Chapter 6
Extremophilic (Hemi)cellulolytic Microorganisms and Enzymes

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Abstract The *second generation* bioethanol represents a main challenge in global efforts to utilize renewable resources rather than fossil fuels. However, the close association of cellulose and hemicelluloses to lignin in the plant cell wall makes it difficult to degrade lignocellulose into fermentable sugars. Consequently, pretreatments are necessary to make the polysaccharides more accessible to the enzymes, but the high temperature and extreme pH conditions required give rise to problems when using conventional enzymes in the saccharification step (Galbe and Zacchi 2002). Microorganisms thriving in habitats characterized by harsh conditions, and the enzymes derived therein, represent a helpful tool in the development of bioethanol production processes. In fact, they allow bioconversions at non-conventional conditions under which common biocatalysts are denatured. The use of high operational temperatures allows energy savings by reducing the cooling cost after high temperature pretreatments, and, in ethanol production, thermophilic conditions permit ethanol evaporation allowing harvest during fermentation.

Contents

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6.1 Extremophilic Microorganisms

The study of extremophiles and extremozymes started in the late 1960s. In the following sections we will summarize the state-of-the-art on (hyper)thermophiles, halophiles and alkaliphiles involved in (hemi)cellulose degradation.

6.1.1 (Hyper)thermophiles

Depending on their optimal growth temperature, thermophilic microorganisms are grouped in thermophiles (45–80 °C) and hyperthermophiles (80–122 °C). The latter are dominated by the Archaea domain, but some Bacteria, such as *Thermotoga* and *Aquifex,* tolerate temperatures around 100 °C. Degradation of cellulosic and hemicellulosic substrates among thermophiles is mostly due to Bacteria species with few Archaea species.

6.1.1.1 (Hyper)thermophilic Bacteria

The major rate-limiting step in the conversion of lignocellulose is represented by the hydrolysis of cellulose, since in plant biomass it has a high order of crystallinity and is scarcely accessible to microbial or enzymatic attack. Not many microorganisms are able to degrade pure crystalline cellulose, and two main concepts are considered for its microbial degradation: free cellulases and large multi-enzyme complexes (cellulosomes). Crystalline cellulose degradation via cellulosomes was firstly described in *C. thermocellum* (T 60 °C). This microorganism solubilized to an extent of 95 % the cellulose microcrystals from the alga *Valonia ventricosa*, whose crystallinity is close to 100 % (Boisset et al. 1999).

The order *Thermoanaerobacteriales* includes several species that utilize (hemi)cellulose as growth substrates. The most thermophilic species belong to the genus *Caldicellulosiruptor*, but, while all species hydrolyze hemicellulose, not all degrade crystalline cellulose. *C. kristjanssonii* (T 78 °C) and *C. bescii* (T 80 °C), which is the most thermophilic cellulolytic bacterium characterized to date, can grow on crystalline cellulose and unprocessed plant biomasses (Bredholt et al. 1999; Yang et al. 2009, 2010). Recently, a novel cellulolytic bacterium has been isolated from Obsidian Pool in Yellowstone National Park. The microorganism,

designated *C. obsidiansis* sp. nov. (T 78 °C), exhibits fermentative growth on arabinogalactan, xylan, Avicel, filter paper, dilute acid-pretreated switchgrass, and poplar, whereas is unable to grow on lignin (Hamilton-Brehm et al. 2010).

Decomposition of lignocellulose by complex microbial communities represents a promising alternative for biomass conversion. In particular, thermophilic consortia are a potential source of enzymes adapted to harsh reaction conditions. Wongwilaiwalin et al. (2010) obtained from a high-temperature bagasse compost a stable thermophilic lignocellulolytic microbial consortium highly active on cellulosic biomass. A microbial consortium including anaerobic bacteria of genera *Clostridium* and *Thermoanaerobacterium,* efficiently degraded rice straw, corn stover, and industrial eucalyptus pulp sludge. In a recent study, compost-derived microbial consortia were adapted on switchgrass at 60 °C: high abundance of thermophilic bacteria as *Rhodothermus marinus* and *Thermus thermophilus* were observed (Gladden et al. 2011).

In addition to traditional bioethanol production processes, consolidated bioprocessing (CBP), which combines in one step saccharification with fermentation using a whole cells-based approach, represents an alternative method with outstanding potential for low-cost processing of lignocellulosic biomass (Lynd et al. 2005). No ideal CBP microbe able to degrade efficiently lignocellulose and, at the same time, to utilize the released sugars to produce ethanol is currently available. A newly discovered thermophilic microorganism, *Geobacillus* sp. R7 (T 60 °C), is a facultative anaerobic bacterium isolated from soil samples of the Homestake gold mine, South Dakota. It produces a thermostable cellulase when grown on extrusion-pretreated agricultural residues such corn stover and prairie cord grass, and ferments lignocellulosic substrates to ethanol in a single step (Zambare et al. 2011).

Xylan represents the second most abundant polysaccharide in lignocellulose; however, the number of characterized thermophilic microorganisms that utilize xylan exceeds the number of cellulose-degrading ones. Xylan-utilizing microorganisms are widely distributed within the order *Thermoanaerobacteriales*. *T. zeae* (T 68 °C), isolated from industrial environments, utilizes cracked corn and xylan, but not cellulose, and produces ethanol as the main product after glucose fermentation (Cann et al. 2001). Xylan-degrading bacteria have also been found in the genera *Thermotoga*. *T. hypogea* sp. nov. (T 70 °C) produces trace amounts of ethanol during xylan fermentation (Fardeau et al. 1997).

In comparison with the anaerobic thermophilic bacteria few aerobic have been described to produce cellulases and xylanases. The aerobic thermophiles *R. marinus* produces a highly thermostable cellulase (Cel12A), and three glycoside hydrolases belonging to family GH10 of the Carbohydrate Active enZyme (CAZy) database (http://www.cazy.org) (Alfredsson et al. 1988; Cantarel et al. 2009). *Aquifex aeolicus*, isolated in the Aeolic Islands in Sicily (Italy), represents one of the most thermophilic bacteria since its growth temperature can reach 95 °C (Deckert et al. 1998). From this source, a single cellulase, able to hydrolyze CMC but not Avicel, has been reported to date. Table 6.1 summarizes other bacteria not mentioned in the text.

Table 6.1 Thermophilic (hemi)cellulolytic bacteria

Microorganism	Growth T (°C)	Growth conditions	References
Acidothermus cellulolyticus	55	Aerobic	Mohagheghi et al. (1986)
Alicyclobacillus acidocaldarius	60	Aerobic	Wisotzkey et al. (1992)
Clostridium stercorarium	65	Anaerobic	Madden (1983)
Dictyoglomus thermophilum	73	Anaerobic	Saiki et al. (1985)
Moorella strain F21	60	Anaerobic	Karita et al. (2003)
Spirochaeta thermophila	70	Anaerobic	Aksenova et al. (1992)
Thermoanaerobacter cellulolyticus	75	Anaerobic	Taya et al. (1988)
Thermotoga neapolitana	80	Anaerobic	Jannasch et al. (1988)
Thermotoga petrophila	80	Anaerobic	Takahata et al. (2001)

6.1.1.2 Hyperthermophilic Archaea

Among Archaea, the genus *Pyrococcus* and *Sulfolobus* have been found to produce cellulases and xylanases (Maurelli et al. 2008). Microorganisms belonging to the genus *Pyrococcus* have been isolated from hydrothermally heated sea vents with T 90–100 °C. The genomes of *P. furiosus* (Fiala and Stetter 1986), *P. horikoshii* (Gonzalez et al. 1998), *P. abyssi* (Cohen et al. 2003) and *Thermococcus kodakaraensis* KOD1 (Fukui et al. 2005), encode a variety of cellulases, however, none of these microorganisms grows on crystalline cellulose.

Within the order *Sulfolobales*, *Sulfolobus* species are commonly isolated from acidic thermal pools (T up to 90 °C). A specific strain *S. solfataricus* (Oα) can grow on xylan as sole carbon source (Cannio et al. 2004), but crystalline cellulose is not a growth substrate for any species of *Sulfolobus* so far reported. Recently, Perevalova et al. (2005) demonstrated that *Desulfurococcus fermentans,* an obligately anaerobic archaeon isolated from a freshwater hot spring of the Uzon caldera (Kamchatka Peninsula, Russia) growing optimally at 82 °C, is capable of growing on crystalline cellulose.

As reported above, few archaea species are known to be able of degrading lignocellulose. To identify new species able to decompose biomasses at high temperatures, analyses of 16S rRNA genes in DNA samples from terrestrial hot springs and deep-sea vents may reveal hyperthermophilic microbes recalcitrant to culture and with potential unknown hydrolyzing properties. Kublanov and coworkers (2009) identified many hot springs (T 68–87 °C) in Kamchatka Peninsula for in situ enrichment on microcrystalline cellulose of thermophilic species. Denaturing gradient gel electrophoresis analysis of 16S rRNA gene fragments obtained after PCR with Archaea-specific primers, revealed the presence of uncultivated microrganisms. They were closely related to uncultured organisms from Iceland and Kamchatka hot springs belonging to the *Crenarchaeota* phylum "unknown *Desulfurococcales*". Recently, a sediment collected from a 94 °C geothermal pool in northern Nevada, USA, was used as inoculum for enrichment trials in laboratory. After two consecutively enrichments with Avicel and filter

paper, an anaerobic consortium consisting of three archaeal species able of growth on cellulose sources was identified (Graham et al. 2011). These data represent a new benchmark in searching for new microbial species capable of degradation of lignocellulose at high temperatures.

6.1.2 Halophiles

Saline and hypersaline environments represented by saline lakes and other water systems as well as saline soils, are widely distributed on the Earth (Oren 2002). Such organisms, called halophiles, are found in all three domains of life: they are widespread in the bacterial and archaeal kingdoms and eukaryotic halophilic microorganisms, such as fungi and algae, are also known (Gunde-Cimerman et al. 2009).

The groups containing halophilic representatives seldom include solely halophiles and only a few phylogenetically consistent groups are composed entirely of halophiles and many genera, families and orders show different salt requirements and tolerance (Oren 2002, 2008). On the basis of the hypersaline conditions needed for growth, halophiles are classified as slight, moderate and extreme halophiles (requiring 2–5, 5–20 or 20–30 % NaCl, respectively). On these basis of a good and simple operative definition suggested by Oren, the halophiles grow optimally at salt concentrations $\geq$50 g/l and tolerate at least 100 g/l salt (Oren 2008). Moreover, halotolerants are able to grow at moderate salt concentrations, even though they grow best in the absence of NaCl (Ventosa et al. 1998).

In Euryarchaeota the most important salt-requiring microorganisms were found within the archaeal order of *Halobacteriales* and among the *Methanothermea*, in the order *Methanosarcinales*. No halophiles have yet been identified within the *Crenarchaeota* kingdom. The Bacteria domain contains many types of halophilic and halotolerant microorganisms, widespread over a large number of phylogenetic subgroups (for a review see Ventosa et al. 1998) including *Proteobacteria*, *Cyanobacteria*, the *Cytophaga-Flavobacterium* branch, the *Spirochaetes*, and the *Actinomycetes*. Within Gram-positive Bacteria (*Firmicutes*), halophiles are found both in the aerobic (*Bacillus* and related organisms) and in the anaerobic branches. Halophiles are scarcely present in Eukarya. In fact, the only eukaryal microorganism of importance, but almost ubiquitously present in high-salt environments, is the green alga *Dunaliella* (Oren 2005).

A cellulose-utilizing, extremely halophilic bacterium was first reported by Bolobova et al. (1992). An obligate anaerobic organism named *Halocella cellulolytica* is able to utilize cellulose as a sole carbon source. Another work has shown that many cellulose-utilizing extremely halophilic Archaea are present in subsurface salt formation (Vreeland et al. 1998). A preliminary work on extracellular hydrolytic enzymes of halophilic microorganisms from subterranean rock salt revealed the presence of cellulases and xylanases. The isolated strains producing these enzyme activities are Gram-negative rods and can grow at 120 °C. These microorganisms are unable to thrive in the presence of various antibiotics such as

neomicin, penicillin, anysomicin and erythromycin, and are tolerant to salt concentrations of up to 3 M NaCl (Cojoc et al. 2009).

The purification and properties of two new halotolerant xylanases with stability and activity in NaCl concentrations in the range 0–5 M from the extremely halophilic archaeon *Halorhabdus utahensis* have been reported (Wainø and Ingvorsen 2003; Wejse et al. 2003). This microorganism was isolated from sediment of Great Salt Lake of Utah, USA and grows with 27 % w/v NaCl and only a few carbohydrates.

In comparison with the thermophilic and the alkaliphilic extremophiles, halophilic microorganisms have found relatively few biotechnological applications yet. Nevertheless, these microorganisms produce unique enzymes stable and active in conditions in which their "conventional" counterparts could not be functional (e.g. high salinity, low water activities and presence of organic solvents) (Litchfield 2011). Moreover, the recent availability of new halophiles genome sequences will allow the identification of novel (hemi)cellulolytic strains useful for biotechnological purposes.

6.1.3 Alkaliphiles

Over the years, an increasing number of alkaliphilic microorganisms and related enzymes have been extensively investigated and exploited for industrial applications. Various definitions for the alkaliphiles have been used. Generally this term is applied to the microorganisms that grow optimally at pH $\geq$ 9.0–9.5. The bacteria that have their optimum growth at pH 7.0, and that can also tolerate pH 9.0 but cannot thrive at pH higher than 10.0, are defined alkali-tolerant. The extreme alkaliphiles can be further subdivided into facultative alkaliphiles, which show optimal growth at 10.0 or above but can grow well at neutral pH, and obligate alkaliphiles that optimally growth at pH $\geq$ 10.0 but not below 8.5–9.0.

A number of cellulolytic and xylanolytic microorganisms that thrive at high pH have been isolated from a variety of natural environments such as geothermal areas, carbonate laden soils, soda deserts and soda lakes. (Hemi)cellulolytic bacteria growing at pH $>$ 6.5 have also been isolated from additional sources such as kraft pulp, pulp and paper industry wastes, decomposing organic matter, insect intestinal tract, plant sources, soils, and even from neutral environments where they are found coexisting with neutrophilic microorganisms.

The group of the alkaliphiles exhibit a wide taxonomical diversity ranging from eubacteria belonging to genera *Bacillus, Micrococcus*, *Pseudomonas*, and *Streptomyces* to archaea such as *Natronobacterium* spp.. A huge number of xylanolytic and cellulolytic alkaliphiles known so far belongs to *Bacillus* spp and *Bacillus*-like genera (Subramaniyan and Prema 2000). These bacteria were also isolated from neutral soils but numerous species come from more alkaline environments. The occurrence of alkaliphiles in conventional ecosystems represents a peculiarity of this group of microorganisms with respect to hyperthermophiles and psychrophiles

adapted and distributed predominantly in selected environments. Presumably, alkaline microenvironments in the soil allow the growth of these extremophiles (Horikoshi 2006).

Several (hemi)cellulolytic bacteria belonging to *Bacillus* sp. (Horikoshi et al. 1984; Shikata et al. 1990; Nakamura et al. 1993; Blanco et al. 1995; Ito 1997; Subramaniyan et al. 1997; Takahashi et al. 2000; Kim et al. 2005), *Gracilibacillus* sp.strain TSCPVG (Giridhar and Chandra 2010), and *B. sphaericus* JS1 (Singh et al. 2004), have been isolated from soil. Generally, most of the alkaliphilic *Bacillus* spp. are facultative alkaliphiles and are distributed in a wider range of soil types compared with obligate alkaliphilic one. For example, the *Paenibacillus* strain Q8 isolated from the acid mine drainage of Carnoulès (France) is even able to grow under both acidic (pH 5.0) and alkaline (pH 9.0) conditions. Function-based screening of a Q8 DNA-library allowed the detection of three clones with xylan-degrading activity that was confirmed and measured in the crude extracts of Q8 grown in liquid medium (Delavat et al. 2012).

The majority of the truly alkalophilic species have been isolated from specific environments such as alkaline soda lakes characterized by the most stable conditions (e.g., pH > 11.5 and sodium bicarbonate/carbonate, and chloride concentrations ranging from about 5 to >15 % w/v). There, the surrounding flora is the possible source of allochthonous cellulose. The majority of the alkaliphiles isolated herein grows in the presence of high salinity and are among the best sources of enzymes with good potential for application in bioethanol production processes. Due to the high levels of halophilicity, halostability and, in several cases, thermostability, these biocatalysts can be added to plant biomasses directly after different types of pretreatment. Thus, the need for costly pH and temperature readjustment or solvent removal before the saccharification process can be completely avoided. Several (hemi)cellulolytic bacteria such as *Amphibacillus xylanus* (Niimura et al. 1987), *Bacillus* sp. (Gessesse and Gashe 1997; Gessesse 1998; Shah et al. 1999; Aygan and Arikan 2008; Roy and Belaluddin 2004; Zhang et al. 2012), *Micrococcus* sp.AR-135 (Gessesse and Mamo 1998), *B. halodurans* S7 (Mamo et al. 2006), *C. alkalicellum* (Zhilina et al. 2005) have been reported.

Another interesting source for alkaliphilic (hemi)cellulolytic bacteria is the intestinal tract of herbivorous insects. Here, different microorganisms carry on the degradation of the biomass facilitating insect phytophagy. Different parts of the intestinal tract show different pHs ranging from slightly acidic values in the foregut to neutral values in the midgut. However, in most larvae midgut high alkaline pH of 10–12 are detected. As reported by Anand et al. (2009) several xylanolytic and cellulolytic isolates were obtained from the digestive tract of *Bombix mori* larvae. In particular, isolates of *Aeromonas* sp, *B. circulans*, *Citrobacter freundii*, *Serratia liquefaciens*, *Proteus vulgaris*, *Klebsiella pneumoniae* and *Enterobacter* sp. were endowed with cellulase activities being able to digest both amorphous and crystalline cellulose while *B. circulans, Aeromonas sp.* and *S. liquefaciens* also showed xylanase activity. Moreover, species of the genus *Micrococcus* have been found in termites and have been reported to display CMCase and xylanase activity (Saxena et al. 1991). Species of the genera *Bacillus*

are predominant in the termite gut contents according to Wenzel et al. (2002) where *Comamonas* species are also found (Kudo 2009). *Micrococcus paraoxydans* has been isolated from the mosquito *Culex quinquefasciatus* midgut (Pidiyar et al. 2004). *Pseudomonas* spp isolates were found in the gut of *Holotrichia parallela* larvae (Huang et al. 2012) and of the ground beetle *Poecilus chalcites* (Lehman et al. 2008). Finally, the (hemi)cellulolytic *Promicromonospora pachnodae*, which can produce xylanase and endoglucanase activities under both aerobic and anaerobic conditions, was recovered from the hindgut of *Pachnoda marginata* (Cazemier et al. 2003).

Numerous (hemi)cellulolytic alkaliphiles that are able to utilize several lignocellulosic biomasses including agro-residues as carbon sources, have also been isolated from other various environments. As reported by Sanghi et al. (2010) the xylanolytic *B. subtilis* ASH 7414 isolated in enrichments from soil sample at pH 7.0–11.0 can utilize wheat bran as carbon source. This strain was exploited for the production of high levels of xylanase (8,964 U/g dry wheat bran) in solid state fermentation. High titer of xylanase were also reached by *B. pumilus* SV-85S, isolated from soil at moderate alkaline pHs and grown under submerged fermentation (SmF). Substrates such as wheat bran, rice straw, wheat straw, soybean flakes, rice bran, sugarcane bagasse, saw dust, ground nut shells, have been found to support the bacterial growth and xylanase production with the highest enzyme amounts obtained with wheat bran, probably beacause of its high xylan content (Nagar et al. 2010). The alkalo-thermo-anaero-bacterium, *Tepidimicrobium xylanilyticum* strain BT14, isolated from soil, has been reported to grow on corn hulls at pH 9.0 and 60 °C under anaerobic conditions (Paripok et al. 2010). Saccharification experiments on pretreated plant biomasses carried out with crude extracts of this bacterium, revealed a marked release of reducing sugars from substrates such as corn cob, rice straw, rice bran, and sugarcane bagasse. These cells also bind to Avicel, xylan, and corn hull and produce a cellulolytic and xylanolytic enzyme complex. Evidence of a cohesin-like domain sequences in the genome of *T. xylanilyticum* BT14 could indicate the presence of a cellulosome. Finally, the ability of the strain to grown on corn hull in anaerobic conditions at pH 9.0 and 60 °C, to produce ethanol and acetate, makes the microorganism exploitable for bioethanol production.

6.2 Extremophilic Enzymes

Hyperthermophilic enzymes are produced naturally by microbes living at temperatures higher than 80 °C. Because extreme heat and very low pH in the current processes for the pretreatment of lignocellulose are known to produce toxic by-products such as furfural (Heer et al. 2009; Mamman et al. 2008), adjustment to less extreme conditions with supplementation of a thermophilic and acidophilic enzyme may be suitable for improving the current pretreatment processes for ethanol production (Miller and Blum 2010). This paragraph summarizes the

state-of-the-art on extremozymes that mediate saccharification of cellulose and hemicellulose of relevance to biomass processing. In particular, 'primary' cellulolytic enzymes are defined as those that contain a catalytic domain and carbohydrate-binding domains (CBM), while 'secondary' enzymes lack a carbohydrate binding domain and/or multiple catalytic domains (Blumer-Schuette et al. 2010). Typically, hyperthermophiles have secondary cellulolytic enzymes whose optimum temperatures and thermal stability are superior to enzymes from thermophilic microorganisms. This is important for robust technological processes for the degradation of polysaccharides and, therefore, attracted interest.

6.2.1 Cellulases

Enzymatic deconstruction of crystalline cellulose can be achieved by three activities: cellobiohydrolase (exocellulase, E.C. 3.2.1.91 and E.C. 3.2.1.−), endoglucanase (endocellulase, E.C. 3.2.1.4), both acting on cellulose, and β-glucosidase (E.C. 3.2.1.21). A short survey of enzymes involved in the cellulose hydrolysis is reported in Table 6.2.

Cellobiohydrolases catalyse the release of cellobiose from either the non-reducing end or the reducing end of cellulose, depending on the enzyme. They belong to GH (glycoside hydrolase) families 5, 6, 7, 9 and 48. A hyperthermophilic cellobiohydrolase was isolated from *Thermotoga* sp. strain FjSS3-B.1 with an optimal temperature for its activity of 105 °C and an half-life of 70 min at 108 °C, making it one of the most thermostable cellobiohydrolases currently known (Ruttersmith and Daniel 1991).

Endoglucanases catalyse the endohydrolysis of (1,4)-β-D-glucosidic linkages in cellulose. They belong to families GH 5, 10, 12, 16, 18, 19, 26, 44, 45, 48, 51, 74 and 124. Several thermophilic bacteria contain 'free-acting' endoglucanases that are not part of a cellulosome complex (for a review see Blumer-Schuette et al. 2008). These include *C. saccharolyticus* (Rainey et al. 1994) and one of the most thermophilic cellulose-degrading organism known to date, *A. thermophilum* (Svetlichnyi et al. 1990). The cellulases of these two hyperthermophiles are multi-domain and multi-functional (VanFossen et al. 2008; Gibbs et al. 2000). They contain CBMs of different families, often duplicated, and in some cases two catalytic domains of different function and/or activity. For example, the *C. saccharolyticus* genome encodes a putative bifunctional cellulase CelB (Csac1078), which is composed of a N-terminal endoglucanase catalytic domain of family GH10, a triplet of CBM3, and a C-terminal exocellulase catalytic domain of family GH5. CelA of the same organism (Csac1076) has a similar domain arrangement: a GH9 endocellulase domain, a triplet of CBM3s, and a GH48 exocellulase (Te'o et al. 1995).

The genomes of many hyperthermophilic microorganisms encode enzymes that are, or appear to be, related to cellulose conversion, but most lack CBMs and/or multiple catalytic domains. For example, the bacterium *T. maritima,* although not growing on cellulose, shows in its genome several β-1,4-glucanases (Cel5A,

Table 6.2 Survey of thermophilic enzymes involved in the hydrolysis of cellulose

Organism	Enzyme	Name	GH family	EC number	Temp opt (°C)	pH opt.	References
C. saccharolyticus	Cellulase	CelB	GH10/GH5	3.2.1.4	80	4.7–5.5	Te'o et al. (1995)
	Cellulase	CelA	GH9/GH48	3.2.1.4	80	6.0	Te'o et al. (1995)
P. furiosus	endo-1.4-glucanase	EglA	GH12	3.2.1.4	100	7.0	Bauer et al. (1999)
	β-glicosidase	CelB	GH1	3.2.1.4	102–105	5.0	Pouwels et al. (2000)
T. maritima	Exoglucanase	cellulose II	n.d.	3.2.1.91	95	6.0–7.5	Bronnenmeier et al. (1995)
	β-1.4-glucanase	Cel5A	GH5	3.2.1.4	80	6.5	Bauer et al. (1999)
S. solfataricus	Endoglucanase	CelS-SSO2534	GH12	3.2.1.4	65	5.8	Limauro et al. (2001)
	Endoglucanase	SSO1949	GH12	3.2.1.4	80	1.8	Huang et al. (2005)
	β-glycosidase	LacS	GH1	3.2.1.21	95	6.5	Pouwels et al. (2000)
	aryl-β-glucosidase/ β-xylosidase	SSO1353	GH116	3.2.1.21	65	5.5	Cobucci-Ponzano et al. (2010)
A. acidocaldarius	Endoglucanase	Cel9A	GH9	3.2.1.4	70	5.5	Eckert et al. (2002)
	Endoglucanase	CelB	GH51	3.2.1.4	80	4	Eckert and Schneider (2003)
A. thermophilum	Endoglucanase	CelA	GH9/GH48	3.2.1.4	85–95	5–6	Zverlov et al. (1998)

Cel5B, Cel12A, Cel12B) that lack CBMs. Similarly, the archaeon *P. furiosus*, which grows optimally near 100 °C but does not grow on cellulose or xylan, contains an endo-1,4-glucanase (EglA) which is mostly active on cellooligosaccharides and CMC but not on insoluble cellulose (Bauer et al. 1999). Also the archaeon *S. solfataricus* possesses genes for the degradation of *β*-linked polysaccharides belonging to GH12 that have been characterized as endoglucanases. CelS (ORF SSO2534) was similar to CelB from *Thermotoga* species and EglA from *P. furiosus* (Limauro et al. 2001). SSO1949, which is similar to the GH12 enzyme Cel12A from *R. marinus* (Crennell et al. 2002; Huang et al. 2005), was extremely acidophilic and thermophilic, with optimum activity at pH 1.8 and T 80 °C. The adaptation of SSO1949 to hot and acidic environment, makes it a good candidate for the exploitation in the pretreatment of lignocellulosic biomass.

An exoglucanase, called cellulase II, was also reported from *T. maritima*. The enzyme has maximal activity at 95 °C, with a half-life of 30 min at that temperature and acted on crystalline cellulose (Bronnenmeier et al. 1995). Among thermophilic endoglucananses, Cel9A from *A. acidocaldarius* (GH9), has an optimum temperature of 70 °C at pH 5.5 and its primary role seems to be the degradation of short, soluble oligosaccharides imported into the cell (Eckert et al. 2002). In addition CelB, also from *A. acidocaldarius,* was expressed during growth on oat spelt xylan, birchwood xylan, CMC and cellobiose and is a cell-associated enzyme (Eckert and Schneider 2003). CelB had an optimal pH of 4.0, an optimal temperature of 80 °C and maintained its stability from pH 1.0 to 7.0. This endoglucanase, together with CelF from *Fibrobacter succinogenes* (Eckert and Schneider 2003), belongs to GH51, which actually consists largely of α-L-arabinofuranosidases (EC 3.2.1.55) (for more details see Miller and Blum 2010).

As described above, the order *Clostridiales* utilize crystalline cellulose, as well as hemicellulose, as growth substrates. The thermostable cellulase CelA from *A. thermophilum* contains an N-terminal GH9 domain, a triplet of CBMs and a C-terminal GH48 domain (Zverlov et al. 1998). Multiple CBMs and both endo- and exoacting domains are required for the efficient hydrolysis of the crystalline substrate (for a review see Blumer-Schuette et al. 2008).

β-Glucosidases complete the degradation of cellulose by acting on soluble cello-oligosaccharides from the terminal non-reducing *β*-D-glucosyl residues. These widespread enzymes belong to families GH 1, 3, 5, 9, 30 and 116. Two hyperthermophilic *β*-glucosidases, belonging to family GH1, from *P. furiosus* and *S. solfataricus* were reported, namely CelB and LacS, respectively. CelB shows an optimal temperature of 102–105 °C and an optimal pH of 5.0, instead LacS shows an optimal temperature of 95 °C and an optimal pH of 6.5 (Pouwels et al. 2000).

Recently, in *S. solfataricus* an aryl-*β*-glucosidase/*β*-xylosidase (ORF SSO1353), that was grouped in the new GH116 family, was identified and characterized (Cobucci-Ponzano et al. 2010). The *sso1353* gene lies downstream of genes encoding endoglucanases, and, in *S. solfataricus*, this gene arrangement occurs twice. Presumably, this *β*-glycosidase activity is involved, in combination with the secreted endoglucanase, in the degradation of exogenous glucans used as carbon energy source as mentioned above (Cobucci-Ponzano et al. 2010).

6.2.2 Hemicellulases

Xylans are the most abundant class of hemicelluloses, with glucuronoarabinoxylan being the main target for enzymatic saccharification for renewable bio-feedstock production. Glucuronoarabinoxylan (e.g. from corn stover) is composed of a β-(1,4)-linked D-xylose polymer backbone (xylan) with L-arabinose and glucuronic acid side chains (Templeton et al. 2010). Extensive acetylation may occur and the L-arabinose side chains can be esterified with ferulic acid. Heterogeneity of glucuronoarabinoxylan requires six distinct enzyme activities for complete saccharification: endoxylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), α-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.131), acetylxylan esterase (EC 3.1.1.72) and ferulic acid esterase (EC 3.1.1.73). The complete description of these enzymatic activities goes beyond the aims of this book (for a review see Jordan et al. 2012), here, a short survey of enzymes from thermophilic source involved in the degradation of several hemicelluloses is reported in Table 6.3.

Many industrially relevant endoxylanase genes have been cloned from a wide array of bacteria (for a review see Collins et al. 2005). The majority of these enzymes are classified into GH10 and GH11 families. Of the extremophilic xylanases, the thermophilic, alkaliphilic and acidophilic ones have been extensively studied, while cold-adapted xylanases have been much less investigated.

A family 10 xylanase, XynA from *Thermotoga* sp. is one of the most thermostable xylanases reported to date with an apparent optimum temperature for activity of 105 °C (Simpson et al. 1991). While less frequent, GH11 thermophilic xylanases have also been isolated, with those from *Caldicellulosiruptor* sp. Rt69B.1, *Dictyoglomus thermophilum*, and *Bacillus* strain D3 being the most thoroughly investigated. In addition a number of xylanases producing hyperthermophilic archaea have also been recently reported, including *Thermococcus zilligii*, *P. furiosus*, *Pyrodictium abyssi* and a number of *Thermofilum* strains (for a review see Collins et al. 2005 and references therein). A xylanase activity (ORF SSO1354) was isolated from a strain of *S. solfataricus* capable of utilizing oat spelt xylan as the sole carbon source (Cannio et al. 2004; Maurelli et al. 2008; Girfoglio et al. 2012). The enzyme is most active at 95 °C at pH 4.0, with a half-life of 53 min at that temperature making it suitable, like SSO1949 mentioned above, to contribute toward glucose production from lignocellulosic biomass in the bioethanol industry.

Even though cold-temperature environments are the most abundant on earth, only a small number of psychrophilic xylanases have been identified in bacteria, such as *Pseudoalteromonas haloplanktis* TAH3a, *Flavobacterium frigidarium* sp. nov. and *Clostridium* strain PXYL1 (see Collins et al. 2005). The first report of a xylanase produced by an alkaliphilic microorganism was as early as 1973 for a xylanase from *Bacillus* sp. C-59-2 (Horikoshi and Atsukawa 1973). Since this initial finding a number of xylanases have been isolated from various acidophilic and alkaliphilic microorganisms, including GH10 and GH11 xylanases from a

Table 6.3 Survey of thermophilic (hemi)cellulolytic enzymes

Organism	Enzyme	EC number	Name	GH family	Temp opt (°C)	pH opt.	References
Bacillus sp. AR-009	Xylanase	3.2.1.8	XylB	10	70–75	9.0–10.0	Gessesse (1998)
C. cellulovorans	Xylanase	3.2.1.8	XynA	10	70	6.0	Sunna et al. (2000)
G.s stearothermophilus	Xylan 1,4-*β*-D-xylosidase	3.2.1.8		3	70	6.0	Nanmori et al. (1990)
G. stearothermophilus T-6	Xylanase	3.2.1.8	XynA	10	75	6.5	Khasin et al. (1993)
	α-D-glucuronidase	3.2.1.139	AguA	67	65	5.5	Zaide et al. (2001)
S. solfataricus	Xylanase	3.2.1.8	SSO1354	10	95	4.0	Cannio et al. (2004)
	β-D-xylosidase/ α-L-arabinosidase	3.2.1.37 3.2.1.55	XarS	3	80	6.5	Morana et al. (2007)
T. ethanolicus	*β*-D-xylosidase/ α-L-arabinosidase	3.2.1.37 3.2.1.55	XarB	3	93	5.8-6.0	Shao and Wiegel (1992)
T.maritima	Xylanase	3.2.1.8	XynA	10	105	6.2	Simpson et al. (1991)
	Xylan 1,4-*β*-D-xylosidase	3.2.1.37		3	95	6.1	Xue and Shao (2004)
	α-D-glucuronidase	3.2.1.131 3.2.1.139	AguA	67	85	6.3	Ruile et al. (1997)

number of *Bacillus* sp. and *Acidobacterium* sp. Quite unexpectedly, many of the xylanases from alkaliphiles showed pH optima in the near neutral region, but relatively high activity was retained in alkaline conditions. One the most alkaliphilic xylanases reported to date is XylB from *Bacillus* sp. AR-009, which has a pH optimum of pH 9.0–10.0 (Collins et al. 2005).

Xylan 1,4-*β*-D-xylosidases (EC 3.2.1.37), catalysing the hydrolysis of single D-xylose units from the non-reducing end of xylo-oligosaccharides, have been classified in CAZy under families GH1, 3, 30, 39, 43, 51, 52, 54, 116 and 120. At present, the preponderance of characterized extremophilic *β*-xylosidases belongs to GH43; instead, α-arabinofuranosidases (EC 3.2.1.55) classified in CAZy under GH3, 43, 51, 54 and 62, catalyse the hydrolysis of terminal non-reducing L-arabinose side chains from the xylan backbone, such as the GH43 enzyme from *R. marinus* (Gomes et al. 2000). Interestingly, the gene *xarS* (ORF SSO3032) from *S. solfataricus*, belonging to GH3, encodes a bifunctional enzyme with both *β*-D-xylosidase and α-L-arabinosidase activities. The optimal conditions for both activities are 80 °C and pH 6.5. Oat spelt xylan that was converted to xylobiose and xylotriose by *S. solfataricus* SSO1354 xylanase (see above) was further converted to xylose after addition of XarS (Morana et al. 2007).

α-Glucuronidases (EC 3.2.1.131), hydrolysing the 1,2-linked glucuronosyl side chains from xylan (de Wet and Prior 2004), are categorized as GH67 and remove only the glucuronosyl group that is attached to the terminal residue at the non-reducing end of xylo-oligosaccharides. The α-glucuronidase from *T. maritima* is the enzyme with the highest reported temperature optimum (85 °C) (Ruile et al. 1997).

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