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ORIGINAL ARTICLE

Insights into the hydrolysis of *Eucalyptus dunnii* bark by xylanolytic extracts of *Pseudozyma* sp.

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Abstract

Transforming lignocellulosic biomass into C5 and C6 sugars suitable to produce biofuels, building blocks, and high-value-added compounds is a key aspect of sustainable strategies and is central to the biorefinery concept. Xylan is found acetylated and bound to cellulose and lignin forming an insoluble complex in nature, and its degradation involves a collection of enzymes acting together. To gain a better understanding of this process, the present study focuses on the elucidation of the main products resulting from the hydrolysis of delignified *Eucalyptus dunnii* bark by an enzymatic extract from *Pseudozyma* sp. with xylanase and acetylxylan esterase activities but no cellulase activity. Scanning electron microscopy (SEM) studies of the insoluble fraction after hydrolysis revealed cracking on the surface of the material. The enzymatic activity of the crude yeast extract was evidenced by TLC and HPLC analysis of the hydrolysate, which allowed us to detect xylose, acetylxylobiose, and acetic acid. Finally, the principal low molecular weight products obtained from this process were characterized by nuclear magnetic resonance (NMR) spectroscopy as xylose and 3-*O*-acetylxylobiose. Based on these spectroscopic and chromatographic results, it was possible to estimate a 4:1 ratio of xylose to 3-*O*-acetylxylobiose. These results highlight the importance of using an enzymatic system for effective xylan degradation.

Keywords 3-*O*-Acetylxylobiose · *Eucalyptus dunnii* bark · *Pseudozyma* sp. · Xylanolytic extract · Xylose

1 Introduction

The Uruguayan pulp production industry reported exports of 1.7 billion dollars in 2018 [1], making it one of the most important sectors in the economy of the country. As part of the pulping process, the tree bark, leaves, and branches are removed and left in the field for mineral recovery. Taking just eucalyptus into account, these residues amount to more than 200,000 tons per year in Uruguay alone [2]. Known as lignocellulosic biomass, this material constitutes, together with pastures and other residues from agriculture, the most important renewable organic resource on the planet [3]. Because lignocellulosic biomass does not compete with food resources and can be obtained locally, it is a very attractive raw material for the production of high-value-added compounds.

Obtaining marketable products and renewable fuels from lignocellulosic biomass is a key aspect of sustainable strategies and is central to the biorefinery concept [4]. The first step in its use involves the separation of its constituents, which include cellulose, hemicellulose, and lignin [5]. Given the complex intertwined network formed by these three polymers, different pretreatment schemes are required to render the polysaccharide fractions susceptible to enzymatic hydrolysis [6].

Sometimes referred to as glucuronoxylan, *O*-acetyl-(4-*O*-methylglucurono)xylan is the most abundant hemicellulose in hardwoods. They present a main chain formed by β-(1 → 4)-linked D-xylopyranoside units [7], which in most hardwoods is partially acetylated and has 4-*O*-methyl-D-glucuronic acid

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(MeGlcA) residues linked through α-(1 → 2) bonds as side groups. On average, there is a MeGlcA residue every 6-11 xylose units in *Eucalyptus* hemicellulose, with acetyl groups generally in positions C2 and C3 and throughout 50% of the polysaccharide [8–10]. In order to develop a C5 sugar-based platform for the production of value-added products from xylan, it is necessary to hydrolyze it to monomeric sugars using chemical or enzymatic methods. Enzymatic hydrolysis methods offer the potential for higher sugar yields, higher selectivity, lower energy costs, and milder operating conditions when compared with chemically based conversion technologies [11]. Xylan backbone hydrolysis requires two types of enzymes, endo-β-1,4-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37). The former hydrolyze the polysaccharide chains into small oligosaccharides, which are then broken down to xylose monomers by the later [12].

Over the past decades, the mode of action of these enzymes has been studied on model xylan substrates, leading to a detailed understanding of the degradation mechanisms of these polymers [13–17]. In nature, however, xylanases attack polysaccharides that are partially acetylated as well as substituted with MeGlcA. Regardless of their structure, these substrates contain fewer sites for productive enzyme binding than deacetylated polysaccharides extracted from plant cell walls under alkaline conditions [18]. As a result, endoxylanases need to act synergistically with several accessory enzymes to achieve efficient polymer degradation, including α-glucuronidases and acetylxylan esterases [16, 19, 20]. However, there are few reports detailing the effects of xylanolytic extracts or cocktails of commercial enzymes on native xylans [21–23], and fewer describing the structure of the hydrolysis products obtained under these conditions [6, 18].

We recently reported the isolation and characterization of *Pseudozyma* sp. strain EBV 97-87, a yeast that produces a cellulase-free xylanolytic extract [24]. While not completely characterized, the objective of this work was to evaluate the activity of an enzymatic extract obtained from this *Pseudozyma* strain on partially delignified lignocellulose material, using delignified *Eucalyptus dunnii* bark residue as substrate. Scanning electron microscopy (SEM) reveals cracks on the surface of the plant tissue after enzymatic action. Nuclear magnetic resonance (NMR) analyses indicate that the two low molecular weight hydrolysis products obtained under the conditions evaluated are xylose and 3-*O*-acetylxylobiose. Our results show that xylose can be produced from bark hemicelluloses using a xylanolytic extract, an essential aspect in the valorization of this abundant source of lignocellulosic biomass. Furthermore, these findings highlight the importance of using an enzymatic system for effective xylan degradation.

2 Materials and methods

2.1 Plant material and reagents

Eucalyptus dunnii bark was provided by UPM Forestal Oriental S.A., a forestry and wood supplying company located in Río Negro, Uruguay (32° 50′ 53.3″ S 57° 57′ 24.3″ W). Plant material was dried at 45 °C and milled to pass a 5-mm screen in a Retsch® SK 100 cross-beater mill.

Beechwood xylan, xylose, 3,5-dinitrosalicylic acid, orcinol, bovine serum albumin (BSA), and D_2O were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Xylooligosaccharides with a degree of polymerization (DP) 2-6 were obtained from Megazyme (Bray, Ireland). Culture media were purchased from Fisher Scientific (Waltham, MA, USA). All other chemicals were of analytical grade unless otherwise stated.

2.2 *E. dunnii* bark delignification

Milled extractive-free milled *E. dunnii* bark [25] was delignified with peracetic acid [8]. Briefly, 10 g of sample was incubated in 500 mL of 10% peracetic acid solution (pH 4) for 30 min at 85 °C. The process was quenched by cooling and dilution with 500 mL of distilled water, and the treated bark suspension was hot-filtered at 40 °C. The solid fraction was then washed continuously with distilled water until the elimination of excess acid, and the recovered delignified bark was dried at 30 °C for 24 h. The resulting samples were stored at room temperature in sealed bags. The composition of the material before and after treatment is presented in Table S1.

2.3 Xylanase production

A pure culture of *Pseudozyma* sp. EBV 97-87 was grown in 50 mL of modified Czapek Dox medium (CDm) prepared in a 250-mL flask under submerged fermentation conditions. CDm was composed of $NaNO_3$ (7.65 g/L), KH_2PO_4 (3.04 g/L), $MgSO_4{\cdot}7H_2O$ (1.52 g/L), and KCl (1.52 g/L) and was supplemented with 0.1% yeast extract, 0.5% peptone, and 1% beechwood xylan. A cell suspension in sterile 0.9% NaCl solution was used as inoculum, reaching a final concentration of 10^5 cells/mL in the culture media. Flasks were incubated in an orbital shaker at 150 rpm and 28 °C for 96 h, and xylanase activity was determined as detailed in the following section.

2.4 Protein concentration

The crude cell extract (50 mL) was centrifuged at 4 °C and 5000 rpm, and the supernatant containing the xylanolytic enzymes was subjected to a 70% ammonium sulfate precipitation and left overnight at 4 °C. The resulting precipitate was collected by centrifugation at 10,000 rpm for 15 min.

Precipitates were then resuspended in a minimum volume of 50 mM citrate buffer (pH 4.5) at 30 °C and 150 rpm for 1 h. Xylanase activity and protein concentration were determined before and after concentration. These samples were used for *E. dunnii* bark hydrolysis (vide infra).

2.5 Total protein quantitation and xylanase activity

Protein concentrations were determined in crude and partially purified extracts according to the Bradford method using a Bio-Rad reagent kit (Munich, Germany) and BSA as standard.

Xylanase assay was performed according to the method of Bailey and coworkers with minor modifications [26]. A reaction mixture of 1.5 mL of a 2.0% suspension of beechwood xylan and 0.5 mL of the enzyme extract (55 mg/mL of soluble protein), both in 50 mM sodium citrate buffer (pH 4.8), was incubated at 30 °C for 15 min. Reducing sugars were determined by adding 3.0 mL of a 0.5% 3,5-dinitrosalicylic acid solution, incubating the mixture at 95 °C for 5 min, and measuring the absorbance at 540 nm [27]. One unit of the enzyme was defined as the amount of enzyme catalyzing the release of 1 μmol of reducing sugars as xylose per minute under these conditions.

2.6 Hydrolysis of delignified *E. dunni* bark

Dry delignified *E. dunnii* bark (200 mg) was dispersed in citrate buffer (50 mM, pH 4.8) and treated with *Pseudozyma* xylanase extract (200 U/mL) in a final volume of 5 mL and incubated for 72 h at 30 °C and 150 rpm. The residual solid and liquid fractions were analyzed as described in the following sections.

2.7 Solid fraction analysis

SEM was performed on enzymatic treated and untreated delignified bark. The materials were dried at 40 °C for 2 days and adhered to a metal support. Gold coating was performed in a Denton Vacuum DeskII chamber for 120 s. Samples were analyzed in a Jeol JSM-5900LV SEM that acquires and displays the signal through the X-Stream image system (Jeol Ltd., Tokyo, Japan).

2.8 Liquid fraction analysis

The hydrolysis products were analyzed by HPLC using a Shimadzu LC-20AT fitted with a Supelcogel^{TM} C610H column and a Shimadzu RID-10AT refractive index detector (Shimadzu Corp., Kyoto, Japan). The column temperature was 55 °C and 0.005 N aqueous sulfuric acid at a flow rate of 0.5 mL/min was used as eluent.

Hydrolysis products were also analyzed by thin-layer chromatography (TLC). A sample of the reaction mixture was applied to a silica gel 60 F_{254} TLC plate (Merck, Darmstadt, Germany) and developed with a butanol/ethanol/H_2O mixture (10:8:5), using a xylose, xylobiose, xylotriose, and xylotetraose mixture as standard (2 mg/mL). Compounds were visualized by treating the plate with a 5% (v/v) H_2SO_4 solution in ethanol containing 0.5% (w/v) orcinol, followed by heating.

Bidimensional TLC was performed according to Biely and coworkers [6]. After elution in the conditions detailed previously, the plate was allowed to dry and exposed to NH_4OH vapors in a closed chamber overnight. Following the evaporation of ammonia, the plate was rotated 90° and developed in the second dimension after the addition of standards. Compound visualization was performed as described above.

2.9 Isolation of hydrolysis products

The liquid fraction obtained after enzymatic hydrolysis of delignified *E. dunnii* bark was lyophilized and resuspended in 250 μL of ultrapure H_2O. Using the conditions described in the previous section, preparative TLC was used as one step in the isolation procedure to identify hydrolysis products. The largest band with an Rf similar to the xylose standard was scraped off with a spatula and sonicated for 30 min in MeOH, the resulting suspension was filtered, and the filtrate was evaporated under vacuum to afford the material used for characterization.

2.10 NMR spectroscopy

The solid obtained by preparative TLC as described in the previous section was dissolved in 600 μL D_2O containing sodium 2,2,3,3-d_4-3-(trimethylsilyl) propionate (TSP) as internal standard and transferred to a NE-HL5-7 5 mm NMR tube (New Era Enterprises Inc., Vineland, NJ, USA). NMR spectra were recorded at 25 °C on a Bruker AVANCE III 500 NMR spectrometer operating at ^{1}H and ^{13}C frequencies of 500.13 MHz and 125.76 MHz, respectively, and equipped with a 5-mm *z*-gradient TXI probe (Bruker Corp., Billerica, MA, USA). 1D ^{1}H spectra were acquired with presaturation of the water signal. 1D-TOCSY ^{1}H spectra were obtained using a water-suppressed gradient-enhanced sequence, Gaussian inversion selective pulses, and a spinlock of 200 ms [28]. A spectral width of 10 kHz and a data size of 32 K was employed for all 1D spectra. HSQC and TOCSY-HSQC experiments were carried out using standard pulse sequences provided with the spectrometer. Chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz.

3 Results and discussion

It is well documented that, along with other factors, lignin content has a significant impact on enzymatic hydrolysis of lignocellulosic material [29]. As indicated in these reports, including a delignification step improves the efficiency of

enzymatic hydrolysis of forest biomass. Therefore, the hydrolyzing activity of the xylanolytic extract produced by *Pseudozyma* strain EBV 97-87 was evaluated on delignified *E. dunnii* bark. The composition of the material before and after delignification demonstrates that the process leads to selective removal of lignin without significant hemicellulose loss (Table S1).

In order to gain insights on the mode of action of this crude enzyme extract, both the solid and liquid fractions obtained after the hydrolysis process were analyzed.

3.1 Solid fraction analysis

Based on results obtained for similar systems [30, 31], we employed SEM to evaluate changes caused by the xylanolytic system on the material surface. In delignified bark without enzymatic treatment, fibers are uniform and a smooth surface is observed (Fig. 1a, b). On the other hand, fibers treated with hydrolytic enzymes show a rough and heterogeneous surface consistent with a peeling process. Indeed, fiber separation is clearly observed (Fig. 1c), and a striated appearance is evident at higher magnification (Fig. 1d). These findings are in agreement with SEM results reported by Xiong and coworkers [30], which indicate that tree barks inoculated with the fungus *Trichoderma reesei* presented pronounced cell degradation. Similar changes in surface morphology were also observed in sugarcane bagasse treated with purified xylanase from *Lichtheimia ramosa* [31].

3.2 Liquid fraction analysis

TLC of the hydrolysate supernatant showed a major spot with an Rf similar to that of the xylose standard. The bluish-purple color of this and other spots after development with ethanolic H_2SO_4/orcinol mixture also indicates that the xylanotic extract generated pentose-based products from the xylan-cellulose matrix (Fig. S1) [32]. Furthermore, the absence of glucose oligomers indicates that there are no cellulose-active enzymes present in the extract (Fig. S1) [24].

HPLC analysis of the delignified bark hydrolysate allowed us to quantify 5.2 mg/mL of the major product, expressed as xylose, as well as 0.9 mg/mL of acetic acid. Consistent with results from TLC, glucose was not detected by HPLC (Fig. S2). The presence of acetic acid hints to acetylxylan esterase activity in the *Pseudozyma* sp. extract. This enzyme is part of the group of auxiliary enzymes in the degradation of xylan [33]. A direct correlation between the enzymatic release of xylose and concomitant deacetylation of xylan has been proposed for purified beech- and birchwood xylans [34, 35]. Since acetylation of the xylan chain leads a polymer that is more recalcitrant to hydrolysis, and taking also into account that 50% of xylan in *E. dunni* is acetylated [8], the presence of this enzyme in the crude extract is critical to achieve a more extensive degradation of the lignocellulosic material obtained from this source.

The presence of acetylated products in the *E. dunni* bark hydrolysate was further demonstrated using bidimensional TLC [36]. Following the development in the first dimension

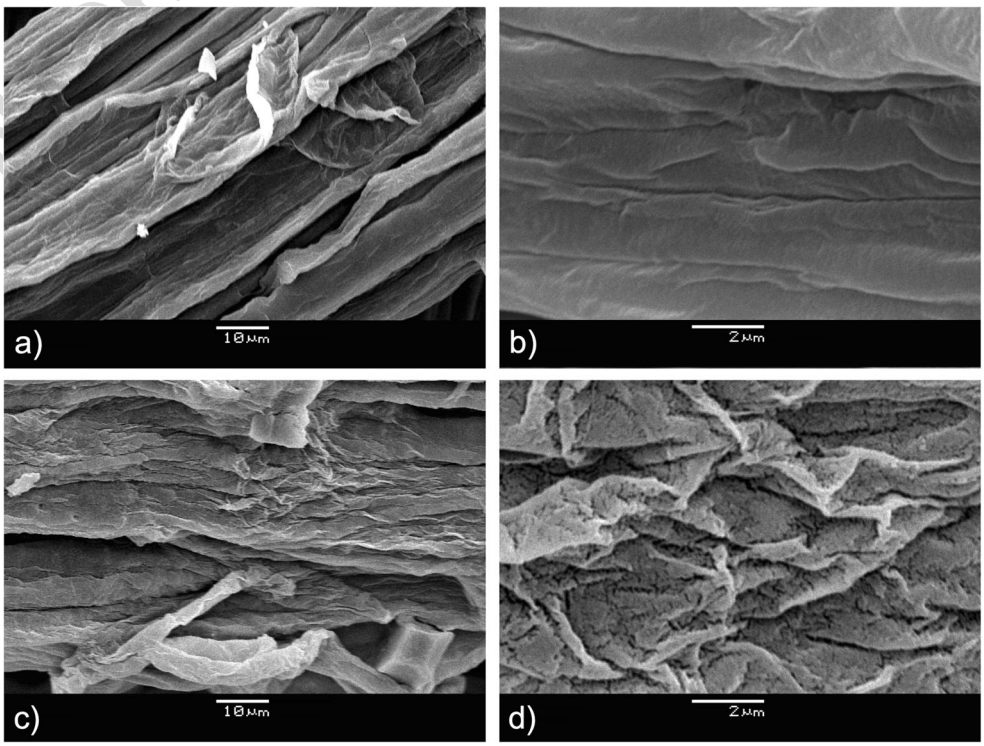

Fig. 1 SEM images of untreated delignified *E. dunnii* bark at × 1400 (**a**) and × 9500 (**b**) magnification, and corresponding images obtained for material treated with *Pseudozyma* xylanolytic extract (**c**, **d**)

and treatment with ammonia vapor, the spot corresponding to the major hydrolysis product described above separated into two new spots with different Rf when developed in the second dimension (Fig. 2). The largest of the two new spots sat on the diagonal of the TLC plate and had an Rf comparable with that of xylose, while the Rf of the smaller spot below the diagonal was similar to that of xylobiose. Since compounds which were originally acetylated show slower migration and appear below the diagonal after deacetylation [6], our results corroborate that hydrolysis with the xylanolytic extract produced a mixture of xylose and acetylxylobiose as the major product. While much weaker, spots with Rf values corresponding to those of xylotriose and xylotetraose are observed below the diagonal of the TLC plate, indicating that these acetylated xylose oligomers are also present in the hydrolysate.

3.3 Structural characterization of hydrolysis products

To determine the structure of the main components in the hydrolysate, the material was isolated using preparative TLC and subjected to NMR analysis.

Several ^{1}H and ^{13}C resonances corresponding to both xylose anomers could be readily assigned through inspection of the ^{1}H and HSQC spectra and comparison with the reported data [37] (Fig. 3). In addition, other ^{1}H signals in the carbohydrate region can be observed. Of these, the ^{1}H resonances at δ_H 5.14, 4.56, and 4.49 correlate to ^{13}C signals at δ_C 92.1, 95.8, and 101.5 in the HSQC spectrum and help identify the anomeric centers of the xylobiose derivative (Fig. 3 and Table S2). Based on their chemical shifts and coupling constants, the signal at lower fields is consistent with sugar in α configuration, while the other two correspond to β anomers. Their relative integrations also suggest that the signals at δ_H 5.14 and 4.56 correspond to the H-1 proton of the reducing end residue, while that at δ_H 4.49 to the non-reducing end H-1 proton. Of the remaining signals, the ^{1}H resonance at δ_H 4.83 is particularly instrumental to determine the position of the acetate fragment in the dimer. First, it correlates to ^{13}C signals

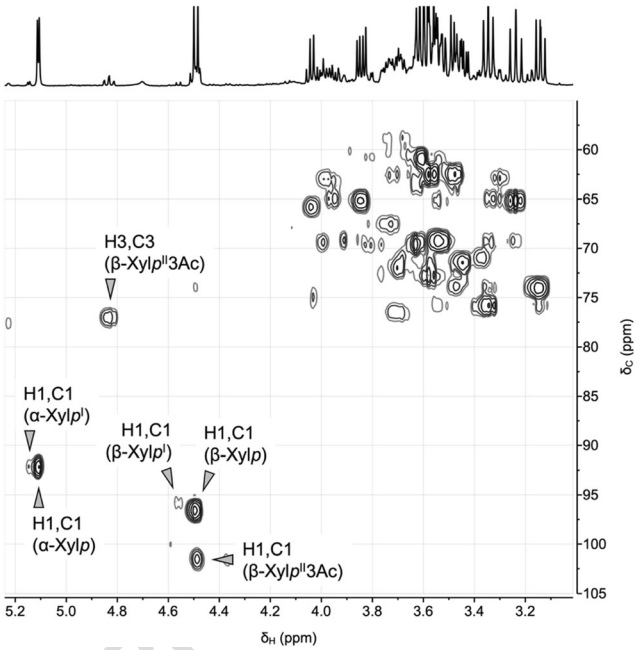

Fig. 3 ^{1}H and HSQC spectra of the hydrolysate major product mixture. Relevant correlations for xylose (Xyl*p*) and 3-*O*-acetylxylobiose (β-Xyl*p*^{II}3Ac-(1 → 4)-Xyl*p*^{I}) are annotated

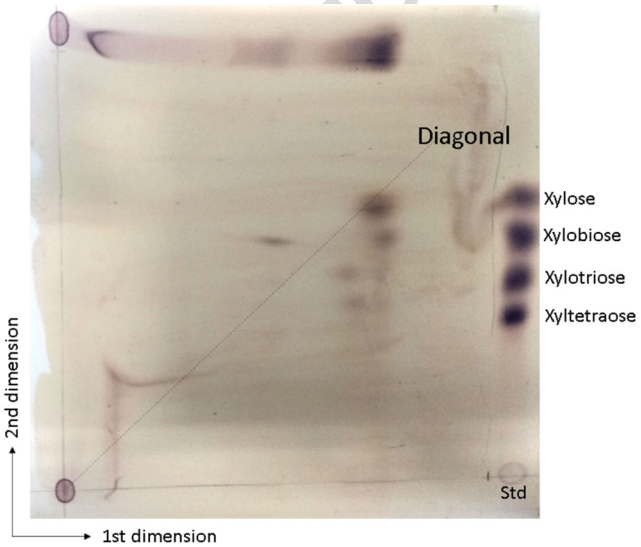

Fig. 2 Bidimensional TLC of the hydrolysate obtained from delignified *E. dunnii* bark with the *Pseudozyma* xylanolytic extract. Xylooligosaccharide standards were applied before the TLC plate was developed in the second dimension

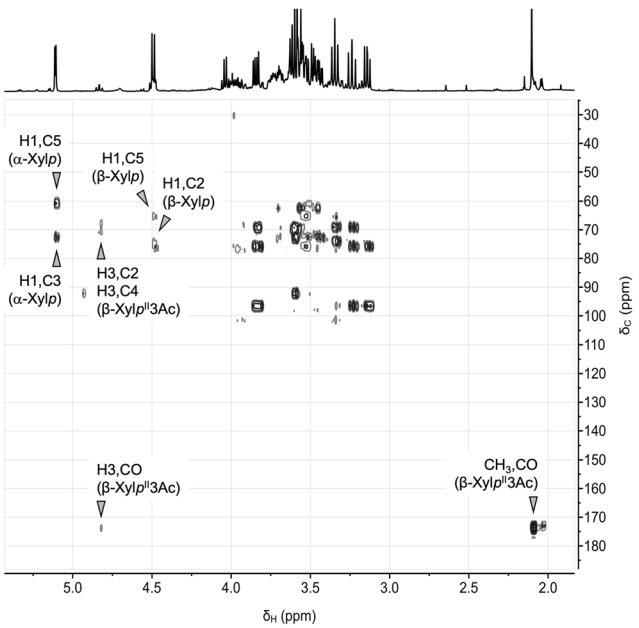

Fig. 4 ^{1}H and HMBC spectra of the hydrolysate major product mixture. Relevant long-range correlations for xylose (Xyl*p*) and 3-*O*-acetylxylobiose (β-Xyl*p*^{II}3Ac-(1 → 4)-Xyl*p*^{I}) are annotated

at δ_C 77.0 and 173.7 in the HSQC and HMBC spectra (Figs. 3 and 4), respectively, indicating that it is attached to an acetoxylated carbon. Furthermore, its integration relative to the anomeric protons and to the acetate proton signal at δ_H 2.10 locates it on the non-reducing end xylopyranose residue. Finally, the signal is a doublet of doublets with J values of 9.6 Hz and thus antiperiplanar to protons on neighboring carbons. These observations suggest that this resonance corresponds to the H-3 proton and allowed us to elucidate the structure of the dimer as 3-*O*-acetylxylobiose. Furthermore, based on the 4:1 ratio of xylose to 3-*O*-acetylxylobiose determined through integration of the corresponding anomeric proton signals and results from the HPLC analysis, it was possible to estimate that concentration of these two sugars in the hydrolysate is approximately 4.0 and 1.0 mg/mL, respectively.

Most of the remaining signals could be assigned with the aid of the HSQC and HMBC spectra (Table S2), and several of these assignments corroborated using 1D-TOCSY experiments. For example, selective excitation of the xylose H-1 signals at δ_H at 5.11 and 4.49 reveals the ^{1}H spin systems for both anomers (Fig. 5a, b). Selection of the signal at δ_H 4.83, which corresponds to the H-3 proton on the non-reducing end of 3-*O*-acetylxylobiose, reveals correlations to the remaining protons in the residue, including H-1, H-2, H-4, H-5$_{eq}$, and H-5$_{ax}$ at δ_H 4.49, 3.37, 3.74, 3.95, and 3.31, respectively (Fig. 5c). Finally, selective excitation of the H-1 resonances at δ_H 5.14 and 4.56 helps corroborate the assignments of ^{1}H signals for both anomers of the reducing end residue (Fig. 5d, e). Overall, the data reported here for 3-*O*-acetylxylobiose are in good agreement with that reported for this and similar compounds [38–42].

The results presented here are in agreement with previous reports which describe the synergistic action of different hydrolytic enzymes to promote hemicellulose saccharification. In particular, Puchart and coworkers analyzed oligosaccharides released from milled *Eucalyptus* wood by xylanases GH10, GH11, and GH30,

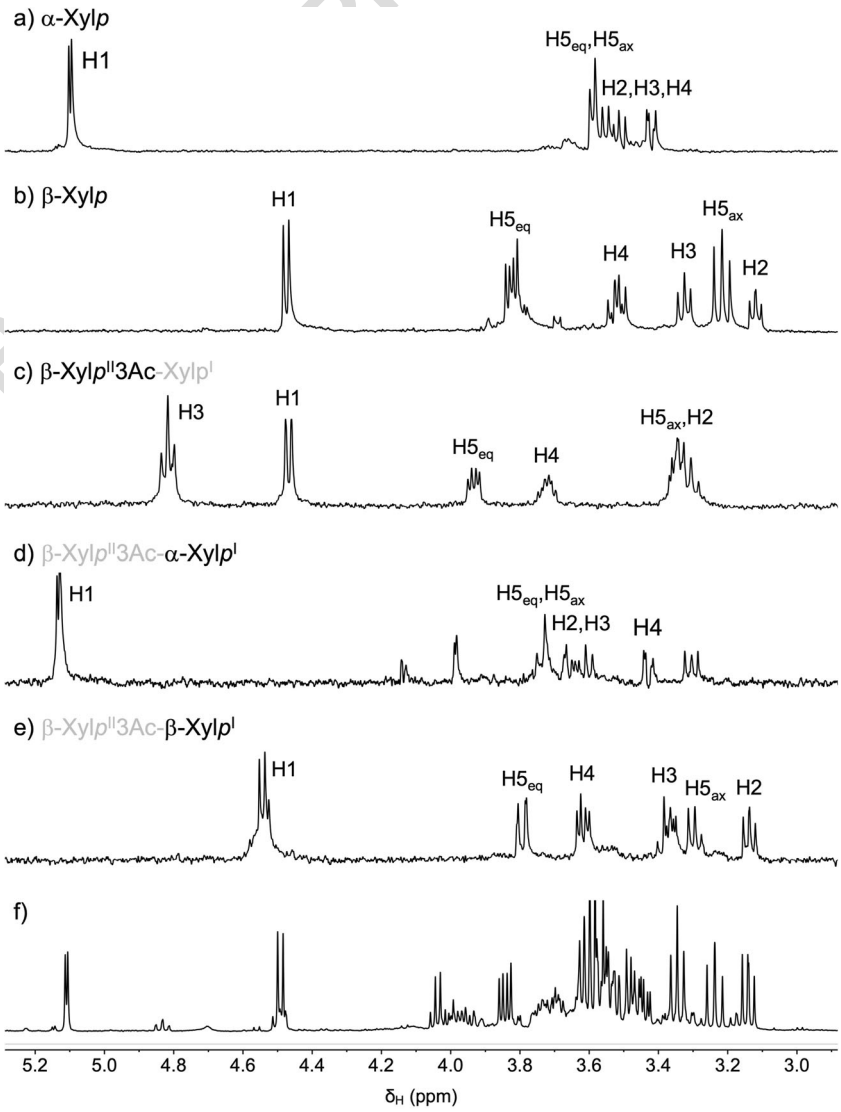

Fig. 5 1D-TOCSY experiments with selective excitation at δ_H 5.11 (**a**), 4.49 (**b**), 4.83 (**c**), 5.14 (**d**), and 4.56 (**e**), and reference ^{1}H spectrum (**f**)

in the presence and absence of acetylxylan esterase OCE6 [18]. All three xylanases led to a variety of neutral and acidic oligosaccharides with up to 9 xylose residues and differing in the degree of acetylation. Furthermore, the presence of OCE6 in the xylanotic mixture resulted in the formation of shorter oligosaccharides that were mostly monoacetylated or diacetylated. When xylanase GH10 and acetylxylan esterase OCE6 were used in combination, a monoacetylated xylobiose was obtained, but the position of the acetyl group was not reported.

The mechanism of action proposed for these enzymes could explain the position of the acetyl group in the product. Acetylxylan esterases from different families are capable of hydrolyzing acetate groups from the C-2 and C-3 positions, but deacetylation at the former site is faster than at the latter. This was the case of OCE6 from *Orpinomyces* sp., and OCE4 from *Streptomyces lividans* which attacks faster the C-2 position in mono-*O*-acetylated Xyl*p* residues in acetylglucuronoxylan. This fact was observed when evaluating the intensity of the resonance corresponding to 2-*O*-acetylated and 3-*O*-acetylated Xyl*p* residues after the action of these enzymes [39]. Also, the presence of a β-xylosidase in the xylanotic *Pseudozyma* extract cannot be excluded. Indeed, hydrolysis of xylooligosaccharides standards ranging from dimer to hexamer produced xylose as the major product at different time intervals (data not shown), and the existence of this enzyme was already reported for *Moesziomyces* (formerly *Pseudozyma*) spp. [43].

4 Conclusions

The use of a xylanolytic extract obtained from *Pseudozyma* sp. EVB97-87 in the enzymatic hydrolysis of delignified *Eucalyptus dunnii* bark was evaluated, constituting to our knowledge the first report on the degradation of xylan attached to cellulose in an insoluble matrix by enzymes from this yeast genus. The action of this hydrolyzing mixture, which displays xylanase and acetyl esterase activity, leads to xylose and 3-*O*-acetylxylobiose as the main products.

While future work is required to determine if other xylanolytic enzymes, such as α-glucuronidases, are present in the *Pseudozyma* sp. extract, the findings presented here help improve our understanding of the enzymatic mechanism for effective xylan degradation in complex xylan-cellulose matrices found in hardwood barks. Furthermore, and since the xylose produced from this abundant source of lignocellulosic biomass can be converted into important building blocks such as levulinic acid, furfural, and xylitol [44], these results are instrumental to reach current and future biorefinery objectives.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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