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Enzymatic production of xylooligosaccharides from beechwood xylan: effect of xylanase preparation on carbohydrate profile of the hydrolysates

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Abstract

Xylooligosaccharides (XOS) are non-digestible food ingredients with beneficial effects on human health. In the present work, three different xylanase preparations from filamentous fungi were characterised and used in the enzymatic conversion of beechwood xylan into XOS. The degree of polymerisation (DP) and yield of XOS, besides the release of xylose in the hydrolysate, are influenced by the xylanolytic activities of xylanase preparation and biochemical properties of endo-1,4-β-xylanases. Enzymatic hydrolysis of beechwood xylan using β-xylosidase-free xylanase from *Thermomyces lanuginosus* produced more than 90% (w/v) XOS in the hydrolysate, equivalent to 9.18 mg/mL in 24 h of reaction time. The XOS obtained in highest amounts were xylobiose (DP 2, 66.46%) followed by xylotriose (DP 3, 25.10%), and a small amount of xylose (4.97%) was simultaneously obtained. These findings suggest that this type of xylanase preparation is very promising for obtaining high-pure XOS with lower DP in food industries.

Introduction

Xylooligosaccharides (XOS) are xylose-based oligosaccharides that may have variable proportions of substituents like acetyl groups and uronic acids, depending on the xylan-structure of original feedstock and production process (Kabel *et al.*, 2002). XOS are usually used as prebiotics and functional foods. The non-digestibility of these compounds contributes to the maintenance of intestinal health by stimulating the growth of beneficial bacteria such as bifidobacteria and lactobacilli (Moure *et al.*, 2006; Carvalho *et al.*, 2013; Samanta *et al.*, 2015). XOS with degrees of polymerisation (DP) from 2 to 5 are preferred in functional foods production since they are consumed by probiotic bacteria (Kiran *et al.*, 2013), of which, xylobiose (DP 2) and xylotriose (DP 3) have greater prebiotic effects (Jiang *et al.*, 2004; Gullón *et al.*, 2011). The sweetness of xylobiose is 30% that of sucrose, although it is a sugarless compound allowing its use in anti-obesity diets (Goldman, 2009).

XOS production by autohydrolysis or hydrothermal processing of lignocellulosic biomass results in hydrolysates containing a large variety of undesirable components, such as xylose and lignin, which can make a complex purification step necessary (Zhu *et al.*, 2006). Alternatively, XOS can be produced by enzymatic or acid hydrolysis of different xylans extracted from the lignocellulosic biomass (Akpinar *et al.*, 2010; Samanta *et al.*, 2015). Enzymatic hydrolysis is generally preferable in the food industry because it does not produce undesirable by-products and requires no special equipment to operate at high temperatures and pressure in contrast to the autohydrolysis (Akpinar *et al.*, 2009).

Enzymatic production of XOS is catalysed by the endo-xylanases, which hydrolyse the β-1,4 glycosidic linkages in xylan. Xylanase preparations with a low exo-xylanase and/or β-xylosidase activity are preferred to avoid the release of xylose that can inhibit the endo-xylanase activity (Vázquez *et al.*, 2000), and should not be present in prebiotic formulations (Escarnot *et al.*, 2012). Furthermore, endo-1,4-β-xylanases should have a good activity under the hydrolysis conditions. The knowledge about the xylanolytic activities, optimum pH and temperature, thermal stability and kinetic parameters of a commercial xylanase preparation is important when considering its use in xylan hydrolysis, in order to have a good XOS yield and to make a large-scale production feasible.

The food industry still spends a considerable amount of time and money to produce XOS, and their market value is very high. The rapid development of

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the functional ingredient market and the large number of oligosaccharide applications increases the interest in investigating different microbial xylanases, types of xylan and new technologies to obtain high-pure XOS with lower DP (Moure *et al.*, 2006; Vegas *et al.*, 2008; Samanta *et al.*, 2015).

In this context, the objective of the present work was therefore to evaluate the influence of commercial xylanase preparations from filamentous fungi on the carbohydrate profile of beechwood xylan hydrolysates. The main xylanolytic activities and biochemical properties of the endo-xylanases were assayed to a better understanding of the hydrolytic performance of different xylanase preparations on beechwood xylan. Hydrolysates containing high amounts of low-DP XOS were prioritised due to its greater prebiotic potential.

Materials and methods

Material

Commercial xylanase preparations; Shearzyme® from *Aspergillus oryzae* and Spring Mono® from *Thermomyces lanuginosus* were supplied by Novozymes A/S (Bagsvaerd, Denmark), and Hemicellulase Amano® from *Aspergillus niger* was supplied by Amano Enzymes Inc. (Nagoya, Japan). Beechwood xylan, p-nitrophenyl-β-D-xylopyranoside (pNPX), p-nitrophenyl-α-L-arabinofuranoside (pNPA), p-nitrophenyl-β-D-glucopyranoside (pNPG) and xylose standard were purchased from Sigma-Aldrich (St. Louis, USA). XOS (xylobiose, xylotriose, xylotetraose and xylopentaose) standards were purchased from Megazyme (Bray, Ireland).

Xylanolytic activities of the xylanase preparations

Xylanase preparations were characterised primarily in terms of the xylanolytic activities. Levels of endo-1,4-β-xylanases, β-xylosidases, α-L-arabinofuranosidases and β-glucosidases were measured in order to better evaluate their hydrolytic performances on structure of beechwood xylan.

Xylanolytic activities assays

Endo-1,4-β-xylanase activity was measured using 1.0% (w/v) beechwood xylan as the substrate (Bailey *et al.*, 1992). The reducing sugars released were quantified by the dinitrosalicylic acid (DNS) method (Miller, 1959), using xylose as standard. One enzyme activity unit (U) is defined as the amount of enzyme that releases 1 µmol xylose per min under the assay conditions. β-xylosidase, β-glucosidase and α-L-arabinofuranosidase activities were measured using pNPX (0.2%, w/v), pNPG (0.2%, w/v) and pNPA (0.1%, w/v) as the substrates, respectively (Tan *et al.*, 1987). One enzyme activity unit (U) is defined as the amount of enzyme that releases 1 µmol of pNP per min under the assay conditions.

Biochemical characterisation of endo-1,4-β-xylanases

Effects of pH and temperature on the activity

The influence of pH on endo-1,4-β-xylanase activity was determined at pH values ranging from 3.5-7.0 (pH 3.5-5.5: 0.05 M sodium citrate buffer, pH 6.0-7.0: 0.05 M sodium phosphate buffer) using beechwood xylan as the substrate at 50°C. The effect of temperature on endo-1,4-β-xylanase activity was determined at temperatures ranging from 35-90°C using beechwood xylan as the substrate at pH 5.3. The relative activity was expressed as a percentage of the maximum activity under experimental conditions.

Thermal stability

Thermal stability was studied by incubating endo-1,4-β-xylanases at 50°C (temperature used in hydrolysis experiments) in the absence of the substrate. At periodic intervals, aliquots were withdrawn and cooled in an ice bath prior to the assay. The residual activity was expressed as a percentage of the initial activity. The inactivation rate constants (Kd) were calculated from a semilogarithmic plot of residual activity versus time and half-lives were estimated using Equation 1. The half-life ($t_{1/2}$) is defined as the time taken for the residual activity to reach 50%.

$$t_{1/2} = \frac{-\ln(0.5)}{K_d} \quad (1)$$

Determination of the kinetic constants

The kinetics of most reactions catalysed by endo-1,4-β-xylanases follow the Michaelis-Menten model (Equation 2). The kinetics constants (Vmax and K_m) were calculated from a Lineweaver-Burk double reciprocal (1/v versus 1/S) plot (Equation 3) using beechwood xylan as the substrate (1-20 mg/mL) at pH 5.3.

$$v = \frac{v_{max}[S]}{K_m + [S]} \quad (2)$$

$$\frac{1}{v} = \frac{K_m}{v_{max}} \times \frac{1}{[S]} + \frac{1}{v_{max}} \quad (3)$$

Enzymatic hydrolysis of xylan and production of XOS

XOS were produced by enzymatic hydrolysis of beechwood xylan using commercial food grade xylanases. The hydrolysis were carried out in mixing reactors containing 75 mL reaction mixture, consisting of 3.0% (w/v) xylan in 0.05 M sodium citrate buffer at pH 5.3 and an endo-xylanase loading of 200 U/g substrate. The enzymatic reactions were maintained at 50°C and 180 rpm for 36 h. Aliquots were withdrawn at periodic intervals and the reaction was stopped by keeping the reaction mixture in a boiling water bath for 5 min. Samples were centrifuged at 10,000 g for 10 min and filtered through a polyvinylidene fluoride membrane of 0.22 µm pore size (Durapore, Millipore, Cambridge, USA). The hydrolysates were characterised using high-performance liquid chromatography (HPLC) to determine the DP and yield of the XOS and the amount of xylose released. All reactions were performed in duplicate.

HPLC analysis

The hydrolysis products were analysed using an HPLC system (Prominence, Shimadzu, Kyoto, Japan) equipped with a refraction index detector (RID-10A) and a column oven (CTO-20A). Aliquots of 20 µL were automatically injected (SIL-20AHT) into the system and eluted with ultra-pure water (Milli-Q, Millipore, Cambridge, USA) at a flow rate of 0.4 mL/min as the mobile phase for a carbohydrate separation column (Aminex HPX-42A, Bio-Rad Laboratories, Hercules, USA) at 50°C. The run time was about 40 min. Xylose and XOS were used as external standards.

Scanning electron microscopy (SEM) analysis

Dried hydrolysates were ground by pestle and mortar to a soft powder and filtered through a sieve with size of 300 µm (50 mesh). All samples were coated with gold before analysis. Specimens were observed under SEM (JSM-6610LV, Jeol, Tokyo, Japan) operating at 15 kV. SEM photomicrographs were taken at a magnification of 700×.

Statistical analysis

The results of total XOS were statistically analysed using analysis of variance (ANOVA) and Tukey's test at a significance level of 5% ($p < 0.05$).

Results and discussion

Xylanolitic activities of the xylanase preparations

Commercial xylanase preparations from filamentous fungi presented different levels of xylanolytic activities. Endo-1,4-β-xylanase activity was about 4,436.63 ± 59.57 U/mL for Shearzyme®, 21,961.89 ± 558.73 U/g for Spring Mono® and 34,895.74 ± 1,465.33 U/g for Hemicellulase Amano®. The β-xylosidase activity was detected in Shearzyme® (28.57 ± 0.41 U/mL) and Hemicellulase Amano® (0.38 ± 0.01 U/g), while α-L-arabinofuranosidase and β-glucosidase activities were only detected in considerable levels in Amano® (9.68 ± 0.28 and 79.54 ± 0.49 U/g, respectively). These results could be explained by the fact that due to the complex structure of the xylans, many microorganisms produce multiple forms of xylanases simultaneously to get extra assimilable monosaccharides for their growth and/or maintenance (Kiran *et al.*, 2013). Therefore, the xylanase preparations contain a mixture of xylanolytic enzymes acting differently on different xylan structures. A good knowledge about the xylanolytic activities in the commercial xylanase preparations enables an enhanced evaluation of their hydrolytic performances on xylan structure and XOS production.

The major enzyme for xylan depolymerisation is endo-1,4-β-xylanase because it attacks the main chain generating unsubstituted or branched XOS, while the β-xylosidase hydrolyses XOS to xylose. Auxiliary enzymes, such as α-L-arabinofuranosidases and β-glucosidases, are responsible for the release of substituents from the xylan backbone (Biely, 2003; Collins *et al.*, 2005), thereby increasing the accessibility of xylanases to the main chain (Kiran *et al.*, 2013). Depending on the xylan type, various auxiliary enzymes are necessary for full xylan degradation (Aro *et al.*, 2005). For enzymatic production of prebiotic XOS, xylanase preparations with low exo-xylanase and/or β-xylosidase activities are preferred to avoid the release of xylose into the hydrolysate (Vázquez *et al.*, 2002; Chen *et al.*, 2009). Although the xylanase preparations contained endo-1,4-β-xylanase and β-xylosidase activities, the ratios values of β-xylosidase/endo-xylanase were less than 0.01; therefore, it has the potential to hydrolyse beechwood xylan to XOS with a minimal xylose production. Compared to other techniques for XOS production from xylans, enzymatic hydrolysis with xylanases offers various advantages, such as mild reaction conditions, better specificities, higher yields and no generation of by-products (Sadaf and Khare, 2014).

Biochemical characterisation of endo-1,4-β-xylanases

For the enzymatic production of XOS, endo-1,4-β-xylanases should be active under the hydrolysis

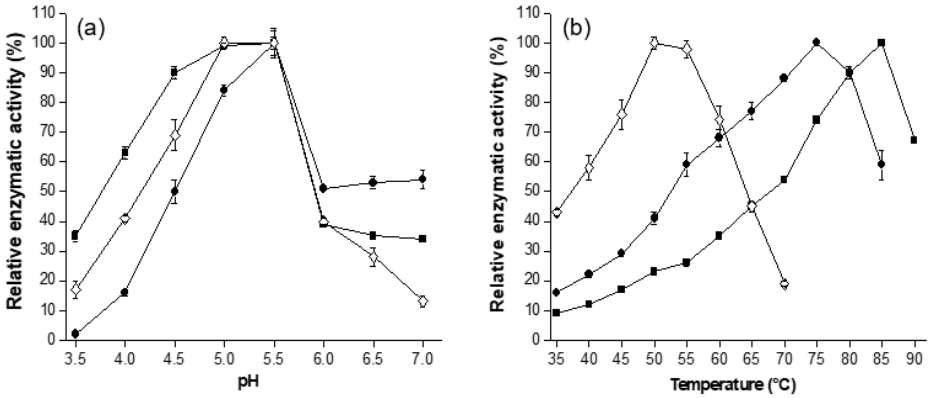

Figure 1. Effect of pH (a) and temperature (b) on enzymatic activity of endo-1,4-β-xylanases: Shearzyme® (■), Spring Mono® (●) and Hemicellulase Amano® (◊). Results are presented as mean ± standard deviation (n = 3)

conditions, and therefore it is very important to determine their biochemical properties. Endo-1,4-β-xylanases exhibits an optimum activity in the pH range of 5.0-5.5 (Figure 1a). These findings are consistent with literature reports indicating that optimum pH of most of the fungal xylanases is between 5.0 and 6.0 (Chidi *et al.*, 2008; Pal and Khanum, 2011; Gonçalves *et al.*, 2012). Moreover, in most researches, a significant increase in XOS production was observed in the pH range of 5.0-6.0 (Zhu *et al.*, 2006; Aachary and Prapulla, 2009; Peng *et al.*, 2012).

The xylanases showed different behaviours with respect to temperature (Figure 1b). Endo-1,4-β-xylanases Shearzyme® and Spring Mono® exhibited optimum activity at 85 and 75°C, respectively, while the endo-1,4-β-xylanase Amano® was more active in the temperature range of 50-55°C. The high catalytic activity at high temperatures suggests that xylanases Shearzyme® and Spring Mono® are thermostable. There are studies that also report a high optimum temperature for fungal xylanases (Vázquez *et al.*, 2002; Kocabas *et al.*, 2015). Moreover, commercial xylanase preparations generally contain preservatives and/or stabilisers included, which contribute to the maintenance of their enzymatic properties under adverse conditions.

The xylanases showing its optimum temperature well above 50°C can be used in the hydrolysis of beechwood xylan at around 50°C, since these enzymes remain active and maintain partial activity at this temperature. The cellulase-free xylanases active and stable at high temperatures are also applied in pulp and paper bleaching at around 60°C (Beg *et al.*, 2001).

The thermal stability of endo-1,4-β-xylanases at 50°C was determined due to promising results for XOS production that have been obtained at this temperature (Aachary and Prapulla, 2009). Endo-β-xylanase Shearzyme® displayed a high thermostability, retaining more than 80% of its initial activity at 50°C during 240 h; therefore, it was not possible to determine the half-life ($t_{1/2}$) of this enzyme. Endo-xylanase Spring Mono® was also very stable at 50°C and its $t_{1/2}$ was 210 h. In comparison, endo-1,4-β-xylanase Amano® showed a low thermal stability at 50°C, having lost more than 50% of its initial activity in 0.3 h. The xylanases thermal stability is an important parameter to be considered in the enzymatic hydrolysis of xylan to XOS, particularly when considering its longer reaction time.

Kinetic parameters analysis was performed to further understanding the affinity of the endo-1,4-β-xylanases towards the substrate xylan. Among the xylanases evaluated, the K_m value of endo-xylanase Shearzyme® was the lowest (8 mg/mL) followed by Spring Mono® (15 mg/mL) and Amano® (20 mg/mL), which indicates the higher affinity of xylanase Shearzyme® to the beechwood xylan. K_m is inversely proportional to the affinity of the enzyme for its substrate; and therefore, a low value of K_m refers to a high affinity for a substrate, and it means that a low substrate concentration is needed to reach half-maximal velocity.

The activity of enzymes are influenced by environmental factors such as pH, temperature and reaction medium, which can strongly affect the specific three-dimensional structure and spatial conformation of the proteins, thereby directly interfering in the enzymatic process yield (Jurado *et al.*, 2004). Hence, the characterisation of endo-1,4-β-xylanase is essential to determine the best conditions for its hydrolytic performance on beechwood xylan, making it possible to produce high levels of XOS with a low content of xylose.

Enzymatic hydrolysis of xylan and production of XOS

The profile of carbohydrate and production of total XOS (X2-X5) obtained using the xylanase

Table 1. XOS and xylose production from beechwood xylan using xylanase preparation Shearzyme® from A. oryzae

Reaction time (h)	Carbohydrate profile (%) of the hydrolysate					Total XOS (mg/mL)
	Xylose	X2	X3	X4-X5	Total XOS	
2	3.29	55.80	22.78	18.14	96.71 ± 0.01	11.76 ± 0.05^{b}
4	7.23	58.93	18.13	15.72	92.77 ± 0.03	12.13 ± 0.12^{ab}
6	9.47	60.21	16.08	14.24	90.53 ± 0.10	12.33 ± 0.05^{ab}
8	10.98	61.79	13.80	13.43	89.02 ± 0.02	12.33 ± 0.11^{ab}
10	11.60	64.54	12.04	11.82	88.41 ± 0.35	12.04 ± 0.20^{ab}
12	11.92	66.54	10.99	10.54	88.08 ± 0.02	11.82 ± 0.12^{b}
14	12.91	66.68	10.06	10.35	87.10 ± 0.31	12.08 ± 0.35^{ab}
16	13.59	65.44	10.02	10.95	86.41 ± 0.03	12.59 ± 0.16^{ab}
20	14.49	68.55	9.11	7.85	85.51 ± 0.09	12.25 ± 0.18^{ab}
24	14.93	66.97	8.49	9.60	85.07 ± 0.04	12.62 ± 0.01^{ab}
36	16.49	70.47	6.23	6.82	83.51 ± 0.02	12.74 ± 0.09^{a}

X2: xylobiose, X3: xylotriose, X4: xylotetraose, X5: xylopentaose. Mean ± standard error. Mean values of total XOS that have different superscripts letters are significantly different ($p < 0.05$).

preparation Shearyzme® are shown in Table 1. At the end of 2 h, the amount of total XOS release was 11.76 mg/mL, which corresponded to 96.71% of the total carbohydrates in the hydrolysate, while xylose represented no more than 3.29%. XOS with DP 3-5 (X3-X5) significantly decreased over time clearly indicating that they were partially hydrolysed to xylobiose (X2) and xylose. The significant increase in xylose release could be explained by the fact that the xylanase preparation contains β-xylosidase activity, which hydrolysed XOS to xylose.

Kiran *et al.* (2013) using a similar xylanase preparation (Shearzyme® 500L) also verified its ability to efficiently hydrolyse a xylan mixture to XOS (69 mg xylobiose/g xylan). However, the enzyme β-xylosidase present in the xylanase preparation released higher amounts of xylose (25 mg/g xylan) as compared to that released by Veron 191® (15 mg xylose/g xylan) without β-xylosidase. With the use of the Shearzyme® in the hydrolysis of beechwood xylan, a high yield of low-DP XOS (226 mg xylobiose/g xylan) and a low yield of xylose (13 mg/g xylan) were achieved in only 2 h of hydrolysis. After 36 h, the total XOS (X2-X5) was decreased by 83.51%, and xylose increased up to 16.49% (84 mg/g xylan); therefore, a longer reaction time (above 4 h) is not advantageous to obtain a XOS mixture containing low content of xylose.

Goldbeck *et al.* (2014) used hemicellulolytic enzymes – endo-1,4-β-xylanases (GH10 and GH11), α-L-arabinofuranosidases (GH51 and GH54), and β-xylosidase (GH43) – in combinatorial assays to analyse the synergistic and antagonistic effects of enzyme interactions on degradation of pretreated sugarcane bagasse (PSB) and wheat xylan (WA) in target biotechnological applications. Based on the results, it was possible to conclude that the presence of GH43 enzyme in enzymatic combinations, such as GH11+GH51 and GH10+GH11+GH51, increased the xylose production from WA and PSB, respectively. This was expected since the β-xylosidase cleaves the XOS to free xylose, which is a hindrance if the goal is to produce XOS.

XOS production using xylanase preparation Spring Mono® is shown in Table 2. After 2 h, the total XOS released was 6.70 mg/mL, corresponding to 99.11% of the total sugars in the hydrolysate. Similarly as in the previous hydrolysis, XOS of DP 3-5 (X3-X5) was also found to decrease as time increased, due to its partial hydrolysis to low-DP XOS and xylose. After 36 h, the amount of total XOS (X2-X5) was 9.55 mg/mL, and the xylose production was less than 0.70 mg/mL (23 mg/g xylan), since the β-xylosidase was absent in xylanase preparation.

Xylobiose (X2) and xylotriose (X3) were produced in higher amounts in hydrolysate, however, the X2 content increased (2.59-6.96 mg/mL) and that of X3 slightly decreased (2.73-2.27 mg/mL) over time. It is important to emphasise that total XOS comprised more than 90% of the carbohydrates in the hydrolysate for all reaction times. However, considering that there was no significant difference in the total XOS of 24 h and 36 h, the reaction time of 24 h appeared to be more suitable for XOS production.

There are few reports on conversion of xylan to XOS with a high purity using xylanase preparations from filamentous fungi. The reported XOS mixtures formed from different xylans using xylanase preparations are generally composed of a high level of monosaccharides. According to Reddy and

Table 2. XOS and xylose production from beechwood xylan using xylanase preparation Spring Mono® from T. lanuginosus

Reaction time (h)	Carbohydrate profile (%) of the hydrolysate					Total XOS (mg/mL)
	Xylose	X2	X3	X4-X5	Total XOS	
2	0.89	38.27	40.41	20.43	99.11 ± 0.00	6.70 ± 0.01^{h}
4	1.45	48.69	37.32	12.55	98.55 ± 0.01	7.50 ± 0.07^{g}
6	1.90	54.02	34.60	9.49	98.10 ± 0.03	7.76 ± 0.14^{fg}
8	2.26	57.63	33.39	6.72	97.74 ± 0.06	8.01 ± 0.01^{ef}
10	2.62	59.75	31.69	5.94	97.38 ± 0.01	8.19 ± 0.04^{def}
12	2.97	61.49	30.42	5.12	97.03 ± 0.06	8.34 ± 0.01^{de}
14	3.31	62.79	29.22	4.68	96.69 ± 0.01	8.47 ± 0.02^{cd}
16	3.60	63.74	28.38	4.28	96.40 ± 0.02	8.56 ± 0.04^{cd}
20	4.11	65.57	26.48	3.84	95.89 ± 0.05	8.86 ± 0.12^{bc}
24	4.97	66.46	25.10	3.47	95.03 ± 0.21	9.18 ± 0.03^{ab}
36	6.47	68.15	22.24	3.13	93.53 ± 0.08	9.55 ± 0.17^{a}

X2: xylobiose, X3: xylotriose, X4: xylotetraose, X5: xylopentaose. Mean ± standard error. Mean values of total XOS that have different superscripts letters are significantly different ($p < 0.05$).

Krishnan (2016), the absence of xylose in prebiotic XOS mixture is an advantage since purification is not necessary to obtain high concentration of XOS. Therefore, the use of xylanase preparation Spring Mono® would be economically viable since a low-DP XOS mixture could be obtained without purification for application as high-pure XOS in functional foods.

The total XOS produced with the use of xylanase preparation Amano® was 7.31 mg/mL after 2 h of hydrolysis, which corresponded to 96.95% of the total carbohydrates in the hydrolysate (Table 3). The percentage of total XOS decreased over time, mainly due to the degradation of XOS with DP 4-5 (X4 and X5) to xylose. The xylobiose (X2) yield gradually increased (2.69-6.44 mg/mL) over time and less changes were observed in xylotriose (X3) production (2.93-3.83 mg/mL). After 36 h of hydrolysis, the xylose content was found to be 1.73 mg/mL (58 mg/g xylan), since the xylanase preparation had β-xylosidase. Although the ratio of β-xylosidase/endo-1,4-β-xylanase in Hemicellulase Amano® was lower than that in Shearzyme®, the sugar profile confirms that the action of β-xylosidase generated xylose from XOS. Based on the results, a longer reaction time (above 8 h) is not advantageous to obtain a XOS mixture containing low content of xylose.

As expected, the hydrolysis level of the beechwood xylan and carbohydrate profile of the hydrolysates varied with each commercial xylanase preparation, since they exhibited a specific composition and action mechanism. Xylanase Shearzyme® showed the highest ratio of β-xylosidase/ β-xylanase and consequently it produced more xylose; however,

Table 3. XOS and xylose production from beechwood xylan using xylanase preparation Hemicellulase Amano® from A. niger

Reaction time (h)	Carbohydrate profile (%) of the hydrolysate					Total XOS (mg/mL)
	Xylose	X2	X3	X4-X5	Total XOS	
2	3.05	35.70	38.82	22.43	96.95 ± 0.08	7.31 ± 0.21^{d}
4	4.35	40.99	38.93	15.74	95.65 ± 0.03	8.15 ± 0.07^{cd}
6	5.33	43.87	38.43	12.37	94.67 ± 0.02	8.61 ± 0.30^{bcd}
8	6.77	45.04	37.38	10.80	93.23 ± 0.04	9.02 ± 0.15^{abcd}
10	8.10	46.10	36.72	9.08	91.90 ± 0.07	9.36 ± 0.19^{abc}
12	9.29	47.28	37.26	6.17	90.71 ± 0.09	9.33 ± 0.25^{abc}
14	10.38	47.89	36.35	5.38	89.62 ± 0.09	9.67 ± 0.26^{abc}
16	11.26	48.24	35.15	5.35	88.74 ± 0.02	9.70 ± 0.34^{abc}
20	11.80	49.47	34.26	4.47	88.20 ± 0.00	9.98 ± 0.42^{ab}
24	12.44	50.32	33.18	4.06	87.56 ± 0.03	10.24 ± 0.43^{ab}
36	13.96	51.96	30.90	3.19	86.04 ± 0.06	10.67 ± 0.55^{a}

X2: xylobiose, X3: xylotriose, X4: xylotetraose, X5: xylopentaose. Mean ± standard error. Mean values of total XOS that have different superscripts letters are significantly different ($p < 0.05$).

its endo-xylanase with the lowest K_m was able to efficiently convert xylan into low-DP XOS. Xylanase Spring Mono® that had no β-xylosidase activity released low levels of xylose and produced good amount of XOS with DP 2-3. It is possible that synergistic action of auxiliary enzymes and endo-1,4-β-xylanases also had an influence on the hydrolysis of beechwood xylan.

The potential industrial applications of XOS are defined based on the carbohydrate profile of the hydrolysate, being important to restrict the content of digestible monosaccharides in the production of prebiotic XOS (Escarnot *et al.*, 2012). Furthermore, the low-DP XOS such as xylobiose (DP 2) and xylotriose (DP 3) should be a priority due to their faster consumption by probiotic bacteria (Van Loo *et al.*, 1999; Reddy and Krishnan, 2016). For this purpose, the xylanase preparations were evaluated in relation to their ability to produce a XOS mixture with DP 2-3 (X2-X3) and little or no xylose.

The maximum xylobiose production was attained using the xylanase Shearzyme®. Xylanases Spring Mono® and Hemicellulase Amano® showed a similar behaviour for X2, while the xylanase Amano® produced more xylotriose than all other xylanases evaluated. The amount of xylose released was in the following order: Shearzyme® > Hemicellulase Amano® > Spring Mono®, according to their β-xylosidase activity. However, the xylanase Spring Mono® is promising for application in prebiotic XOS production with a low xylose content, which is an essential feature for its incorporation into growth medium of probiotics. Xylanases Shearyzme® and Amano® could also be used for the production of XOS carried out in a short time period to avoid the release of xylose.

In comparison to other studies, hydrolysis of beechwood xylan with endo-1,4-β-xylanases from filamentous fungi yielded good results. Aachary and Prapulla (2009) used endo-xylanase from *Aspergillus oryzae* to produce XOS from alkali-pretreated corncob, obtaining a percentage of 81% XOS (10.2 mg/mL) with 73% of xylobiose. Brienzo *et al.* (2010) reported that endo-xylanase from *Thermoascus aurantiacus* produced 2.8-6.4 mg/mL of XOS (14-32% xylobiose) by the hydrolysis of pretreated sugarcane bagasse. The endo-xylanase from *Trichoderma viridae* was used for the hydrolysis of xylan from grass extracted by alkaline hydrolysis, and it produced approximately 2.8 mg/mL of XOS, mainly consisting of xylobiose (1.7 mg/mL) and xylotriose (1.1 mg/mL) (Samanta *et al.*, 2012).

Scanning electron microscopy (SEM)

SEM shows the microstructure of original beechwood xylan and beechwood xylan after having been subjected to enzymatic hydrolysis by endo-1,4-β-xylanases from filamentous fungi (Figure 2). As can be seen in Figure 2a, the original beechwood xylan had an intact structure and the xylan after enzymatic treatments shows very important structural changes (Figure 2b, 2c and 2d) due to the action of xylanolytic enzymes. These results support that all xylanases were highly active in the hydrolysis of beechwood xylan, consequently releasing high levels of XOS. Kocabas *et al.* (2015) also observed significant structural changes in the corncob xylans after enzymatic treatment using xylanase from *Scytalidium thermophilum,* and related it to the level of XOS generated during the hydrolysis.

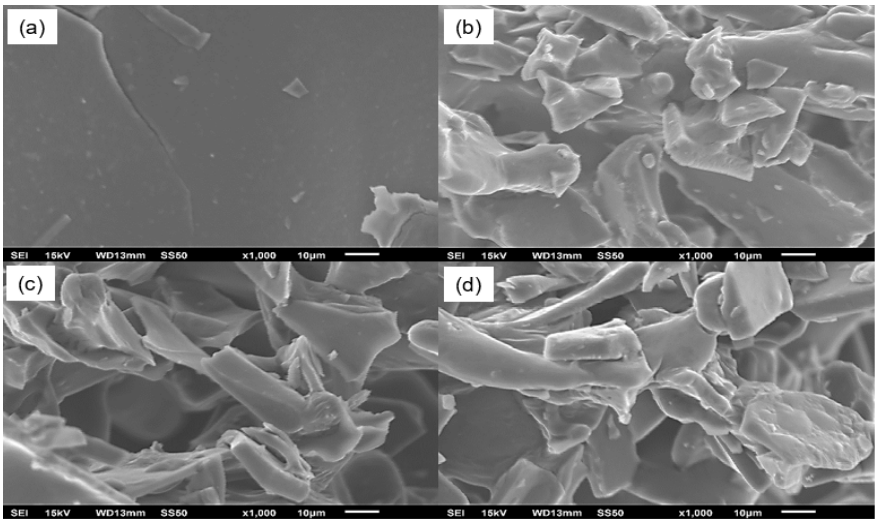

Figure 2. SEM photomicrographs of original beechwood xylan (a), and beechwood xylan after hydrolysis with xylanase preparations: Shearzyme® (b), Spring Mono® (c) and Hemicellulase Amano® (d)

Conclusion

The enzymatic hydrolysis of beechwood xylan using different xylanase preparations from filamentous fungi enabled production of XOS mixture with a DP distribution of 2-5. Xylobiose (DP 2) and xylotriose (DP 3) were found to be the major components of the XOS mixtures. Xylanase preparation without β-xylosidase activity and biochemical properties of the endo-1,4-β-xylanase are the main factors for the production of a high content of low-DP XOS and a low content of xylose from xylans. Hydrolysate predominantly containing xylobiose and xylotriose as well as a negligible amount of xylose was obtained using xylanase from T. lanuginosus, making this approach a promising alternative to produce high-pure XOS from beechwood xylan. The low-DP XOS have a great prebiotic potential and it could be incorporated into functional foods.

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References

Aachary, A. A. and Prapulla, S. G. 2009. Value addition to corncob: production and characterization of xylooligosaccharides from alkali pretreated lignin-saccharide complex using *Aspergillus oryzae* MTCC 5154. Bioresource Technology 100(2): 991–995.

Akpinar, O., Erdogan, K. and Bostanci, S. 2009. Enzymatic production of xylooligosaccharide from selected agricultural wastes. Food and Bioproducts Processing 87(2): 145–151.

Akpinar, O., Erdogan, K., Bakir, U. and Yilmaz, L. 2010. Comparison of acid and enzymatic hydrolysis of tobacco stalk xylan for preparation of xylooligosaccharides. LWT - Food Science and Technology 43(1): 119–125.

Aro, N., Pakula, T. and Penttila, M. 2005. Transcriptional regulation of plant cell wall degradation by filamentous fungi. FEMS Microbiology Review 29(4): 719–739.

Bailey, M. J., Biely, P. and Poutanen, K. 1992. Interlaboratory testing of methods for assay of xylanase activity. Journal of Biotechnology 23(3): 257–270.

Beg, Q., Kapoor, M., Mahajan, L. and Hoondal, G. 2001. Microbial xylanases and their industrial applications: a review. Applied Microbiology and Biotechnology 56(3): 326–338.

Biely, P. 2003. Xylanolytic enzymes. In Whitaker, J. R., Voragen, A. G. J. and Wong, D. W. S. (Eds). Handbook of Food Enzymology, p. 879-916. New York: Marcel Dekker, Inc.

Brienzo, M., Carvalho, W. and Milagres, A. M. 2010. Xylooligosaccharides production from alkali-pretreated sugarcane bagasse using xylanases from *Thermoascus aurantiacus*. Applied Biochemistry and Biotechnology 162(4): 1195–1205.

Carvalho, A. F. A., Neto, P. O., Silva, D. F. and Pastore, G. M. 2013. Xylo-oligosaccharides from lignocellulosic materials: chemical structure, health benefits and production by chemical and enzymatic hydrolysis. Food Research International 51(1): 75–85.

Chen, L. L., Zhang, M., Zhang, D. H., Chen, X. L., Sun, C. Y., Zhou, B. C. and Zhang, Y. Z. 2009. Purification and enzymatic characterization of two β-endoxylanases from Trichoderma sp. K9301 and their actions in xylooligosaccharide production. Bioresource Technology 100(21): 5230–5236.

Chidi, S. B., Godana, B., Ncube, I., Rensburg, E. J. V., Cronshaw, A. and Abotsi, E. K. 2008. Production, purification and characterization of celullase-free xylanase from Aspergillus terreus UL 4209. African Journal of Biotechnology 7(21): 3939–3948.

Collins, T., Gerday, C. and Feller, G. 2005. Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiology Reviews 29(1): 3–23.

Escarnot, E., Aguedo, M. and Paquot M. 2012. Enzymatic hydrolysis of arabinoxylans from spelt bran and hull. Journal of Cereal Science 55(2): 243–253.

Goldbeck, R., Damásio, A. R. L., Gonçalves, T. A., Machado, C. B., Paixão, D. A. A., Wolf, L. D., ... and Squina, F. M. 2014. Development of hemicellulolytic enzyme mixtures for plant biomass deconstruction on target biotechnological applications. Applied Microbiology and Biotechnology 98(20): 8513–8525.

Goldman, N. 2009. Methods for optimizing enzymatic hydrolysis of xylan to improve xylooligosaccharide yield. Basic Biotechnology Journal 5: 31–36.

Gonçalves, T. A., Damásio, A. R. L., Segato, F., Alvarez, T. M., Bragatto, J., Brenelli, L. B., … and Squina, F. M. 2012. Functional characterization and synergic action of fungal xylanase and arabinofuranosidase for production of xylooligosaccharides. Bioresource Technology 119: 293–299.

Gullón, P., González-Muñoz, M. J. and Parajó, J. C. 2011. Manufacture and prebiotic potential of oligosaccharides derived from industrial solid wastes. Bioresource Technology 102(10): 6112–6119.

Jiang, Z. Q., Deng, W., Zhu, Y. P., Li, L. T., Sheng, Y. J. and Hayashi, K. 2004. The recombinant xylanase B of Thermotoga maritima is highly xylan specific and produces exclusively xylobiose from xylans, a unique character for industrial applications. Journal of Molecular Catalysis B: Enzymatic 27(4-6): 207–213.

Jurado, E., Camacho, F., Luzón, G. and Vicaria, J. M.

2004. Kinetic models of activity for β-galactosidases: influence of pH, ionic concentration and temperature. Enzyme and Microbial Technology 34 (1): 33–40.

Kabel, M. A., Carvalheiro, F., Garrote, G., Avgerinos, E., Koukios, E., Parajó, J. C., ... and Voragen, A. G. J. 2002. Hydrothermally treated xylan rich by-products yield different classes of xylo-oligosaccharides. Carbohydrate Polymers 50(1): 47–56.

Kiran, E. U., Akpinar, O. and Bakir, U. 2013. Improvement of enzymatic xylooligosaccharides production by the co-utilization of xylans from different origins. Food and Bioproducts Processing 91(4): 565–574.

Kocabas, D. S., Guder, S. and Ozben, N. 2015. Purification strategies and properties of a low-molecular weight xylanase and its application in agricultural waste biomass hydrolysis. Journal of Molecular Catalysis B: Enzymatic 115: 66–75.

Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry 31(3): 426–428.

Moure, A., Gullón, P., Domínguez, H. and Parajó, J. C. 2006. Advances in the manufacture, purification and applications of xylo-oligosaccharides as food additives and nutraceuticals. Process Biochemistry 41(9): 1913–1923.

Pal, A. and Khanum, F. 2011. Purification of xylanase from *Aspergillus niger* DFR-5: individual and interactive effect of temperature and pH on its stability. Process Biochemistry 46(4): 879–887.

Peng, F., Peng, P., Xu, F. and Sun, R. C. 2012. Fractional purification and bioconversion of hemicelluloses. Biotechnology Advances 30(4): 879–903.

Reddy, S. S. and Krishnan, C. 2016. Production of high-pure xylooligosaccharides from sugarcane bagasse using crude β-xylosidase-free xylanase of Bacillus subtilis KCX006 and their bifidogenic function. LWT - Food Science and Technology 65: 237–245.

Sadaf, A. and Khare, S. K. 2014. Production of Sporotrichum thermophile xylanase by solid-state fermentation utilizing deoiled Jatropha curcas seed cake and its application in xylooligosaccharide synthesis. Bioresource Technology 153: 126–130.

Samanta, A. K., Jayapal, N., Kolte, A. P., Senani, S., Sridhar, M., Suresh, K. P. and Sampath, K. T. 2012. Enzymatic production of xylooligosaccharides from alkali solubilized xylan of natural grass (Sehima nervosum). Bioresource Technology 112: 199–205.

Samanta, A. K., Jayapal, N., Jayaram, C., Roy, S., Kolte, A. P., Senani, S. and Sridhar, M. 2015. Xylooligosaccharides as prebiotics from agricultural by-products: production and applications. Bioactive Carbohydrates and Dietary Fibre 5(1): 62–71.

Tan, L. U. L., Mayers, P. and Saddler, J. N. 1987. Purification and characterization of a thermostable xylanase from a thermophilic fungus *Thermoascus aurantiacus*. Canadian Journal of Microbiology 33(8): 689–692.

Van Loo, J., Cummings, J., Delzenne, N., Englyst, H., Franck, A., Hopkins, M., ... and van den Heuvel, E. 1999. Functional food properties of non-digestible oligosaccharides: a consensus report from the ENDO project (DGXII AIRII-CT94 -1095). British Journal of Nutrition 81(2): 121–132.

Vázquez, M. J., Alonso, J. L., Domínguez, H. and Parajó, J. C. 2002. Enzymatic processing of crude xylooligomer solutions obtained by autohydrolysis of Eucalyptus wood. Food Biotechnology 16(2): 91–105.

Vázquez, M. J., Alonso, J. L., Domínguez, H. and Parajó, J. C. 2000. Xylooligosaccharides: manufacture and applications. Trends in Food Science & Technology 11(11): 387–393.

Vegas, R., Alonso, J. L., Domínguez, H. and Parajó, J. C. 2008. Enzymatic processing of rice husk autohydrolysis products for obtaining low molecular weight oligosaccharides. Food Biotechnology 22(1): 31–46.

Zhu, Y., Kim, T. H., Lee, Y. Y., Chen, R. and Elander, R. T. 2006. Enzymatic production of xylooligosaccharides from corn stover and corn cobs treated with aqueous ammonia. Applied Biochemistry and Biotechnology 130(1): 586–598.