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Complex xylo-oligosaccharides identified from hydrothermally treated *Eucalyptus* wood and brewery's spent grain

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Abstract

Hydrolysates from two hydrothermally treated xylan-rich agrobased materials, *Eucalyptus* wood and brewery's spent grain were fractionated by anion-exchange chromatography and size-exclusion chromatography. Hereby, several pools were obtained and they were characterised by their sugar composition. Additionally, the oligosaccharides in the pools described were further identified by high-performance anion-exchange chromatography and mass spectrometry. The hydrothermally treated brewery's spent grain resulted in three pools of which two contained relatively high molecular weight xylan, singly and doubly branched with arabinose [X_nA_m], separated from a pool of xylo-oligosaccharides less branched with arabinose. The fractionation of the hydrothermally treated *Eucalyptus* wood resulted in a neutral pool, mainly consisting of a series of (acetylated) xylo-oligosaccharides [X_nAc_m], and three acidic pools. Two of these acidic pools contained a series of (acetylated) xylo-oligosaccharides including *one* 4-*O*-methylglucuronic acid [$X_n(GlcA_{me})_1Ac_m$], while the third acidic pool contained (acetylated) xylo-oligosaccharides substituted with *two* 4-*O*-methylglucuronic acids [$X_n(GlcA_{me})_2Ac_m$]. Additionally, a series of xylo-oligosaccharides containing both 4-*O*-methylglucuronic acid and a hexose, most likely galactose, was detected in the acidic *Eucalyptus* pools [$X_n(GlcA_{me})_{1\text{ or }2}Ac_mH$]. Information was obtained about the number of acetyl-groups linked to the (4-*O*-methylglucurono-) xylo-oligosaccharides. Finally, it is demonstrated with an example that the different substituents to the xylo-oligosaccharides present are of relevance for the fermentability of the xylo-oligosaccharides by human faecal samples.

Keywords: *Eucalyptus* wood; Brewery's spent grain; Preparative chromatography; Xylo-oligosaccharides

1. Introduction

Agro-industrial and forest by-products, such as brewery's spent grain and *Eucalyptus* wood are rich in cellulose and hemicelluloses. Currently, more and more effort is directed towards the utilisation of such by-products, considering economic values and environment. From this point of view, hydrothermal treatment is of interest because it will result in a selective fractionation of the hemicellulose and cellulose from the by-products, to be used for different product applications (Garrote, Dominguez, & Parajo, 1999; Koukios, Pastou, Koullas, Sereti, & Kolosis, 1999).

Recently, we described a first characterisation of the hydrolysates obtained after hydrothermal treatment of brewery's spent grain and *Eucalyptus* wood (Kabel et al., 2002). It was shown that the hydrolysate from brewery's spent grain contains about 48% w/w sugar; 5% w/w as monomeric arabinose, 1% w/w as monomeric xylose and about 42% w/w as polymeric/oligomeric material mainly composed of arabinose (18 mol%), xylose (56 mol%) and glucose (16 mol%). Glucose and glucose-oligomers most likely originate from the degradation of starch, while xylo-oligosaccharides substituted with arabinose are released from the arabinoxylan, described to be present in barley and brewery's spent grain (Han, 2000; Vietor, Angelino, & Voragen, 1992).

The hydrolysate from *Eucalyptus* wood contains about 71% w/w sugar, of which 2% w/w is monomeric arabinose, 8% w/w is monomeric xylose and 61% w/w are oligosaccharides mainly composed of xylose (68 mol%) and uronic acid (17 mol%). Furthermore, in addition to acetic acid released during hydrothermal treatment, many of the acetyl-groups present in the native *Eucalyptus* wood xylan resist the treatment (6% w/w) resulting in a variety of acetylated (4-*O*-methyl-glucurono-) xylo-oligosaccharides as identified by matrix assisted laser desorption/ionisation-time-of-flight mass spectrometry (MALDI-TOF MS)

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(Kabel, Schols, & Voragen, 2001; Kabel et al., 2002). Such considerable preservation of acetyl-groups was also observed during steam explosion treatments of birchwood (Korte, Offermann, & Puls, 1991).

From a nutritional point of view, xylo-oligosaccharides usually are considered as non-digestible oligosaccharides and enhance growth of bifidobacteria in the large bowel, which may affect the human gastrointestinal tract beneficially (Campbell, Fahey, & Wolf, 1997; Fooks, Fuller, & Gibson, 1999; Van Laere, Hartemink, Bosveld, Schols, &Voragen, 2000). However, the influence of several types of substitution of xylo-oligosaccharides on the ability and rate of fermentation by the human intestinal flora and on the production of short chain fatty acids is still unknown. Thus, these questions strengthened the importance of further studies on the structural features of xylo-oligosaccharide in hydrothermal treated substrates, for better understanding the mechanism of xylan-breakdown in addition to studying the fermentability of the xylo-oligosaccharides formed.

In this paper, we propose a method to fractionate hydrolysates from brewery's spent grain and *Eucalyptus* wood into several series of differently substituted xylo-oligosaccharides, which are characterised in more structural detail and submitted to fermentation studies.

2. Experimental

2.1. Hydrothermal treated samples

Brewery's spent grain was supplied from the Brewery Central de Cervejas, Vialonga (Portugal). The hydrolysates were kindly provided by Carvalheiro/Gírio of INETI (Lisboa, Portugal). Three hydrolysates, prepared from brewery's spent grain at conditions which resulted in a maximum release of oligosaccharides, were equally mixed and further mentioned as the hydrolysate from brewery's spent grain. Chips of *Eucalyptus* wood were obtained from ENCE Complejo Industrial de Pontevedra Puentenolinos s/n Lourizan (Spain, July 1998). The hydrolysates from *Eucalyptus* wood were received gratefully from Garrote/Parajó of the University of Vigo (Vigo, Spain). Five hydrolysates, all prepared to reach maximal oligosaccharide-concentrations (Garrote et al., 1999), were equally mixed and further mentioned as the hydrolysate from *Eucalyptus* wood.

2.2. Separation of the hydrolysates from brewery's spent grain and Eucalyptus wood by anion-exchange chromatography

Preparative anion-exchange chromatography was performed using a Biopilot system (APB), equipped with a Source 15Q (APB) fine line column (1.2 L). The column was activated with a solution of 1 M sodium acetate (pH 5; 5 column volumes (CV)). The excess of (unbounded) ions was removed by elution with water (~10 CV). The sample (~3.9 g) was applied to the column and the neutral oligosaccharides were separated from the acidic oligomers using the following gradient (25 ml/min): 0–1250 ml → only water; 1250–1750 ml → 0–17 mM sodium acetate buffer (pH 5); 1750–2250 ml → 17–30 mM of buffer (pH 5); 2250–3000 ml → isocratic 30 mM of buffer (pH 5) and 3000–6000 ml → 100 mM of buffer (pH 5). Also in case a gradient was used for elution, the eluent was detected by a Shodex RI-detector (Shodex RI se-72), operable at high flow-rates.

2.3. Size-exclusion chromatography

To remove the monomers and dimers from the neutral fractions and to desalt the fractions eluted with a salt gradient from the Source 15Q-column, a Biopilot system (APB) equipped with a Superdex 30 (APB) column (5 L) was used. Elution was performed with water and from 1000 to 4000 ml fractions were collected for every 100 ml. The eluent was detected by a Shodex RI-detector (Shodex RI se-72).

2.4. Degradation by endoxylanase I

Samples (4 mg) were dissolved in 50 mM sodium acetate buffer pH 5 (1 ml) and incubated with endo-(1,4)-β-D-xylanase I (0.2 μg/ml) for 24 h at 30 °C. The purification and mode of action of the used endo-(1,4)-β-D-xylanase I from *Aspergillus awamori* is described by Kormelink, Gruppen, Vietor, and Voragen (1993). After inactivation of the enzyme, the digests were analysed by high-performance anion-exchange chromatography (HPAEC) and high-performance size-exclusion chromatography (HPSEC), calibrated with a well-characterised digest of wheat arabinoxylan degraded by the endo-(1,4)-β-D-xylanase I (Gruppen et al., 1992; Kormelink et al., 1993).

2.5. Fermentation of the pool Euc NI A by human intestinal bacteria

Fermentation of *Euc* NI A and saponified *Euc* NI A was determined using faecal inocula. Faecal inocula were prepared from fresh faeces in buffered peptone water with cysteine·HCl (0.5 g/l) in approximately 10-fold dilution. A medium consisting of 1 g/l of neutralised bacterial peptone (Oxoid), 8 g/l of sodiumchloride (Merck) and 0.5 g/l of L-cysteine·HCl was adjusted to a pH of 6.7 using a 6N NaOH solution (Hartemink, 1999). In an anaerobic chamber (atmosphere 80% N_2, 10% CO_2, 10% H_2), the 10-fold diluted faeces was diluted further (10 000×) with the medium described. A sterile solution of 0.5% (w/v) *Euc* NI A in thioglycollate broth (sugarfree; Oxoid, CM391) was inoculated with 20% (v/v) of the 10 000× diluted faecal inocula at 37 °C in an anaerobic chamber. Samples were taken at time 0, 1, 4, 10, 24, 48, 72 h and stored at −80 °C. Of each sample, enzyme activity was inactivated (5 min, 100 °C), centrifugated and the supernatants were diluted 12 times

with H_2O before analysis of the reaction products by HPAEC (Van Laere et al., 2000).

2.6. Neutral sugar composition

The neutral sugar composition was determined by gas chromatography according to Englyst and Cummings (1984) using inositol as an internal standard. The samples were treated with 72% w/w H_2SO_4 (1 h, 30 °C) followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C and the constituent sugars released were analysed as their alditol acetates.

2.7. Uronic acid content

The uronic acid content was determined as anhydrouronic acid by an automated *m*-hydroxydiphenyl assay (Blumenkrantz & Asboe-Hansen, 1973; Thibault, 1979) using an autoanalyser (Skalar Analytical BV, Breda, The Netherlands).

2.8. Acetic acid content

The degree of acetylation was determined on a Spectrophysics apparatus (Thermo Separation Products, USA), using an Aminex HPX column (Voragen, Schols, & Pilnik, 1986). The content of acetyl-groups was corrected for the free acetic acid in the samples.

2.9. HPSEC

HPSEC was performed on three TSKgel columns (7.8 mm ID × 30 cm per column) in series (G4000 PWXL, G3000 PWXL, G2500 PWXL; Tosohaas) in combination with a PWX-guard column (Tosohaas). Elution took place at 30 °C with 0.2 M sodium nitrate at 0.8 ml/min. The eluate was monitored using a refractive index detector. Calibration was performed using dextrans.

2.10. HPAEC at pH 12

High-performance anion-exchange was performed on a Dionex system equipped with a CarboPac PA-1 column (4 mm ID × 250 mm) in combination with a CarboPac PA guard column (3 mm ID × 25 mm) and PAD-detection (Lee, 1996). Elution (1 ml/min) of the oligomers in the pools was performed with a combination of linear gradients of 0–150 mM sodium acetate in 100 mM NaOH during 10 min, then 150–450 mM sodium acetate in 100 mM NaOH during 25 min. The oligosaccharides in the samples taken during the fermentation were eluted (1 ml/min) with a combination of linear gradients of 50–90 mM sodium acetate in 100 mM NaOH during 0–5 min, 90–130 mM sodium acetate in 100 mM NaOH during 10 min, followed by a linear gradient to 520 mM sodium acetate in 100 mM NaOH in 15 min. Each elution was followed by a washing and equilibration step.

2.11. MALDI-TOF mass spectrometry

For MALDI-TOF MS, a Voyager-DE RP Biospectrometry workstation (PerSeptive Biosystems Inc., Framingham, MA, USA) was used, operated as described by Daas, Meyer-Hansen, Schols, Ruiter, and Voragen (1999). The mass spectrometer was calibrated with a mixture of maltodextrines (mass range 365–2309).

The samples were mixed with a matrix solution (1 μl of sample in 9 μl of matrix), after desalting the samples using H^+-Dowex AG 50W X8 (Biorad). The matrix solution was prepared by dissolving 9 mg of 2,5-dihydroxybenzoic acid and 3 mg 1-hydroxyisoquinoline in a 1-ml mixture of acetonitrile/water (300:700 μl). Of the prepared (sample + matrix) solutions, 1 μl was put on a gold plate and allowed to dry at room temperature.

2.12. Nanospray mass spectrometry

Dynamic nanospray was performed on a LCQ Ion-trap (Finnigan MAT 95, San Jose, CA) equipped with a nanosource. Sample was running through a transferring capillary (100 μm ID) and a spraying capillary with an ID of 20 μm at a flow rate of 0.3 μl/min. MS analysis was carried out in the positive mode using a spray voltage of 2 kV and a capillary temperature of 200 °C. The capillary voltage was set at 45 kV and the tube lens voltage at 35 kV. MS^2 and higher was performed using a window of 1.5–2 *m/z* and a 30–35% relative collision energy. The apparatus and the data were controlled by Xcalibur software. The accuracy of the mass determinations is ±0.3 Da.

Prior to analysis on the LCQ Ion-trap, the saponified pools *Euc* AII#, *Euc* AIII# and *Euc* AII# (3 mg/ml) were desalted using H^+-Dowex AG 50W X8 (Biorad).

3. Results and discussion

Separation of neutral (acetylated) xylo-oligosaccharides from acidic (acetylated) xylo-oligosaccharides was performed by preparative anion-exchange chromatography using a Source 15Q-resin. A similar application has been described by Teleman et al. (1996), who separated an acidic oligosaccharide from neutral ones.

The neutral oligosaccharides present in the hydrolysate of *Eucalyptus* wood were recovered in pool *Euc* NI (neutral I) and were eluted with water only. In this neutral pool, 71% of the applied sugars was accumulated. Subsequently, the charged oligomers were collected into the four pools *Euc* AI (Acidic I; 17–30 mM sodium acetate-buffer (pH 5)), *Euc* AII (30 mM sodium acetate-buffer), *Euc* AIII (30–50 mM sodium acetate-buffer) and *Euc* AIV (50–100 mM sodium acetate-buffer) with a yield of 13, 7, 4 and 3% of the applied sugar, respectively. In pool *Euc* AI, oligosaccharides were combined eluting with a relatively low ratio of uronic acid to xylose (<0.18). This pool *Euc* AI was not subjected to further purification and analysis, since we were mainly

Table 1
Sugar composition (mol%) of the oligosaccharides-pools obtained from hydrothermal treated *Eucalyptus* wood and brewery's spent grain by anion-exchange and size-exclusion chromatography

	Total[a] sugars	Molar composition							Ara/Xyl[b]	UA/Xyl[b]	Ac/Xyl[b]
		Rha	Ara	Xyl	Man	Gal	Glc	UA			
Eucalyptus wood hydrolysate	58 (8)	2	0	79	0	8	0	11	0	0.14	0.59
Pools											
Euc NI A	78 (11)	1	0	86	5	4	4	0	0	0	0.51
Euc AII#	68 (9)	0	0	85	0	0	0	15	0	0.18	0.53
Euc AIII#	59 (7)	1	1	66	0	0	0	32	0.02	0.48	0.60
Euc AIV#	53 (8)	0	0	72	0	5	0	23	0	0.32	0.67
Brewery's spent grain hydrolysate	43 (1)	1	17	57	0	4	15	6	0.30	0.11	0.12
Pools											
BSG IA	88 (1)	0	23	52	0	3	22	0	0.44	0	0.05
BSG IB	83 (1)	0	14	58	0	2	26	0	0.24	0	0.07
BSG IC	72 (3)	0	12	68	0	1	19	0	0.18	0	0.12
BSG II#	44 (1)	0	22	63	0	3	4	8	0.35	0.13	0.10

[a] Neutral sugars + uronic acids (UA) and between parentheses, the total content of acetyl-esters expressed as weight percentage of each fraction (dm).
[b] Ratio mol/mol.

interested in highly substituted xylo-oligosaccharides. The pools *Euc* AII, AIII and AIV, containing oligosaccharides with a rather high ratio of uronic acid to xylose, were desalted by preparative size-exclusion chromatography prior to further characterisation (*Euc* AII#, AIII# and AIV#). Pool *Euc* NI was subjected to preparative size-exclusion chromatography as well, mainly to remove monomers and dimers. All oligosaccharides (>DP2) from pool *Euc* NI were pooled together (*Euc* NI A) representing about 55% w/w of the applied neutral pool.

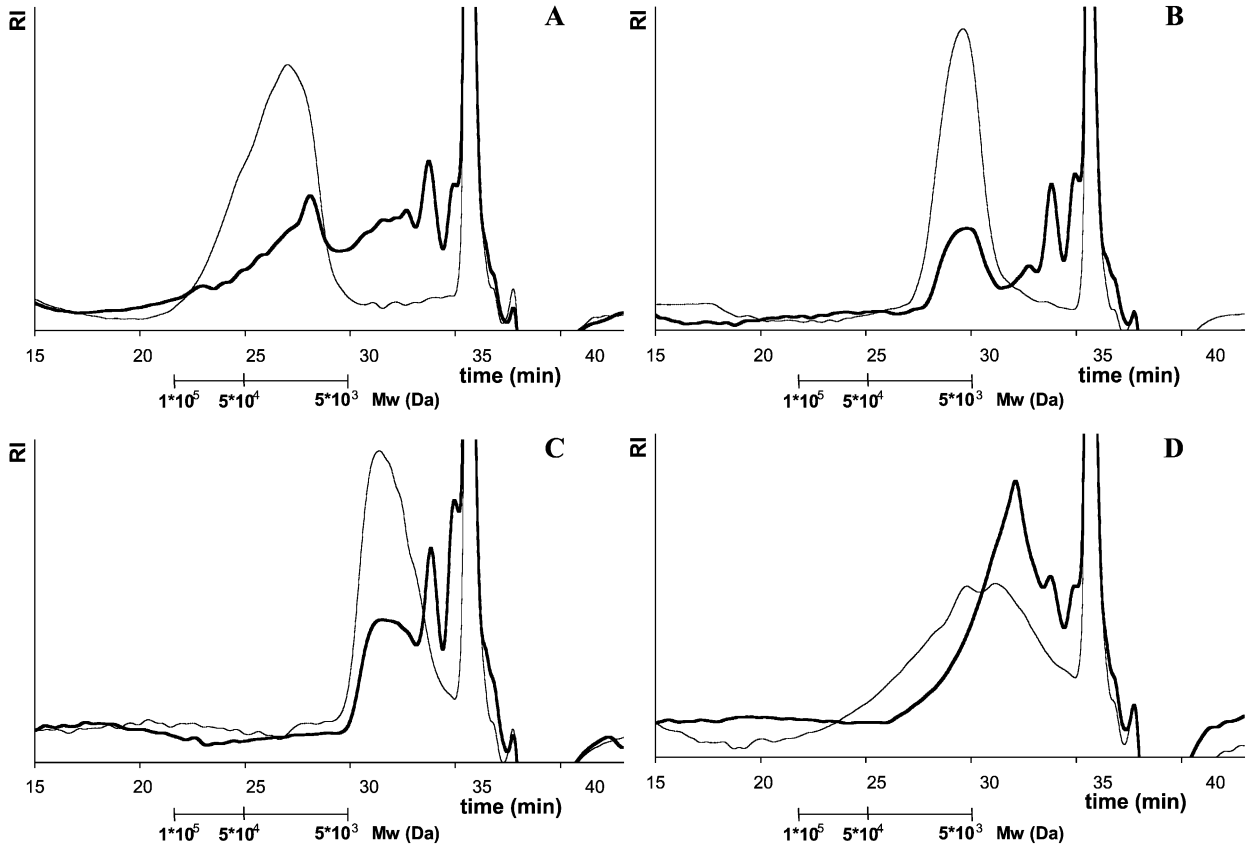

Fig. 1. HPSEC elution profiles of the pools (A) BSG IA, (B) BSG IB, (C) BSG IC, and (D) BSG II, obtained from the hydrothermal treated brewery's spent grain, before (thin line) and after (bold line) enzymatic degradation by endoxylanase I.

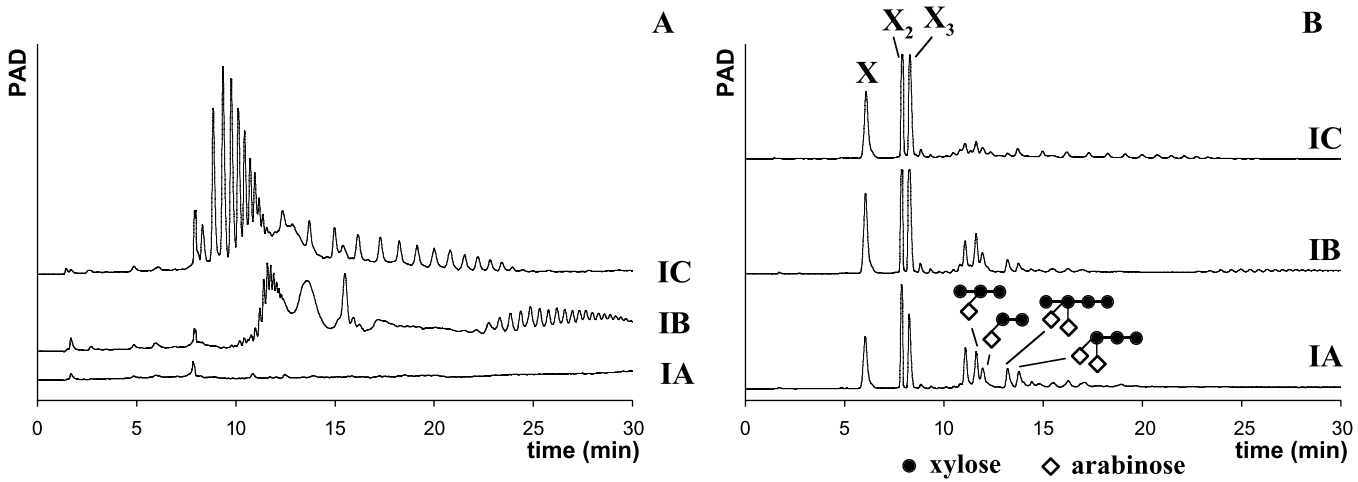

Fig. 2. HPAEC elution profiles of the oligosaccharides-pools (IA, IB and IC), obtained from the hydrothermal treated brewery's spent grain, (A) before and (B) after enzymatic degradation (X = xylose).

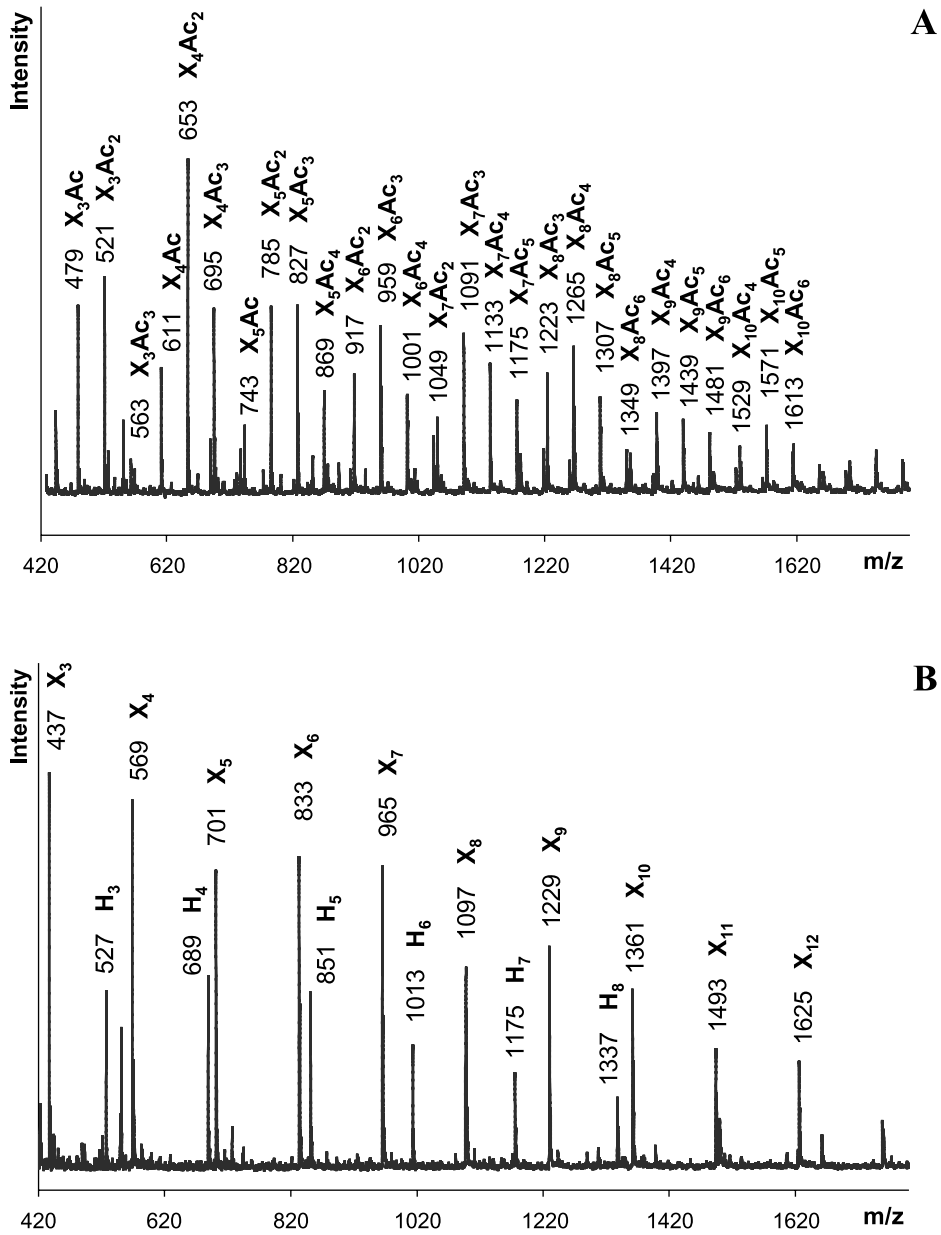

Fig. 3. MALDI-TOF mass spectra of the neutral xylo-oligosaccharides (sodium-adducts) obtained from the *Eucalyptus* wood hydrolysate (pool *Euc* NI A), (A) before and (B) after saponification (X = xylose; Ac = acetyl-group; H = hexose).

Similar fractionations were performed with the hydrolysate from brewery's spent grain. By anion-exchange chromatography besides a neutral pool (BSG I), all fractions collected during the gradient with the sodium acetate-buffer were combined into *one* pool (BSG II), since we have already observed that only few oligomers in this hydrolysate would bind to the column (Kabel et al., 2002). The yield of sugar after this fractionation of the hydrolysate from brewery's spent grain was 90 and 83% in BSG I, and 7% in BSG II, respectively.

The pools BSG I and II were purified further using preparative size-exclusion chromatography to remove monomers and dimers from BSG I and to desalt BSG II. The SEC elution-pattern of the neutral pool BSG I confirmed our results reported previously that the hydrolysate from brewery's spent grain still possess oligosaccharides with a relatively high molecular weight (M_w) (Kabel et al., 2002). Therefore, pool BSG I was fractionated according to size and the oligosaccharides eluted were combined into three pools (BSG IA, BSG IB and BSG IC). BSG IA contained the highest M_w material and BSG IC the smaller oligomers. Each of the three pools contained about 11% w/w of the applied neutral pool.

All pools obtained by anion-exchange and size-exclusion chromatography of both hydrolysates were analysed for their sugar composition (Table 1). The composition of the crude hydrolysates is presented as well.

Pool *Euc* NI A mainly consisted of xylose-residues, while

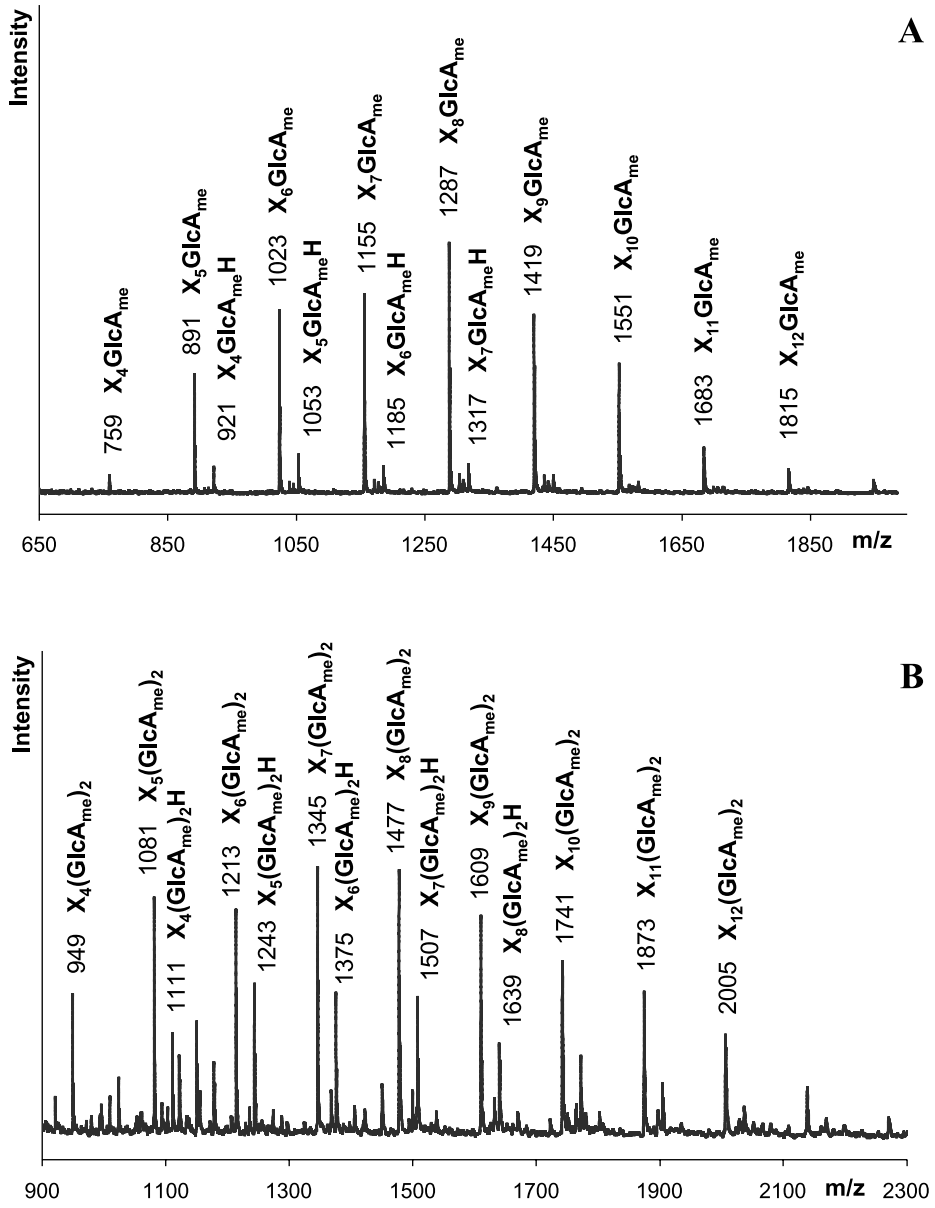

Fig. 4. MALDI-TOF mass spectra of the acidic xylo-oligosaccharides (sodium-adducts) obtained from the *Eucalyptus* wood hydrolysate, pool (A) *Euc* AII# and (B) *Euc* AIV#, both saponified (X = xylose; Ac = acetyl-group; H = hexose; $GlcA_{me}$ = 4-*O*-methylglucuronic acid).

also a rather high level of acetyl-groups was found (Ac/Xyl = 0.51). Also in the acidic pools of the hydrolysate from *Eucalyptus* wood (*Euc* AII#, AIII# and AIV#), a rather high amount of acetyl-groups was determined. Furthermore, as expected, these pools contained quite some uronic acids. The uronic acid-residues were present as 4-*O*-methylglucuronic acid side-chains (vide infra), as in most (purified) xylans from hard woods (Gabrielii, Gatenholm, Glasser, Jain, & Kenne, 2000; Puls, Tenkanen, Korte, & Poutanen, 1991; Timell, 1964, 1965).

The neutral oligosaccharides (DP > 2) from the hydrolysate from brewery's spent grain (pool IA, IB and IC) were mainly constituted of xylose-, arabinose- and glucose-residues. The glucose-residues most likely originated from starch degradation products as already mentioned previously. Comparing the arabinose to xylose-ratios of the pools BSG IA, IB and IC, it was remarkable that the material eluting first from the size-exclusion column having a relatively high M_w, also had the highest degree of branching as derived from the ratio of Ara/Xyl (0.44). From these data, it is assumed that in the hydrothermal treatment of brewery's spent grain first arabinose was removed prior to a further hydrolysis of these unsubstituted segments.

To study the structural features of the higher M_w material in the four pools from brewery's spent grain in more detail, a purified and well-characterised endoxylanase was used (Gruppen et al., 1992; Kormelink et al., 1993). The degradation was followed by HPSEC and the elution-patterns are presented in Fig. 1. The high M_w material in the pools BSG IA, IB and IC appeared to be degradable quite well by the

endoxylanase used. On the other hand, the material present in pool BSG II# was more difficult to degrade, which resulted in only a small decrease of molecular weight. In the latter pool, most likely highly branched (glucurono-)(arabino-)xylan-like material was present, being more difficult to be degraded by the endoxylanase used. The oligosaccharides formed upon degradation of the pools BSG IA, IB and IC by endoxylanase I were further analysed by HPAEC and compared with the oligosaccharides present before the enzymatic degradation (Fig. 2). Apparently, the xylan-like material in pool BSG IA possessed a too high molecular weight to be eluted by HPAEC, while from pool BSG IB and IC some lower M_w material and oligosaccharides were eluted. After endoxylanase-treatment, mainly xylose (X_1), xylobiose (X_2) and xylotriose (X_3) were released in all pools. In pool BSG IC, relatively more X_1, X_2, X_3, were released compared to the amount of (branched) oligosaccharides, which were eluted after 10 min in the HPAEC elution-pattern. This indicated the presence of less branched material in pool BSG IC compared to pool BSG IB and BSG IA. In the endoxylanase digest of pool BSG IA, xylo-oligosaccharides eluted with the same retention time as xylo-oligosaccharides singly or doubly branched with arabinose (Vietor et al., 1994). Therefore, the material originally present in this pool BSG IA was expected to contain more arabinose side-chains than the material in pool BSG IC, as has already been noticed in discussing the sugar composition of these pools.

Further analysis of the oligosaccharides present was performed by MALDI-TOF MS, since this technique has proven to be a powerful method in the analysis of oligosaccharides (Brull, Huisman, Schols, Voragen, & Critchley, 1998; Harvey, 1996; Kabel et al., 2001; Van Alebeek, Zabotina, Beldman, Schols, & Voragen, 2000). The pools BSG IA, BSG IB, BSG IC and BSG II# were all subjected to MALDI-TOF MS. Only for pool BSG IC, a clear mass spectrum could be obtained by MALDI-TOF MS (figure not shown), indicating the presence of a homologous series of pentoses (xylose and/or arabinose (Table 1)) and hexoses; both ranging from DP 3 to 10.

The pools obtained from the *Eucalyptus* wood hydrolysate were subjected to MALDI-TOF MS as well. The fact that the pools consisted for more than 90% of xylose, uronic acid and acetyl-groups (Table 1), made the interpretation of the MALDI-TOF mass spectra rather easy.

The mass spectra of the oligosaccharides present in pool *Euc* NI A, before and after removal of alkali-labile esters, are presented in Fig. 3(A) and (B), respectively. In the *Eucalyptus*' neutral pool NI A, a large variety of acetylated xylo-oligosaccharides were present [X_nAc_m]. The corresponding series of xylo-oligosaccharides without acetyl-groups [X_n] remained after saponification (Fig. 3(B)). Subsequently, to confirm that the oligomers were built from xylose-residues only, endoxylanase I was used to hydrolyse the (saponified) xylo-oligosaccharides. The masses of the series of xylo-oligosaccharides (not acetylated) disappeared, leaving only the masses of X_2, X_3 and hexose-oligomers, which confirmed the structures proposed in Fig. 3(B).

The MALDI-TOF mass spectra of the oligosaccharides present in the acidic pool *Euc* AII#, which were saponified first, are shown in Fig. 4(A). In this pool, a series of xylo-oligosaccharides branched with one 4-*O*-methylglucuronic acid [$X_n(GlcA_{me})_1$] was detected, in accordance with the sugar composition of this pool (Table 1). Additionally, in Fig. 4(A) traces of a series of xylo-oligosaccharides containing both one 4-*O*-methylglucuronic acid and one hexose (H) were observed, most likely corresponding with the 4-*O*-methyl-α-D-glucuronic acid substituted at O-2 with α-D-galactose as described by Shatalov, Evtuguin, and Neto (1999). This assumption was confirmed by our results obtained in dynamic nanospray MS. In the MS^2 spectra of the xylo-oligosaccharides, most likely containing both a 4-*O*-methylglucuronic acid and a hexose-residue, indeed fragments were observed of these oligosaccharides minus the mass of a hexose-residue (162 Da). Subsequently, in the MS^3 spectra of the fragments minus the hexose-residue fragments were released lacking the mass of a 4-*O*-methylglucuronic acid residue (190 Da). Furthermore, no evidence was found for the presence of two glucuronic acid residues (mass 176 Da each), which would also account for the masses of the series of $X_n(GlcA_{me})_1(H_1)$. The presence of galactoses could not be seen from Table 1 since the amount of galactoses present is most likely below the detection level of the sugar analysis method used. The same structures as described for the saponified pool were detected in the MALDI-TOF mass spectrum of the non-saponified pool *Euc* AII#, with this difference that each structure was present having one or more acetyl-groups in addition [$X_n(GlcA_{me})_1Ac_m(H_1)$].

In pool *Euc* AIII#, which was more strongly bound to the anion-exchange column, xylo-oligosaccharides were detected having the same structural features as the oligosaccharides present in pool *Euc* AII#, but of a lower DP (DP 2–4 xyloses). The xylo-oligosaccharides all contained one 4-*O*-methylglucuronic acid or a 4-*O*-methylglucuronic acid plus a hexose, and one or more acetyl-groups (results not shown). Again, the presence of both a 4-*O*-methylglucuronic acid and a hexose, most likely galactose (vide infra), was confirmed by nanospray tandem MS. Thus in this pool, the charge-density per oligomer was rather high, explaining why these oligomers were more strongly bound to the column. The higher charge-density also was reflected in the ratio of uronic acid to xylose (Table 1).

Pool *Euc* AIV#, which was bound most strongly to the anion-exchange column, was analysed by MALDI-TOF MS as well (Fig. 4(B)). It was shown that after saponification, a series of xylo-oligosaccharides substituted with *two* 4-*O*-methylglucuronic acid residues [$X_n(GlcA_{me})_2$] was present. Additionally, a series of similar oligosaccharides having an additional hexose-residue, most likely galactose (vide infra), attached was detected in the mass spectrum (Fig. 4(B)). Again, the same structures as described to be present

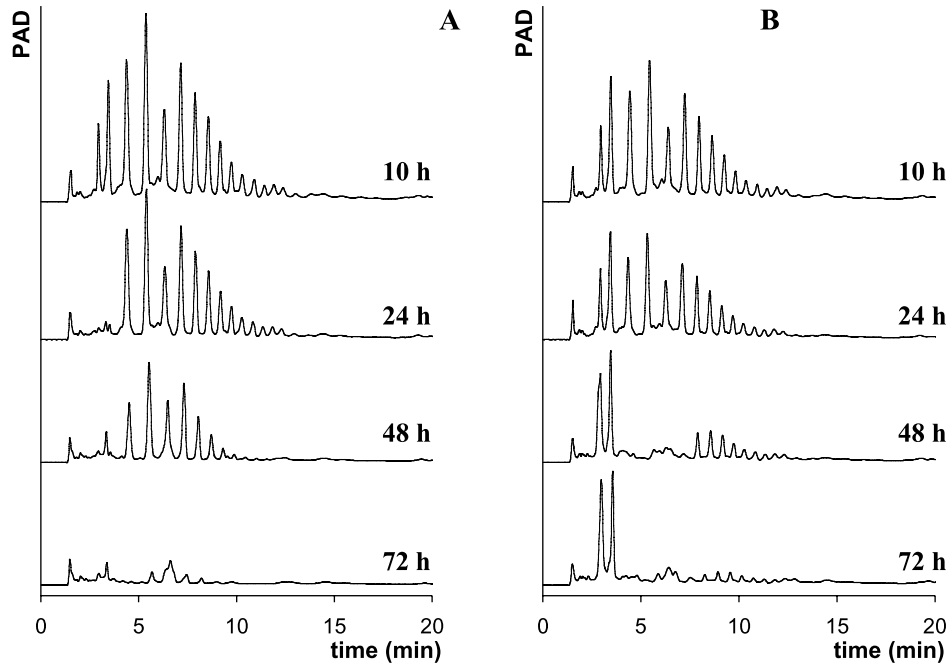

Fig. 5. HPAEC elution profiles of acetylated xylo-oligosaccharides (A) and non-substituted xylo-oligosaccharides (B) during fermentation (72 h) by a human faecal sample.

in the saponified pool were detected in the MALDI-TOF mass spectrum of the oligosaccharides present in the non-saponified pool *Euc* AIV#, having one or more acetyl-groups in addition $[X_n(GlcA_{me})_2Ac_m(H_1)]$.

In conclusion, separation of the hydrolysates from two hydrothermal treated by-products resulted in several pools of xylo-oligosaccharides differing in the degree and type of branching. Obviously, still a mixture of oligosaccharides was present in each pool to be separated further when complete structural elucidation is needed. Further structural characterisation of these oligosaccharides will be helpful in studying and improving the mechanism of hydrothermal treatment in the release of xylose and xylo-oligosaccharides.

The neutral acetylated xylo-oligosaccharides from the *Eucalyptus*' hydrolysate (*Euc* NI A) have already been subjected to reversed phase chromatography or a TSK-gel amide-column. The acetylated xylo-oligosaccharides were rather well separated based on the number of acetyl-groups per oligomer (Schols et al., 2000).

Furthermore, the pools containing xylo-oligosaccharides with various side-chains are very suitable to be used to study the structure-depending effect of xylo-oligosaccharides in several biological activity tests and during fermentation by the human intestinal flora. An example of the fermentation by a human faecal sample of acetylated xylo-oligosaccharides (*Euc* NI A) and non-substituted xylo-oligosaccharides (saponified *Euc* NI A) is presented in Fig. 5(A) and (B), respectively. This figure shows the pattern of oligosaccharide digestion analysed by HPAEC and illustrates the potential of the human intestinal flora to ferment (acetylated) xylo-oligosaccharides in vitro. Also, it was demonstrated that non-substituted xylo-oligosaccharides were fermented divergently as compared to the acetylated ones. The complete results of the fermentation studies will be published in the near future.

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