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Current Opinion in Biotechnology

Extremely thermophilic microorganisms for biomass conversion: status and prospects

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Many microorganisms that grow at elevated temperatures are able to utilize a variety of carbohydrates pertinent to the conversion of lignocellulosic biomass to bioenergy. The range of substrates utilized depends on growth temperature optimum and biotope. Hyperthermophilic marine archaea ($T_{opt} \geq 80$ °C) utilize α- and β-linked glucans, such as starch, barley glucan, laminarin, and chitin, while hyperthermophilic marine bacteria ($T_{opt} \geq 80$ °C) utilize the same glucans as well as hemicellulose, such as xylans and mannans. However, none of these organisms are able to efficiently utilize crystalline cellulose. Among the thermophiles, this ability is limited to a few terrestrial bacteria with upper temperature limits for growth near 75 °C. Deconstruction of crystalline cellulose by these extreme thermophiles is achieved by 'free' primary cellulases, which are distinct from those typically associated with large multi-enzyme complexes known as cellulosomes. These primary cellulases also differ from the endoglucanases (referred to here as 'secondary cellulases') reported from marine hyperthermophiles that show only weak activity toward cellulose. Many extremely thermophilic enzymes implicated in the deconstruction of lignocellulose can be identified in genome sequences, and many more promising biocatalysts probably remain annotated as 'hypothetical proteins'. Characterization of these enzymes will require intensive effort but is likely to generate new opportunities for the use of renewable resources as biofuels.

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Current Opinion in Biotechnology 2008, **19**:210–217

This review comes from a themed issue on
Energy Biotechnology
Edited by Lee R. Lynd

Available online 2nd June 2008

0958-1669/$ – see front matter

DOI 10.1016/j.copbio.2008.04.007

Introduction

Conversion of lignocellulosic biomass to fermentable sugars represents a major challenge in global efforts to utilize renewable resources in place of fossil fuels to meet rising energy demands [1••]. Thermal, chemical, biochemical, and microbial approaches have been proposed, both individually and in combination, although none have proven to be entirely satisfactory as a stand alone strategy. This is not surprising. Unlike existing bioprocesses, which typically encounter a well-defined and characterized feedstock, lignocellulosic biomasses are highly variable from site to site and even season to season. The most attractive biomass conversion technologies will be those that are insensitive to fluctuations in feedstock and robust in the face of biologically challenging process-operating conditions. Given these specifications, it makes sense to consider microorganisms that grow at extreme temperatures, and enzymes derived from them, for key roles in biomass conversion processes. A number of extreme thermophiles, here defined as those microorganisms growing optimally at 70 °C and above, are currently available that grow in pure culture on simple and complex carbohydrates. Corresponding genome sequence information suggests an extensive glycoside hydrolase inventory [2], although in many cases the gene functions remain 'putative'. One particular challenge is to understand more about the synergistic contributions of extremely thermophilic enzymes to biomass deconstruction *in vivo*, since nature has optimized multi-enzyme processes for growth substrate acquisition.

Thermophilic microorganisms and their physiological characteristics

In natural settings, microorganisms evolve and adapt their physiology so as to develop the communal capacity to use available growth substrates in their environs through fortuitous mutations and lateral gene transfer. Thus, in searching for microorganisms and enzymes capable of deconstructing lignocellulosic biomass, corresponding habitats rich in these materials would seem to be the most productive sites. However, at temperatures of 70 °C and above, known photosynthetic processes do not proceed. Yet, many heterotrophic extreme thermophiles utilize glucans and hemicelluloses as growth substrates (Table 1).

Thermococcales

The most thermophilic heterotrophs known, with some growing above 100 °C, are members of the archaeal

Table 1

Thermophilic microorganisms capable of growth on cellulolosic or hemicellulosic substrates

Organism	Location	GC content (%)	T_{opt} (°C)	Source	Genome sequenced[a]	Size of genome (Mb)	Biomass substrates[b]	Reference
Thermophilic archaea								
P. abyssi	North Fiji basin	44	96	Sea water	2001	1.77	α, β	[6]
P. furiosus	Vulcano Island, Italy	41	100	Marine sediments	2002	1.91	α, β, T	[4,7]
P. horikoshii	Okinawa Trough	42	98	Sea water	2001	1.74	β[c]	[11,65]
T. kodakaraensis	Kodakara Island, Japan	52	85	Marine sediments	2007	2.09	α, T	[7]
S. solfataricus	Pisciarelli Solfatara, Italy	36	85	Solfataric hot spring	2007	2.99	α, β, H, P	[15,16, 18•,66]
Thermophilic bacteria								
T. maritima	Vulcano Island, Italy	46	80	Marine sediments	1999	1.86	α, β, H, P	[2,20]
T. neapolitana	Lucrino, Italy	41	80	Marine sediments	In progress	N.D.[d]	α, β, H, P	[2,67]
T. lettingae	Netherlands	39	65	Sulfate-reducing bioreactor	2007	2.14	α, β, H, P	[30]
T. naphthophila	Niigata, Japan	46	80	Kubiki oil reservoir	In progress	N.D.	α	[29]
Thermotoga sp. RQ2	Ribeira Quente, the Azores	46	76–82	Marine sediments	2008	1.88	N.D.	[68]
T. petrophila	Niigata, Japan	47	80	Kubiki oil reservoir	2007	1.82	α	[29]
T. elfii	Africa	39	66	African oil field	None	N.D.	α	[28]
Ca. saccharolyticus[e]	Taupo, New Zealand	35	70	Wood from hot spring	2007	2.97	α, β, C, H, P	[37]
A. thermophilum	Valley of Geysers, Russia	37	75	Plant residues from hot spring	In progress	N.D.	α, β, C, H	[32,34]
C. thermocellum	Louisiana, USA	39	60	Cotton bale	2007	3.84	C	[33]

[a] http://www.genomesonline.org.
[b] Biomass substrates are abbreviated as follows: α: α-linked glucans; β: β-linked glucans; C: crystalline cellulose; T: chitin; H: hemicellulose; P: pectin.
[c] *P. horikoshii* is listed as degrading β-linked glucans on the basis of cloned and characterized β-glucanases from genome.
[d] N.D., not determined.
[e] *Ca. saccharolyticus* was formerly referred to as *Caldocellum saccharolyticum*.

order Thermococcales. These fermentative anaerobes are typically isolated from hydrothermally heated sea vents where photosynthetic-based biomass would seem to be lacking. The most studied species, *Pyrococcus furiosus* (T_{opt} 100 °C), metabolizes α-linked glucosides, such as starch and pullulan, and β-linked glucosides, such as laminarin, barley glucan and chitin generating hydrogen, carbon dioxide, and acetate as primary fermentative products [3,4]. The closely related species, *Pyrococcus horikoshii*, reportedly does not grow α- or β-linked glycosides, yet produces several glycoside hydrolases [5]. The genome sequences of *P. furiosus*, *P. horikoshii*, and two other members of the Thermococcales, *P. abysii* and *Thermococcus kodakaraensis* KOD1 [6–10] encode a variety of glucosidases and glucanases, some of which have been biochemically characterized [11–14]. However, none of these microorganisms grow on crystalline cellulose.

Sulfolobales

Within the archaeal order Sulfolobales, the genus *Sulfolobus* includes aerobic, extremely thermoacidophilic heterotrophs capable of growth on peptides, monosaccharides, and α-linked polysaccharides [15,16]. *Sulfolobus* isolates are commonly isolated from acidic thermal pools where plant biomass may be found. There are reports of *Sulfolobus solfataricus* producing xylanolytic enzymes, including an endoxylanse from strain Oα [15], and a bifunctional β-xylosidase/α-L-arabinosidase enzyme from strains Oα and P2 [17,18•]. In principle, extracellular glycosidases from members of the Sulfolobales would be of particular interest in biomass deconstruction, given

that such enzymes would be functional in hot acid, a milieu used to pre-treat lignocellulose before enzymatic deconstruction. However, crystalline cellulose does not serve as a growth substrate for any species of *Sulfolobus* so far reported.

Thermotogales

The bacterial order Thermotogales includes anaerobic heterotrophic thermophiles capable of fermenting simple and complex sugars to H_2, CO_2, and acetate [19••]. The type strain and the most studied, *Thermotoga maritima* (T_{opt} of 80 °C), is the most thermophilic member of this order, has and devotes roughly 7% of the predicted coding sequences in its genome [20] to the metabolism of monosaccharides and polysaccharides [21,22]. Enzymes that can degrade β-glucan, hemicelluloses, and pectin have been studied from *T. maritima* [23–27]. Other *Thermotoga* species, isolated from geographically diverse locales, including methanol-degrading bioreactors (*Thermotoga lettingae*) and oil fields (*Thermotoga elfii*, *Thermotoga naphthophila*, *Thermotoga petrophila*), are able to degrade simple sugars [28,29], and *T. lettingae* is also able to degrade cellobiose, pectin, xylose, and xylan [30]. However, a *Thermotoga* species has yet to be reported that can utilize crystalline cellulose as a growth substrate.

Clostridiales

In contrast to the hyperthermophilic organisms described above, with $T_{opt} \geq 80$ °C, the order Clostridiales, which is much less themophilic, includes numerous species that utilize crystalline cellulose, as well as hemicellulose, as growth substrates, and these organisms are of great importance to biomass deconstruction. The most thermophilic species are *Caldicellulosiruptor kristjanssonii* [31] (T_{opt} 78 °C) and *Anaerocellum thermophilum* [32] (T_{opt} 75 °C), while the most extensively studied is *Clostridium thermocellum* (T_{opt} 60 °C) [33]. These organisms grow on crystalline cellulose, yielding lactate, ethanol, acetate, H_2, and CO_2 [32–34]. *C. thermocellum* was originally isolated from a cotton bale and is able to grow on a wide array of purified cellulose substrates and will slowly degrade natural untreated cellulosic material [33]. Recently, it was determined that the energy costs of cellulose hydrolysis in *C. thermocellum* are such that the ATP gain from hydrolysis of cellodextrins exceeds the ATP cost of cellodextrin uptake, making cellulose degradation favorable over monosaccharide assimilation [35]. In addition, a higher efficiency of cellulose degradation was demonstrated in *C. thermocellum* cultures as compared with cell-free cellulase mixtures [36••]. In contrast to *C. thermocellum*, *A. thermophilum* utilizes a broader spectrum of substrates, such as glucose, galactose, and arabinose, allowing for synergy with other plant biomass degrading microorganisms [32]. *Ca. kristjanssonii* has not been well studied [31], but the closely related thermophile, *Ca. saccharolyticus* (T_{opt} 70 °C) also utilizes a range of simple sugars as well as hemicelluloses as sole carbon sources, including xylan, xylose, pectin, and arabinose [37]. Fermentation of glucose, xylose or paper sludge in batch cultures of *Ca. saccharolyticus* demonstrated the xylanolytic and cellulolytic capacity of this bacterium to convert biomass to H_2 [38,39], preferentially using the Embden–Meyerhof pathway for glycolysis when fermenting glucose [40].

Carbohydrate transport and regulation in extreme thermophiles

The mechanisms of carbohydrate transport or catabolite repression, if it exists, in anaerobic thermophiles are mostly unknown, although it is clear that sugars profoundly affect their transcriptomes [21,22,41•]. Carbohydrate uptake seems to occur primarily by ATP-binding cassette (ABC) transporters, many of which were initially annotated as peptide transporters [19••,42•]. The regulation of carbohydrate transport is just beginning to be understood. *T. kodakaraensis*, for example, contains a bacteria-like glycolytic regulator (Tgr) that functions as both an activator and repressor to control the expression of 30 genes encoding enzyme related to glycolysis and gluconeogenesis, the largest archaeal regulon described to date [43••]. Activation and repression of the TGM regulon in *T. kodakarensis* is determined by binding of Tgr to the TGM *cis*-element under gluconeogenic conditions or complexing with maltotriose and disassociating from the TGM *cis*-element under glycolytic conditions. A similar regulon has also been described in *P. furiosus*, where the TrmB family of transcriptional regulators repress transcription of the trehalose/maltose [44,45•], and maltodextrin ABC transporter operons via TrmB [46], and also globally regulate repression of carbohydrate transport and metabolism via TrmBL1 [47••].

Cellulolytic enzymes from thermophilic microorganisms

Complete enzymatic hydrolysis of crystalline cellulose to monosaccharides requires several enzymes and may vary in different organisms [35,48]. They include various endoglucanases that cleave cellulose chains internally, releasing shorter fragments and/or cleave the smaller fragments to G2-G4 saccharides and cellobiohydrolases that release disaccharide cellobiose units from either non-reducing or reducing ends of cellulose. These enzymes are usually referred to in the literature as 'cellulases'. Further degradation of cellobiose in bacteria and fungi occurs by action of β-glucosidase producing glucose from cellobiose, and cellobiose phosphorylase, which produces glucose-1-phosphate and glucose. Of these, the endoglucanases containing binding domains, or primary cellulases, are the most crucial in enabling their host organism to efficiently utilize crystalline cellulose. However, the definition of a primary or 'true cellulase' varies widely in the literature. Here, primary cellulolytic enzymes are defined as those that contain a catalytic domain and carbohydrate-binding domains (CBMs; see Carbohydrate Active-Enzymes Database; URL: http://www.cazy.org),

and can efficiently hydrolyze crystalline cellulose. Such enzymes often contain a second catalytic domain of different activity, and/or specificity and domains of unknown function may also be present [48]. While many 'cellulases' have been reported from thermophilic organisms, very few are of the primary type. Two different types of primary cellulolytic enzyme are currently known and both are found in thermophilic anaerobes: those contained in the cellulosome and the free-acting cellulases.

The cellulosome

Many anaerobic bacteria and fungi secrete a high molecular mass multi-protein complex called the cellulosome, initially discovered in *C. thermocellum* [48,49,50••]. The key protein is an enzymatically inactive scaffoldin (CipA) composed of nine highly similar type I cohesin domains (cohI), a C-terminal type II cohesin domain (cohII) that interacts with cell surface proteins, and an internal family CBM3 that binds the cellulosome to cellulose. Assembly of the complex occurs by a specific high-affinity interaction between CohI domains and special dockerin domains (DD), present as part of the catalytic subunits. The cellulosome is simultaneously bound to cellulose and to the cell wall providing efficient consumption of hydrolysis products. The mechanism of incorporation of catalytic subunits into the cellulosome is not clear and different types of cellulosomes are produced. For example, the genome of *C. thermocellum* encodes over 70 DD-containing components with several overlapping activities [51], in addition to 20 non-cellulosomal glycosyl hydrolase-type enzymes [52•]. Nevertheless, the cellulosome is very efficient in hydrolysis of crystalline cellulose. Its components display significant synergism since the individual proteins have low activity against cellulose, presumably because they lack the CBMs necessary to bring catalytic sites into close proximity to the insoluble substrate. Cellulosomes are produced by anaerobic bacteria and anaerobic fungi [48], but are also in some aerobic microorganisms [53–55]. However, cellulosomes have not been identified in bacteria (or eukarya) that grow above 65 °C, and have not been identified in the archaea. Curiously, no member of the archaea is known to degrade crystalline cellulose.

Primary cellulolytic enzymes from thermophiles

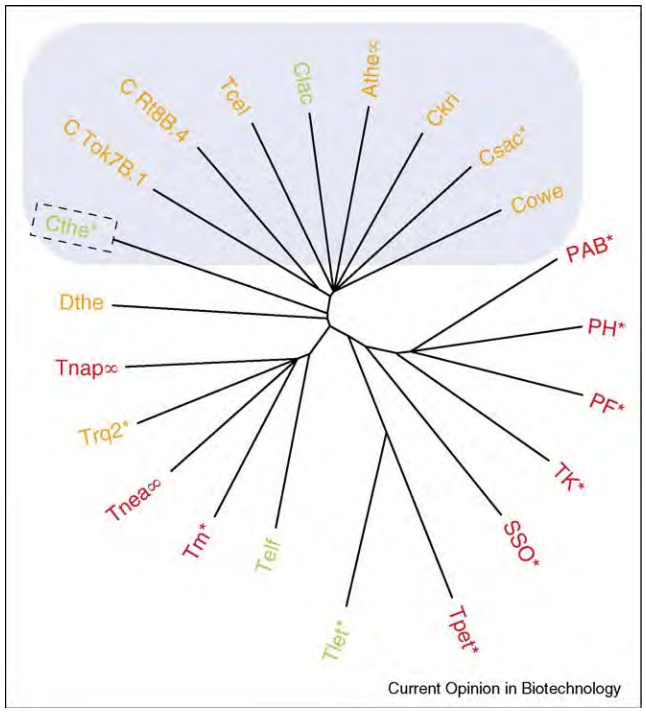
Several thermophilic bacteria contain 'free-acting' cellulases that are not part of a cellulosome complex. These include *Ca. saccharolyticus* (T_{opt} 70 °C) [37], the genome of which was recently sequenced, and the most thermophilic cellulose-degrading organism known to date, *A. thermophilum* (T_{opt} 75 °C) [32]. The cellulases of these two extreme thermophiles are multi-domain and multi-functional [2,56]. They contain CBMs of different families often duplicated and in some cases two catalytic domains of different function and/or activity. For example, the *Ca. saccharolyticus* genome encodes a putative bifunctional cellulase CelB (Csac1078), which is composed of an N-terminal endoglucanase catalytic domain of family GH10, a triplet of CBM3, and a C-terminal exocellulase catalytic domain of family GH5. This molecular complex combines all necessary enzymatic components and CBMs needed to hydrolyze crystalline cellulose and a family CBM3 needed to bind to cellulose. CelA of this organism (Csac1076) has a similar domain arrangement: a GH9 endocellulase domain, a triplet of CBM3s, and a GH48 exocellulase; the recombinant version displayed endoglucanase activity [57]. The presence of CelA and CelB in *C. saccharolyticus* is thought to be responsible for its growth on crystalline cellulose. Other putative cellulolytic-type enzymes include Csac0678, which is composed of GH5 catalytic domain, a CBM and three SHL repeats, and Csac1080 GH5, the catalytic domain of which is attached to duplicated CBMs. It is important to note that none of these putative primary cellulases has been isolated and characterized from the native organism, leaving open the possibility that post-translational processing plays a role in generating the mature enzyme.

A. thermophilum is closely related to *Ca. sacharolyticus* by 16S rRNA analysis (see Figure 1) but differs in having a slightly higher growth temperature and the unusual ability to process untreated biomass [32]. The genes encoding two cellulases and two xylanases of *A. thermophilum* were cloned and expressed into *Escherichia coli*, and the corresponding cell-free extracts showed activity against crystalline cellulose and xylans [34]. One cellulolytic enzyme (CelA) has been isolated from the *A. thermophilum*, and it is very similar in domain structure to CelA of *Ca. saccharolyticus*. This cellulase contains an N-terminal GH9 domain, a triplet of CBMs and a C-terminal GH48 domain [58]. It was highly thermostable and able to bind to and efficiently hydrolyze crystalline cellulose. A truncated version composed of only the GH9 domain and a CBM was also isolated, but it was much less active on crystalline cellulose, although it hydrolyzed soluble carboxymethyl cellulose (CMC) at the same rate as the holoenzyme. This suggests that multiple CBMs and both endoacting and exoacting domains are required for efficient hydrolysis of the crystalline substrate.

Secondary cellulolytic and hemicellulolytic enzymes from thermophiles

The genomes of many extremely thermophilic microorganisms encode enzymes that are, or appear to be, related to cellulose conversion, but most lack CBMs and/or multiple catalytic domains. For example, *T. maritima* (T_{opt} 80 °C) does not grow on cellulose, but its genome encodes several simple β-1,4-glucanases (Cel5A, Cel5B, Cel12A, Cel12B) that lack CBMs. By contrast, xylan is a very good growth substrate for *T. maritima*, and its genome encodes two xylanases (XynA and XynB) with CBMs [19••,59]. Deletion analysis of a xylanase (*xynA*) from *T. maritima* highlighted the subtle effects of carbohydrate-binding modules upon substrate affinity [60•]. In addition

Figure 1



Phylogeny tree of thermophilic microorganisms containing primary and secondary celluloytic enzymes. rRNA sequence data were aligned and drawn as an unrooted tree using Phylip v3.6 [69], http://mobyle.pasteur.fr/. Those capable of growth on crystalline cellulose are boxed in light blue. All are bacteria, all have $T_{opt} < 75$ °C and all but one of them have free primary cellulases (the exception, Cthe, is boxed, see below). Other thermophiles that possess glucanases or xylanases and are discussed herein are also included in the tree. The abbreviations for archaeal species are capitalized. Those species denoted with an asterisk (*) have sequenced genomes, while sequencing is in progress for those denoted with an infinity sign (∞) [http://www.genomesonline.org]. Optimum growth temperature (T_{opt}) is denoted by the font color: red ($T_{opt} > 80$ °C), orange ($T_{opt} > 70$ °C) and green ($T_{opt} > 60$ °C). Species are abbreviated as follows (their T_{opt} value is indicated): Athe: *A. thermophilum* (75 °C); CRt8B4: *Caldicellulosiruptor* sp. Rt8B4 (70 °C); Csac: *Ca. saccharolyticus* (70 °C); C Tok7B.1: *Caldicellulosiruptor* sp. Tok7B.1 (70 °C); Cthe: *C. thermocellum* (60 °C); Ckri: *Ca. kristjanssonii* (78 °C); Clac: *Ca. lactoaceticus* (68 °C); Cowe: *Ca. owensensis* (75 °C); Dthe: *Dictyoglomus thermophilum* (70 °C) [70]; PAB: *P. abyssi* (96 °C); PF: *P. furiosus* (100 °C); PH: *P. horikoshii* (98 °C); Tcel: *Thermoanaerobacter cellulolyticus* (70 °C) [71]; Telf: *T. elfii* (66 °C); Trq2: *Thermotoga* sp. RQ2 (80 °C); Tlet: *T. lettingae* (65 °C); Tm: *T. maritima* (80 °C); Tnea: *T. neapolitana* (80 °C); Tna: *T. naphthophila* (80 °C); Tpet: *T. petrophila* (80 °C); SSO: *S. solfataricus* (85 °C); TK: T. *kodakarensis* (85 °C).

XynA is located in the outer membrane ('toga') of *T. maritima*, but it is also detected in the supernatant as a mature secreted protein [61••]. Membrane-bound XynA is unique in that it is anchored to the toga by a hydrophobic N-terminal signal peptide, an unusual mechanism for outer-membrane-bound proteins.

The hyperthermophilic archaeon *P. furiosus*, which grows optimally near 100 °C but does not grow on cellulose or xylan, contains only one endo-1,4-glucanase (EglA), and this also lacks a CBM. The recombinant EglA was mostly active with cellooligosaccharides and CMC but, as might be expected, it did not efficiently hydrolyze insoluble forms of cellulose [12]. *P. furiosus* produces two chitinases (ChiA and ChiB), and these contain CBMs (CBM2 and CBM5, respectively). A related hyperthermophilic archaeon, *T. kodakaraensis*, produces a similar multi-domain chitinase [62,63]. However, neither archaeon contains a β-1,4-glucanase with a CBM according to their genome sequences. The related organism, *P. horikoshii* (T_{opt} 100 °C) produces a β-glucosidase [11] that has limited activity toward crystalline cellulose [14], and efforts to enhance this feature by fusing a CBM to the enzyme have been described [64•]. However, this too is not a primary cellulase, and the organism does not metabolize crystalline cellulose.

Conclusions

The evidence to date indicates that true or primary free-acting cellulases are produced only by a few anaerobic thermophilic bacteria that grow optimally at or below 78 °C, and accordingly these are the most thermophilic organisms known that are able to grow efficiently on crystalline cellulose. They are represented by the genera *Anaerocellum*, *Thermoanaerobacter*, and *Caldicellulosiruptor*. Conversely, large multi-enzyme complexes known as cellulosomes are produced by thermophilic bacteria growing up to about 65 °C. This observation is, of course, based on limited information. Perhaps with the greater emphasis on lignocellulosic biomass conversion to biofuels, the driving force for examining high temperature biotopes for cellulose and hemicellulose degraders will yield interesting and valuable results.

The reason for the absence of primary cellulases, both free-acting and cellulosomal, in the archaea domain, regardless of growth temperature, is unclear. Why such enzymes are also not present in organisms that grow close to, or even above, the normal boiling point of water, also remains a mystery. The genomes of many of these hyperthermophilic organisms, represented by the bacterial genus *Thermotoga* and the archaeal genus *Pyrococcus*, contain an array of glycoside hydrolases [2], and these organisms utilize a range of polysaccharides and oligosaccharides as growth substrates. Consequently, they have great potential utility in biomass conversion systems. However, their inability to utilize crystalline cellulose is due to the lack of a primary cellulase. CBMs are essential for the efficient degradation of crystalline cellulose and are a defining feature of the primary cellulases. Such domains are present in chitinases in *P. furiosus* and *T. kodakaraensis* and could be utilized to create novel primary cellulases for *in vitro* biomass conversion or engineered into higher temperature organisms to confer the capability to degrade crystalline cellulose. However, at present, a primary cellulase has yet to be found in an

organism that grows near, let alone above, the normal boiling point. Whether this has a profound biological basis or whether diverse thermal habitats have yet to be examined thoroughly enough remains to be seen.

Acknowledgements

This work was supported by grants from the US National Science Foundation (to MWWA and RMK) and the Bioenergy Science Center (BESC), Oak Ridge National Laboratory, a US Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science.

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- • of special interest
- •• of outstanding interest

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