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Kenaf xylan – A source of biologically active acidic oligosaccharides

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Abstract

Two xylose-rich hemicellulose fractions were obtained from kenaf wood (*Hibiscus cannabinus* L.) through a series of sequential extractions which dissociated xylans from other cell wall components. These fractions were subsequently used as substrates for the production of biologically active aldouronic acids. Incubation of the xylans with a family 10 *Thermoascus aurantiacus* endoxylanase resulted in the isolation of an aldotetrauronic acid as the main acidic oligosaccharide in the hydrolysis products. Enzymic hydrolysis with a family 11 *Sporotrichum thermophile* endoxylanase instead resulted in the isolation of a aldopentauronic acid as the main acidic oligosaccharide. The identity and purity of both xylans and aldouronic acids were assessed with solid-state FT-IR, solid-state and solution ^{13}C NMR spectroscopy.

Keywords: Kenaf; Hemicellulose; Extraction; Endoxylanase; Xylan; Uronic acid; Oligosaccharide; FT-IR spectroscopy; ^{13}C NMR spectroscopy

1. Introduction

Kenaf (*Hibiscus cannabinus* L.) is an annual herbaceous plant which has attracted considerable attention as a renewable source of cellulosic fibres. Such fibres are essential to the pulp and paper industry, which traditionally relies on exhaustible resources such as tree wood. A pilot study is already underway for the large scale cultivation of kenaf in south European countries as a "non-food" fibre, energy and feedstock crop (EU KA5 project QLRT-2001-01729, 2003). Kenaf fibre may soon be a standard raw material in the manufacture of paper, particleboards, animal feeds, textiles and fuel.

Far from these "traditional" applications, kenaf biomass may also be used in the preparation of growth media with regulatory effects on plant growth and development. For example, seeds of edible plants, such as cucumber and tomato, were exposed to kenaf extracts from non-weathered and weathered kenaf fibres (Russo, Webber, & Myers, 1997). Extracts from non-weathered kenaf inhibited germination and post-germination development, whereas extracts from weathered kenaf fibres actually benefited germination and growth. Extractions were performed with distilled water, which suggests the presence of a water-soluble regulatory element in kenaf biomass. Furthermore, the inclusion of raw kenaf biomass in soil has been suggested as a method for the production of compact plants by Tsakonas, Stergiou, Polissiou, Akoumianakis, and Passam (2005). Growth of lettuce and pepper plants was reversibly inhibited by the kenaf-based substrates.

Such a prolonged but reversible inhibition of vegetable growth may only be possible if the regulatory element is retained in the soil, in spite of irrigation. Hemicelluloses, which are not washed away, as part of the cell wall matrix, may well be responsible for the observed phenomena.

The major hemicellulose from kenaf consists of a β-(1 → 4)-D-xylopyranose backbone with 4-*O*-methy-

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l-glucuronic acid side chains at the 2-position of many xylose units (Duckart, Byers, & Thompson, 1988; Neto et al., 1996). It is, in many ways, a typical hardwood xylan (see review by Ebringerova & Heinze, 2000). The exact uronic acid-to-xylose ratio varies according to the isolation procedure. Hydrolysis of this xylan with the appropriate enzymes yields neutral and acidic xylooligosaccharides.

The interest in xylooligosaccharides is motivated by their biological activities and their technological applications. They facilitate the growth of *Bifidobacterium* spp. in gastrointestinal tract exhibiting prebiotic activity (Roberfroid, 1997) and they also reduce the risk of colon cancer, as recently shown (Hsu, Liao, Chung, Hsieh, & Chan, 2004). Xylooligosaccharides can be used as ingredients in food, cosmetics, pharmaceuticals or agricultural products (Vasquez, Alonso, Dominguez, & Parajo, 2000). Acidic xylooligosaccharides, products of 4-*O*-methyl-D-glucurono-D-xylan hydrolysis, also exhibit interesting biological activities. They were shown to function as plant growth regulators, acclimatisation bioregulators (Katapodis et al., 2003) and antibacterial agents (Christakopoulos et al., 2003). Aldotetrauronic acid (MeGlcA-Xyl_3) induced callus and somatic embryogenesis in culture explants of common mallow (*Malva silvestris* L.) and cotton (*Gossypium hirsutum*) (Katapodis et al., 2002). Small quantities of acid hydrolysate of xylan from *Betula platyphylla* wood stimulated rooting of conifer cuttings in hydroponic culture solution (Ishihara, Nagas, & Shimadzu, 1995).

Endo-β-1,4-xylanases (EC 3.2.1.8, EXs) randomly cleave xylan main chain and generate variously substituted xylooligosaccharides. EXs have been classified into families GH-10 and GH-11 of glycoside hydrolases, based on hydrophobic cluster analysis and similarities in their amino acid sequences (Davies & Henrissat, 1995; Gilkes, Henrissat, Kilburn, Miller, & Warren, 1991). Members of two families, GH-10 and GH-11, differ in their physico-chemical properties (molecular mass, isoelectric point), as well as in their three-dimensional structure. The action of EXs towards polysaccharides is limited by substitution of xylopyranosyl units. EXs of GH-10 are capable to cleave glycosidic linkages in xylan main chain closer to the substituents. As a result, these EXs release products which are shorter than products of EXs of GH-11. In the case of glucuronoxylan, GH-10 EXs liberate aldotetrauronic acid (MeGlcA-Xyl_3) as the shortest acidic fragment, while GH-11 EXs release aldopentauronic acid (MeGlcA-Xyl_4) (Biely, Vrsanska, Tenkanen, & Kluepfel, 1997).

Uronic acid-containing xylooligosaccharides may thus account for the kenaf-associated phenomena already described. The cell wall material of kenaf core and bast is rich in their precursor. Thus, we decided to explore the isolation of hemicelluloses from kenaf wood and the identify fractions suitable for the efficient enzymic production of aldouronic acids.

2. Experimental

2.1. Hemicellulose isolation from kenaf wood

As shown in Fig. 1, 50 g of finely ground kenaf wood were suspended in 300 mL of 1.5% (w/v) SDS/0.01% (w/v) $Na_2S_2O_5$ and the mixture was homogenized for 10 min. The solids were filtered and washed two times with 300 mL of 0.5% (w/v) SDS/0.01% (w/v) $Na_2S_2O_5$. The solids were then resuspended in 500 mL of 0.5% (w/v) SDS/0.01% (w/v) $Na_2S_2O_5$ and mechanically stirred for 20 h at room temperature. The cell wall material was then filtered, washed with de-ionized water and suspended in 400 mL (2:1:1 w/v/v) phenol–acetic acid–water (PAW) for 20 min. The solids were washed with plenty of de-ionized water and subjected twice to a 750 mL of 0.4 M EDTA/1 M NaOH 24 h treatment. The cell wall material was then processed two times 24 h with 750 mL of 0.05 M Na_2CO_3/0.02 M $NaBH_4$. Lignin was removed from the residual solid by stirring the solids in 400 mL of 5%

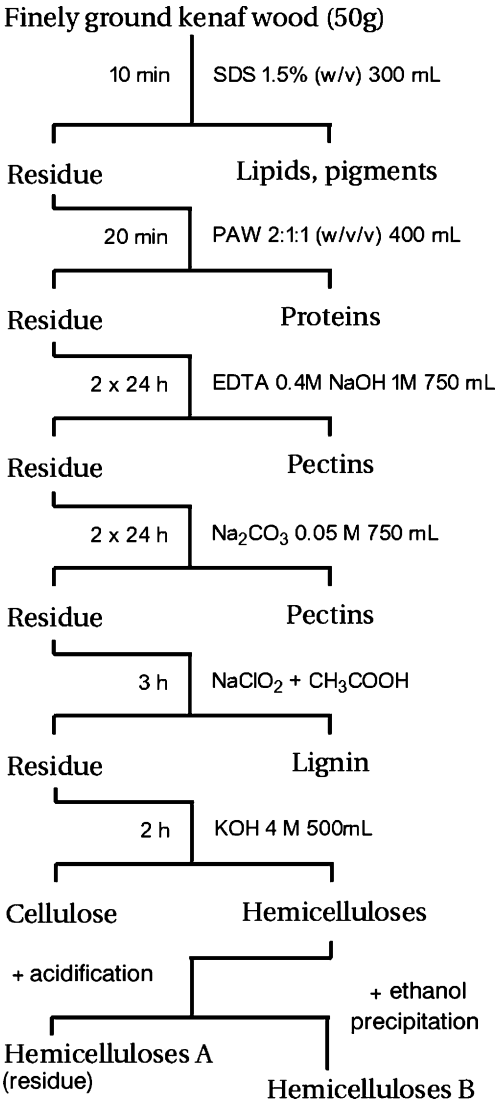

Fig. 1. Isolation of hemicelluloses from kenaf wood.

(w/v) $NaClO_2$ for 3 h, having added 1.2 mL glacial CH_3COOH to the mixture at the start of the first hour and 2 g $NaClO_2$ and 2 mL glacial CH_3COOH at the start of the second and third hours. The suspension was filtered, cell wall solids were collected and washed with de-ionized water. Hemicelluloses were then released into solution after treatment with 500 mL of 4 M KOH/10 mM $NaBH_4$ under N_2 for 2 h. The solution was acidified with HCl and centrifuged for 20 min (18,000*g*, 6 °C). Insoluble hemicelluloses were retained as fraction A. Two volumes of CH_3CH_2OH were added to the supernatant, which was centrifuged in identical conditions. The precipitate was labelled fraction B. Excessive salt was removed from the precipitates by dialysis. Hemicelluloses were then freeze-dried and stored.

2.2. Neutral sugar analysis

In order to study the neutral sugar composition in our hemicellulose fractions with gas chromatography-mass spectrometry (GC-MS), these were hydrolyzed with sulfuric acid to afford monosaccharides, which were reduced by sodium borohydride to alditols. Then the alditols were thoroughly acetylated in sulfuric acid to afford alditol acetates (Kitayama et al., 2000). In the same manner, alditol acetates of xylose, arabinose, glucose and galactose as standard sugars were prepared. GC-MS analysis of the isolated hemicelluloses was subsequently performed on a Hewlett-Packard 5890 II gas chromatograph equipped with a Hewlett-Packard 5972 mass selective detector in the electron impact mode (70 eV) and a HP-5 ms capillary column (bonded and cross-linked 5% phenyl-methylpolysiloxane 30 m × 0.25 mm i.d., film thickness 0.25μm). Injector and MS transfer line temperatures were set at 220 and 290 °C, respectively. The oven temperature was held at 120 °C for 5 min, then programmed to 250 °C at a rate of 5 °C/min and held for 5 min. Helium was used as the carrier gas, at a flow rate of 1 mL/min. Samples of 1.0 μL were injected manually in the splitless mode. Relative percentage amounts were calculated from TIC electronically. The components were identified based on the comparison of their relative retention time and mass spectra with those of standards.

2.3. Quantitative determination of uronic acids

Uronic acids cannot be determined by the aforementioned GC-MS method. Uronic acid content of our hemicellulose fractions was, thus, separately determined using a modification of the UV *m*-hydroxydiphenyl method of Blumenkrantz and Asboe-Hansen (1973) as described by Filisetti-Cozzi and Carpita (1991). Measurements were performed with a UV–Vis HACH DR/2010 spectrophotometer.

2.4. Production and isolation of xylanases

A family 10 endoxylanase was produced by *Thermoascus aurantiacus* Mieche, IMI 216529 in a solid-state culture with wheat straw as carbon source and purified as previously described (Kalogeris, Christakopoulos, Kekos, & Macris, 1998). A family 11 endoxylanase was produced by *S. thermophile* ATCC 34628 in a 20 L stir tank bioreactor with corn cobs as the carbon source and purified as previously described (Katapodis et al., 2003).

2.5. Enzymic hydrolysis of xylan, separation and determination of products

Hydrolysis of kenaf xylan 1% (w/v) in deionized water (100 mL) was performed using endoxylanase (1 U/mL, where 1 U is the amount of xylanase required to release 1 μmol xylose min^{-1} reducing equivalent) for 20 h at 50 °C with constant stirring.

The hydrolysis products were analyzed at different time intervals by thin-layer chromatography (TLC) on microcrystalline cellulose (DC Alufolien Cellulose; Merck, Darmstud, Germany) in the solvent system ethylacetate–acetic acid–water (3:2:1, by vol). Reducing sugars ware visualized by the aniline-hydrogen phthalate reagent. Quantitative data was obtained by HPLC with pulse amperometric detection (HPAEC-PAD) using an HPLC system (Jasco PU-1580I, Jasco Corporation, Tokyo, Japan) and a Dionex ED40 electrochemical detector (Dionex, Sunnyvale, CA, USA). Samples (0.02 mL) were injected onto a CarboPac PA1 column (Dionex) and eluted at 1 mL/min. Gradient elution was applied using two solutions: 16 mM NaOH (A) and 16 mM NaOH containing 300 mM NaOAc (B). The total run time per sample was 50 min. The elution began with 100% A for 20 min followed by a linear gradient from 0% to 30% solution B in solution A for 20 min and maintained at 30% solution B for 5 min. The column was then washed for 5 min with 100% solvent A.

2.6. Isolation of acidic oligosaccharides

Column chromatography (47 × 1.5 cm) of the enzymic hydrolysis products was performed with a Dowex 1 (acetate form), anion-exchange resin (Bio-Rad Laboratories, Hercules, CA, USA) equilibrated with distilled water. Samples (10 mL) were eluted using a two-step purification procedure. First, the resin was washed with distilled water at a flow rate of 90 mL/h until no more neutral sugars were found in the eluate. Then the remaining acidic oligosaccharides were eluted with 3 M acetic acid. This fraction was further purified by size-exclusion chromatography (SEC). SEC was performed on two sequentially connected BioGel P-2 columns (160 × 2.5 cm). Five milliliter of sample were applied and eluted with distilled water at a flow rate of 60 mL/h. Five milliliter fractions were collected and analyzed by pulsed amperometry, as described above.

2.7. FT-IR spectroscopy

The infrared spectra of birchwood xylan, kenaf hemicelluloses A and B, and the acidic oligosaccharide fractions

from the enzymic hydrolysis were recorded. The spectra were collected with a Nicolet Magna 750 FT-IR spectrophotometer equipped with a deuterated triglycine sulfate (DTGS) detector at 4 cm^{-1} resolution and 100 scans per sample, using a Spectra Tech microcup Diffuse Reflectance Infrared Fourier Transform (DRIFTS) accessory. The FT-IR spectra were smoothed and their baselines were corrected automatically using the built-in software of the spectrophotometer (OMNIC 3.1).

2.8. ^{13}C NMR spectroscopy

Solid-state ^{13}C NMR spectra were recorded with a Bruker MSL 400 MHz instrument using a ^{1}H decoupler and magic angle rotation. Spectra were obtained at 25 °C using the cross-polarization technique, a 5000 Hz rotational frequency and 1200 scans. Scan repeat time was adjusted to 60 s, as the relaxation time of carbon atoms is rather long. The duration of the 90° pulse was 9 u and the interaction time between the ^{1}H and ^{13}C nuclei was 2 ms. Adamantane presents two peaks with chemical shifts of 29 and 37 ppm from tetramethylsilane (TMS) and was used as the reference compound. The D_2O solution ^{13}C NMR spectra of the acidic xylooligosaccharides from the enzymic hydrolysis were obtained with a Bruker AC 300 MHz instrument at 47 °C with a rotational frequency of 2500 kHz.

2.9. Mathematical model

A rate equation describing enzyme-dependent hydrolysis rates was proposed by Bailey (1989). Sattler, Esterbauer, Glatter, and Steiner (1989) and Nidetzky, Steiner, Hayn, and Claeyssens (1994), used a similar approach to describe hydrolysis of cellulose by a crude cellulase preparation and by purified cellulases, respectively. Vardakou et al. (2003) and Vardakou et al. (2004) used this model to describe the hydrolysis of water-unextractable arabinoxylan by family 10 and 11 endoxylanases.

$$V(\mathrm{E}) = V_{\max}\left[\frac{\mathrm{E}}{(K_{\mathrm{E}} + \mathrm{E})}\right],$$

where $V(\mathrm{E})$ is the initial rate (enzyme–concentration-dependent) (g of xylose h^{-1} L^{-1}), $V_{\max}$ is the maximal hydrolysis rate (at substrate concentration) (g of xylose h^{-1} L^{-1}), E is the initial enzyme concentration (nM) and K_{E} is the half-saturation constant (nM).

3. Results and discussion

3.1. Isolation of hemicelluloses

The presence of lignin drastically reduces xylan yield because of the ester and ether links which bind lignin to polysaccharides and obstruct the detachment of xylan chains from the cell wall matrix (Ebringerova & Heinze, 2000; Gubitz, Stebbing, Johansson, & Saddler, 1998) Total hemicellulose yield was improved from ~5% to ~30% (w/v) after incorporation of the lignin removal step before hemicellulose release into solution.

3.2. Sugar analysis of hemicellulosic fractions

The GC-MS analysis resulted in the identification of xylose, arabinose, glucose and galactose as the main neutral sugars of the obtained hemicelluloses. In all samples, xylose was the predominant monosaccharide, its content ranging from 92.5% to 94.2% (Table 1) Commercial birchwood xylan (Sigma) was used as reference material. The monosaccharide composition of kenaf hemicelluloses was very similar to the respective profile of birchwood xylan (Table 1). Determination of uronic acid content was separately performed.

3.3. Quantitative determination of uronic acids

Uronic acid content in the obtained fractions of hemicelluloses was estimated at approximately 4% for hemicelluloses A and 14% for hemicelluloses B. These results were also verified by FT-IR spectroscopy (Batsoulis et al., 2004).

3.4. Enzymic hydrolysis of xylan, separation of acidic oligosaccharides

Under the conditions used and for each of the two enzymes studied, initial hydrolysis rates of insoluble fraction proved to be linear (first 60 min) when D-xylose was measured. Adsorption of xylanases to the surface of their insoluble substrate assumed to be a prerequisite step for hydrolysis. Therefore, it seemed appropriate to analyse initial-rate data of kenaf xylan hydrolysis at various enzyme concentrations and constant substrate concentration (Figs. 3 and 4) and to use equations deviating from classical Michaelis–Menten kinetics. Kinetic constants $V_{\max}$ and K_{E} were estimated by nonlinear regression analysis (Tables 2 and 3). The resulting "catalytic efficiencies" point 6-fold higher value for the XYLI compared with that for XYLA. The maximal hydrolysis rates were 0.6 and 0.3 (g of xylose h^{-1} L^{-1}) for XYLI and XYLA, respectively (Tables 2 and 3).

For optimal hydrolysis of insoluble (A) and soluble (B) kenaf xylan 100 U thermostable endoxylanase were used at 50 °C for 20 h. Acidic oligosaccharide yield from

Table 1
Neutral sugar composition in the hemicellulose fractions obtained from kenaf and in birchwood xylan (Sigma)

Sugar[a]	% of total neutral sugars		
	Hemicelluloses A	Hemicelluloses B	Birchwood xylan
Arabinose	0.8	2.4	3.0
Xylose	92.5	94.2	94.2
Glucose	1.2	1.2	0.4
Galactose	–[b]	0.6	1.1

[a] As eluted from HP-5 ms column.
[b] Not detected.

hemicellulosic fraction A reached 3.5% (w/w) and 7.8% (w/w) of the initial substrate concentration, with xylanase from the thermophilic fungus *S. thermophile* and *T. aurantiacus*, respectively. Acidic oligosaccharide yield from hemicellulosic fraction B reached 13.6% (w/w) and 26.2% (w/w) of the initial substrate concentration, with xylanase from the thermophilic fungus *S. thermophile* and *T. aurantiacus*, respectively (Fig. 2). These acidic oligosaccharide yields varied in accordance with the uronic acid content of the hemicellulosic fractions.

The enzymic hydrolysates (90 mL) were concentrated by anion exchange chromatography. Application of distilled water to the column led to the elution of approximately 35–50% of the neutral oligosaccharides. However, no acidic oligosaccharides were detected in this fraction. The acidic oligosaccharides were eluted using 3 M acetic acid. This fraction represented 50–65% of the total oligosaccharides. Further purification by SEC led to the isolation of a major acidic oligosaccharide-containing fraction, in both *S. thermophile* and *T. aurantiacus* cases. These fractions were freeze dried and their content characterized by ^{13}C NMR and FT-IR spectroscopy.

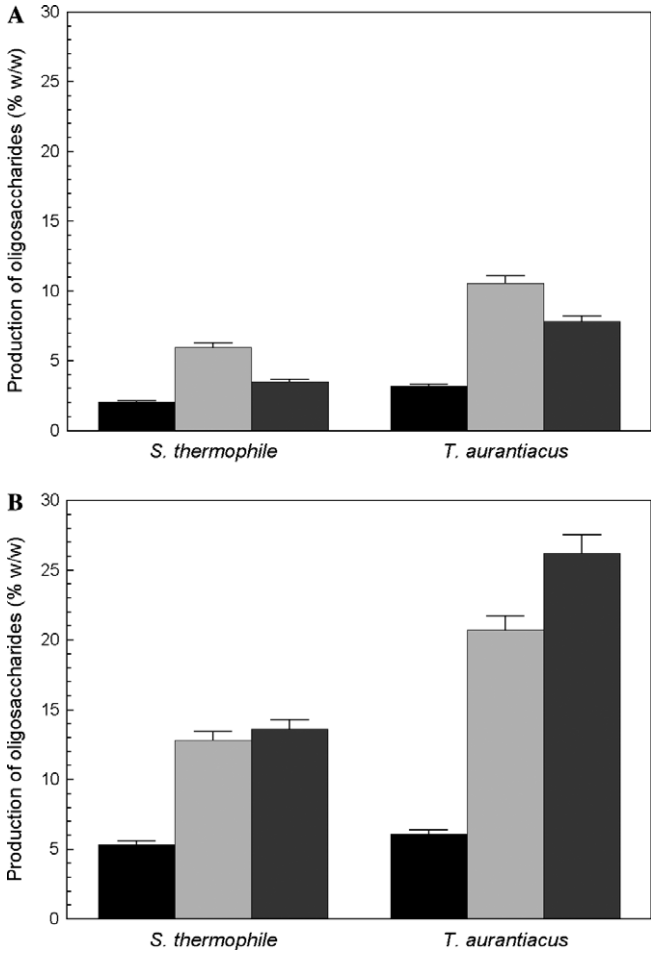

Fig. 2. Xylose, neutral and acidic oligosaccharides released from enzymic hydrolysis of hemicellulose B and hemicellulose A from kenaf with pure xylanases from *S. thermophile* and *T. aurantiacus*. Xylose (black bars), xylobiose (light gray bars), aldouronic acids (dark gray bars).

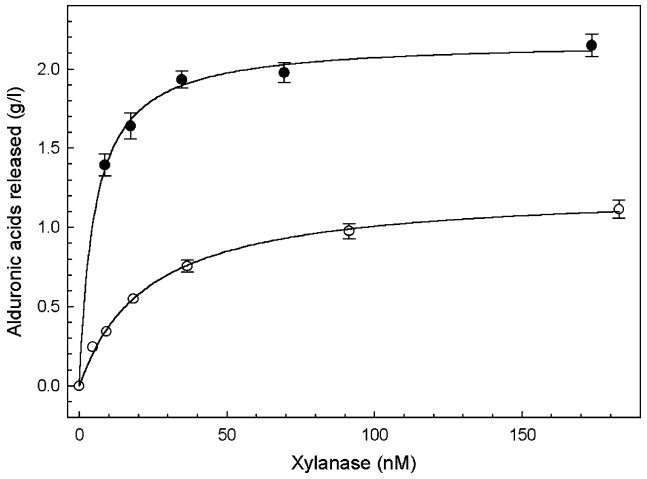

Fig. 3. Release of aldouronic acids from kenaf hemicellulose A 1% (w/v) by different concentrations of XYLI (black circles) and XYLA (white circles).

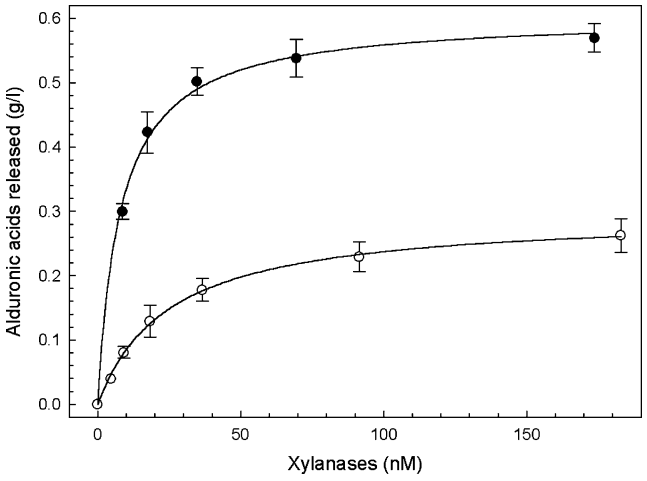

Fig. 4. Release of aldouronic acids from kenaf hemicellulose B 1% (w/v) by different concentrations of XYLI (black circles) and XYLA (white circles).

3.5. FT-IR spectroscopy of hemicellulosic fractions and acidic xylooligosaccharide fractions

The 1700–1250 and 1250–800 cm^{-1} regions of the FT-IR spectra are shown in Figs. 5 and 6, respectively. They are characterized by the contributions of the xylan backbone, glucuronic acid and small traces of phenolics. Absorbance peaks in the region of 1600–1615 cm^{-1} are attributed to the presence of carboxyl groups in their ionized state. The same region is also affected by the presence of residual lignin, which may be deduced by the most characteristic marker band at 1504 cm^{-1}. Absorbance peaks in the regions 1660, 1463–1461 and 1426–1418 cm^{-1} (C–H bending, in-plane), may also be attributed to lignin (Sun, Lawther, & Banks, 1996). Humidity may account for the shoulders at 1660–1643 cm^{-1} in our reference sample (birchwood xylan). Concealed peaks in the region 1383–1336 cm^{-1} correspond to the bending vibrations of

Table 2
Kinetic parameters regarding the liberation of aldouronic acids from kenaf hemicellulose A by XYLI and XYLA

Enzyme	Catalytic efficiency			Ratio (A)/(B)
	K_E (nM)	V_{max} (g L^{-1} h^{-1})	V_{max}/K_E (mg nM^{-1} L^{-1} h^{-1})	
XYL I	8.0 ± 0.6	0.60 ± 0.01	75 ± 5 (A)	6.3
XYL A	24.8 ± 1.2	0.30 ± 0.01	12 ± 1 (B)	

Table 3
Kinetic parameters regarding the liberation of aldouronic acids from kenaf hemicellulose B by XYLI and XYLA

Enzyme	Catalytic efficiency			Ratio (A)/(B)
	K_E (nM)	V_{max} (g L^{-1} h^{-1})	V_{max}/K_E (mg nM^{-1} L^{-1} h^{-1})	
XYL I	5.1 ± 0.5	2.18 ± 0.04	427 ± 38 (A)	7.8
XYL A	22.7 ± 1.5	1.24 ± 0.03	55 ± 4 (B)	

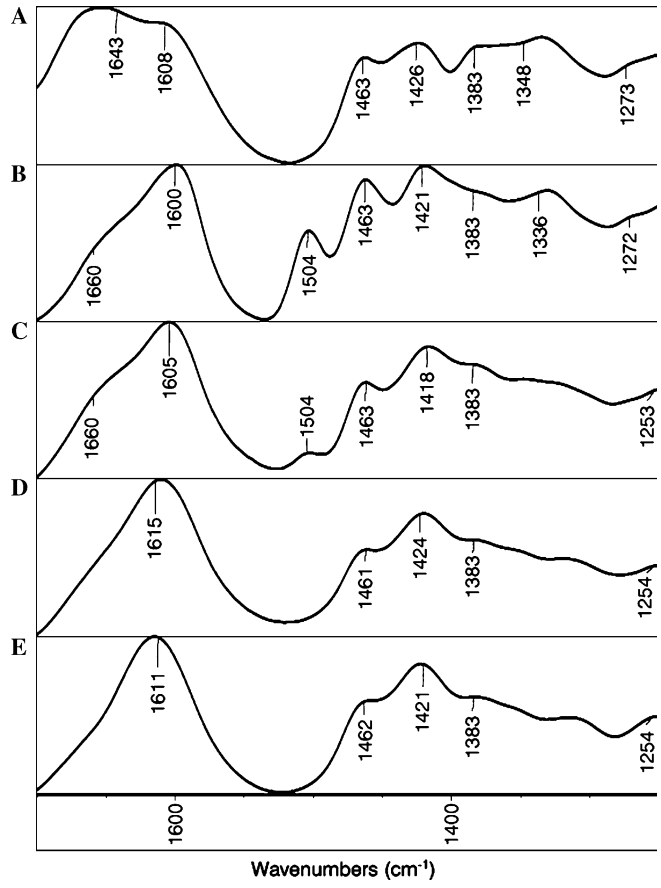

Fig. 5. 1700–1250 cm^{-1} region of the FT-IR spectra of (A) birchwood xylan, (B) kenaf hemicellulose A, (C) kenaf hemicellulose B, (D) acidic oligosaccharide fraction from the *S. thermophile* xylanase hydrolysis, (E) acidic oligosaccharide fraction from the *T. aurantiacus* xylanase hydrolysis.

the C–H and O–H bonds in the sugars. Absorbance noted in the regions 1171–1159, 1132–1121 and 1095–1093 cm^{-1} is due to C–O–C and C–O bending vibrations in the carbohydrate rings. This region is very sensitive to carbohydrate

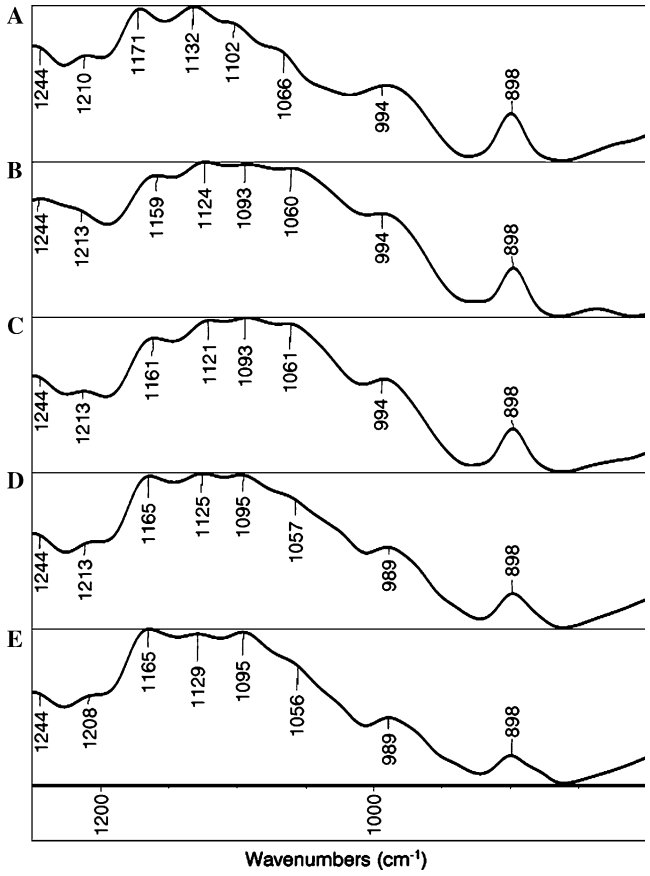

Fig. 6. 1250–800 cm^{-1} region of the FT-IR spectra of (A) birchwood xylan, (B) kenaf hemicellulose A, (C) kenaf hemicellulose B, (D) acidic oligosaccharide fraction from the *S. thermophile* xylanase hydrolysis, (E) acidic oligosaccharide fraction from the *T. aurantiacus* xylanase hydrolysis.

composition and even carbohydrate conformation (Kacurakova et al., 1999). A strong xylose contribution accounts for the 1005–1093 and 1060–1056 cm^{-1} peaks, whereas the absorbance at 994–989 cm^{-1} is the result of C–O stretching, O–H bending and ring deformation motions. A very characteristic peak at 898 cm^{-1} indicates the dominance of β-glycosidic links in the polysaccharide backbone (Kacurakova, Capek, Sasinkova, Wellner, & Ebringerova, 2000).

Absorbance at 1505 cm^{-1} provides an estimate of lignin content in the fractions studied and is higher in the case of hemicelluloses A. The fraction of hemicelluloses A may be, however, contaminated with lignin, which was solubilized during the alkaline extraction and co-precipitated during the acidification step.

Both hemicellulose A and B, however, exhibit strikingly similar FT-IR profiles to the spectrum of commercially obtained birchwood xylan. Their spectra differ slightly in accordance with neutral sugar composition of the samples (Table 1), with prominent contributions from the β-glycosidic bonds, the xylopyranose units and the glucuronic acid units.

The FT-IR spectra of the acidic xylooligosaccharides from the enzymic hydrolysis have also been included in

Fig. 5. The intensity of xylose absorbance at 1055 cm^{-1} is slightly decreased in comparison to the neighboring peaks. This observation is in accordance with the higher uronic acid-to-xylose ratio of the oligosaccharide samples with respect to the hemicellulose fractions. A strong carboxylate contribution is observed at about 1615–1611 cm^{-1}, as expected. These spectra show a high degree of similarity between them. Significant differences between the spectra of these acidic oligosaccharides were only observed with ^{13}C NMR spectroscopy in D_2O solution.

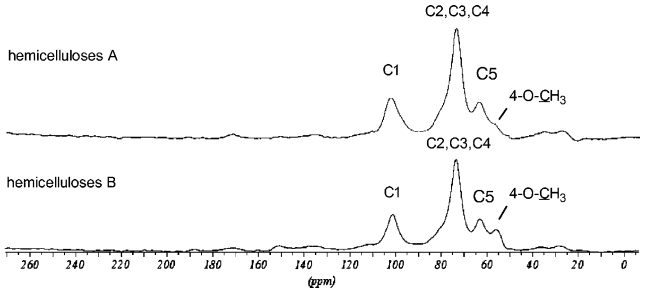

Fig. 7. Solid-state ^{13}C NMR spectra of kenaf hemicelluloses A and B.

3.6. ^{13}C NMR characterization

3.6.1. Solid-state ^{13}C NMR spectra of hemicelluloses

In the solid-state ^{13}C NMR spectra of hemicelluloses, shown in Fig. 7, the chemical shifts observed at 104.9 and 65.7 ppm correspond to C_1 and C_5 from β-D-xylose, the main constituent of the samples. The 78–76 ppm region contains chemical shifts from β-D-xylose C_4, C_3 and C_2 atoms. Contributions from the 4-*O*-methyl group of glucuronic acids may be observed in the region 60–55 ppm in both spectra (Sun, Sun, Liu, Fowler, & Tomkinson, 2002). A stronger 4-*O*-methyl contribution may be observed in the spectrum of hemicellulose B, which contains a higher percentage of uronic acids. No other significant contributions were observed in the spectra. Solid-state ^{13}C NMR spectroscopy, therefore, indicates the prevalence of 4-*O*-methyl-glucuronoxylan in the isolated hemicelluloses.

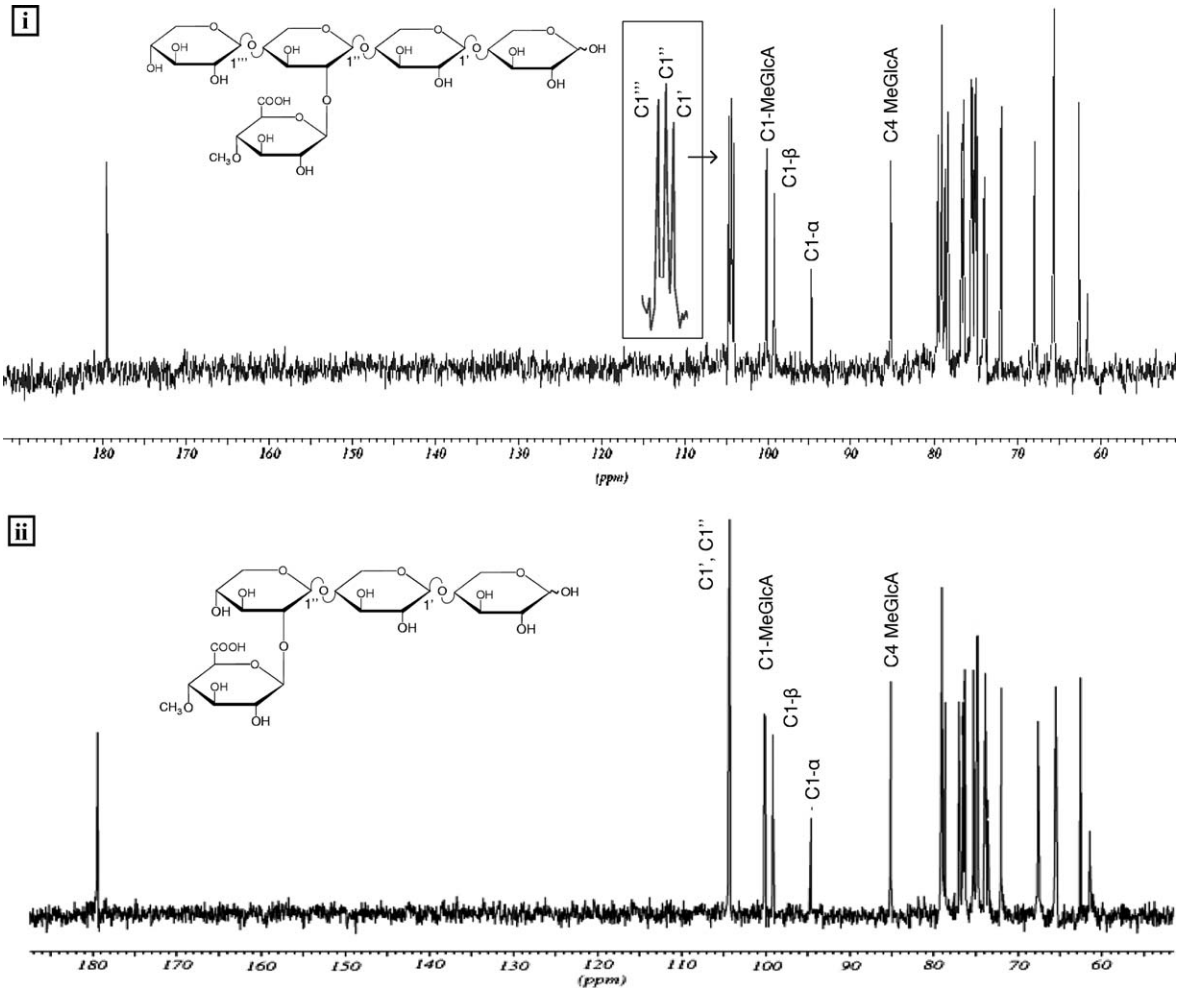

Fig. 8. D_2O Solution ^{13}C NMR spectra of the acidic oligosaccharides from the enzymic hydrolysis. (i) acidic product of the *S. thermophile* hydrolysis (aldopentauronic acid), (ii) acidic product of the *T. aurantiacus* hydrolysis (aldotetrauronic acid).

3.6.2. Solution ^{13}C NMR spectra of the acidic products of the enzymic hydrolysis

The *S. thermophile* xylanase products consisted mainly of xylose, xylobiose and acidic oligosaccharides. The D_2O solution ^{13}C NMR spectrum of the acidic oligosaccharide fraction from the *S. thermophile* enzymic hydrolysis is shown in Fig. 8. It was found consistent with the structure of an aldopentauronic acid, Xyl‴-β-1,4-[MeGlcA-α-1,2-]Xyl″-β-1,4-Xyl′-β-1,4-Xyl, as it is characterized by three chemical shifts in the region 104.6–104 ppm which correspond to the Xyl‴, Xyl″ and Xyl′ C_1 signals. The uronic acid C_1 chemical shift may be observed at 100.1 ppm, whereas the chemical shifts at 99.1 and 94.7 ppm correspond to the C_1 atom of the last Xyl unit in its two anomeric forms, β and α, respectively.

The *T. aurantiacus* xylanase liberated mainly xylose, xylobiose, and acidic oligosaccharides. The solution ^{13}C NMR spectrum of the acidic oligosaccharide fraction from the *T. aurantiacus* enzymic hydrolysis is also shown in Fig. 8. It was found consistent with the structure of an aldotetrauronic acid, MeGlcA-α-1,2-Xyl″-β-1,4-Xyl′-β-1,4-Xyl, as the signals from the C_1 atoms of Xyl′ and Xyl″ present the same chemical shift at 104.3 ppm. The uronic acid C_1 chemical shift may be observed at 100.1 ppm, whereas the chemical shifts at 99.1 and 94.7 ppm correspond to the C_1 atom of the last Xyl unit in its two anomeric forms (β and α, respectively).

Already published spectra of aldopentauronic and aldotetrauronic acids (Biely et al., 1997) exhibit similar chemical shifts in both in terms of the xylopyranose C_1 signals and the C_1, C_4 uronic acid signals. The chemical shift profile in 105–82 ppm region of the previously published spectra is identical, with a slight difference of 1–2 ppm upfield from the chemical shifts of our spectra.

4. Conclusions

Using a series of sequential extractions, we were able to obtain two hemicellulosic fractions from kenaf wood. Hemicellulose A and hemicellulose B were both 4-*O*-methyl-glucuronoxylans, with a 92–94% xylose content and a similar spectral profile to birchwood xylan, but contained a different percentage of uronic acids. They were subsequently used as substrates for the enzymic production of aldopentauronic acid (using *S. thermophile* xylanase) and aldotetrauronic acid (using *T. aurantiacus* xylanase).

The FT-IR and ^{13}C NMR spectra of our hemicelluloses and acidic hydrolysates confirmed the purity of the substrates and the identity of the products. FT-IR profiles, in particular, were very sensitive to uronic acid content and neutral sugar composition of the polysaccharide samples. Solution ^{13}C NMR spectroscopy in D_2O solution was able to discriminate aldopentauronic and aldotetrauronic acid spectra.

Acidic oligosaccharide yields were considerably higher when hemicellulose B was used as a substrate, and the higher yields may be accounted for by the higher uronic acid and the lower lignin content of fraction B. Hemicellulose B, as isolated with the proposed method, seems to be better suited for the enzymic production of aldouronic acids with a regulatory role. Our results, finally, suggest another possible application for kenaf wood, alongside traditional fibre uses.

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References

Bailey, C. J. (1989). Enzyme kinetics of cellulose hydrolysis. *The Biochemical Journal, 262*, 1001–1002.

Batsoulis, A. N., Nacos, M. K., Pappas, C. S., Tarantilis, P. A., Mavromoustakos, T., & Polissiou, M. (2004). Determination of uronic-acids in isolated hemicelluloses from kenaf using diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) and the curve-fitting deconvolution method. *Applied Spectroscopy, 58*(2), 199–202.

Biely, P., Vrsanska, M., Tenkanen, M., & Kluepfel, D. (1997). Endo-β-1,4-xylanase families: Differences in catalytic properties. *Journal of Biotechnology, 57*, 151–166.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry, 54*, 484–489.

Christakopoulos, P., Katapodis, P., Kalogeris, E., Kekos, D., Macris, B. J., Stamatis, H., et al. (2003). Antimicrobial activity of acidic xylo-oligosaccharides produced by family 10 and 11 endoxylanases. *International Journal of Biological Macromolecules, 31*, 171–175.

Davies, G., & Henrissat, B. (1995). Structures and mechanisms of glycosyl hydrolases. *Structure, 3*, 853–859.

Duckart, L., Byers, E., & Thompson, N. S. (1988). The structure of a xylan from kenaf. *Cellulose Chemistry and Technology, 22*, 29.

Ebringerova, A., & Heinze, T. (2000). Xylan and xylan derivatives – biopolymers with valuable properties, 1: Naturally occuring xylans structures, isolation procedures and properties. *Macromolecular Rapid Communications, 21*, 542–556.

EU KA5 project QLRT-2001-01729. (2003). URL: <http://europa.eu.int/comm/research/quality-of-life/ka5/en/projects/qlrt_2001_0_1729_en.htm>.

Filisetti-Cozzi, T. M., & Carpita, N. C. (1991). Measurement of uronic next term acids without interference from neutral sugars. *Analytical Biochemistry, 197*(1), 157–162.

Gilkes, N. R., Henrissat, B., Kilburn, D. G., Miller, R. C., Jr., & Warren, R. A. (1991). Domains in microbial β-1, 4-glycanases: Sequence conservation, function and enzyme families. *Microbiology and Molecular Biology Reviews, 55*(2), 303–315.

Gubitz, G. M., Stebbing, D. W., Johansson, C. I., & Saddler, J. N. (1998). Lignin-hemicellulose complexes restrict enzymatic solubilization of mannan and xylan from dissolving pulp. *Applied Microbiology and Biotechnology, 50*, 390–395.

Hsu, C. K., Liao, J. W., Chung, Y. C., Hsieh, C. P., & Chan, Y. C. (2004). Xylooligosaccharides and fructooligosaccharides affect the intestinal microbiota and precancerous colonic lesion development in rats. *The Jouranl of Nutrition, 134*, 1523–1528.

Ishihara, M., Nagas, Y., & Shimadzu, K. (1995). Physiological activities of xylooligosaccharides. *Proceedings of the International Symposium on Wood Pulping Chemistry* (Vol. 2, pp. 17–22). Finland: Helsinky.

Kacurakova, M., Capek, P., Sasinkova, V., Wellner, N., & Ebringerova, A. (2000). FT-IR study of plant cell wall model compounds: Pectic polysaccharides and hemicelluloses. *Carbohydrate Polymers, 43*, 195–203.

Kacurakova, M., Wellner, N., Ebringerova, A., Hromadkova, Z., Wilson, R. H., & Belton, P. S. (1999). Characterisation of xylan-type polysaccharides and associated cell wall components by FT-IR and FT-Raman spectroscopies. *Food Hydrocolloids, 13*, 35–41.

Kalogeris, E., Christakopoulos, P., Kekos, D., & Macris, B. J. (1998). Studies on the solid-state production of thermostable endoxylanases from *Thermoascus aurantiacus*: Characterization of two isozymes. *Journal of Biotechnology, 60*, 155–163.

Katapodis, P., Kavarnou, A., Kintzios, S., Pistola, E., Kekos, D., Macris, B. J., et al. (2002). Production of acidic xylo-oligosaccharides by a family 10 endoxylanase from *Thermoascus aurantiacus* and use as plant growth regulators. *Biotechnology Letters, 24*, 1413–1416.

Katapodis, P., Kintzios, S., Konstas, J., Kekos, D., Macris, B. J., & Christakopoulos, P. (2003). Enzymic production of aldopentauronic acid and use as a bioregulator in plant airlift bioreactors. *Journal of Bioscience and Bioengineering, 95*(6), 630–632.

Kitayama, T., Morimoto, M., Takatani, M., Tamura, Y., Takumi, M., & Okamoto, T. (2000). Isolation of hemicellulose from a sorghum, Andropogon sorghum Brot, Kumadake no. 263, and determination of its constituent sugars. *Carbohydrate Research, 325*, 230–232.

Neto, C. P., Seca, A., Fradinho, D., Coimbra, M. A., Domingues, F., Evtugin, D., et al. (1996). Chemical composition and structural features of the macromolecular components of *Hibiscus cannabinus* grown in Portugal. *Industrial Crops and Products, 5*, 189–196.

Nidetzky, B., Steiner, W., Hayn, M., & Claeyssens, M. (1994). Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: A new model for synergistic interaction. *The Biochemical Journal, 298*, 705–710.

Roberfroid, M. B. (1997). Health benefits of non-digestible oligosaccharides. *Advances In Experimental Medicine And Biology, 427*, 211–219.

Russo, V. M., Webber, C. L., III, & Myers, D. L. (1997). Kenaf extract affects germination and post-germination development of weed, grass and vegetable seeds. *Industrial Crops and Products, 6*, 59–69.

Sattler, W., Esterbauer, H., Glatter, O., & Steiner, W. (1989). The effect of enzyme concentration on the rate of the hydrolysis of cellulose. *Biotechnology Bioengineering, 33*, 1221–1234.

Sun, R. C., Lawther, J. M., & Banks, W. B. (1996). Fractional and structural characterization of wheat straw hemicelluloses. *Carbohydrate Polymers, 29*, 325–331.

Sun, R. C., Sun, X. F., Liu, G. Q., Fowler, P., & Tomkinson, J. (2002). Structural and physicochemical characterization of hemicelluloses isolated by alkaline peroxide from barley straw. *Polymer International, 51*, 117–124.

Tsakonas, A., Stergiou, V., Polissiou, M., Akoumianakis, K., & Passam, H. C. (2005). Kenaf (*Hibiscus cannabinus* L.) based substrates for the production of compact plants. *Industrial Crops and Products, 21*, 223–227.

Vasquez, M. J., Alonso, J. L., Dominguez, H., & Parajo, J. C. (2000). Xylooligosaccharides: Manufacture and applications. *Trends in Food Science and Technology, 11*, 387–393.

Vardakou, M., Katapodis, P., Samiotaki, M., Kekos, D., Panayotou, G., & Christakopoulos, P. (2003). Mode of action of family 10 and 11 endoxylanases on water-unextractable arabinoxylan. *International Journal of Biological Macromolecules, 33*, 129–134.

Vardakou, M., Katapodis, P., Topakas, E., Kekos, D., Macris, B. J., & Christakopoulos, P. (2004). Synergy between enzymes involved in the degradation of insoluble wheat flour arabinoxylan. *Innovative Food Science and Technology, 5*(1), 107–112.