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MINI-REVIEW

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Xylanases from fungi: properties and industrial applications

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Abstract Xylan is the principal type of hemicellulose. It is a linear polymer of β-D-xylopyranosyl units linked by (1–4) glycosidic bonds. In nature, the polysaccharide backbone may be added to 4-*O*-methyl-α-D-glucuronopyranosyl units, acetyl groups, α-L-arabinofuranosyl, etc., in variable proportions. An enzymatic complex is responsible for the hydrolysis of xylan, but the main enzymes involved are endo-1,4-β-xylanase and β-xylosidase. These enzymes are produced by fungi, bacteria, yeast, marine algae, protozoans, snails, crustaceans, insect, seeds, etc., but the principal commercial source is filamentous fungi. Recently, there has been much industrial interest in xylan and its hydrolytic enzymatic complex, as a supplement in animal feed, for the manufacture of bread, food and drinks, textiles, bleaching of cellulose pulp, ethanol and xylitol production. This review describes some properties of xylan and its metabolism, as well as the biochemical properties of xylanases and their commercial applications.

Introduction

Xylans are hemicelluloses and the second most abundant natural polysaccharide (Collins et al. 2005). These compounds are present in the cell wall and in the middle lamella of plant cells. This term covers a range of non-cellulose polysaccharides composed, in various proportions, of monosaccharide units such as D-xylose, D-mannose, D-glucose, L-arabinose, D-galactose, D-glucuronic acid and D-galacturonic acid. Classes of hemicellulose are named according to the main sugar unit. Thus, when a polymer is hydrolyzed and yields xylose, it is a xylan; in the same way, hemicelluloses include mannans, glucans, arabinans and galactans (Whistler and Richards 1970; Viikari et al. 1994; Uffen 1997; Ebringerova and Heinze 2000). In nature, wood hemicelluloses hardly ever consist of just one type of sugar. Usually they are complex structures made of more than one polymer, the most common being glucuronoxylans, arabinoglucuronoxylans, glucomannans, arabinogalactans and galactoglucomannans (Haltrich et al. 1996; Sunna and Antranikian 1997; Kulkarni et al. 1999b; Subramaniyan and Prema 2002). The amount of each component varies from species to species and even from tree to tree. Therefore, hemicellulose is not a well-defined chemical compound, but a class of polymer components of plant fibres, with properties peculiar to each one. Hemicelluloses mainly comprise xylans, which are degraded by xylanolytic enzymes. Fungal xylanases have many commercial uses, such as in paper manufacturing, animal feed, bread-making, juice and wine industries, xylitol production, etc. This review shall deal with recent advances in the understanding of fungal xylanases and in the industrial application of these enzymes.

Xylan structure

Arabinoxylans have been identified in wheat, rye, barley, oat, rice, sorghum, as well as in some other plants: pangola grass, bamboo shoots and rye grass. Although these polysaccharides are minor components of entire cereal grains, they constitute an important part of plant cell walls (Izydorczyk and Biliaderis 1995). Glucuronoxylans and glucuronoarabinoxylans are located mainly in the secondary wall and function as an adhesive by forming covalent and non-covalent bonds with lignin, cellulose and other

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polymers essential to the integrity of the cell wall. Xylans are the principal class of hemicelluloses in angiosperms contributing 15–30% of the total dry weight, but are less abundant in gymnosperms which contain 7–12% xylans (Haltrich et al. 1996).

Glucuronoxylans are linear polymers of β-D-xylopyranosyl units linked by (1–4) glycosidic bonds (xylose), as illustrated in Fig. 1a. This polysaccharide backbone has 4-*O*-methyl-α-D-glucuronopyranosyl units, D-glucuronosyl units methylated at position 4 and joined to position 2 or 3 of the β-D-xylopyranosyl. Angiosperm (hardwood) glucuronoxylans also have a high rate of substitution (70–80%) by acetyl groups, at position 2 and/or 3 of the β-D-

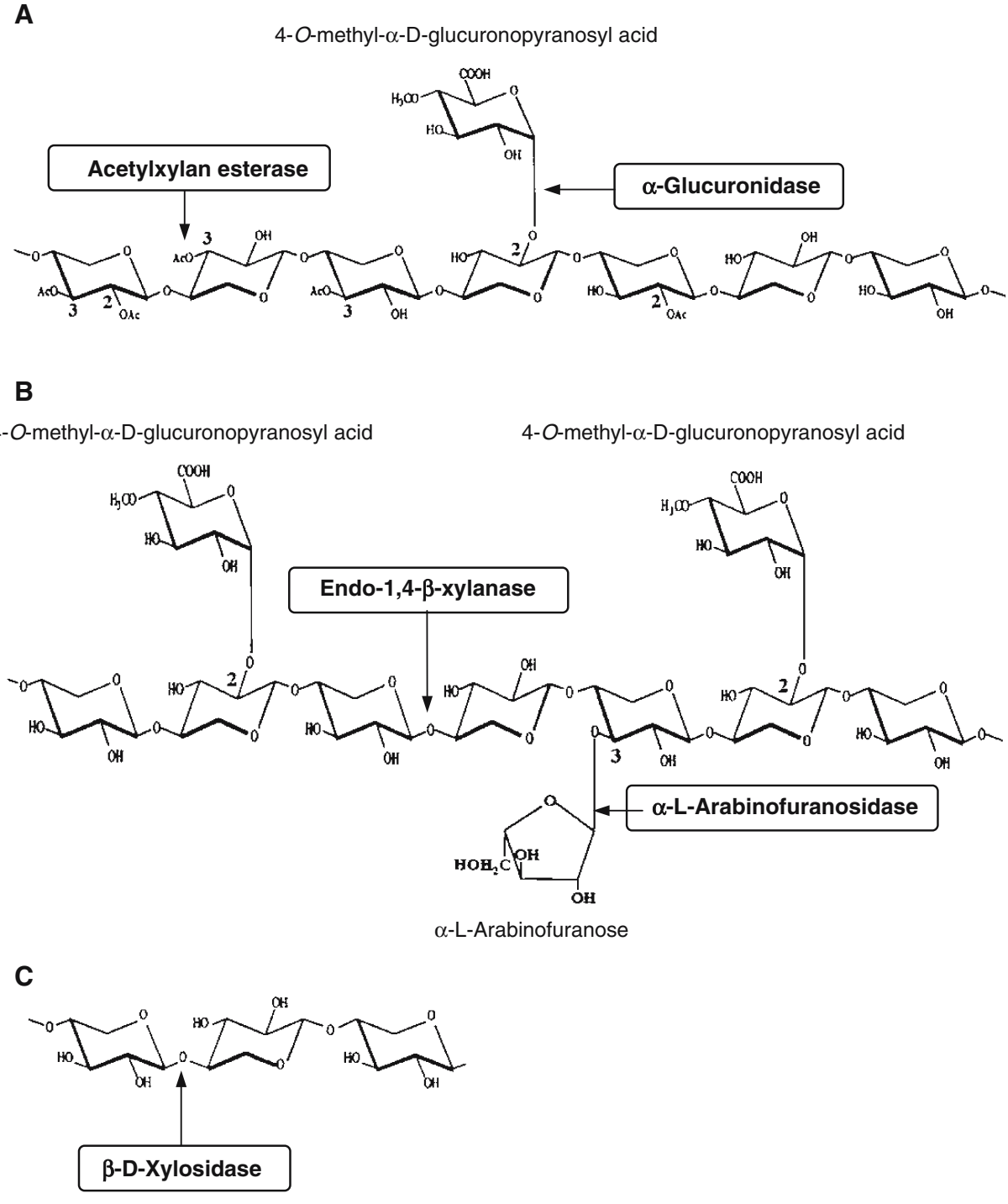

Fig. 1 Structure of the *O*-acetyl-4-*O*-methylglucuronoxylan (**a**), of hardwood and of the arabino-4-*O*-methylglucuronoxylan (**b**), of soft wood. Xylanolytic enzymes involved in the degradation of the xylan: acetylxylan esterase, α-glucuronidase, endoxylanase and α-L-arabinofuranosidase. Hydrolysis realized by β-xylosidase (**c**). The *numbers* indicate carbon atoms to which group substitutions are bound. *Ac* Acetyl group

xylopyranosyl, conferring on the xylan its partial solubility in water (Coughlan and Hazlewood 1993).

Glucuronoarabinoxylans, typically found in softwood, have the same xylan backbone, but in each, ten β-D-xylopyranosyl units are substituted by α-L-arabinofuranosyl (Fig. 1b). Softwood has a higher content of 4-*O*-methyl-α-D-glucuronopyranosyl units than hardwood. These xylans are not acetylated and, because of their furanoside structure, the arabinose side-groups are readily hydrolyzed by acid. Both glucuronoxylans and glucuronoarabinoxylans will be referred to as "xylans" from here on, following established practice (Ferreira-Filho 1994; Sunna and Antranikian 1997).

The xylanolytic complex

Xylanases catalyze the hydrolysis of xylans. These enzymes are produced mainly by microorganisms and take part in the breakdown of plant cell walls, along with other enzymes that hydrolyze polysaccharides, and also digest xylan during the germination of some seeds (e.g. in the malting of barley grain). Xylanases also can be found in marine algae, protozoans, crustaceans, insects, snails and seeds of land plants (Sunna and Antranikian 1997). Among microbial sources, filamentous fungi are especially interesting as they secrete these enzymes into the medium and their xylanase levels are very much higher than those found in yeasts and bacteria. Xylanase genes have been isolated from microorganisms of various genera and expressed in *Escherichia coli*. In bacteria xylanases are not only produced at lower activity levels than in fungi, but are also restricted to the intracellular or periplasmic fractions. Furthermore, enzymes expressed in bacteria are not subjected to post-translation modifications, such as glycosylation. Kulkarni et al. 1999a and Horikoshi 1996 reported the expression of extracellular xylanases in *E. coli* recombinants that express genes from alkalophilic *Aeromonas* and *Bacillus* species and alkalophilic and thermophilic *Bacillus* species. The heterologous expression of the gene *xyn* A, encoding an endoxylanase from *Bacillus*, in the yeast *Saccharomyces cerevisiae*, has also been described (Nuyens et al. 2001). Among fungi, the cloning and expression of endoxylanase genes from *Trichoderma reesei* and *Aspergillus kawachii* has been achieved in *S. cerevisiae* (Dalboge 1997).

Owing to its heterogeneous structure, xylan degradation needs not just one enzyme, but an enzyme complex. The components of this system that have been most extensively studied are the endoxylanases and the β-xylosidases. Ferulic acid esterase, *p*-coumaric acid esterase, acetylxylan esterase and α-glucuronidase were discovered only at the end of the 1980s, probably because of the difficulty of obtaining suitable substrates. These enzymes are present in fungi and bacteria, but until now few have been purified and analysed in terms of their physical and chemical properties. A description of these enzymes follows below.

Xylanolytic enzymes

Endo-1,4-β-xylanase

Endo-1,4-β-xylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) cleaves the glycosidic bonds in the xylan backbone, bringing about a reduction in the degree of polymerization of the substrate (Fig. 1b). Xylan is not attacked randomly, but the bonds selected for hydrolysis depend on the nature of the substrate molecule, i.e. on the chain length, the degree of branching, and the presence of substituents (Reilly 1981; Puls and Poutanen 1989; Li et al. 2000). Initially, the main hydrolysis products are β-D-xylopyranosyl oligomers, but at a later stage, small molecules such as mono-, di- and trisaccharides of β-D-xylopyranosyl may be produced. The hydrolysis of xylan by an endoxylanase may be written as follows:

$$H(C_5H_8O_4)_nOH + H_2O \Rightarrow H(C_5H_8O_4)_{n-p}OH + H(C_5H_8O_4)_pOH$$

This equation shows the stochiometry of a single hydrolytic event in a xylan molecule; such a reaction may occur at many points in the chain.

The endoxylanases have been classified in several ways. Wong et al. (1988) split them into two types, according to the end-products of the reaction: non-debranching enzymes, which do not hydrolyze at the 1,3-α-L-arabinofuranosyl branch-points of arabinoxylans, and thus do not liberate arabinose, and debranching enzymes, which do hydrolyze these side-branches, liberating arabinose. Each of these types has been found separately in a number of fungal species. There are, however, some fungi capable of producing both types of xylanases, resulting in a more efficient hydrolysis of xylan. Wong et al. (1988) also suggested a correspondence between classes of microbial endoxylanases and their physicochemical properties, such as molecular weight (MW) and isoelectric point (*pI*). They were divided into two groups: basic enzymes, with MW <30 kDa, and acid endoxylanases, with MW >30 kDa. It should be stressed, though, that this relation only works for about 70% of cases, the exact opposite being true of many known endoxylanases. Other classifications into various families, proposed for the glycosidases, was arranged by Henrissat and Bairoch (1993), and by Törrönen and Rouvinen (1997), as will be described later.

In general, the endoxylanases show peak activity between 40 and 80°C, and between pH 4.0 and 6.5, but optimal conditions have been found outside these ranges (Tables 1, 2). Individual fungi and bacteria can exhibit a multiplicity of endoxylanases; in some cases three or more enzyme activities have been separated from a single culture (Rizzatti et al. 2004). A number of factors may be responsible for the multiple forms often detected for endoxylanases. These include differential processing of

Table 1 Characteristics of some xylanases produced by different microorganisms. *ND* Not determined

Microorganisms	Molecular weight (kDa)	Optimal temperature (°C)	Optimal pH	Reference
Aspergillus aculeatus	18, 26, 52	50, 50, 70	4.0, 4.0, 5.0	Fujimoto et al. (1995)
Aspergillus awamori	39, 23, 26	45–55	4.0–5.5	Kormelink et al. (1993)
Aspergillus fischeri	31	60	6.0	Raj and Chandra (1996)
Aspergillus fumigatus	19, 8.5	55	5.5	Silva et al. (1999)
Aspergillus kawachii	35, 26, 29	60, 55, 50	5.5, 4.5, 2.0	Ito et al. (1992)
Aspergillus nidulans	22, 34	62, 56	5.5, 6.0	Fernández-Espinar (1994)
Aspergillus nidulans KK-99	ND	55	8.0	Taneja et al. (2002)
Aspergillus oryzae	35	60	5.0	Kitamoto et al. (1999)
Aspergillus sojae	33, 36	60, 50	5.0, 5.5	Kimura et al. (1995)
Aspergillus sp.	26	50	5.0	Khanna et al. (1995)
Aspergillus sydowii	33	50	4.0	Ghosh and Nanda (1994)
Aspergillus terreus	ND	50	7.0	Ghanen et al. (2000)
	ND	45	4.5	Ghareib and El Dein (1992)
Aspergillus versicolor	19	55	6.0	Carmona et al. (1998)
Acrophialophora nainiana	22	55	7.0	Salles et al. (2000)
Aureobasidium pullulans	25	54	4.4	Li et al. (1993)
Bacillus sp.	99	75	6.0	Bataillon et al. (2000)
Chaetomium cellulolyticum	25, 47, 57	50	5.0–7.0	Baraznenok et al. (1999)
Cryptococcus sp.	22	40	2.0	Iefuji et al. (1996)
Fusarium oxysporum F3	20.8, 23.5	60, 55	6.0	Christakopoulos et al. (1996)
H. grisea var. *thermoidea*	23	70	5.5	Monti et al. (1991)
Myceliophthora sp.	53	75	6.0	Chadha et al. (2004)
Penicillium brasilianum	31	ND	ND	Jørgensen et al. (2003)
Penicillium capsulatum	22	48	3.8	Ryan et al. (2003)
Penicillium sp.	25	50	2.0	Kimura et al. (2000)
Streptomyces sp.	24.5, 37.5, 38	55–60	6.0–8.0	Georis et al. (2000)
Thermoascus aurantiacus	ND	70–75	4.0–5.0	Kalogeris et al. (1998)
Thermomyces lanuginosus	24.7	70	6.0–6.5	Singh et al. (2000)
Trichoderma harzianum	20	50	5.0	Tan et al. (1985)
	60	70	4.0–4.5	

mRNA, post-translational modifications such as glycosylation and self-aggregation, and proteolytic digestion. Multiple endoxylanases can also be expressed by distinct alleles of one gene, or even by completely separate genes (Sung et al. 1995; Segura et al. 1998; Chavez et al. 2002).

β-D-Xylosidases

β-D-Xylosidases (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37) can be classified according to their relative affinities for xylobiose and larger xylooligosaccharides. Xylobiases and exo-1,4-β-xylanases can be recognized as distinct entities, as proposed by Biely (1985, 1993), but will be treated as xylosidases, that hydrolyze small xylooligosaccharides and xylobiose, releasing β-D-xylopyranosyl residues from the non-reducing terminus (Fig. 1c). Purified β-xylosidases usually do not hydrolyze xylan; their best substrate is xylobiose and their affinity for xylooligosaccharides is inversely proportional to its degree of polymerization. They are able to cleave artificial substrates such as *p*-nitrophenyl- and *o*-nitrophenyl-β-D-xylopyranoside. Transxylosilation activity has also been detected in fungi, resulting in products of higher MW than the original substrates (Kurakabe et al. 1997). An important role attributed to β-xylosidases comes into play after the xylan has suffered a number of successive hydrolyzes by xylanase. This reaction leads to the accumulation of short oligomers of β-D-xylopyranosyl, which may inhibit the endoxylanase. β-xylosidase then hydrolyzes these products, removing the cause of inhibition, and increasing the efficiency of xylan hydrolysis (Andrade et al. 2004; Zanoelo et al. 2004). Regarding the localization of β-xylosidases, those from filamentous fungi may be retained within the mycelium, only being detected in cell extracts, or liberated into the growth medium (extracellular). For instance, the β-xylosidases from *Humicola grisea* var. *thermoidea* (Almeida et al. 1995) and *Aspergillus phoenicis* (Rizzatti et al. 2001) were purified, respectively, from the cell extract and the culture medium. Those produced by bacteria and yeasts, however, are mainly associated with the cell. Fungal β-xylosidases are often monomeric glycoproteins, but some have been reported to possess two or three subunits (Sunna and Antranikian 1997). Generally these proteins

Table 2 Commercial xylanases produced by microorganisms. *SbmF* Submerged fermentation, *SSF* solid substrate fermentation, *n.c.* not cited

Commercial name	Distributors	Microorganism	Fermentation	Optimal pH	Optimal temperature (°C)	Application
Allzym PT	Alltech	*Aspergillus niger*	SbmF	5.3	65	Animal feed improvement
Amano 90	Amano Pharmaceutical		SSF wheat raw	4.5	50	Pharmaceutical analysis, food industry
Bio-Feed Plus	Novo Nordisk	*Humicola insolens*	SbmF	n.c.	n.c.	Animal feed
Resinase	A/S	n.c.	n.c.	n.c.	n.c.	Cellulose and paper industry
Bleachzyme	Biocon, India	n.c.	n.c.	6.5–7.0	40–50	
Cartazyme	Clariant, UK	*Termomonospora fusca*	n.c.	5.0	45–55	
EcopulpX-200	Primalco	*Trichoderma reesei*	SbmF	5.0–6.0	50–55	Cellulose pulp bleaching
Ecosane	Biotec		SbmF	n.c.	n.c.	Animal feed
Ecozyme	Thomas Swan, UK	n.c.	n.c.	7.0	50	Cellulose and paper industry
Grindazym GP e GV	Danisco Ingredients	*A. niger*	SbmF	n.c.	n.c.	Bird and pig feed
Irgazyme 40	Nalco-Genencor, Ciba, -Geigy	*Trichoderma longibrachiatum*	SbmF	n.c.	n.c.	Paper industry and animal feed
Multifect XL	Genencor	*T. longibrachia-tum*	SbmF	5.0–5.5	55–60	Food industry
Pulpzyme	Novozymes, Denmark	*Bacillus* sp.		9.5	50	Cellulose and paper industry
Solvay pento-nase	Solvay Enzymes	*T. reesei*	SbmF	5.3–5.5	55	Starch and bread-making industries
Sternzym HC 46	Stern-Enzym		SSF	n.c.	n.c.	Bread-making
Sumizyme X	Shin Nihon	*Trichoderma koningii*	SSF wheat raw	5.0	55	Manufacture of mushrooms and vegetables extracts, bread-making, enzymatic peeling of cereals, animal feed
Xylanase	Seikagaku	*Trichoderma* sp.	SbmF	n.c.	n.c.	Carbohydrate structural studies
Xylanase	Granotec do Brazil	n.c.	n.c.	n.c.	n.c.	Weight decreasing in Cream-Crackers, better texture and taste, Wafer's uni-formity improvement
Xylanase GS35	Iogen	*T. reesei*	SbmF	4.5	40	Cellulose pulp bleaching, animal feed
Biobrite		n.c.	n.c.	5.0–6.0	55	Cellulose and paper industry

have relatively high MWs, between 60 and 360 kDa. Although a wide range of pH optima have been observed, most lie between 4.0 and 5.0. The optimum temperature can vary from 40 to 80°C, but most β-xylosidases give best assay results at 60°C. Their thermostability is highly variable and depends on the organism in question. A good example of a stable enzyme is that from *Aspergillus phoenicis*, which retained 100% of its activity after 4 h at 60°C or 21 days at room temperature (Rizzatti et al. 2001).

Acetylxylan esterase

Acetylxylan esterase (EC 3.1.1.6) removes the *O*-acetyl groups from positions 2 and/or 3 on the β-D-xylopyranosyl residues of acetyl xylan (Fig. 1a). This enzyme was a late discovery, probably because the alkaline extraction frequently employed with highly acetylated xylans, like those in hardwoods, tends to strip the acetyls from the xylan (Shao and Wiegel 1992; Blum et al. 1999; Caufrier et al. 2003). Acetylxylan plays an important role in the hydrolysis of xylan, since the acetyl side-groups can interfere with the approach of enzymes that cleave the backbone, by steric hindrance, and their elimination thus facilitates the action of endoxylanases.

Arabinase

Arabinase removes L-arabinose residues substituted at positions 2 and 3 of the β-D-xylopyranosyl. There are two types with distinct modes of action: exo-α-L-arabinofuranosidase (EC 3.2.1.55) which degrades *p*-nitrophenyl-α-L-arabinofuranosides and branched arabinans (Fig. 1b),

and endo-1,5-α-L-arabinase (EC 3.2.1.99) which only hydrolyzes linear arabinans (Kaneko et al. 1993; de Vries et al. 2000). Most arabinases investigated so far are of the exo type.

α-Glucuronidase

α-Glucuronidase (EC 3.2.1.-) hydrolyzes the α-1,2 bonds between the glucuronic acid residues and β-D-xylopyranosyl backbone units found in glucuronoxylan (Fig. 1a). Some microorganisms exhibit their maximum activity only in the presence of short glucuronoxylan substrates. However, the substrate specificity varies with the microbial source, and some glucuronidases are able to hydrolyze the intact polymer (Puls and Schuseil 1993; Tenkanen and Siika-aho 2000). It has also been noted that acetyl groups close to the glucuronosyl substituents can partially hinder the α-glucuronidase activity.

Ferulic acid esterase (EC 3.1.1.-) and *p*-coumaric acid esterase

Ferulic acid esterase (EC 3.1.1.-) and *p*-coumaric acid esterase (EC 3.1.1.-) cleave ester bonds on xylan; the first one cleaves between arabinose and ferulic acid side-groups, while the second one cleaves between arabinose and *p*-coumaric acid (Christov and Prior 1993; Williamson et al. 1998; Crepin et al. 2004).

Synergism between the enzymes of the xylanolytic complex

Synergistic and cooperative effects among the xylan-degrading enzymes enhance the susceptibility of the heteropolymeric xylan to be attacked by endoxylanases (van Peij et al. 1997; de Vries et al. 2000). Thus, adding acetylxylan esterase to xylan results in the liberation of acetic acid and a less acetylated xylan, affording greater accessibility for endoxylanase hydrolysis. On the other hand, small acetylated polymers, produced by endoxylanase, are the preferred substrates of the esterases. Complex substrates such as wheat bran, which contains quite large amounts of arabinoxylan, cannot easily be degraded by endoxylanases without being treated beforehand with α-arabinofuranosidase. Such enzymes, in association with endoxylanases, enhance the saccharification of arabinoxylan. As already mentioned, β-xylosidases may be responsible for removing xylooligosaccharides, the product inhibition of xylanase, allowing a more efficient hydrolysis of xylan. Therefore, for biotechnological purposes, the ideal microorganism would be one that produces an adequate amount of each of the enzymes of the xylanolytic complex.

The term "cellulosome" has been suggested for multienzyme complexes in bacteria, which are associated with the surface of the cell in various cellulolytic species (Bayer et al. 1994; Doi et al. 1994, 1998; Doi and Tamaru 2001; Doi et al. 2003; Doi and Kosugi 2004). The cellulosome is an entity that may mediate the adhesion of the bacterium to cellulose, allowing a more concentrated attack on the polymer substrate. By analogy, the term "xylanosome" is applied to structures observed as protein aggregates of many subunits. These complexes have a very high MW (500–600 kDa) and can consist of more than ten proteins with xylanolytic activity, some of which are endoxylanases. In a few microorganisms, a cellulosome may be associated with a xylanosome, forming large complexes responsible for the hydrolysis of both cellulose and xylan (Sunna and Antranikian 1997; Beg et al. 2001).

Xylanases from mesophilic and thermophilic sources

Research on microorganisms that utilize xylan, and on the enzyme systems involved, is becoming more and more relevant in ecological and economic terms. Xylanases are synthesized by mesophiles and thermophiles (Smith et al. 1991). Among the mesophilic fungi, the genera *Aspergillus* and *Trichoderma* are pre-eminent in xylanase production. In recent years, a lot of effort has been put into the isolation of thermophilic and even extremophilic microorganisms, since they produce enzymes of greater stability (Lasa and Berenguer 1993; Harris et al. 1997; Ishihara et al. 1997; Kalogeris et al. 1998; Andrade et al. 1999; Niehaus et al. 1999; Puchart et al. 1999; Maheshwari et al. 2000; Rizzatti et al. 2001; Bruins et al. 2001, Monti et al. 2003). Noted thermophilic fungi include *Chaetomium thermophile*, *Humicola insolens*, *Humicola lanuginosa*, *Humicola grisea*, *Melanocarpus albomyces*, *Paecylomyces variotii*, *Talaromyces byssochlamydoides*, *Talaromyces emersonii*, *Thermomyces lanuginosus* and *Thermoascus aurantiacus*. The xylanases from these fungi possess optimum temperatures between 60 and 80°C and are very stable in this range. These enzymes are usually glycoproteins and most show highest activity at an acid pH (4.5–6.5). They exist in a multiplicity of forms and the majority exhibit variable MWs in the range 6–38 kDa. Many endoxylanases from thermophiles have some degree of structural homology with those from mesophiles. A number of authors have tried to explain the thermostability observed in enzymes from thermophiles in terms of extra disulphide bridges, an N-terminal proline residue causing a reduction in conformational freedom, salt bridges and presence of hydrophobic side-chains (Turunen et al. 2001). Hakulinen et al. (2003) describes also some minor modifications responsible for the increased thermal stability of xylanases: (1) higher Thr/Ser ratio; (2) increased number of charged residues, especially Arg, resulting in enhanced polar interactions; and (3) improved stabilization of secondary structures involving a higher number of residues in the beta-strands, and stabilization of the alpha-helix region. Some xylanases improve their stability by compacting the protein structure with a higher number of ion pairs or aromatic residues on the protein surface, resulting in enhanced interactions. However, no definite conclusion has

been reached, since a phenomenon seen in one microorganism may not occur in another.

Xylanase production

Xylanases may be industrially produced in submerged liquid culture or on a solid substrate. Tables 1 and 2 summarize data on various commercial xylanases produced in these forms of culture, but it should be noted that about 80–90% of all xylanases are produced in submerged culture. In cultures on solid substrate, wheat bran and rice are regarded as inducers. Alternative substrates for enzyme production have also been reported, such as sugarcane bagasse, rice husks and wood pulp (Kadowaki et al. 1995; Damaso et al. 2000; Medeiros et al. 2000; Pandey et al. 2000; Singh et al. 2000; Anthony et al. 2003). In liquid culture, xylanase is produced in response to xylans from various sources (Gomes et al. 1994; Liu et al. 1999; Rani and Nand 2000). β-D-Xylopyranosyl residues can also act as an inducer of the xylanolytic complex (Ghosh and Nanda 1994; Rizzatti et al. 2001), but in some microorganisms this can give rise to discriminatory control, leading to catabolite repression of endoxylanases (Flores et al. 1996; Mach et al. 1996). Another compound often used as a potent inducer is β-methyl xyloside, a non-metabolizable structural analogue of xylobiose that can be made at low cost (Morosoli et al. 1987; Simão et al. 1997a,b). Induction of the xylanolytic system by other synthetic compounds, such as 2-*O*-β-D-xylopyranosyl D-xylose (Xylβ1-2Xyl), 3-*O*-β-D-xylopyranosyl D-xylose (Xylβ1-3Xyl) and 2-*O*-β-D-glucopyranosil D-xylose (Glcβ1-2Xyl), has also been described (Hrmová et al. 1991). The xylobioses, which are homodisaccharides (Xylβ1-2Xyl e Xylβ1-3Xyl), are potent inducers of endo-1,4-β-xylanase but fail to induce an enzyme of the cellulolytic complex, endo-1,4-β-glucanase. The opposite was true for the heterosaccharide Glcβ1-2Xyl. Such synthetic substrates serve as hybrid inducers, promoting the synthesis of both the enzyme complexes. The results of these studies suggest the existence of separate regulatory systems for the synthesis of cellulases and xylanases.

Specific xylanases are synthesized when microorganisms are cultured on xylan, whereas on cellulose the organisms produce cellulases in association with xylanases, perhaps because the cellulose substrate contains traces of hemicelluloses. In the textile and paper industries, it is important to be able to obtain xylanases free of cellulase activity, as it is necessary to extract hemicellulose from the natural fibres, without damaging the cellulose. Polysaccharides, such as xylan, are known to induce the activity of enzyme complexes in microorganisms (Biely 1985, 1993). This effect was questioned by many researchers for some time, as these macromolecules are unable to make contact with sites within the cell, in order to influence the regulation of gene expression. For induction of the xylanolytic enzymes to occur, there has to be physical contact between part of the regulatory machinery of the cell and the inducer; which suggests the existence of some recognition site on the cell surface. Constitutive xylanases, at relatively low levels of activity, are supposed to be responsible for the initial hydrolysis of xylan, producing small β-D-xylopyranosyl oligosaccharides such as xylobiose and xylotriose, among others (Kulkarni et al. 1999a,b, Biely 1985). Some authors then consider the xylobiose formed to be the true inducer of endoxylanase synthesis (Haltrich et al. 1996, Sunna and Antranikian 1997). With the help of β-xyloside permeases (Fig. 2), these oligosaccharides are transported into the cell, where they trigger the expression of the xylanolytic system genes. The permease activity of the in-

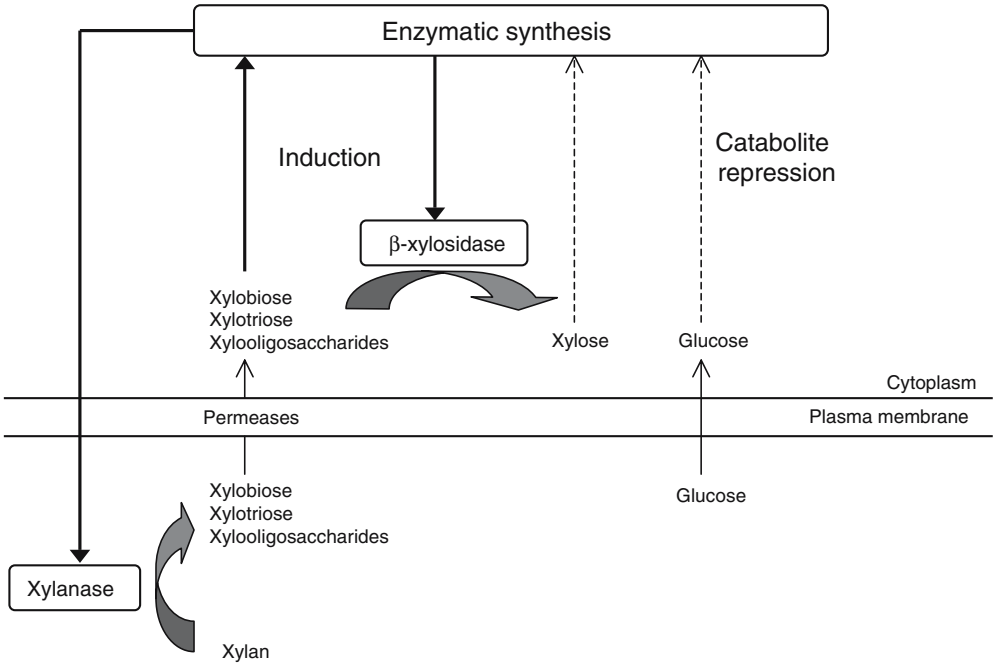

Fig. 2 Hypothetical scheme of the regulation of the xylanolytic complex involving endoxylanase and β-xylosidase. Constitutive endoxylanases degrade xylan to the xylooligosaccharides that through permeases enter the cytoplasm uncoupling the transcription of genes responsible for the production of endoxylanases and β-xylosidases

duced cells diminishes in the presence of glucose, but it is very efficient in the presence of the xylanolytic inducers. In filamentous fungi, carbon catabolite repression is mediated by the protein CREA (transcription repressor) (Dowzer and Kelly 1991; Fernández-Espinar et al. 1994; Piñaga et al. 1994; de Graaff et al. 1994).

Applications of xylanases

In recent years, the biotechnological use of xylans and xylanases has grown remarkably (Bhat 2000; Aristidou and Pentillä 2000; Subramaniyan and Prema 2000, 2002; Beg et al. 2000, 2001; Techapun et al. 2003). The end-products of xylan degradation of considerable importance in commercial applications are furfural and xylitol (Parajó et al. 1998). Xylan can be converted to β-D-xylopyranosyl and its oligosaccharides via two types of hydrolysis: acid or enzymatic. Acid hydrolysis is often preferred because it is faster, but it is accompanied by the formation of toxic compounds that may hinder subsequent microbial fermentation. Furthermore, in the long run, it can lead to corrosion of the metallic equipment that comes in contact with the acid. Recently, some industrial companies have shown interest in the development of efficient enzymatic processes to be used instead of acid hydrolysis in the treatment of material containing hemicellulose.

Commercial xylanases are industrially produced, for example, in Japan, Finland, Germany, Republic of Ireland, Denmark, Canada and the USA. The microorganisms used to obtain these enzymes are *Aspergillus niger*, *Trichoderma* sp. and *Humicola insolens*. Nevertheless, commercial xylanases can also be obtained from bacteria. Xylanase began to be used in the 1980s: initially in the preparation of animal feed and later in the food, textile and paper industries. Currently, xylanase and cellulase, together with pectinases, account for 20% of the world enzyme market. Table 2 summarizes some of the properties, sources and eventual applications of commercial xylanases.

Xylanases in animal feed

The use of enzymes in the production of feed is an important sector of agribusiness, with an annual world production exceeding 600 million tons and a turnover of >50 billion dollars. Xylanases are used in animal feed along with glucanases, pectinases, cellulases, proteases, amylases, phytase, galactosidases and lipases. These enzymes break down arabinoxylans in the ingredients of the feed, reducing the viscosity of the raw material (Twomey et al. 2003). The arabinoxylan found in the cell walls of grains has an anti-nutrient effect on poultry. When such components are present in soluble form, they may raise the viscosity of the ingested feed, interfering with the mobility and absorption of other components. If xylanase is added to feed containing maize and sorghum, both of which are low-viscosity foods, it may improve the digestion of nutrients in the initial part of the digestive tract, resulting in a better use of energy. The joint action of the rest of the enzymes listed produces a more digestible food mixture. Young fowl and swine produce endogenous enzymes in smaller quantities than adults, so that food supplements containing exogenous enzymes should improve their performance as livestock. Moreover, this kind of diet is found to reduce unwanted residues in the excreta (phosphorus, nitrogen, copper and zinc), an effect that could have a role in reducing environmental contamination.

Manufacture of bread, food and drinks

Xylanases may be employed in bread-making, together with α-amylase, malting amylase, glucose oxidase and proteases. The xylanases, like the other hemicellulases, break down the hemicellulose in wheat-flour, helping in the redistribution of water and leaving the dough softer and easier to knead. During the bread-baking process, they delay crumb formation, allowing the dough to grow. With the use of xylanases, there has been an increase in bread volumes, greater absorption of water and improved resistance to fermentation (Maat et al. 1992; Harbak and Thygesen 2002; Camacho and Aguilar 2003). Also, a larger amount of arabinoxylooligosaccharides in bread would be beneficial to health. In biscuit-making, xylanase is recommended for making cream crackers lighter and improving the texture, palatability and uniformity of the wafers.

The juice and wine industries make up a good part of the enzyme market. The production of fruit and vegetable juices requires methods of extraction, clearing and stabilization. In the 1930s, when the manufacture of citrus fruit juices began, the yields were low and problems were encountered in the filtration of the juice, owing to its turbidity. The increase in knowledge of the chemical constituents of fruits and the use of microbial enzymes helped to solve these problems. Nowadays, xylanases, in conjunction with cellulases, amylases and pectinases, lead to an improved yield of juice by means of liquefaction of fruit and vegetables; stabilization of the fruit pulp; increased recovery of aromas, essential oils, vitamins, mineral salts, edible dyes, pigments etc., reduction of viscosity, hydrolysis of substances that hinder the physical or chemical clearing of the juice, or that may cause cloudiness in the concentrate. Xylanase, in combination with endoglucanase, takes part in the hydrolysis of arabinoxylan and starch, separating and isolating the gluten from the starch in the wheat flour. This enzyme is also used in coffee-bean mucilage (Wong et al. 1988; Wong and Saddler 1993). The main desirable properties for xylanases for use in the food industry are high stability and optimum activity at an acid pH.

With the advances in the techniques of molecular biology, other uses of xylanases are being discovered. Recently, a recombinant yeast of wine was constructed with the gene for xylanase of *Aspergillus nidulans,xlnA*, resulting in a wine with a more pronounced aroma than is conventional (Ganga et al. 1999). During the manufacture of beer, the cellular wall of the barley is hydrolyzed re-

leasing long chains of arabinoxylans which increase the beer's viscosity rendering it "muddy" in appearance. Thus, xylanases are used to hydrolyze arabinoxylans to lower oligosaccharides diminishing the beer's viscosity and consequently eliminating its muddy aspect (Debyser et al. 1997; Dervilly et al. 2002).

Pharmaceutical and chemical applications

Xylanase and xylan are little used in the pharmaceutical industry. Xylanases are sometimes added in combination with a complex of enzymes (hemicellulases, proteases and others) as a dietary supplement or to treat poor digestion, but few medicinal products can be found with this formulation.

Hydrolytic products of xylan, such as β-D-xylopyranosyl residues, can be converted into combustible liquids (ethanol), solvents and artificial low-calorie sweeteners. The first steps are the delignification of hemicellulose material rich in xylan, followed by hydrolysis by xylanases and hemicellulases, to produce sugars such as β-D-xylopyranosyl units. Next, the products are fermented, mainly by yeasts (*Pichia stipitis* and *Candida shehatae*), as outlined in Fig. 3, to produce xylitol or ethanol (Shapack et al. 1987; Screenath and Jeffries 2000). Among the sugars used in the production of ethyl alcohol, β-D-xylopyranosyl residues represent between 5 and 20%. Xylitol is a polyalcohol with a sweetening power comparable to that of sucrose (Parajó et al. 1998). It is a non-cariogenic sweetener, suitable for diabetic and obese individuals and recommended for the prevention of osteoporosis and respiratory infections, lipid metabolism disorder, kidney and parenteral lesions. A variety of commercial products containing xylitol, such as chewing gum, can be found on the market. Although the enzymatic hydrolysis of xylan is a promising method of obtaining β-D-xylopyranosyl units, at present commercial xylitol is produced on a large scale by chemical catalysis. This is considered a high-cost process, mainly because the xylose has to be purified initially in several steps. Besides this, the chemical reactions often produce by-products toxic to fermentation; indeed, in the decomposition of lignocellulosic material, besides the liberation of sugars, products may be formed that are derived from the degradation of glucose (hydroxymethylfurfural), xylose (furfural) and lignin (aromatic and phenolic compounds and aldehydes). Substances liberated from the lignocellulose structure, such as acetic acid and extracted material (e.g. terpenes and their derivatives, tropolones and phenolic compounds such as flavonoids, stilbenes, quinones, lignans and tannins), or from the equipment (iron, chromium, nickel and copper), can be powerful inhibitors of microbial activity. The development of a more appropriate technology for xylitol production has generated great hope of its wider use in the food, pharmaceutical and odontological industries.

Textiles

The xylanolytic complex can be used in the textile industry to process plant fibres, such as hessian or linen. For this purpose, the xylanase should be free of cellulolytic enzymes. One process consists of incubating dried ramee (China grass) stems with xylanase to liberate the long cellulose fibres intact. After using this method, there is no need to use the strong bleaching step, since the lignin does not undergo oxidation, which would lead to darkening of the fibres (Prade 1995; Brühlmann et al. 2000; Csiszár et al. 2001). Relatively little research has been done on the enzymatic preparation of textile fibres, and yet this appears to be a promising market demanding the development of new techniques.

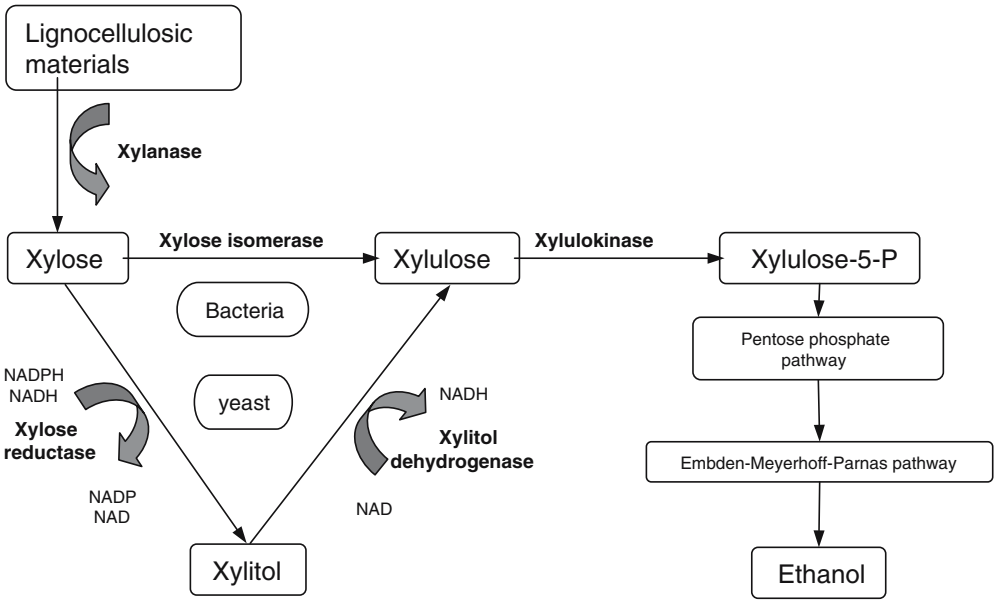

Fig. 3 Simplified scheme of xylitol and ethanol production by bacteria and yeasts from lignocellulosic materials

Cellulose pulp and paper

The main industrial application of the xylanases is in the bleaching of cellulose pulp. Enzymes began to be used in this sector during the last two decades, ever since peroxidases were applied to the degradation of lignin (Viikari et al. 1991; Wong and Saddler 1993; Araújo et al. 1999; Tenkanen et al. 1997; Bajpai 1999, Christov et al. 1999, 2000; Whitmire and Miti 2002, Sandrim et al. 2004). At present, in many countries, including Brazil, the chemical process, rather than enzymatic hydrolysis, is employed in paper manufacture. The usual method is known as the Kraft process, the name signifying strength or force in German. Three species of *Eucalyptus* (*E. grandis*, *E. saligna* and *E. urophylla*) are particularly favoured as the raw material.

The method starts with the pretreatment of wood shavings (Fig. 4a) with a combination of two reagents, sodium hydroxide and sodium sulphide, under 8 kgf/cm^2 and at 165°C, in a digestor. The two reagents in the cooking liquor serve to accelerate the delignification, with recovery of the cellulose fibres. At this stage, the cellulose pulp is known as brown mass, appearing dark in colour because of the black liquor (Fig. 4b). It can be assumed that 90–95% of the hemicellulose and lignin are dissolved and partially degraded during this process. Summarizing, we have:

- Wood shavings (fibres and lignin)+reagents= "cellulose" and lignin.
- Using industrial terminology: wood+white liquor ($NaOH+NaS_2$)="cellulose"+black liquor.

The deposited lignin confers a dark colour to the pulp and, after the brown mass is washed, pre-bleaching is carried out, consisting of the removal of minor impurities and a part of the lignin remaining from the cooking. Oxygen is used in this process (Fig. 4c), with the aim of reducing the cost of other reagents employed in the bleaching process. The pre-bleached pulp has a pale yellow colour and is not yet suitable for the production of good-quality paper for printing or writing. This staining is caused by residual lignin on the walls of the fibres. In the subsequent bleaching step, the paste attains a pure white colour, through the removal of light-absorbing substances (chromophores) formed by products of the breakdown of lignin. The bleaching process can be divided into three stages. In the first, ozone and chlorine dioxide are used (Fig. 4d) and in the second stage, sodium hydroxide, oxygen and hydrogen peroxide. In the last stage another treatment with chlorine dioxide is carried out (Fig. 4e).

The main advantage of the Kraft process is the possibility of recovering the chemical products from the black liquor. However, not all industries recover the sodium hydroxide and other organic materials present in the black liquor. On the other hand, the disadvantages are the high initial costs, the strong smell of gases emitted by the process, low yield (40–50%) and the high cost of bleaching. It can be seen that in the Kraft process polluting reagents are used in large amounts. The use of chlorine results in the production of organochlorines from the degradation products of lignin, which are highly toxic and mutagenic, requiring treatment of the effluents from the paper-making plant. Environmental regulations have restricted the use of chlorine compounds in bleaching processes in the paper and cellulose industries, especially in Western Europe and North America. Special attention has been given to using xylanase in pre-bleaching, which would lower the amount of chlorine compounds used by up to 30%, so that a 15–20% reduction in organochlorines in the effluents could be achieved. The utilization of xylanases could lead to the replacement of 5–7 kg of chlorine dioxide per ton of Kraft pulp and an average fall of 2–4 units in the kappa number, a measure of the lignin content in the cellulose pulp. Xylanases employed in paper technology do not need to be purified, but must be active at high temperatures and alkaline pH, and must not contain cellulolytic enzymes in order to preserve the cellulose fibres. The efficiency of microbial xylanase in the bleaching process has been studied for *Streptomyces thermoviolaceus* (Garg et al. 1996); *Streptomyces roseiscleroticus* (Patel et al. 1993); *Streptomyces* sp. (Beg et al. 2000; Georis et al. 2000); *Streptomyces galbus* (Kansoh and Nagieb 2004); *Bacillus* sp. (Kulkarni and Rao 1996; Shah et al. 1999); *Bacillus pumilus* (Bim and Franco 2000; Duarte et al. 2003); *Bacillus circulans* (Dhillon et al. 2000); *Aspergillus kawachii* (Tenkanen et al. 1997); *Aspergillus oryzae* (Christov et al. 1999); *Aspergillus niger* (Zhao et al. 2002); *Aspergillus nidulans* (Taneja et al. 2002); *Aspergillus fumigatus* (Lenartovicz et

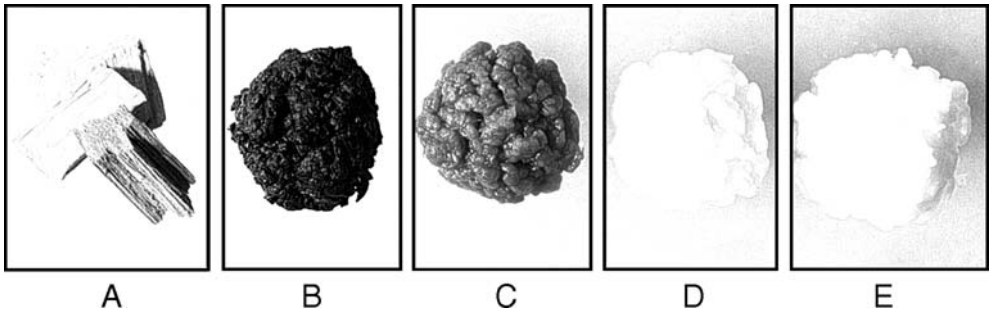

Fig. 4a–e Stages of the bleaching of cellulose pulp for paper production. Wood shavings (**a**), aspect of the cellulose pulp after the Kraft process (**b**), stage of pre-bleaching through the use of oxygen (**c**), cellulose pulp bleached with ozone and chlorine dioxide (**d**), stage using chlorine dioxide only, after the period of treatment with sodium hydroxide, hydrogen peroxide and oxygen (**e**)

al. 2002); *Chaetomium cellulolyticum* (Baraznenok et al. 1999); *Thermomyces lanuginosus* (Haarhoff et al. 1999); *Trichoderma reesei* (Oksanen et al. 2000); *Acrophilophora nainiana* and *Humicola grisea* (Salles et al. 2004).

There are two hypotheses about the role of xylanases in cellulose pulp bleaching. In the first, the xylanases would act on the xylan precipitated on the lignin (Viikari et al. 1994). This xylan is precipitated due to lowering of the pH at the end of the cooking stage. Its removal by the action of xylanases would leave the lignin more exposed to the compounds employed in the bleaching of cellulose pulp. The second hypothesis is based on the ability of lignin to form complexes with polysaccharides such as, for example, xylan, and the fact that some of the bonds are alkali-resistant and might not have been hydrolyzed during the Kraft process (Buchert et al. 1992). The xylanases act by cleaving the remaining bridges between the lignin and xylan, opening the structure of the cellulose pulp and leading to the fragmentation of xylan and subsequent extractions of the fragments (Paice et al. 1992). Treatment with xylanase renders the pulp more permeable to subsequent chemical extraction of the residual brown lignin and lignin-carbohydrate from the fibres.

Xylanases as protein families

Xylanases are glycosyl hydrolases that degrade xylan. They basically comprise two families of glycosyl hydrolases, called F and G or 10 and 11 (Henrissat and Bairoch 1993; Sapag et al. 2002). Xylanase from both families are known to occur in bacteria and eukaryotes, and are assumed to have different physiological roles (Biely et al. 1997). If they were paralogous sister molecule families, this would point to at least one early event of xylanase duplication in the evolution of life, before the separation between both putative basal clades of life, Eubacteria and (Archaea+Eukaryota). However, these two families of xylanases seem to have evolutionary origins different from other endoglucanases, i.e. family 10 more related to family 5, and family 11 more related to family 12 (Collins et al. 2005). Considering Biely et al.'s (1997) study, it seems more cautious to suggest that family 11 is more modified than family 10 in relation to an original, more versatile xylanase. Even though the characterization of families 10 and 11 of xylanases is largely phenetic, each family seems to correspond to well-defined clades. The structures of molecules of both families have been determined and compared (Murzin et al. 1995; Sato et al. 1999; Vardakou et al. 2003). Family 10 xylanases have a catalytic (which is a TIM barrel domain) and substrate-binding domains. The extensive research of Collins et al. (2005), however, shows that endoglucanases of families 5, 7, 8 and 43 also have some kind of, although less important, activity on xylans.

The family 10 xylanases found in species of different groups of bacteria and eukaryotes are not so dissimilar to each other, even though a number of subfamilies have been characterized (Jun et al. 2003). Family 11 xylanases, however, include highly specific and low MW xylanases that vary considerably between groups, with different *pI*, thermostability, pH profiles, structure, and catalytic properties (Törrönen and Rouvinen 1997). This resulted in a further classification of the xylanases into classes such as A, B, C, D, 1, 2, 3, I, II, III (Kulkarni et al. 1999a,b). This implies that phylogenic inferences made when using family 11 xylanases have to consider the high risks of using paralogous molecules, with potentially disastrous results in terms of correct phylogenetic inferences.

Chen et al. (1996) already detected problems of homology with regards to family 11 xylanases. Sapag et al.'s (2002) is the most comprehensive study to date of this problem; they show that there are at least ten subfamilies of xylanases, some of which are restricted to fungi (xylanases Ia, Ib, Ic, II, IIIa, IIIb, IV), and others to bacteria (A, B, C). Some genera of fungi and bacteria show more than one subfamily of xylanases. This means that the duplication of family 11 xylanases may have occurred before the divergence between Eubacteria and (Archaea+Eukarya), so more than one family 11 xylanase would be present in the same genome. However, it is possible that problems in the classification of xylanases may be due to noise in the analysis, the outcome of alignment problems and/or association of species based on algorithms that do not generate reliable phylogenetic reconstructions. In the recent study of Degefu et al. (2004) of a family 11 xylanase of the fungus *Helminthosporium turcicum*, a careful alignment and a parsimony analysis resulted in reliable results, in which all fungal xylanases fall together and reveal a pair of paralogous xylanases 11 within the fungus. Phylogenetic reasoning in applied biology is particularly important since it allows a much more efficient search for clades that share features of economic interest. In xylanase research, this refers to the search, in the appropriate clades, for new species with extremophilic properties and for homolog proteins with best efficiency in an applied sense.

Concluding remarks

Considering the world market in enzymes, we can see that dramatic changes will be necessary in the future in order to make national enzyme production more competitive. Great hopes are placed on technological advances, but there is also a search for new microorganisms, within the great biodiversity of this planet, that may possess better physiological characteristics in relation to temperature, pH of the medium and adaptability to low-cost substrates, which have been until now hardly exploited, such as sugarcane bagasse of which massive amounts are produced in Brazil. The enormous potential of extremophiles should be emphasized, considering their impact in several areas of application. It is worth remembering the impact of the discovery of Taq polymerase in molecular biology. Nevertheless, proteins from thermophilic fungi, as well as from extremophiles, are not yet being produced on an industrial scale, but we believe that this is one of the aspects of enzyme production that will be improved in the next few

decades. On the other hand, we cannot forget the undeniable contribution of recombinant DNA technology. In Brazil, a likely area in which xylanases will be used is in the paper industry. As mentioned above, large-scale Brazilian industries do not yet use xylanolytic enzymes in cellulose pulp bleaching. We believe that in the near future there will be a change in this, and that this stage in the production of paper will be rendered less harmful to nature, with a reduction in the liberation of pollutants in the effluents and consequently better preservation of our fauna and our flora.

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