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Bioactive xylo-oligosaccharides from wheat bran soluble polysaccharides

Chithra Manisseri, Muralikrishna Gudipati*

Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore 570020, Karnataka, India

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ABSTRACT

Soluble polysaccharides (SP, 35 g) isolated from wheat bran (100 g) consisted mainly of arabinose and xylose with minor quantities of rhamnose, mannose, galactose and glucose. Wheat bran SP was subjected to purified endoxylanase (from 96 h ragi malt) treatment to obtain xylo-oligosaccharides (0.3 g/1 g wheat bran). The oligosaccharide mixture was purified on Biogel P-2 column into four major peaks designated as WO-1, WO-2, WO-3 and WO-4. Individual oligosaccharide purity was ascertained by HPLC and their composition was determined by GLC. The purified oligosaccharides were characterized by ESI-MS and ^{1}H NMR analysis. WO-1 and WO-2 were identified as arabinose containing xylotetraose and xylotriose, respectively, whereas WO-3 and WO-4 were identified as unsubstituted xylotriose and xylobiose, respectively. *In vitro* studies carried out using *Bifidobacterium* spp and *Lactobacillus* spp suggested the prebiotic nature of the crude as well as purified xylo-oligosaccharides as revealed by growth characteristics such as high O.D. of the culture broth, decrease in its pH, increase in cell mass and the resultant fermentation products. β-xylanase, β-xylosidase, α-arabinofuranosidase, α/β-galactosidases and acetyl esterase activities were determined in 24 h old culture broth and xylanase activity (440–830 μU/ml) was found to be the most preponderant among all of them.

1. Introduction

Arabinoxylans (AX), one of the major cell wall non-starch polysaccharides of cereals are highly heterogeneous consisting of a linear β-(1 → 4) linked xylopyranosyl (*Xylp*) backbone, which are either unsubstituted, monosubstituted at O-3 or disubstituted at O-3 or O-2 with α-L-arabinofuranosyl units (Barron, Robert, Guillon, Saulnier, & Rouau, 2006; Grootaert et al., 2007; Gruppen et al., 1992). They are designated as water extractable or water unextractable based on their extractability (Brijs et al., 2004; Trogh, Courtin, & Delcour, 2004). The difference in their extractability accounts for various physicochemical properties. Water unextractable nature is due to the involvement of non-covalent interactions and covalent bonds with adjacent arabinoxylans and other cell wall components such as proteins, lignins, etc (Courtin & Delcour, 2002). Studies have been carried out on water extractable and total arabinoxylan content in six barley malts (Debyser, Derdelinckx, & Delcour, 1997). A chemical and histological study on the effect of endoxylanase on wheat bran has been reported and it shows that xylanase attacks arabinoxylans of low degree of substitution (Benamrouche, Cronier, Debeire, & Chabbert, 2002). Barley water unextractable arabinoxylans consist of a backbone of β-1, 4-D-xylopyranosyl residues at O-3 or both at O-2 and O-3 (Vietor, Angelino, & Voragen, 1992). AX from wheat, rye, and barley on average have a relatively low degree of substitution. They contain higher proportions of unsubstituted xylose residues and lower levels of monosubstituted xylose residues than the more highly branched AX from rice and sorghum. The highest levels of doubly substituted xylose have been reported from wheat pericarp AX (Maes & Delcour, 2002).

The complete enzymatic degradation of the arabinoxylans requires the action of a set of enzymes such as endo-β-(1 → 4) xylanase (EC 3.2.1.8), α-L-arabinofuranosidase (EC 3.2.1.55) and β-D-xylosidase (EC 3.2.1.37). Endoxylanase is the key enzyme in the metabolism of arabinoxylans resulting in the alteration of their physicochemical properties and are increasingly used in various industrially relevant processes (Coughaln & Hazlewood, 1993; Tuncer & Ball, 2003). Enzymatic conversion of natural resources is one of the important method having wide applications in functional food research. Endoxylanase hydrolyzes the xylan backbone in a random manner producing xylo-oligosaccharides of degree of polymerization varying from 2 to 10. The increasing commercial importance of the xylo-oligosaccharides is due to their potential health benefits, particularly the prebiotic activity (Crittenden & Playne, 1996; Rycroft, Jones, Gibson, & Rastall, 2001). Apart from their prebiotic effect, xylo-oligosaccharides which come under the broad definition of non-digestible oligosaccharides (NDOs) are believed to alleviate disease symptoms such as diabetes, arteriosclerosis and colon cancer (Hsu, Liao, Chung, Hsieh, & Chan, 2004; Swennen, Courtin, & Delcour, 2006).

* Corresponding author. Tel.: +91 821 251 4876; fax: +91 821 251 7233.
E-mail address: krishnagm2002@yahoo.com (M. Gudipati).

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The human gastrointestinal tract represents a complex ecosystem and carbohydrates are found to be the first limiting nutrient for many bacterial species in the intestinal tract and thus the type of carbohydrates available influence the growth of the gut microflora. A prebiotic is defined as 'a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health' (Gibson & Roberfroid, 1995). Most of the interest in the development of new prebiotic ingredients is focused on non-digestible oligosaccharides (NDOs). These are oligomeric carbohydrates, whose glycosidic bond allows resistance to intestinal digestive enzymes in the upper gastrointestinal tract to become 'colonic nutrients' (Delzenne, 2003) for the selective growth of the beneficial microflora. *Bifidobacteria* and *Lactobacilli* are the most important beneficial microbes inhabiting the human gut. So the present study is mainly focused on the *in vitro* fermentation studies of xylo-oligosaccharides by these two bacterial genera. *Bifidobacterium* strains are largely described as capable of efficiently fermenting xylose-based oligo and polysaccharides (Crittenden et al., 2002). A wide range of *Bifidobacteria* is also found to be utilizing fructans of low degree of polymerization (Hidaka, Eida, Takizawa, Tokunaga, & Tashiro, 1986; Mitsuoka, Hidaka, & Eida, 1987). They produce specific hydrolases which convert these oligosaccharides into short-chain fatty acids (SCFA) by colonic fermentation. Acetate, propionate and butyrate are the major SCFA liberated due to carbohydrate fermentation which play a significant role in physiological level.

Prebiotic activity of xylo-oligosaccharides has not yet been effectively exploited compared to fructo and trans-galacto oligosaccharides. Wheat is the most important cereal crop in the world and in the present study wheat bran, the major by product of wheat industry, was used as the biosource to obtain prebiotic oligosaccharides. In this manuscript, we report the purification and structural characterization of oligosaccharides liberated from wheat bran soluble polysaccharides by ragi (Finger millet; *Eleusine coracana*, Indaf-15) malt xylanase (Chithra & Muralikrishna, 2008) by ESI-MS and ^{1}H NMR analysis. The prebiotic activity of the crude as well as the purified oligosaccharides is also tested in the present study.

2. Materials and methods

2.1. Materials

Wheat bran used for the present study was a gift from Flour Milling Baking & Confectionary Technology (FMBCT) Department, CFTRI, Mysore. HPLC (μ-Bondapak aminopropyl) and GLC (OV-225 and PEG-20 M) columns were obtained from Shimadzu Corporation, Kyoto, Japan. Termamyl (E.C. 3.2.1.1; from *Bacillus licheniformis*; *product code A3403*) and glucoamylase (E.C. 3.2.1.3; from *Aspergillus niger*; *product code A7420*) were purchased from Sigma Chemical Company, MO, USA. Microbial cultures were procured from National Dairy Research Institute (NDRI), Karnal. Microbiological culture media and media ingredients were obtained from HiMedia, Mumbai, India. All other chemicals and solvents were of analytical grade.

2.2. Isolation of soluble (SP) and insoluble polysaccharides (IP) from wheat bran

Wheat bran (100 g) was extracted with acetate buffer (400 ml, 0.05 mol/L, pH 5.0) for 2 h and digested with termamyl (500 μl) at 95 °C for 2 h followed by glucoamylase (2 ml) at 55 °C for 48 h to remove the associated starch. After the enzymatic hydrolysis of wheat bran the starch content was determined by glucose oxidase method (Trinder, 1969) using glucose assay kit (Cayman chemical company, USA). The extract was centrifuged (10000 *g* for 10 min) to obtain residue (designated as acetate buffer unextractable polysaccharide) and supernatant consisting of glucose (emanated from starch) as well as acetate buffer extractable polysaccharides. The supernatant (330 ml) was concentrated to 50 ml by flash evaporation and precipitated with three volumes of ethanol (95 ml/100 ml). Precipitate thus obtained was separated out from the glucose-containing supernatant by centrifugation followed by overnight dialysis (dialysis bag cut off, 5000 Da) against double distilled water and lyophilized to obtain acetate buffer extractable soluble polysaccharides (SP). The acetate buffer unextractable polysaccharide obtained after centrifugation was dried by solvent exchange, i.e., graded ethanol (80, 90 and 95 ml/100 ml) followed by methanol (99.9 ml/100 ml) and diethyl ether (99.9 ml/100 ml). The dried sample obtained after this series of solvent exchange was designated as insoluble polysaccharides (IP) (Rao & Muralikrishna, 2006).

2.3. Chemical composition of polysaccharides (SP & IP) and oligosaccharides by GLC

Wheat bran polysaccharides (10 mg) were hydrolyzed with 1 ml of sulphuric acid (72 ml/100 ml) at ice-cold temperature and finally diluted to 8 ml with water and the acid hydrolysis was carried out in a boiling water bath at 100 °C for 10–12 h. Purified oligosaccharides liberated from wheat bran SP were dissolved in water (5 mg/0.2 ml) and hydrolyzed with 2 mol equi/L sulphuric acid for 8–10 h. The above mixtures were neutralized with barium carbonate (solid) and filtered. To the filtrate obtained, Amberlite IR 120 H^{+} resin was added and kept for 6–8 h with intermittent mixing. Amberlite IR 120 H^{+} resin was removed by filtration and the deionized filtrate was concentrated by evaporation. The concentrated filtrate containing the monosaccharides was taken in a stoppered tubes and Sodium borohydride (20 mg) was added. The tubes were kept at room temperature for 4–6 h. Acetic acid (2 mol equi/L) was added to remove the excess borohydride and the resultant boric acid was removed with methanol (1 ml × 4). After the methanol wash, the tubes were immediately kept in the dessicator. Acetic anhydride and pyridine (1:1) were added to the dry glycitols and the mixture was kept at 100 °C for 2 h. After acetylation, the excess reagents were removed with water and toluene wash (4 × 1 ml each). The alditol acetates were extracted with chloroform, filtered through glass wool and dried by flushing nitrogen. The derivatives were taken in known amount of chloroform (Sawardekar, Slonekar, & Jeanes, 1965) and analyzed by Shimadzu GLC system (GC-15 A) fitted with flame ionization detector and CR4-A monitor and OV-225 stainless steel column (8 ft × 1/8∈).

2.4. Uronic acid estimation in wheat bran non-starch polysaccharides

Uronic acid content in wheat bran non-starch polysaccharides was determined by carbazole method (Knutson & Jeanes, 1968). Concentrated sulphuric acid (3 ml, 98%) was added to the wheat bran non-starch polysaccharides (10 mg/0.5 ml, kept in ice-cold temperature) and the solutions were thoroughly mixed. The above mixture was kept in a boiling water bath for 20 min, cooled and then added carbazole solution (0.1 ml, 0.1 ml/100 ml – prepared by dissolving recrystallized carbazole in alcohol), kept in dark for 2 h and the O.D. was read at 530 nm. Uronic acid content was determined by referring to the standard graph prepared by using D-glucuronic acid.

2.5. Protein determination

Protein concentration of the purified ragi xylanase was determined according to the dye binding method of Bradford (Bradford, 1976) with bovine serum albumin (BSA) as standard.

2.6. Hydrolysis of wheat bran soluble polysaccharides (SP) by purified ragi xylanase and their purification

Wheat bran soluble polysaccharide (20 mg/500 μl 0.1 mol/L acetate buffer, pH 5.0) was hydrolyzed with purified ragi xylanase (240 μg/50 μl 0.1 mol/L acetate buffer, pH 5.0) (Chithra & Muralikrishna, 2008) at 50 °C for 150 min. The reaction was stopped by the addition of three volumes of ethanol (70 ml/100 ml) and the hydrolyzate containing the oligosaccharides liberated from wheat bran SP were separated out from the undegraded polysaccharides by centrifugation (10000 *g* for 10 min).

The hydrolyzate containing the oligosaccharides was concentrated by flash evaporation (1 ml) at 30 °C and fractionated on Biogel P-2 column (0.9 × 105 cm). Water (triple distilled) was used as the eluent at a flow rate of 6 ml h^{-1} (Guillon et al., 2004). The oligosaccharide concentration in individual fractions were determined by phenol sulphuric acid method and the procedure is as follows.

An aliquot of the oligosaccharide solution was taken and made upto 0.5 ml with deionized water. To the solution, Phenol (0.3 ml, 5 ml/100 ml) was added followed by concentrated sulphuric acid (H_2SO_4, 1.8 ml, 100%). The absorbance was read at 480 nm after 20 min incubation at room temperature. Xylose (5–25 μg) was taken as reference sugar for preparing the standard curve (Mckelvy & Lee, 1969).

The oligosaccharides purified on Biogel P-2 were passed through a Millipore filter (0.2 μ) and purified on μ-Bondapak-NH_2 column (4.1 mm × 30 cm) fitted to Shimadzu HPLC system equipped with refractive index detector. Acetonitrile:water (75:25) was used as the eluent system at a flow rate of 0.7 ml/min.

2.7. Characterization of purified oligosaccharides by electrospray ionization mass spectrometry (ESI-MS) and ^1H NMR

The purified oligosaccharides (1 mg/ml in double distilled water) were injected directly to the Alliance, Waters 2695 mass spectrometer and mass spectra were recorded using positive mode electrospray ionization with the following operational conditions, i.e., capillary voltage 3.5 kV, core voltage 100 V, source temperature 80 °C, disolvation temperature 150 °C, core gas (Argon) 35 lt h^{-1} and disolvation gas (Nitrogen) 500 lt h^{-1}.

The ^1H NMR spectra of purified oligosaccharides (1.5 mg in D_2O) were recorded using Bruker 500 spectrometer operating at 500 MHz at 27 °C with tetramethyl silane (TMS) as the internal standard. A total of 16 pulses were collected with pulse retention time of 5 s and pulse angle 30°.

2.8. In vitro fermentation experiments

Bifidobacterium adolescentis NDRI 236, *Bifidobacterium bifidum* 229 ATCC 29521, *B. bifidum* NCDO 2715, *Lactobacillus brevis* 01 NDRI strain RTS, *Lactobacillus plantarum* 020 NDRI strain 184, *Pediococcus pentosaceus* 035 NCDO 813, *P. pentosaceus* ATCC 8081 were the microorganisms used for the present study. *B. adolescentis* NDRI 236, *B. bifidum* 229 ATCC 29521, *B. bifidum* NCDO 2715 were grown in MRS broth containing cysteine–HCl (0.05 ml/100 ml, 100 μl/10 ml MRS broth) at 37 °C in an anaerobic chamber for 48 h. All other microorganisms used for the present study such as *L. brevis* 01 NDRI strain RTS, *L. plantarum* 020 NDRI strain 184, *P. pentosaceus* 035 NCDO 813, *P. pentosaceus* ATCC 8081 were grown in MRS broth at 37 °C for 48 h. All the microbial cultures were stored at 4 °C after 48 h of incubation and sub-cultured within 7 days interval.

The bacterial cultures grown individually in MRS broth were subjected to centrifugation (3000 *g* for 15 min at 15 °C) after 24 h of incubation at 37 °C. The resultant cells were suspended in 0.85 ml/100 ml saline. Serial dilutions were prepared to get the requisite cell population (10^{-6}). All the experiments were carried out under sterile conditions.

Crude as well as purified xylo-oligosaccharides liberated from wheat bran SP were used in the present study to determine their prebiotic nature *in vitro*. The xylo-oligosaccharides liberated from wheat bran by purified ragi xylanase treatment were filtered through membrane filter (0.22 mμ, Millipore) and added at 5 mg/2 ml level to MRS broth (without dextrose, 2 ml) and inoculated with 100 μl of culture suspension giving 200 CFU (Colony Forming Unit) and incubated at 37 °C for 48 h. With respect to *Bifidobacterium* cultures, Cysteine–HCl (0.05 ml/100 ml, 20 μl) was added to the culture broth and incubation was carried out in an anaerobic chamber. MRS without any sugar supplement is taken as the control media. Growth characteristics were monitored by measuring pH and absorbance (600 nm) of culture broth after 48 h of incubation. Microbial cultures were taken out after 48 h of incubation, centrifuged (3000 ×*g* for 20 min at 15 °C) and oven dried to determine the dry cell mass. The resultant supernatants were analyzed for short-chain fatty acids (SCFA).

2.9. Enzyme assays

Xylanase, β-D-xylopyranosidase, α-L-arabinofuranosidase and α/β-D-galactopyranosidase activities were determined in the culture broth (24 h old) of tested microorganisms.

To determine xylanase activity 1 ml of larchwood xylan (5 mg/ml in acetate buffer, pH 5.0, 0.1 mol/L) was incubated with 20 μl of sample at 50 °C for 1 h. The reaction was stopped by the addition of DNS (1.0 ml) and the reducing sugar liberated was quantified by DNS (dinitrosalicylic acid) method (Miller, 1959). Xylanase activity is expressed as micromoles of xylose released under assay conditions. One unit of activity is defined as the amount of enzyme required to release 1 μmol of xylose/min under the experimental conditions. β-D-xylopyranosidase, α-L-arabinofuranosidase, α-D-galactopyranosidase and β-D-galactosidase activities were determined by monitoring the release of p-nitrophenol from their respective p-nitrophenol glycosides (4-Nitrophenyl β-D-xylopyranoside – Product code N2132; 4-Nitrophenyl α-L-arabinofuranoside – Product code N3641; 4-Nitrophenyl β-D-galactopyranoside – Product code N1252; 4-Nitrophenyl α-D-galactopyranoside – Product code N0877) and acetate (4-Nitrophenyl acetate – Product code N8130, Sigma Chemical Company, MO, USA).

Substrate (0.5 ml, 2 mmol in sodium phosphate buffer, 0.1 mol/L, pH 5.7) was incubated with sample (20 μl) for 1 h at 37 °C (Beldman, Osuga, & Whitaker, 1996). The reaction was stopped by the addition of saturated solution of sodium tetraborate (0.5 ml) and absorbance was read at 400 nm. One unit of activity is defined as the amount of enzyme required to liberate 1 μmol of p-nitrophenol/min under assay conditions. P-nitrophenyl acetate was used as the substrate to determine acetyl esterase activity. Substrate (0.5 ml, 2 mmol in sodium phosphate buffer 0.1 mol/L, pH 6.5) was incubated with sample (20 μl) for 1 h at 25 °C. The reaction was stopped by the addition of saturated solution of sodium tetraborate (0.5 ml) and absorbance was read at 400 nm. One unit of activity is defined as the amount of enzyme required to liberate 1 μmol of p-nitrophenol/min under assay conditions.

2.10. SCFA analysis

The culture broth obtained after 48 h of incubation was centrifuged (3000 ×g, for 20 min at 15 °C) to separate out the culture supernatant from the bacterial cell mass. The culture supernatant was acidified with sulphuric acid (50 ml/100 ml) and extracted with diethyl ether (Karppinen, Liukkonen, Aura, Forssell, & Poutanen, 2000) and analyzed for SCFA by GLC on PEG-20 M (also called carbo Wax, length – 3 mt, Id – 3 mm) with column, injector and detector temperatures of 120, 220 and 230 °C, respectively (Silvi, Rumney, Cresci, & Rowland, 1999). Nitrogen (40 ml/min) was used as the carrier gas. Acetate (Product code 71251), propionate (Product code 81910) and butyrate (Product code 19215) were used as the standard SCFAs (Sigma Chemical Company, MO, USA).

3. Results and discussion

3.1. Isolation of soluble polysaccharides (SP) from wheat bran

The major problem associated with the isolation of soluble polysaccharides from wheat bran is starch contamination. Termamyl followed by glucoamylase treatment efficiently removed starch (~63 g/100 g) and yielded wheat bran SP (35 g/100 g). Studies have been carried out on the water extractable non-starch polysaccharides from native and malted ragi and rice (Rao & Muralikrishna, 2006). It is reported that wheat flour contains ~25 g/100 g of the water extractable arabinoxylans (Barron et al., 2006). The neutral sugar composition of wheat bran SP consisted of arabinose and xylose in a ratio of 1:0.86 in the present study. In addition to arabinose (41.8 g/100 g) and xylose (48.6 g/100 g), rhamnose (0.72 g/100 g), mannose (0.44 g/100 g), galactose (5.6 g/100 g) and glucose (2.9 g/100 g) were found in minor amounts in wheat bran SP. Previous report shows that arabinose to xylose ratio of arabinoxylans from barley and malt were close to 1:0.65 (Dervilly et al., 2002). Water insoluble polysaccharides (IP) of wheat bran consisted mainly of arabinose and xylose in a ratio of 1:0.78 with trace amounts of rhamnose (0.44 g/100 g), galactose (0.84 g/100 g) and glucose (19.6 g/100 g). The uronic acid content of wheat bran SP and IP were found to be 1.0 and 3.5 g/100 g, respectively. A large portion of cereal arabinoxylans is reported as water unextractable (Maes & Delcour, 2002).

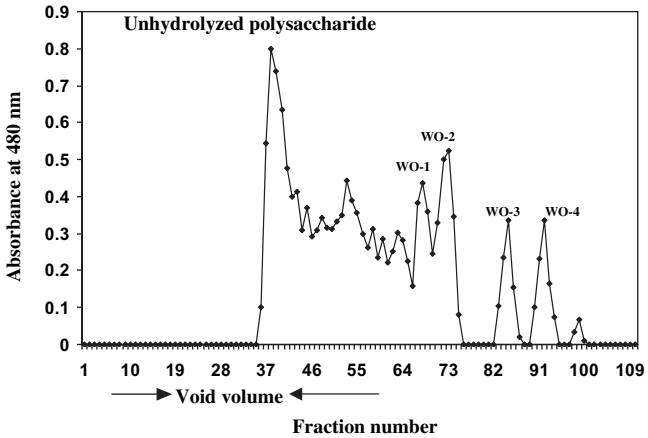

Fig. 1. Elution profile of oligosaccharides liberated from wheat bran SP on Biogel P-2. WO-1, WO-2, WO-3, and WO-4 represent the four major oligosaccharides liberated from wheat bran soluble polysaccharides. Water was used as the eluent at a flow rate of 6 ml h^{-1}.

3.2. Liberation of xylo-oligosaccharides from wheat bran SP and their purification

Soluble polysaccharides isolated from wheat bran were subjected to purified ragi xylanase treatment in order to obtain xylo-oligosaccharides (0.3 g/1 g). The crude oligosaccharides (25 mg/ml distilled water) were fractionated on Biogel P-2 column (Fig. 1). Undegraded or partially degraded polysaccharides were eluted out in the void volume, whereas the oligosaccharides eluted in the bed volume were separated out into four major peaks designated as WO-1, WO-2, WO-3 and WO-4. The purity of the individual

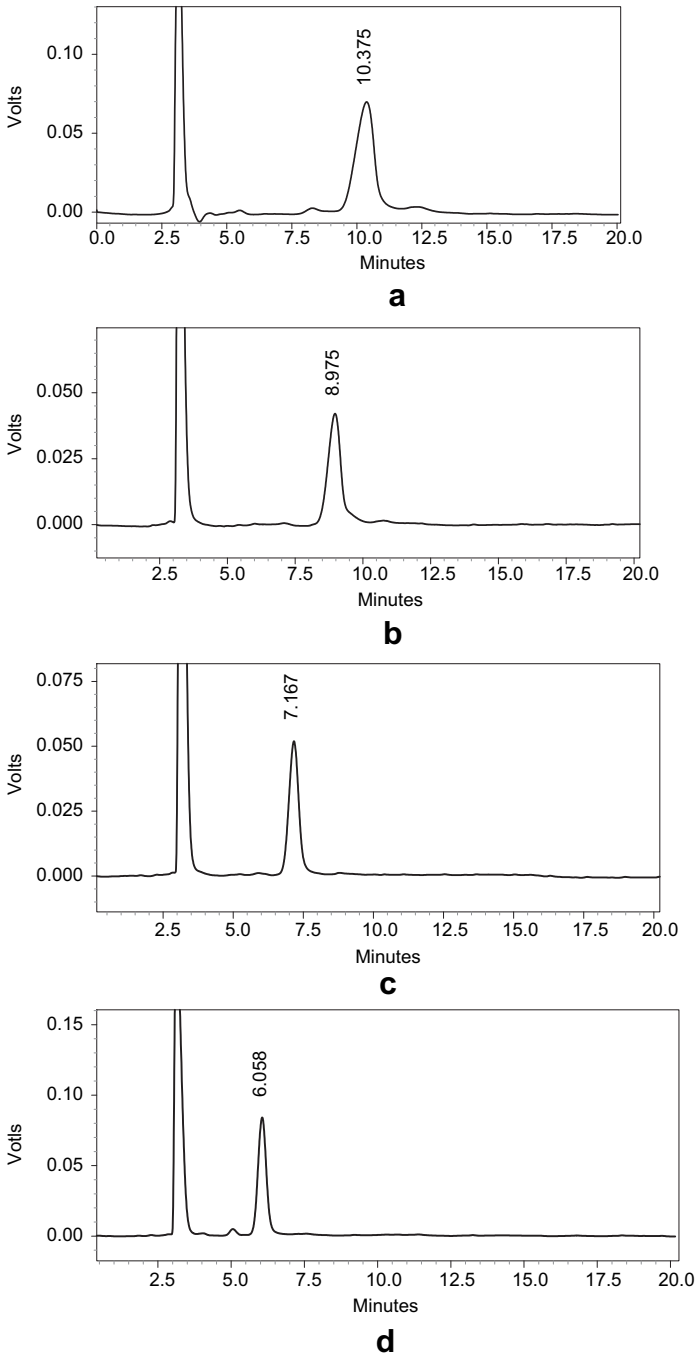

Fig. 2. HPLC profile of purified oligosaccharides liberated from wheat bran SP: (a) WO-1; (b) WO-2; (c) WO-3; (d) WO-4. Eluent system used is acetonitrile:water (75:25) at a flow rate of 0.7 ml/min. WO-1, WO-2, WO-3 & WO-4 represent the four major oligosaccharides liberated from wheat bran soluble polysaccharides.

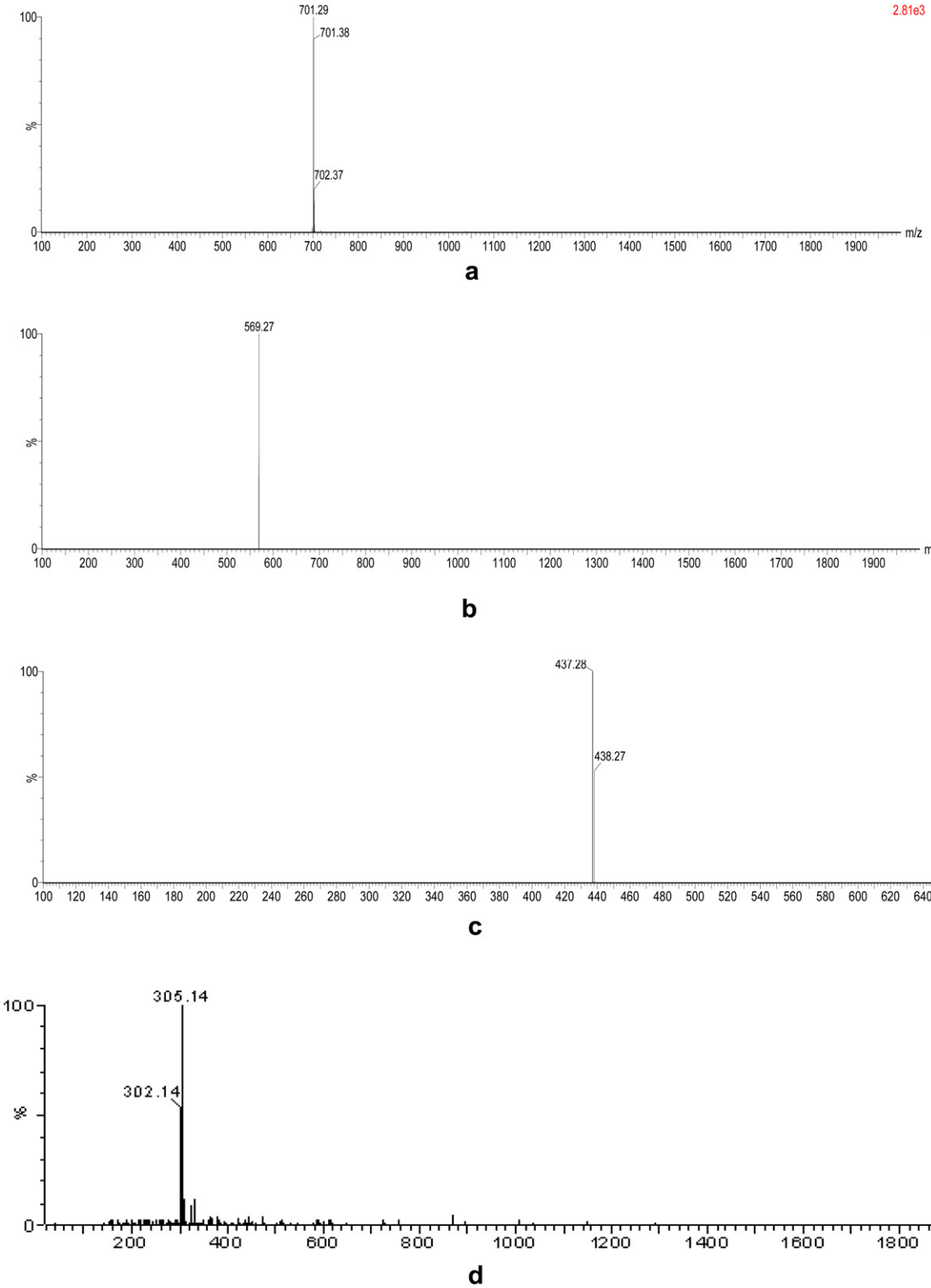

Fig. 3. ESI-MS of purified oligosaccharides:(a) WO-1; (b) WO-2; (c) WO-3; (d) WO-4. WO-1, WO-2, WO-3 & WO-4 represent the four major oligosaccharides liberated from wheat bran soluble polysaccharides.

oligosaccharide recovered from Biogel P-2 column was confirmed by HPLC as it showed a single peak (Fig. 2) WO-1 consisted of arabinose (31 g/100 g) and xylose (69 g/100 g) as analyzed by GLC. Similarly, Arabinose (42 g/100 g) and xylose (58 g/100 g) were found as the constituents of WO-2, whereas WO-3 and WO-4 consisted exclusively of xylose (100%).

3.3. ESI-MS

The HPLC-purified oligosaccharides were identified as cationised molecules $[M+ Na]^+$ in the ESI mass spectrum (Fig. 3). WO-1 showed preponderantly the presence of ion at *m/z* 701.29 which was identified as a pentasaccharide [678 + 23 (sodium adduct) = 701]. The ions observed at *m/z* 569.27 for WO-2 corresponds to a tetrasaccharide (546 + 23 = 569). Similarly, the signal at *m/z* 437.28 (WO-3) and 305.14 (WO-4) were identified as trisaccharide (414 + 23 = 437) and disaccharide, respectively (282 + 23 = 305). ESI mass spectra will not provide the unequivocal identification of the oligosaccharides since it fails to differentiate the mass losses due to arabinose and xylose having same molecular mass (Fernandez, Obel, Scheller, & Roepstorff, 2004). Hence, ^{1}H NMR studies have been carried out for the complete structural elucidation of the purified oligosaccharides.

3.4. ^{1}H NMR

The ^{1}H NMR spectra of the purified oligosaccharides liberated from wheat bran SP are shown in Fig. 4. With respect to WO-1, the

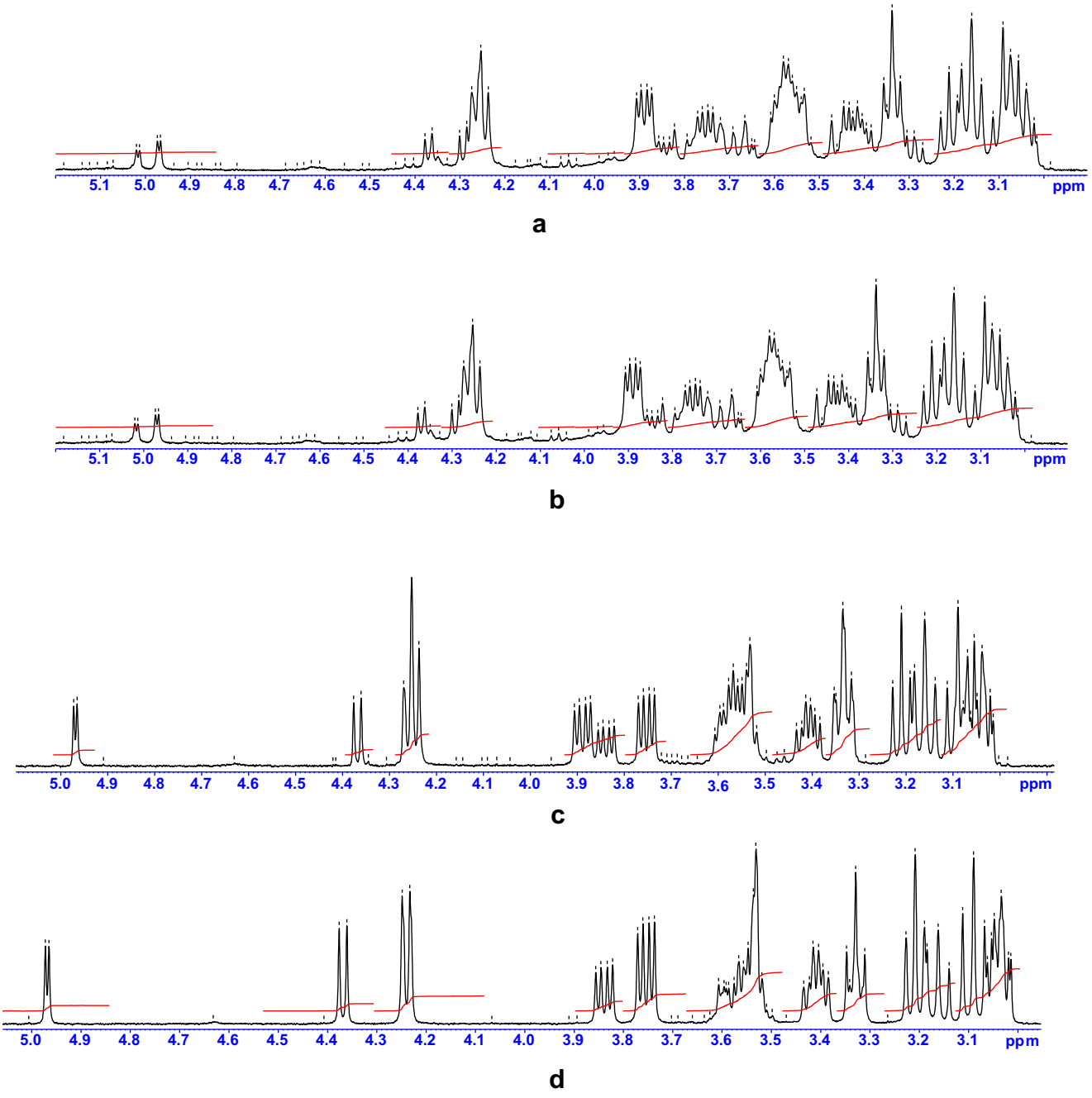

Fig. 4. ^{1}H NMR spectra of purified xylo-oligosaccharides: (a) WO-1; (b) WO-2; (c) WO-3; (d) WO-4. Purified oligosaccharide (1.5 mg) was dissolved in D_2O and Tetramethyl silane (TMS) was used as the internal standard. WO-1, WO-2, WO-3 & WO-4 represent the four major oligosaccharides liberated from wheat bran soluble polysaccharides.

chemical shifts of anomeric proton at 3.41, 3.583, 3.67, 3.95 correspond to H-2, H-3, H-4 and H-5, respectively, of *β-xylp-3*^{II}. Chemical shifts at 3.957, 4.2, and 3.82 correspond to H-3, H-4 and H-5 of arabinofuranose linked to xylopyranose (*α-Araf-A*^{3x3}) (Gruppen et al., 1992; Vietor et al., 1994). Oligosaccharide, WO-2 was identified as xylotetraose with characteristic chemical shifts of anomeric proton at 3.349, 3.748, 3.8226 and 4.1212 indicates that of H-2, H-3, H-4 and H-5 of *β-xyl-p-2*^{II}. Apart from these signals, the spectra showed signals at 4.1497 (H-2), 3.907 (H-3), 4.2739 (H-4) and 3.7944 (H-5) indicating the presence of arabinofuranose unit linked to O-3 of xylopyranose residue.

WO-3 was identified as xylotriose with chemical shifts of 3.2, 3.5494 and 3.77 corresponding to H-2, H-3 and H-4 of *β-xyl-p-1*. The chemical shift observed for WO-4 at 3.2, 3.547 and 3.771 corresponds to that of H-2, H-3 and H-4 of *β-xyl-p-1*.

The proton resonances corresponding to acetyl residue (2.22 ppm) and 4-O-methyl-D-glucuronic acid (H1-5.28 ppm) were not observed in the ^{1}H NMR spectra of any of the oligosaccharide liberated from wheat bran SP.

Probable structures of the purified xylo-oligosaccharides obtained from wheat bran SP are as follows:

WO-1

4 3 2 1

β-Xyl*p*- (1→4) β-Xyl*p*- (1→4) β-Xyl*p*-(1→4) - β-Xyl*p)*

↓

α- Ara *f*- (1→3)

A^{3X3}

WO-2:

3 2 1

β-Xyl*p*- (1→4) β-Xyl*p*- (1→4) β-Xyl*p)*

↓

α - Ara *f*- (1→3)

A^{3X2}

WO-3: Xyl*p*- (1→4) β-Xyl*p*- (1→4) β-Xyl*p*

WO-4:

Xyl*p*- (1→4) β-Xyl*p*

However, the arabinose to xylose ratio of wheat bran SP does not fall into the proposed structure of the oligosaccharide. This may be due to arabinose in furanosidic being labile sugar might have destroyed during hydrolysis and hence we are not getting the exact quantification.

3.5. Prebiotic (In vitro) activity of xylo-oligosaccharides liberated from wheat bran SP

3.5.1. Growth characteristics

The growth characteristics pattern of *Bifidobacteria* and *Lactobacilli* spp grown on crude as well as purified xylo-oligosaccharides proved their prebiotic nature *in vitro* compared to the control media having no sugar supplement. SCFA are produced as a result of the fermentation of NDOs resulting in the reduction of pH of the culture broth. The pattern of fermentation and the proportion of the SCFA liberated depend on the nature of the oligosaccharides (Berggren, Bjorck, & Nyman, 1993; Delzenne, 2003). A decrease in the pH of the culture broth and increase in O.D were observed for all the strains grown on crude as well as purified xylo-oligosaccharides after 48 h of incubation (Table 1). Maximum O.D was observed with respect to *B. adolescentis* NDRI 236 and *L. plantarum* NDRI strain 184. Purified individual oligosaccharides showed, moreover, the same growth pattern *in vitro*. The bifidogenic effect of the xylo-oligosaccharides was further confirmed by an increase in the dry cell mass of the tested microorganisms after 48 h of incubation period compared to the control. It is observed that xylobiose (WO-4) was efficiently utilized by the beneficial bacterial species compared to the crude oligosaccharides indicating slightly higher O.D. and dry cell mass. It has been reported that xylo-oligosaccharides are more effective for the gastrointestinal health compared to fructo-oligosaccharides (Hsu et al., 2004). The growth was found to be comparatively low in the case of *L. brevis* and *P. pentosaceus* NCDO 8081 indicating their lower efficiency to utilize the xylo-oligosaccharides liberated from wheat bran SP. The growth of the microorganism on particular oligosaccharide may be strain specific (Holtl, Miller-Fosmore1, & Cote, 2005). *B. bifidum* NCDO 2715 showed increased growth compared to *B. bifidum* ATCC 29521. Similarly, *P. pentosaceus* ATCC 8081 were more effectively grown on the xylo-oligosaccharides than *P. pentosaceus* NCDO 813. The bifidogenic nature of xylo-oligosaccharides has been reported in human by Rycroft et al. (2001) and in rats by Campbell, Fahey, and Wolf (1997).

3.5.2. Enzyme activity

The hydrolytic enzymes produced by the microorganisms help in the digestion of the xylo-oligosaccharides liberated from wheat bran SP which resist digestion by the gastrointestinal enzymes. The 24 h old cultures showed β-D-xylanase, β-D-xylosidase, α-L-arabinofuranosidase, α/β-galactosidases and acetyl esterase activities. High activity of xylanase (440–694 μU ml^{-1}) was detected in the culture broth of all the tested microorganisms grown on crude as well as purified oligosaccharides (Table 2). Arabinofuranosidase activity was found to be higher than xylosidase, whereas α/β-galactosidase activity was almost in the same range. Acetyl esterase activity was found to be negligible (0.24–0.36 μU ml^{-1}).

3.5.3. In vitro fermentation and SCFA analysis

Lactobacilli and *Bifidobacteria* ferment carbohydrates through a pathway mediated by the glycolytic enzymes in which the main end products are SCFA (Grootaert et al., 2007). Acetic, propionic and butyric acids are the major SCFA produced during fermentation of carbohydrates in the large bowel (Ruppin, Bar-Meir, Soergel, Wood, & Schmitt, 1980). Acetate was the chief SCFA released by the microorganisms due to fermentation of both crude as well as purified xylo-oligosaccharides. This is in accordance with the earlier report (Smiricky-Tjardes et al., 2003). Butyrate was detected only in the culture broth of *B. bifidum* ATCC 29521, whereas propionate was present in the culture broth of *P. pentosaceus* ATCC 8081 and *P. pentosaceus* NCDO 813. But, the amount of butyrate and propionate liberated as a result of *in vitro* fermentation was found to be very less. Lactate concentration is not determined in the present study. SCFA liberated due to *in vitro* fermentation resulted in the reduction of pH of the culture broth. Such decrease in pH correlates with the population growth of the beneficial microbes (Berggren et al., 1993). Moreover, the shift in intestinal pH due to the acidic metabolites as a result of carbohydrate fermentation inhibits the growth of the undesirable pathogenic bacteria (Gibson & Wang, 1994). Oligosaccharide fermentation patterns obtained *in vitro* might be used

Table 1
Growth characteristics of microorganisms grown on crude as well as purified oligosaccharide(s) liberated from Wheat bran SP after 48 h of incubation.

Microorganism	Xylo-oligosaccharide	O.D.	pH	Cell mass (mg/1.5 ml broth)
Bifidobacterium adolescentis NDRI 236		0.070 ± 0.006[a]	7.2 ± 0.4	1.3 ± 0.1
	Crude	2.38 ± 0.05	5.1 ± 0.3	10.0 ± 0.5
	WO-1	2.32 ± 0.03	5.3 ± 0.2	10.0 ± 0.5
	WO-2	2.41 ± 0.06	5.1 ± 0.4	11.0 ± 0.3
	WO-3	2.48 ± 0.04	5.0 ± 0.3	11.0 ± 0.4
	WO-4	2.48 ± 0.05	5.0 ± 0.3	11.0 ± 0.2
Bifidobacterium bifidum		0.068 ± 0.005[a]	6.9 ± 0.3	1.0 ± 0.07
	Crude	0.773 ± 0.05	5.8 ± 0.25	6.0 ± 0.52
ATCC 29521	WO-3	0.79 ± 0.04	5.8 ± 0.22	6.0 ± 0.5
Bifidobacterium bifidum, NCDO 2715		0.06 ± 0.003[a]	7.5 ± 0.3	1.1 ± 0.08
	Crude	1.892 ± 0.1	5.5 ± 0.24	8.0 ± 0.4
	WO-3	2.0 ± 0.08	5.3 ± 0.2	8.0 ± 0.33
Lactobacillus plantarum NDRI strain 184		0.039 ± 0.003[a]	6.9 ± 0.4	1.0 ± 0.05
	Crude	2.408 ± 0.05	5.1 ± 0.22	10.5 ± 0.3
	WO-1	2.4 ± 0.05	5.2 ± 0.2	11.0 ± 0.32
	WO-2	2.42 ± 0.03	5.2 ± 0.3	11.0 ± 0.4
	WO-3	2.46 ± 0.04	5.2 ± 0.26	11.0 ± 0.35
	WO-4	2.46 ± 0.05	5.2 ± 0.3	11.0 ± 0.37
Lactobacillus brevis 01 NDRI strain RTS		0.04 ± 0.002[a]	7.1 ± 0.2	1.4 ± 0.07
	Crude	0.420 ± 0.01	6.4 ± 0.18	4.0 ± 0.37
	WO-3	0.44 ± 0.03	6.4 ± 0.14	4.0 ± 0.43
Pediococcus pentosaceus, NCDO 813		0.031 ± 0.003[a]	7.5 ± 0.2	1.0 ± 0.04
	Crude	0.417 ± 0.02	6.2 ± 0.14	4.0 ± 0.25
	WO-3	0.45 ± 0.025	6.0 ± 0.2	4.0 ± 0.3
P.pentosaceus, ATCC 8081		0.35 ± 0.002[a]	6.9 ± 0.15	1.2 ± 0.05
	Crude	0.924 ± 0.03	5.8 ± 0.1	7.4 ± 0.36
	WO-3	0.963 ± 0.05	5.8 ± 0.12	7.5 ± 0.4

[a] Represents control (media without sugar supplement); WO-1, WO-2, WO-3 & WO-4 represent the four major oligosaccharides liberated from wheat bran SP; The values are represented with standard error, $n = 3$.

to predict behavior *in vivo*. Short-chain fatty acids have been implicated in a number of important physiological events. These include utilization of butyrate as the preferred energy substrate and prevention of colon cancer in humans (Rycroft et al., 2001). Acetate is mainly metabolized in human muscle, kidney, heart and brain. Propionate has been suggested to spare amino acids that would be used in gluconeogenesis in the postabsorptive state (Demigne & Remesy, 1991).

Table 2
Enzyme activities (μU/ml) in 24 h old culture broth of microorganisms grown on crude as well as purified xylo-oligosaccharides.

Microorganism	Xylo-oligosaccharide	Xylanase	Xylosidase	Arabinofuranosidase	α-galactosidase	β-galactosidase	Acetyl esterase
Bifidobacterium adolescentis NDRI 236	Crude	560 ± 12	5.4 ± 0.4	9.6 ± 0.3	5.1 ± 0.3	5.1 ± 0.27	0.24 ± 0.02
	WO-1	570 ± 15	5.5 ± 0.36	9.0 ± 0.35	5.0 ± 0.36	5.2 ± 0.3	0.24 ± 0.03
	WO-2	570 ± 13	5.7 ± 0.35	9.0 ± 0.4	5.1 ± 0.4	5.2 ± 0.3	0.24 ± 0.02
	WO-3	780 ± 16	6.6 ± 0.2	11.0 ± 0.12	5.1 ± 0.38	5.2 ± 0.4	3.5 ± 0.18
	WO-4	830 ± 17	7.2 ± 0.32	11.0 ± 0.15	5.1 ± 0.35	5.2 ± 0.36	3.5 ± 0.2
Bifidobacterium bifidum ATCC 29521	Crude	500 ± 10	8.9 ± 0.3	9.6 ± 0.2	5.4 ± 0.28	7.7 ± 0.25	0.24 ± 0.05
	WO-3	510 ± 12	8.9 ± 0.29	11.5 ± 0.15	5.4 ± 0.3	7.7 ± 0.3	0.46 ± 0.02
Bifidobacterium bifidum NCDO 2715	Crude	666 ± 8	7.0 ± 0.2	10.0 ± 0.08	5.4 ± 0.23	6.6 ± 0.3	0.24 ± 0.03
	WO-3	680 ± 5	8.4 ± 0.3	11.0 ± 0.1	5.1 ± 0.28	7.7 ± 0.41	0.32 ± 0.02
Lactobacillus plantarum NDRI strain 184	Crude	440 ± 13	2.0 ± 0.02	7.7 ± 0.24	2.7 ± 0.03	2.6 ± 0.02	0.2 ± 0.04
	WO-1	500 ± 16	2.3 ± 0.02	7.7 ± 0.3	2.7 ± 0.04	2.5 ± 0.04	0.28 ± 0.03
	WO-2	500 ± 13	2.5 ± 0.03	8.1 ± 0.22	2.7 ± 0.02	2.5 ± 0.04	0.28 ± 0.02
	WO-3	666 ± 15	2.6 ± 0.02	8.99 ± 0.2	2.9 ± 0.03	3.6 ± 0.03	0.36 ± 0.05
	WO-4	694 ± 12	2.6 ± 0.04	12 ± 0.5	3.0 ± 0.02	3.6 ± 0.02	0.36 ± 0.04
L. plantarum NDRI strain 184	Crude	666 ± 14	2.3 ± 0.04	12.5 ± 0.1	2.7 ± 0.02	5.1 ± 0.02	0.2 ± 0.02
	WO-3	694 ± 10	2.4 ± 0.03	12.0 ± 0.15	3.0 ± 0.04	4.9 ± 0.02	0.24 ± 0.02
Lactobacillus brevis 01 NDRI strain RTS	Crude	666 ± 14	2.3 ± 0.04	12.5 ± 0.1	2.7 ± 0.02	5.1 ± 0.02	0.2 ± 0.02
	WO-3	694 ± 10	2.4 ± 0.03	12.0 ± 0.15	3.0 ± 0.04	4.9 ± 0.02	0.24 ± 0.02
Pediococcus pentosaceus NCDO 813	Crude	690 ± 13	3.3 ± 0.05	8.99 ± 0.24	2.7 ± 0.04	3.9 ± 0.03	0.35 ± 0.05
	WO-3	694 ± 15	3.3 ± 0.03	11.0 ± 0.4	2.7 ± 0.05	3.9 ± 0.02	0.36 ± 0.05
P. pentosaceus ATCC 8081	Crude	560 ± 15	2.5 ± 0.03	7.7 ± 0.2	2.7 ± 0.03	3.6 ± 0.02	0.38 ± 0.03
	WO-3	680 ± 13	2.5 ± 0.02	7.7 ± 0.23	2.9 ± 0.03	3.9 ± 0.03	0.4 ± 0.02

WO-1, WO-2, WO-3 & WO-4 represent the four major oligosaccharides liberated from wheat bran SP. The values are represented with standard error, $n = 3$.
The control (without XOS) did not give any enzyme activity, hence not shown in the table.

4. Conclusions

Wheat bran soluble polysaccharides were treated with purified ragi xylanase in order to obtain prebiotic xylo-oligosaccharides (0.3 g/1 g SP). The purified oligosaccharides *viz*, WO-1 and WO-2, were identified as arabinose containing xylotetraose and xylotriose, respectively, whereas WO-3 and WO-4 were identified as unsubstituted xylotriose and xylobiose, respectively, by ESI-MS and ^{1}H NMR studies. The *in vitro* studies carried out on *Bifidobacteria* and *Lactobacilli* sp. suggest the prebiotic nature of xylo-oligosaccharides. The growth characteristic pattern showed that *B. adolescentis* NDRI 236 and *L. plantarum* NDRI strain 184 were able to efficiently utilize the xylo-oligosaccharides liberated from wheat bran SP compared to other tested microorganisms. Eventhough no significant difference is observed between crude and purified oligosaccharides consumption by the microorganisms, a slight increase in bacterial growth was noticed for oligosaccharide having low degree of polymerization (xylobiose & xylotriose). *L. brevis* and *P. pentosaceus* NCDO 8081 were less efficient in utilizing the xylo-oligosaccharides among all the tested microorganisms. The utilization of the oligosaccharides by the bacterial strain was found to be strain specific wherein, *B. bifidum* NCDO 2715 showed increased growth compared to *B. bifidum* ATCC 29521. Similarly, *P. pentosaceus* ATCC 8081 grew more effectively on the oligosaccharides than *P. pentosaceus* NCDO 813. Maximum xylanase activity was detected in the culture broth of *B. adolescentis* NDRI 236. Arabinofuranosidase activity was found to be higher than xylosidase, whereas α/β-galactosidase activity was almost in the same range. Acetate was the chief SCFA liberated due to *in vitro* fermentation of xylo-oligosaccharides.

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