AMINO ACIDS

Composition and Structure of Non-Starch Polysaccharide in Cereal Fiber and their Degrading Enzymes

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Abstract

A diverse range of cereal grains and their by-products are commonly used in the animal feed industry. They not only provide nutrients but also contain some anti-nutritional factors. In animal nutrition as "non-starch polysaccharides (NSP)" are considered polysaccharides, including cellulose, hemicellulose (arabinoxylans, beta-glucans and glucomannan) and pectin, it is convenient to us to get the information of composition and content of these NSPs from analysis reports of research findings. However, enzyme application is not only dependent on the number of NSPs in these cereals, but the structure of NSPs is also a key point. Differentiating from cellulose, the structure of xylan and mannan in cereals is more complex. Xylans are the most abundant hemicelluloses in nature and can constitute up to 50% of the biomass in cereal fiber, but their botanical source strongly determines the specific features with respect to type, amount, position, and distribution of substituents over the xylan backbone. There are four types of mannan in plants, and glucomannan is the major type in cereals. This paper reviews and presents information on NSPs structure and their degrading enzymes in order to gain a better understanding of NSPs' hydrolysis and utilisation.

Background

Approximately two billion tonnes of cereal grains and 140 million tonnes of legumes and oil seeds are produced throughout the world each year, which yield an estimated 230 million tonnes of fibrous material as part of a variety of by-products. The fiber component of the grain consists primarily of non-starch polysaccharides (NSP) which in cereals form part of the cell wall structure.

In animal nutrition as "NSP" are summarized as polysaccharides, which cannot be degraded by endogenous enzymes and therefore reach the colon almost undigested. The harmfulness of NSPs in monogastric diets includes: (a) the soluble NSP elicits anti-nutritive effects, and (b) the utilisation of NSP as feed material in monogastrics is very poor. Exogeneous NSP enzymes have been a key tool to eliminate these adverse factors and improve the utilization efficiency of fibrous ingredients. It is necessary to fully understand the following aspects:

(1) The composition and structure of NSPs;

(2) The enzymes needed to degrade these structures (NSPase)

(3) The source of NSP degrading enzymes (NSPase)

1. Composition of non-starch polysaccharide in cereal fiber

The term NSP covers a large variety of polysaccharide molecules excluding α-glucans (starch). NSPs are mainly present in the cell walls of the endosperm but also in the bran. They are composed predominantly of cellulose, hemicellulose (arabinoxylans, beta-glucans and glucomannan), and pectin, and can also be divided according to their nutritional characteristic into water-soluble, and water-insoluble fractions (Knudsen, 2014).

The levels and types of NSP in cereals and their by-products are shown in Table 1 (Choct, 2006). Cereals contain between 5 and 17% of NSP. Levels of NSP in sorghum (4.8%) and corn (8.1%) are very low, whereas substantial amounts of both soluble and insoluble NSP are found in wheat (11.4%), rye (13.2%) and barley (16.7%). Rye, triticale, wheat, corn, and sorghum are all rich in arabinoxylan (AX), whereas barley and oats contain a high level of beta-glucan. The AX from rye, wheat, and triticale and beta-glucan from barley and oats are to a large extent soluble, whereas the solubility of AX found in corn and sorghum is lower than the other cereals. Cereal by-products consist of higher levels of cell wall components and contain more NSP. For example, rice bran contains approximately 20% NSP which consists mainly of insoluble non-cellulosic polysaccharides and cellulose (Casas et al., 2019)

Tabel 1. The types and levels of NSP present in some cereal grains and cereal by-products (% DM)

		Arabinoxylan	beta- Glucan	Cellulose	Man	Gal	Uronic Acid	Total
Wheat	Soluble	1.8	0.4		0.0	0.2	0.0	2.4
	Insoluble	6.3	0.4	2.0	0.0	0.1	0.2	9.0
Barley	Soluble	0.8	3.6		0.0	0.1	0.0	4.5
	Insoluble	7.1	0.7	3.9	0.2	0.1	0.2	12.2
Rye	Soluble	3.4	0.9		0.1	0.1	0.1	4.6
	Insoluble	5.5	1.1	1.5	0.2	0.2	0.1	8.6
Triticale	Soluble	1.3	0.2		0.0	0.1	0.1	1.7
	Insoluble	9.5	1.5	2.5	0.6	0.4	0.1	14.6
Sorghum	Soluble	0.1	0.1		0.0	0.0	0.0	0.2
	Insoluble	2.0	0.1	2.2	0.1	0.2	0.0	4.6
Corn	Soluble	0.1	0.0		0.0	0.0	0.0	0.1
	Insoluble	5.1		2.0	0.2	0.6	0.0	8.0
Rice (pearled)	Soluble	0.0	0.1		0.0	0.1	0.1	0.3
	Insoluble	0.2		0.3	0.0	0.0	0.0	0.5
Rice bran (defatted)	Soluble	0.2	0.0		0.0	0.2	0.0	0.5
	Insoluble	8.3		11.2	0.4	1.0	0.4	21.3
Wheat pollard	Soluble	1.1	0.4		0.0	0.1	0.1	1.7
	Insoluble	20.8		10.7	0.4	0.7	1.0	33.6

2. Structure and degrading enzyme of the polysaccharides in different cereals

2.1.Cellulose

Cellulose is a linear water-insoluble fibre composed of long, unbranched chains of β-1,4-linked glucose units. In some cereal grains and cereal by-products, the proportion of cellulose reaches 10-50% of NSP (Choct, 2006; Table 1). Each glucose is inverted with respect to its neighbour, resulting in a linear chain of at least 500 glucose residues that are covalently linked to one another to form a ribbon-like structure, which is stabilized by hydrogen bonds. The hydroxyl groups present in the cellulose macromolecule can be involved in intra- and intermolecular hydrogen bonds, resulting in bundles of about 40 cellulose molecules that aggregate into microfibrils. The microfibrils form a highly ordered crystalline arrangement, or a less ordered amorphous region (M. Paloheimo et al., 2010; Figure 1).

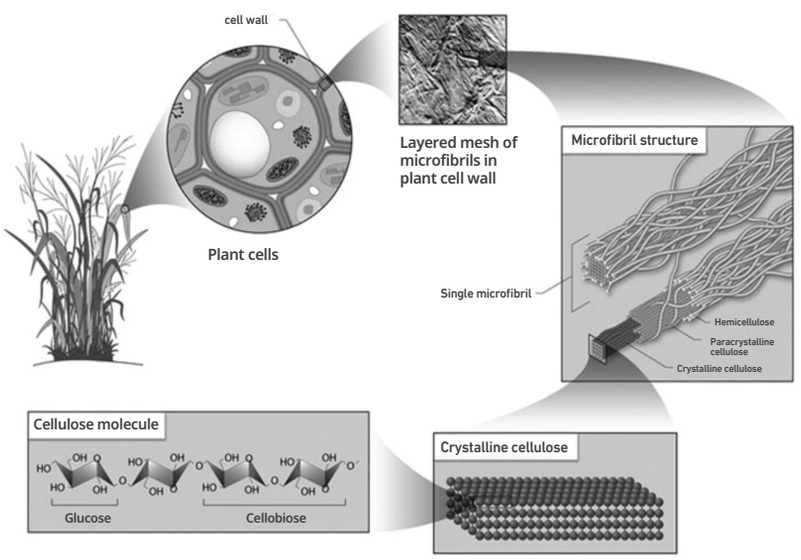

Figure 1. Schematic presentation of cellulose structure

Traditionally, a system of three main enzymatic activities has been reported to be indispensable for cellulose hydrolysis, including endo-β-1,4-glucanases (EC 3.2.1.4), cellobio-hydrolases (EC 3.2.1.91 and EC 3.2.1.176), and β-glucosidases (EC 3.2.1.21) (Teeri, 1997; Juturu and Wu, 2014; Tiwari and Verma, 2019). Classically, cellulose hydrolysis, of which the Hypocrea jecorina (formerly Trichoderma reesei) system is the archetype, is viewed as a synergistic process; endo-acting cellulases create new ends from which the exo-acting cellobiohydrolases can release cellobiose from either the reducing (GH7 and GH48) or nonreducing (GH6) end of the cellulose chains, β-glucosidases that do not have a carbohydrate-binding module (CBM) hydrolyze soluble cellodextrins and cellobiose to glucose, other carbohydrate-active catalytic domains and proteins (non hydrolytic), known as lytic polysaccharide mono-oxygenases (LPMOs), swollenins/expansins and CBMs have been shown to contribute to the overall degradation of cellulose employing increasing cellulose accessibility (Tiwari and Verma, 2019; Figure 2). This model, however, is inconsistent with several features of cellulose degradative systems. Thus, biochemical and structural data indicate that

GH6 cellobiohydrolases are not, exclusively, exo-acting (Amano et al., 1996). Meanwhile, it was evident that although the enzymes are substrate specific, it is apparent that some endo-glucanases may have dual functions due to their processive nature, thus, they display both endo- and exo-glucanase activity (Thoresen et al., 2021).

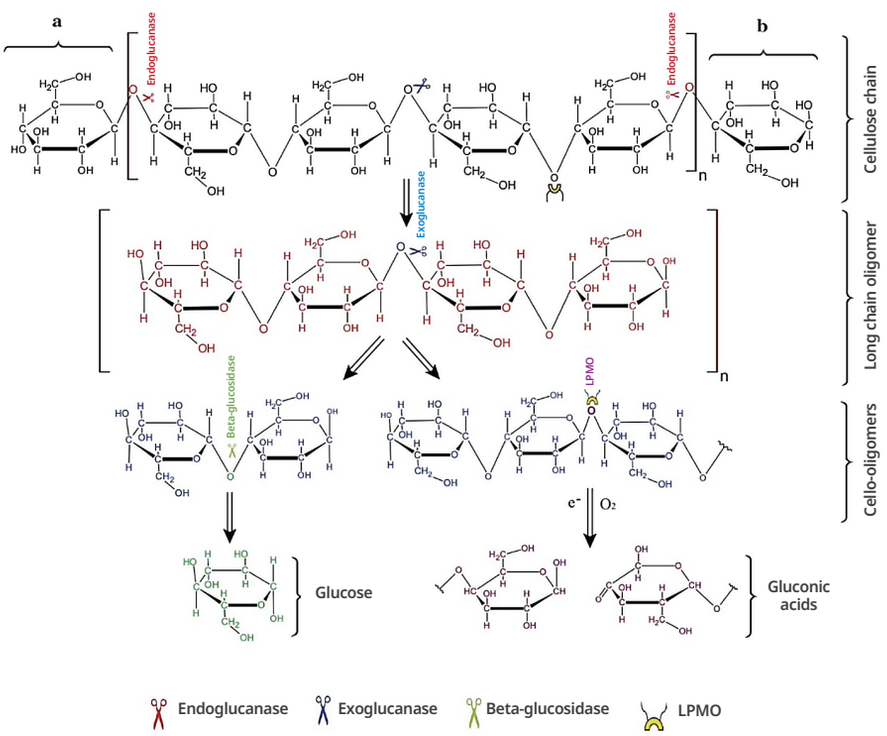

Figure 2. Cellulase hydrolysis theory. a The non-reducing end; b the reducing end.

2.2. Hemicellulose

2.2.1. Xylan

The most abundant hemicelluloses in nature are xylans, which can constitute up to 50% of the biomass in cereal fiber (Choct, 2006). Xylan seems to interact with cellulose only by means of a non-specific surface adsorption mechanism (Martínez-Sanz et al., 2017). The structure of xylan is quite variable, ranging from linear backbones constituted of β-1,4-linked poly-xylose residues denoted as homo-xylans to branched hetero-xylans, whereby the prefix 'hetero' denotes the presence of branching sugar residues other than D-xylose, including a- L-arabinofuranose, 4-O-methyl-a-D-glucuronopyranosyl acid, D- or L-galactose or D-glucuronic acid (Tiwari and Verma, 2019; Figure 3). The botanical source strongly determines the specific features with respect to type, amount, position and distribution of substituents over the xylan backbone (Kabel et al., 2007). For example, homoxylans, glucuronoxylans, arabinoxylans (AX) and glucuronoarabinoxylans (GAX) are commonly found in seaweeds, hardwoods, non-lignified and lignified tissue of grasses and cereals, respectively (El Enshasy et al., 2016). The arabinose to xylose (A/X) ratio is an important parameter to characterize the structure of AX/GAX, which can vary in different grains and different parts

of a grain (Izydorczyk and Biliaderis, 1995). It is suggested that highly substituted GAX acts as a spacer, keeping cellulose microfibrils apart, whereas low substitution GAX is more localized in plant cell walls and promotes cellulose bundling (Shrestha et al., 2019). In addition, the degree of the A/X ratio as well as the average molecular weight is relative to the solubility of xylan in cereals (Izydorczyk and Biliaderis, 1995).

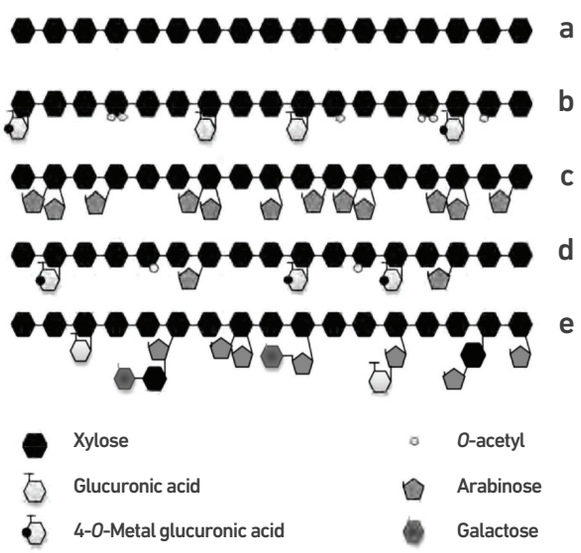

Figure 3. **Structural models of xylans. a) homoxylan; b) glucuronoxylan; c) arabinoxylan; d)glucuronoarabinoxylan; e) heteroxylan**

The most complex xylan is from corn fiber, over 70% of their xylose backbone residues have one or more arabinose, 4-Omethylglucuronic acid, or other side chains (Nghiem et al., 2011). In addition, some of the Xylp residues are acetylated and Araf side chains are not only esterified with ferulic acid, but further substituted at position 2 with β-linked Xylp or disaccharide 2-O-α-L-galactopyranosyl-Xylp or trisaccharides α-D-xylopyranosyl-(1→3)-α-L-galactopyranosyl-(1→2)- β-D-xylopyranose and α-Dgalactopyranosyl-(1→3)-α-L-galactopyranosyl-(1→2)-β-Dxylopyranose (β-D-xylopyranosyl-(1→2)-5-O-(trans-feruloyl)-Larabinofuranose side chain) (Allerdings et al., 2006; Figure 4).

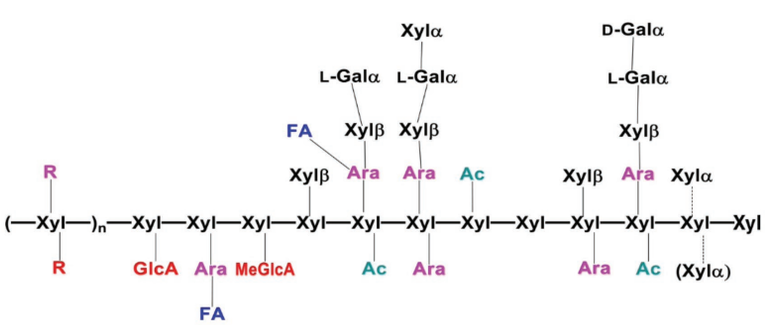

Figure 4. **highly branched corn fiber glucuronoarabinoxylan**

An up-to-date general scheme was proposed, showing how xylanolytic enzymes mechanistically degrade hetero-xylans (i.e. GAXs) in a synergistic fashion (Malgas et al., 2019; Figure 5). Firstly, GH11 xylanases preferably cleave unsubstituted regions of the xylan backbone or insoluble xylans (Pollet et al., 2010).
In this process, GH11 xylanases may be sterically hindered by the presence of acetyl groups on the xylopyranosides constituting the xylan backbone, so CE1 acetyl xylan esterases are necessary to remove these groups on the xylan backbone to allow xylanase action to proceed (Adesioye et al., 2016). Secondly, GH10 xylanases cleave highly decorated GAX backbones or the soluble Xylo-oligosaccharide (XOS) produced by GH11 Xylanase and release XOS which contain arabinose or glucuronic side chains at the non-reducing end (Karlsson et al., 2018). Similarly, The GH115 Agu action removes the methyl-glucuronic acid residues from non-terminal positions of both XOS and polymeric xylans, otherwise, these substitutes would have sterically hindered GH10/11 Xyn activity during xylan degradation (Rhee et al., 2016). Thirdly, The GH8 Rex can hydrolyse xylan from the reducing ends and on the GH10/11 Xyn-produced XOS, leading to the production of shorter XOS and xylose (Malgas and Pletschke, 2019).
However, arabinofuranosidases and XOS-specific glucuronidases may be required for debranching the GH10-produced XOS before Rex can act on them. Finally, GH43 xylosidases (Xyl) release xylose from the non-reducing ends of XOS, and as a result, alleviate product inhibition to xylanases by their products (XOS). Despite all this, enzymatic hydrolysis of some complicated xylan (eg. Corn fiber xylan), xylanase, β-xylosidase and α-L-arabinofuranosidase required consorted action with acetyl xylan esterase and feruloyl esterase, the efficiency of hydrolysis was still not satisfactory (Agger et al., 2010). Fortunately, two novel endo-acting corn fiber xylan-specific xylanase was discovered in Bacteroides ovatus and Bacteroides xylanisolvens (Rogowski et al., 2015)

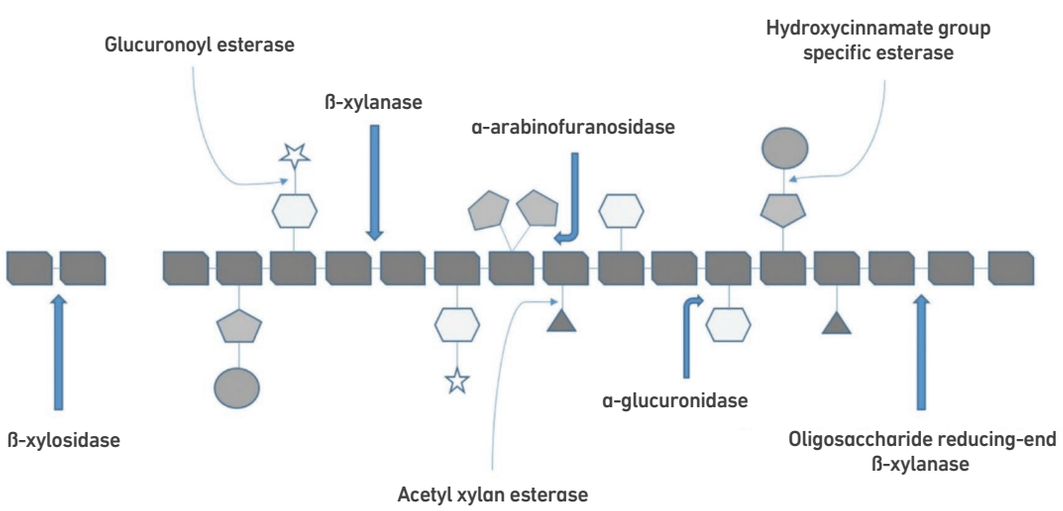

Figure 5. **An illustration of the enzyme sites of the enzymes required to completely degrade hetero-xylans**

2.2.2. (1→3, 1→4)- β-glucan

Mixed-linked β-glucans (MLGs) are also referred to as (1,3 or 1,4)- β- D -glucans or cereal β-glucans. They occur particularly in the walls of the aleurone and starchy endosperm, and vary widely among and within different species. The MLGs also occur in the walls of families related to the Poaceae ('core Poales'), the monilophyte genus Equisetum, some bryophytes, some green and red algae, some lichens, one fungus and a chromalveolate (Harris and Fincher, 2009). The MLGs are linear, unbranched β-glucans usually containing about 30% (1→3) and 70% (1→4) linkages, with the (1→3) linkages occurring singly and more than 90% of the (1→4) linkages occurring in groups of two and three (Figure 6) (Harris and Smith, 2006). The MLGs are thought to form a gel-like matrix in cell walls, between the reinforcing cellulose microfibrils.

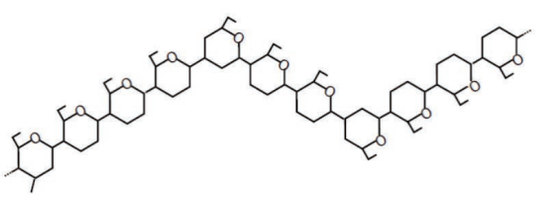

Figure 6. Mixed linkage β-glucan

Endoglucanases that depolymerize β-1,3-1,4-D-glucans can be classified into four main categories:

(a) β-1,4-D-glucanases or cellulases catalyze the hydrolysis of cellulose involving synergy between all cellulase activities: endoglucanases (EC.3.2.1.4), cello- biohydrolase (EC3.2.1.91) and β-glucosidases (EC.3.2.1.21).

(b) β-1,3-D-glucanases or laminarinases are extracellular enzymes that hydrolyze the β-1,3-D-glucan. These enzymes are active on the β- (1,3)-D-glucan as laminarin of Laminarina digitana, the laminarin from Eisenia bicyclis, curdlan, paramylon, and pachyman and are very weakly active on the β-1,3-1,4-D-glucan (Planas, 2000). The total hydrolysis of the β-D-glucan is accomplished by the synergistic action between endo and exo form: exo β-1,3-D-glucanase (EC.3.2.1.58) hydrolyzes bonds β-1,3 at the non-reducing chain of β-glucan; and endo β-1,3-D-glucanase (EC.3.2.1.39) hydrolyses randomly the β-1,3 bonds within the chain of β-glucan (Bamforth, 1980).

(c) β-1,3 (4)-glucanases are able to hydrolyze either the β- linkages (1,3) or β- linkages (1,4) of the β-D-glucan which means that they are not specific. These are endohydrolases (EC 3.2.1.6), which belong to the family 16 glycoside hydrolases. The hydrolysis products generated by this type of enzyme on liche- nan and laminarin are different: they are mono and disaccharides when it is a laminarinases and they are tri and tetrasaccharides when dealing with real lichenases.

(d) Specific β-1,3-1,4-D-glucanases or true lichenases (β-1,3-1,4-D-glucan-4-glucanohydrolase or lichenase; EC 3.2.1.73) which exhibits a strict substrate specificity for cleavage of β-1,4 glycosidic bonds in 3-O-substituted glucopyranose units.

2.2.3. Glucomannan

Mannans are also heterogeneous polymers and the second most abundant hemicellulose. As shown in Figure 7, they are grouped into four subfamilies: linear mannan, glucomannan, galactomannan, and galactoglucomannan (Malgas et al., 2015). In cereals, glucomannans are minor constituents of the walls of most aleurone and starchy endosperm cells (Table 1), but in certain indica rice cultivars the endosperm walls contain up to 17% glucomannan (Fincher and Stone, 2004). Structurally, these polysaccharides consist of a linear backbone of either β-1,4-linked D-mannose or D-mannose and D-glucose in glucomannans. The last polymers have a mannose-to-glucose ratio of around 3:1 and have a high polymerization degree.

Mannan-degrading enzymes include GHs and carbohydrate esterases, being the key enzymes β-mannanases (EC 3.2.1.78), β-mannosidases (EC 3.2.1.25) and β-glucosidases (EC 3.2.1.21). For some complex mannans, other auxiliary enzymes acting on branches are aα-galactosidases (EC 3.2.1.22) and acetyl xylan esterases (EC 3.2.1.72).

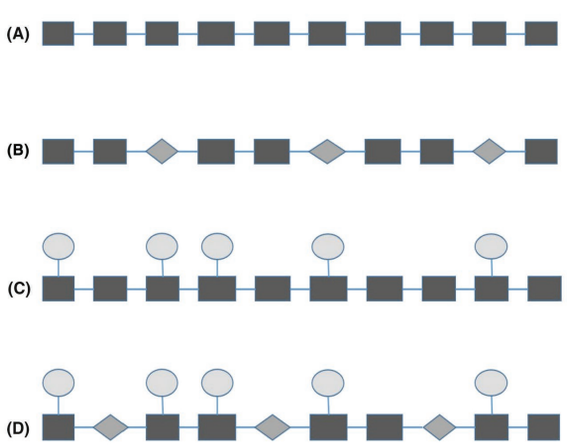

Figure 7. General structure of mannan and heteromannans

3. A toolbox of Trichoderma reesei to address the complexity of cell walls in cereals

Traditionally, the feed industry reduces the risks associated with inherent variation of grain nutrient composition with the application of exogenous non-starch polysaccharide enzymes. Enzymatic preparations with the highest enzyme activities are therefore regarded as the best tools to ensure the consistency of nutrient supply. However, learnings from nature itself point out a different way whereby cell wall polysaccharides are degraded by adopting the right set and balance of enzymatic activities (Rangel Pedersen et al., 2021). The filamentous fungus Trichoderma reesei (T. reesei) is equipped with a unique secretome that acts in synergy to degrade polysaccharides present in cell walls of both monocot and dicot grains (Gong et al., 2015). By analysis of secretomes of T. reesei across five carbon sources (Table 2), it is found that an array of enzyme activities to hydrolyze arabinoxylan, β-glucan, cellulose, xyloglucan, and mannan (Arntzen et al., 2020).

Tabel 2. Identification of CAZymes in Trichoderma reesei

Substrate/enzyme category	Enzyme class	CAZy family	T	Ba	B	S	C
Cellulose	Endoglucanases	GH5, 7,12, 45	13/5	4	2	5	5
	Cellobiohydro- lases	GH6, 7	3/3	2	2	3	3
	LPMOs	AA9	3/2	2	1	2	2
	Oligosaccharide oxidases	AA7	22/3				3
Xylan	Xylanases	GH10, 11 AA14	22/3	2	3	2	2
	LPMOs		1/0				
Arabinan	Endoarabi- nanases	GH43	3/0				
	Exo-arabinanases/arabinofuranosidases	GH51, 62	1/1			1	
Mannan	Endomannanases	GH5, 26	8/2	2	1	2	2
Soluble	β-glucosidases	GH1	2/1	1	1		
oligosaccharides	Mannosidases	GH2	7/0				
	Xylosidases/β- glucosidases	GH3	13/3	2	3	3	2
Monosugars	PDHs	AA12	1/0				
Pectin	Polygalacturo- nases	GH28	4/1			1	
	Rhamnosidases	GH78	1/0				
	Pectin lyases	PL1, 3, 4, 20	3/0				
Lignin	Laccases	AA1	4/0				
	Peroxidases	AA2	6/0				
	Oxidoreduc- tases	AA3_2-4	9/0				
	Vanillyl-alcohol oxidases	AA4	4/0				
	Glyoxal oxidases (GLOX)	AA5_1	1/1	1			1
	1,4-benzoqui- none reductases	AA6	1/0				
	Noncarbohy- drate-active esterases	CE10	30/1				1
	Feruloyl/p-cou- maroyl/acetyl esterases	CE1	12/1	1	1	1	1
Esterases	Acetyl esterases	CE2-5, 12, 16	14/1				1
	4-O-methyl-glucoronoyl esterases	CE15	1/1	1	1	1	1
Binding modules	CBM only	CBMx	13/2	2	2	2	2

The table shows a subset of CAZymes most relevant for lignocellulose deconstruction and how these are expressed in each fungus, categorized by enzyme class and (expected) substrate of action. In column 'T', the first number shows the total number of predicted CAZymes found in the whole genome using dbCAN while the second number shows the total number of proteins detected in the secretomes across all substrates. The columns labeled Ba: sugarcane bagasse, Bi: birch, S: spruce, C: cellulose, G: glucose, show the number of proteins detected in the secretome during growth on these respective substrates, including if the protein was identified in a single biological replicate, given that it was found in at least two replicates on another carbon source

Conclusions

Unlike cellulose, the structure of some hemicelluloses (xylan, mannan, and glucan) in cereals is more complex, structural complexity of these NSPs is determined by their botanical source. Therefore, not only the amount of NSP in the formula but also the origin of the ingredient should be considered when choosing an enzyme cocktail. The diversity of NSP structure in these ingredients accounts for the complexity of their degrading enzyme. Fortunately, T. reesei is a toolbox of the enzymes to address the complexity of cell walls in cereals.

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