Co-expression of a β-D-xylosidase from *Thermotoga maritima* and a Family 10 xylanase from *A. cellulolyticus* significantly improves the xylan degrading activity of the *Caldicellulosiruptor bescii* exoproteome

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Abstract

Caldicellulosiruptor species are hyperthermophilic, Gram-positive, anaerobes and the most thermophilic cellulolytic bacteria so far described. They have been engineered to convert switchgrass to ethanol without pretreatment and represent a promising platform for the production of fuels, chemicals and materials from plant biomass. Xylooligomers such as xylobiose and xylotriose that result from the breakdown of plant biomass more strongly inhibit cellulase activity than do glucose or cellobiose. High concentrations of xylobiose and xylotriose, are present in *C. bescii* fermentations after 90 h incubation and removal or breakdown of these types of xylooligomers is crucial to achieve high conversion of plant biomass to product. In previous studies the addition of exogenous β-D-xylosidase substantially improved the performance of glucanases and xylanases *in vitro*. β-D-Xylosidases are, in fact, essential enzymes in commercial preparations for efficient deconstruction of plant biomass. In addition, the combination of xylanase and β-D-xylosidase is known to exhibit synergistic action on xylan degradation. In spite of its ability to grow efficiently on xylan substrates, no extracellular β-D-xylosidase was identified in the *C. bescii* genome. Here we report that the co-expression of a thermal stable β-D-xylosidase from *Thermotoga maritima* and a xylanase from *Acidothermus cellulolyticus* in a *C. bescii* strain containing the *A. cellulolyticus* E1 endoglucanase significantly increased the activity of the exoproteome as well as growth on xylan substrates. The combination of these enzymes also resulted in increased growth on crystalline cellulose in the presence of exogeneous xylan.

47

48 **Importance (150 words, nontechnical)**

49 *Caldicellulosiruptor* species are bacteria that grow at extremely high temperature, more
50 than 75 degrees centigrade, and are the most thermophilic bacteria so far described
51 capable of growth on plant biomass. This native ability allows the use of unpretreated
52 biomass as a growth substrate, eliminating the prohibitive cost of
53 preprocessing/pretreatment of the biomass. They only grow under strictly anaerobic
54 conditions and the combination of high temperature and the lack of oxygen reduces
55 the cost of fermentation and contamination by other microbes. They have been
56 genetically engineered to convert switchgrass to ethanol without pretreatment and
57 represent a promising platform for the production of fuels, chemicals and materials
58 from plant biomass. In this study we introduced genes from other cellulolytic bacteria
59 and identified a combination of enzymes that improves growth on plant biomass. An
60 important feature of this study is that it measures growth, validating predictions made
61 from adding enzyme mixtures to biomass.

62

63 **Keywords:** consolidated bioprocessing; biomass deconstruction; xylanase; β-D-
64 xylosidase; *Caldicellulosiruptor*

65

66 **Introduction**

Members of the genus, *Caldicellulosiruptor*, are hyperthermophilic anaerobes and the most thermophilic cellulolytic bacteria so far described. Unlike most cellulolytic species of the *Clostridium* genus that rely on complex protein structures, called cellulosomes, *Caldicellulosiruptor* species secrete primarily free multifunctional enzymes into the exoproteome. One such cellulase, CelA, is the most abundant enzyme secreted by *C. bescii* (1, 2), and has been shown to outperform mixtures of commercially available exo- and endoglucanases *in vitro* (3). Moreover, *C. bescii* uses both xylans and glucans simultaneously and has the ability to grow well on xylan (1, 4). While most strain engineering of *C. bescii* has focused on improving cellulolytic activity (5-7), improving hemicellulolytic activity is essential to make consolidated bioprocessing by *C. bescii* an industrially relevant process (8). Hemicelluloses, particularly in the form of xylobiose and xylotriose, strongly inhibit cellulase activity even more so than glucose or cellobiose (9). A previous study showed that high concentrations of xylobiose and, to a lesser extent, xylotriose, accumulate in *C. bescii* fermentations after 90 h incubation (1). Complete removal or breakdown of xylooligomers is crucial to achieve higher conversion of plant biomass, and in fact, β-D-xylosidases are essential in commercial enzyme preparations for efficient deconstruction of plant biomass as the activity of cellulases and hemicellulases in these mixtures has been shown to be inhibited by xylobiose. Previous studies also showed that the addition of exogenous β-D-xylosidase substantially improved the performance of glucanases and xylanases *in vitro* (8, 10, 11). In spite of its ability to grow efficiently on xylan substrates, no extracellular β-D-xylosidase was identified in the *C. bescii* genome (4, 5). To investigate whether the addition of a secreted β-D-xylosidase would improve growth, xylan utilization and

cellulose utilization even in the presence of xylan by *C. bescii*, we cloned and expressed a thermal stable β-D-xylosidase from *T. maritima* (Tm_0076, GenBank accession number AAD35170) in *C. bescii* using the CelA signal sequence for protein export. This gene was chosen because the enzyme was reported to be maximally active at 90°C (12). We then examined the effect of co-expression of this β-D-xylosidase with a Family 10 xylanase (Acel_0180) from *A. cellulolyticus* on the activity of the exoproteome in a strain containing the *A. cellulolyticus* E1 endoglucanase, previously shown to act synergistically to improve the cellulolytic activity of the *C. bescii* exoproteome (6). The combination of xylanase and β-D-xylosidase is known to act synergistically on xylan degradation (10). We selected the xylanase based on a previous study showing that introduction of this enzyme including the tandem carbohydrate binding modules (CBM2 and CBM3) located at the C-terminus significantly improved the ability of *C. bescii* to utilize xylan (13). The combination of these enzymes increased the overall activity of the *C. bescii* exoproteome, improved growth on xylan as well as growth on crystalline cellulose even in the presence of exogenous xylan in the growth medium.

Results and Discussion

Heterologous expression and secretion of a β-D-xylosidase from *T. maritima* in a *C. bescii* strain containing the *A. cellulolyticus* endoglucanase E1. To construct an expression vector for Tm_0076 in *C. bescii* (Figure S1), the gene was amplified from *T. maritima* gDNA and cloned into a shuttle vector, pSKW28, under the transcriptional control of the *C. bescii* S-layer promoter (14, 15). The native Tm_0076 signal peptide was replaced with the CelA signal sequence as CelA is the most abundant extracellular

113 enzyme produced by *C. bescii* (1, 2) and previous work showed that it can be used to
114 drive secretion of other heterologous proteins including the secretion of cellobiose
115 phosphorylase from *T. maritima* in *C. bescii* (7). The plasmid was introduced into a *C.*
116 *bescii* strain, JWCB52, that contains the E1 protein from *A. cellulolyticus,* a
117 thermostable endo-1,4-β-glucanase (GH5) with a family 2 carbohydrate-binding module,
118 inserted into the *C. bescii* chromosome at the chromosomal integration site one (CIS1)
119 (15). Strain JWCB52 also contains a deletion of *pyrF* and transformants were selected
120 for uracil prototrophy to generate JWCB95 which was grown at 65°C to accommodate
121 the expression of *C. thermocellum pyrF* gene used for complementation and plasmid
122 selection. To verify the presence of the plasmid, primers (DC460 and DC228) were
123 used to amplify the portion of the plasmid containing the open reading frame of the
124 targeted proteins, but also annealing to regions of the plasmid outside the gene to avoid
125 amplification of sequences residing on the chromosome (Figure S2A). Total DNA from
126 strain JWCB95 was also back-transformed into *E. coli*, and two different restriction
127 endonuclease digests performed on plasmid DNA purified from three independent back-
128 transformants resulted in identical digestion patterns to the original plasmid (Figure
129 S2B). These results indicated that the plasmid was successfully transformed into *C.*
130 *bescii* and structurally stable during transformation and replication in *C. bescii* and back-
131 transformation into *E. coli.*
132 For detection of β-D-xylosidase activity, the extracellular protein (ECP) fraction from
133 JWCB95 (Figure 1) culture supernatants was first separated in a SDS-PAGE gel (Figure
134 2A) and then renatured in the same gel followed by infusion with 4-methylumbelliferyl β-
135 D-xylopyranoside (MUX). As shown in Figure 2B, two protein bands exhibiting β-D-

xylosidase activity were detected in the parent and E1 expression strain. The *C. bescii* genome contains only two genes annotated as potential extracellular xylosidases, Cbes_2371 (34 KDa) and Cbes_0182 (152 KDa) both containing GH43s, known to exhibit xylosidases (16, 17). Protein bands consistent with a molecular weight of ~160 and ~110 kDa were detected and these species most likely represent the intact Cbes_0182 and functional truncations. Note that we have also observed similar functional truncations of cellulases in the *C. bescii* exoproteome. The predicted molecular weight of a *T. maritima* β-D-xylosidase dimer (Tm_0076) would be ~174 kDa which is close to the molecular weight of Cbes_0182, making it difficult to separate the two. However, comparison of the band intensities from the zymogram showed that the activity corresponding to this molecular weight was in JWCB95 more than twice that in JWCB82 (the parental strain) or JWCB73 (the E1 expression strain).

To comfirm the expression of Tm_0076 and remove the background from native *C. bescii* CAZymes, we performed zymogram analysis using 4-methylumbelliferyl β-D-glucopyranoside (MUG) a substrate specific for β-D-glucosidase activity as Tm_0076 (GH3) should exhibit activity on this substrate but not Cbes_0182 (GH43).

A clearing zone corresponding to a protein of molecular weight 160 kDa was obtained from the JWCB95 strain, but was not present in the JWCB73 strain (Figure 2C). We conclude from these analyses that the *T. maritima* β-D-xylosidase was expressed, secreted, and functional in *C. bescii*. We believe that this band corresponds to a dimer of Tm_0076 (~174 Kda).The maintenance of multimers of thermostable proteins in SDS-PAGE gels is well documented. For example, the β-D-glucosidase from *Pyrococcus furiosus* (BGLPf) appears to form a dimer that is stable in the presence of

159 sodium dodecyl sulfate and this dimer migrated in reducing SDS–PAGE even after
160 incubation at 95°C (18).
161 **Heterologous expression and secretion of a xylanase from *A. cellulolyticus* in a**
162 ***C. bescii* strain containing the *A. cellulolyticus* endoglucanase E1.** To generate a
163 strain containing the *A. cellulolyticus* xylanase (Acel_0180) in the *C. bescii* strain
164 containing E1, the xylanase expression cassette was amplified by PCR from a
165 replicating shuttle vector, pSKW10 (13), and cloned into an integrational expression
166 vector, pSKW23 (Figure 3A). The $P_{S\text{-}layer}$ - Acel_0180 cassette was flanked by two 1-kb
167 DNA regions of homology from the intergenic region between Cbes2199 and Cbes2200
168 (CIS2), previously determined to be available without affecting growth or resulting in a
169 detectable phenotype (19). JWCB52 contained the *A. cellulolyticus* E1 gene inserted
170 into the intergenic region between Cbes0863 and Cbes0864, designated CIS1 (5).
171 Uracil prototrophic transformants were serially passaged as described (14) to allow
172 segregation of merodiploids containing a mixture of the integrated Acel_0180
173 expression cassette and the parental strain (JWCB52) genomes. This resulted in strain
174 JWCB87 (Δ*pyrFA ldh::ISCbe4* Δ*cbe1* CIS1::$P_{S\text{-}layer}$Acel0614(E1) CIS2::$P_{S\text{-}layer}$Acel0180
175 (xylanase)) (Table 1). Verification of insertion of the xylanase gene into the JWCB87
176 chromosome was performed using PCR amplification with primers SK65 and SK66
177 (Figure 3B), and sequencing the PCR products. The parental strain, JWCB52, produced
178 the expected wild type 2.2 kb band, whereas amplification of JWCB87 produced 4.4 kb,
179 indicating an insertion of the xylanase expression cassettes within the targeted region
180 (Figure 3B). Expression and secretion of the *A. cellulolyticus* xylanase in *C. bescii* was
181 confirmed using zymogram analysis (Figure S3).

Co-expression of the *T. maritima* β-D-xylosidase and the *A. cellulolyticus* xylanase in the *C. bescii* strain containing E1 was synergistic for xylan degradation. To first test β-D-xylosidase activity in this strain, cells were grown at 65°C and the extracellular protein fraction from JWCB82 (the parental strain), JWCB73 (containing E1), JWCB95 (containing E1 and the *T. maritima* β-D-xylosidase), JWCB102 (containing E1 and the *A. cellulolyticus* xylanase), and JWCB103 (containing E1, the β-D-xylosidase and the xylanase) were compared. Expression and secretion of the Acel_0180 xylanase and Tm_0076 β-D-xylosidase were confirmed by zymogram analysis (Figure S3). The extracellular protein fraction was then assayed for β-D-xylosidase activity at 65°C and 75°C, on *p*-nitrophenyl β-D-xylopyranoside (pNP-X), a substrate specific for β-D-xylosidase activity. The stability and activity of native *C. bescii* xylan degrading enzymes are known to decrease significantly at temperatures higher than 85°C (13). While the optimal temperature for growth of *T. maritima* is 80°C (20), the optimal temperature for activity of the β-D-xylosidase was reported to be 90°C (12). For the strains expressing β-D-xylosidase there was, as expected, an increase in β-D-xylosidase activity. Culture supernatants from the JWCB95 showed a 70% (P_{value}=0.019) increase over the parental strain at 65°C and a 46% (P_{value}=0.009) higher β-D-xylosidase activity at 75°C. Culture supernatants from the JWCB103 showed a 41% (P_{value}=0.003) increase at 65°C and a 27% (P_{value}=0.035) increase at 75°C (Figure 4A).

Xylobiose is a known inhibitor of xylanase activity, and studies have shown that the exogenous addition of β-D-xylosidase markedly improved the performance of some xylanases (10, 11). To examine whether expression of the β-D-xylosidase enhanced the

xylan degrading activity of the exoproteome, enzyme assays were performed at 65°C using oat spelts and birchwood xylans as substrates using the same three strains. Oat spelts xylan is a complex arabinoxylan, branched with arabinose residues. Birchwood xylan is a simpler, primarily unsubstituted xylose polymer with traces of uronic acids as side groups (more than 90% β-1,4-linked xylose residues) (21, 22). As previously reported (13), the exoproteome from a xylanase expressing strain (JWCB102) showed 23% (P_{value} = 0.026) higher activity on oat spelt xylan and 24% (P_{value} = 0.008) higher activity on birchwood xylan compared to the parental strain, JWCB82 (Figure 4B). Increased activity of the concentrated culture supernatants from the β-D-xylosidase expressing strain was observed for both oat spelts and birchwood xylans compared to the parental strain, indicating that increasing the extracellular β-D-xylosidase does, in fact, increase xylan hydrolysis. In JWCB102 the β-D-xylosidase most likely aids the native *C. bescii* xylanases but an even greater synergy is observed in JWCB103 which also expresses the *A. cellulolyticus* xylanase. The activity of the exoproteome from JWCB95 (the β-D-xylosidase expression strain without the *A. cellulolyticus* xylanase) on oat spelts and birchwood xylans increased 16% (P_{value} = 0.009) and 23% (P_{value} < 0.001), the activity from JWCB103 (the β-D-xylosidase and *A. cellulolyticus* xylanase co-expression strain) increased 45% (P_{value} < 0.001) and 79% (P_{value} < 0.001), respectively (Figure 4B). Total xylanase activity increased at 75°C, (Figure 4C) but the pattern was the same (Figure 4C). These results suggest that the *T. maritima* β-D-xylosidase acts synergistically with both the native and *A. cellulolyticus* xylanases to deconstruct xylans more efficiently.

Co-expression of the *T. maritima* β-D-xylosidase and the *A. cellulolyticus* xylanase in the *C. bescii* strain containing E1 resulted in an increase in the ability of *C. bescii* to grow on xylan substrates. To examine the effect of the expression of the heterologous β-D-xylosidase and xylanase on the growth of the *C. bescii* strain containing E1, growth was first measured on the soluble substrate, cellobiose as sole carbon source, a disaccharide that does not require the activity or either a xylanase or cellulase. As shown in Figure 5A, growth of the JWCB82 (the parental strain), JWCB73 (the E1 expression strain), JWCB95 (the E1 expression strain containing the *A. cellulolyticus* xylanase), JWCB102 (the E1 expression strain containing the *T. maritima* β-D-xylosidase), and JWCB103 (the E1 expression strain containing both the *A. cellulolyticus* xylanase and *T. maritima* β-D-xylosidase) strains was virtually identical. While doubling time of the JWCB102 was slightly shorter than that of the JWCB82, it was not significant (P_{value} = 0.81). This result indicates that expression of these heterologous enzymes had no obvious effect on growth in general.

As previously reported (13) and as shown in Figure 5B, growth of JWCB102 (the E1 expressing strain containing the *A. cellulolyticus* xylanase) on birchwood xylan was 23.0 (P_{value}=0.008) fold higher than that of the JWCB82 parental strain. JWCB95, containing E1 and the Tm_0076 β-D-xylosidase resulted in a dramatic increase in the ability of this strain to grow on xylan, a 2.7 (P_{value}=0.076) and 6.7 (P_{value}=0.039) fold increase on oat spelts and birchwood xylans, respectively, over that of the JWCB82 parental strain (Figure 5B). Growth of JWCB103 (the E1 expression strain containing both the *A. cellulolyticus* xylanase and *T. maritima* β-D-xylosidase) was even more dramatic, a 5.7 (P_{value}=0.052) and 32.1 (P_{value}=0.009) fold increase on oat spelts and

birchwood xylans, respectively, over that of the JWCB82 parental strain. We suggest that in both JWCB102 and JWCB103, Acel_0180 may help release more xylobiose or xylotriose in the media and further reduce the degree of polymerization of the deconstructed xylans rendering them readily transported and used as a carbon source. This is apparently more pronounced in the case of the simpler substrate (birchwood xylans) than the more complex branched substrate (oat spelts xylans). On oat spelts xylan, the activity of Acel_0180 as well as other xylanases is likely limited by increased branching that reduces accessibility. In addition the released xylans, most likely branched, may not be as readily transported or utilized by the microorganism. In JWCB103, the increased growth is most likely due to decreased inhibition by xylobiose or xylotriose, resulting from the action of the β-D-xylosidase, on xylanases including Acel_0180, leading to increased overall solubilization. This is also demonstrated by the difference in growth between JWCB95 and JWCB73 where the additional β-D-xylosidase is augmenting the native xylanases in the *C. bescii* exoproteome therefore leading to higher growth.

The combination of the β-D-xylosidase and xylanase substantially improves the activity of the exoproteome on cellulose even in the presence of exogenous xylan. To test whether the Tm_0076 β-D-xylosidase and Acel_0180 xylanase, do in fact, aid in cellulose utilization by relieving inhibition of xylobiose or xylotriose accumulating in the media, growth was measured on Avicel with and without the addition of xylan. The strains JWCB073, JWCB082, and JWCB103 were grown on both 5 g/L Avicel alone and 5 g/L Avicel + 2.5 g/L oat spelts xylan at 65°C. Oats spelts xylan was chosen because it is a poor substrate for growth (Figure 5B), should be inhibitory

because it contains branched arabinose residues, and its deconstruction is less affected by the presence of Tm_0076 β-D-xylosidase and Acel_0180 (Figure 4B). On Avicel alone, the combination of E1, the Tm_0076 β-D-xylosidase and the Acel_0180 xylanase (JWCB103) resulted in significantly better growth than the parent strain (JWCB82) or the E1 containing strain (JWCB73), a 43% increase compared to the parent strain JWCB082 at 60 hours (Figure 5C). These results indicate that the combination of the β-D-xylosidase and xylanase allows *C. bescii* to more readily utilize the negligible (~5%) xylan content in Avicel. Additionally, this increase could also be due to the fact that the GH3 in Tm_0076 possesses β-D-glucosidase activity (12), or that the fibronectin like domain might aid in the deconstruction of Avicel as shown for other biomass degrading enzymes (23). Growth of JWCB103 on Avicel in the presence of exogenous oat spelts xylan resulted in significant differences in both the timing and overall amount of growth. Perhaps the most striking difference was the almost total elimination of a lag phase for JWCB103 and a reduced lag time for JWCB73 and JWCB82 (Figure 5D) compared to growth on Avicel alone. The overall growth was also less than on Avicel alone. This might be explained by the fact that *C. bescii* can utilize the easily accessible xylan, a preferred carbon source, in oat spelts xylan, resulting in increased biomass and the production of complex biomass degrading enzymes earlier in the fermentation. We also believe that part of this increase is due to the xylan content in Avicel but is not sufficient to explain that level of increased growth. While this is true for both JWCB73 and JWCB82, the inhibition of oat spelts xylan during fermentation resulting in less biomass production is more clear.

297 **Conclusions**

298 The ability of *C. bescii* to deconstruct non pretreated plant biomass, its ability to grow
299 anaerobically at high temperature and its ability to use both C5 and C6 sugars
300 simultaneously make it of special interest for use in consolidated bioprocessing to
301 produce fuels, chemicals and materials from this sustainable substrate. *C. bescii* also
302 represents a model for understanding the fundamentals of plant cell wall deconstruction
303 given its unusual cellulolytic activity. In previous studies the supplementation of its
304 secretome with heterologous CAZyme cassettes led to significant increases in
305 cellulolytic activity and growth on complex substrates. In this study, we examined
306 CAZyme cassettes with specific predicted synergy, a β-D-xylosidase thought to relieve
307 substrate inhibition and a xylanase while also likely to be susceptible to inhibition by
308 xylooligomers (in this case xylobiose and xylotriose) might augment the native *C. bescii*
309 xylanases. Taken together, the data presented support those predictions. Significant
310 increases in the enzymatic activity of the exoproteome as well as dramatic effects on
311 growth were observed suggesting synergistic interactions between CAZymes *in vivo*.
312 We further suggest that this kind of study will facilitate the optimization and the synergy
313 within and with these heterologous CAZyme cassettes to further improve thermophilic
314 consolidated bioprocessing in other microbes. Finally, we suggest that the results
315 shown in this study represent an important step towards addressing the inhibition of
316 xylooligomers in consolidated bioprocessing at industrially relevant (high) solids
317 loadings.

318

Materials and methods

Strains, media, and culture conditions. *E. coli* and *C. bescii* strains used in this study are listed in Table 1. *C. bescii* strains were grown anaerobically at 65°C on solid or in liquid low osmolarity defined (LOD) medium (24), as described, with 5 g/L maltose or cellobiose as sole carbon source for routine growth and transformation experiments (25). For growth of uracil auxotrophs, the medium contained 40 µM uracil. *E. coli* DH5α was used as host for plasmid DNA construction and preparation using standard techniques. *E. coli* cells were cultured in LB broth containing apramycin (50 µg/mL). Plasmid DNA was isolated using a Qiagen Miniprep Kit (Qiagen, Valencia, CA, USA). Chromosomal DNA from *C. bescii* strains was extracted using the Quick-gDNA MiniPrep (Zymo, Irving, CA) as described (26).

Construction of a shuttle vector for β-D-xylosidase expression. Q5 High-Fidelity DNA polymerase (New England BioLabs, Ipswich, MA, USA) was used for all PCR reactions. Restriction enzymes (New England BioLabs, Ipwich, MA, USA) and the Fast-link DNA ligase kit (Epicentre Biotechnologies, Madison, WI, USA) were used for plasmid constructions according to the manufacturer's instructions. To construct pSKW28, a 2.3 kb DNA fragment containing the coding sequence of Tm_0076 was amplified using primers SK74 (with an XmaI site) and SK75 (with an AvrII site) using *T. maritima* MSB8 gDNA as template. In addition, an 8.1 kb DNA fragment containing the *C. bescii* replication origin from BAS2, an apramycin resistance gene cassette (Apr^R), a *C. thermocellum pyrF* expression cassette, the regulatory region of Cbes2303 (S-layer protein), the signal CelA signal sequence, a C-terminal 6X Histidine-tag, and a Rho-

342 independent transcription terminator was amplified with primers SK21 (with XmaI site)
343 and DC700 (with AvrII site) using pSKW10 (13) as template. These two linear DNA
344 fragments were digested with XmaI and AvrII and ligated to construct pSKW28 (Figure
345 S1B). Primers used are listed in Table 2.
346
347 **Construction of a vector for insertion of the Family 10 xylanase (Acel_0180) from**
348 ***Acidothermus cellulolyticus* into the *C. bescii* chromosome.** To construct pSKW23,
349 the 2.3 kb Acel_0180 expression cassette, containing the regulatory region of
350 Cbes2303 (S-layer protein), the CelA signal sequence, a C-terminal 6X Histidine-tag,
351 and a Rho-independent transcription terminator, was amplified by PCR with primers
352 DC460 (with PvuI site) and DC461 (with NotI site) using pSKW10 (13) as template. The
353 6.0 kb DNA fragment containing the 5′ flanking region (1.0 kb) and the 3′ flanking region
354 (1.0 kb) of the targeted insertion site (an intergenic region between Cbes2199 and
355 Cbes2200) in the *C. bescii* genome was amplified with primers SK61 (with PvuI site)
356 and SK62 (with NotI site) using pSKW22 (19) as template. These two linear DNA
357 fragments were digested with PvuI and NotI and ligated to construct pSKW23 (Figure
358 3A). Primers used are listed in Table 2.
359
360 **Transformation, screening, purification, and sequence verification of engineered**
361 ***C. bescii* strains.** Constructed plasmids were introduced into *E. coli* DH5α by
362 electroporation in a 1-mm-gap cuvette at 1.8 kV and transformants were selected for
363 apramycin resistance. All plasmids were sequenced by Automatic Sequencing
364 (Genewiz, South Plainfield, NJ, USA). Electrotransformation of *C. bescii* cells was

365 performed as previously described (27). After electro-pulse with plasmid DNA (~0.5 µg),
366 cultures were recovered in low osmolarity complex (LOC) medium (24) at 75°C and
367 transferred into liquid LOD medium (24) without uracil to select uracil prototrophy. For
368 selection of shuttle vectors, cultures were plated on solid LOD media to obtain isolated
369 colonies, and total DNA was extracted for PCR confirmation. Taq polymerase (Sigma,
370 St. Louis, MO, USA) was used for PCR reactions. PCR amplification was done with
371 primers (DC460 and DC228) outside the gene cassette on the plasmid to confirm the
372 presence of gene insertion. To insert Acel_0180 into the *C. bescii* chromosome,
373 recovery cultures were transferred into liquid LOD medium (24) without uracil to select
374 uracil prototrophic transformants, and transformants were inoculated into nonselective
375 liquid defined medium, with 40 µM uracil, and incubated overnight at 65 °C to allow
376 loop-out of the plasmid. The cultures were then plated onto 5-FOA (8 mM) containing
377 solid medium and transformants containing the insertion were purified by two additional
378 passages under selection on solid medium and screened a second time by PCR. The
379 insertion of the Acel_0180 expression cassette in the targeted region was confirmed by
380 PCR amplification using primers (SK65 and SK66) outside the homologous regions
381 used to construct the insertion, generating JWCB87, and the PCR product was
382 sequenced. Primers used are listed in Table 2.

383

384 **Preparation of extracellular protein and zymogram analysis.** To collect the
385 extracellular protein (ECP) fraction, *C. bescii* cells were grown in 2 L of LOD medium
386 with 40 mM MOPS in closed bottles at 65°C with shaking at 150 rpm to an OD_{680} of

0.25-0.3. Culture broth was centrifuged (6,000 × g at 4°C for 15 min), filtered (glass fiber, 0.7 µm), to separate cells. The 2 L of ECP was loaded onto a hollow fiber cartridge with 3 kDa molecular weight cut off (GE healthcare, Buckinghamshire, UK) and eluted with 50 mL buffer 20 mM MES/2 mM β–mercaptoethanol (pH 5.5). The 50 mL EP was concentrated (~25 times) with a Vivaspin column (10 kDa molecular weight cut off, Sartorius, Goettingen, Germany). Protein concentrations were determined using the Bio-Rad protein assay kit with bovine serum albumin (BSA) as the standard. ECP samples (10 µg) were electrophoresed in 4-20% gradient Mini-Protein TGX gels (BIO-RAD) and protein bands were visualized by staining with Coomassie Brilliant Blue G-250. For detection of in-gel β-D-xylosidase activity, ECP samples (15 µg) were electrophoresed in 4-20% gradient Mini-Protein TGX gels (BIO-RAD). After soaking the gel for 1 h in 2.5% (v/v) Triton X-100 solution to remove the SDS, the zymogram gel was incubated at 75°C for 20 min in reaction buffer containing 0.3 mM 4-methylumbelliferyl β-D-xylopyranoside, 20 mM MES (pH 5.5), 1 mM dithiothreitol (DTT), 1 mM $CaCl_2$, and 1 mM $MgCl_2$. The presence of fluorescent reaction product was visualized under UV light using a gel document system. Detection of in-gel β-D-glucosidase activity was performed similarly to that of β-D-xylosidase activity, but the substrate was 5 mM 4-methylumbelliferyl β-D-glucopyranoside. For the zymogram analysis of xylanase, ECP samples (15 µg) were electrophoresed in 12% polyacrylamide gel with a 5% stacking gel containing 0.1% birchwood xylan. After removing SDS and incubating the gel in the reaction buffer as described above, the gel was submerged in 0.1%(w/v) Congo red solution for 30 min and destained with 1 M NaCl until pale-red hydrolysis zones appeared. The reaction was stopped by dipping the

gel into a 5% acetic acid solution. Quantification of band intensity was carried out using densitometry software (Total Lab 1.01, Nonlinear Dynamics Ltd.)

Enzyme activity assays. The reaction mixture for β-D-xylosidase activity, contained 750 µL distilled water, 100 µL of 200 mM MES buffer (pH 5.5), 10 µL of 100 mM dithiothreitol (DTT), 10 µL of 100 mM $CaCl_2$, 10 µL of 100 mM $MgCl_2$, and 20 µL of the crude enzyme solution, and was preheated at 75°C for 10 min. The absorbance change at 65°C and 75°C and 405 nm wavelength was monitored using a Jenway Genova spectrophotometer after adding 100 µL of 50 mM *p*-nitrophenyl β-D-xylopyranoside (pNP-X, Sigma, USA). One unit (U) of β-D-xylosidase activity was defined as the amount of enzyme needed to release 1 µmol *p*-NP (*p*-nitrophenol) from pNP-X per min. Specific enzyme activity (U/mg protein) was estimated by dividing the enzyme activity by the total protein concentration. Protein concentrations were determined using the Bio-Rad protein assay kit. Enzyme activity on xylan substrates was measured using 10 g/L of either oat spelts or birchwood xylan (Sigma, USA) in MES reaction buffer (pH 5.5) as previously described (28). Cells were grown in a 2 liter volume of LOD medium with 40 mM MOPS and 5 g/L maltose as carbon source. 25 µg/mL of the extracellular protein fraction was added to each reaction and incubated at 65°C and 75°C for 12 h. Reducing sugars in the supernatant were measured using dinitrosalicylic acid (DNS). Samples and standards (xylose) were mixed 1:1 with DNS reaction solution, boiled for two minutes and measured at OD_{575}. Activity was reported as mg/mL of sugar released.

Growth of recombinant strains on cellobiose and xylan. To measure growth on cellobiose, cells were sub-cultured twice in LOD medium with 5 g/L maltose as sole carbon source and used to inoculate media with cellobiose (1% total volume for all experiments) as sole carbon source to a final concentration of 5 g/L in 50 mL LOD medium with 40 mM MOPS, and incubated at 65°C with shaking at 150 rpm. Cell growth on cellobiose was measured by optical density (OD) at 680 nm using a Jenway Genova spectrophotometer. To measure growth on oat spelts and birchwood xylans, both the sub-culture and the initial culture were performed in LOD medium with 5 g/L oat spelts and birchwood xylans. Colony-forming units (CFU) were measured by plating cells on LOC medium.

Growth of recombinant strains on Avicel with and without the addition of xylan. Frozen cells were revived and then sub-cultured twice in LOD medium with 5 g/L maltose as sole carbon source and with 40 mM MOPS. The second sub-culture was grown to mid log-phase and used to inoculate 50 mL of LOD medium supplemented with 40 mM MOPS with either 5 g/L of Avicel or 5 g/L Avicel + 2.5 g/L oat spelts xylan. A 0.2% v/v inoculum was used and cultures were incubated at 65°C while shaking at 150 rpm. Growth was measured CFU of serially diluted samples and plating on LOC medium with 5 g/L maltose. Plates were incubated anaerobically at 65°C for four days.

Acknowledgements

453 Funding was provided by the BioEnergy Science Center (BESC) and the Center for
454 Bioenergy Innovation (CBI), from the U.S. Department of Energy Bioenergy Research
455 Centers supported by the Office of Biological and Environmental Research in the DOE
456 Office of Science. This work was authored in part by Alliance for Sustainable Energy,
457 LLC, the Manager and Operator of the National Renewable Energy Laboratory for the
458 U.S. Department of Energy (DOE) under Contract No. DE-AC36-08GO28308. The
459 views expressed in the article do not necessarily represent the views of the DOE or the
460 U.S. Government.

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574

575 **Figure Captions**

576 **Figure 1. Strains construction flow-chart.** The *A. cellulolyticus* E1 and Acel_0180
577 genes were inserted into the JWCB18 chromosome at the chromosomal integration site
578 one (CIS1) and CIS2, respectively. Then, the plasmids with (pSKW28) and without
579 (pJGW07) the TM_0076 gene from *T. maritima* were introduced into the JWCB18,
580 JWCB52, and JWCB87 strains.

581 **Figure 2. Confirmation of β-D-xylosidase expression and activity in *C. bescii*.** (A)
582 SDS-PAGE analysis of concentrated extracellular proteins (10 µg). (B) Zymogram
583 analysis of concentrated extracellular proteins (15 µg) using 0.3 mM MUX as a
584 substrate for detecting protein bands with β-D-xylosidase activity. (C) Zymogram
585 analysis of concentrated extracellular proteins (15 µg) using 5 mM MUG as a substrate
586 for detecting protein bands with β-D-glucosidase activity. M, Pre-stained SDS-PAGE
587 standards, Broad range (Bio-Rad Laboratories); 1, JWCB82 (parental strain); 2,
588 JWCB73 (E1 expressing strain); 3, JWCB95 (E1 + Tm_0076 expressing strain).

589 **Figure 3. Chromosomal integration of the Acel_0180 xylanase gene into the *C.***
590 ***bescii* genome.** (A) A depiction of the chromosomal location and integration event of
591 the Acel_0180 expression cassette: SP, signal peptide; GH10, a family 10 glycoside
592 hydrolase; CBM3, a family 3 carbohydrate-binding module; CBM2, a family 2
593 carbohydrate-binding module. (B) Agarose gel showing PCR products amplified using
594 primers SK65 and SK66 annealing to regions outside the site of integration in the parent
595 strain, JWCB52 (*ΔpyrFA* + E1), 2.2 kb (*lane 1*) and the newly constructed strain

596 JWCB87 (*ΔpyrFA* + E1 + Acel_0180), 4.4 kb (*lane 2*); no template PCR control (*lane 3*);
597 NEB 1 kb DNA ladder (*lane M*).

598 **Figure 4. Effects of expression of Tm_0076 β-D-xylosidase and Acel_0180**
599 **xylanase on the activity of the *C. bescii* exoproteome.** (A) The enzyme was
600 incubated at 65°C or 75°C for 10 min in reaction buffer containing 5 mM pNP-X, 20 mM
601 MES buffer (pH 5.5), 1 mM DTT, 1 mM $CaCl_2$, and 1 mM $MgCl_2$. (B and C) Relative
602 enzymatic activity of the extracellular fraction of *C. bescii* strains on oat spelts and
603 birchwood xylans. Activity of extracellular protein (25 µg/mL concentrated protein) on
604 oat spelts and birchwood xylans was measured after 12 h incubation at 65°C (B) or
605 75°C (C). JWCB82, the parent strain used in these experiments; JWCB73, the E1
606 expression strain; JWCB95, the E1 expression strain containing Tm_0076; JWCB102,
607 the E1 and Acel_0180 expression strain; JWCB103, the E1 and Acel_0180 expression
608 strain containing Tm_0076. Results are the mean of triplicate experiments and error
609 bars indicate standard deviation.

610 **Figure 5. Growth of *C. bescii* strains on cellobiose (A), xylan substrates (B),**
611 **Avicel (C), or Avicel + oat spelts xylan (D).** (A) Growth as measured by OD at 680
612 nm. (B) Viable cell numbers after 36 h cultivation on xylan substrates. (C and D) Growth
613 of recombinant strains on Avicel without (C) and with the addition of xylan (D). JWCB82,
614 the parent strain used in these experiments; JWCB73, the E1 expression strain;
615 JWCB95, the E1 expression strain containing Tm_0076; JWCB102, the E1 and
616 Acel_0180 expression strain; JWCB103, the E1 and Acel_0180 expression strain

617 containing Tm_0076. Results are the mean of duplicate experiments and error bars

618 indicate standard deviation.

619

Table 1. Strains and plasmids used in this study

Name	Description	Reference
E. coli		
JW532	DH5α containing pSKW23 (Apramycin^R)	This study
JW536	DH5α containing pSKW28 (Apramycin^R)	This study
C. bescii		
JWCB18	Δ*pyrFA ldh::ISCbe4* Δ*cbe1*(*ura*$^-$/5-FOA^R)	(25)
JWCB52	Δ*pyrFA ldh::ISCbe4* Δ*cbe1* CIS1::$P_{S\text{-}layer}$Acel0614(E1) (*ura*$^-$/5-FOA^R)	(5)
JWCB87	Δ*pyrFA ldh::ISCbe4* Δ*cbe1* CIS1::$P_{S\text{-}layer}$Acel0614(E1) CIS2::$P_{S\text{-}layer}$Acel0180 (*ura*$^-$/5-FOA^R)	This study
JWCB73	JWCB52 containing pJGW07 (*ura*$^+$/5-FOA^S)	(13)
JWCB82	JWCB18 containing pJGW07 (*ura*$^+$/5-FOA^S)	This study
JWCB95	JWCB52 containing pSKW28 (*ura*$^+$/5-FOA^S)	This study
JWCB102	JWCB87 containing pJGW07 (*ura*$^+$/5-FOA^S)	This study
JWCB103	JWCB87 containing pSKW28 (*ura*$^+$/5-FOA^S)	This study
Plasmids		
pJGW07	*E. coli*/*C. bescii* shuttle vector containing the *C. thermocellum pyrF* gene (Apramycin^R)	(27)
pSKW10	Source of the Acel_0180 expression cassette	(13)
pSKW22	Integrational vector for *C. bescii* CIS2 (Apramycin^R)	(19)
pSKW23	Integrational vector containing the Acel_0180 expression cassette ($P_{S\text{-}layer}$Acel0180) (Apramycin^R)	This study
pSKW28	Expression vector containing $P_{S\text{-}layer}$Tm0076 (Apramycin^R)	This study

Table 2. List of primers used in this study. The italicized sequences indicate the recognition sites of the corresponding restriction enzymes.

Name	Sequence (5' → 3')	Restriction enzyme	Description
SK74	CCG*CCCGGG*ATGGAACTGTACAGGGATCCTTC	XmaI	To construct pSKW23
SK75	AGA*CCTAGG*CTCCTCGCAGGCTTCCGT	AvrII	
SK21	CCG*CCCGGG*AAACGAACCAGCCCTAACCTCT	XmaI	To construct pSKW23
DC700	AGA*CCTAGG*CATCACCATCACCATCACTAATAAT	AvrII	
DC460	AGAGAG*CGATCG*ACAGTTTGATTACAGTTTAGTCAGAGCT	PvuI	To construct pSKW28
DC461	AGAAGAAG*GCGGCCGC*TTGGTTCCTTAAATCTAAGAGGTATGA	NotI	
SK61	AGAGAG*CGATCG*AGTGTTTTAAAAAGTGGCTAAAGATTAGAAGC	PvuI	To construct pSKW28
SK62	AGAAGAAG*GCGGCCGC*AGGTAAGTCTAAACTATTTAGCTGGTTGAG	NotI	
DC460	AGAGAG*CGATCG*ACAGTTTGATTACAGTTTAGTCAGAGCT	PvuI	To confirm transformants containing pSKW28
DC228	ATCATCCCCTTTTGCTGATG	-	
SK65	ATTAACTTGCTCAAAAACCTTGGCA	-	To verify the targeted insertion of the Acel_0180 expression cassette
SK66	TTGCAGCAGTGAGAAAACCTATG	-	

	Integration of the expression cassette	
	CIS1	CIS2
JWCB18	-	-
JWCB52	E1	-
JWCB87	E1	AceI_0180