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Xylanases: from Biology to BioTechnology

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Summary

Xylan is the main carbohydrate found in the hemicellulosic fraction of plant tissues and accounts for one third of all renewable organic carbon available on earth. Xylanase, the major component of an enzymatic consortium, acts in nature by depolymerizing xylan molecules into monomeric pentosan units that are used by bacterial and fungal populations as a primary carbon source. Xylanase producers have been isolated from all ecological niches where plant material is deposited, and microorganisms often contain multiple loci encoding overlapping xylanolytic functions. The numerical excess of genes and the extensive sharing of structural features within β-glycanase families suggests that extensive gene duplication and conversion events have occurred during xylanase evolution. Hydrolysis of β-glycosidic linkages is sponsored by a general acid catalytic reaction common to all glycanases, whereas substrate recognition is specified by subsites that interact with adjacent glycosyl units. Under natural conditions xylanases are inducible by the products of their own action and subject to carbon catabolite repression. Bleaching paper pulps with xylanases is the first successful commercial application for these enzymes. The recovery of cellulosic textile fibers is the next logical application and bioconversion of biomass into fuels and chemicals, remains the ultimate target. Recent developments have shown that metabolic pathways can be transferred from one organism to another and proteins can be modified to gain conformational stability, suggesting that naturally occurring systems can be custom engineered to the situation in the fermentation tank. Thus, biotechnologies developed to transform biomass into marketable products that gradually substitute materials derived from non-renewable resources are becoming commercially worthwhile.

Abbreviations: ATP, adenosine 5'-triphosphate; bp, base pair; EC, Enzyme Commission; HPLC, high performance liquid chromatography; kb, kilobase pair; kDa, kilodalton; P_i, inorganic phosphate; ORF, open reading frame.

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Introduction

Microorganisms are an assorted group of simple but versatile and well-adapted organisms that affect human life in many different ways. Some are beneficial because they are key components in phytomass decay and carbon recycling in terrestrial ecosystems, and others because they provide nutritional benefits to ruminants through symbiotic associations. More directly microorganisms are used for antibiotic synthesis, preparation of foods, beverages, large-scale production of fuels (e.g., ethanol), additives (e.g., citric acid) and chemicals. Microorganisms also have a detrimental impact because, they are the origin of numerous bacterial and fungal infections in humans and other animals, they are the cause of agricultural loss through crop infections and spoilage of post-harvest produce, and they are responsible for toxic contamination. In addition, since microorganisms are simple biological systems, they have been the subject of important discoveries and intensive research has focused on a few representative model organisms (e.g., *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Aspergillus nidulans* and *Neurospora crassa*). As a consequence, the molecular make-up of these model organisms is becoming fairly well understood, and genetic engineering has become a powerful tool to redesign natural metabolic pathways and proteins.

Biomass is the only alternate natural resource for chemical feedstuffs that has a replacement cycle short enough to sustain the demand for the chemical, biochemical and fuel world markets. Xylan is a hemicellulosic sub-component of biomass that accounts for roughly one third of all the renewable organic carbon on earth. Therefore development of inexpensive technologies where hemicellulose is the primary substrate is essential. The consequences of a world-wide shortage of natural materials can be partially appreciated from two political episodes in the 1970s, where the demand for crude oil was artificially controlled. Ultimately, even if not at present, xylan in combination with cellulose will supply most of the global demand for raw materials. Thus, coal and crude oil are likely to be significantly substituted by phytomass in the next 50 years. Such a trend is clearly shown in *Figure 1* where the global demand for raw materials was analyzed over a 250 year period (Goheen, 1981).

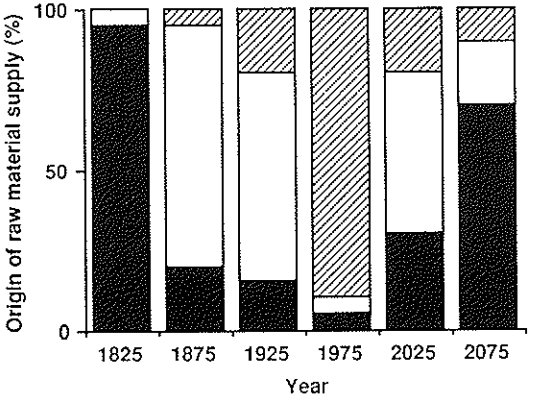

Figure 1. Relative and itemized demand projections for industrial chemical feedstuffs based on a 250 year economic period. The amounts are estimated percentages of petrochemical, (shaded box) coal (white box) and biomass (black box) feedstuffs utilized and do not indicate total quantities. Redrawn with permission (Goheen, 1981).

In this article a comprehensive review of xylanase research is presented and is arranged in two sections: First, recent developments in xylanase biology are summarized and ordered into a logical order that reflects natural selection and biological diversity. Second, current efforts to use these enzymes at the industrial scale are highlighted. Several excellent reviews on related topics complement this work (Biely, 1985; Wong, Tan and Sadler, 1988; Linko, Poutanen and Viikari, 1989; Eriksson, 1990; Saddler, 1992; Wallace, 1992; Biely, 1993; Gilbert and Hazlewood, 1993; Kubicek *et al.*, 1993a; Béguin and Aubert, 1994; Hahn-Hägerdal *et al.*, 1994).

Xylan structure, degradation and D-xylose metabolism

Hemicellulose is a multi-component fraction derived from lignocellulosic plants composed of heteropolysaccharides with various degrees of polymerisation that are always lower than that of cellulose. The majority of components are amorphous and possess branched structures with neutral or acidic side chains (Timel, 1967; Northcote, 1972, 1989). In plant cells, xylan is one of the three major structural polysaccharides and is localized in the cell wall matrix. The relative distribution of lignocellulosic components in cell walls is dependent on the plant species and on the stage of growth and development (Timel, 1967; McNeil *et al.*, 1986; Northcote, 1989; Minoru and Harada, 1992). Xylans form an interface between lignin and other polysaccharides and are mainly found in the secondary cell wall. It is likely that xylan molecules make covalent links with lignin-phenolic residues (Eriksson, Goring and Lindgren, 1980; Overend and Johnson, 1991) and interact with other polysaccharides such as pectin and glucans as well (Selvendran, 1985). Thus, xylans have adhesive functions mediated by covalent and non-covalent interactions with lignin, cellulose and other polymers that are essential to maintain plant cell wall integrity (Kato, 1981).

Xylan is found in large quantities not only in hard (15%–30% of the cell wall content) and softwoods (7%–10%) but also in annual plants (up to 30%), such as maize, ramie and sugar cane (Wilkie, 1959; Timel, 1967; Viikari *et al.*, 1993). In its simplest form xylans are linear homopolymers that contain only D-xylose monomers linked through β-1,4-glycosyl bonds. In nature however, they are often partially substituted (side chains) with acetyl, 4-*O*-methyl-D-glucuronosyl and L-arabinofuranosyl residues, forming complex heterogeneous and polydispersed polymers (Kato, 1981; Smith and Hartley, 1983; Kato and Nevins, 1985). Because the isolation of xylans without loss of original structure and associations with other components is difficult, and because enzymes that cleave the heteropolymer at specific bonding patterns into smaller fragments have not been found, there are many structural aspects that remain unclear. The exact substitution pattern of different xylans and covalent interactions with other plant cell wall polymers are only two important aspects that need to be addressed.

Complete enzymatic degradation of a native xylan molecule requires the action of several enzymes. To recover D-xylose from β-1,4-xylan molecules, at least two discrete enzyme activities are necessary. Depending upon biological origin, one or more endo-1,4-β-xylanase (1,4-β-D-xylan-xylanohydrolase, EC 3.2.1.8) isoforms cleave the xylan β-1,4-bonded backbone into smaller fragments with accumulation of xylobiose. Xylobiose is converted into D-xylose through the action of a β-xylosidase (β-D-xyloside-xylohydrolase, EC 3.2.1.37). Because in nature xylan is a heteropolymer

cross-linked with other plant cell wall components, enzymes that eliminate specific substituted residues and resolve cross-links have been found. Among these auxiliary enzymes the most common activities are, phenolic acid esterases, acetyl esterases, α-glucuronidases and α-L-arabinofuranosidases (Biely *et al.*, 1986; Puls, Schmidt and Gransow, 1987; Poutanen *et al.*, 1990; Faulds and Williamson, 1991; Kormelink *et al.*, 1991; Borneman *et al.*, 1992; Biely, 1993; Ferreira *et al.*, 1993).

Xylose, the main degradation product of xylan, is a primary carbon source that can substitute for glucose in many ways. In most microorganisms, xylose is actively transported across the cell membrane, converted into xylulose directly or through a xylitol intermediate, metabolised by the pentose phosphate pathway to a product that finally merges with glycolysis (Schneider, 1989). The severe redox imbalance caused by the transformation of xylose into xylulose is barely overcome in the absence of oxygen, and results in poor ethanol yield during fermentation (Hahn-Hägerdal *et al.*, 1994).

Ecology

A large number of microorganisms that produce xylanases and other glycosidases have been found in extremely diverse natural habitats. Moreover, microorganisms use these activities to achieve a wide range of goals. For example, some microorganisms utilize biomass-hydrolytic enzymes as alternate pathways to increase the number of options for a primary carbon source, whereas others use these activities as part of a genetic program specifically directed to infect and colonize plant cells. In both cases the understanding of the biochemistry and genetics that underlies these processes are important. This is so, not only because they cause infections that destroy large proportions of the crop production world-wide, but also because biomass is the only renewable source that contains a primary group of compounds that chemically substitutes non-renewable resources.

Microorganisms that are biochemically capable of assimilating biomass components as a primary carbon source belong to remarkably diverse taxa, often occur in mixed populations with non-xylan degrading organisms, and have been found in all environments where plant material accumulates and deteriorates. Phytomass-hydrolytic enzymes provide all the degrading functions required for wood decay. Xylanases are commonly isolated from bacterial and fungal strains that colonise aerobic and anaerobic ecosystems, enduring both mesophilic and thermophilic environments. The photosynthetically fixed carbon contained in glucose and xylose molecules is recycled by metabolic elimination of water and carbon dioxide under aerobic conditions and methane under anaerobic situations.

Abundant accumulation of plant components is found in topsoil, and decay rates are correlated with the lignin content of the deposited material. Lignin is a stable polymer composed mostly of methoxylated phenylpropanoic units (Dean and Eriksson, 1992) and delignifying enzymes are not common among microorganisms. Wood degradation usually takes place immediately after the plant tissue dies. Fungi, mostly Basidiomycetes, are common among wood decomposing microorganisms and are grouped into white-rot, brown-rot and soft-rot fungi, according to the macroscopic aspects of the deteriorating tissue. White-rot fungi are the most important ones, and *Phanaerochaete chrysosporium* provides a paradigm for lignin degradation (Eriksson, 1978). For more information on lignin and wood degradation the extensive and

comprehensive work by Eriksson, Blanchette and Ander (1990) is useful.

Plants are a major part of the diet of many mammals. Ruminants are dependent upon microorganisms, including bacteria and fungi, for the digestion of starch, cellulose, xylans, pectins and other plant polysaccharides. The bacterial population of the rumen is a complex ecosystem composed of many different species, and *Butyrivibrio fibrisolvens*, *Bacteroides ruminicola*, *Ruminococcus flavefaciens* and *Ruminococcus albus* are the predominant genera (Hespell and Whitehead, 1990). This symbiotic relationship enables ruminants to utilize feedstuffs that are not digestible by other mammals and therefore animal production is largely dependent upon the efficiency of the processes conducted by microorganisms. Because we know that these processes are inefficient, there is considerable interest in the manipulation of ruminal fermentation processes (Whitehead, Cotta and Hespell, 1991). Most of the saprophytic fungi that degrade fibers in the rumen of herbivores are classified as obligate anaerobic zoosporic fungi (Bauchop, 1989; Teunissen and Op-den Camp, 1993; Wubah, Akin and Borneman., 1993). Thirteen species grouped into five genera have been assigned to Chytridiomycetes (Wubah, Akin and Borneman., 1993). These fungi produce a wide range of hydrolytic enzymes that provide livestock with the necessary biochemical functions to decompose efficiently dietary plant cell walls. Cellulose, xylans and lignin are efficiently degraded and the resulting monomers, glucose and xylose (but not lignin monomers) are readily adsorbed by the host (Akin and Benner, 1988; Borneman, Akin and Ljungdahl, 1989). These hydrolytic enzymes are detected free in the media or in association with rhizoids and rhizomycelia (Lowe, Theodorou and Trinci., 1987; Mountfort and Asher, 1989). These fungi also secrete auxiliary enzymes, such as acetyl xylan esterases, that release ferulic and *p*-coumaric acids, indicating complete breakdown of xylan under such conditions (Borneman *et al.*, 1990, 1992).

The plant cell wall is a major barrier for a plant pathogen. Most, if not all, fungal plant pathogens produce and secrete enzymes that degrade plant cell wall polysaccharides (Deising and Mendgen, 1991; Heiler, Mendgen and Deising., 1993; Mendgen and Deising, 1993). These activities are induced during the morphogenetic process that produces differentiated infective structures and function by softening the region of penetration by partially degrading cell wall structures (*Figure 2*). Although

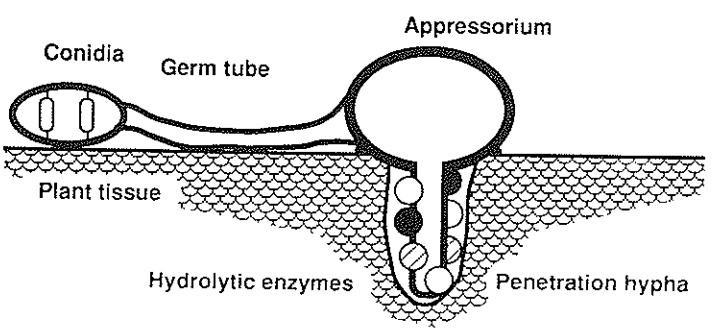

Figure 2. Infection apparatus of a typical fungal plant pathogen. Fungi that penetrate plant cell tissues produce differentiated cell types with specialized biochemical functions. Cell-wall degrading enzymes (white, black and dashed circles) are specifically induced during the infection process and function in softening the plant tissue by degrading cell wall components.

it is certain that cellulases, xylanases, pectinases and cutinases are important functional elements of the penetration process, an essential role in phyto-pathogenesis has not yet been established. For example, the inactivation (disruption) of a cutinase gene in *Nectria heamatococca* (Stahl and Schäfer, 1992), endopolygalacturonase (Scott-Craig *et al.*, 1990) or xylanase (Apel *et al.*, 1993) in *Cochliobolus carbonum*, did not affect pathogenicity in any of the recombinants, indicating that these enzymes are not essential for infection. Thus, it appears that these enzymes are not essential by themselves, and it is conceivable that because of low substrate specificity the deficiency of one activity is compensated by functional redundancy of the hydrolytic system as a whole.

Regulation of expression

The now classic Sternberg and Mandels (Sternberg and Mandels, 1979) model has become generally accepted not only to explain regulation of cellulases, but xylanases as well. The model addresses general concerns regarding cellular recognition and signal transduction in the presence of such a large and complex substrate molecule. Cryptic expression of cellulase activity under repressive conditions is the key to this model where small amounts of degradation products, such as cellobiose and its positional isomer sophorose, are capable of triggering massive production of enzymes (Sternberg and Mandels, 1979; El-Gogary *et al.*, 1989). Some aspects of the Sternberg-Mandels paradigm have raised discussion, mainly because it accounts poorly for the inducing effects of sophorose, a β-1,2 linked glucobiose formed in glycosyl transfer reactions from cellobiose, in the fully induced state (Kubicek *et al.*, 1993a; Kubicek *et al.*, 1993b; Béguin and Aubert, 1994). Because an alternative model has not yet been proposed, regulation is probably best discussed by addressing induction (recognition of substrate and signal transduction) and control of transcription (activation and repression of transcription) independently. A generic regulatory model for xylanases and cellulases is shown in *Figure 3.*

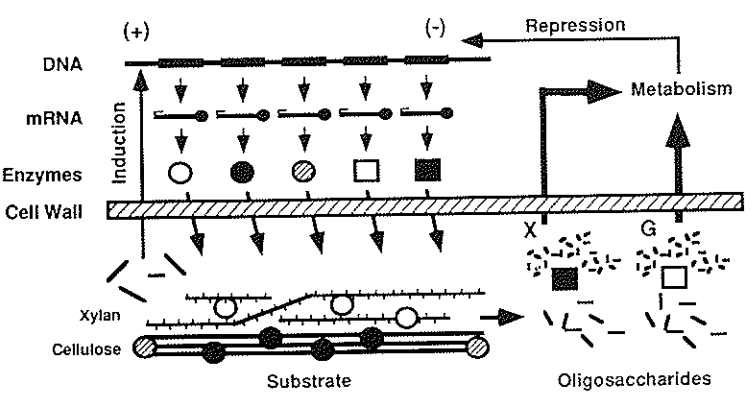

Figure 3. Generic regulatory model for xylanases and cellulases in microorganisms. Transcription is induced (+) by specific molecules that signal the presence of extracellular substrates and repressed (–) by the intracellular excess of degraded substrate (glucose and/or xylose). Symbols: Open circles, endo-β-xylanase; black circles, endoglucanase; dashed circles, cellobiohydrolase; open squares, β-glucosidase; black squares, β-xylosidase.

For xylanases, a similar substrate recognition and induction concept has emerged from the work initiated by the group of Peter Biely with the yeast *Cryptococcus albidus*. It is now generally accepted that induction of xylanases is also mediated by the products of their own action (Biely, 1985; Defaye *et al.*, 1992). In *C. albidus*, three components are necessary to assimilate xylan completely as a carbon source: an extracellular endo-1,4-β-xylanase, an β-xyloside permease to transport xylobiose and other xylo-oligomers and an intracellular β-xylosidase to produce xylose from xylo-oligomers (Biely *et al.*, 1980). Xylanase synthesis is induced by natural and certain artificial positional isomers of β-1-4-xylo-oligosaccharides among which, xylobiose is the only natural component that is not degraded extracellularly, suggesting that it is the natural inducer (Biely *et al.*, 1980; Biely and Petráková, 1984b). Interestingly, xylobiose synthetic positional isomers induce xylanase production only if converted by endo-1,4-β-xylanase into β-1,4-xylobiose, contrasting with the opposite situation observed with sophorose in the induction of cellulases (Biely and Petráková, 1984a).

In a wild *Streptomyces* isolate Godden and colleagues (Godden *et al.*, 1989) report that xylanase and β-xylosidase but not cellulase activities were induced by xylan, xylobiose, kraft lignin and syringic acid (lignin derivative). Carboxymethylcellulose, cellotetraose and cellotriose induced cellulolytic enzymes but not xylanases, indicating that both enzymatic systems are subjected to independent controls (Godden *et al.*, 1989). Independent regulation of xylan- and cellulose-degrading enzymes has also been observed in *Trichoderma reesei*, *Aspergillus terreus* and *Aspergillus niger* by using high-definition inducers of natural and synthetic origin (Hrmová, Biely and Vrsanská, 1986, 1989; Hrmová, Petráková and Biley, 1991). Earlier studies have indicated that cellulases and xylanases are coinduced, apparently because the available inducers were of natural origin and with low chemical definition (Eriksson and Goodell, 1974; MacKenzie *et al.*, 1987; Poutanen *et al.*, 1987; Steiner *et al.*, 1987; Bailey and Poutanen, 1989; Linko, Poutanen and Viikari, 1989).

Streptomyces lividans has three genes that encode xylanases (*xlnA*, *xlnB* and *xlnC*) that are expressed in the presence of xylans and xylan hydrolyzates (Shareck *et al.*, 1991). The polypeptides translated from different loci show different substrate-degradation patterns, suggesting a sequential action of the gene products (Biely *et al.*, 1993). XlnB and XlnC cleave xylan into long xylo-oligomers, whereas XlnA cleaves xylan and long xylo-oligomers mainly into xylobiose and xylotriose (Kluepfel *et al.*, 1990). Inactivation of *xlnA* does not affect the expression of *xlnB* and *xlnC* and its expression is not affected by the inactivation of *xlnB* or *xlnC*, indicating independent regulation. However, disruption of *xlnB* or *xlnC* affects the expression of *xynC* and *xynB*, respectively. Enzymatic degradation products that have not been treated with XlnA and contain xylo-oligomers with 11 or more sugar residues, obtained through the action of either XlnB or XlnC, stimulate the expression of *xlnC*, *xlnB* and *xlnA*, suggesting that substrate-specific induction is mediated by an xylo-oligomer at least 11 residues long (Arhin *et al.*, 1994). It is important to stress though that both, *xynB* and *xynC* are expressed in a wild type strain when xylobiose or xylotriose are provided as inducers. In *xynB* and *xynC* disruptants, wild-type biochemistry is restored in the presence of the inducer xylobiose only when the inactivated gene product is added to the medium (Arhin *et al.*, 1994). In addition transglycosylation products that are longer than the original substrate has been observed for XlnA (Moreau *et al.*, 1994a,b). As a result of these observations, Morosoli and cols. (Arhin *et al.*, 1994) propose a

model, similar in several ways to the Sternberg-Mandels paradigm, where the inducer is not transported into the cell, but recognised by a receptor that through a signal transduction pathway, activates specific gene expression.

In *B. subtilis* β-xylanase (*xynA*) is produced constitutively and cannot be repressed by the addition of glucose (Lindner, Stülke and Hecker, 1994). In contrast, β-xylosidase (*xynB-xynBC* operon) D-xylose isomerase (*xylAB* operon), xylulokinase (*xylAB* operon) and a putative xyloside permease (*xynC-xynBC* operon) are induced by xylose or xylan and repressed by glucose (Gärtner, Geissendörfer and Hillen., 1988). Furthermore catabolite repression is abolished in a *ccpA* (catabolite control protein) mutant suggesting that *xynCB* and *xylAB* operons are under the control of the same regulon that operates *amyE* and *bglS*, α-amylase and β-glucanase, respectively (Krüger, Stülke and Hecker, 1993; Lindner, Stülke and Hecker, 1994). Three components have so far been identified in carbon catabolite repression (i) the promoter of most of the genes known to be negatively regulated by glucose, contain a characteristic catabolite responsive element (CRE); (ii) the gene product of *ccpA* belongs to the GalR/LacI family (helix-turn-helix) of transcriptional regulators and binds (only at high concentrations) to CRE's and (iii) CcpA appears to interact specifically and directly with the phosphorylated form of the heat stable protein, HPr(Ser_{46}-P). This protein is phosphorylated by an ATP-dependent kinase activated by fructose-1,6-diphosphate (and other glycolytic intermediates) and inhibited by inorganic phosphate (P_i). In addition, a P_i-activated phosphatase is able to dephosphorylate HPr(Ser_{46}-P), suggesting that this protein is phosphorylated when glycolytic activity is high and dephosphorylated when cells are under stress (e.g., starvation) conditions (Gärtner, Geissendörfer and Hillen, 1988; Hueck and Hillen, 1995). Thus, a tempting explanation is that sensing of glycolytic activity is mediated through phosphorylation and formation of a heterodimeric protein complex that represses transcription of genes that contain CRE elements (Hueck and Hillen, 1995).

In most fungi, xylanases and cellulases are under carbon catabolite repression. In *Aspergillus tubigensis* (Degraaff *et al.*, 1994) the presence of biologically active *creA* binding sites, suggests that repression is *creA*-dependent and mediated via a double lock mechanism, similar to the ethanol regulon in *A. nidulans* (Kulmburg *et al.*, 1993). In *A. nidulans*, *creA* encodes a C_2H_2 zinc-finger type transcription factor that requires two adjacent and divergent CREA binding sites for *in vivo* carbon catabolite repression to occur (Cubero and Scazzocchio, 1994). With similar molecular features, the *T. reesei xyn1* promoter also supports the two adjacent and divergent CreA-dependent carbon catabolite repression model, however, in this case the evidence supports only a single lock mechanism where the inactivation of the functional CREA binding site abolishes repression but not induction (Strauss *et al.*, 1995). Interestingly, *T. reesei* has a second xylanase locus (*xyn2*) that has several CREA binding sites that do not mediate carbon catabolite repression *in vivo* (Strauss *et al.*, 1995).

Molecular classification of xylanases

Amino acid sequences of a large number of glycosyl hydrolases have been used to create a comparative classification system based on sequence similarities, and alignments based on hydrophobic-cluster analysis (HCA). HCA is a comparative method designed to predict protein folding based on hydrophobic patterns and is used to

correlate distantly related members of a given protein family (Gaboriaud *et al.*, 1987). In the original study, six cellulase-xylanase families were identified (Henrissat *et al.*, 1989). In a second study, families were upgraded to eleven (Gilkes *et al.*, 1991), and in a third update to 45+, including 482 glycosyl hydrolase amino acid sequences (Henrissat and Bairoch, 1993). Xylanase families are subdivided into two distinct groups; acidic high molecular weight (>30 kDa) and basic low molecular weight (Wong, Tan and Saddler., 1988; Törrönen, Kubicek and Henrissat, 1993a).

Amino acid residues of related proteins are usually not linearly conserved and substitutions are more likely to be silent at the polypeptide backbone than on the hydrophobic core or on the catalytic domain. Because functional elements (essential) should be more conserved within conserved proteins than non-functional (dispensable) regions, and HCA families are primarily classified according to hydrophobicity similarities, these families are mainly established based on regions that encode essential domains. One such conserved domain is certainly the catalytic domain. Xylanases, cellulases and other glycanases are presumed to follow a general acid catalysis mechanism that predicts two possible stereochemical configurations of the newly formed reducing end. In one case, hydrolysis results in net inversion of the anomeric configuration and the other net retention (Sinnott, 1990). Gebler and colleagues. (Gebler *et al.*, 1992) tested 16 purified cellulases and xylanases belonging to 6 HCA families for inversion or retention of configuration and found that enzymes from one HCA family follow only one configuration (either retention or inversion), and both configurations were present throughout the families. The HCA family concept has also successfully survived an enzyme specificity challenge. Claeyssens and Henrissat (1992), classified 15 cellulases into six HCA compatible families by substrate-hydrolysis-specificity mapping using chemically synthesised chromophoric-glycoside substrates.

Genetic molecular architecture

Xylanase and xylanase auxiliary genes are found in conjunction with cellulases in the genome of a large number of microorganisms, each of which hydrolyzes unique biochemical substrates (cellulose and hemicellulose), even though catalysis follows a common mechanism. In addition, functionally distinct β-glycanases do not share strong amino acid sequence similarities. Thus, it is likely that individual biochemical functions (cellulase and xylanase) were derived from that of a common low affinity ancestor and evolved independently (Gilkes *et al.*, 1991; Gilbert *et al.*, 1992; Henrissat and Bairoch, 1993). However, these apparent independent groups of loci share many architectural aspects in gene structure and protein function that results in widespread observation of genetic multiplicity and biochemical redundancy.

Genetic multiplicity (redundancy of genetic information) has been observed in at least three different ways. First, regardless of origin, (anaerobic or aerobic, bacterial or fungal), genomes of many microorganisms maintain several non-polycistronic functional copies of loci that encode proteins with overlapping functions (MacKenzie *et al.*, 1989; Shareck *et al.*, 1991; Carter *et al.*, 1992; Tsujibo *et al.*, 1992; Ali *et al.*, 1995). In a few cases, especially in eukaryotes, detection of redundant xylanase polypeptides is the result of differential post-translational processing (Biely, 1985), but in most cases it seems to be the product of genetic multiplicity (Gilbert *et al.*, 1987,

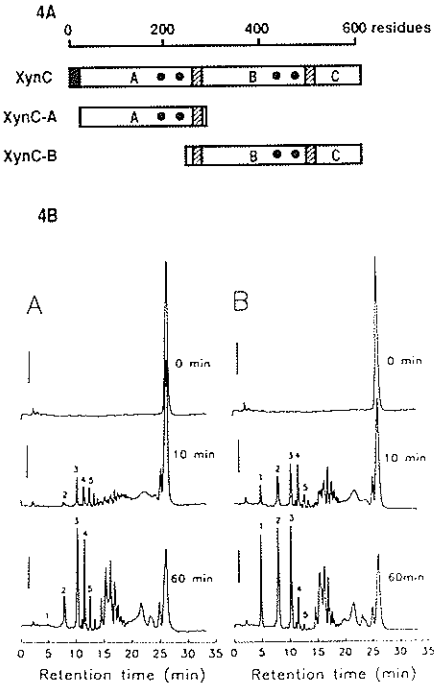

Figure 4. The *xynC* locus of *F. succinogenes* S85 encodes one polypeptide containing two xylanase catalytic domains with discrete degradation patterns. **A** Locus structure and diagram of truncated *xynC-A* and *xynC-B* versions are shown. The full-length XynC polypeptide contains a signal peptide (black box) and three domains (A, B and C) separated by two serine rich linker regions (shaded boxes). Closed circles indicate putative catalytic residues. **B** HPLC of xylan enzymatic degradation products with purified, XynC-A (panel A) and XynC-B (panel B) truncated polypeptides. The numbers, 1, 2, 3, 4 and 5 identify peaks representing xylose, xylobiose, xylotriose, xylotetraose and xylopentaose, respectively. Reprinted with permission (Zhu *et al.*, 1994).

1988; Hazlewood *et al.*, 1988). Second, in several cases a single gene product encodes multiple catalytic domains. The *Fibrobacter succinogenes* S85 *xynC* gene coding region translates into a single polypeptide with two defined xylanase catalytic domains. As shown in *Figure 4*, both domains can be differentiated biochemically because they have distinct substrate-cleavage specificities (Zhu, Martin and Flint, 1994). A similar situation has also been described for *R. flavefaciens* 17 (Zhang and Flint, 1992) and *Neocallimastix patriciarum* (Gilbert *et al.*, 1992) where *xyn* loci encode polypeptides containing two catalytic domains each. Multiple domains do not necessarily arise by duplications, but also by the merger of functional domains. In *N. patriciarum* the *celD* gene translates into a tri-functional polypeptide that encodes endoglucanase, cellobiohydrolase and xylanase domains (Xue, Gobius and Orpin, 1992). All three activities can be detected independently in transformants that carry truncated gene versions (Black *et al.*, 1994). In a similar fashion the *xynD* gene of *R. flavefaciens* encodes a single polypeptide with xylanase and β-(1,3-1,4)-glucanase (lichenase) activities secreted into the medium (Flint *et al.*, 1993). Third, in many microorganisms multiple copies of redundant or related loci are polycistronic or physically clustered (Hall *et al.*, 1989; Kellett *et al.*, 1990; Gosalbes *et al.*, 1991;

Gilbert *et al.*, 1992; Xue, Gobius and Orpin., 1992; Zhang and Flint, 1992; Flint *et al.*, 1993; Baba, Shinke and Nanmori, 1994). In *Caldocellum saccharolyticum* a 6 kb DNA fragment was found that encodes 5 open reading frames encoding a xylanase (*xynA*), a β-xylosidase (*xynB*), an acetyl-esterase (*xynC*) and two ORF's with unknown functions (Lüthi *et al.*, 1990). Lüthi *et al.* suggest that at least one of the unknown ORFs might represent a pseudogene because evidence that this gene is transcribed is absent and because considerable amino acid sequence homology with *xynA* is present. In a similar situation three activities, xylanase, β-xylosidase and α-arabinofuranosidase were found in a 3.8 kb DNA fragment in *Bacteroides ovatus* (Whitehead and Hespell, 1990). Two of the activities (β-xylosidase and α-arabinofuranosidase) appear to be linked to one polypeptide. In *Bacillus polymyxa* the *xynD* (xylanase) and *gluB* (lichenase) loci are separated by 155 bp. In addition, the *xynD* polypeptide, appears to express xylanase and α-arabinofuranosidase activity (Gosalbes *et al.*, 1991). Another example is the *Pseudomonas fluorescens* subsp. *cellulosa XynB* and *XynC* genes that are clustered in tandem, separated by 148 bp (Kellett *et al.*, 1990).

The observed pattern of genetic multiplicity may point to a two-step evolutionary process, where initially primitive proteins with low substrate recognition specificity (recognize both cellulose and xylan) are duplicated and evolve independently (Jensen, 1976). This idea is supported by the fact that β-glycanases follow the same reaction mechanism, as well as consistent observations of gene clustering and multiple copies of redundant domains. In the second step, gene conversion of differentiated catalytic domains is sustained by the finding of multifunctional polypeptides. A two-step evolutionary proposal presumes selective advantages for both types of events. In fact, it is tempting to imagine that gene duplication and domain fusion events are likely to provide microorganisms with improved abilities to access a highly diversified and complex carbon source like phytomass, by supplying a stoichometrically balanced spectrum of biochemical activities (Arhin *et al.*, 1994).

Non-catalytic substrate-enzyme interactions

Microorganisms that degrade xylans are of common occurrence in nature and most of them contain an overlapping set of genes that is expressed in response to the presence of certain substrates. Depending upon the organism, these enzymes are usually transported across the cell membrane where they associate into a multiprotein complex, or diffuse away from the cell into the medium. In *Clostridium thermocellum* large molecular aggregates that interact with cellulose and adhere to the outer cell wall layer were originally found by electron microscopy (Bayer and Lamed, 1986). These aggregates, also known as cellulosomes, are in fact multiprotein complexes that exhibit a wide spectrum of cell wall degrading activities (Lamed and Bayer, 1987; Béguin, Millet and Aubert, 1992; Felix and Ljungdahl, 1993). The discovery of a polypeptide (CipA) that is essential for crystalline cellulose degradation but does not exhibit any enzymatic activity lead to the conclusion that this polypeptide functions as a scaffolding protein (Wu, Orme-Johnson and Demain, 1988). The core protein CipA co-ordinates the assembly of at least one cellobiohydrolase, 15 endoglucanases, two xylanases, two β-glucosidases and one lichenase into a multifunctional complex through non-covalent interactions (Béguin and Aubert, 1994). Even though *C. thermocellum* is unable to grow on xylan as the sole carbon source, a cell associated

β-xylosidase and several cellulosome associated xylanases have been cloned and purified (MacKenzie *et al.*, 1989; Morag, Bayer and Lamed., 1990). The biological advantage of having a cellulosome is not completely clear, mainly because we do not know if polypeptides (enzymes) interact with CipA randomly or are assisted by a specific mechanism (Béguin and Aubert, 1994). Multienzyme complexes have been found in at least five strains of *Clostridium* (Morag, Bayer and Lamed., 1990; Foong and Doi, 1992), in several mesophilic anaerobes (Shoseyov and Doi, 1990) and in at least one aerobic organism (Lamed and Bayer, 1987).

Multienzyme complexes have not been recognised in *P. fluorescens* subsp. *cellulosa* (Hazlewood *et al.*, 1992), *Cellulomonas fimi* (Gilkes *et al.*, 1988), *F. succinogenes*, *N. frontalis*, *R. flavefaciens* (Poole *et al.*, 1991) and *T. reesei* where the enzymes appear to be secreted into the media (Béguin, 1990). *P. fluorescens* subsp. *cellulosa* has been suggested as an alternate paradigm to that of *C. thermocellum* with its cellulosomes, because cellulases and xylanases are secreted into the media, where they are found in close association with cellulose molecules (Hazlewood *et al.*, 1992). As the gene products of *celA* (cellulase), *celB* (cellulase), *xynA* (xylanase), *xynB* (xylanase), *xynC* (α-arabinofuranosidase) and *xynD* (esterase) are secreted, they diffuse away from the cell surface and come across cellulose molecules with which they interact through specific non-catalytic interactions. All of these polypeptides contain a similar non-catalytic domain (Gilbert *et al.*, 1987, 1988, 1990; Ferreira *et al.*, 1990, 1993). Furthermore, deletion from the original polypeptide or grafting of these non-catalytic domains to alkaline phosphatase, result in recombinant proteins that are unable to interact with cellulose but retain catalytic activity, or bind to cellulose and exhibit phosphatase activity, respectively (Kellett *et al.*, 1990; Poole *et al.*, 1991; Tomme *et al.*, 1994). Domains that interact with cellulose have been referred to as cellulose binding domains (CBD), and similar domains that bind specifically to xylan (XBD) have recently been found (Black *et al.*, 1995). The XBD from *C. fimi* XylD interacts specifically with xylans even though it shares 65% identity with the CBD domain (Black *et al.*, 1995). These substrate-interacting domains have been identified in other organisms and are consistently alluded to as non-catalytic elements that do not obstruct hydrolysis (Gilkes *et al.*, 1988, 1989, 1991; Stahlberg, Johansson and Pettersson, 1988; Coutinho *et al.*, 1992; Sakka *et al.*, 1993; Black *et al.*, 1994; Zhang, Martin and Flint, 1994). CBDs in xylanases are usually located on the amino or carboxy terminus, are about 100 amino acids long, contain an excess of hydroxyamino acids, conserved tryptophans, asparagines, glycines and cysteines and, based on amino acid sequence alignments, can also be grouped into related families (Gilkes *et al.*, 1991). In particular, tryptophan residues appear to be relevant because it has been shown that conserved residues are essential for the interaction with cellulose (Gilbert *et al.*, 1990; Poole *et al.*, 1993; Millward-Sadler *et al.*, 1994).

The ecological niches in which xylanolytic microorganisms survive and make themselves comfortable are as diverse as is the occurrence of phytomass, regardless of environmental conditions. Degradation processes are the result of overlapping enzymatic functions that dismount xylan molecules to their basic building blocks that are assimilated and metabolised by the cell. Simple secretion of enzymes appears to be insufficient, even if provided in multiple copies, to supply the needs of a primary carbon source. As a result, improved polypeptides have evolved with novel non-

catalytic functions that through non-covalent interactions improve the overall efficiency of the phytomass degradation process.

Enzyme-substrate recognition

Xylanases and cellulases, like all known β-1-4-glycan hydrolases follow a general acid mechanism of catalysis that is sponsored in its essence by two acidic amino acid residues (Bray and Clarke, 1990). Thus, for specific recognition of the substrate, these enzymes require additional subsites, besides the catalytic center, that interact non-covalently with monomer units of the substrate to coordinate substrate-specific binding and stereochemical alignment (Chipman and Sharon, 1969; Robyt and French, 1970; Allen and Thoma, 1978).

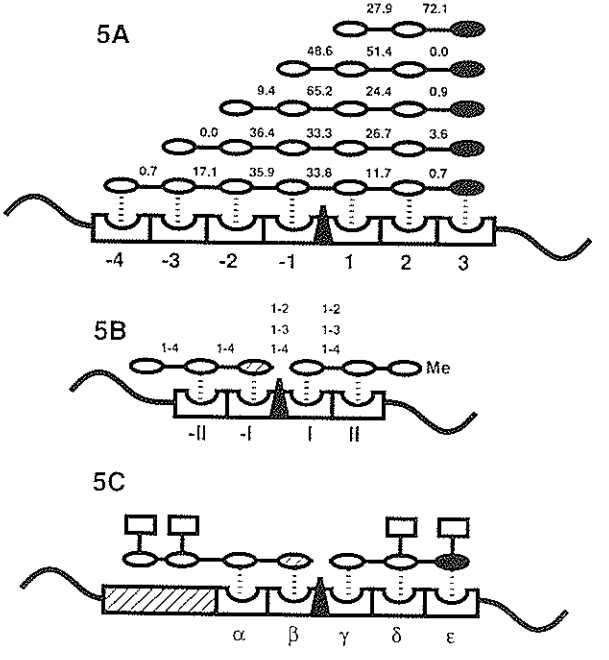

Figure 5. Non-catalytic specificity of xylanase/substrate interactions. The figure is a schematic representation summarizing three experiments that indicate the degree of specificity in xylanase/substrate interactions. **A** The distribution of seven substrate-enzyme recognition subsites (–4, –3, –2, –1, 1, 2 and 3), asymmetric location of the catalytic site and the preferential hydrolysis of specific β-1,4 glycosyl bonds by *S. commune* xylanase A are shown. Numbers indicate bond-cleavage frequencies of enzymatic hydrolysis expressed as percentage of total cleavage events. Redesigned and condensed with permission from the authors (Bray and Clarke, 1992). **B** The distribution of four substrate-enzyme recognition subsites (-II, -I, I and II), symmetric location of the catalytic site and the recognition of xylo-oligomers containing other than β-1,4-linkages by *C. albidus* endo-β-D-xylanase are shown. Numbers indicate the possible β-bonding substitutions accepted by the corresponding subsite. Redesigned and condensed with permission from the authors (Vrsanská *et al.*, 1990). **C** The distribution of five substrate-enzyme recognition subsites (α, β, γ, δ, and ε), asymmetric location of the catalytic site and the recognition of substituted regions of 4-*O*-methyl-D-glucuronoxylan by *C. thermolacticum* xylanase I are shown. Redesigned and condensed with permission from the authors (Debeire et. al, 1990). Symbols indicate non-reducing terminii or intramolecular xylose residues (open circles); reducing-end xylose residues (closed circles); reducing-end created by hydrolysis (shaded circles); β-1,4-glycosyl bond, unless otherwise specified (lines); substrate-enzyme recognition subsite (boxes with cavity); catalytic site (arrowheads); side chain 4-*O*-methyl-D- glucuronic acid residues (squares), non-covalent intermolecular interactions (dashed lines) and Me, methyl group.

Three sets of elegant experiments illustrate the importance and specificity of these subsites (*Figure 5*). First, the *Schizophyllum commune* xylanase A (endo-1,4-β-D-xylan xylanhydrolase), is unable to cleave xylobiose and has low activity on xylotriose and xylotetraose (*Figure 5A*). Oligomers with a higher degree of polymerisation are cleaved with increased efficiency and affinity constants decrease with chain length and turnover numbers, indicating that the substrate recognition domain spans about seven xylose units (Bray and Clarke, 1992). In addition, the action of this enzyme on reduced substrates suggests that the catalytic site is located asymmetrically within the binding domain. Second, the binding subsites and catalytic center of the yeast *C. albidus* endo-β-D-xylanase (*Figure 5B*) is organized into four binding subsites (-II, -I, +I and +II), and the catalytic center in this case is located in the middle, between binding site -I and +I (Biely, 1981; Biely, Vrsanská and Gorbacheva, 1983). Using specific synthetic substrates that alternate β-1-4, β-1-3 and β-1-2 linked bonds at specific positions on the oligomeric substrate strongly indicates that different subsites have specific requirements regarding accommodation of β-linkages. For example, subsites -I and -II have a strict requirement for xylopyranosyl residues with β-1-4 linkages and sites +I and +II also accept β-1-2 and β-1-3 linked xylobiosyl oligomers (Vrsanská *et al.*, 1990) and third, xylanase I from *Clostridium thermolacticum* appears to contain 5 binding subsites (α, β, γ, δ and ε) with the catalytic site positioned in an asymmetric position (*Figure 5C*). Subsites δ and ε accept substituted xylose residues and subsites α, β and γ are unable to bind substituted residues (Debeire *et al.*, 1990).

Since the evidence strongly suggests that enzyme-substrate specificity is the result of the non-covalent interactions between the substrate and several specific subsites of the enzyme (usually located around the catalytic center), endo-cleaving enzymes are likely to be more specific than exo-cleaving enzymes. Furthermore, the stringency of the substrate-enzyme interaction is not uniform throughout xylanase families, and in many cases these enzymes also recognize and cleave a second substrate but with much lower specific activity (e.g., carboxymethylcellulose).

Hydrolysis

Significant progress has been made in recent years in the understanding of the sequence of events that take place at the catalytic center of xylanases. The initial drive originated from the observation that xylanases not only share amino acid sequence similarities with the hen's egg lysozyme (Morosoli, Roy and Yagushi., 1986) but also show a lysozyme-type pattern of action (Biely, Krátky and Vranská, 1981), indicating that xylanases might follow a similar general acid/base catalysis. Lysozyme is the model enzyme used to infer the mode of action of glycanases in general. Glycanases are grouped into two classes, based on a mechanistic distinction. The so called retaining enzymes (lysozyme) retain anomeric configuration by utilizing a double displacement (also double inversion of configuration) mechanism involving an oxicarbonium intermediate. An glycosyl-enzyme intermediate has also been suggested and cannot be ruled out (McCarter and Withers, 1994). The inverting class hydrolyzes glycosidic bonds with net inversion of anomeric configuration by a direct displacement of the leaving group by water (same as single nucleophilic substitution). At the active site, both classes require a pair of carboxylic acids; the inverting type uses one as a general acid and the other as a general base, and the retaining group uses one

group as a general acid/base and the other acts as a nucleophile and a leaving group. There has been considerable discussion regarding the catalytic events of hydrolysis, particularly the resubmission of an opening ring mechanism (Franck, 1992), and a number of recent reviews are available (Sinnott, 1990; Svensson and Sogaard, 1993; McCarter and Withers, 1994).

The amino acid sequence similarity that placed xylanases into the lysozyme family is strengthened by several biochemical observations: first, Tavobilov *et al.* (1981) indicate that ionizable groups participate in the catalysis in a xylanase from *A. niger* and several reports show that carboxy groups are involved (Bray and Clarke, 1990; Zhu *et al.*, 1994); second, several studies with the functionally related enzyme, cellulase, suggest the involvement of catalytic carboxy groups (Hurst, Sullivan and Shepherd, 1977; Yaguchi *et al.*, 1983; Paice *et al.*, 1984; Clarke and Yaguchi, 1985; Høj *et al.*, 1989; Chauvaux, Beguin and Aubert, 1992) and third, several groups succeeded in mapping both essential carboxy amino acid residues by utilizing a number of independent experimental approaches (Okada, 1989; Tull *et al.*, 1991; Chauvaux, Beguin and Aubert, 1992; Keskar, Rao and Deshpande, 1992; Ko *et al.*, 1992; Lee *et al.*, 1993; Bray and Clarke, 1994; Macleod *et al.*, 1994; Miao *et al.*, 1994; Törrönen, Harkki and Rouvinen, 1994; Moreau *et al.*, 1994a; Wakarchuk *et al.*, 1994a).

The first indication that a xylanase has been crystallized with sufficient resolution to suggest a three-dimensional structural model comes from work with *Bacillus pumilus* (Okada, 1989; Ko *et al.*, 1992), and predicts two structural parts with a cleft region about 3 nm (30 Å) long and 1.5 nm (15 Å) in diameter. The size of the cleft is large enough to accommodate a xylan fiber of about 1.1 nm. (11 Å) in diameter, and two appropriately located Glu residues (93 and 182) essential for hydrolysis were determined by site-directed mutagenesis.

The *T. reesei* XynII belongs to the family of low molecular weight xylanases (Törrönen, Kubicek and Henrissat, 1993; Törrönen *et al.*, 1993; Törrönen, Harkki and Rouvinen, 1994). The primary structure of XynII contains 190 amino acid residues and the secondary structure indicates that this protein is a single-domain polypeptide, containing two β-sheets (A and B) and one α-helix. Both β-sheets coil and bend to form a cleft on one side of the protein and the hydrophobic faces are wrapped against each other in parallel forming the hydrophobic core of the protein. The overall structure has the shape of a 'right hand' where the two β-sheets form the fingers and the twisted part of β-sheet B together with the α-helix forms the palm. A loop of nine amino acids forms the thumb that closes the cleft, and an unusual loop of 12 amino acids forms a cord that partially closes one side of the cleft. The residues Glu86 and Glu177 are the most likely candidates for catalysis because they are located at appropriate positions and are conserved throughout the protein family. An interesting observation is that the surrounding region of Glu86 is far more conserved than Glu177. Thus, if Glu177 initiates the reaction by donating a hydrogen, the pK of this ionizable side chain will depend on the adjoining amino acids, and as a consequence, different xylanases show discrete optimal pHs (Törrönen, Harkki and Rouvinen, 1994). Moreover, structural comparisons between *T. reesei* XynI and XynII indicate that the different pH optima between these two enzymes are the result of amino acid sequence variations surrounding Glu177 (Törrönen and Rouvinen, 1995).

The *Bacillus circulans* xylanase is a retaining type of enzyme that has also been

crystallized and a three-dimensional model is available (Wakarchuk *et al.*, 1994**a**). This enzyme is also from the low molecular weight family and resembles many of the structural features of the *T. reesei* XynII xylanase (Törrönen, Harkki and Rouvinen, 1994). In this study modelling of crystallographic data is combined with site-directed mutagenesis experiments to provide strong evidence for catalytic and substrate-subsite-recognizing amino acids. Mutations in Glu78 (nucleophile) and Glu172 (acid/base catalyst) do not cause drastic conformational changes but induce complete loss of catalytic activity.

The xylanase A from *S. lividans* belongs to the high molecular weight xylanase family and has a structural makeup different from the two xylanases discussed earlier (Derewenda *et al.*, 1994). The XlnA molecule shows a tertiary fold that is typical of an $(\alpha/\beta)_8$ barrel, first described for triose-phosphate isomerase (also α-amylase and cyclodextrin glucanotransferase), and consists of a 'salad bowl' shape. Although the $(\alpha/\beta)_8$ pattern is the most common fold among enzymes (Farber and Petsko, 1990), it has been found neither in cellulases nor in xylanases. In XlnA, Glu128 and Glu236 have been implicated in catalysis through site-directed mutagenesis experiments (Moreau *et al.*, 1994a,b), and both residues are located within a shallow depression, equivalent to lysozyme. Thus, it is likely that the substrate binds to the shallow groove along the top face of the molecule and Glu128 and Glu236 promote catalysis (Derewenda *et al.*, 1994).

Protein design

Engineering of natural proteins by *in vitro* mutagenesis has become a straightforward process that allows almost any desired modification to be constructed in the laboratory. Moreover, domain grafting, deletion, duplication and site directed point mutation techniques are widely used molecular tools and allow almost any protein modification to be made with absolute precision. Thus, the problem of targeted protein design is primarily related to the accuracy by which one can make functional and conformational predictions. For example, the introduction of disulfide cross-links into proteins to protect them from unfolding, requires the creation of cysteine residues that form disulfide bonds spontaneously in solution and do not obstruct functional domains. In *B. circulans* mutant xylanase proteins, with several artificially introduced cysteine residues, disulfide cross-links form spontaneously without loss of activity. In this case, disulfide bridges confer thermoprotection; the best result was a 15°C increase in thermostability, based on residual enzyme activity assays (Wakarchuk *et al.*, 1994b). Artificial disulfide cross-links may prove to be useful denaturation protection tools in the adaptation of xylanases to biotechnologies where physico-chemical properties differ from the natural conditions (e.g., bleaching of pulps). Even though the creation of disulfide bonds is a direct way to achieve protection against folding perturbations caused by denaturing agents, its use is restricted to conditions where disulfide bonds are stable. Interestingly, many thermophilic enzymes in nature, particularly hyperthermophiles, do not use the disulfide cross-link strategy for conformational protection. Stability in hyperthermophiles appears to be context dependent and may be the result of the reduction of water-accessible hydrophobic surfaces (Adams, 1993; Meng, Bagdasarian and Zeikus, 1993). In fact, xylanases without disulphide bonds that are more thermostable than

equivalent enzymes that are S-S cross-linked have been found (Oku *et al.*, 1993). Thus, even though thermoprotection, conformational stability and enzymatic activity can be engineered, much remains to be learned from natural thermophilic enzymes before we can guarantee precise molecular predictions.

Metabolic engineering

For low-cost high-volume commodities, such as ethanol, it is economically important that all sugars, hexoses and pentoses, are utilized as substrates. Microorganisms that ferment both monosaccharides at acceptable yields have not been found in nature (Jeffries and Kurtzman, 1994). In addition, ethanol produced from xylose is an inefficient pathway in most natural organisms because of a strong redox imbalance imposed by the pentose phosphate shunt. To bypass these natural barriers two similar but opposite metabolically engineered strains have been constructed. In one set of metabolic designs, the ethanologenic genes from *Zymonomas mobilis*, pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*), were transferred to *E. coli* to complement the host deficiencies for ethanol production from xylose. Recombinant strains showed high ethanol production yields using both xylose and glucose as substrates (Alterthum and Ingram, 1989; Ohta, Alterthum and Ingram, 1990; Ohta *et al.*, 1991a,b; Barbosa *et al.*, 1992; Takahashi, Carvalho and Alterthum., 1994). In a similar metabolic engineering design, *Klebsiella oxytoca* received the ethanologenic genes from *Z. mobilis* (*pdc* and *adhB*) along with the *xynZ* (xylanase from *C. thermocellum*) and *xylB* (xylosidase and arabinofuranosidase from *B. fibrisolvens*) genes. Recombinant strains were able to convert xylan into ethanol in a two-stage fermentation process, metabolizing not only xylose but xylobiose and xylotriose as well (Burchhardt and Ingram, 1992; Bothast *et al.*, 1994). In a different set of experiments, two operons encoding xylose assimilation and pentose phosphate pathway genes were constructed and transformed into *Z. mobilis*. The resulting recombinant strain was able to grow on xylose and use this sugar in anaerobic fermentations combining the phosphate pentose and Entner–Doudoroff pathways, thereby achieving high ethanol production yields using xylose and glucose as substrates (Zhang *et al.*, 1995).

Engineering metabolic pathways, to adjust microorganisms to the conditions required for industrial operations of biotransformation processes, has been a primary concern, and the results obtained by engineering *E. coli* with *Z. mobilis* genes and *Z. mobilis* with xylose metabolic pathway genes, are strong indications that such technologies will soon be commercially attractive.

The xylanase marketplace

Plants are the only group of photoautotrophic biosystems and therefore do not depend upon the supply of organic nutrients from the environment. It is difficult to determine world-wide annual biomass production rates, and estimates range from 10^{10} to 10^{11} tons, where at least half of it is produced by forests (Blazej and Kosik, 1993). The major carbohydrates from lignocellulosics belong to two families. Cellulosic components, composed exclusively of glucose monomers, account for approximately half, whereas hemicelluloses, composed of xylose, arabinose and other pentosans, make up

to 30% of the total dry weight of terrestrial plants (Linko, Poutanen and Viikari, 1989; Viikari *et al.*, 1993). Thus, the complete depolymerization of lignocellulosic carbohydrates roughly produces hexoses and pentoses in a 6:4 ratio, respectively. The interest in hemicellulases as enzymes used to process biomass has increased sharply in recent years because of the successful application of these enzymes within the pulp and paper industry.

Biomass as an organic resource is obtained in many different ways and logs, paper- and textile-fiber products, grains, starch, cassava, cotton, potato, sugar cane, beet molasses, bagasse- and straw-surplus and municipal solid waste are just a few examples. The large-scale consumption of the various metabolically active carbon sources derived from biomass share the advantage of being rapidly replaced. However, in many cases they have to be obtained by degradation of polysaccharides that are not easily accessible. Thus, even though the physico-chemical properties of wood are advantageous for many of its products (e.g., timber, boards), there is growing concern (environmental protection) for certain chemical processing techniques (e.g., kraft pulping and bleaching) in the production of fibers (e.g., paper, rayon fibers, cellophane) and chemicals (e.g., lignosulphonates, dimethyl sulphoxide).

Enzymatic processing of biomass is usually applied in two distinct ways: bulk enzymatic preparations are used to degrade cellulose and xylans to recover glucose and xylose for further use in fermentations, or specific enzymes are used to degrade completely one kind of polysaccharide and recover the other without chemical modification. Examples of these two cases are ethanol production through fermentation of lignocellulosics and production of paper from wood, respectively. These enzymatic processes are accompanied by several complications associated with the complex biochemical interactions among the plant components (Saddler, 1992). Since many of the sources of lignocellulosic materials are hard and compact, diffusion and access of enzymes are inefficient. Thus, woody materials often need to be reduced in size (e.g., sawdust, wood chips) and subjected to chemical and physical pre-treatments before an enzymatic process can be applied. The most common pre-treatment is high-pressure steaming, with or without rapid decompression (Saddler, Ramos and Breuil, 1993). High-pressure steam treatments with rapid decompression (steam explosion) increases the accessibility of enzymes to their substrates and is the result of mechanical action during decompression (Kling *et al.*, 1987; Ramos, Breuil and Saddler, 1992). Lignocellulosic materials undergo extensive chemical modifications during most of the high-pressure steam protocols. The sharp reduction in length of xylan polymers in steam exploded material is the most obvious indication of such changes. Impregnation of hardwoods with sulfuric acid before steam explosion increases the yield of xylose recovered after steam explosion, suggesting extensive hydrolysis of the hemicellulosic fraction (Schell *et al.*, 1991). Mild acid treatments of wood to recover xylose leads to the co-extraction of fermentation inhibitors that include compounds such as furfural, acetic acid, acetates and lignin derived phenols (Beck, 1993). Pre-treatments are designed to offer easy access of enzymes to one or more components of the lignocellulosic material and are useful in applications where complete hydrolysis of one or more polysaccharides is desired (Saddler, 1992).

Most of the current experiments are aimed at the conversion of cellulose to ethanol, involving sequential or simultaneous processes of saccharification and fermentation (Hayn *et al.*, 1993). Xylanases have not been used extensively in hydrolysis and

fermentation processes for two main reasons: pre-treatments of lignocellulosic materials partially hydrolyzes xylans (Hahn-Hägerdal *et al.*, 1991), and xylose fermentation processes are metabolically inefficient despite the progress in fermentation techniques (Hahn-Hägerdal *et al.*, 1994; Jeffries and Kurtzman, 1994; Preez, 1994; Zhang *et al.*, 1995). Thus, the use of xylanases on an industrial scale is primarily dependent upon process improvements and molecular interventions aimed to bypass current pitfalls imposed by the substrate and the metabolic machinery.

Paper

Purified plant polysaccharides are extensively used in traditional markets such as fibers, films and adhesives and are predominantly prepared by chemical methods. Cellulose is a high molecular weight linear molecule with strong intermolecular hydrogen bonding that confers considerable tensile strength. In contrast, hemicelluloses are highly substituted (branched) and therefore are not suitable as fibers and films. The natural fiber market is divided into two groups: (1) fiber production through pulping of wood, produces paper and paper derived products, and (2) natural fibers such as cotton, flax and ramie are recovered for their use in the textile industry.

A common chemical process to recover cellulosic fibers from hardwood is kraft-pulping. Hemicelluloses and lignin are dissolved and partially degraded during the heating process. In a subsequent phase of the process the pH drops sharply because of the discharge of xylan side groups and xylan precipitates with readsorption of lignin on top of the cellulosic microfibrils. Lignin is colored during kraft-pulping and as a consequence, cellulosic fibers become darkly stained. Usually one or more bleaching sequences are needed to remove the dark color caused by the deposition of lignin. Xylanases have been successfully used as additives in the bleaching step of kraft pulps (Eriksson, 1989, 1990; Viikari, Sundquist and Kettunen, 1991; Buchert *et al.*, 1992; Viikari *et al.*, 1993). Chemical bleaching technologies are harmful to the environment and need to be substituted by environment compatible procedures. The use of xylanases to facilitate pulp bleaching is a natural solution that lowers chlorine consumption and toxic discharges (Senior and Hamilton, 1992; Senior *et al.*, 1992; Tolan and Canovas, 1992). The general notion is that xylanases cleave and solubilize reprecipitated xylan and lignin located on the surface of the microfibrils. The effectiveness of xylanase treatments has been evaluated in at least two aspects: first by determining the amount of solubilized sugars after enzyme incubations, where 0.5%–1.0% of the pulp carbohydrate content is liberated and second, by observing increased bleachability with conventional methods after xylanase treatments (Viikari *et al.*, 1993). In addition, Yang and Eriksson (1992) report the release of lignin-carbohydrate complexes by xylanase treatments of kraft pulps.

For applications in cellulosic fiber recovery, xylanases are only useful if the cellulosic fiber is not affected by the presence of cellulolytic activities (Eriksson, 1990; Viikari *et al.*, 1993). Preparation of cellulase-free xylanases can be obtained through several, not always cost effective methods, like removal by purification or enzymatic inactivation of cellulases, or by production from microorganisms that fail to produce detectable amounts of cellulase activity (Biely *et al.*, 1980; Gomes *et al.*, 1993; Milagres, Lacis and Prade, 1993). Selective induction using chemically defined inducers (Biely, Vrsanká and Kratky, 1980; Hrmová, Petráková and Biely, 1991),

production by cellulase negative mutants (Eriksson and Goodell, 1974; Mondou *et al.*, 1986) and production by genetically engineered strains can also be used. In addition, since xylanases are used as bleaching additives in the processing of chemical or thermomechanical pulps, it would be desirable that the enzyme meets the requirements of the process rather than the process meeting the needs of the enzyme. Thus, the perfect enzyme for a bleaching process should be active at high temperatures and pHs (Wakarchuk *et al.*, 1994b).

Textile fibers

The major difference between xylanases used in the recovery of textile fibers and paper fibers, is that textile fibers are extracted from plants that are more accessible to enzymes. Flax and ramie fibers are recovered from stems that are soft and have a lower lignin content than hardwoods and softwoods. For example, incubation of dried ramie stems with xylanases releases intact cellulosic fibers that do not require extensive bleaching treatments (Milagres and Prade, 1994). Although the mechanisms by which xylanases affect lignin removal are not completely understood, there is no doubt that xylanases are useful in the disassembling processes (*Figure 6*) in which fibers are

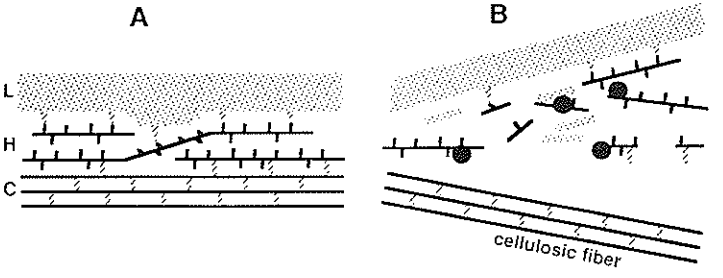

Figure 6. Simple schematic representation of a plant tissue disassembly process observed in treating ramie stems with xylanase. **A** Non treated plant stems and **B** plant stems treated with xylanases. L, lignin; H, hemicellulose; C, cellulose. Black circles indicate xylanase molecules and dashed line non-covalent interactions.

separated from the matrix of plant cell walls (Paice and Jurasek, 1984; Viikari *et al.*, 1990; Archibald, 1992; Paice *et al.*, 1992; Yang and Eriksson, 1992). Enzymatic treatment of cellulosic fibers from intact materials has the additional advantage that lignin is not oxidized during the process, thus eliminating fiber staining. Indications are that such an enzymatic process may explain the recovery of non-stained cellulosic fiber bundles from traditional natural treatments (retting). Relatively little attention has been given to the enzymatic preparation of textile fibers, even though they are a complete and ideal model system for molecular biochemistry. They would also be the perfect pilot-scale market for commercial biotechnological developments (Milagres and Prade, 1994).

Conclusions

As a result of natural genetic variability, functional redundancy and the intensive research conducted in recent years, public databases and genebanks are filled with

information on DNA fragments that constitute a rich, unique and useful resource of biological material. Catalytic domains that hydrolyze individual biomass components are available from a large selection of loci and cover a wide spectrum of biochemical conditions. Non-catalytic domains that reversibly bind to cellulose and xylan are also available and constitute a valuable resource for situations where close associations between and within substrate molecules is desired. In addition, scaffoldins, proteins that assemble many individual proteins with unique functions into a large multi-functional agglomerate of activities can also be engineered to meet the complex biochemical requirements of certain applications. Thus, even though biomass bioconversion is a complex and inaccessible biotechnological problem, our present genetic and biochemical assets (DNA fragments) represent an appealing opportunity to address the current pitfalls in process development. Moreover, it is important to add that in addition to the availability of natural genetic resources, metabolic pathways can be transferred from one organism to another and proteins can be modified to gain conformational stability. Thus, it is now possible to custom design natural occurring biochemical properties to fit the situation in the fermentation tank and comply with the demands of an industrial process as a whole. However, we should keep in mind that current limitations, such as for example the apparent context-dependency of thermoprotection in hyperthermophiles, indicate that much useful information still remains to be learned from naturally occurring biological systems.

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