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Xylooligosaccharides production from tobacco stalk xylan using edible acid

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In the present study, a process was developed to hydrolyse xylan from tobacco stalks into xylooligosaccharides (XOS) by applying tartaric acid, an edible acid. The tobacco stalks contained approximately hemicelluloses (16.99%), cellulose (50.8%) and lignin

(15.6%). Use of 8% KOH or NaOH under overnight incubation resulted in almost complete recovery of xylan from the tobacco stalks. Application of 1 M tartaric acid at 90°C hydrolysed xylan into XOS and the highest (0.357 mg/ml) yield of XOS was recorded after 120 minutes. Hence, prebiotic XOS could be produced from the tobacco stalk xylan by applying tartaric acid.

Keywords: Edible acid, tobacco stalk, xylan, xylooligosaccharides.

LIGNOCELLULOSIC wastes are generated from different crop production systems. These are considered as an abundant carbon-neutral renewable reservoir of the earth that can be utilized for the production of multiple value-added products for various industrial applications such as food, feed, pharmaceutical, polymer, fuel, etc. Currently, there is global attention for utilizing agricultural wastes as raw materials for producing numerous valuable products, including prebiotics. According to an estimate¹, the cost of lignocellulosic biomass was approximately US\$ 23 per tonne compared to the crude oil price of US\$ 50. Despite that, efficient bio-refining processes for translating agricultural waste into value-added products are lacking². Amongst the list of agricultural wastes, tobacco (*Nicotiana tabacum*) stalks occupy significant niche because they are grown in almost 4.3 million hectares of agricultural land distributed among 124 countries. After harvesting of leaves, the stalks are generally incorporated in the soil or burned in field as they carry meagre economic significance³. The tobacco stalk is primarily composed of cellulose and lignin in addition to hemicelluloses. Therefore, partitioning of biomolecules from the tobacco stalks is noteworthy in the direction of environmental protection as well as effective utilization of agricultural wastes. Xylan represents a significant fraction of hemicelluloses present in the tobacco stalks. Therefore, it can be used for the production of numerous finer molecules, including prebiotic xylooligosaccharides (XOS).

In current days, prebiotics attracted the attention of global researchers for health and well being as its principle of action relies on 'prevention is better than cure'. Out of the several classes of prebiotics, XOS is presumed to be an emerging one which possesses several beneficial roles. It selectively stimulates the growth of beneficial gut bacteria, produces short-chain volatile fatty acids, boosts immunity, enhances mineral absorption and lowers blood cholesterol, colonic pH and pro-carcinogenic enzymes in hind gut⁴. Additionally, XOS also alleviates the symptoms of diabetes, cancer, stress, etc.⁴⁻⁶. The production of XOS is environmentally caring because it generates value-added products from the abundantly available agricultural wastes unsuitable for human consumption. Xylan is the precursor for XOS. The main backbone of xylan is formed by xylose monomers, linked by β -1,4-xylosidic bonds and often replaced with arabinose or acetyl or methyl groups⁷.

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In view of the growing demands of prebiotics coupled with disposal difficulty of the over produced agricultural wastes, the XOS production is being attempted from the several agricultural wastes namely natural grass², almond shell⁸, oil palm fronds⁹, garlic straw¹⁰ and vine shoots¹¹. It seems limited efforts are being made on the exploration of tobacco stalk as a raw material despite its abundance in more than 124 countries. Previously, attempts have been made to extract xylan from tobacco stalks for the production of XOS applying inorganic acid or xylanase enzyme^{3,12}. Although enzymatic production of XOS is highly safe and consumer-friendly, the application of inorganic acid (sulphuric acid) is a debatable issue³. The current study focused on maximizing the yield of xylan from tobacco stalks. The study also encompasses the conversion of xylan into XOS using edible acid.

Following removal of leaves, tobacco stalks were collected from the farmer's field (Hunsur, Mysore district, Karnataka), air-dried and chopped. The samples were dried in a hot air oven ($60 \pm 2^\circ\text{C}$) and grounded uniformly (particle size <1 mm). The organic matter, total ash and crude protein of tobacco stalks were estimated¹³. Cellulose, hemicelluloses and lignin contents of the tobacco stalks were also analysed¹⁴. All the chemical analysis of tobacco stalks were performed in triplicates.

Xylan from the tobacco stalks (2 g) was extracted with the application of various concentrations (2%, 4% and 8%) of NaOH and KOH as follows: incubation for overnight (16 h) in room temperature (25°C) or steam (120°C and pressure 15 lbs) treatment for 45 min. The ratio of tobacco stalks to alkali solution kept at 1 : 10 (W/V). Nylon bag (pore size <100 μm) was used to recover the alkali solubilized xylan from the tobacco stalks. The pH of the xylan solution was brought down to 5.0 through the addition of glacial acetic acid and the volume was measured. Thereafter, the xylan was precipitated following the addition of three volumes of precooled rectified spirit. Finally, it was recovered through centrifugation at a speed of 8000 rpm, for 10 min at 4°C . The pellets obtained were subjected to drying in a hot air oven, till constant weight. The weight of the dried xylan was recorded to ascertain its yield. Further, it was grounded and stored in an airtight container (room temperature) for other analysis and XOS production.

The dried and powdered xylan was first subjected to analysis for reducing sugar contents using xylose as standard¹⁵. Fourier transform infrared spectrophotometer (FT-IR) analysis of the xylan (5 mg) was performed in the spectral range of $4000\text{--}400$ cm^{-1} , equipped with KBr beam splitter and DTGS detector^{2,16}.

The xylan (200 mg) of tobacco stalks was subjected to hydrolysis applying 10 ml of 1 M organic acids at different temperatures (60°C and 90°C) in a shaking water bath. The organic acids used in this study were acetic acid, citric acid and tartaric acid. The samples from the acid hydrolysate were taken out at 15, 30, 60, 120 and

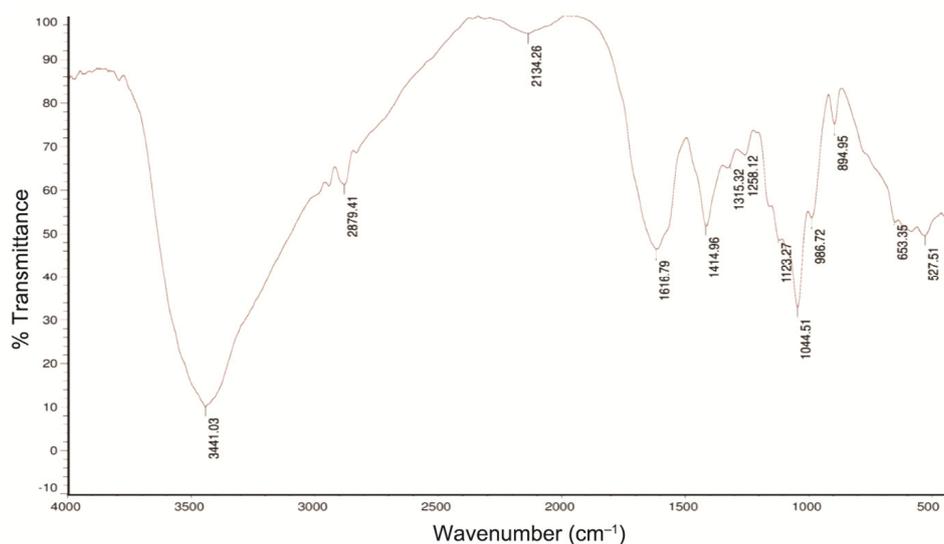
180 min. As XOS is also categorized as reducing sugars, the hydrolysis products of xylan were first analysed for the presence of reducing sugars¹⁵. Further, samples were analysed in HPLC (fitted with refractive index detector, Agilent, USA) to ascertain the components of XOS². In brief, the hydrolysis products were subjected to membrane filtration (0.2 μm) before HPLC analysis. Aliquots of 20 μl was injected in HPLC through a manual injector. The components of XOS were separated in HPLC after passing through carbohydrate analysis column (Agilent) applying the mobile phase containing acetonitrile and water (63 : 37) with the flow rate of 1 ml/min. The concentration of the individual component of the XOS was calculated based on the peak areas of individual standard, namely xylobiose and xylotriose (Wako chemicals, Japan). The total concentration of XOS in each hydrolysate was calculated after summing the concentration of each XOS component.

Compositional analysis of tobacco stalks has been reported to see its potentiality as feedstock for the generation of products with higher values applicable to the second-generation bio-refinery^{17,18}. As XOS production relies on xylan, we performed the fibre analysis of tobacco stalks to underpin its appropriateness as a starting material. It indicated the presence of organic matter $93.12 \pm 0.81\%$, total ash $6.87 \pm 0.81\%$, neutral detergent fibre $83.43 \pm 0.6\%$, acid detergent fibre $66.44 \pm 0.39\%$, and crude protein $6.31 \pm 0.09\%$. Essentially, the primary constituent of the tobacco stalk was cellulose ($50.81 \pm 0.42\%$), trailed by hemicellulose ($16.99 \pm 0.65\%$) and acid detergent lignin ($15.63 \pm 0.05\%$). According to earlier reports³, tobacco stalks contained cellulose $44.3 \pm 2.9\%$, lignin $22.7 \pm 1.0\%$, xylan $21.8 \pm 1.2\%$ and ash $6.65 \pm 0.57\%$. In fact, the proportion of chemical components (cellulose, xylan and lignin) in agricultural waste varies widely depending upon species, harvesting time and maturity¹⁹. Tobacco stalks used in the present study contained a lower (15.63% versus 22.7%) level of acid detergent lignin as compared to previous reports. In the present study, lower content of lignin in tobacco stalks was advantageous as it permeated higher recovery of xylan.

During last few decades, xylan has received noticeable attention from scientists in academia and the industry, because it is the starting material for the formation of numerous high valued products including bio-based materials (xylan composite, hydrogels), chemicals (ethanol, lactic acid, furfural, xylitol) and functional foods (XOS comprising of xylobiose, xylotriose, xylo-tetrose and xylopentose)²⁰. NaOH, KOH, $\text{Ca}(\text{OH})_2$ and NH_4OH are commonly used for the extraction of xylan²¹. Fractionation of xylan from its structural complex encompasses a two step process: (i) disintegration of ester bonds and solubilization into alkali medium, (ii) precipitation with solvent²². The yield of xylan from the agricultural waste depends upon hydrogen bonding, ester and ether linkages,

Table 1. Effect of alkali and extraction condition on yield of xylan from tobacco stalks

Extraction conditions	Concentration of alkali (%)		
	2	4	8
KOH followed by overnight incubation	8.24 ± 0.17	12.54 ± 0.23	18 ± 0.86
KOH followed by steam application	7.45 ± 0.12	11.16 ± 0.59	12.2 ± 1.14
NaOH followed by overnight incubation	9.41 ± 0.43	14.77 ± 0.40	19.54 ± 0.26
NaOH followed by steam application	6.88 ± 0.07	12.78 ± 0.28	18.65 ± 0.40

**Figure 1.** FT-IR analysis of xylan extracted from tobacco stalks.

concentration of alkali and extraction conditions^{23,24}. The current endeavour was taken to fractionate the xylan from the tobacco stalks with different levels of NaOH and KOH following incubation for overnight and steam treatment. Table 1 gives the actual yield of xylan from the tobacco stalks. Increasing the concentration of alkali resulted in higher recovery of xylan regardless of the extraction settings. In the present study, both 8% KOH (incubation for overnight) and 8% NaOH (incubation for overnight and steam treatment) enabled nearly complete retrieval of xylan. The actual yield of xylan from agricultural waste varies widely; ranging from 6.2 (Lespedeza stalk) to 52.7 (corn bran)^{25,26}.

The agricultural activities on the earth generate about 5 billion tonnes of biomass residues each year²⁰ and a significant portion of it is thrown at the dumping grounds for natural decomposition; else burned. Nevertheless, these wastes could be considered as ideal raw materials for partitioning of biomolecules followed by their translation into high valued biological products²⁷. For the suitable usages of agricultural wastes, an appropriate process for the extraction of xylan is imminent. At this point, fractionation of xylan through the application of alkali is considered an advantageous process because it is cost-effective and involves less intense operating conditions^{4,23}. Hence, we endeavoured to obtain the xylan from

tobacco stalks applying conventional alkali, i.e. NaOH and KOH. The steam application⁶ is reported to enhance xylan yield from green coconut husks, pigeon pea stalks, natural grass. However, we recorded higher recovery of xylan at all levels of alkali subjected to extraction performed through room temperature incubation. It indicates that the source of raw material is also one of the significant factors for determining the xylan yield. Further, the xylan extraction protocol, applying 8% KOH and incubation for overnight, was followed for bulk production. The presence of reducing sugars in the extracted xylan was negligible.

FT-IR is a spectroscopic analysis for identification of molecule based on the absorption pattern of the infra-red light²⁸. It is considered as an effective means for understanding the physicochemical and conformational characteristics of a molecule as it is fast, delicate and inexpensive^{24,29}. It represents the presence or absence of functional groups conforming to the signature molecule and thereby evaluates the purity of molecule relying on absorption band outlines³⁰. The FT-IR analysis of extracted xylan from tobacco stalks is presented in Figure 1.

The wide and deep spectral band recorded at 3441 cm⁻¹ is ascribed to extending vibrations of OH groups that exist in the alkali extracted xylan of tobacco stalks¹⁷. The small vibration represented at 2879 cm⁻¹ originated

owing to the presence of aliphatic C–H group^{31,32}. The disappearance of vibration bands near the spectral range of 1734 cm⁻¹ substantiates the absence of acetyl group in the xylan of tobacco stalks. The small absorption bands at the spectral wavelength of 1258 cm⁻¹ embody C–O stretching of xylan³³. The absorption band noted at wave number 1616 cm⁻¹ seems to have appeared because of the presence of absorbed water by the xylan²⁹. The spectral bands appearing at 1414 and 1315 cm⁻¹ represent C–H deformation of carbohydrate and syringyl alcohol (lignin) respectively¹⁷. Even though lignin is not precipitated by the ethanol²², the appearance of low dip band at 1315 cm⁻¹ indicated the presence of lignin in xylan. The vibration noticed at around 1000 cm⁻¹ might indicate the presence of C–O of alcohol³⁴. The β -xylosidic linkages between the xylose units of xylan molecule are evidenced by the presence³⁵ of a band at spectral range of 894 cm⁻¹. The additional vibration bands observed at 653, 527 and 418 cm⁻¹ might represent the presence of C–C–H or C–O–C stretching or bending²⁹.

Out of the several chemical processes of XOS production, application of edible acid is preferred over inorganic acids because it does not generate toxic compounds and no sophisticated high-end instrument is required. Additionally, it addresses the consumer mindset. Hence, in the present study, the xylan was subjected to hydrolysis by 1 M edible acids, namely, tartaric acid, citric acid and acetic acid at a temperature of 60°C and 90°C. Surprisingly, none of the above acid hydrolysed xylan into XOS except tartaric acid, at 90°C. The concentration of reducing sugars in the acidic hydrolysate is presented in Table 2. It reflected time dependent increase in the concentration of reducing sugars and reached up to 1.06 mg/ml

Table 2. Effect of tartaric acid on xylooligosaccharides production from xylan of tobacco stalks

Duration of hydrolysis (min)	Concentration of reducing sugars (mg/ml)	Total XOS (mg/ml)
15	0.17 ± 0.04	0.16 ± 0.02
30	0.22 ± 0.01	0.18 ± 0.05
60	0.37 ± 0.01	0.23 ± 0.04
120	0.64 ± 0.02	0.37 ± 0.02
180	1.06 ± 0.01	0.37 ± 0.04

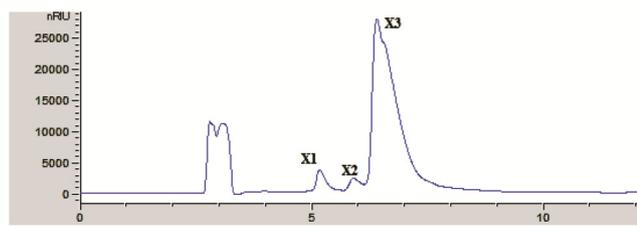


Figure 2. HPLC analysis of xylooligosaccharides generated from xylan of tobacco stalks. X1, Xylose; X2, Xylobiose; X3, Xylootriose.

after 180 min of hydrolysis. The presence of reducing sugars in the hydrolysate indicates xylan hydrolysis into short-chain oligosaccharides under the influence of 1 M tartaric acid. According to earlier reports, the reducing sugars concentration increases with time³. As the reducing sugars included both xylose and XOS, the acidic hydrolysate was further analysed by HPLC to precisely quantify the XOS.

The total XOS concentration in the hydrolysate ranged from 0.16 to 0.37 mg/ml in the time intervals of 15 to 180 min (Table 2). The total XOS concentration increased with the duration of hydrolysis till 120 min, following which it remained constant. The hydrolysate beyond 120 min reflected higher reducing sugar concentration without changing the concentration of total XOS. It indicated that further hydrolysis of already produced XOS leads to a higher concentration of reducing sugars. The xylan was hydrolysed by tartaric acid to short-chain oligosaccharides, namely xylobiose and xylootriose in addition to monomer xylose (Figure 2). Several attempts have been made to produce XOS by applying various inorganic acids^{3,36}. A report³⁷ suggests that higher yield of XOS from commercial xylan is possible with 20% acetic acid at 140°C for 20 min with concomitant generation of furfural and xylose. From the present study, it was also clear that reaction time is also a significant factor that influences the XOS concentration. Generally, enzymatic process of XOS production is preferred over acidic hydrolysis because the former is consumer-friendly. However, the application of edible acid (tartaric acid) is equally potential to address the issues of consumer concerns.

The purpose of the present study was to produce XOS by applying edible acid from the xylan of tobacco stalk, an abundantly available agricultural waste. The compositional analysis of tobacco stalks revealed moderate contents (approximately 17%) of xylan; which was almost completely recoverable through 8% KOH under overnight incubation or 8% NaOH under overnight incubation followed by steam treatment. FT-IR analysis of xylan indicated its quality. Among the three edible acids tested, only tartaric acid is the one that hydrolyses xylan into XOS at a temperature of 90°C and highest yield (0.37 mg/ml) was recorded after 120 min of hydrolysis.

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