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MINI-REVIEW

Fungal enzyme sets for plant polysaccharide degradation

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Abstract Enzymatic degradation of plant polysaccharides has many industrial applications, such as within the paper, food, and feed industry and for sustainable production of fuels and chemicals. Cellulose, hemicelluloses, and pectins are the main components of plant cell wall polysaccharides. These polysaccharides are often tightly packed, contain many different sugar residues, and are branched with a diversity of structures. To enable efficient degradation of these polysaccharides, fungi produce an extensive set of carbohydrate-active enzymes. The variety of the enzyme set differs between fungi and often corresponds to the requirements of its habitat. Carbohydrate-active enzymes can be organized in different families based on the amino acid sequence of the structurally related catalytic modules. Fungal enzymes involved in plant polysaccharide degradation are assigned to at least 35 glycoside hydrolase families, three carbohydrate esterase families and six polysaccharide lyase families. This mini-review will discuss the enzymes needed for complete degradation of plant polysaccharides and will give an overview of the latest developments concerning fungal carbohydrate-active enzymes and their corresponding families.

Keywords Polysaccharides · Plant biomass · Fungal enzymes · *Aspergillus* · *Trichoderma*

Introduction

Plant polysaccharides have applications in many industrial sectors, such as biofuel, pulp and paper, and food and feed. Cellulose, hemicelluloses, and pectin are the main components of plant cell walls representing up to 70% of the biomass (Jorgensen et al. 2007). Of the three, cellulose is the least complex polymer with a linear structure of β-1,4-linked D-glucose residues. The long glucose chains are tightly bundled together in microfibrils and are non-covalently linked together by hemicelluloses (Kolpak and Blackwell 1976; Carpita and Gibeaut 1993).

Hemicelluloses are classified according to the main sugar in the backbone of the polymer, i.e., xylan (β-1,4-linked D-xylose), mannan (β-1,4-linked D-mannose), or xyloglucan (β-1,4-linked D-glucose). The backbone of hemicelluloses has many branches composed of monomers such as D-galactose, D-xylose, L-arabinose, and D-glucuronic acid. The precise composition of hemicellulose is strongly dependent on the plant species and tissue (Scheller and Ulvskov 2010). For instance, hard wood xylans often have D-glucuronic acid attached to their backbone, whereas L-arabinose is the most common branching residue in cereal xylans (de Vries and Visser 2001). Moreover, hemicelluloses are often acetylated and to a lesser extent ester-linked with feruloyl or *p*-coumaroyl residues (Ebringerova et al. 1990; Xu et al. 2010).

Pectin is less prominently present in most plant biomass compared to cellulose and hemicellulose. However, some plant biomass types (e.g., citrus peels) are very rich in pectin (Angel Siles Lopez et al. 2010; Ridley et al. 2001; Grohmann and Bothast 1994). The backbone of pectin consists mainly of alpha-1,4-linked D-galacturonic acid residues that can be methyl-esterified or substituted with acetyl groups. Pectins are classified in three general groups, homogalacturonan (linear polymer), xylogalacturonan (branched by β-1,3-linked D-xylose), and rhamnogalacturonan (Ridley et al. 2001; Wong 2008; Caffall and Mohnen 2009). The latter polysaccharide is the most complex pectin structure. Its backbone consists of alternating L-rhamnose

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and D-galacturonic acid residues, while branches with β-1,4-linked D-galactose and different α-linked L-arabinose residues are connected to the L-rhamnose residues (Ridley et al. 2001; Wong 2008).

In nature, fungi play a central role in the degradation of plant biomass. Plant-biomass-degrading fungi produce an extensive set of carbohydrate-active enzymes specifically dedicated to degrade plant polysaccharides. However, these sets differ between fungal species. For instance, *Trichoderma reesei* has a highly efficient set of enzymes involved in cellulose degradation (Martinez et al. 2008; Kubicek et al. 2011), while *Aspergillus* species produce many enzymes to degrade pectin (Martens-Uzunova and Schaap 2009). The industrial importance of polysaccharide-degrading enzymes and the availability of many fungal genomes have strongly deepened our understanding of fungal biodiversity with respect to plant cell wall degradation. This mini-review will give an overview of the latest developments and insights into fungal enzymes involved in plant polysaccharide degradation.

Dedicated fungal toolboxes for the degradation of specific plant polysaccharides

Carbohydrate-active enzymes can be organized in different families based on amino acid sequence of the structurally related catalytic modules (www.cazy.org) (Cantarel et al. 2009; Henrissat 1991). Fungal enzymes involved in plant polysaccharide degradation are assigned to at least 35 glycoside hydrolase (GH) families, three carbohydrate esterase (CE) families, and six polysaccharide lyase (PL) families (Battaglia et al. 2011; Coutinho et al. 2009). Even though enzymes within the same family share sequence similarity, some families can contain multiple activities. For example, GH5 contains many catalytic activities, including exoglucanases, endoglucanases, and endomannanases (Dias et al. 2004). In addition, a specific enzyme activity can be present in several CAZy families. This is important for efficient degradation of plant polysaccharides as enzymes of each family have often complementary substrate specificity. For instance, endoxylanases in GH10 have lower substrate specificity and can degrade xylan backbones with many substitutions, while GH11 endoxylanases have higher substrate specificity with preference for unsubstituted xylan chains (Pollet et al. 2010; Biely et al. 1997).

Annotation of carbohydrate-active enzymes has been done for many fungal genomes (Pel et al. 2007; Espagne et al. 2008; Battaglia et al. 2011; Ohm et al. 2010; Martinez et al. 2004; Martinez et al. 2008). As an illustration, Table 1 shows a comparison of carbohydrate-active enzymes involved in plant polysaccharide degradation of 13 fungal genomes, including industrial fungi such as *Aspergillus oryzae*, *Aspergillus niger*, *Penicillium chrysogenum*, *T. reesei*, and *Saccharomyces cerevisiae*. Most apparent from this table is the correlation between habitat and the amount of carbohydrate-active enzymes. For example, the Saccharomycete *S. cerevisiae* does not require extracellular enzymes for polysaccharide degradation to survive in its natural niches like surfaces of rotting fruits (Liti et al. 2009; Cherry et al. 1997). This fungus has therefore hardly any carbohydrate-active enzymes. Another Saccharomycete *Pichia stipitis* can be found, among other places, in the guts of termites that inhabit and degrade white-rotted hardwood (Jeffries et al. 2007). The genome of this fungus contains only a few β-glucosidases and β-mannosidases to degrade glucan and mannan oligosaccharides, which are present in the termite guts (Jeffries et al. 2007). In contrast to both Saccharomycetes, the filamentous fungi in Table 1 have a lifestyle involving degradation of plant biomass and feeding from plant polysaccharides. The genomes of these fungi therefore contain many more genes encoding carbohydrate-active enzymes. For example, the saprobe *A. niger* has 178 putative GH enzymes, nine putative CE enzymes, and 13 putative PL enzymes involved in plant biomass degradation (Coutinho et al. 2009). However, there is also variation within this group of fungi. For instance, Aspergilli have a large number of enzymes involved in pectin degradation, in contrast to the cellulose-degrading specialist *T. reesei* and the lignin-degrader *Phanerochaete chrysosporium*. The Zygomycete *Rhizopus oryzae* has a different set of carbohydrate-active enzymes compared to the other filamentous fungi (Battaglia et al. 2011). For instance, the difficulty of this fungus to grow on xylan substrates is reflected in its absence of genes required for xylan degradation. This fungus has therefore also been described as a fast grower on easily accessible and digestible substrates (Richardson 2009).

To have a better impression of the latest developments regarding fungal carbohydrate-active enzymes, the following sections will discuss the enzymes needed for each of the main polysaccharides: cellulose, hemicellulose, and pectin. Furthermore, as an illustration of a fungal enzyme set, each section will show the genes encoding characterized and putative polysaccharide-degrading enzymes of *A. niger*.

Cellulose degradation

Cellulose degradation requires three classes of enzymes, β-1,4-endoglucanases (EGL), exoglucanases/cellobiohydrolases (CBH), and β-glucosidase (BGL), which are divided over eight GH families (Fig. 1; Vlasenko et al. 2010; de Vries et al. 2011). For example, *A. niger* has five EGLs within GH families 5 and 12, four CBHs in families 6 and 7, and 13 BGLs in families 1 and 3 (Table 2). In comparison, one of the

Table 1 Number of putativegenes involved plant polysaccharide degradation of 13 fungal genomes based on the CAZy database (http://www.cazy.org)

Total putative genes involved in plant polysaccharide degradation				AN	An	Ao	PC	Fg	Nc	Pa	Tr	Ps	SC	Pc	Sc	Ro
Glycoside hydrolases (GHs)				194	178	207	158	190	119	161	113	33	29	140	183	70
Carbohydrate esterases (CEs)				10	9	15	7	17	10	17	6	2	1	11	14	9
Polysaccharide lyases (PLs)				20	13	20	9	20	3	7	0	0	0	0	13	0
Substrate	Enzyme activity	Code	CAZy fam.	AN	An	Ao	PC	Fg	Nc	Pa	Tr	Ps	SC	Pc	Sc	Ro
Cellulose	β-1,4-endoglucanase	EGL	GH5,7,12,45	20 (7)	15 (6)	20 (5)	18	20	14	22 (10)	13	4 (0)	5 (0)	31 (5)	22 (4)	12 (5)
	Cellobiohydrolase	CBH	GH6,7	5 (4)	4 (4)	4 (3)	3	3	8	10 (6)	3	0 (0)	0 (0)	10 (7)	3 (3)	0 (0)
	β-1,4-glucosidase	BGL	GH1,3	22 (19)	20 (15)	21 (18)	20	24	10	12 (9)	15	5 (4)	0 (0)	13 (9)	15 (12)	6 (5)
Xyloglucan	Xyloglucan β-1,4-endoglucanase	XEG	GH12,74	3	4	4	3	5	2	3	3	0	0	6	2	0
	α-arabinofuranosidase	ABF	GH51,54	4	5	4	4	3	2	1	2	0	0	2	2	0
	α-xylosidase	AXL	GH31	10	7	10	11	8	5	5	4	3	1	6	4	3
	α-fucosidase	AFC	GH29,95	3	3	3	1	3	0	0	4	0	0	1	3	0
	α-1,4-galactosidase	AGL	GH27,36	7	7	6	3	5	1	3	10	0	0	3	1	6
	β-1,4-galactosidase	LAC	GH2,35	14 (7)	11 (5)	14 (8)	8	14	7	8 (4)	8	2 (1)	0 (0)	5 (2)	8 (4)	1 (1)
Xylan	β-1,4-endoxylanase	XLN	GH10,11	5	5	8	4	8	6	14	5	1	0	7	6	0
	β-1,4-xylosidase	BXL	GH3,43	37 (11)	28 (6)	38 (11)	31	38	16	21 (5)	15	5 (1)	0 (0)	15 (2)	31 (2)	8 (0)
	α-arabinofuranosidase	ABF	GH51,54	4	5	4	4	3	2	1	2	0	0	2	2	0
	Arabinoxylan arabinofuranohydrolase	AXH	GH62	2	1	2	1	1	0	2	1	0	0	0	1	0
	α-glucuronidase	AGU	GH67,115	2	1	5	1	3	2	4	2	1	0	1	2	0
	α-1,4-galactosidase	AGL	GH27,36	7	7	6	3	5	1	3	10	0	0	3	1	6
	β-1,4-galactosidase	LAC	GH2,35	14 (7)	11 (5)	14 (8)	8	14	7	8 (4)	8	2 (1)	0 (0)	5 (2)	8 (4)	1 (1)
	Acetyl xylan/feruloyl esterase	AXE/FAE	CE1	4	5	5	2	5	7	14	3	1	1	5	9	0
Galactomannan	β-1,4-endomannanase	MAN	GH5,26	18 (8)	11 (2)	14 (3)	14	13	8	13 (2)	8	4 (0)	5 (0)	20 (4)	19 (2)	7 (1)
	β-1,4-mannosidase	MND	GH2	10 (3)	6 (3)	7 (3)	6	11	5	7 (1)	7	2 (1)	0 (0)	2 (2)	4 (3)	0 (0)
	β-1,4-galactosidase	LAC	GH2,35	14 (7)	11 (5)	14 (8)	8	14	7	8 (4)	10	2 (1)	0 (0)	5 (2)	8 (4)	1 (1)
	α-1,4-galactosidase	AGL	GH27,36	7	7	6	3	5	1	3	8	0	0	3	1	6
Pectin	Endo-/exo-(rhamno)galacturonases		GH28	10	22	20	5	6	2	0	4	0	1	4	3	18
	α-rhamnosidase	RHA	GH78	9	8	8	5	7	0	1	1	0	0	1	3	0
	α-arabinofuranosidase	ABF	GH51,54	4	5	4	4	3	2	1	2	0	0	2	2	0
	Endoarabinanase	ABN	GH43	18 (4)	11 (5)	20 (5)	14	17	7	10 (5)	2	0 (0)	0 (0)	4 (2)	19 (12)	2 (2)
	Exoarabinanase	ABX	GH93	2	1	3	2	2	2	3	0	0	0	0	2	0
	β-1,4-endogalactanase	GAL	GH53	1	1	1	1	1	1	1	0	0	0	1	1	0
	Unsaturated glucuronyl hydrolase	UGH	GH88	3	1	3	0	1	0	0	0	0	0	1	1	0
	Unsat.-rhamnogalacturonan hydrolase	URH	GH105	4	2	2	2	3	1	0	1	0	0	0	3	0
	β-1,4-xylosidase	BXL	GH3,43	37 (11)	28 (6)	38 (11)	31	38	16	21 (5)	14	5 (1)	0 (0)	15 (2)	31 (2)	8 (0)
	β-1,4-galactosidase	LAC	GH2,35	14 (7)	11 (5)	14 (8)	8	14	7	8 (4)	8	2 (1)	0 (0)	5 (2)	8 (4)	1 (1)
	Pectin lyase	PEL	PL1	9	6	12	5	9	1	4	0	0	0	0	5	0
	Pectate lyase	PLY	PL1,3,9	15	10	16	6	17	2	6	0	0	0	0	10	0

Table 1 (continued)

Total putative genes involved in plant polysaccharide degradation			AN	An	Ao	PC	Fg	Nc	Pa	Tr	Ps	SC	Pc	Sc	Ro
	Rhamnogalacturonan lyase	RGL	PL4,11	5	3	4	3	3	1	1	0	0	0	0	3	0
	Pectin methyl esterase	PME	CE8	3	1	5	2	6	1	1	0	0	0	2	2	6
	Rhamnogalacturonan acetyl esterase	RGAE	CE12	2	2	4	2	3	1	1	0	0	0	0	2	0
	Feruloyl esterase	FAE	CE1	4	5	5	2	5	7	14	3	1	1	5	9	0
Starch	α-amylase	AMY	GH13	13	16	17	16	8	10	9	5	5	9	9	13	4
	glucoamylase	GLA	GH15	2	2	3	3	3	2	3	2	1	1	2	3	6
	α-1,4-glucosidase	AGD	GH31	10	7	10	11	8	5	5	4	3	1	6	4	3
Inulin	Inulinase	INU	GH32	2	4	4	7	5	1	0	0	0	1	0	1	0

Total values obtained per enzyme activity correspond to the theoretical maximum number of active enzymes that could be obtained by adding up all the members of the concerned families. The values between brackets indicate a more precise estimate as taking into consideration the functional assignments based on sequence similarities to biochemically characterized enzymes. These values are to be taken as a maximum rather than as absolute values. AN=*Aspergillus nidulans* A4 (Coutinho et al. 2009), An=*Aspergillus niger* CBS513.88 (Coutinho et al. 2009), Ao=*Aspergillus oryzae* RIB4 (Coutinho et al. 2009), PC=*Penicillium chrysogenum* Wisconsin 54–1255 (van den Berg et al. 2008), Fg=*Fusarium graminearum*/*Gibberella zeae* PH-1 (Cuomo et al. 2007), Nc=*Neurospora crassa* OR74A (Galagan et al. 2003), Pa=*Podospora anserina* S mat+(Espagne et al. 2008), Tr=*Trichoderma reesei*/*Hypocrea jecorina* QM6A (Martinez et al. 2008), Ps=*Pichia stipitis* CBS6054 (Jeffries et al. 2007), SC=*Saccharomyces cerevisiae* S288C (Cherry et al. 1997), Pc=*Phanerochaete chrysosporium* RP78 (Martinez et al. 2004), Sc=*Schizophyllum commune* H4-8 (Ohm et al. 2010), Ro=*Rhizopus oryzae* 99–880 (Battaglia et al. 2011)

most efficient cellulose-degrading fungi *T. reesei* has five characterized EGLs within GH families 5, 7, 12, and 45, two highly expressed CBHs in families 6 and 7, and two characterized BGLs in families 1 and 3 (Martinez et al. 2008; Kubicek et al. 2011). Although *T. reesei* does not have the biggest number of cellulases, its set of enzymes is very efficient in breaking down cellulose by acting synergistically (Ward et al. 1993). The family numbers are also used in the nomenclature of hydrolytic enzymes (Henrissat et al. 1998). For example, the first three letters of endoglucanase Cel5A of *T. reesei* refer to its substrate cellulose, the number to glycoside hydrolase family 5 and the last capital letter indicates that this was the first enzyme reported of this family with this activity. This review will mainly refer to the original functionally based nomenclature. We will therefore use for instance EGII instead of Cel5A, based on the consideration that these names were mainly used in database depositions and give a clearer indication of function.

EGI (GH7) and EGII (GH5) are the most abundantly produced of the five EGLs of *T. reesei* (Foreman et al. 2003; Vlasenko et al. 2010). Both enzymes have a carbohydrate-binding module which greatly enhances the efficiency in degradation of cellulose microfibrils by binding to cellulose (Beckham et al. 2010; Guillen et al. 2010). EGIII, from GH12, is expressed at a lower level than EGI and EGII, but has a broad activity against cellulose, β-1,3-1,4-glucan, xyloglucan, and xylan (Sprey and Bochem 1993; Eriksson et al. 2002). Two proteins from GH61 were expressed in the presence of cellulose and activity has been measured against β-glucan (Saloheimo et al. 1997; Foreman et al. 2003). However, the enzymes within GH61 have recently shown not to be glycoside hydrolases (Harris et al. 2010). Although they are involved in improving the efficiency of degrading tightly packed cellulose microfibrils, the precise function of GH61 enzymes is still unknown (Harris et al. 2010; Vaaje-Kolstad et al. 2010).

The two highly expressed CBHs of *T. reesei*, CBHI and CBHII, belong to families 7 and 6, respectively. These are considered to work synergistically and have preference for the reducing or non-reducing end, respectively, of the cellulose chains (Nutt et al. 1998). CBHs are also considered to be important for the hydrolysis of the crystalline parts of cellulose (Liu et al. 2011). These CBHs of *T. reesei* are highly sensitive to product inhibition, in particular by cellobiose, which might explain the need for a high amount of CBHs in an effective fungal cellulase enzyme mix (Bezerra and Dias 2005; Holtzapple et al. 1990; Kristensen et al. 2009).

After endo- and exo-cleaving of cellulose, BGLs belonging to GH families 1 and 3 degrade the remaining oligosaccharides to monomeric glucose. BGLs are a group with very diverse properties and cellular location, although

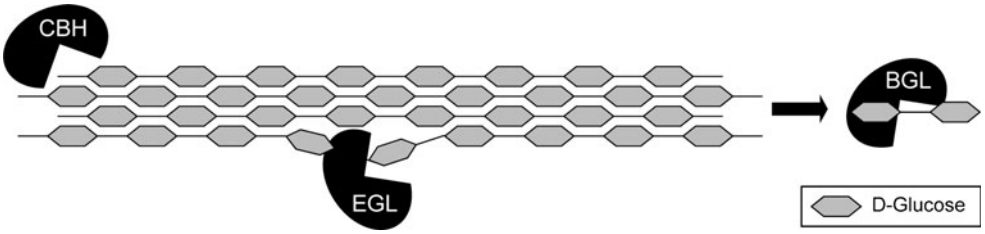

Fig. 1 Schematic structure of cellulose with cellulolytic enzymes. *BGL* β-glucosidase, *CBH* cellobiohydrolase, *EGL* β-1,4-endoglucanase

most BGLs belong to GH3 and have a similar retaining mechanism (Decker et al. 2001; Saloheimo et al. 2002). The two known BGLs of *T. reesei*, BGI and BGII, are produced at low levels (Reczey et al. 1998) and subject to strong product inhibition (Chen et al. 1992). These characteristics hinder the function of *T. reesei* for extensive in vitro saccharification of cellulose. Therefore, in industrial applications, *T. reesei* cellulase mixtures are often supplemented with BGLs from Aspergilli, which are highly expressed and more glucose tolerant (Decker et al. 2000; Reczey et al. 1998; Riou et al. 1998). Another strategy to convert cellulose into a sustainable product is to express BGLs in the fermentation host like *S. cerevisiae* (Li et al. 2010; Ha et al. 2011). This way, oligosaccharides like cellobiose are directly fermented into fuels or chemicals.

Hemicellulose degradation

Hemicellulose, the second most abundant plant polysaccharide, is a group of complex structures composed of different residues with three kinds of backbones and many different branches (Fig. 2). The three backbones of the corresponding group of hemicelluloses are hydrolysed by a specific set of dedicated carbohydrate-active enzymes: β-1,4-endoxylanase and β-1,4-xylosidase for xylan, xyloglucan-active β-1,4-endoglucanase and β-1,4-glucosidase for xyloglucan, and β-1,4-endomannanase and β-1,4-mannosidase for (galacto-) mannan (Table 3) (de Vries and Visser 2001).

The fungal β-1,4-endoxylanases belong to GH families 10 and 11 (Polizeli et al. 2005). Similar to enzymes within families GH1, GH2, and GH5, endoxylanases of GH10 have a TIM-barrel fold at their catalytic domain in contrast to GH11 which has a β-jelly roll structure at its catalytic domain (Henrissat and Bairoch 1993; Pollet et al. 2010). As a consequence, the two groups of endoxylanases differ from each other in substrate specificity (Biely et al. 1997). GH10 endoxylanases have in general a broader substrate specificity then endoxylanases of family GH11. Specifically, GH10 enzymes not only degrade linear chains of 1,4-linked D-xylose residues, but also xylan backbones with a high degree of substitutions and smaller xylo-oligosaccharides (Biely et al. 1997; Vardakou et al. 2003; Pollet et al. 2010). GH10 endoxylanases are therefore important for the complete degradation of substituted xylans. Although, in principal, less accessory enzymes are required with a higher amount of GH10 endoxylanases, fungal genomes have no clear correlation between the amount of GH10 endoxylanases and number of accessory enzymes.

The released xylo-oligosaccharides are degraded by β-xylosidases. Most fungal β-xylosidases belong to GH3 (Mozolowski and Connerton 2009), but several putative β-xylosidases are assigned to GH43 (e.g., in *Penicillium herquei* and *A. oryzae* (Ito et al. 2003; Machida et al. 2005)). GH3 is a conserved family containing mainly BGLs. As shown in *T. reesei* and bacteria, β-xylosidases of GH3 contain the conserved Asp-311 residue but miss the other active-site Asp-120 residue of β-glucosidases, which might explain the

Table 2 Genes encoding characterized and putative enzymes of *Aspergillus niger* CBS513.88 involved in cellulose degradation (Coutinho et al. 2009)

Enzyme class	Code	CAZy families	Genes of characterized and putative enzymes	Reference
β-1,4-endoglucanase	EGL	GH5	An07g08950 (*eglB*), An01g11670, An16g06800	(van Peij et al. 1998)
		GH7 & GH45	–	
		GH12	An14g02670 (*eglA*), An01g03340, An03g05380	(van Peij et al. 1998)
Cellobiohydrolase	CBH	GH6	An08g01760, An12g02220	
		GH7	An07g09330 (*cbhA*), An01g11660 (*cbhB*)	(Gielkens et al. 1999)
β-1,4-glucosidase	BGL	GH1	An11g02100, An04g03170, An03g03740, An02g08600	
		GH3	An18g03570 (*bgl1*), An07g09760, An08g08240, An11g00200, An14g01770, An17g00520, An03g05330, An06g02040, An15g01890, An11g06090, An15g04800	(Dan et al. 2000; Pel et al. 2007)

The genes with names between brackets are biochemically characterized and their references are given in the last column

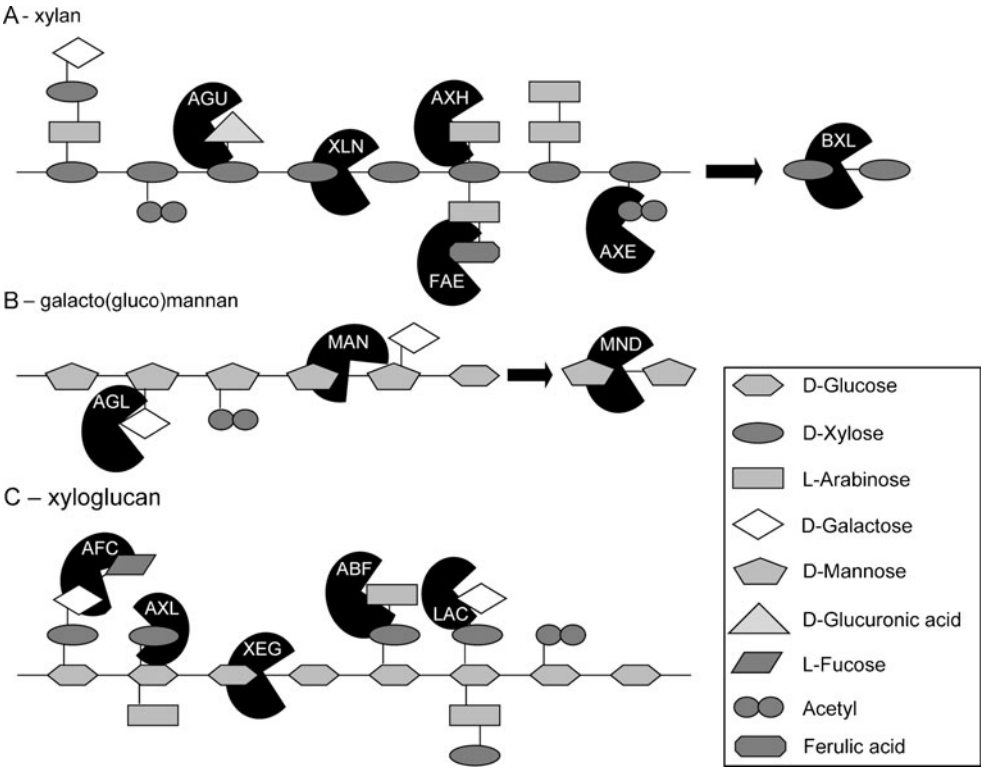

Fig. 2 **a**–**c** Schematic structure of three hemicelluloses, xylan, galacto(gluco)mannan, and xyloglucan, with hemicellulolytic enzymes. *ABF* α-arabinofuranosidase, *AFC* α-fucosidase, *AGL* α-1,4-galactosidase, *AGU* α-glucuronidase, *AXE* acetyl (xylan) esterase, *AXH* arabinoxylan α-arabinofuranohydrolase, *AXL* α-xylosidase, *BXL* β-1,4-xylosidase, *FAE* feruloyl esterase, *LAC* β-1,4-galactosidase, *MAN* β-1,4-endomannanase, *MND* β-1,4-mannosidase, *XEG* xyloglucan-active β-1,4-endoglucanase, *XLN* β-1,4-endoxylanase

discrimination between β-xylosidases or β-glucosidases within this family (Margolles-Clark et al. 1996; Dodd et al. 2010).

Xyloglucan-active endoglucanases, also referred to as xyloglucanases, belong to GH families 12 and 74 (Grishutin et al. 2004). The main difference between enzymes of GH12 and GH74 is their retaining and inverting mechanism, respectively (Gilbert et al. 2008). The specific modes of action of the different xyloglucanases were recently elucidated (Desmet et al. 2007; Powlowski et al. 2009; Yaoi et al. 2009; Master et al. 2008). For instance, in contrary to the GH74 xyloglucanase from *T. reesei*, the GH12 enzyme from *A. niger* does not cleave at branched glucose residues and prefers xylogluco-oligosaccharides containing more than six glucose residues with, at least, one non-branched glucose residue (Master et al. 2008; Desmet et al. 2007).

Endomannanases, involved in the degradation of mannan polysaccharides, belong to GH families 5 and 26. However, fungal endomannanases are predominately present in GH5. The GH5 endomannanases from *A. niger* and *T. reesei* both show substrate specificity towards manno-oligosaccharides with more than three D-mannose residues (Tenkanen et al. 1997; Do et al. 2009). Like many other fungal carbohydrate-active enzymes, some endomannanases have a carbohydrate-binding module (mainly CBM1) which promotes the

Table 3 Genes encoding characterized and putative enzymes of *Aspergillus niger* CBS513.88 involved in the degradation of the three hemicellulose backbones (Coutinho et al. 2009)

Enzyme class	Code	CAZy families	Genes of characterized and putative enzymes	Reference
Xyloglucan-active	XEG	GH12	An14g02670 (*eglA*), An01g03340, An03g05380	(van Peij et al. 1998)
β-1,4-endoglucanase		GH74	An01g01870 (*eglC*)	(Hasper et al. 2002)
β-1,4-endoxylanase	XLN	GH10	An03g00940 (*xynA*)	(Krengel and Dijkstra 1996)
		GH11	An01g00780 (*xynB*), An01g14600, An14g07390, An15g04550	(Levasseur et al. 2005)
β-1,4-xylosidase	BXL	GH3	An01g09960 (*xynD*), An17g00300	(van Peij et al. 1997)
		GH43	An11g03120, An02g00140, An08g10780, An08g01900	
β-1,4-endomannanase	MAN	GH5	An05g01320 (*manA*)	(Ademark et al. 1998)
		GH26	An15g07760	
β-1,4-mannosidase	MND	GH2	An11g06540 (*mndA*), An12g01850, An01g06630	(Ademark et al. 2001)

The genes with names between brackets are biochemically characterized and their references are given in the last column

association of the enzyme with the substrate (Herve et al. 2010; Pham et al. 2010; Boraston et al. 2004). The released mannobiose and mannotriose are further degraded by β-1,4-mannosidases belonging to GH family 2 (Ademark et al. 2001).

To completely degrade hemicellulose, all substitutions on the hemicellulose backbones have to be released. This requires at least nine different enzyme activities divided over at least 12 GH families and four CE families (Table 4).

L-Arabinose, a common residue in hemicellulose, is cleaved from arabinose-substituted xyloglucan and (arabino-) xylan by α-arabinofuranosidases and arabinoxylan arabinofuranohydrolases. Fungal α-arabinofuranosidases are mainly found in GH families 51 and 54, although some bifunctional enzymes from GH3 and GH43 were described to have α-arabinofuranosidase activity (Saha 2000; Ravanal et al. 2010). The difference in substrate specificity between GH51 and GH54 enzymes is illustrated by the two main arabinofuranosidases of *A. niger*. AbfA (GH51) releases L-arabinose from arabinan and sugar beet pulp, while AbfB (GH54) also releases L-arabinose from xylan (de Vries and Visser 2001). Arabinofuranosidases within GH54 are also described to have a carbohydrate-binding module (CBM 42) with specific binding to arabinofuranose side chains of hemicellulose (Miyanaga et al. 2004; Miyanaga et al. 2006). The arabinoxylan arabinofuranohydrolases from GH62 act specifically against the α-1,2- or α-1,3-linkage of the L-arabinose residues of arabinoxylan (Verbruggen et al. 1998), but are also sensitive to the substitutions of adjacent D-xylose residues. For instance, AxhA from *A. niger* is not able to release arabinobiose from xylan or substituted L-arabinose from D-xylose residues adjacent to D-glucuronic acid residues (Verbruggen et al. 1998; Sakamoto et al. 2011).

D-Xylose residues with α-linkages are released from the xyloglucan backbone by α-xylosidases. Of two α-xylosidases in *Aspergillus flavus*, AxlI hydrolyzes xyloglucan oligosaccharides and AxlII is most active on *p*-nitrophenyl α-L-xylose residues and does not hydrolyze xyloglucan (Yoshikawa et al. 1993, 1994). α-Xylosidases were suggested to belong to family GH31 based on genome analysis within Aspergilli which was proven by overexpression in *Pichia pastoris* (Bauer et al. 2006; de Vries et al. 2005). This GH31 family contains mainly enzymes with α-glucosidase activity and has only a limited number of characterized α-xylosidases (de Vries et al. 2005).

L-Fucose residues in xyloglucan branches are released by α-fucosidases belonging to GH29 and GH95. Several α-fucosidases of plants have been identified to degrade xyloglucan (Leonard et al. 2008; Ishimizu et al. 2007; Minic and Jouanin 2006). Nevertheless, only *A. niger*, *Aspergillus nidulans*, and *Penicillium multicolor* have been reported to produce an α-fucosidase which release L-fucose residues similar to the fucose-linkages in xyloglucan (Ajisaka et al. 1998; Ajisaka and Shirakabe 1992; Bauer et al. 2006).

Alpha-linked D-galactose residues are released from hemicellulose, e.g., xylan and galactomannans, by α-galactosidases belonging to GH27 and GH36. The α-

Table 4 Genes encoding characterized and putative enzymes of *Aspergillus niger* CBS513.88 involved in the degradation of the substitutions on the hemicellulose backbones (Coutinho et al. 2009)

Enzyme class	Code	CAZy families	Genes of characterized and putative enzymes	Reference
α-arabinofuranosidase	ABF	GH51	An01g00330 (*abfA*), An08g01710, An09g00880	(Flipphi et al. 1993c)
		GH54	An15g02300 (*abfB*)	(Flipphi et al. 1993b)
α-xylosidase	AXL	GH31	–	
α-fucosidase	AFC	GH29	An13g02110	
		GH95	An16g02760, An16g00540	
α-1,4-galactosidase	AGL	GH27	An06g00170 (*aglA*), An02g11150 (*aglB*), An01g01320, An14g01800, An11g06330	(den Herder et al. 1992; de Vries et al. 1999)
		GH36	An09g00260 (*aglC*), An04g02700, An09g00270	(Ademark et al. 2001)
β-1,4-galactosidase	LAC	GH2	–	
		GH35	An01g12150 (*lacA*), An01g10350, An07g04420, An06g00290, An14g05820	(Kumar et al. 1992)
Arabinoxylan α-arabinofuranohydrolase	AXH	GH62	An03g00960 (*axhA*)	(Gielkens et al. 1997)
α-glucuronidase	AGU	GH67	An14g05800 (*aguA*)	(de Vries et al. 2002c)
		GH115	–	
Acetyl (xylan) esterase	AXE	CE1	An12g05010 (*axeA*)	(van Peij et al. 1998)
Feruloyl/p-coumaroyl esterase	FAE	CE1	An09g00120 (*faeA*), An12g10390 (*faeB*), An12g02550	(de Vries et al. 1997; de Vries et al. 2002d)

The genes with names between brackets are biochemically characterized and their references are given in the last column

galactosidases of GH27 and GH36 both act via a double-displacement mechanism and are considered to have a common evolutionary origin (Rigden 2002). Some enzymes of GH27 also showed α-*N*-acetylgalactosaminidase activity and it is therefore argued that some GH27 α-galactosidases are not involved in hemicellulose degradation (Kulik et al. 2010). The GH36 α-galactosidases are often larger in size and are active against mono- and oligosaccharides, such as melibiose and raffinose (Ademark et al. 2001). The presence of terminal β-linked D-galactose residues in some hemicelluloses, e.g., xylan, xyloglucan, and galactoglucomannans, suggested that β-galactosidases (GH2 and GH35) also play a role in degradation of hemicelluloses (Sims et al. 1997). This was proven by the increased release of D-galactose residues from wheat flour after supplementing LacA of *A. niger* to an enzyme cocktail (de Vries et al. 2000). Bga1, a β-galactosidase of *T. reesei*, showed broad substrate specificity with also activity against polymeric galactans (Gamauf et al. 2007). Nevertheless, the fast majority of studies concerning β-galactosidases are focused on their activity against lactose.

D-Glucuronic acid residues from polymeric xylan are released by α-glucuronidases belonging to GH67 and the newly identified family GH115 (Ryabova et al. 2009; Chong et al. 2011). Like many other carbohydrate-active enzyme families, the difference between α-glucuronidases from both families lays in their substrate specificity. GH67 α-glucuronidases are active on short oligosaccharides, while some of the GH115 α-glucuronidases are active on polymeric xylan (Chong et al. 2011; Tenkanen and Siika-aho 2000). The GH115 α-glucuronidase from *Pichia stipitis* showed however higher activity against short oligosaccharides, which corresponds with its ability to degrade oligosaccharides within their environment (Kolenova et al. 2010). Remarkably, GH67 α-glucuronidases are found exclusively within Ascomycetes, while GH115 α-glucuronidases are present in Ascomycetes and Basidiomycetes (Chong et al. 2011).

Acetyl residues from xylan chains are released by acetylxylan esterases belonging to CE families 1, 4, 5, and 16 (Biely et al. 2011). The presence of acetylxylan esterases is essential for efficient degradation of the xylan backbone by endoxylanases. For instance, only in the presence of *A. niger* acetylxylan esterase, birchwood xylan can be degraded by the three endoxylanases and the β-xylosidase of *A. niger* (Kormelink et al. 1993). The main difference between the CE families is their preference for hydrolyzing the different *O*-linked acetyl groups. CE families 1, 4, and 5 have a strong preference for 2-*O*-linked residues, the most common linkage in hemicellulose, while CE16 prefers 3-*O*- and 4-*O*-linked residues (Li et al. 2008; Biely et al. 2011). There has also been a description of acetyl esterase of *A. oryzae* active against acetyl residues attached to galactomannan chains (Tenkanen et al. 1995). However, the gene encoding this specific enzyme has not yet been identified nor characterized in other fungi.

p-Coumaric acid and ferulic acid, the two cinnamic acids present in xylan, are removed by feruloyl/*p*-coumaroyl esterases. Most of these esterases have not been assigned to CE families. However, several classifications have been reported for these enzymes based on sequence similarity and substrate specificity (Crepin et al. 2004; Olivares-Hernandez et al. 2010; Benoit et al. 2008). One particular group of esterases, belonging to *Aspergillus* and *Penicillium* sp., has preference for substrates with methoxy substituents (Koseki et al. 2009; Kroon et al. 1997). For example, FaeA of *A. niger* prefers substrates with a methoxy group at position three, such as ferulic acid (Benoit et al. 2007; Kroon et al. 1997). FaeB of *A. niger* belongs to another group of esterases with preference for substrates containing one or two hydroxyl substitutions, such as *p*-coumaric acid (de Vries et al. 1997; Koseki et al. 2009; Kroon et al. 1997).

Pectin degradation

The degradation of pectin backbones (Fig. 3) requires two classes of enzymes: glycoside hydrolases and polysaccharide lyases (PL; Table 5). A large part of the fungal glycoside hydrolases involved in the degradation of the pectin backbone belongs to GH family 28 (Martens-Uzunova and Schaap 2009). These GH28 enzymes can be divided in groups according to the specific pectin region they attack: endo- and exo-polygalacturonase (GH28) cleave the backbone of the smooth regions, while the more intricate, hairy regions are further attacked by endo- and exo-rhamnogalacturonase (GH28), xylogalacturonase (GH28), α-rhamnosidases (GH78), unsaturated glucuronyl hydrolases (GH88), and unsaturated rhamnogalacturonan hydrolases (GH105).

Endo- and exo-polygalacturonases of GH28 in general cleave the α-1,4-glycosidic bonds between the α-galacturonic acids. The genome of *A. niger* contains seven endopolygalacturonases, each of them exhibiting distinct kinetic properties, substrate methylation sensitivity and mode of action (Martens-Uzunova and Schaap 2009; Benen et al. 2000; Bussink et al. 1991; Parenicova et al. 1998, 2000a). For instance, although the structures of PgaI and PgaII are highly similar, only PgaI has enzyme processivity due to a narrower substrate binding cleft and the presence of an arginine at position 96 (van Santen et al. 1999; van Pouderoyen et al. 2003). The genome of *A. niger* contains four potential exopolygalacturonases: PgaX, PgxA, PgxB, and PgxC (Martens-Uzunova et al. 2006). Of these, PgxB prefers homogalacturonan as a substrate, while PgxC has high activity against homogalacturonan and xylogalacturonan (Martens-Uzunova et al. 2006). PgxA has a low

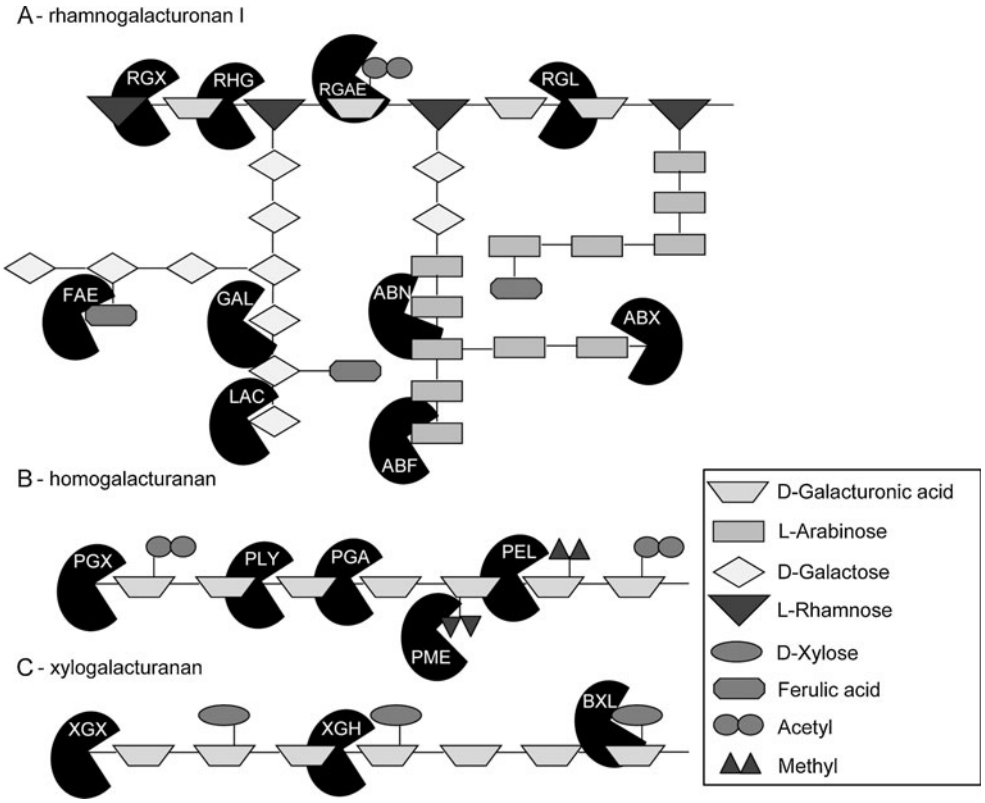

Fig. 3 **a**–**c** Schematic structures of three pectins, rhamnogalacturonan I, homogalacturonan, xylogalacturonan, with pectinolytic enzymes. *ABF* α-arabinofuranosidase, *ABN* endoarabinanase, *ABX* exoarabinanase, *BXL* β-1,4-xylosidase, *FAE* feruloyl esterase, *GAL* β-1,4-endogalactanase, *LAC* β-galactosidase, *PEL* pectin lyase, *PLY* pectate lyase, *PGA* endopoly-galacturonase, *PGX* exo-polygalacturonase, *PME* pectin methyl esterase, *RGAE* rhamnogalacturonan acetyl esterase, *RGL* rhamnogalacturonan lyase, *RHG* endorhamnogalacturonase, *RGX* exorhamnogalacturonase, *XGH* endoxylo-galacturonase, *XGX* exoxylogalacturonase. α-Rhamnosidase (RHA), unsaturated rhamnogalacturonase (URH), and unsaturated glucuronyl hydrolase (UGH) are not depicted in this figure

activity against homogalacturonan and is more active against xylogalacturonan. Therefore, PgxA very likely represents a new kind of exoxylogalacturanase (Martens-Uzunova et al. 2006). An endo-acting xylogalacturanase, XghA, was already described to specifically degrade the xylogalacturonan present in the hairy regions (van der Vlugt-Bergmans et al. 2000). Also within *R. oryzae*, 15 of the 18 putative GH28 polygalacturonases showed different substrate specificity for polygalacturonan, trigalacturonan, and digalacturonan, and their activity against polygalacturonan ranged from less than 1 to 200 μmol/min/mg protein (Mertens and Bowman 2011). These different characteristics between polygalacturonases of *A. niger* and *R. oryzae* shows the requirement for a large number of isoenzymes to concertedly act on complex plant polysaccharides.

Another important group of GH28 are the rhamnogalacturonan hydrolases, which employ either an endo- or exolytic mechanism to cleave the α-1,2-glycosidic bonds formed between D-galacturonic acid and L-rhamnose residues in the hairy regions (Kofod et al. 1994; Suykerbuyk et al. 1995). *A. niger* has two characterized (RhgA and RhgB) and four putative endorhamnogalacturonanases (Martens-Uzunova and Schaap 2009; Suykerbuyk et al. 1997). The pattern of reaction products produced after degradation of modified hairy regions with either RhgA or RhgB is quite different, suggesting that each enzyme acts on a structurally different region of the substrate (Suykerbuyk et al. 1997). Exorhamnogalacturonases release D-galacturonic acid residues from the non-reducing end of rhamnogalacturonan chains but not from homogalacturonans (Mutter et al. 1998). Sequence analysis of the *A. niger* genome indicates the presence of three genes, RgxA, RgxB, and RgxC, encoding putative exorhamnogalacturonases (Martens-Uzunova et al. 2006).

Hydrolysis of the pectin backbone also requires enzymes from other GH families: α-rhamnosidases (GH78), unsaturated glucuronyl hydrolases (GH88), and unsaturated rhamnogalacturonan hydrolases (GH105). As several of these enzymes and families have only recently been described, little biochemical characterization has been performed on them (see Table 5 for the putative genes of *A. niger*). For example, characterization of unsaturated glucuronyl hydrolases and unsaturated rhamnogalacturonan hydrolases is lacking in fungi, although several putative enzymes have been identified.

Pectin and pectate lyases both cleave, via a β-elimination mechanism, the α-1,4-linked D-galacturonic acid residues within the smooth regions of pectin (Lombard et al. 2010). A comparison between the structures of pectin and pectate lyases has indicated that both lyases most likely descended from a common ancestor enzyme (Mayans et al. 1997; Vitali et al. 1998). However, both types of lyases have important differences in their active site. As a consequence, pectin lyases attack preferentially heavily methyl-esterified substrates and have their optimum pH around 5.5 (Mayans et al. 1997). In contrast, pectate lyases favor lower degrees of esterification, have their optimum

Table 5 Genes encoding characterized and putative enzymes of *Aspergillus niger* CBS513.88 involved in the degradation of pectin (Coutinho et al. 2009; Martens-Uzunova and Schaap 2009)

Enzyme class	Code	CAZy families	Genes of characterized and putative enzymes	Reference
Endopoly-galacturonase	PGA	GH28	An16g06990 (*pgaA*), An02g04900 (*pgaB*), An05g02440 (*pgaC*), An09g03260 (*pgaD*), An01g14670 (*pgaE*), An01g11520 (*pgaI*), An15g05370 (*pgaII*)	(Parenicova et al. 2000b; Parenicova et al. 2000a; Bussink et al. 1992; Parenicova et al. 1998; Kester and Visser 1990)
Exopoly-galacturonase	PGX	GH28	An11g04040 (*pgxA*), An03g06740 (*pgxB*), An02g12450 (*pgxC*), An12g07500 (*pgaX*)	(Martens-Uzunova et al. 2006)
Endorhamno-galacturonase	RHG	GH28	An12g00950 (*rhgA*), An14g04200 (*rhgB*), An06g02070, An11g06320, An11g08700, An07g01000	(Suykerbuyk et al. 1997)
Exorhamno-galacturonase	RGX	GH28	An01g14650 (*rgxA*), An03g02080 (*rgxB*), An18g04810 (*rgxC*)	(Martens-Uzunova et al. 2006)
Endoxylo-galacturonase	XGH	GH28	An04g09700 (*xghA*)	(van der Vlugt-Bergmans et al. 2000)
α-rhamnosidase	RHA	GH78	An15g04530, An01g06620, An10g00290, An08g09140, An12g05700, An07g00240, An04g09070, An18g04800	
Endoarabinanase	ABN	GH43	An09g01190 (*abnA*), An16g02730, An02g01400, An07g04930, An02g10550	(Flipphi et al. 1993a)
Exoarabinanase	ABX	GH93	49311[a]	
β-1,4-endogalactanase	GAL	GH53	An18g05940 (*galA*), An16g06590	(de Vries et al. 2002b)
Unsaturated glucuronyl hydrolase	UGH	GH88	An01g01340	
Unsaturated rhamnogalacturonase	URH	GH105	An14g05340, An14g02920	
Pectin methyl esterase	PME	CE8	An03g06310 (*pmeA*),An04g09690, An02g12505	(Khanh et al. 1991)
Rhamnogalacturonanacetyl esterase	RGAE	CE12	An09g02160 (*rgaeA*), An04g09360	(de Vries et al. 2000)
Pectin lyase	PEL	PL1	An14g04370 (*pelA*), An03g00190 (*pelB*), An11g04030 (*pelC*), An19g00270 (*pelD*), An15g07160 (*pelF*)	(Harmsen et al. 1990; Kusters-van Someren et al. 1992; Gysler et al. 1990; de Vries et al. 2002a)
Pectate lyase	PLY	PL1	An10g00870 (*plyA*)	(Benen et al. 2000)
		PL3 and PL9	–	
Rhamnogalacturonan	RGL	PL4	An14g01130 (*rglA*), An11g00390	(de Vries et al. 2002a)
Lyase		PL11	–	

The genes with names between brackets are biochemically characterized and their references are given in the last column

[a] Exoarabinanase has only been identified in *A. niger* isolate ATCC1015

pH around 8.5, and require Ca^{2+} for their activity (Mayans et al. 1997). Currently, all characterized pectin lyases belong to the PL1 family, while the fungal pectate lyases belong to PL1, PL3, and PL9. As an example, six pectin lyases and only one pectate lyase have been identified and partially characterized in *A. niger* (Benen et al. 2000; Harmsen et al. 1990; Gysler et al. 1990). In contrast, *A. nidulans* has only two pectin lyases, five identified pectate lyases, and another six putative pectate lyases (Galagan et al. 2005; Bauer et al. 2006), suggesting significant differences between these fungi (Coutinho et al. 2009).

Rhamnogalacturonan lyases differ substantially in their structure from pectin and pectate lyases, and cleave within the hairy regions of pectin. This group of lyases belongs to two families, PL4 and PL11, where PL4 lyases have a much lower optimum pH then PL11 lyases (Jensen et al. 2010). The PL4 rhamnogalacturonan lyase from *Aspergillus aculeatus* showed that cleavage preferably occurs four residues from L-rhamnose at the reducing end and is severely affected by the presence of acetyl groups in the backbone of rhamnogalacturonan (Mutter et al. 1998; de Vries et al. 2000). Therefore, the cooperative action of rhamnogalacturonan acetyl-esterases is required for efficient degradation (de Vries et al. 2000).

The pectin structures xylogalacturonan and rhamnogalacturonan also require accessory enzymes to remove the side chains and provide access for the main-chain hydrolysing pectinolytic enzymes. Of these, α-arabinofuranosidases (GH51 and GH54), β-galactosidases (GH2 and GH35), and β-xylosidases (GH3 and GH43) are also needed for hemicellulose degradation, while endoarabinanases (GH43), exoarabinanases (GH93), β-endogalactanases (GH53), and several

esterases (CE8, CE12, and CE13) are specific for pectin degradation (Martens-Uzunova and Schaap 2009).

Future prospects

Many industrial processes make use of relative easy accessible sugar residues within cellulose or other plant polysaccharides. In industrial processes, the monomeric sugars within these structures are released from the plant material by a limited set of enzymes, utilizing only a small fraction of the available biodiversity of fungal enzymes. For instance, a minimal mixture of cellobiohydrolases (CBHI and CBHII) and endoglucanase (EGI) from *T. reesei* and β-glucosidase from *A. niger* is sufficient to efficiently degrade pure cellulose (Sternberg et al. 1977; Rosgaard et al. 2007a). Exploitation of the available sugars within the intricate fractions of plant biomass involves a more elaborate process. For example, plant polysaccharides have to be separated from the present lignin compounds by chemical, physical, or biological pretreatment (Hendriks and Zeeman 2009; Martinez et al. 2009). And, as described in the previous sections, releasing the sugars from the different plant polysaccharides requires a more complex set of carbohydrate-active enzymes. In addition to a number of cellulases, efficient enzymatic hydrolysis of pretreated plant biomass also needs synergistic activity of, at least, several endoxylanases, β-xylosidase, α-arabinofuranosidase, and acetyl esterase (Kormelink et al. 1993; Alvira et al. 2011; Gao et al. 2010). The precise mixture of hydrolytic enzymes also depends on the method of pretreatment and type of plant substrate (Rosgaard et al. 2007b; Saha and Cotta 2010). Nearly complete hydrolysis of specific xylan and pectin fractions can still be achieved with a relative small sets of enzymes (de Vries et al. 2000), but this is not the case for more crude biomass fractions. For this reason, a better understanding of plant polysaccharide degradation will help to design an enzyme mixture which can efficiently degrade a wide range of substrates.

The division of carbohydrate-active enzymes into different families based on modules of amino acid conservation (www.cazy.org) can help with a better understanding of the enzymatic repertoire of different fungi. In recent years, new families have been described and new activities have been assigned to known families (Martens-Uzunova et al. 2006; Cantarel et al. 2009; Li et al. 2008; Chong et al. 2011; Harris et al. 2010). Nevertheless, more knowledge is required to fully profit from this large database of information. The biggest limitation is the low coverage of biochemical information on specific enzyme classes. This is illustrated by the small amount of enzymatically characterized proteins that has been added to the CAZy database in the last decade in contrast to the large quantity of putative carbohydrate-active enzymes of sequenced fungal genomes (Cantarel et al. 2009). As a consequence, some enzyme activities are putatively assigned to families, but do not have biochemical support. For example, none of the many putative fungal unsaturated glucuronyl hydrolases (GH88) and unsaturated rhamnogalacturonan hydrolases (GH105) have been biochemically characterized. Another challenge is the large carbohydrate-active enzyme families containing enzymes with different enzyme classes. With increasing biochemical information, some families like GH2, GH3, GH5, GH28, or GH43 could be split up in smaller and better defined subfamilies according to their hydrolytic function. This will allow a better predictive value of future fungal genome annotations, and thereby will lead to an even larger toolbox to access for industrial purposes.

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