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Xylan degradation: a glimpse at microbial diversity

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The key to taking the measure of biodiversity lies in a downward adjustment of scale. . .Most of the Earth's largest species—mammals, birds, and trees—have been seen and documented. But microwildernesses exist in a handful of soil or aqueous silt collected almost anywhere in the world. . .Bacteria, protistans, nematodes, mites and other minute creatures swarm all around us, an animate matrix that binds the Earth's surface.
Edward O Wilson (1994. *Naturalist*. Island Press, Washington, DC)

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Introduction

As suggested by Edward O Wilson, the birds, the trees, and the terrestrial plants that inhabit the Earth's surface are largely known. The demise of animal species, such as the Dodo bird and the passenger pigeon, at the hand of man and the threatened extinction of other groups of animals, even insects, are widely recognized. What we know virtually nothing about is the extent of *microbial* life—and death. And this despite the fact that the survival of life on Earth depends upon the health and well-being of our microbial population. The bacteria alone operate to reintroduce dinitrogen into the *bio*-cycle, without which life as we know it would cease to exist. Microbial cells also act to oxidatively cycle metal ions and carbon in soils and aqueous environments, and to degrade, or mineralize, all plant and animal materials back to the elemental carbon, hydrogen, nitrogen and oxygen from which they were formed. In addition to 'cleaning' the planet, the microbial world can delight human appetites with foods, such as cheeses, yogurt, and other dairy products, with 'pickled' cabbage, cucumber and vegetable products, and with fine wines and other alcohol beverages from fruits and grains. Conversely, microbes can also destroy and kill by causing animal and plant disease. Surprisingly with this awareness of the importance of microorganisms, we still know very little about 'who is doing what and how' in the microbial world. Our work to find answers to these questions defines the current study of microbial biodiversity.

In this minireview, I will attempt to illustrate one facet of the remarkable biodiversity that exists in our environment by discussing the range of microbes that function to degrade, to bioconvert, or to mineralize the plant product, xylan. In addition, I will survey some of the conditions under which the microbial xylan-degrading enzymes operate in order to illustrate their functional diversity. The detailed physical properties, chemical composition, and molecular mechanisms of the enzymes will not be considered. The present review is intended to stimulate the desire to explore the microbial biosphere, and to encourage the desire to make new discoveries and develop new microbial-based technology for use in industry.

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Why study xylan degradation?

Xylan (a major component of hemicellulose) and cellulose account for more than 50% of all plant biomass. Consequently, in total, both polymers together constitute the most abundant organic carbon resource on the planet [3,8,14,15,21,40,44]. They are products of photosynthesis and, as such, constitute an inexhaustible renewable resource. Coughlan [3] has suggested that the energy content of both xylan and cellulose, based upon estimates of the total global plant biomass, is equivalent to almost 640 billion tons of oil.

As major components of plant biomass, xylans play an important role in ruminant animal and insect nutrition where the polymer is bioconverted into small digestible molecules [2,19,20,25,38,42,43]. Likewise, development of biotechnological methods for polymer conversion *via* xylose (from xylan) into acetate, propionate, lactate, or succinate by using microbial fermentation technologies [4,25,27], could provide a fully renewable resource of feedstock molecules for the chemical and pharmaceutical industries [21]. With acetate, or with hydrogen and carbon dioxide (from polymer mineralization), methanogenic bacterial reactors could be used to produce inexhaustible supplies of methane, as a nonpolluting fuel source.

Our current knowledge of microbial action on xylans has already led to suggestions about new technologies that are ready for development in both agriculture and in industry [8,21,24,40,46]. Some examples of these applications suggested in a review by Gilbert and Hazlewood [8] were that: pretreatment of forage crops with xylanases and associated polymer degradation enzymes may be used to improve digestibility of ruminant feeds and to aid in composting; addition of xylan-degrading enzymes to pig and poultry cereal diets may improve nutrient utilization and intestinal absorption resulting in greater animal weight gain; and enzymatic saccharification of xylan in agricultural, industrial, and municipal wastes may be applied to obtain sugar supplements for human and animal consumption, or for

producing specialty products, such as artificial sweeteners. Maat *et al* [24] reported that treatment of poor grade wheat flour with *Aspergillus niger* xylanases improved dough texture and handling, and the quality and the flavor of the final baked product. Finally, results of numerous studies [6,21,26–28,47] have shown that pretreatment of paper pulps to partially degrade xylans aids in brightening the paper product. Treatment with xylanase appears to loosen lignin surrounding cellulose fiber bundles and thereby reduces use of environment-polluting chlorine in paper pulp bleaching processes [6,28,34,39].

Xylans

Xylans are major structural heteropolysaccharides in plants where they can represent up to 30% of the dry weight of the cell walls of monocots and hardwoods [41]. The polymer is second only to cellulose in abundance on Earth, and is thus a major reserve of reduced carbon in the environment where roughly 10^{10} metric tons are turned over annually. Unlike cellulose, xylans constitute a group of complex structural polymers collectively referred to as 'hemicelluloses'. They are described as alkali-soluble, linear or branched polysaccharides, precipitable from aqueous solution by alcohol, and more easily hydrolyzed by mineral acids than cellulose [44]. Isolated xylans are typically polydispersed heteropolysaccharides and comprise a backbone of β-1,4-linked D-xylopyranosyl residues. The xylopyranosyl backbone is substituted at positions C-2, C-3, and C-5 to varying degrees depending upon the plant and the stage of development of the plant when the polymer was obtained [16,46]. In monocots, at the C-2 position 1→2-linked α-D-glucuronic acid or 4-*0*-methyl-α-D-glucuronic acid might occur, while at C-3 of xylopyranose, one frequently finds 1→3-linked α-L-arabinofuranose. In some xylans, particularly in hardwoods, xylopyranose residues may be *0*-acetylated at the C-2 or (more commonly) the C-3 positions. Additionally, a small, but important amount of phenolic components, such as ferulic and *p*-coumaric acids (associated with lignin), may be esterified to xylan *via* their carboxyl groups to C-5 of arabinose branches [17].

In plants, xylans or the hemicelluloses are situated between the lignin and the collection of cellulose fibers underneath. Consistent with their structural chemistry and side-group substitutions, the xylans seem to be interspersed, intertwined, and covalently linked at various points with the overlying 'sheath' of lignin, while producing a coat around underlying strands of cellulose [2,15] *via* hydrogen bonding [16]. The xylan layer with its covalent linkage to lignin and its noncovalent interaction with cellulose may be important in maintaining the integrity of the cellulose *in situ* and in helping protect the fibers against degradation by cellulases.

Microbial biodiversity

Many bacteria and fungi are able to degrade xylan. Some of these microbes are saprophytic, free-living soil or aquatic cells, some grow anaerobically while others grow aerobically, some grow at room temperature (mesophilic conditions) while others grow thermophilicly, and some follow a host-specific lifestyle in ruminant animals or in the intestines of wood-eating insects.

Under mesophilic growth conditions, xylanolytic activity has been reported in a wide variety of different genera and species of fungi and yeast [5,10,46]. For example, xylan degradation occurs in certain strains of *Aspergillus niger*, *Aspergillus funigatus*, *Neurospora crassa*, *Trichoderma harzianum*, *Trichoderma reesei*, *Trichoderma viride*, *Penicillium janthinellum*, *Penicillium wortmanni*, *Penicillium capsulatum* [7], *Pichia stipitis*, *Aureobasidium pullulans*, *Candida shehatae*, and *Fusarium oxysporum* [2,46]. Élisashvili [5] describes and references a long list of additional xylanolytic fungi. Thermophilic fungi that degrade xylans include *Humicola lanuginosa*, *Thermoascus aurantiacus*, *Sporotrichium thermophile*, and *Talaromyces byssochlamydoides* [35].

As among the fungi and yeasts, xylanolytic degradation also extends across bacterial generic lines involving both Gram-positive and Gram-negative staining aerobic and anaerobic microbes, including cell types that live in extreme environments.

Gram-positive staining, spore-forming bacteria are ubiquitous soil microbes that play important roles in plant and animal biomass turnover. These spore-forming bacteria either respire and grow aerobically, or grow under anaerobic conditions and ferment xylan with production of volatile fatty acid and gas products. Among aerobic, or facultative anaerobic species, xylanolytic activity has been reported in *Bacillus subtilus*, *Bacillus circulans*, *Bacillus pumilus*, and *Bacillus polymyxa* [11,46]. *Streptomyces* species with xylanolytic activity include *Streptomyces exfoliatus*, *Streptomyces flavogriseus*, *Streptomyces lividans*, *Streptomyces xylophagus*, and *Streptomyces halstedii* JM8 [33,46]. Strictly anaerobic, fermenting microbes, which grow under mesophilic conditions, have also been reported, such as *Clostridium acetobutylicum*, *Clostridium stercorarium*, and *Clostridium papyrosolvens* C7 [27,46].

The Gram-negative staining, aerobic, non-spore-forming soil microbe, *Pseudomonas fluorescens* subsp *cellulosa*, has also been shown to degrade xylans [10,18]. Other pseudomonas-type xylanolytic Gram-negative staining bacteria seem to reside principally in ruminant animals. These cells represent a large, fastidiously anaerobic group of cells that include *Butyrivibrio succinogenes*, *Butyrivibrio fibrisolvens*, *Bacteroides ruminicola*, *Bacteroides ovatus*, and *Ruminococcus albus* [10,25,38,42,43]. *Aeromonas caviae* ME-1 was recently isolated from the intestine of a herbivorous insect [19,20]. Finally, *Cytophaga xylanolytica* sp nov [12], a new cytophaga species which grows anaerobically warrants additional comment.

The cytophagas are normally aerobic, Gram-negative, non-fruiting, rod-shaped bacteria that exhibit gliding motility and are commonly found on forest litter where they degrade a diverse collection of plant and insect biopolymers, including xylans, cellulose, and chitin. *C. xylanolytica* grows luxuriously under anaerobic conditions with xylan and other mono-, di- and polysaccharides (but not with cellulose) as sole carbon and energy source [13]. Its specificity for xylan and its anaerobic lifestyle makes it an interesting candidate for use for methane formation in xylan-driven anaerobic methanogenic biodigesters.

In addition to *meso*-temperature conditions and environments near neutral pH values, a host of unidentified bacteria inhabit *extreme* environmental conditions where they thrive and grow at temperatures above 50°C, at pH values 9.0 or greater [9,23,27,35,45,46], and/or in high ionic strength aqueous systems containing salt approaching saturating concentrations [46,47]. *Bacillus stearothermophilus* [9] is one of the aerobic, thermophilic bacteria shown to actively degrade plant polymers, including xylan. *B. stearothermophilus* is a Gram-positive staining, aerobic, spore-forming microbe. Anaerobic, spore-forming, thermophilic cells include *Clostridium thermocellum*, *Clostridium thermohydrosulfuricum* and *Clostridium thermosaccharolyticum* [2]. Additional anaerobic cells that grow and thrive at high temperature with xylan include *Thermoanaerobacter ethanolicus*, *Thermoanaerobacter acetigenum* [27], *Thermoanaerobium brockii*, *Thermoanaerobacterium* sp strain JW/SL-YS485 [35], and *Thermobacteroides* species [2].

Use of microorganisms at temperatures above 50°C and in alkaline conditions is especially desirable for kraft pulp treatment in the paper industry [6,28,47]. For this purpose, hyperthermophilic eubacteria have been isolated that grow anaerobically at temperatures above 80°C. These microbes include *Thermotoga maritima* MSB8 [45], *Thermotoga* sp strain FjSS3-B.1 [34], *Caldocellum saccharolyticum* [23], *Dictyoglomus* species [26,36], and *Rhodothermus marinus* [4]. During growth on xylan these cells produce fermentation products such as acetate, sometimes lactic acid (depending on the cell type), ethanol, H_2 and CO_2. Xylanolytic enzymes in these hyperthermophilic cells operate around neutral pH. Yang *et al* [47] recently published the results of studies on a *Bacillus* sp that degrades birch wood xylan around pH 9. However, xylanolysis by this cell occurred under modest thermophilic conditions at 50°C.

Xylanases in microorganisms

As suggested in previous discussion, microorganisms are primarily responsible for xylan degradation in nature. The complex chemical structure of xylan has been described as a linear polymer of repeating xylopyranosyl groups substituted at various carbon positions with different sugars and/or acidic compounds. As a result, complete and efficient enzymatic hydrolysis of the complex polymer requires that the microbial cell produce an array of enzymes with diverse specificity and modes of action. Some of the enzymes known to be involved in xylan degradation are: endo-1,4-β-xylanases, which hydrolyze the β-1,4-linked xylose backbone; β-xylosidases, which hydrolyze xylobiose and other short xylooligosaccharides resulting from action of endoxylanase; debranching enzymes such as α-glucuronidase and α-L-arabinofuranosidase; and esterases, such as acetyl- and xylan-acetylesterases and arylesterases which act to remove acetyl and phenyl side groups, respectively [1,2,46].

Because of the complex chemical nature of plant xylans, it is not surprising for xylan-degrading cells to produce an arsenal of polymer-degrading proteins. How this collection of enzymes occurs and how they interact together in a cell, or in a cell population, to degrade xylans is poorly understood. Some of the complexities the cell(s) must deal with, however, may become evident from a discussion of the diverse biochemical properties of two important types of xylan-degradation enzymes; endo-β-1,4-xylanase (henceforth referred to as xylanase) and α-L-arabinofuranosidase.

The xylanases

During searches to find the 'ideal' enzyme for use in specific commercial processes, more than 50 microbial xylanases have been isolated and studied [35]. Broadly based upon results of these studies, the molecular weights and the isoelectric point (pI) of the different proteins suggested that the enzymes might be divided into two groups. One group was comprised of enzymes with an MW less than 30 kDa and which had alkaline pI values ranging from 8.5 to 10.0. The larger enzymes of the second group with MW $\geqslant$45 kDa, generally exhibited pI values ranging from 4.5 to 7 [39,46]. Using this designation, for simplicity's sake, one could hope that a xylan-degrading microbe be equipped with one type of enzyme or the other. However, it was not long before neither this idea, nor this division of xylan-degrading enzymes appeared useful. For example, *C. stercorarium* was found to produce three 'diverse' xylanases with MW values of 44.0, 62.0, and 72.0 kDa, respectively, with all three proteins exhibiting pI values of 4.4–4.5. Another example of this diversity occurred in the fungus, *T. reesei*. *T. reesei* produced two xylanases with MW 19 and 21 with pI values of 5.2 and 9.0, respectively [37,39]. In addition to synthesizing a diverse collection of xylanolytic enzymes with different MWs and pI values, microbial cells also degraded the plant polymer under a variety of different environmental conditions.

The ordinary temperature optimum of both fungal and bacterial xylanases ranged from 45° to 60°C. However, since it is desirable to operate at even higher temperatures in many industrial applications, studies were undertaken to discover *hyper*-thermophilic bacteria (which grow above 80°C) that also metabolize xylans. As a result, a xylanase gene was obtained from the gene bank of the hyperthermophile *Thermotoga* sp strain FjSS3-B.1. The *Thermotoga* gene expressed in *Escherichia coli* attacked pine kraft pulp at 95°C, but the enzyme operated best at pH around 6.3, below a preferred pH value around 9.0 [34]. Other enzymes have also been examined from hyperthermophilic bacteria. However, in these cases, the enzymes, once removed from cells, were more active at temperatures lower than cell growth temperatures and at pH values around 7.0 [27,28,36].

Xylanase multiplicity and mixed function

During studies to isolate microorganisms with the ability to degrade xylans and to extract and purify their xylan-degrading enzymes, it became apparent that both fungal and bacterial cells produce a multiplicity of enzymes that may belong to the same functional class and which sometimes also exhibit broad plant polymer specificity similar to the cellulases [10,46]. One example of this enzyme multiplicity was studied in the fungus, *T. reesei* [46]. Results suggested that *T. reesei* produced four xylanases [46], each one with different MW and pI values. The question whether these enzymes, all with the same apparent function, were different gene products was examined by Törrönen *et al*

[37]. This research team successfully cloned two *T. reesei* genes, *xyn1* and *xyn2*, that appeared to encode separate products, XYL1 and XYL2. XYL1 and XYL2 exhibited similar MWs (19 and 21 kDa, respectively) but had pI values of 5.2 and 9.0. Both xylanases operated at pH around 4.5, but the XYL2 exhibited a V_{max} 16-fold faster than XYNI and XYNII.

In analogous studies using bacteria, Gosalbes *et al* [11] cloned genes *xynD* and *gluB* from *B. subtilis*. Data suggested that the *xynD* gene encoded a xylanase (XYND) and *gluB* encoded an endo-β-(1,3)-(1,4)-glucanase (GLUB). Both gene products exhibit xylanase activity. In this microbial system, however, XYND seemed to be multifunctional and also acted as an α-L-arabinofuranosidase. In a similar study, the single *B. fibrisolvens xylB* gene also encoded a *bi*functional protein with both β-D-xylosidase and α-L-arabinofuranosidase activities [38].

In her review, Thomson [36] presented an interesting discussion about the diversity of xylan-degrading enzymes and the role multiple xylanases may play in the cell. She suggested various mechanisms that could account for the multiplicity of function and specificity of the xylan-degrading enzymes. 'Multiple' proteins could, for example, arise from post-translational modification of a gene product through glycosylation and/or proteolysis, or the appearance of secondary, minor xylanases could be 'artifacts' produced during protein purification [36]. On the other hand, multiple enzymes with broad range specificity might be products of separate genes on the same, or on different operons coordinately controlled by 'global' regulation. According to Thomson, this arrangement might serve the cell as a mechanism of change in response to different types of xylans confronting it.

Nevertheless, the molecular basis for xylanase multiplicity and 'mixed' specificity in a single cell is unknown. In a recent study, Ruiz-Arribas *et al* [33] cloned a xylanase gene from *S. halstedii* JM8 that appeared to be transcribed to produce two xylanases, Xys1L and Xys1S. Xys1L and Xys1S exhibited similar reaction characteristics but the MW values of the proteins were 45 and 35 kDa, respectively. As a result, the researchers suggested that post-transcriptional modification of the gene product resulted in the two different xylanases. However, their suggestion needs to viewed with caution, since, at that time, they had not determined base sequence of the cloned gene.

Xylanase delivery systems

With the aim of developing effective xylan-degrading technology using whole cells in industry, it is important to understand the mechanism(s) of release of xylan-degrading enzymes into the system. At present, much of our understanding of xylanolytic enzyme action comes from studies on aerobic fungi and yeasts where xylan-degrading proteins are secreted by the cells. More recently, because of the remarkable diversity of enzyme systems in the bacteria, enzyme release from bacteria is also being explored.

As in fungi and yeast systems, bacteria also release xylan-degrading enzymes to their surroundings, but the mechanism may be quite different. In bacteria, for example, some cells appear to 'release' xylan-degrading enzymes in the form of 'protein complexes' or 'xylanosomes' [36], while other cells localize xylan-degrading enzymes on their outside surface.

The suggestion that certain bacteria produce structured enzyme aggregates, or xylanosomes [36], is analogous to the formation of cellulosomes in some cellulose-degrading clostridia [22]. It was first reported [36] in *B. fibrisolvens* cultures where it was released into the culture medium as an insoluble 'structure' exhibiting a molecular mass greater than 669 kDa. The *B. fibrisolvens* xylanosome appeared to consist of at least 11 xylanolytic active proteins ranging in size from MW 45 to 180 kDa. A related multiprotein complex was also reported to occur in the supernatant culture fluid of *C. papyrosolvens* C7 cells growing with cellulose [29]. Although neither xylanosome nor cellulosome was used to describe the *C. papyrosolvens* protein aggregates, they ranged in size from 500 to 660 kDa, containing both cellulolytic and xylanolytic activities.

In a different bacterial system, Shao *et al* [35] reported the localization of xylanase in the S-layer fraction of the anaerobic, thermophilic microbe, *Thermoanaerobacterium* sp strain JW/SL-YS 485. This appears to be the first report of xylanase association with the S-layer of a cell, although in an earlier study from a different laboratory [30], some xylanolytic activity was reported associated with growing *Cellulomonas uda* cells. A second example of a specific cell-wall associated xylan-degrading system has been suggested in the anaerobic, gliding bacterium *C. xylanolytica* [31]. In *C. xylanolytica*, an enzymatic cell association seems particularly appropriate to the lifestyle of the microbe as it glides over the surface of plant material and digests xylan underneath.

Role of α-L-arabinofuranosidase

Fungi, yeast, and bacteria with xylanolytic activity, in addition to enzymes that hydrolyze the xylan 'backbone' polymer into xylose, xylobiose, xylotriose and other short xylooligosaccharides, also form a roster of ancillary enzymes to cleave off polymer side groups. An example of one such enzyme is α-L-arabinofuranosidase (ARAF), which attacks α-L-arabinosyl side-chains of xylans and releases arabinose.

Ancillary enzymes that hydrolyze xylan side-chains exhibit biochemical biodiversity similar to the xylanases. As noted above, *bi*functional xylanase/ARAF enzymes have been reported [38] and enzyme multiplicity also occurs, albeit observed to a lesser extent than among the xylanases or the β-xylosidases. ARAF also comes in a wide range of MW values. The enzyme isolated and characterized from *B. stearothermophilus* T-6 has an MW of 256 kDa. It is comprised of four identical protein subunits, and operates best at 70°C and at pH 5.5–6.0 [9]. *C. xylanolytica* strain XN3 appears to produce a single similar ARAF. This enzyme has a molecular mass of 210 kDa comprising subunits of 56 kDa. At 45°C, *C. xylanolytica* ARAF exhibits maximal activity at pH 5.8, but the enzyme is stable over a pH range from 4.0 to 10.0. Finally, *P. capsulatum* produces two ARAF enzymes [7], Ara I and Ara II. Fungal Ara I and Ara II enzymes exhibit an MW of 64.5 and 62.7, respectively. Both enzymes operate maximally at pH 4.0 at 55–60°C.

The combined action of ARAF and other side chain-spe-

cific enzymes in the microbial xylan-degrading arsenal may work together with multiple xylanases and β-xylosidases to improve the efficiency of polymer metabolism. Studies suggesting that this is the case are cited in earlier reviews [1,2,36,46]. In 1994, Viikari *et al* [39] published the results of studies suggesting the cooperative action of two *T. reesei* xylanases on kraft pulp. However, as yet experiments have not provided a clear picture of how either the molecular or the biochemical basis for this cooperative action may occur. On this note, it is also interesting that the presence of multiple xylan-degrading enzymes may not be beneficial. For example, Maat *et al* [24] reported that treatment of poor quality flour with *A. niger* var *awamori* xylanase significantly improved the quality of dough and baked goods. However, fractionated fungal enzyme extracts specifically lacking ARAF activity produced the best results. Was it the absence of ARAF that made the difference and why?

Conclusion

In this review I have attempted to provide a glance at the remarkable variety of cells and the diversity of biochemical systems present in the microbial world that have evolved to degrade xylan. Clearly, during evolutionary history, microorganisms followed many different routes to grow at the expense of xylan. The array of different schemes and different enzymes for this purpose is in part a response to the complex structure of xylan itself. The resulting array of enzymes with their different physical properties, their multiplicity in a single cell, and their broad catalytic specificities contribute to our understanding of extraordinary microbial versatility and biochemical 'flexibility' that occurs in the biosphere.

The question of what new microbes will be found and what special traits they might possess will only be answered as we continue to explore the diversity of the microbial world [32].

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