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Impact and efficiency of GH10 and GH11 thermostable endoxylanases on wheat bran and alkali-extractable arabinoxylans

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Abstract—The results of a comparative study of two thermostable (1→4)-β-xylan endoxylanases using a multi-technical approach indicate that a GH11 xylanase is more useful than a GH10 xylanase for the upgrading of wheat bran into soluble oligosaccharides. Both enzymes liberated complex mixtures of xylooligosaccharides. ^{13}C NMR analysis provided evidence that xylanases cause the co-solubilisation of β-glucan, which is a result of cell-wall disassembly. The simultaneous use of both xylanases did not result in a synergistic action on wheat bran arabinoxylans, but instead led to the production of a product mixture whose profile resembled that produced by the action of the GH10 xylanase alone. Upon treatment with either xylanase, the diferulic acid levels in residual bran were unaltered, whereas content in ferulic and *p*-coumaric acids were unequally decreased. With regard to the major differences between the enzymes, the products resulting from the action of the GH10 xylanase were smaller in size than those produced by the GH11 xylanase, indicating a higher proportion of cleavage sites for the GH10 xylanase. The comparison of the kinetic parameters of each xylanase using various alkali-extractable arabinoxylans indicated that the GH10 xylanase was most active on soluble arabinoxylans. In contrast, probably because GH11 xylanase can better penetrate the cell-wall network, this enzyme was more efficient than the GH10 xylanase in the hydrolysis of wheat bran. Indeed the former enzyme displayed a nearly 2-fold higher affinity and a 6.8-fold higher turnover rate in the presence of this important by-product of the milling industry.

Keywords: Xylanases; Wheat bran; Cell wall; Arabinoxylans; Hydroxycinnamic acids

1. Introduction

Wheat bran is an abundant, xylan-rich by-product of the milling industry that is mainly used as feed for livestock. Bran is also a potential feedstock for the production of nonfood products (bioenergy, bulk chemicals, etc.). To this end, previously developed transformation processes based on chemical hydrolysis using dilute or strong acid solutions are applicable to wheat bran. However, taking into account the high arabinoxylan (AX) content of wheat bran (40% dry weight of destarched wheat bran), enzymatic strategies for bran

Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; AMAC, 2-aminoacridone; AX, arabinoxylans; DiFA, diferulic acid; DP, degree of polymerisation; DWB, destarched wheat bran; FA, ferulic acid; GH, glycosyl hydrolase; HCA, hydroxycinnamic acid; HPAEC, high-pressure anion-exchange chromatography; PACE, polysaccharide analysis using carbohydrate gel electrophoresis; pCA, *p*-coumaric acid; RWB, residual wheat bran; XYL10, *Thermobacillus xylanilyticus* family 10 endo-1,4-β-xylanase; XYL11, *Thermobacillus xylanilyticus* family 11 endo-1,4-β-xylanase; WI-AX_i, WI-AX_r, water-insoluble arabinoxylan isolated from initial and XYL11 residual wheat bran; WS-AX_i-50, WS-AX_i-80, 50%- and 80%-ethanol precipitate of water soluble arabinoxylans isolated from initial wheat bran; WS-AX_r-50, WS-AX_r-80, 50%- and 80%-ethanol precipitate of water soluble arabinoxylans isolated from XYL11 residual wheat bran.

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upgrading may constitute viable alternatives to such chemical methods.

The major prerequisite for the development of an enzymatic upgrading strategy is the identification of suitable enzymes. Enzyme suitability can be defined using multiple criteria that include appropriate specificity, high catalytic efficiency and good intrinsic stability. For the enzymatic hydrolysis of AX-rich substrates such as bran, the major enzyme requirement is that of a depolymerising xylanase capable of hydrolysing the β-(1→4)-linkages between xylopyranoside residues in xylans.[1]

Based on the structural and sequence classification[2,3] of glycoside hydrolases, two major xylanase (EC 3.2.1.8) families (GH10 and GH11) that differ both in structure and in catalytic properties have been distinguished.[4] The members of both families have been extensively studied and certain enzymes, most frequently from GH11, have been incorporated into industrial processes.[5–7] The GH10 family members all possess a catalytic domain, which exhibits $(\beta/\alpha)_8$ architecture and displays an average molecular mass of approximately 40 kDa. The GH11 members are generally smaller (approximately 20 kDa) and display a β-jelly roll structure. The enzymes from both families hydrolyse β-(1→4)-linkages between adjacent xylopyranoside residues, which are accommodated within subsites (−1) and (+1). Additionally, the enzymes from both families display endo-action and thus exhibit extended active site clefts that are generally composed of three, five[8] or more subsites.[9] Endoxylanases may also harbour carbohydrate binding domains that would presumably facilitate enzyme fixation on insoluble substrate.[10] Although GH11 xylanases are most frequently chosen for industrial processes, an increasingly large amount of data reveal that GH10 enzymes display certain enzymological characteristics, which could theoretically make them better candidates for enzymatic upgrading of lignocellulosic biomass. First, GH10 xylanases are more permissive in terms of substrate specificity. Xylanases from GH11 do not tolerate the presence of arabinose decorations on either the O-2/O-3 positions of the xylose residues present in the (−1) and (+1) subsites or on the O-2/O-3 of the xylose residue present in the (−2) subsite.[4,11] In contrast, enzymes from GH10 appear to tolerate arabinose-decorated xylose residues in either the (−3), (−2), or (+1) subsites.[4,12] Likewise, Fujimoto et al.[13] suggested that some GH10 members might also accommodate arabinose-decorated xylose residues in the (+2) subsite. Secondly, unlike GH11 xylanases, GH10 xylanases have also been shown to be unaffected by the presence of TAXI-like proteinaceous inhibitors, which occur in cereals.[14] However, importantly GH11 are most active against insoluble polymeric xylans, whereas GH10 xylanases are preferentially active against soluble substrates and can readily hydrolyse small xylooligosaccharides such as xylotriose.[4,15,16]

Previously, we have identified and characterised two thermostable xylanases (one from GH11 and one from GH10) from the thermophilic bacterium *Thermobacillus xylanilyticus*[17,18] (designated as XYL11 and XYL10, respectively) that exhibit similar physicochemical characteristics. So far, studies mainly using the GH11 enzyme have shown that wheat bran is partially degraded, with the aleurone and nucellar layer being the main targets.[19] Furthermore, analysis of the residual fraction has revealed that the arabinoxylans therein are highly substituted with arabinose, indicating that, like other GH11 enzymes, XYL11 is unable to attack such substrates. Accordingly, in addition to cellulose and lignin, pericarp is known to contain AX, which are highly substituted with glucuronic acid and phenolic acids, in addition to cellulose and lignin.[20] Moreover, hydroxycinnamic acids (HCA), notably ferulic acid (FA) and its diferulic form (DiFA) are known to play a significant role in covalently interconnecting cell-wall polymers.[21] Noncovalent interactions between xylans and polymers like β-glucans or cellulose also contribute to the cohesiveness of the wall network.[22] The intimately interconnected cell-wall network would then possibly impair AX susceptibility to endoxylanase by limiting enzyme contact and mobility.[23,24]

Therefore, in this current study we have sought to address two major questions. The first concerns the role of arabinose substitution of arabinoxylans in limiting xylanase activity. The second concerns the limiting effects of cell-wall network encountered in wheat bran on the ability of xylanases to access and hydrolyse their appropriate substrates. Studies on both destarched wheat bran (DWB) and on various AX fractions isolated from DWB or XYL11 residual bran (RBW) were thus undertaken. Finally, these questions have been correlated to the type of xylanase used, in order to elucidate the relative usefulness of GH10 and GH11 xylanases for industrial processes.

2. Results

2.1. Impact of XYL10 and XYL11 on wheat bran carbohydrates

After a 24 h treatment, the action of XYL11 upon DWB resulted in the release of 49% of AX, whereas XYL10 action released only half that amount (Table 1). Moreover, XYL11 action was rapid since the yield of (Ara + Xyl) was near maximal after a 1 h incubation period, whereas XYL10 action was more progressive. Additionally, in the water control experiments, a very few amount of AX was released (3.0% and 3.9% of the initial content in DWB sample after 1 and 24 h, respectively), and accounted for nearly 60% and 80% of the soluble neutral carbohydrate at 1 and 24 h incubation

Table 1. Kinetics of the release of soluble arabinoxylans from DWB by XYL10 and/or XYL11 treatments

Treatment (h)	Ara + Xyl (%) removed[a]	Ara/Xyl	Ara + Xyl (%) of solubilised sugar[b]
XYL10			
1	13.6 ± 0.5	0.49 ± 0.02	87.1 ± 2.7
6	23.5 ± 0.7	0.39 ± 0.02	88.7 ± 3.4
24	25.5 ± 0.7	0.43 ± 0.02	85.7 ± 1.0
XYL11			
1	46.0 ± 1.1	0.23 ± 0.01	97.4 ± 2.2
6	48.4 ± 0.6	0.23 ± 0.01	95.3 ± 2.5
24	49.0 ± 1.4	0.23 ± 0.01	90.2 ± 1.2
XYL10 + XYL11			
1	48.1 ± 1.4	0.23 ± 0.02	92.8 ± 2.2
6	49.4 ± 1.8	0.24 ± 0.01	91.2 ± 2.5
24	50.7 ± 1.5	0.24 ± 0.01	89.4 ± 1.3

[a] % of (Ara + Xyl) removed with respect to initial (Ara + Xyl) content of DWB sample.
[b] (Ara + Xyl) content of the soluble fractions was expressed as a percentage of total neutral carbohydrate content (Gal + Glc + Xyl + Ara = 100%).

time; moreover, values of the Ara/Xyl ratio did not vary during incubation and were closed to 0.44. The Ara/Xyl ratio of carbohydrates solubilised by XYL11 remained constant from 1 to 24 h time period while that of carbohydrates released by XYL10 decreased after 1 h incubation. At the end of the 24 h period, carbohydrates solubilised by XYL11 or XYL10 exhibited an Ara/Xyl ratio of 1:4 and 1:2.5, respectively. When XYL10 and XYL11 were combined, the rate and extent of hydrolysis was similar to that observed when XYL11 was used alone. However, analysis of the fraction from 24 h hydrolyses involving XYL11 or XYL11 + XYL10 revealed that RWB showed a higher Ara/Xyl ratio (1.00) compared to that obtained from the XYL10-derived RWB (0.71) or water-treated control bran (0.58).

AX represented the major part of the soluble fraction released by both xylanases from DWB, although an increasing co-solubilisation of other carbohydrates was noticeable after prolonged enzyme incubation (Table 1). These carbohydrates were mainly composed of glucose and, to a lesser extent, of galactose. To further investigate their nature, the XYL11-solubilised (24 h treatment) wheat bran fraction was concentrated. During the volume reduction, some precipitation occurred; the precipitate was isolated by centrifugation and examined by ^{13}C NMR spectroscopy (Fig. 1). Specific high intensity resonances at 103.7 ppm, C-1 β-(1→3); 87.2 ppm, C-3 β-(1→3); 102.8 ppm, C-1 β-(1→4) and 80.2 ppm, C-4 β-(1→4) indicated the presence of (1→3)(1→4)-β-glucan.[22,25] After 24 h treatment by XYL11 and XYL10, non-AX carbohydrates accounted for approximately 10% and 15% (dry weight), respectively, of the soluble fractions. Since XYL10 was an impure enzyme preparation, a control reaction using XYL10-free *E. coli* lysate was performed in order to ascertain whether XYL10 action was directly responsible for the greater removal of glucose-containing carbohydrates. The results of this control revealed that a small amount of glucose monomer was obtained, but this could not account for the 5% difference described above.

Oligosaccharides released from DWB after treatment with XYL10 and/or XYL11 were studied by polysaccharide analysis using carbohydrate gel electrophoresis (PACE).[26] Two fluorophores, ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid) and AMAC (2-aminoacridone) are used to study the oligosaccharides. ANTS (a charged compound) is used to study both uncharged and charged oligosaccharides (Fig. 2A), whereas AMAC

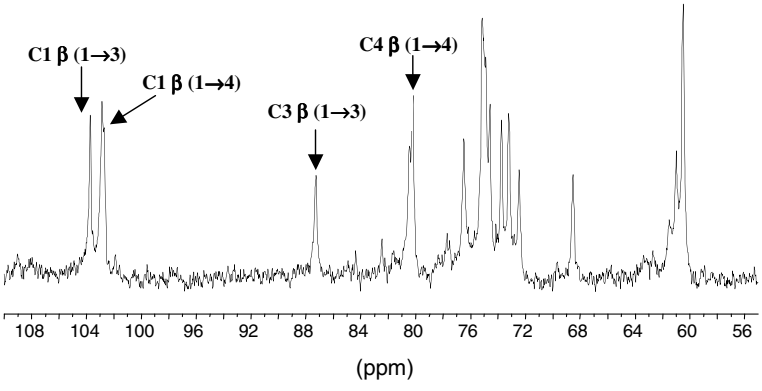

Figure 1. ^{13}C NMR spectrum of the precipitate isolated from the XYL11 soluble fraction.

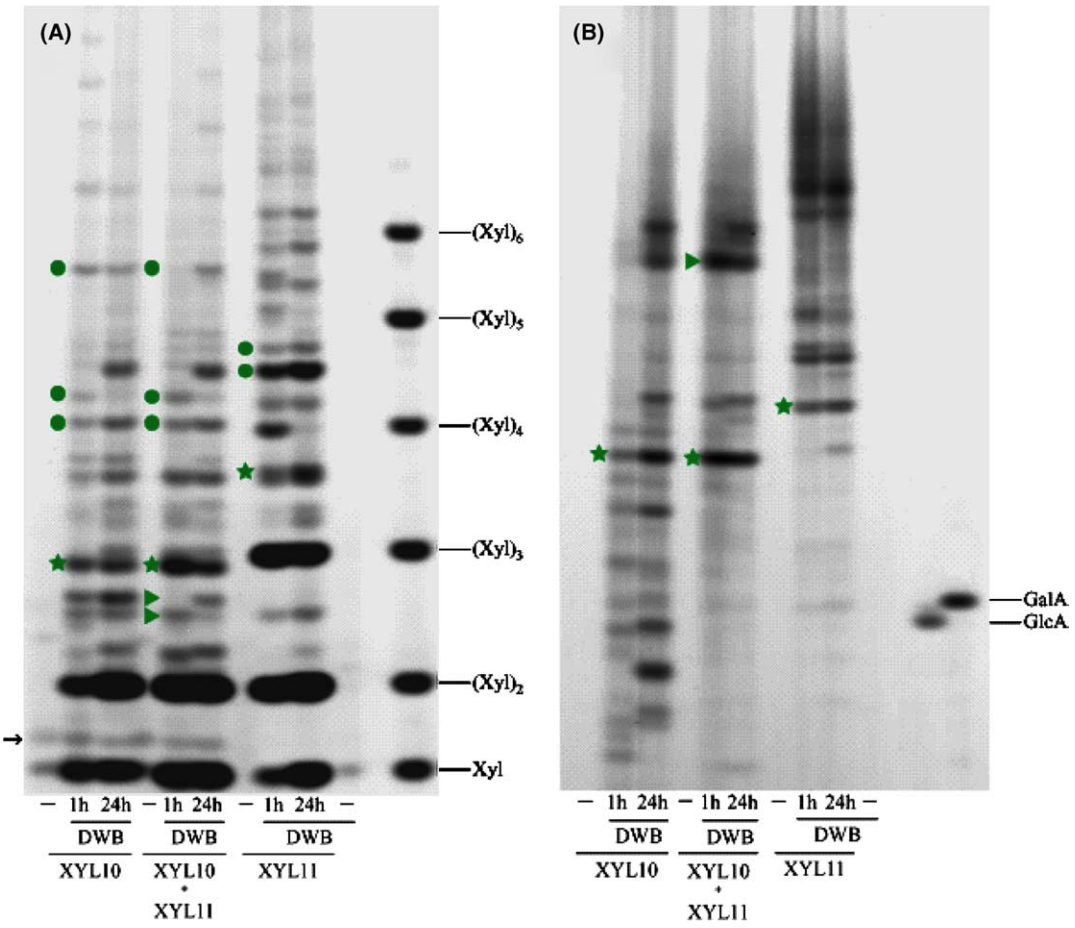

Figure 2. Fingerprints of oligosaccharide products released from DWB following treatment by XYL10 and/or XYL11. (A) Neutral and charged oligosaccharides observed following derivatisation with ANTS. (B) Charged oligosaccharides observed following derivatisation with AMAC. All reactions were performed at pH 7 and 60 °C for 1 or 24 h. A standard xylo-oligosaccharides (DP 1–6) mixture was used to characterise the faster migrating species. Unspecific bands from the hydrolase and/or DWB controls are indicated by an arrow. DWB-hydrolysis products that co-migrated with those obtained from commercial xylan and arabinoxylan hydrolysed with the same enzyme(s) are indicated with stars or circles (glucuronoxylo- or arabinoxylo-oligosaccharides, respectively). Specific DWB-hydrolysis products with differential occurrence are indicated with triangles.

(an uncharged compound) is used to study charged oligosaccharides only (Fig. 2B). Controls were performed using the same conditions as for the assays but with the absence of either xylanase(s) or polysaccharide in order to detect compounds present in the absence of enzyme (see arrows in Fig. 2A). XYL10 and/or XYL11 action on DWB produced complex fingerprints with mainly small DP xylooligosaccharides being released (Fig. 2A and B). XYL10 produced mainly xylose monomer (Xyl) and xylobiose (Xyl_2), whereas XYL11 was also able to produce xylotriose (Xyl_3). Additionally, bands corresponding to higher DP components (>Xyl_6) were also observed; a higher intensity being displayed in the case of XYL11-soluble products. Other observed oligosaccharides migrated either between or above the xylooligosaccharide standards. When compared to the oligosaccharides generated by the same enzymes using commercial xylans (data not shown) and to results reported elsewhere,[27,28] some of the products released from DWB were oligo-glucuronoxylans or oligo-arabinoxylans indicated by either stars or circles (Fig. 2A and B). The remaining unassigned bands might represent xylooligosaccharides containing varying degrees of substitution. Indeed, some charged species were observed using the AMAC fluorophore (Fig. 2B), which constitute oligoxylans substituted with uronic acids and/or arabinose conjugated with phenolic acids.

Prolonged incubation (24 h vs 1 h) resulted in the decrease in bands intensity of very large DP along with the appearance of new bands with smaller DP species and increase in the bands intensity of small DP (Fig. 2A). Using the ANTS conditions, the simultaneous hydrolysis of DWB by both enzymes produced a fingerprint, which was highly similar to that produced by XYL10 alone than XYL11. A few marked differences were observed such as the absence/presence of bands in the 1 h/24 h incubation times (respectively) when compared to the same times with XYL10 alone (triangles in Fig. 2A). Analysis of the same samples using the AMAC conditions clearly indicated that the simultaneous action

of both enzymes on DWB resulted in a much simpler and different fingerprint of charged oligosaccharides when compared to those obtained with either enzyme alone (Fig. 2B). As previously found with ANTS, differences in band occurrence were also observed (with AMAC). Likewise, the band indicated by a triangle in Fig. 2B, which was only detected after 24 h incubation when XYL10 was used, was present after only 1 h incubation when both enzymes were employed.

2.2. Impact of XYL10 and XYL11 on wheat bran hydroxycinnamic acids

Ferulic acid (FA), *p*-coumaric acid (pCA) and diferulic acids (DiFA) were quantified both in the DWB and RWB samples (Table 2). Recovery yields of phenolic acids from RWB were calculated with respect to the extent of (Ara + Xyl) removal and were expressed as a percentage of the initial hydroxycinnamic acid (HCA) content in water-treated DWB (100%). In DWB, pCA was detected as a minor component when compared to FA; sinapic acid was only detected at trace levels. With regard to DiFA, three different forms were detected. The 8-*O*-4′ form was the most abundant, followed by the 5-5′ one and then the 8-5′ form. In the both XYL10- and XYL11-generated RWB, both FA and pCA were decreased (Table 2). Indeed, nearly 40% and 30% of the initial pCA content and 50% and 36% of initial FA content were removed by XYL11 and XYL10. However, DiFA content in RWB samples remained unaltered compared to DWB, irrespective of the enzyme used (the maximal 9% variations in the amount of residual DiFA are very close to experimental error).

Table 2. Impact of XYL10 and XYL11 on wheat-bran phenolic acids: recovery yields of ferulic acid, *p*-coumaric acid and diferulic acids (DiFA) in xylanase RWB

Phenolic acids	Water-DWB[a,b]	XYL10-RWB[b]	XYL11-RWB[b]
p-Coumaric acid	183	71	61
Ferulic acid	6663	64	50
DiFA			
5,5′	236	109	109
8-*O*-4′	255	108	109
8,5′	133	107	99

[a] Expressed as μg per g of dry matter mass.
[b] Phenolic acid in RWB expressed as a percentage of the values determined for the water-treated DWB (control).

2.3. Extraction and partial characterisation of AX fractions

The yield and composition of AX fractions arising from DWB and from XYL11-RWB are shown in Table 3. Apart from WS-AX_r-50 for which (Ara + Xyl) content and recovery yields were feeble, all other fractions contained a high proportion of AX and were obtained in sufficient yields for further enzymatic assays. The DWB-derived fractions, each of which represented an approximately equal proportion of DWB, showed variability in Ara/Xyl ratio. WS-AX_i-80 was highly substituted, WS-AX_i-50 less so and WI-AX_i was poorly substituted with approximately one arabinose for six xylose residues. In contrast, XYL11-RWB was essentially composed of one major fraction (WS-AX_r-80) that was highly substituted and a minor fraction (WI-AX_r) that displayed Ara/Xyl ratio of 0.85. The various AX fractions contained differing amounts of protein, glucose, galactose and uronic acids, which together accounted for 15–43% of the total weight of the fraction. In spite of their small amount, uronic acids with glucuronic acid being dominant were found in higher proportions in the most arabinosyl-substituted fractions. Structural information of AXs in each fraction was provided by methylation analysis; arabinose and xylose were the only sugar considered herein. The major differences between samples concerned the relative molar percentage of: (i) arabinose di-substituted xylose (referred as 'Xyl' in Table 4); (ii) terminal xylose (2,3,4-Me_3-Xyl) and; (iii) nonsubstituted xylose (2,3-Me_2-Xyl). Based on chemical features and glycosyl linkage patterns, the AX fractions were distinguishable, with the exception of WS-AX_i-80 and WS-AX_r-80 that both displayed high Ara/Xyl ratios and similar structural profiles.

Table 3. Composition and recovery yield of alkali-extractable arabinoxylans fractions from DWB and XYL11-RWB

Extraction	Yield[a]	Protein[b]	TC[b]	UA[c]	Glc[c]	Gal[c]	Ara + Xyl[c]	Ara/Xyl
WI-AX_i	10.5	8.03	64.1	1.74	8.57	0.65	89.0	0.16
WS-AX_i-50	8.26	15.1	59.3	2.09	44.6	0.89	52.3	0.40
WS-AX_i-80	8.29	8.80	68.6	4.98	3.31	2.08	88.8	1.13
WI-AX_r	4.44	22.5	69.6	2.27	19.5	3.97	74.1	0.85
WS-AX_r-50	0.40	nd	66.5	4.13	89.2	4.16	5.50	1.25
WS-AX_r-80	17.1	17.3	61.9	6.54	7.53	4.56	81.3	1.12

Abbreviations used: TC, total carbohydrate (UA + Glc + Gal + Ara + Xyl); UA, uronic acids.
[a] Expressed as weight percentage of native or residual wheat bran dry matter.
[b] Expressed as weight percentage of fraction.
[c] Expressed as weight percentage of total carbohydrates of fraction.

Table 4. Glycosyl linkage pattern in arabinoxylan extracts from DWB and XYL11-RWB

Methylated sugar[a]	Relative mol% in each fraction				
	WI-AX_i	WS-AX_i-50	WS-AX_i-80	WI-AX_r	WS-AX_r-80
2,3,4-Me_3-Xyl	5.6	6.2	11.9	20.2	12.9
2,3-Me_2-Xyl	71.4	42.8	15.2	18.4	15.7
2-Me-Xyl	10.8	9.3	12.1	8.3	14.2
Xyl		9.5	16.9	18.2	20.4
2,3,5-Me_3-Ara	12.2	28.9	32.1	27.3	25.4
3,5-Me_2-Ara		1.8	3.9	4.1	3.8
2,5-Me_2-Ara		1.5	7.9	3.5	7.6

[a] 2,3,4-Me_3-Xyl = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-xylitol, etc.

2.4. Kinetic parameters of XYL10 and XYL11 on soluble AX fractions and DWB

The kinetic parameters $K_m^{(app)}$, V_{max} and k_{cat} for hydrolyses of soluble AX fractions or DWB by XYL10 and XYL11 are shown in Table 5. Several of the reactions described were complicated by the relative insolubility of the substrates. This was particularly true for birchwood glucuronoxylan and WI-AX_i. With these substrates the highest attainable concentration was never higher than $2 \times K_m$. Therefore, the data presented here must be interpreted with prudence. Similarly, the poor affinity (high $K_m^{(app)}$) of XYL11 for WS-AX_i-80 and WS-AX_r-80 precluded the determination of kinetic parameters. Overall, when kinetic parameters could be determined, XYL10 appeared to be a better enzyme for AX hydrolysis, although the $K_m^{(app)}$ values for both enzymes increased and k_{cat} values decreased with increasing Ara/Xyl ratio of the AXs (Fig. 3). In most cases XYL10 displayed lower $K_m^{(app)}$ values and higher k_{cat} values than XYL11. The major exception to this trend was XYL11 activity on DWB. In this case, when compared to XYL10, the XYL11 displayed an approximately twofold lower K_m value and a nearly sevenfold higher k_{cat} value. Therefore, on DWB XYL11 was approximately 11 times more efficient than XYL10 ($k_{cat}/K_m^{(app)} = 127$ for XYL11 and 11.5 for XYL10). Likewise, when compared to XYL10, XYL11 displayed an approximately twofold higher k_{cat} on WI-AX_r although the $K_m^{(app)}$ value was also much higher. Finally, it is important to note that the use of an appropriate quantity of XYL10-free *E. coli* lysate as a control did not give rise to any detectable production of reducing sugars so the measures were specific of the xylanase actions.

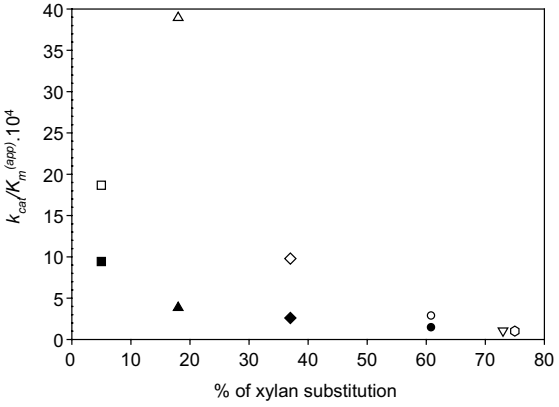

Figure 3. Comparison of the relationship between $k_{cat}/K_m^{(app)}$ and the degree of arabinose substitution for reactions involving XYL10 or XYL11 and various AX fractions. XYL11- and XYL10-catalysed reactions are indicated by black and white symbols, respectively. The different substrates are indicated as follows: birch glucuronoxylan (squares), WI-AX_i (triangles), WS-AX_i-50 (diamonds), WI-AX_r (circle), XYL10-induced hydrolysis of WS-AX_i-80 (inverse triangle) or WS-AX_r-80 (hexagon).

Table 5. Kinetic parameters of XYL10 and XYL11 related to soluble and insoluble AX sources

	XYL10			XYL11		
	$K_m^{(app)}$ (g/L)	V_{max} (μmol/min/mL)	k_{cat} (s^{-1})	$K_m^{(app)}$ (g/L)	V_{max} (μmol/min/mL)	k_{cat} (s^{-1})
WI-AX_i	0.4 ± 0.03	0.09 ± 0.002	2400	0.8 ± 0.1[c]	0.07 ± 0.003	520
WS-AX_i-50	1.0 ± 0.1	0.23 ± 0.01	1550	1.1 ± 0.1	0.07 ± 0.004	500
WS-AX_i-80	5.3 ± 1.5[c]	0.14 ± 0.02	930	nd	nd	nd
WI-AX_r	3.4 ± 0.6	0.24 ± 0.01	1600	14 ± 2.9[b]	0.45 ± 0.06	3180
WS-AX_r-80	5.5 ± 1.0[b]	0.14 ± 0.01	940	nd	nd	nd
Birch xylan	0.5 ± 0.06[c]	0.05 ± 0.003	1470	1.6 ± 0.1[a]	0.36 ± 0.003	2550
DWB	40 ± 9[c]	0.14 ± 0.01	470	25 ± 8[c]	0.22 ± 0.02	3170

Abbreviation used: nd = not determined.
$K_m^{(app)}$ and V_{max} are given ± their respective standard error.
[a] Measured for only one $K_m^{(app)}$ value.
[b] Measured for only upper of twofold $K_m^{(app)}$ value.
[c] Measured for only upper of threefold $K_m^{(app)}$ value.

3. Discussion

The aim of this study was to compare the activities of two similarly thermostable xylanases, belonging to two distinct enzyme families (GH10 and GH11), on different substrates. The use of two enzymes, whose native forms are produced by the same thermophilic bacterium, was attractive since these enzymes share several common characteristics. Both enzymes are thermostable and exhibit very similar temperature (for thermoactivity) and pH optima, thus allowing reactions to be performed in identical conditions.

3.1. Influence of the substrate structure and solubility on xylanase activity

To study the impact of the arabinose substitution on the efficiency of thermostable xylanases, AX fractions having distinct Ara/Xyl ratio were used as substrates. To this end, AX fractions were isolated from both DWB and XYL11-RWB using an alkaline extraction procedure; consequently, these fractions were almost free of esterified hydroxycinnamic acids and acetate moieties in contrast to DWB. Furthermore, the simple three-step fractionation procedure efficiently gave rise to several distinct AX fractions whose cumulative extraction yields (approximately 35% of Ara + Xyl content in DWB or RWB) were comparable to those obtained from wheat bran using other procedures.[29,30]

Using the alkali-extractable AX fractions as substrates for the two xylanases revealed for both enzymes an inverse relationship between catalytic efficiency and arabinose substitution. In the case of XYL11 this relationship was very clear, whereas the behaviour of XYL10 did not completely comply with this trend. The catalytic efficiency of XYL10 towards birchwood xylan, the least substituted was lower than that towards WI-AX_i. However, in the recent study by Courtin and Delcour,[15] it was suggested that GH10 xylanases should be most active against AXs exhibiting an intermediate level of substitution, a suggestion that is coherent with the high activity of XYL10 towards WI-AX_i. Moreover, the chain length may have an impact on catalytic activity of xylanases.[31] The superiority of XYL10 upon isolated arabinoxylan fractions and birchwood xylans is certainly due to the preference exhibited by XYL10 for soluble substrates,[18] which is coherent with the observations made by others using other GH10 xylanases and other substrates.[15,32] Similarly, the use of comparable amounts (IU) of XYL10 and XYL11 for the hydrolysis of DWB also revealed significant differences in catalytic efficiency. Only a 12-fold difference in efficiency ($k_{cat}/K_m^{(app)}$) of XYL11 on DWB and birchwood xylan was measured, whereas XYL10 was 260-fold more efficient on birchwood xylan than DWB. In the presence of DWB, an intrinsically insoluble substrate, XYL11 solubilised approximately twice as much AX than XYL10. Furthermore, combining the two enzymes did not enhance AX solubilisation, indicating the absence of synergistic action on DWB. Once again, these findings highlight the superior efficiency of GH11 xylanases toward insoluble substrates and confirm their suitability for industrial applications that require the treatment of insoluble substrates.

Other factors may also determine enzyme efficiency towards complex substrates such as wheat bran. A heterogeneous distribution of arabinose substituents on xylans is one likely candidate. Such a phenomenon is partially illustrated by the fraction WI-AX_r that was composed of almost as much arabinose as xylose. Upon this fraction, XYL11 activity was measurable. Taking into account the fact that GH11 enzymes cannot hydrolyse bonds adjacent to substituted xylose residues, this observation suggests that some regions of the substrate were devoid of substitution. Concordantly, structural analysis of WI-AX_r revealed a high proportion of disubstituted xylose residues that must be clustered in such a way that several successive xylose residues are unsubstituted. Finally, although substrate solubility and structural features are important factors for enzyme activity, it is noteworthy to recall that WI-AX_r is an alkaline-extracted fraction of RWB, which itself is the residue of XYL11 hydrolysis of intact bran. Therefore, within RWB, WI-AX_r was not accessible to XYL11, indicating that the cell-wall network is also a major determinant of enzyme activity.

3.2. Impact of the cell-wall network on xylanase efficiency

Within the different tissues that constitute wheat bran, cell walls are composed of a complex interconnected network in which AXs, cellulose and lignin are closely associated. Among the key elements that contribute to this network are the hydroxycinnamic acids. Compared to other graminaceous tissues, bran is particularly rich in cell-wall-bound phenolics. The FA monomer represents the predominant phenolic species, the minority species being pCA and to a much lower level, sinapic acid. FA is one of the main actors involved in lignin and polysaccharide cross-linkage in graminaceous cell walls,[20] notably through the formation of dimers (DiFA). In wheat bran, these are mainly present as 5,5′, 8-*O*-4′ and 8,5′ derivatives.[33] In maize, DiFA is known to be a major limiting factor for enzymatic hydrolysis.[34] In wheat bran, this has not been demonstrated, although such a role for DiFA has been recently suggested.[35] Analysis of the residual bran fractions has shown that both XYL10 and XYL11 are able to release HCA from bran cell-wall polysaccharides in the form of cinnamoyl-oligosaccharides.[36] XYL11 degraded DWB-AX and removed HCA to a greater extent than XYL10. Accordingly, higher proportions of FA and pCA were removed from DWB.

However, taking into account the fact that XYL11 displayed a twofold higher activity on bran AX, the removal of HCA by this enzyme was not twofold greater than that of XYL10. Therefore, if one assumes that xylanases had provided a similar yield of AX degradation, one can calculate that XYL10 would have presumably liberated approximately 40% more FA and pCA compounds than XYL11. In contrast, DiFA were not altered by treatment with either xylanase, suggesting that both XYL10 and XYL11 attacked similar cell walls. This is in agreement with the findings of Maes et al.[16] who speculated that GH10 and GH11 enzymes might use the same AX subpopulations in wheat bran. Moreover, in this context it is relevant to recall that in a previous study GH11 xylanases were found to be the preferred synergistic partners for feruloyl esterase action.[37] Concordantly, on the basis of our present data, we propose that GH11 xylanases are better partners because they hydrolyse more AXs, but GH10 xylanases degrade more substituted regions of AXs and produce a higher relative yield of FA substituted products. Finally, we can conclude that, while the poorer performance of XYL10 on wheat bran cannot be correlated with the presence of phenolic compounds, DiFA would constitute a limiting factor for both types of xylanase.

To hydrolyse bran AXs, enzymes must be able to penetrate the cell-wall network and thus gain access to their substrates. However, XYL10 and XYL11 should have distinct action patterns on DWB since the enzyme-solubilised AX fractions obtained in our study clearly exhibited different Ara/Xyl ratios. This supposition is supported by the results of the electrophoretic analysis of the xylooligosaccharides solubilised by XYL10 and XYL11 that showed distinct profiles for each type of hydrolysis. In this respect, PACE proved to be very suitable in providing size analysis of the heterogeneous populations, hence allowing tentative identification of certain species.[26] Consistent with the high efficiency of GH10 on soluble substrates, XYL10 produced smaller oligosaccharides than XYL11, which is in agreement with the conclusions drawn from previous studies in which size determination of xylanase-soluble was performed by high-performance size exclusion chromatography.[15,16,38] Indeed, after initially generating intermediate-size oligosaccharides, XYL10 action led to their progressive hydrolysis over a 24 h period.

In the case of XYL11, previously obtained data has revealed that this enzyme initially penetrates wheat bran from the subaleurone and then gradually progresses via aleurone cell walls towards the nucellar layer, causing disintegration of the cell walls along its path.[24] In this work, we have shown that AX depletion by xylanases is accompanied by β-glucan release, consistent with the degradation of the aleurone cell walls, which are known to contain such polymers.[20] This finding also supports previous data, which indicated the existence of intermolecular interactions between glucans and arabinoxylans.[22] Finally, the detection by PACE of oligosaccharides probably containing aldouronic acid[38] and/or hydroxycinnamic acid,[36] is also indicative of XYL11-induced disassembly of the wall network. With regard to XYL10 action, taking into account the similarity of Ara/Xyl ratios displayed by the products released by either XYL10 or by the combined action of XYL11 + XYL10, like Maes et al.[16] we conclude that the GH10 enzyme does not act upon tissues other than those degraded by XYL11. Indeed, because (i) the extent of AX degradation by XYL11 was not enhanced by the presence of XYL10 and (ii) the PACE profile of oligosaccharides generated by XYL10 was not dramatically changed upon the combined action of XYL11 and XYL10, it is probable that when used with XYL11, the XYL10 acts preferentially upon the soluble products of XYL11. This finding, which is in agreement with previous data,[15] underlines the fact that XYL10 does not attack the more branched XYL11-resistant AX population present in the pericarp, even though the data for the XYL10-catalysed hydrolysis of a similarly substituted, extracted AX population would suggest the contrary. Accordingly, preliminary microscopic observations of XYL10-treated bran appear to confirm this conclusion and show enzyme-induced disintegration of part of the aleurone (data not shown). Furthermore, during XYL10 hydrolysis of DWB, the average Ara/Xyl ratio of soluble products was subject to a time-dependent decline that suggests that the initial target of hydrolysis was more substituted than the subsequent ones. Overall, it is clear that DWB cannot be equated to water-unextractable AXs. Instead, it must be considered as a heterogeneous, xylan-rich substrate.

Finally, with regard to the slower penetration of bran tissues by XYL10 compared to XYL11, which is evidenced by the slower kinetics of the former enzyme in the presence of bran, size might be an important factor. The pore radius in wheat cell walls ranges from 1.5 to 4 nm, with smaller pores being reported for the pericarp and larger ones in the aleurone.[39] Assuming that in solution both XYL10 and XYL11 are in a monomeric state, one can estimate that XYL11 (20 kDa) will have an approximate volume of 24 nm^3, whereas that of XYL10 (40 kDa) will be approximately 40 nm^3, suggesting that XYL10 protein would have more difficulty to penetrate the bran tissues.

In conclusion, we have compared the hydrolytic ability of two naturally thermostable xylanases towards an economically relevant agricultural by-product. Based on our analyses, although the GH10 xylanase is more efficient on alkaline-extractable AXs, it is probable that the smaller size and the higher efficiency of the GH11 xylanase towards insoluble substrates make it a better enzyme for wheat bran hydrolysis. Therefore, taken together with previous data, we conclude that GH11

xylanases should be better tools for the initial stage of processing. Nevertheless, GH10 xylanases, such as XYL10, might constitute better candidates for further by-product refining. Indeed, XYL10 displayed higher efficiency towards soluble AXs and produced smaller arabinoxylosaccharide products. Finally, despite the absence of synergy between the two enzymes on wheat bran, the simultaneous use of both GH10 and GH11 xylanases could be advantageous for the production of certain specific arabinoxylosaccharide products. Indeed, the growing interest in xylanases as tools for the processing of AX-rich materials is highlighted by a recent report by Palackal et al.[28] that described the use of sophisticated enzyme engineering technology for the improvement of thermostability in a GH11 xylanase.

4. Experimental

4.1. Specialist reagents

Birchwood xylan was purchased either from Sigma–Aldrich S.A.R.L. (Saint-Quentin Fallavier, France) or from Carl Roth GmbH (Karlsruhe, Germany). Wheat arabinoxylan and xylooligosaccharides were purchased from Megazyme (Megazyme Corp. Wicklow, Ireland). The neutral monosaccharides L-arabinose, L-fucose, D-xylose and D-galactose were obtained from Sigma–Aldrich S.A.R.L. (Saint-Quentin Fallavier, France) and D-glucose was from Carl Roth GmbH (Karlsruhe, Germany). D-Glucuronic and D-galacturonic acids were purchased from Sigma–Aldrich S.A.R.L. (Saint-Quentin Fallavier, France). The fluorescent probes 2-aminoacridone (AMAC) and 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) were purchased from Sigma–Aldrich S.A.R.L. (Saint-Quentin Fallavier, France) and Molecular Probes (Leiden, The Netherlands), respectively. Polyacrylamide for PACE containing a ratio of acrylamide/*N*,*N*′-methylenebisacrylamide (29:1) was obtained from Severn Biotech Ltd. (Worcs, UK).

4.2. Production of xylanases

The GH11 xylanase (XYL11) used in this study is a β-(1→4)-endo-xylanase (EC 3.2.1.8) that was purified to homogeneity from *Thermobacillus xylanilyticus* culture medium using a previously established protocol.[40] The degree of purity of XYL11 was checked by SDS-PAGE. The GH10 xylanase (XYL10) is recombinant protein produced by the plasmid pBADXYL. Briefly, to construct pBADXYL the previously isolated xylanase encoding *T. xylanilyticus* genomic DNA[41] was inserted between the Nco I and Hind III sites of the expression vector pBABMycHisC (Invitrogen, Amsterdam, Netherlands). For expression, precultured pBADXYL-harbouring *Escherichia coli* TOP 10 cells were diluted (1:1000) in Erlenmeyer flasks containing LB broth and ampicillin (100 μg/mL). The cultures were incubated at 37 °C with shaking until an $OD_{600\,nm}$ of approximately 0.5 was reached. To induce expression of recombinant xylanase, an aq soln of L-arabinose was added to achieve a final concentration of 0.2% (w/v). Incubation was continued for 4 h, after which the *E. coli* cells were harvested by centrifugation (6000*g*, 15 min, 4 °C) and re-suspended in 1 mM EDTA, 1 mM DTT, 20 mM Tris–HCl, pH 7.5. Cell lysis was achieved by sonication and the xylanase-containing supernatant was recovered after centrifugation (12,000*g*, 20 min, 4 °C). Finally, to provide a crude purification of the xylanase, a 30 min heat treatment (60 °C) and centrifugation (12,000*g*, 20 min, 4 °C) were performed. To extend the shelf-life of the recombinant XYL10, ethylene glycol (10% v/v final concentration) was added to the supernatant, which was then stored at 4 °C. In addition, a control expression using pBADMycHisC-harbouring *E. coli* TOP 10 cells was also performed in order to provide a xylanase-free *E. coli* lysate, which was used as an experimental control in certain assays. The specific activity values of XYL11 and XYL10 were 2000 and 960 IU/mg protein, respectively.[18,40] One IU is the amount of xylanase required to release 1 μmol of xylose reducing equivalent from birchwood xylan per min at 60 °C.

4.3. Xylanase treatment of destarched wheat bran

Destarched wheat bran (DWB provided by ARD, Pomacle, France) was hydrated in distilled water (3% w/v) for 16 h at 60 °C. After, an aliquot of XYL10 or XYL11 (10 IU/mL) was added and incubation at 60 °C with constant stirring was continued. Experiments in which both xylanases were employed simultaneously were performed using equal amounts (5 IU/mL) of each enzyme. All experiments were performed in duplicate. After 1, 6 or 24 h of incubation, the supernatant (soluble fraction) was separated from the solid, residual wheat bran (RWB) by centrifugation (4000*g* for 15 min) and the enzymes were denatured by heating at 100 °C for 10 min. The supernatant and the residual RWB were retained for analysis; the supernatant was further centrifuged at 6050*g* for 15 min prior to carbohydrate analysis by HPAEC. For XYL10 hydrolyses, a control experiment using a xylanase-free *E. coli* TOP 10 lysate was included in order to evaluate any cell-wall hydrolysis provoked by endogenous *E. coli* glycoside hydrolases. Before carbohydrate or phenol analyses, residual bran fractions were freeze-dried and ground using a ball crusher (MM 2000, Retsch GmbH, Haan, Germany). Another control experiment consisted of incubating DWB in distilled water at 60 °C in the absence of xylanase. The supernatant and residue from this control were prepared and analysed as before. In a separate experiment, a larger amount of DWB was treated with

XYL11 using the conditions described above and the RWB was then retained for alkali-extraction of three enzyme-resistant AX fractions, WI-AX_r, WS-AX_r-50 and WS-AX_r-80 (see Section 4.4).

4.4. Extraction method for AX-enriched fractions

Alkaline extraction of AX fractions from native DWB or RWB after a 24 h XYL11 treatment (see Section 4.2) was achieved according to Zinbo and Timell.[42] Samples (15 g) were immerged in 300 mL of $NaBH_4$ 1% (w/v)/KOH 24% (w/v) for 3 h with agitation at room temperature. After centrifugation, EtOH (6 vol) 95% (v/v) was added to the supernatant. The pH was adjusted to neutral by the addition of AcOH. During overnight incubation at 4 °C, hemicellulose precipitation occurred. Hemicelluloses were then recovered by centrifugation (30 min, 9000*g*, 4 °C) and washed vigorously in EtOH 80%. The hemicellulose-rich fractions were suspended in distilled water. After 12 h at 4 °C, the water-insoluble arabinoxylan (WI-AX) fraction was recovered by centrifugation and designated as either WI-AX_i (initial water-insoluble arabinoxylan) or WI-AX_r (residual water-insoluble arabinoxylan). Water soluble arabinoxylans (WS-AX) were further fractionated by a two step ethanol precipitation procedure. First the WS-AX was adjusted to 50% (v/v) EtOH and incubated at 4 °C for 12 h. The resulting precipitates were designated either WS-AX_i-50 or WS-AX_r-50. After the final EtOH concentration in the remaining supernatant was raised to 80% (v/v), incubation at 4 °C was repeated. This gave rise to a second precipitate, which was designated either WS-AX_i-80 or WS-AX_r-80. All fractions were then freeze-dried prior to further analysis.

4.5. Activities of XYL10 and XYL11 on AXs and DWB

The activities of XYL10 or XYL11 towards isolated AX fractions were determined by the quantification of reducing sugar liberation using the ferricyanide-based method described by Kidby and Davidson.[43] The assay mixture (1 mL) consisted of various concentrations of substrate (0.05–3% w/v) in 50 mM sodium acetate, pH 5.8. Before the assay, both the alkali-extractable AXs and commercial birchwood xylan were stirred and vigorously homogenised before incubation at 60 °C in the presence of XYL10 or XYL11 (0.025–0.1 IU/mL final). Following enzyme addition, aliquots were removed at 2 min intervals for reducing sugar quantification. Additionally, in order to ascertain the level of nonspecific hydrolysis catalysed by *E. coli*-encoded glycoside hydrolases, a control experiment using xylanase-free *E. coli* lysate was performed for all AXs tested. For such controls, each substrate was incubated with an aliquot of lysate whose volume was equivalent to that needed to obtain 0.1 IU/mL of XYL10 in the other experiments.

To determine the kinetic parameters $K_m^{(app)}$, V_{max} and k_{cat}, xylanase assays were also performed in initial rate conditions using DWB (0.05–20% w/v in distilled water). Reactions were performed at 60 °C with stirring in the presence of either XYL10 or XYL11 at concentrations of 0.2 and 0.05 IU/mL, respectively. Each experimental condition was reproduced in triplicate and the progression of hydrolysis was determined after 10 min by monitoring the liberation of reducing sugars. Data were analysed using the Enzyme Kinetics software (SPSS Science Inc., USA). $K_m^{(app)}$ and V_{max} were expressed ± standard error.

4.6. Chemical analysis

4.6.1. Carbohydrate analysis. The identification and quantification of neutral and acidic carbohydrates was carried using HPAEC. The various AX fractions (see Section 4.4) were hydrolysed (5 mg) using 12 M H_2SO_4 acid for 2 h at room temperature, then diluted at 1.5 M for again 2 h at 100 °C. The same procedure was used for the carbohydrate analysis of both DWB and RWB. Soluble fractions (500 μL) released by xylanase from DWB were hydrolysed for 2 h at 100 °C in the presence of 1 M H_2SO_4. All samples were then filtered (PTFE, 0.22 μm) before injection onto a CarboPac PA-1 anion exchange column (4 × 250 mm, Dionex). Detection was performed by pulsed amperometry (PAD 2, Dionex) and samples were eluted using the following conditions: A (Milli-Q water) 95–0% with B (0.1 M NaOH in Milli-Q water) 5–100% for 19 min; then 100–0% B with C (0.3 M AcONa; 0.1 M NaOH in Milli-Q water) 0–100% until 49 min, and finally D 100% (0.3 M NaOH in Milli-Q water) for 6 min. A post-column addition of 0.3 M NaOH was used. Monosaccharide composition was analysed and quantified using both L-fucose as the internal standard and standard solutions of neutral carbohydrates (L-arabinose, D-glucose, D-xylose, D-galactose) and uronic acids (D-galacturonic and D-glucuronic acids). AX content was expressed as the sum of the amounts of xylose and arabinose. Variation SD in the analytical measurements was ⩽5%.

4.6.2. Structural analysis of arabinoxylan-enriched fractions. All alkali-extracted AX fractions, except WS-AX_r-50, which was not obtained in sufficient quantities, were subjected to structural analysis. Briefly, 4 mg of each AX were methylated twice (to ensure complete methylation) as previously described.[44] The dry extract of the methylated sample was then hydrolysed with formic acid (0.8 mL, 90% at 100 °C by mg of samples) for 1 h. After drying, further hydrolysis with trifluoroacetic acid (1 mL, 2 M, 100 °C) was performed for 3 h. Samples were then reduced with a $NaBH_4$ solution for 16 h at room temperature. Borate compounds were eliminated by MeOH/HCl 1% treatment. The reduced samples were

acetylated with acetic anhydride/pyridine (v/v) at 100 °C for 1 h.[45] Alditol acetate derivatives were analysed using a Hewlett-Packard 5890A gas chromatography system equipped with a flame-ionisation detector (GC/FID) and a SP2380 macrobore column (0.53 mm × 30 m) (Sigma–Aldrich Chimie S.A.R.L, Saint-Quentin Fallavier, France). Appropriate methylated alditol carbohydrates were used as standards. The carrier gas was high-purity nitrogen and the injector port and detector were heated at 260 and 280 °C, respectively. For sample separation the following conditions were applied: 3 min at an initial temperature of 165 °C followed by an incremental increase (2.5 °C/min) to a final of 225 °C during 3 min. Complementary analyses were performed using a GC–MS system (Delsi GC coupled to a Nermag R10-10C mass analyser) using the chromatographic protocol described above.

4.6.3. Determination of bran hydroxycinnamic acids. Esterified ferulic acid (FA), *p*-coumaric (pCA) and diferulic acid (DiFA) were released from the ball crushed RWB (40 mg) and the water-treated DWB (control) (see Section 4.3) by alkali extraction using 2 M NaOH (10 mL) for 2 h at 35 °C. Phenolic acids were quantified by HPLC using a Kromasil 5μ (RP-18, 250 mm × 4.6 mm) A.I.T Chromato column and an MeCN/MeOH/water gradient for elution as previously described.[35] Measurements were performed in triplicate and the variation was estimated to be 5%.

4.7. Protein estimation

The protein content in each of the extracted AX fractions was calculated by determining in duplicate the total N content (N × 5.7) using approximately 3 mg of freeze-dried sample and an elementary analyser (NA 1500, Carlo Erba) coupled to a mass spectrometer (Fisons Isochrom). The experimental error was less than 5%.

4.8. Analysis of oligosaccharides by PACE

Prior to PACE analysis, enzyme-produced oligosaccharides (0.5 mg/mL^{-1}) and standard xylooligosaccharides and monosaccharides (20 μmol) were vacuum air-dried. Derivatisations using the ANTS or AMAC fluorophores and polyacrylamide gel electrophoresis were carried out as described previously.[28] Under ANTS conditions used, arabinose migrates very close to xylose and its position was not annotated. Gels were scanned using a Master-Imager CCD camera system (Amersham, Bucks, UK) equipped with an excitation and detection filters at 400 and 530 nm, respectively. Captured gel images (resolution 100 μm) were exported in an 8 bit file to Microsoft PowerPoint.

4.9. ^{13}C NMR spectroscopy

The ^{13}C NMR spectrum of the glucose-rich fraction that was solubilised by the action of XYL11 on wheat bran was recorded in Me_2SO-d_6 (12 mg/0.5 mL) at 75 MHz using a Bruker AM 300 FT spectrometer operating at 323 K. The chemical shifts were referenced to Me_2SO-d_6 at 39.6 ppm and are reported relative to TMS.

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