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3 **S-layer homology (SLH) domain proteins Csac_0678 and Csac_2722 implicated in**
4 **plant polysaccharide deconstruction by the extremely thermophilic bacterium**
5 ***Caldicellulosiruptor saccharolyticus***

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31 **ABSTRACT**

32 The genus *Caldicellulosiruptor* contains extremely thermophilic bacteria that grow on plant
33 polysaccharides. The genomes of *Caldicellulosiruptor* species reveal certain surface layer
34 homology (SLH) domain proteins that have distinguishing features, pointing to a role in
35 lignocellulose deconstruction. Two of these proteins in *Caldicellulosiruptor saccharolyticus*
36 (Csac_0678 and Csac_2722) were examined from this perspective. In addition to three
37 contiguous SLH domains, Csac_0678 encodes a glycoside hydrolase family 5 (GH5) catalytic
38 domain and a family 28 carbohydrate-binding module (CBM); orthologs to Csac_0678 could be
39 identified in all genome sequenced *Caldicellulosiruptor* species. Recombinant Csac_0678 was
40 optimally active at 75°C and pH 5.0, exhibiting both endoglucanase and xylanase activity. SLH
41 domain removal did not impact Csac_0678 GH activity, but deletion of the CBM28 domain
42 eliminated binding to crystalline cellulose, and rendered the enzyme inactive on this substrate.
43 Csac_2722 encodes the largest ORF in the *C. saccharolyticus* genome (predicted M_r of 286,516
44 kDa) and contains two putative sugar-binding domains, two Big4 domains, bacterial domains
45 with an immunoglobulin (Ig)-like fold and a cadherin-like (Cd) domain. Recombinant
46 Csac_2722, lacking the SLH and Cd domains, bound to cellulose, and had detectable CMC
47 hydrolytic activity. Antibodies directed against Csac_0678 and Csac_2722 confirmed that these
48 proteins bound to the *C. saccharolyticus* S-layer. Their cellular localization and functional
49 biochemical properties indicate roles for Csac_0678 and Csac_2722 in recruitment and
50 hydrolysis of complex polysaccharides and the deconstruction of lignocellulosic biomass.
51 Furthermore, these results suggest that related SLH domain proteins in other
52 *Caldicellulosiruptor* genomes may also be important contributors to plant biomass utilization.
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55 **INTRODUCTION**
56

57 Members of the extremely thermophilic genus *Caldicellulosiruptor* have potential as
58 consolidated bio-processing (CBP) microorganisms because of their capacity to convert plant-
59 based polysaccharides directly into a biofuel (i.e., hydrogen) in a growth-associated manner (6,
60 7, 25, 54). The first member of this genus to be studied in detail, *Caldicellulosiruptor*
61 *saccharolyticus* (43), has been examined with respect to its genome sequence (53), sugar
62 transport (56), bioenergetics (14, 54), utilization of cellulose in comparison to other members of
63 the genus (7), capacity to degrade plant biomass (55), and for its biotechnological potential (33).
64 Looking forward, further insights into how *C. saccharolyticus* functions as a CBP microorganism
65 will help in the ultimate goal of designing microbial systems, thermophilic or otherwise, for direct
66 plant biomass conversion to biofuels.

67 For microorganisms that convert insoluble forms of cellulose and other recalcitrant plant
68 polysaccharides to fermentable sugars, the synergistic action of a variety of glycoside
69 hydrolases (GH) must be coordinated with overall growth physiology. One strategy is to produce
70 a cellulosome, a novel biological structure that packages many GHs and accessory proteins into
71 a single unit (5, 18); cellulosomes have been described in the *Clostridia* (3, 4) and other bacteria
72 and fungi (2). Within the cellulosome are enzymes endowed with carbohydrate binding modules
73 (CBM) that serve to anchor the biocatalyst to the substrate surface, thereby placing the active
74 site in close proximity to the substrate (36). The CBM can also play a role in destabilizing the
75 insoluble substrate, such that enzymatic activity is enhanced or made possible (8, 35). Other
76 cellulose-degrading microorganisms, such as *Trichiderma reeseii*, hydrolyze the insoluble
77 substrate through the direct action of several GHs, not associated with a cellulosome (37). This
78 is also the case for the cellulolytic, extremely thermophilic *Caldicellulosiruptor* species. For
79 example, the genome of *C. saccharolyticus* encodes at least a dozen multi-domain GHs, ten of
80 which have identifiable signal peptides (55). These ten multi-domain GHs presumably play key

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81 roles in CBP, since they can interact directly with plant polysaccharides. Several of these
82 extracellular GHs (either from *C. saccharolyticus* or related orthologs in *C. bescii*) have been
83 characterized biochemically: Csac_1076 (CelA) (50), Csac_1078 (CelB) (48) (55), Csac_2410
84 (XynE) (55), and Csac_2411 (XynF) (55). In addition, the secretome of *C. saccharolyticus*,
85 grown on glucose, contained the GHs encoded by Csac_1076-1079, suggesting a constitutive
86 role in carbohydrate utilization (1).

87 *C. saccharolyticus* has eleven S-layer homology (SLH) domain proteins, presumably to
88 enable binding to the S-layer. SLH domains, 50-60 amino acid long, have been identified at the
89 amino terminal region of S-layer proteins from various organisms (45) and at the carboxy
90 terminal end of cell-associated extracellular enzymes (45, 47). S-layer motifs specifically
91 recognize pyruvylated secondary cell wall polymers (SCWP) as the anchoring structure (17, 47).
92 SLH domains seemingly play a contributing role in plant polysaccharide degradation. For
93 example, cellulosomes contain proteins that have SLH domains (16). The anchoring mechanism
94 of the *Clostridium thermocellum* cellulosome to the cell surface involves several proteins with
95 repeating SLH domains: OlpA, OlpB, ORF2p, and SdbA (34, 45). Many studies, both *in vivo*
96 and *in vitro*, involving extracellular enzymes containing both catalytic domains and SLH domains
97 connected through a linker region, have showed that the SLH motif anchors the enzyme to the
98 cell surface (9, 12, 31, 34, 38, 51). The linker region likely provides a certain degree of flexibility,
99 facilitating attack on the substrate (38). Apparently, SLH domains neither contribute to
100 enzymatic activity nor are they required for substrate binding (9). These domains are implicated
101 in the binding of enzymes to the cell surface so that release of hydrolysis products are in close
102 proximity to, and can be readily transported into, the cell (40, 63).

103 In the *C. saccharolyticus* genome, two SLH domain-containing proteins (Csac_0678 and
104 Csac_2722) could be distinguished from others by the presence of putative binding domains
105 and, in the case of Csac_0678, a glycoside hydrolase (CAZy [http://www.cazy.org]) (10)
106 belonging to family 5 glycoside hydrolases (GH5) which are reported to have a common $(\beta/\alpha)_8$

107 TIM barrel fold (24). For Csac_0678, the presence of the SLH domains suggests that this
108 protein associates with the S-layer and may play a specific role in utilization of insoluble
109 substrates by *C. saccharolyticus* and other *Caldicellulosiruptor* species. In addition to the SLH
110 domain, Csac_0678 also has a family 28 CBM; this CBM family (type B) has a cleft shape to
111 accommodate cellooligosaccharides (52) and belongs to the β-jellyroll fold subfamily (23). No
112 identifiable GH catalytic domain is present in Csac_2722, the largest ORF in the *C.*
113 *saccharolyticus* genome. However, this protein contains putative binding motifs, which suggests
114 some involvement in polysaccharide utilization. Furthermore, the cadherin-like domains may
115 play a role in protein-protein interactions, binding to bacterial cell surfaces (19, 20); these
116 domains were shown to bind carbohydrates, such as chitin and pectin (19). In this report,
117 biochemical characteristics of Csac_0678 and Csac_2722 are examined with an eye towards
118 the role of these proteins in plant biomass deconstruction.
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121 **MATERIALS AND METHODS**
122

123 **Bacterial strains and culture conditions**. *E. coli* NovaBlue and *E. coli* Rosetta™ (DE3) 124 (Novagen, Madison, WI) were used as cloning and expression hosts, respectively. The *E. coli* 125 strains were cultivated in Luria-Bertani (LB) medium, supplemented with kanamycin (Fisher 126 Bioreagents) (30 µg/ml) and/or chloramphenicol (Sigma) (34 µg/ml). *C. saccharolyticus* DSM 127 8903 cultures were grown anaerobically at 70°C in modified DSM640 medium (DSMZ 128 [http://www.dsmz.de]) containing NH_4Cl (0.9 g/l), NaCl (0.9 g/l), $MgCl_2.6H_2O$ (0.4 g/l), KH_2PO_4 129 (0.75 g/l), K_2HPO_4 (1.5 g/l), yeast extract (1 g/ml), trace element solutions SL-10 (1 ml/l), 130 cellobiose (5 g/l), and $Na_2S.9H_20$ (0.05% w/v). Cells were visualized by epifluorescence 131 microscopy (Nikon Intensilight C-HGFI, Lewisville, TX) as previously described (55).

132

133 **Cloning, expression, and purification of Csac_0678, Csac_0678-TM1, Csac_0678-** 134 **TM2, and Csac_2722**. Standard molecular cloning techniques (46) were used in this work. 135 Genomic DNA from *C. saccharolyticus* was isolated, as described previously (22). ORFs 136 encoding these genes were amplified by PCR from *C. saccharolyticus* genomic DNA by using 137 the primers listed in Table 1. The primers were designed in a way that a C-terminal histidine 138 (His)-tag was added to all three versions of Csac_0678 constructions and an N-terminal His-tag 139 was added to Csac_2722. Gene constructions used to express intact proteins and truncation 140 mutants are shown in Figure 1. The insertions and the plasmid, pET-28b(+) (Novagen, Madison, 141 WI), were digested by the restriction endonucleases (New England Biolabs, Beverly, MA). DNA 142 fragments ranging from 990-2134 bp were ligated into the cut vector. The plasmids, which were 143 confirmed to contain the insertion, were isolated from the cloning host by using a Qiagen kit 144 (QIAprep, Valencia, CA). The nucleotide sequences of both strands of the DNA insertions were 145 determined at the Duke University Health System DNA Analysis Facility. *E. coli* Rosetta™ (DE3) 146 cells, containing pET-28b(+) under the control of T7lac promoter (pET System Manual), were

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147 used for gene expression. Expression of the target genes was induced by the addition of IPTG
148 to Rosetta cells when OD_{600} reached 0.8. The cells were then harvested after 4 hours of
149 induction by centrifugation at 10,000 *x g* for 10 minutes. The cell pellet was re-suspended with 5
150 ml of 50 mM sodium phosphate pH 8.0, 100 mM NaCl, Nonidet P40 (0.1% (v/v), 100 μg/ml
151 lysozyme, and 1 μg/ml DNase for every gram of wet cell pellet. The cells were lysed by
152 sonication (S-4000, Misonix Ultrasonic Liquid Processors, Farmingdale, NY) for 10 minutes with
153 10 sec off/on pulses. The suspension was heat-treated at 60°C for 20 minutes in order to
154 remove *E. coli* proteins. The cell extract was obtained after centrifuging the lysed and heat-
155 treated cells at 20,000 *x g* for 20 minutes. The intact Csac_0678 (without the signal peptide) and
156 the two truncation mutants (Csac_0678-TM1, which lacked the SLH domains, and Csac_0678-
157 TM2, missing the SLH domains and CBM) were present in the cell extract in a soluble form,
158 whereas Csac_2722 was found in the insoluble fraction, forming inclusion bodies. A protein
159 refolding kit (Novagen, Madison, WI) was used to re-solubilize recombinant Csac_2722. HiTrap
160 HP (GE Life Sciences, Piscataway, NJ), and a Resource Q (GE Life Sciences, Piscataway, NJ)
161 column were used to purify the recombinant proteins on a Biologic DuoFlow FPLC (BioRad,
162 Hercules, CA). Purity of the recombinant intact Csac_0678, Csac_0678-TM1, Csac_0678-TM2,
163 and Csac_2722 was evaluated by SDS-PAGE. Size exclusion chromatography was carried out
164 to determine the oligomeric state of Csac_0678 using a Superdex-75 HiLoad 16/60 column (GE
165 Healthcare Lifesciences), which was calibrated with the following protein standards: cytochrome
166 c (12.4 kDa), carbonic anhydrase (29 kDa), albumin (66 kDa), alcohol dehydrogenase (150
167 kDa), and β-amylase (200 kDa). The column was equilibrated with 50 mM Tris-HCl (pH 8.0),
168 100 mM NaCl. The protein sample was applied at 2 mg/ml in 0.5 ml of equilibration buffer.
169 Protein concentration was determined by the Bradford protein assay (Biorad, Hercules, CA),
170 using bovine serum albumin as the standard (Sigma).
171

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172 **Biochemical characterization of Csac_0678**. The optimal pH for recombinant
173 Csac_0678 was determined at 70°C in buffers containing 2% CMC at pH 2.5 to 9 (50 mM
174 sodium acetate buffer, pH 2.5-6, and 50 mM sodium phosphate buffer, pH 6-9). The optimal
175 temperature for Csac_0678 activity was determined by 3,5-dinitrosalicylic acid (DNS) assay
176 between 40°C and 90°C at the optimal pH. The melting temperature of Csac_0678 was
177 obtained using differential scanning calorimetry (Calorimetry Sciences Corporation, Provo, UT).
178 Csac_0678 was prepared at 1 mg/ml in PBS buffer and scanned between 25°C and 125°C,
179 using a rate of 1°C·min^{-1}. Thermoinactivation of Csac_0678 was examined by incubating the
180 enzyme without the substrate in the reaction buffer at 70, 77, 80, and 85°C for 48 hours.
181 Aliquots were taken at different time intervals and residual activity was assayed on CMC.

182 The DNS reducing sugar assay, which was adapted to utilize a miniaturized 96-well
183 microplate format (61, 62), was used to detect both five- and six-carbon reducing sugars. DNS
184 reagent was prepared as previously described (39). Avicel (PH-102, 100 μm average particle
185 size, Sigma), xylan oat spelt (Sigma, St. Louis, MO), xylan birchwood (Sigma), barley glucan
186 (Megazyme International Ireland Ltd., Wicklow, Ireland), (konjac root) glucomannan (Jarrow
187 Formulas, Los Angeles, CA), (amyloid) xyloglucan (Megazyme), (ivory nut) mannan
188 (Megazyme), laminarin (from *Laminaria digita*) (Sigma, St. Louis, MO) switchgrass (*Panicum*
189 *virgatum* -20/+80 mesh fraction; dilute acid pretreatment was performed at the National
190 Renewable Energy Laboratory (44)), lichenan (Megazyme), arabinoxylan (Megazyme) and
191 carboxymethylcellulose (CMC, Sigma) were prepared at 1% (w/v) in 50 mM sodium acetate, pH
192 5. Single discs of filter paper with a radius of 5 mm and total weight of 3 mg (Whatman No. 1,
193 Kent, UK) were used in the same buffer. Phosphoric acid swollen cellulose (PASC) was
194 prepared from Sigmacell (Type 20, 20 μm average particle size, Sigma), as described
195 previously (65) and was used at 1% (w/v) final concentration in each reaction. Bacterial
196 microcrystalline cellulose (BMCC) (provided by David B. Wilson, Cornell University (Ithaca, NY))
197 at 0.5% (w/v) suspended in 50 mM sodium acetate (pH 5) was used for activity analysis. The

198 reactions took place at 75°C and 500 RPM in a thermomixer (Eppendorf) with 2.7 mg/ml
199 Csac_0678. Xylose and glucose standards were used to convert absorbance readings to
200 reducing sugar concentrations. One unit (U) of enzyme activity is defined as the amount of
201 enzyme required for producing 1 μmol of glucose or xylose per minute. The kinetic parameters
202 (V_{max} and K_m) were calculated by using *p*-nitrophenyl-β-D-cellobioside (pNPC, Sigma).
203 Substrate concentrations were varied between 0.5 and 10 mM. After adding 2 M Na_2CO_3, pH
204 10, the absorbance was measured at 420 nm. The concentration of the product was calculated
205 by using a standard curve of 4-nitrophenol (Sigma) in the same buffer. For V_{max} and K_m
206 calculation, one unit of enzyme activity is defined as the amount of enzyme required for
207 producing 1 μmol of 4-nitrophenol per minute.

208 Hydrolysis products were determined by HPLC refractive index detection (2414 RI
209 detector, Waters). Shodex KS-801 and KS-802 (Showa Denko K.K., Kanagawa, Japan)
210 columns operated at 80°C with a flow rate of 0.6 ml/min. Hydrolysis products were also viewed
211 on thin-layer chromatography (TLC) by using silica gel 60 TLC sheets (Fisher), following
212 protocols described previously (27). EtOAc-CH_3COOH-H_2O (3:2:1, by volume) was used to
213 develop the TLC plates; reducing sugars were detected by the orcinol reagent (1% orcinol in
214 10% H_2SO_4 dissolved in ethanol). Cellooligosaccharides and xylooligosaccharides (Sigma) were
215 used as standards.

216

217 **Activity of Csac_0678 on filter paper.** The processivity was determined by using a
218 modified protocol of Irwin et al (29). Single discs of filter paper with a radius of 5 mm and total
219 weight of 3 mg were incubated with 9 μM purified Csac_0678 at 75°C in 50 mM sodium acetate
220 buffer, pH 5 for 16 hours. After the incubation, the supernatant (100 μl), which contained the
221 soluble reducing sugars, was removed. The filter papers were washed with 1 ml of 50 mM
222 sodium acetate buffer three times. After the washing step, 100 μl of the same buffer was added

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223 to the filter paper. DNS reagent (200 µl) was added to the supernatant and to the filter paper
224 tubes. The DNS reaction was run as described above. Reducing sugars were estimated using a
225 glucose standard curve. The ratio of soluble sugars (found in the supernatant fraction) to
226 insoluble sugars (found on the filter paper) was calculated. All the reactions were run in
227 triplicates.
228
229 **CMC viscosity reduction assay.** CMC viscosity reduction was carried out by following
230 a modified version of a protocol described elsewhere (64). Medium viscosity CMC (1%) (Sigma)
231 in 50 mM sodium acetate buffer (pH 5) was incubated with 1.5 µM purified Csac_0678 for one
232 hour at 75°C and 300 rpm. After incubation, the samples were boiled for 5 minutes, and then
233 diluted ten-fold with distilled water. The viscosity of each sample was measured in triplicates by
234 an AR-G2 rheometer (TA instruments, New Castle, DE).
235
236 **Carbohydrate affinity assay**. Affinity of recombinant Csac_0678, Csac_0678 TM2, and
237 Csac_2722 to insoluble polysaccharides was assessed by following a protocol described
238 elsewhere (32). Avicel cellulose gel (20 mg), PH-102 (FMC Corporation) with a 100 µm-average
239 particle size was washed with distilled water and with 50 mM sodium acetate (pH 5.0) three
240 times. Protein (40 µg) was added to the insoluble polysaccharide in the buffer at a final volume
241 of 250 µl. The tubes were incubated at room temperature for 45 minutes. The sample-
242 polysaccharide mixtures were centrifuged at 15,800 *x g* for 5 minutes. The supernatant
243 contained the unbound fraction. The pellet was washed with 1 ml of 50 mM sodium acetate (pH
244 5.0) three times to remove the remaining unbound protein. The bound protein was separated
245 from the insoluble polysaccharides in 100 µl of elution buffer (2% (w/v) SDS and 5% (v/v) β-
246 mercaptoethanol) by incubating in a boiling water bath for 10 minutes. These samples were
247 then centrifuged at 15,800 *x g* for 5 minutes such that the supernatant contained the bound
248 proteins. The bound and unbound fractions were concentrated to 20 µl and compared by SDS-

249 PAGE. Densitometry analysis was done using a Gel Logic 212 Pro with Carestream MI software
250 v5 (Carestream Molecular Imaging,Woodbridge, CT).

251 Quantitative binding assays were done following a previously described protocol (57).
252 Protein (40 µg) was incubated in a 1% (w/v) Avicel solution in 50 mM sodium acetate buffer at
253 pH 5 and at 4°C for one hour on a Labquake Tube Shaker/Rotator (Barnstead/Thermolyne).
254 After the incubation, tubes were centrifuged. The optical density of the supernatant at 280 nm
255 was measured by using Synergy Mx microplate reader (Biotek, Winooski, VT) to determine the
256 concentration of the unbound protein. The extinction coefficients of the proteins were estimated
257 by Expasy ProtParam tool (ExPASy Bioinformatics Resource Portal
258 [http://web.expasy.org/protparam/]). The bound protein fraction was determined by subtracting
259 the unbound protein from the initial amount of protein added.

260

261 **Localization of Csac_0678 and Csac_2722 in *C. saccharolyticus* - extraction of the**
262 **S-layer**. Four liters of *C. saccharolyticus* cells grown on cellobiose were harvested at early
263 stationary phase by centrifugation at 12,100 *x g* for 10 minutes. Cell wall fractions were
264 prepared by following the steps described earlier (34), but using a different buffer. Cell pellets
265 were washed twice with 100 ml of 50 mM Tris-HCl (pH 7.4), and cells were lysed by sonication
266 in 30 ml of the same buffer. Intact cells were separated by centrifuging twice at a lower speed,
267 1,940 *x g* for 5 minutes. The resulting supernatant was centrifuged at 39,200 *x g* for 20 min. The
268 pellet, re-suspended in 5 ml of 50 mM Tris-HCl (pH 7.4), contained the cell wall fraction. The S-
269 layer was purified from the cell wall fraction using a modification of a previously described
270 method (49). The cell wall fraction (24 mg/ml) was treated with lysozyme (150 µg/ml) for 3 hours
271 at room temperature on a shaker at 100 rpm. The mixture was centrifuged for 30 minutes at
272 25,000 *x g*. The pellet, which contained the S-layer fraction, was washed three times with 50
273 mM Tris-HCl (pH 7.4) and re-suspended in distilled water.

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275 **Protein immunoblotting**. Proteins in the S-layer fraction and recombinant Csac_0678
276 and Csac_2722 were resolved by SDS-PAGE using Nu-Page 4-12% Bis-Tris (Invitrogen) pre-
277 cast gels. The proteins were transferred to a nitrocellulose membrane (Whatman) by using
278 Fisher Biotech Semi-Dry blotting unit FB-SDB-2020. The blot was incubated with nonfat dry milk
279 to block non-specific binding of the antibodies. Polyclonal rabbit antibodies (GeneTel
280 Laboratories LLC, Madison, WI), generated against recombinant Csac_0678-TM1 or
281 Csac_2722, both lacking the SLH domains, were incubated with the blot for two hours. After
282 rinsing to remove the unbound rabbit antibodies, the blot was exposed for an hour to goat anti-
283 rabbit antibodies conjugated to horseradish peroxide, HRP Goat Anti-Rabbit IgG (H+L)
284 (Invitrogen). Then, the blot was washed to remove any unbound secondary antibody.
285 SuperSignal West Femto Max Sensitivity chemiluminescent substrate (Thermoscientific) was
286 used for detection of peroxide activity from HRP-conjugated secondary antibody. A Kodak 1500
287 Gel Logic system (Carestream Molecular Imaging) was used to image the blot.
288
289 **RESULTS and DISCUSSION**
290
291 **Csac_0678 is a bifunctional endoglucanase/xylanase that binds to the S-layer.** The
292 sequenced *Caldicellulosiruptor* genomes encode ORFs representing putative glycoside
293 hydrolases that are associated with S-layer homology domains (see Table 2). The *C.*
294 *saccharolyticus* DSM 8903 genome, in particular, encodes at least 57 identifiable glycoside
295 hydrolases (GHs), 16 of which have one or more catalytic domains linked to one or more non-
296 catalytic domains (53, 55), but only Csac_0678 has SLH domains, in addition to glycoside
297 hydrolase (GH5) and sugar-binding (CBM28) domain.
298 Orthologs of Csac_0678 exist (Table 2) in all eight sequenced *Caldicellulosiruptor*
299 species; in 6 species, including *C. sacharolyticus*, the ORF encodes a 755-756 aa protein (85
300 kDa), while in *C. owensensis* and *C. obsidiansis*, the ortholog is 566-567 aa (64 kDa),

301 differentiated from the longer version by a truncated CBM28 domain. Since Csac_0678 and its
302 orthologs are so highly conserved (Csac_0678 is at least 65% identical at the amino acid
303 sequence level to each of the orthologs), they are a defining feature of *Caldicellulosiruptor*
304 species.

305 Csac_0678 encodes a signal peptide at the N-terminus (SignalP 3.0 Server,
306 [http://www.cbs.dtu.dk/services/SignalP/]) and potentially associates with the cell surface via the
307 SLH domains (see Figure 1A). The molecular assembly of recombinant Csac_0678 (M_r of 81
308 kDa without the signal peptide), as determined by size exclusion chromatography, was found to
309 be monomeric. Optimum temperature and pH of Csac_0678 were determined to be 75°C and
310 5.0, respectively. Csac_0678 demonstrated high thermostability, retaining 50% of its original
311 activity after 48 hours of incubation at 75°C (Figure 2). The half-life at 77°C was found to be
312 approximately 19 hours at 77°C, less than 3 hours at 80°C, and less than 30 min at 85°C.
313 Differential scanning calorimetry (DSC) of Csac_0678 with the SLH domains showed a single
314 unfolding transition with a peak at 81°C. To examine cellular localization, polyclonal rabbit
315 antibodies raised against Csac_0678 lacking the SLH domains (Csac_0678-TM1) were used to
316 probe for possible Csac_0678 attachment to the S-layer in cells grown on cellobiose. By using
317 anti-Csac_0678-TM1 antibodies, hybridization to SLH domains was avoided. Immunoblot
318 analysis (Figure 1C) showed that anti-Csac_0678-TM1 antibodies bound specifically to the S-
319 layer fraction. Purified recombinant Csac_0678 was used as the positive control. An unrelated
320 protein sample from *Sulfolobus solfataricus* and Benchmark protein ladder (Invitrogen) were
321 used as negative controls. Anti-Csac_0678 antibodies recognized the enzyme localized on the
322 cell surface.
323

324 **Biochemical characterization of Csac_0678.** Recombinant Csac_0678 was tested for
325 activity towards a range of complex carbohydrates (Table 3). Activity on barley glucan and
326 lichenan indicated a preference towards soluble substrates with mixed β-1,4 and β-1,3

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327 glycosidic linkages. Csac_0678 exhibited moderate activity on CMC and PASC, which are at
328 least 20-fold more accessible than Avicel (66). Csac_0678 hydrolyzed glucomannan but not
329 mannan, presumably attacking β-(1→4)-linked D-glucose units in glucomannan. It was
330 interesting that Csac_0678 also degraded polymers composed of β-(1→4)-linked xylose units,
331 such as xylan and arabinoxylan. But, in comparison with XynA (306 U·mg^{-1}) and XynB (4,600
332 U·mg^{-1}) from *Thermotoga maritima* (60), Csac_0678 xylanase activity was very low. However, it
333 was comparable to thermophilic xylanases from *Bacillus* sp. (NCIM59) (0.0172 and 0.742
334 μM·min^{-1}·mg^{-1}) (15). Csac_0678 hydrolyzed pNPC, indicating that the active site of Csac_0678
335 can accommodate cellobiose moieties. In addition, low but measurable activity was detected on
336 insoluble substrates, such as BMCC, filter paper, Avicel and switchgrass. Although specific
337 activity on switchgrass could not be directly measured, HPLC analysis showed small amounts of
338 glucose and xylose as hydrolysis products.

339 Table 3 shows that Csac_0678 exhibited bifunctional xylanase and endoglucanase
340 activity, despite the fact that it contains only a single catalytic domain (GH5). A *Thermotoga*
341 *maritima* endoglucanase Cel5A, comprised of only GH5 domain, had dual activity against both
342 glucan- and mannan-based polysaccharides (41). The specific activity of Cel5A on CMC was
343 616 U·mg^{-1}, but no activity detected on xylan (birch wood) (13). Cel5A activity on Avicel was not
344 reported. Another endoglucanase, Cel5B from *Thermobifida fusca*, containing both GH5 and
345 CBM3 domains, had specific activities of 121.4 and 3.9 U·mg^{-1} against CMC and Avicel,
346 respectively (42), but no activity observed either on xylan or on mannan. RuCelA, cloned from a
347 metagenomic library of yak rumen microorganisms, encoded a GH5 enzyme possessing
348 xylanase (264.1 U·mg^{-1}) and endoglucanase (54.3 U·mg^{-1}) activity, but with no CBM (11).
349 Additionally, RuCelA, had no activity on Avicel, but measurable activity on filter paper.

350 The crystalline cellulose hydrolyzing capability of endoglucanases such as *T. maritima*
351 Cel5A (41) and Csac_0678 likely arises from the presence of a CBM. However, Cel5F, an
352 endoglucanase from *Saccharophagus degradans* contains only a GH5 domain, yet has

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353 measurable activity towards Avicel (6.6×10^{-4} U·mg^{-1}) and filter paper (6.26×10^{-4} U·mg^{-1}) (58).
354 Hydrolysis products from Csac_0678 on Avicel, determined by TLC and HPLC, indicated that
355 the enzyme generated predominantly cellobiose and some glucose (Figure 3). Although
356 cleaving cellobiose from crystalline cellulose is a cellobiohydrolase-trait, longer oligosaccharides
357 may have been initially released by Csac_0678 which were then converted to the disaccharide.
358 X-ray diffraction analysis of Avicel incubated with Csac_0678 indicated that crystallinity of Avicel
359 decreased after the hydrolysis (data not shown). Furthermore, Csac_0678 appears to produce
360 cellotriose, cellobiose and small amounts of glucose from cellohexaose but no cellotetraose
361 detected (data not shown). Interestingly, Csac_0678 also cleaved xylan at multiple sites,
362 generating xylobiose, xylotriose, and xylotetraose (see Figure 4) and, after prolonged
363 incubation, small amounts of xylose. This supports the premise that Csac_0678 acts via an
364 endo-type mechanism. Csac_0678 activity followed typical Michaelis-Menten kinetics for *p*-
365 nitrophenyl β-D-cellobioside hydrolysis; at pH 5.0 and 75°C, V_{max} and K_m were 6.1 U·mg^{-1} and
366 0.65 mM, respectively.

367 The processivity of Csac_0678 on filter paper examined to determine if the enzyme
368 functions as an exoglucanase or an endoglucanase, or a processive endoglucanase (28, 29).
369 Exoglucanases cleave cellobiose moieties from the ends of cellulose molecule, whereas
370 endoglucanases attack the cellulose molecule at any accessible point, randomly cutting the β-
371 1,4-linkages and they dissociate leaving reducing sugars on the substrate (28, 29). Therefore,
372 the ratio of soluble to insoluble reducing sugars is large for exo-acting enzymes and small for
373 endo-acting enzymes. The distribution of reducing sugars on filter paper compared to the
374 supernatant was measured. The soluble/insoluble reducing sugar ratio of Csac_0678 was
375 0.6±0.05, indicating that it is an endoglucanase. In a previous study, soluble/insoluble reducing
376 sugar ratios for classical endo-acting GH5 enzymes from *S. degradans*, Cel5B, Cel5C, CelD,
377 Cel5E, and Cel5F, were reported to be between 0.096-1.42 (58). The same study showed that
378 the ratios for processive endo-acting GH5 enzymes, Cel5G, Cel5H, and Cel5J, were between

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379 4.04 and 4.59. Exocellulases have larger ratios of soluble/insoluble reducing sugars. For
380 example, 96% of the reducing sugars produced by an exocellulase, Cel48A from *Thermobifida*
381 *fusca* were found to be soluble (soluble to insoluble reducing sugar ratio is 24) (30).

382 CMC viscosity before and after GH addition can also be used to distinguish between
383 endo- and exo-glucanase activity (29). As a soluble form of cellulose, CMC, is a good substrate
384 for endo-acting glucanases, as they randomly bind CMC cleaving β-1,4-linkages and then they
385 dissociate from the cellulose molecule (59). This leads to a reduction in the viscosity of CMC
386 solution. Exoglucanases do not reduce the viscosity of CMC solution, although they have low
387 activity on CMC (59). Cellobiohydrolases, Cel7A (formerly CBHI) and Cel6A (formerly CBHII),
388 which are exo-acting enzymes from *Trichoderma reesei*, did not significantly reduce the
389 viscosity of a CMC solution, although they degraded 6% of CMC (29, 30). Processive
390 endoglucanases decrease the viscosity of CMC solution as they attack the cellulose chain in a
391 similar way to classical endoglucanases. But they differ from other endoglucanases and
392 exoglucanases since they processively cleave cellobiose units from the cellulose chain (58, 59).
393 Cel48, an exocellulase from *T. fusca*, reduced the viscosity of a CMC solution only by a few
394 percent (64), while Cel5B from *Clostridium phytofermentas* decreased the viscosity of the CMC
395 ~65%. Here, Csac_0678 reduced the viscosity of 1% (w/v) CMC solution by 40% in one hour,
396 further supporting the contention that it is an endoglucanase.
397

398 **Biochemical analysis of Csac_0678 truncation mutants**. Three different recombinant
399 (C-terminal His tag) versions of Csac_0678, lacking the N-terminal signal peptide, were
400 produced to examine the significance of the SLH and CBM domains (refer to Figure 1A): intact
401 version with SLH domains, CBM and GH5, Truncation Mutant 1 (TM1) with GH5 and CBM28
402 domains (60 kDa) but no SLH domains, and Truncation Mutant 2 (TM2) with only the GH5
403 domain and neither CBM nor SLH domains (34 kDa). While intact Csac_0678 could hydrolyze
404 Avicel, PASC, BMCC, xylan, and filter paper, the removal of the CBM28 domain virtually

405 eliminated the activity of the enzyme (i.e., ~98% decrease in specific activity) on these
406 substrates as well as on CMC (i.e., ~ 95% decrease in specific activity). CBMs assist hydrolysis
407 by targeting, binding, and disrupting the cellulosic substrates (8). Binding of Csac_0678 to
408 insoluble polysaccharides was determined by incubating the intact enzyme and Csac_0678-
409 TM2 with Avicel. Figure 5A and 5C show intact Csac_0678 bound to Avicel to a greater extent
410 than Csac_0678-TM2; by densitometry, 48 wt% and 13 wt% of Csac_0678 and Csac_0678-
411 TM2 bound to Avicel, respectively. Quantitative binding study results agreed with the
412 densitometry Figure 5C. Eliminating SLH domain did not affect the activity of Csac_0678 (data
413 not shown) indicating that SLH domains do not play a role in activity.
414
415 **Csac_2722 encodes a large polypeptide that binds to cellulose.** The process by
416 which cellulolytic microorganisms attack and hydrolyze crystalline cellulose is complex and likely
417 involves steps not limited to hydrolytic biocatalysis. While most SLH-domains lacking GH
418 domains in *Caldicellulosiruptor* species appear to function as structural components of the S-
419 layer, several contain putative binding domains (see Table 4) (26). These proteins potentially
420 represent an element of the complex carbohydrate recruitment strategy used by these bacteria.
421 For example, the *C. kristjanssonii* and *C. lactoaceticus* genomes encode homologous 575 aa
422 proteins (Calkr_0834 and Calla_1498, respectively) with SLH domains at the N-terminus, and
423 which contain fibronectin type 3 (FN3) at the C-terminus (21). *C. bescii* (formerly *Anaerocellum*
424 *thermophilum*) and *C. kronotskyensis* genomes also encode SLH + FN3 domain proteins,
425 Athe_0012 and Calkro_0014, respectively; these are each approximately 3,000 aa, the two
426 largest ORFs in the *Caldicellulosiruptor* genomes sequenced to date. FN3 domains have been
427 implicated in lignocellulose degradation. For example, Cel9A-90, a processive endoglucanase
428 from *Thermobifida fusca* has a GH9 catalytic domain, and a CBM3c, followed by a fibronectin 3-
429 like domain and a CBM2 (67). Deletion studies showed that the activity of Cel9A-90 on BMCC
430 decreased (43% reduction in activity compared to the wild type) when the fibronectin domain

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431 was deleted (67). There are also SLH-domain proteins in *Caldicellulosiruptor* species that
432 encode CBMs: Calkr_1989 in the *C. kristjanssonii* genome encodes a 326 aa protein, with three
433 SLH domains at the N-terminus followed by a CBM20 domain, while in *C. obsidiansis*, COB47-
434 _0167 is a 886 aa protein with three CBM27 domains upstream of the three SLH domains.
435 Whether any of these SLH domain proteins in *Caldicellulosiruptor species* participate directly or
436 indirectly in plant biomass deconstruction has not been determined.
437 Several *Caldicellulosiruptor* species (*C. kronotskyensis, C. lactoaceticus* and *C.*
438 *owensensis*) contain SLH-domain proteins at the C-terminus and also Big domains; these
439 domains may play a role in biomass attachment, since proteins containing Ig-like domains from
440 other microorganisms were reported to play roles in cell-cell adhesion, binding, and extracellular
441 hydrolysis (21). Csac_2722 represents the largest ORF in the *C. saccharolyticus* genome
442 comprised of 2,593 amino acids, and one of the largest annotated ORFs in *Caldicellusiruptor*
443 species. Csac_2722 has no discernible catalytic domains but does contain two Big4 domains
444 arranged in tandem with two galactose-binding domain-like domains (GBD), one cadherin-like
445 domain and three SLH domains. Cadherin-like domains appeared to contribute to protein-
446 protein interactions and carbohydrate binding (19, 20). Polyclonal rabbit antibodies raised
447 against a recombinant version of Csac_2722 lacking the SLH and cadherin-like domains
448 recognized Csac_2722 attachment to the S-layer and cell membrane fraction in cells grown on
449 switchgrass (data not shown). Pull-down experiments with Avicel as bait showed that
450 Csac_2722 binds to Avicel (~32% bound) (Figure 5B). Csac_2722 also exhibited activity on
451 CMC (2.8 mM glucose equivalent of reducing sugars were released in 3 hours), although no
452 identifiable GH domain could be found in the Csac_2722 amino acid sequence. The fact that
453 Csac_2722 bound to cellulose and that it is associated with the outer cell envelope suggested
454 that it, and perhaps some or all of the putative proteins listed in Table 4, contribute to
455 lignocellulose conversion in *Caldicellulosiruptor* species.
456

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457 **Conclusion.** Consolidated bioprocessing (CBP) describes the comprehensive capacity
458 of a microbial system to not only significantly deconstruct lignocellulose but also to convert the
459 hydrolysis products to a biofuel. This metabolic capability no doubt involves subtle but important
460 contributions which are more than enzymatic hydrolysis and involves factors not yet fully
461 appreciated in CBP microorganisms. Certain SLH domain-containing proteins likely play a role
462 in lignocellulose-degrading microorganisms that goes beyond providing structural integrity to the
463 cell envelope. Two such proteins in the extremely thermophilic cellulolytic bacterium
464 (Csac_0678 and Csac_2722) have the capacity to bind to crystalline cellulose and, in the case
465 of Csac_0678, hydrolyze this substrate. Figure 6A shows the attachment of *C. saccharolyticus*
466 cells to switchgrass and Figure 6B illustrates how Csac_0678 and Csac_2722 contribute to the
467 deconstruction of plant biomass. Contributions from non-catalytic, carbohydrate binding
468 proteins, which can associate with the S-layer, such as Csac_2722, may be important to CBP
469 microorganisms and this issue merits further attention. There is much still to understand about
470 the complex process by which lignocellulose is degraded and utilized in natural environments
471 that can be translated to current efforts to produce biofuels from renewable feedstocks.
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494

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TABLES and FIGURES

Table 1. Primers used for cloning Csac_0678 and Csac_2722 genes

Clone	F/R	Primer
Csac_0678	F	GGCGGCCCATGGATAATACTGCGTATGAAAAGGATAAGTATCCACAC
	R	GGCGCCTCGAGCATCTTTCCTGTAAGTTCTAAAATTTTGTA
Csac_0678-TM1	F	GGCGGCCCATGGATAATACTGCGTATGAAAAGGATAAGTATCCACAC
	R	GGCGGCCTCGAGATTTGTAAATCTTACATTGT
Csac_0678-TM2	F	GGCGGCCCATGGATAATACTGCGTATGAAAAGG
	R	GGCGGCCTCGAGAGCACCAGAAGTCTCATT TT
Csac_2722	F	GGCGGCGCTAGCCTGTTCATAAAGAGTACA
	R	GAAGACCTCGAGCTATTGAGCCTGTCCATAGGTGGC

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Table 2. SLH-domain proteins in *Caldicellulosiruptor* genomes that contain sugar binding domains and Glycoside Hydrolase (GH) catalytic domains		
DOMAINS[a]	**ORF**	**# Amino Acids**
GH5-CBM28-SLH-SLH-SLH	Csac_0678	755
	Calkro_2036	
	Calla_0352	756
	Calhy_2064	
	Calkr_2007	
	Athe_0594	
	COB47_0546	568
	Calow_0459	567
(CBM22)-CBM22-CBM22-GH10-CBM9-CBM9-CBM9-(CE15)-SLH-SLH	Calow_1924	1,625
	Calkro_0402	1,672
	Calla_0206	1,593
	Calkr_2245	2,159
(SLH-SLH-SLH-CBM54-GH16)[b]-CBM4-CBM4-CBM6-CBM4-CBM4-CBM4	Calkro_0072	1,732
	Calhy_0060	
	COB47_0076	
	Csac_2548-2549[b]	1,648[c]
SLH-SLH-SLH-CBM54-FN3-GH16-FLD-FN3-FN3-FLD-GH55-CBM32-(CBM32)	Calkro_0111	2,435
	Calkro_0121	2,229
SLH-SLH-SLH-GH43-CBM54	Calhy_1629	1,440
CBM35-GH87-FN3-SLH-SLH	Calhy_2383	2,007

[a]Abbreviations used are as follows: SLH (S-layer homology domain); FN3 (Fibronectin III domain); Big4 (Ig-like domain); CBM (cellulose binding domain, family #); FLD (Fascin-like domain).

[b]Csac_2548 and Csac_2549 are homologous to the ORFs listed and were most likely created by a deletion event that truncated the GH16 domain and split the ORF into two separate proteins.

[c]Total size of Csac_2548 and Csac_2549.

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Table 3. Hydrolytic activity of Csac_0678 on complex carbohydrates

Substrate	Linkage	Specific activity* ($U \cdot mg^{-1}$ enzyme)
Barley glucan	β-1,3/4-glucan	28.2
Lichenan	β-1,3/4-glucan	17.4
CMC	β-1,4-glucan	8.94
pNPC	β-1,4-glucan	5.52
Glucomannan	β-1,4-glucan/mannan	4.21
PASC	β-1,4-glucan	2.43
Xylan Oat Spelt	β-1,4-xylan	0.709
Xylan Birchwood	β-1,4-xylan	0.570
Arabinoxylan	β-1,4-xylan	0.500
BMCC	β-1,4-glucan	0.227
Filter paper	β-1,4-glucan	0.0518
Avicel	β-1,4-glucan	0.0113
Xyloglucan	β-1,4-glucan/β-1,6-xylan	low activity
Mannan	β-1,4-mannan	ND
Laminarin	β-1,3/6-glucan	ND

*Specific activities are based on triplicate analysis for each substrate and account for abiotic hydrolysis.

All standard deviations were less than ± 10%

ND = No detectable activity
CMC = Carboxymethyl Cellulose
PASC = Phosphoric Acid Swollen Cellulose
BMCC = Bacterial Microcrystalline Cellulose
pNPC = *p*-nitrophenyl-β-D-cellobioside

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Table 4. SLH-domain proteins in *Caldicellulosiruptor* genomes that contain binding domains but no Glycoside Hydrolase (GH) catalytic domains		
DOMAINS[a]	**ORF**	**# Amino Acids**
SLH-SLH-SLH-FN3-FN3-vWFA-vWFA-SH3	Athe_0012	3,027
	Calkro_0014	2,994
SLH-SLH-SLH-Big-(Big)	Athe_1839	575
	Calow_1583	
	Csac_2381	
	Calkro_0875	576
Big4-SLH-SLH-(SLH)	Calkro_0550	1,789
	Calkro_0550	
	Calow_1771	1,790
(SLH-SLH)-SLH-Big1-Tg	Calhy_0047	1,626
	Calla_2324	1,779
	COB47_0063	1,774
	Calkr_2463	1,179
SLH-SLH-SLH-FN3-FN3	Calla_1498	575
	Calkr_0834	
(Big)-(CBM20-CBM20)-SLH-SLH-SLH-CBM20	Calla_0367	1,097
	COB47_0564	
	Calow_0484	1,097
	Calkr_1989	326
(CBM27)-CBM27-CBM27-CBM27- SLH-SLH-SLH	Calla_2176	1,088
	COB47_0167	886
SLH-SLH-SLH-CBM54-Tg	Calow_0034	1,774
Big3- Big3- Big3- Big3-PL9-SLH-SLH-SLH	Calow_2109	1,711
GBD-Big4-GBD-Big4-Cd-SLH-SLH-SLH	Csac_2722	2,593

[a]Abbreviations used are as follows: SLH (S-layer homology domain); FN3 (Fibronectin III domain); vWFA (von Willebrand domain); SH3 (src Homology-3 domain); GBD (Galactose Binding Domain-like); Big1, Big4 (Ig-like domains); CBM (cellulose binding domain, family 6,20,27) RB (Ricin-like domain); PL (Pectate Lyase fold); Tg (Transglutiminase-like domain); Cd (Cadherin-like domain)

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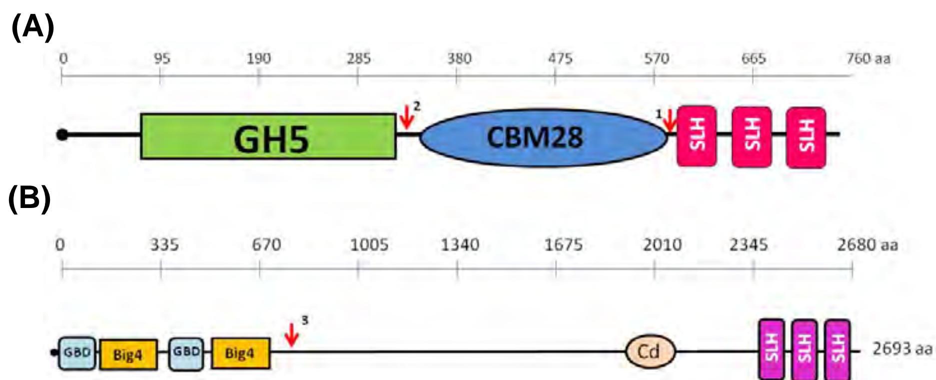

Figure 1. Csac_0678 and Csac_2722 conserved domains and truncation points for deletion mutants. **(A)** Recombinant intact Csac_0678 (727 aa), lacking only the signal peptide. Csac_0678-TM1 (575 aa) and Csac_0678-TM2 (317 aa) were constructed by truncating at the points 1 and 2, respectively. **(B)** Recombinant Csac_2722 (810 aa), without the signal peptide, was truncated at point 3. LEGEND - GH5: Glycoside hydrolase family 5, CBM28: Carbohydrate binding module family 28, SLH: Surface layer homology domain, GBD: Galactose binding domain-like, Big4: Family 4 bacterial immunoglobulin-like domain, Cd: Cadherin-like domain, ●: Signal peptide; aa: amino acid. **(C)** Western Blot showing the localization of Csac_0678 in the S-layer for *C. saccharolyticus* grown on cellobiose. The lane labeled as "Csac_0678" had purified recombinant Csac_0678. The lane labeled as the "S-layer" is the S-layer fraction extracted from native *C. saccharolyticus* cells. Negative controls with an unrelated protein did not have any bands (not shown).

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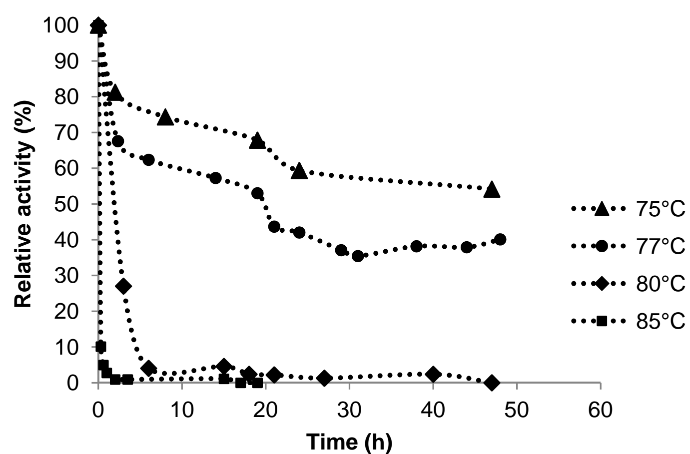

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741 **Figure 2.** Comparison of thermostability of Csac_0678 at different incubation temperatures.
742 Half-life at 75°C was approximately 48 hours, and 19 hours at 77°C. At 80° and 85°C,
743 Csac_0678 lost 50% of initial activity in less than three and in less than 30 min, respectively.
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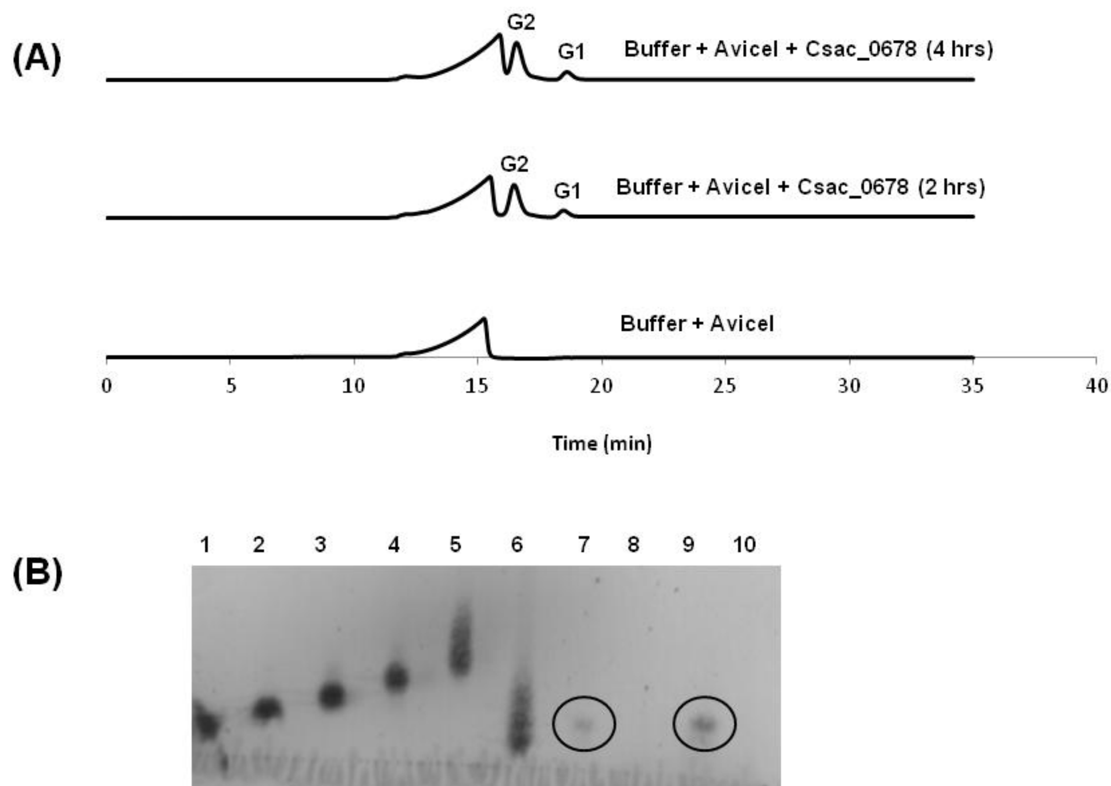

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759 **Figure 3**. Hydrolysis of cellulose by Csac_0678 at 70°C. **(A)** HPLC (RI) analysis shows
760 glucose and cellobiose produced after 2 and 4 hour incubations of enzyme with Avicel (100
761 µm). **(B)** TLC for incubation of Csac_0678 for 4 hrs on two different particle sizes of
762 cellulose (20 and 100 µm). TLC Standards, 1: Glucose, 2: Cellobiose. 3: Cellotriose, 4:
763 Cellotetraose, 5: Cellopentaose, 6: Xylooligosaccharides, Incubation with Csac_0678 with
764 7: Cellulose (100 µm) and 9: Cellulose (20 µm). Controls were 8: Cellulose (100 µm)
765 and10: Cellulose (20 µm), in each case with no added enzyme.
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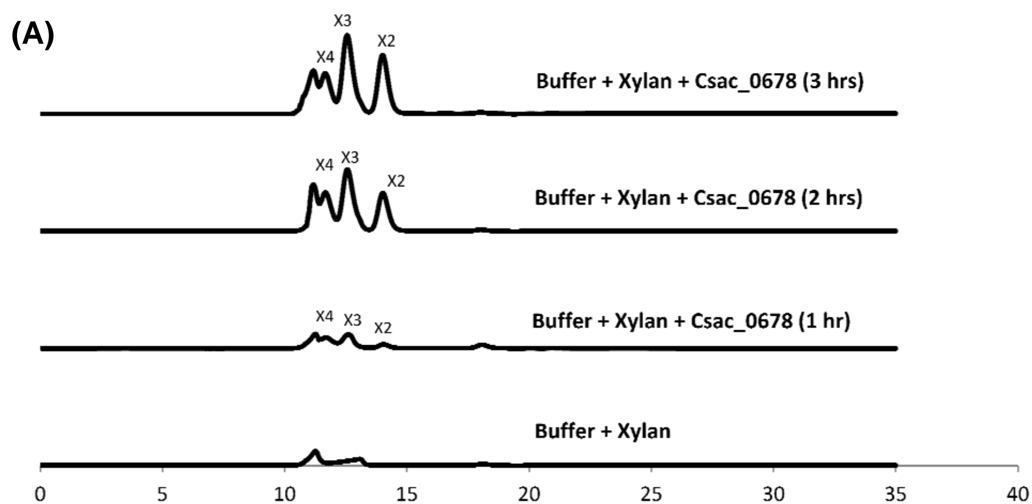

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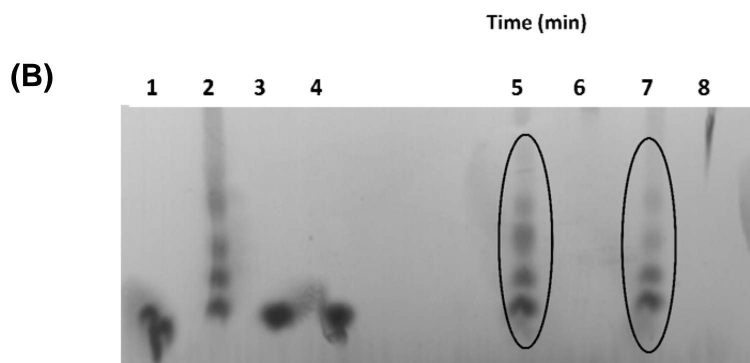

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776 **Figure 4**. Hydrolysis of xylan by Csac_0678 at 75°C. **(A)** HPLC (RI) analysis shows
777 xylobiose, xylotriose and xylotetraose released upon incubation with the enzyme. **(B)** TLC
778 for incubation of xylan with the enzyme for 4 hours. Standards, 1: Xylose, 2:
779 Xylooligosaccharides, 3: Arabinose, 4: Glucose. Reactions with Csac_0678 on 5: Xylan
780 birchwood and 7: Xylan oat spelt. Controls were 6: Xylan birchwood and 8: Xylan oat spelt,
781 in each case with no enzyme added.
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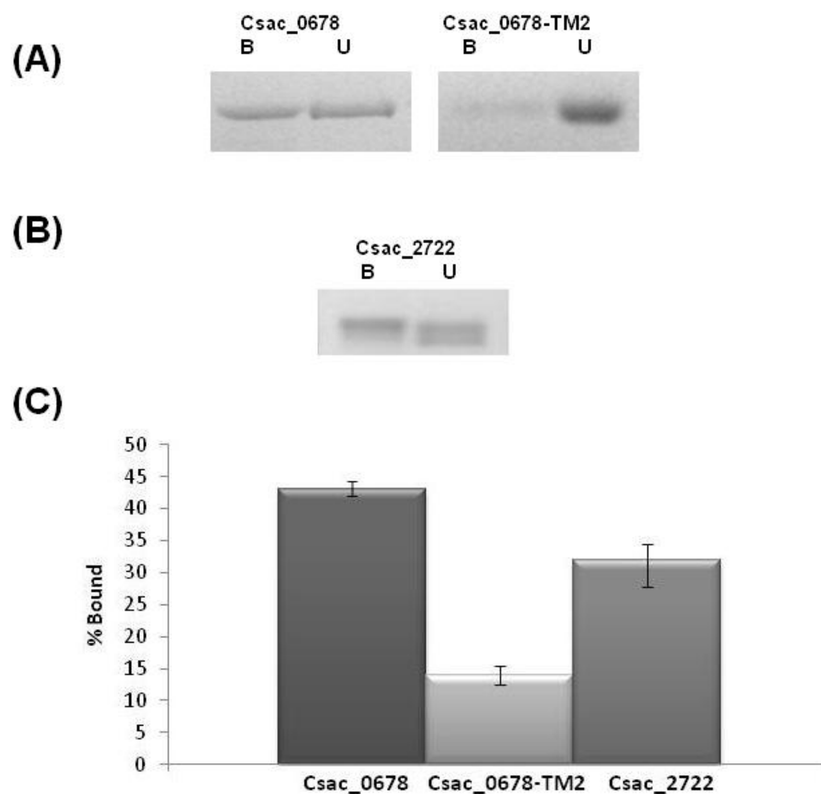

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794 **Figure 5**. Csac_0678 binding to Avicel was decreased with the deletion of the CBM28 domain.
795 (A) and (B) Shown are SDS-PAGE with bound (B) and unbound (U) fractions of Csac_0678 with
796 and without CBM domains and Csac_2722, respectively from carbohydrate (Avicel) affinity
797 experiment. (C) Binding of Csac_0678, Csac_0678 without the binding domain, and Csac_2722
798 to Avicel.
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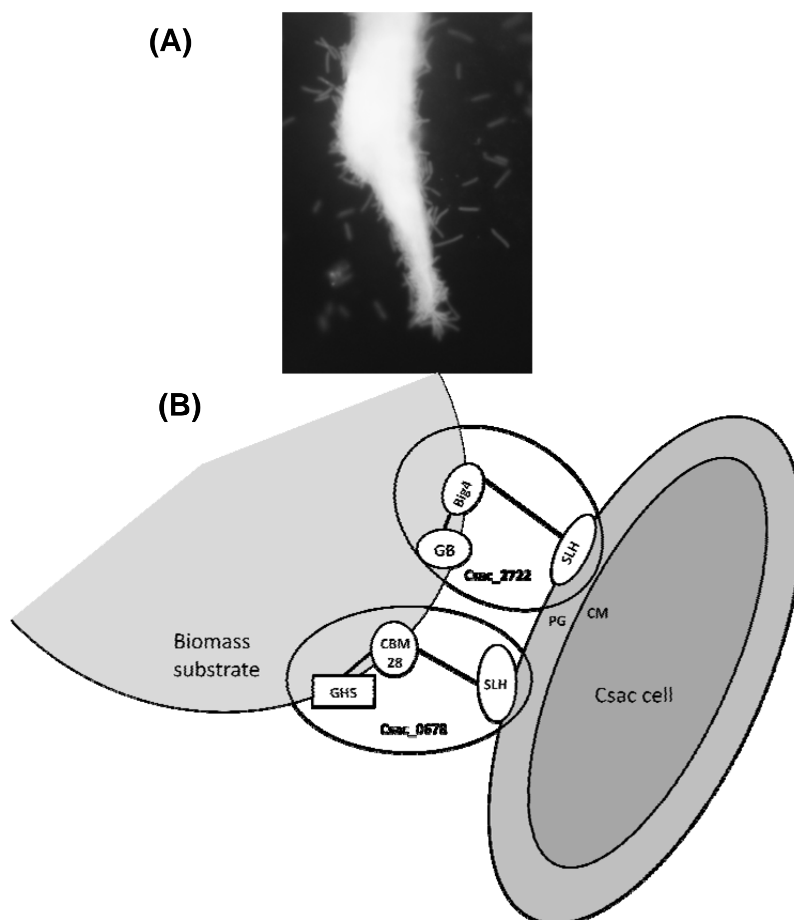

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805 **Figure 6**. Proposed role of Csac_0678 and Csac_2722 in plant biomass deconstruction by *C.*
806 *saccharolyticus*. **(A)** Epifluorescence micrograph (acridine orange stain) showing *C.*
807 *saccharolyticus* (rods) attachment to acid pre-treated switchgrass particle. **(B)** Schematic
808 representation of cell wall and carbohydrate attachment of Csac_0678 and Csac_2722.
809 LEGEND- PG: Peptidoglycan layer; CM: Cell membrane; GB: Galactose binding domain like;
810 GH5: Glycoside hydrolase family 5; CBM28: Carbohydrate binding module family 28; SLH:
811 Surface layer homology domain.

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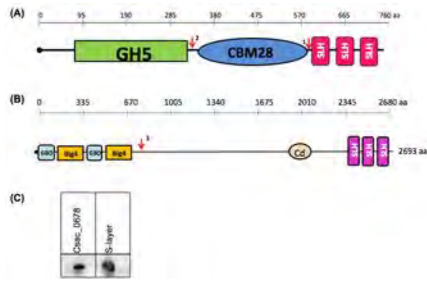

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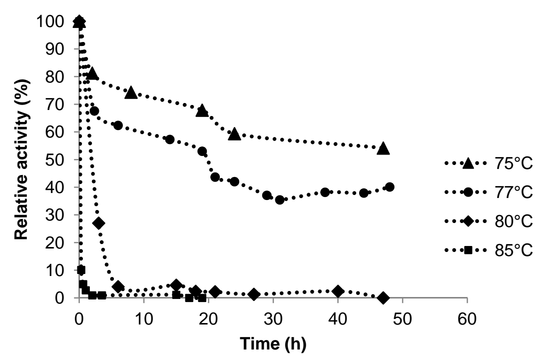

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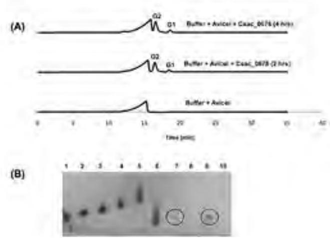

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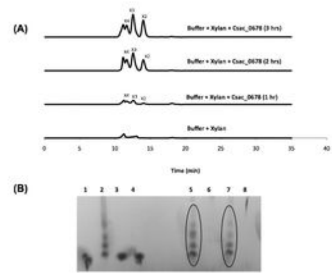

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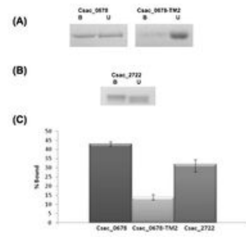

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