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2 **TITLE PAGE**

3 ***Miscanthus x giganteus* Xylooligosaccharides: Purification and Fermentation**

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ABSTRACT

A procedure was developed to recover xylooligosaccharides (XOS) from *Miscanthus x giganteus* (MxG) hydrolyzate. MxG hydrolyzate was prepared using autohydrolysis, and XOS rich fractions were acquired using activated carbon adsorption and stepwise ethanol elution. The combined XOS fractions were purified using a series of ion exchange resin treatments. The end product, MxG XOS, had 89.1% (w/w) total substituted oligosaccharides (TSOS) composed of arabinose, glucose, xylose and acetyl group. *Bifidobacterium adolescentis* and *Bifidobacterium catenulatum* (health promoting bacteria) were cultured *in vitro* on MxG XOS and a commercial XOS source, which was used as a comparison. *B. adolescentis* grew to a higher cell density than *B. catenulatum* in both XOS cultures. Total xylose consumption for *B. adolescentis* was 84.1 and 84.8%, respectively for MxG and commercial XOS cultures; and for *B. catenulatum* was 76.6 and 73.6%, respectively. The xylobiose (X2), xylotriose (X3) and xylotetraose (X4) were almost utilized for both strains. Acetic and lactic acids were the major fermentation products of the XOS cultures.

Key words: *Miscanthus x giganteus*; Autohydrolysis; Xylooligosaccharides; Fermentation; Acetic acid; Lactic acid

40 **Chemical compounds studied in this article:**

41 D-Xylose (PubChem CID: 135191), Xylobiose (PubChem CID: 439538), Xylotriose (PubChem
42 CID: 10201852), Xylotetraose (PubChem CID: 10230811), Xylopentaose (PubChem CID:
43 10146542), Xylohexaose (PubChem CID: 74539951), D-Glucose (PubChem CID: 5793), L-
44 Arabinose (PubChem CID: 5460291), Acetic acid (PubChem CID: 176), Lactic acid (PubChem
45 CID: 612)

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1. Introduction

Miscanthus x giganteus (MxG) is a warm season perennial rhizomatous grass being developed as a bioenergy crop for production of fuel. Bred by crossing *M. sinensis* and *M. sacchariflorus*, MxG is a sterile triploid hybrid. MxG grows to 3.5 m tall and requires less nutrient application than comparable perennial grasses (Lewandowski, Clifton-Brown, Scurlock, & Huisman, 2000). Total biomass yield is 60% more than a well fertilized maize and can reach 30 MT/ha/year (Somerville, Youngs, Taylor, Davis, & Long, 2010). High yield and rich carbohydrate contents make MxG an attractive bioenergy crop for biochemical production of sustainable fuels and chemicals (Brosse, Dufour, Meng, Sun, & Ragauskas, 2012).

In earlier studies, investigators have reported on processing MxG to sugars and ethanol (Brosse, Sannigrahi, & Ragauskas, 2009; Khullar, Dien, Rausch, Tumbleson, & Singh, 2013). While technically feasible, there remains a need for value added coproducts. We used the xylan portion of MxG to produce xylooligosaccharides (XOS) for use as a prebiotic.

Autohydrolysis (hot water pretreatment) is a widely employed pretreatment method and has been shown to be efficient and save costs because it uses only water. The process involves treating biomass with liquid hot water (160 to 220°C) for a period of time to partially extract and hydrolyze hemicellulose. During the reaction, acetic acid forms from acetyl groups released from hemicellulose that lower the pH. The hydronium ions catalyze the xylan cleavage and result in the production of xylose oligomers and monomers. Autohydrolysis can remove up to 80% of hemicellulose from plant cell wall and enhance enzymatic saccharification of cellulose microfibrils (Alvira, Tomas-Pejo, Ballesteros, & Negro, 2010).

Xylooligosaccharides (XOS) are sugar oligomers with xylose backbones. Structures consist of xylose units connected by β 1,4 glycosidic bonds and substituted with α 1,2 or α 1,3 glycosidic bonds connecting other sugars or glucuronic acids. The hydroxyl group on C2 and C3 often are esterified with acetyl groups (Oteino & Ahring, 2012). The degree of polymerization (DP), branching and substituted acids determine XOS solubility in water; XOS structure varies depending upon the source of biomass and method used to extract the xylan (Vázquez, Alonso, Domínguez, & Parajó, 2000). The term “oligosaccharide” refers to a DP from 3 to 10, but for XOS the definition has been broadened to include from 2 to 20 (Moure, Gullón, Domínguez, & Parajó, 2006). The interest in producing XOS from lignocellulosic material originates from their health promoting benefits, including selective utilization by probiotic microorganisms, improvement in gastrointestinal function, production of short chain fatty acids (SCFA), lower blood cholesterol concentration, reduction of stomach ulcer lesions and prevention of colorectal cancer (Carvalho, Neto, da Silva, & Pastore, 2013). Nutritionally, XOS act as nondigestible oligosaccharides (NDO), which are not digested by gastrointestinal enzymes or low pH gastric fluids, but can be metabolized by microbiota in the large bowel. XOS can stimulate growth of beneficial *Bifidobacterium* spp. and *Lactobacillus* spp. in the gut (Mussato & Mancilha, 2007).

Recovering XOS after autohydrolysis is challenging because the biomass hydrolyzate is a complex solution (Vázquez, Alonso, Domínguez, & Parajó, 2000). There is a variety of compounds derived from plant extractives, acid soluble lignin, carbohydrates, protein and lipids that are present along with XOS in the hydrolyzate. Pigments, furfural, phenolics, free fatty acids and amine related derivatives can be removed through combinations of solvent extraction, membrane filtration and chromatography. Food grade XOS has a purity of 75 to 95% (Aachary & Prapulla, 2011).

The technique of purifying XOS from biomass hydrolyzate is evolving. Vázquez et al. (2005, 2007) recovered XOS from *Eucalyptus globulus* with the combination of vacuum evaporation, solvent precipitation and solvent extraction and reported total substituted oligosaccharides (TSOS) of 70.6 to 82.2% purity. TSOS is the total amount of arabinose, glucose, xylose oligomers and substituted acetyl groups in XOS sample. Vegas et al. (2006) used a combination of nanofiltration, solvent extraction and ion exchange chromatography to obtain 90.7% TSOS purity. Gullón et al. (2010) improved the method from their previous report (Gullón et al., 2008) and used a combination of diafiltration, concentration, ultrafiltration and ion exchange chromatography to recover 90.7% TSOS purity.

The objectives of this study were to investigate the purification procedure for high purity XOS production from autohydrolyzed MxG and to elucidate MxG XOS utilization by *Bifidobacterium* spp. The selected two strains are known for their abilities to utilize XOS as a carbon source (Pastell, Westermann, Meyer, Tuomainen, & Tenkanen, 2009). Previously, we demonstrated ethanol fractions from activated carbon adsorption and ethanol/water elution recovered 47.9% (w/w) of XOS from MxG hydrolyzate (Chen et al., 2014). While the study optimized reaction conditions for extraction of XOS and demonstrated its recovery, the product was crude and did not support microbial growth of gut bacteria. In this study, the collected ethanol fractions were treated with ion exchange resins to remove heavy metal ions, negatively charged organic compounds and pigments. The purified MxG XOS was cultured with *B. adolescentis* and *B. catenulatum* *in vitro* for 24 h. Cell growth, specific growth rate, total xylose consumption, oligomer utilization and SCFA production were monitored and compared with commercial XOS.

2. Material and methods

2.1. Raw materials, chemicals and *Bifidobacteria* strains

MxG was planted in the Energy Bioscience Institute Experimental Farm at the University of Illinois (40.06°N, 88.19°W). The average annual temperature and precipitation of the Urbana-Champaign area were 11°C and 104 cm, respectively; soil was classified as fine-silty, mixed, and mesic Typic Endoaquoll (Arundale et al., 2014). The biomass was harvested in two consecutive years (2011 and 2012), and it was oven dried at 49°C, sliced, and ground using a hammer mill (model MHM4, Glen Mills, Clifton, NJ) equipped with a screen (250 µm openings), and it was stored at 4°C. The moisture contents (NREL TP 510 42621) of ground MxG were 8.2 and 5.0%, respectively. The MxG 2011 sample was used previously in the autohydrolysis investigation and was applied as a reference of initial material for this study (Chen et al., 2014), while the MxG 2012 sample was used for XOS preparation. Commercial XOS (Wako XOS) was purchased from Wako Chemicals USA (Richmond, VA) as a comparison. The Wako XOS were composed mainly of xylobiose (X2) and xylotriose (X3). Purified xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohexaose (X6) were purchased from Megazyme International (Wicklow, Ireland) and used as chromatographic standards. Ion exchange resins Amberlite IR120Na, Amberlite IR96 and Amberlite FPA90Cl were purchased from Dow Chemical (Midland, MI). *Bifidobacterium adolescentis* DSM 20083 and *Bifidobacterium catenulatum* DSM 16922 were obtained from the DSMZ culture collection (DSMZ, Braunschweig, Germany). All chemicals and laboratory reagents, unless stated otherwise, were analytical grade and supplied from Fisher Scientific (Springfield, NJ).

2.2. Autohydrolysis, MxG hydrolyzate and preparation of ethanol eluted XOS fractions

Two kilograms of MxG powder were pretreated at a water:solids ratio of 7:1 (12.5% w/w solids) using sixteen 500 ml stainless pipe reactors. The reactors were heated in a fluidized sand bath to 200°C and incubated for 20 min (Chen et al., 2014). The reaction was quenched by submerging the reactors in room temperature tap water. Pretreated solids and liquids were separated by vacuum filtration through glass fibers filters (Whatman GF/D, Fisher Scientific, Springfield, NJ); 4.2 L of the pretreated liquid was collected for XOS recovery. An aliquot at 0.5 ml of the pretreated liquid (MxG hydrolyzate) was filtered through a 0.2 µm membrane and analyzed for sugars. A second aliquot at 1 ml was subjected to a quantitative posthydrolysis with 4% H₂SO₄ at 121°C for 60 min (NREL LAP 014), for end hydrolysis of the XOS.

Two liters of the MxG hydrolyzate, a concentrated source of XOS, was placed in a 5 L plastic vessel. Two hundred grams of activated carbon powder (100 mesh size powder; Darco G60, Sigma Aldrich, St. Louis, MO) was added to 10% (w/v) of the liquid volume. The vessel containing the carbon mixture was placed in an agitated water bath set at 30°C for 60 min. Every 15 min, the whole mixture was stirred manually using a spatula. The mixture was vacuum filtered to recover the carbon powder. The XOS enriched carbon cake was washed with 2 L deionized (DI) water and eluted sequentially using 2 L ethanol solution at concentrations of 5, 30, 50, 70 and 95% (v/v). The eluted ethanol fractions were incubated in an 80°C water bath for 2 h to evaporate ethanol and concentrated in a static 49°C oven until constant weight was achieved. The recovery of XOS in each ethanol/water fraction was calculated as equation (1):

$$\text{Recovery (\%)} = \left(\frac{W_f}{W_p} \right) \times 100 \quad (1)$$

Where W_f was the mass of XOS in each fraction, W_p was the mass of XOS in the 2 L of the pretreated liquid.

2.3. XOS purification, colorant removal, filtration, drying and comminution

The dried 30, 50 and 70% (v/v) ethanol eluted XOS fractions were resuspended in DI water and combined into a XOS solution at a concentration of 2.74 g XOS/100 ml (Step 1, 670 ml). The XOS solution was treated in sequence with Amberlite IR120Na (Step 2, a strong cation exchange resin), Amberlite IR96 (Step 3, a weak anion exchange resin) and Amberlite FPA90Cl (Step 4, a strong anion exchange resin) to remove impurities. Each resin was added at the mass ratio of resin/liquor of 1:10 (10 g resins in 100 ml). Resins were incubated with XOS solution at ambient temperature for 24 h and separated from the solution using gravity filtration with a glass fiber filter (Whatman GF/D, Clinton, NJ). While new resins were used in the process, the used resins could be regenerated following manufacture's instruction and reused in the process.

XOS sample color intensity was measured with a UV-VIS spectrophotometer (Evolution 60s, Thermal Scientific, Waltham, MA) by measuring optical density (OD) between the wavelengths 200 and 500 nm. One ml of the XOS sample at each purification stage was added into a 1 cm wide cuvette (ThermoElectron, Madison, WI) for OD measurement. The crude XOS solution was diluted 10x to ensure it was within the linear measurement range.

The refined XOS solution was filtered through a 0.22 μ m vacuum filter unit (EMD Millipore Steriflip, Millipore Corp, Bedford, MA) to obtain a clear and pathogen free solution. The filtered Step 4 solution was oven dried at 45°C to constant weight and milled by pestle and mortar to form a fine powder (17.0 g from 517 ml filtered Step 4 solution). The XOS powder was named MxG XOS. A patent has been filed on the purification process in United States Patent and Trademark Office (USPTO) and assigned a serial number 62/188,429.

2.4. *In vitro* fermentation of XOS

191 *B. adolescentis* and *B. catenulatum* were cultured anaerobically in a modified RGM
192 medium (mRGM) which was supplemented with 0.5% (w/v) XOS sample as the carbon source.
193 The mRGM removed short chain volatile fatty acids (VFA) from the original formula and the
194 amounts of yeast extract and trypticase were reduced by half (Hespell, Wolf, & Bothast, 1987;
195 Cotta, 1993). XOS samples were dissolved in boiled distilled water, to remove gases, to form
196 20% (w/v) stock solutions. Stock solutions were cooled under N₂ gas, filter sterilized and stored
197 in sterile anaerobic bottles that were flushed with N₂. To prepare culture medium with 0.5%
198 substrates, 0.25 ml of stock solution was added into 10 ml mRGM medium in tubes under
199 anaerobic conditions.

200 Two *Bifidobacterium* strains were cultured at 37°C for 24 h with 0.5% respective XOS
201 substrate. Each culture tube was inoculated with 0.5 ml for overnight growth. Three replicates
202 were prepared for each combination of bacterium and XOS sample. Fermentation was carried out
203 at 37°C and sampled at 0, 1, 2, 3, 4, 5, 6, 7, 8 and 24 h. Bacterial growth was determined by
204 monitoring OD at 660 nm using a Vis spectrophotometer (Spectronic 21, Milton-Roy Co.,
205 Rochester, NY). The specific growth rate (μ) was calculated based on logarithm values recorded
206 of OD during bacterial exponential growth stage. At each sampling point, one ml aliquot was
207 centrifuged to remove cells; supernatant was used for total xylose determination, HPAEC-PAD
208 analysis and SCFA quantification.

209 2.5. Analytical methods for sugars, oligomers and metabolites

210 The XOS composition and metabolites produced during *in vitro* fermentation were
211 quantified by two HPLC systems coupled with refractive index detectors (Model 2414, Waters
212 Corporation, Milford, MA) and 100 μ L sample loops. Monosaccharides (arabinose, galactose,

glucose and xylose) were analyzed using a Micro-Guard De-Ashing precolumn (Bio-Rad Laboratories Inc., Hercules, CA) and a Bio-Rad Aminex HPX-87P column with B-pure purified water as mobile phase flowing at 0.6 ml/min and 85°C. The short chain fatty acids (acetic, propionic and butyric acids), lactic acid and ethanol were analyzed using a Micro-Guard Cation H precolumn and a Bio-Rad Aminex HPX-87H column with 0.005M H₂SO₄ as mobile phase flowing at 0.6 ml/min and 50°C. Peak area integration and quantification was performed by Breeze 2 software (Waters Corporation, Milford, MA).

The oligomer composition in the XOS solution was determined following National Renewable Energy Laboratory standard procedure TP 510 42623 (Sluiter et al., 2008a). The oligomer sample was hydrolyzed in 4% H₂SO₄ (w/w) at 121°C for 60 min. The HPLC analysis was described as previous paragraph, an increase in the concentration of monosaccharides and acetyl group was used to calculate the mass of oligosaccharides and the percentage of oligosaccharides in XOS sample. The total substituted oligosaccharides (TSOS) were used to represent the total amounts of arabinose, glucose, xylose and substituted acetyl groups; and to represent the purity of MxG XOS. The oligomer mass, composition and TSOS were calculated as following equation (2), (3) and (4).

$$Oligomer\ mass = (C_h - C_i) \times V \times A \quad (2)$$

$$Composition\ (\%) = \frac{Oligomer\ mass}{Total\ solid\ content} \quad (3)$$

$$TSOS\ (\%) = O_A(\%) + O_G(\%) + O_X(\%) + SA(\%) \quad (4)$$

Where *Oligomer mass* represented the mass of oligosaccharides in a fixed volume of XOS solution; C_h was the concentration of monosaccharides or acetic acid after dilute acid hydrolysis and corrected with sugar recovery standard; C_i was the initial concentration; V was the volume of

the XOS solution; A was the anhydro correction factor for oligomers (0.88 for C5 sugars and 0.90 for C6 sugars). *Composition* represented the ratio of the oligosaccharides in the XOS sample; the total solid content of the XOS solution was determined by adding XOS solution in aluminum can and sequentially dried in a 45°C and 105°C oven until reaching a constant weight. O_A , O_G , O_X , SA were the ratio of arabinose oligomer, glucose oligomer, xylose oligomer and substituted acetyl groups in the XOS sample, respectively.

XOS utilization during *in vitro* fermentation was determined by orcinol assay. Orcinol reagent was prepared using 1 g orcinol dissolved in 100 ml of concentrated HCl containing 0.5 g $FeCl_3$ (Schneider, 1957). The colorimetric assay was quantified at OD 660 nm; a standard curve was developed using xylose (Hespell, Wolf, & Bothast, 1987; Cotta, 1993).

Refined XOS solution and fermented media were analyzed for DP of oligomers by high performance anion exchange chromatography with a pulsed amperometric detection system, HPAEC-PAD (Dionex ICS 3000, Sunnyvale, CA). Twenty five microliters of each sample (diluted 1:200 with milliQ water) were analyzed by HPAEC-PAD utilizing a Dionex PA-100 column (4x 250mm, Dionex). The system was operated at 1 ml/min running 100% 100 mM NaOH (mobile phase A) initially with a linear gradient program to 12% 100 mM NaOH containing 1 M sodium acetate (mobile phase B) over 20 min followed by reequilibration in 100 mM NaOH for 15 min. Standard curves of X1 to X6 were plotted for peak area vs concentration; oligomer amount was calculated by applying sample peak area to standard curves (Rantanen et al., 2007; Bowman, Dien, O'Bryan, Sarath, & Cotta, 2011).

2.6. Statistical analysis

Experiments were performed in duplicates for XOS purification and were performed in triplicates for *in vitro* fermentation. The data were expressed as mean $\pm$ standard deviation. Fisher's least significant difference (LSD) was performed to test the statistical difference between the means with $\alpha = 0.05$ using SAS software (version 9.4, SAS, Cary, NC).

3. Results and discussion

3.1. Purification of XOS from autohydrolyzed MxG

MxG harvested in 2012 had (%w/w, db): 39.4% glucan, 22.3% xylan, 0.7% acid soluble lignin, 17.0% acid insoluble lignin, and 8.0% extractives, as measured using NREL standard procedures TP 510 42618 (Sluiter et al., 2008b) and TP 510 42619 (Sluiter, Ruiz, Scarlata, Sluiter & Templeton, 2005). Compared with MxG harvested from the same plot in 2011, the amount of water and ethanol extractives was reduced from 14.7 to 8.0%, whereas, the compositions on an extractive free basis were statistically equivalent in glucan (42.8 vs 42.9%), xylan (24.1 vs 24.2%), and lignin (19.3 vs 19.2%) ratio for the MxG 2011 and 2012 samples, respectively. Le Ngoc Huyen et al. (2010) demonstrated that the portion of extractives (soluble components) in MxG was affected by harvest date and may be attributed to loss of leaves. Hodgson et al. (2011) analyzed MxG samples harvested in November 2005 and February 2006; there was no significant difference between cell wall compositions. Reported MxG carbohydrate contents in this study corresponded to those previously reported (El Hage, Chrusciel, Desharnais, & Brosse, 2010; Le Ngoc Huyen, Rémond, Dheilly, & Chabbert, 2010).

Pretreated liquid was collected after autohydrolysis at 200°C for 20 min. The pretreated liquid had 1.02 g/100 ml xylose oligomers and 0.18 g/100 ml xylose (DP1). XOS were recovered

by activated carbon adsorption 10% (w/v) and eluted stepwise with 5, 30, 50, 70 and 95% (v/v) ethanol/water solutions. The fractionation resulted in 2.7% XOS of pretreated liquid eluted in 5% ethanol fraction (fraction dry weight 1.94 ± 0.06 g); 19.1% XOS of pretreated liquid eluted in 30% ethanol fraction (fraction dry weight 7.07 ± 0.06 g); 16.2% XOS of pretreated liquid eluted in 50% ethanol fraction (fraction dry weight 5.35 ± 0.08 g); 6.4% XOS of pretreated liquid eluted in 70% ethanol fraction (fraction dry weight 2.22 ± 0.01 g) and 1.0% XOS of pretreated liquid eluted in 95% ethanol fraction (fraction dry weight 0.55 ± 0.02 g). Most of xylose (X1) was eluted into the 5% ethanol fraction and nearly 2% (w/w) xylose monomers (3.9% of the amount in the pretreated liquid) existed in 30% ethanol fractions. The 30, 50 and 70% fractions from several batches were dried, dissolved in water and combined to form a semipurified XOS solution (Step 1).

The semi purified XOS solution (Step 1) was treated with ion exchange resins sequentially for further purification. As shown in Fig. 1, step 1 solution had 67.2% (w/w) xylose oligomers and 80.7% (w/w) TSOS. With the incubation of strong cation exchange resin IR120Na for 24 h (Step 2), the percentages of XOS and TSOS in dried XOS solution increased to 68.3 and 81.9% (w/w), respectively. Through the use of weak anion resin IRA96 (Step 3), the ratio of XOS and TSOS increased to 74.9 and 89.4% (w/w), respectively. Further treatment with polishing resin FPA90Cl (Step 4) resulted in XOS at 74.9% (w/w) and TSOS at 89.1% (w/w); no significant differences ($p > 0.05$) were found in the XOS and TSOS values between Step 3 and Step 4 using Fisher's LSD test. There was 8.7% (w/w) loss of xylose oligomers during the three-stage resin treatments.

Heavy metals were removed from XOS solution using Amberlite IR120 Na (Step 2). This is a gel type strong cation exchange resin with polystyrene matrix and sulfonic ligand. IR120 Na

is a general purpose cation exchange resin, which is used for water softening. It exchanges heavy metal ions with sodium ions. Since metal ion exchange was the target for Step 2, it was not unexpected that the overall purity (TSOS) only increased from 80.7 to 81.9%.

Organic acids were removed using Amberlite IRA96 (Step 3). This is a weak anion exchange resin with styrene divinylbenzene matrix and tertiary amine ligand. IRA96 is used primarily for acid removal in water purification and has been tested for XOS purification from rice husk and *Eucalyptus globulus* (Vegas et al., 2006; Vázquez, Alonso, Domínguez, & Parajó, 2007; Gullón et al., 2010). When Vázquez et al. (2007) compared the use of IRA96 and IRA400 (a strong anion exchange resin with polystyrene divinylbenzene matrix and quaternary ammonium ligand) for eucalyptus XOS purification, IRA400 and IRA96 produced similar quality XOS solutions but the loss of XOS was less using IRA400. However, when testing these two anion ion exchange resins, we found the performance of IRA96 was better than IRA400 in treating MxG XOS solution (XOS purity 72 vs. 66%, respectively).

Color agents were removed using Amberlite FPA90Cl (Step 4). This is a food grade strong anion exchange resin with polystyrene matrix and quaternary ammonium ligand often used in sugar and pharmaceutical industry for color removal. FPA90Cl is suitable for trapping high molecular weight organic color bodies. The strong anion exchange resins are noted for adsorbing phenolic compounds, which are predicted to be major colorants because they are derived from the lignin (Alexandratos, 2009). The Amberlite FPA90Cl did not significantly increase the XOS and TSOS ratio, a possible explanation was that some of the oligomers have been bound to lignin fragments and removed by FPA90Cl as colorants.

Fig. 1. Purity of XOS solutions and XOS recovery during purification.

While crude XOS solution was dark brown in color, the carbon/ethanol elution lightened the color to yellow and the sequential ion exchange chromatography reduced the color to pale yellow. The optical density (OD) of XOS liquids from 200 to 500 nm wavelengths are shown in supplementary data 1. Amount of impurities and color pigments were monitored at UV 230 and 280 nm. Treatment with the resins reduced OD at 230 and 280 nm from 4.44 to 1.84 and from 3.67 to 1.51, respectively.

Detailed composition of MxG XOS and commercial Wako XOS were performed by acid hydrolysis and analyzed by HPLC (Table 1). The sample was hydrolyzed in 4% H₂SO₄ to quantify sugar monomers and organic acids. Besides 74.9% of xylose oligomers, there were 3.0% arabinose oligomers, 4.5% glucose oligomers and 6.7% bound acetyl group. The composition of MxG XOS was compared with a commercially marketed XOS product (Wako). The amounts of xylose oligomers (74.9 vs 77.8%) were not statistically different ($p > 0.05$), but MxG XOS had fewer arabinose and glucose oligomers (3.0 vs 9.4%; 4.5 vs 6.2%) and higher bounded acetyl groups (6.7 vs 0.0%). Purities of MxG XOS and Wako XOS were determined to be 89.1 and 93.4%, respectively (Table 1).

Oligomers for MxG XOS and Wako XOS are listed in Table 2. The ion exchange resins treatments (Step 1 to Step 4) did not change the oligomer composition (supplementary data 2). The MxG XOS product was high in X2 (xylobiose), X3 (xylotriose), X4 (xylotetraose) and X5 (xylopentaose) (Table 2). The commercial product Wako XOS was high in X2 and X3 (Table 2).

Compared with other methods reported for XOS purification from crude biomass hydrolyzate, our method utilized carbon adsorption to substitute for the energy and capital intensive membrane filtration or vacuum evaporation steps; the ethanol used in elution can be recovered and reused; the final purity (TSOS) of the MxG XOS was similar to other reported purities (Table 3).

Table 1

Composition of MxG XOS and Wako XOS.

Composition	MxG XOS ^a	Wako XOS
% (w/w) of dry weight		
Xylose oligomer	74.9 ± 2.4	77.8 ± 0.7
Arabinose oligomer	3.0 ± 1.9	9.4 ± 0.1
Glucose oligomer	4.5 ± 0.2	6.2 ± 0.1
Acetyl group in oligomer	6.7 ± *	ND ^b
TSOS	89.1	93.4

^{*}Standard deviation was less than 0.05.^aNo free glucose or acetic acid was detected. pH of the MxG XOS sample is 6.3.^bND = Not detected.**Table 2**

Oligosaccharides composition of MxG XOS and Wako XOS.

Composition	MxG XOS ^a	Wako XOS
% (w/w) of dry weight		
Xylose (X1)	1.7 ± 0.4	ND ^a
Xylobiose (X2)	8.9 ± 0.2	18.5 ± 0.8
Xylotriose (X3)	11.3 ± 0.5	6.5 ± 0.5
Xylotetraose (X4)	8.8 ± 0.1	1.5 ± 0.4
Xylopentaoose (X5)	9.0 ± 0.3	ND
Xylohexaoose (X6)	5.8 ± 0.5	3.2 ± 0.1
Total (X1~X6)	45.4	29.7

^aND=Not detected.

Table 3

Comparison of MxG XOS with other published reports.

Raw material	TSOS ^a % (w/w) of dry weight	Monosaccharide	Purification strategy	Source
<i>Miscanthus x giganteus</i>	89.1	1.7	Carbon adsorption, ethanol elution, ion exchange chromatography	This study
<i>Eucalyptus globulus</i>	70.6 to 82.6	2.8	Vacuum evaporation, precipitation, solvent extraction,	Vázquez et al. (2005)
Rice husks	90.7	2.0	Nanofiltration, solvent extraction, ion exchange chromatography	Vegas et al. (2006)
<i>Eucalyptus globulus</i>	72.8	16.3	Evaporation, solvent extraction, ion exchange chromatography	Vázquez et al. (2007)
Rice husks	74.9	1.2	Nanofiltration, enzymatic hydrolysis, ion exchange chromatography	Gullón et al. (2008)
Rice husks	90.7	0.6	Diafiltration, concentration, ultrafiltration, ion exchange chromatography	Gullón et al. (2010)

^aTSOS, total substituted oligosaccharides.

TSOS%= % of (glucopoligosaccharides + xylooligosaccharides + arabinoxyloligosaccharides + acetyl groups + uronic acids).

3.2. Growth of *B. adolescentis* and *B. catenulatum* on MxG XOS and Wako XOS

XOS were evaluated as a carbon source for gut associated bacteria. Both strains, *B. adolescentis* and *B. catenulatum*, were able to grow on mRGM media with 0.5% (w/v) MxG XOS (Fig. 2). *B. adolescentis* grew faster and reached a maximum OD of 0.55 within 24 h and a specific growth rate (μ) of 0.515 h^{-1} (supplementary data 3). *B. catenulatum* growth was slower and the final OD less than that for *B. adolescentis*. The maximum OD of 0.38 was reached at 24 h and the specific growth rate was 0.296 h^{-1} .

Similarly, *B. adolescentis* grew faster than *B. catenulatum* when cultured with Wako XOS (Fig. 2). *B. adolescentis* reached an OD of 0.73 at 24 h and had a specific growth rate (μ) of 0.474 h^{-1} (Supplementary data 3). In contrast, *B. catenulatum* reached an OD value of 0.56 at 24 h, and the specific growth rate was 0.578 h^{-1} .

Palframan et al. (2003) tested *B. adolescentis* DSM 20083 and *B. catenulatum* DSM 20103 for growth on glucose, xylose, XOS and xylan. *B. adolescentis* DSM 20083 grew faster on glucose, but had similar growth on XOS and xylose; *B. catenulatum* DSM 20103 produced similar cell density on xylose and glucose and had higher cell growth on XOS than fructooligosaccharides (FOS). Moura et al. (2007) cultured *B. adolescentis* DSM 20083 with commercial XOS (Xylo-Oligo95P, Suntory, Osaka, Japan) and attained a maximum growth of OD 0.7 at substrate concentration of 0.5% (w/v) that was similar to Wako XOS fermentation in this study. Pastell et al. (2009) used Wako XOS and arabinoxylan derived oligosaccharides to culture with *B. adolescentis* ATCC 15703 (DSM 20083). *B. adolescentis* ATCC 15703 was not able to grow on arabinose but could grow on XOS. However, the growth rate became slow with the presence of xylose monomers. Our results were similar to Palframan et al. (2003); the slower growth of *B. catenulatum* may indicate strain differences in XOS utilization.

Fig. 2. Growth of *Bifidobacterium adolescentis* and *Bifidobacterium catenulatum* in nutrient base medium supplemented with MxG XOS and Wako XOS.

3.3. XOS consumption during fermentation by *B. adolescentis* and *B. catenulatum*

Total XOS consumption for the *B. adolescentis* culture is shown in Fig. 3. Residual XOS concentration was expressed on a xylose equivalent basis. Beginning with 5.5 mg/ml of xylose in MxG XOS, the total xylose concentration decreased from 2 to 6 h corresponding to exponential cell growth. At the end of fermentation, 0.9 mg/ml of the xylose (15.9% of the initial amount) remained in the medium. The Wako XOS culture started with a lower initial xylose at 4.5 mg/ml and decreased from 3 to 8 h. At the end of fermentation, 0.7 mg/ml of xylose (15.2% of the initial amount) remained in the medium.

Total XOS utilization, cultured with *B. catenulatum*, is shown in Fig. 3. Starting from 4.9 mg/ml of initial xylose, the total xylose concentration decreased continuously from 0 to 24 h. At the end of fermentation, 1.2 mg/ml of xylose (23.4% of the initial amount) remained in the medium. The Wako XOS medium started with a lower concentration of 4.4 mg/ml and decreased from 4 to 8 h. At 24 h, 1.1 mg/ml of xylose (26.4% of the initial amount) remained in the medium.

From the growth and substrate consumption during fermentation, *B. adolescentis* produced more cell mass in the Wako XOS than the MxG XOS cultures. The specific growth rate was similar for both XOS samples. The growth phase of *B. adolescentis* on MxG XOS (6 h) was shorter than for the Wako XOS (8 h). Total xylose consumption paralleled the growth curve. *B. catenulatum* grew slowly in the MxG XOS but appeared to grow well on the Wako XOS. Total sugar utilizations were similar between two XOS samples but this was not reflected in cell growth either because of the presence of non pentose growth factors and/or growth on MxG required the cell to expend further energy for nongrowth related activities.

Fig. 3. Total xylose consumption during culture with *B. adolescentis* and *B. catenulatum*.

For *B. adolescentis*, the concentration of X2, X3 and X4 decreased rapidly at the beginning of fermentation and was almost depleted at 8 h (Fig. 4). The concentration of X1 increased from 200 to 309 $\mu\text{g/ml}$ from 0 to 6 h and decreased to 96 $\mu\text{g/ml}$ at 24 h. X5 and X6 concentrations decreased more slowly compared with X2 to X4 oligomers. At the end of fermentation, concentrations of XOS were 94.9% X2, 97.5% X3, 95.0% X4, 39.9% X5 and 34.1% X6 lower than at inoculation. The shorter XOS (X2, X3 and X4) were completely used by 24 h but longer XOS (DP, X5, X6, X7 and X8) were utilized more slowly (Supplementary data 4). For *B. catenulatum*, X3 concentration decreased rapidly and was followed by X2 and X4. X1 continued to accumulate in the medium after 2 h and reached 887 $\mu\text{g/ml}$ at 24 h. X5 and X6 were utilized more slowly compared with X2 to X4 oligomers. At the end of fermentation, 90.4% X2, 97.7% X3, 87.2% X4, 50.4% X5 and 52.8% X6 were lower than at inoculation. Depletion of X2, X3 and X4 with residual concentrations of X5, X6, X7 and X8 and the accumulation of X1 were detected by chromatograph (Supplementary data 4).

Fig. 4. Time course of MxG XOS oligomers consumed during fermentation with *B. adolescentis* and *B. catenulatum*.

Unlike MxG XOS, the Wako XOS was composed mainly of X2 (900 to 948 µg/ml) and X3 (300 to 349 µg/ml) and only minor amounts of X4 and X5 (Fig. 5). For *B. adolescentis*, the concentration of X2 and X3 decreased rapidly after 2 h, which paralleled exponential cell growth. X2 and X3 were depleted within 8 h. Accumulation of X1 was observed for 0 to 6 h and then decreased to 73.8 µg/ml by 24 h. At the end of fermentation, 97.7% X2 and 95.6% X3 were utilized. Unexpected increases in X5 and X6 concentrations were observed at the end of fermentation; the X5 and X6 peaks appeared after 8 h of fermentation (Supplementary data 4). For *B. catenulatum*, the concentration of X2 decreased immediately after inoculation. The utilization of X2 and X3 slowed at 8 h. At the end of fermentation, 96.0% X2 and 96.2% X3 were utilized. Accumulation of X1 was observed and maintained at high concentration to the end of fermentation. Increases of X4, X5 and X6 concentrations were found at 24 h (Supplementary data 5).

Fig. 5. Time course of Wako XOS oligomers consumed during fermentation with *B. adolescentis* and *B. catenulatum*.

X2, X3 and X4 XOS were almost utilized by both *Bifidobacteria* strains. In MxG XOS, the decrease in concentration of X3 was faster than for X2 (Figs. 4 & 5). Gullón et al. (2008) assessed the fermentability of XOS from rice husk by *B. adolescentis* CECT5781. Rapid decreases of X2 and X3 and slower degradations of X4 and X5 were found. Xylose was observed to increase in concentration, suggesting that xylose was not a preferred sugar, perhaps because monosaccharides are absent in the colon. Pastell et al. (2009) tested utilization of arabinoxylan derived XOS by *B. adolescentis* ATCC 15703 (DSM 20083); quick consumption of X2, X3 and X4 were reported. Pastell et al. (2009) hypothesized that X2, X3 and X4 were consumed rapidly by *B. adolescentis* because it was able to transport these into the cell while longer XOS needed to be hydrolyzed externally. Pastell et al. (2009) claimed xylose was a possible substrate for *B. adolescentis*; but this was in disagreement with Gullón et al. (2008). Wang et al. (2010) cultured *B. adolescentis* with wheat bran derived XOS. Consumptions of 88.0% X3, 79.4% X2, 75.0% X4, 73.5% X5, 59.5% X6 and 50.9% X7 were recorded. They stated X3 was the preferred substrate for consumption by *B. adolescentis*. Our results were consistent with Gullón et al. (2008), Pastell et al. (2009) and Wang et al. (2010).

3.4 Short chain fatty acid production

Concentrations of short chain fatty acids produced during *in vitro* fermentation were quantified; acetic and lactic acids were the major metabolites and their concentrations increased along with XOS utilization (data not shown). Production of other fatty acids were either minor or below detectable limits. MxG XOS were converted into 289 mg/g SCFA (194 mg/g acetic acid and 95 mg/g lactic acid) by *B. adolescentis* and into 273 mg/g SCFA (214 mg/g acetic acid and 59mg/g lactic acid) by *B. catenulatum*; Wako XOS were converted into 296 mg/g SCFA (179 mg/g acetic acid and 117 mg/g lactic acid) by *B. adolescentis* and into 258 mg/g SCFA (193mg/g

acetic acid and 65mg/g lactic acid) by *B. catenulatum*. In addition, an amount of formic acid at 59 mg/g MxG XOS and 63 mg/g Wako XOS produced by *B. catenulatum* was detected at the end of fermentation.

Bifidoabcterium spp. expresses a set of carbohydrate modifying enzymes both intra and extracellularly that enable it utilize complex carbohydrates. XOS are hydrolyzed into monomers, phosphorylated and deacetylated to yield ATP and converted into acetate, lactate, formate and ethanol (de Vries & Stouthamer, 1968; Pokusaeva, Fitzgerald, & van Sinderen, 2011). The ratio of the metabolites varies among the strains and carbohydrate sources (Palframan, Gibson, & Rastall, 2003). Gullón et al. (2008) cultured XOS from rice husk with *B. adolescentis* CECT5781, they observed the major metabolites were acetic, lactic and formic acids. Wang et al. (2010) used XOS extracted from wheat bran to grow *B. adolescentis* and reported production of acetic, lactic, butyric and propionic acids. Madhukumar and Muralikrishna (2012) evaluated XOS from Bengal gram husk with *B. adolescentis* NDRI 236, and found that acetic acid was the dominant metabolite. In our study, acetic acid production was detected earlier in Wako XOS cultures than MxG XOS cultures, but the latter produced higher final titer for both *Bifidobacteria* strains (Supplementary data 6). In the MxG XOS cultures, acetic acid could also in part originate from released acetyl groups.

4. Conclusions

A method has been described for purifying XOS from crude MxG hydrolyzate. With a recovery of 45.4% XOS from carbon adsorption and elution, and 91.3% XOS from serial ion exchange resin treatments; the final product MxG XOS had 89.1% (w/w) TSOS and 74.9% (w/w)

xylose oligomers. The MxG XOS was composed of 8.9% X2, 11.3% X3, 8.8% X4, 9.0% X5 and 5.8% X6. The utilization of the MxG XOS by *Bifidobacterium spp.* was comparable with a commercial source of XOS. The rates of disappearance of XOS were from faster to slower: X3, X2, X4, X5 and X6. Acetic and lactic acids were the dominant metabolites during fermentation.

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References

1. Aachary, A. A., & Prapulla, S. G. (2011). Xylooligosaccharides (XOS) as an emerging prebiotic: microbial synthesis, utilization, structural characterization, bioactive properties, and application. *Comprehensive Reviews in Food Science & Food Safety*, 10(1), 2-16.
2. Alexandratos, S. D. (2009). Ion-exchange resins: a retrospective from Industrial and Engineering Chemistry Research. *Industrial & Engineering Chemistry Research*, 48(1), 388-398.
3. Alvira, P., Tomas-Pejo, E., Ballesteros, M., & Negro, M. J. (2010). Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. *Bioresource Technology*, 101(13), 4851-4861.

- 542 4. Arundale, R. A., Dohleman, F. G., Heaton, E. A., Mcgrath, J. M., Voigt, T. B., & Long, S. P.
543 (2014). Yields of *Miscanthus x giganteus* and *Panicum virgatum* decline with stand age in
544 the Midwestern USA. *GCB Bioenergy*, 6(1), 1-13.
- 545 5. Bowman, M. J., Dien, B. S., O'Bryan, P. J., Sarath, G., & Cotta, M. A. (2011). Selective
546 chemical oxidation and depolymerization of switchgrass (*Panicum virgatum* L.) xylan with
547 oligosaccharide product analysis by mass spectrometry. *Rapid Communications in Mass*
548 *Spectrometry*, 25(7), 941-950.
- 549 6. Brosse, N., Dufour, A., Meng, X., Sun, Q., & Ragauskas, A. (2012). Miscanthus: a fast-
550 growing crop for biofuels and chemicals production. *Biofuels, Bioproducts & Biorefining*,
551 6(5), 580-598.
- 552 7. Brosse, N., Sannigrahi, P., & Ragauskas, A. (2009). Pretreatment of *Miscanthus x giganteus*
553 using the ethanol organosolv process for ethanol production. *Industrial & Engineering*
554 *Chemistry Research*, 48(18), 8328-8334.
- 555 8. Carvalho, A. F. A., Neto, P. D. O., da Silva, D. F., & Pastore, G. M. (2013). Xylo-
556 oligosaccharides from lignocellulosic materials: chemical structure, health benefits and
557 production by chemical and enzymatic hydrolysis. *Food Research International*, 51(1), 75-
558 85.
- 559 9. Chen, M. -H., Bowman, M. J., Dien, B. S., Rausch, K. D., Tumbleson, M. E., & Singh, V.
560 (2014). Autohydrolysis of *Miscanthus x giganteus* for the production of
561 xylooligosaccharides (XOS): kinetics, characterization and recovery. *Bioresource*
562 *Technology*, 155, 359-365.
- 563 10. Cotta, M. A. (1993). Utilization of xylooligosaccharides by selected ruminal bacteria.
564 *Applied & Environmental Microbiology*, 59(11), 3557-3563.

- 565 11. De Vries, W., & Stouthamer, A. H. (1968). Fermentation of glucose, lactate, galactose,
566 mannitol, and xylose by Bifodobacteria. *Journal of Bacteriology*, 96(2), 472-478.
- 567 12. El Hage, R., Chrusciel, L., Desharnais, L., & Brosse, N. (2010). Effect of autohydrolysis of
568 *Miscanthus x giganteus* on lignin structure and organosolv delignification. *Bioresource*
569 *Technology*, 101(23), 9321-9329.
- 570 13. Gullón, P., González-Múñoz, M. J., van Gool, M. P., Schols, H. A., Hirsch, J., Ebringerová,
571 A., & Parajó, J. C. (2010). Production, refining, structural characterization and
572 fermentability of rice husk xylooligosaccharides. *Journal of Agricultural & Food Chemistry*,
573 58(6), 3632-3641.
- 574 14. Gullón, P., Moura, P., Esteves, M. P., Girio, F. M., Domínguez, H., & Parajó, J. C. (2008).
575 Assessment on the fermentability of xylooligosaccharides from rice husks by probiotic
576 bacteria. *Journal of Agricultural & Food Chemistry*, 56(16), 7482-7487.
- 577 15. Hespell, R. B., Wolf, R., & Bothast, R. J. (1987). Fermentation of xylans by
578 *Butyrivibrio fibriolvens* and other ruminal bacteria. *Applied & Environmental Microbiology*,
579 53(12), 2849-2853.
- 580 16. Hodgson, E. M., Nowakowski, D. J., Shield, I., Riche, A., Bridgwater, A. V., Clifton-Brown,
581 J. C., Donnison, I. S. (2011). Variation in *Miscanthus* chemical composition and
582 implications for conversion by pyrolysis and thermos-chemical bio-refining for fuels and
583 chemicals. *Bioresource Technology*, 102(3), 3411-3418.
- 584 17. Khullar, E., Dien, B. S., Rausch, K. D., Tumbleson, M. E., & Singh, V. (2013). Effect of
585 particle size on enzymatic hydrolysis of pretreated *Miscanthus*. *Industrial Crops & Products*,
586 44, 11-17.

- 587 18. Le Ngoc Huyen, T., Rémond, C., Dheilly, R. M., & Chabbert, B. (2010). Effect of
588 harvesting date on the composition and saccharification of *Miscanthus x giganteus*.
589 *Bioresource Technology*, 101(21), 8224-8231.
- 590 19. Lewandowski, I., Clifton-Brown, J. C., Scurlock, J. M. O., & Huisman, W. (2000).
591 *Miscanthus*: European experience with a novel energy crop. *Biomass & Bioenergy*, 19(4),
592 209-227.
- 593 20. Madhukumar, M. S., & Muralikrishna, G. (2012). Fermentation of xylo-oligosaccharides
594 obtained from wheat bran and Bengal gram husk by lactic acid bacteria and bifidobacteria.
595 *Journal of Food Science & Technology*, 49(6), 745-752.
- 596 21. Moura, P., Barata, R., Carneiro, F., Girio, F., Loureiro-Dias, M. C., & Esteves, M. P.
597 (2007). *In vitro* fermentation of xylo-oligosaccharides from corn cobs autohydrolysis by
598 *Bifidobacterium* and *Lactobacillus* strains. *LWT-Food Science and Technology*, 40(6), 963-
599 972.
- 600 22. Moure, A., Gullón, P., Domínguez, H., & Parajó, J. C. (2006). Advances in the manufacture,
601 purification and applications of xylooligosaccharides as food additives and nutraceuticals.
602 *Process Biochemistry*, 41(9), 1913-1923.
- 603 23. Mussatto, S. I., & Mancilha, I. M. (2007). Non-digestible oligosaccharides: a review.
604 *Carbohydrate Polymers*, 68, 587-597.
- 605 24. Otieno, D. O., & Ahring, B. K. (2012). A thermochemical pretreatment process to produce
606 xylooligosaccharides (XOS), arabinooligosaccharides (AOS) and mannoooligosaccharides
607 (MOS) from lignocellulosic biomasses. *Bioresource Technology*, 112, 285-292.

- 608 25. Palframan, R. J., Gibson, G. R., & Rastall, R. A. (2003). Carbohydrate preference of
609 *Bifidobacterium* species isolated from human gut. *Current Issues in Intestinal Microbiology*,
610 4, 71-75.
- 611 26. Pastell, H., Westermann, P., Meyer, A. S., Tuomainen, P., & Tenkanen, M. (2009). *In vitro*
612 fermentation of arabinoxylan-derived carbohydrates by bifidobacterium and mixed fecal
613 microbiota. *Journal of Agricultural & Food Chemistry*, 57(18), 8598-8606.
- 614 27. Pokusaeva, K., Fitzgerald, G. F., & van Sinderen, D. (2011). Carbohydrate metabolism in
615 *Bifidobacteria*. *Genes & Nutrition*, 6(3), 285-306.
- 616 28. Rantanen, H., Virkki, L., Tuomainen, P., Kabel, M., Schols, H., & Tenkanen, M. (2007).
617 Preparation of arabinoxylobiose from rye xylan using family 10 *Aspergillus aculeatus* endo-
618 1,4- β -D-xylanase. *Carbohydrate Polymers*, 68(2), 350-359.
- 619 29. Schneider, W. C. (1957). Determination of nucleic acids in tissues by pentose analysis.
620 *Methods in Enzymology*, 3, 680-684.
- 621 30. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, D. (2008a).
622 Determination of sugars, byproducts, and degradation products in liquid fraction process
623 samples. In: NREL Laboratory Analytical Procedure, National Renewable Energy
624 Laboratory, Golden, CO. <http://www.nrel.gov/docs/gen/fy08/42623.pdf>.
- 625 31. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, D.
626 (2008b). Determination of structural carbohydrates and lignin in biomass. In: NREL
627 Laboratory Analytical Procedure, National Renewable Energy Laboratory, Golden, Co.
628 <http://www.nrel.gov/biomass/pdfs/42618.pdf>.

32. Sluiter, A., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, D. (2005). Determination of extractives in biomass. In: NREL Laboratory Analytical Procedure, National Renewable Energy Laboratory, Golden, CO. <http://www.nrel.gov/biomass/pdfs/42619.pdf>.
33. Somerville, C., Youngs, H., Taylor, C., Davis, S. C., & Long, S. P. (2010). Feedstock for lignocellulosic biofuels. *Science*, 329(5993), 790-792.
34. Vázquez, M. J., Alonso, J. L., Domínguez, H., & Parajó, J. C. (2000). Xylooligosaccharides: manufacture and applications. *Trends in Food Science & Technology*, 11(11), 387-393.
35. Vázquez, M. J., Garrote, G., Alonso, J. L., Domínguez, H., & Parajó, J. C. (2005). Refining of autohydrolysis liquors for manufacturing xylooligosaccharides: evaluation of operational strategies. *Bioresource Technology*, 96(8), 889-896.
36. Vázquez, M. J., Alonso, J. L., Domínguez, H., & Parajó, J. C. (2007). Production and refining of soluble products from *Eucalyptus globulus* glucuronoxylan. *Collection of Czechoslovak Chemical Communications*, 72(3), 307-320.
37. Vegas, R., Luque, S., Alvarez, J. R., Alonso, J. L., Domínguez, H., & Parajó, J. C. (2006). Membrane-assisted processing of xylooligosaccharide-containing liquors. *Journal of Agricultural & Food Chemistry*, 54(15), 5430-5436.
38. Wang, J., Sun, B., Cao, Y., & Wang, C. (2010). *In vitro* fermentation of xylooligosaccharides from wheat bran insoluble dietary fiber by Bifidobacteria. *Carbohydrate Polymers*, 82(2), 419-423.