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Xylooligosaccharides from Hardwood and Cereal Xylans Produced by a Thermostable Xylanase as Carbon Sources for *Lactobacillus brevis* and *Bifidobacterium adolescentis*

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Supporting Information

ABSTRACT: To compare xylans from forestry with agricultural origins, hardwood xylan (birch) and cereal arabinoxylan (rye) were hydrolyzed using two variants of the xylanase *Rm*Xyn10A, full-length enzyme and catalytic module only, from *Rhodothermus marinus*. Cultivations of four selected bacterial species, using the xylooligosaccharide (XOS) containing hydrolysates as carbon source, showed selective growth of *Lactobacillus brevis* DSMZ 1264 and *Bifidobacterium adolescentis* ATCC 15703. Both strains were confirmed to utilize the XOS fraction (DP 2−5), whereas putative arabinoxylooligosaccharides from the rye arabinoxylan hydrolysate were utilized by only *B. adolescentis*. *Escherichia coli* did not grow, despite its capability to grow on the monosaccharides arabinose and xylose. It was also shown that *Pediococcus parvulus* strain 2.6 utilized neither xylose nor XOS for growth. In summary, *Rm*Xyn10A or its catalytic module proved suitable for high-temperature hydrolysis of hardwood xylan and cereal arabinoxylan, producing XOS that could qualify as prebiotics for use in functional food products.

KEYWORDS: *birch xylan, rye arabinoxylan, GH10 xylanase, Lactobacillus brevis, Bifidobacterium adolescentis, xylooligosaccharide*

INTRODUCTION

Biomass utilization is a topic of wide interest today, due to future needs in the fields of energy and chemicals, as well as food production. It is thus of interest to develop technologies that improve use of currently underutilized resources in biomass, such as the hemicelluloses. Xylans are the most common types of hemicelluloses and are heterogeneous polymers built with a linear β-1,4-linked xylose backbone with various degrees of short side chains; their structure and composition differ depending on origin, part, and age of the plant, as well as the processing method for their purification.[1−3] In hardwood, the predominant hemicellulose is with little variation *O*-acetyl-4-*O*-methylglucurono-β-D-xylan, sometimes called acetylglucuronoxylan.[2] The heteroxylans from grasses, including cereals, have the same backbone of β-1,4-linked xylose residues, but are generally more branched. Moreover, the side chains contain large proportions of L-arabinose residues, which may occur in combination with other sugars, for example, xylose and galactose, or in combination with ferulic or coumaric acid.[1,2] These substituents occur randomly in the polymers, leaving stretches of nonsubstituted xylan. In the evaluation of the environmental impact of processing of xylans, enzyme fragmentation, for example, by xylanases, is favorable due to the possibility to control the catalysis, compared to chemical agents, limiting the formation of side products and lacking the need to use harsh chemicals in line with a green chemistry perspective.[4] Xylanases are classified as glycoside hydrolases and are industrially attractive as they are generally easy to handle and do not require cofactors.[5] Xylanases (EC 3.2.1.8) are classified under different glycoside hydrolase (GH) families, for example, GH5, GH7, GH8, GH10, GH11, and GH43, depending on sequence and structure similarities between the enzymes.[6] GH10 and GH11 are dominated by enzymes with endo-1,4-β-xylanase activity, catalyzing random hydrolysis of 1,4-β-D-xylosidic linkages in the backbone. Several GH families, including GH43, also host exoacting enzymes, such as xylosidases (EC 3.2.1.37), that catalyze successive hydrolysis of the terminal xylose residue at the nonreducing end of the xylan polymer, or exoxylanase (EC 3.2.1.156), found in GH8, that catalyzes removal of the terminal xylose in the reducing end of oligomeric xylan. Previous studies have shown that carbohydrate binding modules (CBMs) carried by xylanases can have a significant effect on the hydrolysis of lignocellulosic substrates,[7,8] likely dependent on enzyme and type and complexity of substrate. To obtain oligosaccharides, only endoxylanases are of interest as their more random action on the xylan polymer will result in a spectrum of oligosaccharide products. If the reaction is run above ambient temperatures, a thermostable endoxylanase can be used. Having a process at higher temperatures is advantageous as such reaction conditions lead to increased solubility and reduced viscosity of the substrates, and when processing is taken into account, it will also reduce costs of cooling before an enzyme treatment step as well as decrease the risk of microbial contamination in the process.[6,9] Thermostable enzyme variants are advantageous in processing of resources such as agricultural

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crops or wood, as high temperatures promote enzyme penetration and cell-wall degradation.[5,10−12]

Arabinoxylooligosaccharides (AXOS) and xylooligosaccharides (XOS) produced upon hydrolytic processing of cereal xylan, especially those originating from wheat, have recently been studied as novel nondigestible oligosaccharides. These oligosaccharides have been extensively evaluated and are regarded as safe for intake and proven to be health beneficial in animal and human studies.[13] Several studies have proved their prebiotic potential by in vitro and in vivo fermentation experiments,[14−16] showing that the growth rate of probiotic bacteria is stimulated by their fermentation.

Probiotic bacteria are defined as microorganisms that survive the passage into the colon, where they exert health-beneficial effects to their host and have been proved to modulate the host immune system by increasing the secretion of antimicrobial substances to balance the intestinal microbiota.[17,18] *Lactobacillus* and *Bifidobacterium* spp. are well recognized as representative genera of probiotics. Suppression of nonwanted or harmful (pathogenic) bacteria can be achieved by their presence, reducing the risk of many colonic diseases such as inflammatory bowel diseases (IBD), acute ulcerative colitis, and Crohn's disease.[18,19] Moreover, overall health status has been claimed to be affected by colonic health.[20] Several recent studies have shown the ability of selected strains of *Lactobacillus* and *Bifidobacterium* to ferment xylan-derived oligosaccharides.[21,22] In the case of *Bifidobacterium adolescentis* the specific growth rate was higher on XOS than on the constituent monosaccharide xylose, proving the great growth stimulatory effects of XOS.[23] Many studies have shown that *Bifidobacterium* can also utilize AXOS,[24,25] and several enzymes able to cleave the arabinose side chain have been characterized.[26] However, it is still unclear if some lactobacillus species actually can ferment AXOS. There is also still an interest in identifying new strains of lactic acid bacteria that can utilize XOS and to find new resources for making XOS. When it comes to growth studies, there are not many studies in which the uptake of individual oligosaccharides in complex hydrolysis mixtures have been analyzed.

In this work two types of model xylans, originating from hardwood (birch) and cereal (rye), were utilized as the starting material for enzymatic hydrolysis using a thermostable xylanase (*Rm*Xyn10A) to obtain a range of xylooligosaccharides. The materials were treated with *Rm*Xyn10A, active at temperatures above 80 °C, of family GH10 to obtain XOS and AXOS from hardwood and cereal xylan, respectively. Two variants of the xylanase were evaluated as biocatalysts for the hydrolysis, one comprising the complete native enzyme full length (FL), composed of five modules including two CBMs that bind mainly to xylan,[27,28] and the other comprising the isolated catalytic module of the enzyme (CM). The hydrolysate obtained after the enzymatic treatment was used as carbon source in the growth trials. As stated above, AXOS have been proposed to have a prebiotic potential, whereas XOS hydrolysates of xylan from hardwoods have been less investigated. The three probiotic/putative probiotic strains tested for growth on the hydrolysates in this study were *Pediococcus parvulus*, *B. adolescentis*, and *Lactobacillus brevis*. *P. parvulus* 2.6 was chosen on the basis of its production of putatively health-promoting exopolysaccharides[29] as well as results showing lowered cholesterol in humans after consumption of oatmilk containing cocultures of *P. parvulus* and *Bifidobacterium* spp.[30] As a pathogen bacteria control, the less desired bacterium in the intestinal flora, *Escherichia coli*, was used.

MATERIALS AND METHODS

Preparation of Thermostable Endoxylanase. Xylanase Xyn10A from *Rhodothermus marinus* and the isolated catalytic module (CM) from the same enzyme (for cloning, see Abou-Hachem et al.)[31] were produced in batch cultivations in *E. coli* BL21(DE3) in Luria−Bertani (LB) medium containing ampicillin and induced by IPTG in the midlog phase.[31] Cell pellets were resuspended in binding buffer and lysed by ultrasonication on ice for 5 × 5 min in 5 min intervals. The lysed cells were centrifuged at 5500*g* for 20 min and then heat treated at 70 °C in a water bath for 30 min to partially purify the thermostable xylanase. The supernatant after heat treatment, containing the His-tagged xylanase, was purified by immobilized metal ion affinity chromatography (AKTA Prime, Uppsala, Sweden) as described elsewhere.[27,32] Imidazole was used as elution buffer. Eluted protein was dialyzed 20 kDa cutoff (SpectrumLab, Los Angeles, CA, USA) in 20 mM sodium phosphate buffer, pH 7.5, at 4 °C overnight. The purity of the xylanase solution was determined by SDS-PAGE (Bio-Rad, Stockholm, Sweden) with 12.5% acrylamide. Total concentration and enzyme activity of xylanase was determined by the bicinochoninic protein assay (Sigma, Steinheim, Germany) and dinitrosalicylic acid stopping method[33] using the reported conditions.[34] The xylanase activity was calculated from a standard curve, using xylose standards of defined concentrations dissolved in a solution containing 1% xylan in 20 mM sodium phosphate buffer, pH 7.5. The xylanase activity was expressed in katals (mol/s) of released reducing xylose residues per mole of xylanase.

Hydrolysis of Xylan and Arabinoxylan. Xylan from birchwood X-0502 (Sigma, Steinheim, Germany) with >90% xylose and arabinoxylan from rye flour P-RAXY (Megazyme, Wicklow, Ireland) with 90% carbohydrates (arabinose 38%, xylose 59%, 3% other sugars) were hydrolyzed by the purified endoxylanase variants. A time study of the hydrolysis reaction was done at 70 °C under stirring by using 60 nM xylanase in a 10 g/L solution of birchwood xylan or rye flour arabinoxylan dissolved in sodium phosphate buffer, pH 7.5. The reaction was followed by sampling at 2 h intervals during the first 6 h and then again after 24 h. The samples were analyzed with HPAEC-PAD as described below. The XOS hydrolysates, used for the batch cultivation experiments, was prepared in the same way but in Milli-Q water, and in this case the reaction was terminated after 4 h. To inactivate the endoxylanase, the samples were immediately immersed in boiling water for 30 min and were then kept at room temperature to cool, after which they were transferred to round-bottom flasks for freeze-drying.

Analysis of Hydrolyzed Xylan and Arabinoxylan. Oligosaccharides present in hydrolyzed birchwood xylan and rye flour arabinoxylan were analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Dionex, Sunnyvale, CA, USA) using a 250 mm × 4 mm i.d., 8.5 μm, CarboPac PA100 column and guard column, 50 mm × 4 mm, of the same material (Dionex), a mobile phase (1 mL/min) of constant 100 mM NaOH, and a gradient of sodium acetate 0−20 min of 10−160 mM and 20−25 min of 160−400 mM. Standards of arabinose (Fluka, Buchs, Switzerland), xylose (Merck, Darmstadt, Germany), xylobiose, xylotriose, xylotetraose, and xylopentaose (Megazyme) were used to identify the peaks in the chromatograms using 10 μL injections. Samples were diluted in Milli-Q water before analysis and compared with the standards.

Bacterial Strains and Growth Media. The bacterial strains used to test the fermentability of the hydrolyzed xylans were *B. adolescentis* ATCC 15703, *L. brevis* DSMZ 1264, *P. parvulus* 2.6,[35] and *E. coli* BL21 (Novagen, Madison, WI, USA). *B. adolescentis*, *L. brevis*, *P. parvulus*, and *E. coli* were all precultivated twice using glucose as carbon source.

B. adolescentis was inoculated in *Bifidobacterium* medium at 37 °C. The medium contained 12.5 g of casein peptone, tryptic digest, 6.25 g of yeast extract, 6.25 g of meat extract, 6.25 g of bacto soytone, 2.5 g of K_2PO_4, 0.25 g of $MgSO_4 \cdot 7\ H_2O$, 0.0625 g of $MnSO_4 \cdot\ H_2O$, 6.25 g of

NaCl, and 1.25 mL of Tween 80 per liter, respectively. To this solution was added 5 mL of solution with resazurin (25 mg/100 mL) together with 50 mL of salt solution containing 0.25 g of $CaCl_2 \cdot H_2O$, 0.5 g of $MgSO_4 \cdot 7\ H_2O$, 1 g of K_2HPO_4, 1 g of KH_2PO_4, 10 g of $NaHCO_3$, and 2 g of NaCl per liter, respectively. The medium was subsequently boiled followed by cooling under N_2 gas. Cysteine was added to a concentration of 0.625 g/L and adjusted to pH 6.8 using NaOH.

L. brevis was grown anaerobically in MRS broth (pH 6.7) under anaerobic condition at 37 °C.

P. parvulus was grown anaerobically in MRS broth (pH 5.2) at 30 °C. In this case, inoculum cultures were grown in the presence of erythromycin for maintaining an exopolysaccharide producing plasmid (EPS plasmid).

E. coli was first streaked on LB agar and incubated at 37 °C. A single colony was then transferred to fresh defined mAT medium with glucose as carbon source[36] and incubated at 37 °C aerobically. Cells were then transferred to fresh mAT medium, and when the cells had reached early stationary phase, they were used for the anaerobic cultivations at 37 °C in the presence of different carbon sources.

The respective carbon sources arabinose, glucose and xylose were filter sterilized through a 0.22 μm filter, whereas rye flour arabinoxylan (RAX), hydrolyzed rye flour arabinoxylan (H-RAX), birchwood xylan (BX), and hydrolyzed birchwood xylan (H-BX)) were autoclaved at 121 °C for 15 min separately. At the start of the cultivation the sterile carbon source was added to the respective medium, prepared without carbon source, at a concentration of 5 mg/mL based on total sugar or hydrolysate concentration. Medium without carbon source was for all strains also used as a negative control.

All media for the cultivation experiments, broth as well as agar, were autoclaved at 121 °C for 15 min. All cultivation media used for anaerobic growth were deaerated by replacing the oxygen in the anaerobic tubes with nitrogen gas. Then, all tubes were closed with metal caps and autoclaved at 121 °C for 15 min. Afterward, they were kept at 4 °C before being used in the experiments.

Batch Fermentation Experiment. *B. adolescentis*, *L. brevis*, and *P. parvulus* were all preinoculated for 24 h and *E. coli* was preinoculated for 7 h in the respective medium before the culture was transferred to fresh medium for the anaerobic batch fermentation experiment. The cell pellets from the inoculum cultures were harvested by centrifugation at 3200*g* for 15 min and washed twice with 0.9% saline solution.

The total volume of the inoculum was 2% (v/v) except for *P. parvulus*, which required 4% (v/v) of culture. Anaerobic cultivations of *B. adolescentis*, *L. brevis*, and *E. coli* were done at 37 °C for 72 h, and *P. parvulus* was cultivated at 30 °C, which is the optimal growth temperature for this strain, for 72 h. In each fermentation experiment duplicate tubes were used for every carbon source, and there was one control tube without carbon source. The total volume of each tube was 10 mL: 8 mL of prepared medium and 2 mL of added carbohydrate substrate or sterile water for the control.

Samples from the cell cultures were collected over a time period of 72 h with 24 h time intervals, for analysis of growth, pH, and utilization of the carbon source. The cell density was used as a measure of bacterial growth and was determined by measuring the optical density (OD) at 620 nm. A microplate was used for measuring the growth using 200 μL samples of *B. adolescentis*, *L. brevis*, and *E. coli* cultures, respectively, whereas a Biowave II UV−visible spectrophotometer (Biochrom, Cambridge, UK), with a 1 mL cuvette, was used to measure the growth rate of *P. parvulus*. Serial dilution and total plate count (CFU/mL) were used to confirm the growth yield of *P. parvulus* at 72 h. pH values were measured to estimate the production of acid in the cultures. The carbohydrate utilization ability of the bacteria on the different carbohydrate sources was determination by HPAEC-PAD.

RESULTS AND DISCUSSION

Xylanase Activity Measurements. *Rm*Xyn10A is a modular GH10 xylanase that originates from the thermophilic marine bacterium *R. marinus*.[31] The purified *Rm*Xyn10A variants FL, that is, the full-length enzyme, and CM, that is, a construct with only the catalytic module of the enzyme, were first analyzed to compare their activity optima, as well as specific activity on the birchwood xylan and rye flour arabinoxylan substrates.

The temperature optima at pH 7.5 of the xylanase variants were determined using birchwood xylan as substrate. Both full-length and catalytic module of *Rm*Xyn10A had temperature optima for activity at 80 °C (Figure 1) in accordance with previous measurements using the catalytic module only.[34]

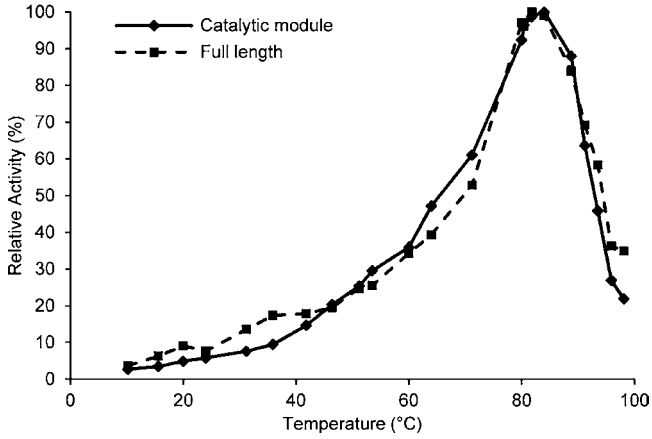

Figure 1. Temperature optima for full-length and catalytic module of *Rm*Xyn10A at pH 7.5.

Previous thermal deactivation experiments had shown that the catalytic module had a half-life of 100 min at this temperature, while being completely stable at 70 °C for 24 h.[34] To allow long incubations with xylan substrates without loss of activity, it was decided to use 70 °C for the current experiments. In addition, it was proven that the methodology used to deactivate the enzyme, in this case the CM, after hydrolysis of the xylan (boiling for 30 min, followed by autoclaving), resulted in complete deactivation. After the boiling step, approximately 15% of the activity was left, whereas autoclaving completely inactivated the xylanase.

Specific activity on the hardwood xylan and cereal arabinoxylan was determined for both full-length enzyme (FL) and the CM at 70 °C and was in the same range on both substrates. The activity was slightly higher for the FL (106 ± 3 kat/mol on birchwood xylan and 109 ± 4 kat/mol on rye flour arabinoxylan) compared to the CM (71 ± 2 kat/mol on birchwood xylan and 93 ± 3 kat/mol on rye flour arabinoxylan). The activity differences for the respective substrate were, however, small, indicating that the presence of CBMs in the FL gives a limited contribution. Despite the slightly higher activity of FL, we decided to continue working only with CM because of the easier production and higher thermostability.

Hydrolysis Product Profiles and Preparation of XOS Hydrolysates. The CM and FL produced identical hydrolysis product patterns in the low degree of polymerization (DP) range (data not shown), motivating continued use of only one of the enzyme variants. In further experiments, only the CM was used.

Time course studies using two concentrations of the CM were thus performed to monitor the production of oligosaccharides. As can be seen in Figures 2A and 3A xylotetraose (X_4) and xylopentaose (X_5) are first formed and

later degraded further into xylotriose (X_3), xylobiose (X_2), and xylose (X).

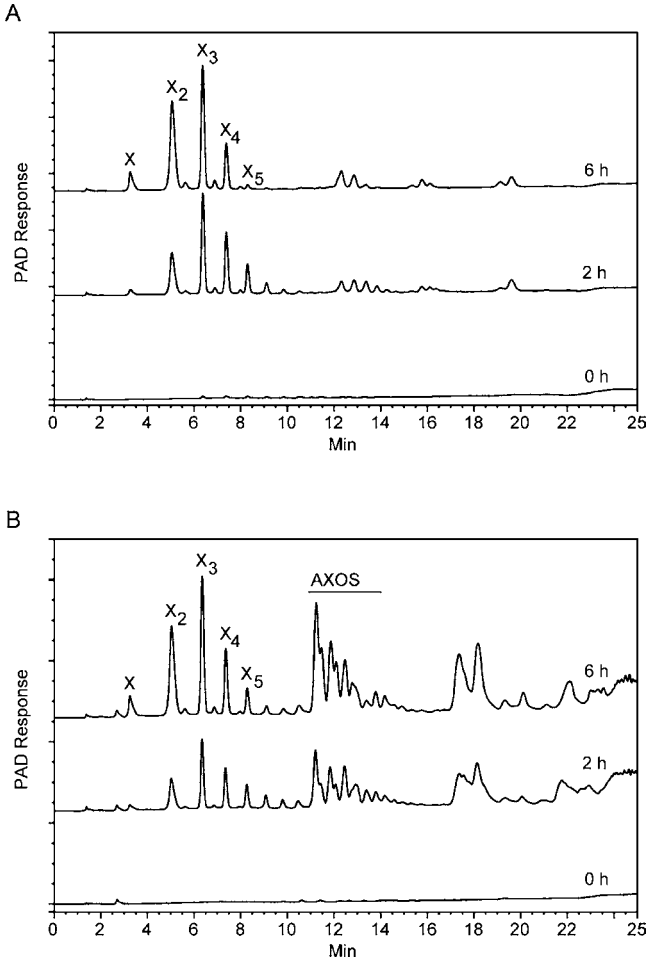

Figure 2. HPAEC-PAD chromatogram shows hydrolysis pattern of (A) birchwood xylan and (B) rye flour arabinoxylan for 0, 2, and 6 h using *Rm*Xyn10A catalytic module. X, xylose; X_2, xylobiose; X_3, xylotriose; X_4, xylotetraose; X_5, xylopentaose; AXOS, arabinoxylooligosaccharides.

This shows that X_2 is the end product of birchwood xylan hydrolysis and that a limited amount of X is produced as a consequence of slow degradation of X_3 to X_2 and X. This is in accordance with previous data on the enzyme, which showed that the smallest hydrolyzable XOS substrate is X_3.[34] Worth noting are also the peaks appearing between 12 and 15 min in Figure 2A, which most likely are XOS substituted with 4-*O*-methylglucuronic acids.[37] Rye flour arabinoxylan is hydrolyzed into a mixture of both XOS and AXOS (Figures 2B and 3B). No significant free arabinose could be detected after hydrolysis (Figure 3B), indicating that the enzyme cannot hydrolyze the arabinose side chains on the rye flour arabinoxylan. Instead, some arabinose is released due to the incubation at elevated temperatures. On the basis of the formation of xylose in the time course of hydrolysis data (Figure 3), 4 h was chosen as the reaction time for making the hydrolyzed substrates for the growth trials.

The quantity of oligosaccharides in each fraction of the 4 h hydrolysates used for the growth experiments was analyzed by HPAEC-PAD (Table 1). In hydrolyzed birchwood xylan the total XOS content is 20% compared to 3.3% in hydrolyzed rye flour arabinoxylan; however, arabinose-substituted AXOS (Figure 2B) have not been quantified due to lack of standards. This means that the total oligosaccharide content in hydrolyzed rye flour arabinoxylan is underestimated.

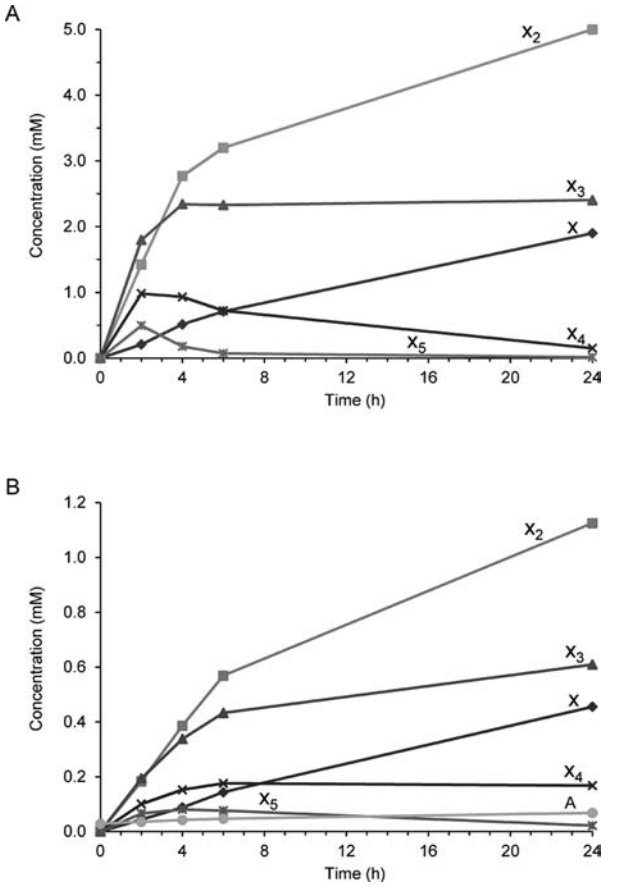

Figure 3. Concentration of xylooligosaccharides present in the reaction mixture of (A) birchwood xylan for 0, 2, 4, 6 and 24 h and (B) rye flour arabinoxylan, using *Rm*Xyn10A catalytic module.

Table 1. Quantity (% w/w) of Monosaccharides and Xylooligosaccharides Present in Substrates for Growth Experiments

	hydrolyzed xylans[a]	
sugar	birchwood	rye flour
arabinose	0	0.1
xylose	0.2	0.1
xylobiose	3.6	1.0
xylotriose	8.2	1.4
xylotetraose	5.2	0.6
xylopentaose	2.9	0.3

[a]Values are means, $n = 2$. SD not more than 3%.

Selection of a temperature that is 10 °C below the highest activity for the enzyme allowed the hydrolysis to proceed for several hours without deactivation of the enzyme (Figure 1), resulting in a lower enzyme load, which can contribute to reduced costs for the process.

The hydrolysates produced were in accordance with previously reported oligosaccharide profiles, for example, obtained after hydrolysis by a family 10 xylanase from *Aspergillus aculeatus*.[38] *Rm*Xyn10A thus specifically hydrolyzed the xylan backbone and did not modify the substituent

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Table 2. Relative Growth Compared with Glucose and pH of Four Different Intestinal Strains with Seven Different Substrates after a Period of 48 h[a]

	B. adolescentis		*L. brevis*		*P. parvulus*		*E. coli*	
substrate	growth	pH	growth	pH	growth	pH	growth	pH
arabinose	++	4.7	−	6.5	+ /−[b]	5.1	++	5.1
glucose	++	4.3	++	6.6	++	4.4	++	4.8
xylose	+	4.7	+++++	5.8	+ /−	5.1	+	5.4
RAX[c]	−	5.9	−	6.7	−	5.2	−	6.9
H-RAX[d]	+	5.1	++	6.6	+ /−	5.2	−	6.9
BX[e]	−	6.2	−	6.7	+ /−	5.2	−	7.0
H-BX[f]	+	5.3	+++++	5.9	+ /−	5.2	−	6.9
control[g]	−	6.1	−	6.7	−	5.2	−	6.9

[a]Growth relative growth on glucose: −, no growth; +, 1−50% growth; ++, 51−100% growth; +++, 101−150% growth; ++++, 151−200% growth; + ++++, >201% growth. Growth experiments were done in duplicates except control which was grown only a single time. [b]+/− increase in OD without uptake of sugars confirmed by HPAEC-PAD. [c]Rye flour arabinoxylan. [d]Hydrolyzed rye flour arabinoxylan. [e]Birchwood xylan. [f]Hydrolyzed birchwood xylan. [g]No added carbohydrate substrate.

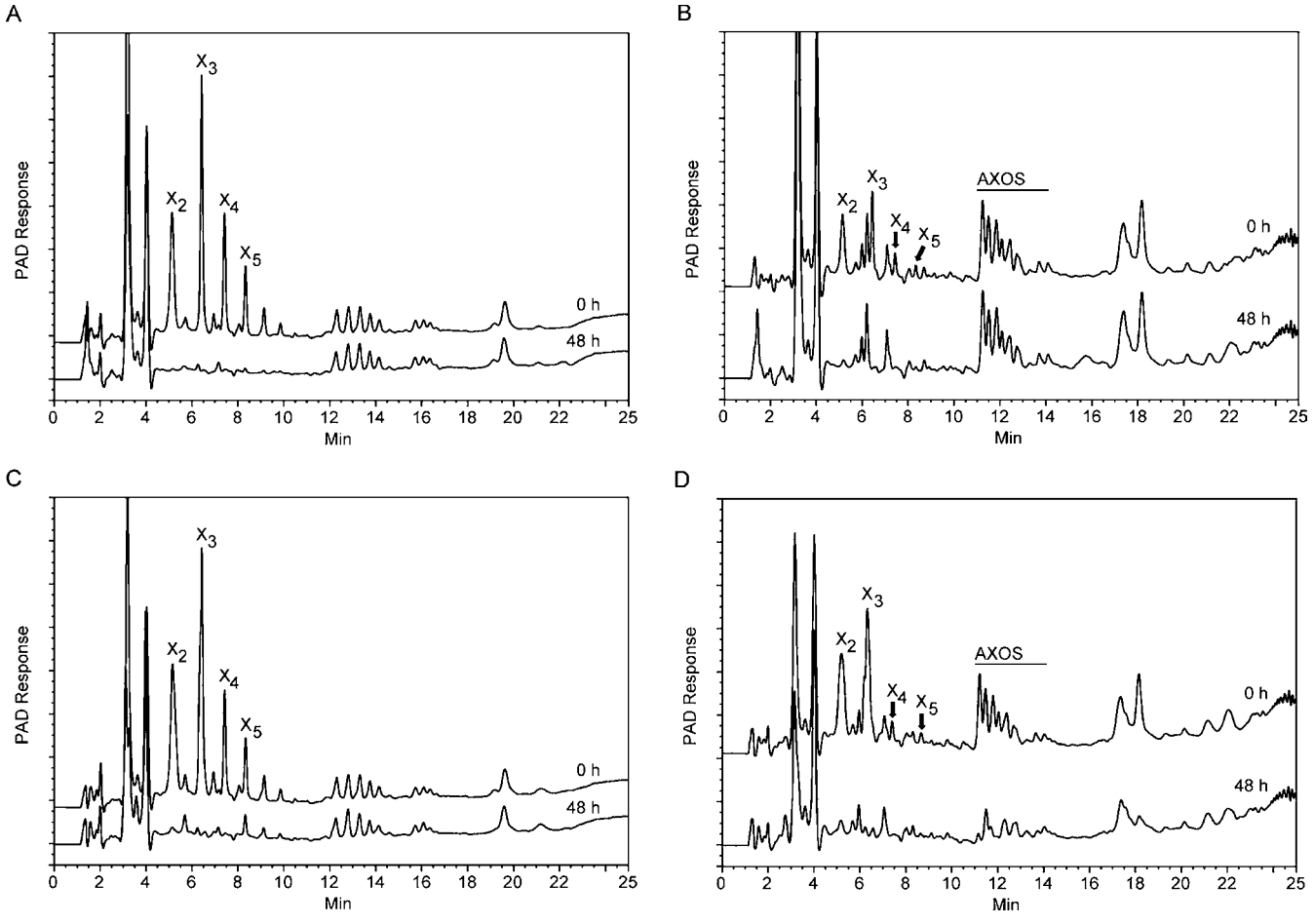

Figure 4. HPAEC-PAD chromatograms showing oligosaccharides present in broth before (upper chromatogram) and after (lower chromatogram) 48 h of fermentation. (A, B) *L. brevis* and (C, D) *B. adolescentis* on hydrolyzed birchwood xylan and hydrolyzed rye flour arabinoxylan, respectively. X_2, xylobiose; X_3, xylotriose; X_4, xylotetraose; X_5, xylopentaose; AXOS, arabinoxylooligosaccharides (only in panels B and D).

arabinose residues. Indeed, previous work has shown that most family 10 xylanases can attack the xylosidic linkage on the nonreducing end of a substituted residue but cleave only at the third xylosidic linkage after the substituted residue.[39] For *Rm*Xyn10A, it was also shown that reducing sugars in the same range were obtained for both the rye flour arabinoxylan and birchwood xylan, indicating that the enzyme had access to the xylan backbone to approximately the same extent in the two substrates, but the higher yield of XOS from birch xylan shows that this substrate to a much larger extent consists of nonsubstituted xylose residues. Another advantage of using *Rm*Xyn10A is the relatively low amount of the monosaccharide xylose produced in the hydrolysis, limiting the loss of oligomeric xylose in the hydrolysate. Because both *Rm*Xyn10A variants FL and CM produced similar product profiles and rate of hydrolysis using both rye and birch xylan as substrate, it appears that the CBM of *Rm*Xyn10A does not contribute to the outcome of the hydrolytic process.

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Growth of Selected Intestinal Bacteria on the XOS Hydrolysates. Results from the batch fermentation experiments showed that the XOS hydrolysates were fermented by both *B. adolescentis* and *L. brevis*, shown by both an increase in OD and a decrease in pH, whereas nontreated polymeric xylans were not fermented by any of the strains (Table 2) in accordance with previous studies.[21] In a study using the same strain of *B. adolescentis* they achieved higher growth on XOS than on glucose, which can be explained by the fact that they had a higher concentration of XOS in their fermentation experiment.[40] *E. coli* did, as expected, ferment neither hydrolyzed nor polymeric xylan, confirming that the probiotic strains were selected over *E. coli* on the XOS hydrolysates. *E. coli* could ferment the monosaccharides arabinose and xylose, but despite this did not grow on the XOS hydrolysate as neither arabinose nor xylose was present in significant amounts (Table 1). All strains tested fermented glucose, the carbon source used as positive control, and *P. parvulus* did not show significant growth on any other carbon source.

Carbohydrate utilization measured by using HPAEC-PAD after 48 h of fermentation (Figure 4 and Table 4) confirms the results from Table 2 that *B. adolescentis* and *L. brevis* consume the XOS present in the hydrolyzed xylans, whereas *P. parvulus* cannot (Table 4).

Table 3. Monosaccharides Present after 48 h of Fermentation in Media Containing Single Pure Monosaccharides

	monosaccharides[a] (g/L)		
bacterial strain	arabinose	glucose	xylose
B. adolescentis	4.15	0.05	2.66
L. brevis	4.94	4.78	3.74
P. parvulus	4.98	0	5.14

[a]Starting concentrations were 5.0 g/L. Values are means, $n = 2$. SD not more than 3%.

Table 4. Xylooligosaccharides Present after 48 h of Fermentation on Hydrolyzed Birchwood Xylan

	xylooligosaccharides[a] (g/L)			
bacterial strain	xylobiose	xylotriose	xylotetraose	xylopentaose
starting concn	0.18	0.41	0.26	0.15
B. adolescentis	0.00	0.00	0.00	0.03
L. brevis	0.00	0.00	0.00	0.00
P. parvulus	0.18	0.43	0.30	0.16

[a]Values are means, $n = 2$, except for *P. parvulus* for which a single determination was done to verify that no XOS were utilized based on the growth data. For sample analyzed in duplicate the SD was not more than 4%.

The chromatograms from HPAEC-PAD analysis also show that there is a difference between the fermentation pattern of AXOS present in hydrolyzed rye flour arabinoxylan between *L. brevis* and *B. adolescentis* (Figure 4B,D). Although both strains used the nonsubstituted XOS, only *B. adolescentis* used the arabinose-substituted AXOS. *B. adolescentis* could also utilize the arabinose monosaccharide, which was not fermented by *L. brevis* (Table 3). Interestingly for *L. brevis* is that both xylose and XOS stimulated growth to a higher degree than glucose, whereas other studies have shown that glucose is fermented to the same or even to a higher degree than xylose when added together in the same mixture.[41]

Genomic data on *P. parvulus* are currently not available. Screening of genes encoding GHs in currently available genomes in the genus *Pediococcus* (*P. acidilactici*, *P. pentosaceus*, and *P. claussenii*), however, showed that genes that could putatively encode xylanases or xylosidases were present only in *P. acidilactici* (GH3 and GH43), indicating that it may be more common not to utilize than to utilize XOS in this genus.

The same statement may also be valid for the genus *Lactobacillus*. Only some *Lactobacillus* strains have been found to utilize XOS, and in a study conducted previously,[14] it was reported that except for *L. brevis*, used in the current work, none of the tested lactobacilli showed XOS utilization during fermentation. Our data showed that growth of *L. brevis* was promoted in both the rye hydrolysate (3.3% XOS) and birch hydrolysate (20% XOS) but that the putative AXOS fraction was not utilized. These results show the importance of analyzing which oligosaccharides are truly utilized by the tested strain when using xylan hydrolysates as it has previously been reported that *L. brevis* showed growth on hydrolysates containing AXOS.[22] However, we believe that *L. brevis* in the previous study consumed only the XOS fraction, presumably also present in the hydrolysates. The AXOS fraction in the rye flour arabinoxylan hydrolysate was, however, used by *B. adolescentis*, which also, on the basis of previous papers, was expected to grow on this substrate.[25] This difference in the two substrates used points in the direction that the nonsubstituted XOS would stimulate a broader range of probiotic bacteria than the AXOS.

To date, no model of the uptake of XOS is presented for *L. brevis*, whereas for *Bifidobacterium* a mechanism has been proposed. Both *B. adolescentis* and *B. animalis* subsp. *lactis* BB-12[42−44] are predicted to transport XOS (and AXOS) across the cell membrane by an ATP-binding cassette transporter (ABC) type sugar transport system capable of importing a variety of oligosaccharides, which are further hydrolyzed by intracellular enzymes. Intracellular hydrolysis of the oligosaccharides is corroborated by the fact that the relevant characterized enzymes from *B. adolescentis* appear to lack signal peptides.[43]

Only a few xylanolytic enzymes are to date characterized in *L. brevis*,[45] and the lack of hydrolysis of AXOS in *L. brevis* is not immediately clear as genes that could putatively encode both xylanase/xylosidase (GH3 and GH43) and arabinofuranosidase (GH51) encoding genes are present in the genome. Confirmation of specificity, however, requires characterization of the gene products, as the activity profiles of individual enzymes in both GH3 and GH43 may vary. At present, it can only be speculated that the lack of consumption of AXOS in *L. brevis* is either a consequence of differences in the uptake system or a consequence of substrate specificity of *L. brevis* enzyme candidates. Future research into the GH in *L. brevis* would be of interest to explain their xylooligosaccharide fermenting abilities.

■ ASSOCIATED CONTENT

Supporting Information

Overview of the domain structure of the xylanase *Rm*Xyn10A. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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