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ORIGINAL PAPER

Deconstruction of plant biomass by a *Cellulomonas* strain isolated from an ultra-basic (lignin-stripping) spring

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Abstract

Plant material falling into the ultra-basic (pH 11.5–11.9) springs within *The Cedars*, an actively serpentinizing site in Sonoma County, California, is subject to conditions that mimic the industrial pretreatment of lignocellulosic biomass for biofuel production. We sought to obtain hemicellulolytic/cellulolytic bacteria from *The Cedars* springs that are capable of withstanding the extreme alkaline conditions wherein calcium hydroxide-rich water removes lignin, making cell wall polysaccharides more accessible to microorganisms and their enzymes. We enriched for such bacteria by adding plant debris from the springs into a synthetic alkaline medium with ground tissue of the biofuel crop switchgrass (*Panicum virgatum* L.) as the sole source of carbon. From the enrichment culture we isolated the facultative anaerobic bacterium *Cellulomonas* sp. strain FA1 (NBRC 114238), which tolerates high pH and catabolizes the major plant cell wall-associated polysaccharides cellulose, pectin, and hemicellulose. Strain FA1 in monoculture colonized the plant material and degraded switchgrass at a faster rate than the community from which it was derived. Cells of strain FA1 could be acclimated through subculturing to grow at a maximal concentration of 13.4% ethanol. A strain FA1-encoded β-1, 4-endoxylanase expressed in *E. coli* was active at a broad pH range, displaying near maximal activity at pH 6–9. Discovery of this bacterium illustrates the value of extreme alkaline springs in the search for microorganisms with potential for consolidated bioprocessing of plant biomass to biofuels and other valuable bio-inspired products.

Keywords Biofuel · Bioprospecting · Lignocellulose degradation · Serpentinization · Endoxylanase

Introduction

The structural polysaccharides cellulose and hemicellulose are shielded from degradation by the lignin matrix within the plant cell wall. Treatment with alkaline solutions, including calcium hydroxide, removes lignin thereby giving deconstructing enzymes access to these cell wall polysaccharides (Mosier et al. 2005). Microorganisms that could hydrolyze polysaccharides and ferment the resulting sugars and oligosaccharides

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under such lignin-stripping alkaline conditions are desirable candidates for consolidating the bioprocessing of plant biomass to biofuels and other valuable products because they would obviate the need for pH adjustment following the pretreatment step. Along this rationale, we sought to isolate such organisms from the calcium hydroxide-rich ultra-basic springs found within *The Cedars* (Sonoma County, California, USA), a rare environment undergoing active serpentinization (Barnes and O'Neil 1969; Morrill et al. 2013).

Most studies of biomass processing to biofuel primarily focus on the conversion of cellulose, since *Saccharomyces cerevisiae*, the primary commercial microorganism for production of ethanol, ferments solely glucose among the monosaccharides that compose the cell wall structural polysaccharides cellulose and hemicellulose (Liu and Qureshi 2009). However, the biofuel crop *Panicum virgatum* L. (switchgrass), for example, contains on average 33.0% cellulose and 26.0% hemicellulose (Keshwani and Cheng 2009). Thus, the energy extraction strategies that only target cellulose waste a substantial portion of the biomass energy embodied in hemicellulose. Several microorganisms, including bacteria of the genus *Cellulomonas*, have the metabolic capacity to degrade both cellulose and hemicellulose. Furthermore, *Cellulomonas* species are facultative anaerobes, having the flexibility to grow via aerobic or anaerobic respiration or fermentatively, producing a variety of end products, including ethanol (Stackebrandt and Schumann 2015).

The enzymes that hydrolyze large polymers like hemicellulose must be secreted in the environment and, therefore, may show adaptations to extreme conditions tolerated by the microorganism that produces them (Bai et al. 2015). A major component of hemicellulose is xylan, which is composed of β-1, 4-linked D-xylopyranose units that can be substituted with side chains made of other sugars and organic acids (Bowman et al. 2015). The xylooligosaccharides released through the enzymatic degradation of xylan by β-1, 4-endoxylanase can be used in the production of many commodities, including foods, pharmaceuticals, paper, cosmetics, and, potentially, biofuels (Motta et al. 2013). Thus, xylanases that are stable in alkaline conditions and high temperatures have a multitude of potential industrial applications.

Here we describe *Cellulomonas* sp. strain FA1 (NBRC 114238) isolated from *The Cedars* that can withstand extreme alkaline conditions while subsisting on plant cell wall polysaccharides, with a focus on a β-1, 4-endoxylanase that displays enzymatic activity across a broad range of pH values.

Materials and methods

Environmental sampling and establishment of switchgrass enrichment cultures

Decaying plant materials from four alkaline springs within *The Cedars* were sampled into a sterile 100-mL container along with approximately 80 mL of spring water on May 21, 2011 (Fig. S1). Two enrichment cultures were initiated, one on heat-sterilized switchgrass with a 2 mL combined inoculum of *The Cedars* plant debris rinsate (termed "CI" for *Cedars* inoculum), the other on unsterilized switchgrass without external inoculum (termed "NI" for no inoculum) in 125-mL Erlenmeyer flasks containing 0.125 g ground switchgrass in 25 mL of a synthetic medium. The medium was formulated based on the composition of water at Barnes Springs in *The Cedars* (Barnes and O'Neil 1969) to contain 2.2 mM NaCl, 12.3 μM $MgCl_2$, 30.7 μM KOH, and 1.32 mM $Ca(OH)_2$, added after autoclaving (final pH 11.5). Where specified, some cultures were supplemented with 5 mM KNO_3. Cultures were maintained at 37 °C with 50 rpm shaking and the medium was replaced once to twice weekly. Subcultures of the CI and NI cultures were carried out at 2 to 4-week intervals by transferring 0.5 mL of a mixed suspended culture to fresh medium containing new sterilized switchgrass.

Isolation and characterization of *Cellulomonas* sp. strain FA1

To isolate anaerobic cellulose-metabolizing bacteria the following manipulations and incubations were carried out in an anoxic chamber maintained at 30 °C. Samples from the 11th CI enrichment subculture were serial diluted and 1 mL aliquots transferred into septum-sealed test tubes, each containing 10 mL of an N_2-sparged basal liquid medium containing 2 g/L carboxymethylcellulose (CMC) Na^+ salt, 1.0 mM $MgSO_4$, 5.0 mM NH_4NO_3, 2.0 mM K_2HPO_4, 0.1 g/L yeast extract, 1.32 mM $Ca(OH)_2$, and 0.5% (w/v) crystalline cellulose (Avicel®), brought to a final pH 10.3 by adding 1 M KOH. After 9 days, the septum of each tube was punctured with a needle connected through its hub to tubing immersed at the other end in water. Samples from tubes from which gas production was apparent as bubbling were streaked onto basal medium (without Avicel®) containing 15 g/L agar and incubated under anaerobic conditions at 30 °C. Release of CMC-degrading cellulases was visualized as a zone of clearing on plates flooded with 0.1% Congo red followed by flooding with 1 M NaCl (Sazci et al. 1986). Using this procedure strain FA1 was isolated from one of the many uniform

light yellow, glossy CMC-degrading colonies. The strain was deposited in the National Institute of Technology and Evaluation (NITE), Biological Resource Center (Tokyo, Japan) under the accession number NBRC 114238.

Analyses of switchgrass degradation

Axenic cultures of strain FA1 in synthetic medium with switchgrass were initiated using a starting cell density of 10^8 cells/mL and maintaining the cultures as described for the enrichment cultures. At specified times following initiation of switchgrass substrate subculture of strain FA1, suspended plant material (~ 1 mg dry mass) was harvested for chemical composition analysis using synchrotron radiation-based Fourier-transform infrared (SR-FITR) spectral microscopy. Briefly, spectra of the treated samples and controls were acquired using a Thermo Nicolet 6700 FTIR spectrometer equipped with a Nicolet Continuum IR VIS microscope at the Berkeley Synchrotron Infrared Structural BioImaging (BSISB) infrared beamline 5.4 at the Lawrence Berkeley National Laboratory's Advanced Light Source. The IR spectra between 800 and 4000 cm^{-1} at 4 cm^{-1} spectral resolution were recorded with 64 co-added scans. A total of 350 spectra were acquired for each treatment sample, control, and their corresponding replicates. Lignin quantity was determined by the absorption band intensity at 1510 cm^{-1}, whereas the cellulose content was estimated using the C–O–C band that ranges from 1000 to 1200 cm^{-1}.

Dry mass analysis cultures were harvested by filtration onto Whatman GF/A glass microfiber filters and dried at 62 °C to constant mass. Ash-free dry mass was calculated by subtracting the mass of non-combustible material remaining after heating at 550 °C from the dry mass.

Culture samples were prepared for scanning electron microscopy (SEM) by fixing with 2% glutaraldehyde following standard methods. Fixed material was mounted on a silicon wafer, washed twice with deionized water, and allowed to dry under air. The material was dehydrated by a graded series of ethanol washes and sputtered with 136 Å gold film (Polaron SC7640, Quorum Technologies Ltd., East Sussex, United Kingdom). SEM was carried out with a Zeiss Gemini Ultra-55 analytical scanning electron microscope at the LBNL Molecular Foundry.

Exogenous expression and characterization of a β-1, 4-endoxylanase

Whole genome shotgun sequencing (WGS) with initial characterization of the *Celluomonas* sp. strain FA1 genome was previously described (GenBank accession number NZ_LBMY01000001; Cohen et al. 2015). From the genomic sequence, we designed PCR primers flanking a gene encoding a putative 36.2 kDa β-1, 4-endoxylanase (referred to as FA1Xyl1 herein). The amplified product was ligated to a fragment encoding for a C-terminal poly-histidine tag (Hisx8-tag) and cloned into the *Nco*I/*Hind*III site of the bacterial expression vector pTrcHis (Thermo Fisher). The protein was expressed in *E. coli* DH5α and purified from lysate by binding to Ni^{2+}-nitrilotriacetic acid resin beads (Thermo Fisher) and eluted with 300 mM imidazole. Since imidazole can affect the activity of glycosyl hydrolases (Caramia et al. 2017), the imidazole in the eluent was separated from the purified protein using a 10 K MWCO protein concentrator (Thermo Fisher). The concentrate was added to an equal volume of sterile glycerol and aliquoted for storage at − 80 °C.

The identity of purified protein was confirmed by immunoblotting. Purified protein was applied to a 10% polyacrylamide SDS-PAGE gel and electrophoretically transferred to a nitrocellulose membrane. The blot was blocked with 5% (*w*/*v*) dry milk in Tris-buffered saline to prevent non-specific binding of antibodies, and probed with an anti-His primary antibody complexed with horseradish peroxidase that was detected by enhanced chemiluminescence (Thermo Fisher).

Xylanase activity was quantified by measuring the amount of reducing sugar released from beechwood xylan (Megazyme) using 3, 5-dinitrosalicylic acid (DNS) reagent (Bailey et al. 1992). Reactions were initiated by adding 5 µL of crude enzyme solution to 195 µL buffered 1% (*w*/*v*) beechwood xylan solution and incubated at 50 °C. Control reactions were carried out using 5 µL dH_2O in place of enzyme solution. Reactions were terminated by boiling for 10 min. After cooling the mixtures were measured at 540 nm in a spectrophotometer.

Bioinformatic analyses

NCBI's protein–protein BLAST was used to identify sequence homologs. Alignments for sequence comparisons were generated using the constraint-based multiple species alignment tool (COBALT) and SmartBLAST to find closely related sequence homologs among other members of the genus *Cellulomonas* (both NCBI). SignalP version 3.0 from DTU Bioinformatics (https://www.cbs.dtu.dk/services/SignalP-3.0/) was used to predict the location of the glycosyl hydrolase family 11 signal sequence and the hidden Markov model (HMM) to detect the cleavage site.

The obtained xylanase protein sequences were aligned with MUSCLE (https://doi.org/10.1093/nar/gkh340) and the alignment was manually curated. The aligned sequences were examined with maximum likelihood (ML), distance matrix (DM), and maximum parsimony (MP) methods, using PAUP version 4.0b10 (Swofford 2002). ML was performed using the neighbor joining (NJ) search option. The DM was calculated using mean distances, and the distance tree was constructed using the NJ method. MP was performed using the heuristic search option with random

addition of sequences (1000 replicates) and a branch-swapping algorithm (TBR). The three methods yielded very similar trees, and, therefore, only the ML tree was subjected to bootstrap analysis (Felsenstein 1985). Full heuristic search option with random 1000 replicates and a nearest neighbor interchange (NNI) swapping algorithm was employed.

Results and discussion

Degradation of plant material in situ and establishment of switchgrass enrichment cultures

The Cedars is an actively serpentinizing geologic outcrop in which calcium hydroxide-rich ultra-basic springs approximate industrial conditions used to remove lignin from plant biomass. To confirm that the conditions within the *The Cedars* alkaline springs are indeed lignin-stripping, an immersed decaying shoot of the stream orchid *Epipactis gigantea* Hook. f. *rubrifolia* PM Br. was collected from a spring and subjected to chemical composition analysis by synchrotron radiation-based Fourier-transform infrared (SR-FTIR) spectroscopy. As expected, compared to shoot tissue of senescent plants sampled nearby the spring, the spring-immersed tissue had a lower content of lignin relative to carbohydrates (Fig. S2).

A combined sample of plant debris from alkaline springs within *The Cedars* was the source of microbial inoculum for enrichment cultures in a synthetic alkaline medium with ground switchgrass (*Panicum virgatum* L.), a biofuel crop, as the sole source of carbon. We established two switchgrass enrichment cultures, one inoculated with decaying plant material sampled from within alkaline springs at *The Cedars* (termed "CI" for cedars inoculum) and one with switchgrass only (termed "NI" for no inoculum), that were utilized for subsequent experiments to isolate cellulolytic facultative anaerobic bacteria and to determine the rate and nature of degradation of the plant biomass.

Isolation and characterization of the cellulolytic *Cellulomonas* sp. strain FA1

A single bacterium, termed strain FA1, was isolated from the CI enrichment culture under anaerobic conditions on a medium with CMC and cellulose as the sole carbon sources and was identified as a *Cellulomonas* species by genomic sequencing (Cohen et al. 2015) and microbiological characterization (Table S1).

To confirm the presence of strain FA1 in the source CI culture and to check for its existence in the control enrichment culture, we carried out PCR amplification on genomic DNA extracted from the cultures using a *Cellulomonas*-specific 16S rRNA gene primer pair and a FA1Xyl1 xylanase gene-specific primer pair. The expected products were amplified from the CI source culture but not from the equally treated control culture (Fig. S3). Cells of strain FA1 were able to grow on switchgrass and other cellulosic materials, including rice husks, filter paper, and crystalline cellulose, as sole carbon sources in a mineral alkaline medium. Strain FA1 exhibited extensive colonization of autoclave-sterilized switchgrass (Fig. 1). Scanning electron microscopy of

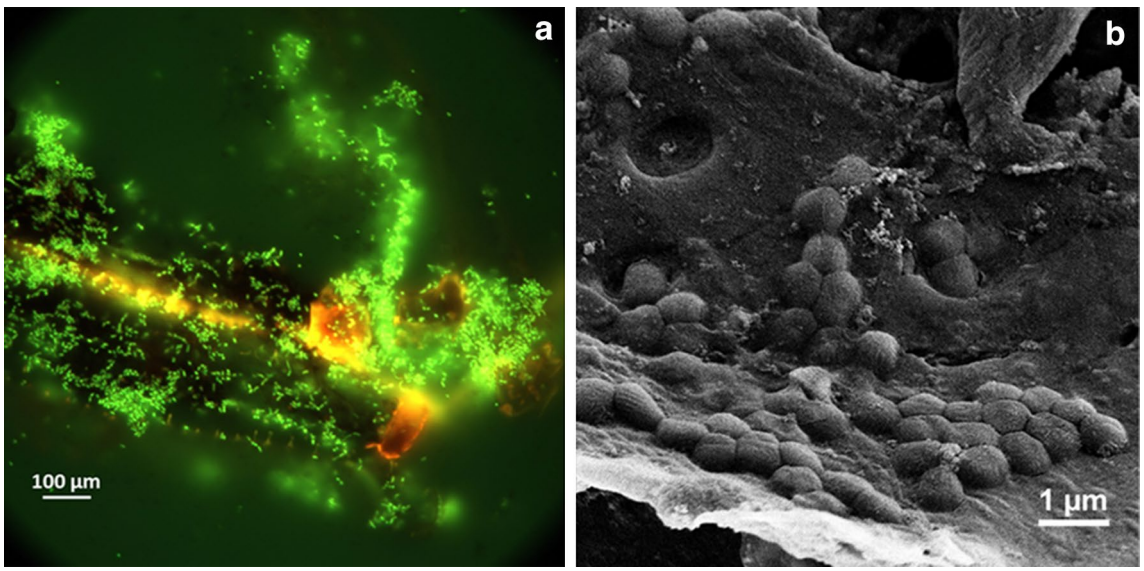

Fig. 1 Micrographs of switchgrass colonized with *Cellulomonas* sp. strain FA1 in alkaline medium with 5 mM KNO_3. **a** Fluorescence microscopy of sample. Bacteria, stained with acridine orange, are green and switchgrass is red–orange owing to autofluorescence of its naturally-occurring pigments, e.g. chlorophyll. **b** Scanning electron micrograph of plant material colonized by strain FA1 cells

colonized material revealed areas of apparent pitting along with bacterial cells surrounded by irregular interconnected capsules (Fig. 1). Strain FA1 degraded switchgrass at a faster rate than the CI heterogeneous enrichment from which it was isolated. It also outperformed the control culture that only contained microbes derived from the switchgrass itself (Fig. 2a). This result was unexpected because plant cell wall degradation typically occurs most efficiently through the activity of microbial consortia (Wang and Chen 2009; Wei et al. 2009). SR-FTIR analyses indicated a significantly greater decline in carbohydrate signal from switchgrass incubated for 9 days in the FA1 culture compared to the CI culture (Fig. 2b). Lignin signals in the cultures with a starting pH of 11.5 declined at rates that were not significantly different (Fig. 2c); strain FA1 does not grow on lignin as a sole C source (Table S1). Microorganisms show two different strategies for deconstructing C-rich plant biomass, increasing output of polysaccharide-degrading enzymes either (1) when plentiful external N is provided to thereby obtain C at a stoichiometric proportion to support growth, or (2) when N is limiting, as a way to extract or "mine" N embedded in the biomass (Chen et al. 2014). Under laboratory cultivation conditions *Cellulomonas uda* was shown to release cellulolytic enzymes in accordance with a N-mining strategy (Young et al. 2012). However, consistent with a stoichiometric metabolism strategy, we found strain FA1 degraded the switchgrass at a faster rate when the culture was supplemented with nitrate (Fig. S4). Biochemical characterization of strain FA1 revealed that, in addition to cellulose, it catabolizes the acidic heteropolysaccharide pectin, hemicellulose, and monosaccharides common in most hemicelluloses: xylose, mannose, arabinose, and galactose (Table S1). Therefore, this strain has the capacity to metabolize the three major plant cell wall polysaccharides cellulose, hemicellulose, and pectin. Strain FA1 grew in media with initial pH values from pH 6 to 9 and survived at up to pH 12.2 (Fig. S5). Members of genus *Cellulomonas* in general, appear to be well-adapted to persisting under highly alkaline conditions (Marschoun et al. 1987; Trujillo-Cabrera et al. 2013).

Cellulomonas species have been shown to ferment a variety of carbohydrates to ethanol (Stackebrandt and

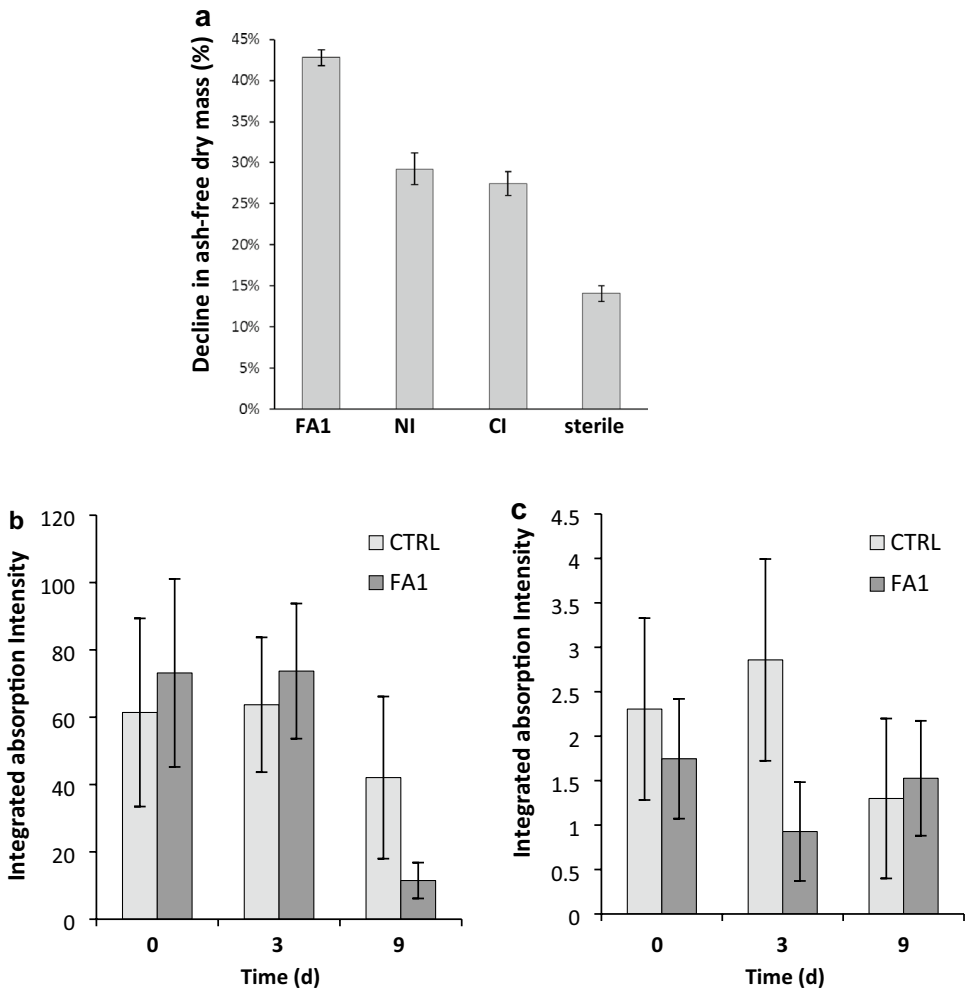

Fig. 2 Characteristics of NI and CI cultures during incubation in alkaline medium (initial pH 11.5). **a** Decline in switchgrass ash-free dry mass after 9-day incubation of autoclaved switchgrass in 5 mM KNO_3-supplemented medium inoculated with NI culture, CI culture, or strain FA1. Changes in **b** carbohydrate and **c** lignin content of switchgrass monitored by SR-FTIR spectroscopy in a CI switchgrass subculture compared to sterile switchgrass (CTRL) after 9 days of incubation (means ± SE, $n = 3$)

Kandler 1980; Marschoun et al. 1987; Poulsen et al. 2016) and under anaerobic conditions to co-occur with other ethanol-producing microorganisms on decaying lignocellulosic substrates. The FA1 genome encodes homologs of three Zn-alcohol dehydrogenases (WP_046528611, WP_046530663, WP_046530044), one Fe-alcohol dehydrogenase (WP_046529978), and a bifunctional acetaldehyde-CoA/alcohol dehydrogenase (WP_052748132) that could potentially function in catabolism or production of ethanol. By serial transfer of cultures to media with successively higher concentrations of ethanol over the course of 6 months, we found that strain FA1 could tolerate up to 13.4% ethanol (Fig. S6). However, the developed tolerance was lost after sub-culturing to ethanol-free medium. In other microorganisms, acclimation to ethanol has been shown to occur through changes in cell membrane phospholipid composition and other alterations to the cell envelope (Liu and Qureshi 2009).

Characterization of a β-1, 4-endoxylanase of strain FA1

To better understand the basis for its ability to degrade lignocellulosic materials under alkaline conditions, we characterized a β-1, 4-endoxylanase of strain FA1 (GenBank accession number WP_046529867) hereafter referred to as FA1Xyl1. The deduced 341 amino acid length sequence of FA1Xyl1 has a 41 amino acid N-terminal secretion signal peptide, a 174-amino acid glycosyl hydrolase family 11 (GH11) domain, and a 64-amino acid carbohydrate binding module 2b (CBM2b) superfamily domain specific for xylan (Simpson et al. 1999). The gene was amplified using PCR primers designed according to the genomic sequence (Cohen et al. 2015), cloned to carry the C-terminal His-tag peptide used for purification, and was found to confer xylanase activity when expressed in *E. coli* cells (Fig. S7). Since the protein was purified from lysate it retained the signal peptide that would otherwise be removed during the secretion process (Fig. S7). All assays of xylanase activity reported herein were made using preparations of the cloned FA1Xyl1 enzyme.

The enzyme had optimal activity at 60 °C and remained active at up to 80 °C (Fig. S8). The activity of FA1Xyl1 at 50 °C could be increased by pre-heating the enzyme at 64 °C for 1 h immediately prior to conducting the assay (unpublished results). A similar such heat activation phenomenon was observed in a GH11 xylanase from *Bacillus subtilis* (Miyazaki et al. 2006). The primary products of hydrolysis of beechwood xylan by FA1Xyl1 were three- to six-unit oligomers of xylose with minor amounts of xylobiose (Fig. S9), which are the xylooligosaccharides typically released by GH11 xylanases (Liu and Liu 2008).

Typical of alkaline tolerant GH11 xylanases (Mamo et al. 2006; Bai et al. 2015), the purified FA1Xyl1 enzyme displayed a broad pH activity profile with a double peak at pH 6 and pH 8 (Fig. 3). For the vast majority of enzymes with alkaline optima, it has been noted that the optimal pH for activity is higher than that for the structural stability of the enzyme (Talley and Alexov 2010). Thus, higher rates of denaturation under alkaline conditions may be offset by higher enzymatic activity, leading to a broader pH activity range.

Based on the measured pH activity profile (Fig. 3), FA1Xyl1 xylanase would function at < 20% of its maximal activity under the ultra-basic conditions found in *The Cedars* spring from which strain FA1 was isolated. However, the actual pH within the microenvironment where cells are in contact with their lignocellulosic substrate would presumably be lower than the pH of the ambient environment because the primary fermentation products of *Cellulomonas* species, including strain FA1 (data not shown), are small organic acids, especially acetic acid (Stackebrandt and Schumann 2015). Release of these acids lowers the pH in the vicinity of the cell and thus serves a selective advantage beyond that of maintaining intracellular pH homeostasis under extremely basic conditions (Mamo 2019).

Sixty-eight GH11 xylanases from other microorganisms were classified by Bai et al. (2015) into three phylogenetic groupings according to their pH optima: acidic (Cluster B2), near-neutral (Cluster B1), and alkaline (Cluster A). We regenerated this phylogenetic grouping with the addition of the FA1Xyl1 xylanase and a recently characterized GH11 xylanase from *Cellulomonas flavigena* that has a pH activity profile similar to that of FA1Xyl1 (Lisov et al. 2017). We found these two xylanases to comprise a distinct sub-cluster within Cluster B1 (Fig. 4). Further phylogenic analysis shows the closest matching xylanase sequences to

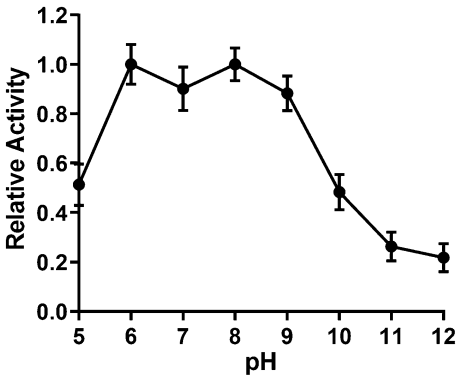

Fig. 3 Effect of pH on the activity of the FA1Xyl1 β-1, 4-endoxylanse of *Cellulomonas* sp. strain FA1. The enzyme was incubated at 50 °C with 1% (*w*/*v*) beechwood xylan in 0.5X Britton–Robinson universal buffer (values are means ± SE); for pH 5, 9, 10, 11 and 12, $n = 7$; for pH 6, 7 and 8, $n = 8$

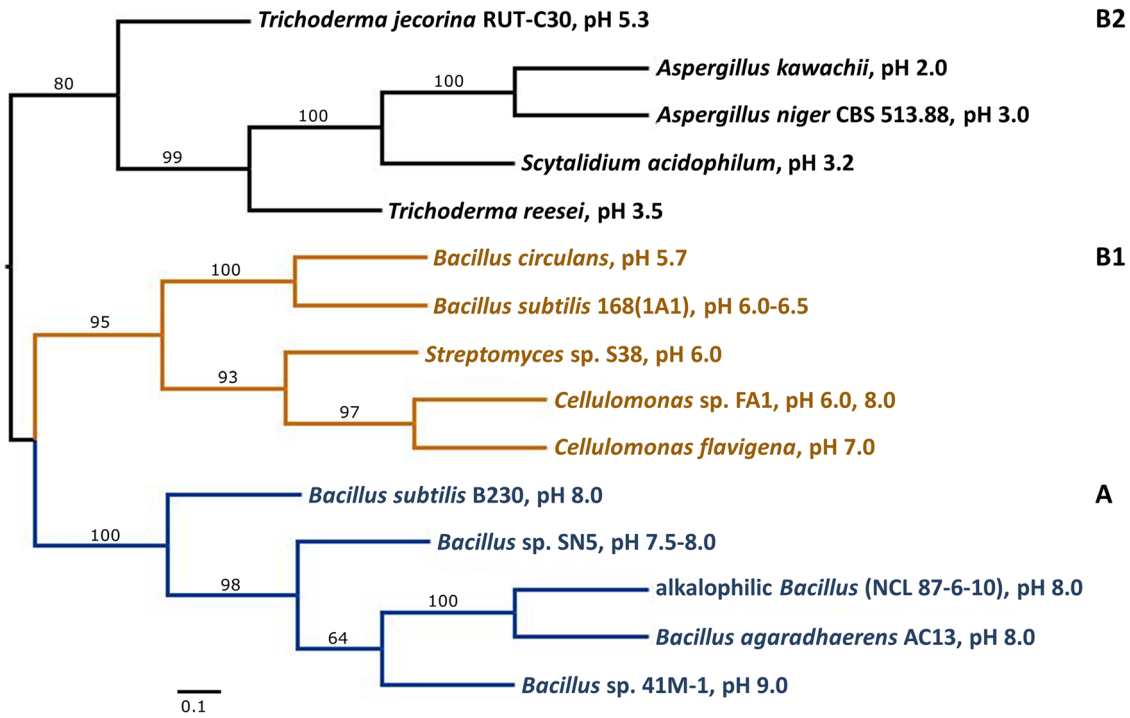

Fig. 4 Phylogenetic tree of FA1Xyl1 β-1, 4-endoxylanase and other glycosyl hydrolase 11 family xylanase sequences for which pH optima have been determined. All optimal pH values listed after each species name are those determined by Bai et al. (2015), except for the two *Cellulomonas* strains. Clusters A, B1 and B2 labeled according to by Bai et al. (2015). The scale bar represents 0.1 substitutions per site. Numbers at nodes indicate bootstrap values

FA1Xyl1 lack a deacetylase domain, while in other branches the presence of this domain varies (Fig. S10). Sequence similarity-based search/analysis suggests that in strain FA1 the xylan deacetylating function is likely to be carried out by a putative 337-amino acid protein (WP_046529866), having a polysaccharide deacetylase domain and CBM2b domain, encoded immediately downstream of the FA1Xyl1 gene (Cohen et al. 2015).

Conclusions and future prospects

Our results demonstrate that *The Cedars* represents a rare environment in which alkaline-mediated abiotic lignin dissolution occurs simultaneously with biological plant cell wall polysaccharide deconstruction. Exploitation of microorganisms and enzymes which can tolerate these conditions could potentially allow for consolidation of chemical lime pretreatment of lignocellulose crops with biological depolymerization and fermentation. Currently, the cost feasibility of lignocellulosic crops as feedstocks for biofuel production is greatly diminished by the requirement to change conditions during processing that includes a pretreatment to unbind lignin from the cell wall, followed by polysaccharide hydrolysis, and finally fermentation. The goal of "consolidated bioprocessing" is to gain efficiency by combining processing steps. With its possession of numerous desirable characteristics, *Cellulomonas* sp. strain FA1 could potentially be honed through future metabolic engineering for use in consolidated bioprocessing of plant biomass.

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