PAPER

Colloid-based multiplexed screening for plant biomass-degrading glycoside hydrolase activities in microbial communities†

W. Reindl,[ab] K. Deng,[ac] J. M. Gladden,[ad] G. Cheng,[ac] A. Wong,[ac] S. W. Singer,[ae] S. Singh,[ac] J.-C. Lee,[f] C.-H. Yao,[f] T. C. Hazen,[ae] A. K. Singh,[ac] B. A. Simmons,[ac] P. D. Adams[a] and T. R. Northen*[ab]

The enzymatic hydrolysis of polysaccharides into fermentable sugars is a crucial step in the conversion of biomass to lignocellulosic biofuels. An efficient hydrolysis is highly dependent on the identification and characterization of optimal glycoside hydrolases. However, existing techniques for characterizing activity are limited by the range of reaction conditions that can be used, sample complexity, and throughput. The method we present is a multiplexed approach based on nanostructure-initiator mass spectrometry (NIMS) that allows for the rapid analysis of several glycolytic activities in parallel under diverse assay conditions. By forming colloids, it was possible to perform aqueous reactions in tubes and microwell plates despite the substrate analogs' hydrophobic perfluorinated tags. This method was validated by analyzing standard enzymatic parameters (temperature, pH, and kinetics) of β-glucosidase and β-xylosidase in separate setups. The multiplexity of this assay system was demonstrated by the simultaneous analysis of β-glucosidase and β-xylosidase activities, which was then used to profile environmental samples. Enzymes secreted by microbial communities within these samples were extracted by washing the sample with buffer. Enzymatic activities were directly detected within this crude extract without any further sample pretreatment steps. The multiplexed analysis of β-glucosidase, exo-/endoglucanase, and xylanase activities was applied for a detailed characterization of previously unknown glycoside hydrolase activities. The results show the suitability of the described method for the rapid screening of crude environmental samples under a wide range of conditions to determine enzyme activities from the microbial communities present within these samples.

[a]*Joint BioEnergy Institute (JBEI), Physical Biosciences Division, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, CA, 94720, USA. E-mail: TRNorthen@lbl.gov; Fax: +1 (510) 486-4545; Tel: +1 (510) 486-5240*
[b]*Department of Bioenergy/GTL & Structural Biology, Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA*
[c]*Biotechnology and Bioengineering & Biomass Science and Conversion Technology Departments, Sandia National Laboratories, Livermore, CA, USA*
[d]*Physical and Life Sciences Directorate, Lawrence Livermore National Laboratory, Livermore, CA, USA*
[e]*Department of Geochemistry & Department of Ecology, Earth Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA*
[f]*Institute of Biotechnology and Pharmaceutical Research, National Health Research Institutes, Zhunan Town, Miaoli County, Taiwan*
† Electronic supplementary information (ESI) available: Supplementary figures, tables, and description of substrate synthesis. See DOI: 10.1039/c1ee01112j

Broader context

Today's energy sector is highly dependent on petroleum based fuels. However, the world's oil resources are limited and emissions from fossil fuels lead to increased production of carbon dioxide, causing climate changes and global warming. Recently, there is growing interest in the production of biofuels from lignocellulosic biomass (*e.g.* switchgrass or *Miscanthus*) which is a highly abundant renewable resource that can be converted into potentially carbon-neutral transportation fuels. The basis for this approach is the deconstruction of plant cell walls into sugar monomers which can be fermented into ethanol. The efficiency of the breakdown of (hemi)celluloses is closely connected to the availability of optimal (hemi)cellulases. Fungi and other microorganisms are a valuable source for these classes of enzymes. The identification of such enzymatic activities is highly dependent on enzyme assays. This work describes a new approach to screen for glycoside hydrolase activities involved in plant cell wall degradation. Our assay system can be used directly with crude environmental samples and under extreme reaction conditions (high temperatures, extreme pH values, presence of ionic liquid). Therefore, it presents a novel method for high throughput screening and detection of novel secreted (hemi)cellulolytic activities in microbial communities. Ultimately, this approach may potentially aid in finding a more efficient way to convert biomass into lignocellulosic biofuels.

Introduction

Glycoside hydrolases play important roles in a multitude of biological processes, both in eukaryotes (*e.g.* processing of glycans in glycoproteins)[1] and prokaryotes (*e.g.* utilization of sugar polymers as carbon source).[2] This enzyme class has become critical for the development of biofuels from lignocellulosic biomass:[3] Here, long-chain polysaccharides from plant cell walls are enzymatically hydrolyzed and the resulting sugar monomers are fermented into ethanol or advanced biofuels.[4] The three major components forming plant cell walls that must be deconstructed are the polysaccharides cellulose and hemicellulose, and the highly phenolic macromolecule lignin. Cellulose is comprised of linear chains of β-1,4-linked D-glucose units, while hemicellulose consists mainly of mixtures of pentoses with D-xylose and D-arabinose being the most abundant.[5] Cellulose is hydrolyzed into glucose through the concerted action of at least three known classes of enzymes: endoglucanases that randomly produce free ends from cellulose microfibrils that are subsequently hydrolyzed by exoglucanases that release cellobiose, which in turn is hydrolyzed by β-glucosidases into glucose.[6] Hemicelluloses are degraded by a complex class of multi-domain enzymes known as hemicellulases.[7] Lignin gets broken down by so-called ligninases, *e.g.* laccases or lignin peroxidases.[8]

Microbial communities (*e.g.* fungi or bacteria) capable of growing on lignocellulose have gained increasing attention as sources for discovering these types of glycoside hydrolases.[6] Their identification and characterization is dependent on enzyme activity assays, most of which are based on changes in the spectroscopic properties (*e.g.* absorbance, fluorescence, *etc.*) of a substrate analog upon hydrolysis.[9] However, there are various inherent limitations to these assays. For example, the overlap of absorption or emission spectra of fluorescent labels greatly complicates the analysis of more than one reaction at a time; the optical density of complex microbial samples interferes with spectroscopic measurements; substrate analogs often have only limited thermal or temporal stability. Due to its high resolving power, sensitivity, and specificity, mass spectrometry is capable of detecting large numbers of enzymatic reaction products in parallel. However, the complexity of crude cell lysates complicates analysis and often requires time-consuming chromatographic steps that lower sensitivity and throughput. Recently, surface-based mass spectrometry techniques have gained attention as they allow for rapid analysis. For example, a nanostructure-initiator mass spectrometry (NIMS)[10] based enzymatic activity assay[11] was successfully applied for the detection of β-1,4-galactosidase enzymatic activity; self-assembled monolayers for matrix assisted laser desorption/ionization time-of-flight (SAMDI-TOF) mass spectrometry was used for the analysis of glycosyl transferase and β-1,4-galactosidase activities on gold surfaces;[12,13] recently, glycan arrays on aluminum oxide-coated glass slides using fluorous-phase-chemistry were used to study glycoside hydrolases.[14] However, the reaction conditions for surface-based techniques are often complex to handle and hard to control, especially at high temperatures.

Here, we report a new method based on self-assembled colloids and NIMS for the multiplexed identification and characterization of glycoside hydrolase activities in a wide range of samples ranging from isolated enzymes to complex environmental samples. Due to colloid-formation of the amphiphilic substrate analogs, all reactions could be carried out in solution in tubes or microwell plates. Using this method, we were able to study isolated β-glucosidase and β-xylosidase activities in separate and multiplexed enzyme assays. Additionally, we simultaneously characterized β-glucosidase, exoglucanase, endoglucanase, and hemicellulase activities directly from crude environmental samples. Overall, this method is well suited for the identification of new enzymes involved in the deconstruction of plant biomass.

Results

NIMS analysis of glycoside hydrolase activity

Glycoside hydrolase assays were performed with amphiphilic substrate analogs and cleavage reactions were subsequently analyzed using NIMS (Fig. 1). The substrates comprise a polar sugar head group and a highly hydrophobic perfluorinated tail (F17). The sugar groups used in this study were formed by hexose monomers, such as cellobiose (CB) and cellotetraose (CT), or pentose monomers, such as xylobiose (XB) (Fig. S1†). Due to their amphiphilic character, the substrates spontaneously formed colloids, most likely micelles (Fig. 1a and 2). Thus, despite containing a large hydrophobic moiety, substrates could be dissolved in aqueous solutions, allowing enzymatic reactions to be performed in standard buffers and labware with one or multiple sugar substrates in parallel (Fig. 1a). After enzymatic cleavage, samples were spotted onto a NIMS surface without any further sample preparation steps, even if they contained crude environmental extracts with visible particles (Fig. 1b). This is due to the fact that the substrates selectively bind to the surface *via* fluorous-phase-interactions, while other sample components (*i.e.* enzymes, cleaved off sugar units, salts) remain in the aqueous phase. After a short incubation period the samples were removed leaving only the substrates on the chip (Fig. 1b), which enhances signal intensities.[15] Next, samples were analyzed by NIMS in a MALDI mass spectrometer, where laser irradiation leads to vaporization of the initiator liquid on the chip surface and subsequent transfer of applied samples into the gas phase (Fig. 1c). Acquired spectra show pairs of substrate and product signals (Fig. 1d), such that enzymatic activities can always be measured as product-to-substrate ratios, which is completely independent from total signal intensities. Substrates and products were shown to have equal ionization efficiencies under the experimental conditions used in this study (Fig. S2†).

Colloid-formation was analyzed for the cellobiose substrate (CB-F17) by small-angle neutron scattering (SANS) and dynamic light scattering (DLS) (Fig. 2). Both techniques showed a consistent radius for the detected main particles of 27.5 Å (polydispersity 14%) for SANS (Fig. 2a) and 30.0–33.0 Å (polydispersity 12–14%) for DLS (Fig. 2b). Additionally, SANS measurements revealed a spherical shape for the aggregates (Fig. 2a). With an estimated molecule size of ~24 Å for CB-F17, the recorded diameters of ~60 Å and the spherical shape suggest the formation of micelles, with the substrate extended into the aqueous phase and accessible to enzymes. A small portion of the particles measured had sizes greater than 1000 Å, probably due to aggregation (Fig. 2a and 2b). These colloids may reflect more
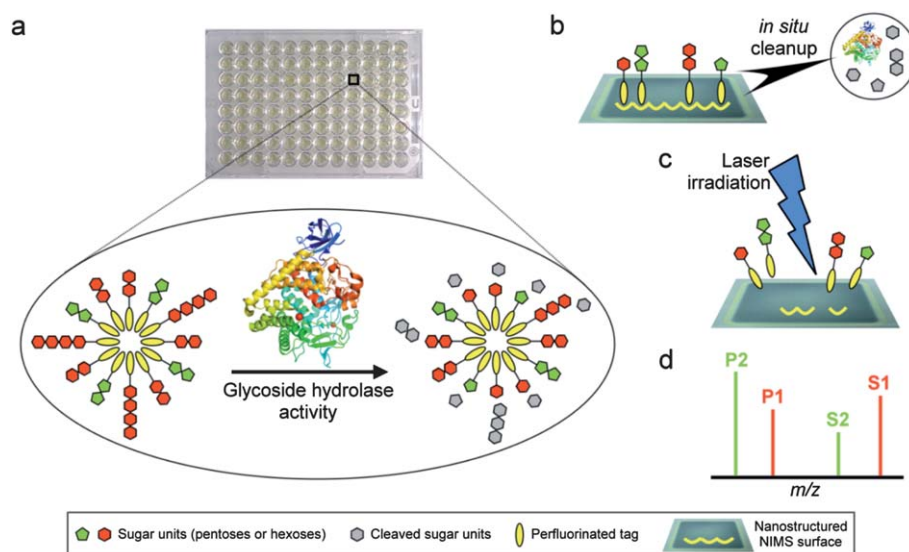
Fig. 1 NIMS analysis of colloid-based glycoside hydrolase assays. (a) Substrates consisting of a sugar head group coupled to a hydrophobic perfluorinated tag self-assemble into colloids under the used aqueous buffer conditions. Enzymatic reactions are performed in tubes or multiwell plates. Hydrolysis by glycoside hydrolases leaves at least one sugar monomer bound to the fluorous tag. (b) After enzymatic cleavage, samples are spotted onto a NIMS chip. Sugar substrates bind to the chip surface, which is coated with a perfluorinated initiator liquid, *via* fluorous-phase-interactions. All other sample components (*e.g.* enzymes, cleaved off sugar units, salts) can be washed away *in situ*. (c) Laser irradiation causes vaporization of the initiator, effectively transferring applied samples into the gas phase. (d) Ions of reaction products and non-cleaved substrates are detected by mass spectrometry, enzymatic activities can be determined as product-to-substrate ratios. Several enzymatic reactions can be measured in parallel, as long as the used substrates and occurring products differ in mass.

natural reaction conditions in comparison to completely soluble substrate molecules often used in activity screening as many glycoside hydrolases act on insoluble solid substrates (*e.g.* cellulose microfibrils).[6,7]

Two substrates were utilized to validate and illustrate the range of assays that can be prepared using this method. Cellobiose (CB-F17) or xylobiose (XB-F17) were used to analyze β-glucosidase or β-xylosidase activity of commercially available enzymes in separate setups (Fig. 3). We first characterized the temperature and pH optima of these enzymes. For the β-glucosidase an optimal reaction temperature of 50–60 °C (Fig. 3a) and a pH optimum at around pH 4.0 (Fig. 3b) were detected, while the β-xylosidase showed highest activity at a temperature of 35 °C (Fig. 3a) and at around pH 7.5 (Fig. 3b). These data match
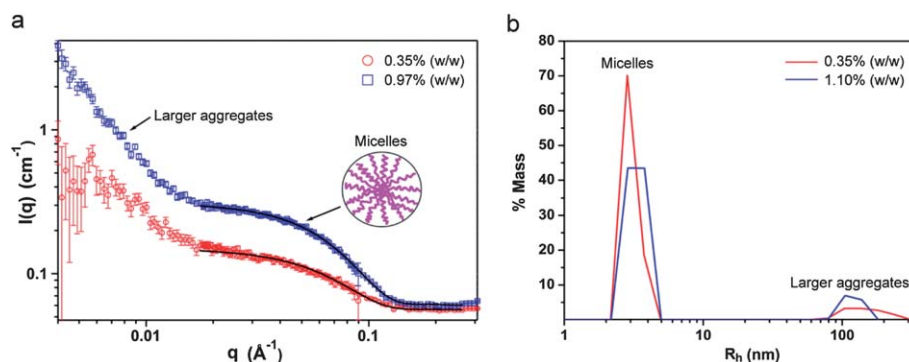
Fig. 2 Detection of colloid/micelle formation. (a) Small-angle neutron scattering (SANS) curves were acquired for 0.35% (w/w) and 0.97% (w/w) cellobiose (CB-F17) in D_2O. Scattering intensity is proportional to solution concentration. The upturn in the low q range of $0.004 < q < 0.017$ Å^{-1} indicates the presence of large aggregates. Following the low q upturn, a plateau is clearly observed over a wide q range, which indicates that clusters have a minor influence on the scattering in the higher q regime and only a small fraction of the mass belongs to the larger aggregates. The scattering in the intermediate q range of $0.017 < q < 0.15$ Å^{-1}, corresponds to the scattering from individual colloids/micelles whereas in the high q range of $0.15 < q < 0.3$ Å^{-1} it comes from incoherent background scattering of the solutions. A form factor of a uniform sphere was used to fit the SANS data in the intermediate q range (solid lines).[33] The main particle radius was determined to be 27.5 Å (polydispersity 14%). The cartoon depicts a schematic micelle. (b) Size distribution of the hydrodynamic radius (R_h) determined by dynamic light scattering (DLS) for 0.35% (w/w) or 1.10% cellobiose (CB-F17) in D_2O shows a R_h of 30.0 Å (polydispersity 12%) for the detected main particles and a small amount of larger aggregates with a radius of ~148 nm (polydispersity 36%) for 0.35% CB-F17 and a R_h of 33.0 Å (polydispersity 13.8%) for the detected main particles and a small amount of larger aggregates with a radius of ~119 nm (polydispersity 14.7%) for 1.10% CB-F17.

This journal is © The Royal Society of Chemistry 2011

the known reaction optima for these enzymes as specified by the manufacturers. Kinetic studies were performed by analyzing aliquots of each reaction sample at specific time points (Fig. 3c). A kinetic competition study using the β-glucosidase and a combination of CB-F17 and natural cellobiose showed that the perfluorinated tag does not interfere with activity for the enzymes in this study (Fig. S3†).

Multiplexed glycoside hydrolase assays

It is difficult to discriminate between several products in simultaneous reactions using conventional spectroscopic methods since these reactions typically yield the same outputs and there are only a few unique absorption or emission patterns that can be resolved separately. In contrast, mass spectrometry can resolve thousands of ions and therefore is well suited for the parallel detection of large numbers of reaction products as long as the substrates and occurring products differ in mass. Both β-glucosidase and β-xylosidase activities were detected simultaneously utilizing an enzyme cocktail comprised of equal amounts of CB-F17 and XB-F17 (Fig. 4). In the negative control only strong signals for cellobiose and xylobiose were detectable (Fig. 4a). Incubation of the two substrates with a β-glucanase/xylanase enzyme cocktail leads to complete cleavage of both substrates (Fig. 4b). After incubation with β-glucosidase, cellobiose was almost completely converted to glucose (Fig. 4c). As expected, for β-xylosidase there was conversion of xylobiose to xylose and no enzymatic cleavage of cellobiose (Fig. 4d). These data demonstrate that this method can be applied to analyze several glycoside hydrolase activities in parallel.

Multiplexed detection of glycoside hydrolase activities in environmental samples

The multiplexed colloid-based assay system was used for screening for potential glycoside hydrolase activities in environmental samples. The following workflow was applied for obtaining these samples (Fig. 5a): soil samples were collected at various sites with different soil characteristics; a small amount of each sample was used to inoculate minimal growth medium with switchgrass as sole carbon source; after one week of incubation the remaining soil particles were removed by centrifugation and the resulting supernatants containing secreted enzymes

(secretome) from the present microbial community were used for further analysis. The secretomes of the environmental samples were incubated with a mixture of equal amounts of CB-F17 and XB-F17 for 1 h at 50 °C and analyzed for conversion of these substrates. This approach proved to be very effective, and at least one of the targeted activities was detected in the majority of samples tested, while several samples showed clear substrate conversion for both targeted enzymatic activities (Fig. 5b). In total, a broad range of activities was exhibited by the samples. The Jepson Prairie (JP) compost sample was the most effective in terms of overall liberation of monomeric sugars with 87.1% ± 2.1% cellobiose and 92.3% ± 0.4% xylobiose conversion (Fig. 5b). We would like to note that this assay detects the total activity in a sample and cannot distinguish between samples with high enzyme loads or with enzymes of high specific activity. Specific activity measurements would require fairly pure proteins, and the secretomes studied are very complex mixtures that also contain non-glycoside hydrolase enzymes of varying amounts and there are likely multiple redundant glycoside hydrolases of unknown concentrations, thus making it very hard to obtain comparable crude sample specific activities. Additionally, these samples contain varying amounts of humics that can interfere with protein measurements and further confound the quantitative interpretation of the results. Due to these factors, we decided to limit the assay to comparison of total activity produced per volume by communities grown on the same feedstock.

Profiling of an environmental sample

As the JP compost sample showed highest activities, it was further used to generate switchgrass-adapted thermophilic consortia grown on microcrystalline cellulose (JP MC). The JP MC secretome was used for a detailed multiplexed characterization. To this end the extract was incubated with a mixture of XB-F17 and CT-F17 for 30 min at 50 °C or 80 °C at pH 3, 5, 7, and 9, and in the presence of 0%, 10%, 20%, and 30% ionic liquid (IL; ethyl-3-methyl imidazolium (EMIM) acetate). The IL reagent is used for pretreatment of biomass, and IL-tolerance is highly desirable for enzymes and microbes involved in biomass degradation.[16] The β-xylosidase activity showed a clear preference for higher temperatures under every reaction condition tested, suggesting that the β-xylosidase(s) present in the JP secretome are
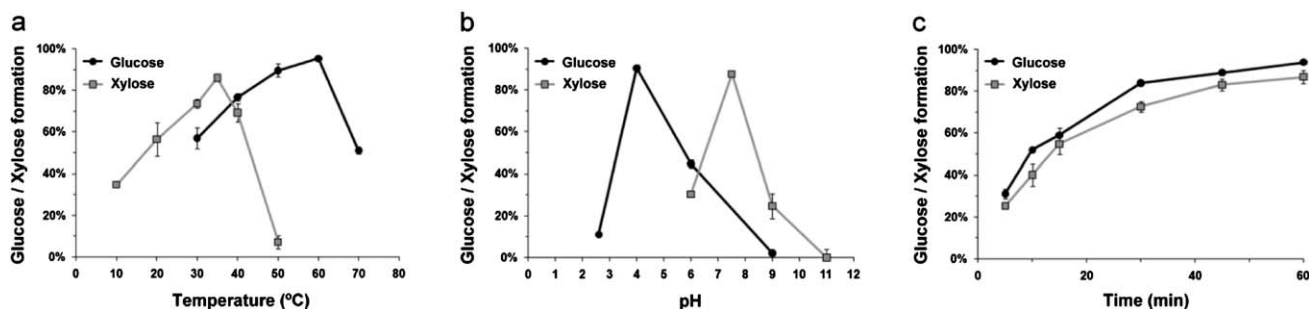
Fig. 3 Separate assays for β-glucosidase and β-xylosidase activity. (a) Determination of the temperature optima for the used β-glucosidase (black) and β-xylosidase (gray). (b) Determination of the pH optima for the used β-glucosidase (black) and β-xylosidase (gray). (c) Monitoring β-glucosidase (black) and β-xylosidase (gray) activity over time. All activities were corrected by the values of negative control samples without enzyme. Error bars represent standard deviation of three independent experiments.
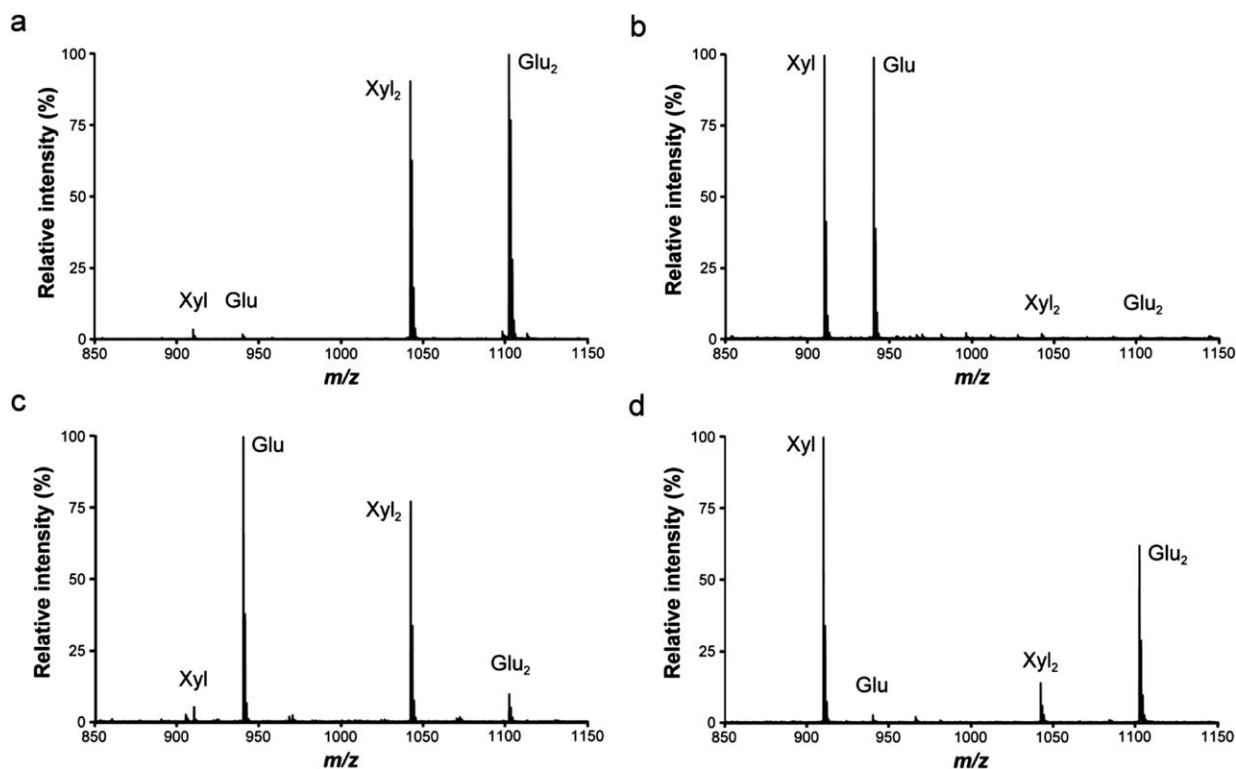
Fig. 4 Multiplexed assay for the parallel analysis of β-glucosidase and β-xylosidase activity. Mass spectra show signals for xylose (Xyl; m/z 910), glucose (Glu; m/z 940), xylobiose (Xyl$_2$; m/z 1042), and cellobiose (Glu$_2$: m/z 1102). (a) No enzyme control (ratios: Glu:Glu$_2$ = 2.9%:97.1% ± 1.1%; Xyl:Xyl$_2$ = 5.0%:95.0% ± 1.2%). (b) β-glucanase/xylanase enzyme cocktail showed both activities (ratios: Glu:Glu$_2$ = 98.7%:1.3% ± 0.1%; Xyl:Xyl$_2$ = 98.0%:2.0% ± 0.3%). (c) β-glucosidase cleaves cellobiose only (ratios: Glu:Glu$_2$ = 91.3%:8.7% ± 0.5%; Xyl:Xyl$_2$ = 3.6%:96.4% ± 0.7%). (d) β-xylosidase is specific for cleavage of xylobiose (ratios: Glu:Glu$_2$ = 4.3%:95.7% ± 3.0%; Xyl:Xyl$_2$ = 86.8%:13.2% ± 3.2%). Assays were performed in triplicates with one representative spectrum shown. In a few cases where only small amounts of product are formed, somewhat larger standard deviations can be seen as a result of the difficulty in quantifying low intensity peaks due to background noise effects.

thermophilic enzymes (Fig. 6a and b). Essentially no β-xylosidase activity was detected at pH 3, while XB-F17 was converted at pH 5, 7, and 9. At 80 °C more than 90% of XB-F17 was degraded at pH 5, 7, and 9. The 50 °C data show a pH optimum at around pH 7 (Fig. 6a). The IL profile of β-xylosidase activity revealed that these enzymes have a surprisingly strong IL-tolerance. Even at 30% IL 32.3% ± 4.2% of XB-F17 was hydrolyzed at 80 °C (Fig. 6b).
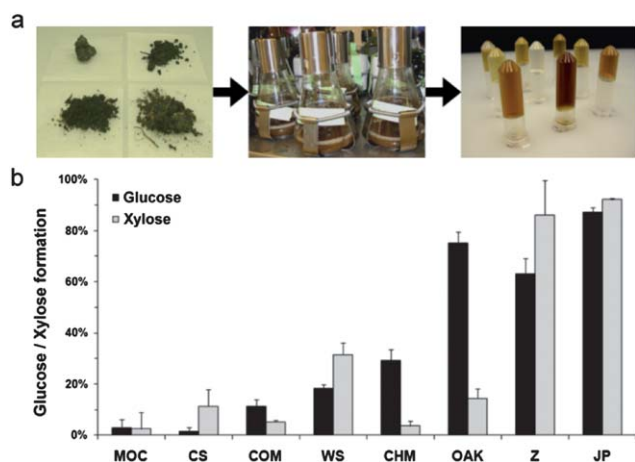
The cellulase activity in the secretome was also highest at 80 °C (Fig. 6c), showing that the cellulase enzymes are thermophilic, as well. Strong cellobiose and glucose signals suggest that the JP secretome is composed primarily of exo- and endoglucanases, as it showed an activity pattern comparable to exo- and endoglu-canase control enzymes (Fig. S4†). However, as no triose formation was observed for the control exo- and endoglucanases, but for control β-glucosidases (Fig. S4†) and a significant amount of triose was detected for the JP secretome (Fig. 6c), this indicates that the secretome also contains β-glucosidases. The pH optimum for cellulase activity was determined to be at pH 5 (Fig. 6c). The cellulases showed only weak IL-tolerance. At 50 °C, there was hardly any detectable cellulase activity at 30% IL, with 89.6% ± 5.1% of the CT-F17 substrate remaining (Fig. 6d). The greater IL-tolerance of the xylanases relative to the cellulases is most likely due to the inherent properties of these enzymes alone and not an assay or substrate artifact, as it would have been observed for both the C5 and C6 substrates.

Surprisingly, the limited IL-tolerance of the cellulases was greater at 50 °C than 80 °C. We hypothesize that the particular reaction leading to the inactivation of the affected glycoside hydrolase(s) must have been kinetically favored at higher temperatures. Information on enzyme structures would help to understand the underlying mechanism.

Discussion

We report an integrated method utilizing substrate colloids coupled with NIMS for the simultaneous analysis of multiple glycoside hydrolase activities. This method was shown to correctly determine the optimal reaction conditions for already known enzymes, and to be suitable for the detection of activities in complex environmental samples. It is compatible with conventional microwell plate formats and has several benefits over standard assays.

Multiple enzymatic activities can easily be assayed in parallel, the only requirement is a difference in mass. This is the case, *e.g.* when using pentose- and hexose-based substrates at the same time. The hydrolysis of polysaccharides with identical masses (*e.g.* cellobiose and maltose) can even be analyzed in the same run by using different tags, *e.g.* different chemical structures in the linker or perfluorinated tags of varying length. Thus, potentially dozens of enzymatic conversions can be tested simultaneously using this method. The ability to conduct rapid

2888 | *Energy Environ. Sci.*, 2011, **4**, 2884–2893

This journal is © The Royal Society of Chemistry 2011

Fig. 5 Multiplexed detection of glycoside hydrolase activities in environmental samples. (a) Workflow for enzyme extraction: Environmental samples (soil and compost; left) were used to inoculate minimal growth medium with switchgrass as sole carbon source and incubated for one week (middle). After removal of soil particles by centrifugation, the supernatant containing the enzymes secreted by microbial communities in the soil samples (secretome; right) was tested for glycoside hydrolase activity. (b) Eight environmental samples (mixed organic compost (MOC), clay-rich soil (CS), cow manure (COM), a sample from light woodland (WS), chicken manure (CHM), soil mixed with leafs from under an oak tree (OAK), Zamora compost (Z), Jepson Prairie compost (JP)) were analyzed for β-glucosidase (black) and β-xylosidase (gray) activity by determining the conversion of cellobiose to glucose and xylobiose to xylose. Reactions were performed at 50 °C and pH 5. Activities were corrected by the values of a negative control sample without enzyme. Error bars represent standard deviation of three independent experiments.

parallel analysis of several enzyme reactions in combination with low sample volumes (less than 1 μL spotted per sample) makes this approach well suited for high throughput screens for novel enzymes that can be used to deconstruct biomass and aid in the development of lignocellulosic biofuel technologies.

Another major advantage of this technique is that there is no need for highly processed and clean samples. Even crude environmental samples containing visible soil particles (Fig. 5a, middle) did not interfere with the mass spectrometric analysis. Due to the unique fluorous-phase-interactions, substrates selectively bind to the chip surface while all other sample components including soil particles or cell debris can simply be washed away *in situ*. Our assay system comprises the benefits of sample purification, while requiring only a minimal fraction of time in comparison to standard chromatography techniques, *e.g.* liquid chromatography coupled mass spectrometry (LC-MS). Particles present in a sample would strongly interfere with any traditional spectrophotometric analysis due to multiple light scattering effects. Additionally, some of the environmental samples used in this study were strongly colored (Fig. 5a, right) and would also potentially generate false positives in spectrophotometry-based assays. Furthermore, once spotted onto the chip surface our NIMS-based assay is stable for weeks, while the substrates used in absorption or fluorescence measurements sometimes are often only stable for minutes.

Our method can be used under a broad range of assay conditions. Reactions can be performed at basically any pH,

a contrast to commonly used chromogenic or fluorogenic substrate analogs that are only functional over a smaller pH range and cannot be used at extreme pH values. For example, the common glycoside hydrolase substrates p-nitrophenol-β-D-cellobioside and 4-methylumbelliferyl-β-D-cellobioside are not usable at acidic pH, while resorufin-β-D-cellobioside is not applicable at basic pH.[17–20] Additionally, as the usage of analogs like p-nitrophenol-β-D-cellobioside is based on the hydrolysis of a glyconic rather than naturally occurring glycosidic bonds, determined activities only yield approximations of enzyme activities,[9,21] whereas the natural substrates and bonds are utilized in our substrates. An advantage of the aforementioned methods is that the spectroscopic characterization currently allows for a higher throughput than mass spectrometry based read-outs. The presence of ionic liquid did not interfere with the assay, which is particularly important for biofuel technologies under development which are using these green solvents for biomass pretreatment. It should be noted that the method presented here has the same types of limitations as any method based on substrate analogs in that it requires chemical synthesis of specific substrates and is susceptible to the same concerns over reaction specificity when compared to natural substrates. However, competition studies using perfluorinated (CB-F17) and natural cellobiose (Fig. S3†) indicate that there is no interference of the tag with the activity of the used enzymes.

A similar assay system has been described for β-galactosidase activity.[11] However, these assays were performed directly on a chip-surface with immobilized substrates. For surface-based assays it can be very difficult to keep conditions constant during the reaction, *e.g.* to avoid evaporation and the accompanying change in concentration when using submicroliter volumes at high temperatures. Additionally, the use of enzymes directly on a Teflon-coated surface can decrease activity.[14] As the substrates used in this study formed micelles, all assays could be performed in solution, which allowed for complete control of reaction conditions. Assays were carried out in standard tubes or sealed plates and reactions were stopped in the tubes by adding methanol before spotting samples onto the chip for analysis, ensuring that the chip surface could not have any negative effects on the enzymatic activity. However, one big advantage of surface-based techniques is their high throughput. Still, the throughput of the described solution-based method can be increased by coupling the assay to an automated liquid handling and spotting system or can also be performed directly on-surface with the previously described setup.[11] As reactions are performed in solution, only soluble substrates can be used in the described setup. In this study, samples with up to four glycan units were used. The limit of solubility should be reached for seven or more glycan units. Chemical modifications of the tag moiety might increase solubility.

Regarding the reaction mechanism, we anticipate that the assay works with both monomeric and colloidal/micellar forms of the substrates, whereas the main fraction of substrate forms larger micellar structures, as seen in the DLS measurements (Fig. 2b). The formation of colloids should not interfere with enzyme activities, as enzymes bind the perfluorinated substrates with a comparable affinity to the natural substrates (Fig. S3†). We suggest, that on the one hand micelles may diffuse slower through solution increasing the number of enzyme-micelle
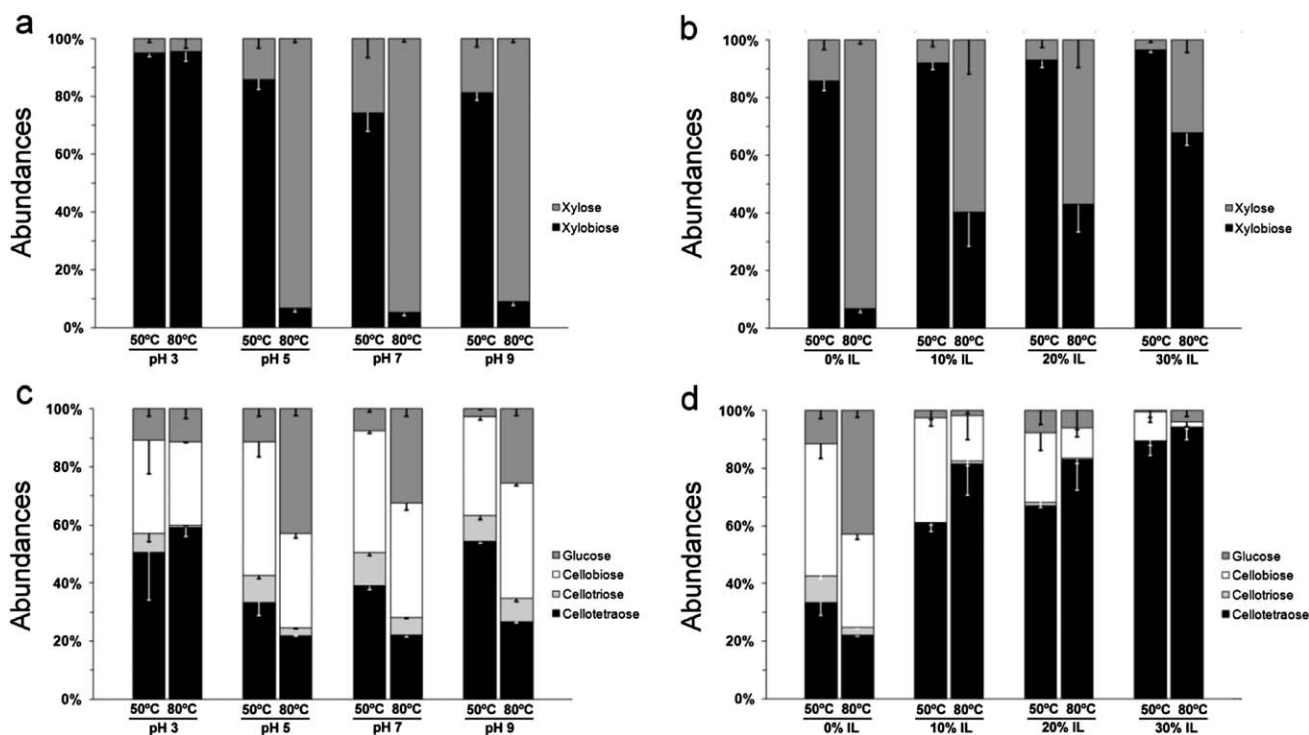
Fig. 6 Profiling of an environmental sample. Xylanase (a, b) and cellulase (c, d) activities present in the secretome of the Jepson Prairie compost sample grown on microcrystalline cellulose (JP MC) were analyzed under a broad range of reaction conditions: (a) cleavage of xylobiose at different pH, (b) cleavage of xylobiose at various concentrations of ionic liquid (IL) at pH 5, (c) cleavage of cellotetraose at different pH, (d) cleavage of cellotetraose at various concentrations of ionic liquid (IL) at pH 5. All activities were corrected by the values of negative control samples without enzyme. Error bars represent standard deviation of three independent experiments.

interactions, and that on the other hand the relatively bulky perfluorinated tail prevents the formation of tightly packed micelles, so that the sugar moieties should be rather extended into the solution and easy to access by the enzymes. We speculate that longer oligomers, as long as they are still soluble, would still form micelles. However, there will likely be a shift in the critical micelle concentration.

In summary, we hypothesize that the described method can most efficiently be used for the rapid initial screening and relative comparison of glycoside hydrolase activities in large sample sets (e.g. clone libraries or environmental sample collections). It has to be noted that the multiplexed assay platform cannot distinguish between several separate or promiscuous enzyme activities. Therefore, for a detailed characterization of the activities detected in microbial communities, the respective enzymes would have to be isolated and identified with the help of proteomic and meta-genomic analyses, followed by additional mechanistic studies. The presented experimental setup is suitable for the identification of secreted microbial (hemi)cellulolytic activities. However, not all glycoside hydrolases are secreted, e.g. cellulases in *Fibrobacter succinogenes* are immobilized within the cell membrane.[22] Still, the detection of non-secretory enzymes should be feasible by performing the enzymatic reaction in the presence of cells, followed by the described *in situ* sample cleanup step.

Conclusion

Using this method, both crude and purified enzymes representing some of the major classes of lignocellulolytic enzymes

(β-glucosidases, exo-/endoglucanases, and β-xylosidases) were characterized for activity optima and stability in the presence of inhibitory compounds (i.e. ionic liquid). For a more comprehensive analysis of plant cell wall degradation, it would be interesting to use several more substrates to target additional enzymatic activities. Longer β-1,4-linked glucose chains would help to study endoglucanases in more detail, longer xylose chains or also other sugars, such as arabinose or mannose, would aid in the identification and characterization of hemicellulases, and substrates containing phenolic molecules (e.g. coumaryl alcohol or coniferyl alcohol) could be used to study lignin degradation. While the method presented here is focused on enabling biofuel development, it can potentially be used for a wide range of other glycan assays including medical applications, e.g. screening for viruses based on profiles of neuraminidase activity.

Materials and methods

Substrates

The substrates used in this study were cellobiose, cellotetraose, and xylobiose, all attached to a perfluorinated tag (Fig. S1†). Oligosaccharides were purchased from the following sources: cellobiose from Sigma-Aldrich (St. Louis, MO), cellotetraose from Toronto Research Chemicals (North York, Canada), and xylobiose from TCI America (Portland, OR). Substrate synthesis is described in detail in the ESI† and Figures S5–9†. Briefly, a $(CH_2)_5$-linker was coupled to the reducing end of each sugar molecule by Schmidt imidate chemistry. Hydrogenation by Pd/C

removed the carbobenzyloxy (Cbz) protection group to give a primary amine. The heptadecafluoro-1,1,2,2-tetrahydrodecyl (F17) tag was attached to a dimethyl-arginine by an amide bond forming reaction. Finally, a peptide coupling reaction linked the sugar moiety with the fluorous tag to yield the desired substrate.

Measuring colloid formation

Small-angle neutron scattering (SANS). SANS experiments were conducted at Oak Ridge National Laboratory (ORNL) on the CG2 (GP-SANS) instrument with a neutron wavelength of $\lambda = 4.8$ Å ($\Delta\lambda/\lambda \sim 0.14$). Liquid solutions of 0.35% (w/w) and 0.97% (w/w) CB-F17 in D_2O were put into 1 mm quartz cells. Measurements were carried out at room temperature. Two sample-detector distances were used (4.0 and 14 m with a 40 cm detector offset), which resulted in a range of 0.004 Å^{-1} < q < 0.3 Å^{-1} for the scattering vector q ($=4\pi \sin\theta/\lambda$). The data was corrected for instrumental background as well as detector efficiency and put on absolute scale (cross section I(q) per unit volume in units of cm^{-1}) by means of pre-calibrated secondary standard.[23]

Dynamic light scattering (DLS). DLS experiments were performed at 25 °C with a 0.35% (w/w) and a 1.10% (w/w) solution of CB-F17 in D_2O on a DynaPro plate reader (Wyatt Technology; Santa Barbara, CA) with a wavelength of 832.6 nm and a detection angle of 158°. The size distribution was obtained by analyzing the auto correlation function using regularization analysis.[24]

Enzymes

β-glucosidase from *Aspergillus niger* (An_BG) and 1,4-β-D-xylosidase from *Bacillus pumilus* were purchased from Megazyme (Wicklow, Ireland). The β-glucanase/xylanase mixture (NS22002) was part of the 'Biomass Kit' from Novozymes (Davis, CA). Two different expression versions of an exoglucanase from *Caldicellulosiruptor saccharolyticus*[25] (Cs_GH5), an endoglucanase from *Prevotella ruminicola*[26] (Pr_GH5), Cel5A from *Thermotoga maritima*[27] (Tm_Cel5A), and Cel9A from *Alicyclobacillus acidocaldarius*[28] (Ac_Cel9A) were kindly provided by Joshua Park and Supratim Datta (Joint BioEnergy Institute, Emeryville, CA). An additional undisclosed β-glucosidase (UBG) was also included.

Enzymatic activity assays

Separate analysis of β-glucosidase and β-xylosidase activity. The separate characterization of β-glucosidase and β-xylosidase activity was carried out in reaction volumes of 20 or 50 μL, by mixing 0.1 mM CB-F17 with 2 ng (in 20 μL) of β-glucosidase from *Aspergillus niger* in 50 mM sodium acetate buffer (pH 4.0), or 0.1 mM XB-F17 with 130 ng (in 20 μL) or 330 ng (in 50 μL) of 1,4-β-D-xylosidase from *Bacillus pumilus* in 50 mM potassium phosphate buffer (pH 7.5) with 1 mg/mL BSA. Other buffers used for the determination of the pH optima were McIlvaine's citrate/phosphate buffer (pH 2.6 and 6.0),[29] 50 mM Tris buffer (pH 9.0) for the β-glucosidase, and 50 mM potassium phosphate buffer (pH 6.0), 50 mM Tris buffer (pH 9.0), 50 mM sodium borate buffer (pH 11.0), all with 1 mg/mL BSA for the β-xylosidase. Samples were incubated for 1 h at 35 °C (β-xylosidase) or 50 °C

(β-glucosidase), for determination of temperature optima samples were additionally incubated at the given temperatures. Reactions were quenched by adding one sample volume of ice-cold methanol. For monitoring the enzymatic reactions over time, small aliquots were taken out of a bigger reaction volume at the given time points, quenched by adding one aliquot volume of methanol, and kept on ice until further analysis. The same setup was used for the competition study, but instead of 0.1 mM CB-F17 a mixture of 0.02 mM CB-F17 and 0.08 mM natural cellobiose was used.

Multiplexed β-glucosidase/β-xylosidase assay. For the simultaneous analysis of β-glucosidase and β-xylosidase activity a mixture of 0.1 mM CB-F17 and 0.1 mM XB-F17 was incubated with 2 ng of β-glucosidase from *Aspergillus niger* in 50 mM sodium acetate buffer (pH 4.0), with 6.5 μg β-xylosidase in 50 mM potassium phosphate buffer (pH 7.5) with 1 mg/mL BSA, or with 2.4 mg NS22002 in 50 mM sodium acetate buffer (pH 6.0) in a total reaction volume of 20 μL each. Samples were incubated for 1 h at 35 °C (β-xylosidase) or 50 °C (An_BG and NS22002). Reactions were quenched by adding one sample volume of ice-cold methanol.

Characterization of β-glucosidase, exoglucanase, and endoglucanase activity. To assess the spectrum of potentially catalyzed reactions by the three different groups of cellulose degrading enzymes, two β-glucosidases, two exoglucanases, and three endoglucanases were incubated for 15 min with 0.02 mM CT-F17 in a total reaction volume of 25 μL. The used enzymes, buffers and temperatures are shown in Table S1†. All enzymes were used at their optimal reaction temperatures. Reactions were quenched by adding one sample volume of ice-cold methanol.

Environmental samples

Two commercially available compost samples and four soil samples collected at various sites in Berkeley, CA or Walnut Creek, CA were used: cow (COM) and chicken (CHM) manure, soil mixed with leafs from under an oak tree (OAK), clay-rich soil (CS), mixed organic compost (MOC), and a sample from light woodland (WS). These samples were manually fragmented with a hammer. Additionally two samples collected from two different green waste compost sites in northern California were included: Zamora (Z) and Jepson Prairie (JP). Zamora compost collection and processing has been described previously.[30] The latter compost sample was collected at the Jepson Prairie Organics facility (Vacaville, CA). This facility processes municipal green waste in turned and watered windrows. Compost was collected from 7, 30, and 60 day windrows. The 7 day windrow was in the mesophilic stage of composting and slightly warm to the touch. The 30 and 60 day windrows were in the thermophilic composting stage, hot to the touch, and steaming. The top 12 inches of each windrow was removed with a spade and the exposed biomass underneath was packed into 50 ml Falcon tubes, stored at room temperature during transport, and then frozen at −80 °C.

Cultivation and extraction of environmental samples

200 mg of small fragments (<3 mm³) of each of the eight environmental samples were used to inoculate 50 mL liquid cultures

containing 1 g of extracted switchgrass (washed exhaustively with water and ethanol in a soxhlet apparatus) in M9 minimal medium with trace elements (M9TE).[31] Cultures were incubated at 60 °C with shaking at 200 rpm for one week. Two mL of each sample was then collected: each sample was placed in a 2 mL tube, spun at 21,000 × g for 5 min, the supernatant containing secreted enzymes (secretome) was aliquoted to a new tube, and stored at 4 °C till further use in enzymatic activity assays.

The Jepson Prairie (JP) compost sample was further used to generate switchgrass-adapted thermophilic consortia. Briefly, JP was used to inoculate liquid cultures containing switchgrass as the sole carbon source and grown at 60 °C. The compost-derived microbial communities were allowed to adapt to switchgrass in liquid culture for a total of 32 weeks (16 × 2 week passages). 2 ml of passage #15 of the JP switchgrass-adapted culture were used to inoculate a culture containing 50 mL of M9TE and 0.5 g of microcrystalline cellulose (MC; Sigma-Aldrich; St. Louis, MO). This culture was incubated for two weeks at 60 °C with shaking at 200 rpm. Ten ml of the culture was placed into five 2 mL tubes, spun at 21,000 × g for 5 min, the supernatant (JP MC secretome) was collected and filtered through a 0.2 μm filter, and stored at 4 °C until used in enzyme assays. We found that feeding the switchgrass-adapted community MC induced the community to produce substantially more cellulase activity, while only marginally reducing hemicellulase activity. Therefore, adaptation to MC produces higher titers of both types of enzymes and since cellulases have a higher impact factor in biomass conversion technologies, we focused on samples with high cellulase activity.

Direct analysis of enzyme activity in environmental samples

Screening for β-glucosidase/β-xylosidase activity. For the detection of glycoside hydrolase activity a mixture of 0.02 mM CB-F17 and 0.02 mM XB-F17 were mixed with 75% (v/v) per environmental sample secretome in 50 mM sodium acetate buffer (pH 5.0). The total reaction volume was 30 μL. Samples were incubated for 1 h at 50 °C and reactions quenched by adding four reaction volumes of ice-cold methanol.

Enzymatic profiling of the JP MC environmental sample. For analysis of the activity profile of cellulose-degrading glucoside hydrolase(s) and β-xylosidase(s) of the JP MC sample a mixture of 0.006 mM CT-F17 and 0.01 mM XB-F17 were mixed with 42% (v/v) of the JP MC secretome. For determination of the pH profile reactions were carried out in McIlvaine's citrate/phosphate buffer (pH 3.0, 5.0, 7.0)[29] or 10 mM Tris buffer (pH 9.0). For determination of the ionic liquid (IL) tolerance 0%, 10%, 20%, or 30% (v/v) ethyl-3-methyl imidazolium (EMIM) acetate (Sigma-Aldrich; St. Louis, MO) were added to the sample in McIlvaine's citrate/phosphate buffer (pH 5.0).[29] All samples were incubated for 30 min at 50 °C and 80 °C. Reactions were quenched by adding one sample volume of ice-cold methanol.

Fabrication of NIMS chips

The production of NIMS chips has been described in extensive detail elsewhere.[32] In brief, a 4″ silicon wafer (single-sided polished P/Boron, orientation <1-0-0>, resistivity 0.01–0.02 Ω cm, thickness 525 ± 25 μm) obtained from Silicon Quest International (Santa Clara, CA) was cut into a 70 × 70 mm square and cleaned thoroughly with methanol, followed by anodic etching with 25% hydrofluoric acid in ethanol in a custom made Teflon etching chamber using extreme caution. A current of 2.4 A was applied for 15 min. After etching, chips were coated by adding 400 μL of the initiator liquid bis(heptadecafluoro-1,1,2,2-tetrahydrodecyl)tetramethyl-disiloxane (Gelest; Morrisville, PA) for 20 min. Excess initiator was blown off with nitrogen.

Nanostructure-initiator mass spectrometry (NIMS)

In each case 0.5–1 μL per quenched reaction sample was spotted onto the NIMS surface and removed after an incubation of ~30 s. A grid drawn manually on the NIMS chip using a diamond-tip scribe helped with spotting and identification of sample spots in the spectrometer. Chips were loaded using a modified standard MALDI plate. NIMS was performed on a 4800 MALDI TOF/TOF mass analyzer from Applied Biosystems (Foster City, CA). In each case signal intensities were identified for the ions of the used substrates and occurring products. Enzyme activities were determined by forming product-to-substrate ratios.

References

1 A. Helenius and M. Aebi, *Science*, 2001, **291**, 2364–2369.
2 R. H. Doi and A. Kosugi, *Nat. Rev. Microbiol.*, 2004, **2**, 541–551.
3 H. W. Blanch, P. D. Adams, K. M. Andrews-Cramer, W. B. Frommer, B. A. Simmons and J. D. Keasling, *ACS Chem. Biol.*, 2008, **3**, 17–20.
4 E. J. Steen, Y. Kang, G. Bokinsky, Z. Hu, A. Schirmer, A. McClure, S. B. Del Cardayre and J. D. Keasling, *Nature*, 2010, **463**, 559–562.
5 M. Pauly and K. Keegstra, *Curr. Opin. Plant Biol.*, 2010, **13**, 305–312.
6 L. R. Lynd, P. J. Weimer, W. H. van Zyl and I. S. Pretorius, *Microbiol. Mol. Biol. Rev.*, 2002, **66**, 506–577.
7 D. Shallom and Y. Shoham, *Curr. Opin. Microbiol.*, 2003, **6**, 219–228.
8 M. Dashtban, H. Schraft and W. Qin, *Int. J. Biol. Sci.*, 2009, **5**, 578–595.
9 K. R. Sharrock, *J. Biochem. Biophys. Methods*, 1988, **17**, 81–105.
10 T. R. Northen, O. Yanes, M. T. Northen, D. Marrinucci, W. Uritboonthai, J. Apon, S. L. Golledge, A. Nordstrom and G. Siuzdak, *Nature*, 2007, **449**, 1033–1036.
11 T. R. Northen, J. C. Lee, L. Hoang, J. Raymond, D. R. Hwang, S. M. Yannone, C. H. Wong and G. Siuzdak, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 3678–3683.
12 J. Su and M. Mrksich, *Angew. Chem., Int. Ed.*, 2002, **41**, 4715–4718.

13 L. Ban and M. Mrksich, *Angew. Chem., Int. Ed.*, 2008, **47**, 3396–3399.

14 S. H. Chang, J. L. Han, S. Y. Tseng, H. Y. Lee, C. W. Lin, Y. C. Lin, W. Y. Jeng, A. H. Wang, C. Y. Wu and C. H. Wong, *J. Am. Chem. Soc.*, 2010, **132**, 13371–13380.

15 W. Reindl and T. R. Northen, *Anal. Chem.*, 2010, **82**, 3751–3755.

16 S. Datta, B. Holmes, J. I. Park, Z. W. Chen, D. C. Dibble, M. Hadi, H. W. Blanch, B. A. Simmons and R. Sapra, *Green Chem.*, 2010, **12**, 338–345.

17 M. V. Deshpande, K. E. Eriksson and L. G. Pettersson, *Anal. Biochem.*, 1984, **138**, 481–487.

18 V. M. Chernoglazov, A. N. Jafarova and A. A. Klyosov, *Anal. Biochem.*, 1989, **179**, 186–189.

19 D. J. Coleman, M. J. Studler and J. J. Naleway, *Anal. Biochem.*, 2007, **371**, 146–153.

20 D. J. Coleman, D. A. Kuntz, M. Venkatesan, G. M. Cook, S. P. Williamson, D. R. Rose and J. J. Naleway, *Anal. Biochem.*, 2010, **399**, 7–12.

21 M. Dashtban, M. Maki, K. T. Leung, C. Mao and W. Qin, *Crit. Rev. Biotechnol.*, 2010, **30**, 302–309.

22 J. Gong and C. W. Forsberg, *J. Bacteriol.*, 1993, **175**, 6810–6821.

23 G. D. Wignall and F. S. Bates, *J. Appl. Crystallogr.*, 1987, **20**, 28–40.

24 B. Chu, *Laser light scattering: basic principles and practices*, Academic Press, Boston, 1991.

25 J. I. Park, M. S. Kent, S. Datta, B. M. Holmes, Z. Huang, B. A. Simmons, K. L. Sale and R. Sapra, *Bioresour. Technol.*, 2011, **102**, 5988–5994.

26 R. G. Gardner, J. E. Wells, M. W. Fields, D. B. Wilson and J. B. Russell, *Curr. Microbiol.*, 1997, **35**, 274–277.

27 S. R. Chhabra, K. R. Shockley, D. E. Ward and R. M. Kelly, *Appl. Environ. Microbiol.*, 2002, **68**, 545–554.

28 K. Eckert, F. Zielinski, L. Lo Leggio and E. Schneider, *Appl. Microbiol. Biotechnol.*, 2002, **60**, 428–436.

29 T. C. McIlvaine, *J. Biol. Chem.*, 1921, **49**, 183–186.

30 M. Allgaier, A. Reddy, J. I. Park, N. Ivanova, P. D'Haeseleer, S. Lowry, R. Sapra, T. C. Hazen, B. A. Simmons, J. S. VanderGheynst and P. Hugenholtz, *PLoS One*, 2010, **5**, e8812.

31 K. M. DeAngelis, J. M. Gladden, M. Allgaier, P. D'haeseleer, J. L. Fortney, A. Reddy, P. Hugenholtz, S. W. Singer, J. S. Vander Gheynst, W. L. Silver, B. A. Simmons and T. C. Hazen, *BioEnergy Res.*, 2010, **3**, 146–158.

32 H. K. Woo, T. R. Northen, O. Yanes and G. Siuzdak, *Nat. Protoc.*, 2008, **3**, 1341–1349.

33 A. Guinier and G. Fournet, *Small-angle scattering of X-rays*, Wiley, New York, 1955.