

Hydrolysis of Cellulose by Soluble *Clostridium Thermocellum* and *Acidothermus Cellulolyticus* Cellulases

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ABSTRACT

The goal of this work was to clone, express, characterize and assemble a set of soluble thermostable cellulases capable of significantly degrading cellulose. We successfully cloned, expressed, and purified eleven *Clostridium thermocellum* (Cthe) cellulases and eight *Acidothermus cellulolyticus* (Acel) cellulases. The performance of the nineteen enzymes was evaluated on crystalline (filter paper) and amorphous (PASC) cellulose. Hydrolysis products generated from these two substrates were converted to glucose using *beta*-glucosidase and the glucose formed was determined enzymatically. Ten of the eleven Cthe enzymes were highly active on amorphous cellulose. The individual enzymes all produced <10% reducing sugar equivalents from filter paper. Combinations of Cthe cellulases gave higher conversions, with the combination of CelE, CelI, CelG, and CelK converting 34% of the crystalline cellulose. All eight Acel cellulases showed *endo*-cellulase activity and were highly active on PASC. Only Acel_0615 produced more than 10% reducing sugar equivalents from filter paper, and a combination of six Acel cellulases produced 32% conversion. Acel_0617, a GH48 *exo*-cellulase, and Acel_0619, a GH12 *endo*-cellulase, synergistically stimulated cellulose degradation by the combination of Cthe cellulases to almost 80%. Addition of both Acel enzymes to the Cthe enzyme mix did not further stimulate hydrolysis. Cthe CelG and CelI stimulated cellulose degradation by the combination of Acel cellulases to 66%.

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Introduction:

Cellulose is the most prevalent biopolymer on the planet, and its effective and low cost enzymatic conversion of cellulose to glucose is essential for production of biofuels and other products from biomass. However, the crystalline nature of cellulose [1] is a major impediment to this conversion. The original and most studied system for enzymatic degradation of cellulose is that of the *Trichoderma reesei* system of soluble cellulolytic enzymes [2]. In the *T. reesei* system, a minimum set of four enzymes, two exo-glucanases (CBHI and CBHII) acting on the reducing and non-reducing ends of cellulose chains respectively, an *endo*-glucanase (EG-1) and a *beta*-glucosidase (Bgl1) are sufficient for essentially complete degradation of purified cellulose [3, 4] as well as cellulose contained in pre-treated biomass [5]. Analyses of the genomes of cellulolytic bacteria shows no simple analogue to this fungal system; rather, a number of different paradigms are utilized to degrade cellulose [6]. In addition to the different modalities, these cellulolytic bacteria possess many more annotated cellulases from many different Glycosyl Hydrolase (GH) families.

In the 1980s, a new paradigm for cellulose degradation was discovered in *Clostridium thermocellum* [7, 8]. *Clostridium thermocellum* (Cthe) utilizes complex, high molecular weight (estimated at 2.1 million daltons) [8] structures termed cellulosomes that are anchored to the Cthe cell wall [9-11]. Cthe cellulosomes rapidly degrade cellulose [7, 8, 12, 13] *in vivo*. Reports have indicated that, on a weight basis, isolated cellulosomes were significantly more cellulolytic than the *T. reesei* set of enzymes when evaluated on autoclaved, but not pre-treated biomass [14]. Cellulosomes have been fractionated into their individual protein components [15] and many of the individual components have been isolated and characterized. Cellulosomes contain two major classes of proteins, scaffoldins: which are large non-catalytic proteins that anchor enzymes to the bacteria [16-19] and the enzymes that degrade biomass. Among these biomass-degrading enzymes, there are 29 potential cellulases including ten Glycosyl Hydrolase [20] family 5 (GH5) family members, 1 GH8 family member, 16 GH9 family members, and 2 GH48 family members; the

genome codes for no GH6, GH7, or GH12 family member cellulases that are present in other cellulolytic bacteria and fungi. Many of the Cthe cellulases have been cloned, expressed, and characterized including CelA [21], CelC [22], CelD [23], CelE [24], CelG [25], CelH [26], CelI [27], CelJ [28], CelK [29], CelL [30], CelN [31], CelO [32], CelR [33], CelS [34], and CbhA [35]. While a few of these cloned enzymes have been used to assemble synthetic mini-cellulosomes [16, 36-38], these cellulases have never been assembled into a set of soluble cellulases for evaluation. To help understanding the mechanism of cellulose degradation by Cthe, we have cloned, expressed, and purified eleven of the Cthe cellulases and characterized their performance both individually and in groups. In addition, we have cloned, expressed, and purified eight potential *Acidothermus cellulolyticus* strain 11B (Acel) cellulases. The Acel cellulases were tested for synergies with each other and the Cthe cellulases.

Material and Methods

Materials

C. thermocellum bacterial cell concentrate was a kind gift of Dr Paul Weimer, United States Department of Agriculture, Agricultural Research Service, United States Dairy Forage Research Center, WI, USA. *Acidothermus cellulolyticus* (Acel) strain 11B DNA was purchased from the American Type Culture Collection (ATCC® Number: 43068D-5™) and used without further purification. BL21(DE3) chemically competent *E. coli* cells and Taq DNA polymerase were obtained from Lucigen, Middleton, WI. pET28a vector was obtained from Merck Chemicals, San Diego, CA. Azurine cross-linked-labelled HE Cellulose (AZCL-HEC) *beta*-glucan, and D-Glucose (GOPOD Format) Assay Kits were obtained from Megazyme International (Wicklow, Ireland). 4-methylumbelliferyl-b-D-cellobioside (MUC) was obtained from Research Products International Corp. (Mt. Prospect, IL). *Alicyclobacillus acidocaldarius* LAA1 *beta*-glucosidase was obtained from C5-6 Technologies LLC (Fitchburg, WI). Sigmacell cellulose 20 μ m, Whatman 1 filter paper and Avicel PH-101 were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals were of analytical grade. Phosphoric acid swollen cellulose (PASC) was prepared by completely dissolving Sigmacell cellulose in concentrated

phosphoric acid, followed by precipitation into 10 volumes of deionized water [39]. YT plate media (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl and 16 g/l agar) was used in all molecular biology screening experiments. Luria-Bertani Broth (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) was used for liquid cultures. For enzyme production, 4.0 g/l was glycerol added after autoclaving to increase cell mass and enzyme production.

Genomic DNA Purification, Cellulase Cloning, and Protein Purification

The *C. thermocellum* cell concentrate was lysed using a combination of SDS and proteinase K, and the genomic DNA was purified using a phenol/ chloroform extraction methodology [40]. *A. cellulolyticus* DNA product was used without additional purification. The Cthe and Acel cellulases were cloned by polymerase chain reaction (PCR) amplification of the desired genes from *C. thermocellum* genomic DNA as described elsewhere [41]. Purified PCR products were ligated into pET28a and the resulting vectors were used to transform BL21 (DE3) chemically competent cells. Clones containing the genes from *C. thermocellum* genomic DNA and *A. cellulolyticus* genomic DNA for cloned cellulases were sequenced to verify the absence of mutations in the sequence. Clones expressing CelA and CbhA were obtained from B. Fox at University of Wisconsin, Madison, WI.

Cultures of recombinant *E. coli* cells were grown in LB medium containing 0.4% glycerol and 30 micrograms/ml kanamycin at 37°C. Protein production was induced by addition of 1 mM IPTG at log phase (absorbance 0.8 to 1.0). Cultures were harvested after overnight expression, lysed by sonication, clarified, and heat-treated at 70°C for 30 minutes to precipitate native *E. coli* proteins. The heat-treated extracts were then clarified by centrifugation. Enzymes labeled with C-terminal His6 tags were purified by chromatography on Ni NTA resin while enzymes without C-terminal His6 tags were purified by chromatography on Q-Sepharose resin. Purity was determined by SDS PAGE followed by Coomassie Brilliant Blue G staining. The details of the Cthe cellulases cloned and expressed are shown in Table 1, and the details of the Acel cellulases cloned and expressed are shown in Table 2.

Enzyme Activity

Enzyme-specific activity was measured using the modified Somogyi method for reducing sugars [42] using 1% β -glucan as substrate. One unit of activity was defined as one micromole of reducing sugar formed per minute. The *endo*-glucanase specificity of cellulases was determined in 0.50 ml of 50 mM acetate buffer, pH 5.8, containing 0.2% azurine cross-linked-labeled insoluble substrate (AZCL-HEC) and 1.0 μ g of enzyme protein. Assays were performed at 70°C, with shaking at 1000 rpm, for 20 minutes in a Thermomixer R (Eppendorf, Hamburg, Germany). Tubes were clarified by centrifugation and absorbance values determined using a Bio-Tek EL_x800 plate reader. The *exo*-glucanase specificity of cellulases was determined by spotting 1.0 μ g of enzyme directly on agar plates containing 10 mM 4-methyl umbelliferyl substrate (MUC). Plates were incubated in a 70°C incubator for 60 minutes; after incubation, the plates were examined using a hand-held UV lamp and compared to negative and positive controls. Cellulose hydrolysis experiments were conducted at 60°C (unless otherwise noted) in a final volume of 1.0 ml of 50 mM acetate buffer, pH 5.8, containing 5 mM CaCl₂ and 3.4 mg of Whatman 1 filter paper. Thermostable *beta*-glucosidase (*beta*-glucosidase 1, C5-6 Technologies LLC) was added to all reactions to convert cellodextrin products to glucose. Glucose formed was determined using the Megazyme D-Glucose (GOPOD Format) Assay Kit according to the manufacturer's directions in triplicate.

Results

Cl. Thermocellum Cellulases

Cloning, Expression and Purification of Cl. Thermocellum Cellulases

Nine cellulosomal and two non-cellulosomal Cthe cellulolytic enzyme (Table 1) were cloned, expressed and purified to >90% homogeneity. To improve stability and yield, all enzymes were cloned without their signal peptides, and CelG, CelD, CelH, CelK and CelR were cloned without their dockerin domains. CelE was cloned without its lipase and dockerin domains. CelI and CelC do not naturally contain dockerin domains. Clones for CelA and CbhA with their dockerin domains were obtained from B. Fox

Table 1. Cthe enzymes cloned, expressed and purified

Enzyme	Gene Locus Cthe_	A.A. Seq. Cloned	GH	CBM	Reported Activity	Specific Activity (u/mg)
CelA	0269	8-466	GH8		N [21]	95
CelC	2807	1-343	GH5		E [22]	50
CelD	0825	42-580	GH9		E [23]	967
CelE	0797	30-409	GH5		E [24]	25
CelG	2872	37-512	GH5		E [25]	75
CelH	1472	36-832	GH26, GH5	CBM11	E [26]	47
CelI	0040	56-887	GH9	CBM3 CBM3	N [27]	58
CelK	0412	28-895	GH9	CBM4	R [29]	2.0
CelO	2147	34-589	GH5	CBM3	E [32]	58
CelR	0578	28-736	GH9	CBM3	N [33]	22
CbhA	0413	28-1230	GH9	CBM3 CBM4	R [35]	1.7

E, *endo*-cellulase; N, non-reducing end *exo*-cellulase; R reducing end *exo*-cellulase; A.A. amino acid. Specific activity values determined as described in methods.

Table 2. Acel enzymes cloned, expressed and purified

Acel Gene	GH	CBM	Specific Activity (u/mg)
0135	GH6		22
0614	GH5	CBM2*	23
0615	GH6 GH12	CBM3 CBM2	173
0616	GH5	CBM3 CBM2	1
0617	GH48	CBM3 CBM2	0.04
0619	GH12	CBM2	306
0970	GH9	CBM3 CBM2	47
1701	GH9	CBM3 CBM3 CBM2	17

Specific activity values determined as described in methods. *not expressed in *E. coli*.

and used for enzyme production and purification. Of these eleven cellulases, six are annotated *endo*-cellulases, three are annotated non-reducing end *exo*-cellulases, and two reducing-end *exo*-cellulases (Table 1). The eleven cellulases come from three GH families; five are GH5 family members, five are GH9 family members and one is a GH8 family member.

Cthe Cellulase Exo-Activity and Endo-Activity

Cthe cellulases were evaluated for *endo*-activity or *exo*-activity. AZCL-HEC cellulose, an insoluble substrate that generates a soluble blue hydrolysis product, was used to determine the presence of *endo*-cellulase activity. As expected, the five, annotated *endo*-cellulases showed *endo*-activity in this assay. Among the five annotated *exo*-cellulases, four cellulases, CelA, CelI, CelR, and CbhA, were positive in this assay; only CelK showed no *endo*-cellulase activity. Reducing-end *exo*-activity was assayed using 4-methylumbelliferyl- β -D-cellobioside (MUC). All enzymes except CelI and CelR were able to cleave MUC, indicative of reducing-end *exo*-activity. There is no chromogenic substrate for determining non-reducing-end *exo*-activity, which CelI and CelR are reported to possess.

The specific activities of the 11 enzymes were determined using *beta*-glucan as substrate. The specific activities of the five GH5 cellulases fall in the range of 25 to 75 u/mg (Table 1). CelA, the GH8 family member had a specific activity of 95, similar to GH5 cellulases. The five GH9 cellulases showed a much broader range of specific activities, with members having both the highest and lowest specific activities. CelD has the highest specific activity of the eleven cellulases, 967 u/mg, that is >10-fold higher than the GH5 cellulases. CelK and CbhA, have specific activities of approximately 2 u/mg, 10-fold lower than the GH5 cellulases.

Cellulase Hydrolysis by Individual Cthe Cellulases

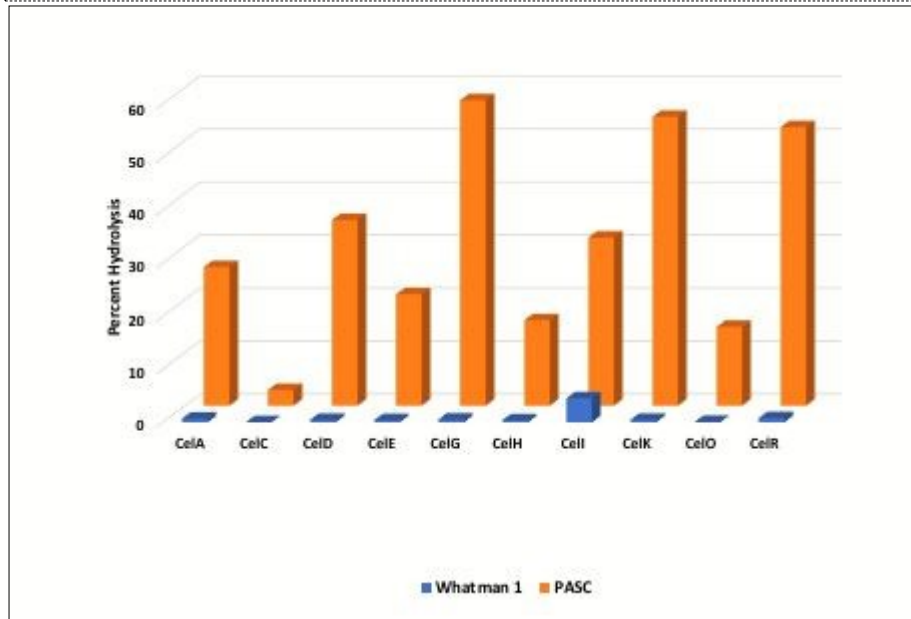
Cellulose hydrolysis by Cthe cellulases was evaluated using amorphous (PASC) and crystalline (Whatman 1 and Avicel) celluloses in the presence of exogenous *beta*-glucosidase (Figure 1). After 22 hours, all cellulases except CelC showed significant activity against PASC; extending the incubation to 67 hours

increased the conversions only slightly. Each enzyme appeared to approach a different maximum percentage of PASC conversion. At 67 hours, CelG, CelK and CelR plateaued at over 50% of the PASC, CelI and CbhA plateaued between 40% and 50%, and CelA, CelD, CelH and CelO plateaued between 10% and 40%. There was no relationship between the specific activities measured on *beta*-glucan and the extent of PASC degradation; CelK and CbhA, with the lowest specific activities on *beta*-glucan, showed excellent degradation of PASC. CelD, with an almost 500-fold higher specific activity than CelK and CbhA, gave substantially lower conversion. With the exception of CelG, a GH5 family enzyme, the GH9 family enzymes showed superior performance to the GH5 and GH8 family enzymes in hydrolysis of PASC.

While all the enzymes except CelC showed significant activity on the PASC, most Cthe cellulases were unable to hydrolyse more than 3% of the crystalline cellulose samples (Figure 2). Generally, the enzymes showed similar but lower conversions with Avicel microcrystalline cellulose versus Whatman 1 filter paper. Only CelI, the non-cellulosomal enzyme, showed significant hydrolysis of these two substrates, and had slightly better performance on Avicel. Surprisingly, the presence of CBM modules again did not improve the performance of the cellulosomal enzymes. The performance of CelH, CelK, CelR, CbhA and CelO (CBM-containing) was not significantly different from the performance of CelA, CelC, CelD, CelE, and CelG (non-CBM).

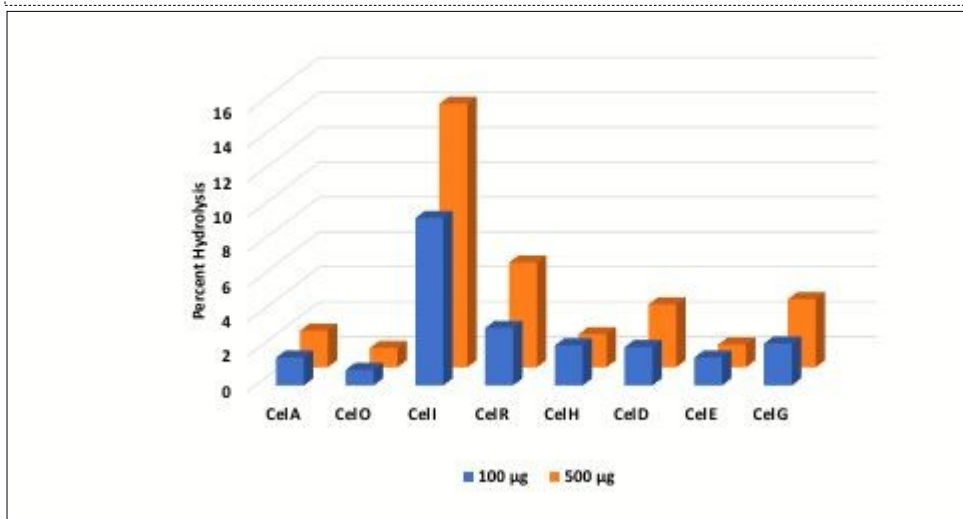
The observed low crystalline cellulose conversions could be due to a number of non-substrate related factors including low turnover number, enzyme denaturation during incubation, or non-productive binding to cellulose during the course of the reaction. To determine if any of these factors were responsible for the poor performance on Whatman 1 the Cthe cellulases were evaluated at dosages of 100 mg or 500 mg for a short (18 hr) incubation to minimize enzyme denaturation during incubation. At the 5x higher dosage, (Figure 2) only slight increases in conversion were noted for the individual enzymes, indicating that enzyme concentration or inactivation is not limiting conversion. This further suggests that the conversion of

Figure 1. Hydrolysis of PASC and Cellulose by Cthe enzymes



PASC hydrolysis was carried out using 3.7 mg dry weight PASC and 20 mg of pure enzyme at 60°C, pH 5.8, for 22 hrs. Cellulose hydrolysis was carried out using 3.4 mg filter paper and 50 mg of pure enzyme at 60°C, pH 5.8, for 67 hours as described in Methods.

Figure 2. Hydrolysis of filter paper by high and low dosages of Cthe enzymes



Cellulose hydrolysis was carried out using 3.4 mg Whatman 1 filter paper and either 100 mg or 500 mg of pure enzyme for 18 hr at 60°C, pH 5.8, as described in Methods.

cellulose from crystalline to non-crystalline may be the limiting the rate and extent of conversion.

Cellulose Hydrolysis by Combinations of Cthe Cellulases

Cellulose hydrolysis was evaluated using combinations of three, four, five or six Cthe cellulases (plus *beta*-glucosidase) (Figure 3). When combinations of three cellulases were evaluated, the combinations containing a reducing-end *exo*-glucanase (RC), a nonreducing-end *exo*-glucanase (NC), and an *endo*-glucanase (EC) (RC + EC + NC) performed significantly better (Samples 3, 4, 5, and 6) than either combinations of two reducing-end *exo*-glucanase and an *endo*-glucanase (RC + EC + RC) (Samples 1 and 2) or the combination of reducing-end *exo*-glucanase (RC), and two *endo*-glucanases (RC + EC + EC) (Sample 7). Among the RC + EC + NC sets of three Cthe cellulases, the combination of CelI, CelG, and CelK (Sample 3) performed best, yielding 26.6% cellulose hydrolysis after 43 hours. Addition of a second *endo*-glucanase (Sample 9) (RC + EC + NC + EC) increased cellulose hydrolysis to 30.7% after 43 hours. Increasing the number of cellulases in the mixture to five led to a slight increase in conversion to 34.0% (Sample 11). Further increasing the number of cellulases in the mixture to six resulted in no further increase in conversion, suggesting that the availability of non-crystalline cellulose may be limiting hydrolysis under these conditions.

C. thermocellum Cel48S, a reducing-end *exo*-glucanase, is the most abundant protein in the cellulosome [43], and is reported to have a key role in cellulose hydrolysis by the cellulosome. To determine if soluble Cthe Cell48S would improve conversion by our best soluble set of Cthe enzymes, we attempted to clone and express the enzyme in *E. coli*. Since we were unable to achieve satisfactory expression of the gene, known to produce only low levels of protein in *E. coli* [44], we utilized the *Clostridium cellulolyticum* Cel48S, a cellulosomal *exo*-glucanase with no CBM modules. Being from a mesophilic organism, the *C. cellulolyticum* Cel48S is not thermostable and requires a reducing agent for activity. Assays were run at 40°C in the presence of 1 mM dithiothreitol (DTT). *C. cellulolyticum* Cel48S did not stimulate cellulose conversion by the combination CbhA, CelI, CelK, and CelG (data not

shown), indicating a lack of GH48 cellulase was not responsible for the limited conversion.

A. cellulolyticus Cellulases

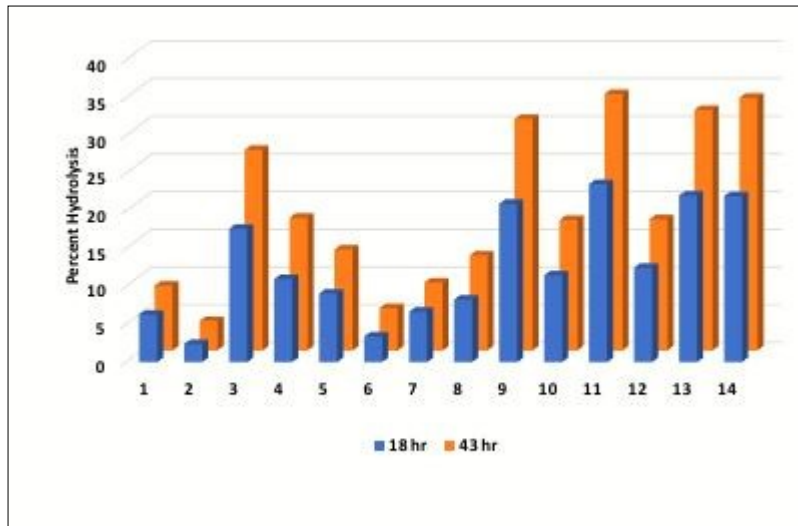
Cloning, Expression and Purification of A. Cellulolyticus Cellulases

The slow and incomplete hydrolysis of cellulose by the soluble *C. thermocellum* cellulases suggested that some components of the cellulosomal system were absent from the consortium of Cthe enzymes tested. To determine if this was the result of converting normally cellulosomal enzymes into soluble analogues, we searched for a cellulolytic organism that matched the temperature and pH profile of *C. thermocellum* and produced only soluble cellulases. *Acidothermus cellulolyticus* (Acel) closely matches the pH and temperature of *C. thermocellum*. In addition, the genome of *A. cellulolyticus* codes for eight potential cellulases (Table 2) and no cellulosomal components, making it an excellent candidate for this experiment. All eight *A. cellulolyticus* enzymes were cloned without their signal peptides. Seven of the eight potential cellulases were expressed as the expected full-length proteins. A truncated form of Acel_0614 containing only the GH5 domain was the predominant expression product in *E. coli*. This cloned, truncated form is also known as *A. cellulolyticus* Endoglucanase E1 [45-47] or Endoglucanase E1cd [48]. All enzymes were purified using a combination of heat treatment and chromatography on NiNTA resin and were greater than 90% pure as determined by SDS gel electrophoresis.

Acel Cellulase Exo-Activity and Endo-Activity

The classification of the Acel cellulases into reducing end and non-reducing *exo*-acting and *endo*-acting is not described in the literature. Acel cellulases were evaluated for their specificity in performing *endo*- or *exo*-cleavage using AZCL-HEC cellulose. Unlike the Cthe cellulases, all eight enzymes showed strong *endo*-activity in this assay. Three of the eight enzymes (0614, 0615, and 0619) are strongly active on MUC, and two (0616 and 0970) are weakly active on MUC, indicative of reducing-end *exo*-activity. The specific activities of the eight enzymes were determined using *beta*-glucan as substrate. The specific activities of most cellulases fall in the range of 17 to 47 u/mg (Table 2), similar to the values observed with the

Figure 3. Hydrolysis of filter paper by combinations of Cthe enzymes



Cellulose hydrolysis was carried out using 3.4 mg Whatman 1 filter paper at 60°C, pH 5.8 for 43 hours, as described in Methods. Combinations tested were: 1, 100 mg each of CbhA, CelK and CelG; 2, 100 mg each of CbhA, CelE and CelK; 3, 100 mg each of CelI, CelK, and CelG; 4, 100 mg each of CelI, CelE, and CelG; 5, 100 mg each of CelR, CelK, and CelG; 6, 100 mg each of CelR, CelE, and CelK; 7, 100 mg each of CelE, CelK, and CelG; 8, 100 mg each of CbhA, CelE, CelK, and CelG; 9, 100 mg each of CelI, CelE, CelK, and CelG; 10, 100 mg each of CelR, CelE, CelK, and CelG; 11, 100 mg each of CelI, CelR, CelE, CelK, and CelG; 12, 100 mg each of CbhA, CelI, CelE, CelK, and CelG.

Cthe cellulases. The two GH12 cellulases have significantly higher specific activities. Acel_0619 has a specific activity of 306 u/mg, while Acel_0615, which possesses both GH12 and GH6 carbohydrase modules, has a specific activity of 173 u/mg. Acel_0616 has a specific activity of 1 u/mg using *beta*-glucan, but 6 u/mg using mannan, indicating Acel_0616 is most likely a GH5 mannanase [20, 49]. Acel_0617, A GH48 family member has a very low specific activity of 0.04 u/mg, but this low specific activity is similar to values reported for the Cthe Cel48S [50].

Cellulose Hydrolysis by Acel Cellulases

The six Acel cellulases were evaluated for their ability to degrade amorphous (PASC) and crystalline (Whatman 1) celluloses in the presence of exogenous *beta*-glucosidase. All six cellulases hydrolysed PASC with Acel_0614 and Acel_0615 degrading 40 to 50% of the PASC under the conditions tested (Figure 4). Similar to the Cthe cellulases, there was no relationship between the specific activities of the Acel cellulases measured on *beta*-glucan and the extent of PASC degradation under the tested conditions. The results of Whatman 1 hydrolysis were again significantly different from the results obtained with PASC. While all the enzymes showed significant activity on the PASC, only Acel_0615 was able to hydrolyse more than 10% of the more crystalline cellulose sample. The combination of six Acel cellulases (Acel_0135, Acel_0614, Acel_0615, Acel_0617, Acel_0619, and Acel_0970) produced 32% conversion of the crystalline cellulose, similar to the sum of the individual activities on crystalline cellulose. This result is similar to the 34% conversion produced by the best combination of Cthe enzymes.

Cellulose Hydrolysis by Cellulases from Both Species

Individual Cthe cellulases were added to the combination of six Acel cellulases (Acel_0135, Acel_0614, Acel_0615, Acel_0617, Acel_0619, and Acel_0970) to determine if any improved the performance of the Acel enzyme mixture. Of the eight cellulases tested two cellulases, CelI and CelG stimulated cellulose conversion (Figure 5), improving conversion to 66% and 65% respectively.

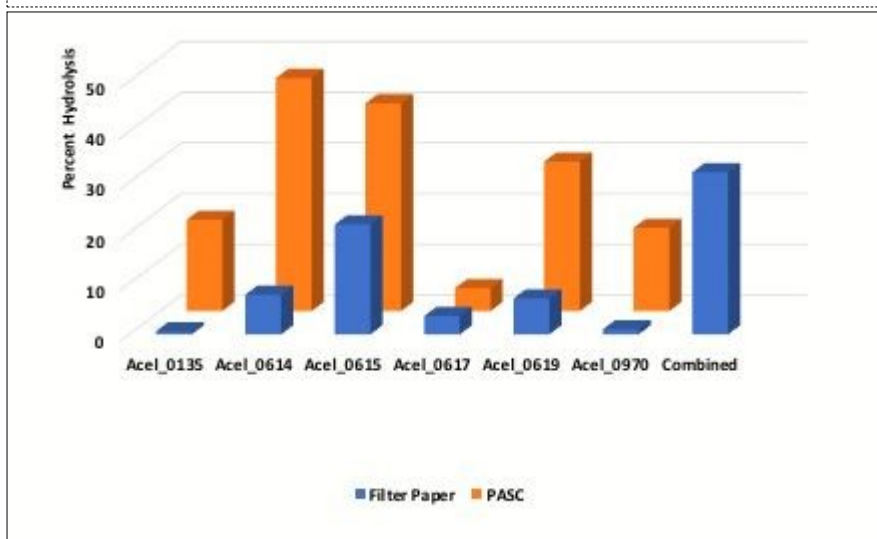
To determine if an Acel cellulase could substitute for a "missing" cellulosomal component, Acel_0135, Acel_0614, Acel_0615, Acel_0617,

Acel_0619, Acel_0970 and Acel_1701 were added individually to a mixture of Cthe enzymes. Two cellulases, Acel_0617 (GH48 CBM3 CBM2) and Acel_0619 (GH12 CBM2) stimulated cellulose conversion when added to the Cthe soluble set of CbhA, CelI, CelG, and CelK (Figure 6). Combining both Acel_0617 and Acel_0619 with this Cthe soluble set only slightly increased the conversion of cellulose further. To determine if the improvement in cellulose hydrolysis is additive (the sum of the activities of the Cthe and Acel enzymes) or truly synergistic, the experiment was repeated comparing the sum of hydrolysis obtained using Cthe set, Acel_0617 and Acel_0619 separately, and the yield resulting from the combination of the Cthe set and Acel_0617 and Acel_0619. After 136 hr, the Cthe set hydrolyzed 38% of the cellulose, while Acel_0617 and Acel_0619 hydrolyzed less than 9% (Figure 7). The combination of the Cthe set and Acel_0617 and Acel_0619 hydrolyzed 74% of the cellulose, almost double that seen with the Cthe set alone. These results indicate that the Acel and Cthe enzymes are acting synergistically to degrade the cellulose.

Discussion :

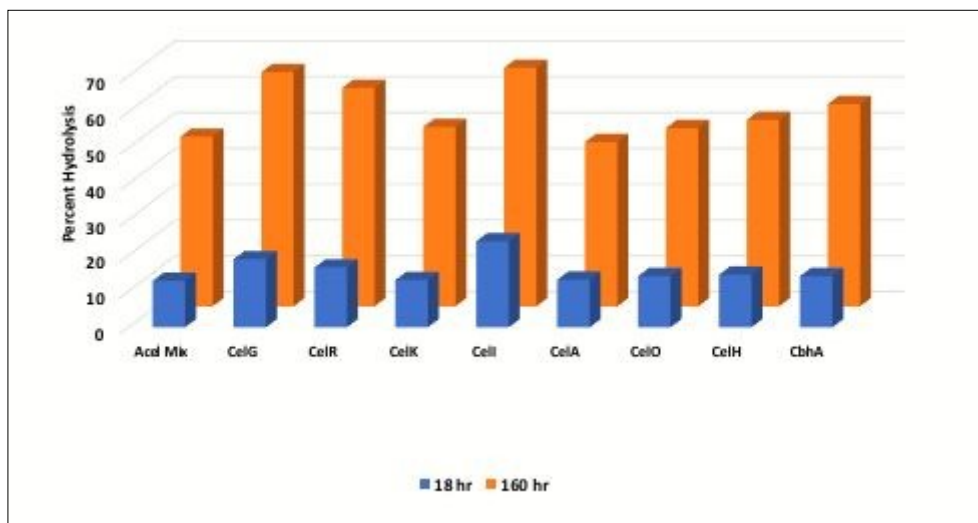
The Cthe genome codes for 29 potential cellulases in the four GH families 5, 8, 9, and 48 [51] that form part of a supramolecular structure, the cellulosome [11], while the genome of *A. cellulolyticus* codes for eight potential cellulases and no cellulosomal components. It remains unclear why Cthe possess significantly more cellulases than Acel or any other cellulose-degrading bacteria [6]. To address this question, we successfully cloned, expressed and purified eleven Cthe cellulases and six Acel cellulases. Individually, most of the cellulases show strong activity on *beta*-glucan as well as amorphous cellulose (PASC). Performance in PASC hydrolysis was unrelated to activity measured on *beta*-glucan, with the individual cellulases hydrolyzing from 3% to greater than 60% of the PASC. In contrast to the PASC results, most of the enzymes showed poor performance on crystalline cellulose. While the individual cellulases all produce at least some reducing sugar equivalents from crystalline cellulose substrates, Acel_0615 was able to individually degrade >10% of the crystalline celluloses. The limited hydrolysis was not the result of enzyme inactivation but

Figure 4. Hydrolysis of PASC and filter paper by Acel enzymes



Cellulose hydrolysis was carried out using either 3.4 mg Whatman 1 or 2.0 mg dry weight PASC and 20 mg of cellulase at 60°C, pH 5.8, as described in Methods. The combined sample contained 20 mg of each of the 6 cellulases. PASC hydrolysis was performed for 19 hours and Whatman 1 hydrolysis for 63 hours.

Figure 5. Hydrolysis of filter paper by Acel enzymes supplemented with Cthe cellulases



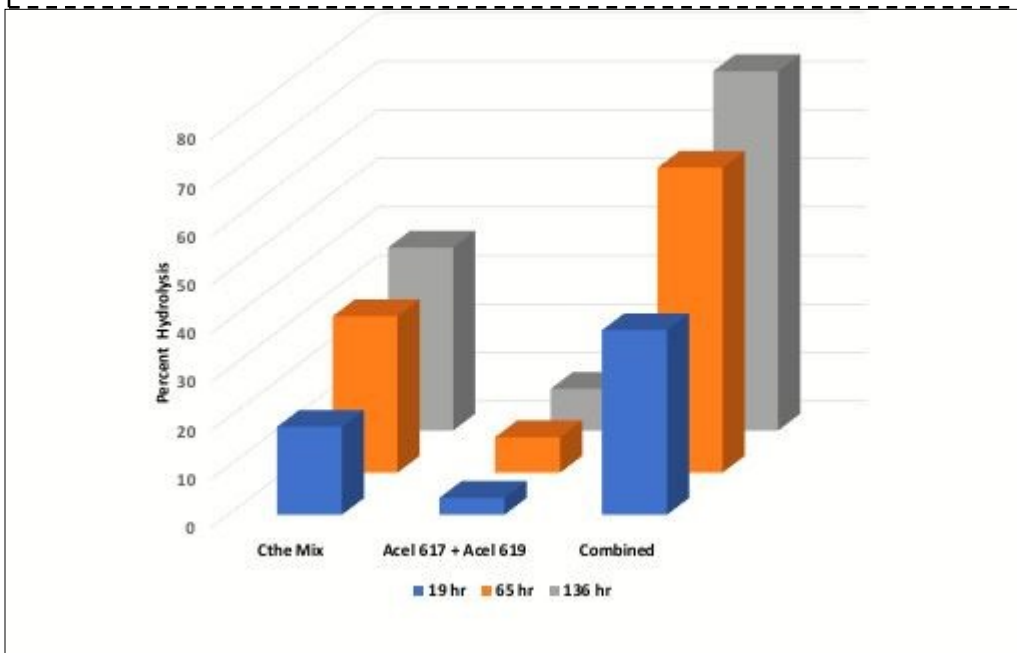
Cellulose hydrolysis was carried out using 3.4 mg Whatman 1 filter paper and 100 mg each of CbhA, CelI, CelK, and CelG (Cthe Mix), 25 mg of Acel_0617, and 100 mg each of CbhA, CelI, CelK, and CelG (Acel_0617) 70 mg of Acel_0619, and 100 mg each of CbhA, CelI, CelK, and CelG (Acel_0619) or 25 mg of Acel_0617, 70 mg of Acel_0619, and 100 mg each of CbhA, CelI, CelK, and CelG (Both) at 60°C, pH 5.8, as described in Methods.

Figure 6. Hydrolysis of filter paper by Cthe enzymes supplemented with *Acidotherrmus* cellulases



Cellulose hydrolysis was carried out using 3.4 mg Whatman 1 filter paper and 100 mg each of CbhA, CelI, CelK, and CelG named Cthe mix, (Cthe Mix); 25 mg of Acel_0617, and Cthe Mix (Acel_0617); 70 mg of Acel_0619, and Cthe Mix (Acel_0619); or 25 mg of Acel_0617, 70 mg of Acel_0619, and Cthe Mix (Both) at 60°C, pH 5.8, as described in Methods.

Figure 7. Hydrolysis of filter paper by Cthe, Acel, and the combination of cellulases



Cellulose hydrolysis was carried out using 3.4 mg Whatman 1 filter paper and 100 mg each of CbhA, CelI, CelK, and CelG (Cthe Mix), 25 mg of Acel_0617, and 70 mg of Acel_0619 (617 + 619 or 25 mg of Acel_0617, 70 mg of Acel_0619, and 100 mg each of CbhA, CelI, CelK, and CelG (Combined) at 60°C, pH 5.8, as described in Methods.

appears to be the result of slow conformational changes in the cellulose allowing access to enzyme binding sites. Same-species combinations of cellulases showed significant improvement over single cellulases in crystalline cellulose hydrolysis, raising cellulose conversions up 34%. Mixed species combinations showed additional improvements in cellulose hydrolysis, raising cellulose conversions to approximately 80%. These results approach the results obtained with *T. reesei* cellulase mixtures [4] and isolated cellulosomes [52], though a much higher enzyme loading and longer incubation were required. Additional research is needed to determine how the soluble set can be further improved, including how cellulose decrystallization can be speeded up.

There appears to be no clear relationship between GH family and performance in cellulose hydrolysis. Of the two GH48 cellulases tested, addition of *C. cellulolyticum* Cel48S did not significantly stimulate cellulose conversion, while addition of *A. cellulolyticus* Cel48 (Acel_0617) more than doubled the conversion of cellulose by the set of four Cthe cellulases. This stimulation in cellulose degradation was not specific to Acel_0617. Similar increases were seen when Acel_0619, a GH12 *endo*-acting cellulase, was substituted from Acel_0617 in the set of soluble enzymes. No major increase in conversion was seen when both Acel_0617 and Acel_0619 were added, suggesting both enzymes acted at similar sites and performed similar functions. With the set of Acel enzymes, CelI (GH9 with two CBM3) and CelG (GH5 with no CBM) gave similar stimulation of hydrolysis. The results obtained here with soluble enzymes suggest a mechanism for the rate enhancement for the cellulosomal structures of *C. thermocellum* that is missing from the soluble set of enzymes. The cellulosome may decrystallize cellulose, freeing individual chains for hydrolysis by cellulosomal cellulases [51]. This decrystallization of cellulose may represent the rate-limiting step for cellulose hydrolysis by the soluble enzymes [6]. A rate-limiting decrystallization step is consistent with the lack of a dosage response for cellulose hydrolysis by enzymes, as well as the lower conversion and slower rates than seen with cellulosomes [52]. Further work is needed to investigate how non-

catalytic cellulosome proteins and carbohydrate binding modules facilitate cellulose decrystallization and hydrolysis.

Conclusion

We successfully cloned, expressed, and purified eleven *C. thermocellum* cellulases and eight annotated *A. cellulolyticus* cellulases. The most effective combinations of Cthe enzymes, CelE, CelR, CelI, CelG, and CelK, resulted in 34% cellulose conversion. Addition of one of two *Acidothermus cellulolyticus* enzymes, Acel_0617 or Acel_0619 significantly improved the conversion to almost 80%. These results approach the results obtained with commercial *T. reesei* cellulase mixtures and isolated cellulosomes, and suggest that production of a thermostable, bacterial cellulase cocktail may be feasible.

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Competing Interests

PB is founder and CEO of C5-6 Technologies LLC, a company started to make the biomass-degrading enzymes developed by the GLBRC available to researchers at a nominal charge. The company was formed after the completion of the work presented here, and had no involvement in the study design, collection, analysis and interpretation of data.

Authors Contributions

PB designed the study, analyzed the data, purified the enzymes and wrote the first draft of the manuscript. DX performed all cellulose degradation studies. LA cloned the *C. thermocellum* and *A. cellulolyticus* cellulases. DM managed the enzyme cloning and expression, as well as all DNA sequencing used in the cloning and revised the manuscript. All authors read and approved the final manuscript.

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