Bioresource Technology 245 (2017) 283–288

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Xylooligosaccharides production by crude microbial enzymes from agricultural waste without prior treatment and their potential application as nutraceuticals

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ARTICLE INFO

Keywords:
Xylooligosaccharides
Agricultural residue
Prebiotic
Antioxidant
Nutraceuticals

ABSTRACT

Aspergillus fumigatus R1, on submerged fermentation using agricultural residues as carbon source produced extracellular xylanase (152 IU/ml after 96 h of incubation at 37 °C with constant shaking at 100 rpm). A maximum yield of 1 gm% Xylooligosaccharides (XOS) mixture was obtained after 12 h by enzymatic hydrolysis of xylan rich wheat husk without any prior pretreatment using the crude enzyme without any purification. HP-TLC data confirmed the presence of an array of XOS for its prebiotic properties by carrying out studies on ten standard probiotic cultures. Six of ten probiotic cultures were able to utilize XOS produced from agricultural wastes and showed remarkable growth in the media containing XOS as the sole source of carbon. XOS mixture also exhibited concentration dependent anti-oxidant activity. Thus, the results showed that XOS produced from agricultural residues have great prebiotic potential and good antioxidant activity; therefore, it can be used in food-related applications.

1. Introduction

In recent years there has been a spurt of interest in research on lignocellulosic materials (LCM) as the efficient bioconversion of lignocellulosics to liquid and gases is one of the forthcoming approaches for sustainable biofuels, biochemical and biomaterials (Collins et al., 2005) which is impeded by recalcitrant nature of polymers present in LCM and are required to be removed by pretreatment (Juturu and Wu, 2012). Pretreatments are necessary to break the bonds that link hemicellulose, lignin and cellulose so that they are more accessible to enzyme action. Lignocellulose wastes contain 30–40% of hemicellulose. Numerous pretreatment technologies contribute to the overall production cost and each one has its own set of advantages and disadvantages. Moreover, the pretreatment technologies exert effects on the treated biomass (Vazquez et al., 2000). Xylan, the heterogeneous biomolecule and the major component of hemicellulose, is depolymerized to oligosaccharides and monosaccharides by enzymatic action of xylanases (Nacos et al., 2006).

"A prebiotic is a non-viable food component that confers health benefit on the host associated with modulation of microbiota" as defined by Food and Agricultural Organization (FAO, 2007). Non-digestible oligosaccharides (NDOs) are low molecular weight carbohydrate oligomers; non-cariogenic and have a low calorific value. NDOs are believed to benefit the host (Mussatto and Mancilha, 2007) by selectively stimulating the growth of beneficial bacteria in the colon (Sanchez et al., 2009; Makelainen et al., 2010) mainly the Bifidobacteria species (Reddy and Krishnan, 2016) and hence can be considered as prebiotics (Qiang et al., 2009). Xylooligosaccharides (XOS) are the NDOs that can be utilized as the nutraceuticals (Moure et al., 2006) because they have the ability to improve the biological availability of calcium, lower the cholesterol level, possess the antioxidant and anti-inflammatory (Singh et al., 2015), immunomodulatory, antidiabetic (Yang et al., 2015) and anti-cancer activities (Maeda et al., 2012; Kajihara et al., 2000; Achary et al., 2015). Clinical trials have reported the advantages of XOS with no adverse effects; however, research shows that in healthy men and elderly subjects, XOS is strongly bifidogenic (Chung et al., 2007).

Considering the tremendous health benefits XOS offer, research on it though has relevance; it is a challenge to make it available at economic price to large number of consumers particularly in developing countries. For this reason every step in product preparation that contributes to the total price needs to be thoughtfully evaluated. As commercially available highly pure xylan is costly, there is a paradigm shift in producing XOS from readily available agricultural wastes which are rich in hemi cellulosic biomass (Parajó et al., 2004). Possible lignocellulosic raw materials for XOS production include corn cobs

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http://dx.doi.org/10.1016/j.biortech.2017.08.174
Received 14 July 2017; Received in revised form 24 August 2017; Accepted 29 August 2017
Available online 01 September 2017
0960-8524/

(Chapla et al., 2013; Gowdhaman and Ponnusami, 2015), hardwoods (Huang et al., 2016), straws (Kallela et al., 2015; Gullón et al., 2011; Monize et al., 2016), bagasse (Jaypal et al., 2013; Mandelli et al., 2014), bamboo (Xiao et al., 2013) and bran (Otieno and Ahring, 2012; Immerzeel et al., 2014). The work on XOS reported till now by many independent researchers involved the pretreatment of agricultural residues using different methods such as microwave irradiation (Kumar and Satyanarayana, 2015), Alkali treatment (De Figueiredo et al., 2017; Brienzo et al., 2010), non-isothermal process (Ho et al., 2014), ammonia pretreatment (Bowman et al., 2012) or a combination of the other methods (Moura et al., 2007). Moreover, partially purified or recombinant xylanase enzyme has been employed in such pretreatment technologies.

In the present study, we have reported the production of XOS by crude xylanase produced by *Aspergillus fumigatus* R1 from agricultural residues without prior pretreatment and its further application as nutraceuticals.

2. Materials and methods

All the reagents, media and chemicals used in this study were of analytical grade (SRL, Hi-media). Birchwood Xylan and para-Nitrophenyl β-D-xylopyranoside (pNPX) were procured from Sigma, Germany. Standard xylooligosaccharides (xylobiose, xylotriose, xylotetraose, xylopentaose) were purchased from Megazyme, Ireland. TLC plates of silica gel 60 F_{254} and Centricon tubes were obtained from Merck, Germany. An Agro waste like wheat husk was procured from local farmers. It was sieved to separate the fine particles, dried in sunlight and stored in an airtight container.

2.1. Standard microbial cultures

Aspergillus fumigatus R1 was isolated in the laboratory. The bacterial cultures viz. *Bifidobacterium bifidum*, *Bifidobacterium adolescentis*, *Lactobacillus fermentum*, *Lactobacillus rhamnosus* and *Lactobacillus paraplantarum* were procured from NCDC-NDRI, Karnal, India. Bacterial cultures *Alcaligenes faecalis* and *Lactobacillus brevis* were obtained from MTCC, IMTECH, Chandigarh, India.

2.2. Preparation of crude xylanase and enzyme assays

2.2.1. Preparation of crude xylanase

Aspergillus fumigatus R1 was cultivated by submerged fermentation (SmF) in Erlenmeyer flasks (250 mL) containing 50 mL Mineral Salts Medium (MSM) supplemented with 1% Wheat husk and Ammonium Chloride as the carbon and nitrogen source respectively (Deshmukh et al., 2016). The culture medium was inoculated with 1×10^5 spores/mL. Spores were counted by microscopy in a Neubauer chamber. The flasks were incubated at the 37 °C in an orbital shaker incubator at 100 rpm for 96 h. The culture media was filtered through Whatman No. 1 filter paper, subsequently the filtrate was centrifuged at 5000 rpm for 10 min at 4 °C. The cell free supernatant containing crude Xylanases was used as enzyme source for all experimental work described in this study. All the experiments were performed in triplicates for statistical analysis of the results.

2.2.2. Xylanase assay

The xylanase activity was assayed by measuring the amount of reducing sugars released from the Birchwood xylan according to Deshmukh et al. (2016). The reaction mixture contained equal volumes of 1% Birchwood xylan and the suitably diluted enzyme solution with 50 mM phosphate buffer (pH 7) at 37 °C for 30 min. The amount of reducing sugar liberated was determined by Dinitrosalicylic (DNS) acid method using Xylose as the standard.

2.2.3. Cellulase assay

The exo and endo- cellulase activity of the cell free supernatant was estimated at pH 7 by DNS method using avicel and carboxymethyl cellulose as substrate respectively under the assay conditions mentioned above.

2.2.4. Pectinase assay

1% pectin was used as substrate under the assay conditions mentioned above and enzyme assay was carried out by DNS method. One unit of pectinase activity is the amount of pectinase that releases 1 µmol of galacturonic acid per minute under the assay conditions.

2.2.5. β-Xylosidase assay

β-Xylosidase (E.C. 3.2.1.37) was assayed by measuring the release of *p*-nitrophenol (pNP) from the hydrolysis of para-Nitrophenyl β-D-xylopyranoside (pNPX). The reaction mixtures (1.5 mL) containing 0.125 mL suitably diluted crude enzyme and 0.125 mL of 0.25 mM pNPX substrate in phosphate buffer (0.1 M, pH 7) were incubated at 37 °C for 30 min. 1 mL of 2 M Na_2CO_3 was added to terminate the reaction. The released pNP was quantified by measuring absorbance at 410 nm. One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmol of pNP per minute under the standard assay conditions.

2.3. Enzymatic hydrolysis of wheat husk for production of XOS

Since there is no β-xylosidase activity, formation of high-pure XOS from xylan is possible. Xylose will be formed only after complete subsequent hydrolysis of XOS generated due to enzyme action. The sun-dried clean Wheat husk and Birchwood Xylan were dissolved separately in 50 mM phosphate buffer (pH 7) in 50-mL conical flasks to a concentration of 2% (w/v). Crude xylanase was added at a concentration of 75 U g^{-1} (Yang et al., 2015). Hydrolysis reaction was carried out at 37 °C at a constant rotation of 100 rpm for 2, 4, 6, 8, 10, 12, 14, 16, and 20 h, respectively. After incubation for the desired time, 0.2 mL of XOS-containing liquids was withdrawn from the incubation mixture, the solution containing XOS was filtered through sieve to remove the husk particles. The filtrate was centrifuged at 6000 rpm for 30 min at 4 °C, where the clear supernatant consisted of XOS mixture. XOS mixture was further concentrated by ultrafiltration with centrifugation speed of 8500 rpm for 15 min using 10 kDa ultra filtration centrifuge tubes (Millipore, cut off range-10 KDa). The concentrated sample was lyophilized for further analysis. Since 12 h indicated the optimal hydrolysis time for maximum XOS production; hence, enzymatic hydrolysis was repeated several times to obtain sufficient XOS containing liquor. For *in vitro* growth fermentation experiments, the XOS was filtered through 0.22 µm filter (Millex) and stored at 4 °C until further use.

2.4. Effect of different parameters on XOS production

Different concentrations of wheat husk (1%, 5% and 10%) were taken as suspensions in phosphate buffer (0.1 M, pH 7.0), and the crude xylanase (30 U g^{-1} of substrate) was added to the reaction mixture of 5.0 ml. The effect of different enzyme doses was evaluated by hydrolysis of wheat husk with different titers of the crude xylanase (30, 35, 40, 42, 45, and 50 U g^{-1} substrate) for 12 h.

2.5. Analysis of XOS

2.5.1. Quantification of total sugar by phenol-sulphuric acid (PSA) method

The amount of total sugar content in XOS mixture produced after enzymatic hydrolysis was quantified by PSA method (Dubois et al., 1956).