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Value addition of orange fruit wastes in the enzymatic production of xylooligosaccharides

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Xylooligosaccharides (XOS) are sugar oligomers made up of xylose units with the chain length of 2 to 10 and are considered non-digestible food ingredients. It is mainly produced from xylan hydrolysis. XOS have a characteristic of prebiotic by promoting the growth of probiotic organisms. They have various physiological effects such as reducing cholesterol, maintaining the gastrointestinal health and improving the biological availability of calcium, animal feed, anti-cancerous. The production of XOS from agricultural residues offers great scope to the nutraceutical and pharmaceutical industries as the raw material is cheap and abundantly available. The driving force of this study was to produce XOS from the organic wastes such as orange fruit wastes. These wastes are rich in xylan which can be used as a renewable material for producing XOS. XOS can be obtained by chemical or enzymatic method, but due to the yield of toxic by-product, enzymatic production is preferred. In the enzymatic extraction method, acetic acid was used to prepare pellets from dried orange peels powder followed by xylanase enzyme degradation performed at 2, 4, 6 and 8 h. Samples containing XOS were chromatographed on HPLC system having a fluorescence detector (Ex320 nm, Em420 nm). The column used was Agilent C18 of length 250 mm and 4.6 mm internal diameter.

Key words: Xylooligosaccharide, xylobiose, xylotriose, prebiotic, xylan, enzymatic extraction, HPLC.

INTRODUCTION

The oligosaccharides with low degree of polymerization (DP 2-20 monomers) are considered as potential non-digestible sugars known for their benefits as dietary fibers. Recently, the significance of non-digestible oligosaccharides (NDOs) have been realized mainly due to their properties like sweeting ability water binding capacity, fat replacement value and notably their confrontation to digestion in higher region of gastrointestinal tract and maximum possibility of fermentation in the large bowel. The non-digestible oligosaccharides can be incorporated into processed food and could be promising functional ingredients in nutraceutical products (Gibson et al., 2004; Nyangale et al., 2012; Rossi et al., 2011). The utility is not the only parameters for these nutraceutical products; it is also economically and environmentally feasible as well as offers an opportunity for agriculture and food industries to produce value added products from agriculture or fruit

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wastes. As per the Stowell classification, the xylooligosaccharides (XOS) is categorized under emerging prebiotics whereas several other common prebiotics has different origin and chemical properties such as inulin, fructooligosacchrides (FOS), galactooloigosacchrides (GOS), lactulose and polydextrose, etc (Stowell, 2006).

According to a survey conducted on 1000 randomly selected consumers by the International Food Information Council (IFIC) in Washington, D.C. in 1998, it was observed that more than 95% of people believe that certain foods have various health assistances which completely depends on quality on rudimentary nutrition and can have a chance to reduce the risk of disease; whereas 92% people believe that they can control their own health by changing the life style, 78% of people reportedly name a particular food or component completely associated with various health benefits. Various food materials and green vegetables such as broccoli, carrots, fish and fish oil, oranges and orange juice, garlic, ginger, cucumber and milk were mentioned mostly by consumers, in that order (AsiaOne, 2015).

In this sense, orange have been shown to be a promising good food due to having variety of phytochemicals such as carotenoids which includes (beta-carotene, lutein and beta-cryptoxanthin), flavonoids in form of abundant volatile organic compounds producing orange aroma mainly including aldehydes, esters, terpenes, alcohols, and ketones (Elaine, 2011; Ensminger et al., 1986).

Orange fruit wastes mainly contain soluble sugars and pectin. According to Rivas et al. (2008), the orange fruit wastes constituted highest percentage of soluble sugars with 16.9% wt, followed by starch (3.75% wt), fiber which includes cellulose (9.21% wt), hemicelluloses (10.5% wt), lignin (0.84% wt) and pectines (42.5% wt). The high percent of ashes (3.50% wt) and proteins (6.50% wt). The least amount of fats, 1.95% wt is present in orange fruit wastes. The total cellulose content in orange fruit wastes ranged from 12.7 to 13.6% and hemicellulose from 5.3 to 6.1%. After all this consideration, the cost of materials becomes about 45% lower than that of conventional pulp of other fruits. Moreover, the dried orange fruit wastes showed lower fat concentration which will increase further the prebiotics components (Rivas et al., 2008; Beg et al., 2001). In addition, the recent studies showed that due to presence of hemicellulose and pectin the orange peels can be utilized to produce prebiotics materials such as xylooligosacchrides, fructooligosacchrides and pectin (Ma et al., 1993; Grohmann et al., 1995).

Though the orange fruit wastes contain maximum amount of pectic oligosaccharides, it has low prebiotic potential as compared to XOS and even FOS. Also, the cost of producing prebiotic materials such as XOS becomes 45% lower than that of other conventional fruit pulp industries as shown in the earlier research (Olano-Martin et al., 2002). Hence, the current research prefers XOS production from orange fruit wastes.

The pre-treatment of hemicellulose is one of the most important steps in the process to recover xylan from dried orange fruit wastes powder. The most common NaOH alkaline pre-treatment methods is used to separate hemicellulose content from other cellulosic waste materials such as lignin, pectines, fat substances and protein materials (Samanta et al., 2012a, b).

In order to produce the maximum xylan content, high alkaline concentration is required. Hence in the current research work the alkaline concentration was taken in varying concentration with water such as 2, 4, 6, 8, 10 and 12%, respectively. The main goal of pre-treatment alkaline or enzymatic hydrolysis is to modify or eliminate the structural and compositional inhibitions of waste materials present in hemicellulose other than desired materials to increase the yields of intended products such as XOS (Hendriks and Zeeman, 2009).

MATERIALS AND METHODS

Xylan extraction and production of XOS from extracted xylan were two major important things in the current research work.

Phase 1: xylan extraction

Xylan extraction involves the treatment of powdered orange fruit wastes with alkaline solution based on the use of NaOH (Sigma, India) followed by steam extraction. Based upon earlier investigation as well as trial and error analysis, 5 g of sample was chosen initially to extract xylan. To evaluate and optimize the condition, minimum of three trials were performed at each parameter using different concentrations (2, 4, 8 and 12%) of NaOH followed by steam treatment (120°C, 15 lbs pressure for 45 min). The solid to liquid ratio was 1:10. The alkali-solubilized xylan was centrifuged at 5,000 rpm for 20 min and filtered first by zero filter paper followed by Whatman filter paper 40. By using glacial acetic acid at pH 5.0, the acidified condition was maintained to supernatant. On addition of 3 vol. of ice-cold, 70% ethanol xylan was extracted. Later, the solution centrifuged at 8,000 rpm for 10 min at room temperature and xylan was precipitated and dried in hot air oven at 60°C till it reached persistent weight. LastLY, the dried pellets were stoked in powder form at room temperature for further analyses (Samanta et al., 2012a, b; 2013; Hsiao, 2006). The extracted xylan was characterized using Fourier Transform – Infrared Spectra (FTIR) and the true yield of xylan was calculated using the following formula:

$$\text{True yield (\%)} = \frac{\text{Dry weight of extracted xylan (g)} \times 100}{\text{Weight of the sample (g)}}$$

Production of XOS from extracted xylan

The production of XOS was performed using enzymatic hydrolysis on extracted xylan pellets obtained from dried orange fruit wastes powder. The enzymatic hydrolysis steps were carried out using endoxylanase enzyme extracted from *Trichoderma viride* procured from Sigma, India. In order to carry out enzymatic hydrolysis process, first enzyme buffer solution was prepared. Therefore,

Table 1. Variables for condition optimization.

Variables	XOS concentration	Ideal condition to maximize the response
pH		
4.5	9.48071 E-2±.0004	
5.5	6.303752 E-1 ± .0009	pH: 5.5
6.5	9.20759 E-2± .0003	
P value	2.99E-06	
Temperature		
30	1101479±.0007	
40	630.3752 ± .0009	Temperature: 40
50	0493784±.0004	
P value	2.58E-05	
Enzyme dose		
2.65	3.7040E-4±.0002	
5.30	1.51320 E-2±.0003	
7.95	8.47607 E-2 ±.004	Enzyme concentration 10.60
10.60	630.3752 ± .0009	
13.25	1.03005 E-1 ± .004	
P value	4.68E-05	
Alkaline concentration		
2% (5 g in 250 ml)	6.10963 E-2±.0007	
4% (10 g in 250 ml)	5.73150 E-2±.0004	
8% (20 g in 250 ml	2.13.2163 E-1±.0004	Alkaline concentration: 12%
12% (30 g in 250 ml)	630.3752 ± .0009	
15% (37.5 g in 250 ml)	7.30759 E-2 ± .0006	
P value	0.0007	
Incubation period (h)		
2	0.1700659 ± .008	
4	0.0817579 ± .005	
6	630.3752 ± .0009	Incubation period: 6 h
8	0.04159974 ± .006	
10	0.0055916± .001	
12	0.01565706± .002	

initially, 10 ml of sodium citrate buffer was taken in round neck conical flask. The initial substrate concentration (2%) was chosen as per the previous research (Akpinar et al., 2009). The pH (4.5, 5.5, 6.5), temperature (30–50°C) and enzyme dose (2U, 4U, 6U 8U and 10U when 1U= 2.65) were varied to optimise the conditions, in order to produce maximal conversion of xylan to XOS. The flasks, containing the reaction mixtures, were put in the incubator having shaking speed of 150 rpm. Aliquots were taken at fixed time intervals (2, 4, 6, 8, 10 and 12 h, respectively) and assayed. The enzymatic reaction was stopped by boiling it for 10 min before being subjected to the various analyses. Duplicate samples for enzyme hydrolysis were set up for each of the experiments. To quantify the final product, first reducing sugars, followed by HPLC analysis were considered. For HPLC analysis, the refractive index detector having ZORBAX carbohydrate column (Agilent, USA) were used (Samanta et al., 2012a, b; 2013; Hsiao, 2006).

Statistical methods

The statistical approach was required to enhance the production of XOS under different variables used in the current investigation. The main purpose of the statistical analysis was to increase the XOS production (X1-X5). The variables were identified based upon earlier investigation and optimized mainly based upon trial and error analysis. The statistical analysis was performed using MS Excel 2010 statistical add on tools in order to justify the maximum significant variation in XOS production. Table 1 shows the variables identified for condition optimization.

RESULTS AND DISCUSSION

FTIR analysis

In the current investigation, the FTIR spectroscopy analysis was applied to the 12% alkaline concentration xylan pellets obtained after the treatment of dried orange peels powder with 12% alkaline solution prepared from NaOH followed by steam treatment (120°C, 15 lbs

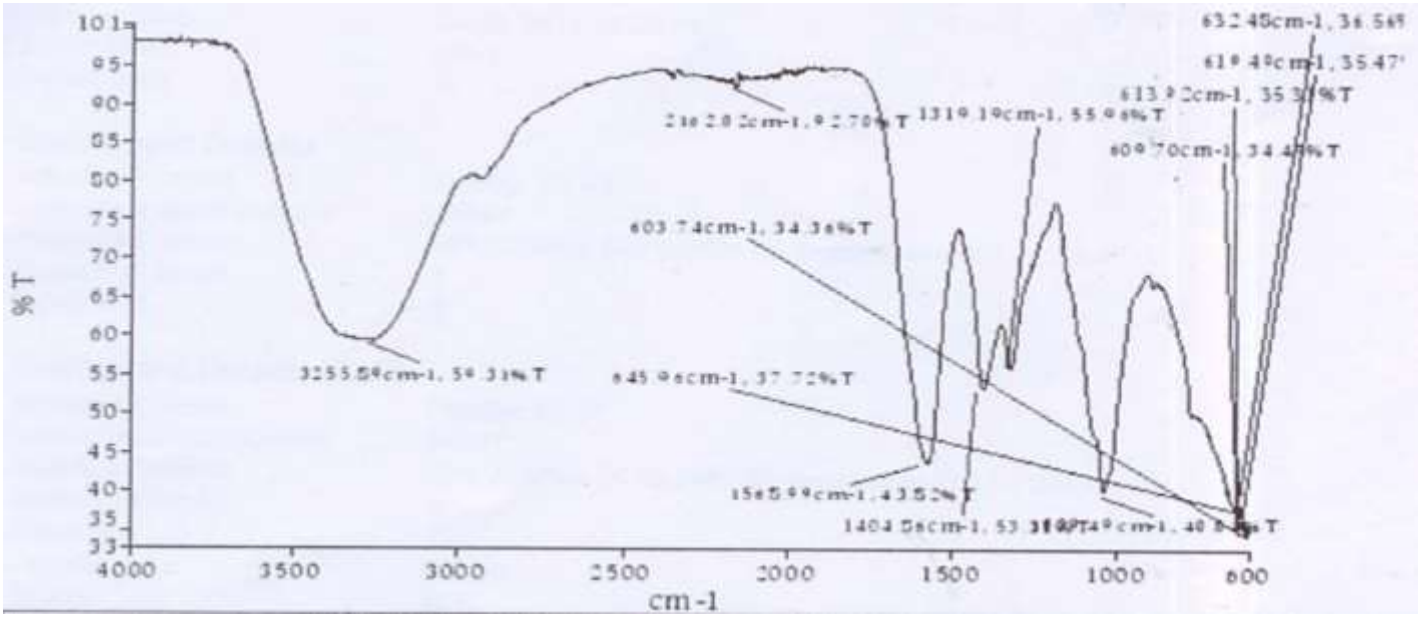


Figure 1. FT-IR spectra of the xylan obtained from dried orange peels powder treated 12% NaOH solution followed by steam application for 45 min.

pressure for 45 min). The solid to liquid ratio was 1:10. The alkali-solubilized xylan was centrifuged at 5,000 rpm for 20 min and filtered first by zero filter paper followed by Whatman filter paper 40. By using glacial acetic acid at pH 5.0 the acidified condition was maintained to supernatant. On addition of 3 volume of ice-cold 70% ethanol, xylan was extracted (Griffiths and De Haseth, 2007).

Figure 1 shows the observed absorption bands at 3000-3300 = 3255 cm^{-1} which recognizably extended the H-bonded OH groups existing in extracted xylan. Hence, it is confirmed that there was removal of acetyl, uronic acid, or ester groups in the alkaline treatment because no band at 1,736 cm^{-1} was observed. Although it is difficult, it is possible to identify removal of all lignocellulosic-based impurities in the extracted xylan using FTIR technique. However, in one study it has been reported that the xylan has got strong affinity towards water molecules which was not shown in band but may be due to xylan being prepared from completely dried orange peels powder (Samanta et al., 2013). The current FTIR was operated at 4000–650 cm^{-1} spectral range having a resolution of 0.9 cm^{-1} using a MIR source and KBr beam splitter and LiTa03 Detector (7800 to 350 cm^{-1}) (Software version CPU32 main 00.09.9951).

From the FTIR spectra, it can be said that the presence of the C–H and C–O or OH are confirmed because the intense bands at 1505 cm^{-1} were observed. The C–H and C–O or OH comprises bending vibrations in xylan. Similarly, the C–O, C–C stretching, or C–OH bending was confirmed in the xylan due to observation found at 1,027 cm^{-1} in form of vibrations. Two additional absorption bands were also seen in the FTIR spectra of current investigation at the range of 645 and 600 cm^{-1}. It may be due to possible stretching or bending of C–C–H or C– O–C (Samanta et al., 2012a, b; 2013; Hsiao, 2006).

Dinitrosalicylic (DNS) acid method

The current research work was conducted at fixed condition on precisely quantifying the enzyme activities of endoxylanase on extracted xylan pellets in order to measure the reducing sugar amount. The investigation was based upon different concentration of standards taken in increasing volumes of concentrations of 1 mg/100 ml along with samples of increasing volumes of concentrations of 2, 4, 8, and 12%. Incubation period was varied in 2, 4, 6, 8, 10 and 12 h, respectively.

The varying sample determination was chosen based upon a method explained by Nelson-Somogyi (also known as arsenomolybdate assay) (Somogyi, 1952). Each experiment was done in triplicate to avoid any ambiguity in OD values. The xylose concentration was calculated using the equation below:

Concentration of reducing sugar = Extrapolation on the X axis x Dilution factor

The graph was plotted using MS Excel 2007 and the R value was measured (Somogyi, 1952; Akpinar et al., 2007). For each different concentration of standards and samples, ANOVA was analyzed separately using MS

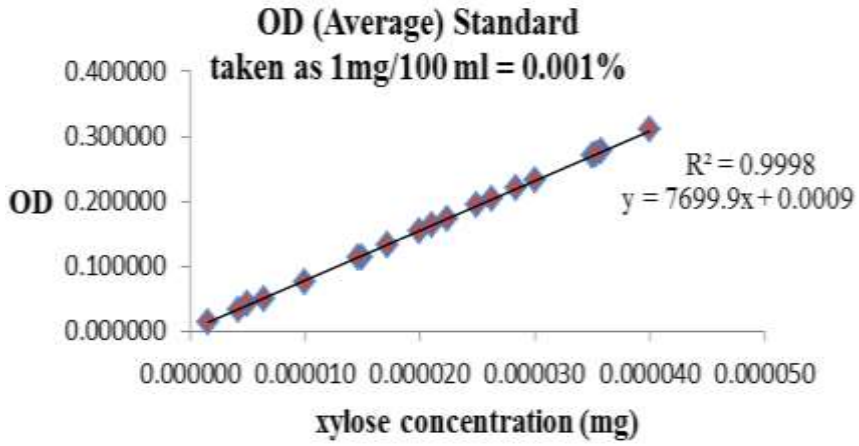


Figure 2. Determination of xylose by using DNS assay method. Standard taken as 1 mg/100 ml = 0.001%.

Excel 2007 version.

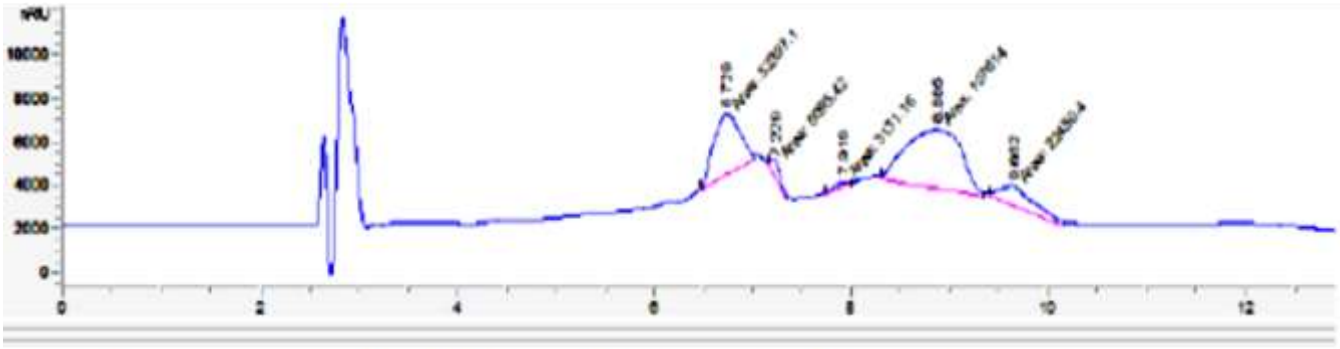
Figure 2 shows the xylose as standard (1 mg/100 ml = 0.001% carried out) and the concentration of reducing sugar was found to be 3.58E-05 and 3.54E-05 in 2 and 4 ml respectively for 12% unknown sample solution. The result was analyzed using ANOVA and interpreted mainly based on the F value and lowest P value. With this criteria, the P value was observed to be P = 1.06E-09*** which is lower than P =0.005. Also, F value is higher than P and Fcrit which was also found in earlier investigations (Samanta et al., 2012b, 2013; Hsiao, 2006; Akpinar et al., 2009) (*< = 0.5, ** <= 0.01 to 0.05 and *** <= 0.001-0.005).

High performance liquid chromatography analysis

The present investigation carried out HPLC analysis in order to perform qualitative and quantitative analysis of XOS based on their variable DP ranges. The HPLC system (Agilent, USA) was equipped with refractive index detector and the mobile phase used for XOS elution comprising acetonitrile and water in the proportion of 63:37. The flow rate was maintained at 0.5 ml/min. During the investigation the ZORBAX carbohydrate column (Agilent, USA) was used and the column temperature was fixed at 25°C (Samanta et al., 2012b). The sample was filtered using 0.2 µm membrane (Minigen USA- MG-25020PVDF) and around 20 ul samples was injected in the column using manual injector. The total HPLC investigation for one sample was achieved in around 30 min. The quantification of sample XOS was based upon comparison of average peak areas compared with the standards XOS (X1–X5= (xylose, xylobiose, xylotriose, xylotetrose and xylopentose). The quantification was expressed in ng/ml and converted to mg/ml for further analysis (Samanta et al., 2012b; Akpinar et al., 2007). All standards were provided and analysis was performed at National Institute of Animal Nutrition and Physiology Bangalore.

The Figure 3 displayed the quantification of HPLC result at 12% alkaline concentration and varying incubation period which showed that the 6 h incubation period has produced maximum amount of XOS (X1-X5) 630.38 ng/ul. Hence 1 g of xylan will produce 0.63 mg of XOS in given condition which is maximum in all other incubation periods. Therefore, the 6 h incubation period was chosen to best carry out further analysis.

Orange fruit wastes are becoming one of the largest amount of organic waste which are dumbed with other garbage and litters and since these waste are unprocessed, therefore the chance for development of air borne disease, bad odor and air pollutions are very high (Wu et al., 2004; Peng et al., 2009; Sukri et al., 2014; Marín et al., 2007). In this context few study research showed that around 50-60% of the total orange fruits are converted into peel waste (Wu et al., 2004; Peng et al., 2009) which gives a consequence that the continuous increasing in peel waste day to day especially near to fruits processing industries. Due to unawareness towards disposal method and the need to recycle these wastes, these organic wastes are becoming a serious environmental issues and health associated disorders considerations (Sukri et al., 2014; Marín et al., 2007). Among the different agro industrial residues, the usages of orange peels for XOS production have given new organic waste sources which were not mentioned in the earlier investigations. The present study demonstrated the application of alkali and steam treatment for extraction and recovery of xylan from orange peels waste. Extraction with sodium hydroxide in combination with steam application was used for achieving higher recovery of xylan. The enzymatic hydrolysis of xylan using the commercial endoxylanase enzyme from *T.*



6.729 MM	5.23971e4	2.23558e-3	117.13796	Xylotriose
7.919 MM	3131.16040	2.20727e-3	6.91133	Xylotetrose
8.885 MM	1.07614e5	4.70500e-3	506.32586	Xylopentose

Figure 3. HPLC Chromatogram -Condition fixed at 12% alkaline concentration 6 h incubation period.

viride enabled production of XOS in the DP range of 1-5, that is, xylose and xylopentose.

Though the XOS production techniques uses both chemical and enzymatic classically, in the current investigation, the enzymatic method is preferred to avoid any toxic material production and to improve edibility performance which is significantly higher in terms of toxicity and lower edibility. Moreover, the pH is much more balanced in enzymatic method of XOS production. The statistical methods employed defined the point wherein maximum production of XOS could be achieved which was further proved experimentally.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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