VALORIZATION OF RICE STRAW *VIA* PRODUCTION OF MODIFIED XYLANS AND XYLOOLIGOSACCHARIDES FOR THEIR POTENTIAL APPLICATION IN FOOD INDUSTRY

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The present study attempted to valorise rice straw, one of the most abundant, renewable and economic agricultural wastes, *via* production of modified xylans and xylooligosaccharides (XOS), which were evaluated for their total antioxidant capacity. The percent contents of extractives, cellulose, hemicelluloses, lignin and ash (silica) in rice straw were found to be 20, 41, 24, 9 and 6, respectively. Hemicellulosic xylan was extracted from straw by reaction with 11% (*w/v*) sodium hydroxide for about 3 h at 80 °C, so that a yield of about 19.4% was obtained, followed by spectral and thermal analyses. Xylan was then subjected to chemical modifications using acetic anhydride, benzyl chloride and sodium monochloroacetate, and the modifications of functional groups were confirmed by FT-IR and NMR spectral techniques. Controlled acid hydrolysis of xylan was carried out using dilute sulphuric acid (0.1, 0.25, 0.5 and 1.0M) at 121 °C, 15 psi for 10 min to produce XOS-rich hydrolysate. Xylan, modified xylans and XOS exhibited significant concentration dependent AAE values, XOS exhibiting the highest AAE value, followed by xylan, benzylated xylan, carboxymethylated xylan and acetylated xylan. Therefore, rice straw based modified xylans and XOS with potential antioxidant properties may serve as potential candidates for use in food industries as packaging films and functional food ingredients.

Keywords: rice straw, xylan, modified xylans, xylooligosaccharides, total antioxidant capacity

INTRODUCTION

Agricultural waste biomass constitutes a major portion of lignocellulosic biomass; it includes both the crop residues left in the field after the harvest, *i.e.* stalk and straw, or the residues obtained after processing, *i.e.* husks, seeds, bagasse, molasses, roots *etc*.[1] The most important and abundant agricultural biomass feedstocks comprise rice straw, corn cobs, wheat straw, sugar crops bagasse, *etc*.

The straw of rice (*Oryza sativa*), also called paddy straw in northern India, is stalk obtained as by-product after harvesting rice grains. In India, the rice-wheat cropping system is the most frequently adopted one, but the short window period between harvesting of rice and cultivation of wheat makes the management of rice straw a major issue. Farmers burn approximately 70-80 million tonnes of paddy straw per year to reduce the turnaround time between harvesting and sowing for the second crop.[2] The burning of straw triggers global warming, damages bulk minerals in the soil, causes environmental pollution and hence respiratory problems;[3] therefore, the valorization of rice straw becomes a major issue.

Rice straw, a lignocellulosic material, is composed of three structural biopolymers, *i.e.* cellulose, hemicelluloses and lignin, and small amounts of non-structural compounds, *i.e.* extractives and ash. Although straw contains up to 30% hemicelluloses, its application potential is yet to be fully exploited;[4] therefore, the fraction of interest in the present study is hemicellulose. The predominant hemicellulose type in rice straw is xylan; the parent chain of xylan contains xylose sugar units linked by *β*-1,4 glycosidic bonds, along with certain side chain sugar residues, such as arabinose, glucuronic acid, acetyl groups etc., based upon the plant source from which it is extracted. Various methods for extracting

hemicelluloses include alkaline extraction,[5] alkaline peroxide extraction,[6] organic solvent extraction,[7] ultrasound-assisted extraction,[8] steam explosion,[9] autohydrolysis,[10] microwave-assisted extraction[11] and ionic liquid extraction.[12] The extracted hemicelluloses can be chemically modified to improve its properties, such as thermal stability, solubility, mechanical strength, viscosity *etc.*; the common modifications include esterification, etherification, oxidation, reduction and partial hydrolysis.[13] Significant research has been carried out on extraction, characterization and modifications of hemicelluloses from various hardwood and softwood species, as well as agricultural feedstocks; however, little literature is available on the extraction and chemical modifications of rice straw hemicelluloses.

Although xylan and its derivatives find diverse applications in the fields of agriculture, medicine, chemistry and technology, their film forming properties and biodegradability aroused interest for their application as food packaging materials.[14,15] With the changing lifestyle, people are more inclined towards the use of packaged food and drinks; therefore, food preservation has become a major target for food industries. Food packaging is the process of enclosing food to protect it from physical, chemical or biological spoilage. Oxidation is the major cause for food spoilage, which causes a decrease in nutritional value and energy content of food, results in off-flavour and sometimes colour change; therefore, the food industry is focusing on antioxidant-based food packaging materials.

Antioxidants are defined as the substances that inhibit, delay or control oxidation processes both in foods and the body, when used in small amounts.[16] Natural antioxidants are incorporated into the polymer matrices of active antioxidant polymer packaging material enclosing the food product. The polymer packaging would interact with food and absorb the unwanted substances from the environment, as well as from the food, thereby promoting its increased shelf life.[17] Synthetic polymers used as food packaging materials offer advantages in terms of physical properties, mechanical strength and low cost, but their non-biodegradability and non-renewability arouse environmental concerns and sustainability issues. As a result, a shift has been observed from fossil fuel-based polymers to biomass-based polymers. Xylan and modified xylan-based food packaging films, with inherent potential antioxidant activity, may be instrumental to the food packaging industry in preventing food spoilage.

Xylooligosaccharides (XOS), obtained by chemical and/or enzymatic hydrolysis of xylan, are non-digestible oligomers consisting of 2-9 xylose sugar units associated by usual β-1,4 glycosidic linkages. They have attracted the attention of researchers for they possess significant biological properties, such as anti-allergic, anti-inflammatory, anti-cancer, anti-microbial, antioxidant, antiviral activities *etc*.[18,19] Apart from this, they are also known to exhibit prebiotic potential and cytotoxic and immunomodulatory properties.[20] The prebiotic potential of XOS has attracted much research attention as well, due to their ability to stimulate the growth of human gut bacteria to a larger extent than existing prebiotic fructooligosachharides.[21,22] XOS based hydrogels or XOS derived nanoparticles find use in drug delivery for curing gastrointestinal and cardiovascular diseases.[23] The antioxidant properties of XOS extracted from rice straw have not been fully exploited to date.

Therefore, the present study intended to achieve the extraction of hemicellulosic xylan from rice straw, its structural characterization and chemical modifications; followed by the evaluation of total antioxidant capacity of modified xylans and xylooligosaccharides (XOS) for application in the food industry.

EXPERIMENTAL

Raw material

Rice straw of the variety Pusa Basmati 1121 was dried (40-50 °C), milled and passed through a 1 mm sieve to obtain a homogeneous powdered material, which was stored in plastic bags, at room temperature, in an air-tight container until further use.

Reference standards and other materials

Xylose and *L*-ascorbic acid were purchased from Sigma-Aldrich, St. Louis, MO, USA. All other reagents, chemicals and solvents (analytical research grade) employed in the study were purchased from Molychem (Mumbai, India). Filtrations were carried out using Whatman Grade 91 filter paper obtained from Whatman International Ltd., Maidstone, England.

Partial delignification of rice straw and alkaline extraction of xylan

The chemical composition of rice straw, *i.e.* percent contents of cellulose, hemicelluloses, lignin, extractives and acid insoluble ash (silica), were determined by the Detergent System method.[24] Finely powdered rice straw was treated with warm distilled

water to remove any dirt and water-soluble impurities. The dirt-free material was partially delignified by treatment with a mixture of NaOH (1, 2 and 4% *w/v*) and 96% ethanol (3:7, *v/v*) with a solid to liquid ratio of 1:30 (*w/v*) in accordance with the method proposed by Farhat *et al*.[5] The surface morphology of untreated and partially delignified rice straw samples was recorded on a Hitachi S-3400N Scanning Electron Microscope (SEM). Xylan was extracted from rice straw by the alkaline process already optimized using response surface methodology in our previous research.[25]

Structural characterization and thermal analysis of extracted xylan

Fourier transform infrared (FT-IR) spectra of xylan were recorded on a Perkin-Elmer FTIR Spectrophotometer. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra of xylan were recorded on a Bruker Avance Neo 500 MHz NMR spectrometer, using tetramethylsilane (TMS) as an internal reference standard. Thermal gravimetric analysis of xylan was carried out on a Perkin Elmer TGA-4000.

Chemical modifications of xylan

Hemicellulosic xylan extracted from rice straw was subjected to chemical modifications *via* acetylation, benzylation and carboxymethylation reactions.

Acetylation

Powdered xylan was dissolved in a minimum amount of DMSO and a mixture of acetic anhydride: pyridine (1:1, *v/v*) was added to it. The resulting mixture was placed for 72 hours in the dark at 27 °C. It was neutralized with acetic acid and precipitated by addition of an excess of cold ethanol. The precipitates were filtered and washed thoroughly with methanol. The washed precipitates were dissolved in a small amount of chloroform and re-precipitated by the addition of ethanol. The resulting xylan acetate was left to dry overnight and then oven dried for about 4 hours.

Benzylation

Powdered xylan was dissolved in distilled water by heating at 80 °C with continuous stirring, followed by dropwise addition of sodium hydroxide solution for about 5 minutes. Then, 60% ethanol was added to the above mixture, resulting in heterogeneous reaction medium. After that, benzyl chloride was added and the contents were refluxed for about 9 hours at 70 °C. Finally, the mixture was allowed to reach room temperature, neutralized with acetic acid, and precipitated by the addition of excess ethanol. The precipitates were filtered, air-dried and oven-dried in hot air oven at 100 °C.

Carboxymethylation

Powdered xylan was mixed in isopropanol by continuous stirring at room temperature. Then, sodium hydroxide solution was added to the above mixture with stirring at 80 °C for about 1 hour. After that, sodium monochloroacetate was added with continuous stirring for about 5 hours at the same temperature, with timely addition of isopropanol, when required. Finally, the mixture was allowed to cool to room temperature, neutralized with acetic acid, and precipitated with excess of ethanol. The precipitates were washed several times with methanol or ethanol until they no longer reacted with silver nitrate. The precipitates were filtered, air-dried and oven-dried in hot air oven at 100 °C.

Characterization of modified xylans
Physical characterization

The modified xylans were physically characterized using weight percent gain (WPG) analysis and solubility analysis. WPG analysis, a measure to calculate the extent of reaction, was determined by the difference in weight of oven-dried sample before (W_o) and after (W_o') modifications, as given by the following equation:

$$WPG\ (\%) = \frac{W_o' - W_o}{W_o} \times 100 \tag{1}$$

The nature of the polymers (hydrophillic or hydrophobic) was determined by checking their solubility in water and organic solvents, *i.e.* ethanol, toluene, chloroform, dimethyl sulfoxide (DMSO) and dichloromethane.

Structural characterization

The modified xylans were characterized by FT-IR, ^{1}H NMR and ^{13}C NMR spectral techniques.

Controlled acid hydrolysis of xylan to xylooligosaccharides (XOS)

Xylan powder was suspended in dilute sulfuric acid (0.1, 0.25, 0.5 and 1.0 M) in a solid to liquid ratio of 1:50 for 10 min at 121 °C and 15 psi to carry out hydrolysis. The mixture was cooled to room temperature and calcium carbonate was added to neutralize the excess acid. The mixture was filtered to obtain xylan hydrolysate.

A fine spot of xylan hydrolylsate was applied on activated silica gel-G coated glass plates (10 cm × 20 cm). The plates were developed using a solvent system of acetonitrile: water (9:1, *v/v*) as mobile phase. The spots were visualized by spraying with orcinol spray agent (0.2% *w/v*) prepared in methanol: sulphuric acid (9:1, *v/v*), followed by incubation in the oven at 100 °C for 10 minutes to obtain blue spots, indicating XOS presence. The degree of polymerization of XOS present in the xylan hydrolysate was determined using Waters, Q-TOF MICROMASS (ESI-MS).

Determination of total antioxidant activity of xylan, modified xylans and XOS

Total antioxidant capacity was determined by the

phosphomolybdate assay using ascorbic acid as standard.[26] The reagent for the assay was prepared by mixing 100 mL of each 4 mM ammonium molybdate, 28 mM sodium phosphate and 0.6 M sulphuric acid. Firstly, different concentrations (1.0 to 7.0 mg/mL) of 0.2 mL of sample were shaken with 0.6 mL of the reagent solution and placed at 95 °C for 1.5-2 h. Then, the solutions were allowed to cool and their absorbance was measured at 695 nm against the blank. The antioxidant capacity was expressed as ascorbic acid equivalents (AAE), *i.e.* μg ascorbic acid per mg sample. The analysis of variance (ANOVA), appropriate in a completely randomized design, was employed to the data using CPCS1, and CD (5%) was calculated.

RESULTS AND DISCUSSION
Determination of chemical composition of rice straw

The amounts of cellulose, hemicelluloses, lignin, extractives and silica in rice straw were found to be 41 ± 0.4%, 24 ± 0.6%, 9 ± 0.5%, 20 ± 0.8% and 6 ± 0.3%, respectively. The results were in accordance with Khandanlou *et al.*,[4] who reported rice straw to be composed of 32-47% cellulose, 19-32% hemicelluloses, 5-24% lignin and 13-17% ash.

Partial delignification of rice straw

The presence of lignin affects the extraction yield and purity of the resulting hemicellulosic xylan; therefore, partial delignification of rice straw was carried out, prior to alkaline extraction, using a mixture of NaOH and ethanol, and the effect was studied through SEM imaging. The surface morphologies of untreated and partially delignified rice straw samples are shown in Figure 1. The SEM image of untreated rice straw displays compact fibrous bundles with a thick lining (Fig. 1(a)). This thick lining is basically lignin encapsulating the carbohydrate polymers.
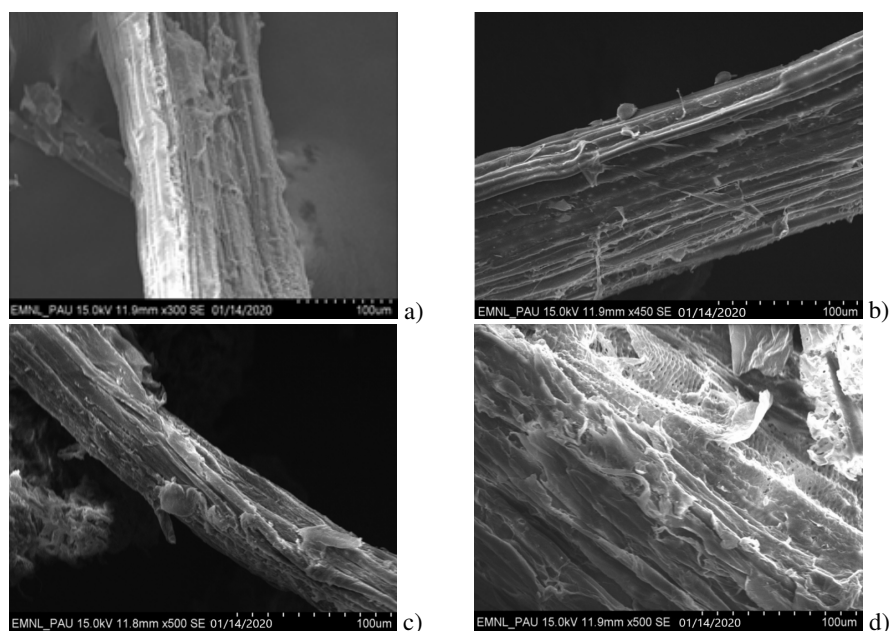

Figure 1: Scanning electron microscopy images of untreated (a) and partially delignified rice straw samples: (b) treated with 1% NaOH and ethanol; (c) treated with 2% NaOH and ethanol; (d) treated with 4% NaOH and ethanol

The treatment with 1% NaOH results in lignin flaking off from rice straw, so that a relatively smoother surface is obtained (Fig. 1(b)). When the obtained straw was sequentially treated with 2% NaOH, a larger portion of lignin began to shed off from its surface due to cleavage of ester linkages between the lignin and the hemicellulosic portion of straw (Fig. 1(c)). When the concentration of NaOH reached 4%, the removal of lignin was associated with opening of the intact fibrous bundle, indicating the swelling of cellulosic microfibrillar structure and weakening of the intermolecular bonds between hemicelluloses and cellulose (Fig. 1(d)).

Extraction of hemicellulosic xylan from rice straw

The alkaline extraction method was selected

for it is the most well-studied and efficient method for extracting the hemicellulosic fraction of biomass, due to its lower operation cost, comparatively milder reaction conditions and lower structural degradation of the obtained hemicelluloses.[5,27] Alkali causes swelling of cellulose, thus decreasing its crystallinity, breaks the intermolecular hydrogen bonds between cellulose and hemicelluloses, and cleaves ester linkages between lignin and hemicelluloses; thereby, causing dissolution of hemicelluloses, lignin and silica into the solution. Silica, responsible for the recalcitrance of straw, is also removed *via* the alkaline extraction method, as insoluble silica particles are converted to soluble sodium silicate by decreasing the pH to less than 9 using 6M HCl.[28]

$$SiO_2 + 2NaOH \rightarrow Na_2SiO_3 + H_2O \qquad (2)$$
$$Na_2SiO_3 + 2HCl \rightarrow SiO_2 + 2NaCl + H_2O \qquad (3)$$
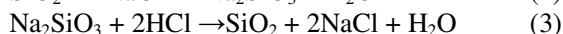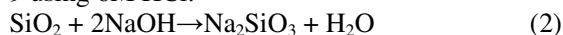
As a result, 194 mg of xylan was produced per g rice straw through the alkaline extraction and ethanol precipitation method, so that the recovery of about 80.83% was attained.

Structural characterization and thermal analysis of extracted xylan

Spectral analysis of hemicelluloses, extracted from rice straw, was carried out for determining the predominant xylan type. The spectral analysis of extracted xylan is shown in Figure 2 (a, b, c) and the corresponding data are summarized in Table 1. The assignment of bands in the FT-IR spectrum was supported by Xie *et al.*,[29] Carvalho[30] and Sun *et al.*[31] The observed chemical shift values for ^{1}H-NMR and ^{13}C-NMR analyses were in accordance with the values reported by Wei *et al.*,[32] Palaniappan *et al.*[33] and Konduri and Fatehi.[34] Therefore, the structure can be defined as *L*-arabino (4-*O*-methylglucurono) xylan, as shown in Figure 3.
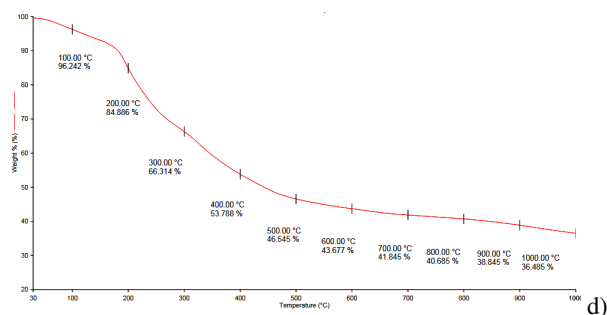

a)

b)

c)
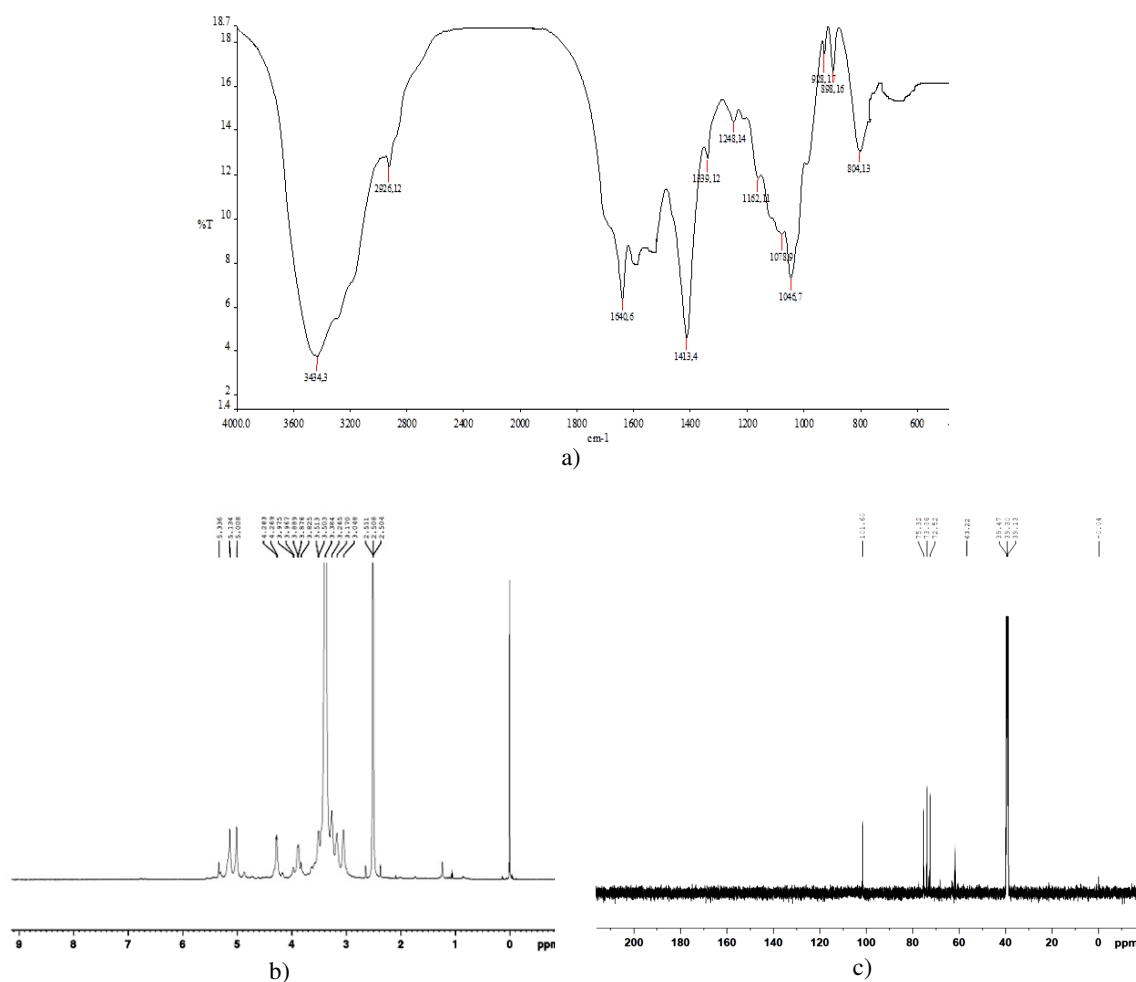
Figure 2: (a) FT-IR, (b) ^{1}H-NMR, (c) ^{13}C-NMR spectra and (d) TGA curve of xylan

Table 1
Spectral analysis of hemicellulosic xylan

| FT-IR	
Wavenumber ($\bar{\upsilon}$, cm^{-1})	Assignment
3434	O-H stretching of hydroxyl group
2926	C-H symmetric stretching of methylene group
1640	O-H bending due to absorbed water molecules
1560	COO symmetric stretching of carboxylate group of 4-*O*-methylglucuronic acid side chain
1413 and 1339	CH$_2$ bending in methylene group in xylopyranose ring
1248	O-H bending of hydroxyl group
1210	C-C bending in xylan skeleton
1162 and 928	C-O stretching in *α*-(1,3) linked glycosidic bond (characteristic of arabinofuranose)
1080	C-O stretching of C-OH group of xylose subunit
1046 and 898	C-O stretching in *β*-(1,4) linked glycosidic bond (characteristic of xylan)
^{1}H-NMR	
Chemical shift (δ, ppm)	Assignment
3.1-3.9	Axial protons of xylose subunit in xylan backbone
4.0-4.3	Equatorial protons of xylose subunit in xylan backbone
4.2	*β*-glycosidic linkage
4.8	Anomeric proton of *β-D*-xylopyranose
5.1	Anomeric proton of *α-L*-arabinofuranose
5.3	Anomeric proton of 4-*O*-methylglucuronic acid
2.9-3.2	Methoxy group of 4-*O*-methylglucuronic acid
^{13}C-NMR	
Chemical shift (δ, ppm)	Assignment
72-76 (C2-C4), 63 (C5)	Ring carbons of *β-D*-xylopyranose
101.6	Anomeric carbon of *β-D*-xylopyranose
106.1	Anomeric carbon of *α-L*-arabinofuranose
59.0	Methoxyl group of 4-*O*-methylglucuronic acid
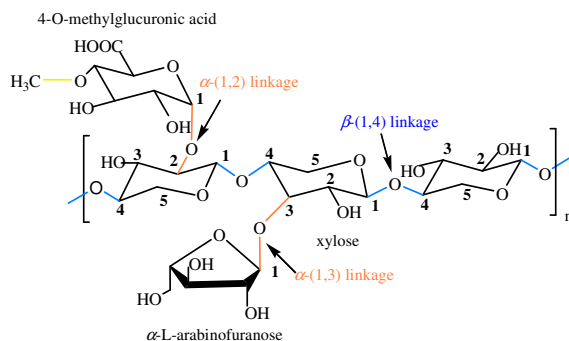
Figure 3: Predicted structure of hemicellulosic xylan

Table 2
WPG (%) and solubility analysis of chemically modified xylans

Polymer	WPG (%)	Solubility				
		Distilled water	DMSO	Chloroform	Toluene	Ethanol
Xylan	-	Partially soluble	Soluble	Insoluble	Insoluble	Insoluble
Acetylated xylan	18.2 ± 0.05	Insoluble	Soluble	Soluble	Insoluble	Insoluble
Benzylated xylan	34.4 ± 0.6	Insoluble	Soluble	Insoluble	Insoluble	Insoluble
Carboxymethylated xylan	26.6 ± 0.12	Soluble	Partially soluble	Insoluble	Insoluble	Insoluble

The thermal properties of xylan were studied using the thermogravimetric analysis in the temperature range of 0-1000 °C (Fig. 2d). The percent weight loss of xylan with temperature could be divided into three stages, *i.e.* moisture loss (up to 185 °C), pyrolysis (186-400 °C) and charring (above 400 °C). Further, small weight loss stages from 500 to 1000 °C can be attributed to the decomposition of smaller residues, as no appreciable percent weight loss was observed.[35]

Chemical modifications of xylan
Physical characterization
The modifications of xylan resulted in replacement of one or both hydroxyl groups of the xylose subunit with acetyl, benzyl or carboxymethyl groups. Therefore, WPG (%) was calculated by comparing the weight of xylan before and after modifications. The modified xylans were dried in the oven at 100 °C for about 2 hours and then WPG (%) was calculated. The results of WPG (%) of xylan after modifications, *i.e.* acetylation, benzylation and carboxymethylation, are summarized in Table 2. Akkus *et al.*[36] calculated WPG (%) for acetylated xylan synthesized at different reaction times, temperatures and molar ratio of reactants ranging from 0.2 ± 0.1 to 23.5 ± 0.7.

Solubility analysis (Table 2) suggested that the acetylation and benzylation reactions increased the hydrophobicity of xylan, while the carboxymethylation reaction resulted in a hydrophilic polymer. Carvalho *et al.*[37] observed the solubility of acetylated xylan (with different

Up to 100 °C, a weight loss of about 3.758% occurred. As the temperature was increased, the weight loss (%) was found to increase as well, so that up to the fast pyrolysis stage, 46.22% of mass had been lost. From 400 to 500 °C, a mass loss of about 7.2% was observed. Further increase in temperature caused only a small weight percent loss of hemicellulosic xylan, thus, constant weight percent of 36.42% was attained up to 1000 °C.

degrees of substitution) in DMSO (at 80 °C) and chloroform, whereas the unacetylated xylan was soluble in water and DMSO. Mattos *et al.*[38] reported the solubility of carboxymethylated xylan in water, while Junli *et al.*[39] found the benzylated product to be insoluble in water, ethanol and acetone and soluble in DMSO.

### *Reaction mechanisms and structural characterization*
Acetylation
The hydroxyl group of xylan reacted with acetic anhydride in the presence of pyridine to form xylan acetate (Scheme 1). This reaction resulted in hydrophobic xylan, where one or more hydroxyl groups (-OH) of the xylan subunit were replaced by the –OCOCH$_3$ group.

The mechanism involves the nucleophilic attack by the alcohol group of xylan on the carbonyl carbon of acetic anhydride, followed by deprotonation by pyridine and finally the removal of the leaving group, *i.e.* acetate ion, to form acetylated xylan (Scheme 2).
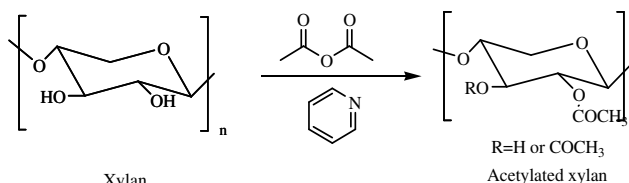
Scheme 1: Acetylation of xylan
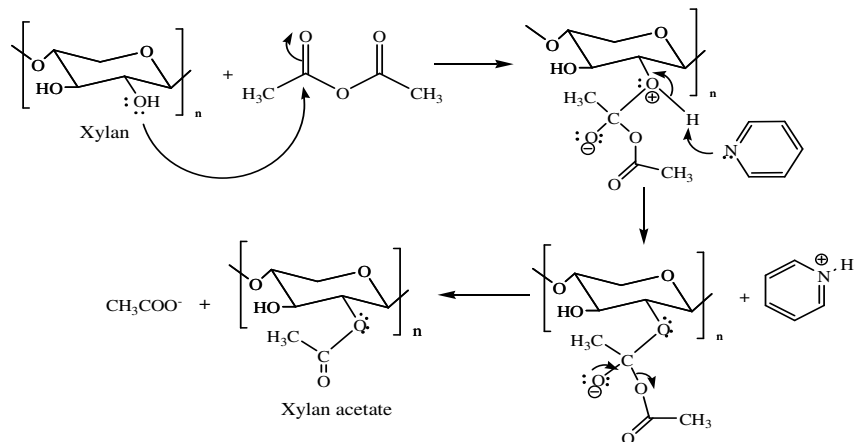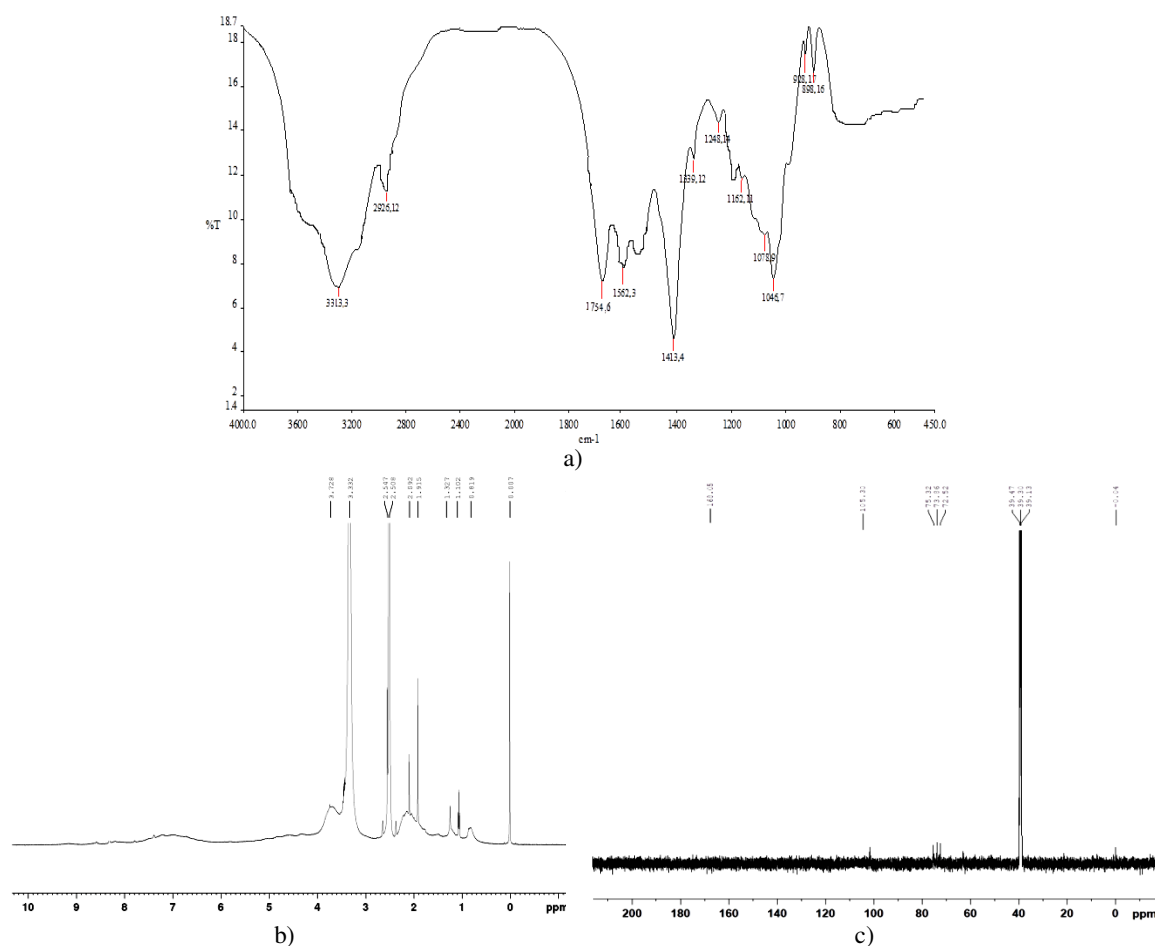
Scheme 2: Mechanism for acetylation of xylan

a)

b)   c)

Figure 4: (a) FT-IR, (b) ^{1}H-NMR and (c) ^{13}C-NMR spectra of acetylated xylan

The FT-IR spectrum of acetylated xylan is shown in Figure 4 (a). The replacement of hydroxyl groups with acetyl groups resulted in a decrease in intensity of the O-H stretching band at 3313 cm^{-1} of xylan and an increase in intensity of 2 additional bands associated with the ester bond in acetylated xylan, *i.e.* C=O stretching band at 1752 cm^{-1} and C-O stretching band at 1210 cm^{-1}.[40] An increased intensity of the band at 2926 cm^{-1} indicated C-H stretching in methyl group of acetylated xylan. The rest of the bands of native hemicelluloses were common to acetylated xylan.

Also, the absence of a band in the region of 1840-1760 cm^{-1} and at 1700 cm^{-1} indicated that the product was free of unreacted acetic anhydride, as well as of the acetic acid by-product.[41]

The ^1H NMR spectrum of acetylated xylan is shown in Figure 4 (b). One or both of the C-2(OH) and C-3(OH) groups of the xylopyranose unit upon acetylation resulted in the signal around 0.8-2 ppm.[40]

The ^{13}C NMR spectrum of acetylated xylan is shown in Figure 4 (c). Acetylated xylan was characterized by the presence of a signal at 169 ppm, corresponding to the carbonyl group of the ester linkage.[36]

Benzylation

Benzylation was carried out by the reaction of xylan with benzyl chloride in ethanol/water, in the presence of NaOH (Scheme 3). It this reaction, one or more hydroxyl groups (-OH) of the xylan subunit were replaced by -$OCH_2C_6H_5$ and hydrophobic polymer was obtained.

The benzylation of xylan is a typical Williamson ether reaction (nucleophilic substitution), where the deprotonated alcohol of xylan subunit acts as a nucleophile and attacks the organic halide (benzyl chloride) *via* the SN_2 reaction to form ether. The deprotonation of the alcoholic group was achieved by carrying out the reaction in presence of a base, *i.e.* NaOH (Scheme 4).

The FT-IR spectrum of benzylated xylan is shown in Figure 5 (a). The benzylation of xylan introduced benzyl groups into the structure, which resulted in a decrease in intensity of the O-H stretching band at 3413 cm^{-1}. The appearance of bands at 3090 cm^{-1} and 1514 cm^{-1} indicated C-H stretching and C=C stretching in the benzene ring.[39] The bands observed at 800 and 643 cm^{-1} corresponded to C-H bending in the monosubstituted benzene ring. A band in the region of 1162-1078 cm^{-1} was due to C-O stretching corresponding to the O-CH_2 group of the carboxymethyl unit.

The ^1H NMR spectrum of benzylated xylan is shown in Figure 5 (b). Benzylated xylan showed signals between 7.2-7.4 ppm, corresponding to the ring protons of benzene, while two methylenoxy protons of the benzyl group showed peaks between 3.8-4.2 ppm.[39]

The ^{13}C NMR spectrum of benzylated xylan is shown in Figure 5 (c). The C-13 spectrum of benzylated xylan showed a signal at 150 ppm, corresponding to ring carbons of the benzene ring.[42]
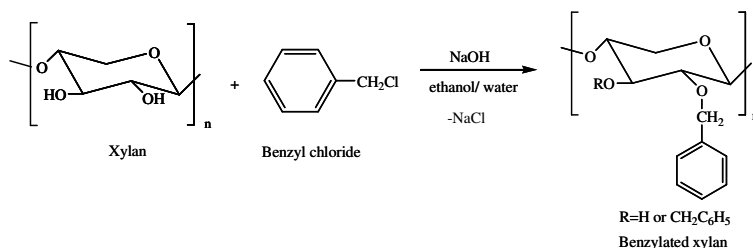
Scheme 3: Benzylation of xylan
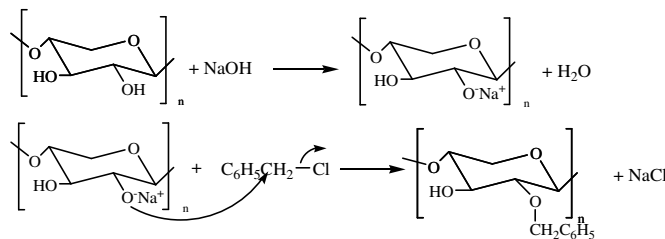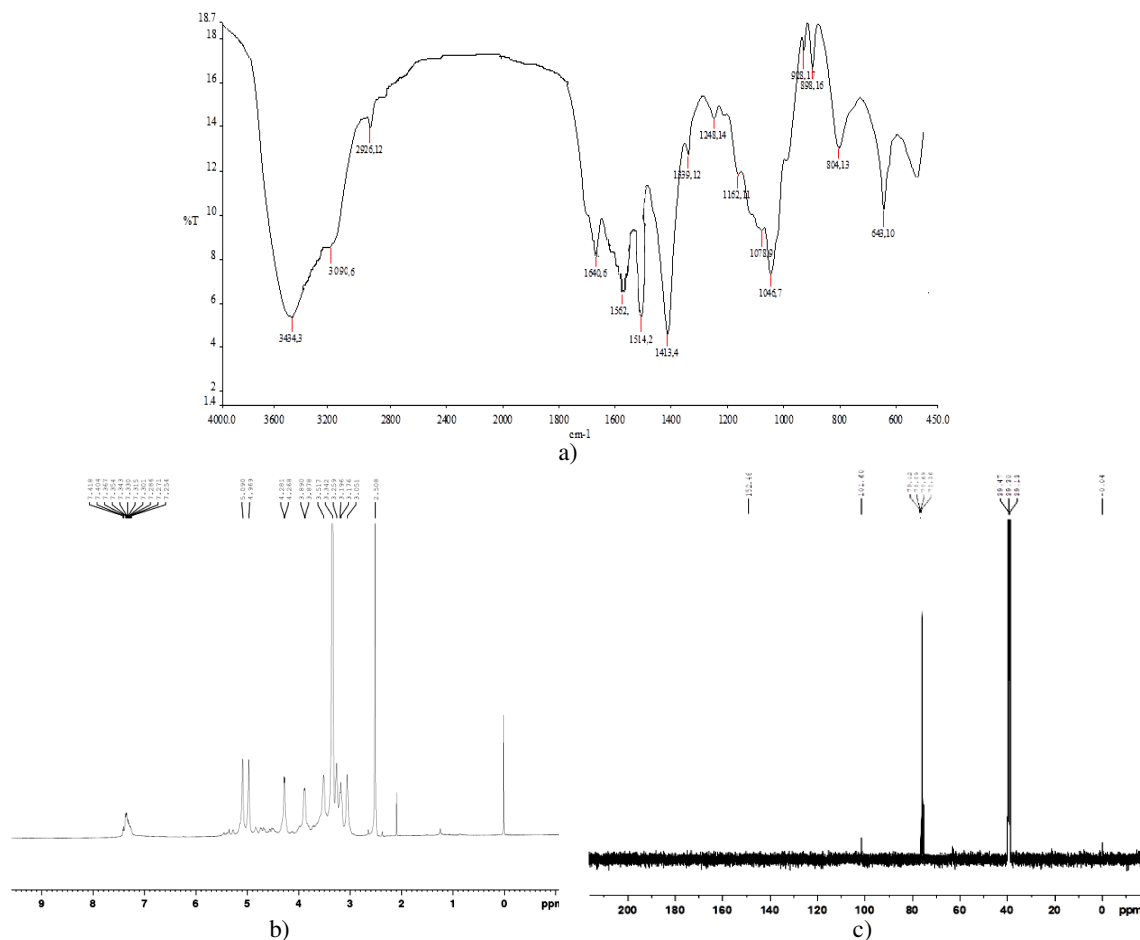

Scheme 4: Mechanism for benzylation of xylan

Figure 5: (a) FT-IR, (b) ^{1}H-NMR and (c) ^{13}C-NMR spectra of benzylated xylan

Carboxymethylation

Carboxymethylation of xylan was carried out in the presence of NaOH, by the reaction of xylan with sodium monochloroacetate in isopropanol (Scheme 5). In this reaction, one or both hydroxyl groups (-OH) of the xylan subunit was replaced by the -OCH$_2$COO$^-$ group and hydrophilic polymer was obtained. Carboxymethylation also follows the Williamson ether mechanism, where the deprotonated alcohol of the xylan subunit attacks sodium monochloroacetate *via* the SN$_2$ reaction.

The FT-IR spectrum of carboxymethylated xylan is shown in Figure 6 (a). Carboxymethylation of xylan resulted in the replacement of the hydroxyl group of xylan with the OCH$_2$COO$^-$ group. As a result, there was a decrease in intensity of the O-H stretching band (3400-3200 cm^{-1}) and an increase in intensity of the band at 1562 cm^{-1} due to carboxylate group stretching.[43] A band in the region of 1162-1078

cm^{-1}, due to C-O stretching corresponding to the O-CH$_2$ group of the carboxymethyl unit, was observed.

The ^{1}H NMR spectrum of carboxymethylated xylan is shown in Figure 6 (b). Sharp peaks of methylene protons of the carboxymethyl group were observed at 4.00 and 4.06.[34]

The ^{13}C NMR spectrum of carboxymethylated xylan is shown in Figure 6 (c). Carboxymethyl xylan showed a signal at 186 ppm, corresponding to the carboxylate group.

Controlled acid hydrolysis of xylan to XOS

Autohydrolysis of alkali extracted xylan, in the presence of dilute acid, results in the production of the XOS rich hydrolysate (Fig. 7).

The TLC plate showing spots of standard xylose and the four different hydrolysates treatments obtained with different concentrations of sulphuric acid, *i.e.* 0.1M, 0.25M, 0.5M and 1.0M, is shown in Figure 8. T1 was considered

302

the best treatment since XOS with up to 6 degree of polymerization were visible on the plate. As the acid concentration was increased, degradation of higher XOS to xylose was observed.
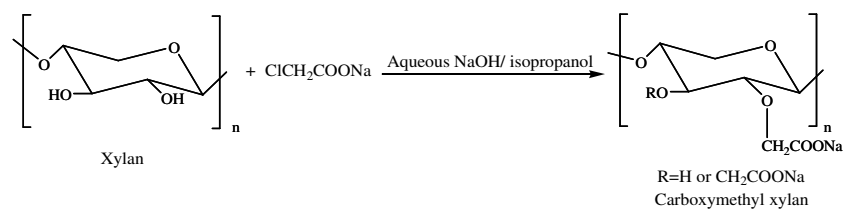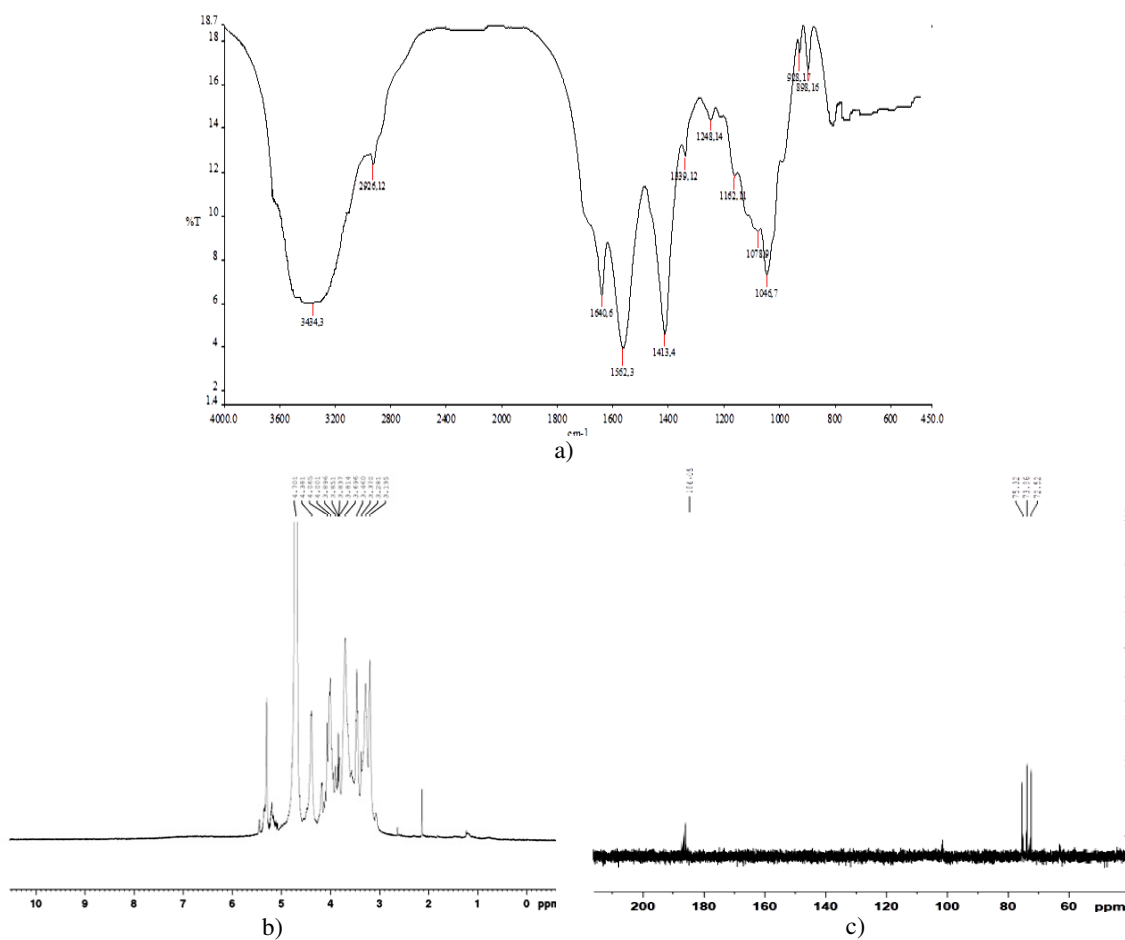

Scheme 5: Carboxymethylation of xylan

Figure 6: (a) FT-IR, (b) ^{1}H-NMR and (c) ^{13}C-NMR spectra of carboxymethylated xylan
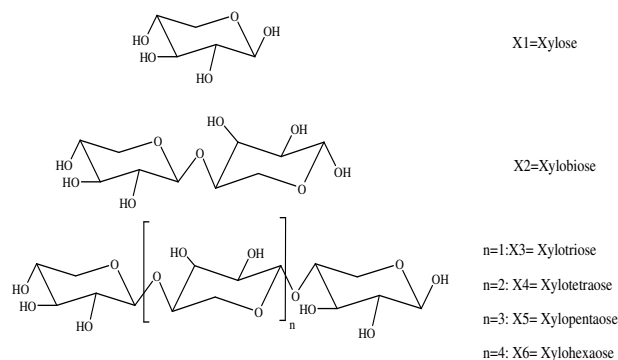
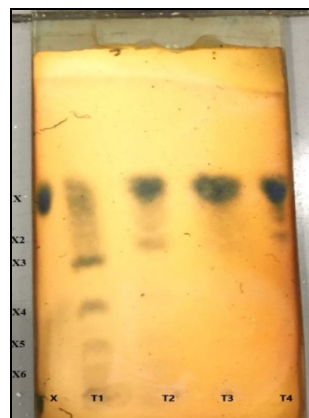Figure 7: Xylooligosaccharides obtained by autohydrolysis of xylan

Figure 8: TLC plate with spots for standard xylose (X), and the four treatments T1-0.1M, T2-0.25M, T3-0.5M and T4-1.0M sulphuric acid
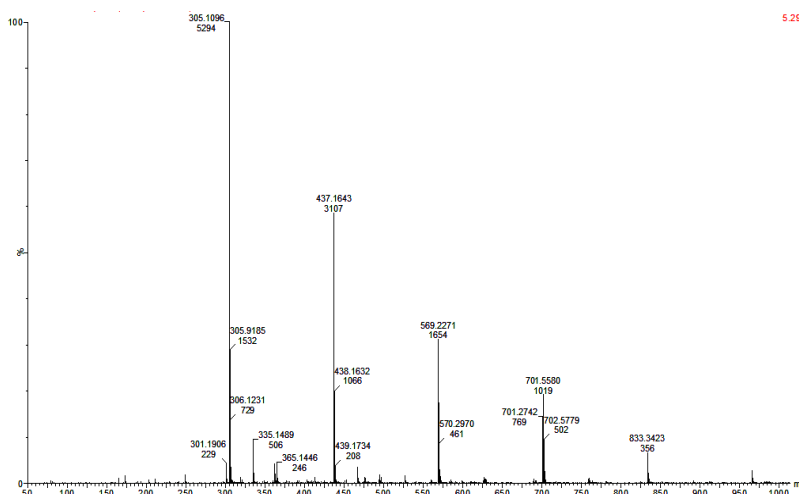

Figure 9: Mass spectrum of xylan autohydrolysate

T1 resulted in dissolution of 35% of the 100 mg of xylan in the autohydrolysate, indicating that 35% xylan was degraded into oligomers (up to 6 degree of polymerization), monomers or by-products, so that the concentration of autohydrolysate was 7 mg/mL.

The MS-ESI spectrum of the T1 hydrolysate showed the presence of neutral xylooligosaccharides with a degree of polymerization up to 7 (Fig. 9). The base peak or the most abundant peak observed at a *m/z* value of 305 represented xyobiose. Other peaks of decreasing abundance at *m/z* values of 437, 569, 701 and 833 corresponded to xylotriose, xylotetraose, xylopentaose and xylohexaose, respectively. The parent ion peak at the *m/z* value of 965, with the lowest abundance, showed the presence of xyloheptaose.[44]

Evaluation of total antioxidant capacity of xylan, modified xylans and XOS

The phosphomolybdate assay, for the determination of total antioxidant capacity, is based on the single electron transfer (SET) mechanism that measures the capacity of an antioxidant to reduce an oxidant, which changes colour when reduced.[45] It is a simple, effective and inexpensive quantitative method, where the antioxidant capacity of a compound is measured in terms of reduction of molybdate ions, *i.e.* Mo (VI) to Mo (V), which forms the bluish green phosphomolybdenum complex, showing maximum absorption at 695 nm.

The calibration curve of ascorbic acid (y = 0.0054x + 0.05; R² = 0.9983) was prepared by plotting different concentrations of ascorbic acid against the total antioxidant capacity for ascorbic acid or absorbance measured at 695 nm.

The reduction ability or antioxidant activity of the sample was expressed in terms of ascorbic acid equivalents (AAE), *i.e.* µg of ascorbic acid/mg material used. Figure 10 shows a graphical representation of AAE values calculated (from the calibration curve) corresponding to different concentrations of xylan, modified xylans and XOS. The CD (5%) values for xylan, acetylated xylan, benzylated xylan, carboxymethylated xylan and XOS were calculated to be 0.498, 0.121, 0.346, 0.435, and 0.186, respectively.
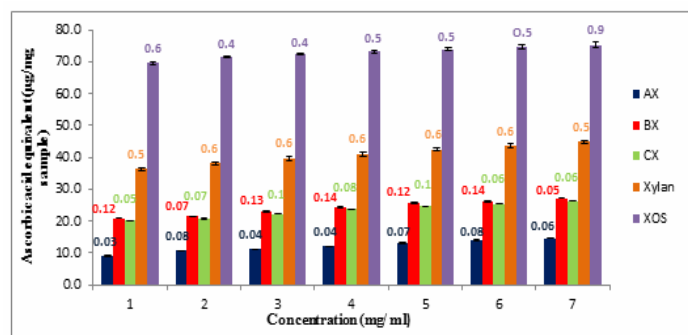

Figure 10: AAE value at different concentrations of materials (AX – acetylated xylan; BX – benzylated xylan; CX – carboxymethylated xylan)

All the materials were found to exhibit concentration-dependent antioxidant activity, as XOS showed the highest AAE values, followed by xylan and modified xylans. The higher value of the AAE value for XOS (in xylan hydrolysate), in comparison with xylan, could simply be attributed to its lower degree of polymerization and consequently greater reducing sugar content. Bian *et al.*[46] also reported concentration-dependent antioxidant activity for XOS extracted from sugarcane bagasse hemicelluloses *via* enzymatic hydrolysis. Zhou *et al.*[47] observed XOS concentration playing an important role in absorbance by the phosphomolybdenum complex.

This neutral XOS–rich hydrolysate with good antioxidant potential could be incorporated into food products (as natural antioxidant) after purification; therefore, it can serve global nutraceuticals, while also providing a solution for the waste disposal issue associated with straw.

The AAE value of xylan at 7 mg/mL concentration was 44.9, *i.e.* 44.9 µg of ascorbic acid/mg xylan. The results were in corroboration with those of Melo-Silveira *et al.*,[26] who reported the total antioxidant capacity of 1 g xylan being equivalent to 48.5 mg ascorbic acid. Modified xylans (ester and ether) exhibited lower AAE values than xylan, because of the presence of electron withdrawing groups. Although benzylated and carboxymethylated xylans exhibited comparable total antioxidant capacity, acetylated xylan had significantly lower value of AAE. This may be explained by a decrease in electron density on oxygen because of the presence of the electron withdrawing acetyl group in conjugation with it.

Acetylated and benzylated xylan based hydrophobic films have already been reported with improved thermal stability, mechanical strength, barrier properties, *i.e.* lowered water vapours and gas permeability than xylan.[15,48,49] Alekhina *et al.*[50] reported carboxymetylated birch wood xylan based-biodegradable films, with significant oxygen barrier properties, thermal stability and mechanical strength, to be used as coating material for fruits and vegetables. Although the modified xylans have lower antioxidant capacity than xylan, the introduction of benzyl, carboxymethyl and acetyl moieties may make them potential candidates for use as sustainable food packaging materials with inherent antioxidant potential.

CONCLUSION

Alkali extracted rice straw hemicelluloses, *i.e.* *L*-arabino (4-*O*-methylglucurono) xylan, exhibited total antioxidant activity corresponding to 44.9 µg of ascorbic acid equivalent/mg of xylan. Chemically modified xylans produced *via* acetylation, benzylation and carboxymethylation reactions had lower AAE values than xylan, but the inherent antioxidant potential of functionalized xylans might offer better film formation. The functionalized xylans may be crosslinked with polymers, such as polyvinyl alcohol, polyethylene glycol, gelatin *etc.* to form

biodegradable and biocompatible film materials, holding immense potential for the food packaging and storage industries. Neutral XOS with a degree of polymerization of up to 7, produced by autohydrolysis of xylan using 0.1M sulphuric acid, also have a great potential to be included as a functional ingredient into nutraceuticals.

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