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PharmTech

International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304
Vol.7, No.3, pp 488-496, 2014-2015

Extraction of Xyloloigosaccharides by using *Aspergillus niger* from orange wastes

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Abstract: Xylan is an important structural component of plant biomass left in agricultural wastes. It is the most abundant of all of the hemicelluloses materials in the world. A number of economically valuable products such as xylose and xylitol can be made from Xylan, but one category of emerging importance is Xylooligosaccharides. Xylooligosaccharides are short chain polymers made of monomeric units of xylose. It plays an important in role in food industries; it has a prebiotic effect which helps in stimulating healthy micro flora in the gut. In addition, XO offer an array of other dietary benefits such as fiber-like properties, improving uptake of calcium, reducing cholesterol, and acting as antioxidant which can be incorporated into many food products. XO seem to exert their nutritional benefit in various animal species, which by definition have an intestinal tract populated by a complex, bacterial intestinal ecosystem. XO can be obtained by chemical or enzymatic method, but due to the yield of toxic by product enzymatic production is preferred XO. We are using orange peels as substrate and the source of enzyme is *Aspergillus niger*. The driving force of this study was to produce XO from the organic wastes including orange peels which are usually discarded as a waste from food and beverage industries. These wastes are usually left to rot, as they are rich in xylan which can be used as a renewable material for producing XO. Utilization of agricultural biomass as raw material provides economic and ecological benefits because it reduces the amount of waste and makes possible to recover high value added compounds. In this work, the chemical composition of selected agricultural wastes are explored.
Keywords: Xyloloigosaccharides, Aspergillus niger, Orange wastes.

Introduction

Xylan is an important structural component of plant biomass left in agricultural waste[1]. It is the most abundant of all the cellulosic materials in the world, comprising over 30% of renewable organic carbon on this planet[1,2]. Xylan is a polysaccharide made up of D-xylose units connected by β-1,4-glycosidic bonds. It is often branched with predominantly acetyl, arabinofuranosyl and glucuronic side chains attached to the xylose backbone. A number of economically valuable products such as xylose and xylitol can be made from xylan, but one category of emerging importance is xylooligosaccharides (XO)[1-3].

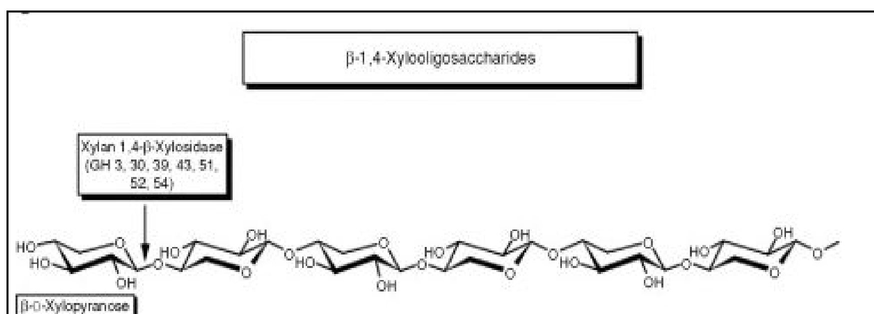


Fig.1 Structure of xylooligosaccharides.

Xylooligosaccharides are sugar oligomers made up of xylose units. These are naturally present in fruits, vegetables, bamboo, honey and milk and can be produced at industrial scale from xylan-rich materials. Of particular interest are those sources of residual origin, such as forestal, agricultural or industrial wastes of lignocellulosic nature[1,2]. Processing of residual vegetable biomass as raw material offers economic and ecological benefits, since it is a bio renewable, widely distributed and abundant resource. The growing commercial importance of these non-digestible oligosaccharides is based on their beneficial health properties, particularly the prebiotic activity. The XO are stable over a wide range of pH and temperatures have the ability of producing lower available energy and for achieving significant biological effects at low daily intakes. As food ingredients, XO have an acceptable odour and have low caloric value allowing their utilization in anti-obesity diets. These are non-cariogenic, save insulin secretion from the pancreas and stimulate intestinal mineral absorption. Moreover, the XO cause prebiotic effects when ingested as part of the diet through the modulation of colonic micro flora[4,5].

There are currently two processes used to break xylan into XO: hydrolysis by acid or by enzymes. The yield of XO is minimal with acid hydrolysis because acid prefers to cleave xylan into individual xylose units rather than XO, and it yields several toxic compounds including furfural[6,7].

Where enzymatic hydrolysis with xylanases does not produce toxic by-products. Hence in this work we are concentrating on enzymatic production[8,9].

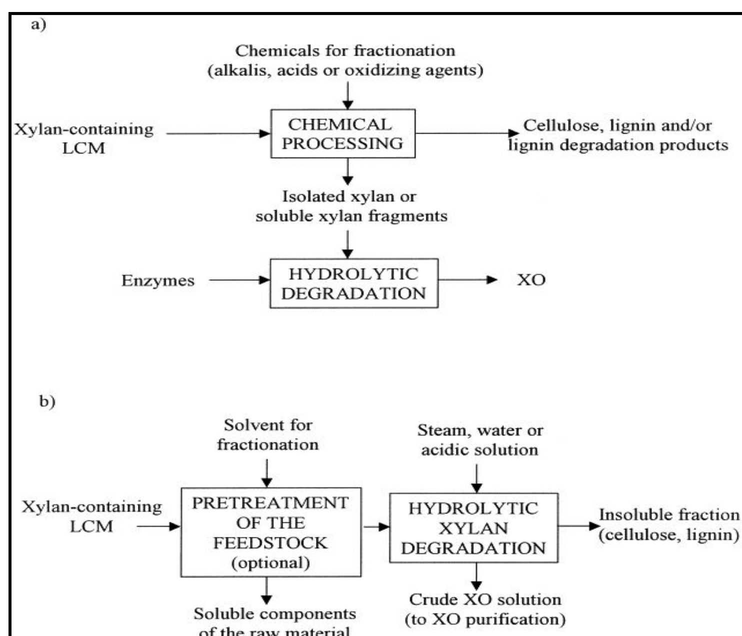


Fig. 2 (a) General idea of XO manufacture by chemical-enzymatic methods. (b) Procedure for XO manufacture by hydronium-catalyzed process.

Desired Enzymatic Reactions

There are several enzymes that comprise the xylanase family, with each one cleaving xylan in a different way to yield different products. While both the enzyme used and the structure of the xylan substrate will determine which compounds are made[7], only the former can be readily tailored to give desired outcomes. Three main enzyme groups exist: endo-1,4-β-D-xylanases, or endoxylanases, that break xylan at random to make smaller polymers; β-D-xylosidases, or xylosidases, that remove a single xylose from the non-reducing end of a polymer; and a number of debranching enzymes that cleave specific groups from the backbone (Figure 3)[7]. XO with degrees of polymerization (DP) ranging from 2 to 7 are typically desired for functions in food[10,11]. To cleave XOs units without producing xylose, a high endoxylanase to xylosidase ratio is needed[7,12].

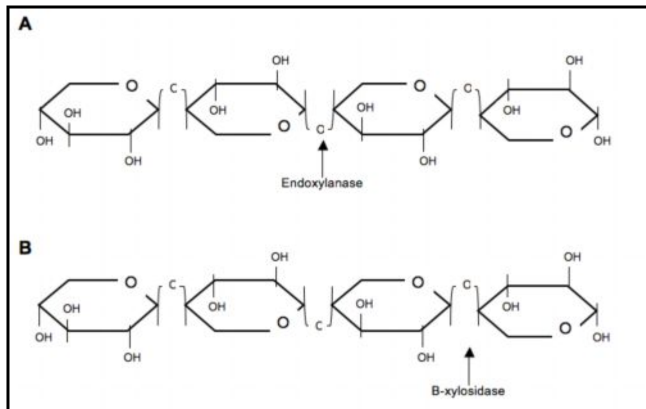


Figure 3: Point of cleavage on xylan by A) endoxylanase and B) β-xylosidase. This figure shows the ability of endoxylanase to form two separate XOS products while β-xylosidase will break off monomeric units of xylose.

Materials and Methodology:

Preparation of the substrates:

Citrus sinensis (orange) peels were chosen as the substrate and procured from local household and juice shops situated in Bangalore India. The substrate were washed under the running tap water 3 – 5 times to remove the dust and the mud deposited on the surface of the peels followed by shade dried for 3 days and finely powdered. Also the difference in the production of xylan was analyzed by freeze drying the substrate at -52°C for 18hrs. It was sieved using a mesh of a uniform pore size. The size of the substrate was determined by analysis in the Particle Size Analyzer and it was found to be 6.00µm for both the substrates.

Fig 4 Sun dried sample of orange peel

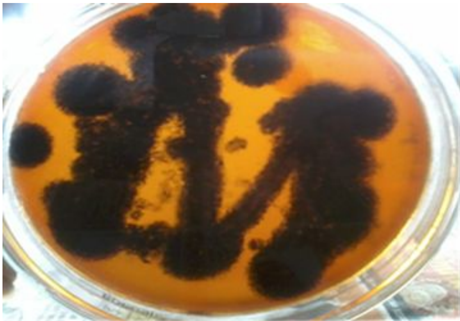
Fig 5 Freeze drying of orange sample

Fig 6 : Powdered sample of Orange peel

Fig 7: powdered sample of orange peel

Culturing of *Aspergillus niger* for xylanase production:

For growing *Aspergillus Niger* (MTCC 281) PDA (Potato dextrose agar) (Sigma Aldrich India). was prepared by dissolving 3.9gm of the mixture in 100ml of distilled water. The pH was adjusted to 3.5 by using 1M HCl and 1M NaOH (Sigma Aldrich India). The media was autoclaved at 121°C for 15min.The media was aseptically poured onto the Petri plates under the Laminar Air Flow Hood and allowed to solidify. *Asp.Niger* was inoculated and the culture was grown for 96 hrs at 36°C in a bacteriological Incubator and was stored at -



4°C for further use.

Figure 8: Plate containing PDA on which *Aspergillus niger* is cultured

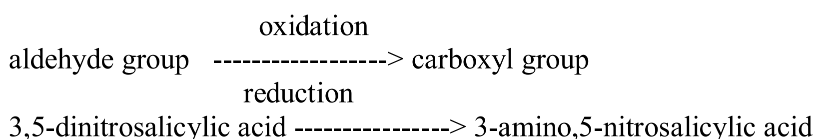
Enzymatic hydrolysis of substrates:

About 1lt of PDB was prepared in conical flask, the substrate was added as the sole carbon source i.e 1gm/10ml and it was autoclaved at 121°C for 15min. *Asp. Niger* was aseptically inoculated into the flask and it was incubated in a rotary incubator for 5 days at 40°C.The contents where filtered using Watman 1 filter paper. The filtrate was autoclaved at 121psi for 15min to remove the suspended spores of *Asp. Niger* and was used for further analysis.

Figure 9: Filtration of the substrate using Watman 1 filter paper

Estimation of reducing sugars (xylose) using DNS method.

This method tests for the presence of free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in, for example, glucose and the ketone functional group in fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions[13]:



Because dissolved oxygen can interfere with glucose oxidation, sulfite, which itself is not necessary for the color reaction, is added in the reagent to absorb the dissolved oxygen. The above reaction scheme shows that one mole of sugar will react with one mole of 3,5-dinitrosalicylic acid. However, it is suspected that there

are many side reactions, and the actual reaction stoichiometry is more complicated than that previously described. The type of side reaction depends on the exact nature of the reducing sugars.

Ultrafiltration:

Ultrafiltration helps in concentrating XOS in the permeate solution hence it was carried out using the equipment in our laboratory.[9, 10,14] Ultrafiltration was done only for the sample in which the substrate was orange peel. The membrane (Millipore) used was 1kDa to separate components other than XOS, whereas XOS passes through this membrane (i.e recovered in permeate). It was carried out at 1.5bars pressure and flow rate of 0.77ml/sec. The equipment was run for about half an hour. About 500ml of retentate was recovered out of 750ml of starting solution. Both the retentate and permeate was collected in different containers which was stored at 4ºC until further analysis. Qualitative confirmatory analysis of XOS produced where assayed by TLC and HPLC.

Figure 10 : Ultrafilteration unit

Results and Discussion:

1.Estimation of reducing sugars by DNS method: As a result of xylan hydrolysis xylose was obtained in the liquid media. Analysis was done for the substrates which was sun dried and freeze dried the samples which was taken in duplicates and was repeated for 2 trials.

Table 1: DNS method observation for 2 different substrate in sun dried and freeze dried condition

Sl No.	Standard XOS (in ml)	Distilled Water (in ml)	DNS (in ml)		OD 540nm	Concentration of xylose(µg/ml)
1.	0	1	1		0	0
2.	0.2	0.8	1		0.17	200
3.	0.4	0.6	1		0.35	400
4.	0.6	0.4	1		0.49	600
5.	0.8	0.2	1		0.60	800
6.	1	0	1		0.71	1000
7. Ss1	0.5	0.5	1	Incubation in boiling water bath for 10 min.	0.07	90
8. Ss2	0.5	0.5	1		0.09	95
9. Os1	0.5	0.5	1		0.26	280
10. Os2	0.5	0.5	1		0.30	300
11. Sf1	0.5	0.5	1		0.13	155
12. Sf2	0.5	0.5	1		0.14	165
13. Of1	0.5	0.5	1		0.25	315
14. Of2	0.5	0.5	1		0.28	325

Concentration of xylose(µg/ml) :

Ss1,Ss2 are the solution containing sweet lime peel which were sun dried sample and Os1,Os2 are the solution containing orange peel as substrate which were sun dried . Whereas Sf1,Sf2 are the sample solution containing sweet lime peel which were freeze dried and Of1 and Of2 are the solution containing orange peel as the substrate which were freeze dried The amount of Xylose in sun dried samples was found to be 185±5µg/ml Sweet lime peel and 580 ±20µg/ml for orange peel. The amount of Xylose in the freeze dried sample was found to be 320±5 µg/ml for Sweet lime peel and 625±5 µg/ml for Orange peel. Hence it can be observed that on hydrolysis of xylan into xlyose and XOS is produced which is determined by DNS method.

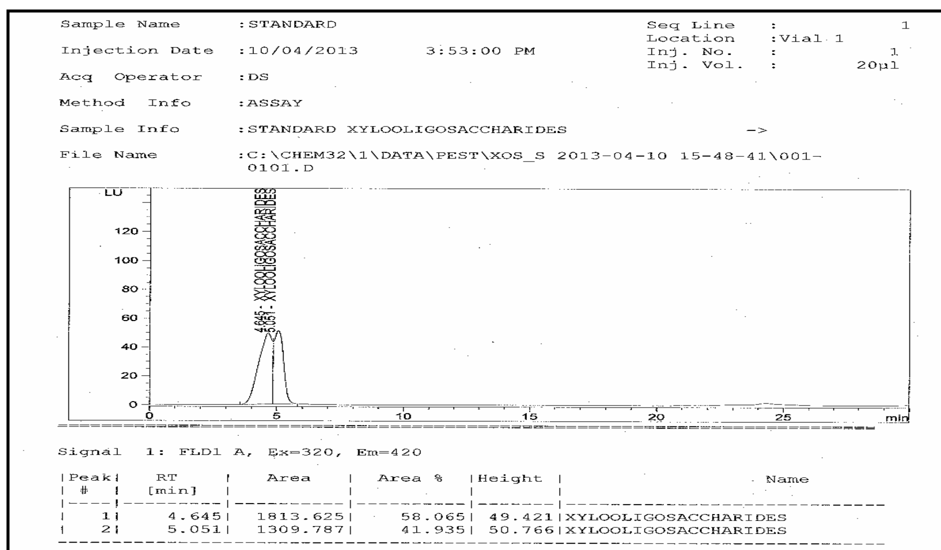
Table 2: Comparative study of the Xylose obtained by different modes of drying techniques in Sweet lime peel and Orange peel.

	Orange peel when sun dried	Sweet lime peel when sun dried	Orange peel when freeze dried	Sweet lime peel when freeze dried
Concentration in µg/ ml of substrate sol.	580±20	185±5	625±5	320±5

2.HPLC analysis of xylooligosacchride:

Samples containing XOS were chromatographed on HPLC system having a fluorescence detector (Ex320nm, Em420nm). The column used was Agilent C18 of length 250mm and 4.6mm internal diameter. About 20µl of the sample was injected into the column, XOS where eluted using aqueous methanol (80%) containing 0.02% trifluoro acetic acid (0-5min) followed by a gradient to 100% methanol over 45min [9,10,15].

At first the peak of standard XOS was obtained the area obtained was 1813.625 and 1309.787, the concentration of standard XOS was 1gm/10ml. Therefore 20µl of the standard contains 2mg of XOS.



Hence considering the area covered by the peak by taking the mean of areas obtained we get an area of 1561.706 which contains 2mg of XOS.

Sample Name :SAMPLE 2
Injection Date :10/04/2013 8:16:25 PM
Acq Operator :DS
Method Info :ASSAY
Sample Info :AU/2013/04/0044 ->
File Name :C:\CHEM32\1\DATA\PEST\XOS_S 2013-04-10 15-48-41\003-0301.D

Seq Line : 3
Location :Vial 3
Inj. No. : 1
Inj. Vol. : 20µl

4.629 - XYLOOLIGOSACCHARIDES

Signal 1: FLD1 A, Ex=320, Em=420

Peak #	RT [min]	Area	Area %	Height	Name
1	4.629	2280.608	100.000	42.486	XYLOOLIGOSACCHARIDES
2	0.000	0.000	0.000	0.000	XYLOOLIGOSACCHARIDES

Analysis of Sample 2: Orange peel as substrate in PDB before ultrafiltration

Sample Name :SAMPLE 3
Injection Date :10/04/2013 10:00:45 PM
Acq Operator :DS
Method Info :ASSAY
Sample Info :AU/2013/04/0045 ->
File Name :C:\CHEM32\1\DATA\PEST\XOS_S 2013-04-10 15-48-41\004-0401.D

Seq Line : 4
Location :Vial 4
Inj. No. : 1
Inj. Vol. : 20µl

4.628 - XYLOOLIGOSACCHARIDES

Signal 1: FLD1 A, Ex=320, Em=420

Peak #	RT [min]	Area	Area %	Height	Name
1	4.628	8721.811	100.000	150.411	XYLOOLIGOSACCHARIDES
2	0.000	0.000	0.000	0.000	XYLOOLIGOSACCHARIDES

Analysis of Sample3: Orange peel as substrate in PDB after ultrafiltration.

Comparative results of HPLC:

Sl. No.	Sample name	Retention time (min)	Concentration of XOS
1.	Standard XOS	4.645	100 mg / ml
2.	Retentate with orange peel as substrate	4.629	146mg/ml

3.	Permeate with orange peel as substrate	4.628	558 mg/ml

Conclusion

By HPLC analysis the amount of XO in the sweet lime peel and orange peel (retentate and permeate) obtained was 333 mg/ml, 146 mg/ml and 558 mg/ml. Out of orange peel and sweet lime peel, when orange peel was freeze dried it showed the maximum yield of XOS i.e 558mg/ml of the liquid media when analyzed by HPLC. When the process of drying used was freeze drying it showed 2 fold increase in xylose from 185±5mg/ml to 625±5mg/ml of in sweet lime peel. Hence it can be concluded that orange peel is the best out of the two substrates for producing XO.

Dry extraction method can be attempted where in *Aspergillus niger* can be allowed to grown on the peels directly to release xylans or XO[11,12]. While this research has successfully demonstrated the feasibility of using organic waste like orange peels as a feedstock for XO, more of research work is required before this technology can be implemented at a large scale. This enzymatic degradation technique requires further refinement like condition optimization (pH and temperature conditions) to obtain maximum activity of the xylanase which will improve XOS yield and reaction efficiency should be employed for better results[11,14,16,17].

Acknowledgement:

We would like to acknowledge the financial support of R.V College of Engineering, Bangalore and encouraging team members for their constant support and related help.

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