probes were diluted 1:10, 1:1000, and 10 μ g/ml, respectively, in MPBS. Afterward arrays were washed thoroughly in PBS and incubated for 2 h with anti-rat, anti-mouse, or anti-His tag secondary antibodies conjugated to alkaline phosphatase (Sigma) diluted 1:5000 (anti-rat and anti-mouse) or 1:1500 (anti-His tag) in MPBS. Once washed in PBS and deionized water, microarrays were developed in a solution containing 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium in alkaline phosphatase buffer (100 mM NaCl, 5 mM MgCl_2 , 100 mM diethanolamine, pH 9.5).

Data Analysis—Developed arrays were scanned at 2400 dots/inch (CanoScan 8800F, Søborg, Denmark) and converted to TIFFs; afterward, probe signals were quantified using appropriate software (Array-Pro Analyzer 6.3, Media Cybernetics, Rockville, MD). Maximal mean spot signal intensity was set to 100, and all other values were normalized accordingly. A cutoff of 5 was applied (thus, values lower than 5 were converted to 5). To present the data, -fold change heat maps were produced by calculating the ratio of signal from the control/signal from the enzyme-treated sample. Therefore, ratios >1 indicate detected substrate degradation.

AZCL Assay—AZCL assays were performed in a 96-well plate (PP Microplate, V-shape, catalogue number 651201,

TABLE 2**Specificities of monoclonal antibodies and CBMs used in this study**

G, glucose; X, xylose.

Probe	Recognized epitope	Ref.
JIM5	Partially methyl-esterified/de-esterified HG	44
JIM7	Partially methyl-esterified HG	44
LM18	Partially methyl-esterified/de-esterified HG	45
LM19	Partially methyl-esterified/de-esterified HG	45
LM20	Partially methyl-esterified HG	45
INRA-RU1	Rhamnogalacturonan I backbone	46
INRA-RU2	Rhamnogalacturonan I backbone	46
LM5	(1→4)-β-D-Galactan	47
LM6	(1→5)-α-L-Arabinan	48
LM12	Feruloylated polymers	49
LM21	Heteromannan	50
BS-400-4	(1→4)-β-D-Mannan	51
BS-400-2	(1→3)-β-D-Glucan	52
BS-400-3	(1→3)(1→4)-β-D-Glucan	53
LM15	Xyloglucan (XXXG motif)	54
LM25	Xyloglucan (XXXG/galactosylated)	49
LM10	(1→4)-β-D-Xylan	55
LM11	(1→4)-β-D-Xylan/arabinoxylan	55
JIM8	Arabinogalactan protein	56
JIM13	Arabinogalactan protein	57
CBM3a	Cellulose (crystalline)	58
CBM30	(1→4)-β-Glucopolymers	59
CBM42A	Arabinose-containing polymers	60

Greiner Bio-One) format. All combinations of substrate and enzyme as well as controls without enzyme were performed in triplicate. Solutions of 135 μ l of 0.1 M buffer (sodium acetate or phosphate depending on the pH used as specified above) containing a defined unit/ml enzyme and 15 μ l of AZCL substrate (10 mg/ml) were incubated (Ecotron) at 100 rpm for a specific time and temperature (specified in the figure legends). Then the solutions were spun down at 4000 rpm for 5 min, and 100 μ l of supernatant of each well was transferred to a new plate. The absorbance at 590 nm was detected by using a plate reader (SpectraMax M5, Molecular Devices).

Reducing Sugar Assay—Reducing sugar assays (modified from Ref. 29) were performed by 5-min incubation of 1 mg/ml substrate in 50 mM sodium acetate buffer, pH 5.5 at 50 °C with varied enzyme load followed by cooling. Next a solution containing parahydroxybenzoic acid hydrazide (15 mg/ml), sodium potassium tartrate (50 mg/ml), and NaOH (20 mg/ml) was added followed by 10-min incubation at 95 °C. Subsequently the absorbance at 405 nm was read and compared with standard curves of the substrate monosaccharides.

Validation of Specificities by Matrix-assisted Laser Desorption/Ionization Time-of-flight (MALDI-TOF) Mass Spectrometry (MS) and High Performance Anion-exchange Chromatography (HPAEC)—MALDI-TOF MS was conducted essentially as described (30) except that Dowex 50 treatment and sodium chloride doping were omitted. HPAEC was run as described previously (31).

RESULTS

Development of Microarray-based Enzyme Screening Technology—The method exploits the fact that the activities of enzymes that degrade or modify polysaccharides can be detected by the abolition or diminution of mAb or CBM binding to the particular molecular structure (epitope) affected by enzyme activity (Fig. 1A). Enzyme(s) and substrate(s) are combined in wells of 384-microwell plates, and for each reaction, there is a corresponding no-enzyme buffer control (Fig. 1B).

Each reaction and control are performed in triplicate (in separate wells on the same plate). After incubation, multiple identical microarrays are printed from the microwell plates onto nitrocellulose (Fig. 1C). Degradation of polysaccharide substrates is detected by probing each array with separate mAbs or CBMs (Fig. 1D). Antibody binding is detected using a secondary antibody conjugated to an enzyme and used in conjunction with a substrate that yields a colored product. Mean spot antibody signal intensities are then calculated and the difference (-fold change) in signal between the no-enzyme control and the enzyme reaction provides information about enzyme activity (Fig. 1E). A -fold change of “1” indicates that there is essentially no difference in the binding of the probe with or without the enzyme present, and hence the enzyme has no activity against the epitope recognized by that probe. A -fold change >1 indicates that the binding of the probe has been diminished with the enzyme present, and hence that enzyme has activity against the epitope recognized. Diminished probe binding can be caused either directly by the degradation/modification of the epitope or because the polysaccharide has been reduced in size such that it is no longer capable of immobilizing onto the nitrocellulose membrane. -Fold changes can conveniently be displayed as heat maps in which the -fold change values are correlated to color intensity, and this format is used throughout this study. Examples of arrays together with heat maps of mean spot signal intensities and the corresponding -fold changes are shown in Fig. 2. This general approach can be adapted extensively with different permutations of mixtures, enzymes types, and digestion conditions.

Comparison with Other Enzyme Screening Methods—We analyzed the same set of four purified enzymes eNZ1, eNZ2, eNZ3, and eNZ4 (for enzyme details, see Table 1) using the new technique, by measuring the production of reducing ends, and with AZCL substrates (Fig. 3, A–C). All three techniques confirmed that eNZ1 and eNZ2 have xylanase activity, eNZ3 has β-glucanase activity, and eNZ4 has mannanase activity. None of the enzymes showed activity against galactan, which was used as a negative control.

In the case of GHs, it could be possible that a decrease in mAb and CBM binding to a polysaccharide may be caused not by the degradation of the polysaccharide by the catalytic module but rather by the binding of an appended CBM, which could mask the polymer toward the molecular probe. To test this, we incubated arabinoxylan, hydroxyethylcellulose, and xyloglucan with GHs with specific activities against these substrates and with or without CBMs that bound to them (Fig. 4). No differences in -fold changes were observed between samples that had been incubated with both CBM and GH compared with GH alone. Also, no -fold changes >1 were generated when incubating the polysaccharides with their specific CBMs. These data indicate that for these samples at least the detected reductions in mAb and in CBM binding were indeed due to degradation rather than masking of epitopes (Fig. 4B). To provide information about probe binding *per se* (rather than the indirect effects of changes in binding resulting from epitope degradation), the experimental data were also presented as a heat map of absolute binding values (Fig. 4A). The binding of the anti-arabinoxylan mAb LM11 to arabinoxylan was not inhibited by the presence

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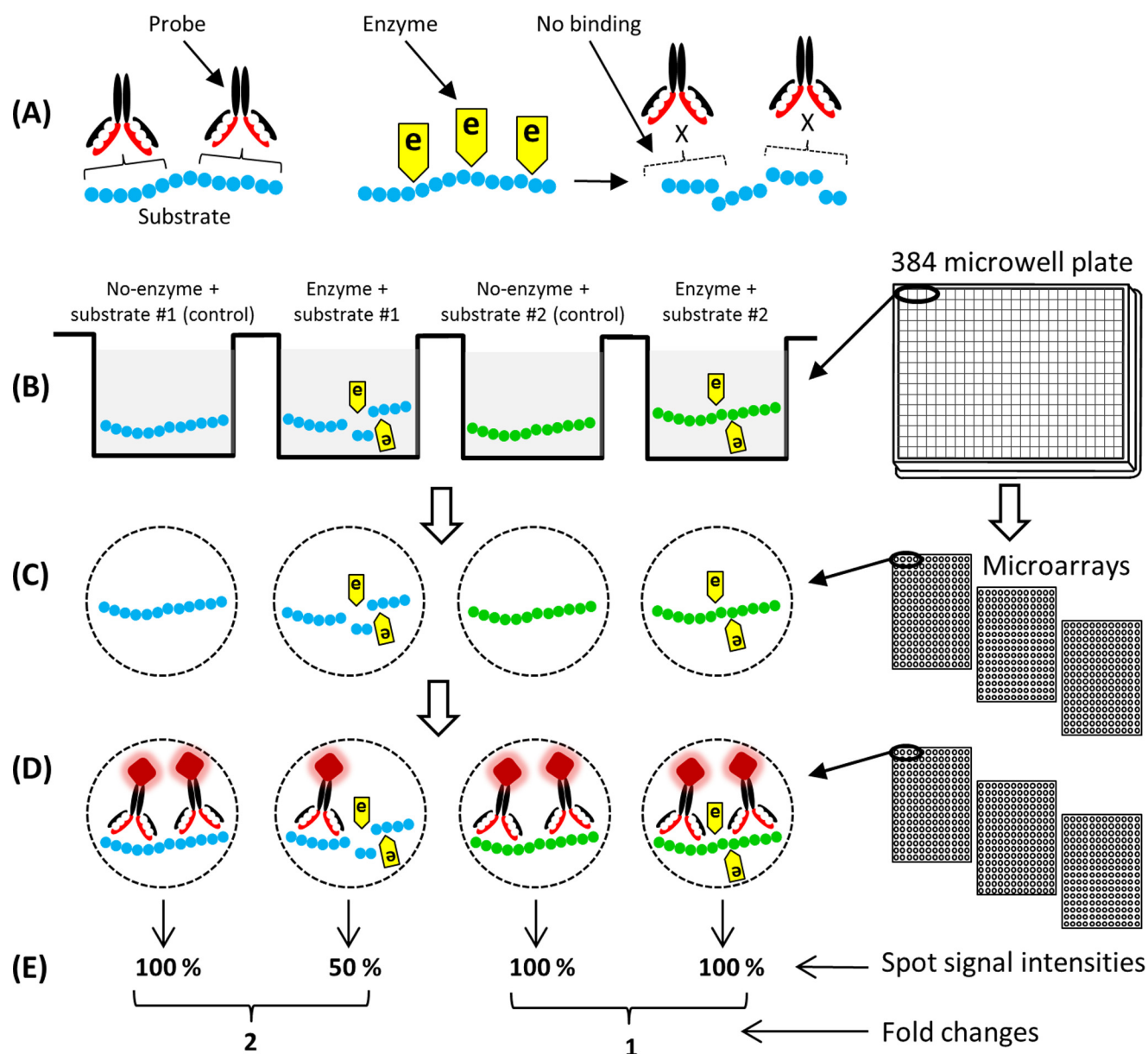


FIGURE 1. Schematic overview of the technique. *A*, the method is based on the principle that a particular mAb or CBM binds to an epitope on a polysaccharide substrate and that binding is reduced if that polysaccharide is degraded or modified by an enzyme. *B*, polysaccharide substrates and enzymes are mixed in wells of 384-microwell plates in an appropriate buffer, and for each reaction, there is a corresponding no-enzyme control. Each reaction is performed in triplicate. *C*, after incubation, the digestion mixtures are printed directly from the 384-microwell plate onto nitrocellulose membranes using a microarray robot. Multiple identical arrays are produced from each plate, corresponding to the number of probes subsequently used. *D*, microarrays are probed individually with mAbs and/or CBMs, and binding of probes is detected using enzyme-linked secondary antibodies used in conjunction with a substrate that yields a colored product. *E*, mean spot signal intensities are calculated for each reaction and corresponding control. -Fold changes are then calculated by dividing the mean signal intensities from no-enzyme controls by the mean signal from the enzyme-treated samples.

of the arabinoxylan-binding CBM42A (as evidenced by comparing *rows 1* and *3* in Fig. 4A). Similarly, the binding of the probes recognizing hydroxyethylcellulose and xyloglucan was not inhibited by the presence of CBMs recognizing the corresponding polysaccharides (compare *rows 1* and *3* of each sub-heat map). Taken together, these data provide evidence that when a CBM is present during the enzyme reaction step this does not affect the outcome of the subsequent probing step. Nevertheless, it is formally possible that in some cases competitive binding of probes *versus* appended CBMs could be problematic, and if this were suspected a protease treatment step after the enzyme reaction could be included.

Detection Limits and Reproducibility—Detection limits of the method were assessed by performing a series of digestions using progressively lower concentrations of substrates and enzymes in various combinations (Fig. 5). An example of the raw array output from one such experiment is shown in Fig. 5A in which the substrate was galactomannan, the enzyme was endo-1,4- β -mannanase, and the detecting mAb was LM21 (anti-mannan). The binding of mAb LM21 clearly decreases as the enzyme concentration increases (Fig. 5A). The dosage responses of four enzymes were tested in each case using a single substrate and an appropriate molecular probe for detection of that substrate (Fig. 5B). In the case of endo-1,3- β -gluca-

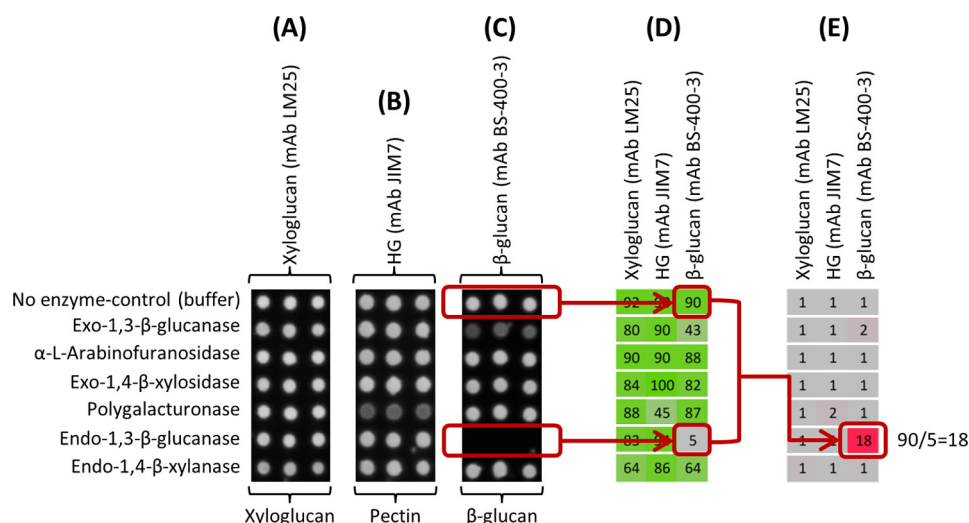


FIGURE 2. Generation of -fold change heat maps from raw data. A–C, representative examples of arrays produced from a series of reactions involving the incubation (5 h at 30 °C) of several substrates at 0.1 mg/ml including xyloglucan, pectin (DE = 81%), and β -glucan barley (listed at the bottom) with a set of GHs (listed to the left) used at 1 unit/ml and probed with antibodies with specificity for xyloglucan (mAb LM25) (A), HG (mAb JIM7) (B), and (1 \rightarrow 3)(1 \rightarrow 4)- β -glucan (mAb BS-400-3) (C). D, heat map showing the mean spot signal intensities derived from the triplicates of each reaction. The highest value in the data set is set to 100, and all other values are normalized accordingly. E, -fold change heat map derived from D. -Fold changes are calculated by dividing mean signal intensities from control reactions by mean signal intensities from enzyme-containing reactions. A -fold change > 1 indicates that the binding of the probe has been reduced when the enzyme was present in the reaction. In this case the -fold change "18" reveals digestion of the β -glucan (as detected by mAb BS-400-3) by the endo-1,3- β -glucanase. Details of enzymes and probes used are provided in Tables 1 and 2, respectively.

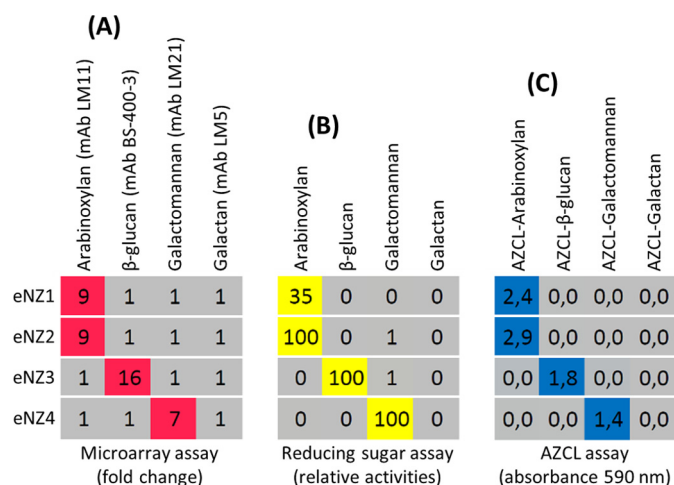


FIGURE 3. Comparison with reducing sugar assay and AZCL substrates. Four purified enzymes (listed to the left) were tested individually against four substrates (arabinoxylan, β -glucan barley, galactomannan, and galactan) using the new method (A), by a reducing sugar assay (B), and using insoluble AZCL substrates (C). Heat maps show mAb-fold change values, relative reducing end abundance, and absorbances at 590 nm, respectively. All three methods confirmed that eNZ1 and eNZ2 are xylanases, eNZ3 is a β -glucanase, and eNZ4 is a mannanase. None of the enzymes had detectable galactanase activity. Reactions were performed at 50 °C with 1-h incubation for the microarray and AZCL analyses and for 5 min for the reducing sugar assay. Details of enzymes and probes used are provided in Tables 1 and 2, respectively. For details of the reducing sugar assay, see "Experimental Procedures."

nase and xyloglucanase (detected using mAbs BS-400-3 and LM15, respectively), the assay was able to detect activity (*i.e.* -fold change > 1) when the enzymes were used at 0.0001 unit/ml (which corresponds to an enzyme concentration of 0.007 μ g/ml for the endo-1,3- β -glucanase and 0.0009 μ g/ml for the xyloglucanase). In the case of cellulase (detected by CBM30) and endo-1,4- β -mannanase (detected by mAb LM21), the enzyme doses required for detection of activities were 0.0005 and 0.001 unit/ml (equivalent to 0.007 and 0.002 μ g/ml),

respectively. We compared these detection limits with those obtained using corresponding AZCL substrates with the same enzymes under the same conditions (Fig. 5C). The lowest enzyme concentrations at which color release was detectable were 0.01 unit/ml for endo-1,3- β -glucanase and xyloglucanase (corresponding to 0.7 and 0.09 μ g/ml, respectively) and 0.05 unit/ml for cellulase and endo-1,4- β -mannanase (0.7 and 0.1 μ g/ml, respectively). These data indicate that the technique described has an improved detection limit (about 2 orders of magnitude enhanced) compared with that typically obtained using AZCL substrates.

We also tested the activities of two enzymes, xyloglucanase and polygalacturonase, when used in combination with their respective substrates at a range of concentrations to determine the effect of substrate concentration on detection limits (Fig. 5, D and E). In the case of xyloglucanase, there was a clear improvement in detection limit with decreasing substrate concentration (Fig. 5D). When the xyloglucan was used at 0.8 mg/ml, a xyloglucanase concentration of 0.0001 unit/ml (which corresponds to 0.0009 μ g/ml) was required for detection of activity, but when used at 0.05 mg/ml, activity was still detectable at a xyloglucanase concentration of 0.000001 unit/ml (0.000009 μ g/ml) (Fig. 5D). For polygalacturonase, the effects of reducing the substrate concentration were less pronounced with detection of activity using 0.01 unit/ml (corresponding to 0.04 μ g/ml) enzyme when the pectin substrate was used at 0.8 mg/ml and detection at 0.0001 unit/ml (0.0004 μ g/ml) when the pectin substrate was used at 0.05 mg/ml (Fig. 5E). These data are consistent with the fact that reduction in antibody binding is likely to be more pronounced as the number of epitopes present in the substrate become limiting. These data also reflect the different avidities of the probes used such that each substrate/probe has a specific relative response to enzyme activity. Although there are clearly variations in the optimal

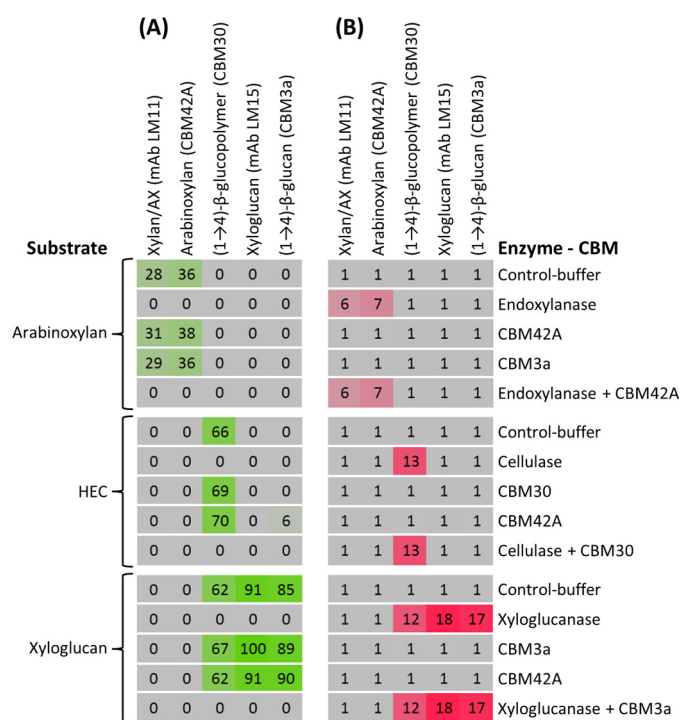


FIGURE 4. The effects of CBM and GH incubation on polysaccharides probe binding. A, heat map showing the results of incubating three polysaccharide substrates (arabinosyloxylan, hydroxyethylcellulose (HEC), and xyloglucan) at 0.1 mg/ml with a GH and/or a CBM with specificity toward them. A CBM with no specificity toward the substrate was included as a negative control. GHs and CBMs (shown to the right) were analyzed with the same protein concentration (GHs at 0.1 unit/ml and CBMs at the corresponding $\mu\text{g}/\text{ml}$ equivalent to 0.1 unit/ml GH, 1.3, 1.47, and 0.92 $\mu\text{g}/\text{ml}$ for CBMs compared with endoxylanase, cellulase, and xyloglucanase, respectively). In each sub-heat map, the first to fifth rows show the results of incubating the polysaccharide with buffer, GH specific for the polysaccharide, CBM specific for the polysaccharide, CBM with no specificity for the polysaccharide, and GH and CBM specific for the polysaccharide simultaneously (both used at the same concentration as separately), respectively. After plate incubation and printing, the resulting microarrays were probed with the mAbs and CBMs shown at the top. The data shown in the heat map are mean probe signal intensities derived from the triplicates of each reaction. The highest value in the data set was set to 100, and all other values were normalized accordingly. B, -fold change heat map derived from A. A cutoff of 5 was applied (thus, values lower than 5 were converted to 0 in A and to 5 in B to create the -fold change heat map). All reactions were performed for 1 h at 40 °C. AX, arabinosyloxylan. Details of enzymes and probes used are provided in Tables 1 and 2, respectively.

substrate and enzyme concentrations necessary to achieve maximum sensitivity, we found that in most cases a substrate concentration of 0.1 mg/ml and an enzyme concentration of 0.01 unit/ml achieved a good compromise between detection and sensitivity.

Reproducibility of the technique was tested by performing a series of identical analyses and comparing the data from each replicate (Fig. 6). One such example was done by analyzing enzyme activities on crude fungal broths (24 samples from six different species grown at four different conditions). Each sample was incubated separately with a series of different polysaccharide substrates (Fig. 6, A–D). These reactions were duplicated in the same microwell plate, all the reactions were then printed together as microarrays, which were probed with appropriate antibodies, and signal intensities were quantified. Signal intensities from the duplicated incubations were then plotted against each other, and R^2 values were calculated to determine correlation coefficients (Fig. 6, A–D). For all four

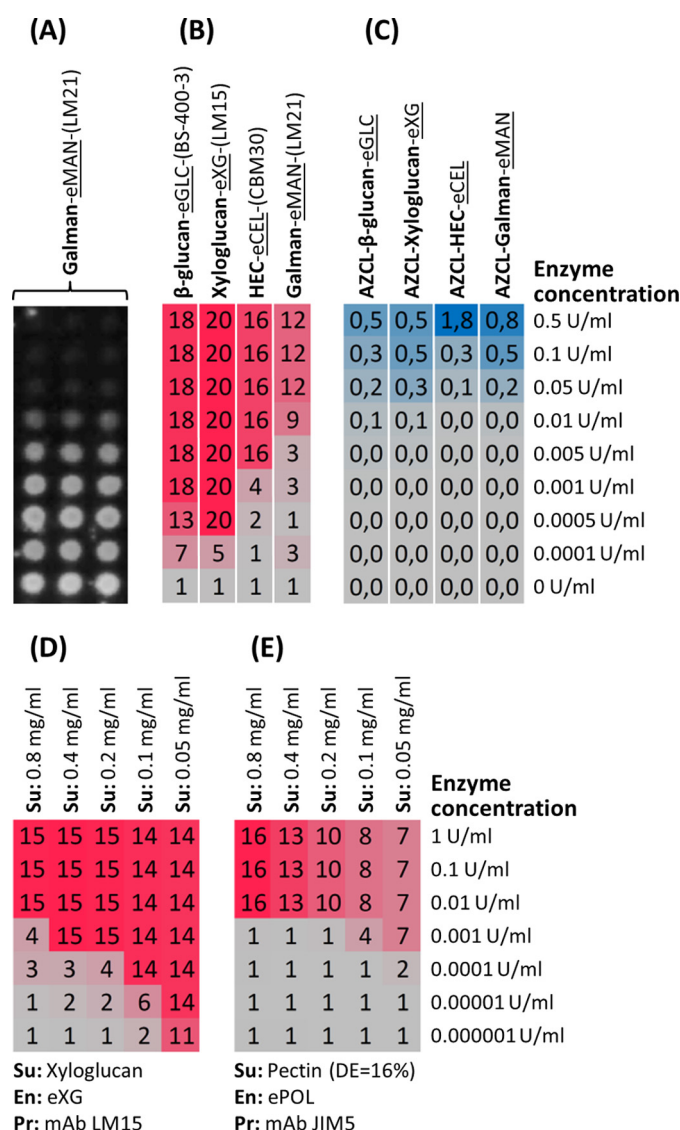


FIGURE 5. Detection limits and substrate concentration effects. Note that in A–C the substrate used is shown in **bold text**, the enzyme is underlined, and the detecting probe is in *parentheses* (all indicated at the top). A, a microarray produced from a reaction involving the digestion of galactomannan substrate (0.1 mg/ml) with endomannanase used at a range of concentrations (listed to the right) and probed with the anti-mannan mAb LM21. This array shows how epitope deletion progressively increases with increasing enzyme concentration. B, -fold change heat map showing the result of the digestion of four different substrates (0.1 mg/ml) with four different GHs used at a range of concentrations (listed to the right). The substrate-enzyme-probe combinations used are shown at the top. C, the same enzymes at the same concentrations were used to digest four AZCL substrates, and the color of the supernatants was measured at 590 nm. Values in the heat map (C) correspond to absorbance units. All reactions were performed for 1 h at room temperature. D and E, -fold change heat maps showing the effect of substrate concentration for two enzyme-substrate combinations: xyloglucanase + xyloglucan (D) and polygalacturonase + pectin (E). In both cases, enzymes were used at a range of concentrations from 0.000001 to 1 unit/ml (listed to the right), and substrates were used at a range of concentrations from 0.05 to 0.8 mg/ml (listed at the top). Note that the detection limit improves with decreasing substrate concentration. Reactions were performed for 2 h at 40 °C. β-Glucan used in B and C was from barley. Galman, galactomannan; HEC, hydroxyethylcellulose; Su, substrate; En, enzyme; Pr, probe. Details of enzymes and probes used are provided in Tables 1 and 2, respectively.

different permutations of substrate and antibody, the R^2 values were >0.97 , indicating a high level of reproducibility. We also assessed reproducibility between experiments by analyzing the

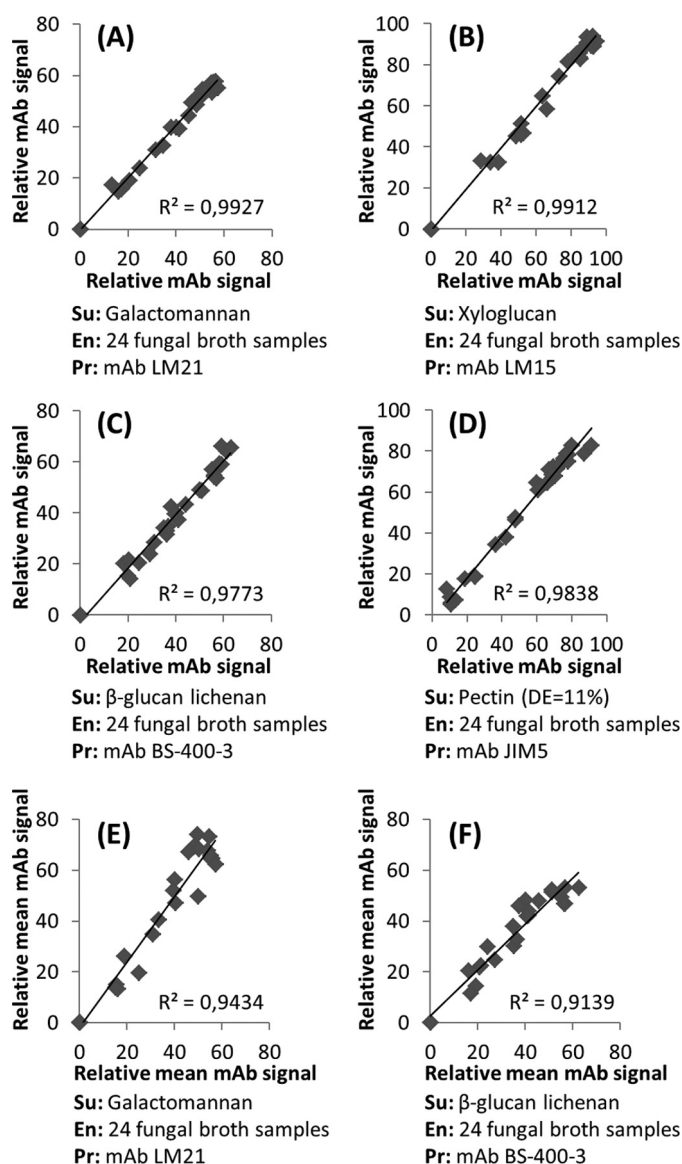


FIGURE 6. Reproducibility. A–D, reproducibility within an experiment. 24 crude fungal culture broths from six different species (details under “Experimental Procedures”) were incubated with different polysaccharides to test the reproducibility. Duplicates of each reaction were performed in different wells of the same microwell plate, and plate content was printed in the same microarray. Once the data analysis was performed, the antibody signals of the two screening replicates for each of the 24 samples were plotted, and R^2 were calculated. A low level of variability was detected in the replicates of the same analysis showed by $R^2 > 0.97$. Axes on the graphs represent relative antibody signal intensities. E and F, reproducibility between experiments was tested by comparing the results obtained by two fully independent analyses. The same sample set as for A–D was analyzed in two separate events using newly prepared polysaccharide substrates (galactomannan and β -glucan lichenan) and performing the incubation, printing, probing, and developing on different days. E and F show the plotted mean antibody signals obtained in the two experiments when analyzing each of the 24 samples, and R^2 values >0.90 indicate low variability between analyses. Axes on the graphs represent relative mean (three replicates) spot mAb signals. In all the analyses, substrate concentrations were 0.2 mg/ml, fungal broth samples were 2-fold diluted, and reactions were performed for 2 h at 50 °C. Su, substrate; En, enzymatic samples; Pr, probe. Details of mAbs used are provided in Table 2.

same sample set in two different experiments (incubations were performed in different microwell plates, printed on different microarrays, performed on different days, and performed using newly prepared substrates). In both experiments, each reaction

was performed in triplicate, and the mean signal intensity of each sample obtained from each experiment was plotted against each other (Fig. 6, E and F). In this case, the R^2 values were lower but still >0.90 . The high R^2 values in the first set of experiments indicate that the technical variation of the technique is low, and the lower R^2 values of the completely independent experiments are most likely due to slight variations in the condition of enzyme or substrate and variations introduced during microarray printing, probing, and developing. All the data presented in this study were produced from experiments in which each reaction and corresponding control were performed in triplicate in the same microwell plate and printed on the same array.

Using the Technique under Different pH Conditions—Because all digestion reactions are performed as liquids in microwell plate wells, it is possible to vary the assay conditions of each digestion as required. The effect of pH on enzyme activity was investigated using an endo-1,3- β -glucanase, a cellulase, and a xyloglucanase at a pH range of 2–10 (Fig. 7A). The analysis indicated that the endo-1,3- β -glucanase and cellulase were most active at a pH between 3.5 and 5.5 and between 3.5 and 5, respectively. The xyloglucanase had a much broader activity range with high activity observed between pH 4 and 10. One interpretation for the apparent high activity of the xyloglucanase even at the highest tested pH could be that the substrate was degraded by the alkaline conditions rather than by the enzyme; however, this could be discounted by direct observation of the arrays themselves, which clearly showed antibody binding to controls that lacked enzyme at pH 10 (Fig. 7B).

Analysis of Lyase, Esterase, Exo-acting Glycoside Hydrolase, and Lytic Polysaccharide Monooxygenase (LPMO) Activities—The essential conditions required for the technique are that the substrate will immobilize into nitrocellulose in its native form and that a mAb or CBM is available with specificity such that its binding to the substrate is altered by the activity of the enzyme in question. With this in mind, we explored the possibility of using this technique for investigating enzyme classes other than endo-acting GHs (Fig. 8). Pectate lyase activity was readily detectable using a pectin substrate with a low DE in combination with the anti-homogalacturonan (HG) mAb LM19 (Fig. 8A). A feruloyl esterase was tested using feruloylated arabinoxylan as substrate. Its activity was detected using mAb LM12, which has specificity for feruloylated polymers (Fig. 8B). As expected, no activity was observed when non-feruloylated arabinoxylan was used as substrate as detected by the anti-xylan/arabinoxylan mAb LM11 (Fig. 8B). An exoxylosidase was assessed using an arabinoxylan substrate, and activity was detected with a 4-fold change similar to that obtained using an endoxylosidase (Fig. 8C). The unexpected capacity of the exoxylosidase to degrade arabinoxylan is most likely attributable to its reported α -L-arabinofuranosidase side activity (Table 1), which may remove the arabinosyl substitutions, thus creating new cleavage sites for the exoxylosidase, which would normally not hydrolyze substituted xylose residues. We also investigated whether this approach could be used to assess LPMO activity (Fig. 8D). An LPMO from *Neurospora crassa* (NcLPMO9C) has recently been shown to have activity against xyloglucan and other polysaccharides containing (1 \rightarrow 4)- β -D-glucan (32), and

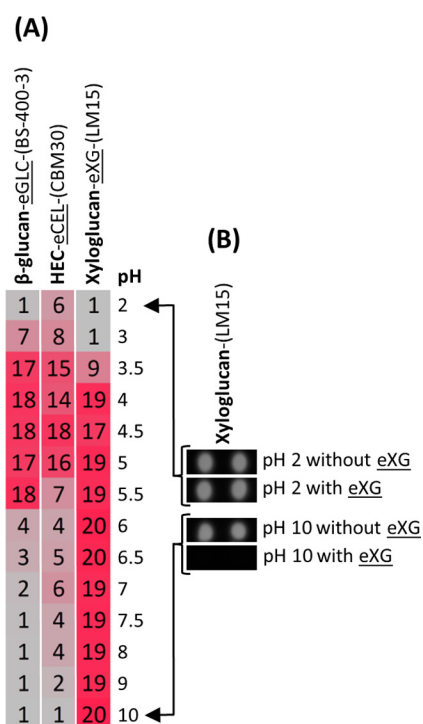


FIGURE 7. Analysis of pH effect on activity. A, -fold change heat map resulting from analyzing single polysaccharides (0.1 mg/ml) with GHs (0.01 unit/ml) in a range of different pH values (listed to the right). The substrates used are shown in **bold text**, the enzymes are underlined, and the detecting probes are in *parentheses* (all indicated at the top). B, an example of signals obtained (raw data) when xyloglucan was treated with and without xyloglucanase (eXG) at pH 2 and 10 and the microarray was probed with mAb LM15. All reactions were performed for 1 h at room temperature. The β -glucan source was barley. HEC, hydroxyethylcellulose. Details of enzymes and probes used are provided in Tables 1 and 2, respectively.

consistent with this, we detected activity against xyloglucan but not pachyman ((1 \rightarrow 3)- β -D-glucan). Low activity was observed when the digestion was performed without reductant, implying that the degradation was indeed the result of oxidative activity (Fig. 8D).

Multiplexed Analysis Using Mixed Polysaccharide Substrates—Using this technique, substrates can be mixed together (*i.e.* each reaction well may contain mixtures of several polysaccharides), and the activities of individual enzymes can then be resolved by the degradation of epitopes specific to one substrate. This multiplexing capacity is significant not only with regard to increasing the throughput of analysis but also because it enables enzyme and substrates to be combined in mixtures that may more closely resemble the natural operating environment of enzymes.

Examples of multiplexing are shown in Fig. 9A in which three enzymes (xyloglucanase, endo-1,3- β -glucanase, and polygalacturonase) and an enzyme mixture (Cellic Ctec2) were each tested against two different mixtures of substrates. When screening against mixture 1 (containing arabinoxylan, galactomannan, β -glucan barley, pectin, and xyloglucan), xyloglucanase activity was detected using the anti-xyloglucan mAb LM15. Activity of the endo-1,3- β -glucanase was detected in both mixtures by the -fold changes in binding of the anti-(1 \rightarrow 3)(1 \rightarrow 4)- β -glucan mAb BS-400-3, showing that both (1 \rightarrow 3)(1 \rightarrow 4)- β -glucan barley and (1 \rightarrow 3)(1 \rightarrow 4)- β -glucan

lichenan were effective substrates for this enzyme. When the polygalacturonase was tested against mixture 2, activity toward the low DE pectin was detected using the anti-HG mAbs JIM5 and JIM7. When this enzyme was screened against mixture 1, a relatively low -fold change of 2 was observed in the binding of mAb JIM5, whereas a higher -fold change of 10 was observed with mAb JIM7, which binds preferentially to high DE pectin. These data suggest that the high DE pectin from mixture 1 contained sufficient non-esterified regions to allow hydrolysis by this polygalacturonase. As expected, Cellic Ctec2 had a broad range of activities evidenced by -fold changes in the binding of multiple mAbs. Especially high (>10) -fold changes were observed for mixture 1 in the binding of JIM5 (HG), LM21, BS-400-4 (mannan), BS-400-3 ((1 \rightarrow 3)(1 \rightarrow 4)- β -glucan), and LM15 (xyloglucan) and for mixture 2 in the binding of LM21 (mannan) and BS-400-3 ((1 \rightarrow 3)(1 \rightarrow 4)- β -glucan) (Fig. 9A).

We also tested the potential of the technique for screening activities in recombinant culture broths. As shown in Fig. 9B, a crude recombinant *A. nidulans* endo-1,4- β -mannanase culture broth was used in combination with a substrate mixture. As expected, when the culture broth was used as a 4-fold dilution, the dominant activity was mannanase (detected by high -fold changes in the binding of the anti-mannan mAbs LM21 and BS-400-4). Apparent side activities against HG, galactan, β -glucan, and arabinoxylan (evidenced by -fold changes >1 in the binding of mAbs JIM5, JIM7, LM5, BS-400-3, and LM11, respectively) were detected, presumably originating from background activities in the host or from components in the growth medium. When used at a 40-fold dilution, only xylanase and minor β -glucanase side activities were detected, but when used at a 4000-fold dilution, only mannanase activity was detectable (Fig. 9B). These data indicate that when analyzing crude broths care is needed to perform assays at a range of broth concentrations to help discriminate between main and side activities. Taken together these data demonstrate that using this technique individual enzyme activities can indeed be resolved when diverse substrates are mixed.

Multiplexed Analysis Using Plant Cell Wall Biomass Substrates—We further extended the scope and complexity of our screening by using cell wall extracts as substrate mixtures. Polysaccharides were extracted from *A. thaliana*, willow, corn stover, barley, and horsetail by sequential extractions using CDTA and NaOH. After neutralizing the pH, the extracts were used as potential substrates for a polygalacturonase, a xyloglucanase, an endo-1,3(4)- β -glucanase, and the enzyme mixture Cellic Ctec2 (Fig. 10). Pectins would be expected to be extracted with CDTA, and this was reflected in -fold changes in the anti-HG mAbs JIM5, JIM7, LM18, and LM19 when CDTA fractions were treated with Cellic Ctec2 and polygalacturonase. The highest activity against HG was noted for *A. thaliana*, willow, and horsetail, consistent with the relative abundance of pectin in horsetail and in the type I cell walls of the other two species (2, 33, 34). Activity of the xyloglucanase, evidenced by -fold changes in mAb LM15, was highest when using NaOH fractions from horsetail and *A. thaliana* with lower levels of activity from willow and no activity from barley and corn stover. We also observed that the xyloglucanase had some side activity against other polysaccharides with β -1,4-D-linkages including

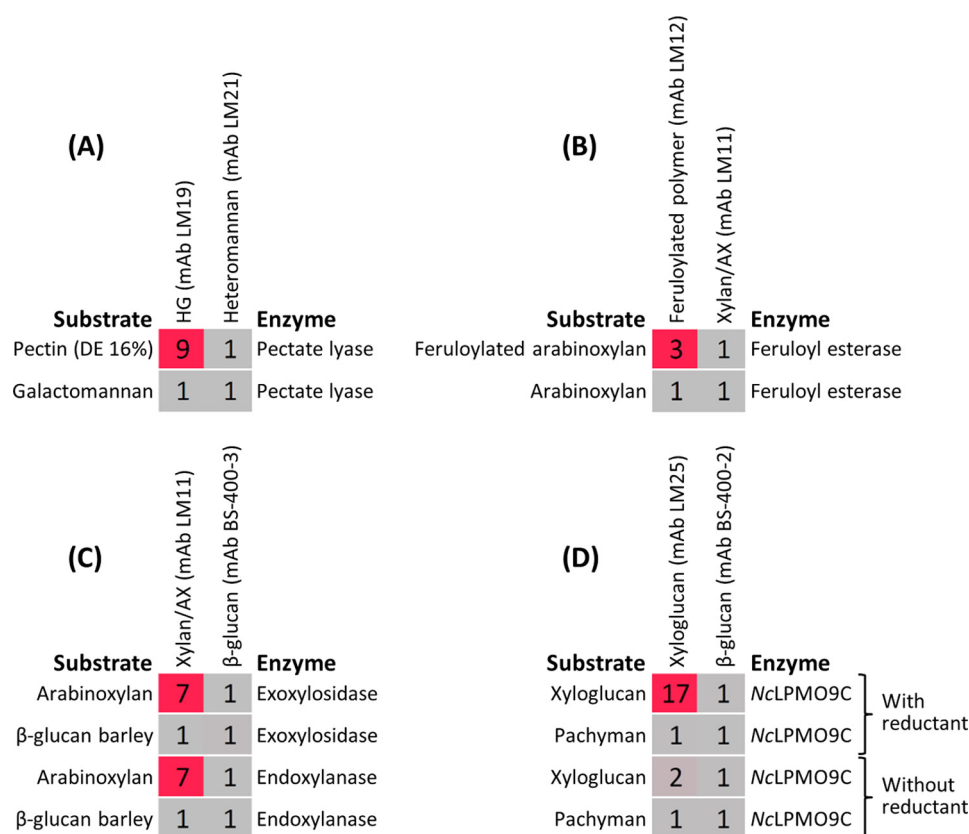


FIGURE 8. **Analyzing lyase, esterase, exo-acting GH, and LPMO activities.**—Fold change heat maps showing the use of the method to detect lyase (1 unit/ml) (A), esterase (2 unit/ml) (B), exo- and endo-acting GHs (both at 0.1 unit/ml) (C), and LPMO (5 μ g/ml) (D) activities. The reductant used for LPMO analysis was ascorbic acid. All incubations had substrate concentrations of 0.1 mg/ml and were performed for 1 h at 40 °C except for LPMO for which the incubation was performed for 2 h at 50 °C. AX, arabinoxylan. Details of enzymes and probes used are provided in Tables 1 and 2, respectively.

(1 \rightarrow 3)(1 \rightarrow 4)- β -glucan (as shown by the -fold change in mAb BS-400-3). These data are consistent with the side activities of this enzyme as described by the manufacturer (Megazyme). As expected, the endo-1,3(4)- β -glucanase (lichenase) was active on hemicellulosic fractions (NaOH extract) from corn stover, barley, and horsetail (revealed by -fold changes in the binding of mAb BS-400-3), and this is consistent with the presence of this polysaccharide in horsetail and in the type II cell walls of corn stover and barley (2, 33–35). It is worth noting that xylanase activity (as detected by -fold changes in mAbs LM10 and LM11) was detected in Cellic Ctec2 with corn stover and barley NaOH fractions as substrates, reflecting the presence of glucuronoarabinoxylans in these species (2, 36). Xylanase activity was not detected when the *A. thaliana* and horsetail NaOH fractions were the substrate but was observed when the willow NaOH fraction was used. This is consistent with the fact that the secondary cell walls of the mature, woody willow tissue are richer in xylan than the predominantly primary walls of the *A. thaliana* material (33, 37).

Uncharacterized Putative Glycoside Hydrolases from *P. militaris*—Six purified enzyme candidates from the gut microbiome of *P. militaris*, a fungus-growing termite, were analyzed against a substrate mixture (Fig. 11A). The results indicated that proteins *Pm12*, *Pm23*, and *Pm25* were xylanases (evidenced by -fold changes in mAbs LM10 and LM11) and *Pm83*, *Pm84*, and *Pm85* were β -glucanases (-fold changes in mAb BS-400-3) (Fig. 11A). Based on amino acid sequence, the xyla-

nases *Pm12* and *Pm25* were previously classified in CAZy glycoside hydrolase family GH10 (27). *Pm23* (which is the N-terminal module of a putative multimodular xylanase, GH11) could be attributed from BLAST analysis to the DUF303 superfamily, which is a family of unknown function but with putative acetyltransferase activities. However, *Pm23* cannot at present be classified in any existing CAZy family and thus represents a module of “unknown” function. The three β -glucanases were annotated as GH8 (27). Therefore, apart from *Pm23*, *P. militaris* microbiome protein CAZy classification was consistent with the activities we recorded. Furthermore, substrate specificities detected by our method were confirmed with parallel reactions (same incubation conditions and same enzyme and substrate mixture concentrations as those in Fig. 11A) analyzed by MALDI-TOF MS (Figs. 11B and 12A) and HPAEC (Figs. 11C and 12B). Essentially, *Pm12*, *Pm23*, and *Pm25* proteins released a combination of non-acetylated neutral and acidic xylooligosaccharides, which would be expected from xylanase treatment (38), whereas products from *Pm83*, *Pm84*, and *Pm85* treatments contained peaks implying activity on the β -glucan substrate.

Screening Enzyme Activities in Plant Pathogenic and Saprotrophic Fungi—We also assessed the technique for analyzing enzyme activities from a wood-decaying fungus (*T. versicolor*) and two additional plant pathogenic fungi (*C. acutatum* and *P. expansum*) grown in medium containing apple pomace. Culture broths were collected at 2–7 days after inoculation, and

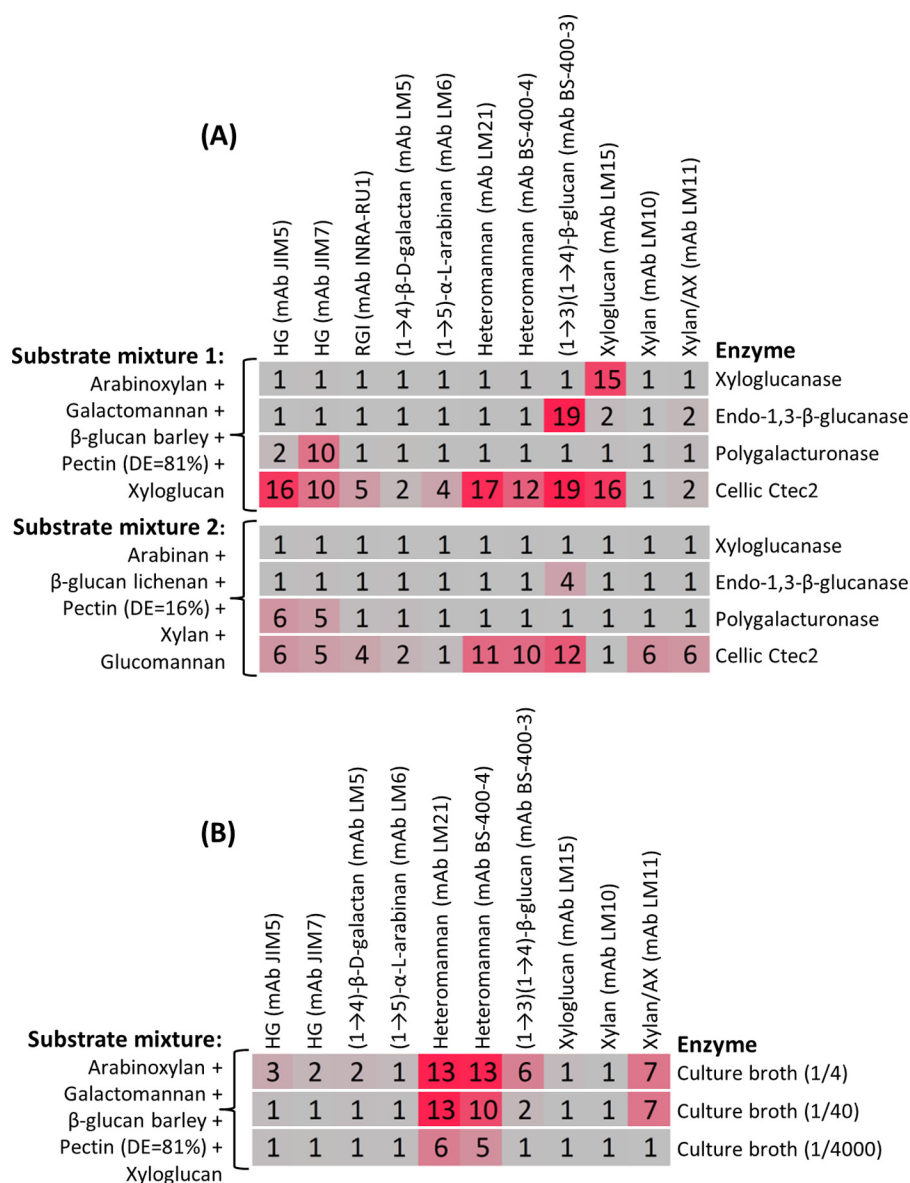


FIGURE 9. **Resolving activities against specific epitopes within complex substrate mixtures.** A, three enzymes (xyloglucanase at 0.001 unit/ml, endo-1,3-β-glucanase at 0.01 unit/ml, and polygalacturonase at 0.01 unit/ml) and an enzyme mixture (Cellic Ctec2 used at 1:20 dilution) listed to the right were tested against the two mixtures of substrates shown to the left, and activities were detected with the probes listed at the top of the heat map. The -fold change values in the heat map show that individual activities can be resolved from the substrate mixtures by the specific binding of the probes. Reactions were performed for 1 h at 40 °C. B, similarly, a crude recombinant *A. nidulans* endo-1,4-β-mannanase culture broth was also tested against a mixture of polysaccharide substrates. Note that side activities progressively diminished with decreasing broth concentration (shown to the right). Reactions were performed for 1 h at 30 °C. A and B, substrate mixtures contained 0.1 mg/ml (per polysaccharide). RGI, rhamnogalacturonan I; AX, arabinoxylan. Details of enzymes and probes used are provided in Tables 1 and 2, respectively.

their activities were tested using two different substrate mixtures (Fig. 13). *T. versicolor* displayed a limited repertoire of activities mostly against HG and xylan and to a lesser extent against (1→3)(1→4)-β-glucan. In contrast, both *C. acutatum* and *P. expansum* produced a wide range of cell wall-degrading enzymes, and we detected degradation of multiple epitopes including ones associated with pectin, mannan, xylan, xyloglucan, and (1→3)(1→4)-β-glucan. Control incubations were also performed using the apple pomace-containing medium inoculated with sterile distilled water, and some degradation of HG was detected. This activity most likely originated from the medium. Some clear trends in activity levels were observed over the time course of the experiment. For example, in broths from

P. expansum, activity against the low DE pectin epitopes (mixture 2) recognized by mAbs LM19 and JIM7 was high initially and then decreased, whereas activity against mannan recognized by mAbs LM21 and BS-400-4 was lower initially but increased rapidly by day 3.

DISCUSSION

The effective and sustainable use of plant biomass is of vital importance for reducing dependence on fossil fuels, and carbohydrate-degrading and -modifying enzymes are important tools in this effort. The semiquantitative technique described here combines the high throughput capacity of microarrays with the specificity of mAbs and CBMs and has several distinct

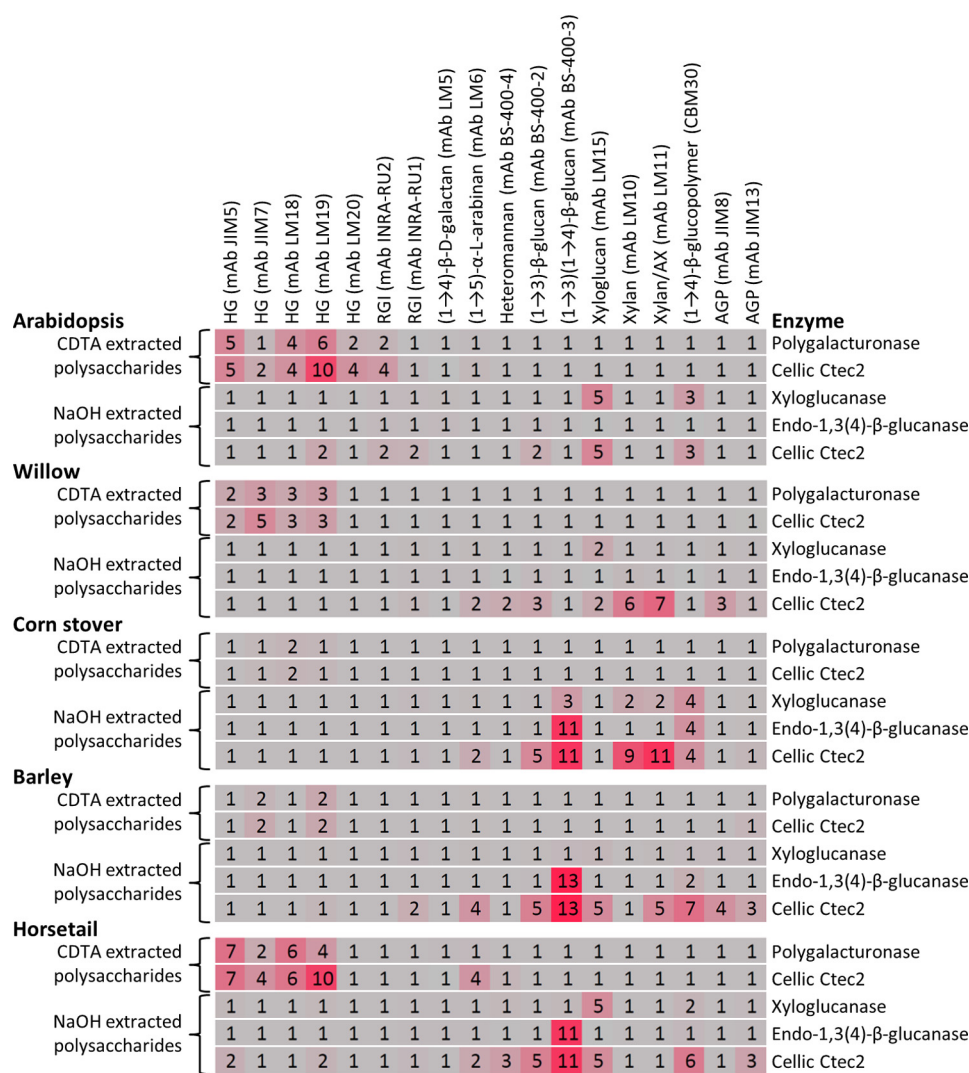


FIGURE 10. Resolving activities against specific epitopes present within extracts of plant polysaccharides. The activities of polygalacturonase (0.01 unit/ml), xyloglucanase (0.001 unit/ml), endo-1,3(4)-β-glucanase (lichenase; 0.1 unit/ml), and Cellic Ctec2 (1:30 diluted) were tested against crude mixtures of polysaccharides extracted with CDTA and NaOH from *A. thaliana*, willow (*S. alba*), corn stover (*Z. mays*), barley (*H. vulgare*), and horsetail (*E. arvense*). The heat map values indicate activities against individual epitopes as detected by the probes listed at the top. Reactions were performed for 1 h at 40 °C. RGI, rhamnogalacturonan I; AX, arabinoxylan; AGP, arabinogalactan protein. Details of enzymes and probes used are provided in Tables 1 and 2, respectively.

advantages. Substrates are unmodified and are free to interact with enzymes in solution under varied conditions of pH, temperature, and buffer type. The same procedure may be used to screen for endo- and exo-acting GHs, polysaccharide lyases, carbohydrate esterases, and LPMOs. Furthermore, the degradation of individual glycan structures can be detected within complex mixtures of polysaccharides and when multiple enzyme activities are present. This is significant because both in nature and in industrial applications enzymes are often applied as mixtures to complex biomass substrates. The method is high throughput (in 48 h, the activity of at least 200 enzymes or broths can be analyzed in triplicate against 15 polysaccharides) and requires low amounts of enzyme and substrate (such an experiment would require typically <0.0005 unit per enzyme and <1 mg per substrate). The method has high reproducibility and sensitivity with a detection limit in the range of 0.001–0.000001 unit/ml enzyme, which could be optimized by adjustment of the substrate concentration. Because reactions are performed in 384-microwell plates that are loaded directly into

microarray robots, we envisage that the technique would potentially be well suited to full or partial automation and would be complementary to associated technologies for colony picking of enzyme-expressing microbes and high throughput protein expression methods. Most of the work described here used commercially available enzymes with well characterized specificities with which our data were consistent. Furthermore, the robustness of our approach was confirmed by parallel analyses of enzymes using AZCL, reducing sugar assay, MALDI-TOF MS, and HPAEC.

The high throughput capacity of microarrays allows multiplexed analysis involving multiple sets of enzymes, substrates, and time points. The potential of this was demonstrated by following the progression of enzyme activity in broths of three fungi growing on apple pomace medium, which revealed clear differences in their secretomes and their temporal regulation (Fig. 13). Our data are consistent with the known natural ecologies of the fungal species tested. *T. versicolor* typically grows on dead or decaying wood, which contains a limited set of poly-

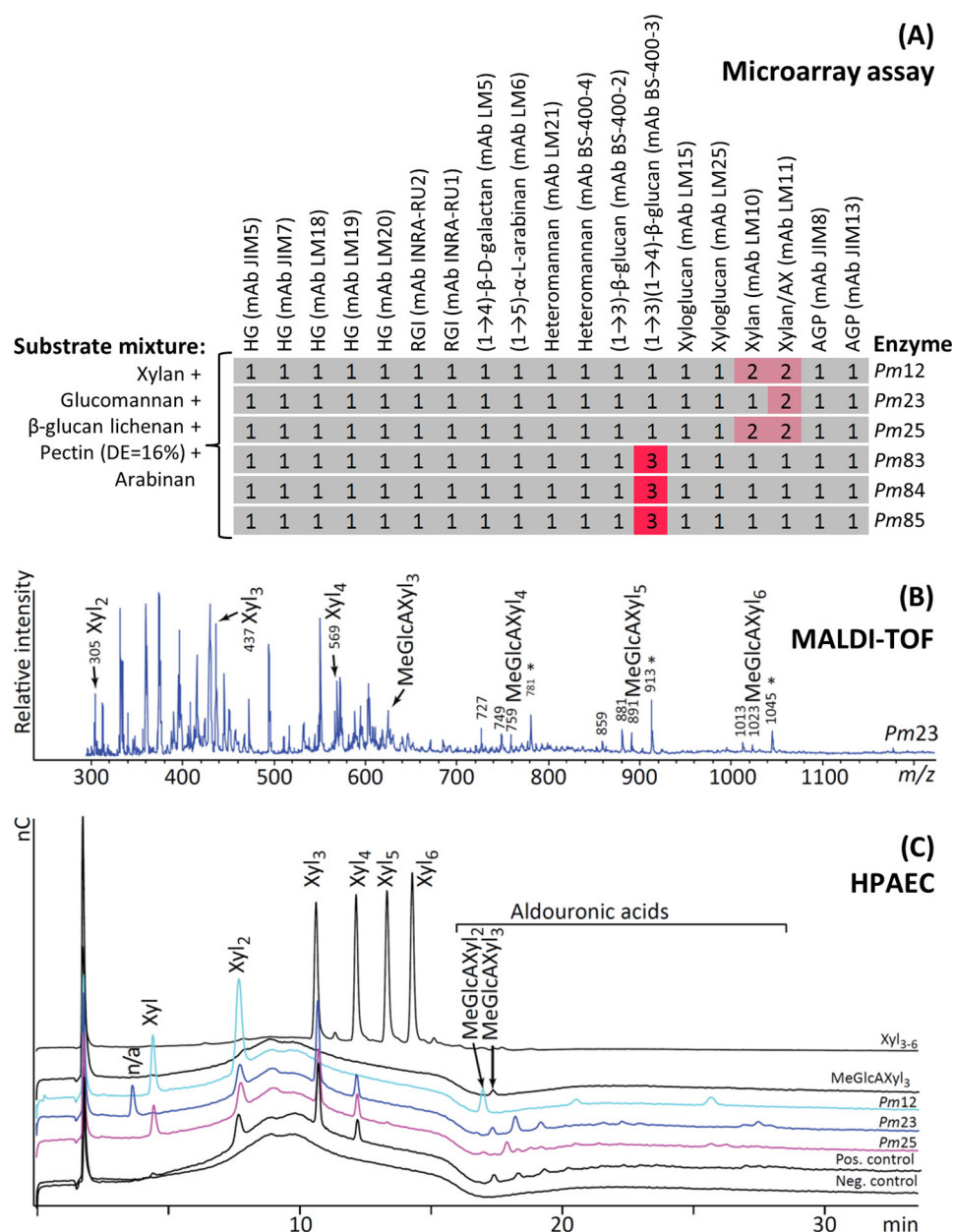


FIGURE 11. Activities of uncharacterized *P. militaris* microbiome proteins. A, the specificities of six purified and uncharacterized *P. militaris* microbiome proteins (5 μ g/ml) shown to the right were analyzed by incubating (overnight at 30 °C) the proteins with a substrate mixture (shown to the left; all components at 0.1 mg/ml) and activities evidenced by the binding of the probes listed at the top. The microarray assay indicated that proteins Pm12, Pm23, and Pm25 were xylanases and proteins Pm83, Pm84, and Pm85 were β -glucanases. B, Pm23 parallel reaction (parallel to A; substrate mixture + Pm23) analyzed by MALDI-TOF MS confirming its xylanase activity (represented as sodium adducts). Please note that the rather intense signals in the region from 300 to 600 m/z are mainly due to matrix peaks. C, external standards (Xyl₃₋₆ and MeGlcAXyl₃), Pm12, Pm23, and Pm25 parallel reactions (parallel to those analyzed in A), positive control (Pos. control; substrate mixture + eXYL from Table 1 at 0.01 unit/ml) and negative control (Neg. control; substrate mixture with no enzyme added) were analyzed by HPAEC. The positive control showed essentially the same profile as Pm23. nC, nanocoulomb; *, the corresponding sodium salt of sodium adducts often observed for carbohydrates containing carboxylic acids (30, 43); RGI, rhamnogalacturonan I; AX, arabinoxylan; AGP, arabinogalactan protein; Xyl, xylooligosaccharide; MeGlcAXyl, 4-O-methylglucuronoxyloligosaccharide (aldouronic acid); n/a, not available. Details of probes used are provided in Table 2.

mers, mostly cellulose, lignin, and xylan (39). *C. acutatum* and *P. expansum* are some of the most important fungal pathogens of fruits including apples (40, 41) and are therefore well adapted to grow on apple pomace medium. Fruits contain a rich variety of polysaccharides in primary and secondary walls, and this is reflected in the diverse array of plant cell wall-degrading enzymes secreted by these two fungal species. Our findings suggest that this technique has the potential to aid the screening of the secretomes of lignocellulose-degrading microbes and facili-

itate the identification of novel microbial sources of cell wall-degrading enzymes.

A previously described functional metagenomics approach (27) was used to investigate the gut microbiome of *P. militaris* for identification of hemicellulases. In function-driven metagenomics, only clones that respond positively to the screen are selected and further analyzed, making this an attractive alternative to less targeted conventional global metagenomic studies that typically generate vast data sets that are cumbersome to

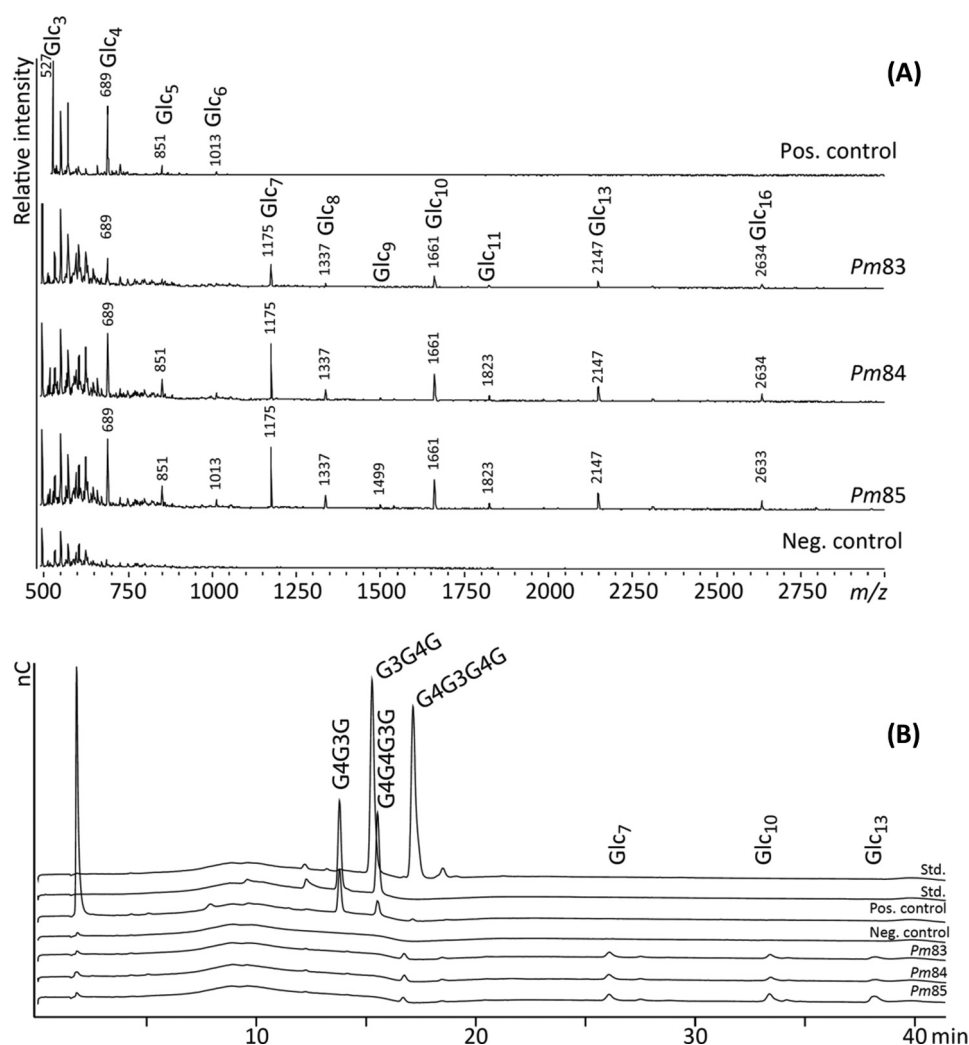


FIGURE 12. **MALDI-TOF MS and HPAEC analysis of uncharacterized *P. militaris* microbiome proteins.** A, *Pm83*, *Pm84*, and *Pm85* parallel reactions of Fig. 11A (same incubation conditions and same enzyme and substrate mixture concentrations as those analyzed in Fig. 11A), positive control (Pos. control; substrate mixture + eGLC from Table 1 at 0.01 unit/ml) and negative control (Neg. control; substrate mixture with no enzyme added) analyzed by MALDI-TOF MS confirming *Pm83*, *Pm84*, and *Pm85* β -glucanase activity. B, external standards (Std.) (mixed linkage glucan trimers (G4G3G and G3G4G) and tetramers (G4G4G3G and G4G3G4G)) and the same positive control, negative control, and *Pm83*, *Pm84*, and *Pm85* parallel reactions as those analyzed in A were analyzed by HPAEC. The peak annotations Glc₇, Glc₁₀, and Glc₁₃ are based on the major peaks observed on the same samples in the MALDI-TOF MS spectra and represent mixed linkage glucan oligosaccharides with degrees of polymerization 7, 10, and 13, respectively. nC, nanocoulomb. The substrate mixture contained pectin (DE = 81%), galactomannan, β -glucan barley, gum arabic, and xyloglucan.

mine for specific activities (42). Identification of the specificities of novel *P. militaris* microbiome proteins illustrates the potential of the method for enzyme discovery (Fig. 11), and our data were supported by both MALDI-TOF MS and HPAEC analyses. Our findings on *Pm23* are particularly intriguing because although we demonstrated this protein has xylanase activity *Pm23* cannot presently be assigned to any existing CAZy family. Therefore the possibility exists that *Pm23* represents a putative new GH family, although the veracity of this depends on further characterization of this protein and associated bioinformatics analysis.

Although primarily designed as a tool for enzyme specificity screening, the method can also provide information about substrate availability in biomass samples (Fig. 10). The ability to use complex plant materials as substrates is significant because it means that in contrast to other enzyme screening methods an enzyme activity can be detected even if a pure example of the substrate is not available.

One limitation of the technology in its present form is that it is necessary that the material printed onto membranes is soluble. This is because the fine bore piezo electric printing robot we used would be clogged by insoluble material. However, the method could in principle also be used to examine enzyme activities against insoluble crystalline polysaccharides, such as cellulose, if a solenoid-driven wider bore microarray printing robot was used. Although the scope of the technique is constrained by the availability of mAbs and CBMs, the development of high throughput microarray-based screening methods in antibody production and the emergence of CBMs as specific polysaccharide probes have the potential to provide large repertoires of new probes. It is worth noting that the mAb and CBM probes we used are publically available. This together with the decreasing cost of microarray robots means that the technology we have described is feasible for many researchers engaged in enzyme characterization. In conclusion, we believe that the

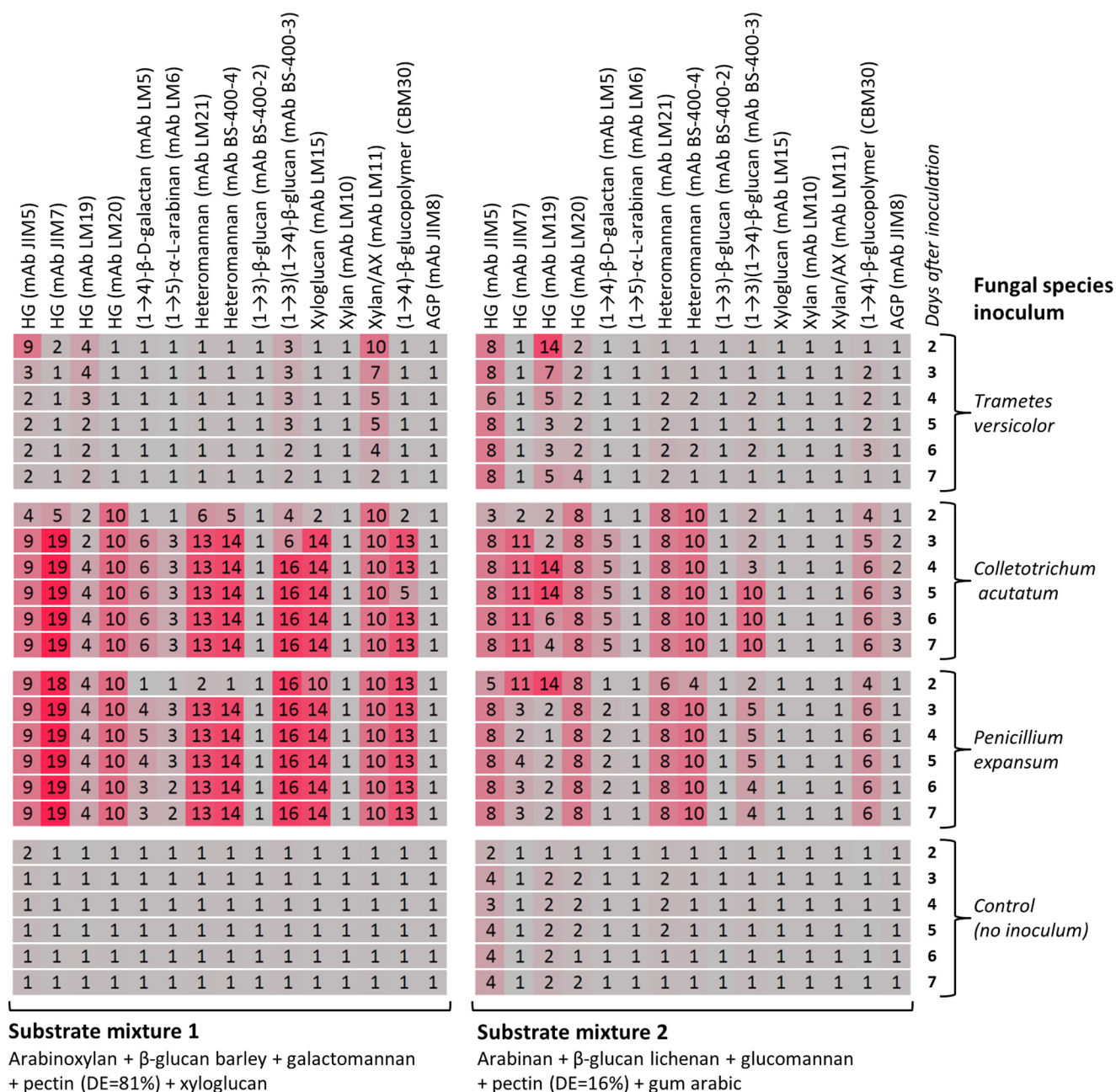


FIGURE 13. **Screening of enzymes secreted by three fungal species grown in apple pomace medium.** Two plant pathogenic (*C. acutatum* and *P. expansum*) and one saprotrophic (*T. versicolor*) fungi were grown in apple pomace medium. Culture broths were collected 2–7 days after inoculation. Culture broths from apple pomace medium (inoculated with water) were collected as controls. Broth supernatants (listed to the right) were incubated without further dilution with two mixtures of polysaccharides (listed at the bottom). The enzyme activities detected by the probes (listed at the top) are shown in the -fold change heat map. All reactions were performed overnight at 25 °C. The concentration of each polysaccharide in the substrate mixtures was 0.1 mg/ml. AX, arabinoxylan; AGP, arabinogalactan protein. Details of probes used are provided in Table 2.

method we have developed will make an important contribution to the discovery of enzymes and the empirical screening of their activities.

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A New Versatile Microarray-based Method for High Throughput Screening of Carbohydrate-active Enzymes

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