Microwave-assisted Enzymatic Hydrolysis to Produce Xylooligosaccharides from Rice Husk Alkali- soluble Arabinoxylan

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Abstract

This study aimed to produce xylooligosaccharide (XOS) and arabino-xylooligosaccharide (AXOS) from rice husk (RH) using microwave treatment combined with enzymatic hydrolysis and evaluate their prebiotic properties. The RH was pretreated by a microwave heating process at 140, 160 and 180°C for 5, 10, and 15 min to obtain crude arabinoxylan (AX). The results emphasized that increasing microwave pretreatment time increased sugar content. The crude AX was then extracted with 2% (w/v) sodium hydroxide at 25°C for 24 h. The obtained AX was then used as a substrate for XOS production by commercial xylanases. The results showed that the oligosaccharide produced by Pentopan Mono BG and Ultraflo Max provided xylobiose and xylotriose as the main products. Interestingly, AXOS was also present in the oligosaccharide product. Furthermore, the oligosaccharides obtained were able to promote the growth of *Lactobacillus* spp. and to resist degradation more than 70% after exposure to simulated human digestion.

1. Introduction

Rice husks (RH) are a sheath that protects the rice seed during the growth period. As a by-product of rice production, the milled white rice process results in approximately 28% of RH during milling. RH products are mostly used as solid fuel, carbonised after burning, and the remaining ash after combustion. Nevertheless, previous studies have reported that RH is the remaining lignocellulosic material, which consists of cellulose and hemicellulose at 36% and 29%, respectively (Worasuwannarak et al., 2007). The hemicellulose structure of RH contains long chain xylose units as the backbone, called xylan. Xylan is a group of hemicelluloses, which are composed of β-1,4-linked xylose residues with or without side branches of α-arabinofuranose and α-glucuronic acids (Kulkarni et al., 1999). Arabinoxylans (AX) are one of the four main families of xylans, which consists of a backbone of β-(1,4)-linked xylose residues, substituted with arabinose residues at the C(O)-2 and/or C(O)-3 positions (Dornez et al., 2009).

The conversion of AX by enzymatic hydrolysis into XOS and AXOS has gained considerable interest because of the mild conditions used and the specific products obtained. XOS is more effective than other oligosaccharides with some properties. XOS hold a status for ‘intestinal regulation’, and effects on bowel habit are associated with improvement of gut microbiota. Especially, the growth of bifidobacteria includes *B. breve*, *B. lactis*, *B. adolescentis*, and *B. bifidum* which have been demonstrated to ferment XOS *in vitro* and *in vivo* (Stahl et al., 2007). XOS and AXOS production is usually performed by endo-xylanases, which are negligible in the presence of β-xylosidase and exo-xylanase activities to prevent high xylose production. This end-product might affect the enzyme activity and overall efficiency of XOS and AXOS production (Akpinar et al., 2009). This research mainly focused on two xylanases that belong to glycoside hydrolase families, particularly families 10 and 11. GH11 xylanases preferentially cleave the unsubstituted regions of the AX backbone, whereas GH10 enzymes are less hampered by the presence of substituents along the xylan backbone, cleave the decorated regions (Biely et al., 1997). Therefore, it can be mentioned here that the difference in substrate specificity has important implications in the deconstruction of xylan in biomass (Dodd & Cann, 2009).

In recent years, there have been many techniques used for AX extraction. Extraction through fermentation or solvent can reduce the loss of important substances compared to the heating process. However, these methods are time consuming and produce a number of chemical wastes. Microwave extraction may overcome these disadvantages because of its short processing time and low amount of solvent (Aguilar-Reynosa et al., 2017). Microwave irradiation of biomass is a promising pretreatment process because it utilises thermal and specific effects generated by microwaves in aqueous environments (Keshwani & Cheng, 2010). Other heating processes such as hydrothermal treatment using high temperatures for a longer time may cause the loss of important substances including large amounts of monosaccharides. Gissibl et al. (2018) recently revealed that microwave pretreatment at 170°C for 2 min could enhance the enzymatic production of soluble β-1,3-glucans. Using microwave at 200°C for 5 min significantly increased the oligosaccharides extraction yield from Spruce (Palm & Zacchi, 2003). Moreover, Coelho et al. (2014) reported that microwave superheated water at 210°C with dilute alkali could recover 43% of AX and AXOS from brewers' spent grain extraction. The former study report that the cleavage of alkali-labile linkages between hemicellulose and other associated constituents, depending on its performance nature (Kundu et al., 2018).

A prebiotic is defined as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Gibson et al., 2010). AX can be transformed into XOS and AXOS. These substrates are considered to be functional foods due to their potential prebiotic properties. XOS and AXOS approach the colon intact and serve as a carbon source for bacteria, including *Bifidobacterium* and *Lactobacillus* (Grootaert et al, 2007). Probiotic fermentation helps in the development of short chain fatty acids such as acetate, propionate, and butyrate, which provide the host with metabolic energy and help in intestinal acidification (Swennen et al., 2006).

This study aimed to investigate the effects of temperature and time of microwave pretreatment on RH-derived AX extraction in order to produce XOS and AXOS by GH10 and GH11 xylanases. And to evaluate the growth promotion effect on lactic acid bacteria and digestion-resistance in *in vitro* human digestion simulation to emphasis on emerging prebiotic potential of the XOS and AXOS. This research will maximize the use of rice husk by-product as an alternative prebiotic source.

2. Materials And Methods

2.1. Materials

The RH used in this study was kindly provided by a local rice milling plant in Phitsanulok province, Thailand. A milling process gave approximately 28% rice husk as a by-product. RH was dried in a hot-air oven at 40°C until the moisture content was lower than 10% (w/w) then crushed with a blender and sieved through a 40 μm mesh-screen. RH powder was stored in a zip lock bag at ambient temperature. The methods of association of official analytical chemists was used for determination of moisture, crude fiber, protein, fat, ash and carbohydrate of RH (AOAC, 2000).

Commercial xylanases, Ultraflo Max (700 U/mL from *Aspergillus oryzae* and *Trichoderma reesei*), and Pentopan Mono BG (2,500 U/g from *Thermomyces lanuginosus*) were purchased from Novozyme Co. Ltd., Denmark. All chemicals and solvents used in this research were of analytical grade. Xylose (Merck, Germany), arabinose (Sigma, Germany), mannose (Merck, Germany), galactose (Sigma, Germany), and glucose (Sigma, Germany) were used as standards for determination of carbohydrate composition. A mixture of arabinose (A1) (Sigma, USA), xylose (X1) (Merck, Germany), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), xylohexaose (X6), (Wako, Japan), 2^3-α-L-arabinofuranosyl-xylotriose (A2XX), 3^2-α-L-arabinofuranosyl-xylobiose (A3X), 3^3-α-L-arabinofuranosyl-xylotetraose (XA3XX), and 2^3,3^3-di-α-L-arabinofuranosyl-xylotriose (A2,3XX) (Megazyme, Ireland) were used as standards for the determination of oligosaccharides. Commercial prebiotics were used to compare the prebiotic properties with obtained oligosaccharide, 95% commercial XOS (XOS95P) was purchased from AWBIO, Taiwan, Resistant maltodrextin (RMD) and inulin were purchased from Blenntag Ingredients, Thailand. Simulated human digestion enzymes, α-amylase from *Aspergillus oryzae* (40,000 U/mL), pepsin from porcine gastric mucosa (3200 U/mg), and pancreatin from porcine pancreas (8X USP) (Sigma, Germany) were used as simulated human digestion enzymes.

2.2. Pretreatment of RH

One gram of previously prepared RH powder was soaked in 20 mL of acetone and ethanol mixture in the ratio of 1:2 (v/v) at ambient temperature for 24 h. The RH residue was filtered through Whatman No. 1 filter paper and washed with boiling water After washed again with distilled water, it was dried at 45°C for 24 h in a hot air oven to obtain extractive-free RH and used in microwave pretreatment.

2.3. Determination of Carbohydrates composition

The determination of structural carbohydrates of extractive-free RH and RH-WUAX were modified from Jaichakan et al. (2019a). Briefly, 0.4 g of extractive-free RH was pre-hydrolysed with 4.5 mL of 72% sulphuric acid and mixed for 30 min in a mortar. Upon completion of pre-hydrolysis, the slurry was diluted to a final acid concentration of 4% by adding 84 mL distilled water and autoclaved for 1 h at 121°C. After completion of the autoclave cycle, an approximately 10 mL aliquot was transferred and neutralised to pH 5–6 with calcium carbonate. This aliquot was used to determine structural carbohydrates by the high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD, Dionex ICS-5000 Ion Chromatography, Thermo Scientific, Bellefonte PA, USA) with a Dionex CarboPac PA-1 column (250 mm × 4 mm) and a guard column (50 mm × 4 mm) at a flow rate of 1.0 mL/min. The post-column pump operated at a flow rate of 0.5 mL/min with 300 mM sodium hydroxide. A stepwise linear gradient was applied over 20 min with 100% distilled water and was applied over 16 min by mixing solutions of 200 mM sodium hydroxide and 200 mM sodium acetate in 170 mM sodium acetate. Eluted oligosaccharides were monitored by PAD detection using gold electrode. The mixture of xylose, arabinose, mannose, galactose, and glucose were used as a calibration standard.

The sugar profile of the XOS-containing liquor was determined by HPAEC with a Dionex CarboPac PA-200 column (250 mm × 4 mm) and a guard column (50 mm × 4 mm) at a constant flow rate of 0.4 mL/min.

The sample pumps gradient elution of the neutral carbohydrate was performed as described by McCleary et al. (2015). Oligosaccharides were identified using A1, X1-X6, A2XX, A3X, XA3XX, and A2,3XX as standards.

2.4. Microwave pretreatment of extractive-free RH

A closed-vessel microwave digestion system equipped with a 10 position rotor and capable of delivering 1600 W of Power (ETHOS 1600, Milestone Inc., Sorisole, Italy) was used for sample pretreatment. The temperature of all samples was directly controlled by this easyTEMP contactless sensor and the rotor used was a high-pressure and high-temperature rotor (SK-15 easyTEMP high pressure rotor, Milestone Inc., Sorisole, Italy) with a capacity of up to 15 vessels. The vessels used with this machine are modified polytetrafluoroethylene (PTFE) vessels with 100 mL volume. Each vessel contained 45 mL of sample. During each run, 15 vessels were fit in all position. A 1.5 g of the extractive-free RH was suspended in 45 mL of distilled water in the PTFE closed vessel. The heating program was performed at the microwave irradiation power of 600, 1100 and 1600 W to reach 140, 160, and 180°C, respectively in 5 min (come up time) and then held for the predetermined time of 5, 10, and 15 min for each temperature. Temperature and pressure sensors were used in all treatments. After completion, the reactant was immediately cooled to 25°C in a cold water bath. Microwave-pretreated residues were separated by vacuum filtration. The pretreated RH residue was washed with 95% ethanol, twice with distilled water, and dried overnight at 45°C in a hot air oven to obtain RH water-unextractable AX (RH-WUAX) for further processing of the RH alkali-soluble AX (RH-AX) extraction.

2.5. Microstructure analysis of RH after microwave pretreatment

A scanning electron microscope (SEM, EDS 6610LV, JEOL Ltd., Tokyo, Japan) was used to observe the surface morphology of the microwave pretreated RH. The samples were dried at 65°C for 24 h in a hot air oven and then ground and passed through 40 mesh sieves. The dried samples were mounted on aluminium, coated with gold, and then viewed at an accelerating 15 kV with a magnification factor of 200 to 1000. The diameter of the final beam spot on the sample was 40 nm.

2.6. Extraction of alkali-soluble RH-AX

One gram of RH-WUAX was suspended in 25 mL of 2% (w/v) sodium hydroxide at ambient temperature with continuous shaking at 180 rpm for 24 h of extraction time. Subsequently, the alkali-soluble AX liquor was collected by centrifugation at 9000 rpm and 25°C for 15 min. The liquor was adjusted to pH 6.0 with 37% hydrochloric acid and then 95% ethanol was added to a final ethanol concentration of 80% (v/v) to precipitate RH-AX. The precipitate was centrifuged at 9000 rpm at 4°C for 10 min, washed with acetone, and dried for 24 h at 45°C in a hot air oven to obtain crude RH-AX. Total sugar and reducing sugar contents were analysed using the phenol-sulphuric method (Dubois et al., 1956) and dinitrosalicylic acid method (DNS) (Miller, 1959), respectively.

2.7. Enzymatic hydrolysis of XOS and AXOS production from RH-AX

Hydrolysis of RH-AX was performed with two commercial xylanases, namely Pentopan Mono BG and Ultraflo Max. Briefly, 2% (w/v) RH-AX was suspended in 100 mM sodium phosphate buffer at pH 5.0. Then, each xylanase was separately added at enzyme concentrations of 50, 150, and 300 U/g substrate, and incubated at 50°C in a water bath shaker at 170 rpm for 24 h. The samples were periodically taken at the pre-set time and the reaction was stopped by boiling for 5 min. Then, the samples were dried with a freeze dryer (FreeZone 18 Liter Console Freeze Dryer, Labconco Corp., USA) to obtain an RH oligosaccharide (RH-XOS). Total reducing sugar content was measured by the DNS method, and the sugar profile was screened by thin layer chromatography (TLC). The XOS composition was qualitatively checked by TLC according to a previously described method (Jaichakan et al., 2019b). In brief, TLC silica gel 60 (Merck, Germany) was used as the stationary phase. The mobile phase consisted of an n-butanol:acetic acid:water solution in a ratio of 2:1:1 by volume. The TLC sheet was sprayed with 10% sulphuric acid in ethanol solution containing 0.2% orcinol. The bands were developed once by heating in a hot air oven at 110°C. The mixed XOS (X1-X6) (Wako, Japan) was used as the standard. Furthermore, HPAEC was used to identify the profile of XOS and AXOS.

2.8. Lactic acid bacteria growth promotion of RH–XOS

The RH-XOS and commercial prebiotic utilisation of lactic acid strains were evaluated in 96-well microplates. *Lactobacillus plantarum* JCM1149T, *Lactobacillus sakei* JCM1157, and *Lactobacillus bulgalicus* JCM1002 were purchased from the Japan Collection of Microorganisms. *Lactobacillus brevis* TISTR860 was purchased from the Thailand Institute of Scientific and Technological Research (Bangkok, Thailand). *Lactobacillus johnsonii* KUNN19-2, *Lactobacillus reuteri* KUB-4C5, *and Lactococcus lactis* KA-FF-1-4 were obtained from Kasetsart University, Thailand. The strains were pre-cultured in De Man, Rogosa and Sharpe (MRS) broth at 37°C for 18 h. MRS broth was reconstituted without glucose according to Nakphaichit et al. (2011) as follows: peptone 1%, beef extract 1%, yeast extract 0.5%, dipotassium hydrogen phosphate 0.2%, sodium acetate, ammonium monohydrogen citrate 0.2%, magnesium sulphate 0.01%, manganese sulphate 0.005% (w/v), and tween 80 0.1% (v/v). The initial pH of the medium was adjusted to 6.5 by 1 M sodium hydroxide or 1 M Hydrochloric acid and autoclaved at 121°C for 15 min. RH-XOS, XOS95P, RMD, and glucose were each partially dissolved in modified MRS and filtered through a sterile filter 0.2 µm and added to the media to a final concentration of 2%. The microplates were inoculated with cultured lactic acid bacteria at a final concentration of 1×10^4 CFU/mL, and sterile sealing tape was used to prevent vaporisation and contamination. The samples were incubated at 37°C for 24 h. Growth parameters were monitored using a microplate reader (UV-Vis SpectraMax 190 Microplate Reader, Molecular Devices, Sunnyvale, CA, USA) at 600 nm.

2.9. Continuous in vitro digestion of RH–XOS

The method of *in vitro* digestion used in this study was described by Minekus et al. (2014). Briefly, RH-XOS, XOS95P, RMD, and inulin were added into simulated salivary fluid (SSF), simulated gastric fluid

(SGF), and simulated intestinal fluid (SIF) to study the sugar release of the sample. Samples were first added with SSF, and two percentages of sample solutions were mixed with SSF electrolyte stock solution at a ratio of 50:50 (v/v). Amylase solution was added to achieve 75 U/mL in the final mixture at pH 7.0, incubated for 5 min. The oral bolus sample was mixed with SGF electrolyte stock solution at a final ratio of 50:50 (v/v). Porcine pepsin was added to achieve 2000 U/mL in the final mixture at pH 3.0, and incubated for 2 h. Lastly, gastric chyme was mixed with SIF electrolyte stock solution at a final ratio of 50:50 (v/v). Pancreatin solution was added to achieve 200 U/mL in the final mixture at pH 7.0, incubated for 2 h, and bile salts were added to give a final concentration of 10 mM in the final mixture.

Total sugar and reducing sugar contents of samples in each phase were determined using phenol-sulphuric acid method and DNS method, respectively. The percentage of hydrolysis was calculated as described by Korakli et al., (2002):

$$\text{Digestion (\%)} = \frac{\text{(final reducing sugar content - initial reducing sugar content)}}{\text{total sugar content - initial reducing sugar content}} \times 100$$

2.10. Statistical analysis

Three independent trials were conducted for each treatment. The mean values and standard deviations of the data were calculated. Statistical analyses were carried out using the SPSS 11 software. Duncan's one-way multiple comparisons were performed to determine significant differences ($p < 0.05$).

3. Results

3.1. Chemical and carbohydrate composition of RH

RH contained mainly fibre of 43.58 g, followed by ash moisture and protein of 15.56 g, 6.78 g and 2.05 g, respectively. The results showed that RH has a total carbohydrate as much as 75.08 g. This is because RH has lignocellulosic fibres, which are formed by cellulose, hemicellulose, and lignin. In addition, it is highly siliceous, which is different from other biomass materials. According to previous reviews, RH ranged from 15 to 20% of ash content. However, it depends on the state of prevention of RH degradation after harvesting (Ismail & Waliuddin, 1996). RH is composed of three primary components, including cellulose, hemicelluloses, and lignin. Di Blasi et al. (1999) demonstrated that rice husk is a good source of lignocellulosic materials, comprising 28.6% hemicellulose, 28.6% cellulose, 24.4% lignin, and 18.4% extractive matter (Fig. 1A). The cellulose and hemicellulose components are both sugar polymers that can be converted to functional sugars. The main hemicellulose compound in RH is xylan with a made up of substituted arabinoxylan which can be used as a precursor for XOS and AXOS production (Garrote et al., 1999: Vegas el al., 2004).

Lignocellulosic fibre of RH was divided into the polymeric sugar, which consisted of 1.71% arabinan, 0.70% galactan, 29.91% glucan, and 10.93% xylan. However, extractive free RH after pretreatment with acetone and ethanol consisted of 1.58% arabinan, 0.64% galactan, 32.53% glucan, and 11.57% xylan

(Fig. 1B). Glucose is a sugar with six carbon atoms, found in most plant structures. However, glucose can be absorbed in the upper gastrointestinal tract and enhances the growth of pathogens in human microbiota, exhibiting no prebiotic properties. Interestingly, RH also contains a high amount of xylose as 5 carbon atom sugar that enhances the growth of probiotics while most pathogens cannot use it as a carbon source (Desai et al., 2016). Therefore, oligosaccharide consisting of xylose oligomers in RH is an interesting functional ingredient as a prebiotic compound. However, hemicellulose is a complex component of the plant cell wall which is associated with cellulose and lignin in a hetero-matrix form. Hemicellulose forms ether and ester bonds with lignin, while hydrogen bonds form with cellulose (Harmsen et al., 2010). Pretreatment of lignocellulosic materials is required to increase biological conversion yields by disrupting the interpolymer linkage and allowing the lignocellulose fractions to separate.

3.2. Effects of microwave treatment on AX extraction

Hemicellulose extraction with an alkaline reagent and subsequent hydrolysis in the presence of acid or enzyme is preferred, due to its high XOS output yield from sugarcane bagasse (Brienzo et al., 2010). Furthermore, AX alkaline extraction can be assisted by microwave, where the energy is uniformly distributed throughout the material, as opposed to conventional heating. Process time can thus be reduced, resulting in a more efficient and homogeneous process (Bastos et al., 2018; Coelho et al., 2014). The effects of microwave treatment can cause fragmentation and swelling, leading to lignin and hemicellulose degradation in biomass and improving the pentose yield (Chen et al., 2011). Table 1 showed that after microwave pretreatment during 140–180°C for 5–15 min, increasing temperatures and processing times, AX content was slightly decreased. However, at 180°C, the highest AX content of 8.94 g/100 was provided after 15 min. This is in accordance with previous study revealing that at higher temperature and longer time of microwave treatment, a higher efficiency was found to degrade the lignocellulosic structure of a biomass then release the extractive free and lignin free fraction (Keshwani et al., 2007).

Table 1

Arabinoxylan content after microwave processing.

Temperature (°C)	Time (min)	Content (wt%)			A/X ratio
		A1	X1	AX content	
140	5	2.08 ± 0.12	8.61 ± 0.54	9.01 ± 0.30^{a}	0.24
	10	1.88 ± 0.08	7.79 ± 0.25	8.51 ± 0.24^{bc}	0.24
	15	1.81 ± 0.24	8.15 ± 0.20	8.76 ± 0.18^{b}	0.22
160	5	1.80 ± 0.16	6.88 ± 0.30	8.31 ± 0.23^{cd}	0.26
	10	1.84 ± 0.09	7.60 ± 0.11	7.64 ± 0.07^{e}	0.24
	15	1.75 ± 0.14	7.29 ± 0.04	7.96 ± 0.05^{cd}	0.24
180	5	1.68 ± 0.20	7.70 ± 0.18	8.25 ± 0.21^{cd}	0.22
	10	1.68 ± 0.04	6.89 ± 0.15	7.54 ± 0.10^{e}	0.24
	15	1.69 ± 0.15	8.47 ± 0.08	8.94 ± 0.15^{a}	0.20

The values with different superscript letters in a column are significantly different ($p < 0.05$).

Figure 2A and 2B showed that reducing sugar and total sugar content increased significantly with increasing temperature during microwave treatment. Microwave treatment at 180°C for 15 min gave the highest reducing sugar and total sugar contents of 20.75 and 88.3 mg/g, respectively. The operating parameters that affect the microwave conversion efficiency of sugar-biomass assisted pretreatment have recently been discussed by Ethaib et al. (2015). They reported that loading biomass, the power level of the microwave, and time of irradiation are factors affecting the efficiency of microwave pretreatment, which could convert the biomass into sugar. Accordingly, microwave-assisted pretreatment of lignocellulosic biomass immersed in alkaline glycerol has recently been shown to increase enzyme hydrolysis of corn straw and rice husk to sugar production (Diaz et al., 2015). The AX concentration in brewer's spent grains was maximized under pretreatment conditions of microwave heating at 172°C and 0.38 M sodium hydroxide (López-Linares et al., 2020). Therefore, the optimal condition for hemicellulose extraction was 180°C for 15 min, providing 3.5% AX yield of RH (Fig. 2C). For XOS and AXOS production, the extracted RH-AX was further hydrolysed by commercial xylanase.

The preliminary study compared the activity of the endoxylanases in hydrolysis of untreated and microwave-treated RH. The results showed that in microwave-treated RH, the reducing sugar content exhibited more than 2 folds compared with the untreated sample (data not shown). SEM was used to investigate the surface morphology of RH before and after microwave pretreatment. SEM images of

untreated and treated RH showed remarkable changes in the surface structure. As shown in Fig. 3A, the untreated RH surface had a packed structure with the composition of the plant cell wall and some convex structure. According to the baseline data of raw RH, the outer surface of the raw RH consisted mainly of carbon, oxygen, nitrogen, and silica. The complex lignocellulosic structure of RH has very low lignocellulosic-degrading enzyme permeability. Microwave pretreatment was expected to degrade the cell wall sufficiently and increase the surface area of the RH structure to allow direct contact with the degrading enzyme. Figure 3B shows that the surface morphology of the treated RH varied remarkably. The results showed that the thermal effects can lead to fragmentation and swelling, leading to lignin and hemicellulose degradation in biomass (Puligundla et al., 2016).

Alkaline pretreatment enhances enzymatic hydrolysis by selectively removing lignin without damaging carbohydrates and increasing porosity and surface area (Kim et al., 2016). Binod et al. (2012) reported the crystallinity index of native sugarcane bagasse was lower than other pretreated samples, according to the X-ray diffraction profile of native and microwave pretreated sugarcane bagasse. The crystalline size of native sugarcane bagasse was found to be greater than that of pretreated sugarcane bagasse. This was due to the degradation of the linkages between lignin and hemicellulose and the subsequent removal of lignin, which increased the surface area. Furthermore, non-thermal effect of exposure might be important to degrade the lignocellulose structure, which is other physical parameters can cause fluctuations and vibration of the charged particles and tissues (Belyaev, 2005). Wang and Lu (2013) reported that the effect of microwave on XOS production from wheat bran with xylanase was characterised by rapid increase in the reducing sugar almost 3–4 folds compared with wheat bran that had not been pretreated. Microwave alkaline extraction of AX has been reported for various lignocellulosic residues, including barley husks (Roos et al., 2009), and corn bran (Jiang et al., 2019).

3.3. Effects of commercial xylanases on XOS and AXOS production

The oligosaccharides profile was observed by TLC during 0–24 h of incubation, as compared to mixtures of X1, X2, X3, and X4 as standards (Fig. 4). Before the incubation of RH-AX with xylanases, the sugar profiles demonstrated a degree of polymerisation (DP) greater than 5. Incubations using Pentopan Mono BG and Ultraflo Max could hydrolyse long-chain oligomers into short-chain oligosaccharides. The determination of sugar pattern by TLC confirmed that both enzymes gave similar sugar patterns, including X1, X2, X3 X4, and X5. However, Ultraflo Max seemed to hydrolyse the substrate to a small DP and produced predominantly monosaccharides more than Pentopan Mono BG. XOS produced by Pentopan Mono BG provided various kinds of oligosaccharides from X2 to X5.

Furthermore, the oligosaccharide hydrolysates of RH-AX produced by both commercial xylanases were analysed by HPAEC for sugar pattern analysis. The results demonstrated that the Pentopan Mono BG produced oligosaccharide hydrolysate with the highest total oligosaccharide content at 150 U/g of enzyme concentration, after 12 and 24 h of incubation (Fig. 5A). The Ultraflo Max produced oligosaccharide hydrolysate with the highest total oligosaccharide content at 50 U/g of enzyme

concentration, after 8 h of incubation (Fig. 5B). Nevertheless, increasing enzyme concentration and incubation time increased the total monosaccharide content. The maximum reaction rate increased significantly due to the increase in enzyme concentration, resulting in the hydrolysis of the short-chain oligomer into monosaccharide. Furthermore, the longer an enzyme was incubated with its substrate, the greater the hydrolysis activity and the amount of the smallest product that will be formed.

As shown in the HPAEC chromatograms showed that both commercial enzymes provided xylobiose and xylotriose as the main oligosaccharide found in the hydrolysates of RH-AX, followed by xylose and xylotriose. Interestingly, AXOS was found in both hydrolysates (Fig. 6A and B). The quantitative analysis of XOS and AXOS produced by both commercial xylanases were present in Supplementary Tables 1 and 2. The oligosaccharide produced by Pentopan Mono BG exhibited A2XX, A3X, XA3XX and A2,3XX as 55.96, 784.68, 171.20 and 687.55, respectively. While the oligosaccharide produced by Ultraflo Max exhibited A2XX, and XA3XX as 670.72 and 590.46 ppm, respectively. In this study, oligosaccharide produced by Pentopan Mono BG was then used to evaluate the prebiotic properties, due to it was less providing monosaccharide and contained various kind of oligosaccharide.

In the carbohydrate-active enzyme (CAZY) database, xylanases are classified based on primary structure comparisons of the catalytic domains and grouped in families of related sequences. Members of the same family have similar protein folds, the same catalytic mechanism, and anomeric carbon retention or inversion. Although enzymes from glycoside hydrolase families 5, 7, and 8 contain a catalytic domain with endo-1,4 xylanase activity, research has primarily focused on xylanases from glycoside hydrolase families 10 and 11 (Collins et al., 2014). Pentopan Mono BG and Ultraflo Max belong to the glycoside hydrolase family 10 and 11, showing some differences in sugar profiles (Cheng et al., 2014). Family 11 xylanases hydrolyse only xylan and generally exhibit a preference for internal xylan bonds and unsubstituted xylan chains, acting primarily on the xylose unit at the centre of the oligosaccharide. While, family 10 xylanases tend to show preference for groups at the end of the xylan bonds, especially favouring the reduction end, have low substratum specificity, and can degrade xylan backbones with several replacements by hydrolysing at the side branch location (Morgan et al., 2017). Pentopan mono BG produced XOS from *Phoenix dactylifera* L. seed higher amounts of X3 than X2 (Ataei et al., 2020). Falk et al. (2014) reported that GH10 xylanases produce a higher amount of short XOS from rye bran than GH11. According to the datasheet of the enzymes, GH10 and GH11 produce lower DP and higher amounts of DP XOS, respectively.

3.4. Growth promotion of lactic acid bacteria by RH–XOS

Accordingly, a prebiotic is a selectively fermented ingredient that allows for specific changes in the composition and/or activity of the gastrointestinal microflora, which confers benefits. It is necessary to establish clear criteria for classifying a food ingredient as a prebiotic (Roberfroid, 2008). XOS and AXOS possess promising functional properties as they can be specifically fermented by intestinal commensals such as bifidobacteria and lactobacilli (Neyrinck et al., 2012). XOS has a high prebiotic potential and can be incorporated into a wide range of food products. Seven strains of lactic acid bacteria were used to evaluate the ability of RH-XOS to promote growth compared to xylose and commercial prebiotics,

including XOS95P, RMD, and inulin. Table 2 showed the utilisation of RH-XOS and commercial prebiotics by lactic acid bacteria strains. XOS95P and RMD promoted the growth of all the strains, whereas RH-XOS did not promote *L. Lactis*. Xylose promoted only 2 strains, including *L. plantarum* and *L. brevi*s. Inulin and RH-XOS promoted 6 strains except *L. Brevis*. And *L. lactis* could be promoted by RH-XOS. A previous study revealed that inulin partially promoted *L. brevis* strains (Kariyawasam et al., 2021). However, *L. brevis* displayed high growth and consumption in XOS (Moura et al., 2007). The results of this study also demonstrated the ability of probiotic bacteria to utilise prebiotics varies even within the same species. Iliev et al. (2020) reported that the ability of XOS with a different DP could stimulate the growth of some heterofermentative *Lactobacillus* strains. Approximately 30 strains were identified and screened for XOS utilization with the possibility of three enzymes, included β-xylosidase, exo-oligoxylanase, and α-L-arabinofuranosidase, altering the end-products and morphology of Lactobacillus strains growth by XOS.

Table 2

RH-XOS utilization by lactic acid bacteria.

Microorganisms	**Carbohydrate sources**				
	X1	**XOS95P**	**RMD**	**inulin**	**RH-XOS**
Lactobacillus johnsonii	-	+	+	+	+
Lactobacillus plantarum	-	+	+	+	+
Lactobacillus reuteri	-	+	+	+	+
Lactobacillus bulgaricus	-	+	+	+	+
Lactobacillus sakei	-	+	+	+	+
Lactococcus lactic	-	+	+	+	-
Lactobacillus brevis	+	+	+	-	+

+ : Carbohydrates can promoted growth of lactic acid bacteria
- : Carbohydrate cannot promoted growth of lactic acid bacteria

3.5. In vitro simulation of human digestibility of RH-XOS

Human-simulated digestion typically includes the oral, gastric, and small intestinal phases. These phases were performed to study the digestion-resistance property of RH-XOS compared with commercial prebiotics. Figure 6 showed that the carbohydrate used in this study was significantly hydrolysed in the gastric phase and small intestinal phase. Commercial XOS and RMD were not digested in the oral phase because commercial XOS mostly contain short chain oligomers, which are less than DP 4. Alpha-amylase is a glycoside hydrolase family 13, which catalyses the hydrolysis of (1–4)-α-d-glucosidic linkages in polysaccharides. Substrates are not digested by α-amylase, such as α-limit dextrin, and small linear

oligomers, along with larger α-glucans (Boron & Boulpaep, 2012). In contrast, inulin and RH-XOS were digested in small amounts in the oral phase by 1.01% and 7.08%, respectively. In the gastric phase, the percentage of digestion increased in all samples, especially inulin and RH-XOS by (19.05% and 21.30%, respectively) as the stomach plays an enhanced role in hydrolysis by hydrochloric acid (Boron & Boulpaep, 2012). Commercial XOS was digested in the gastric phase and small intestinal phase at the same digestion level of 12.21% and 11.23%, respectively. RMD was the most digested in the intestinal phase, (23.24%). However, inulin and RH-XOS were less digested in the intestinal phase (Fig. 7). This study found that the obtained RH-XOS was more than 70% resistant to human simulated digestion, which is the same as commercial prebiotics.

According to previous research, XOS are resistant to hydrolysis by enzymes and/or the low pH found in human saliva, gastric, and pancreatic juices, and are not absorbed during transit through the small intestine. These are exo-glucosidases that act on the non-reducing end of glucose oligomers and catalyse not only the hydrolysis of α-(1,4) bonds, but also to a lesser extent α-(1,6) branch bonds, ensuring further degradation of nonlinear oligosaccharides (Boron & Boulpaep, 2012). Eventually reaching the colon and serving as fermentable substrates for certain members of the resident.

4. Conclusion

The results of this study showed that microwave pretreatment is a promising method for lignocellulosic-degradation of RH. Commercial xylanases, Pentopan Mono BG, and Ultraflo Max were able to hydrolyse RH-AX into XOS and AXOS. Nevertheless, Pentopan Mono BG provided less monosaccharide and various kinds of oligosaccharides compared to Ultraflo Max. RH-XOS prepared by Pentopan Mono BG was able to promote the growth of six of the seven lactic acid bacterial species and was resistant to human simulated digestion by more than 70%, indicating the high potential of prebiotic properties.

Declarations

Acknowledgement

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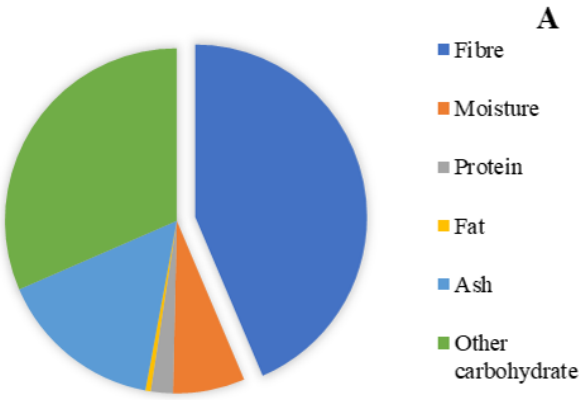
Figures



B

Polymeric sugar	Content (wt%)	
	RH	Extractive free RH
Arabinan	1.71±0.05	1.58±0.06
Galactan	0.70±0.04	0.64±0.05
Glucan	29.91±0.87	32.53±0.75
Xylan	10.93±0.35	11.57±0.27
Mannan	Not detected	Not detected
AX content	12.64±0.35	13.15±0.29

Figure 1

Chemical composition of RH (A) and polymeric sugar content of RH and extractive free RH (B).

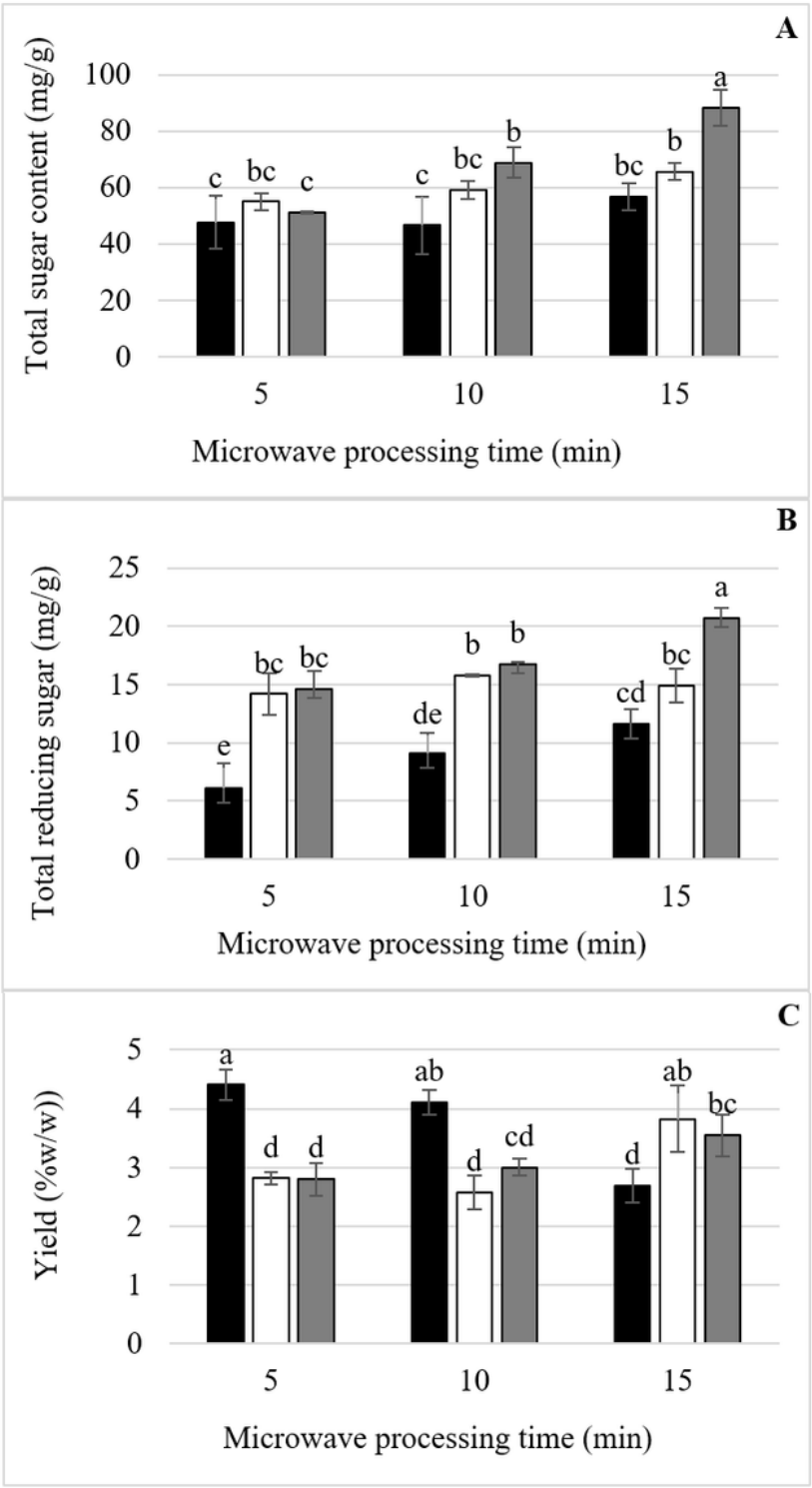


Figure 2

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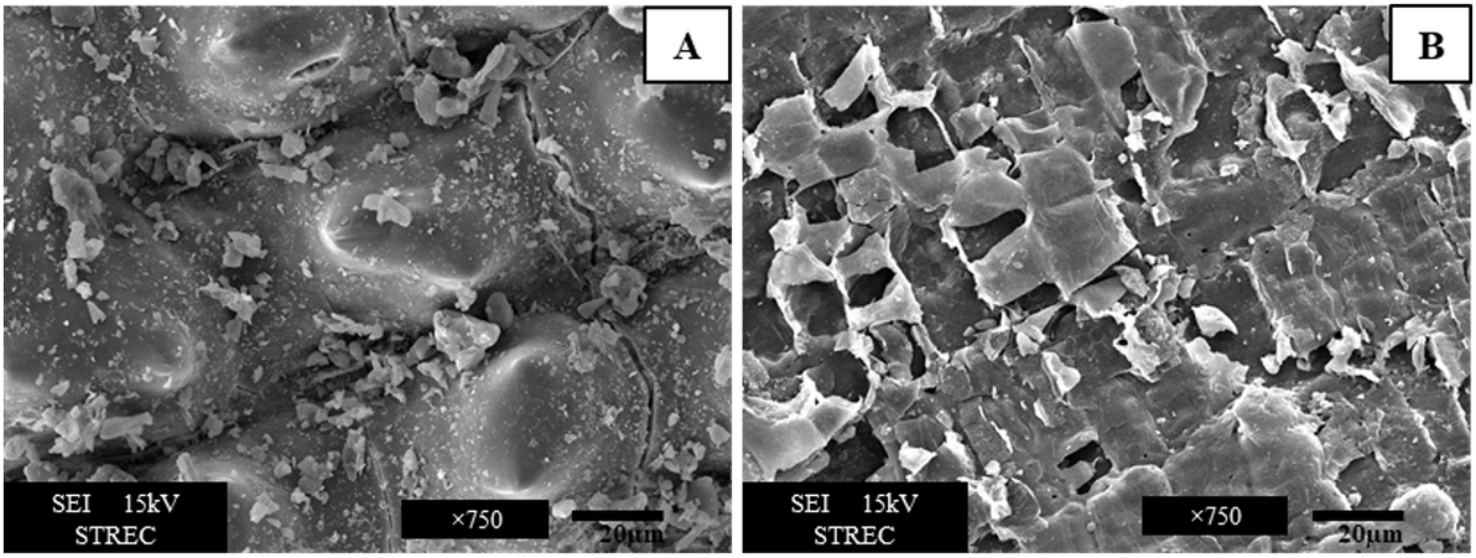

Figure 3

Scanning electron microscopy (SEM) analysis of untreated RH (A) and the pretreated RH (B) with microwave at 180 °C for 15 min.

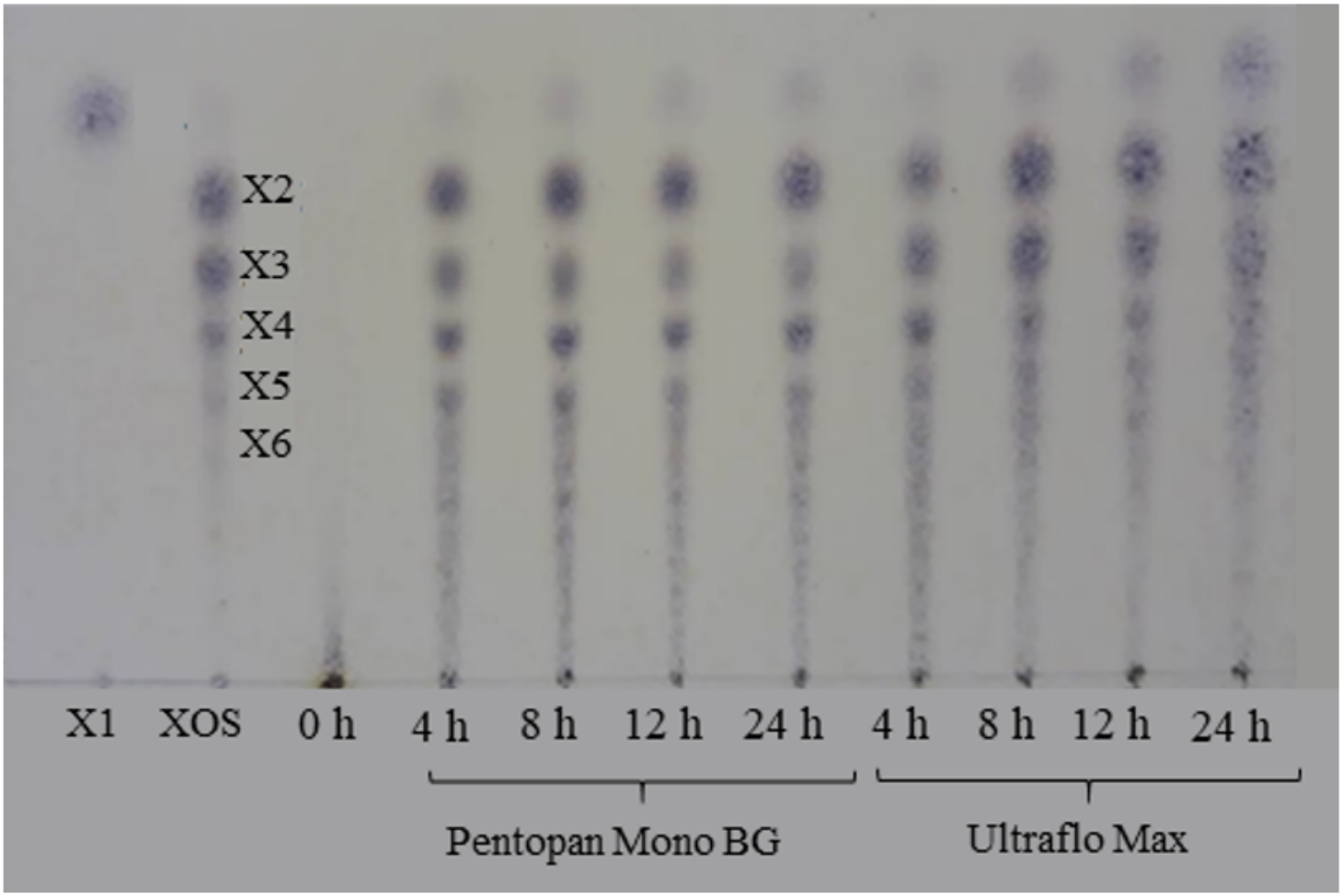

Figure 4

TLC chromatograms of XOS obtained from RH-AX after hydrolyzed with difference commercial xylanases at 50 U/g, 50 °C, pH 6.0 for 0-24 h. The mixture of xylose (X1), xylobiose (X2), xylotriose (X3) and, xylotetraose (X4) were used as standard.

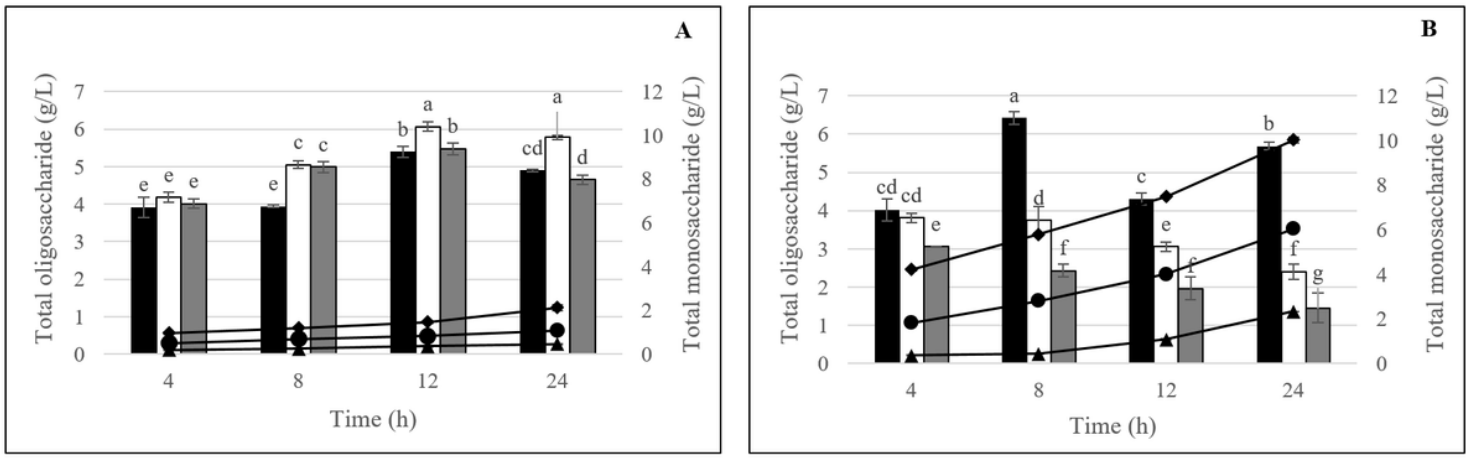

Figure 5

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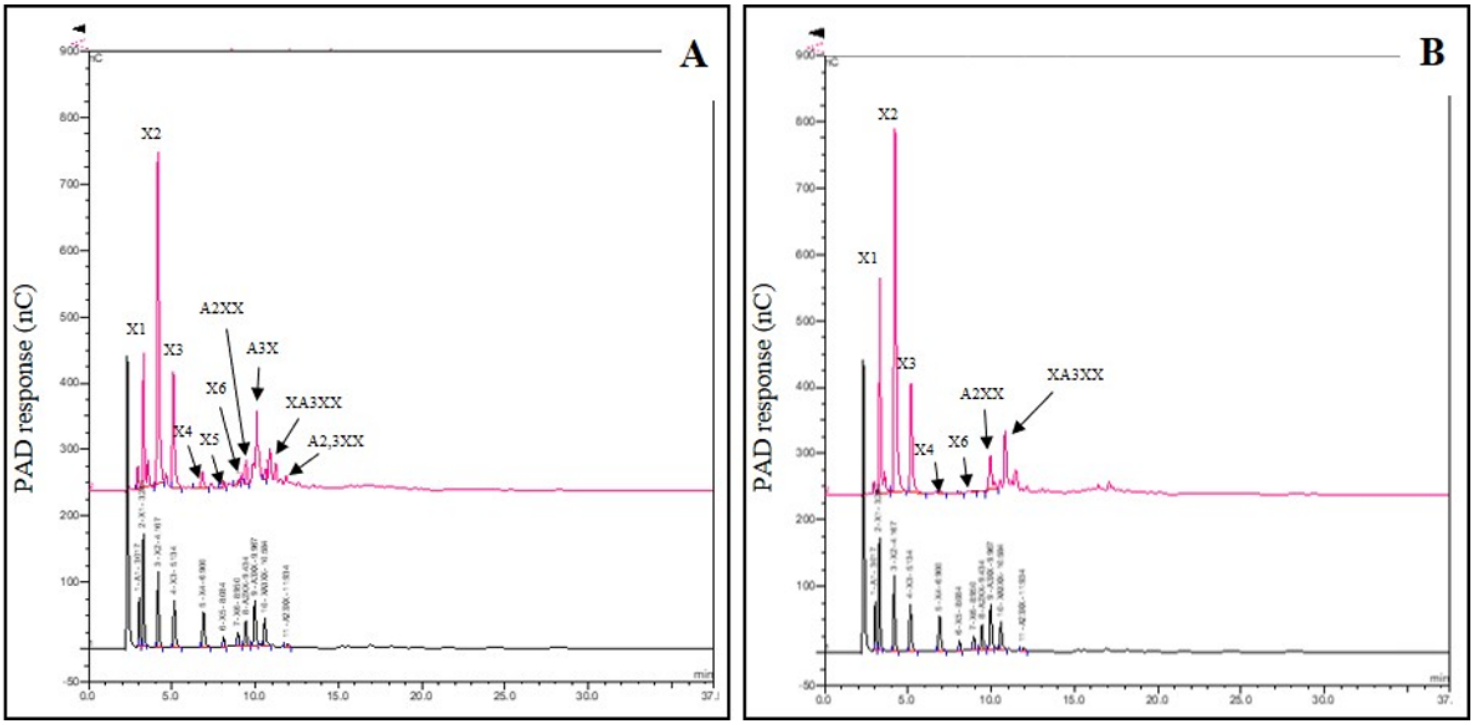

Figure 6

HPAEC chromatograms of RH-AX treated with Pentopan Mono BG (C) at 150 U, 50 °C for 12 h and Ultraflo Max (D) at 50 U, 50 °C for 8 h.

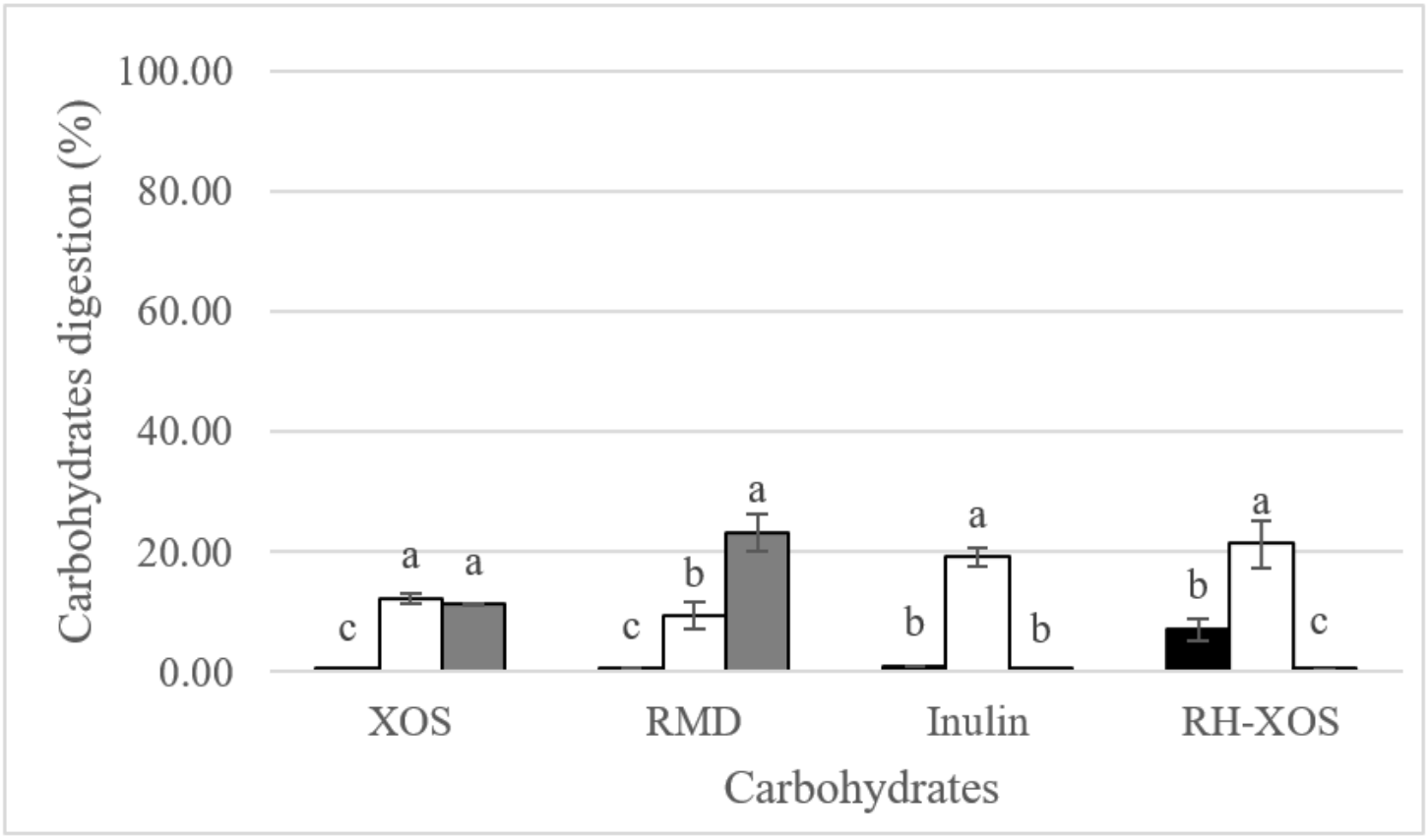

Figure 7

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