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Carbohydrate and lignin are simultaneously solubilized from unpretreated switchgrass by microbial action at high temperature†

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The three major components of plant biomass, cellulose, hemicellulose and lignin, are highly recalcitrant and deconstruction involves thermal and chemical pretreatment. Microbial conversion is a possible solution, but few anaerobic microbes utilize both cellulose and hemicellulose and none are known to solubilize lignin. Herein, we show that the majority (85%) of insoluble switchgrass biomass that had not been previously chemically treated was degraded at 78 °C by the anaerobic bacterium *Caldicellulosiruptor bescii*. Remarkably, the glucose/xylose/lignin ratio and physical and spectroscopic properties of the remaining insoluble switchgrass were not significantly different than those of the untreated plant material. *C. bescii* is therefore able to solubilize lignin as well as the carbohydrates and, accordingly, lignin-derived aromatics were detected in the culture supernatants. From mass balance analyses, the carbohydrate in the solubilized switchgrass quantitatively accounted for the growth of *C. bescii* and its fermentation products, indicating that the lignin was not assimilated by the microorganism. Immunoanalyses of biomass and transcriptional analyses of *C. bescii* showed that the microorganism when grown on switchgrass produces enzymes directed at key plant cell wall moieties such as pectin, xyloglucans and rhamnogalacturonans, and that these and as yet uncharacterized enzymes enable the degradation of cellulose, hemicellulose and lignin at comparable rates. This unexpected finding of simultaneous lignin and carbohydrate solubilization bodes well for industrial conversion by extremely thermophilic microbes of biomass that requires limited or, more importantly, no chemical pretreatment.

Broader context

The three major components of plant biomass are cellulose (a glucose polymer), hemicellulose (a polymer of xylose and a variety of other sugars) and lignin (a complex polymer of aromatic units). The sugar polymers are potential feedstocks for the production of biofuels by anaerobic microorganisms. However, plant biomass is highly recalcitrant and harsh and inefficient chemical treatments are required to solubilize the biomass and release the sugars. Moreover, no anaerobic microorganism is known that can degrade the highly recalcitrant lignin. Herein it is shown that switchgrass, a model plant for bioenergy production, can be degraded at moderate temperatures (78 °C) by an anaerobic bacterium that solubilizes lignin as well as cellulose and hemicellulose. The microorganism produces a range of both known and as yet uncharacterized enzymes that degrade at comparable rates all of the major components of the plant cell wall. Such thermophilic microbes could potentially be developed to enable the direct conversion of plant biomass to biofuels without the need for any chemical pretreatment.

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Introduction

Plant biomass is a potential renewable feedstock for biofuel production but its three major components, cellulose, hemicellulose and lignin, are highly recalcitrant and efficient deconstruction remains a major challenge.[1,2] The recalcitrance problem can be overcome to some extent by thermal and chemical pretreatments to solubilize the plant biomass and release cellulose and various soluble sugars, but such processes are generally expensive and not efficient.[3,4] Microbial conversion of biomass-derived carbohydrates from chemically pretreated biomass is a potential solution, but few anaerobic microbes utilize both cellulose and hemicellulose and none are known to solubilize lignin.[5] Nevertheless, the microbial conversion of the carbohydrates derived from chemically pretreated biomass to biofuels, termed consolidated bioprocessing (CBP), has been extensively investigated.[2,6,7] In particular, the use of anaerobic microorganisms at high temperature has several significant advantages over processes at ambient temperature, including reduced contamination issues, high metabolic activity and low cell biomass yields.[7,8] In terms of cellulose utilization, species of *Clostridia* have been well studied, particularly with regard to the cellulose-degrading cellulosome complex.[9,10] However, such species typically utilize a limited range of carbohydrates as growth substrates and degrade either cellulose or hemicellulose but not both.[11]

Clearly, breakthroughs in biomass conversion processes using chemical and/or biological approaches are needed if the potential of renewable plants as sources of biofuels is to be realized. One particularly promising avenue for overcoming biomass recalcitrance stems from our recent report that an anaerobic thermophilic bacterium, *Caldicellulosiruptor bescii*, can grow on plant biomass that has not been previously chemically pretreated to release the cellulose component.[12,13] Members of the genus *Caldicellulosiruptor* degrade a broad range of polysaccharides and represent the most thermophilic cellulose- and hemicellulose-utilizing organisms known,[13–21] although uncharacterized organisms were recently reported to be able to degrade cellulose at even higher temperatures.[22] *Caldicellulosiruptor* species are therefore prime candidates for efficient CBP approaches for cellulose and hemicellulose converion,[2,23] particularly in light of the recent report of a potential genetic system with *C. bescii*.[24]

Herein we focus on the cellulolytic and xylanolytic *C. bescii*, which grows to high cell densities at 78 °C on switchgrass. We have applied a spectrum of analytical and imaging technologies to evaluate structural and chemical changes in the plant biomass during the microbial degradation process, coupled with molecular analyses of the microorganism to gain insight into the types of enzyme involved in the deconstruction process. The unexpected finding is that all major parts of the biomass are solubilized simultaneously, including both the lignin and the carbohydrate components. The organism appears to overcome recalcitrance by exploiting the thermally induced changes in the plant material using enzymes directed at key plant cell wall groups and specifically rhamnogalacturonans, arabinogalactans and homogalacturonans.

Experimental

Growth of microorganism

C. bescii DSM 6725 (formerly *Anaerocellum thermophilum* strain DSM 6725) was obtained from the DSMZ (http://www.dsmz.de/index.htm).[13] Untreated switchgrass (Alamo, sieved 20/80-mesh fraction) was obtained from Dr Brian Davison, Oak Ridge National Laboratory, Oak Ridge, TN. The switchgrass was washed by shaking (150 rpm) in hot water (78 °C) for 18 h. The insoluble material was filtered using filtering crucibles with 40–60 μm porosity, washed with 78 °C water, and then dried to give the washed unspent material (wSG). *C. bescii* was grown in modified 516 medium with hot water washed switchgrass (wSG) or spent versions of it (see below) at a concentration of 0.5% (w/v) as the primary carbon and energy sources as described previously.[12] The medium also contained yeast extract (0.05% w/v). To investigate biomass conversion, all controls and cultures were incubated at 78 °C with shaking (150 rpm) for 5 days unless otherwise stated. The cells were removed by filtering and the residual biomass was dried as described above and used where indicated as the carbon and energy sources for a subsequent *C. bescii* culture. The conversion of the switchgrass was calculated based on weight before and after incubation with correction on moisture content as described previously.[12]

Analytical pyrolysis of switchgrass

The biomass samples (~4 mg) were placed into 80 μL stainless steel sample cups of a commercially available auto sampler of double shot pyrolyzer (PY-2020iD, Frontier Ltd). They were then pyrolyzed at 500 °C with helium as the carrier gas and an interface temperature of 350 °C. Each pyrolysis reaction was completed in 1.2 min with a total pyrolysis time of 2 min. The residues were analyzed using a custom built Super Sonic Molecular Beam Mass Spectrometer (Extrel Model MAX-1000) that had been modified by the addition of the auto sampler. Mass spectral data from m/z 30–450 were acquired on Merlin Automation Data System version 3.3. Multivariate analysis was performed using Unscrambler software version 10.1 (CAMO). The intensities of the lignin peaks were summed and averaged in order to estimate the lignin contents in the sample.[34] Syringyl to guaiacyl (S/G) ratios were also determined and lignin values were corrected to approximate Klason lignin values provided by switchgrass standards from NREL.

Analysis of soluble aromatics

Cultures were centrifuged and filtered through fritted glass. High molecular-weight fractions were prepared by using Centriprep devices (Millipore, Ireland) with 50 kDa cut-off filters. Acetyl bromide-soluble lignin analysis was performed as previously described.[35] The switchgrass samples were filtered (40–60 μm porosity) and the resulting cell- and biomass-free supernatant samples were stored at −80 °C until analyzed for aromatic compounds by GC-MS following trimethylsilylation (TMS).[36] Aromatic compounds associated with lignin were targeted for analysis and relative quantitation. A user-defined database of electron impact ionization fragmentation patterns

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of TMS-derivatized metabolites (>1800 signatures) enabled identification of aromatic metabolites and characteristic mass-to-charges (m/z) were used for their subsequent quantitation.

Carbohydrates and lignin analysis of residual switchgrass

Samples for carbohydrate and acid-insoluble lignin analysis were prepared from milled switchgrass using a two-stage acid hydrolysis protocol based on TAPPI methods T-222 om-88 with a slight modification.[37] The first stage utilized a severe pH and a low reaction temperature (72 vol% H_2SO_4 at 30 °C for 1 h). The second stage was performed at much lower acid concentration and higher temperature (3 vol% H_2SO_4 at 121 °C for 1 h) in an autoclave. The resulting solution was cooled to room temperature and filtered using G8 glass fiber filter (Fisher Scientific, USA). The remaining residue was oven-dried and weighed to obtain the Klason lignin content. The filtered solution was analyzed for carbohydrate constituents of the hydrolyzed biomass samples determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using Dionex ICS-3000 (Dionex Corp., USA).

Carbon balance

A 10 L culture of *C. bescii* was grown on 5 g L^{-1} wSG in a 20 L fermenter with pH control (pH 7.0), continuous mixing and removal of the gas headspace. The culture was harvested in the late stationary growth phase after 152 h after acid production stopped. Lactic acid was determined by using the Megazyme l-lactic assay kit (Megazyme, Wicklow, Ireland). Acetate was determined by high-performance liquid chromatography (HPLC) on a model 2690 separations module (Waters, Milford, MA) equipped with an Aminex HPX-87H column (300 mm by 7.8 mm; Bio-Rad, Hercules, CA) and a photodiode array detector (Model 996; Waters).

Solid state NMR

Holocellulose was isolated from Wiley-milled biomass. The resulting sawdust was treated with $NaClO_2$ (1.30 g per 1.00 g lignocellulosic dry solids) in acetic acid (375 mL of 0.14 M) at 70 °C for 2 h. The samples were then collected by filtration and rinsed with an excess of DI filtered water. This procedure was repeated to ensure complete removal of the lignin component. Isolated cellulose was prepared from the holocellulose (1.00 g) by hydrolysis for 4 h in HCl (100.0 mL of 2.5 M) at 100 °C. The isolated cellulose was then collected by filtration and rinsed with an excess of DI filtered water, and dried in the fume hood. Solid-state NMR measurements were carried out on a Bruker Avance-400 spectrometer operating at frequencies of 100.55 MHz for ^{13}C in a Bruker double-resonance MAS probe head at spinning speeds of 10 kHz. CP/MAS experiments utilized a 5 μs (90°) proton pulse, 2.0 ms contact pulse, 4 s recycle delay and 4–8 K scans.

Gel permeation chromatography (GPC) of cellulose

Isolated cellulose was prepared from the holocellulose sample (1.00 g) by extraction with a 17.5% NaOH solution (50.0 mL) at 25 °C for 30 min. 50 mL of deionized filtered water was then added to the NaOH solution. The extraction was continued with the 8.75% NaOH solution (100 mL) at 25 °C for an additional 30 min. The isolated α-cellulose samples were then collected by filtration and rinsed with 50 mL of 1% acetic acid, an excess of deionized filtered water, and air-dried. The number-average molecular weight (M_n) and weight-average molecular weight (M_w) were determined by GPC after tricarbanilation of cellulose.[38]

Stimulated Raman microscopy

The stimulated Raman scattering images each containing two channels (cellulose and lignin channel) were loaded in a user-written Matlab program. The cell wall regions were chosen by the user and the program then created a binary mask image that specified the cell region and the background region.[26] The average background value was calculated and subtracted, and the intensities distribution was built upon the intensities on the cell wall region. Percentage values are calculated based on pixel intensity of whole image.

Glycome profiling and quantitation of ELISA data

Switchgrass samples were subjected to sequential extraction to give soluble fractions that are enriched in particular constituents (given in parentheses): (1) ammonium oxalate (xyloglucans, pectic polysaccharides, arabinogalactans), (2) sodium carbonate (branched pectic polysaccharides, arabinogalactans), (3) 1 M KOH (xyloglucans, xylans, pectic arabinogalactans), (4) 4 M KOH (xyloglucans, xylans, pectic arabinogalactans), (5) sodium chlorite (delignification, pectic polysaccharides), and (6) post chlorite 4 M KOH.[39] The residual pellets were not further analyzed. All of the extracts were dialyzed against four changes of 4 L de-ionized water, lyophilized, dissolved in de-ionized water and total sugars were determined using the phenol–acid method.[40] All extracts were diluted to the same sugar concentration (60 μg sugar per well) and loaded onto 96-well ELISA plates (Costar 3598) at 50 μl per well. The extracts were evaporated to dryness at 37 °C overnight. ELISAs were performed as described[28] using a series of 149 monoclonal antibodies (AB) directed against different plant cell wall glycan epitopes (Table S3†). ELISA data are presented as a heat map in which the antibody order is based on a hierarchical clustering analysis of the antibody collection that groups the antibodies according to their binding patterns to a panel of diverse plant glycans.[28]

Preparation of alcohol insoluble residue (AIR) cell wall and glycosyl residue composition

Each individual sample was ground under liquid nitrogen with a mortar and pestle and the ground materials were extracted with 80% (v/v) ethanol, 100% ethanol and chloroform : methanol (1 : 1 [v/v]). After centrifugation the AIR was washed with 100% acetone, dried and were treated with thermostable α-amylase (Sigma-Aldrich; 7 units per mL) in 0.1 M ammonium formate (pH 6.0) and 0.02% sodium azide for 48 h at room temperature with constant rotation to remove starch.

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The AIR (cell walls) were subjected to sequential fractionation as described above in glycome profiling. Both total AIR and five fractionated samples from the AIR were used for monosaccharide composition analysis as previously described.[41] The glycosyl residue composition analysis was performed by combined GC-MS of the per-*O*-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the sample by acid methanolysis.[41]

Transcriptional analysis

C. bescii cultures were grown using glucose wSG as the carbon sources. Cells were harvested at the end of exponential growth.[12] Purified RNA samples were converted to fluorescence-labeled cDNA and hybridized to a whole-genome *C. bescii* microarray according to the standard procedures previously described.[42] A spotted whole-genome DNA microarrays was designed for *C. bescii* based on the 2662 protein-coding sequences in the genome. Oligonucleotide probe sequences (60-mers) for each gene were generated using Oligoarray 2.0 and synthesized by Integrated DNA Technologies (Coralville, IA). Arrays were constructed as described previously[43] with five replicates of each probe represented.

Results and discussion

Growth of *C. bescii* on unpretreated switchgrass

C. bescii grows at 78 °C to high cell densities (>10^8 cells per mL) using unpretreated switchgrass (SG) as the sole carbon and energy sources.[12] Although the microorganism's growth medium also contains yeast extract (as a source of cofactors), this supports only minimal growth (Fig. S1, see ESI†). Similar high cell densities were obtained when the organism grew on SG that had been washed with hot water at the growth temperature of the organism (78 °C for 18 h, to give wSG) to remove readily solubilized organic material (sugars, proteins, *etc.*) that might be present within the biomass and serve as growth substrates. Hence, *C. bescii* grows well on insoluble plant biomass. To investigate how the biomass is degraded, *C. bescii* was grown on wSG and the resulting insoluble spent biomass from the first pass, SG1, was collected and used as a growth substrate for a second *C. bescii* culture, thereby generating a second batch of insoluble spent biomass, SG2. SG2 was in turn used to support the growth of a third *C. bescii* culture to produce SG3 (Fig. S2, see ESI†). The wSG biomass was also subjected to three successive incubations at 78 °C in the same medium, but in absence of *C. bescii*, to generate the corresponding insoluble control samples, SG1c, SG2c and SG3c, respectively. The amounts of switchgrass converted by the high temperature treatments, with and without *C. bescii*, are summarized in Fig. 1A. Approximately 85% of wSG was solubilized by *C. bescii* after three incubations at 78 °C (yielding SG3), compared to 17% in the control lacking *C. bescii* (yielding SG3c). Successive high temperature treatments alone, therefore, abiotically solubilize a certain amount of the plant material (17%), but not nearly to the extent observed after incubation with *C. bescii* (85%).

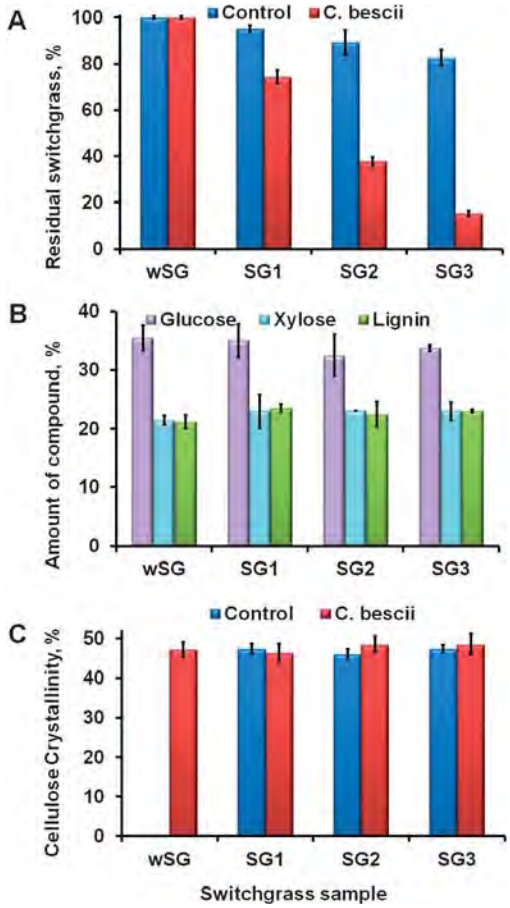

Fig. 1 Switchgrass (SG) conversion by *C. bescii*. (A) Residual biomass remaining (%, w/w) after a hot water wash (wSG) and after three consecutive *C. bescii* treatments (SG1, SG2 and SG3) and corresponding controls treated in the absence of the organism (SG1c, SG2c and SG3c). (B) Amounts (%, w/w) of glucose, xylose and lignin in the same samples of residual biomass determined by analytical pyrolysis. (C) Crystallinity of cellulose in the same samples of residual biomass determined by solid state NMR.

Lignin analyses

SG contains (by weight) approximately 33% cellulose, 26% hemicellulose and 20% lignin.[25] No anaerobic microbe is known that can metabolize polyaromatic lignin. Consequently, it was expected that the relative lignin content of the insoluble switchgrass biomass would dramatically increase after each successive *C. bescii* treatment, and that the final residual biomass (SG3), representing only 15% of the initial switchgrass sample used for microbial conversion (wSG), would be predominantly lignin. Remarkably, however, as shown in Fig. 1B, for all microbially treated samples, including SG3, the ratio of the glucose, xylan and lignin contents of the residual biomass did not change significantly. *C. bescii*, therefore, solubilized lignin at the same rate that it rendered soluble the carbohydrate components of switchgrass.

Imaging of the lignin in the unpretreated (SG) and washed (wSG) samples by Stimulated Raman Scattering (SRS,[26]), before and after treatment with *C. bescii* (SG1), showed no dramatic changes in lignin content or its corresponding plant cell wall morphology, although the data indicated that in the presence of

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the microbe there was more lignin reduction in low-lignin-content cell walls and less reduction in high-lignin-content walls (Fig. S3, see ESI†). Similarly, pyrolysis molecular beam mass spectrometry revealed that the peak intensities of specific lignin components did not vary significantly between any of the residual SG samples (Table S1, see ESI†).

The material that was solubilized by incubating *C. bescii* with wSG was assayed for acetyl bromide-soluble lignin. The analysis of high molecular-weight preparations (>50 kDa) revealed that some lignin was released from wSG without *C. bescii*, but more than double the amount was released when it was incubated with the microorganism (Fig. S4, see ESI†). Furthermore, gas chromatography-mass spectrometry (GC-MS) of the soluble material, following trimethylsilylation, revealed numerous lignin-related monomeric aromatic compounds at significantly increased concentrations in the supernatants after incubation with *C. bescii* compared with the abiotic controls. After short incubation (24 h), these were identified as the monolignols coniferyl and sinapyl alcohols, related degradation products such as guaiacylglycerol and syringylglycerol, and phenolic acids, including ferulic, coumaric, sinapic and caffeic acids (Fig. 2A). It is evident that a degree of solubilization of the cell walls occurred without *C. bescii* as both coniferyl alcohol and a syringylglycerol glycoside (the nature of the glycoside was not determined) are present after 24 h at greater concentrations than those observed in the biomass samples with microbial treatment. The syringylglycerol concentrations differed the most between the biotic and abiotic treatments, suggesting that the microorganism cleaved the aforementioned glycoside for subsequent sugar catabolism, resulting in a higher concentration of the aglycone moiety. After a long term incubation (240 h), the difference in syringylglycerol glycoside concentrations between the two treatments was not statistically significant (0.34 ± 0.06 μg mL^{-1} in abiotic control *vs.* 0.27 ± 0.15 μg mL^{-1} in microbe-treated samples, $P = 0.73$; data not shown in Fig. 2B).

The bulk of the aromatic constituents remaining in solution after a long term incubation were phenolic acids but greatest

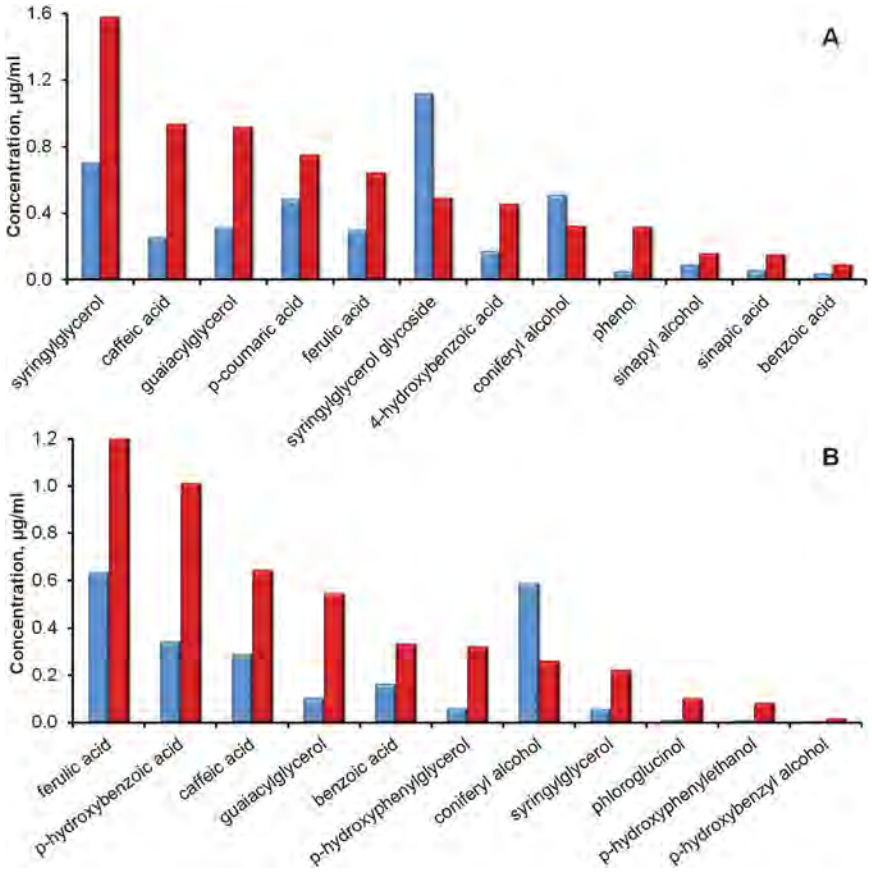

Fig. 2 Release of aromatic constituents by *C. bescii*. Soluble aromatic compounds released from switchgrass after (A) 24 h and (B) 240 h incubation at 78 °C with (red bars) and without (blue bars) *C. bescii*. The aromatic metabolites are listed in order of descending abundance as observed in the *C. bescii*-treated samples, as determined by gas chromatography-mass spectrometry using electron impact ionization, following trimethylsilylation of sample extracts. The targeted aromatic metabolite peaks were integrated using a key selected mass-to-charge (m/z) ratio to minimize integrating co-eluting metabolites. Extracted peaks were quantified by area integration and the areas scaled back up to the total ion current using scaling factors for each metabolite. The concentrations were normalized to the quantity of the internal standard (sorbitol) recovered, amount of sample extracted, derivatized and injected. The mean concentrations (μg mL^{-1} sorbitol equivalents) of five replicates per treatment (4 replicates for the control treatment at 240 h) are shown. All treatment differences are statistically significant at $P \leq 0.05$, as determined by Student's t-tests. After 240 h, the concentrations of gallic acid (4.60 μg mL^{-1} in *C. bescii*-treated samples and 3.16 μg mL^{-1} in control) and *p*-coumaric acid (1.37 and 1.06 μg mL^{-1}, respectively) were not significantly different in the two sample types.

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fold-changes in concentration relative to the abiotic control were observed in aromatic alcohols, identified as *p*-hydroxyphenylethanol (>8-fold), phloroglucinol (>7-fold), guaiacylglycerol and *p*-hydroxyphenylglycerol (both >5-fold), and syringylglycerol and *p*-hydroxybenzyl alcohol (both >3-fold: Fig. 2B). There were smaller (2–3-fold) albeit significant increases in the concentrations of *p*-hydroxybenzoic acid, caffeic acid, benzoic acid, and ferulic acid (Fig. 2B). Gallic acid and *p*-coumaric acid were also identified but their concentrations were not significantly different after the abiotic and microbial treatments (Fig. 2B). It should also be noted that unchanged monolignols are released, albeit at low concentrations, from both biotic and abiotic treatments. Hence, as illustrated in Fig. 2, we conclude that under all conditions the same components were present in the non-microbial abiotic control but typically at much lower concentrations, suggesting that *C. bescii* accelerates the release of the same compounds that are slowly released by abiotic thermal degradation.

Mass balance analysis

To determine if *C. bescii* utilizes the solubilized lignin as carbon and energy sources, the bacterium was grown on wSG and the disposition of substrates and products was determined (Fig. 3). About one-third of the plant biomass (36%) was rendered soluble, thereby releasing both C_6- (equivalent to 30.0 mM carbon) and C_5-based (20.9 mM carbon) sugars. These together with minor amounts of sugar from the yeast extract (2.4 mM carbon) account for 53 mM carbon, which are potential growth substrates for *C. bescii*. This 53 mM total could be quantitatively accounted for in the material balance as products at the end of cell growth in the form of lactate and acetate generated by fermentation, acetate derived from acetylated sugars in the switchgrass, carbon dioxide, soluble carbohydrates present in the growth medium, and *C. bescii* biomass (Fig. 3). The carbohydrate contained in the switchgrass that was solubilized, therefore, appears to be sufficient to support the observed growth of *C. bescii*. Consequently, we conclude that significant amounts of the solubilized lignin-derived aromatic material described above were not assimilated by the microorganism and used as growth substrates.

Carbohydrate analyses

Imaging of the residual plant biomass after *C. bescii* treatment by SRS revealed that the cellulose component was not significantly perturbed by the microbial solubilization process (Fig. S5, see ESI†). Compared with SG, the cellulose content was reduced both in wSG and SG1 and the cellulose distribution in the different cell walls remained essentially unchanged, although *C. bescii* appeared to utilize more cellulose in low-lignin-content walls with less deconstruction in high-lignin-content walls (Fig. S5, see ESI†). The degree of crystallinity within cellulose fibrils determined by NMR (Fig. 1C) also revealed no significant changes throughout any of the treatments. This was the case after washing the switchgrass with hot (78 °C) water (to give wSG), and after three consecutive treatments of wSG, with or without *C. bescii*. Different types of

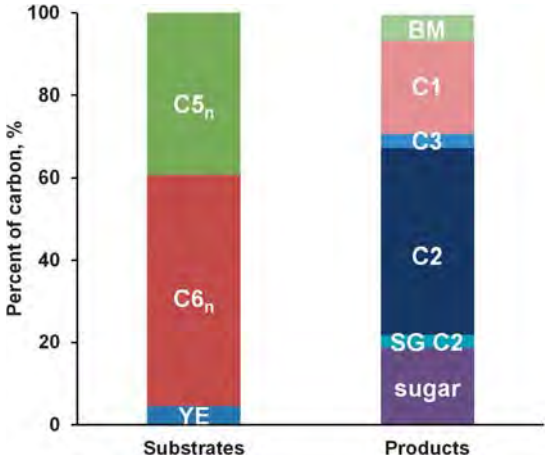

Fig. 3 Mass balance of the solubilized and assimilated carbon. The results are expressed in terms of percentage of total C_1 units solubilized from washed switchgrass (wSG, 5.0 g L^{-1}) by *C. bescii*. The substrates include the cellulose (45.2%, $(C_6)_n$ or 30.0 mM carbon, including glucose, galactose and mannose; see Fig. S9†) and hemicellulose (30.8%, $(C_5)_n$ or 20.9 mM carbon, including xylose and arabinose) present in the switchgrass that were solubilized (1.79 g L^{-1}) together with the carbohydrate (0.4 mM glucose equivalents, or 2.4 mM carbon) available from the yeast extract (containing 132 mg of carbohydrate per g, taken as C_6 sugars). The products include *C. bescii* biomass (BM), acetate (C_2), lactate (C_3), acetate derived from acetylated sugars in the switchgrass (SG C_2), carbon dioxide (C_1), and the soluble carbohydrate measured in the growth medium (sugar). The carbon in the products was calculated as follows: BM, calculated from the measured protein concentration of 41.2 μg mL^{-1}, assuming that 50% (w/w) of the cell content is protein and 50% of the dry weight is carbon; C_2; where the amount of acetate measured (25.9 mM carbon) is assumed to be derived from both fermentation (24.2 mM carbon) and the deacetylation of acetylated hemicellulose (1.7 mM carbon, or 2.9% dry weight of the switchgrass); C_3 (1.7 mM carbon, measured as lactate); C_1 (12.1 mM carbon as CO_2, which was not measured but an acetate/CO_2 ratio of unity was assumed); sugar, measured as 10.0 mM carbon.

cellulose allomorphs and amorphous regions have been shown to be hydrolyzed to a different extent depending on the cellulase enzymes,[27] but from the properties of the cellulose in the various SG samples, it is clear that *C. bescii* effectively solubilizes the complete cellulose fibril within the switchgrass. The molecular weight distribution of cellulose (Fig. S6, see ESI†) and its degree of polymerization (Fig. S7, see ESI†) were also not significantly different after any of the microbial treatments. Consequently, stacked ^{13}C cross polarization magic angle spinning (CP/MAS) NMR revealed only minor changes in the relative proportion of components and chemical nature in all SG samples (Table S2 and Fig. S8, see ESI†). For example, the hemicellulose acetate content decreased with increasing number of heat treatments (Fig. S8†). These results are therefore consistent with highly similar amounts of the major and minor carbohydrates in all samples, including arabinose, mannose and galactose, in addition to glucose and xylose (Fig. 1B, S9 and S10, see ESI†).

Taken together, the results of the cellulose and lignin analyses clearly show that *C. bescii* is able to dramatically accelerate the abiotic thermal processes and cause the simultaneous solubilization of lignin, hemicellulose and cellulose in such a manner that the ratios of these major cell wall components in

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the residual biomass remain unchanged, even after 85% of the plant material has been solubilized.

Glycan immunoanalyses

To obtain insight into how *C. bescii* solubilizes plant biomass, monoclonal antibodies (mAbs) against various plant cell wall glycan epitopes were used to reveal fine changes in structure and composition of the biomass not detectable by other methods.[28,29] The toolkit of 149 mAbs included those against xyloglucans, rhamnogalacturonans, arabinogalactans, galactomannans, polygalacturonans and xylans (Table S3, see ESI†). Each insoluble biomass sample resulting from both microbial and abiotic treatments of SG was sequentially extracted by six chemical treatments of increasing harshness to give six soluble fractions (Fig. S10 and Table S4, see ESI†). The extent of binding of each mAb to each of the solubilized extracts is shown in Fig. 4 (OD range 0 to >1.3, black to yellow). The similarity in the vertical banding patterns for a given extraction method demonstrates that virtually the same set of glycans is extracted in a similar amount regardless of how many treatments with *C. bescii* the biomass had undergone. Moreover, similar groups of glycans are chemically extracted from the residual biomass after each successive hot water treatment without *C. bescii* (compare wSG, SG1c, SG2c and SG3c; Fig. 4) and after each microbial treatment (compare SG1–SG3 and cSG1–cSG3; Fig. 4).

To gain more specific information about which glycan epitopes are primarily affected by heat and/or *C. bescii* incubation, the amounts of each epitope extracted were calculated as a ratio for a given pair of treatments for all chemical extraction methods used (Table S4, see ESI†). While such ratios are characteristically semi-quantitative, there was a significant increase in the extractability of 28 epitopes in the presence of *C. bescii*, 14 of which also increased simply after the high temperature treatment without the organism (Table S5, see ESI†). A combination of *C. bescii* and high temperature, therefore, results in the more facile extractability particularly of pectins (pectic arabinogalactans, RG-1/AG epitopes), homogalacturonan and arabinogalactans (AG-4 epitopes), together with some xylans (xylan-2 and -3 epitopes) and xyloglucans. While these components are present in relatively small quantities (<5%, w/w) in switchgrass,[30] they are apparently important in maintaining the structural integrity of the biomass and contribute to its recalcitrance.

Microbial transcriptional analyses

The increased susceptibility to chemical extraction of specific glycan epitopes suggested that they are likely targets for enzymatic action during switchgrass degradation by the microorganism. To gain insight into such enzymes, genes specifically up-regulated when *C. bescii* grows on switchgrass were identified by a whole genome transcriptomics analysis. Only 94 of the 2666 genes in the *C. bescii* genome[18] were up-regulated more than 4-fold during growth on switchgrass compared to growth on glucose, suggesting that their protein products were directly or indirectly involved in biomass degradation (Table S6, see ESI†). Of the up-regulated genes, 18 were predicted to be involved in carbohydrate metabolism, 16 in membrane transport, 6 in oxidative stress, 5 in cell signaling processes, 25 were grouped as miscellaneous, and 20 were annotated as hypothetical.[18] The most-highly up-regulated genes included those encoding twelve carbohydrate-active enzymes (CAZy,[31] Table 1)

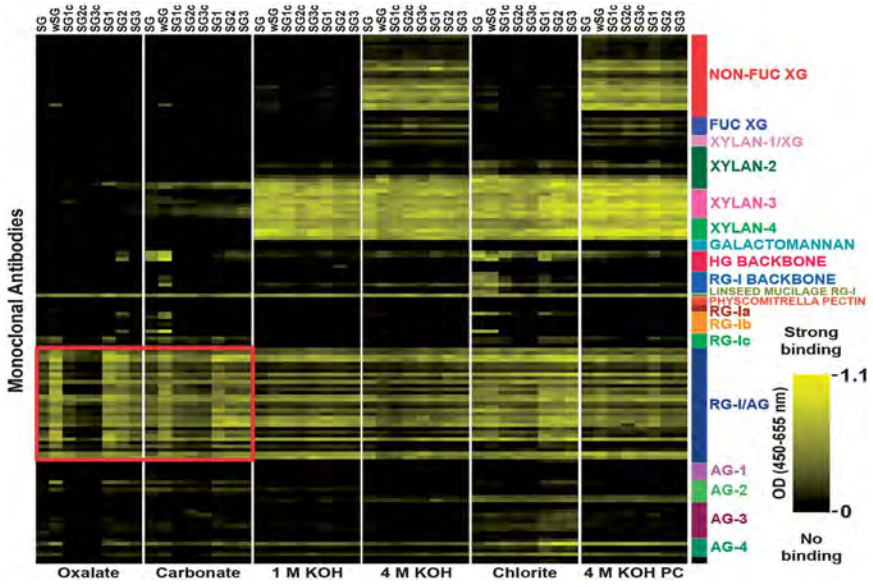

Fig. 4 Glycome profiling of sequential extracts prepared from wSG, SG1–SG3 and SG1c–SG3c switchgrass samples. The presence of cell wall glycan epitopes in each extract was determined by ELISA assays using 149 glycan-directed monoclonal antibodies (mAbs).[28] The data are presented as a heat map. The yellow-black scale indicates the strength of the ELISA signal: bright yellow depicts highest binding and black indicates no binding. The chemical treatments used for extracting the switchgrass biomass are indicated at the bottom of each column. The panel on the right lists the mAbs used grouped according to their glycan binding specificity (Table S3 contains a detailed listing, see ESI†). The red box indicates an example of the banding patterns referred to in the text.

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Table 1 Genes encoding carbohydrate-active enzymes (CAZy;[31]) related to plant biomass degradation that are up-regulated during growth of *C. bescii* on washed switchgrass (compared to growth on glucose). The complete list of up-regulated genes is given in Table S6.† The extent of up-regulation is indicated by the fold-change. The gene annotation indicates the predicted catalytic activity of the gene product based on the type(s) of CAZy module(s) that the enzyme contains. The abbreviations are: PL, polysaccharide lyase; CBM, carbohydrate-binding module; CBM_4_9, putative pectin-binding module; GH, glycoside hydrolase. All genes are predicted to encode extracellular or membrane-associated enzymes with the exception of those with an asterisk, which are predicted to lack signal peptide and encode cytoplasmic enzymes. Superscripts 1 and 2 indicate different catalytic modules present in the same protein

Gene	Predicted catalytic activity	CAZy modular structure	Up-regulation (fold change)
Pectin-degrading enzymes			
Cbes_1853	Rhamnogalacturonan lyase	PL11–CBM3	9.6
Cbes_1854	Pectate lyase	CBM_4_9–PL3	51.0
Cbes_1855	Pectate lyase, exopolygalacturonate lyase	CBM_4_9–PL9	27.9
Cbes_2353*	Pectate lyase, unclassified	Unknown	4.8
Xylan- and cellulose-degrading enzymes			
Cbes_0618	*endo*-1,4-β-Xylanase, *endo*-1,3-β-xylanase	CBM22–CBM22–GH10	4.5
Cbes_0183	*endo*-1,4-β-Xylanase, *endo*-1,3-β-xylanase	CBM22–CBM22–GH10	13.7
Cbes_0184*	β-Xylosidase	GH39	17.3
Cbes_0187*	β-Xylosidase	GH39	5.8
Cbes_1857	[1]*endo*-1,4-β-Xylanase, *endo*-1,3-β-xylanase [2]Cellobiohydrolase, endo-processive cellulase	$GH10^1$–CBM3–CBM3–$GH48^2$	3.9
Cbes_1865	[1]Endoglucanase, cellobiohydrolase, β-glucosidase [2]β-Mannosidase, cellulase, *endo*-β-1,4-xylanase	$GH9^1$–CBM3–CBM3–CBM3–$GH5^2$	22.8
Cbes_1866	β-Mannosidase, cellulase, *endo*-β-1,4-xylanase, xyloglucan-specific *endo*-β-1,4-glucanase	GH5–CBM3–CBM3–CBM3–GH5	6.6
Cbes_1867	[1]Endoglucanase, cellobiohydrolase, β-glucosidase [2]Cellobiohydrolase, endo-processive cellulase	$GH9^1$–CBM3–CBM3–CBM3–$GH48^2$	2.3
Cbes_0234*	β-Mannosidase, cellulase, *endo*-β-1,4-xylanase, xyloglucan-specific *endo*-β-1,4-glucanase	GH5	17.3

that are predicted to bind to and to hydrolyze cellulose, xylan, xyloglucan and mannan. They also include four polysaccharide lyases (Table 1) that, remarkably, are predicted to attack the types of pectin whose epitopes showed increased extractability during switchgrass degradation (Table S5, see ESI†). Presumably, some of these more extractable glycans are recognized by *C. bescii*, leading to up-regulation of CAZy-related genes. These data, therefore, suggest a direct relationship between the polysaccharide lyases and the degradation of rhamnogalacturonan I and homogalacturonan. Furthermore, they support the notion that specific pectin components are major recalcitrance factors, and that their hydrolysis facilitates biomass degradation by *C. bescii*. Homogalacturonans localized at cell–cell interfaces and cell corners (Avci and Hahn, unpublished results) and de-methyl-esterified forms[32] play key roles in tissue stabilization and wall access. Similarly, homogalacturonan and also rhamnogalacturonan are components of a recently characterized plant cell wall proteoglycan that contains pectin and xylan covalently attached to a core arabinogalactan protein, and it has been suggested that this structure may impact strategies to overcome plant cell wall recalcitrance.[33] These various glycan and also protein components are all potential targets of the *C. bescii* enzymes encoded by the up-regulated genes.

These results therefore demonstrate that *C. bescii* specifically up-regulates genes encoding key enzymes that degrade both major and minor components of plant cell walls, and these must also include as yet uncharacterized enzymes that ultimately lead to the solubilization of lignin. Such genes are presumably annotated in the *C. bescii* genome as putative esterases or hypothetical proteins. Interestingly, many genes encoding uncharacterized proteins were up-regulated more than 15-fold during growth of *C. bescii* on switchgrass and several are predicted to be membrane-bound or extracellular, which would enable them to attack the insoluble lignin (Table S6, see ESI†). These as yet unknown enzymes in concert with the CAZy enzymes (Table 1) must simultaneously deconstruct the lignin, cellulose and hemicellulose within the cell walls, as well as hydrolyzing the pectins, which may well be the key to overcoming biomass recalcitrance. The results presented here, therefore, strongly support further initiatives using anaerobic, cellulolytic and extreme thermophilic microbes for the efficient deconstruction of plant biomass to biofuels, and bode well for industrial conversion by extremely thermophilic microbes of biomass that requires limited, or more importantly even no, pretreatment.

Conflict of interest

The authors declare no conflict of interest.

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