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Enzymatic production of xylooligosaccharides from red alga
dulse (*Palmaria* sp.) wasted in Japan

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22 ABSTRACT

23 Red alga dulse (*Palmaria* sp.) has xylan with a small amount of cellulose and less lignin,
24 hence these characteristics may provide some advantages in the enzymatic production of
25 xylooligosaccharides (XOS). To evaluate dulse xylan as XOS source, we prepared xylan rich
26 fraction (DXRF) and attempted XOS production using commercial enzymes. 13.8% of xylan
27 rich fraction (DXRF: containing 52.2% of xylan) was obtained from dried dulse powder. We
28 then evaluated the hydrolysis products of two commercial enzymes, revealing that
29 hydrolysate of Hemicellulase amano 90 contained less xylose. The effective XOS production
30 by the enzyme was evaluated in terms of enzyme dose, DXRF concentration and reaction
31 period, showing that 66.6% of XOS was obtained from DXRF with the hydrolysis rate of
32 82% under the following condition: 54 U Hemicellulase amano 90, 10 mg/ml DXRF, pH 4.5,
33 50 °C for 24 h. These results indicated that red algae xylan is suitable for XOS source.

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35 *Keywords:* Xylooligosaccharides; Red alga; *Palmaria* sp.; Xylan; Hemicellulase

1. Introduction

Xylooligosaccharides (XOS) are the oligomers composed of D-xylose as a major constituent and possess some advantageous features (stable at low gastric pH and heat resistance up to 100 °C) for the uses in food industry [1,2]. XOS have been recognized as non-digestible food ingredients, because they are not hydrolyzed and absorbed in human gastrointestinal tract showing various health beneficial effects on human. XOS has been known to be important in prebiotics [3,4]. Indeed, Sheu et al. [5] reported the effects of XOS (Xylooligo 95P: Suntory Co. Ltd.) on the blood sugar, lipids and oxidative status in type-2 diabetes mellitus. Okazaki et al. [6] found that XOS from birchwood xylan show a favorable effect on human intestinal flora; i.e., the XOS were utilized by bifidobacteria, whereas they were not used by *Escherichia coli* and *Clostridium* spp. *in vitro*, and they (5 g/day) promoted the growth of bifidobacteria *in vivo*. Therefore, XOS have been used as a functional food material; e.g., dietary sweeteners for low-calorie foods [1,7].

The XOS are industrially produced by enzymatic hydrolysis of xylan from terrestrial plants [8]. To date, many researchers have attempted to produce the XOS from various sources, such as corncob, wheat straw, rice hull, pigeon pea, peanut shell and areca nut husk [9-13]. The xylan of terrestrial plants exists in lignocellulose materials as xylan-cellulose-lignin complex which is formed by covalent and non-covalent bonds [7,14]. Consequently, the terrestrial xylan becomes resistant to enzymatic hydrolysis. Therefore, pretreatment with acid or alkali was necessary for the enzymatic degradation of terrestrial xylan [1,15,16]. On the other hand, marine algae contain polysaccharides with no lignin and less cellulose [17]. Due to the bio-architectural feature, marine algae have various advantages in the bioconversion fields [18]. For example, ethanol production from red algal cellulose and agar was reported [19]. Among marine algae, red alga dulse (*Palmaria palmata*) is known to

contain a mix-linked β -(1 $\rightarrow$ 3)/ β -(1 $\rightarrow$ 4)-xylan with a minor amount of cellulose and β -(1 $\rightarrow$ 4)-xylan in the cell wall [20-22]. Unlike terrestrial plants xylan, the mix-linked dulse xylan is primarily held in the cell wall non-covalently and a part of the mix-linked xylan is loosely held in it [23,24]. Thus, the dulse xylan appears to have the certain advantages in enzymatic production of XOS. However, the past studies on dulse xylan mainly focused on the elucidation of its fine structure, and as far as we know there appears to be no research to use dulse xylan as a source of XOS.

Previously, we found that the dulse harvested in Japan is abundant in proteins (approximately 40 g/100 g dried dulse) and the major component of them is phycoerythrin (PE) [25]. Further, we revealed that the peptides generated by thermolysin hydrolysis of PE strongly inhibited angiotensin I converting enzyme (ACE), and the chromophores prepared from PE exhibit high antioxidant activity [25-28]. Recently, we also found that a considerable amount of xylan is contained in the Japanese dulse, similar to dulse species distributed in Europe and North America. In this study, we investigated the potential of Japanese dulse as a XOS source by enzymatic production. We then evaluated the enzyme source and determined the enzymatic condition (enzyme dose, substrate concentration and reaction period). Our results would help to produce XOS from red algae xylan.

2. Materials and methods

2.1. Materials

Dulse (*Palmaria* sp.) was harvested off Usujiri, Hokkaido, Japan in February 2016 and stored at -30 °C until use. Hemicellulase amano 90 (from *Aspergillus niger*, > 90,000 units (U)/g at pH 4.5, 50 °C) was procured from Amano Enzyme Inc. (Aichi, Japan). Sucrase

X (from *Trichoderma longibrachiatum*, > 25,000 U/g at pH 5.0, 40 °C) was procured from Mitsubishi-Chemical Foods Corporation (Tokyo, Japan). Glucose oxidase (GOD, EC 1.1.3.4 from *Aspergillus niger*, 220 U/mg at pH 5.6), peroxidase (POD, EC 1.11.1.7 from horseradish, 100 U/mg at pH 5.0-8.0), xylose, β -(1 $\rightarrow$ 4)-xylobiose (X2) and β -(1 $\rightarrow$ 4)-xylotriose (X3) were purchased from Wako Pure Chemical Industries (Osaka, Japan). β -(1 $\rightarrow$ 4)-Xylotetraose (X4) and β -(1 $\rightarrow$ 4)-xylopentaose (X5) were purchased from Megazyme (Bray, Ireland). Galactose Colorimetric Detection Kit was purchased from Arbor Assays (Ann Arbor, MI, USA). All other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Extraction of dulse xylan

The frozen dulse thalli were lyophilized and homogenized by a Wonder Blender WB-1 (OSAKA CHEMIKAL CO., Osaka, Japan) into powder. The dulse powder was suspended in 50-volumes (v/w) of chloroform-methanol (1:2, v/v) and stirred at room temperature for 30 min. The suspension was filtrated by No.2 filter paper (ADVANTEC, Tokyo, Japan) and the residues were treated by the same solvent. Then, the filtrated residues were treated by 20-volumes (v/w) of acetone twice, and the residues were air-dried at room temperature obtaining defatted dulse powder.

Dulse xylan was extracted as follows: the defatted dulse powder was suspended in 40-volumes (v/w) of distilled water, and the suspension was autoclaved at 121 °C for 20 min. Then, solid urea was added to the autoclaved suspension to make final concentration of 8 mol/l, and xylan was extracted at room temperature for 24 h with stirring. The extract was filtrated through No.2 filter paper, and the filtrate was dialyzed against distilled water with a dialysis tube (molecular weight cut off: approximately 14 kDa, EIDIA Co., Ltd., Tokyo, Japan). The solution was centrifuged at 15,000 $\times$ g for 5 min to remove small amount of

insoluble materials, and the supernatant was lyophilized and used as dulse xylan rich fraction (DXRF). The concentration of protein in the DXRF was determined by measuring the absorbance at 280 nm using bovine serum albumin as standard [29].

2.3. Sugar composition analysis

Ten milligrams of the dulse powder and the DXRF were pre-hydrolyzed with 4 M trifluoroacetic acid (TFA) at room temperature for 10 min and further hydrolyzed with 2 M TFA at 100 °C for 2 h. The monosaccharides were derivatization with 1-phenyl-3-methyl-5-pyrazolone (PMP) [30] and analyzed by HPLC equipped with Mightysil RP-18GP aqua column (4.6×250 mm, Kanto Kagaku, Tokyo, Japan). PMP-monosaccharides were eluted with 0.1 M phosphate buffer-acetonitrile (83:17, v/v) at a flow rate of 1.0 ml/min and detected at 235 nm. PMP-derivatives of xylose, arabinose, ribose, lyxose, galactose and glucose were used as standards.

The quantity of the non-derivatized monosaccharides was determined by HPLC using RI detector. The monosaccharides were applied to HPLC equipped with Sugar-D column (4.6×250 mm, Nakalai Tesque, Kyoto, Japan). The monosaccharides were eluted with acetonitrile-water (4:1, v/v) at a flow rate of 1.0 ml/min. Xylose and glucose were used as standards.

2.4. Enzymatic production and evaluation of XOS

The DXRF (10 mg/ml) was hydrolyzed for 24 h by 1 wt% (9 U) of Hemicellulase amano 90 at 50 °C and 1 wt% (2.5 U) of Sucrase X at 40 °C. The enzyme reactions were stopped by heating at 100 °C for 5 min. The products were lyophilized and suspended in

acetonitrile-water (4:1, v/v). The samples were sonicated for 3 min and centrifuged at 15,000×g, 4 °C for 10 min. The supernatant was subjected to HPLC equipped with Sugar-D column. Xylose and XOS (X2, X3, X4 and X5) were used as standards. Since the retention time of X2 and hexose was the same by the HPLC, the amount of X2 was determined by removing the amount of hexose (glucose and galactose) by colorimetric analysis of GOD-POD (detection limit: 0.05 mg/ml) [31] and galactose detection kit (detection limit: 0.04 mg/ml) (Arbor Assays, Ann Arbor, MI, USA). The amounts of unknown components (U1, U2 and U3) were determined by xylose as standard.

2.5. Production of XOS by Hemicellulase amano 90

Effects of enzyme dose (13.5, 27 and 54 U) and reaction period (2-36 h) were examined in a reaction mixture containing 10 mg/ml of the DXRF at 50 °C. Effects of DXRF concentration (10, 20 and 30 mg/ml) and reaction period (2-36 h) were examined in a reaction mixture containing 54 U of the enzyme at 50 °C. Analysis of hydrolysis products was described in the above paragraph 2.4. The hydrolysis rate (%) was calculated by the formula: (monosaccharides and XOS content/polysaccharides content in DXRF) ×100. The yield of XOS was calculated by the formula: (XOS content/xylan content in DXRF) ×100.

2.6. Molecular mass measurement of XOS

Molecular masses of XOS were measured by Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF MS) using a 4700 Proteomics Analyzer (Applied Biosystems, Carlsbad, CA, USA). The equal volumes of sample and 10 mg/ml of 2,5-dihydroxybenzoic acid in 50% acetonitrile were mixed, and 1 µl

of mixtures was applied to a MALDI-TOF MS.

3. Results and discussion

3.1. Extraction of dulse xylan

The sugar compositions of dulse powder and DXRF were analyzed. Dulse powder contained 21.3% of xylose as a major component and 6.0% of galactose and glucose, whereas the other pentoses were not detected (Table 1). In the procedure of Materials and methods 2.2., DXRF was obtained from dulse powder in the yield of 13.8% containing xylose (52.2%) together with galactose and glucose (10.0%). Xylose content in DXRF increased 2.5-fold with the yield of 33.8%. The non-sugar compounds (37.8%) of DXRF were regarded as proteins (11.7%) and unknown substances (26.1%). Deniaud et al. [23] reported that dulse xylan extracted with 8 M urea contained xylose (55.8%) together with galactose (1.3%), glucose (0.9%) and proteins (0.9%). The rest of 41.1% was unknown substances. Thus, the composition of DXRF was similar to that of their report except for proteins. Although both xylan extractions were performed using 8 M urea, the difference in the amount of proteins might be occurred by measurement methods (absorbance at 280 nm and Bradford method) or samples [*Palmaria* sp. (Japan) and *Palmaria palmata*].

3.2. Evaluation of commercial enzyme for XOS production

To evaluate the enzyme sources, DXRF was hydrolyzed by two kinds of commercial enzymes, Hemicellulase amano 90 and Sucrase X, and the hydrolysates were analyzed by HPLC. The hydrolysate of Hemicellulase amano 90 contained xylose, X2, X3 and unknown

components (U1, U2 and U3 with retention time; 10.80, 16.62 and 26.50 min, respectively); while, the hydrolysate of Sucrase X contained xylose as a main hydrolysis product, and X2 and U1 and the small amount of X3 and U2 were detected (Fig. 1). X4 and X5 were not detected in both hydrolysates. Since hexoses and X2 were eluted in the same retention time, the amount of hexoses was measured by colorimetric methods, showing that the hydrolysate of Hemicellulase amano 90 contained glucose but not galactose (Fig. 2).

3.3. MALDI-TOF MS analysis of unknown components (U1, U2 and U3)

To investigate the structure of U1, U2 and U3, these components were subjected to MALDI-TOF MS analysis (Table 2). Since m/z 437.22 $[M+Na]^+$ and 453.17 $[M+K]^+$ ion peaks were detected in the spectrum of U1, it was regarded as xylotriose. Then, U1 was subjected to sugar composition analysis, methylation analysis and NMR spectroscopy (Fig.S1, Fig. S2 and Table S1). Consequently, U1 was identified as β -xylopyranosyl-(1 $\rightarrow$ 3)- β -xylopyranosyl-(1 $\rightarrow$ 4)-xylopyranoside.

U2 showed m/z 569.29 $[M+Na]^+$ and 585.29 $[M+K]^+$ ion peaks, and U3 showed m/z 701.37 $[M+Na]^+$ ion peak, revealing that U2 and U3 were regarded as xylotetraose and xylopentaose, respectively. HPLC analysis showed that U2 and U3 were eluted earlier than X4 and X5, respectively. Comparing the elution of U1 and X3, U1 was eluted earlier. Therefore, we tentatively identified U2 and U3 as mix-linked β -(1 $\rightarrow$ 3)/ β -(1 $\rightarrow$ 4)-xylotetraose and β -(1 $\rightarrow$ 3)/ β -(1 $\rightarrow$ 4)-xylopentaose, respectively. These specific oligosaccharides were also detected in hydrolysates of xylan from dulse as reported in the past studies [20,32,33].

3.4. Selection of commercial enzyme for XOS production

Hemicellulase amano 90 gave a low xylose yield (19%) in the hydrolysate compared

with Sucrase X (42%). The hydrolysates of Hemicellulase amano 90 and Sucrase X contained 72.5 and 58.0% of XOS, respectively (Fig. 2). Akpinar et al. [34] prepared XOS from tobacco stalk xylan using two xylanases from *T. longibrachiatum* and *A. niger*, resulting that xylanase from *T. longibrachiatum* produced a high yield of xylose. It was reported that xylanase from *T. longibrachiatum* did not produce xylose [35]. The average degree of polymerization (DP) of the hydrolysate of Sucrase X (DP 2.0) was small compared with that of Hemicellulase amano 90 (DP 2.5). We thought that Sucrase X contains a large amount of β -(1 $\rightarrow$ 4)-xylosidase than Hemicellulase amano 90.

The hydrolysate of Hemicellulase amano 90 contained glucose (8.3%). In the product report of Sucrase X, cellulase activity is also included; however, the hydrolysate did not contain glucose. Red algae possess floridean starch as a glucose source, implying that glucose in the hydrolysate of Hemicellulase amano 90 would come from amylase activity of the enzyme. In fact, α -glucan hydrolase enzyme cocktails from *Aspergillus* sp. are sold from many companies.

Taking above things into consideration, we employed Hemicellulase amano 90 for XOS production from DXRF.

3.5. Effect of enzyme dose on XOS production

Since Hemicellulase amano 90 was considered to be advantageous for XOS production, we investigated suitable condition for XOS production from DXRF. Firstly, the effect of enzyme dose (13.5, 27 and 54 U) was investigated (Fig. 3). An increase in the amount of enzyme from 13.5 to 54 U resulted in an increase in hydrolysates for 36 h from 3.5 to 4.8 mg/ml and hydrolysis rate from 60 to 81%. Although the ratio of XOS in hydrolysates decreased from 87 to 79% with the enzyme dose from 13.5 to 54 U, the amount of XOS

increased 1.23-fold (3.31 mg/ml at 54 U). Therefore, we used the enzyme dose of 54 U for the further examination.

3.6. Effect of DXRF concentration on XOS production

The effect of DXRF concentration (10-30 mg/ml) on XOS production was investigated (Fig. 4). An increase in DXRF concentrations from 10 to 30 mg/ml resulted in an increase in the hydrolysate (4.8, 6.7 and 7.1 mg/ml, respectively) for 36 h. However, hydrolysis rate for 36 h decreased from 81 to 40% with the increase in DXRF concentrations (from 10 to 30 mg/ml). This is ascribable to a reduction of water content in the reaction mixture including too much amount of substrate as reported in past studies [36-38]. Therefore, we concluded that 10 mg/ml DXRF is suitable for XOS production.

3.7. Effect of reaction period on XOS production

Finally, reaction period (2-36 h) for XOS production was determined. When 10 mg/ml of DXRF was hydrolyzed by 54 U of Hemicellulase amano 90, hydrolysate concentration reached to 4.7 mg/ml for 24 h, and hydrolysis rate remained constant thereafter (approximately 82% of xylan in DXRF was hydrolyzed) (Fig. 4A). Comparing the composition between 24 and 36 h, U2 and X3 were degraded as U1, X2 and X1, implying that enzyme reaction more than 24 h decreased XOS in the hydrolysate. This may be due to a decrease in the easily accessible hydrolytic sites in substrate molecules or the inhibition of enzyme activity by end products [34,39]. By the following condition: 10 mg/ml of DXRF, 54 U of Hemicellulase amano 90, pH 4.5 at 50 °C for 24 h, 3.5 mg/ml of XOS was obtained with the yield of 66.6% (82% of hydrolysis rate) (Table 3). Yang et al. [40] produced 4.7 and 5.9

mg/ml of XOS from alkali extracted bagasse and corncob xylans, respectively, with 20 mg/ml of substrate concentration. Akpinar et al. [34] also reported that 3.1 mg/ml of XOS was produced from alkali extracted tobacco stalk xylan (20 mg/ml). In the present study, we obtained 3.5 mg/ml of XOS production from dulse without alkaline pre-treatment in a high hydrolysis rate (82%), indicating that xylan from red algae would be superior source for XOS production.

4. Conclusions

In this study, we investigated the enzymatic production of XOS from Japanese dulse (*Palmaria* sp.). First, 13.8% of DXRF (containing 52.2% of xylan) were obtained from dried dulse powder. The reasonable procedure for XOS production from DXRF was concluded as follows: 10 mg/ml of DXRF is hydrolyzed by 54 U of Hemicellulase amano 90, pH 4.5 at 50 °C for 24 h. Under the condition, 66.6% of XOS was obtained from xylan in DXRF. These results indicated that xylan from red algae is a good candidate for XOS production. In addition, XOS from red algae contained a relatively high amount of β -(1→3)/ β -(1→4)-linked XOS (49%). Now, the health benefits of U1 and U2, which had specific structure of β -(1→3)/ β -(1→4)-XOS, are under the investigation. We demonstrated that Japanese dulse protein and the peptides contain antioxidant activity and ACE inhibitory activity. The simultaneous productions of bioactive compounds are also ongoing, leading to the understanding of food-intake mechanism in our bodies.

Supplementary data

Supplementary data are showed in Fig. S1, Fig. S2 and Table S1.

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Table 1

Sugar compositions of the dulse powder and the DXRF.

The sugar compositions were quantified by HPLC analysis of the non-derivatized monosaccharides after hydrolysis.

	Content (wt%)				Galactose + Glucose
	Xylose	Arabinose	Ribose	Lyxose	
Dulse powder	21.3±2.2	n.d.*	n.d.*	n.d.*	6.0±0.6
DXRF	52.2±3.3	n.d.*	n.d.*	n.d.*	10.0±0.2

* not detected.

Galactose + Glucose: the amount of galactose and glucose.

Mean ± data range of two replicate determinations.

Table 2

The m/z in MALDI-TOF MS spectra and structures of U1, U2 and U3.

Peak	[M+Na] ⁺ (m/z)	[M+K] ⁺ (m/z)	linkage	DP [*]
U1	437.22	453.17	β -(1→3)/ β -(1→4)	3
U2	569.29	585.29	— ^{***}	4
U3	701.37	n.d. ^{**}	— ^{***}	5

^{*} degree of polymerization, ^{**} not detected, ^{***} not determined.

Table 3

Composition of hydrolysis products by Hemicellulase amano 90 under the following condition: 54 U enzyme, 10 mg/ml DXRF, pH 4.5, 50 °C for 24 h.

Saccharides	Concentration (mg/ml)
xylose	0.8 ± 0.05 (15%)
glucose	0.4 ± 0.00
X2 [β -(1→4)-xylobiose]	1.1 ± 0.15 (21%)
X3 [β -(1→4)-xylotriose]	0.3 ± 0.06 (6%)
U1 (unknown components 1)	1.1 ± 0.09 (21%)
U2 (unknown components 2)	0.9 ± 0.15 (17%)
U3 (unknown components 3)	0.1 ± 0.04 (2%)
Total (without glucose)	4.3 ± 0.48 (82%*)
XOS (X2, X3, U1-3)	3.5 ± 0.44 (67%)

Mean ± standard deviation of three replicate determinations.

* 100% corresponds to 5.22 mg/ml xylose in DXRF.

Figure Legends

Fig. 1. HPLC chromatograms of the enzyme hydrolysates of DXRF and standards. Hemicellulase amano 90: Ten mg/ml of the DXRF was hydrolyzed with 1 wt% (9 U) of Hemicellulase amano 90 at 50 °C for 24 h. Sucrase X: Ten mg/ml of the DXRF was hydrolyzed with 1 wt% (2.5 U) of Sucrase X at 40 °C for 24 h. The resulting products were analyzed by HPLC using Sugar-D column with isocratic elution of acetonitrile-water (80:20, v/v) at a flow rate of 1.0 ml/min. Eluents were detected using RI detector. X1: xylose, Glc: glucose, X2: β -(1 $\rightarrow$ 4)-xylobiose, X3: β -(1 $\rightarrow$ 4)-xylotriose, X4: β -(1 $\rightarrow$ 4)-xylotetraose, X5: β -(1 $\rightarrow$ 4)-xylopentaose, U1-U3: unknown components 1-3, respectively.

Fig. 2. Saccharide compositions of the enzyme hydrolysates of DXRF. The conditions of enzymatic hydrolysis were the same as in Fig. 1. The concentration of each product was quantified by HPLC. Ten mg of DXRF contains 5.22 mg of xylose. Mono means monosaccharides (xylose and glucose). Gray: xylose, Red: glucose, Blue: X2, Orange: X3, Pink: U1, Green: U2, Purple: U3. Each data of saccharides was an average of three replicate determinations, and error bars show the standard deviation.

Fig. 3. Effect of enzyme dose. The DXRF (10 mg/ml) were hydrolyzed by various concentration of Hemicellulase amano 90 (13.5, 27 and 54 U) at 50 °C for 2-36 h. A: 13.5 U of enzyme, B: 27 U of enzyme, C: 54 U of enzyme. The concentration of each product was quantified by HPLC. Gray: xylose, Dark blue: glucose and X2, Orange: X3, Pink: U1, Green: U2, Purple: U3. Each data of saccharides was average of two replicate determinations, and bars show the data range.

455 **Fig. 4.** Effects of substrate concentration and reaction period. Various concentration of the
456 DXRF (10, 20 and 30 mg/ml) were hydrolyzed by 54 U of Hemicellulase amano 90 at 50 °C
457 for 2-36 h. A: 10 mg/ml of the DXRF, B: 20 mg/ml of the DXRF, C: 30 mg/ml of the DXRF.
458 The concentration of each product was quantified by HPLC. Gray: xylose, Dark blue: glucose
459 and X2, Orange: X3, Pink: U1, Green: U2, Purple: U3. Each data of saccharides was average
460 of two replicate determinations, and error bars show the data range.

461

Fig. 1

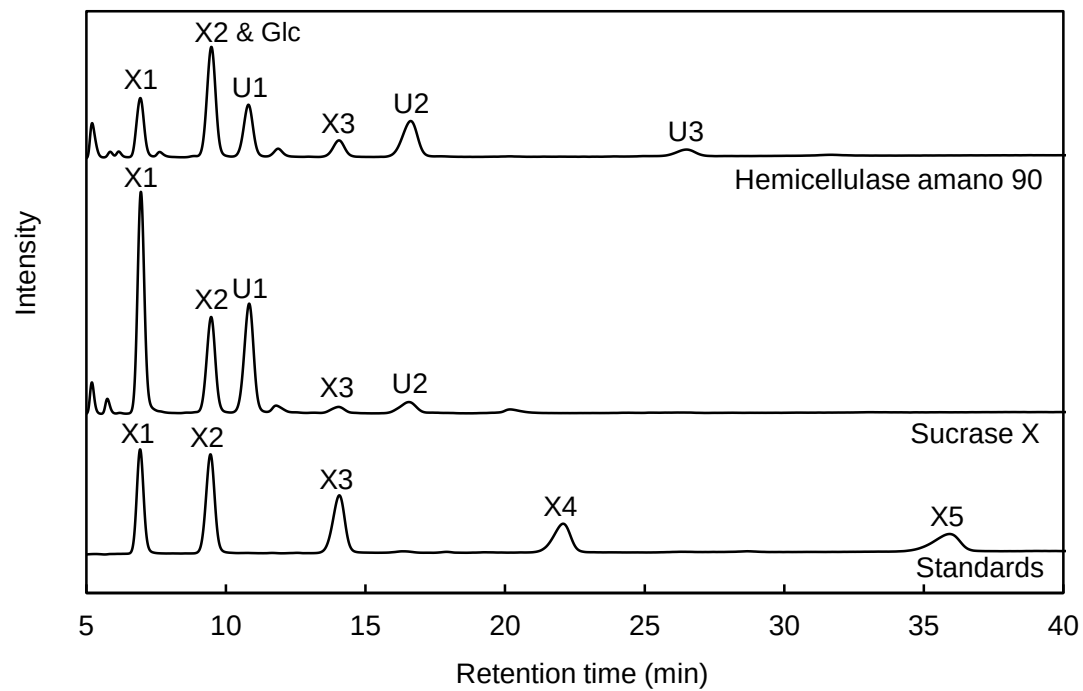


Fig. 2

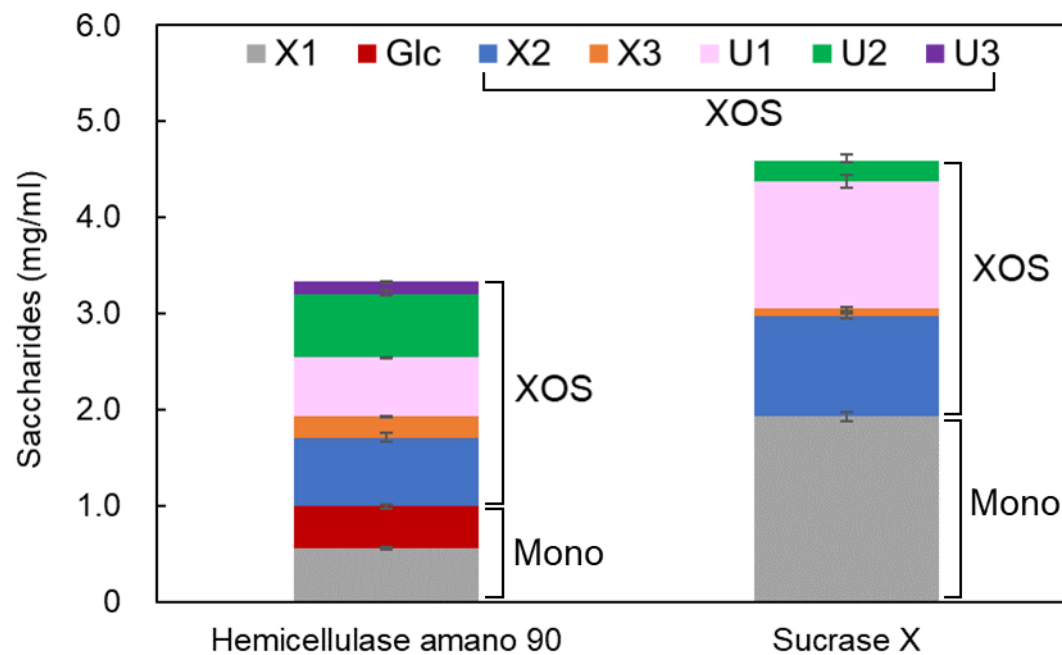


Fig. 3

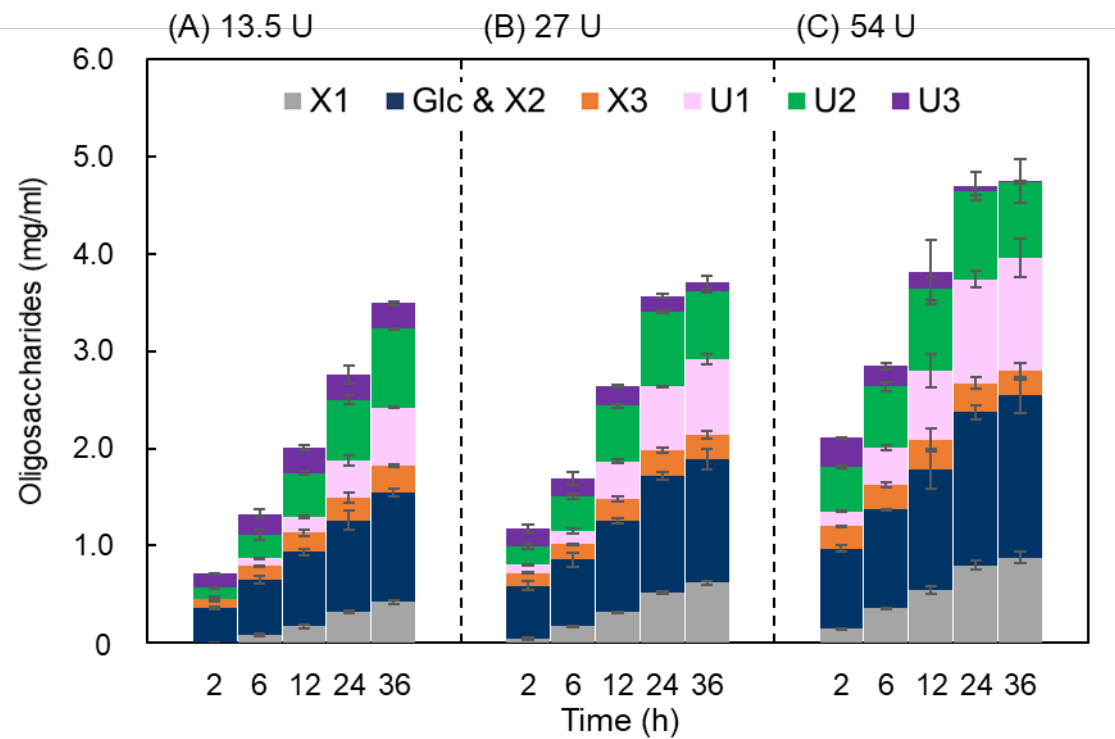
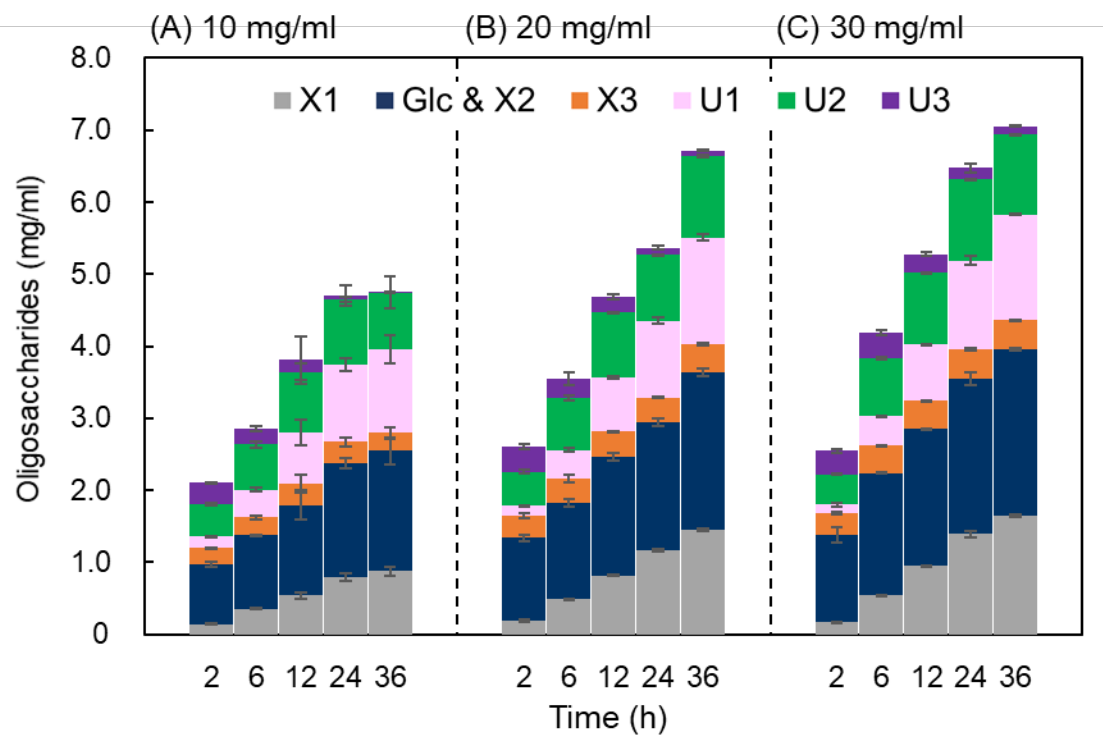


Fig. 4



1 Supplementary data

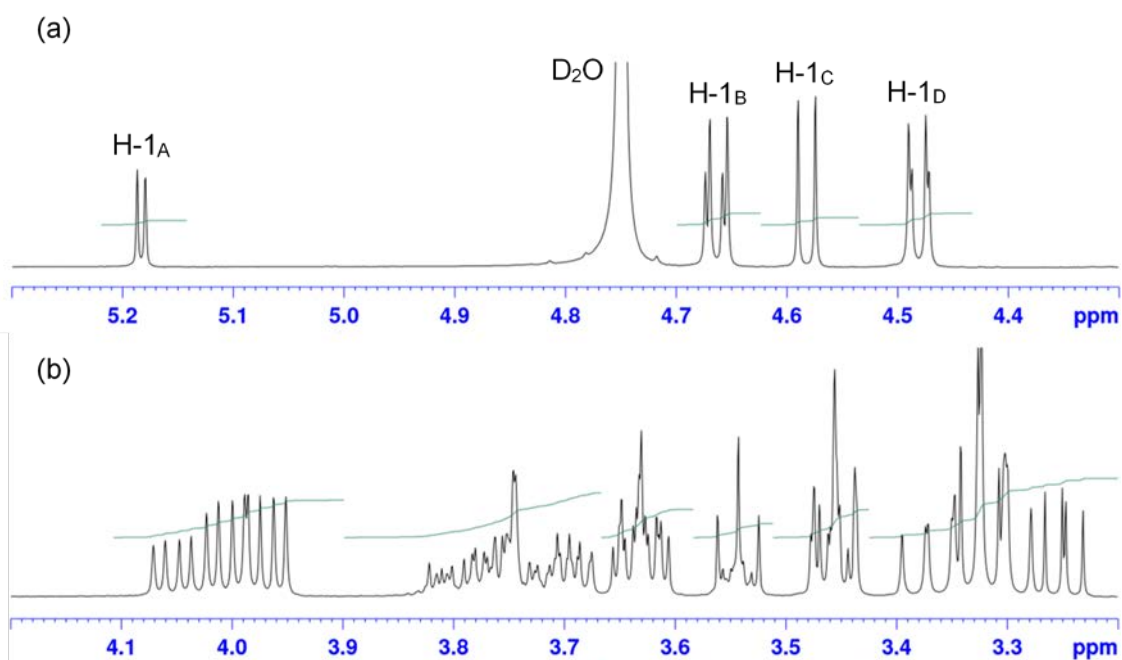


Fig. S1. ¹H NMR spectrum of U1. H-1 signals from our xylose residues were arbitrary labelled as H-1_{A-D}. The exchange of hydrogen to deuterium (D) in U1 was done by twice in D₂O (99.8% D) and then the U1 was dissolved in D₂O (100% D). ¹H NMR experiment was performed at 300 K using a Bruker AMX500 (Bruker, Wissembourg, France) at 500.13 MHz. A and C are reducing end of α- and β-D-xylopyranose (5.18 and 4.58 ppm), respectively. [(a) 4.3-5.3 ppm, (b) 3.2-4.2 ppm)].

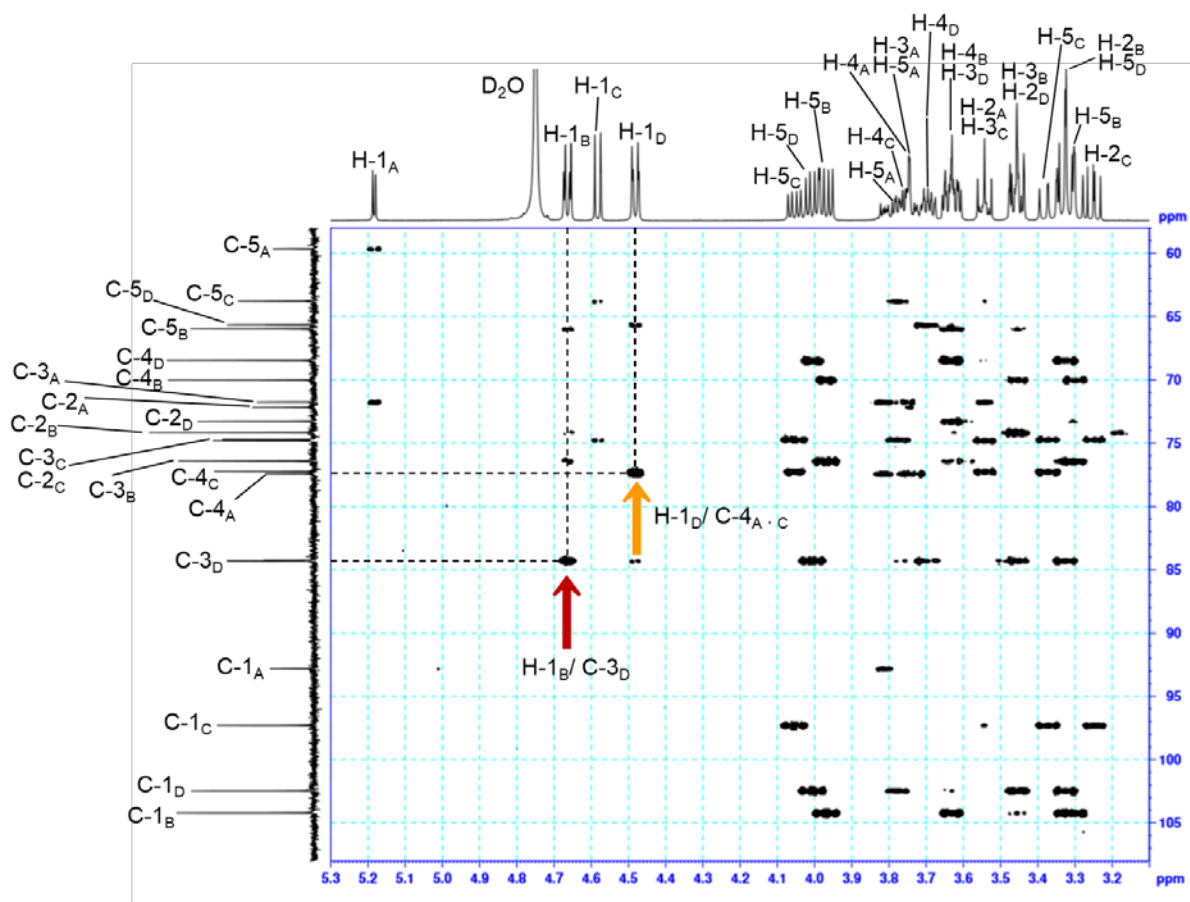


Fig. S2. HMBC spectrum of U1. The glycoside linkages between four xylose residues of U1 (non-reducing terminus, the centrl sugar of saccharide, α -anomer reducing sugar, and β -anomer reducing sugar) were elucidated with HMBC experiment. HMBC experiment was performed at 300K using a Bruker AMX500 (Bruker, Wissembourg, France) at 500.13 and 125.77 MHz for ^1H and ^{13}C NMR analysis, respectively. H-1 signals from four xylose residues were arbitrary labelled as H-1_{A-D}. The red arrow ($\uparrow$) emphasizes ^1H - ^{13}C correlation between H-1_B and C-3_D which indicates that C-1_B and C-3_D are glycosidically linked. The yellow arrow ($\uparrow$) emphasizes ^1H - ^{13}C correlation between the H-1_D/C-4_A and H-1_D/C-4_C, which indicates that the C-1_D and the C-4_A (reducing ends of α anomer), C-1_D and C-4_C (reducing ends of β anomer) are glycosidically linked.

21 **Table S1**

22 Chemical shift assignment of ^1H and ^{13}C resonances of U1.

23 H-1 signals from four xylose residues were arbitrary labelled as H-1_{A-D}.

Residues		¹ H-NMR				¹³ C-NMR	
		H	δ (ppm)	Mult.	<i>J</i> (Hz)		δ (ppm)
A	→4)-α-Xyl	H-1	5.18	d	3.7	C-1	92.8
		H-2	3.55	m		C-2	72.17
		H-3	3.75	m		C-3	71.74
		H-4	3.76	m		C-4	77.4
		H-5	3.82	m		C-5	59.63
		3.75	m				
B	β-Xyl-(1→	H-1	4.66	d	7.9	C-1	104.21
		H-2	3.33	dd	9.4, 7.9	C-2	74.15
		H-3	3.46	dd	9.4, 9.1	C-3	76.41
		H-4	3.63	ddd	10.8, 9.1, 5.5	C-4	70
		H-5	3.97	dd	11.4, 5.5	C-5	65.95
		3.3	dd	11.4, 10.8			
C	→4)-β-Xyl	H-1	4.58	d	7.9	C-1	97.29
		H-2	3.25	dd	9.3, 7.9	C-2	74.78
		H-3	3.54	dd	9.3, 9.3	C-3	74.7
		H-4	3.78	ddd	10.6, 9.3, 5.4	C-4	77.24
		H-5	4.05	dd	11.7, 5.4	C-5	63.78
		3.37	dd	11.7, 10.6			
D	→3)-β-Xyl-(1→	H-1	4.48	d	7.9	C-1	102.44
		H-2	3.45	dd	9.0, 7.9	C-2	73.26
		H-3	3.63	dd	9.3, 9.0	C-3	84.29
		H-4	3.7	ddd	10.1, 9.3, 5.5	C-4	68.45
		H-5	4.01	dd	11.7, 5.5	C-5	65.65
		3.33	dd	11.7, 10.1			

24 d: doublet, dd: double doublet, ddd: double double doublet, m: multiplet.