CHAPTER 1

Thermostable Enzymes as Biocatalysts in the Biofuel Industry

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Advances in Applied Microbiology, Volume 70
ISSN 0065-2164, DOI: 10.1016/S0065-2164(10)70001-0

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Abstract Lignocellulose is the most abundant carbohydrate source in nature and represents an ideal renewable energy source. Thermostable enzymes that hydrolyze lignocellulose to its component sugars have significant advantages for improving the conversion rate of biomass over their mesophilic counterparts. We review here the recent literature on the development and use of thermostable enzymes for the depolymerization of lignocellulosic feedstocks for biofuel production. Furthermore, we discuss the protein structure, mechanisms of thermostability, and specific strategies that can be used to improve the thermal stability of lignocellulosic biocatalysts.

I. INTRODUCTION

With the increase in global energy consumption and expected impending shortages of crude oil, there is a considerable and immediate interest in developing alternative energy sources. Plants harness solar energy at the earth's surface to fix atmospheric carbon dioxide and collectively recycle an estimated 10^{11} tons of carbon annually (Brett and Waldren, 1996). This carbon is utilized in the formation of complex carbohydrates via photosynthesis. Lignocellulose is the most abundant carbohydrate source in plants and has significant potential for conversion into liquid fuels or biofuels. Biofuels provide a means to reduce the dependence on fossil fuels as well as to reduce global emissions of greenhouse gases into the environment. This is because, unlike fossil fuels, biofuels are renewable over more useful time frames. Further, biofuels such as ethanol have higher octane ratings and combust in a cleaner and more efficient manner than gasoline, meaning their atmospheric carbon footprint is inherently low (Demain *et al.*, 2005; Lynd *et al.*, 1991). Consequently, biofuels have the additional potential to reduce CO_2 emissions to the atmosphere.

First-generation biofuels have already resulted in reduced vehicular emissions of greenhouse gases (Hill *et al.*, 2006). However, the production of first-generation biofuels, which are based on the fermentation of corn starch or cane sugar, are neither economically nor ecologically sustainable, as corn and cane require large areas of land for their cultivation and compete with food crops meant for human consumption. Second-generation fuels that utilize lignocellulose, a recalcitrant, but more abundant part of plant material, are therefore more desirable to tackle the looming environmental and social crisis (Tollefson, 2008). The potential energy inherent in plant biomass far exceeds present human usage (Demain *et al.*, 2005).

Cellulosic feedstocks already available from agriculture and other sources are estimated to be approximately a billion tons per year in the USA alone (Corr and Hettenhaus, 2009). Many plants that produce large proportions of lignocellulosic material are capable of growth on less desirable land and require less maintenance (Tollefson, 2008). This means crops available for the production of second-generation biofuels may easily be expanded with little impact. Collectively these factors make second-generation biofuels a cost-effective, plentiful, and renewable energy resource. Accordingly, methods for optimizing the deconstruction of plant cell wall polysaccharides into their component sugars for production of biofuels have garnered considerable attention worldwide.

Lignocellulose consists primarily of three major polymers: cellulose, hemicellulose, and lignin. Cellulose accounts for up to 40% of plant biomass and consequently is the most abundant natural polymer on earth. It comprises a linear polymer of glucopyranose molecules linked by β-1-4 glycosidic linkages that have alternating orientations. Cellulose microfibrils form interstrand hydrogen bonds, which along with van der Waals forces result in a highly crystalline structure. This crystalline form limits enzyme accessibility and, therefore, limits the efficiency of enzymatic hydrolysis. Cellulose hydrolysis is further limited by the intimate associations between cellulose, hemicellulose, pectin, and lignin (Brett and Waldren, 1996; Cosgrove, 2005; Popper and Fry, 2008; Vignon *et al.*, 2004; Zykwinska *et al.*, 2007a,b), which further reduces the accessibility of cellulase enzymes to the cellulose fibers. The hemicellulose fraction of lignocellulose represents a significant source of mostly pentose sugars that are potentially important value-added products for fermentation to biofuels. Indeed, there is an increasing focus on engineering pentose utilization, and even xylan and cellulose saccharification, pathways into ethanologenic microorganisms such as yeast, allowing these organisms to ferment multiple monosaccharide products (Pasha *et al.*, 2007; Ryabova *et al.*, 2003; Voronovsky *et al.*, 2009).

Enzymatic release of monosaccharides from cellulose and hemicellulose is mediated by glycoside hydrolases. Glycoside hydrolases (GHs) are a large class of enzymes that exhibit both broad and stringent substrate specificities. GH enzymes selectively catalyze reactions that produce smaller carbohydrate units from polysaccharides (Kobata, 2001). These enzymes are exquisite catalysts that accelerate the rate of hydrolysis of glycosidic linkages by up to 17 orders of magnitude over the uncatalyzed hydrolysis (Wolfenden *et al.*, 1998). They are applied as biocatalysts in the hydrolysis of natural polysaccharides to mono- and oligosaccharides. GHs are classified into different families based on their amino acid sequences and three-dimensional folds (Cantarel *et al.*, 2009). At present, this system comprises 115 families that have been organized into 14 different clans (CAZy; http://www.cazy.org/). GHs, even within

the same genome, typically exhibit a diverse array of multimodular configurations. Polypeptides associated with plant cell wall hydrolysis commonly harbor a catalytic GH domain and a carbohydrate-binding module (CBM). CBMs are small domains with affinity for specific carbohydrate linkages and consequently act to target the catalytic portion of the enzyme to its cognate substrate (see Shoseyov *et al.*, 2006 for a pertinent review). Despite the enormous variety and remarkable structural diversity of GH enzymes, as exhibited through analyses of their three-dimensional structures, all GHs, except for those in GH family 4 (Yip and Withers, 2006), hydrolyze glycosidic linkages by either a single displacement (inversion), or a double displacement (retention) of stereochemical configuration at the anomeric carbon (C1) center (Dodd and Cann, 2009), the mechanism being uniform within a GH family (Davies and Henrissat, 1995).

Enzymes that catalyze the depolymerization of cellulose are broadly classified as cellulases. However, complete and efficient hydrolysis of cellulose requires the cooperative action of at least three cellulolytic enzyme activities, namely endoglucanase (1,4-β-D-glucan glucohydrolase [EC 3.2.1.4]), exoglucanase (1,4-β-D-glucan cellobiohydrolase [EC 3.2.1.91]), and β-glucosidase (β-D-glucoside glucohydrolase, [EC 3.2.1.21]). By contrast, complete enzymatic hydrolysis of hemicellulose requires the action of a larger repertoire of enzymes due to a broader diversity in chemical linkages inherent in these heteropolymers. These enzymes include endo-β-1,4-xylanases ([EC 3.2.1.8]), xylan 1,4-β-xylosidases ([EC 3.2.1.37]), α-L-arabinofuranosidases ([EC 3.2.1.55]), α-glucuronidases ([EC 3.2.1.139]), acetylxylan esterases ([EC 3.1.1.72]), feruloyl esterases ([EC 3.1.1.73]), mannan endo-1,4-β-mannanases ([EC 3.2.1.78]), β-1,4-mannosidases ([EC 3.2.1.25]), and arabinan endo-1,5-α-L-arabinosidases ([EC 3.2.1.99]).

Current efforts to improve depolymerization of lignocellulose or search for new biocatalysts (bioprospecting) employ a multifaceted approach. The strategy includes a search for novel enzymes with high specific activities and relatively low levels of end-product inhibition. In order to be useful on an industrial scale, care is being afforded to other characteristics including thermal stability and tolerance of solutions that vary in pH, organic solvents, chemical and oxidative reagents, and detergent composition.

In the optimization of biorefinery-scale lignocellulose deconstruction, thermostable enzymes (enzymes that maintain structural integrity above 55 °C) possess a number of important advantages over their mesophilic counterparts: (1) these enzymes typically have a higher specific activity and higher stability, allowing for extended hydrolysis times and decreasing the amount of enzyme needed for saccharification (Shao and Wiegel, 1995, Viikari *et al.*, 2007); (2) these enzymes are more compatible with

nonenzymatic processes designed to decrease the crystallinity of cellulose (Szijarto *et al.*, 2008); (3) the costs associated with process cooling are decreased or eliminated allowing the volatilization of products such as ethanol to be streamlined (Viikari *et al.*, 2007); (4) mass transport costs are decreased due to decreased fluid viscosity; (5) there is an increased flexibility for biorefinery process configurations (Stutzenberger, 1990); (6) microbial contamination risks are significantly reduced; and finally (7) these enzymes can typically be stored at room temperature without inactivation of activity. These advantages are significant because approximately one-half of the projected process costs in biomass conversions are estimated to be associated with enzyme production, and all these benefits attributed to thermostable enzymes will result in an improvement to the overall economy of the process (Haki and Rakshit, 2003).

Various bacteria, archaea, and fungi have received considerable attention as potential sources for thermostable cellulosic enzymes. The breadth of thermophilic microbes with enzymatic characteristics amenable to lignocellulose deconstruction has been reviewed recently (Blumer-Schuette *et al.*, 2008); however, thermostable enzymes are produced both by thermophilic and mesophilic microorganisms. Additionally, the former review largely neglects fungi, which are a valuable source of thermostable enzymes active on lignocellulose. Further, this is a rapidly evolving area that warrants a comprehensive update. The structural and functional characteristics of thermostable enzymes isolated from both mesophilic and thermophilic organisms, including fungi, and their application to improving lignocellulose hydrolysis for the production of second-generation biofuels is the subject of this review. Furthermore, we will evaluate the advantages, and current knowledge regarding the mechanisms, of thermostability. Finally, we will discuss the methods being employed for improving thermostability.

II. THERMOSTABLE CELLULASES

Cellulose-degrading enzymes are widespread in nature and are predominantly produced by microorganisms such as bacteria, archaea, and fungi that harvest energy from decaying plant matter. Efficient cellulose hydrolysis requires the concerted action of three different classes of enzymes, including endoglucanases and exoglucanases, which operate at the solid: liquid interface and β-glucosidases which operate on the soluble degradation products of cellulose. The enzymatic hydrolysis of cellulose by the various cellulase enzymes is diagrammed in Fig. 1.1. Endoglucanases randomly hydrolyze internal glycosidic linkages, resulting in a rapid decrease in polymer length and a gradual increase in the number of

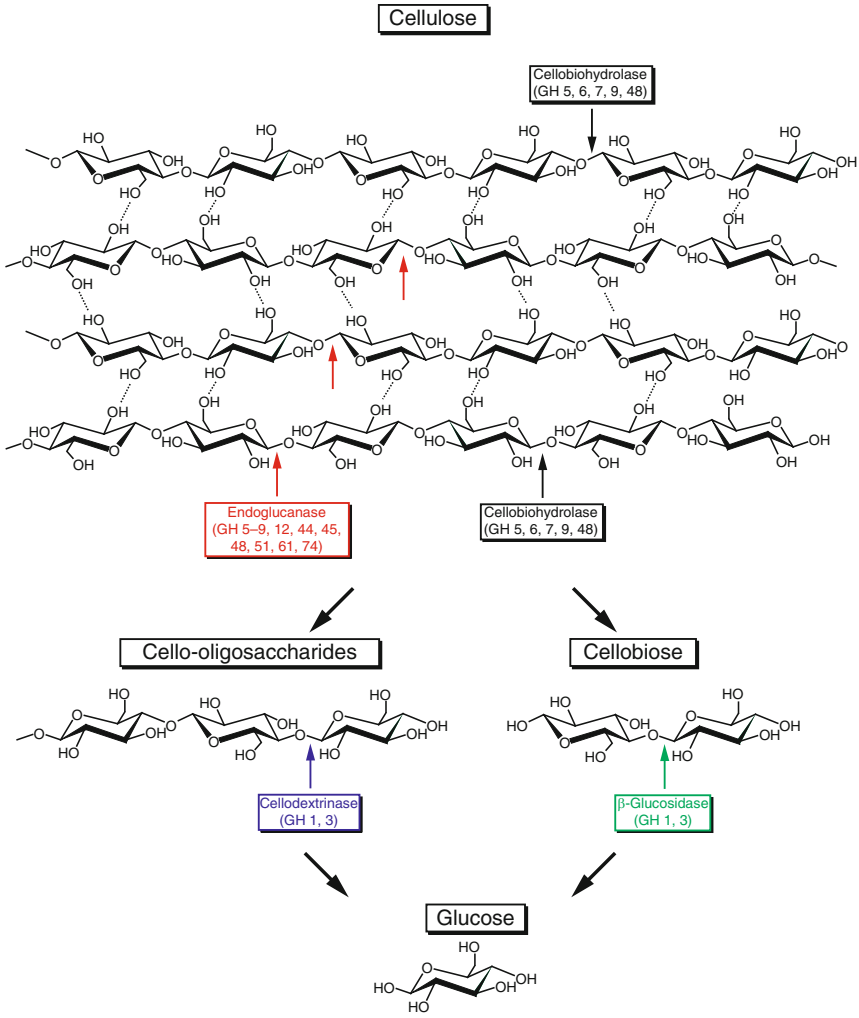

FIGURE 1.1 Enzymatic activities associated with cellulose deconstruction. Endoglucanase enzymes (indicated by red arrows) randomly cleave β-1,4 glucosidic linkages within the backbone of cellulose. Cellobiohydrolase enzymes (also known as exoglucanases) cleave cellobiose from either the reducing end or the nonreducing end of cellulose chains in a processive manner. Oligosaccharides released as a result of these activities are converted to glucose by the action of cellodextrinases, whereas the cellobiose released mainly by the action of cellobiohydrolases is converted to glucose by β-glucosidases.

liberated reducing ends (Sun and Cheng, 2002). Exoglucanases hydrolyze cellulose chains by removing mostly cellobiose from either the reducing or nonreducing ends, resulting in a rapid release of reducing ends but a

less dramatic change in overall polymer chain length (Percival Zhang *et al*., 2006). Cellobiose, the repeating structural unit that makes up the cellulose chain, comprises two β-1,4-linked glucose molecules. Extraction of glucose from cellobiose is primarily achieved through the action of β-glucosidases. These enzymes may also act on cellodextrins and larger cello-oligosaccharides, in which case they are commonly named cellodextrinases (Qi *et al*., 2008). β-Glucosidase activity is critical for the efficient hydrolysis of cellulose because end-product inhibition of endo- and exoglucanases by cellobiose can represent a significant limitation to cellulose hydrolysis (Shen *et al*., 2008).

A. Exoglucanases

β-1,4-Exoglucanases or cellobiohydrolases (CBHs) are of significant importance to cellulolytic systems, facilitating the production of mostly cellobiose which can readily be converted to glucose by β-glucosidases. These enzymes largely derive from the GH families 6, 7, 9, and 48, although several examples are also seen in GH family 5 (Table 1.1). CBHs attach to the ends of cellulose microfibrils and then processively slide down the strands cleaving off cellobiose as they progress. The processive nature of CBHs is mediated by tunnel-like active sites, which can only accept a substrate chain via its terminal regions (Fig. 1.2). These exo-acting CBH enzymes function by threading the cellulose chain through the tunnel, removing cellobiose units in a sequential manner.

Many CBHs have been characterized from bacteria of the genus *Clostridium*. In fact, most thermostable CBHs derive from cellulosomal complexes produced by members of this genus. *C. thermocellum*, in particular, appears to be a significant resource for CBHs with up to four GH family 5, 15 GH family 9, and four GH family 48 CBHs having been described. Clostridial CBHs typically have optimal activity at temperatures ranging from 60 to 75 °C and pH 5.0 to 6.5 (see Table 1.2 for a complete list of thermostable CBHs). To date, the most thermostable CBH has been isolated from the culture supernatant of the thermophilic bacterium *Thermotoga* sp. strain FjSS3-B1. The enzyme has maximal activity at 105 °C and maintains a half-life of 70 min at 108 °C (Ruttersmith and Daniel, 1991). The *Thermotoga* sp. CBH is active on amorphous cellulose and carboxymethyl cellulose (CMC) with cellobiose as the sole product. However, the enzyme exhibits limited activity against filter paper or Sigmacell 20, highlighting the need for this enzyme to work synergistically with an endoglucanase enzyme exhibiting activity on the crystalline substrate. Other examples exist where this need is less evident. For example, a GH family 5 CBH, CelO, from *C. thermocellum* shows a marked preference for crystalline substrate, yet maintains activity on cellodextrins, barley β-glucan, and CMC (Zverlov *et al*., 2002). An exoglucanase from

TABLE 1.1 Characteristics of major GH families

GH family	Fold	Mechanism	Nucleophile/ proton donor	Enzymatic activities[a]
1	$(\beta/\alpha)_8$	Retaining	Glu/Glu	GBA, MOS
2	$(\beta/\alpha)_8$	Retaining	Glu/Glu	MOS
3	$(\beta/\alpha)_8 + (\alpha/\beta)_6$	Retaining	Asp/Glu	GBA, BXL, ABF
5	$(\beta/\alpha)_8$	Retaining	Glu/Glu	CBH, CEL, XYN, MAN, MOS
6	Nd[b]	Inverting	Asp/Asp	CBH, CEL
7	β-Jelly roll	Retaining	Glu/Glu	CBH, CEL
8	$(\alpha/\alpha)_6$	Inverting	Asp[c]/Glu	CEL, XYN
9	$(\alpha/\alpha)_6$	Inverting	Asp/Glu	CBH, CEL
10	$(\beta/\alpha)_8$	Retaining	Glu/Glu	XYN
11	β-Jelly roll	Retaining	Glu/Glu	XYN
12	β-Jelly roll	Retaining	Glu/Glu	CEL
26	$(\beta/\alpha)_8$	Retaining	Glu/Glu	MAN
30	$(\beta/\alpha)_8$	Retaining	Glu/Glu[c]	BXL
39	$(\beta/\alpha)_8$	Retaining	Glu/Glu	BXL
43	Fivefold β-propeller	Inverting	Nd[b]/Nd[b]	XYN, BXL, ARA, ABF
44	$(\beta/\alpha)_8$	Retaining	Glu/Glu	CEL
45	Nd[b]	Inverting	Asp/Asp	CEL
48	$(\alpha/\alpha)_6$	Inverting	Nd[b]/Glu	CBH, CEL
51	$(\beta/\alpha)_8$	Retaining	Glu/Glu	CEL, BXL, ABF
52	Nd[b]	Retaining	Asp/Glu	BXL
54	Nd[b]	Retaining	Nd[b]/Nd[b]	BXL, ABF
61	Nd[b]	Nd[b]	Nd[b]/Nd[b]	CEL
62	Nd[b]	Nd[b]	Nd[b]/Nd[b]	ABF
67	$(\beta/\alpha)_8$	Inverting	Nd[b]/Glu	AGU
74	Sevenfold β-propeller	Inverting	Asp/Asp	CEL
113	$(\beta/\alpha)_8$	Retaining	Glu/Glu	MAN

[a] ABF, α-L-arabinofuranosidase; AGU, α-glucuronidase; ARA, α-L-arabinase; BXL, β-xylosidase; CBH, exo-β glucanase; CEL, endo-β-glucanase; GBA, β-glucosidase; MAN, β-mannanase; MOS, β-mannosidase; XYN, β-xylanase.
[b] Characteristic has not been determined.
[c] No experimental evidence exists but catalytic residue has been inferred bioinformatically.

C. stercorarium, in particular, shows significant biotechnological potential as it is able to maintain a linear rate of hydrolysis on Avicel, a highly crystalline substrate, for three days at 70 °C (Bronnenmeier *et al.*, 1991).

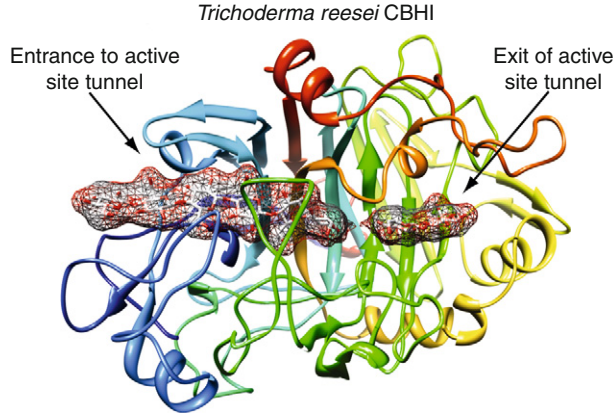

FIGURE 1.2 Crystal structure of *Trichoderma reesei* cellobiohydrolase (CBHI) in complex with cellohexaose (PDB accession no. 7CEL; Divne *et al.*, 1998). A prominent active site tunnel 50 Å in length accommodates the cellulose chain. For CBHI, cellobiose units are cleaved from the reducing end as the enzyme processively slides down the cellulose chain from the reducing end toward the nonreducing end. The structural representation of CBHI was generated with the UCSF Chimera software package.

For biotechnological applications, enzymes exhibiting broad substrate specificity are desirable. In this regard, two CBHs have been isolated from *C. thermocellum* that were found to cleave lichenan, xylan, and *para*-nitrophenyl derivatives of cellobiose and lactopyranose in addition to CMC (Tuka *et al.*, 1990).

Thermostable CBHs also occur widely in fungi, such as *Thermoascus aurantiacus*, *Talaromyces emersonii*, and *Cladosporium* spp. Fungal CBHs are typically composed of a catalytic domain connected to a CBM by a flexible pro/ser/thr-rich peptide linker. A recent comparison of several family 7 GHs found Cel7A from *C. thermophilum* exhibit as much as 11-fold higher specific activity than the others tested on soluble substrates and significantly greater activity on Avicel and phosphoric acid swollen cellulose (PASC; Voutilainen *et al.*, 2008).

GH family 5 and 7 enzymes catalyze the hydrolysis of glycosidic bonds with retention of configuration, while GH families 6, 9, and 48 employ an inverting mechanism for hydrolysis (Khademi *et al.*, 2002; Rouvinen *et al.*, 1990). GH family 6 and 7 enzymes hydrolyze cellulose specifically from the nonreducing and reducing ends of the polymeric chain, respectively (Boisset *et al.*, 2000; Divne *et al.*, 1998), while examples of CBHs active on either reducing or nonreducing ends have been reported for GH family 48 (Bronnenmeier *et al.*, 1991; Irwin *et al.*, 2000; Sanchez *et al.*, 2003). The crystal structures of several thermostable

TABLE 1.2 Thermostable exoglucanases

Microorganism	Optimum		Substrate[a] specificity	Specific[b] activity	References
	Temperature (°C)	pH			
Chaetomium thermophilus	65	5	PNPG	Nd[c]	Li *et al.* (2006b)
Clostridium stercorarium	75	5–6	Cdex, AVI, PASC, OSX	0.6	Bronnenmeier *et al.* (1991)
Clostridium thermocellum	65	6.0	PNPC, CMC, Cdex	Nd[c]	Kataeva *et al.* (1999)
Clostridium thermocellum	65	6.5	CMC	Nd[c]	Cornet *et al.* (1983)
Clostridium thermocellum	65	6.5	LICH, CMC, Xylan[d]	0.54	Tuka *et al.* (1990)
Clostridium thermocellum	65	7	LICH, CMC, Xylan[d]	0.83	Tuka *et al.* (1990)
Streptomyces sp.	60	4	Nd[c]	Nd[c]	Park *et al.* (2001)
Talaromyces emersonii	78	3.6	AVI	24.8	Tuohy *et al.* (2002)
Talaromyces emersonii	66–69	4.1	CNPG, AVI	6.1	Tuohy *et al.* (2002)
Talaromyces emersonii	68	3.8	MLC, AVI	51.6	Tuohy *et al.* (2002)
Thermoascus aurantiacus	65	6	PASC , AVI	0.04	Hong *et al.* (2003)
Thermotoga sp.	100–105	6.8–7.8	CMC, AC[d], WFP, MCC	38	Ruttersmith and Daniel (1991)

[a] AC, amorphous cellulose; AVI, Avicel; Cdex, cellodextrins; CMC, carboxymethylcellulose; CNPG - 2-chloro-4-nitrophenyl-L-cellobioside; LICH, lichenan; MCC, microcrystalline cellulose; MLC, 4-methylumbelliferyl-L-cellooligosaccharides; OSX, oat spelt xylan; PNPG, *p*-nitrophenyl-β-D-glucoside; PASC, Phosphoric acid swollen cellulose; WFP, Whatmann filter paper.
[b] Amount of enzyme releasing 1 μmol product (or equivalent) from first-listed substrate per minute. Substrate selected corresponds to that giving largest reported specific activity.
[c] Not determined (or reported) for purified enzyme.
[d] Substrate source not reported.

GH 7 CBHs, have been solved, both in complex with unmodified substrates or in the uncomplexed form (Divne *et al.*, 1998, 1994; Grassick *et al.*, 2004; Munoz *et al.*, 2001; Parkkinen *et al.*, 2008; Stahlberg *et al.*, 1996). The general structure consists of a β-sandwich, with loops extending from this structure to form an enclosed cellulose-binding tunnel. Site-directed mutagenesis studies of Cel7A (CBHI) from *T. reesei* have shown that there are three catalytic residues in the active site, where Glu^{212} acts as the nucleophile, Glu^{217} as a proton donor, and Asp^{214} is responsible for maintaining the correct positioning and protonation state of Glu^{212} through the hydrogen bond formed between Asp^{214} and Glu^{212} (Divne *et al.*, 1998; Stahlberg *et al.*, 1996). These catalytic residues occur in the long cellulose-binding tunnel, which can accommodate up to 10 glycosyl units (-7 to $+3$ subsites), and are conserved in other family 7 GHs including a glycosylated version from *T. emersonii* (Grassick *et al.*, 2004). The conformations and catalytic residues differ for enzymes from other families with GH family 5 enzymes forming a $(\beta/\alpha)_8$ structure and family 9 and 48 both forming an $(\alpha/\alpha)_6$ structures (Parsiegla *et al.*, 1998; Sakon *et al.*, 1996). GH family 6 enzymes utilize two aspartate residues as the nucleophile and proton donor, while family 9 enzymes use an aspartate and a glutamate, respectively (Khademi *et al.*, 2002, Wolfgang and Wilson, 1999).

B. Endoglucanases

Endo-1,4-β-D-glucanases expedite the cellulolytic process, acting synergistically with CBHs. In the cellulolytic deconstruction of cellulose, endoglucanases attack the cellulose polymer in a random manner, disrupting the regular crystalline nature of the substrate. The CBHs may then bind to these amorphous regions and processively cleave cellobiose units. Endoglucanases are widespread among GH families, with examples described for families 5–9, 12, 44, 45, 48, 51, 61, and 74 (Table 1.1). The majority of thermostable endoglucanases belong to GH family 12, although thermostable endoglucanases belonging to families 5, 8, and 45 have also been reported (Ando *et al.*, 2002; Kim *et al.*, 2000; Park *et al.*, 2001).

Thermostable endoglucanases have been isolated from a number of thermophilic bacteria, and archaea as well as mesophilic and moderately thermophilic filamentous fungi. Recombinant versions of many of these endoglucanases have been heterologously expressed and characterized, and their enzymatic characteristics are summarized in Table 1.3. In addition to enzymes obtained from microbial sources, a thermostable family 45 endoglucanase has also been characterized from the mollusk, *Mytilus edulis* (blue mussel). This enzyme was able to retain enzymatic activity after being heated to 100 °C for 10 min (Xu *et al.*, 2000).

To date several enzymes have been reported that display optimal enzymatic activity at, or above, 100 °C, with the hyperthermophilic

TABLE 1.3 Thermostable endoglucanases

Microorganism	Optimum		Substrate[a] specificity	Specific[b] activity	References
	Temperature (°C)	pH			
Acidothermus cellulolyticus	83	5.0	Nd[c]	Nd[c]	Ding (2006)
Alicyclobacillus acidocaldarius	65	4.0	CMC	Nd[c]	Morana *et al.* (2008)
Anaerocellum thermophilum	95–100	5–6	AVI, PASC, CMC, BBG, OSX	285	Zverlov *et al.* (1998a)
Aquifex aeolicus VF5	80	7.0	CMC	Nd[d]	Kim *et al.* (2000)
Bacillus sp. KSM-S237	45	8.6–9.0	LICH, CMC	104.7	Hakamada *et al.* (1997)
Bacillus sphaericus	60	8.0	LICH, CMC, LAM, AVI, MCC, WFP	89.1	Singh *et al.* (2004)
Bacillus subtilis	60	6.0	CMC	Nd[c]	Yang *et al.* (2009)
Caldocellulosiruptor saccharolyticus	68–70	7.0	CMC, LICH	Nd[c]	Te'o *et al.* (1995)
Chaetomium thermophilum	60	4.0	CMC, PASC, WFP, MCC	38.7	Li *et al.* (2003)
Clostridium stercorarium	90	6.0–6.5	BBG, CMC, PASC, AVI	481	Bronnenmeier and Staudenbauer (1990)
Clostridium thermocellum	70	6.6	C5, C4, CMC	18.5	Fauth *et al.* (1991)
Clostridium thermocellum	70	7.0	PASC, AVI, MCC	42.5	Reverbel-Leroy *et al.* (1997)
Clostridium thermocellum	83	6.6	C5, C4, CMC	18.5	Fauth *et al.* (1991)
Fervidobacterium nodosum	80	5.5	BBG, CMC	~3200	Zheng *et al.* (2009)
Mytilus edulis	30–50	4.6	CMC, PASC, C5, C6	10.4	Xu *et al.* (2000)
Pyrococcus furiosus	100	6.0	BBG, LICH, CMC, WFP, Cdex	58	Bauer *et al.* (1999)

Pyrococcus horikoshii	97	Nd[c]	CMC, AVI, LICH	8.5	Ando *et al.* (2002)
Pyrococcus horikoshii	95	5.5–6.0	CMC, PASC	~1000	Kang *et al.* (2007)
Rhodothermus marinus	95	7.0	CMC, Cdex	1.35	Hreggvidsson *et al.* (1996)
Rhodothermus marinus	100	6–7	GSM,CMC, LICH	2.3	Halldórsdóttir *et al.* (1998)
Sporotrichum sp.	70	4.5–5.5	CMC, AVI	8.1	Ishihara *et al.* (1999)
Streptomyces sp.	60	4	Nd[c]	Nd[c]	Park *et al.* (2001)
Sulfolobus solfataricus	80	1.8	CMC	1.2	Huang *et al.* (2005)
Syncephalastrum racemosum	70	5–6	CMC	Nd[c]	Wonganu *et al.* (2008)
Talaromyces emersonii	80	4.8	BBG, LICH	1651.8	Murray *et al.* (2001)
Thermoascus aurantiacus	75	4.5	Nd[c]	Nd[c]	Gomes *et al.* (2000)
Thermomonospora curvata	70–73	6.0–6.5	CMC	755.6	Lin and Stutzenberger 1995)
Thermotoga maritima	95	6.0–7.5	BBG, CMC	Nd[c]	Bronnenmeier *et al.* 1995)
Thermotoga maritima	95	6.0–7.5	BBG, CMC, AVI	Nd[c]	Bronnenmeier *et al.* (1995)
Thermotoga neapolitana	95	6.0	CMC, PASC, WFP	1219	Bok *et al.* (1998)
Thermotoga neapolitana	106	6.0–6.6	CMC, PASC, WFP, OSX	1536	Bok *et al.* (1998)

[a] AVI, Avicel; BBG, barley β-glucan; C4, cellotetraose, C5, cellopentaose, C6, cellohexaose; Cdex, cellodextrins; CMC, carboxymethylcellulose; GSM, glucosomannan; LAM, laminarin; LICH, lichenan; MCC, microcrystalline cellulose; OSX, oat spelt xylan; PASC, phosphoric acid swollen cellulose; WFP, Whatmann filter paper.
[b] Amount of enzyme releasing 1 μmol product (or equivalent) from first-listed substrate per minute. Substrate selected corresponds to that giving largest reported specific activity.
[c] Not determined (or reported) for purified enzyme.

archaeon, *Pyrococcus furiosus*, possessing a GH family 12 endoglucanase that maintains enzymatic integrity up to 112 °C. This endoglucanase has a signal peptide indicating that it may be exported from the cell and act extracellularly. The enzyme, however, lacks a CBM (Bauer *et al.*, 1999), a feature apparently common to many thermostable cellulases including those described for GH family 7 endo- and exo-glucanases from the ascomycete fungus *Melanocarpus albomyces* (Haakana *et al.*, 2004), and the GH family 8 endoglucanase of *Aquifex aeolicus* (Kim *et al.*, 2000). The absence of a CBM is commonly associated with reduced hydrolytic activity on crystalline substrates (Szijarto *et al.*, 2008).

With respect to biotechnological applications, thermotolerance should be considered alongside the corresponding enzymatic half-life. Typically, the half-life of an enzyme will reduce dramatically as it is shifted beyond the temperature at which it exhibits maximal activity. For example the *Thermotoga neapolitana* endoglucanase, CelB, has a half-life of 130 min at its optimum temperature of 106 °C, yet this reduces to just 26 min at 110 °C (Bok *et al.*, 1998). Although not an exclusive feature of the fungi, numerous glycosylated fungal endoglucanases exist, such as the GH7 family endoglucanase of *T. aurantiacus*. It has been proposed that glycosylation may enhance the thermostability of an enzyme, and consistently the removal of glycosyl-sugar chains has been shown to reduce thermostability (Hong *et al.*, 2003). While a number of examples of fungal endoglucanases have been described, which retain relatively high activity at elevated temperatures, they have yet to match the thermostable attributes of bacterial endoglucanases with the most extreme fungal examples being two GH family 5 endoglucanases purified from *T. aurantiacus* (70–80 °C) and *T. emersonii* (80 °C; Gomes *et al.*, 2000; Murray *et al.*, 2001; Parry *et al.*, 2002), although the endoglucanase of *T. aurantiacus* was able to retain some activity following incubation at 100 °C.

The pH optima of thermostable endoglucanases vary, and examples of acidophilic and alkaliphilic ones have been reported. Extremes in all enzyme classes are typically more evident at the acidophilic side of the scale, for example a GH family 12 endoglucanase from the thermoacidophilic archaeon *Sulfolobus solfataricus* exhibits a half-life of approximately 8 h at 80 °C and pH 1.8 (Huang *et al.*, 2005), while the GH family 5 endoglucanase of *T. aurantiacus* that was able to retain activity following incubation at 100 °C, also maintains structural stability down to pH 2.8 (Murray *et al.*, 2001). Consequently, considerable emphasis has been placed on identifying alkaliphilic enzymes to enable assembling of a set of enzymes that span the pH range used in a biotechnological pipeline.

Several unusual, but potentially useful, activities have been reported for thermostable endoglucanases, including an enzyme from *T. maritima* that exhibits its own inherent exoglucanase activity (Chhabra *et al.*, 2002),

while an endoglucanase from *S. solfataricus* additionally exhibits endoxylanase activity (Maurelli *et al.*, 2008).

Three-dimensional structures have been solved for at least eight GH 12 family enzymes including a thermostable endoglucanase from the hyperthermophile *Rhodothermus marinus* (Crennell *et al.*, 2002). This family of enzymes assembles to form a β-jelly roll and, unlike family 8 and 45 enzymes, utilizes a retaining mechanism for catalysis. Site-directed mutagenesis of a GH family 12 endoglucanase from *Hypocrea jecorina* has shown that two glutamate residues (Glu^{116} and Glu^{200}, respectively) (Okada *et al.*, 2000), conserved through all GH family 12 enzymes, comprise the catalytic nucleophile and the proton donor. In GH family 8 and 45 enzymes, these residues comprise Asp/Glu and Asp/Asp, respectively (Collins *et al.*, 2005; Yennawar *et al.*, 2006).

C. Glucosidases and cellodextrinases

β-Glucosidase enzymes are responsible for the hydrolysis of β-glucosidic linkages in aryl-, amino-, or alkyl-β-D-glucosides, cyanogenic glucosides, and oligo- or disaccharides. In the enzymatic hydrolysis of cellulose, endoglucanases and CBHs are responsible for degrading cellulose to cellobiose, after which β-glucosidases hydrolyze cellobiose to free glucose molecules (Fig. 1.1). β-Glucosidases are generally a rate-limiting factor and are consequently responsible for the regulation of the entire cellulolytic process. This is due to the inhibitory effects typically elicited by cellobiose on both endoglucanase and CBH activities (Bok *et al.*, 1998; Kruus *et al.*, 1995). Therefore, β-glucosidases not only produce glucose from cellobiose but also reduce cellobiose-mediated repression, thereby allowing the cellulolytic enzymes to function more efficiently.

β-Glucosidases may be divided into three groups on the basis of substrate specificity: aryl-β-glucosidases, cellobiases, and broad-specificity β-glucosidases. Aryl-β-glucosidases exhibit an extreme preference toward hydrolysis of aryl-β-glucosides, whereas cellobiases hydrolyze cello-oligosaccharides only (including cellobiose). Members of the third group, termed broad-specificity β-glucosidases, show significant activity on both substrate types and represent the most commonly observed group in cellulolytic microbes (Bhatia *et al.*, 2002). On the basis of amino acid similarities, β-glucosidases are grouped into two GH families, GH1 and GH3 (CAZy; http://www.cazy.org/; Table 1.1). Both families hydrolyze their cognate substrate with net retention of configuration of the anomeric carbon. The crystal structures for a GH family 3 β-D-glucan exohydrolase in complex with a variety of transition-state analogs have been reported (Hrmova *et al.*, 2002, 2004, 2005; Varghese *et al.*, 1999). This enzyme exhibits two domains: an N-terminal $(\alpha/\beta)_8$ TIM-barrel domain and a C-terminal six-stranded β-sandwich domain. Theses enzymes are

known to utilize the catalytic residues aspartate and glutamate as the nucleophile and proton donor, respectively (Paal *et al.*, 2004). Family 1 GHs similarly assemble to form $(\beta/\alpha)_8$ structures but utilize two glutamate residues for catalysis (Moracci *et al.*, 1996; Nijikken *et al.*, 2007).

β-Glucosidases are ubiquitous, occurring in organisms representing all domains of life ranging from bacteria to highly evolved mammals. *Thermotoga* species represent an important source of hyperthermophilic GHs, and many thermophilic β-glucosidases have been obtained from these organisms (Table 1.4). Examples include two GH 1 family β-glucosidases from *T. maritima* and *T. neapolitana* that were both active at 100 °C (Park *et al.*, 2005). However, the most thermostable β-glucosidase reported to date comes from the hyperthermophilic archaeon *P. furiosus*. This β-glucosidase shows optimum activity at 102–105 °C with half-lives of 85 h at 100 °C and 13 h at 110 °C (Kengen *et al.*, 1993). One β-glucosidase with particular biotechnological applicability has been isolated from *Thermus* sp. Z1. This β-glucosidase displays optimal activity at 85 °C, has a broad optimal pH range (4.5–7.0), and exhibits a half-life of 5 days at 75 °C (Takase and Horikoshi, 1988).

Evidence would suggest that fungi are a particularly rich source of thermostable β-glucosidases with reports of these enzymes being isolated and characterized in numerous strains, including mesophilic species such as *Sclerotium glucanicum* and *Aspergillus phoenicis* (Rapp, 1989; Zeng and Zhang, 1989). Both *S. glucanicum* and *A. phoenicis* typically grow at 24–27 °C but produce β-glucosidases with temperature optima and stability limits around 60–75 °C. Only moderate increases in thermal stability are seen in enzymes derived from thermophilic fungi, which typically exhibit optimal catalytic activities at temperatures ranging from 65 to 80 °C. It is unclear why some mesophiles have evolved thermostable enzymes, but such a strategy may enable energy conservation through a decreased need for enzyme synthesis due to increased enzymatic stability. It is also possible that these genes in mesophilic fungi were horizontally acquired from organisms that flourish under high temperature conditions.

Several fungal β-glucosidases have been shown to produce glucose from larger cellodextrins thus having the potential to increase the rate and extent of lignocellulose deconstruction to fermentable sugars. A thermostable example is found in *Aureobasidium pullulans* that displays optimal activity at 75 °C and is active on *para*-nitrophenyl β-D-glucoside, cellobiose, and cellodextrins up to celloheptaose (Saha *et al.*, 1994). A thermostable β-glucosidase of particular biotechnological applicability is found in *Paecilomyces thermophila*. This enzyme, in addition to β-glucosidase activity, displays exoglucanase and transglycosidase activities (Yang *et al.*, 2008).

A factor often overlooked, or rarely reported in the biochemical characterization of β-glucosidases is the degree of glucose-mediated inhibition.

TABLE 1.4 Thermostable β-glucosidases

Microorganism	Optimum		Substrate[a] specificity	Specific[b] activity	References
	Temperature (°C)	pH			
Aureobasidium pullulans	75	4.5	PNPG, C2, SAL, CMC, TRH	124	Saha *et al.* (1994)
Clostridium thermocellum	65	6.0	PNPG, C2	113.3	Ait *et al.* (1979)
Fomitopsis palustris	70	4.5	PNPG, C2	191	Yoon *et al.* (2008)
Microbispora bispora	60	6.2	C2, ESC, ARB, SAL	130	Wright *et al.* (1992)
Monascus purpureus	75	6.2	PNPG, MAL, C2, SAL	84.34	Daroit *et al.* (2008)
Paecilomyces thermophila	75	6.2	PNPG, C2-C5, GTB, SOP, SAL, GEN, DDZ, AMG, LAM, LICH	80.6	Yang *et al.* (2008)
Pyrococcus furiosus	102–105	5.0	C2, SAL, LAM, ARB	470	Kengen *et al.* (1993)
Sclerotium rolfsii	65	4.5	PNPG	3.4	Rapp (1989)
Talaromyces emersonii	71.5	4.02	PNPG, SAL, C2, BBG	512	Murray *et al.* (2004)
Thermoanaerobacter brockii	75	5.5	LA, Cdex, SOP, ARB, SAL	100.3	Breves *et al.* (1997)
Thermoascus aurantiacus	80	4.5	PNPG, C2, LAM	190,000	Parry *et al.* (2001)

(*continued*)

TABLE 1.4 (*continued*)

Microorganism	Optimum		Substrate[a] specificity	Specific[b] activity	References
	Temperature (°C)	pH			
Thermoascus aurantiacus	70	5	PNPG, C2, Cdex	86.7	Hong *et al.* (2007a)
Thermomyces lanuginosus	65	6.0	PNPG, C2, SAL, GTB, MAL	9.65	Lin *et al.* (1999)
Thermotoga neapolitana	95	5–7	C2, TRH, SOP, LAM, GTB	Nd[c]	Park *et al.* (2005)
Thermus nonproteolyticus	90	5.6	PNPG, C2	5358	Xiangyuan *et al.* (2001)
Thermus sp.	85	4.5–6.5	C2, LAM, GTB	1.6	Takase and Horikoshi (1988)

[a] AC, amorphous cellulose; AMG, amygdalin; ARB, arbutin; AVI, Avicel; BBG, barley β-glucan; C2, cellobiose, C3, cellotriose, C4, cellotetraose, C5, cellopentaose, C6, cellohexaose; Cdex, cellodextrins; CMC, carboxymethylcellulose; CNPG, 2-chloro-4-nitrophenyl-L-cellobioside; DDZ, daidzin; ESC, esculin; GEN, genistin; GSM, glucosomannan; GTB, gentiobiose; LA, laminaribiose; LAM, laminarin; LICH, lichenan; MAL, maltose; MCC, microcrystalline cellulose; MLC, 4-methylumbelliferyl-L-cellooligosaccharides; OSX, oat spelt xylan; PNPG, *p*-nitrophenyl-β-D-glucoside; PASC, phosphoric acid swollen cellulose; SAL, salicin; SOP, sophorose; TRH, trehalose; WFP, Whatman filter paper.

[b] Amount of enzyme releasing 1 μmol product (or equivalent) from first-listed substrate per minute. Substrate selected corresponds to that giving largest reported specific activity.

[c] Not determined (or reported) for purified enzyme.

The presence of high concentrations of glucose is known to be inhibitory to many β-glucosidases, blocking the substrate from the active site or preventing the hydrolyzed substrate from leaving (Yoon *et al.*, 2008). Such inhibition would undoubtedly feedback across the entire cellulolytic process. Consequently, evaluation of the resistance of β-glucosidase enzymes to such inhibitory action will be critical for moving forward in the development of effective enzyme cocktails for plant cell wall hydrolysis. A recent report illustrates a sensitive new approach to address these requirements (Krogh *et al.*, 2009).

III. THERMOSTABLE HEMICELLULASES

Hemicellulose is a highly branched mixture of complex polysaccharides, including xylans, glucans, xyloglucans, callose, mannans, and glucomannans. Xylans, the major hemicellulose component of plant cell walls, can vary dramatically in composition, with the major classes including glucuronoxylan, arabinoxylan, and glucuronoarabinoxylan. In a general sense, a xylan polymer comprises a linear backbone of β-1,4-D-xylopyranoside residues which are commonly substituted by acetyl, arabinofuranosyl, and 4-*O*-methyl-glucuronyl groups. In nature, the polysaccharide backbone may be modified with these different substituents in variable proportions, and this substitution pattern is primarily governed by the source of the xylan. The architecture becomes more complex with the various hemicellulose components forming intimate interactions with each other as well as plant cell wall cellulose and pectin (Marcus *et al.*, 2008). In this respect, optimizing the enzymatic conversion of lignocellulose to fermentable sugars must take into account hemicellulose depolymerization. In addition, hemicellulose can account for 37–48% of a plant's primary cell wall (Chesson *et al.*, 1986) and represents an abundant and exploitable source of pentose sugars. Commensurate with the structural complexity of xylan, which in addition to comprising a complex mixture of polysaccharides, is laden with numerous types of glycoside linkages, its complete enzymatic hydrolysis requires the synergistic action of a complex set of enzymes. These include endo-β-1,4-xylanases and β-D-xylosidases acting on the backbone and also debranching enzymes such as α-L-arabinofuranosidases, α-glucuronidases, and esterases (Dodd and Cann, 2009; Fig. 1.3).

A. Xylanases

Almost all xylanases that have been described to date are endo-acting; to our knowledge only two bacteria have been reported to produce exo-acting xylanases, in both cases these enzymes were mesophilic

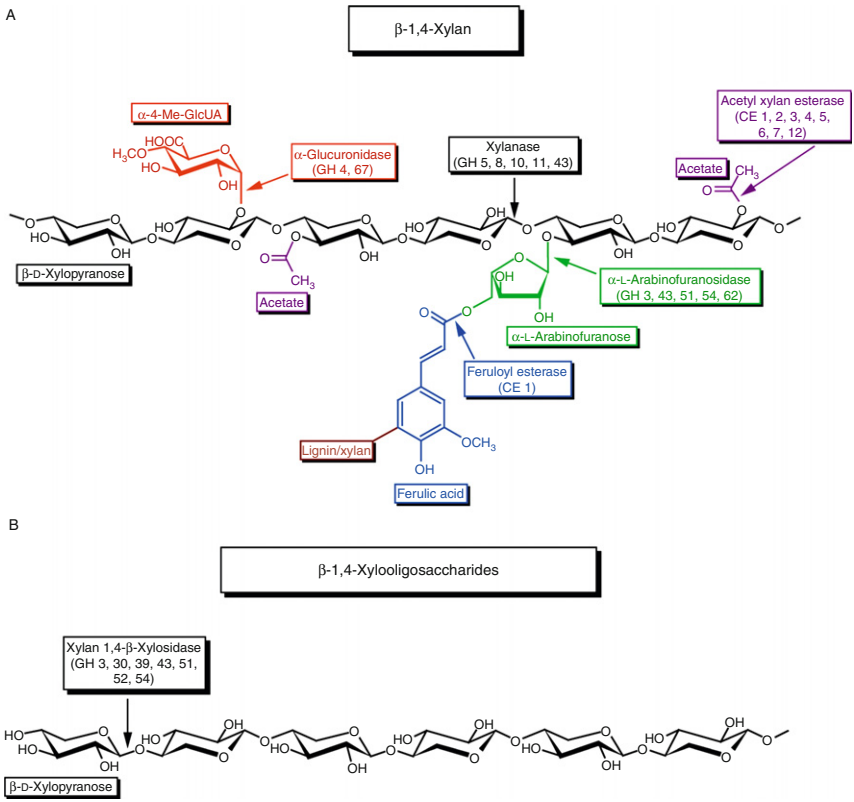

FIGURE 1.3 Enzymatic activities associated with xylan deconstruction. (A) Endoxylanases cleave the backbone of xylan chains to release shorter xylo-oligosaccharides which are further debranched by accessory enzymes. (B) β-Xylosidases release xylose monomers from the nonreducing ends of debranched xylo-oligosaccharides. Adapted from Dodd and Cann (2009).

(Gasparic *et al.*, 1995; Kubata *et al.*, 1994, 1995). Consequently, the term xylanase is often used in the literature (and will be in this review) to specifically describe endo-acting β-1,4-xylanases. Xylanases catalyze the endo-hydrolysis of 1,4-β-D-xylosidic linkages in a seemingly random fashion, although more recent evidence would suggest the cleavage sites used by GH10 and 11 enzymes are influenced by sidechain substituents (Dodd and Cann, 2009; Kolenova *et al.*, 2006; Maslen *et al.*, 2007). Xylanases have long been utilized in the, food, paper, and fine chemical industries and are well recognized as critical components in the deconstruction of lignocellulose for biofuels production (Garcia-Aparicio *et al.*, 2007). Xylanases largely derive from the GH families 10 and 11, although examples are also described for families 5, 8, and 43 (CAZy; http://www.

cazy.org/; Table 1.1). Crystal structures have been solved for numerous xylanases from GH families 10 and 11 (Manikandan *et al.*, 2005), with GH family 10 xylanases forming a $(\beta/\alpha)_8$ TIM-barrel fold and GH family 11 enzymes largely consisting of β-sheets. Despite these inherent differences, xylanase-mediated catalysis from both families occurs via a retention mechanism (Henrissat and Davies, 1997) with two glutamate residues, one acting as the proton donor, and the other acting as a catalytic nucleophile. In the *Geobacillus stearothermophilus* xylanase, XT6, these residues are Glu^{159} and Glu^{265}, respectively (Teplitsky *et al.*, 2004).

Bacteria comprise a rich resource of thermostable xylanases (Table 1.5), with xylanases from this lineage being generally preferred for lignocellulose hydrolysis, as is currently applied to industrial approaches such as the biobleaching of paper pulp (Giordano *et al.*, 2006).

The marine ecosystem, in particular, is an important source with several thermostable xylanases having been isolated from this environment (Giordano *et al.*, 2006). Two interesting examples of thermostable xylanases from GH family 10 have been described from *T. maritima*. The first, XynA, exhibits a multimodular protein structure with an N-terminal signal peptide, two repeated N-terminal CBMs, a catalytic GH 10 xylanase domain, and two repeated C-terminal CBMs (Winterhalter *et al.*, 1995). The C-terminal CBMs were found to bind to microcrystalline cellulose and not xylan. It is thought this property may allow XynA to preferentially engage xylan fragments found closely associated with cellulose. The second *T. maritima* xylanase, XynB, has an optimal temperature of 90 °C but remains stable at temperatures of up to 100 °C. In addition, this enzyme has an extremely broad pH stability range that spans pH 5–11.4 (Jiang *et al.*, 2001). Many other alkaliphilic xylanase examples have been described, including several from mesophilic *Bacillus* spp. (Ahlawat *et al.*, 2007; Kashyap *et al.*, 2003; Mamo *et al.*, 2006). These xylanases typically operate over a broad pH range (pH 6–10) and display maximum catalytic activities at 60–75 °C. *Bacillus halodurans* S7, for example, produces a xylanase with optimum activities at 75 °C at pH 9 and 70 °C at pH 10 (Mamo *et al.*, 2006). Other related thermophilic clades also represent a rich source of alkali-tolerant xylanases, *Geobacillus sp* MT-1, for example, produces a xylanase with a temperature optimum of 90 °C that is active across the pH range 5.5–10 (Wu *et al.*, 2006). Evidence, therefore, would suggest this trait is more prevalent in xylanases than other lignocellulase enzymes.

Despite the current preference for bacterial xylanases in industrial processes, it has been suggested that fungal xylanases may be of greater use due to their stronger catalytic activities (Lee *et al.*, 2009). Indeed the xylanases with the highest reported catalytic rates belong to the thermophilic xylanases of *Aspergillus awamori, Bispora* sp. and *Neurospora crassa*, which exhibit specific activities greater than 10^4 U/mg (Kormelink *et al.*,

TABLE 1.5 Thermostable Xylanases

Microorganism	Optimum		Substrate[a] specificity	Specific[b] activity	References
	Temperature (°C)	pH			
Aspergillus niger PPI	60	4.0	BWX	16	Pandey and Pandey (2002)
Bacillus circulans AB 16	80	6–7.0	Nd[c]	Nd[c]	Dhillon *et al.* (2000)
Bacillus firmus	70	5.0–9.5	BWX	~400	Chang *et al.* (2004)
Bacillus firmus	60	4.5–8.0	BWX	~230	Chang *et al.* (2004)
Bacillus stearothermophilus	65	6.5	OSX	288	Khasin *et al.* (1993)
Bacillus stearothermophilus	60	7.0	OSX	122	Nanmori *et al.* (1990)
Bacillus spp. strain SPS-0	75	6.0	BWX	129.2	Bataillon *et al.* (2000)
Caldibacillus cellulovorans	70	Nd[c]	BWX, BEX, OSX, LWX	Nd[c]	Sunna *et al.* (2000a)
Caldibacillus cellulovorans	60	5.0	BWX, OSX, LICH, LAM, CMC	266	Ali *et al.* (2005)
Clostridium acetobutylicum	70	5.0	BWX, OSX, CMC, PGA, LICH, LAM, BBG, AVI	1436	Ali *et al.* (2004)
Clostridium stercorarium	80	7.0	OSX, CMC	4460	Fukumura *et al.* (1995)
Clostridium thermocellum	75	6.8	RAX, WAX, OSX	16.5	Fontes *et al.* (1995)
Geobacillus sp.	70	7.0	OSX	Nd[c]	Wu *et al.* (2006)

Marasmius sp.	90	4–8	BWX, BEX, CMC, AVI	350	Ratanachomsri *et al.* (2006)
Melanocarpus albomyces	70	Nd[c]	OSX, BWX, BEX, CCX, RSX, WAX, RHX, BX, CMC, LAM	198	Jain *et al.* (1998)
Paecilomyces themophila	75–80	7.0	BWX, BEX, OSX	Nd[c]	Li *et al.* (2006a)
Paenibacillus sp.	60	6.0	BWX	562	Lee *et al.* (2007)
Streptomyces thermoviolaceus	70	7.0	OSX	1460	Tsujibo *et al.* (1992)
Streptomyces thermoviolaceus	60	7.0	OSX	1405	Tsujibo *et al.* (1992)
Sulfolobus solfataricus	90	7.0	RBB, BEX, BWX, OSX	11.4	Cannio *et al.* (2004)
Talaromyces thermophilus	75	4.0–10	BWX	160	Maalej *et al.* (2008)
Thermomonospora alba	80	6.5	sOSX, iOSX, BWX	365	Blanco *et al.* (1997)
Thermomonospora fusca	65	7.0	BWX	490	Irwin *et al.* (1994)
Thermomonospora fusca	50–60	6			Weng and Sun (2005)

(*continued*)

TABLE 1.5 (*continued*)

Microorganism	Optimum		Substrate[a] specificity	Specific[b] activity	References
	Temperature (°C)	pH			
Thermomonospora sp.	80	7.5	OSX	455	George *et al.* (2001)
Thermomyces lanuginosus	75	6.2			Jiang *et al.* (2005)
Thermomyces lanuginosus	75	Nd[c]	BWX	323	Damaso *et al.* (2003)
Thermotoga maritima	90	6.14	RBB	13.3	Zhengqiang *et al.* (2001)
Thermotoga maritima	87	6.5	BWX	Nd[c]	Reeves *et al.* (2000)
Thermotoga maritima	92	6.2	OSX, BWX, LWX, LICH, BBG	306	Winterhalter and Liebl (1995)
Thermotoga maritima	105	5.4	OSX, BWX, LWX, BBG, LICH, LAM	4600	Winterhalter and Liebl (1995)
Thermotoga sp.	105	5.0	OSX	126.3	Simpson *et al.* (1991)

[a] AVI, Avicel; BBG, barley β-glucan; BEX, beechwood xylan; BWX, birchwood xylan; BX, bagasse xylan; CCX, corn cob xylan; CMC, carboxymethylcellulose; LAM, laminarin; LICH, lichenan; LWX, larchwood xylan; OSX, oat spelt xylan (i, insoluble; s, soluble); PGA, polygalacturonic acid; RAX, rye arabinoxylan; RBB, Remazol brilliant blue *R*-D-xylan; RHX, rice husk xylan; RSX, Rice straw xylan; WAX, Wheat arabinoxylan (i, insoluble; s, soluble).

[b] Amount of enzyme releasing 1 μmol product (or equivalent) from first-listed substrate per minute. Substrate selected corresponds to that giving largest reported specific activity.

[c] Not determined (or reported) for purified enzyme.

1993; Luo *et al.*, 2009; Mishra *et al.*, 1984). The *Bispora* sp. xylanase, Xyn10C, in particular appears to be an attractive option for biotechnological adaptation. This enzyme displays optimal activity at 80 °C, higher than any other fungal xylanase, and is active over a broad pH range (pH 1.5–6). In addition Xyn10C was resistant to a number of cations, including Na^+, K^+, Li^+, Co^{2+}, Ni^{2+}, and Mn^{2+} which are inhibitory to many other described xylanases (Luo *et al.*, 2009). Aside from these few enzymes with extreme catalytic potential, there appears to be little difference in the average specific activities between xylanases described for the two lineages (both $\sim 10^3$ U/mg; Sunna and Antranikian, 1997).

B. Xylosidases

β-D-Xylosidases generate D-xylose from xylobiose and other short chain xylo-oligosaccharides. β-D-xylosidases are found in GH families 3, 30, 39, 43, 51, 52, and 54, with all, but family 43 enzymes, exerting their catalytic activities via the retention mechanism (Table 1.1). For those families with which structural information has been determined or inferred, the common structural conformation exhibited is a $(\beta/\alpha)_8$ fold (families 30, 39, and 51), although family 43 enzymes form a fivefold β-propeller. The catalytic residues for families 30, 39, and 51 comprise a pair of glutamate amino acids, while family 52 enzymes utilize a glutamate residue as the catalytic nucleophile and an aspartate as the proton donor (Bravman *et al.*, 2001; Czjzek *et al.*, 2005; Zverlov *et al.*, 1998b; CAZy; http://www.cazy.org/).

In an analogous role to β-glucosidases, found in cellulase systems, β-D-xylosidases are important in the relief of end-product inhibition of xylanases caused by xylobiose. Thermostable β-D-xylosidases have been isolated from a number of fungi and some bacteria. The genus *Aspergillus*, in particular, appears to be a significant resource with reports of thermophilic β-D-xylosidases being characterized from no less than 10 species of this genus (Kiss and Kiss, 2000; Kitamoto *et al.*, 1999; Kitpreechavanich *et al.*, 1986; Kormelink *et al.*, 1993; Kumar and Ramon, 1996; Pedersen *et al.*, 2007; Rizzatti *et al.*, 2001). The optimum temperature for these enzymes ranges from 50 to >75 °C and covers the pH range 3.0–6.5. In another analogy to β-glucosidases, the performance of β-xylosidases is typically inhibited by increasing concentrations of their end-product, xylose. Interestingly, a thermophilic β-xylosidase from the fungus *Scytalidium thermophilum* was, however, found to be immune to xylose-mediated inhibition (Zanoelo *et al.*, 2004). Features such as this are likely to be immensely important to the overall efficiency of biocatalysts in the biofuel industry.

C. Glucuronidases

α-Glucuronidases catalyze the cleavage of the α-1,2-glycosidic bond between 4-*O*-methyl α-glucuronic acid and the terminal nonreducing end xylopyranosyl unit of small xylo-oligosaccharides (Mierzwa *et al.*, 2005; Puls *et al.*, 1987). Their activities on polymeric xylan and *para*-nitrophenyl α-D-glucuronopyranoside are typically negligible, although interestingly, a thermostable α-glucuronidase from the bacterium *T. maritima* was found to hydrolyze *para*-nitrophenyl α-D-glucuronopyranoside, with an optimum temperature of 80 °C (Suresh *et al.*, 2003). α-Glucuronidases cluster into two groups within GH family 67, defined by either bacterial or fungal origin. The α-glucuronidases of the two origins differ in their molecular mass and quaternary structures, but the enzymes share highly conserved active site architectures. Bacterial α-glucuronidases function as homodimers with monomeric molecular weights of about 70 kDa, while fungal α-glucuronidases function as monomeric proteins with a molecular weight of about 90 kDa (De Wet *et al.*, 2006). This discrepancy in the monomeric molecular weights for α-glucuronidases isolated from these two separate sources is likely due to glycosylation of the fungal enzymes. These enzymes utilize an inverting mechanism for catalysis (Fig. 1.4), and although the proton-donating residue is known to be a glutamate, the nucleophile has yet to be determined (Biely *et al.*, 2000; CAZy; http://www.cazy.org/).

Relatively few thermostable α-glucuronidases have been described compared to other lignocellulosic enzymes. Both the fungi *A. pullulans*

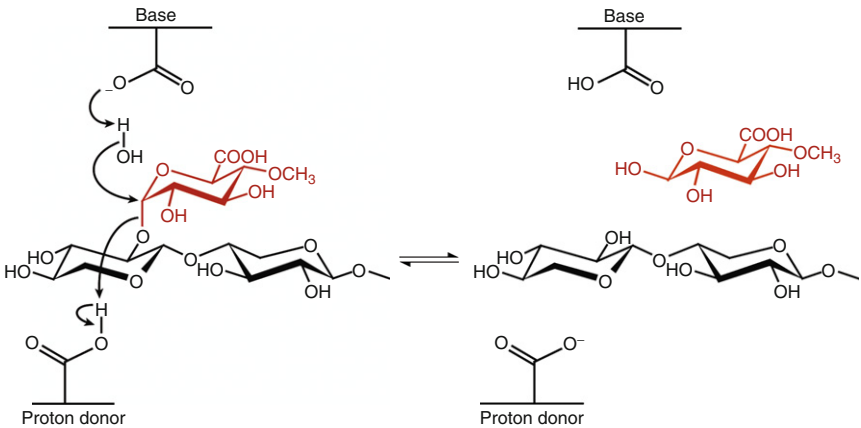

FIGURE 1.4 Catalytic mechanism of α-glucuronidase enzymes. These enzymes bind oligosaccharides with 4-*O*-methyl glucuronic acid side chains substituted at the 2′ hydroxyl group of a terminal xylose residue at the nonreducing end. The catalytic base then activates a water molecule which displaces the attached xylose residue.

and *T. aurantiacus* produce α-glucuronidases, each with optimal catalytic activity at 65 °C (Khandke *et al.*, 1989). Perhaps the most promising α-glucuronidase described to date, however, is AguA from *T. maritima* which displays optimum catalytic activity at 85 °C (Xue *et al.*, 2004).

D. Endoarabinanases

Arabinan comprises a significant portion of plant cell walls where it is widely distributed among hemicellulosic and pectic polysaccharides (Verhertbruggen *et al.*, 2009). The arabinan polymer forms a linear backbone of α-1,5-linked L-arabinofuranosyl residues, which can be substituted with α-1,2- and/or α-1,3-linked L-arabinose side chains (McNeil *et al.*, 1984). The hydrolysis of arabinan is facilitated by the synergy between endo-1,5-α-L-arabinanases and α-L-arabinofuranosidases (Fig. 1.5). Endo-1,5-α-L-arabinanases hydrolyze α-1,5-L-arabinofuranoside linkages between arabinose units in an endo-manner with an inverting catalytic mechanism. All endo-1,5-α-L-arabinanases belong to GH family 43 (CAZy;

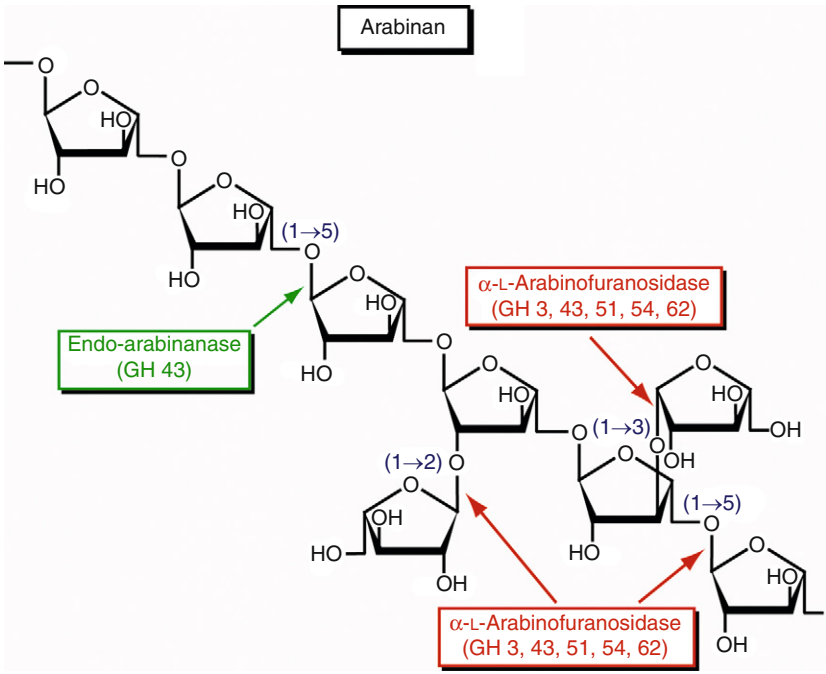

FIGURE 1.5 Enzymatic activities associated with arabinan hydrolysis. Endoarabinanases cleave α-1,5 linkages between main chain arabinose residues. α-L-Arabinofuranosidases release arabinose monomers by cleaving α-1,2, α-1,3, or α-1,5 linked arabinose residues from the nonreducing end.

http://www.cazy.org/), a family for which members catalyze the hydrolysis of glycosidic linkages through an inverting mechanism. A crystal structure has been produced for an endoarabinanase from the thermophilic bacterium, *Bacillus thermodenitrificans* TS-3, showing the enzyme to adopt a five-bladed β-propeller fold. The endo-acting nature of the enzyme is facilitated by an open substrate-binding cleft that would allow random binding of the arabinan substrate. The N-terminal region is thought to play an important role in the thermostability (Yamaguchi *et al.*, 2005).

Only a few examples of thermostable endo-1,5-α-L-arabinanases have been characterized (Table 1.6) and these appear to be exclusively of bacterial origin. Perhaps the best characterized endoarabinanases are those from *Caldicellulosiruptor saccharolyticus* and the previously described enzyme from *B. thermodenitrificans* (Mi-Ri Hong and Deok-Kun, 2009; Takao *et al.*, 2002). The *C. saccharolyticus* enzyme appears particularly thermostable, exhibiting maximum enzyme activity at 75 °C with half-lives of 2440, 254, and 93 h at 65, 70, and 75 °C, respectively.

E. α-L-Arabinofuranosidases

In contrast to α-L-arabinanases, α-L-arabinofuranosidases are exo-acting enzymes that hydrolyze terminal α-1,5-glycosidic linkages to arabinofuranosides in arabinan as well as α-1,2 and α-1,3-linkages to arabinofuranosides of arabinan, arabinoxylan, and arabinogalactan (Matsuo *et al.*, 2000). These enzymes work synergistically with other hemicellulolytic enzymes removing L-arabinose sidechains that would otherwise restrict the activity of the backbone-degrading enzymes (Fig. 1.5).

Arabinofuranosidases are classified into five GH families: 3, 43, 51, 54, and 62 (Table 1.1). The structural conformations and catalytic mechanisms for each of these enzyme families have been described in the preceding sections of this review with the exception of GH family 62 for which no information is currently available regarding structure, catalytic mechanism, or residues important to the active site (CAZy; http://www.cazy.org/). GH 43 arabinofuranosidases act on terminal α-1,5-linked arabinofuranosides. GH 51 and GH 54 family arabinofuranosidases remove both α-1,2 and α-1,3 arabinofuranosyl moieties from arabinan and xylans (Flipphi *et al.*, 1994; Matsuo *et al.*, 2000), while the activity of GH 62 family enzymes appears to be specific to arabinoxylans (Taylor *et al.*, 2006; Tsujibo *et al.*, 2002). To date almost all thermostable arabinofuranosidases described appear to derive from GH family 51. This family of arabinofuranosidases appears especially potent as exemplified by *C. thermocellum*, an organism displaying significant xylanolytic activity with just a single identifiable family 51 α-L-arabinofuranosidase, CtAraf51A (Taylor *et al.*, 2006). The most thermostable α-L-arabinofuranosidase described

TABLE 1.6 Thermostable α-L-arabinases and α-L-arabinofuranosidases

Microorganism	Optimum		Substrate[a] Specificity	Specific[b] activity	References
	Temperature (°C)	pH			
Anoxybacillus kestanbolensis	65	5.5	PNPA, SBA, AX[d], OSX, Adex	1112	Canakci *et al.* (2008)
Aureobasidium pullulans	75	4.0–4.5	PNPA, ARA[d], dARA[d], WAX, RAX, OSX, BWX	21.48	Saha and Bothast (1998)
Bacillus pumilus	55	7.0	PNPA	46.8	Degrassi *et al.* (2003)
Clostridium thermocellum	82	7.0			Taylor *et al.* (2006)
Geobacillus caldoxylolyticus	75–80	6.0	SBA, Adex	Nd[c]	Canakci *et al.* (2007)
Penicillium capsulatum	55	4.0	PNPA, SBA, sOSX, sWAX	4.2	Filho *et al.* (1996)
Penicillium capsulatum	60	4.0	PNPA, SBA, sOSX, sWAX	16.2	Filho *et al.* (1996)
Thermobacillus xylanilyticus	75	5.6–6.2	PNPA, WAX, LWX, OSX	490	Debeche *et al.* (2000)
Thermomicrobia sp.	70	6	bARA[d], dARA[d], AX[d], OSX	0.6	Birgisson *et al.* (2004)

[a] AX, arabinoxylan; Adex, arabinodextrins; ARA, arabinan (b, branched; d, debranched); BWX, birchwood xylan; LWX, larchwood xylan; OSX, oat spelt xylan (i, insoluble, s, soluble); PNPA, *p*-nitrophenyl-α-L-arabinofuranoside; RAX, Rye arabinoxylan; SBA, sugar beet arabinan; WAX, wheat arabinoxylan (i, insoluble; s, soluble).
[b] Amount of enzyme releasing 1 μmol product (or equivalent) from first-listed substrate per minute. Substrate selected corresponds to that giving largest reported specific activity.
[c] Not determined (or reported) for purified enzyme.
[d] Substrate source not reported.

currently is found in *Thermobacillus xylanilyticus* D3. This enzyme is stable up to 90 °C and has significant biotechnological applicability with activity ranging from pH 4 to 12 (Debeche *et al*., 2000).

Thermostable α-L-arabinofuranosidases have also been found in fungi; for example, two arabinofuranosidases exhibiting optimal activity at 60 and 55 °C were purified from solid-state cultures of *Penicillium capsulatum* (Filho *et al*., 1996). In addition, a novel thermostable α-L-arabinofuranosidase from *A. pullulans* was shown to have optimal activity at 75 °C and can hydrolyze arabinan and debranched arabinan but shows no activity against arabinogalactan (Saha and Bothast, 1998).

Crystal structures have been solved for two GH 51 family α-L-arabinofuranosidases (Hovel *et al*., 2003; Taylor *et al*., 2006). In both examples the enzyme appeared to function as a hexamer, with monomeric components assembling to form a $(\beta/\alpha)_8$ catalytic domain linked to a 12-stranded β-barrel C-terminal domain. Similar to other GH 51 family enzymes, catalysis occurs with net retention of the anomeric carbon atom configuration (Pitson *et al*., 1996). The residues Glu^{173} and Glu^{292}, of the thermophilic α-L-arabinofuranosidase, CtAraf51 from *C. thermocellum* act as the acid/base proton donor and nucleophile, respectively (Taylor *et al*., 2006).

F. Esterases

Plant cell wall polysaccharides, particularly those comprising the hemicellulose fraction, such as xylans, mannans, and glucomannans, as well as pectin, are commonly acetylated and on occasion feruloylated, with *O*-bound acetyl groups comprising up to 7% of plant cell walls by dry weight (Brett and Waldren, 1996). Xylans, the major hemicellulosic component, in particular, are typically rich in acetyl and 4-*O*-methyl substituents in the β-1,4-linked backbone. In fact studies of hardwood xylans suggest that as much as 60–70% of xylose residues are esterified with acetic acid. Acetylated xylans are also common to perennial plants (Biely *et al*., 1986, Shao and Wiegel, 1995).

Esterases are enzymes that catalyze the hydrolysis (and formation) of ester bonds. Acetyl xylan esterases catalyze the removal of acetyl ester groups from C2 or C3 positions of D-xylopyranosyl residues (Biely, 2003), while ferulic acid esterases release ferulic acid from the C2 or C5 positions of α-L-arabinofuranosyl side chains (Salnier and Thibault, 1999). Ferulic acids esterified to arabinofuranosyl side chains commonly link with other ferulic acid esters forming various types of diferuloyl bridges resulting in the inter-, and possibly even intralinking of polymers within plant cell walls (Iiyama *et al*., 1994). Consequently, the efficient and complete degradation of acetylated xylans requires the cooperation of esterases with

other xylanolytic enzymes. Furthermore, phenolic acids released through the action of esterases are potential precursors to a variety of value-added products and may be exploited as a means to offset some of the costs currently limiting the feasibility of biorefineries (Faulds *et al.*, 2000; Graf, 1992).

As esterase activity is not directed at glycosidic linkages, these enzymes are not classified within GH families; however, similar to GHs, carbohydrate esterases (CEs) are able to be grouped by the predicted structure of their catalytic domains. Currently there are 16 CE families, with members of each family utilizing conserved catalytic apparatus to execute identical catalytic mechanisms (Correia *et al.*, 2008; CAZy; http://www.cazy.org/). The expanse of CE families appears to be necessary due to the apparently limited substrate specificity typically displayed by these enzymes (Aurilia *et al.*, 2000; Correia *et al.*, 2008; Dalrymple *et al.*, 1997). Thermostable acetyl xylan esterases have been purified and characterized from just a handful of bacteria including the anaerobic bacteria *Thermoanaerobacterium* sp. and *C. thermocellum* (Correia *et al.*, 2008). To date most reported thermostable acetyl esterases have optimal activity at near-neutral pHs and ~70–80 °C, although an acetyl esterase from *Thermoanaerobacterium* sp. displays optimal activity at 84 °C (Shao and Wiegel, 1995). Bacterial examples of thermostable feruloyl esterases appear even rarer. A few examples have been reported from clostridia, including a naturally occurring ferulic acid esterase/xylanase enzyme hybrid from *C. thermocellum* (Kataeva *et al.*, 2001). The bacterium *Clostridium stercorarium* produces an interesting feruloyl esterase that has temperature and pH optima of 65 °C and pH 8.0, respectively, and is active on ester-linked ferulic, coumaric, caffeic, and sinapinic acids (Donaghy *et al.*, 2000). Examples of fungal esterases with thermostable activity on ester-linked acetate and/or ferulate have recently been expanded significantly. Bioprospecting of thermotolerant fungal strains by Ghatora *et al.*, 2006 led to the description of 84 acetyl-xylan and ferulic-acid esterases that appear to have activity optima spanning at least pH 4–9.

Crystal structures have been solved for one or more esterases from most CE families (1, 3–11, 13 and 14) and all, with the exception of those from CE family 4, which exhibit a distorted $(\beta/\alpha)_8$ barrel, have a typical α/β hydrolase fold. Those from CE families 1 and 5–7 are serine esterases that utilize classical Ser-His-Asp catalytic triads (Correia *et al.*, 2008). Site-specific mutational analysis of a CE family 3 acetyl xylan esterase from *C. thermocellum* revealed Ser^{44} to be the nucleophile, His^{208} to be the proton donor and Asp^{205} to play an important role in increasing the basic character of the histidine (Correia *et al.*, 2008).

G. Mannanases, mannosidases, and other auxiliary enzymes

Mannan is a major constituent of hemicellulose where it exists in a variety of forms, including linear mannan, glucomannan, galactomannan, or glucogalactomannan (Fig. 1.6). Each of these polymers comprises a β-1,4-linked backbone of mannose residues that may be substituted up to 33% (or up to 50% in hardwoods) with glucose residues. In the case of galactomannans or glucogalactomannans, galactose residues form α-1,6-linkages to the mannan backbone (Moreira and Filho, 2008). The biodegradation of mannan into oligosaccharides or fermentable sugars involves the synergy of various mannan-degrading enzymes. β-1,4-D-mannanases hydrolyze β-(1,4)-linkages in the backbone of mannan polymers, producing short-chain manno-oligomers. These are further degraded through the action of β-1,4-D-mannosidases. Additional enzymes such as acetyl esterases and α-galactosidases remove side-chain substituents attached at various points along the mannan backbone, thereby creating additional sites for subsequent hydrolysis (Moreira and Filho, 2008) .

β-1,4-D-Mannanases are found in GH families 5, 26, and 113, while β-mannosidases are found in GH families 1, 2, and 5. All β-mannanases and β-mannosidases assemble to form a $(\beta/\alpha)_8$ fold and exhibit catalytic activity via a retaining mechanism, utilizing two glutamate residues as the nucleophile and proton donor (CAZy; http://www.cazy.org/; Table 1.1).

β-Mannanases are common constituents of the plant cell-wall-degrading arsenals of various bacteria and fungi (Table 1.7). One particularly interesting mannanase was described from *Caldanaerobius polysaccharolyticus*. This thermostable mannanase exhibits both endomannanase and endoglucanase activity presumably by virtue of its unusual multidomain structure. This enzyme is fitted with a putative mannanase-cellulase dual-function catalytic domain, two CBMs, and a surface-layer-like protein region (SLH-1, SLH-2, and SLH-3; Cann *et al.*, 1999). Characterization of the two CBMs reveals high specificity toward β-1,4-linked sugars as well as both cello- and mannopolysaccharides (Bae *et al.*, 2008). Given the conformational relationship between glucose and mannose (mannose is a C2 epimer of glucose) the catalytic activity of mannanases may be expected to extend to glucose-based polymers; however, this is rarely the case. Structural comparisons of a GH 5 family mannanase from *Thermomonospora fusca* with GH family 5 cellulases suggest specificity may be achieved through a hydrophobic interaction between the substrate and a conserved valine residue, Val^{263}, which may enable discrimination between the conformational differences occurring at the hydroxymethyl group of mannan and cellulose, along with a specific interaction in the –2 subsite between an aspartate residue, Asp^{259}, and the

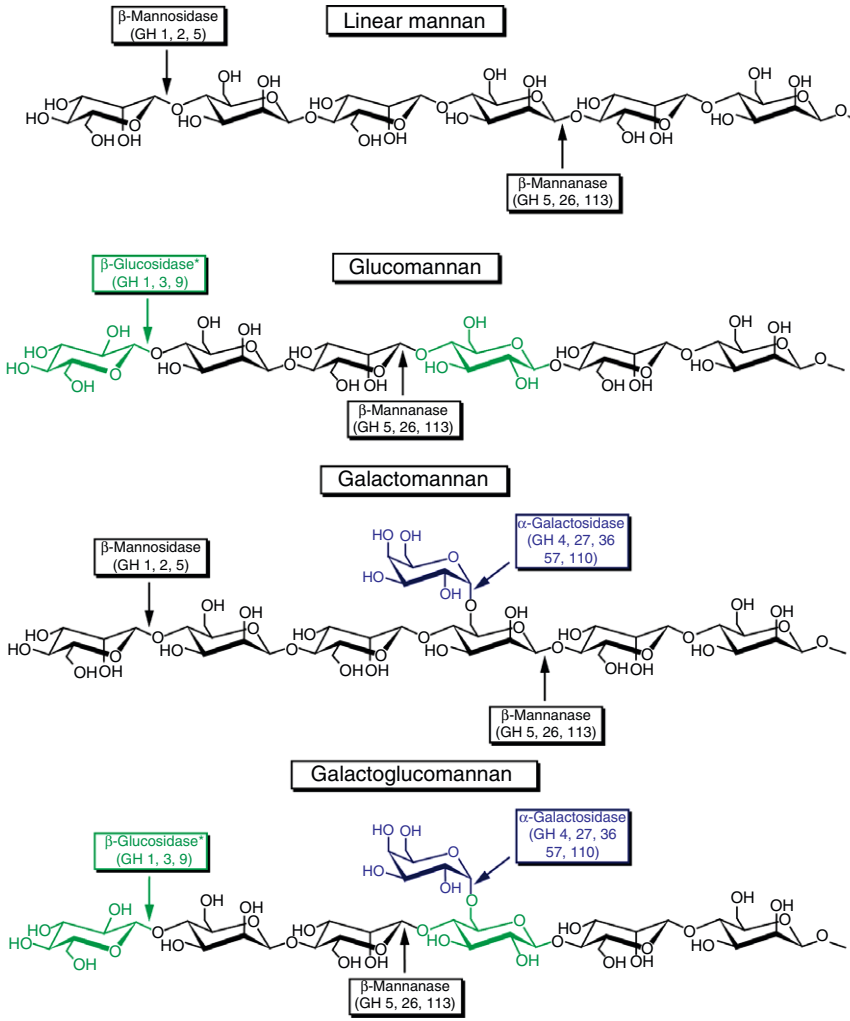

FIGURE 1.6 Enzymatic activities associated with hydrolysis of various mannans. There are four different types of mannan: linear mannan, glucomannan, galactomannan, and galactoglucomannan. In all four types of mannan, β-mannanase enzymes cleave β-1,4 linkages between either mannose and glucose or mannose and mannose sugars within the backbone chain. In galactomannan and galactoglucomannan, α-galactosidase enzymes release galactose residues which are appended at the 6′ hydroxyl groups of main chain mannose or glucose residues. In glucomannan and galactoglucomannan, β-glucosidase enzymes cleave glucose residues from the nonreducing ends of oligosaccharides produced by the action of β-mannanase enzymes.

TABLE 1.7 Thermostable β-mannanases and β-mannosidases

Microorganism	Optimum		Substrate[a] specificity	Specific[b] activity	References
	Temperature (°C)	pH			
Bacillus stearothermophilus	70	5.5–7.5	LBG, GG	100	Talbot and Sygusch (1990)
Caldibacillus cellulovorans	85	6.0	LBG, KGG, GG	1949	Sunna *et al.* (2000b)
Pyrococcus furiosus	105	7.4	PNPM	31.1	Bauer *et al.* (1996)
Rhodothermus marinus	85	5.4	CGM, LBG, GG	Nd[c]	Politz *et al.* (2000)
Thermomonospora fusca	80	Nd[c]	MAN, Xylan[d], CMC	Nd[c]	Hilge *et al.* (1998)
Thermotoga neapolitana	91	7.1	CGM	1.23	McCutchen *et al.* (1996)
Thermotoga neapolitana	90–92	Nd[c]	CGM	3.8	Duffaud *et al.* (1997)
Thermotoga neapolitana	87	Nd[c]	PNPM	34	Duffaud *et al.* (1997)

[a] CGM, carob galactomannan; CMC, carboxymethylcellulose; GG, guar gum; KGG, konjac gum glucomannan; LBG, locus bean gum; MAN, mannan; PNPM, *p*-nitrophenyl-β-mannopyranoside.
[b] Amount of enzyme releasing 1 μmol product (or equivalent) from first-listed substrate per minute. Substrate selected corresponds to that giving largest reported specific activity.
[c] Not determined (or reported) for purified enzyme.

axial hydroxyl group of C2 (Hilge *et al.*, 1998). Examples of significant thermostability are also seen within this group of enzymes, for example a β-mannanase from *B. stearothermophilus* was able to retain near-full activity after 24 h at 70 °C (Talbot and Sygusch, 1990), while a β-mannanase from *Thermotoga neapolitana* exhibited a half-life of 13 h at its temperature optima of 91 °C. In addition, *T. neapolitana* appears to possess a β-mannosidase with comparable attributes (Duffaud *et al.*, 1997; McCutchen *et al.*, 1996). Other β-mannosidases have been reported, including an extremely thermostable GH family 1 enzyme from *P. furiosus*, which has a half-life of >60 h at 90 °C and exhibits maximal activity at 105 °C (Bauer *et al.*, 1996).

Several thermostable α-galactosidases have also been reported from bacteria, archaea, and yeast (Brouns *et al.*, 2006; Carrera-Silva *et al.*, 2006; Shankar *et al.*, 2009). It will be interesting to see, during the optimization of lignocellulosic enzyme cocktails, if auxiliary enzymes such as α-galactosidases affect the economy of the process.

IV. STRUCTURAL BASIS FOR THERMOSTABILITY

Questions regarding the structural basis of thermostability are not new, nor are they confined to studies of enzymes facilitating plant cell wall deconstruction. An enzyme's stability is defined by its capacity to retain its active structural conformation in spite of disruptive forces, such as increases in temperature. The major forces leading to, and maintaining, an enzyme's active conformation are hydrophobic effects and hydrogen bonding. In the native setting, these and other stabilizing forces typically outweigh disruptive forces by as little as 5–10 kcal mol^{-1}. This balance is known as the free energy change, ΔG, of a protein (Pace, 1975). A recent review on thermostable proteins found, relative to mesophilic homologues, that thermostability was most commonly implied through increases in ΔG across all temperatures (resulting from an increase in ΔH–change in enthalpy—at the temperature of maximum stability, without changes to ΔS—change in entropy) and decreases in ΔC_p—the change in heat capacity—rather than increases in the maximum thermal stability (Razvi and Scholtz, 2006). This was particularly evident for enzymes and likely derives from a catalytic intolerance to structural rigidity (Hammel *et al.*, 2004; Jaenicke, 2000). Broad increases in ΔG may be mediated by stabilizing features such as salt or disulfide bridges, ion pairings, and increases in interactions between aromatic residues—particularly in the active site. Comparisons between the numerous crystal structures available for thermophilic- and mesophilic- acting GH enzymes have revealed these features to be common (Ihsanawati *et al.*, 2005). For example the *R. marinus* endocellulase Cel12A, which shows optimal activity at >90 °C,

has a significant increase in ion pairings and an increase in aromatic residues in the active site as compared to its mesophilic counterparts (Crennell *et al.*, 2002). Further, enzyme engineering strategies that have been used to introduce disulfide bridges, aromatic residues, or salt bridges to mesophilic or moderately thermophilic enzymes have succeeded in enhancing thermostability and/or thermophilicity (Georis *et al.*, 2000; Xie *et al.*, 2006). In one example, the introduction of disulfide bridges to a GH family 11 xylanase from *Thermobacillus xylanilyticus* resulted in a 10-fold increase in thermal stability compared to that of the wild-type enzyme with no commensurate loss in specific activity (Paes and O'Donohue, 2006). A decrease in ΔC_p can be mediated by tighter core packing or oligomerization. Structural analysis of a xylanase with enhanced thermostability, produced through random mutagenesis, revealed the introduction of a valine residue that filled the hydrophobic core with an increased number of van der Waals interactions (Xie *et al.*, 2006). Further comparisons of thermostable and mesophilic GHs have identified reductions in the exposure of hydrophobic residues (Ihsanawati *et al.*, 2005) and reductions in the exposed surface area through oligomerization or substrate binding as means to enhance thermostability (Chi *et al.*, 1999). Although less common, increases in the maximum thermal stability brought about through increases in structural rigidity have been surmised from comparative structural analyses particularly in connecting loops (Crennell *et al.*, 2002; Wang *et al.*, 2003). Increases in rigidity may also be brought about through glycosylation or interactions with metal ions (Hong *et al.*, 2003; Yan *et al.*, 2008). Another important difference in the primary structure of thermophilic GHs relative to mesophilic GHs is a reduction in thermolabile residues, such as Asn and Cys (Wang *et al.*, 2003). Understanding the mechanisms of thermal stability and using the structural features, as described earlier, has and will continue to enable targeted approaches to improving the thermostability of GH enzymes.

V. IMPROVING THERMOSTABILITY AND BIOTECHNOLOGICAL APPLICABILITY

In addition to bioprospecting, many groups are moving forward through the manipulation of enzymes already available to researchers. Single or successive rounds of random mutagenesis using methods such as error-prone PCR or DNA shuffling, followed by selection for improvements in desired traits, such as catalytic activity, thermostability, or pH tolerance is becoming a commonly employed tool for optimizing an enzyme's characteristics (Stephens *et al.*, 2009, Wang and Xia, 2008). This process, known as directed evolution, is not new but allows researchers a route forward in

the absence of knowledge regarding the features underpinning these traits. These methods have successfully led to significant increases in catalytic activity, thermostability, and pH stability of a number of xylanases (Chen *et al.*, 2001; Stephens *et al.*, 2009; Stephens *et al.*, 2007; Wang and Xia, 2008). Methods are continuing to be developed to expedite and simplify the selection procedures (Liu *et al.*, 2009). Other more calculated approaches include the structure-guided recombination process, SCHEMA (Meyer *et al.*, 2006). The SCHEMA process produces chimeric proteins by interchanging contiguous blocks of amino acids. SCHEMA is more directed than a random process, using the parental proteins' structural data to define the boundaries of these amino acid blocks so as to minimize the average number of amino acid sidechain contacts that are broken in the library. SCHEMA was recently employed to develop a library of CBHs with improved thermal stability. Chimeric CBHs were produced through recombination of CBH genes from the fungi *Chaetomium thermophilum, Humicola insolens*, and *Hypocrea jecorina* (Heinzelman *et al.*, 2009). Although the authors only studied a small fraction of the library of chimeras (48 from 6561 total chimeras), a number of novel enzymes with improved traits were identified, including HJPlus, which gave a high specific activity across a broad pH range and exhibited a 7–15 °C increase in temperature optimum over the parental enzymes. Given the sampling size, it is predicted that many more enzymes with improved thermal stability, along with other biotechnological attributes, may reside within this chimeric library, and consequently this appears a very valuable approach.

Similarly, the exchange of identifiable modules can lead to commensurate increases in thermostability and hydrolytic activity. An obvious example is the addition of CBMs to enzymes lacking this module, which has regularly been shown to improve performance, particularly against crystalline substrate (Kang *et al.*, 2007; Kittur *et al.*, 2003; Szijarto *et al.*, 2008). Module shuffling between two GH 10 family xylanases with different thermostabilities, Cex (optimum temperature: 40 °C) from *Cellulomonas fimi* and XylA (optimum temperature: 80 °C) from *Thermomonospora alba*, led to a recombinant enzyme that exhibited significantly improved thermal profiles (optimum temperature: 65 °C) compared to one of the parental proteins, Cex (Ahsan *et al.*, 2001). Studies such as these suggest that in some proteins, specific protein domains may exist that confer or enhance thermostability. Consistent with this hypothesis the deletion of particular domains resident in some cellulases and hemicellulases has resulted in decreases in thermostability (Hayashi *et al.*, 1997; Riedel *et al.*, 1998). One such domain, the A-domain, has been identified in the N-terminal regions of xylanases from organisms such as *T. maritima*, *C. thermocellum*, and *T. saccharolyticum* (Fontes *et al.*, 1995; Lee *et al.*, 1993) and has been shown to improve its cognate enzyme's thermostability and substrate-binding capacity (He *et al.*, 2009). This may suggest that a major

source of thermoinstability derives from the binding module. It is evident from these studies that modest changes in an enzymes primary structure can lead to significant improvements in biotechnologically important traits (Stephens *et al.*, 2009), although such changes may also lead to undesirable properties.

The extracellular cellulolytic enzymes of several bacteria, particularly *Clostridia*, assemble as protein complexes or aggregates known as cellulosomes. Cellulosomes have often been attributed with having improved catalytic activities, particularly on crystalline substrate, as compared to the free form (individually acting) enzymes due to the improved synergy afforded by colocalization of complementary enzymatic activities (Fierobe *et al.*, 2005). Consequently significant research has been invested in developing thermostable cellulosomes and optimizing their enzymatic constituents. The rozettazyme, for instance, is a group II chaperonin that derives from the hyperthermo-acidophilic archaeon *Sulfolobus shibatae*, which has been retrofitted with cohesin modules from the *C. thermocellum* CipA protein. This synthetic scaffoldin self-assembles in the presence of ATP and Mg^{2+} ions into a thermostable double-ringed structure capable of aggregating 18 complementary enzymatic activities through interactions between their dockerin domains and the cohesins (Mitsuzawa *et al.*, 2009). In native systems, cellulosome-associated activities are wide ranging and can include cellulases, hemicellulases, pectinases, chitinases, glycosidases, and esterases (Zverlov *et al.*, 2002, 2005a,b,c) demonstrating the heterogeneous and highly associative nature of the substrates that these systems have evolved to degrade (Zverlov *et al.*, 2005a,c). The development of synthetic cellulosomes that are effective in biotechnological applications will require optimization of the enzyme constituents. Recently derived methods such as cohesin-dockerin microarrays will undoubtedly simplify this process (Haimovitz *et al.*, 2008).

An alternative approach for colocalizing synergistic lignocellulase activities is the construction of gene fusions (Hong *et al.*, 2006). Both approaches have shown promising results; however, issues have occasionally emerged with the latter regarding enzyme folding and stability.

VI. DISCUSSION AND FUTURE PROSPECTS

The applicability of thermostable enzymes as biocatalysts for the depolymerization of lignocellulosic feedstock in the production of biofuels is gaining wide industrial and biotechnological interest. Their robust thermostabilities make them better suited for the harsh processing conditions required for efficient deconstruction of lignocellulose to fermentable products. The thermostabilities of these enzymes have been attributed to many factors, such as: (a) amino acid composition, including decreases

in thermolabile residues such as Asn and Cys; (b) hydrophobic interactions; (c) aromatic interactions, ion pairs, and increased salt bridge networks; (d) oligomerization and intersubunit interactions; (e) packing and reduction of solvent-exposed surface area; (f) metal binding; (g) substrate stabilization; and (h) a decrease in number and size of surface loops. It is likely that each of these in the correct context may contribute to thermostability. Researchers have correspondingly developed methods to enhance the thermal stability and activity of lignocellulosic enzymes, using both random and directed approaches.

A considerable number of enzymes applicable to lignocellulose depolymerization have been investigated. With the seemingly continual emergence of potential sources being uncovered (Ghatora *et al.*, 2006; Picart *et al.*, 2007, 2008; Tai *et al.*, 2004), particularly through culture-independent techniques such as metagenomics (Elend *et al.*, 2006; Feng *et al.*, 2007; Ferrer *et al.*, 2005; Grant *et al.*, 2004; Kim *et al.*, 2006; Rees *et al.*, 2003; Voget *et al.*, 2006) and metatranscriptomics (Warnecke and Hess, 2009), it is hard to be sure when, or if, we will realize the complete diversity of these enzymes in nature. This review has considered thermostable enzymes required for the depolymerization of lignocellulosic substrates to fermentable sugars, largely in the context of bioethanol or longer chain biofuel production; however, research is also proceeding into alternate energy sources such as hydrogen, which require further enzymatic activities. The complete conversion of cellulosic substrate to H_2 has been shown using mesophilic systems to be possible with as few as 13 enzymes and a coenzyme, and yield almost 12 H_2 molecules per glucose equivalent (Ye *et al.*, 2009). Commensurate with this work thermostable enzymes important to this expanded process are also starting to be described (Wang and Zhang, 2009).

The hydrolytic performance of most described cellulases and hemicellulases has been well defined; however, just a handful of studies (e.g., see Kambourova *et al.*, 2007) have examined these same enzymes in the context of a multicomponent synergistic mixture, which must be the ultimate goal of this research field. Lignocellulose is a highly complex and rigid substrate. The crystallinity of cellulose, the available surface area, and the distribution therein of lignin and hemicellulose are substrate-related factors limiting the hydrolysis rate of plant cell walls. To enable the production of plentiful amounts of hexose, as well as pentose, or monosaccharides for biofuel production, optimizing thermostable enzyme mixtures for both cellulose and hemicellulose hydrolysis will be essential, as this will reduce the overall cost of production.

Clearly the research area in this field is multifaceted with areas beyond the scope of this review. This includes areas such as the optimization of enzyme expression systems, which may include the engineering of the plants themselves (Kimura *et al.*, 2003; Oraby *et al.*, 2007; Yu *et al.*, 2007) or

the bioengineering of fermentative organisms, such as yeast to produce ''one-stop biorefineries'' with thermotolerant cellulolytic and hemicellulolytic capabilities (Hong *et al*., 2007b). It is, however, clear that regardless of the system employed, optimization of the process will require a better understanding/knowledge of the enzymes facilitating the deconstruction of lignocellulose.

ACKNOWLEDGMENTS

We thank the Energy Biosciences Institute (EBI) for supporting our research on lignocellulose deconstruction. We would also like to thank Shinichi Kiyonari, Shosuke Yoshida, and Michael Iakiviak of the Energy Biosciences Institute for scientific discussions.

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