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Production of xylanolytic enzymes by *Moesziomyces* spp. using xylose, xylan and brewery's spent grain as substrates

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ABSTRACT

Xylanases play a crucial role in the hydrolysis of xylan-rich hemicelluloses and have wide industrial applications in the fuel, food, feed and pulp and paper industries. The production of these enzymes at low cost is of paramount importance for their commercial deployment. *Moesziomyces antarcticus* PYCC 5048^T and *M. aphidis* PYCC 5535^T were screened for their ability to produce xylanolytic enzymes when grown on D-xylose, xylan (beechwood) and brewery's spent grain (BSG). The extracellular crude extracts produced were characterized and tested in xylan hydrolysis. The yeasts produced xylanolytic enzymes without cellulolytic activity on all the substrates tested. The highest xylanase volumetric activity was obtained with *M. aphidis* PYCC 5535^T grown on BSG, reaching 518.2 U/ml, a value 8.4- and 4.7-fold higher than those achieved on xylan and D-xylose, respectively. The xylanase activities were characterized in relation to pH and temperature with optima at 4.5 and 50 °C, respectively. The extracts from both *M. antarcticus* PYCC 5048^{T}and *M. aphidis* PYCC 5535^T were used in xylan hydrolysis, producing D-xylose as the major end product (0.43 and 0.34-0.47 $g_{D\text{-xylose}}/g_{xylan}$, respectively, at 50 °C) and relatively low or no xylobiose accumulation (from no detection to 0.12 $g_{D\text{-xylobiose}}/g_{xylan}$ at 50 °C).

Introduction

Xylan is the major component of hemicelluloses from hardwoods (glucoronoxylans) and agricultural residues (arabinoxylans) and the second most abundant polysaccharide in nature, after cellulose. Xylan is a complex, highly branched heteropolysaccharide, with a backbone formed of D-xylopyranose units (D-xylose) linked through β-1,4 glycosidic bonds, and side-chains composed of acetyl, glucuronosyl, arabinosyl and other groups [1]. The enzymatic hydrolysis of xylan requires the action of several enzymes, of which the most important are endo-β-1,4-xylanase (β-1,4-D-xylan xylanohydrolase, EC 3.2.1.8), that cleaves glycosidic bonds to produce xylooligosaccharides, and β-1,4-xylosidase (β-1,4-D-xyloside xylohydrolase, EC 3.2.1.37), responsible for the final breakdown of xylooligosaccharides into D-xylose [2]. These microbial enzymes can be applied in lignocellulose bioconversion within many biotechnological processes, such as food (e.g. baking), animal feed, pulp and paper, as well as for the bioconversion of lignocellulosic material into biofuels and other added value chemicals [3].

While extensive research on microbial cellulase and xylanase production has been performed with filamentous fungi [4], which are known as efficient enzyme producers, reports on xylanolytic enzymes from yeasts are comparatively limited [5–10]. Among these, ascomycetous and basidiomycetous are described as cellulase and/or xylanase producers, including those belonging to the genera *Cryptococcus*, *Scheffersomyces*, *Candida*, *Trichosporon*, *Dekkera*, *Hanseniaspora*, *Metschnikowia*, *Rhodotorula*, *Sugiyamaella* and *Wickerhamomyces* [6,10–15].

Moesziomyces spp. are anamorphic basidiomycetous yeasts belonging to the *Ustilagomycetes*, a group that includes the smut fungus *Ustilago maydis* [16]. Among the genus *Moesziomyces*, *M. antarcticus,* (formerly known as *Pseudozyma antarctica* and *Candida antarctica*), was found to produce industry-relevant extracellular lipases [17], which were heterologously expressed in different hosts [18–20] and commercialized. The extracellular production of an excellent plastic-

Abbreviations: DNS, 35-dinitrosalicylic acid; pNPX, *p*-nitrophenyl-β-D-xyloside; pNPX2, *p*-nitrophenyl-β-xylobioside; pNP, *p*-nitrophenol; PYCC, Portuguese Yeast Culture Collection; BSG, brewery's spent grain

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degrading enzyme, which degrades poly-butylene succinate and poly-butylene succinate-co-adipate, was also found in the strains of *M. antarcticus* isolated from plant surfaces [21]. Recent studies describe this and other yeasts from the *Ustilaginaceae* family with the ability to produce cellulase-free xylanase when grown in xylan [22,23]. *Moesziomyces antarcticus* and *M. aphidis*, well known producers of the biosurfactant mannosylerythritol lipids (MEL), have been reported to use xylan as sole carbon source [23], thereby producing xylanolytic enzymes.

The present study investigated the production of xylanolytic enzymes by *M. antarcticus* PYCC 5048^T and *M. aphidis* PYCC 5535^T on D-xylose, beechwood xylan and brewery spent grain (BSG), an abundant agro-industrial residue which might be used as an inexpensive substrate for industrial biotechnology processes [24]. The different extracellular crude extracts produced were characterized and further tested for enzymatic hydrolysis of xylan.

Materials and methods

Yeast strains, substrate and cultivation conditions

Moesziomyces antarcticus PYCC 5048^T and *Moesziomyces aphidis* PYCC 5535^T were obtained from the Portuguese Yeast Culture Collection (PYCC), DCV, FCT/UNL, Caparica, Portugal. Yeasts were cultured for 3 days at 25 °C on Yeast Malt Agar (YM–agar) medium (yeast extract, 3 *g*/l; malt extract, 3 *g*/l; peptone, 5 *g*/l; glucose, 10 *g*/l; agar, 20 *g*/l). Stock cultures were prepared by propagation of yeast cells in liquid medium as described below for the inoculum and stored (in 20% v/v glycerol aliquots) at −70 °C for later use. Inocula were prepared by incubation of stock cultures at 28 °C, 140 rpm, for 48 h, in liquid medium containing glucose (40 *g*/l), $NaNO_3$ (3 *g*/l), $MgSO_4$ (0.3 *g*/l), KH_2PO_4 (0.3 *g*/l) and yeast extract (1 *g*/l). The cultivation media, containing 40 *g*/l carbohydrate as carbon source, $MgSO_4$ (0.3 *g*/l), KH_2PO_4 (0.3 *g*/l) and yeast extract (1 *g*/l) as supplement for other elements, was inoculated with 10% v/v of inoculum culture and incubated at 28 °C, 140 rpm, for 10 days. All experiments were carried out at least in biological duplicates. One-ml culture samples were centrifuged at 13,000 rpm, 4 °C, and the supernatants were stored at -20 °C for analysis.

Carbon sources used for enzyme induction included: D-xylose (Sigma-Aldrich, USA), beechwood xylan (Sigma-Aldrich, USA) and brewery's spent grain (BSG). BSG, kindly provided by Sociedade Central de Cervejas (Vialonga, Portugal), was ground with a knife mill to particles smaller than 1.5 mm, homogenized in a defined lot, and stored in plastic containers at room temperature (RT). BSG had 95% dry matter content, with 36.2 g/100 g_{solids} of polysaccharides, consisting of 22.1 g/100 g_{solids} glucan and 14.1 g/100 g_{solids} xylan. BSG was pretreated at 121 °C for 15 min with 0.16 N HCl in a liquid-to-solid ratio of 9 (w/w) (11% (w/v) dry matter) using an autoclave. The pH was adjusted to 5.5 using 4 M NaOH and this pretreated slurry material was subsequently used for cultivations. All the experiments were carried out with 40 g/l of carbohydrates, corresponding to 4% (w/v) of D-xylose and beechwood xylan, and 11% (w/v) of BSG.

Enzyme activity assays

Xylanase activity was assessed through the release of reducing sugars from xylan measured by the 3,5-dinitrosalicylic acid (DNS) method described by Miller [25] with a few modifications. The supernatant culture sample was appropriately diluted with 0.1 ml of 1% beechwood xylan solution in 50 mM potassium phthalate buffer and incubated at 50 °C for 30 min. Subsequently, 0.6 ml of DNS reagent was added to stop the reaction and the solution was boiled for 5 min. After cooling to RT, reducing sugars were estimated using a D-xylose calibration curve, with absorbance of samples and standards measured at 550 nm. Each reaction and its control were run in quadruplicate. One unit (U) of xylanase activity was defined as the amount of enzyme required to release 1 µmol of reducing sugar (D-xylose) equivalent per min.

β-Xylosidase and β-xylobiohydrolase were determined as previously described [26]. The supernatant culture sample was appropriately diluted in a reaction mixture (0.3 ml), containing 5 mM p-nitrophenyl-β-D-xyloside (pNPX, Sigma, USA) or p-nitrophenyl-β-xylobioside (pNPX2, Megazyme, Ireland), respectively, in 50 mM potassium phthalate buffer pH 5.5. After incubation at 50 °C for 30 min, 0.15 ml of 1 M Na_2CO_3 was added to stop the reaction. The p-nitrophenol absorbance (pNP) was measured at 405 nm. One unit (U) of β-xylosidase activity was defined as the amount of enzyme releasing 1 µmol pNP per minute.

Extracellular protein content was assessed using Pierce™ BCA protein assay kit (Thermo Scientific, USA) using 1 ml of culture broth supernatant after centrifugation for 10 min at 13,000 rpm.

Characterization of xylanolytic activities in extracellular crude extracts

Extracellular crude extracts obtained from 10-day cultures were used to assess:

(i) xylanase activity for a pH range from 3.0 to 8.0 at 50 °C and for a temperature range from 20 °C to 72 °C at pH 5.5. Xylanase and β-xylosidase activities were determined as above. For the activity profile as a function of pH, xylanase activity was determined using citrate buffer (pH 3–6) and phosphate buffer (pH6-8).

(ii) stability of xylanolytic activities at pH 5.5 and 28 °C or 50 °C for 48 h in the presence and absence of substrate.

The experiments were performed at least in biological duplicates with at least technical duplicates, i.e. at least four determinations. Some of the data points at specific pH, temperature and time resulted from single biological experiments, but the data points obtained from biological replicates have a variance below 15%.

Comparative analysis of proteins present in extracellular crude extracts by SDS-PAGE

The culture supernatants from cells grown on xylan and BSG (30 µL samples) were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide). Electrophoresis was carried out at 110 V at RT and proteins were stained with Coomassie brilliant blue R-250.

Application of crude extracellular extracts on xylan hydrolysis

Crude extracellular extracts were used for the hydrolysis of 4% (w/v) xylan in 50 mM potassium phthalate, at pH 5.5, 28 °C or 50 °C, for 7 days, in the presence of 0.8% sodium azide to avoid biological contamination. Extracts were used at xylanase dosages close to 300 U/g (corresponding approximately to 1/3 dilution), unless otherwise stated. Commercial hemicellulase was used for benchmarking at 300 U/g. Samples were taken periodically for analysis of enzyme activity and for D-xylose and xylobiose quantification. D-Xylose and xylobiose were estimated using a high performance liquid chromatography (HPLC) system (Merck Hitachi, Darmstadt, Germany), equipped with a refractive index detector (L-7490, Merck Hitachi, Darmstadt, Germany) and an Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad), at 50 °C. Sulfuric acid (5 mM) was used as mobile phase at 0.4 ml/min. Before injection into the HPLC, samples and standards were boiled for 10 min to inactivate extracellular enzymes, then centrifuged at 13,000 rpm for 10 min and passed through a 0.45-µm filter.

Statistical analysis

Statistics were performed by analysis of variance (one-way ANOVA) and p-values of the differences between groups were corrected for simultaneous hypothesis testing according to Tukey's method. The level of significance was set at $p < 0.05$.

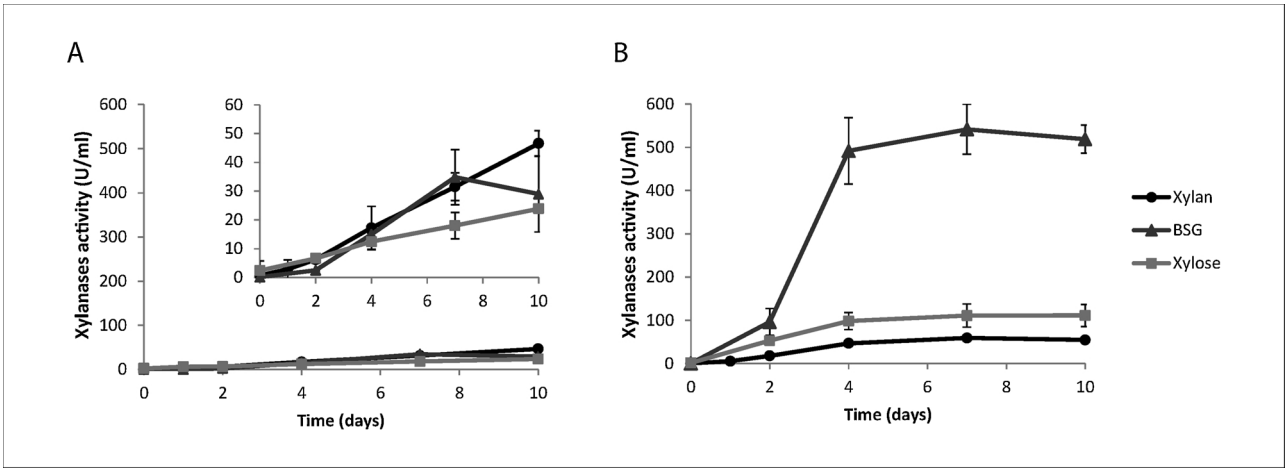

Fig. 1. Time course of xylanase volumetric activity at 50 °C of extracellular crude extracts obtained from *M. antarcticus* PYCC 5048^T (A) and *M. aphidis* PYCC 5535^T (B) cultures incubated for 10 days at 28 °C using (as carbon source): 4% (w/v) beechwood xylan (circles); 4% (w/v) D-xylose (rectangles); or 11% (w/v) BSG (triangles). Mean values are stated and error bars represent standard deviation (n = 4).

Results

Assessment of Moesziomyces antarcticus PYCC 5048^T and Moesziomyces aphidis PYCC 5535^T as producers of xylanolytic enzymes

In order to explore the extracellular xylanolytic potential of *M. antarcticus* PYCC 5048^T and *M. aphidis* PYCC 5535^T, the two strains were grown in media containing D-xylose, beechwood xylan or pre-treated BSG (4% w/v carbohydrate content) for 10 days at 28 °C. The respective crude extracellular extracts were characterized in terms of (endo-1,4-β-) xylanase, β-xylobiohydrolase and β-xylosidase activities at 50 °C. The xylanase activity of extracellular crude extracts from *M. antarcticus* PYCC 5048^T and *M. aphidis* PYCC 5535^T were estimated and compared for the different substrates tested (Fig. 1A and B). Both strains revealed cellulase-free xylanase activity regardless of the substrate tested. In all cases, xylanase activity was detected after 1 day and increased over the following days, at least until day 7. The use of BSG as substrate allowed *M. aphidis* PYCC 5535^T to achieve a maximum xylanase volumetric activity of 518.2 U/ml at day 10. At day 4, the activity was already 492.0 U/ml, which corresponded to a volumetric productivity of 5.1 U/ml/h (Fig. 1A). These results were far superior to those achieved when beechwood xylan or D-xylose were used as substrates (8.4-and 4.7-fold, respectively). Conversely, the xylanase volumetric activity found in *M. antarcticus* PYCC 5048^T cultures did not exceed 50 U/ml regardless of the substrates tested, with a maximum of 46.6 U/ml obtained in beechwood xylan (Fig. 1B).

Detailed information of (endo-1,4-β-)xylanase, β-xylobiohydrolase, β-xylosidase activities and protein content of extracellular crude extracts is shown in Table 1. Interestingly, beechwood xylan induced both higher xylanase volumetric activity and total protein in cultures of *M. aphidis* PYCC 5535^T than in those of *M. antarcticus* PYCC 5048^T, but xylanase specific activities were very similar for both strains (31.1 and 32.4 U/mg$_{prot}$, respectively). However, such correlation was not found when other substrates were used, with both volumetric and specific activities being higher for *M. aphidis* PYCC 5535^T than for *M. antarcticus* PYCC 5048^T (Table 1). While the xylanase volumetric activity in BSG was greater (14.8-fold) in *M. aphidis* PYCC 5535^T cultures than in *M. antarcticus* PYCC 5048^T cultures, the determination of specific activity in BSG can be misleading since this substrate is rich in protein, masking that produced by the yeasts.

When grown in D-xylose medium, *M. antarcticus* PYCC 5048^T showed much lower xylanase volumetric and specific activities (23.9 U/ml and 18.4 U/mg$_{prot}$, respectively) than *M. aphidis* PYCC 5535^T (111.3 U/ml and 123.7 U/mg$_{prot}$, respectively) (Table1).

The highest β-xylosidase activities were 0.32 U/ml for *M. aphidis* PYCC 5535^T in BSG and 0.14 U/ml for *M. antarcticus* PYCC 5048^T in beechwood xylan. Xylose seems to induce xylanase, but not β-xylosidase, production better in *M. aphidis* PYCC 5535^T when compared to xylan induction. The β-xylobiohydrolase volumetric activities estimated followed the same trend as those for xylanase, representing 5–30% of those, 20–30% for *M. antarcticus* PYCC 5048^T and 5–20% for *M. aphidis* PYCC 5535^T (Table 1).

SDS-PAGE analysis confirmed that beechwood xylan and BSG acted as inducing substrates for enzyme production (Figure S1, Supplementary data). Two bands, slightly greater than 30 kDa, were observed in all the extracellular crude extracts from *M. antarcticus* PYCC 5048^T and *M. aphidis* PYCC 5535^T. One of these bands is tentatively assigned to endo-β-1,4-xylanase (GH10), UniProtKB A0A068Q818 and W3VMB4, respectively. The other band, around 30 kDa, can tentatively be assigned to β-xylosidase (GH43), UniProtKB A0A081CDX0 and

Table 1
Effect of carbon sources on xylanase, β-xylobiohydrolase and β-xylosidase volumetric activities and protein content. Volumetric activities (U/ml) were determined at 50 °C, after 10 days incubation at 28 °C in 4% (w/v) beechwood xylan, 4% (w/v) D-xylose or 11% (w/v) BSG medium. Mean values are stated ± SD (n = 4).

	Xylanase (U/ml)	β-xylobiohydrolase (U/ml)	β-xylosidase (U/ml)	Total protein (mg/ml)	Xylanase: β-xylosidase activity ratio
M. antarcticus PYCC 5048^T					
Xylan	46.6 ± 4.4	9.5 ± 2.8	0.14 ± 0.03	1.5 ± 0.3	333
D-xylose	23.9 ± 0.0	6.8 ± 0.9	0.05 ± 0.02	1.3 ± 0.2	478
BSG	34.9 ± 8.7	8.5 ± 0.7	0.03 ± 0.00	6.6 ± 0.7	1163
M. aphidis PYCC 5535^T					
Xylan	61.5 ± 7.0	10.3 ± 2.4	0.09 ± 0.01	1.9 ± 0.2	683
D-xylose	111.3 ± 25.6	13.2 ± 2.5	0.04 ± 0.00	0.9 ± 0.1	2783
BSG	518.2 ± 32.2	99.4 ± 0.4	0.32 ± 0.05	8.0 ± 0.3	1619

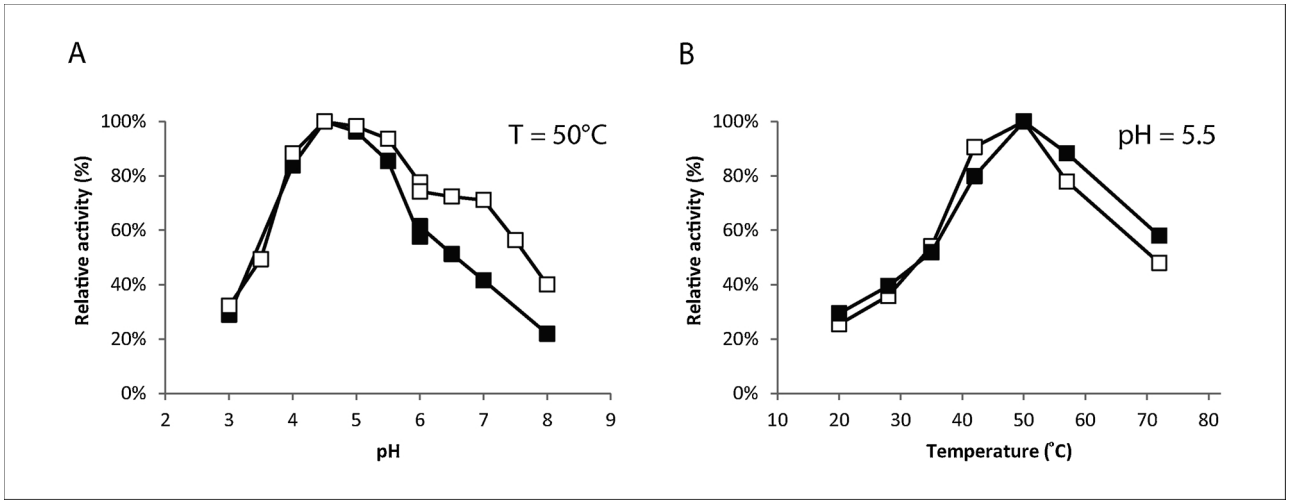

Fig. 2. Effect of pH (A) and temperature (B) on xylanase activity in extracellular crude extracts obtained from *M. antarcticus* PYCC 5048^T (open symbols) and *M. aphidis* PYCC 5535^T (filled symbols) grown in 4% (w/v) beechwood xylan for 10 days. Relative activity is expressed as percentage of the maximal activity. The pH plot was performed at 50 °C, while the temperature plot was performed at pH 5.5.

W3VSV8, in *M. antarcticus* and *M. aphidis*, respectively. Moreover, additional bands of approx. 66 and 20 kDa are found in extracts from *M. antarcticus* PYCC 5048^T, which can tentatively be assigned to other endo-β-1,4-xylanases, respectively from GH10 and GH11 families. The 20 kDa protein from *M. antarcticus* PYCC 5048^T is expressed in xylan but not in BSG, while all the other proteins appeared to be more induced in BSG. The proteins of approx. 30 kDa from *M. aphidis* PYCC 5535^T seem to be strongly induced by BSG, which is in concordance with the higher xylanase and β-xylosidase activities determined in this substrate.

Characterization of pH and temperature profile of xylanase activity in crude extracellular extracts

Xylanase activity was evaluated at different pH values and temperatures using crude extracellular extracts from *M. antarcticus* PYCC 5048^T and *M. aphidis* PYCC 5535^T grown in beechwood xylan (Fig. 2). A pH range between 3.0 and 8.0 was used to study the effect of pH on xylanase activity (Fig. 2A). The optimum pH for xylanase activity in the two crude extracts was estimated at 4.5, exhibiting more than 85% of maximal activity for pH ranging from 4.0 to 5.5. A temperature range between 20 and 72 °C was used to study the effect of temperature (Fig. 2B). The optimum temperature was estimated at 50 °C for both *M. antarcticus* PYCC 5048^T and *M. aphidis* PYCC 5535^T (Fig. 2B), with very similar temperature profiles exhibited in both crude extracts. At 72 °C, both crude extracts retained more than 50% of maximal xylanase activity, while at 20, 28 and 35 °C approximately 25, 40 and 50% of maximal activity was achieved.

Stability of xylanolytic activities in crude extracellular extracts

The stability of the xylanolytic activities in crude extracellular extracts from *M. antarcticus* PYCC 5048^T and *M. aphidis* PYCC 5535^T produced in beechwood xylan were evaluated in terms of xylanase and β-xylosidase activities, at 28 and 50 °C, at pH 5.5 (Fig. 3A and 3B). Both *M. antarcticus* PYCC 5048^T and *M. aphidis* PYCC 5535^T extracts exhibited high stability at 28 °C. After 6 h incubation they exhibited 85% and 76% of initial xylanase activity and, after 48 h, the values fell to 84% and 62%, for *M. antarcticus* PYCC 5048^T or *M. aphidis* PYCC 5535^T, respectively (Fig. 3A). However, at 50 °C, xylanase activity dropped to about 50% of the initial value after 6 h incubation and to 17% or 13%, after 24 h incubation for *M. antarcticus* PYCC 5048^T or *M. aphidis* PYCC 5535^T extracts, respectively. After 48 h incubation at 50 °C, xylanase activity detected in both extracts was residual (Fig. 3B). However, after 48 h in the presence of substrate, the xylanase activities remained over 95% when incubated at 28 °C and around 30% of the initial activity at 50 °C.

The stability of β-xylosidase activity in crude extracellular extracts of *M. antarcticus* PYCC 5048^T and *M. aphidis* PYCC 5535^T was also evaluated at 28 and 50 °C. After 48 h at 28 °C, they retained more than 90% of their initial activity (Fig. 3A) and at 50 °C about 83% and 78% activity was retained by *M. antarcticus* PYCC 5048^T and *M. aphidis* PYCC 5535^T extracts, respectively (Fig. 3B).

Hydrolysis of xylan with crude extracellular extracts from Moesziomyces spp.

The potential application of crude extracellular xylanolytic extracts from *M. antarcticus* PYCC 5048^T and *M. aphidis* PYCC 5535^T was evaluated in the enzymatic hydrolysis of a 40-g/l beechwood xylan solution at pH 5.5 and 28 or 50 °C, the optimal temperatures for yeast growth and enzymatic hydrolysis, respectively. The extracts produced by *M. antarcticus* PYCC 5048^T and *M. aphidis* PYCC 5535^T grown in beechwood xylan and by *M. aphidis* PYCC 5535^T grown in BSG were selected for this study. They were diluted approximately 3 times, resulting in a xylanase dosage of about 300 U/g_{xylan} for beechwood xylan extracts (xylanase ANT-I and APH-I), but 4500 U/g_{xylan} for BSG extract (xylanase APH-II). Taking into account such high xylanase activity in the extract from *M. aphidis* PYCC 5535^T grown in BSG, it was also applied at the same xylanase dosage of about 300 U/g_{xylan}, corresponding to a dilution of 1/45 (xylanase APH-IId). These extracts were compared with a commercial xylanase at 300 U/g_{xylan}.

The release of D-xylose and xylobiose was followed as shown in Fig. 4. Xylanases ANT-I and APH-I showed different maximum D-xylose yields (and final concentrations) reaching 0.42 g/g_{xylan} (16.9 g/l) and 0.29 g/g_{xylan} (11.5 g/l) when incubated at 28 °C for 168 h (Fig. 4A). However, for enzymatic hydrolysis at 50 °C these two extracts reached maximum D-xylose yields of 0.43 g/g_{xylan} (17.1 g/l) and 0.41 g/g_{xylan} (16.3 g/l) for ANT-I and APH-I, respectively (Fig. 4B). Around 70% of the maximum D-xylose released was attained after 24 h at 50 °C, whereas it took 48 h to reach the same yield at 28 °C (Fig. 4A, B). APH-I has a significantly higher xylanase/β-xylosidase activity ratio (at 50 °C) than ANT-I (Table 1), and xylobiose accumulation was higher for longer period with APH-I at 50 °C. Nevertheless, the enzymatic hydrolysis using both ANT-I and APH-I at 28 °C resulted in similar xylobiose accumulation (Fig. 4C, D).

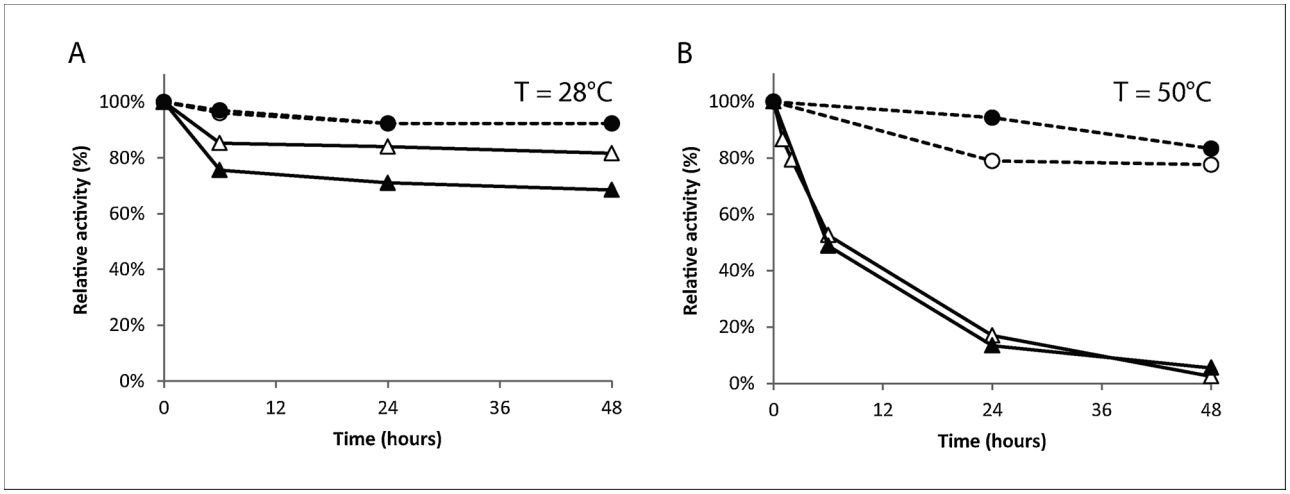

Fig. 3. Effect of temperature, 28 °C (A) or 50 °C (B), on thermal stability of xylanolytic enzymes: xylanase (continuous lines) and β-xylosidase (dashed lines) activities in extracellular crude extracts obtained from *M. antarcticus* PYCC 5048^T (open symbols) and *M. aphidis* PYCC 5535^T (filled symbols) grown in 4% (w/v) beechwood xylan for 10 days. Relative activity is expressed as percentage of the maximal activity.

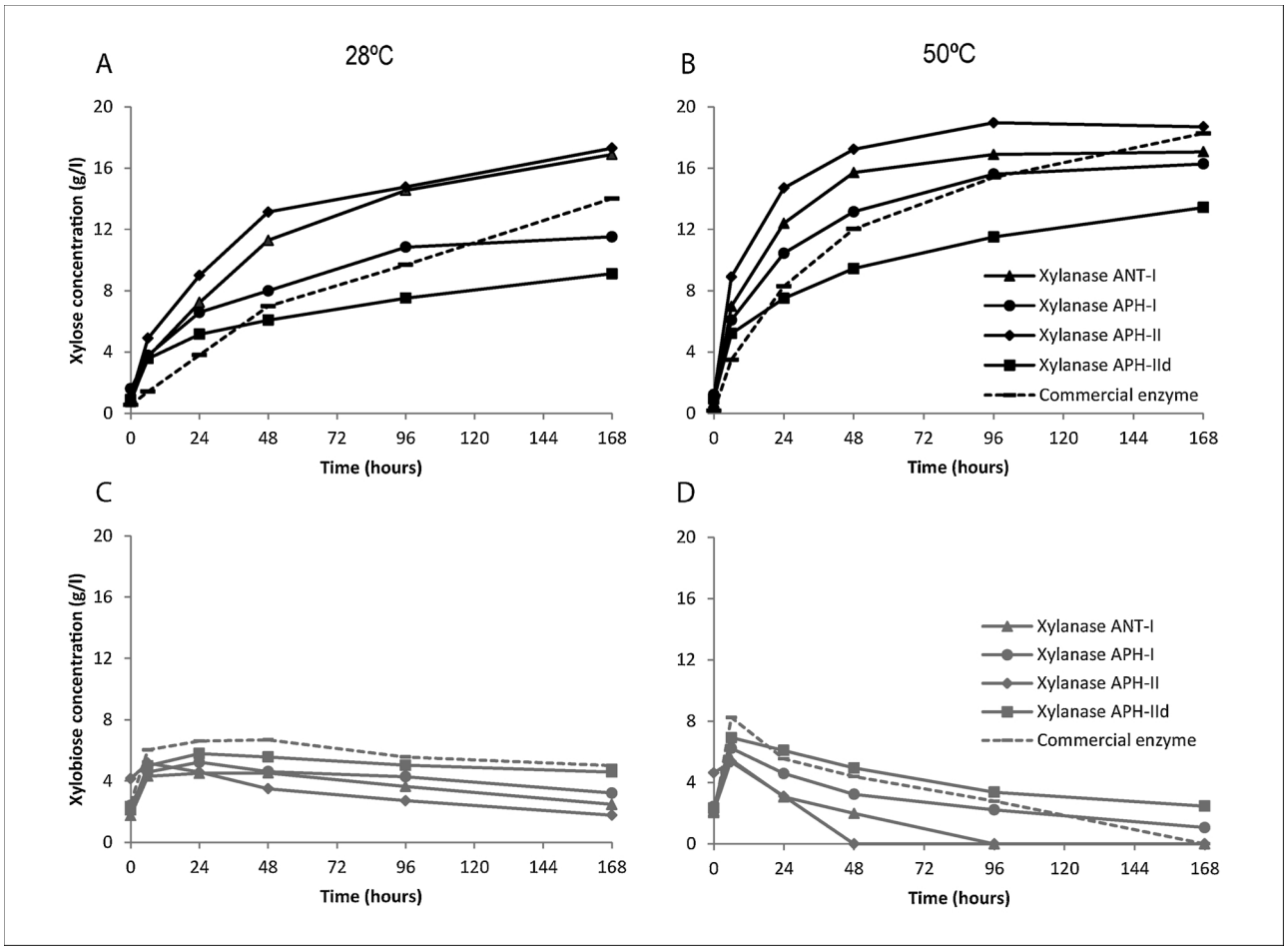

Fig. 4. Time course of 4% (w/v) beechwood xylan hydrolysis using extracellular crude extracts from: *M. antarcticus* PYCC 5048^{T}in xylan, diluted 1:3 (xylanase ANT-I); *M. aphidis* PYCC 5535^T in xylan, diluted 1:3 (xylanase APH-I); *M. aphidis* PYCC 5535^T in BSG, diluted 1:3 (xylanase APH-II) and diluted 1:45 (xylanase APH-IId); and a commercial enzyme. The diluted extracts tested corresponded to xylanase activities of around 300 U/g_{xylan} (xylanase ANT-I, APH-I, APH-IId and commercial enzyme) and 4500 U/g_{xylan} (xylanase APH-II) and 0.6, 1.7, 2.7 and 0.2 U β-xylosidase/g_{xylan} for xylanase ANT-1, APH-I, APH-II and APH-IId, respectively, at 50 °C. D-Xylose (a and b) and xylobiose (c and d).

The D-xylose released with xylanase APH-II was 0.43 g/g_{xylan} (17.3 g/l) and 0.47 g/g_{xylan} (18.7 g/l) at 28 °C and 50 °C, respectively, while with APH-IId only 0.24 g/g_{xylan} (9.7 g/l) and 0.34 g/g_{xylan} (13.4 g/l) were obtained at 28 °C and 50 °C, respectively.

Indeed, APH-II exhibited the best performance towards complete xylan hydrolysis at both 28 and 50 °C, with the highest D-xylose release and lowest xylobiose accumulation. In fact, no xylobiose was detected after 48 h at 50 °C (Fig. 4D) and 77% of the maximal enzymatic hydrolysis yield was already observed after 24 h. Enzymatic hydrolysis of xylan using APH-IId revealed that D-xylose release was 57% of the maximum after 24 h, corresponding to 0.13 g/g_{xylan} and 0.19 g/g_{xylan} at 28 and 50 °C, respectively. In general, the extracts have a similar or better performance than the commercial enzyme used for benchmarking.

Discussion

Xylanolytic enzymes have wide industrial applications in the fuel, food, feed and pulp and paper sectors. These enzymes play a crucial role in xylan hydrolysis and their efficient production from low-cost substrates together with proven effectiveness towards specific applications are highly relevant for their commercialization [34].

While efficient cellulose hydrolysis into glucose is possible with current commercial cellulolytic cocktails, the conversion of hemicellulose into monosaccharides is more challenging due to the heterogeneous nature of this polysaccharide, the composition of which varies among lignocellulosic materials [4,34].

Several commercial xylanolytic cocktails produced by filamentous fungi are available for specific applications [4]. Although filamentous fungi are generally described as the best cellulolytic and hemicellulolytic microorganisms, there are yeasts growing efficiently in xylan [5,6,11], but xylanase volumetric activities are typically 1–2 orders of magnitude lower than for filamentous fungi.. The results presented here for volumetric activity are similar to, or higher than, those found in the yeast-like fungus *Aureobasidium pullulans* [12] and the well-known enzyme producer *Trichoderma reesei* RUT-C30, a filamentous ascomycetous fungus [27].

Both *M. antarcticus PYCC* 5048^T and *M. aphidis* PYCC 5535^T are already established as biosurfactant producers [28,29], but the use of lignocellulose sugars in the production of these chemicals has only recently been reported, including D-glucose [30,31], D-xylose [30], cellulose [32] and xylan [23]. While the use of cellulosic materials by *M. antarcticus* PYCC 5048^T and *M. aphidis* PYCC 5535^T requires supplementation with commercial cellulolytic enzymes [32], the same yeasts were able to grow in beechwood xylan (and produce biosurfactant in the case *M. antarcticus* PYCC 5048^T) [23], showing that these strains are able to produce cellulase-free xylanolytic enzymes.

Both strains assessed were able to grow and produce xylanolytic enzymes using all the substrates tested, showing robust and versatile enzyme production systems when induced by xylose or xylan (note that pretreated BSG contains both xylose and xylan, among other carbohydrates). The presence of glucose in pretreated BSG did not impair the production of the enzymes, probably because this sugar was consumed first during the initial growth phase. The extracellular crude extracts obtained from *M. aphidis* PYCC 5535^T showed higher volumetric and specific xylanase activities than those from *M. antarcticus* PYCC 5048^T when cultivated both in xylose and xylan media. This is in line with the capacity of *M. antarcticus* PYCC 5048^T to produce both xylanolytic enzymes and biosurfactants when growing in xylan as a consolidated bioprocessing (CBP), while *M. aphidis* PYCC 5535^T dedicates its machinery for enzyme production [23]. BSG had a remarkable induction effect in *M. aphidis* PYCC 5535^T, generating high xylanase activity (518.2 U/ml). The much higher volumetric activity found in this substrate can be explained by the higher potential of *M. aphidis* PYCC 5535^T to produce xylanases combined with the presence of protein in BSG (from malting barley) [24], a source of organic nitrogen (amino acids) that can be recycled for the biosynthesis of xylanolytic enzymes. In turn, the protein from BSG reduces the purity of the xylanases in the extracellular crude extract and the highest xylanase specific activity was in turn found in *M. aphidis* PYCC 5535^T crude extracts when cultivated in xylose medium (124 U/mg_{prot}). The β–xylosidase activity was higher in those extracts from cells grown in xylan or, in the case of *M. aphidis* PYCC 5535^T, when grown in BSG and a high β–xylosidase activity is apparently mandatory to complete hydrolysis of xylan (see Table 1 and Fig. 4D).

The analysis of the crude extracts by SDS-PAGE allowed the correlation with the observed xylanolytic profiles. The putative endo-β-1,4-xylanases from family GH10 (UniProtKB A0A068Q818 and W3VMB4, respectively from *M. antarcticus* and *M. aphidis*), of approximately 30 kDa molecular weight and apparently induced in xylan and BSG, are orthologs of the characterized endo-β-1,4-xylanase from *P. hubeiensis* (PhX33, putatively UniPortKB R9P747), reported to have a size of 33.4 kDa [33], and from *U. maydis* (UniPortKB Q4P902) [34]. Other strains of *M. antarcticus* have also shown to have a 33 kDa endo-β-1,4-xylanase (GH10) as major extracellular protein product when induced by xylose [35]. Another band around 30 kDa was tentatively assigned to β-xylosidase from family GH43 (UniProtKB A0A081CDX0 and W3VSV8, respectively from *M. antarcticus* and *M. aphidis*). Those are orthologs of one protein from *U. maydis* UniProtKB A0A0D1C3L0) which is very similar to a β-xylosidase from *Ustilago* sp. SL-2014 (UniProtKB A0A0B5A2 × 5). The additional bands of approx. 66 and 20 kDa found in extracts from *M. antarcticus* PYCC 5048^T were tentatively assigned to endo-β-1,4-xylanases, respectively from GH10 and GH11 families. This is supported by a similar pattern found in other *M. antarcticus* strains when grown in xylose [39], by one protein of approximately 66 kDa with a GH10 conserved domain found in *M. antarcticus* (UniProtKB A0A081CI12) and by *P. hubeiensis* reported to have a second xylanase (PhX20) of 20 kDa belonging to the GH11 family [33]. While the 20 kDa protein from *M. antarcticus* PYCC 5048^T is expressed in xylan but not in BSG, all the other proteins seem to be more induced in BSG. The proteins of approximately 30 kDa, probably one endo-β-1,4-xylanase (GH10) and one β-xylosidase (GH43) from *M. aphidis* PYCC 5535^T seem to be strongly induced by BSG, allowing efficient xylan hydrolysis.

The potential biotechnological application of the produced enzyme extracts depends on their specific features, such as optimal pH and temperature and thermal stability. In the present study, xylanase activities exhibited high activity (> 80% of maximum) at pH and temperature ranges of 4.0–5.5 and 45 and 55 °C, which fall in the range of values reported for most xylanases and specifically for those isolated from other *M. antarcticus* strains – 4.0–6.5 and 40–80 °C, respectively [33,36]. The relatively high activity at low temperatures, around 40% of maximum at 28 °C and around 50% of maximum at 35 °C, may allow the combination of enzymatic hydrolysis and microbial conversion processes through simultaneous saccharification and fermentation (SSF) or consolidated bioprocessing (CBP) for the production of fuels and chemicals [23,32]. According to the different process configurations applied in the bioconversion of lignocellulosic materials, the thermal stability and the production of xylose solutions was assessed at two temperatures using xylan as substrate: 28 °C, to assess the performance for SSF and CBP strategies; 50 °C, to assess the performance under separate hydrolysis and fermentation (SHF), where optimal temperature for enzyme activity can be applied without affecting mesophilic microorganisms. Over a 48 h period, crude extracts retained more than 62% of their xylanase activity and 90% of β–xylosidase activity at 28 °C, but at 50 °C those activities decreased to virtually 0% and 78%, respectively. Xylanases are therefore much more sensitive to high temperatures than β–xylosidases. In the presence of substrate (xylan) thermal inactivation was reduced as the extract retained 30% of xylanase activity after 48 h. Thus, the thermal stability of xylanase might be limiting the efficiency of xylan hydrolysis when applying this extract at high temperatures. Nevertheless, the formulation for

commercial applications can include stabilizers to mitigate this effect. Crude extracts from *M. antarcticus* PYCC 5048^T and *M. aphidis* PYCC 5535^T were, in general, more efficient than the commercial enzyme in the production of xylose solutions from xylan during the first 24–48 hours, reaching after 40/50% hydrolysis yields, in line with the yields achieved with commercial enzyme. These yields reflect the limitation of xylanolytic enzymes at two levels: (i) inhibition by the products of the xylanolytic enzymes, which is commonly seen in non-modified hydrolases; (ii) thermal inactivation of xylanases, as discussed above.

The application of these xylanolytic extracts in lignocellulose conversion processes can thus be optimized by increasing thermal stability of xylanases for SHF processes, by the overexpression of β-xylosidase and by reducing product inhibition effects, namely by applying SSF and CBP processes or at the molecular level by protein engineering.

The enzymes and enzyme extracts produced by *Moesziomyces* spp. have other potential applications such as: (i) food processing, including beer filtration processes and baking, mainly due to their relatively high activity at low temperature; (ii) the pulp and paper industry, for the production of dissolving pulp [37,38], because these are cellulase-free xylanolytic extracts; and (iii) ensiling processes, to selectively release xylose to be used by anaerobic lactic acid bacteria in feed stabilization, where both cellulase-free xylanases and xylanolytic activity at low temperature may be advantageous.

Conclusions

The current study has shown that *M. antarcticus* PYCC 5048^{T}and *M. aphidis* PYCC 5535^T can produce cellulase-free xylanolytic enzymes efficiently from xylose, xylan and BSG, with a remarkably high xylanase volumetric activity obtained from *M. aphidis* PYCC 5535^T when induced by BSG – 518 U/mL. The properties and performance of these extracellular crude extracts in xylan hydrolysis place them as promising for biotechnological applications under SHF, SSF and CBP systems for lignocellulose conversion into bio-based products or as technical enzymes for food, feed or pulp processing (in the pulp and paper industry).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.nbt.2018.11.001.

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