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Xylanases, xylanase families and extremophilic xylanases

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Abstract

Xylanases are hydrolytic enzymes which randomly cleave the β 1,4 backbone of the complex plant cell wall polysaccharide xylan. Diverse forms of these enzymes exist, displaying varying folds, mechanisms of action, substrate specificities, hydrolytic activities (yields, rates and products) and physicochemical characteristics. Research has mainly focused on only two of the xylanase containing glycoside hydrolase families, namely families 10 and 11, yet enzymes with xylanase activity belonging to families 5, 7, 8 and 43 have also been identified and studied, albeit to a lesser extent. Driven by industrial demands for enzymes that can operate under process conditions, a number of extremophilic xylanases have been isolated, in particular those from thermophiles, alkaliphiles and acidiphiles, while little attention has been paid to cold-adapted xylanases. Here, the diverse physicochemical and functional characteristics, as well as the folds and mechanisms of action of all six xylanase containing families will be discussed. The adaptation strategies of the extremophilic xylanases isolated to date and the potential industrial applications of these enzymes will also be presented.

Keywords: Xylanase; Xylanase families; Extremophilic xylanases; Thermophilic xylanases; Cold-adapted xylanases; Xylanase applications

Contents

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1. Introduction

Xylanases are glycosidases (*O*-glycoside hydrolases, EC 3.2.1.x) which catalyze the endohydrolysis of 1,4-β-D-xylosidic linkages in xylan. They are a widespread group of enzymes, involved in the production of xylose, a primary carbon source for cell metabolism and in plant cell infection by plant pathogens, and are produced by a plethora of organisms including bacteria, algae, fungi, protozoa, gastropods and anthropods [1]. First reported in 1955 [2], they were originally termed pentosanases, and were recognized by the International Union of Biochemistry and Molecular Biology (IUBMB) in 1961 when they were assigned the enzyme code EC 3.2.1.8. Their official name is endo-1,4-β-xylanase, but commonly used synonymous terms include xylanase, endoxylanase, 1,4-β-D-xylan-xylanohydrolase, endo-1,4-β-D-xylanase, β-1,4-xylanase and β-xylanase.

In the present review, the diversity of xylanases, their substrate, action and function, their importance in industry, classification into families and adaptation to various extreme environments are discussed. Special emphasis is paid to the 'new' xylanase containing families, highlighting their similarities and differences to the better known family 10 and 11 members, as well as to the peculiarities and interests of these hitherto scantily reviewed enzymes. In addition, the adaptation strategies, characteristics and industrial potential of extremophilic xylanases will be discussed.

2. The substrate: xylan

The substrate of xylanases, xylan, is a major structural polysaccharide in plant cells, and is the second most abundant polysaccharide in nature, accounting for approximately one-third of all renewable organic carbon on earth [1]. Xylan constitutes the major component of hemicellulose; a complex of polymeric carbohydrates including xylan, xyloglucan (heteropolymer of D-xylose and D-glucose), glucomannan (heteropolymer of D-glucose and D-mannose), galactoglucomannan (heteropolymer of D-galactose, D-glucose and D-mannose) and arabinogalactan (heteropolymer of D-galactose and arabinose) [3]. This, together with cellulose (1,4-β-glucan) and lignin (a complex polyphenolic compound) make up the major polymeric constituents of plant cell walls [4]. Within the cell wall structure, all three constituents interact via covalent and non-covalent linkages, with the xylan being found at the interface between the lignin and cellulose where it is believed to be important for fiber cohesion and plant cell wall integrity [5].

Xylan is found in large quantities in hardwoods from angiosperms (15–30% of the cell wall content) and softwoods from gymnosperms (7–10%), as well as in annual plants (<30%) [6]. It is typically located in the secondary cell wall of plants, but is also found in the primary cell wall, in particular in monocots [7]. A complex, highly branched heteropolysaccharide, it varies in structure between different plant species, and the homopolymeric backbone chain of 1,4-linked β-D-xylopyranosyl units can be substituted to varying degrees with glucuronopyranosyl, 4-*O*-methyl-D-glucuronopyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl and/or *p*-coumaroyl side-chain groups [4,8] (Fig. 1). Wood xylan exists as *O*-acetyl-4-*O*-methylglucuronoxylan in hardwoods and as arabino-4-*O*-methylglucuronoxylan in softwoods, while xylans in grasses and annual plants are typically arabinoxylans [4]. Linear unsubstituted xylan has also been reported, e.g., in esparto grass [9], tobacco [10] and certain marine algae [11,12], with the latter containing xylopyranosyl residues linked by both 1,3-β and 1,4-β linkages [12,13]. The degree of polymerisation in xylans is also variable, with, for example, hardwood and softwood xylans generally consisting of 150–200 and 70–130 β-xylopyranose residues, respectively [4].

Due to its heterogeneity and complexity, the complete hydrolysis of xylan requires a large variety of cooperatively acting enzymes [14–16] (Fig. 1). Endo-1,4-β-D-xylanases (EC 3.2.1.8) randomly cleave the xylan backbone, β-D-xylosidases (EC 3.2.1.37) cleave xylose monomers from the non-reducing end of xylo-oligosaccharides and xylobiose while removal of the side groups is catalysed by α-L-arabinofuranosidases (EC 3.2.1.55), α-D-glucuronidases (EC 3.2.1.139), acetylxylan esterases (EC 3.1.1.72), ferulic acid esterases (EC 3.1.1.73) and *p*-coumaric acid esterases (EC 3.1.1.-). Indeed, complete xylanolytic enzyme systems, including all of these activities, have been found to be quite widespread among fungi [17,18], actinomycetes [19] and bacteria [18], and some of the most important xylanolytic enzyme producers include the Aspergilli, Trichodermi, Streptomycetes, Phanerochaetes, Chytridiomycetes, Ruminococci, Fibrobacteres, Clostridia and Bacilli [16,18,20,21]. The ecological niches of these micro-organisms are diverse and widespread and typically

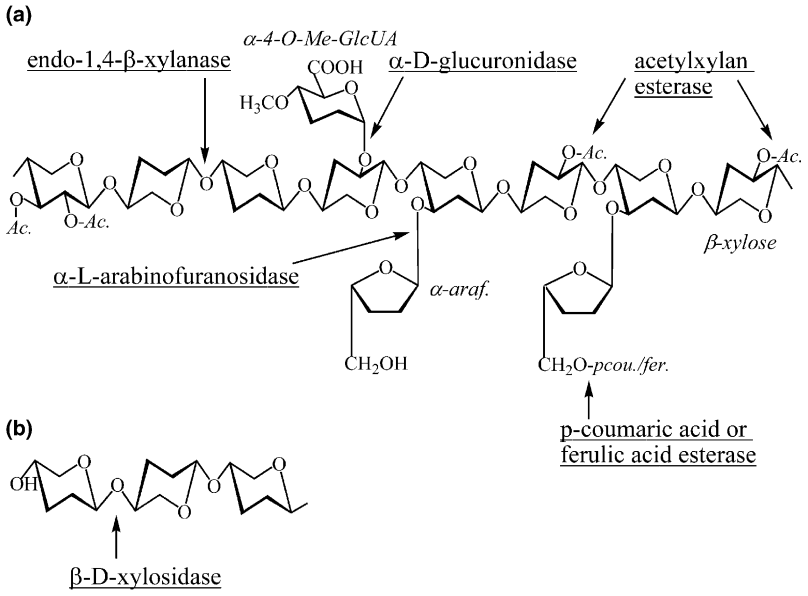

Fig. 1. (a) Structure of xylan and the sites of its attack by xylanolytic enzymes. The backbone of the substrate is composed of 1,4- β-linked xylose residues. Ac., Acetyl group; α-araf., α-arabinofuranose; α-4-*O*-Me-GlcUA, α-4-*O*-methylglucuronic acid; pcou., *p*-coumaric acid; fer., ferulic acid. (b) Hydrolysis of xylo-oligosaccharide by β-xylosidase. Adapted from [18]. Figures were prepared with CS Chemdraw Ultra version 6.0.

include environments where plant material accumulate and deteriorate, as well as in the rumen of ruminants [1,16,22].

3. Xylanases: multiplicity and multiple-domains

In addition to the production of a variety of xylanolytic enzymes, many micro-organisms produce multiple xylanases [23–25]. These may have diverse physicochemical properties, structures, specific activities and yields, as well as overlapping but dissimilar specificities, thereby increasing the efficiency and extent of hydrolysis, but also the diversity and complexity of the enzymes. Typical examples of micro-organisms which produce xylanase isoenzymes include *Aspergillus niger*, which produces fifteen extracellular xylanases [26], and *Trichoderma viride*, which secretes thirteen [26]. This multiplicity may be the result of genetic redundancy [7], but cases of differential post-translational processing have also been reported [15]. The isoenzyme genes may be found as polycistronic or non-polycistronic multiple copies within the genome, and in some cases several xylanases are expressed as a distinct gene product. For example, the xylanase, β-xylosidase and acetyl esterase genes of *Caldocellum saccharolyticum* (now known as *Caldocellulosiruptor saccharolyticus*) are polycistronic [27], while the XynC gene product from *Fibrobacter succinogenes* S85 encodes two different xylanase catalytic domains [28]. Moreover, as well as multiple catalytic domains, many xylanases are also characterized by the presence of various supplementary domains. Examples include xylan binding domains [29], cellulose binding domains [30,31], dockerin domains (implicated in binding to multidomain complexes produced by certain micro-organism, e.g., *Clostridium thermocellum*) [32,33], thermostabilising domains [34] and domains for which the function has not as yet been elucidated. These domains may fold and function in an independent manner [35,36] and are typically separated by short junction segments enriched in hydroxyl amino acids [4].

The vast majority of xylanases are excreted into the extracellular environment as the large size of the substrate prevents its penetration into the cell. In fact, the current belief is that xylanase production is induced by means of the products of their own action [6,15,37]. It is believed that small amounts of constitutively produced enzymes liberate xylo-oligomers which may be transported into the cell where they are further degraded by β-xylosidases, or indeed by intracellular xylanases [38–40], and where they induce further xylanase synthesis.

4. Classification of xylanases

The heterogeneity and complexity of xylan has resulted in an abundance of diverse xylanases with varying specificities, primary sequences and folds, and hence has lead to limitations with the classification of these enzymes by substrate specificity alone. Wong et al. [7] classified xylanases on the basis of their physicochemical properties and proposed two groups: those with a low molecular weight (<30 kDa) and basic p*I*, and those

with a high molecular weight (>30 kDa) and acidic p*I*. However, several exceptions to this pattern have been found [18,21] and approximately 30% of presently identified xylanases, in particular fungal xylanases, cannot be classified by this system.

Later, a more complete classification system was introduced [41] which allowed the classification of not only xylanases, but glycosidases in general (EC 3.2.1.x), and which has now become the standard means for the classification of these enzymes. This system is based on primary structure comparisons of the catalytic domains only and groups enzymes in families of related sequences [42]. The initial classification grouped cellulases and xylanases into 6 families (A–F) [41], which was updated to 77 families in 1999 (1–77) [42] and which continues to grow as new glycosidase sequences are identified. At the time of writing, 96 glycoside hydrolase families exist (see the carbohydrate–active enzyme CAZY server at http://afmb.cnrs-mrs.fr/~cazy/CAZY/ [43]), with approximately one-third of these families being polyspecific, i.e., contain enzymes with diverse substrate specificities. As the structure and molecular mechanism of an enzyme are related to its primary structure, this classification system reflects both structural and mechanistic features. Enzymes within a particular family have a similar three-dimensional structure [42] and similar molecular mechanism [44] and it has also been suggested that they may have a similar specificity of action on small, soluble, synthetic substrates [45]. Furthermore, divergent evolution has resulted in some of the families having related three-dimensional structures and thus the grouping of families into higher hierarchical levels, known as clans, has been introduced [46]. Presently, 14 different clans have been proposed (GH-A to GH-N), with most clans encompassing two to three families, apart from clan GH-A which currently encompasses 17 families.

Within this classification system, xylanases are normally reported as being confined to families 10 (formerly F) and 11 (formerly G) [6,16,18,47–49]. Interestingly, a search of the appropriate databases (e.g., CAZY [43]) using the enzyme classification number EC 3.2.1.8 indicates that enzymes with xylanase activity are also found in families 5, 7, 8, 16, 26, 43, 52 and 62. A closer look at the available literature however, shows that only those sequences classified in families 5, 7, 8, 10, 11 and 43 (Table 1) contain truly distinct catalytic domains with a demonstrated endo-1,4-β-xylanase activity. Those sequences reported for families 16, 52 and 62 appear, in fact, to be bifunctional enzymes containing two catalytic domains; a family 10 or 11 xylanase domain as well as a second glycosidase domain. For example, a *Ruminococcus flavefaciens* enzyme contains an amino-terminal family 11 xylanase and a carboxy-terminal family 16 lichenase and is thus classified in both families 11 and 16 [50]. In addition, those enzymes classified in family 26 appear not to be endo-1,4-β-xylanases, but endo-1,3-β-xylanases. Thus, the current view that enzymes with xylanase activity are solely restricted to families 10 and 11 is not entirely correct and should be expanded to include families 5, 7, 8 and 43.

5. Glycoside hydrolase families 5, 7, 8, 10, 11 and 43

5.1. Catalytic mechanisms

Members of families 5, 7, 8, 10, 11 and 43 differ in their physico-chemical properties, structure, mode of action and substrate specificities. Similarities do however exist, for example, families 5 and 10 are both classified in clan GH-A, thus indicating a similar three-dimensional fold. Furthermore, families 5, 7, 10 and 11 contain enzymes which catalyse hydrolysis with retention of anomeric configuration with two glutamate residues being implicated in the catalytic mechanism in all cases [43]. This indicates a double-displacement mechanism, in which a covalent glycosyl-enzyme intermediate is formed and subsequently hydrolysed via oxocarbenium-ion-like transition states [51–53] (Fig. 2(a)). Two carboxylic acid residues suitably located in the active site (approximately 5.5 Å apart) are involved in the formation of the intermediate; one acts as a general acid catalyst by protonating the substrate, while the second

Table 1
Glycoside hydrolase families containing enzymes with a demonstrated activity on xylan. The fold, mechanism of action and catalytic residues characteristic to each family are given

Glycoside hydrolase family	Members with a demonstrated activity on xylan	Fold	Clan	Catalytic mechanism	General acid/base residue	Nucleophile/general base
5	8	$(\beta/\alpha)_8$	GH-A	Retaining	Glutamate	Glutamate
7	1	β-Jelly roll	GH-B	Retaining	Glutamate	Glutamate
8	4	$(\alpha/\alpha)_6$	GH-M	Inverting	Glutamate[a]	Aspartate[a]
10	127	$(\beta/\alpha)_8$	GH-A	Retaining	Glutamate	Glutamate
11	173	β-Jelly roll	GH-C	Retaining	Glutamate	Glutamate
43	1	5-Blade β-propeller	GH-F	Inverting	Glutamate[a]	Aspartate[a]

[a] Putative catalytic residues only, these have not been conclusively confirmed.

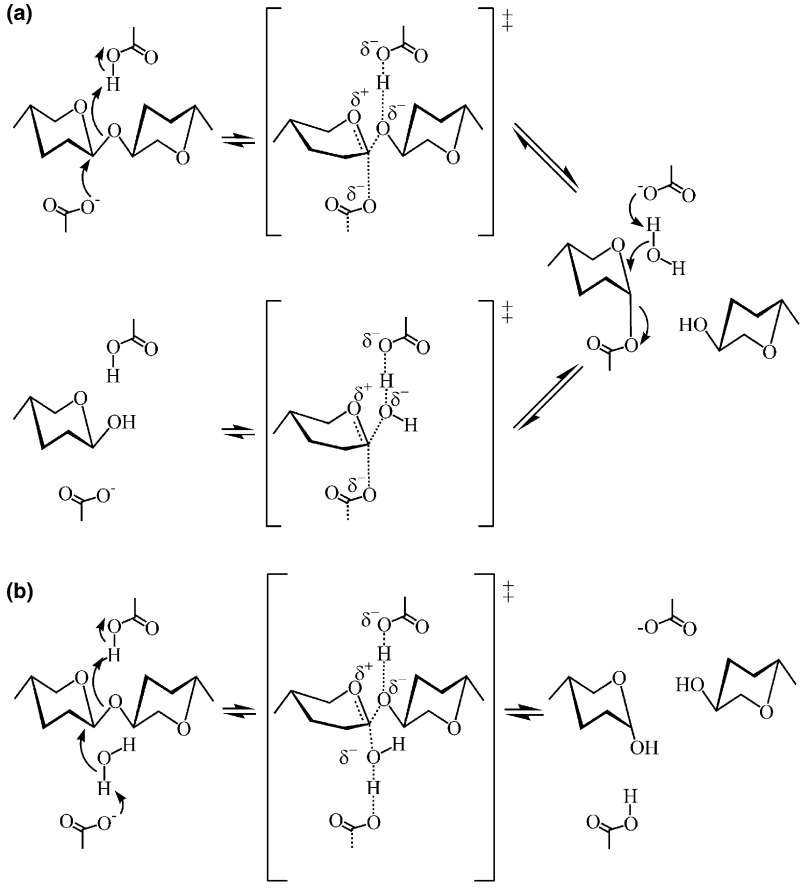

Fig. 2. General mechanisms for (a) retaining and (b) inverting glycosidases. Adapted from [51]. Figures were prepared with CS Chemdraw Ultra version 6.0.

performs a nucleophilic attack which results in the departure of the leaving group and the formation of the α-glycosyl enzyme intermediate (inversion β to α). In the second step, the first carboxylate group now functions as a general base, abstracting a proton from a nucleophilic water molecule which attacks the anomeric carbon. This leads to a second substitution in which the anomeric carbon again passes via an oxocarbenium-ion-like transition state to give rise to a product with the β configuration (inversion α to β). Thus the overall result is a retention of the configuration at the anomeric centre.

In contrast, enzymes in families 8 and 43 typically operate with inversion of the anomeric centre and a glutamate and aspartate are believed to be the catalytic residues [43,54]. Inverting enzymes function by a single displacement reaction (Fig. 2(b)) in which one carboxylate provides for a general acid-catalyzed leaving group departure and the second functions as a general base, activating a nucleophilic water molecule to attack the anomeric carbon, thereby cleaving the glycosidic bond and leading to an inversion of the configuration at the anomeric carbon [51–53]. Typically the distance between the two residues is around 9.5 Å so as to allow for accommodation of the water molecule between the anomeric carbon and the general base [51–53]. However, Alzari et al. [55] and Guérin et al. [56] have shown that this distance is 7.5 Å in the inverting endoglucanase CelA and have suggested that the distance between the two catalytic residues is less constrained in inverting than in retaining enzymes.

5.2. Glycoside hydrolase family 5

At the time of writing, family 5 (formerly family A) of the glycoside hydrolases consists of 467 sequences with varying activities, including: endoglycosylceramidase (EC 3.2.1.123), cellulase (EC 3.2.1.4), licheninase (EC 3.2.1.73), β-mannosidase (EC 3.2.1.25), glucan 1,3-β-glucosidase (EC 3.2.1.58), glucan endo-1,6-β-glucosidase (EC 3.2.1.75), mannan endo-1,4-β-mannosidase (EC 3.2.1.58), cellulose 1,4-β-cellobiosidase (EC 3.2.1.91), endo-1,6-β-galactanase (EC 3.2.1.-), 1,3-β-mannanase (EC 3.2.1.-) and endo-1,4-β-xylanase (EC 3.2.1.8) [43]. This is the largest glycoside hydrolase family and only seven amino acid residues, including the nucleophile and the general acid/base residue, are strictly conserved among all members. It is a rather diverse

Table 2
Organisms producing family 5 enzymes with a demonstrated activity on xylan

Organism	Reference
Prevotella (*Bacteroides*) *ruminocola* 23	[63]
Clostridium cellulovorans	[64]
Fibrobacter succinogenes S85	[65]
Ruminococcus albus SY3	[66]
Trichoderma reesei (*Hypocrea jecorina*)	[67,68]
Erwinia (*Pectobacterium*) *chrysanthemi* D1	[57,70,71]
Erwinia (*Pectobacterium*) *chrysanthemi* SR120A	[57,69,70]
Aeromonas puncata (*caviae*) ME-1	[72]

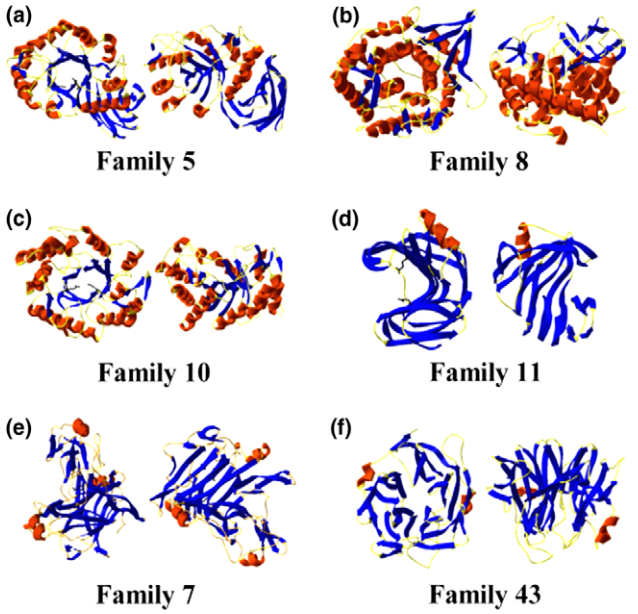

Fig. 3. Representative structures of enzymes from various glycoside hydrolase families. (a) Structure of the family 5 enzyme, XynA, from *Erwinia chrysanthemi*. The $(\beta/\alpha)_8$ barrel structure of the catalytic domain and the β_9-barrel of the small domain are shown [57]. (b) Structure of the family 8 xylanase, pXyl, from *Pseudoalteromonas haloplanktis* TAH3a. The $(\alpha/\alpha)_6$ barrel structure of the catalytic domain is shown in two perpendicular views [82]. (c) Structure of the *Streptomyces lividans* xylanase showing the typical family 10-fold [95]. (d) Structure of the *Trichoderma reesei* family 11 xylanase showing the typical family 11-fold [127]. (e) Structure of the *Trichoderma reesei* family 7 non-specific EGI [131]. (f) Structure of the *Cellvibrio japonicus* family 43 α-L-arabinanase [54]. Figures were prepared with Swiss-Pdb Viewer v3.7b2 [205].

group of enzymes, with structural alignments indicating rms deviations of 1.25 ± 0.12 Å between equivalent residues in its members [57] and a further classification of this family into nine subfamilies has even been suggested [58].

Eight enzymes with a demonstrated activity on xylan have been reported in this family to date (Table 2) and a number of other putative xylanases, identified by sequence similarity during genome sequencing programs, have been identified in *Clostridium acetobutylicum* ATCC 824 (2 putative xylanases) [59], *Leptosphaeria maculans* [43], *Xanthomonas axonopodis pv. citri* str. 306 (2 putative xylanases) [60], *Xanthomonas campestris pv. campestris* str. ATCC 33913 [60], *Bacillus subtilis* str. 168 [61] and *Bacteroides thetaiotaomicron* VPI-5482 [62]. Xylanase activity has also been apparently demonstrated for enzymes from *Aeromonas puncata* (*caviae*) W-61, *Erwinia* (*Pectobacterium*) *chrysanthemi* P860219, *Meloidogyne incognita* and *Ruminococcus albus* 7 [43] but it appears that no literature has been published in direct relation to these. In total, 20 putative xylanolytic entries can be proposed.

Although characterisation of these enzymes is incomplete, large variations in the catalytic properties are already evident, this being in keeping with the poor sequence identity between some of these enzymes. Those enzymes from *Prevotella ruminicola* 23 [63], *Clostridium cellulovorans* [64], *F. succinogenes* S85 [65] and *R. albus* SY3 [66] were all found to have carboxymethyl cellulase as well as xylanase activities, while those from *Trichoderma reesei* (also known as *Hypocrea jecorina*) [67,68], *Erwinia chrysanthemi* D1 [57,69–71] and *E. chrysanthemi* SR120A [69] appear to be specific for xylan. XynD from *Aeromonas punctata* ME-1 was found to be active on xylan but was not apparently tested on other substrates [72]. Furthermore, a search of the InterPRO database (www.ebi.ac.uk/interpro, [73]) indicates that only the four carboxymethyl cellulose active enzymes (i.e., those from *P. ruminicola* 23, *C. cellulovorans*, *F. succinogenes* S85 and *R. albus* SY3) give hits with family 5 signatures; the remaining 16 sequences give hits with family 30 or with other non-glycoside hydrolase family signatures. Moreover, these four enzymes have relatively high amino acid sequence similarity with family 5 members, while the majority of the remaining sequences have homology with both family 5 and family 30 enzymes. This has already been noted for the *E. chrysanthemi* and *A. punctata* ME-1 enzymes where it was suggested that these enzymes be classified into a new family (or subfamily) situated between families 5 and 30 [70,72]. In addition, structural analysis of the family 5 xylanase, XynA from *E. chrysanthemi* showed that, as would be expected for a family 5 enzyme, the catalytic domain displayed a common $(\beta/\alpha)_8$ barrel fold (Fig. 3(a)) [57]. However, while the β-barrels aligned well with those of another family 5 enzyme, the α-helices and loops were altered, showing differences in the positioning, orientation and length. Moreover, structural alignment with a number of family 5 and 10 enzymes showed that this enzyme is nearly as structurally different to the family 5 enzymes as are the family 10 enzymes [57].

Of the four family 5 enzymes exhibiting carboxymethyl cellulase and xylanase activities, only the *P. ruminicola* 23 xylanase was found to have highest activity on xylan, with the carboxymethyl cellulase activity being only 18% of this [63]. In contrast, EngB from *C. cellulovorans* was found to be most active on lichenan, with

approximately 15% activity on carboxymethylcellulose and 14% on xylan. No activity was detected on microcrystalline cellulose, avicel, polygalacturonic acid, mannan, laminaran, *p*-nitrophenyl β-cellobioside, *p*-nitrophenyl β-D-glucoside or *p*-nitrophenyl β-D-xyloside [64]. Unfortunately, as with the *P. ruminicola* 23 xylanase, the endoglucanase isolated from *Fibrobacter succinogenes* S85 was not tested with lichenan, this enzyme was found to have highest activity on carboxymethyl cellulose, as well as activity on oat spelt xylan (71% as compared to CMC), *p*-nitrophenyl cellobioside (5.3%), cellobiose (3.5%), *p*-nitrophenyl lactoside (2.27%) and *p*-nitrophenyl glucoside (0.27%) [65].

The action patterns of the xylan specific family 5 enzymes with sequence similarities to family 30 enzymes also appear to vary from one to another. Only those enzymes from *A. punctata* ME-1 [72], *E. chrysanthemi* D1 [57,70,71] and *T. reesei* [67,68] have been characterized in this respect, and while the first does appear to be an endoxylanase, producing xylotriose and higher xylo-oligosaccharides from birchwood xylan, the second appears to be a type of 'appendage-dependent xylanase' or glucuronoxylanase [74] and the third an exoxylanase. Substrate specificity studies showed that the xylanase from *E. chrysanthemi* D1 requires D-glucuronic acid (or its 4-*O*-methyl derivative) substituents on the xylan backbone to enable cleavage of 1,4-β xylosidic bonds and that it cleaves between the first and second xylose residues on the non-reducing terminal side of the substituted residue [71]. On the other hand, XYN IV from *T. reesei* is active on unsubstituted and substituted xylans and attacks at the first glycosidic linkage from the reducing end, producing mainly xylose, but also xylobiose and low amount of higher xylo-oligosaccharides [67,68]. Furthermore, the activity of this enzyme is affected by substituents on the xylan main chain and it is unable to cleave linkages next to substituted residues. Hydrolysis studies have shown that the shortest substituted fragments formed from glucuronoxylan and arabinoxylan are substituted xylotrioses, with the substitution being found on the internal xylose residue. Indeed these products are shorter than those produced by the family 7 EGI [75] produced by the same micro-organism as well as the family 11 enzymes, but, in agreement with members of this latter family and with the family 5 XynA from *E. chrysanthemi* D1 they contain a single xylose at the non-reducing end.

Of the above mentioned enzymes, only the structure of Xyn A from *E. chrysanthemi* has been determined (1.42 Å resolution) [57] while crystallisation and preliminary X-ray analysis (at 2.2 Å resolution) of the *T. reesei* XYN IV has also recently been reported [76]. Xyn A is composed of two domains, the larger domain contains the catalytic site and displays a $(\beta/\alpha)_8$ barrel fold while the small domain probably functions as a xylan binding domain and has a β_9-barrel fold (Fig. 3(a)). The two domains are connected by two linker peptides as well as 11 hydrogen bonds and hydrophobic interactions [57]. The β-barrel of the catalytic domain of Xyn A is elliptical in shape and the active site is formed by an acidic cleft situated on the carboxy-terminal side of the β-strands near the larger face of the molecule. This 8-fold α/β barrel structure is indeed the most frequently encountered fold and has also been described for members of families 1, 2, 10, 17, 26, 30, 35, 39, 42, 50, 51, 53, 59, 72, 79 and 86 [43]. This type of fold was originally described for triose-phosphate isomerase (TIM barrel) and as a common characteristic of all these structures is the positioning of the glutamate residues implicated in the catalysis on the carboxy-terminal of β-sheets 4 and 7, these were originally termed the 4/7 superfamily. Nowadays they are better known as clan GH-A.

5.3. Glycoside hydrolase family 8

Family 8 (formerly family D) is mainly composed of cellulases (EC 3.2.1.4), but also contains chitosanases (EC 3.2.1.132), lichenases (EC 3.2.1.73) and endo-1,4-β-xylanases (EC 3.2.1.8) [43]. It is a rapidly expanding family; from 18 members, including one xylanase, in 2001, to 61 members, including four xylanases, in 2004 (Table 3). Three of the xylanases have been isolated from *Bacillus* sp. while the fourth is a cold-adapted enzyme isolated from the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAH3a. Xylanase Y from the alkaliphile *Bacillus halodurans* C-125 was identified as part of the sequencing program of the whole genome of this organism [77,78] and the sequence of the xylanase from *B. halodurans* MIR32 is found to be identical to that for this enzyme [43]. Information is minimal and while we have demonstrated xylanase activity by these enzymes [79], little literature has been published in direct relation to these two xylanases. Indeed the means used for their initial classification as xylanases is unclear and was probably only based on their weak isology to the cold-adapted xylanase and xylanase Y from *Bacillus* sp. KK-1. In contrast, the biochemical properties and substrate specificities of these latter two enzymes have been investigated [80–84]. Both have a high molecular weight (~46 and ~45 kDa, respectively) and while the cold-adapted xylanase also has a high p*I* (~pH 9.5), this was not determined for the *Bacillus* sp. KK-1 xylanase.

Table 3
Organisms producing family 8 enzymes with a demonstrated xylanase activity

Organism	Reference
Bacillus halodurans C-125	[77,78]
Bacillus halodurans MIR32	[43]
Bacillus sp. KK-1	[84]
Pseudoalteromonas haloplanktis TAH3a	[79–83]

In addition, both were found to be optimally active at pH 6.5 and are solely active on xylan, being more active on birchwood than oat spelt xylan and being inactive on cellulose, carboxymethylcellulose, starch, lichenan and chitosan (this latter substrate was only tested for the cold-adapted xylanase). The cold-adapted xylanase was found to hydrolyse xylan to principally xylotriose and xylotetraose and was most active on long chain xylo-oligsaccharides. Similar to family 11 xylanases, a large substrate binding cleft containing at least six xylose binding residues, with the catalytic site in the middle, was proposed [80]. However, unlike family 10 and 11 xylanases this enzyme was found to catalyse hydrolysis with inversion of the anomeric configuration and, under the conditions used, was found to be inactive on aryl-β-glycosides of xylose, xylobiose and xylotriose.

This cold-adapted xylanase folds into a distorted $(\alpha/\alpha)_6$ barrel (Fig. 3(b)) formed by six inner and six outer α helices [82,83] and as such can be classified with family 48 in clan GH-M [43]. This topology has also been observed for family 9 endoglucanases, family 15 glucoamylases, family 48 cellobiohydrolases and a family 65 maltose phosphorylase [55,85,86]. However, in contrast to the common $(\alpha/\alpha)_6$ barrel proteins, the cold-adapted enzyme has an extra α-helix near the amino terminus. The globular core has an overall distorted spherical shape with a long acidic cleft running across the molecular surface at the N-terminal end of the inner helices while the proposed catalytic residues (glutamate and aspartate) are located close to each other near the middle of the cleft.

As the three-dimensional structure is believed to be comparable for members of the same family, the $(\alpha/\alpha)_6$ barrel fold described above can probably be extended to the other family 8 xylanases. Furthermore, one would expect these enzymes to operate with inversion of the anomeric configuration [80,87], with an aspartic and glutamic acid residue as proton acceptor and proton donor, respectively [56,83].

5.4. Glycoside hydrolase family 10

This family consists of endo-1,4-β-xylanases (EC 3.2.1.8), endo-1,3-β-xylanases (EC 3.2.1.32) and cellobiohydrolases (EC 3.2.1.91) [43]. The major enzymes of this family are endo-1,4-β-xylanases, however, substrate specificity studies have revealed that these may not be entirely specific for xylan and may also be active on low molecular mass cellulose substrates [88,89], in particular on aryl-cellobiosides [90,91] and certain cello-oligosaccharides [45,88]. In effect, it has been found that the replacement of one or two xylose residues by glucose is normally tolerated by the xylanases of this family, with this generally resulting in a lowered catalytic efficiency [90]. In consonance with family 11 xylanases, but in contrast to the cold-adapted family 8 xylanase, members of this family are also capable of hydrolyzing aryl β-glycosides of xylobiose and xylotriose at the aglyconic bond [90,92,93]. Furthermore, these enzymes are highly active on short xylo-oligosaccharides, thereby indicating small substrate binding sites [90]. In effect, crystal structure analyses, kinetic analyses of activity on xylo-oligosaccharides of various sizes and end product analyses have indicated that family 10 xylanases typically have four to five substrate binding sites [90,94,95]. Hydrolysis studies have also shown that most family 10 xylanases can attack the xylosidic linkage on the non-reducing end of a substituted residue or 1,3-β bond, but can only cleave at the third xylosidic linkage after a substituted residue and the second after a 1,3-β bond [90]. This indicates that the subsites on the non-reducing side (i.e., subsites −1, −2) are more specific than those on the reducing side (subsite +1) of the cleavage site.

Members of this family typically have a high molecular mass, a low p*I* and display an $(\alpha/\beta)_8$ barrel fold [40,43,58,95–105] (Fig. 3(c)) (Table 4). The structure has been likened to a 'salad bowl', with one face of the molecule having a large radius (approximately 45 Å) due to an elaborate loop architecture, while the opposite face, which consists of simple α/β turns, has a radius of approximately 30 Å. This is similar to the fold described for family 5 enzymes and both are members of clan GH-A. Indeed, these two families are quite closely related and in addition to sharing a common fold they have the same type of catalytic mechanism and share several common residues [57,106]. However, the family 10 xylanases are a more closely related family and have a high percentage of spatially equivalent and identical residues as well as much smaller rms deviations between equivalent residues (0.95 ± 0.11 Å) in its members [57].

5.5. Glycoside hydrolase family 11

In contrast to all other families hitherto discussed, this family is monospecific, it consists solely of xylanases. Moreover, these xylanases are 'true xylanases' as they are exclusively active on D-xylose containing substrates. They have a lower catalytic versatility than family 10 xylanases and indeed the products of their action can be further hydrolyzed by the family 10 enzymes [90,92]. Like family 10 xylanases, these enzymes can hydrolyze aryl β-glycosides of xylobiose and xylotriose at the aglyconic bond, but in contrast to this family they are inactive on aryl cellobiosides. Furthermore, substituents or β 1,3 linkages represent a more serious hindrance to their activity, resulting in the production of larger products than family 10 xylanases [90]. Hydrolysis studies indicate that aldopentauronic acid, with an unsubstituted xylose residue at the non-reducing end [90,107], and an isomeric xylotetraose, with the 1,3-β bond at the non-reducing end [108], are the smallest acidic and mixed linkage fragments liberated from heteroxylans and

Table 4
Family 10 xylanases for which structural coordinates are available

Protein	Organism	PDB accession code(s)	Ref.
Xylanase (Xyn 10A)	*Cellulomonas fimi*	1EXP, 1FH7, 1FH8, 1FH9, 1FHD, 1J01, 2EXO, 2HIS, 2XYL	[96]
Xylanase A (Xyn 10A)	*Cellvibrio japonicus*	1CLX, 1E5N, 1XYS	[97]
Xylanase F (Xyn 10C)	*Cellvibrio japonicus*	1US2, 1US3	[99]
Xylanase C (Xyn 10B)	*Cellvibrio mixtus*	1UQY, 1UQZ, 1UR1, 1UR2	[98]
Xylanase Z	*Clostridium thermocellum* NCIB 10682	1XYZ	[100]
Xylanase T-6	*Geobacillus stearothermophilus* T-6	1HIZ, 1R85, 1R86, 1R87	[43,101]
Xylanase (Xyn A2)	*Geobacillus stearothermophilus* T-6	1N82	[40]
Xylanase A (Xyn A)	*Penicillium simplicissimum* BT2246	1B30, 1B31, 1B3V, 1B3W, 1B3X, 1B3Y, 1B3Z, 1BG4	[102]
Xys 1	*Streptomyces halstedii* JM8	1NQ6	[103]
Xylanase A	*Streptomyces lividans*	1E0V, 1E0W, 1E0X, 1XAS, 1OD8	[95]
β-1,4-Xylanase	*Streptomyces olivaceoviridis* E-86	1ISV, 1ISW, 1ISX, 1ISY, 1ISZ, 1ITO, 1XYF	[104]
Xylanase	*Thermoascus aurantiacus*	1FXM, 1GOK, 1GOM, 1GOO, 1GOQ, 1GOR, 1I1W, 1I1X, 1K6A, 1TAX, 1TIX, 1TUX	[58,105]
Xylanase B	*Thermotoga maritima*	1VBR, 1VBU	[43]

Table 5
Family 11 xylanases for which structural coordinates are available

Protein	Organism	PDB accession code(s)	Ref.
Xylanase C	*Aspergillus kawachii*	1BK1	[114]
Xylanase 1	*Aspergillus niger*	1UKR	[115]
Xylanase	*Bacillus agaradhaerens* AC13	1H4G, 1H4H, 1QH6, 1QH7	[116]
Xylanase A	*Bacillus circulans*	1BCX, 1BVV, 1C5H, 1C5I, 1HV0, 1HV1, 1XNB, 1XNC, 2BVV	[117]
Xylanase	*Bacillus subtilis* B230	1IGO	[118]
Xylanase A	*Bacillus subtilis subsp. subtilis* str. 168	1AXK	[119]
Xyn 11A	*Chaetomium thermophilum*	1H1A	[120]
Xylanase XynB	*Dictyoglomus thermophilum* Rt46B.1	1F5J	[121]
Xyn 11A	*Nonomuraea flexuosa*	1M4W	[120]
Xylanase	*Paecilomyces varioti Bainier*	1PVX	[122]
Xylanase	*Streptomyces* sp. S38	1HIX	[123]
Xylanase	*Thermomyces* lanuginosus	1YNA	[124]
Xylanase	*Trichoderma harzianum* E58 (*Hypocrea lixii* E58)	1XND	[125]
Xylanase 1	*Trichoderma reesei* (*Hypocrea jecorina*	1XYN	[126]
Xylanase 2	*Trichoderma reesei* (*Hypocrea jecorina*)	1ENX, 1RED, 1REE, 1REF, 1XYO, 1XYP	[127]

rhodymenan, respectively. Others have, however, suggested that this isomeric xylotetraose may contain a 1,4-β bond at the non-reducing end, with the 1,3-β linkage occurring subsequent to this bond [90,109]. In further contrast to the family 10 xylanases, but in common with the family 8 cold-adapted xylanase, these enzymes are most active on long chain xylo-oligosaccharides and indeed it has been found that they have larger substrate binding clefts, e.g., family 11 xylanases from *Schizophyllum commune* and *A. niger* have at least seven subsites [110,111], while, as has already been stated, family 10 enzymes are reported to have four to five subsites [90,94,95]. Further differences between family 10 and 11 xylanases include their stereochemistry of protonation and the effect of ω-epoxyalkyl glycosides of xylose and xylo-oligosaccharides on their activity; family 10 xylanases are *anti*-protonators and are unaffected by the ω-epoxyalkyl glycosides while family 11 xylanases are *syn*-protonators and are inactivated by the ω-epoxyalkyl glycosides [88,112,113].

Family 11 enzymes are generally characterized by a high p*I*, a low molecular weight, a double displacement catalytic mechanism, two glutamates acting as the catalytic residues and a β-jelly roll fold structure [114–127] (Table 5) (Fig. 3(d)). The structure consists principally of β-pleated sheets formed into a two-layered trough that surrounds the catalytic site. Two [48], or perhaps three [128], β-sheets are present and the hydrophobic faces of these are packed against each other to form the hydrophobic core of the protein. Only one α-helix is present and this is typically packed against the hydrophobic face of the second β-sheet. This type of structure has also been described for family 12 endoglucanases and thus both families have been grouped into the same clan, clan GH-C.

5.6. Glycoside hydrolase families 7 and 43

To date, only one enzyme exhibiting xylanase activity has been identified and studied in each of these families

and thus their importance as xylanase containing families is unclear. In addition, neither enzyme studied is a true xylanase; the family 7 enzyme, EGI (Cel7B) from *T. reesei*, is a non-specific endo-β-1,4-glucanase (EC 3.2.1.4) [75,129–131] and the family 43 XYND from *Paenibacillus polymyxa* has both xylanase and α-L-arabinofuranosidase activities [132].

The family 7 endoglucanase I (EGI) from *T. reesei* is not produced during growth on xylan [75] and while its activity on cellulose (hydroxyethylcellulose) is only slightly higher than that on xylan (beechwood and grass), its activity on cello-oligosaccharides (G_3, G_5) is 10-fold higher than that on xylo-oligosaccharides (X_3, X_5) [130]. Hydrolyses of both these substrates takes place in the same active site but a shift in the bond cleavage frequency appears to occur towards the non-reducing end linkages in xylo-oligosaccharides [75,130]. This enzyme has characteristics in common with both family 10 and 11 xylanases. As for the former family, it has a high molecular weight and low p*I* as well as a small substrate binding site, approximately four subsites, with the catalytic site in the middle. On the other hand, like family 11 xylanases, this enzyme displays a β-jelly roll fold [131] (Fig. 3(e)) and produces aldopentauronic acid and isomeric xylotetraose as the shortest acidic and mixed linkage fragments from glucuronoxylan and rhodymenan, respectively [75]. However, even though the core structures of this enzyme and the family 11 xylanases are similar, differences, such as: variations in the location, length and orientation of the structural elements outside of this core, the presence of four short helical segments as opposed to one in the family 11 xylanases and differences in the type and conformation of the amino acid residues lining the active site, results in family 7 enzymes being classified in clan GH-B with family 16 enzymes and not in clan GH-C with the family 11 enzymes.

The family 43 enzyme (XYND) has a molecular weight of 64 kDa and was found clustered with a lichenase gene, with only 155bp separating the two genes, in *Paenibacillus polymyxa*. Unfortunately however, additional studies of the physicochemical or functional characteristics of this enzyme have not been carried out. Further putative family 43 xylanases in *Caldicellulosiruptor* sp. [133,134], *C. acetobutylicum* [59], *Bifidobacterium longum* [135] and *Bacillus* sp. [61] have also been inferred on the basis of sequence homology to the above enzyme but xylanase activity has apparently not been confirmed by functional analysis. Indeed, members of this family have not been as thoroughly studied as some of the other glycoside hydrolase families and the structure of only one member has been determined, indicating that members of this family may display a five-blade β-propeller fold (Fig. 3(f)). Furtherrmore, a glutamate and aspartate in the centre of a long V-shaped surface groove formed across the face of the propeller have been suggested as the catalytic residues [54]. The family is grouped with family 62 in clan GH-F [43,54] and, as has also been demonstrated for family 8 enzymes, its members are believed to catalyze hydrolysis via the single displacement mechanism.

6. Extremophilic xylanases

The preponderance of xylanases studied are of fungal or bacterial origin and in the majority of cases are found to be optimally active at, or near, mesophilic temperatures (approximately 40–60 °C) [16,18] and neutral (in particular for bacterial xylanases) or slightly acidic (in particular for fungal xylanases) pHs. Nevertheless, xylanases have also been reported which are not only stable, but active, at the extremes of pH and temperature. Indeed, xylanases active at temperatures ranging from 5 to 105 °C [4,80,81], pH's from 2 to 11 [4,114,136] and NaCl concentrations as high as 30% [137,138] have been reported. These are produced by micro-organisms which have colonized environments that may be said to be extreme from an anthropocentric point of view and which produce enzymes adapted to these extreme habitats. Of the extremophilic xylanases, the thermophiles, alkaliphiles and acidophiles have been the most extensively studied while cold-adapted xylanases have been much less investigated.

6.1. Thermophiles

A number of thermophilic (optimal growth at 50–80 °C) and hyperthermophilic (optimal growth at >80 °C) xylanase producing micro-organisms have been isolated from a variety of sources, including terrestrial and marine solfataric fields, thermal springs, hot pools and self-heating decaying organic debris [6,128,139–142]. The majority of the xylanases produced have been found to belong to families 10 and 11, with as yet, no reported studies of thermophilic xylanases belonging to any of the other glycoside hydrolase families. Interestingly, the gene for the thermostable xylanase (half-life of 8 minutes at 100 °C) from the extreme thermophilic archaeon *Thermococcus zilligii* [143] has thus far proven refractory to cloning with family 10 and 11 consensus primers [141], suggesting that this enzyme may belong to one of the other less well studied glycoside hydrolase families described in this paper (i.e., families 5, 7, 8 or 43) or indeed to another as yet unknown xylanase family.

Family 10 xylanases have been isolated from various thermophilic and hyperthermophilic organisms, including *Thermotoga* sp. [34,144], *Caldicellulosiruptor* sp. [145], *Rhodothermus marinus* [146], *Bacillus stearothermophilus* [147], *Thermoascus aurantiacus* [58] and *C. thermocellum* [58]. Indeed, a family 10 xylanase, XynA

from *Thermotoga* sp. strain FjSS3-B.1 is one of the most thermostable xylanases reported to date with an apparent optimum temperature for activity of 105 °C and a half-life of 90 minutes at 95 °C [148]. While less frequent, family 11 thermophilic xylanases have also been isolated, with those from *Thermomyces lanuginosus* [6,149], *Paecilomyces varioti* [122], *Caldicellulosiruptor* sp Rt69B.1. [133], *Dictyoglomus thermophilum* [121], *Chaetomium thermophilum* [120], *Nonomuraea flexuosa* [120] and *Bacillus* strain D3 [128,150] being the most thoroughly investigated. Those from *Nonomuraea flexuosa* and *Dictyoglomus thermophilum* are among the most stable, with apparent temperature optima of 80 and 85 °C, respectively. In addition to the above mentioned xylanase producing bacteria a number of xylanase producing hyperthermophilic archaea have also been recently reported: *Thermococcus zilligii* [143], *Pyrococcus furiosus* [143], *Sulfolobus solfataricus* [140], *Pyrodictium abyssi* [151,152] and a number of *Thermofilum* strains [153].

Crystal structure analyses, sequence alignments and mutagenesis studies have indicated that mesophilic and thermophilic xylanases are very similar and that enhanced stability is probably due to an array of minor modifications, with many xylanases using unique strategies to improve their thermostability. These modifications include: an increase in the number of salt bridges and hydrogen bonds [120,124], an improved internal packing [120], an increased number of charged surface residues [154], the presence, often as tandem repeats, of thermostabilising domains [34,144,155], and/or the introduction of disulphide bridges, in particular at the N- or C- termini or in the α-helix regions [122,156,157]. Recently, the thermostabilising role of calcium on a modular family 10 xylanase was demonstrated [146] while the *Bacillus* D3 xylanase was also shown to use a very unique adaptation strategy. Here a series of surface aromatic residues form clusters or ''sticky patches'' between pairs of molecules and these intermolecular hydrophobic interactions are believed to contribute to the thermostability of this enzyme [128,150]. Collectively, or singly, all of the above mentioned modifications could improve the network of interactions within the protein, thereby leading to a more rigid and stable enzyme.

A number of comprehensive structural studies of thermal adaptation for family 10 and 11 xylanases have allowed identification of specific adaptation strategies for each family. For example, a comparison of the thermophilic xylanases from *Thermoascus aurantiacus* and *C.thermocellum* with mesophilic family 10 xylanases indicated that the thermostability in this family is a consequence of an improved hydrophobic packing, a favorable interaction of charged side chains with the helix dipoles as well as an increased proline content in the N-termini of helices [58]. In contrast, a recent comparative structural analysis of 5 thermophilic and 7 mesophilic family 11 enzymes suggested that a general thermostabilising adaptation in this family is a higher threonine to serine ratio (threonine has a high β-forming propensity), an increased number of residues in the β-strands and frequently an additional β-strand B1 at the N-terminus [120]. It is evident that the structural differences between the families are the basis for this difference in adaptation strategies; family 10 enzymes have a high α-helix content (approximately 40%) [95] while family 11 enzymes have a high β-sheet content (greater than 50%) [120].

6.2. Psychrophiles

Even though cold-temperature environments are the most abundant on earth [158], only a small number of cold-adapted, or psychrophilic, xylanase producers have been identified. These encompass a wide range of organisms; two gram negative bacteria (*Pseudoalteromonas haloplanktis* TAH3a [80–83] and *Flavobacterium frigidarium* sp. nov. [159]), a gram positive bacterium (*Clostridium* strain PXYL1 [160]), a yeast isolate (*Cryptococcus adeliae* [161]), krill (*Euphasia superba* [162]), a number of fungi (*Penicillium* sp., *Alternaria alternata* and *Phoma* sp. 2 [163]) and a number of basidiomycetes (e.g., *Coprinus psychromorbidus* [164]). All have been isolated from the Antarctic environment, but, apart from the bacterial family 8 xylanase from *Pseudoalteromonas haloplanktis* TAH3a (pXyl) and the *Cryptococcus adeliae* family 10 xylanase (X_B), studies of the xylanases produced are minimal. Indeed, only these two xylanases and two xylanases, xylanases A and B, from Antarctic krill have been purified and characterized.

In accordance with most other psychrophilic enzymes investigated to date [165–167], the common features of the psychrophilic xylanases studied are a low temperature optimum, high catalytic activities at low temperatures and poor stability (Fig. 4). Indeed, comparative studies of pXyl and X_B with mesophilic xylanases showed that these enzymes have a higher catalytic activity at low and moderate temperatures, having, respectively, 10 and 3 times higher activity at 5 °C and 3 and 2 times higher activity at 30 °C [80]. Moreover, all psychrophilic enzymes studied display high catalytic activity at low temperatures. At 5 °C, activity of pXyl is 60% of the maximum while xylanases A and B from *Euphasia superba* display, respectively, approximately 30% and 40% of their maximum activity. In comparison, a mesophilic xylanase showed less than 5% of its maximum activity at this temperature [80]. Likewise, the apparent optimal temperatures for activity of pXyl, X_B and the microfungal xylanases, which are, respectively, approximately 25, 9 and 10–30 °C lower than that of the mesophilic reference xylanases used, gives further evidence of the adaptation to cold environments of these enzymes. Poor thermal stability of the psychrophilic xylanases studied is indicated by short half-lives (e.g., at 55 °C

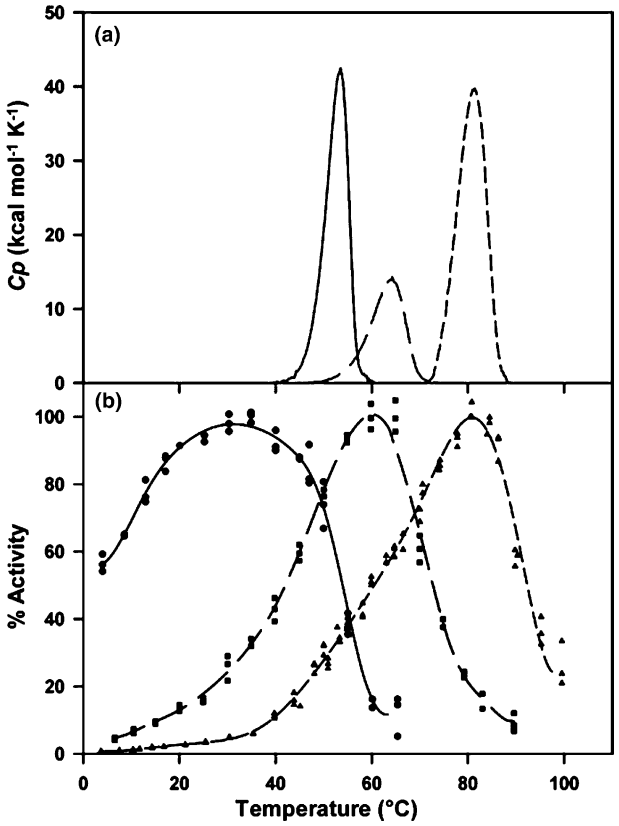

Fig. 4. (a) Unfolding as monitored by differential scanning calorimetry at a scan rate of 1 K min^{-1} and (b) thermodependence of activity of the cold-adapted family 8 xylanase pXyl (circles, solid lines), the mesophilic family 11 xylanase Xyl1 [206] (squares, dashed lines) and the thermophilic family 8 endoglucanase CelA [55,207] (triangles, short dashed lines). Baseline subtracted DSC data have been normalized for protein concentration. The lower molecular weight of the mesophilic enzyme gives rise to the observed low calorimetric enthalpy.

pXyl has a 12 times shorter half-life of inactivation than a mesophilic xylanase) and low denaturation temperatures (pXyl shows a 10 °C and X_B a 14 °C decrease in melting temperature compared to mesophilic reference xylanases) while a lower chemical stability of the cold-adapted family 8 xylanase is demonstrated by short half-lives of guanidine hydrochloride inactivation and unfolding [81].

Fluorescence monitoring of acrylamide quenching indicated that, in addition to a reduced stability and increased low temperature activity, the family 8 cold-adapted xylanase has an increased flexibility compared to a thermophilic homologous enzyme (CelA from *C. thermocellum*) [81]. Indeed, this supports the hypothesis that efficient catalysis at low temperatures is brought about by an increased flexibility of the molecular edifice, thereby allowing the molecular motions necessary for activity in the low temperature, low energy environment but also leading to the observed reduced stability.

At the structural level, it was found that, when compared to a thermophilic and mesophilic homolog, respectively, the family 8 and yeast cold-adapted xylanases are both distinguished by a number of discrete modifications which could give rise to a decrease in the stability, and hence an increase in the flexibility, of the molecular structure. The *Pseudoalteromonas haloplanktis* TAH3a xylanase is characterised by a reduced number of salt-bridges and an increased exposure of hydrophobic residues [82,83], while the family 10 yeast xylanase is characterized by a less compact hydrophobic packing, the loss of one salt bridge and a destabilization of the helix macrodipoles [161]. Indeed it can be seen that these modifications are an extension of those observed between thermophilic and mesophilic xylanases, however, further analysis of psychrophilic xylanases, especially comparative studies with more closely related and better characterised homologs are necessary to better understand temperature adaptation in these enzymes. In particular, further comparative studies of family 10 and 11 psychrophilic xylanases would be most beneficial.

6.3. Alkaliphiles and acidophiles

While the majority of natural environments on earth are essentially neutral, with pH values of between 5 and 9, habitats with extreme pHs are also common, in particular in geothermal regions, carbonate laden soils, soda deserts and soda lakes such as found in Egypt (Wadi Natrun), the African Rift valley (Lakes Magadi and Nakuru in Kenya), Central Asia, Western USA (Yellowstone National Park) and Southern Europe (Vulcano Island, Italy), Indeed, xylanase producing alkaliphilic micro-organisms, which typically grow optimally at pH values above 9, and acidophiles, which grow optimally between pH 1 and 5, have been isolated from these environments [136,168,169] and also from such sources as kraft pulp [170], pulp and paper industry wastes [171], decomposing organic matter [172], faeces [168], plant sources [173], soils [136,174] and even from neutral environments where they are found coexisting with neutrophilic micro-organisms [168].

The first report of a xylanase produced by an alkaliphilic micro-organism was as early as 1973 for a xylanase from *Bacillus* sp. C-59-2 [175] and since this initial finding a number of xylanases have been isolated from various acidophilic and alkaliphilic micro-organisms. These include family 10 and 11 xylanases from a number of *Bacillus* sp. [116,147,174], *Trichoderma* sp. [126,127,176], *Aspergillus* sp. [114,115], *Penicillium* sp. [136], *Acidobacterium* sp. [177] and *Cryptococcus* sp. [178]. In addition, family 8 xylanases have been isolated from alkalophilic *B. halodurans* C-125 [77,78] and *B. halodurans* MIR32 [43], however analysis of the amino-acid sequences indicates that these may be intracellu-

lar enzymes and thus may not be adapted to the environment of their hosts.

Many of the alkaliphilic microorganisms studied have been found to produce xylanases with pH optima in the near neutral region but with relatively high activities being retained in alkaline conditions. In addition, a number of xylanases with more alkaline pH optima have also been isolated and one the most alkaliphilic xylanases reported to date is XylB from *Bacillus* sp. AR-009, which has a pH optimum of pH 9–10 [169]. Other highly alkaliphilic xylanases include xylanase J from *Bacillus* sp. strain 41M-1 [174] and a xylanase from *Bacillus pumilus* 13_a [172], both of which have a pH optimum of 9. Much fewer acidophilic than alkaliphilic xylanases have been studied and the most important of these are the family 10 and 11 members from *T. reesei* [126] *A. niger* [115], *Aspergillus kawachii* [114,179], *Cryptococcus* sp. S-2 [178] and *Penicillium* sp. 40 [136]. The latter three of these are among the most acidophilic of the studied xylanases with a pH optimum of 2 and stability over a broad pH range; the *Penicillium* sp. 40 xylanase is stable from pH 2 to pH 5 [136] and the *A. kawachii* XynC is stable at pH 1–9 [114,179].

The pH activity profiles of enzymes are highly dependent on the pK_as of the catalytic residues which are themselves dependent on the local environment and hence on the nature of the amino-acids in the vicinity of the catalytic residues. A recent study of a family 11 xylanase showed that, in general, residues that contribute positive charges and hydrogen bonds serve to lower the pK_a values with shorter bonds having a more pronounced effect. The chemical nature of the donor is also important, with COOH being more effective than OH and $CONH_2$ [180]. In contrast to this, neighbouring carboxyl groups can either lower or raise the pK_a values of the catalytic glutamic acids depending upon the electrostatic linkage of the residues involved in the interaction [180]. In fact it has been noted that family 11 acidophilic xylanases have an aspartic acid residue hydrogen bonded to the general acid/base catalyst which is replaced by an asparagine in the xylanases active under more alkaline conditions [114,126,181,182]. This residue influences the pH dependence of activity and mutation of this aspartic acid to its amide derivative in the *A. kawachii* acidophilic xylanase resulted in an upward shift of the pH optimum from pH 2 to a pH of 5. Indeed, tertiary structure analysis of this enzyme [114] as well as of the family 11 xylanases from *T. reesei* [126] and *A. niger* [115] indicated that adaptation to low pH is brought about by an increase in negative charge and a substitution and reorientation of residues, in particular aromatic residues, in the active sites. In contrast, a random mutagenesis study of a *Neocallimastix patriciarum* xylanase indicated that an increased negative charge and increased hydrophobicity increased the pH optimum of this enzyme [183].

Stability at the extremes of pH appears to be characterized by a spatially biased distribution of charged residues. The acidophilic and acid stable xylanase from *A. kawachii*, for example, is characterized by a concentration of acidic residues on its surface [114] which are believed to reduce electrostatic repulsion of the positively charged residues at low pHs. In contrast, enzymes stable in alkaline conditions are typically characterized by a decreased number of acidic residues and an increased number of arginines. Furthermore, a recent comparative structural study of family 11 enzymes suggests a correlation between pH activity/stability and the number of salt bridges, with acidophilic xylanases having much less of these interactions than their alkaliphilic homologs [120]. Indeed it was even suggested that adaptation to high pH may occur via a similar mechanism to adaptation to high temperatures [120].

7. Application of xylanases: extremophilic xylanases

Global markets for industrial enzymes grew from €1 billion in 1995 [184] to almost €2 billion in 2001 [185] and continue to increase as new enzymes and applications are discovered. In the grain-processing enzymes sector alone (which currently accounts for approximately 25–28% of total enzyme sales) an increase in market value from €510 million in 2001 to €760 million in 2010 has been forecasted [185]. Presently the technical industries, dominated by the detergent, starch, textile and fuel alcohol industries, account for the majority of the total enzymes market, with the feed and food enzymes together totaling only about 35%. Recently however, sales in some of the major technical industries has stagnated (3% drop in 2001) while sales in both the food and feed industries are increasing, with annual growth rates of approximately 4–5% being forecasted [185].

Hydrolases constitute approximately 75% of the markets for industrial enzymes, with the glycosidases, including cellulases, amylases and hemicellulases, constituting the second largest group after proteases [186]. Xylanases constitute the major commercial proportion of hemicellulases but represent only a small percentage of the total enzyme sales. The sales figures are expected to increase however, as these enzymes have attracted increasing attention due to their potential for use in several applications. In effect, the United States Patent and Trademark Office (http://www.uspto.gov/) lists 468 patents introduced since 2001 with reference to xylanases (search field = all fields).

Xylanases have potential applications in a wide range of industrial processes, covering all three sectors of industrial enzymes markets [186] and some of the most important of these are listed in Table 6. Other less well documented putative applications include: in brewing, to increase wort filterability and reduce haze in the final

Table 6
Potential applications for xylanases

Market	Industry	Application	Function	Reference
Food	Fruit and vegetable processing, brewing, wine production.	Fruit and vegetable juices, nectars and purees, oils (e.g., olive oil, corn oil) and wines	Improves maceration and juice clarification, reduces viscosity. Improves extraction yield and filtration, process performance and product quality.	[186,196,197]
	Baking	Dough and bakery products	Improves elasticity and strength of the dough, thereby allowing easier handling, larger loaf volumes and improved bread texture.	[79,186,198]
Feed	Animal feeds.	Monogastric (swine and poultry) and ruminant feeds	Decreases the content of non-starch polysaccharides, thereby reducing the intestinal viscosity and improving the utilization of proteins and starch. Improves animal performance, increases digestability and nutritive value of poorly degradable feeds, e.g., barley and wheat.	[186,199–201]
Technical	Paper and pulp	Biobleaching of kraft pulps	Reduces chlorine consumption and toxic discharges.	[5,192]
		Bio-mechanical pulping	Facilitates the pulping process and reduces the use of mechanical pulping methods, hence reduces energy consumption.	[186]
		Bio-modification of fibers	Improves fibrillation and drainage properties of pulp, hence improving the process efficiency and the paper strength.	[186]
		Bio-de-inking	Facilitates the de-inking process and reduces the use of alkali.	[186,202]
	Starch	Starch-gluten separation	Reduces batter viscosity, improves gluten agglomeration and process efficiency.	[203]
	Textiles	Retting of flax, jute, ramie, hemp, etc.	Enzymatic retting, reduces/replaces chemical retting methods.	[1,5,204]
	Bioremediation/Bioconversion	Treatment of agricultural, municipal and food industry wastes	Treatment/recycling of wastes. Production of fermentable products, renewable fuel (bioethanol) and fine chemicals.	[1,193,194]

product [187]; in coffee extraction and in the preparation of soluble coffee [7]; in detergents [188]; in the protoplastation of plant cells [4]; in the production of pharmacologically active polysaccharides for use as antimicrobial agents [107] or antioxidants [189]; in the production of alkyl glycosides for use as surfactants [190]; and in the washing of precision devices and semiconductors [191]. The xylanases are frequently utilized alone, but are more commonly used in conjunction with other enzymes and in particular with other hydrolases, but also with proteases, oxidases, isomerases etc.

Many of the xylanases used in industry today appear to be of mesophilic and/or neutrophilic origin, yet enzymes from extremophilic sources may be of tremendous utility in many biotechnological processes. In particular, thermophilic enzymes could be used in applications where a cooling step would be uneconomical or where high temperatures are required to increase the bioavailability and/or solubility of substrates, to reduce viscosity and/or to reduce the risk of contamination. Acidophilic and alkaliphilic enzymes would obviously be beneficial in processes where extreme pH conditions are required or where adjustment of the pH to neutral conditions is uneconomical. On the other hand, cold-adapted xylanases would be beneficial to those processes where heating is economically counterproductive or where low temperatures are required to avoid alteration of ingredient and/or product quality (e.g., flavour, colour etc.), to avoid microbial development and fermentation and/or to avoid product denaturation. In fact, enzymes which combine a number of extremophilic characteristics may be of the most use in industry.

The major current application of xylanases is in the pulp and paper industries where the high temperature (55–70 °C) and alkaline pH of the pulp substrate requires thermo-alkaliphilic enzymes for efficient biobleaching [5,192]. Thermo-alkaliphilic or even thermo-acidophilic xylanases may also be of use in bioconversion processes where a variety of treatments, including hot water and steam explosion, alkaline, solvent or acidic pretreatments may be used prior to or simultaneous to enzyme treatment [193,194]. Alkaliphilic xylanases would also be required for detergent applications where high pHs are typically used [188] while a thermostable xylanase would be beneficial in animal feeds if added to the feeds before the pelleting process (typically carried out at 70–95 °C). In addition, for this latter application the enzyme must be highly active at the temperature (approximately 40 °C) and pH (approximately pH 4.8) of the digestive tract [195]. Cold adapted xylanases, which are most active at low and intermediate temperatures, could offer advantages over the currently used xylanases in many of the low to moderate temperature processes, in particular in the food industry. For example, they would be most suited for use in the baking industry as dough preparation and proofing is generally carried

out at temperatures below 35 °C and indeed it has recently been demonstrated that a cold-adapted family 8 xylanase is more efficient in baking than a commonly used commercial enzyme [79].

8. Concluding remarks

A large variety of enzymes with xylanase activity have been isolated and studied and can be classified in glycoside hydrolase families 5, 7, 8, 10, 11 and 43, with each of these families being characterized by a particular fold and mechanism of action. While extensive studies of family 10 and 11 xylanases have been carried out, leading to an enrichment in the understanding of these enzymes, studies of the xylanase members of the other families are sparse. Only a small number of enzymes with xylanase activity have been identified in families 5, 7, 8 and 43, however, the numbers will increase with further studies and may indeed be extended to additional families as novel enzymes from novel sources are discovered. New approaches, such as genome sequencing programs, functional and/or consensus PCR screening of environmental DNA libraries (known as metagenomics) as well as the study of extremophilic enzymes will, without doubt, further extend the current repertoire, understanding, and applications of xylanases and xylanase families.

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