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Note

Structural analysis of arabinoxylans isolated from native and malted finger millet (*Eleusine coracana*, ragi)☆

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Abstract—Structural elucidation of purified arabinoxylans isolated from finger millet and its malt by methylation, GLC–MS, periodate oxidation, Smith degradation, NMR, IR, optical rotation, and oligosaccharide analysis indicated that the backbone was a 1,4-β-D-xylan, with the majority of the residues substituted at C-3. The major oligosaccharide generated by *endo* xylanase treatment was homogeneous with a molecular weight of 1865 Da corresponding to 14 pentose residues as determined by MALDI-TOF-MS and gel filtration on Biogel P-2. The structural analysis of this oligosaccharide showed that it contained 8 xylose and 6 arabinose residues, substituted at C-3 (monosubstituted) and at both C-2 and C-3 (disubstituted).

Keywords: Arabinoxylans; Finger millet; Malt

Studies have been carried out on hemicelluloses and their constituent polysaccharides such as arabinoxylans, D-glucans of various cereals such as barley,[1] rice,[2] maize,[3] sorghum[4] and wheat.[5] Finger millet (*Eleusine coracana*) also known as '*ragi*' in India, is a rich source of dietary fiber (18%) and calcium (0.34%) compared to most of the cereals, and minor millets.[6] It is used either in its native or processed forms in the preparation of weaning foods, pharmaceutical products and low cost ready-to-eat food items.[7] Studies were carried out on native and malted ragi with respect to (a) free sugars, nonstarch polysaccharides, (b) bound phenolic acids and their antioxidant properties[8–10] and (c) starch/cell wall degrading enzymes.[11]

In the present study, hemicellulose-B isolated from native and malted ragi was purified to homogeneity according to the scheme depicted in Figure 1. In addition to arabinose and xylose, the purified polysaccharides from native ragi and its malt contained 10% of uronic acid. This was reduced to 2.0% and 2.8% in native and malted polysaccharides, respectively (Table 1). Control and carboxyl-reduced samples were permethylated and hydrolyzed and the derivatives were analyzed by GLC–MS (Table 2). The major *O*-methyl sugars obtained were 2,3,5-Me_3 arabinose, 2-Me xylose, 2,3-Me_2 xylose, followed by 2,3-Me_2 arabinose, 2-Me arabinose, along with very small amounts of 2,3,4,6-Me_4 galactose, free arabinose, xylose, in both native and malted conditions before reduction. The presence of 2,3,4-Me_3 xylose indicated that xylose was also present in side chains. The increase in 2,3,4,6-Me_4 glucose (11%) in carboxyl-reduced samples originated from glucuronic acids present in the samples. Methylation analysis of the xylanase-degraded polysaccharide (XDP) indicated high amounts of 2,3,5-Me_3 arabinose, 2-Me xylose along with small amounts of 2,3-Me_2 xylose, free arabinose in the ratios slightly differing than those of untreated samples (Table 2).

The above results indicated the backbone to be made up of (1→4)-linked xylan with arabinose substitutions primarily at C-3 of xylose as indicated by the presence in equal amounts of 2-Me-xylose and 2,3,5-Me_3 arabinose residues. Disubstitutions at C-2 and C-3 of xylose and arabinose are due to the presence of small amounts of free xylose/arabinose residues. Smith degradation

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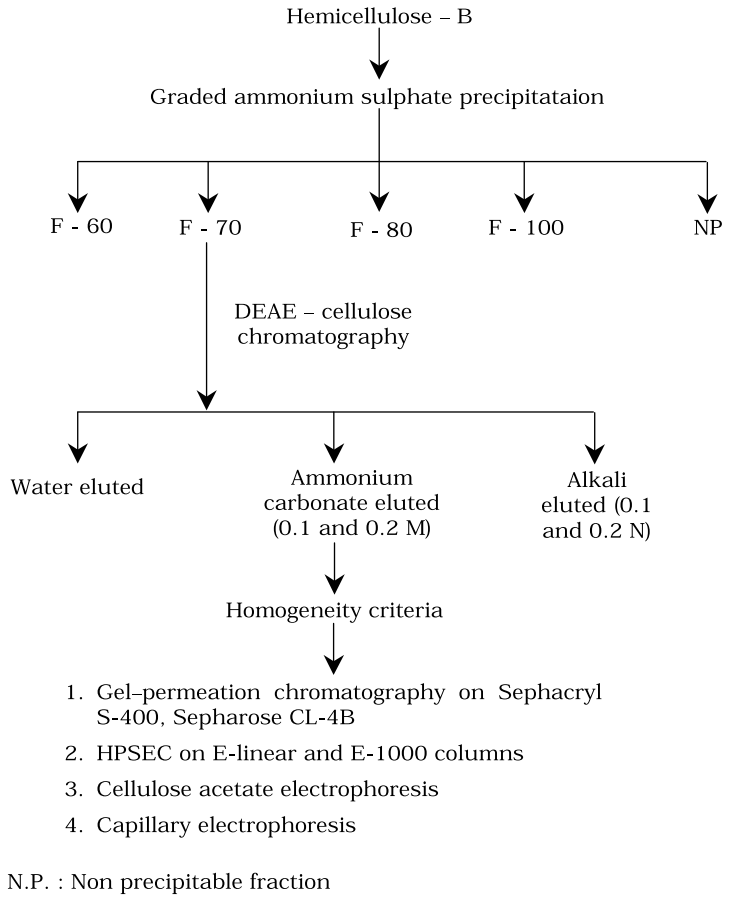

Figure 1. Purification scheme of arabionoxylan of hemicellulose B isolated from native (N) and malted (M) ragi.

Table 1. Sugar composition (%) of purified polysaccharides before and after reduction

Reduction	Sample	Ara	Xyl	Gal	4-O-Me Glc	A:X	U.A.
Before							
	N	43.00	43.30	1.20	—	1.0:1.00	10.00
	M	48.00	40.00	2.00	—	1.0:0.83	9.00
After							
	N	44.00	43.50	1.00	9.50	1.0:0.98	2.00
	M	46.00	40.00	2.20	9.00	1.0:0.86	2.80

experiments of the purified arabinoxylans revealed unattacked arabinose and xylose corroborating the methylation analysis. Uronic acid residues may be linked to xylose residues at C-2 via $1\rightarrow2$ linkage as indicated by the increase in 2,3,4,6-Me_4 glucose in carboxyl-reduced sample. The uronic acid was found to be 4-(O-Me) glucuronic acid as identified by the acetylation of carboxyl-reduced polysaccharides followed by their GLC–MS analysis, which yielded characteristic diagnostic fragments (129, 189, 201 and 261). Similar types of results were reported for arabinoxylans extracted from sorghum and rice bran hemicelluloses.[10]

^{13}C NMR of pure arabinoxylans obtained from native and malted hemicellulose-B is shown in Figure 2. In the present study, signals obtained at 109.337 and 102.949–103.379 ppm can be assigned to α-L-arabinofuranose and β-D-xylopyranose, respectively, and also indicated that the xylose is present in mono/or unsubstituted forms (Table 3). C-1 signals for densely substituted xylose residues were not prominent (100.7 ppm), because of the low amount of disubstituted xylose residues as showed by the methylation analysis (very low amount of free xylose). C-2 to C-4 signals of arabinose and xylose were observed between 72–86 ppm and signals

Table 2. Methylation analysis of purified (before and after reduction) and enzyme-degraded polysaccharides obtained from native (N) and malted (M) ragi hemi-B

O-Methyl ether	RR_T[a]	% Sugar						Diagnostic fragments	Mode of linkage
		Before reduction		After reduction		XDP			
		N	M	N	M	N	M		
2,3,5-Me_3-Ara	0.78	35.50	36.60	35.00	36.00	40.00	43.0	45, 87, 101, 118, 129, 145, 161, 162	Araf-(1→
2,3,4-Me_3-Xyl	0.90	1.50	1.50	2.00	2.00	2.00	2.00	101, 102, 118, 129, 148, 162	Xylp-(1→
2,3,4,6-Me_4-Glc	1.00	N.D.	N.D.	10.20	9.00	N.D.	N.D.	45, 102, 118, 129, 145, 161, 161, 205	Glcp-(1→
2,3,4,6-Me_4-Gal	1.30	2.00	1.50	1.50	1.40	2.20	1.50	45, 102, 118, 129, 145, 161, 161, 205	Galp-(1→
2,3-Me_2-Ara	1.36	3.00	4.00	4.00	4.00	4.00	4.00	87, 102, 118, 129, 189	-5)Araf-(1→
2,3-Me_2-Xyl	1.52	9.00	8.00	9.00	8.00	5.00	5.00	87, 102, 118, 129, 189	-4)Xylp-(1→
2-Me-Ara	1.90	3.00	4.00	3.30	3.30	3.00	3.00	85, 118, 127, 145, 187	-3,5)Araf-(1→
2-Me-Xyl	2.26	28.00	26.00	26.00	25.00	27.20	27.00	85, 118, 127, 145, 187	-3,4)Xylp-(1→
Arabinose	2.46	2.00	3.40	1.00	3.00	2.00	2.00	85, 103, 115, 128, 145, 159, 188, 203	-2,3,5)Araf-(1→
Xylose	3.30	6.00	6.00	5.50	5.50	5.00	4.00	85, 103, 115, 128, 145, 159, 188, 203	-2,3,4)Xylp-(1→
$\frac{2,3-Me_2-Xyl}{2-Me-Xyl+Xyl}$		0.26	0.25	0.28	0.26	0.15	0.16		
$\frac{Xyl}{2-Me-Xyl}$		0.21	0.23	0.21	0.22	0.18	0.15		
Ara:Xyl		1.0:1.02	1.0:0.86	1.0:0.98	1.0:0.87	1.0:0.80	1.0:0.72		

N.D. Not detected.
XDP: Xylanase degraded polysaccharide.
[a] RR_T: with respect to 1,5,di-*O*-acetyl 2,3,4,6-Me_4-glucose.

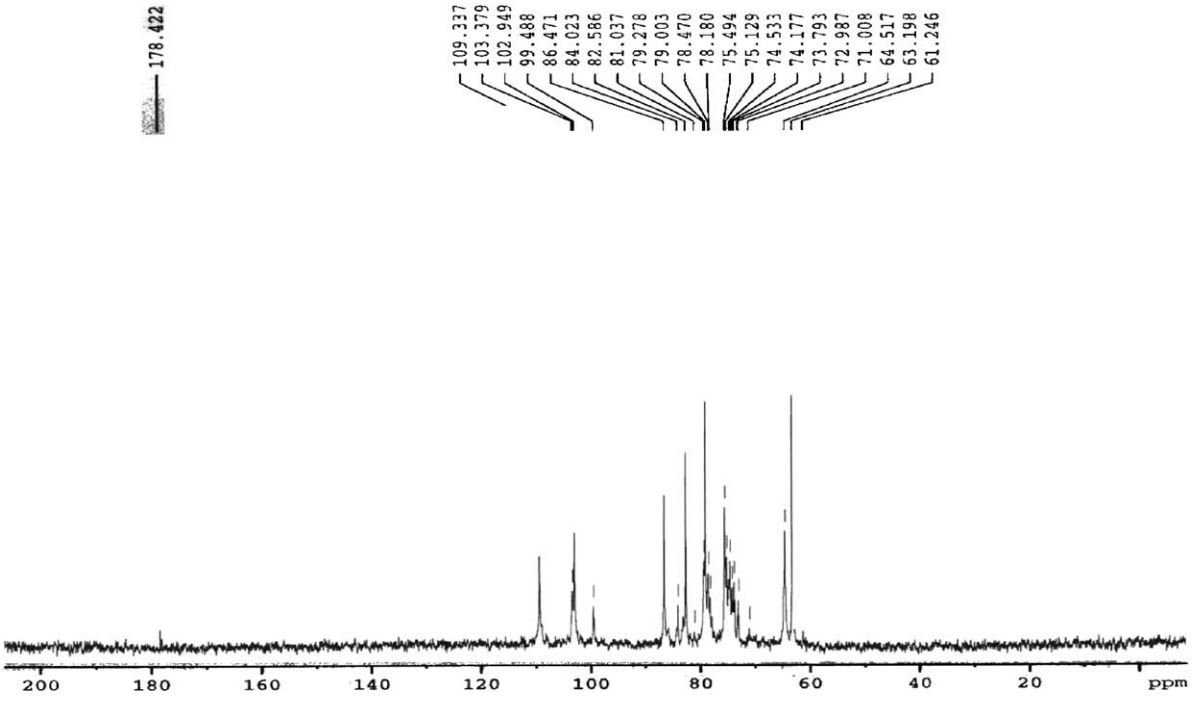

Figure 2. ^{13}C NMR spectrum of purified arabinoxylan isolated from native (N) and malted (M) ragi.

at 64.517 and 63.198 ppm are assigned for C-5 of arabinose and xylose, respectively. Two characteristic signals observed at 99.4 and 178.422 ppm, can be assigned to the carbonyl groups (C=O) of C-1 and C-6 of glucuronic acid.[12]

^1H NMR spectra of purified arabinoxylans indicated a prominent signal at 5.403 ppm, for α-anomeric proton of arabinose substituted at C-3 of xylose backbone (Fig. 3). However, signals for arabinose residues linked to the same xylose unit at C-2 and C-3 (5.22 and 5.28 ppm) were not prominent, indicating low amount of doubly substituted xylose residues. This result agrees with methylation (very less amount of free xylose) and also with ^{13}C NMR (minor signals in the region of 100.7 ppm) data. The signals assigned here are in close agreement with the signals assigned for cold water soluble arabinoxylans obtained from wheat endosperm.[13]

IR spectra indicated high arabinose substitution at C-3 of xylose residues (low intensity shoulder at 990 and 1164 cm^{-1}) and the loss of peak multiplicity in

Table 3. Assignment of ^{13}C NMR signals of purified arabinoxylan obtained from native (N) and malted (M) ragi

Sugar	Chemical shifts (ppm)									
	C1		C2		C3		C4		C5	
	N	M	N	M	N	M	N	M	N	M
A	102.95	102.97	74.53	74.53	79.28	79.27	75.99	75.47	64.52	64.52
B	N.D	N.D	—	—	—	—	—	—	64.52	64.52
C	103.38	103.37	75.13	75.12	75.48	75.49	78.47	78.47	64.52	64.52
D	109.34	109.34	82.59	82.58	79.01	79.01	86.47	—	63.20	63.18
E	109.34	109.34	82.59	82.58	—	—	—	—	63.20	63.18
F	N.D	N.D	84.02	84.03	—	—	—	—	63.20	63.18

Signals for C1 and C6 of 4-*O*-methyl glucuronic acid residues were observed at 99.488 and 178.422, respectively [99.478 (M) 178.420 (M)—(sugar G)].

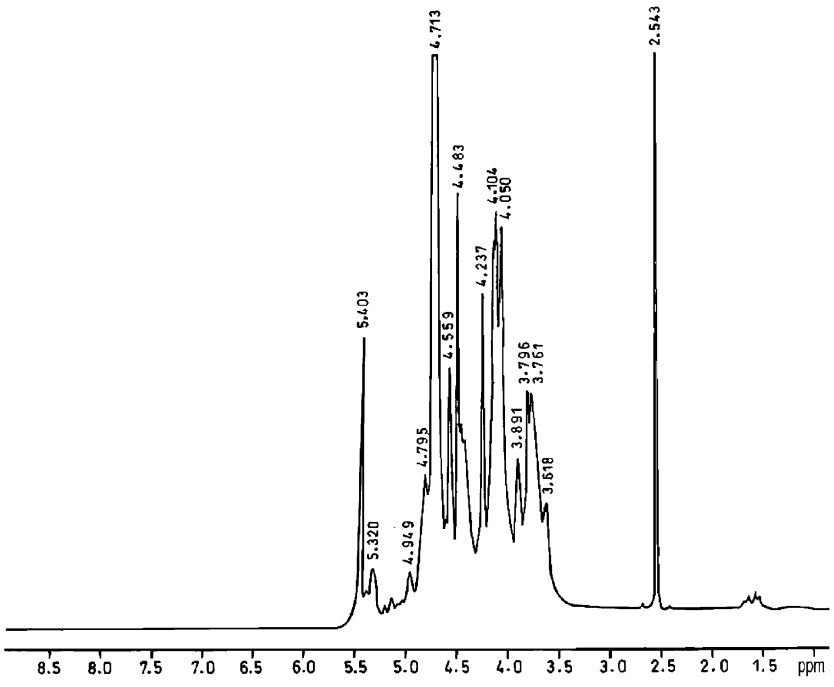

Figure 3. ^{1}H NMR spectrum of purified arabinoxylan isolated from native (N) and malted (M) ragi.

1120–1000 cm^{-1}, is a typical characteristic of highly substituted arabinoxylans. The signal at 1747.11 cm^{-1} can be attributed to the carbonyl group stretching of uronic acid carboxyls and its intensity has decreased upon carboxyl reduction. The remaining signals in the spectra that is at 1461.98, 1377, 1046.17 cm^{-1} were due to $–CH_2$, $–CH_2–OH$, and –C–OH, respectively. Optical rotation values of purified arabinoxylans obtained from native and malted hemicellulose-B fraction were found to be −68° and −70°, respectively, which indicated that the xylan backbone of arabinoxylans was made up of β-linkages.

Two oligosaccharides (major, 10–12% yield, $[\alpha]_D$ −78° and a minor, 2–4% yields, $[\alpha]_D$ −60°) were obtained from purified arabinoxylans by xylanase treatment. They were composed of arabinose and xylose in 1:1.5 and 1:1 ratio, respectively. The major oligosaccharide consisted of 14 pentose residues as indicated by the molecular ion obtained by MALDI-TOF-MS at *m/e* 1865.6 (Fig. 4) and also by the elution profile on precalibrated Biogel P-2 column. Methylation of the unreduced oligosaccharides indicated the presence of mono- and unsubstituted xylose residues along with very small amount of disubstituted residues. Nonreducing terminal arabinose residues were found along with small amounts of mono substituted arabinose as indicated by high amount of 2,3,5-Me_3 and a small amount of 2,3-Me_2 arabinose.

Proton NMR analysis of this oligosaccharide indicated signals at 5.3513, and 5.2947, 5.2603, corresponding to anomeric protons of α-L-arabinofuranoses substituted at O-3 (mono substituted) and at both O-3 and C-2 (disubstituted) of xylose residues, respectively. Signals obtained at 4.5804, 4.5378 and 4.4461 ppm were due to the anomeric protons of β-D-xyloses substituted at C-2 and C-3 (disubstituted), C-3 (monosubstituted)

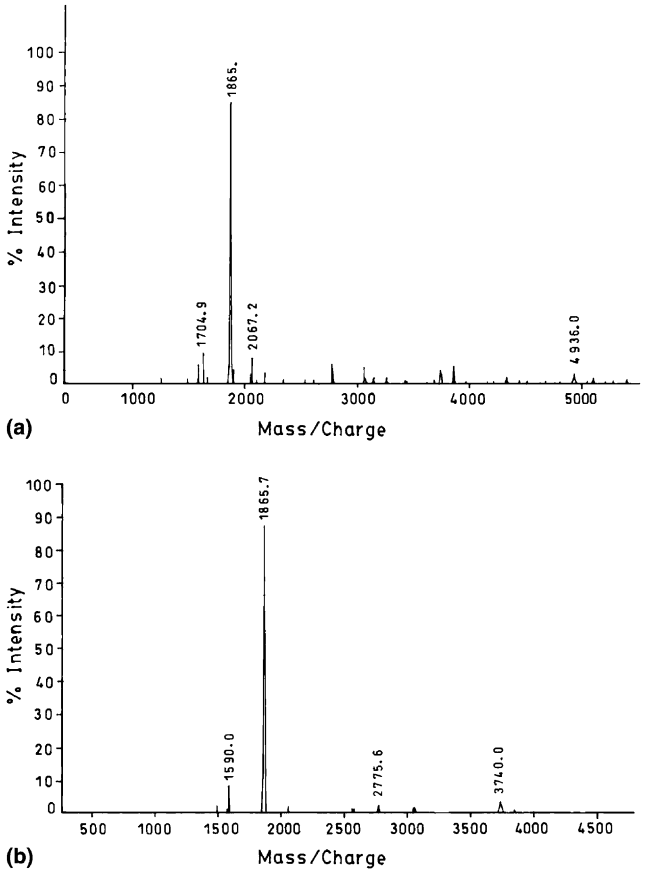

Figure 4. MALDI-TOF-MS analysis of major oligosaccharide obtained by enzymatic treatment from (a) native (N) and (b) malted (M) ragi purified arabinoxylan.

and unsubstituted residues, respectively. A signal obtained at 5.1533 ppm could be due to the α-anomer of unsubstituted xylopyranosyl residues at the reducing end (data not shown). The signals for other protons of arabinose and xylose were observed in the region of 3.2170–4.3117 ppm and were in close proximity with the signals obtained for oligosaccharides generated from wheat water-soluble arabinoxylans.[13]

Based on the above results, a tentative structure can be proposed for purified arabinoxylans from finger millet (Fig. 5). Ragi arabinoxylans resemble maize,[3] sorghum husk[14] and rice bran arabinoxylans, however, they differ from wheat endosperm[15] and bran[16] arabinoxylans in having a more branched structure.

1. Experimental

Ragi (indaf-15) was obtained from V.C. Farm, Mandya, Karnataka, India. Malting of ragi was carried out in a B.O.D. incubator at 25 °C. Hemicelluloses (A and B) were isolated from native and malted ragi.[17] Hemicellulose-B was fractionated by ammonium sulfate into five different fractions,[16] and the major fraction (F-70) was further fractionated on DEAE-cellulose[18] (carbonate form) into five different fractions. The major fraction (0.1 M ammonium carbonate eluted) was purified by gel filtration on Sephacryl S-400 and the homogeneity of the polysaccharide was ascertained on cellulose acetate/capillary electrophoresis and gel filtration on Sepharose CL-4B.

Carboxyl reduction of the purified polysaccharides (100 mg/20 mL) was carried out according to the method of Taylor and Conrad[19] by adding 1-cyclohexyl-2(4-methyl morpholino-ethyl carbodiimide), *p*-toulene sulfonate (1 g) in small portions over a period of 2 h followed by sodium borohydride (2 M, 10 mL) over a period of 4 h, and the samples were dialysed and lyophilized. The purified polysaccharides/oligosaccharide were methylated,[20]

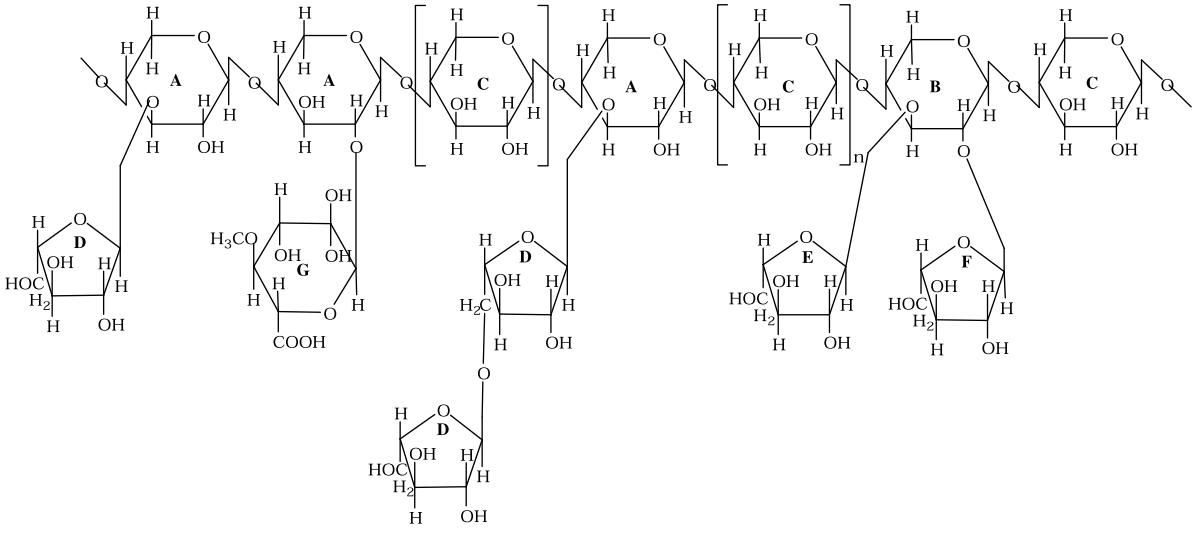

Figure 5. Probable structure of purified arabinoxylan obtained from native (N) and malted (M) ragi.

hydrolyzed and the resultant permethylated alditol acetates were analyzed by GLC–MS.

GC–MS was performed on a Shimadzu GC 17-A QP-5000 system using SP 2330 capillary column (30 M × 0.31 mm i.d.) operating at an ionization potential of 70 eV with a temperature programme mode (180–200 °C, 4 °C raise per min).[21] The mass range was taken between 40 and 400 amu (*m*/*z*) for the analysis. Helium was used as the carrier gas.

FTIR spectra of native and malted samples, before and after carboxyl reduction was recorded between 400 and 4000 cm^{-1} by preparing (a) a smear with nujole and (b) KBr pellets using Perkin–Elmer spectrum 2000. Optical rotation of the poly and oligosaccharides was determined by Perkin–Elmer (Model 243) polarimeter.

The molecular mass of oligosaccharide was determined by MALDI-TOF-MS by using PC Kratos Kompact analytical SEQ MALDI-TOF-MS, UK, with a nitrogen laser of 337 nm wavelength and 5 ns pulse width. The laser beam was focused on to the sample spot (10–30 μm size) at an angle of 45°. Ions were accelerated to the energy of 3 kV before entering the time-of-flight mass spectrometer. At the detector, ions were post-accelerated to a maximum kinetic energy up to 30 kV for more efficient detection.

^{13}C NMR and ^{1}H NMR of polysaccharides/oligosaccharide were carried out by taking samples in D_2O as described[13,22] (2 × 1 mL each, 1 mL). The deuterium resonance was used as a field frequency lock and the shifts were referenced to external TMS. The spectra were recorded in a Bruker AMX 400 MHz NMR spectrometer (5 mm multinuclear probe) operating at 60 °C for 4 h using spectral width of 22,727 Hz with 6000 scans. Periodate oxidation and Smith degradation of the polysaccharides were carried out as reported earlier.[23,24]

The polysaccharide (100 mg) was dissolved in an acetate buffer (pH 4.5, 0.1 M, 10 mL) and incubated with *endo* xylanase (from *Trichoderma viridae*, 30 units) in a constant-shaking water bath for 24 h. After digestion, the reaction was stopped by adding 3 vols of ethanol, the precipitated material was removed by centrifugation (10,000 rpm, for 15 min) followed by dialysis and lyophilization. The resulting supernatant was concentrated to 1 mL and analyzed for total and reducing sugar contents and also for the determination of the degree of polymerization.

The oligosaccharides obtained from the purified polysaccharide (100 mg) by the action of xylanase from *T. viridae* (30 units) were separated on Biogel P-2 column (0.8 cm × 94 cm), precalibrated with malto oligosaccharides (maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose). Triple distilled, degassed water was used as the eluent at a flow rate of 16 mL/h and fractions (1.5 mL) were collected. The carbohydrate containing fractions were pooled and lyophilized for further analysis.

Purified oligosaccharides were subjected to HPLC analysis to confirm their purity. Oligosaccharide samples (2 mg) were dissolved in water (0.2 mL) and 20 μL of it was injected to the μ-Bondapak-NH_2 carbohydrate column (4.1 mm × 300 mm) and eluted at a flow rate of 1 mL/min and detected by using a refractive index detector.[25]

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