Biochemistry and genetics of microbial xylanases
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Xylanases are classified into two major families (10 or F and 11 or G) of glycosyl hydrolases. Both use ion pair catalytic mechanisms and both retain anomeric configuration following hydrolysis. Family 10 xylanases are larger, more complex and produce smaller oligosaccharides; Family 11 xylanases are more specific for xylan. Alkaline-active and extreme-thermophilic enzymes are of particular interest. Such xylanases are being commercialized for bleaching pulps and other applications.

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Current opinion in Biotechnology 1996, 7: 337-342
© Current Biology Ltd ISSN 0958-1669

Abbreviations
HexA 4-deoxyhex-4-enuronic acid
BLAST basic local alignment search tool
MeGlcA 4-O-methylglucuronic acid
PNP p-nitrophenyl

Introduction
Hemicelluloses are widely distributed heteropolysaccharides. The enzymes that degrade them are ubiquitous and diverse. In nature, xylans have L-arabinose, acetyl, glucuronic, 4-O-methylglucuronic, and p-coumaric side chains, as well as ferulic acid cross linkages [1•]. Intraglycosidic hydrogen bonding occurs through the O-3 position, giving unsubstituted xylan a helical twist. Acetylation and substitution, however, disrupt and complicate this structure. Xylans are complexed with cellulose and pectin and are bound to lignin. As esterification and substitution increases, digestibility of the hemicellulose decreases [2]. Removal of the side chains is carried out by acetyl esterase [3], ferulic esterase [4], glucuronosidase [5], or arabinosidase [6], but a discussion of such enzymes is beyond the scope of this review.

Native xylans present a formidable substrate for degradation, but commercial xylans recovered by alkaline extraction have a low degree of substitution. Alkaline extraction increases the yield, but it also de-esterifies the substrate, removes acetyl groups, breaks most of the cross linkages, and increases enzymatic degradability. Most assays and screens use commercial xylans. Therefore, despite the large number of xylanases now known, they represent only a small fraction of the enzymatic repertoire involved in hemicellulose degradation. Screens using substrates closer to native polymers will enable the discovery of new enzymes with novel activities or substrate specificities.

Xylanases are drawing increased attention because of their usefulness in facilitating the bleaching of kraft pulp [7•,8]. They increase the extractability of lignin [9] and release chromophores [10] from pulp. Xylanases also improve the quality of dough and help bread to rise [11]. They might also be used in the bioconversion of lignocellulosic materials to fuels and chemicals. This review summarizes recent progress in understanding structure, catalytic mechanisms and the biochemical characteristics of microbial xylanases. Features useful for bioprocessing applications are emphasized.

Enzyme families
Xylanases can be divided into two major families of glycosyl hydrolases: Family 10 (F) and Family 11 (G) [12••]. The relatedness of enzymes within these families can be demonstrated either by pairwise alignments of the protein sequences or by the basic local alignment search tool (BLAST) to discern sequence similarity. BLAST searches using recognized Family 10 or Family 11 xylanase protein sequences identify sets of enzymes that are mutually exclusive. Using BLAST searches, listings of 77 Family 10 and 88 Family 11 xylanases were retrieved in January 1996. Only one sequence — that of a bifunctional enzyme possessing both Family 10 and Family 11 catalytic regions [13] — was identified using sequences of either Family 10 or Family 11 enzymes.

BLAST searches with Family 10 sequences turn up similarities to \( \beta-(1 \rightarrow 3) \) and \( \beta-(1 \rightarrow 4) \) glucanases. Recently, crystallographic studies have shown that the largest cellulase family (Family 1 or A) has a protein fold and an active site similar to those of Family 10 xylanases [14••]. Members of Family 10 will act on both \( p \)-nitrophenyl (PNP)-xylodiose and PNP-cellobiose; however, the overall catalytic efficiency on PNP-xylodiose is ~50-fold higher [15]. This suggests that Family 10 enzymes act mainly on xylan. Yet, the relatively greater volubility of the xylan substrate and the higher reactivity of the xylan glycosidic linkage can increase the hydrolytic rate for xylan compared with cellulose.

Catalytic sites
The Family 10 catalytic domain is a cylindrical \( \alpha/\beta \) barrel resembling a salad bowl, with the catalytic site at the narrower end, near the carboxyl terminus of the \( \beta \)-barrel [16••, 17••]. There are five xylopyranose binding sites. Catalytic domains of these enzymes belong to a ‘superfamily’ that includes Family A cellulases, \( \beta \)-glucosidase, \( \beta \)-galactosidase, \( \beta-(1 \rightarrow 3) \)-glucanases, and...
Family 11 catalytic domains consist principally of \( \beta\)-pleated sheets formed into a two-layered trough that surrounds the catalytic site [20,21]. Protruding down into the trough, and located toward one side of the protein is a long loop terminating in an isoleucine (see Fig. 1). Tössrönen and Rouvinden [22] have likened the trough to the palm and fingers, and the loop to the thumb, of a right hand. The positions of many amino acids are essentially identical in Family 11 xylanases from bacterial (e.g. *Bacillus circulans*) or fungal (e.g. *Trichoderma harzianum*) origins. The *Trichoderma* enzyme, however, is more complex.

The pH optimum depends on properties of the acid/base catalyst [27]. In Xyn1, this is Glu164; in Xyn2, it is Glu177. Asp33 in Xyn1 makes a strong hydrogen bond (2.9 Å) to Glu164, thereby lowering the pK\(_a\) [22]. In Xyn2, an asparagine residue (Asn44; three-letter amino acid code) takes the place of Asp33; the hydrogen bond is much longer (3.7 Å), and the interaction is weaker. All acidic pH xylanases of Family 11 have an aspartic residue in this position; all basic xylanases have an asparagine residue. The exception to the rule is the *Schizophyllum commune* xylanase [28], which has an acidic pH, but on the basis of amino acid sequence is grouped with the basic enzymes; the enzyme has an asparagine interacting with the acid/base catalyst. In the *B. circulans* xylanase, Arg112 interacts with Glu78 and Glu172 (which corresponds to *Trichoderma* Xyn2 Glu177) to raise the pK\(_a\) of Glu172 to 6.8 [29].

**Xylopyranose-binding subsites**

Xylanases possess three to five subsites for binding the xylopyranose rings in the vicinity of the catalytic site. *T. reesei* Xyn2 has five pyranose binding sites; only three are found in Xyn1. Xyn2 also tends to be more
Reaction mechanism for the hydrolysis of xylan by 1XNB (see Fig. 1). (a) The helical xylan substrate is positioned in the trough formed between Tyr65 and Tyr69. Glu172 is the acid/base catalyst and Glu78 is the nucleophile [20]. (b) The glycone is bound to Glu78. This intermediate is retained during transglycosylating reactions. (c) Water displaces the nucleophile. (d) Dissociation and diffusion of the glycone (xylobose) allows movement of the enzyme to a new position on the substrate. Because the aglycone is released in (b) and the glycone is released in (d), many xylanases of Family 11 exhibit a random endo mechanism, rather than progressivity.

transglycosylating. The binding sites are numbered in either direction from the catalytic site and are assigned positive numbers in the direction of the reducing end of the substrate, which constitutes the leaving group (the aglycone), and negative numbers in the direction of the non-reducing end, which remains bound to the catalytic site. The subsites for binding xylopyranose residues are defined by the presence of tyrosine as opposed to tryptophan [28,30]. Pyranose rings with axial hydroxyls present a hydrophobic surface that interacts with aromatic side chains. Tryptophan is essential for substrate binding in most glycosidases, but is not reported to play a role in xylanases.

Non-catalytic domains
Xylanases do not usually have xylan-specific binding domains [31,32], but one is present in the xylanase (XylD) of *C. fimi* [33•]. Substrate-binding domains are more common in Family 10 xylanases than in Family 11 xylanases. They may play an important role in determining specificity and reactivity in pulp-bleaching operations (see below) [26]. Cellulose-binding domains are found in xylanase, arabinofuranosidase [34] and acetylxylan esterase [35]. The only Family 11 xylanases known to have substrate-binding domains are *Thermomonospora fusca* TfxA and *Streptomyces lividans* XylB [36•]. TfxA binds to both cellulose and insoluble xylan, but the enzyme has
activity against only xylan. The complete enzyme has a low \(K_c\) (1.1 mg ml\(^{-1}\)); the \(K_a\) of the catalytic fragment is higher (2.3 mg ml\(^{-1}\)), indicating that the binding site helps the enzyme ‘scavenge’ for substrate. The \(S.\) \textit{lividans} XylB has 64% identity with \(T.\) \textit{fusca} TfxA. It has been shown to bind to insoluble xylan, but not to cellulose. \(T.\) \textit{fusca} TfxA also has a 21 amino acid glycine/proline-rich hinge region that separates the catalytic domain from a xylan/cellulose-binding region.

Xylanases from \textit{Clostridium thermocellum} and \textit{Thermoanaerobacterium saccharolyticum} contain conserved domains that are responsible for the ability of these enzymes to resist thermal denaturation. XylY from \textit{C. thermocellum} also has a carboxy-terminal protein docking sequence [37]. XylB from the fungus \textit{Neocallimastix} \textit{patriciarum} has a Family 10 catalytic subunit and a non-catalytic linker sequence that consists of 45 tandem repeats of an octapeptide rich in hydroxy amino acids and proline [38]. Such linker sequences are common in modular polysaccharidases, but infrequent in fungi. The segment does not bear any similarity to cellulose-binding domains, but \textit{Neocallimastix} is known to form large cellulase complexes, and this might also constitute a docking sequence.

### Mode of action

All xylanases retain the anomeric configuration of the glycosidic oxygen following hydrolysis. This indicates that they use a double-displacement mechanism in which the reactive intermediate is bound to the enzyme (Fig. 2). This enables them to carry out transglycosylation reactions. In ‘retaining’ glycosidases, distances between the nucleophile and the acid base catalyst are 5.4-5.5 Å [12••]. In ‘inverting’ glycosidases, the corresponding distances are greater. This is because for inversion to come about, it is necessary for water to come between the aglycone and the enzyme.

### Alkaline activity

The use of xylanases in bleaching kraft pulps has spurred an interest in the identification of enzymes with alkaline pH optima. Xylanase J from the alkalophilic \textit{Bacillus} sp. strain 41M-1 [39] has a pH optimum of 9, making it one of the most extreme. Other alkaline-active \textit{Bacillus} xylanases include those from strains N-137 [40] TAR-1 [41] and V1-4 [42]. In most instances, alkaline activity results from a broad pH optimum that extends from 5 to 9.5.

### Thermostability

\textit{Thermotoga maritima} is a hyperthermophilic heterotrophic bacterium that is able to grow at 90°C. It produces two thermostable xylanases, XynA and XynB [43]. XynA has a molecular mass of 120 kDa and appears to belong to Family 10. XynB has an apparent molecular mass of 40 kDa and probably belongs to Family 11. XynA and XynB exhibit optimal activity at 92°C and 105°C, respectively. Both enzymes have acidic pH optima.

A Family 10 xylanase from another \textit{Thermotoga} species has been used to bleach kraft pulps [44]. Thermostabilization of the \textit{B. cirrulans} xylanase has been achieved through the introduction of disulfide bonds [45].

### Substrate interaction and characterization

Xylanases with several different properties have been demonstrated in recent years; for example, several have been reported to produce xylolbiose. Recently, however, an enzyme from \textit{Aeromonas} has been shown to produce xylolbiose exclusively [46]. Another \textit{Aeromonas} xylanase produces only xylotetraose [47].

Interaction of xylanases with their substrates depends upon the substitution of the xylan moiety. If xylan is substituted with arabinose, the hydrolytic products obtained with \textit{Streptomyces} xylanase are slightly different from those obtained when glucuronic or 4-O-methylglucuronic acid substituents are present. This difference apparently does not depend on the sugar charge because when glucuronoxylan is reduced chemically to glucoxylan, the products are similar to those obtained with glucuronoxylan [48]. Products obtained from enzymatic digests of hardwood xylans suggest that this substrate may have (1→2) and (1→3) xylopyranosyl branches [49], but it is also possible that such products arise from transglycosylation reactions.

### Enzymatic bleaching

Kraft cooking converts 4-O-methylglucuronic acid (MeGlcA) side groups into 4-deoxyhex-4-enuronic acid (HexA) groups almost completely. The HexA side groups are degraded by ozone or chlorine dioxide, but MeGlcA and arabinose side groups are relatively stable toward the bleaching chemicals [50]. The interaction of enzymes with kraft pulp depends on the presence of ionized side chains (see above) and the metal counter ions that may be present [51]. Metal-free pulp is poorly hydrolyzed. Xylanases exhibit different abilities to facilitate bleaching, but bleaching enhancement generally correlates with the release of chromophores from pulp [10].

### Conclusions

Elucidation of the tertiary structure and catalytic mechanisms of the two major families of microbial xylanases has enabled a better understanding of what determines the pH optimum and substrate binding sites. Genetic engineering has enhanced thermostability, but not always with the desired activity at the elevated temperature. Further engineering should improve the enzyme properties for commercial bleaching applications. For the field to progress, the nature of the xylan binding and the details of enzyme interaction with side chains needs to be better understood.
References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:
• of special interest
•• of outstanding interest

A comprehensive review of xylan and lignin cross links.


A recent review on the use of xylanase in the pulp and paper industry by pioneers in the field.


An excellent overview of the three-dimensional structures of the major families of glycosyl hydrolases.


The catalytic mechanism and overall structure of C. thermocellum CelIC, a member of Family I cellulases are compared with C. thermocellum XynZ, a member of the Family 10 xylanases.


The crystal structure of the catalytic domain of XlnA is α/β-8 barrel. This paper describes the overall shape and catalytic site residues.


An excellent synthesis of recent crystallographic information on β-glucosidase, β-galactosidase, Family A cellulases, Family F xylanases and glycanoses.


At the pH optimum (5.0) for endo-1,4-β-xylanase, Tyr88 is shown to interact with Tyr77, whereas at pH 6.8, Tyr88 bonds with Glu177, thereby disrupting the catalytic activity. This information represents a significant advance in our understanding of the pH optimum for the enzyme.


The pKa of the acid/base catalytic residue, Glu172, of the Family 11 xylanase from B. circulans is shown to be abnormally high (pKₐ=6.8) as a result the electrostatic interaction with Glu78 and Arg112.


Reports the structure of the Family 10 xylanase XyD from C. limicola. This xylanase is unusual in that it contains two binding domains, one of which is specific for xylan.


Reports the sequence of Txa, a thermostable xylanase from T. fusca. This xylanase is unusual in that it is one of only two Family 11 xylanases known to possess a binding domain. The only other example is also found in an actinomycete xylanase, XyB, from S. lividans.


