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Functional comparison of versatile carbohydrate esterases from families CE1, CE6 and CE16 on acetyl-4-*O*-methylglucuronoxylan and acetyl-galactoglucomannan

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ABSTRACT

Background: The backbone structure of many hemicelluloses is acetylated, which presents a challenge when the objective is to convert corresponding polysaccharides to fermentable sugars or else recover hemicelluloses for biomaterial applications. Carbohydrate esterases (CE) can be harnessed to overcome these challenges.

Methods: Enzymes from different CE families, *An*AcXE (CE1), *Os*AcXE (CE6), and *Mt*AcE (CE16) were compared based on action and position preference towards acetyl-4-*O*-methylglucuronoxylan (MGX) and acetyl-galactoglucomannan (GGM). To determine corresponding positional preferences, the relative rate of acetyl group released by each enzyme was analyzed by real time ^{1}H NMR.

Results: *An*AcXE (CE1) showed lowest specific activity towards MGX, where *Os*AcXE (CE6) and *Mt*AcE were approximately four times more active than *An*AcXE (CE1). *Mt*AcE (CE16) was further distinguished by demonstrating 100 times higher activity on GGM compared to *An*AcXE (CE1) and *Os*AcXE (CE6), and five times higher activity on GGM than MGX. Following 24 h incubation, all enzymes removed between 78 to 93% of total acetyl content from MGX and GGM, where *Mt*AcE performed best on both substrates.

Major Conclusions: Considering action on MGX, all esterases showed preference for doubly substituted xylopyranosyl residues (2,3-*O*-acetyl-Xyl*p*). Considering action on GGM, *Os*AcXE (CE6) preferentially targeted 2-*O*-acetyl-mannopyranosyl residues (2-*O*-acetyl-Man*p*) whereas *An*AcXE (CE1) demonstrated highest activity towards 3-*O*-acetyl-Man*p* positions; regiopreference of *Mt*AcE (CE16) on GGM was less clear.

General significance: The current comparative analysis identifies options to control the position of acetyl group release at initial stages of reaction, and enzyme combinations likely to accelerate deacetylation of major hemicellulose sources.

1. INTRODUCTION

Hemicelluloses constitute approximately 20–30% of the total mass of annual and perennial plants. They are differentiated based on backbone and side group chemistries, which vary depending on the botanical source [1,2]. 4-*O*-methylglucuronoxylans (MGX) are the main hemicellulose in deciduous wood such as eucalyptus trees, and contain xylopyranosyl (Xyl*p*) backbone sugars that are connected through β-(1→4)-linkages and are substituted by α-(1→2)-linked 4-*O*-methylglucopyranosyluronic acid (MeGlc*p*A) (Figure 1). By contrast, galactoglucomannans (GGM) are the main hemicellulose in coniferous wood such as spruce trees, and contain β-(1→4)-linked D-glucopyranosyl (Glc*p*) and β-D-mannopyranosyl (Man*p*) units that are likewise connected through β-(1→4) glycosidic bonds (Figure 1). The galactoglucomannan backbone is also substituted by α-(1→6)-linked-D-galactose side groups, mainly to Man*p* in spruce galactoglucomannans [3].

Glucuronoxylans and galactoglucomannans are both acetylated in their natural forms [2]. Several biological functions have been attributed to the acetylation of these main hemicelluloses, including protection of plant cell walls from degradation by microbial enzymes, along with regulated interaction with cellulose [4,5]. Acetylation may occupy single O-2 or O-3 positions of Xyl*p* or Man*p* subunits in glucuronoxylans and galactoglucomannans, respectively. Xyl*p* in glucuronoxylans may also be di-acetylated [6–8], and Xyl*p* decorated with 2-O-MeGlc*p*A can be acetylated at the O-3 position ((2-*O*-MeGlc*p*A)3-*O*-acetyl-Xyl*p*) [6,7,9]. A summary of reported compositions of acetyl-4-*O*-methylglucuronoxylans and acetyl-galactoglucomannans is provided in Supplemental Table 1.

The carbohydrate active enzyme (CAZy) classification system (www.cazy.org) [10] currently assigns carbohydrate esterases (CEs) into 16 CE families. Deacetylating enzymes with reported activity on xylans and/or corresponding oligosaccharides include acetyl xylan esterases (AcXE; EC. 3.1.1.72) belonging to CE families CE1-7, an unclassified carbohydrate esterase [11], as well as acetyl esterases (AcE; EC. 3.1.1.6) belonging to family CE16 [12–14]. Nevertheless, only AXEs from CE1, CE3-CE6, and CE16 were confirmed to include enzymes with preferred activity towards xylans [15, 42]. By contrast, comparatively few enzymes have been reported to deacetylate galactoglucomannans or corresponding oligosaccharides. Notable examples include: 1) an AcE from *Trichoderma reesei* VTT-D-86271 (Rut C 30) (*Tr*CE16) [16–19] an acetyl glucomannan esterase (AGME) and feruloyl esterase (FE; CE1) from *Aspergillus oryzae* VTT-D-85248 (presently renamed as *Aspergillus tubingensis*) [18–20]; 3) an esterase within a commercial

enzyme preparation from *Aspergillus niger* (Celluzyme) [21,22] and 4) two AcXEs belonging to CE1, one from *Penicillium purpurgenum* [23] and other from *Schizophyllum commune* VTT-D-88362 [24]. Low levels of acetyl group release from oligomers of galactoglucomannans was also detected in culture filtrates of *Aspergillus awamori* VTT-D-71025, *Aureobasidium pullulans* VTT-D-89397 and *Streptomyces olivochromogens* VTT-E-82157 [18].

Considering the impacts of hemicellulose acetylation summarized above, AcEs and AcXEs can be harnessed to promote hemicellulose saccharification [25–27], control the rheology and solubility of hemicelluloses [1,13], and improve thermomechanical pulp (TMP) yield [28]. The applied significance of AcEs and AcXEs, along with the inability to predict enzyme action based on CAZy family assignation alone, has motivated functional comparisons of several CE families [8,13,29–32]. Neumüller et al. [32] propose three distinguishing groups of xylan-active CEs: 1) those acting only on O-2 and O-3 monoacetylated Xyl*p*, which have been identified in families CE2, CE3, and CE4; 2) those with further activity towards 2,3-di-*O*-acetylated Xyl*p*, which have been identified in families CE1, CE5 and CE6, and 3) those with further activity towards 3-*O* acetylated Xyl*p* substituted at O-2 by MeGlc*p*A; which have been identified in family CE16 (e.g. *An*CE16). Puchart et al. [33] subsequently isolated aldo-tetraouronic acid ($Ac^3MeGlcA^3Xyl_3$) to compare the ability of selected CE16s to target 3-*O* acetylated Xyl*p* substituted also at O-2 by MeGlc*p*A. Notably, *Tr*CE16 was the only CE16 esterase able to release acetyl group from both isomers of $Ac^3MeGlcA^3Xyl_3$, containing acetyl group at O-3 and O-4, where acetyl group migration from O-3 to O-4 is possible.

Whereas recent comparative analyses of CEs have considerably advanced our understanding of enzyme regio-selectivity towards acetylated xylans, only one study has been reported that investigates CE action towards specific acetylated positions within mannan substrates [24]. Accordingly, herein we compare the activity and regio-selectivity of three CEs using major, wood-derived hemicelluloses, namely 4-*O*-methylglucuronoxylan (MGX) (herein, from *Eucalyptus globulus*) and acetyl-galactoglucomannan (GGM) (herein, from Norway spruce). The CEs were selected from three different CE families, namely acetyl xylan esterase *An*AcXE (CE1) from *Aspergillus nidulans*, acetyl xylan esterase *Os*AcXE (CE6) from *Orpinomyces* sp., and acetyl esterase *Mt*AcE (CE16) from *Myceliophthora thermophile*. Enzymes from these CE families were selected based on (1) our previous analysis of *An*AcXE (CE1) that confirmed activity on acetylated polysaccharides, including cellulose acetate [34], (2) *Os*AcXE (CE6) being a commercially available enzyme with reported activity on acetylated xylans [29,32], but so far not on mannans, which is notable given that the CE6 family comprises the largest number of

predicted plant acetyl esterases and so may be expected to possess broad substrate specificity and act on both xylans and (gluco)mannans, and (3) few examples where CE16 enzymes have been characterized using natural substrates even though reports to date indicate activity towards typically resistant positions, such as (2-*O*-MeGlc*p*A)3-*O*-acetyl-Xyl*p* in MGX [33], and reported activity towards (oligomeric) GGM [14,17,18].

Briefly, the current comparative analysis showed that positional preference of selected enzymes towards MGX does not predict its positional preference towards GGM, and that enzymes showing similar positional preference towards one substrate may differ when compared using another. The present comparative analysis of CEs on both MGX and GGM underscores the importance of including natural substrates when characterizing enzyme action, and the difficulty to predict enzyme action on GGM based on known activity towards MGX or vice versa.

2. MATERIALS AND METHODS

2.1. Enzymes, substrates, and assay reagents

In-house and commercially available enzymes were used for this study. Specifically, *An*AcXE (AN6093.2) from *Aspergillus nidulans* was recombinantly produced in *Pichia pastoris* at pH 5.0 and 30 °C and then purified based on [35]. The CE6 AcXE from *Orpinpmyces sp.* (AAC14690.1; *Os*AcXE) was purchased from Megazyme (Wicklow, Ireland). The CE16 AcE acetyl esterase from *Myceliophthora thermophila* (AGW01024.1; *Mt*AcE) [8] was produced at DuPont Biosciences (Wageningen, Netherlands) and polished using size exclusion and hydrophobic chromatography (Supplemental Figure 1).

Acetyl-4-*O*-methylglucuronoxylan (MGX) isolated from milled (8 mm) chips of eucalyptus by steam extraction [36] was kindly provided by Prof. J.C. Parajó (University of Vigo, Spain) and acetyl-galactoglucomannan (GGM) obtained from the TMP mill process waters [37] was from Prof. Willför (Åbo Akademi University, Finland). Total acetyl groups available in MGX or GGM were determined as described in [9] and were found to be 15 and 9% of total substrate weight, respectively. Briefly, 1 mg of GGM or MGX was suspended in 200 µL of 0.1 N NaOH and incubated with shaking (120 rpm) for 24 h at room temperature; released acetic acid was neutralized and then measured using the Acetic Acid Assay Kit (K-ACET) purchased from Megazyme (Ireland).

2.2. pH Optima

The pH optimum of each enzyme was evaluated using 4-methylumbelliferyl acetate (4-MUA)[38] (Supplemental Figure 2). Reaction mixtures (400 µL) comprised 100 mM of the selected buffer, 2.5 mM 4-MUA, and 10 µL of the enzyme sample. Enzyme doses were adjusted to ensure that linear rates of reaction were measured, and were 3.6 µg for *An*AcXE (CE1); 0.2 µg for *Os*AcXE (CE6) and 0.05 µg for *Mt*AcE (CE16). Chosen buffers were: sodium citrate buffer (pH 3.0 to pH 5.0), sodium phosphate buffer (pH 6.0 to pH 8.0), and glycine-OH buffer (pH 9.0 to pH 10.0). Following incubation at 40 °C for 10 min, reactions were stopped by adding of 600 µL 50 mM citric acid (pH 2.2), vortexed, and then passed through a 0.2 µm GHP Acrodiscs 13 filter (PALL) to remove insoluble particles prior to measurement of 4-methylumbelliferone at 345 nm. pH optima were also confirmed using MGX and the Acetic Acid kit (K-ACET) (Supplemental Figure 3).

2.3. Enzyme stability at pH 6.0

Because overnight reactions with MGX and GGM were performed at pH 6.0 (see below), enzyme stabilities were also evaluated at this pH condition. In this case, 2.5 µg of *An*AcXE (CE1), *Os*AcXE (CE6), or *Mt*AcE (CE16) were incubated for 24 h in 50 µL of 100 mM sodium phosphate buffer (pH 6.0). Following the incubation period, 3.6 µg of *An*AcXE (CE1), 0.2 µg of *Os*AcXE (CE6), and 0.05 µg of *Mt*AcE (CE16) were transferred to 50 µL reaction mixtures comprising 100 mM sodium phosphate buffer (pH 6.0) and 2.5 mM 4-MUA. Reaction products were then processed and measured as described above.

2.4. Activity measurements using MGX and GGM

To measure specific activities for each enzyme on each substrate, reaction mixtures (200 µL) comprised 100 mM sodium phosphate buffer (pH 7.0), 0.5% (w/v) substrate, and 50 µL of the enzyme sample. Enzyme doses were adjusted to ensure that linear rates of reaction were measured. When using MGX, enzyme doses were 0.5, 1 or 5 µg of *An*AcXE (CE1), 0.5, 1 or 5 µg of *Os*AcXE (CE6), and 0.5 or 1 µg of *Mt*AcE (CE16). When using GGM, enzyme doses were 2.5 or 5 µg of *An*AcXE (CE1), 2.5 or 5 µg of *Os*AcXE (CE6), and 0.05, 0.1 or 0.3 µg of *Mt*AcE. Following incubation at 40 °C for 10 min, reactions were stopped by adding 40 µL of 0.33 M H_2SO_4. Released acetic acid was quantified using the Acetic Acid Assay Kit (K-ACET).

To quantify the extent of acetyl group released by each enzyme after 24 h, reaction mixtures (200 µL) comprising 100 mM sodium phosphate buffer (pH 6.0), 0.5% (w/v) substrate, and 10 µg of

*An*AcXE (CE1), *Os*AcXE (CE6), or *Mt*AcE (CE16) (i.e., 10 mg enzyme/ g substrate) were incubated with shaking (120 rpm) at 40 °C. Overnight reactions were performed at pH 6.0 to minimize the possibility of auto hydrolysis, release of acetyl groups from the substrate, and acetyl group migration [39]. Reaction mixtures without enzyme served as negative controls and were subtracted from test measurements.

2.5. HSQC NMR spectroscopy

Quantitative HSQC NMR was performed to quantify acetyl group release from MGX and GGM. Analyses of MGX were carried out in D_2O, whereas analyses of GGM were carried out in DMSO-d6. All NMR spectra were collected on a 600 MHz Bruker Avance III NMR spectrometer equipped with a QCI H-C/N/P-D cryogenically cooled probe head. The measurements were carried out at either 22 °C (samples dissolved in D_2O) or 60 °C (samples dissolved in DMSO-d6). For the 1D ^{1}H spectra the residual water signal was suppressed by a four second volume selective presaturation (so called 1D-NOESY presaturation). (Neuhaus et al. 1996). The quantitative HSQC spectra were acquired using matched sweep adiabatic pulses optimised for ^{13}C sweep width of 130 – 10 ppm for all 180º ^{13}C pulses in order to compensate the differences in the $^{1}J_{CH}$ coupling constants (Bruker's pulse program hsqcedetgpsisp2.3) (Zwahlen et al. 1997). Matrices of 2048 x 256 data points were collected and zero filled once in F1; a $\pi/2$ shifted squared sine bell weighting function was applied in both dimensions prior to the Fourier transformation.

2.6. HSQC NMR Spectra annotations and quantifications

The chemical shifts for MGX were referenced to the C-1 and H-1 signal of MeGlc*p*A (5.28 ppm, 98.85 ppm); chemical shifts for GGM in D_2O were referenced to C-1 of Glc*p* (103.55 ppm) and H-2 of 2-*O*-acetyl Man*p* (5.42 ppm). In the case of GGM in DMSO, the chemical shifts were calibrated against DMSO-d6 (2.50 ppm, 39.51 ppm). Annotation of MGX spectra were according to [7] and [6]; annotation of GGM spectra were according to [40].

The relative content of the acetylated Xyl*p* in the MGX was calculated from quantitative heteronuclear single quantum coherence (qHSQC) spectra according to [41]. Briefly, the signals of the H-1 and C-1 of substituted and nonsubstituted Xyl*p* were summed to 100%, thereafter the signals of H-2 of 2-*O*-acetyl, H-3 of 3-*O*-acetyl, H-2 of 2,3-*O*-acetyl, and H-3 of (2-*O*-MeGlc*p*A)3-*O*-acetyl-Xyl*p* were integrated separately to calculate the relative content of each

form of *O*-acetyl-Xyl*p* subunit. For the quantitation of relative content of acetylated Man*p* in the GGM, the H-1 and C-1 of the substituted and non-substituted Man*p*, as well as the Glc*p* and Gal*p* were summed as 100%, thereafter the signal of H-2 of 2-*O*-acetyl- and H-3 of 3-*O*-acetyl-Man*p* was integrated separately to calculate the relative content of each form of O-acetyl Man*p*.to 100%, thereafter the signals of H-2 of 2-*O*-acetyl, H-3 of 3-*O*-acetyl, H-2 of 2,3-*O*-acetyl, and H-3 of (2-*O*-MeGlc*p*A)3-*O*-acetyl-Xyl*p* were integrated separately to calculate the relative content of each form of *O*-acetyl-Xyl*p* subunit. For the quantitation of relative content of acetylated Man*p* in the GGM, the H-1 and C-1 of the substituted and non-substituted Man*p*, as well as the Glc*p* and Gal*p* were summed as 100%, thereafter the signal of H-2 of 2-*O*-acetyl- and H-3 of 3-*O*-acetyl-Man*p* was integrated separately to calculate the relative content of each form of *O*-acetyl Man*p*.

2.7. ^{1}H NMR Analyses

Rates of acetyl group release from specific positions within MGX and GGM were monitored by ^{1}H NMR. Both substrates (600 mg) were dissolved in 600 µL of 100 mM sodium phosphate buffer (pH 6.0) following replacement of milliQ water by D_2O through freeze drying. All samples were analyzed in 5.0 mm NMR tubes (Aldrich) using a Bruker Avance III 400 MHz spectrometer equipped with a 5 mm BBFO Plus probe head. A water suppression pulse program (noesygppr1d) with suppression power of 3.1623e-006 W was used for relative quantitative measurements. The following acquisition parameters were applied: 90° pulse with relaxation delay of 4 s and acquisition time of 5.1 s; 8 scans and 4 dummy scans; 65536 data points and spectrum width of 15.979 ppm. Before the reaction was initiated by addition of enzyme, the spectra of native substrates were measured at 40 °C. After enzyme addition, acquisition parameters were adjusted (e.g., to align signals) and then spectra were automatically recorded every 1.8 min over 30 min (Figure 2). When using MGX, enzyme doses per mg of substrate were 5 µg of *An*AcXE (CE1), 1 µg of *Os*AcXE (CE6), and 1 µg of *Mt*AcE (CE16). When using GGM, enzyme doses per mg of substrate were 10 µg of *An*AcXE (CE1), 10 µg *Os*AcXE (CE6), and 1 µg/mg *Mt*AcE (CE16). In all cases, enzymes were prepared in 100 mM deuterated sodium phosphate buffer, spectra were processed and analyzed using TopSpin 3.0 (Bruker).

Sum of integrals was normalized to 100% and signals were then plotted against time using Origin 2016 64 bit (Supplemental Figure 4), and resulting slopes were used to calculate the rate of acetyl groups release from specific positions by each enzyme [32].

3. RESULTS and DISCUSSION

3.1. Establishing reaction conditions for comparative analyses

*An*AcXE (CE1), *Os*AcXE (CE6), and *Mt*AcE (CE16) were similarly active at pH 7.0 or pH 6.0 (Table 1), by showing ≥90% of their maximal activity at pH 7.0 and ≥85% at pH 6.0. Accordingly, reaction rates were measured at pH 7.0 to ensure comparable substrate solubility, whereas overnight reactions were performed at pH 6.0 to minimize the possibility of autohydrolysis, non-enzymatic release of acetyl groups, and acetyl group migration. Notably, *An*AcXE and *Mt*AcE retained over 90% activity after 24 h at pH 6.0 (40 °C), whereas under these conditions, *Os*AcXE activity decreased by 40% after 5 h and 60% after 24 h (Figure 3).

3.2. Extent of carbohydrate esterase action on MGX

Acetic acid measurement and HSQC NMR analyses were performed to compare the deacetylation efficiency of *An*AcXE (CE1), *Os*AcXE (CE6), and *Mt*AcE (CE16) on MGX. In general, slightly higher deacetylation efficacies were calculated from HSQC spectra as compared to acetic acid measurements (Figure 4; Table 2), which likely reflects the relative sensitivity of the analytical methods used.

Following 24 h of incubation, maximal removal of acetyl groups from MGX by *An*AcXE (CE1) was between 80%-90% (Figure 4; Table 2). This result was similar to that reported for other CE1 acetyl xylan esterases, including: (1) a CE1 from *Schizophyllum commune* (~80% deacetylation of DMSO-extracted birchwood MGX) [24]; (2) *An*AXE from *Aspergillus niger* and *Mt*AXE3 from *Myceliophthora thermophila* C1 (76-88% deacetylation of *O*-acetylated neutral xylo-oligosaccharides and 50–60% deacetylation of *O*-acetylated MeGlc*p*A-substituted xylo-oligosaccharides [8]); and (3) *Te*CE1 from *Talaromyces emersonii* (~80% deacetylation of MeGlc*p*A-substituted xylooligosaccharides) [32].

Similar to *An*AcXE (CE1), *Os*AcXE (CE6) released 80-90% of acetyl groups from MGX (Figure 4; Table 2). Earlier analyses of *Os*AcXE (also known as OsCE6) show less than 50% of total acetyl group release from MGX. However, in that case, MGX enriched in acidic xylo-oligosaccharides AcUXOS (i.e., (2-*O*-MeGlc*p*A)-3-*O*-acetyl-Xyl*p*) was used [32], and

Koutaniemi et al. [8] previously showed that enzymatic deacetylation of acidic xylo-oligosaccharides is typically lower than neutral xylo-oligosaccharides. Compared to *An*AcXE (CE1) and *Os*AcXE (CE6), *Mt*AcE (CE16) released the highest extent of acetyl groups from all positions in MGX (~90% total released). By comparison, *An*CE16 from *Aspergillus niger* was reported to release 18% of acetyl groups from AcUXOS derived from *Eucalyptus globulus* [32], whereas *Tr*CE16 from *Trichoderma reesei* was reported to release 10% of acetyl groups from xylo-oligomers with DP of ~10 [16,17]. These results suggested that *Tr*CE16 and *An*CE16 activity is restricted to the non-reducing end of corresponding substrates [19,32]. Different to both *An*CE16 and *Tr*CE16, *Pa*CE16 was able to act on birchwood acetyl-glucuronoxylan leading to substrate precipitation [42]. Thus, given the extent of acetyl groups released from MGX measured in the current study, *Mt*AcE (CE16) likely targets both non-reducing and internal positions within targeted substrates and so in this regard, is more similar to *Pa*CE16 than *Tr*CE16 or *An*CE16.

Quantitative HSQC (qHSQC) was then used to identify acetylated positions in MGX most susceptible to enzyme action. Less than 1% of 2-*O*-acetyl-Xyl*p* positions, and approximately 5% of 3-*O*-acetyl-Xyl*p* and 2,3-O-acetyl-Xyl*p* positions remained following MGX treatment with *An*AcXE (CE1). Similarly, less than 5% 2-*O*-acetyl-Xyl*p* positions in MGX remained intact following treatment of MGX with *Os*AcXE (CE6). Somewhat lower deacetylation efficiencies were measured for *Os*AcXE (CE6) action towards 3-*O*-acetyl-Xyl*p* and 2,3-O-acetyl-Xyl*p* positions, where 10% and 20% of corresponding acetyl groups remained.

By comparison, *Mt*AcE (CE16) effectively targeted the broadest range of acetyl group positions, leaving between 3-5% of 2-*O*-acetyl-Xyl*p*, 3-*O*-acetyl-Xyl*p*, and 2,3-*O*-acetyl-Xyl*p* positions, while also partially removing acetyl groups from (2-*O*-MeGlc*p*A)-3-*O*-acetyl-Xyl*p* positions (Table 2; Supplemental Figure 4B). Low but detectable and reproducible activity of *Mt*AcE towards (2-*O*-MeGlc*p*A)-3-*O*-acetyl-Xyl*p* after 24 h of reaction is consistent with reports of other CE16 enzymes, including *P. anserina* (*Pa*CE16A) [42], which shares approximately 70% of protein sequence identity with *Mt*AcE (CE16), but also *Tr*CE16 from *T. reesei* [14,33,43] and *An*CE16 from *A. niger* [32,33]. It was earlier postulated [8] and later shown [33] that acetyl group migration from O-3 to O-4 could account, at least partially, for *Mt*AcE (CE16) activity towards (2-*O*-MeGlc*p*A)-3-*O*-acetyl-Xyl*p*.

3.3. Extent of carbohydrate esterase action on GGM

All three enzymes were able to act on GGM in addition to MGX. During the 24 h incubation, *Mt*AcE released approximately 90% of acetyl groups in GGM, whereas roughly 80% of acetyl groups were released by *An*AcXE (CE1) and *Os*AcXE (CE6) under the same conditions (Figure 4). *Mt*AcE (CE16) action on GGM was comparable to that of an acetyl mannan esterase (AGME) from *Aspergillus niger* [20,21], an acetyl xylan esterase (CE1) from *Schizophyllum commune* [24], and a feruloyl esterase (CE1) from *Aspergillus oryzae* [24]. In each of these cases, between 90 and 95% of acetyl groups from GGM were released. By comparison, the family CE1 AcXEs from *Penicillium purpurogenum* (30%) and AGME (unclassified CE) from *Aspergillus oryzae* [24] were previously shown to release approximately 70% of acetyl groups from GGM.

Consistent with the expected higher degree of polymerization of GGM (recovered from a TMP process waters) compared to MGX (recovered from a steam explosion process), reaction products from GGM precipitated upon enzyme treatment. As a result, qHSQC analyses could not be performed in D_2O or DMSO-d6. Accordingly, ^{1}H NMR spectra were collected to identify acetyl group positions most resistant to enzyme action (Figure 5). Most notably, *Os*AcXE (CE6) targeted all acetyl groups in GGM, whereas 2(b)-*O*-acetyl-Man*p* was resistant to hydrolysis by *An*AcXE (CE1) and *Mt*AcE (CE16). Positions 2(a)-*O*-acetyl-Man*p* and 2(b)-*O*-acetyl-Man*p* (along with 3(a)-*O*-acetyl-Man*p* and 3(b)-*O*-acetyl-Man*p*) differ in terms of neighboring sugars [40,44]. Retention of 2(b)-*O*-acetyl-Man*p* in GGM therefore reveals that neighboring groups along the polysaccharide backbone influence enzyme accessibility to pendent acetyl groups.

3.4. Specific activity and positional preference of selected carbohydrate esterases

*Os*AcXE (CE6) and *Mt*AcE (CE16) showed similar specific activity towards MGX, which were approximately four times higher than that obtained for *An*AcXE (CE1) (Table 3). By contrast, similar specific activities were measured for *An*AcXE (CE1) and *Os*AcXE (CE6) on GGM, which were lower than corresponding enzyme activities on MGX (Table 3). *Mt*AcE (CE16) was interestingly distinguished by demonstrating 100 times higher activity on GGM compared to *An*AcXE (CE1) and *Os*AcXE (CE6), and five times higher activity on GGM than MGX (Table 3). The specific activity of *Mt*AcE (CE16) towards GGM (~2000 nkat/mg; 120 µg/min/mg) was comparable to that reported for AGME from *Aspergillus niger* (1190 nkat/mg) where 5 times higher GGM concentration was used [20,21]. Similarities between *Mt*AcE (CE16) and AGME suggests that *Mt*AcE (CE16) is likewise an unspecific acetyl mannan esterase [8].

Real time ^{1}H NMR was then performed for each enzyme to unravel potential preferences for

specific positions within MGX and GGM. Positional preference was defined as the ratio of deacetylation rate for a given acetyl group position to the abundance of corresponding acetyl groups in the original substrate. It is important to note here that calculated rates of acetyl group release from 2-*O*-acetyl-Xyl*p* and 3-*O*-acetyl-Xyl*p* may underestimate true values since reaction products after removal of one of the acetyl groups from 2,3-*O*-acetyl-Xyl*p* will contribute to these signals.

Although the specific activity of *An*AcXE on MGX differed from both other enzymes, *An*AcXE (CE1), *Os*AcXE (CE6) and *Mt*AcE (CE16) similarly demonstrated comparatively high activity towards acetyl groups present on doubly substituted Xyl*p* (i.e., 2,3-*O*-acetyl-Xyl*p,* Table 4). Although it was not possible to resolve which of these two acetyl residues were preferentially targeted, preferred action towards 2,3-*O*-acetyl-Xyl*p* positions was previously also reported for *Talaromyces emersonii Te*CE1 [32], *Orpinomyces sp. PC-2* OsCE6 [29,32], and *Aspergillus niger An*CE16A [33].

Considering singly substituted Xyl*p* positions, *An*AcXE (CE1), *Os*AcXE (CE6) and *Mt*AcE (CE16) all revealed preference towards 2-*O*-acetyl-Xyl*p* positions, which is consistent with reported activities for above mentioned *Te*CE1, OsCE6 and *An*CE16A [29,32,33]. *An*AcXE (CE1) and *Os*AcXE (CE6) were similarly active towards 3-*O*-acetyl-Xyl*p*, whereas activity towards this position was not observed for *Mt*AcE (CE16) (Table 4). In contrast to *Mt*AcE (CE16), *Tr*CE16 was shown to preferentially target 3-*O*-acetyl-Xyl*p* and 4-*O*-acetyl-Xyl*p* positions of oligosaccharides of acetyl-glucuronoxylan [43]. However, *An*CE16A rapidly targets 2,3-*O*-acetyl-Xyl*p* positions, followed by 2-*O*-acetyl-Xyl*p* and 3-*O*-acetyl-Xyl*p* positions, of polymeric xylans [33], but poorly targets 2-*O*-acetyl-Xyl*p* positions in methyl β-D-xylopyranoside diacetates and triacetates Puchart et al. 2016), thus showing differing posion specificty with polymeric and monomeric substrates. Similarly, then, the difference in reported positional preference of *Mt*AcE and *Tr*CE16 may be due to differences in the length of substrates used in corresponding analyses.

Whereas *An*AcXE (CE1) and *Os*AcXE (CE6) displayed similar positional preferences in MGX, the positional preferences of *Os*AcXE (CE6) and *Mt*AcE (CE16) were most similar when evaluated using GGM. Specifically, when considering the anomeric region of corresponding ^{1}H NMR spectra where peak signals were clearly resolved, it could be seen that both *Os*AcXE (CE6) and *Mt*AcE (CE16) displayed similar preference for 2-*O*-acetyl-Man*p* positions in GGM, whereas highest rates of *An*AcXE (CE1) activity were towards 3-*O*-acetyl-Man*p* substituents. Notably, however, positional preference within GGM was less clear when considering the acetyl region of corresponding ^{1}H NMR spectra, where peak signals are more intense but also overlapping (Figure

2B, Table 5). Still, the comparably high activity of *Mt*AcE (CE16), as well as *An*AcXE (CE1) and *Os*AcXE (CE6) towards the O-2 acetylated position of both Xyl*p* in MGX and Man*p* in GGM suggests that stereochemistry plays a minor role in substrate recognition by these enzymes. *Tr*CE16 preferentially targets 3-*O*-acetyl-Xyl*p* in oligo-saccharides of MGX; it was also shown to deacetylate oligomers of GGM [18], however, regio-selective activity of *Tr*CE16 or other AcEs from CE16 on oligomers or polymers of GGM has not been characterized.

4. CONCLUSION

The comparison of three CE families using both MGX and GGM uncovered substrate-dependent and enzyme dependent differences in reaction rates, extent of substrate conversion, and regio-selectivity. In particular, the acetyl xylan esterases *An*AcXE (CE1) and *Os*AcXE (CE6) displayed different specific activities towards MGX yet similar regio-selectivity. On the other hand, these enzymes were similarly active towards GGM. Notably, *Mt*AcE (CE16) was set apart from both acetyl xylan esterases by its comparatively high specific activity towards GGM. Nevertheless, comparably high activity of all three enzymes on 2-*O*-acetylated positions in GMX and GGM, which has equatorial orientation in Xyl*p* and axial orientation in Man*p,* suggests that the stereochemistry of the acetyl group has little effect on the activity of these enzymes.

The comparative analysis of three CE families on MGX and GGM underscore the impact of the selected substrate on reported enzyme activity as well as regio-selectivity, further highlighting known challenges associated with predicting enzyme action based on model compounds. Positions within major hemicellulose sources that remain resistant to CE action were confirmed, including (2-*O*-MeGlc*p*A)3-*O*-acetyl-Xyl*p* in MGX, and some 2-*O*-acetyl-Man*p* or 3-*O*-acetyl-Man*p* in GGM, thereby identifying targets for enzyme discovery as well as enzyme combinations that could be harnessed to promote hemicellulose recovery (e.g., through precipitation) versus full saccharification. Finally, earlier reports which tested AXE activity on ground wood powder [45], and fungal AXE expressed in plants [26], show that enzyme action on hemicelluloses embedded with plant fibre could be predicted from enzyme action on extracted hemicelluloses. However, direct comparison of different AXEs on MGX or GGM present in plant fibre remains to be done.

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TABLES

Table 1. Specific activity (µmol/min/mg) of *An*AcXE (CE1), *Os*AcXE (CE6) and *Mt*AcE (CE16) on 4-MUA at pH 6.0 and 7.0.

Enzyme	pH 6.0	pH 7.0
***An*AcXE (CE1)**	24 ± 0	26 ± 1
***Os*AcXE (CE6)**	201 ± 13	237 ± 5
***MtAcE* (CE16)**	506 ± 7	547 ± 28

n=3; errors indicate standard deviation

Table 2. HSQC NMR analysis of enzyme treated acetyl-4-*O*-methylglucuronoxylan. Mole percent remaining (compared to untreated MGX) of specific acetyl group positions following enzyme treatment.

acetyl-4-*O*-methylglucuronoxylan (MGX)	Untreated[a]	*An*AcXE (CE1)	*Os*AcXE (CE6)	*Mt*AcE (CE16)
2-*O*-acetyl-Xyl*p*	24.1 ± 0.2	0.2	1.1	1.1 ± 0.4
3-*O*-acetyl-Xyl*p*	57.6 ± 0.4	2.3	4.9	2.7 ± 1.4
2,3-*O*-acetyl-Xyl*p*	6.5 ± 0.3	0.2	1.2	0.2 ± 0.1
(2-*O*-MeGlc*p*A)3-*O*-acetyl-Xyl*p*	11.7 ± 0.1	8.1	9.0	7.6 ± 0.9
total acetylation (%)	100.0	10.8	16.3	11.6 ± 0.2
reduction acetylation (%)		89.2	83.7	88.4

a. The mole percent of acetyl group positions in the original MGX. n=2 for untreated and *Mt*AcE (CE16), otherwise n=1.

Table 3. Specific activity (µmol/min/mg) of *An*AcXE (CE1), *Os*AcXE (CE6) and *Mt*AcE (CE16) on MGX and GGM. Reactions were performed at pH 7.0 and 40º C; acetyl group release was measured using the acetic acid kit.

Substrate	*An*AcXE (CE1)	*Os*AcXE (CE6)	*Mt*AcE (CE16)
Eucalyptus acetyl-4-*O*-methylglucuronoxylan (MGX)	5.4 ± 0.7	22.8 ± 3	20.1 ± 2
Spruce acetyl- galactoglucomannan (GGM)	1.2 ± 0.1	1.6 ± 0.3	120 ± 20

n=3 (minimum); errors indicate standard deviation

Table 4. Relative activity (%) towards specific acetyl group positions in eucalyptus acetyl-4-*O*-methylglucuronoxylan (MGX) determined using real time ^{1}H NMR.

Enzyme	2-*O*-acetyl-Xyl*p*	3-*O*-acetyl-Xyl*p*	2,3-*O*-acetyl-Xyl*p*	(2-*O*-MeGlc*p*A)3-*O*-acetyl-Xyl*p*
*An*AcXE (CE1)	32	13	55	ND
*Os*AcXE (CE6)	40	15	46	ND
*Mt*AcE (CE16)	45	ND	55	ND

Relative activity was calculated as follows: (slope/mg$_{enzyme}$/(% acetyl groups at the specific position in untreated MGX)/ ∑ (slope/mg$_{enzyme}$/(% acetyl groups at the specific position in untreated MGX) *100. ND, not determined given that corresponding slope values were insignificantly different from zero (Suppl. Fig. 4).

Table 5. **Relative activity (%) towards specific acetyl group positions in spruce acetyl-galactoglucomannan (GGM) determined using real time ^{1}H NMR.**

Enzyme	2-*O*-acetyl-Man*p*	3-*O*-acetyl-Man*p*	2-*O*-acetyl-Man*p*; H2	3-*O*-acetyl-Man*p;* H3
*An*AcXE (CE1)	39	61	31	69
*Os*AcXE (CE6)	56	44	83	17
*Mt*AcE (CE16)	50	50	84	16

Calculation for relative activity: (slope/mg_{enzyme}/(% acetyl groups at the specific position in untreated GGM)/ $\sum$ (slope/mg_{enzyme}/(% acetyl groups at the specific position in untreated GGM) *100.

FIGURE LEGENDS

Fig. 1. Molecular structures of acetyl-4-*O*-methylglucuronoxylan (MGX) (A) and acetyl-galactoglucomannan (GGM) (B). Acetyl group positions are shown in red.

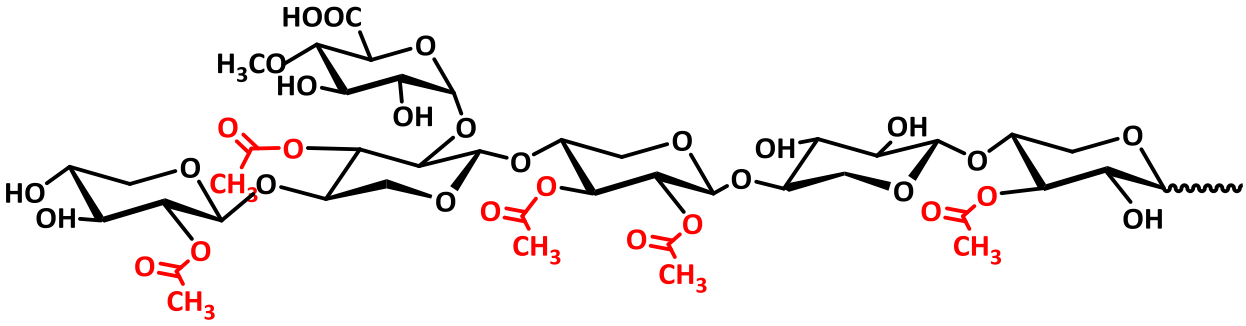
Fig. 2. ^{1}H NMR spectra following enzyme action on acetyl-4-*O*-methylglucuronoxylan (MGX) and acetyl-galactoglucomannan (GGM) in buffered D_2O at 40° C. The first spectrum was collected after 10 min of reaction; subsequent spectra were collected every 1.8 min. (A) MGX before and after treatment with *An*AcXE (CE1) (5 μg/mg MGX), *Os*AcXE (CE6) (1 μg/mg MGX), *Mt*AcE (CE16) (1 μg/mg MGX); (B) GGM before and after treatment with *An*AcXE (CE1) (10 μg/mg GGM), *Os*AcXE (CE6) (10 μg/mg GGM), *Mt*AcE (CE16) (1 μg/mg GGM).

Fig. 3. Enzyme stability at pH 6.0 and 40°C for up to 24 h. Relative activity was measured using 4-MUA. Average values are indicated above each bar. n=3; error bars indicate standard deviation.

Fig. 4. Deacetylation of acetyl-4-*O*-methylglucuronoxylan (MGX; black bars) and acetyl-galactoglucomannan (GGM; grey bars). Percent of acetyl group content released by *An*AcXE (CE1), *Os*AcXE (CE6), and *Mt*AcE (CE16) (10 μg enzyme /1 mg substrate) measured after 24 h at 40°C and pH 6.0, using the Acetic Acid kit from Megazyme. Average values are indicated above each bar. Non-enzymatic release of acetyl groups from MGX and GGM at pH 6.0 after 24 h was 4 % and 2%, respectively. n=3; error bars represent standard deviation.

Fig. 5. HSQC spectra of GGM in DMSO at 60° C. Acetyl-galactoglucomannan (GGM) in DMSO-d6 before and after treatment with *An*AcXE (CE1), *Os*AcXE (CE6), *MtAcE* (CE16) (10 μg enzyme /1 mg substrate) for 24 h at pH 6.0 and 40°C. Scaling for the native substrate was adjusted to show all available peaks. Acetylated O-2 (H2) and O-3 (H3) positions in the anomeric region are shown due to the partial overlap of signals in the acetyl group region between 2.5-2.0 ppm.

A.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H3CO
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CH3
O
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B.

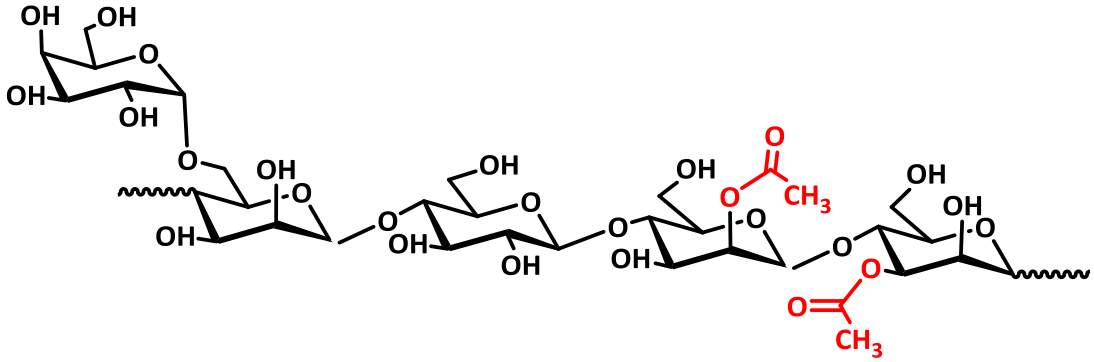
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Fig. 1.

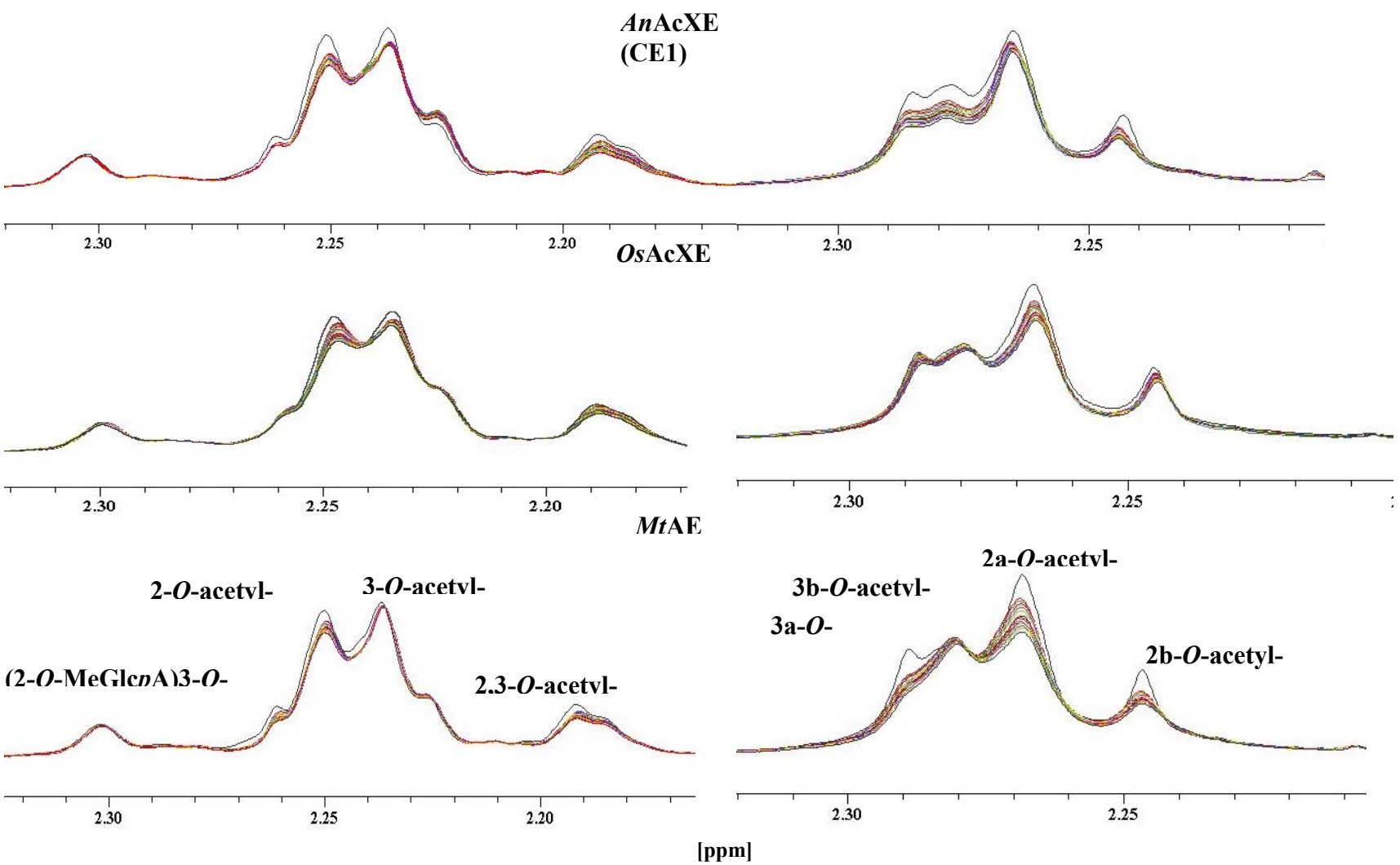

A. [1]H NMR spectra of enzyme treated MGX
B. [1]H NMR spectra of enzyme treated GGM
*An*AcXE
(CE1)
*Os*AcXE
*Mt*AE
2-*O*-acetyl-
3-*O*-acetyl-
(2-*O*-MeGlc*p*A)3-*O*-
2.3-*O*-acetyl-
2a-*O*-acetyl-
3b-*O*-acetyl-
3a-*O*-
2b-*O*-acetyl-
2.30
2.25
2.20
[ppm]

Fig. 2.

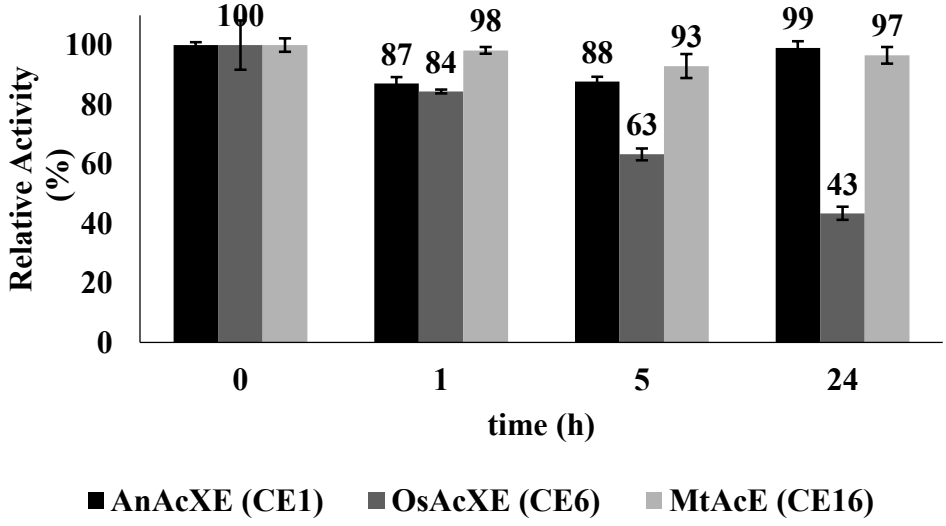

Relative Activity (%)
100
80
60
40
20
0
100
87
84
98
88
63
93
99
43
97
0
1
5
24
time (h)
AnAcXE (CE1)
OsAcXE (CE6)
MtAcE (CE16)

Fig. 3.

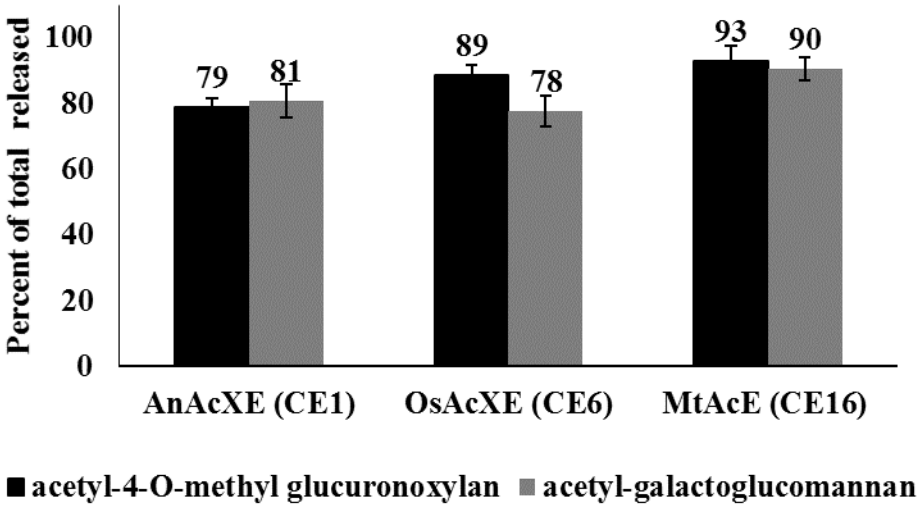

Percent of total released
100
80
60
40
20
0
79
81
89
78
93
90
AnAcXE (CE1)
OsAcXE (CE6)
MtAcE (CE16)
acetyl-4-O-methyl glucuronoxylan
acetyl-galactoglucomannan

Fig. 4

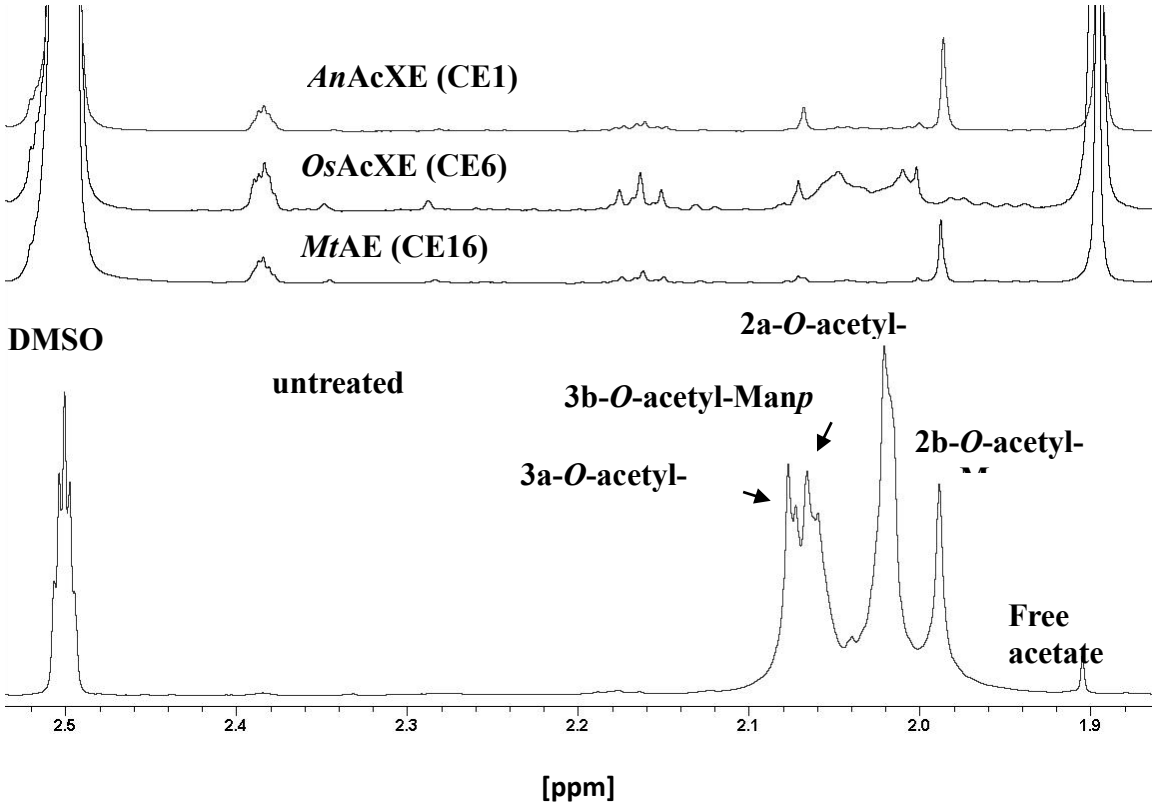

*An*AcXE (CE1)
*Os*AcXE (CE6)
*Mt*AE (CE16)
DMSO
untreated
2a-*O*-acetyl-
3b-*O*-acetyl-Man*p*
2b-*O*-acetyl-
3a-*O*-acetyl-
Free
acetate
2.5
2.4
2.3
2.2
2.1
2.0
1.9
[ppm]

Fig. 5.

SUPPLEMENTAL TABLE

Suppl.Table 1. Reported compositions (mol%) of acetyl-4-*O*-methylglucuronoxylan (MGX), acetyl-galactoglucomannan (GGM), determined using NMR or by acid methanolysis/gas-chromatography (Meth-GC)

Sample	Extraction/ Determination method	β-D- Xyl*p*	Me-Glc*p*A	β-D-Man*p*	β-D-Glc*p*	α-D-Gal*p*	O-Ac (DS)	Ref.
MGX (Birch)	DMSO/ NMR	68	3	-	-	-	29 (0.4)	[9]
MGX (Beech)		70	4	-	-	-	26 (0.3)	
MGX (Aspen)	Microwave/ NMR	60	6	-	-	-	35 (0.6)	[7]
GGM (Birch)	Hot water/ NMR	-	-	68	32	-	(0.2)	[10]
GGM (Spruce) TMP or wood	Meth-GC	2-9	1-2	43-65	7-19	7-20	(0.5)	[3]

DS: degree of substitution

- not determined or not detected

TMP: thermomechanical pulp

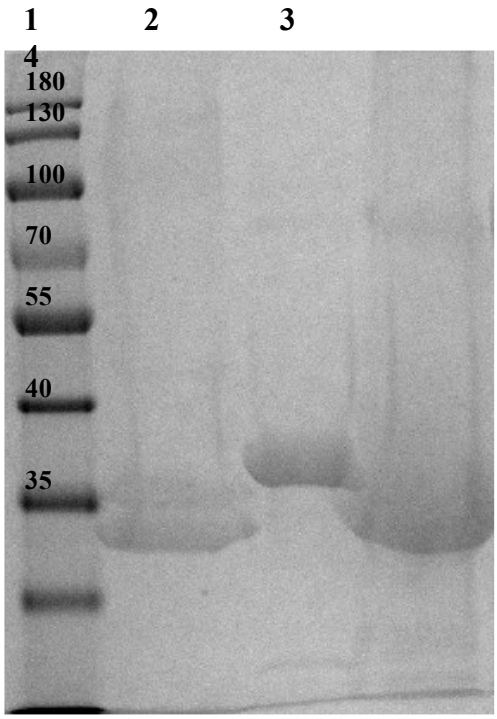
SUPPLEMENTAL FIGURES

Suppl.Fig. 1. SDS-PAGE analysis of purified enzymes (15 µg). Lanes, PageRuler™ Prestained Protein Ladder, 10 to 180 kDa (ThermoFisher Scientific; USA), 2: *An*AcXE (CE1); 3: *Os*AcXE (CE6); 4: *Mt*AE (CE16).

Suppl.Fig. 2. Optimum pH of selected enzymes measured using 4-MUA. (A) *An*AcXE(CE1); (B) *Os*AcXE (CE6), and (C) *Mt*AcE (CE16).

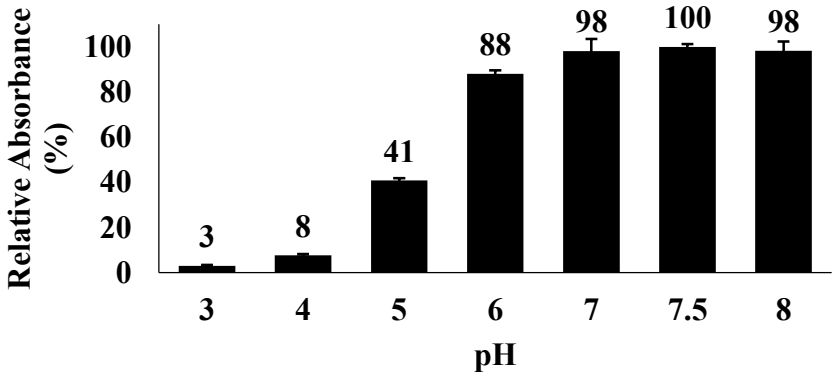
Suppl.Fig. 3. Optimum pH of (A) *An*AcXE (CE1) and (B) *Mt*AcE (CE16) using MGX.

Suppl.Fig. 4. Normalized resonance signals from H^1 NMR plotted against time. (A) Plots showing activity of each enzyme towards each acetyl group in MGX and GGM, (B) Slopes calculated from plots shown in (A). Prob(F) values were obtained using ANOVA (Origin 2017 software); Prob(F) values >0.05 indicate insignificant difference from zero.

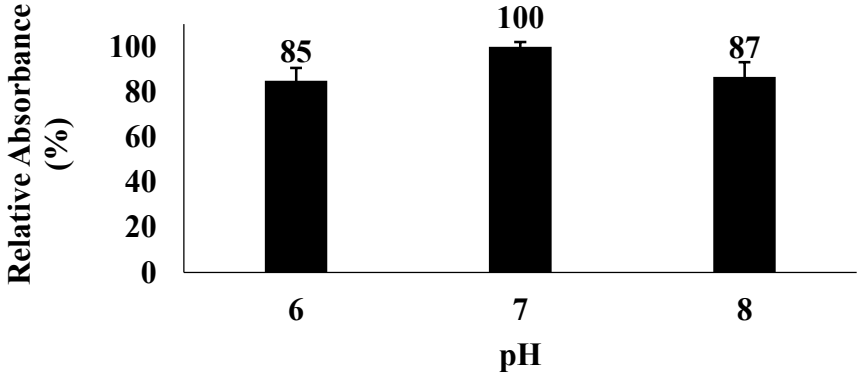


Suppl.Fig. 1

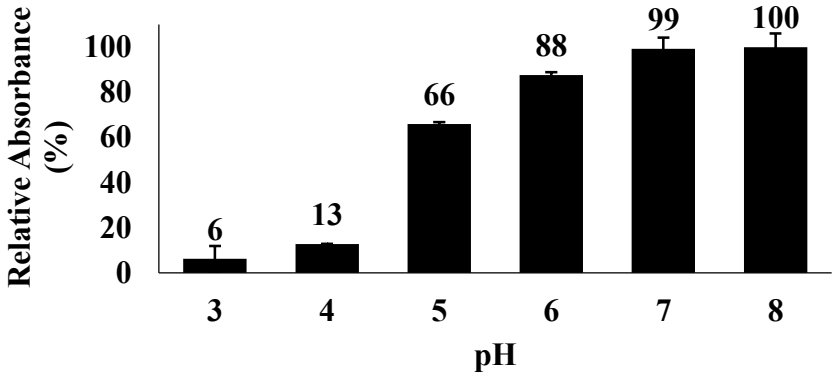
A. *An*AcXE (CE1)



B. *Os*AcXE (CE6)

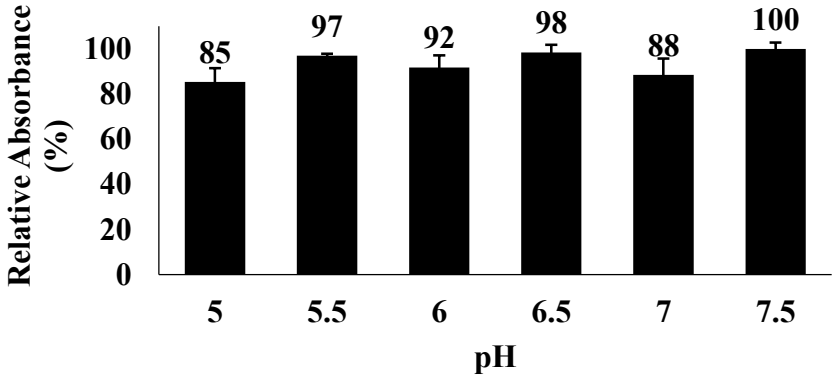


C. *Mt*AcE (CE16)

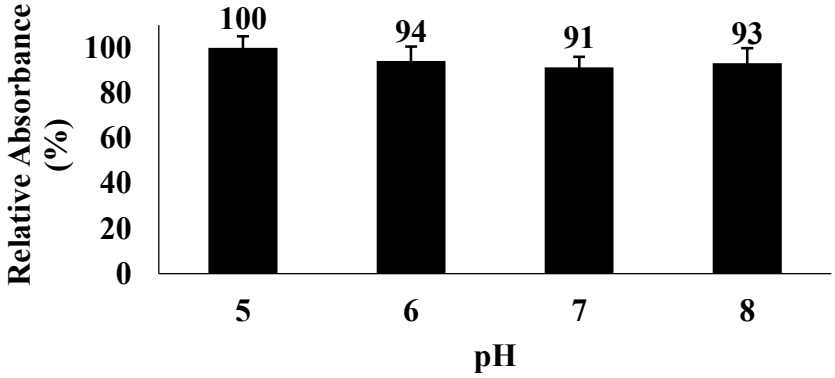


Suppl.Fig. 2

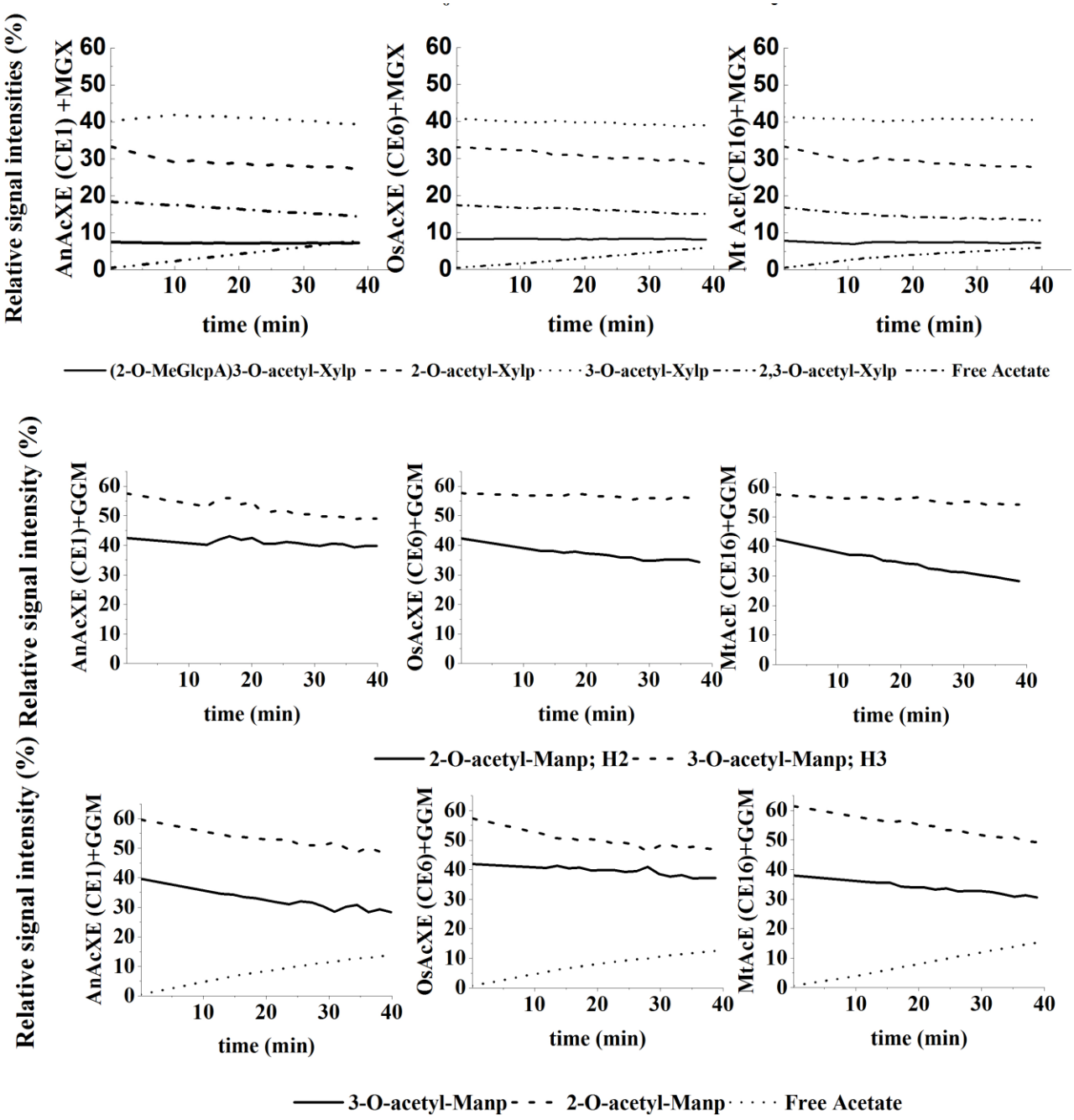
A. *An*AcXE (CE1)



B. *Mt*AcE (CE16)



Suppl.Fig. 3


Relative signal intensities (%)
AnAcXE (CE1) +MGX
OsAcXE (CE6)+MGX
Mt AcE(CE16)+MGX
time (min)
(2-O-MeGlcpA)3-O-acetyl-Xylp
2-O-acetyl-Xylp
3-O-acetyl-Xylp
2,3-O-acetyl-Xylp
Free Acetate
Relative signal intensity (%)
AnAcXE (CE1)+GGM
OsAcXE (CE6)+GGM
MtAcE (CE16)+GGM
2-O-acetyl-Manp; H2
3-O-acetyl-Manp; H3
3-O-acetyl-Manp
2-O-acetyl-Manp
Free Acetate

Suppl.Fig. 4A

Calculated slopes derived from enzyme-treated MGX								
Enzyme	**2-*O*-acetyl-Xyl*p***	**Prob (F)** 2-*O*-acetyl-Xyl*p*	**3-*O*-acetyl-Xyl*p***	**Prob (F)** 3-*O*-acetyl-Xyl*p*	**2,3-*O*-acetyl-Xyl*p***	**Prob (F)** 2,3-*O*-acetyl-Xyl*p*	**(2-*O*-MeGlc*p*A) 3-*O*-acetyl-Xyl*p***	**Prob (F)** (2-*O*-MeGlc*p*A) 3-*O*-acetyl-Xyl*p*
***An*AcXE (CE1)**	0.11 ± 0.02	3.5×10^{-6}	0.06 ± 0.01	7.8×10^{-4}	0.10 ± 0.002	0.0	0.002 ± 0.002	3.1×10^{-1}
***Os*AcXE (CE6)**	0.11 ± 0.01	1.9×10^{-11}	0.05 ± 0.01	3.9×10^{-7}	0.07 ± 0.003	2.1×10^{-12}	0.001 ± 0.002	6.1×10^{-1}
***Mt*AcE (CE16)**	0.11 ± 0.01	4.9×10^{-7}	0.003 ± 0.007	7.1×10^{-1}	0.07 ± 0.01	1.3×10^{-8}	0.01 ± 0.003	6.3×10^{-2}
Calculated slopes derived from enzyme-treated GGM								
Enzyme	**2-*O*-acetyl-Man*p***	**Prob (F)** 2-*O*-acetyl-Man*p*	**3-*O*-acetyl-Man*p***	**Prob (F)** 3-*O*-acetyl-Man*p*	**2-*O*-acetyl-Man*p***; H2	**Prob (F)** 2-*O*-acetyl-Man*p*; H2	**3-*O*-acetyl-Man*p***; H3	**Prob (F)** 3-*O*-acetyl-Man*p*; H3
***An*AcXE (CE1)**	0.25 ± 0.02	1.1×10^{-9}	0.26 ± 0.02	5.3×10^{-9}	0.08 ± 0.02	5.2×10^{-4}	0.24 ± 0.03	8.1×10^{-8}
***Os*AcXE (CE6)**	0.23 ± 0.03	3.9×10^{-7}	0.13 ± 0.02	1.1×10^{-6}	0.19 ± 0.01	2.3×10^{-10}	0.05 ± 0.01	7.7×10^{-5}
***Mt*AcE (CE16)**	0.31 ± 0.01	2.2×10^{-16}	0.19 ± 0.01	3.1×10^{13}	0.36 ± 0.01	0	0.09 ± 0.01	6.3×10^{-8}

Suppl.Fig. 4B