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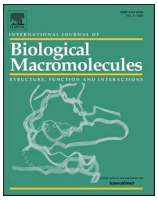

A novel endo-1,4-β-xylanase from *Alicyclobacillus mali* FL18: Biochemical characterization and its synergistic action with β-xylosidase in hemicellulose deconstruction

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ABSTRACT

A novel endo-1,4-β-xylanase-encoding gene was identified in *Alicyclobacillus mali* FL18 and the recombinant protein, named *Am*Xyn, was purified and biochemically characterized. The monomeric enzyme worked optimally at pH 6.6 and 80 °C on beechwood xylan with a specific activity of 440.00 ± 0.02 U/mg and a good catalytic efficiency (k_{cat}/K_M = 91.89 $s^{-1}mLmg^{-1}$). In addition, the enzyme did not display any activity on cellulose, suggesting a possible application in paper biobleaching processes. To develop an enzymatic mixture for xylan degradation, the association between *Am*Xyn and the previously characterized β-xylosidase *Am*βXyl, deriving from the same microorganism, was assessed. The two enzymes had similar temperature and pH optima and showed the highest degree of synergy when *Am*Xyn and *Am*βXyl were added sequentially to beechwood xylan, making this mixture cost-competitive and suitable for industrial use. Therefore, this enzymatic cocktail was also employed for the hydrolysis of wheat bran residue. TLC and HPAEC-PAD analyses revealed a high conversion rate to xylose (91.56 %), placing *Am*Xyn and *Am*βXyl among the most promising biocatalysts for the saccharification of agricultural waste.

1. Introduction

The growing demand for sustainable and eco-friendly technologies aligns with the promising strategies that can promote the transition toward a circular economy [1]. The lignocellulosic biomass is the most abundant, renewable and low-cost feedstock available on the Earth, that can be exploited in biorefineries for food, biofuel, and biomaterial production [2–5]. In this context, non-chemical pretreatments, such as ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and supercritical fluid extraction (SFE) [6], coupled with enzyme saccharification, can be utilized to create the building blocks to produce new biomaterials. In particular, the employment of UAE promotes the solubilization of cellulose, hemicellulose, and lignin and increases the substrate accessibility of enzymes to enhance the overall efficiency of the bioconversion process [7–11]. Microwave energy also dissolves target molecules, without changing their chemical structure, due to the pressure generated by the water vapor in the cell walls, whereas the last green technology, SFE, uses solvents above their critical temperature and pressure [12,13]. Nowadays, to improve biomass hydrolysis, different strategies are developed, such as the use of nanostructured materials coupled with enzymes [14–18] or the use of more performing biocatalysts like enzymes from thermophilic microorganisms [19–21]. Different bacterial species belonging to *Pyrococcus*, *Bacillus*, *Thermotoga*, *Pseudothermotoga*, *Geobacillus*, *Alicyclobacillus*, and *Dictyoglomus* genera have attracted considerable attention as sources of glycoside hydrolases (GHs) that can withstand high temperatures and other inhibitory conditions in biorefineries [22–27]. Indeed, the high operational temperatures of thermozymes (from 50 to 90 °C) are in line with actual feedstock pretreatment procedures, since they promote biomass disorganization, improve substrate and product solubility, and minimize the risk of microbial contaminations [28,29]. Thereby, the development of new thermophilic and thermostable enzymatic cocktails helps in maintaining high operative temperatures that are associated with higher yield of lignocellulose transformation, thus making the

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enzymatic process cost-competitive and reducing the enzyme load dosage [1,30,31]. Several thermozymes have been employed for their exceptional efficiency in breaking down xylan, which represents ~35 % of hemicellulose. Its backbone is constituted of β-1,4-linked xylopyranosyl residues decorated with substituted groups such as arabinose, acetyl, glucuronic acids, and ferulic acid [32,33]. Due to the intrinsic matrix complexity of xylan, enzymatic cocktails consisting of a combination of various enzymes, have now become a powerful tool for breaking down xylan into simpler sugars and producing xylooligosaccharides (XOS), which have numerous applications in food, pharmaceuticals, and biofuels [34]. To achieve complete depolymerization of xylan to xylose monomers the synergistic action of multiple enzymes is required, including *endo*-acting enzymes (endo-1,4-β-xylanases) and *exo*-acting enzymes (β-xylosidases, α-L-arabinofuranosidases, and acetyl xylan esterases) [32]. Among them, endo-1,4-β-xylanases (E.C 3.2.1.8) and β-xylosidases (E.C 3.2.1.37) are the two key enzymes responsible for the hydrolysis of xylan. Endo-1,4-β-xylanases randomly cleave the internal β-1,4 glycosidic linkages to yield XOS [35] and subsequently β-xylosidases catalyze the hydrolysis of β-1,4 linkages of short XOS to xylose monomers, thus preventing the inhibition of xylanases by their end-products [36]. So far, the use of xylanase cocktails to produce XOS and/or xylose has not been well established and the aim of this work was to characterize a novel endo-1,4-β-xylanase in order to set up a performing enzymatic cocktail for hemicellulose degradation.

The bacterial source chosen was *Alicyclobacillus mali* FL18, a thermoacidophilic microorganism isolated from a hot spring in Pisciarelli [23,37]. Based on its genomic annotation previously published, a novel endo-1,4-β-xylanase (*Am*Xyn) belonging to glycosyl hydrolase (GH) family 10 was purified and biochemically characterized for its catalytic properties and tolerance to different solvents and chemical agents. In a previous work, a novel β-xylosidase (*Am*βXyl) from the same microorganism was biochemically characterized, showing hydrolytic activity on xylobiose (X_2) and xylotriose (X_3) [38]. Therefore, a novel enzymatic mixture was set up by using the β-xylosidase (*Am*βXyl) and the newly characterized endo-1,4-β-xylanase (*Am*Xyn). This cocktail efficiently hydrolyzed both commercial beechwood xylan and UAE pretreated wheat bran (WB) residues with high xylose yields, suggesting its potential use as a promising candidate in saccharification processes.

2. Materials and methods

2.1. Expression of AmXyn of A. mali FL18 in E. coli and purification

The gene encoding putative endo-1,4 β-xylanase, *Am*Xyn, (GenBank Accession No. MBF8379065.1) was synthetically produced by GenScript Biotech (Piscataway, NJ, USA) and cloned in pET30 (a+). The codon usage of recombinant gene was optimized for the expression in *Escherichia coli*. 1 L of BL21-Codon-Plus(DE3)RIL/pET*Amxyn* culture was grown to 0.5 OD_{600nm} in Luria-Bertani (LB) medium supplemented with kanamycin (50 μg/mL) and chloramphenicol (33 μg/mL) at 37 °C. *Am*Xyn expression was induced with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) (PanReac AppliChem, Ottoweg, Darmstadt, Germany) for 16 h at 37 °C [39]. The bacterial cells were harvested by centrifugation (5000 ×*g*, 15 min, 4 °C) and the pellet resuspended in 40 mL buffer A (50 mM Tris-HCl pH 7.5, 100 mM NaCl and 20 mM imidazole) supplemented with a protease inhibitor cocktail 1× (Roche, Basilea, Switzerland). The cells were lysed with the following procedure: i) incubation with lysozyme 1 mg/mL for 90 min on an orbital shaker at 37 °C (PanReac AppliChem, Ottoweg, Darmstadt, Germany); ii) incubation with Triton X-100 1 % and DNaseI 5 μg/mL on ice for 40 min; iii) freezing with ethanol and dry ice for 15 min and iv) re-incubation for 15 min at 37 °C. The cell extract was separated by centrifugation at 40,000 ×*g* for 40 min at 4 °C, and the soluble extract underwent thermal precipitation at 60 °C for 10 min followed by centrifugation at 40,000 ×*g* for 40 min at 4 °C. The soluble fraction containing recombinant *Am*Xyn was loaded on IMAC because the recombinant protein was fused with His-tag at the N-terminus. A HisTrapHP column (GE Healthcare, Chicago, IL, USA) was connected to an AKTA system. Protein elution was carried out with buffer A supplemented with a linear gradient of imidazole (0–500 mM), and all the peak fractions were pooled and dialyzed against 50 mM Tris-HCl pH 7.5 and 100 mM NaCl at 4 °C for 16 h. The purity degree of AmXyn was assessed by 12 % SDS-PAGE stained with Coomassie brilliant blue R-250 (Biorad, Hercules, CA, USA) and Bradford assay was used to estimate the purified protein concentration using bovine serum albumin as standard [40].

2.2. Quaternary structure determination of AmXyn

The native molecular weight of the purified *Am*Xyn was analyzed by a size-exclusion chromatography connected to a Mini DAWN Treos spectrometer (Wyatt Instrument Technology Corp.) connected to a triple-angle light scattering detector, equipped with a Quasi-Elastic Light Scattering (QELS) module. One mL of protein (0.5 mg/mL) was loaded on a S200 column (16/60, GE Healthcare), equilibrated in 50 mM Tris-HCl pH 7.5 and 100 mM NaCl. A constant flow of 0.5 mL/min was applied. Data were analyzed using Astra 5.3.4.14 software (Wyatt Technology, Santa Barbare, CA, USA). Determination of molecular masses and hydrodynamic radii are reported as mean values of triplicate experiments.

2.3. Determination of enzymatic activity of AmXyn

The 3,5-dinitrosalicylic acid (DNS) method was employed for the detection of the xylanase activity of the enzyme using beechwood xylan (Megazyme) as substrate [41]. The assay mixture, containing 10 mg/mL beechwood xylan and 50 mM sodium phosphate (pH 6.6) in a volume of 40 μL, was preincubated at 80 °C for 5 min before the addition of 2 μL of purified enzyme (0.25 μg/μL). The reaction was carried out for 1 min at 80 °C and stopped on ice for 5 min. Then, 160 μL of DNS were added to the reaction mixture, which is transferred to a 96-well microplate and placed in a thermomixer (Eppendorf, Hamburg, Germany) at 100 °C for 20 min. After a cooling step at 4 °C for 20 min, the concentration of reducing sugars in solution was determined by measuring A_{540nm} in a 96-well microplate reader (Synergy H4, Biotek, Agilent, Santa Clara, CA, USA). Standard curves were quantified with D-xylose, as described by Kim et al. [42]. One international unit (IU) is defined as the amount of enzyme capable of releasing 1 μmol of reducing sugars per minute at 80 °C and pH 6.6 [43]. Each experiment was performed in triplicate.

2.4. Effect of pH and temperature on AmXyn activity

The optimal pH value for enzymatic activity was evaluated by measuring enzymatic activity at 80 °C in various 50 mM buffer solutions, including citrate buffer (pH 3.0–5.6), sodium phosphate buffer (pH 6.0–8.0) and glycine-NaOH buffer (pH 8.0).

The optimal temperature within the range 30–90 °C was determined at the optimal pH; each experiment was performed in triplicate and results were reported as relative activity (%).

The pH stability of the recombinant *Am*Xyn was tested by preincubating the enzyme in different buffers from pH 3.0 to 9.0 at 4 °C for 16 h; the residual activity was measured under standard assay conditions.

The thermostability of the enzyme was determined by preincubating the enzyme at different temperatures (60, 65, 70 and 75 °C) for multiple time periods ranging from 5 to 120 min. The residual activities were then assessed under the standard conditions as previously reported. All the experiments were performed in triplicate and non-preincubated enzyme was used as a control.

2.5. Effect of cations and chemicals on AmXyn activity

The effect of 8 different metal salts at ion concentrations of 1 and 5

mM ($CuCl_2$, $ZnCl_2$, LiCl, $MgCl_2$, $CaCl_2$, $MnSO_4$, and $NiCl_2$) and metal chelator EDTA on the xylanase activity of *Am*Xyn was evaluated [44]. Moreover, the influence on the enzymatic activity of the surfactants Tween-20, Triton X-100, and sodium dodecyl sulfate (SDS) at a final concentration of 0.5 % (*v*/v) was also monitored.

Lastly, *Am*Xyn activity was tested at 60 °C and optimal pH with the addition of organic solvents (dimethyl sulfoxide (DMSO), ethanol, or methanol) at final concentrations of 10 %, 20 %, 30 %, 40 %, 50 %, and 60 % in the reaction mixture. The enzyme in the absence of metal ions or chemical agents was used as a control.

2.6. Substrate specificity and catalytic properties of AmXyn

The substrate specificity of *Am*Xyn was investigated under optimal assay conditions in the presence of the following substrates: beechwood xylan, oat spelt xylan, carboxymethylcellulose (CMC), and *para*-nitrophenyl-β-D-xylopyranoside (*p*NP-β-xyl). All enzymatic measurements were performed in triplicate. The concentration of released *p*NP was determined by measuring A_{405nm}. Enzymatic parameters K_M, k_{cat}, and k_{cat}/K_M, were determined under optimal assay conditions with different concentrations of beechwood xylan, ranging from 0.25 to 14.00 mg/mL, using GraphPad 8.0 Prism software. Each of the data points was collected in triplicates.

2.7. Expression and purification of β-xylosidase from A. mali FL18 (AmβXyl)

*Am*βXyl from *A. mali FL18* was expressed in recombinant strain BL21 (DE3)RIL/pET30a-*Amxyl* and purified by affinity chromatography [38]. The enzymatic activity was determined using para-nitrophenyl-β-D-xylopyranoside (PNP-β-xyl) as substrate and measuring the concentration of released pNP at 405 nm, under the optimal conditions previously set [38].

2.8. Synergistic effect of AmXyn and AmβXyl on xylan degradation

In order to achieve the complete degradation of beechwood xylan, the synergistic action between *Am*Xyn and *Am*βXyl was evaluated. The reaction mixture was prepared as follows: i) 10 mg/mL of saturating beechwood xylan was dissolved in 50 mM sodium phosphate buffer (pH 6.5), ii) 2 μg of total proteins were added to the reaction mixture in different percentage ratios: a) 1.5 μg *Am*Xyn and 0.5 μg *Am*βXyl corresponding to 75 % – 25 %; b) 1.0 μg *Am*Xyn and 1.0 μg *Am*βXyl, corresponding to 50 % – 50 %; c) 0.5 μg *Am*Xyn and 1.5 μg *Am*βXyl corresponding to 25 % – 75 %; iii) temperatures were 60 °C and 80 °C, and iv) reaction times were 1, 80, and 180 min. Mixtures without any enzyme were included in the analysis as the negative control. All the samples were analyzed for the concentration of reducing sugars by DNS method, using xylose as standard. All the enzyme assays were carried out in triplicate.

The degree of synergy (DS) between the two enzymes was calculated by the following equation:

$$DS = Y_{1+2}/(Y_1 + Y_2) \quad (1)$$

where Y_{1+2} indicates the μmol of reducing sugars released by enzymes when used together simultaneously or sequentially, Y_1 and Y_2 indicate the yield of reducing sugars achieved when the enzymes work separately [45].

2.9. Analysis of beechwood xylan hydrolysis products by TLC and HPAEC-PAD

Beechwood xylan hydrolysis products generated by the best determined synergistic action of *Am*Xyn and *Am*βXyl were qualitatively assessed by thin layer chromatography (TLC) and high-performance anion exchange chromatography equipped with pulsed amperometric detection (HPAEC-PAD). In detail, 75 % *Am*Xyn (1.5 μg) and 25 % *Am*βXyl (0.5 μg) were incubated simultaneously or sequentially with 10 mg/mL of beechwood xylan dissolved in 50 mM sodium phosphate buffer pH 6.5 at 60 °C for 180 min. 10 μL of each reaction and the oligosaccharide mixture standard were spotted on a silica plate (type 60 F254, 0.25 mm, Merck, Darmstadt, Germany), and then developed with a chloroform/acetic acid/ddH_2O (6:7:1 *v/v/v*) solvent system in a covered chamber. The results were visualized by dipping the plates into a solution containing ethanol/sulfuric acid (90:10 *v/v*), and heating for 5 min at 120 °C in an oven.

For HPAEC-PAD analysis 2.5 μL of supernatant containing XOS was injected having the following gradient [46] of eluents (A: MilliQ water; B: NaOH 1 M): from 0 to 11 min 96 % of A and 4 % of B in isocratic flow of 0.250 mL/min, from 11 to 18 min a gradient of B starting from 4 % to 35 %, from 18 min to 20 min eluent B was kept isocratic at 35 %, finally at min 20 the initial conditions at 4 % eluent B were reestablished and kept until end of the run at 30 min.

2.10. UAE pretreatment and enzymatic hydrolysis of WB

A physical pretreatment of biomass was performed as described by Wang et al. [47]. WB was washed and dried at 37 °C for 2 days, and grounded with pestle and liquid nitrogen. An ultrasonic bath (Branson 1510E-DHT Ultrasonic Cleaner) was used to pretreat 1 g dried WB mixed with 20 mL water at a constant ultrasonic frequency of 40 kHz at 60 °C for 40 min. The samples were centrifuged at 5000 ×*g* for 10 min at 4 °C and the supernatants were filtered and stored at −20 °C. The solid fraction was dried in an oven at 40 °C. The chemical composition of native and pretreated WB was analyzed according to the National Renewable Energy Laboratory (NREL) procedure and down scaled for smaller volume as already described [31]. The native WB contained (% of dry mass) 24.70 of glucan, 13.10 of xylan, 6.10 of arabinan, and 14.60 of lignin; the UAE pretreated WB instead resulted having 26.50 of glucan, 20.00 of xylan, 8.10 of arabinan, and 10.60 of lignin. Proteins and water-soluble extractives were not measured.

Enzymatic hydrolysis of UAE pretreated WB (10 % *w/v*) was carried out using *Am*Xyn (90 U/g biomass) and *Am*βXyl (27 U/g biomass) in 50 mM sodium phosphate buffer pH 6.5. Mixtures without any enzyme were included in the analysis as control. 10 mL mixtures were placed under stirring (180 rpm) at 60 °C for 3 h. Samples were centrifuged at 5000 ×*g* for 10 min at 4 °C and the supernatants were filtered and analyzed by HPAEC-PAD as described in the previous paragraph.

3. Results and discussion

3.1. Expression, purification, and quaternary structure of recombinant AmXyn

The *Amxyn* gene was synthetically produced and codon-adapted to *E. coli* genetic system. The gene was cloned in the expression vector pET-30a (+) and the recombinant protein, with His-tag at N-terminus, was expressed in *E. coli* BL21 (DE3) RIL strain. The protein was purified, almost to homogeneity, by heat treatment and His-trap affinity

Table 1
Purification steps of *Am*Xyn. Abbreviations: C.E., cellular extract from IPTG-induced cells; H.T, the crude extract from heat-treated cells; A.C., affinity chromatography.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
C.E.	4160.00	104.00	40.00	100.00	1.00
H.T.	4212.00	70.20	60.00	100.00	1.50
A.C.	2604.00	5.92	440.00	62.00	11.00

chromatography. The purification steps are summarized in Table 1, which shows a yield and purification ratio of 62.00 % and 11.00 times, respectively, after affinity chromatography.

As revealed by SDS-PAGE analysis (Fig. 1a), recombinant *Am*Xyn showed a single band with a molecular mass of ~40 kDa, in line with the molecular weight predicted by Expasy-ProtParam tool ("https://web.expasy.org/protparam/"). The yield of the purified protein was about 6 mg from 1 L of culture.

The native molecular weight of the purified *Am*Xyn was analyzed through size-exclusion chromatography coupled with a triple-angle light scattering QELS. This analysis showed a molecular weight of about 41 kDa ± 0.8 %, indicating that *Am*Xyn forms a monomer in solution (Fig. 1b), similar to other previously characterized *endo*-β-1,4-xylanases, such as Xyn10 from a sugar cane bagasse compost-derived microbial consortia [48] and xynBCA from *Caldicoprobacter algeriensis* [49].

3.2. Biochemical properties

Activity and stability of enzymes at high temperatures are important properties in industrial processes [50]. Thermophilic xylanases find application in paper and pulp industries, during the bio-bleaching process that is carried out at high temperatures [51,52]. Moreover, xylanases are also successfully employed in hot water pretreatment of lignocellulosic waste for biofuel production [53]. Therefore, the effect of temperature on *Am*Xyn activity was examined and the optimal activity was observed at 80 °C (Fig. 2a): the enzyme retained over 80 % of its maximal activity at temperatures ranging from 60 °C to 75 °C and over 60 % in the range 30–50 °C, while enzymatic activity significantly decreased after 80 °C; this latter value can be due to its low thermostability at already 75 °C, as shown below.

The temperature optimum is slightly higher than that of *Cb*Xyn10B and XynA from the thermophilic bacteria *Caldicellulosiruptor bescii* and *Geobacillus* sp. WBI [54,55]. It is worthy of attention that (Fig. 2a), like Xyn10B from *Thermotoga thermarum*, which is active from 60 to 95 °C, with an optimum at 80 °C [56]. This property may be considered useful either for the bioconversion of lignocellulosic biomass and the production of XOS [57]; the latter have important prebiotic properties when added to infant milk powder or beverage products, like tea, coffee, and dairy products [58]. In addition, XOS improve nutrient utilization and digestibility in animals, resulting in positive effects on the growth, intestinal immunity, and microbial composition [59].

High thermostability also offers the economic advantage of reduced enzyme consumption thanks to the higher specific activity and greater rate of hydrolysis [35]. Interestingly, after 120 min at 60 °C the relative activity of *Am*Xyn was about 80 %, while at 70 °C the enzyme kept about 30 % activity (Fig. 2b), showing a noticeably higher thermostability compared to other xylanases. Some examples are the thermophilic xylanases from *Rhodothermaceae* RA [60] and *Melioribacter roseus* [61], that showed a half-life of 60 min and 120 min at 60 °C, respectively.

*Am*Xyn activity was evaluated over a pH range of 3.0–8.0 using beechwood xylan as substrate. As shown in Fig. 3a, the maximal activity was observed at pH 6.6. At pH values of 6.0, 7.0, and 8.0 the enzymatic activity was about 50 %, 90 %, and 60 %, respectively. Most of the GH10 xylanases characterized so far possess conserved acidic residues acting as the catalytic nucleophile and proton donor and are active in near-neutral condition, like Xyn10B from *T. thermarum* [56], which exhibits optimal activity at pH 6.0. The activity at near-neutral pH is a further evidence that *Am*Xyn is an intracellular enzyme, as also suggested by the lack of a signal peptide for secretion (data not shown) ("https://services.healthtech.dtu.dk/services/SignalP-5.0/").

The pH stability of *Am*Xyn was investigated by determining the remaining activity after 16 h of incubation in different buffers. Interestingly, the enzyme was proved to be stable, maintaining about 90 % and 50 % of its original activity, at pH values ranging from 5.5 to 8.0 and pH 9.0, respectively (Fig. 3b).

3.3. Effect of chemical agents on enzyme activity

Metal ions and chemical agents' tolerance is an advantageous property for various industrial processes, including bioethanol production and waste biodegradation [62], therefore their effect on *Am*Xyn activity was evaluated (Table 2). The enzyme exhibited the ability to resist most of the metal ions tested, except for Zn^{+}, Cu^{2+}, and Ni^{2+} which inhibited enzymatic activity at both the concentrations of 1 mM and 5 mM. The inhibition effect of these metal ions has long been reported [63], since their binding to indole rings of tryptophan altering the structure and biochemical function of the enzymes [64]. On the other hand, the addition of EDTA did not affect *Am*Xyn activity, indicating that no metals are needed for the enzymatic reaction.

Interestingly, $LiCl_2$, $MgCl_2$, and $MnSO_4$ exerted positive effects on *Am*Xyn demonstrated by an increased relative activity of 143.12, 136.20, and 113.51 %, was detected, respectively (Table 2). It is known

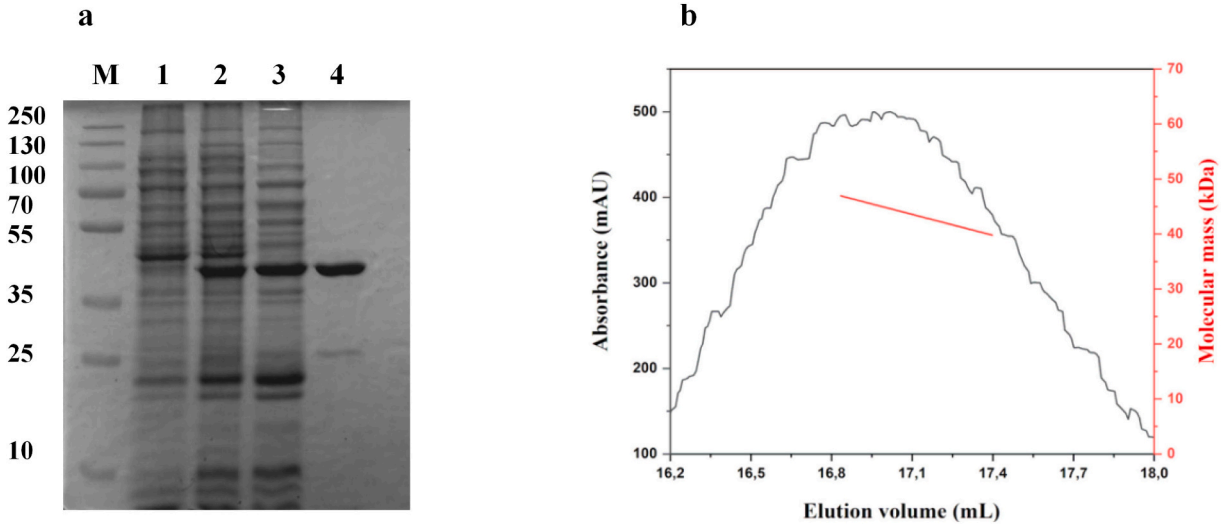

Fig. 1. Analysis of the recombinant *Am*Xyn. a) SDS-PAGE of the purification steps. M) Protein Marker; 1) cellular extract from non-induced cells; 2) cellular extract from IPTG-induced cells; 3) crude extract of induced cells after 10 min at 60 °C; 4) *Am*Xyn after affinity chromatography. b) Analysis of purified *Am*Xyn by gel filtration chromatography coupled with light scattering QELS. The elution profile of *Am*Xyn is shown as a continuous line. The clustered points represent the light scattering data converted to molecular mass.

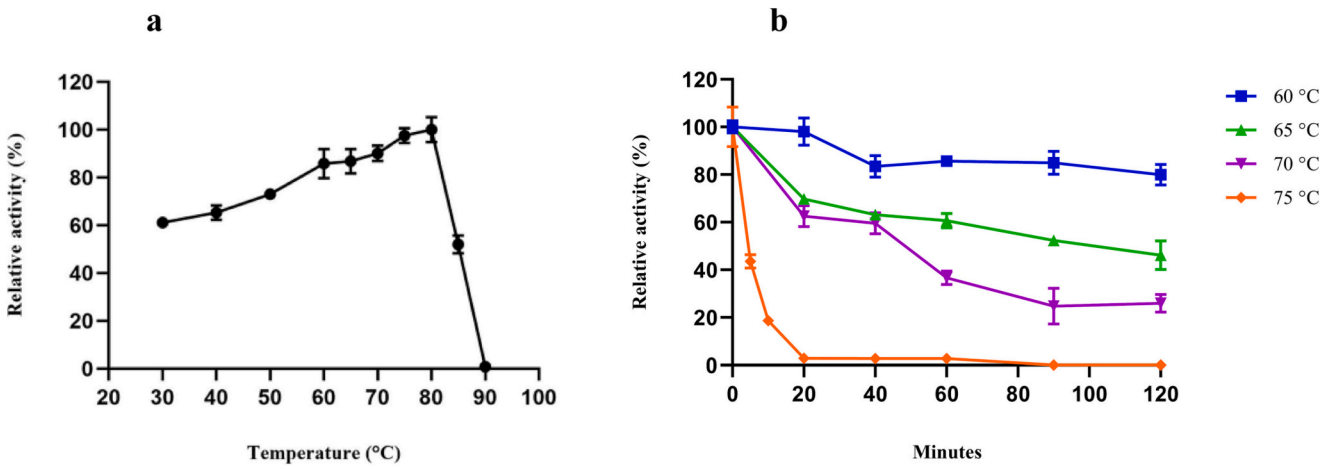

Fig. 2. Effect of temperature on enzymatic activity of *Am*Xyn. a) Temperature optimum was determined by measuring enzymatic activity in the range 30–90 °C. b) Thermostability was evaluated by incubating the enzyme at 60, 65, 70, and 75 °C for different times and then assaying the residual activity under optimal conditions.

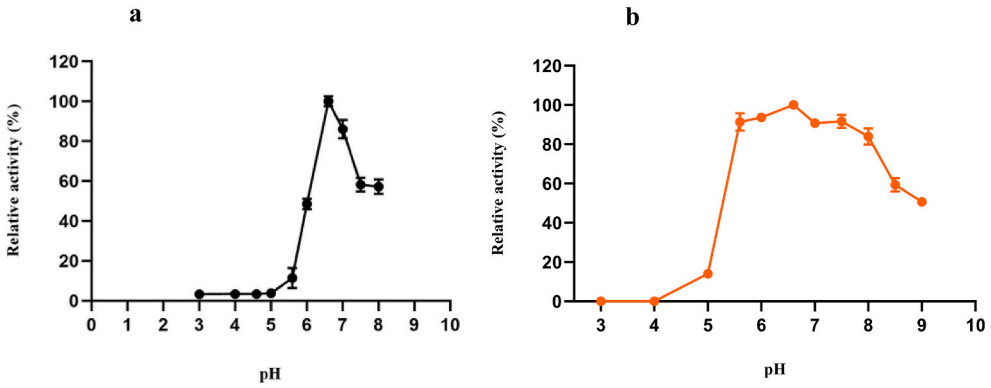

Fig. 3. Effect of pH on the enzymatic activity of *Am*Xyn. a) pH optimum was evaluated in buffers ranging from pH 3.0 to pH 8.0. b) pH stability was determined by incubating *Am*Xyn in various buffers (from pH 3.0 to pH 9.0) for 16 h and assaying residual activity under optimal conditions. The activity of *Am*Xyn was determined using beechwood xylan as substrate.

Table 2
Effect of metal ions and chemicals on *Am*Xyn activity.

Metal Ions or chemical agents	Relative activity (%)			
	1 mM	5 mM	0.1 %	0.5 %
None	100	100	100	100
$CuCl_2$	19.4	12.31		
$ZnCl_2$	32.42	10.80		
LiCl	110.3	143.12		
$MgCl_2$	105.84	136.20		
$CaCl_2$	88.57	95.65		
$MnSO_4$	139.58	113.51		
$NiCl_2$	17.13	10.21		
EDTA	85.48	85.36		
SDS			ND	ND
Triton X-100			72.03	ND
Tween 20			69.53	48.73

that metal ions such as Mg^{2+} and Mn^{2+} could help improve enzyme activity, and the beneficial effect of these bivalent cations may be attributed to their effect of stabilizing the enzyme-substrate complex [51,65]. Conversely, other xylanases from *Rhodothermaceae* RA and *Paenibacillus* sp. HPL-001 were inhibited by $CaCl_2$ and $MgCl_2$ [60,66]. Moreover, *Am*Xyn is also resistant to Ca^{2+} ions that are employed in paper industry to increase the opacity [67] and the filler content of cellulosic paper [68]. The good tolerance of *Am*Xyn to most of the tested metal ions is a notable industrial advantage especially when considering the deconstruction of lignocellulose [23,38]; infact it is reported that biomasses link efficiently metal ions through mechanisms of ion exchange, surface precipitation, complexation and chelation [62]. Interestingly, *A. mali* FL18 has several hydrolytic enzymes that can be applied in the deconstruction of lignocellulose [23,38]; moreover, the microorganism itself, which has been isolated from an arsenic-rich hot spring, is tolerant to nickel, cobalt, mercury and other metals [37].

Addition of surfactants has been reported to facilitate enzymatic hydrolysis of biomass, since they adsorb to the lignin surface and reduce unproductive enzyme binding as well as reduce enzyme denaturation [69]. Therefore, the influence of surfactants on *Am*Xyn activity was evaluated (Table 2). The enzyme was totally inhibited by the anionic detergent SDS, while it retained about 70 % of its original activity in presence of 0.1 % Triton X-100 and Tween-20 and about 50 % activity at 0.5 % Tween-20.

Since organic solvents are often employed for solubilizing hydrophobic substrates during enzymatic treatments of lignocellulosic biomass, the effect of some of them on *Am*Xyn activity was also examined (Fig. 4). *Am*Xyn activity increased about 1.6-fold with 10 % DMSO and 1.4-fold with 20 % DMSO. Moreover, the enzyme retained about 90 % and 60 % of its original activity in presence of 30 and 40 % DMSO, respectively. This stability may be ascribed to the presence of hydrophobic amino acid residues on the protein surface [70]. On the other hand, 20 % methanol led to an inhibition of enzymatic activity, similarly to the xylanase from *Talaromyces thermophilus* that lost activity in the presence of methanol at concentrations ranging from 20 to 40 % [71].

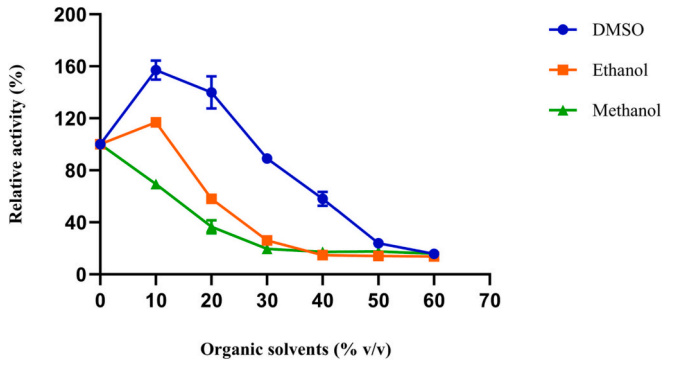

Fig. 4. Effect of organic solvents on *Am*Xyn activity. DMSO (circles), ethanol (squares), and methanol (triangles).

Interestingly, *Am*Xyn was slightly stimulated in the presence of 10 % ethanol (Fig. 4), envisaging its potential application for simultaneous saccharification and fermentation to bioethanol or its use to clarify alcoholic beverages such as wine or in the brewing industry [72].

3.4. Substrate specificity and catalytic properties

The hydrolytic activity of *Am*Xyn was determined on various substrates. The enzyme showed the highest activity on beechwood xylan (440.00 ± 0.02 U/mg), followed by oat spelt xylan (256.57 ± 0.01 U/mg), and it exhibited a slight activity against the synthetic substrate *para*-nitrophenyl-β-D-xylopyranoside (*p*NP-β-xyl) (10.30 ± 0.02 U/mg) (Table 3). It is worth mentioning that, in contrast with most GH10 xylanases [28], *Am*Xyn can be classified as cellulase-free xylanase since no activity was observed toward CMC, suggesting a possible application of the enzyme in the paper pulp industry. Indeed, cellulase-free xylanases facilitate the release of lignin from paper pulp without damaging cellulose, thus reducing the consumption of chlorine as a bleaching agent and enabling the production of bleached pulp of superior quality and brightness [73].

The specific activity and Michaelis-Menten kinetic constants of *Am*Xyn on beechwood xylan were also compared with those of other characterized xylanases belonging to GH10 family. Interestingly, as shown in Table 4, *Am*Xyn exhibited a higher specific activity and catalytic efficiency compared to xylanase from *A. acidocaldarius* ATCC 27009 [33] as well as other thermophilic xylanases from *Streptomyces* SP7 and *Streptomyces* sp. B6.

3.5. Analysis of beechwood xylan and WB waste hydrolysis products

To achieve the complete hydrolysis of xylan, the activity of *Am*Xyn was corroborated by the already characterized *Am*βXyl from *A. mali* FL18 [38]. The two enzymes shared similar pH and temperature optima as well as a good stability at 60 °C [38] and their synergistic effect was evaluated on commercial beechwood xylan.

The effect on beechwood xylan hydrolysis was measured by adding sequentially or simultaneously the two enzymes in different ratios (75 % *Am*Xyn - 25 % *Am*βXyl; 50 % *Am*Xyn - 50 % *Am*βXyl; 25 % *Am*Xyn - 75 % *Am*βXyl), at different temperatures (60 °C and 80 °C), and reaction times (1, 80, and 180 min). DNS assay results indicated that *Am*βXyl alone did not catalyze any xylose release from xylan, as expected. Furthermore, both sequential and simultaneous additions of the two enzymes had significant synergistic effects on xylan degradation. The highest DS (1.35) was obtained when *Am*Xyn and *Am*βXyl (75 %–25 %) were added in sequential manner at 60 °C for 180 min (Fig. 5a); however also in the simultaneous assay there was a significant DS (1.13). These results were similar to those reported previously by Yang et al. [74] and Nguyen et al. [75] and suggested a major role for xylanase in the initial phases of xylan hydrolysis by cleaving the internal β-(1,4) bonds of xylan and generating short XOS for further degradation into xylose by *Am*βXyl [76].

Table 3
Substrate specificity of *Am*Xyn. ND: non detected.

Substrate	Specific activity (U/mg)
Beechwood xylan	440.00 ± 0.02
Oat spelt xylan	256.57 ± 0.01
PNP-β-xyl	10.30 ± 0.02
CMC	ND

Xylan hydrolysis products obtained in the same conditions previously reported were analyzed by TLC and the results showed that xylanase alone digested xylan to form xylose, xylobiose (X_2) and XOS with higher polymerization degree (PD) (Fig. 5b), whereas xylosidase alone was not able to degrade xylan. Most xylanases produce X_2-X_4-X_6 and a low amount of xylose from xylan, such as BsXyn10 from *Bacillus safensis* and Xyn10A from *Flavobacterium johnsoniae* [77,78], or xylanase from *Marinifilaceae bacterium* SPP2 which produces X_2-X_4, but no xylose [79]. Liu et al. identified the hydrolysis products released from beechwood xylan by the xylanase rePBaxA as xylose to xylohexaose (X-X_6), with X_2 (1.86 g/L) and X_3 (1.29 g/L) as the major products and xylose in a minor fraction (0.09 g/L) [80].

To gain insight into xylan hydrolysis products, HPAEC-PAD was performed (Fig. 5c). The analysis revealed that *Am*Xyn alone digested xylan to form mainly xylose (1.18 g/L) and X_2 (0.73 g/L) and in minor fractions XOS (X_3-X_6). The release of xylose as the main hydrolysis product by *Am*Xyn alone suggests that the enzyme has significant hexoglycosidic activity, removing β-xylosyl residues from the nonreducing ends of X_2 and XOS, as suggested also by the slight activity on the synthetic substrate pNP-β-xyl.

Interestingly, X_2 was also produced in high quantities from the hydrolysis of xylan by *Am*Xyn. X_2 has been reported to have the most noticeable prebiotic activity compared to high-DP XOS, promoting the growth of intestinal probiotic strains such as *Bifidobacterium* and *Lactobacillus*, preventing the growth of pathogens, and improving nutrient absorption [81].

Xylose plays a crucial role in biorefinery since it is fermented by *Saccharomyces* yeast strains for producing ethanol [82]. Moreover, xylose is used in biorefinery C5 molasses suitable for cattle feed, since cattle is capable of converting C5 sugars, and also can be utilized as "glue" in pig feed [83]. In this context, HPAEC-PAD analysis (Fig. 5c) revealed that the combination of *Am*Xyn and *Am*βXyl and their synergistic effect led to an increase from 1.18 to 2.07 g/L of xylose, with a conversion yield of 22.62 %, considering that beechwood xylan is composed by ~90 % xylose and ~10 % glucuronic acid according to the supplier.

In order to valorize lignocellulosic wastes as source of fermentable sugars, *Am*Xyn and *Am*βXyl were tested on physically pretreated WB. As shown in Table 5, the combined action of *Am*Xyn and *Am*βXyl released 20.71 g/L xylose from UAE pretreated WB. Fig. 6 showed the % of conversion to xylose, glucose, and arabinose after hydrolysis with the enzymatic cocktail. Noteworthy the yield of conversion for xylose reached up to 91.56 %.

4. Conclusions

The novel endo-1,4-xylanase *Am*Xyn from the thermoacidophilic bacterium *A. mali* FL18 displays peculiar biochemical features, like thermostability and thermophilicity, together with a good catalytic efficiency. Unlike most of the GH10 xylanases, its ability in hydrolyzing exclusively xylan and not cellulose makes it very suitable in the paper and pulp industry for biobleaching process. In addition, differently from other characterized xylanases, the main products of *Am*Xyn are xylose and xylobiose. The latter, thanks to its prebiotic potential in increasing

Table 4
Comparison of specific activity and kinetic constants of *Am*Xyn with other thermophilic xylanases.

Microorganisms	Enzyme	K_M (mg/mL)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}mLmg^{-1}$)	Specific activity (U/mg)	Reference
A. mali FL18	AmXyn	5.29	486.11	91.89	440.00	This work
A. acidocaldarius ATCC27009	GH10-XA	1.81	98.20	54.30	152.00	[10]
Streptomyces SP7	XynST7	3.54	ND	27.98	ND	[53]
Streptomyces sp. B6	XynST10	10.40	149.00	14.30	84.79	[54]
Thermotoga thermarum	Xyn10B	1.80	520.00	289.00	192.00	[35]
Caldicellulosiruptor bescii	CbXyn10B	1.90	378.4	199.2	497.00	[33]

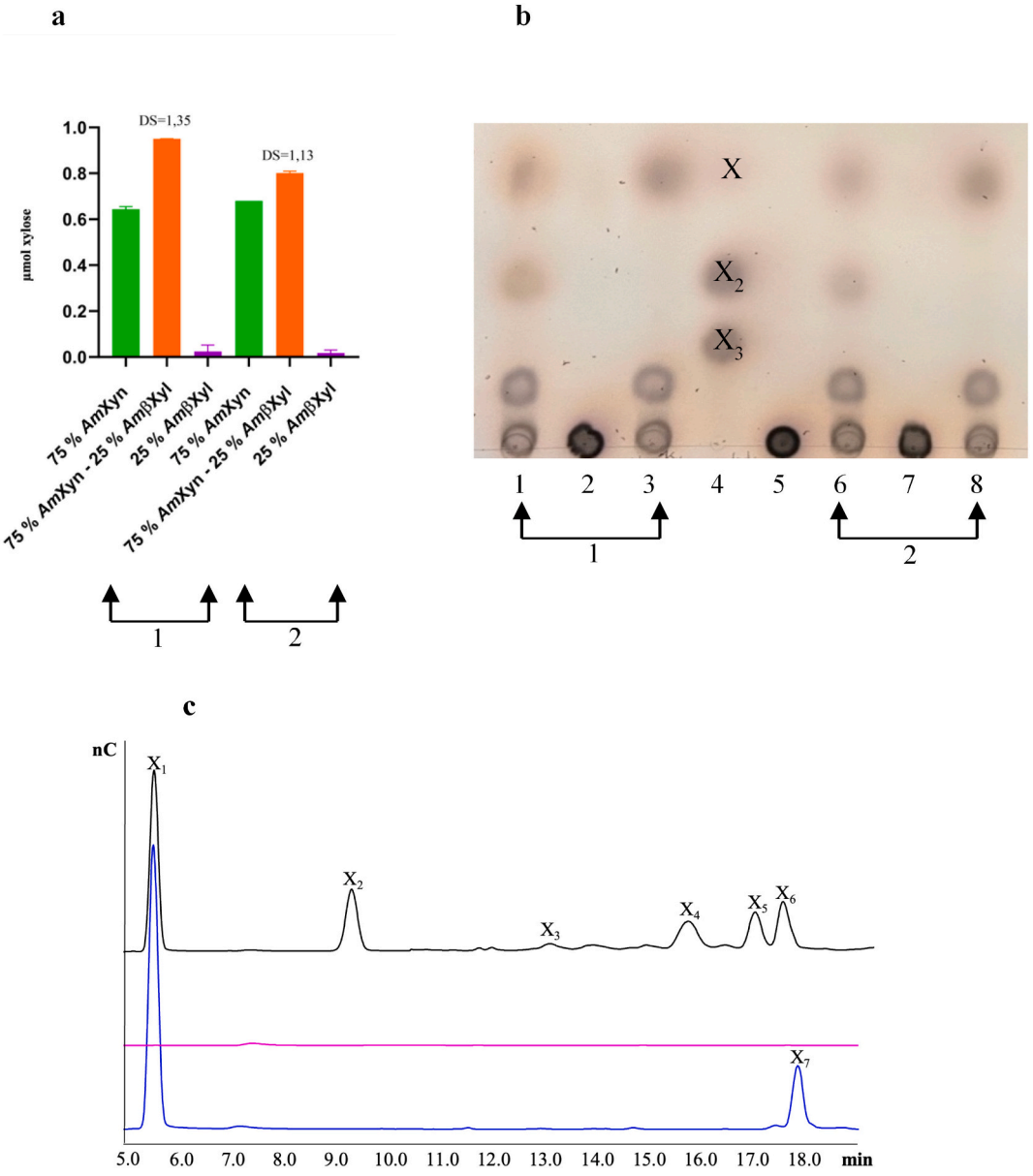

Fig. 5. Analysis of the hydrolytic activity of *Am*Xyn and *Am*βXyl. a) Quantification of µmol xylose released by sequential (1) and simultaneous (2) addition of recombinant *Am*Xyn and *Am*βXyl using 10 mg/mL beechwood xylan. Protein ratio was expressed as relative percentage. Xylose release was determined using DNS assay. Values were presented as mean values ± S.D. ($n = 3$). b) TLC analysis of the hydrolysis products from beechwood xylan by sequential (1) and simultaneous (2) addition of *Am*Xyn and *Am*βXyl: lane 1, hydrolysis products by *Am*Xyn; lane 2, hydrolysis products by *Am*βXyl; lane 3, hydrolysis products by the combination of *Am*Xyn and *Am*βXyl; lane 4, mixture of standards (X, X_2, X_3); lane 5, beechwood xylan; lane 6, hydrolysis products by *Am*Xyn; lane 7, hydrolysis products by *Am*βXyl; lane 8, hydrolysis products by the combination of *Am*Xyn and *Am*βXyl. c) HPAEC-PAD analysis of hydrolysis products from xylan. In black the chromatogram relative to *Am*Xyn alone, in blue *Am*Xyn and *Am*βXyl in sequential addition, in pink *Am*βXyl alone. Peak assignments: 1) xylose, 2) X_2 3) X_3, 4) X_4, 5) X_5, 6) X_6, 7) X_7.

Table 5
HPAEC-PAD analysis of hydrolysis products from WB. The values represent the mean ± standard deviation of three measurements.

	UAE	UAE + *Am*Xyn + *Am*βXyl
Arabinose	1.1 ± 0.05 g/L	3.48 ± 0.22 g/L
Glucose	3.62 ± 1.81 g/L	2.07 ± 1.22 g/L
Xylose	1.52 ± 0.07 g/L	20.71 ± 1.51 g/L

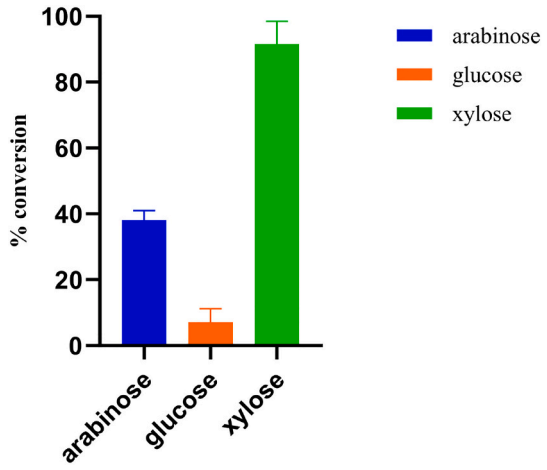

Fig. 6. Conversion percentage of arabinan, glucan, and xylan to monomers arabinose, glucose, and xylose. The values represent the mean ± standard deviation of three measurements.

animal feed digestibility and its anti-inflammatory properties in human, is very attractive for both food and pharmaceutical industries.

Interestingly, *Am*Xyn synergistically works with *Am*βXyl resulting in an increase of released xylose when used on both synthetic substrates and on UAE pretreated WB. This novel strategy of using multiple enzymes from a single thermophilic microorganism makes the process economically feasible, since they act under the same reaction conditions. In this study, the recombinant enzymes *Am*Xyn and *Am*βXyl derived from *A. mali* FL18 have shown great applicability in the formulation of effective enzyme cocktails for the valorization of agricultural waste.

CRediT authorship contribution statement

Flora Salzano: Writing – original draft, Methodology, Data curation. **Martina Aulitto:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Gabriella Fiorentino:** Writing – review & editing, Investigation, Conceptualization. **David Cannella:** Writing – review & editing, Methodology. **Eveline Peeters:** Writing – review & editing, Supervision, Funding acquisition. **Danila Limauro:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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