



Article

Alicyclobacillus mali FL18 as a Novel Source of Glycosyl Hydrolases: Characterization of a New Thermophilic β -Xylosidase Tolerant to Monosaccharides

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Abstract: A thermo-acidophilic bacterium, *Alicyclobacillus mali* FL18, was isolated from a hot spring of Pisciarelli, near Naples, Italy; following genome analysis, a novel putative β -xylosidase, Am β Xyl, belonging to the glycosyl hydrolase (GH) family 3 was identified. A synthetic gene was produced, cloned in pET-30a(+), and expressed in *Escherichia coli* BL21 (DE3) RIL. The purified recombinant protein, which showed a dimeric structure, had optimal catalytic activity at 80 °C and pH 5.6, exhibiting 60% of its activity after 2 h at 50 °C and displaying high stability (more than 80%) at pH 5.0–8.0 after 16 h. Am β Xyl is mainly active on both para-nitrophenyl- β -D-xylopyranoside (K_M 0.52 mM, k_{cat} 1606 s⁻¹, and k_{cat}/K_M 3088.46 mM⁻¹·s⁻¹) and para-nitrophenyl- α -L-arabinofuranoside (K_M 10.56 mM, k_{cat} 2395.8 s⁻¹, and k_{cat}/K_M 226.87 mM⁻¹·s⁻¹). Thin-layer chromatography showed its ability to convert xylooligomers (xylobiose and xylotriose) into xylose, confirming that Am β Xyl is a true β -xylosidase. Furthermore, no inhibitory effect on enzymatic activity by metal ions, detergents, or EDTA was observed except for 5 mM Cu²⁺. Am β Xyl showed an excellent tolerance to organic solvents; in particular, the enzyme increased its activity at high concentrations (30%) of organic solvents such as ethanol, methanol, and DMSO. Lastly, the enzyme showed not only a good tolerance to inhibition by xylose, arabinose, and glucose, but was activated by 0.75 M xylose and up to 1.5 M by both arabinose and glucose. The high tolerance to organic solvents and monosaccharides together with other characteristics reported above suggests that Am β Xyl may have several applications in many industrial fields.

Keywords: β -xylosidase; thermophiles; glycosyl hydrolases; *Alicyclobacillus mali*



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1. Introduction

Efficient utilization and valorization of lignocellulosic biomass are important in the shift to a biobased society in which biochemical processes are regarded as the most sustainable alternatives to physicochemical treatments [1]. Lignocellulose, an organic fibrous material obtained from natural wood and agro-industrial residues, is composed mainly of cellulose, hemicellulose, and lignin [2]. Xylan is the main component of hemicellulose, and it consists of β -(1,4)-linked D-xylopyranose residues that can be decorated with α -L-arabinofuranose, 4-O-D-methyl-glucuronic acids, and acetyl groups in nature [3]. To convert xylan into its constituent sugars, an array of enzymatic activities including endoxylanase (EC 3.2.1.8) and β -xylosidase (EC 3.2.1.37) together with α -glucuronidase (E.C.3.2.1.139), α -arabinofuranosidase (E.C.3.2.1.55), and acetylxylan esterase (E.C.3.1.1.72) is required. Endoxylanase randomly cleaves the internal β -(1,4) linkages of xylan to yield

xylooligosaccharides (XOS) [4]; β -xylosidase, which is an exoglycosidase, removes β -xylosyl residues from non-reducing ends of xylobiose and XOS, being essential to relieve the end-product inhibition of endoxylanase during complete hydrolysis of xylan. However, a synergistic action of the other enzymes is required to completely degrade the polymer [5].

β -xylosidases are classified into the glycoside hydrolase (GH) families 3, 5, 30, 39, 43, 51, 52, and 120 (<http://www.cazy.org/Glycoside-Hydrolases.html>, accessed on 15 May 2022) according to the amino-acid sequence similarities. They operate through a mechanism of inversion (GH family 43) or retention (GH families 3, 30, 39, 43, 51, 52, and 120) of the stereochemical configuration at the anomeric carbon [6]. β -Xylosidases have found application in a wide range of industrial processes, such as bleaching of paper pulp, enhancing the digestibility and nutritional properties of animal feed, manufacturing of beer and wine, clarification of fruit juices, and extraction of coffee [7]. They are also commonly used in biorefinery processes during the saccharification of pretreated agro-industrial wastes to produce biofuels [8–12]. Even now, significant efforts are being made to isolate and characterize increasingly high-performance and resistant enzymes for efficient biomass utilization. Thermophilic and thermostable glycosyl hydrolases from thermophilic microorganisms are generally used to promote faster reactions, increase substrate solubility, and reduce the risk of contamination [13–16]. However, most of the β -xylosidases are known to be inhibited by the hydrolysis products. Considering that, during the saccharification processes, high concentrations of monosaccharides are released, the research of new tolerant GHs is needed to increase the efficiency of substrate hydrolysis and lower the cost of many industrial processes [4,17–20].

In the present work, we performed cloning and heterologous expression of a putative β -xylosidase (Am β Xyl) based on the most recent genomic annotation of *Alicyclobacillus mali* FL18, an interesting thermo-acidophilic microorganism isolated from a hydrothermal hot spring of Pisciarelli, near Naples, in Italy [21]. Additionally, we purified the recombinant enzyme and exhaustively investigated the sequence identity, enzymatic properties, xylose tolerance, and activity against natural XOS new monosaccharides/solvent-tolerant enzymes that improve the saccharification of pretreated lignocellulosic biomass into fermentable sugars, e.g., for the production of value-added products.

2. Results

2.1. Sequence Analysis

According to the analysis of the genome sequence of *A. mali* FL18 (accession No. JADPKZ0000000) [21], the 2360 bp gene encoding putative 1,4 β -xylosidase (Am β Xyl) (Accession No. MBF8378422.1) of 783 amino acids (MW 84.70 kDa and pI 5.13) was highlighted. The putative protein belongs to the GH3 family, a heterogeneous family that includes various GH activities such as β -glucosidase, xylan 1,4- β -xylosidase, β -glucosylceramidase, β -N-acetyl-hexosaminidase, α -L-arabinofuranosidase, glucan 1,4- β -glucosidase, exo-1,3,1,4-glucanase, β -N-acetylglucosaminide phosphorylase, β -1,2-glucosidase, β -1,3-glucosidase, xyloglucan-specific hexo- β -1,4-glucanase/hexo-xyloglucanase, and lichenase/endo- β -1,3,1,4-glucanase. In addition, genome analysis showed two putative ATP-binding cassette (ABC) permeases upstream of the Am β Xyl gene (accession no. WP_230088200.1 and WP_230088182.1), which could be used for the uptake of sugars [22,23].

To express the recombinant protein in *E. coli*, SignalP 6.0 and NetNGlyc bioinformatics analyses were performed. The results showed the absence of a signal peptide and the presence of a single N-glycosylation site (Asn518), conditions that could allow the expression in *E. coli* as a soluble protein.

Multiple sequence alignment and BlastP analyses indicated homology of Am β Xyl with other thermophilic β -xylosidases from *Thermotoga petrophila* (accession No. ABQ46867.1), *Pseudothermotoga thermarum* (accession No. AEH50242.1), *Dictyoglomus turgidum* (accession No. ACK42133.1), and *D. thermophilum* (accession No. WP012548714.1), with 54%, 51%, 50%, and 43% identity, respectively. In addition, I-TASSER and Pfam analyses highlighted some typical structural features of GH3 family: the presence of the amino-acid sequences

of two conserved residues, Asp281 (nucleophile) and Glu519 (acid/base), involved in the retaining reaction mechanism (Figure 1a,b) and the fibronectin type III-like domain (Figure 1c), which is generally found at the C-terminus of enzymes belonging to the GH3 family [7].

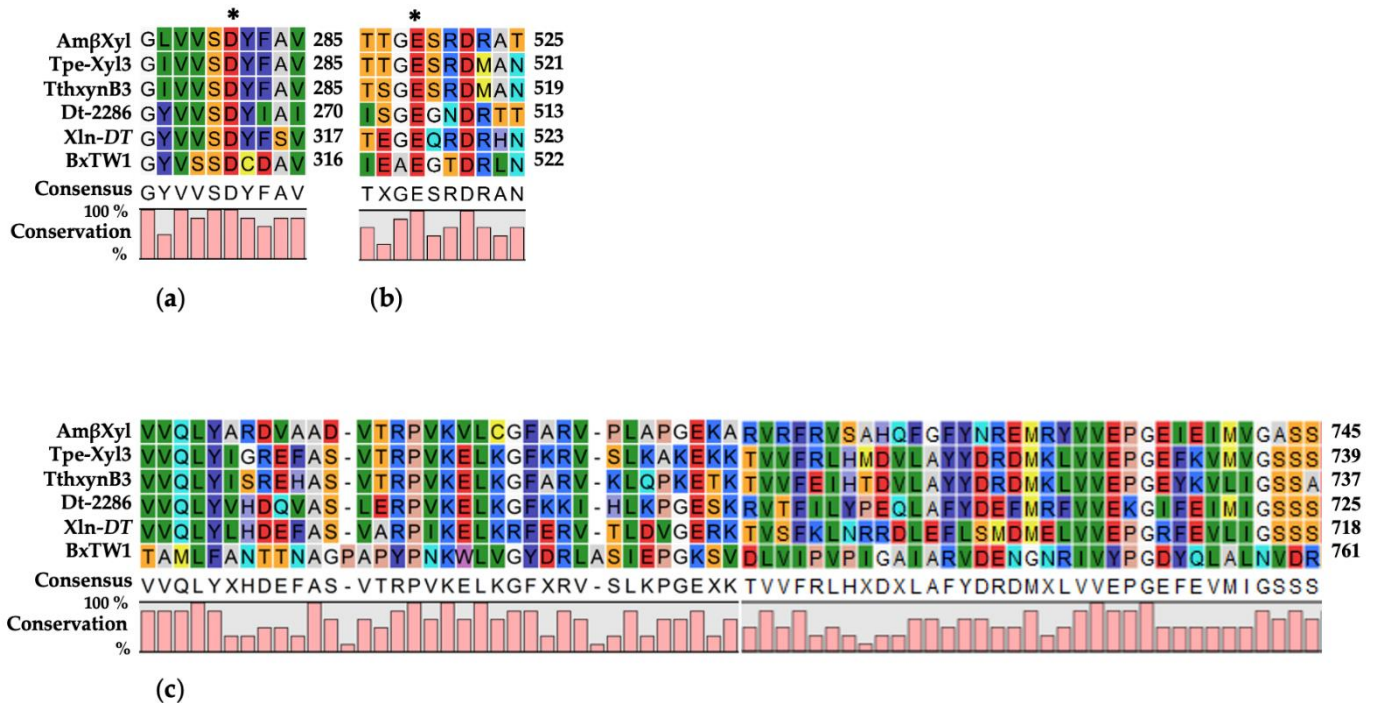


Figure 1. Sequence homology of β-xylosidases from different sources. The amino-acid sequences of *A. mali* FL18, *T. petrophila*, *P. thermarum*, *D. turgidum*, *D. thermophilum*, and *T. amestolkiae* were aligned to optimize the sequence similarity. (a,b) Amino-acid stretches surrounding the catalytic nucleophile (Asp281) and the general acid/base (Glu519) residues, respectively, involved in the catalysis; these residues are marked with an asterisk. (c) Fibronectin type III-like domain. Colors code is automatically generated by CLC Main Workbench in according to the amino-acid residues.

To investigate the evolutionary relationship among thermophilic GH3 β-xylosidases, the phylogenetic tree was constructed using the CLC Main Workbench 22.0.1 tool with the neighbor-joining (NJ) method (Figure 2).

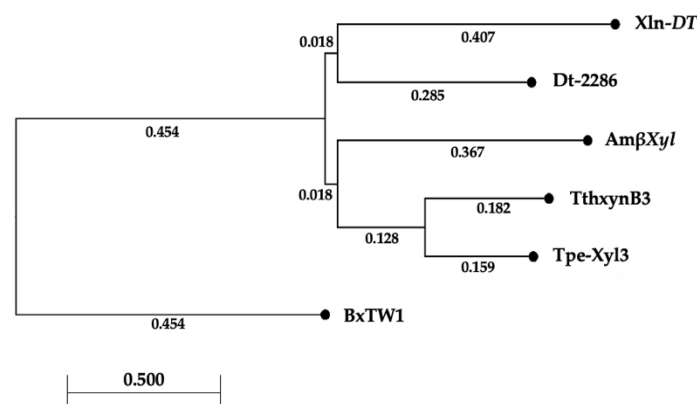


Figure 2. Phylogenetic analysis built on the basis of the multiple alignment of amino-acid sequences (numbers on nodes correspond to percentage bootstrap values for 200 replicates).

The tree showed that AmβXyl has a distant relationship from *P. thermarum* and *T. petrophila*, despite a high sequence identity, suggesting possible different enzymatic features.

2.2. Purification and Quaternary Structure of Am β Xyl

The Am β Xyl gene was synthetically produced and codon-adapted to *Escherichia coli* genetic system. The gene was cloned in pET-30a(+) vector and it was expressed in *E. coli* BL21 (DE3) RIL. The recombinant protein was purified, almost to homogeneity, by His-trap affinity chromatography and anionic exchange chromatography, with a final amount of 2 mg for 1 L of culture. SDS-PAGE analysis showed a single-band with a molecular mass of ~89 kDa, which is in line with the predicted molecular weight (Figure 3a). The purification steps were summarized in Table 1, which shows a yield and purification ratio of 12% and 26.80 times, respectively, after anionic exchange chromatography.

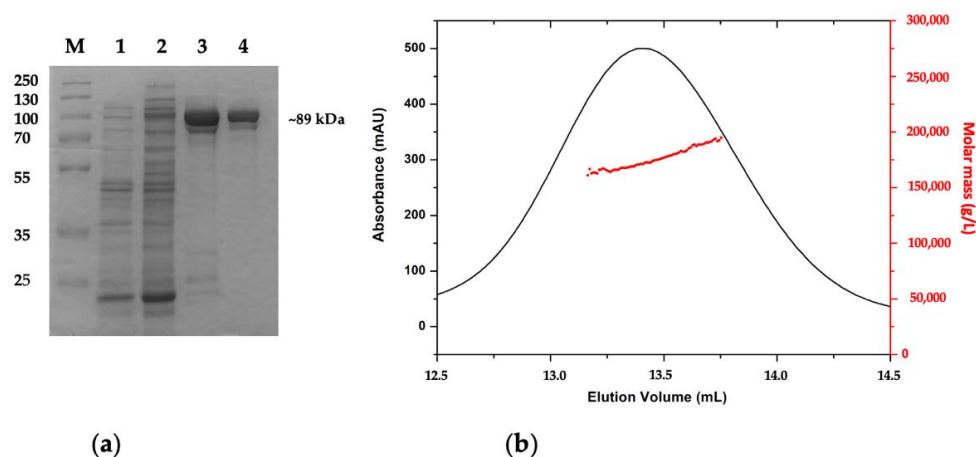


Figure 3. Analysis of the recombinant Am β Xyl expressed in *E. coli* BL21(DE)RIL/pET30a-Am β Xyl. (a) SDS-PAGE of the purification steps. M, molecular mass markers. (1) C.E. from non-induced cells; (2) C.E. from IPTG-induced cells; (3) A.C.; (4) A.E.C. (b) Analysis of purified Am β Xyl by gel filtration chromatography coupled with light scattering QELS. The elution profile of Am β Xyl is shown as a continuous line. The clustered points represent the light scattering data converted to molecular mass.

Table 1. Purification steps of Am β Xyl. Abbreviations: C.E., culture extract; A.C., affinity chromatography; A.E.C., anionic exchange chromatography.

Purification Step	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Fold Purification
C.E.	6300.00	281.29	22.39	100.00	1.00
A.C.	1470.60	3.87	380.00	23.30	16.97
A.E.C.	756.00	1.26	600.00	12.00	26.80

To gain insight into the quaternary structure, purified Am β Xyl was analyzed by size-exclusion chromatography coupled with a triple-angle light scattering QELS. The result (Figure 3b) showed a molecular weight of about 177 kDa \pm 0.1% (Rh = 5.7 nm \pm 2%), indicative of a dimeric structure of Am β Xyl in solution. Within the prokaryotes, many β -xylosidases belonging to GH3 family are characterized, but few quaternary structures are known [7]; among these, β -xylosidase from *Streptomyces* CH7 adopts a dimeric structure, while β -xylosidase from *Streptomyces thermoviolaceus* OPC-520 is monomeric [24,25].

2.3. Characterization of Am β Xyl

2.3.1. Biochemical Properties

The influence of temperature and pH to determine the optimal activity and stability of Am β Xyl was tested on PNP- β -xyl as substrate. Enzymes that work in a wide range of pH and high-temperature values are desirable in biotechnology for various applications, such as acid and alkaline pretreatment or steam explosion to generate bioethanol from plant biomass, such as wheat straw [26].

Am β Xyl was tested in the range of pH values from 3.0 to 8.0, showing an optimal pH at 5.6 and retaining over 70% activity in a range of pH from 5.0 to 6.6 and ~50% activity at pH 4.5 (Figure 4a).

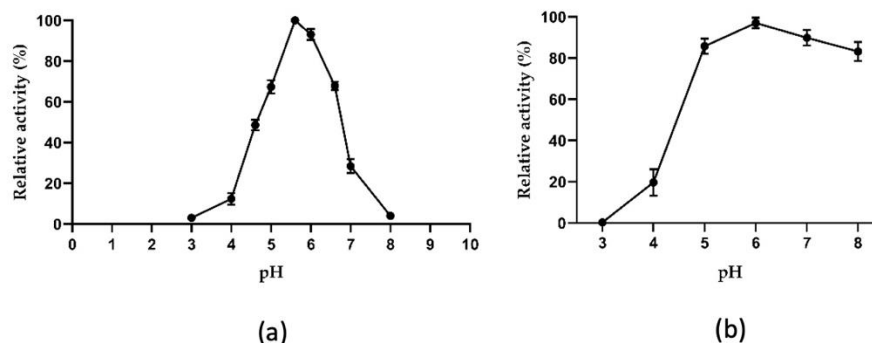


Figure 4. Effect of pH on the enzymatic activity of Am β Xyl. (a) The pH optimum was evaluated in buffers ranging from pH 3.0 to pH 8.0. (b) The pH stability was determined by incubating Am β Xyl in various buffers (from pH 3.0 to pH 8.0) for 16 h and assaying residual activity under optimal conditions. The activity of Am β Xyl was determined using PNP- β -xyl as substrate. The concentration of released pNP was detected by measuring A_{405nm}.

A similar trend in pH profile was observed also for the other thermophilic GH3 β -xylosidases, such as Dt-xyl3 from *D. turgidum*, Dt-2286 from *D. thermophilum*, Tpe-Xyl3 from *T. petrophila*, and TthxynB3 from *T. thermarum*, which exhibit optimal activity in the pH range from 5.0 to 6.0. Approximately 70% of the maximum activity was retained in the pH ranging from 5.0 to 7.0 for these enzymes [27–30]. As shown in Figure 4b, the enzyme was stable in a wide pH range after 16 h of incubation at 4 °C in the various buffers, retaining 95% activity at pH 6.0 and 80% activity in the pH range from 5.0 to 8.0 in line with β -xylosidase from the thermophilic bacterium *C. crescentus*, which has similar pH activity ranges with an optimum close to 6.0 and stability between 5.0 and 10.0 at 4 °C for 24 h [31]. This remarkable pH stability points to Am β Xyl as suitable for hydrolyzing acid or alkaline pretreated lignocellulose.

Activity and stability of catalysts at high temperatures are also properties worthy of attention in industrial processes for various reasons; high temperatures reduce the risk of microbial contamination, increase the solubility of substrates, and reduce the cost of cooling the industrial biorefinery plant, allowing the volatilization of products such as ethanol [32]. Many β -xylosidases, already characterized in the literature, isolated from both fungi and thermophilic bacteria, show a temperature optimum in the range of 50–65 °C, such as PtXyl43 from the fungus *Paecilomyces thermophila* [33] or TtGH39 from the bacterium *Thermoanaerobacterium thermosaccharolyticum* [34]. Only one β -xylosidase from *Alicyclobacillus* genus has been reported with an optimal temperature of 65 °C [35]. Am β Xyl showed a temperature optimum of 80 °C and it also retained more than 50% activity between 65 °C and 85 °C, while a decrease in activity was observed at lower temperatures and at 90 °C (Figure 5a).

Therefore, Am β Xyl is one of the most thermophilic β -xylosidases characterized so far, together with those from *T. petrophila* (90 °C), *T. thermarum* (95 °C) and *D. turgidum* (98 °C) [27,29,30].

The thermal inactivation of the enzyme was also examined; it was stable for 120 min at 40 °C and retained more than 50% activity up to 120 min at 50 °C and 60 min at 60 °C and 65 °C (Figure 5b), whereas there was no activity after 5 min at 70 °C and 80 °C. Am β Xyl was less thermostable than the homologous enzymes of *T. thermarum* and *D. turgidum*, but more thermostable compared to other characterized β -xylosidases, e.g., β -xylosidase from *Aspergillus niger* which is completely inactive after only 40 min of incubation at 65 °C [36], and β -xylosidase from *Enterobacter ludwigii*, which loses completely activity after 20 min at 55 °C [37].

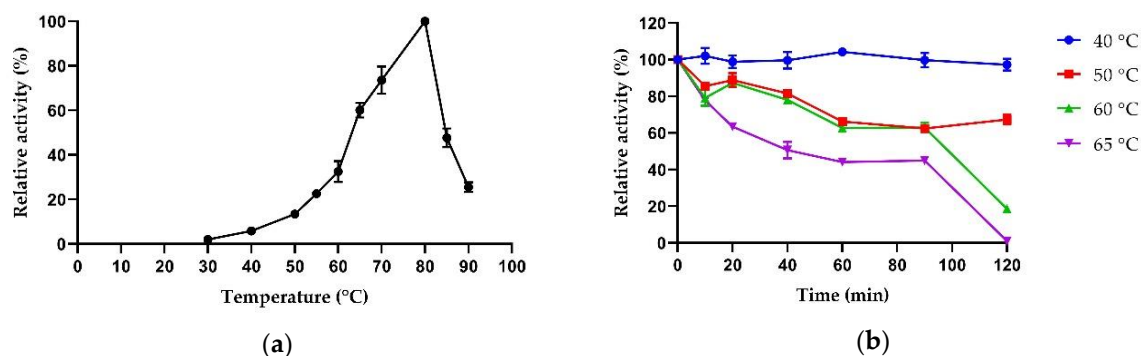


Figure 5. Effect of temperature on the enzymatic activity of Am β Xyl. (a) The optimum temperature was determined by measuring enzyme activity in the range 30–90 °C. (b) Thermostability was determined by incubating the enzyme at 40 °C, 50 °C, 60 °C, and 65 °C for different times and then assaying for residual activity under optimal conditions. The activity of Am β Xyl was determined using PNP- β -xyl as a substrate. The concentration of released pNP was detected by measuring A_{405nm} .

2.3.2. Substrate Specificity

Both β -linked and α -linked artificial glycoside substrates and natural substrates were used to analyze the specificity of Am β Xyl. The enzyme showed higher specific activity toward PNP- β -xyl than PNP- α -ara (Table 2). This bifunctional enzymatic activity, which might be correlated to the spatial similarity between D-xylopyranose and L-arabinofuranose [38], is present mainly in families 3, 43, and 53 [7], and it was found in the homologous GH3 β -xylosidases from *T. petrophila* (TpeXyl3) [30] and *T. thermarum* (TthxynB3) [29]. Enzymatic activity was measured on other synthetic substrates; slight activity was observed on PNP- α -glu (0.19%) and PNP- β -glu (0.73%), while no activity was reported toward PNP- α -gal and PNP- β -gal (Table 2) in comparison to PNP- β -xyl. Furthermore, Am β Xyl was weakly active on the natural substrates beechwood xylan and wheat arabinoxylan (14.59% and 20.86%, respectively), similar to Xyl43A and Xyl43B from *Humicola insolens* [39].

Table 2. Substrate specificity of Am β Xyl. ND: non detected.

Substrate	Relative Activity (%)
PNP- β -xyl	100
PNP- α -ara	59.30
PNP- β -glu	0.19
PNP- α -glu	0.73
PNP- β -gal	ND
PNP- α -gal	ND
Beechwood xylan	14.59
Wheat arabinoxylan	20.86

An important feature of β -xylosidases, for biotechnological application, is their ability to hydrolyze natural substrates such as XOS, since they are often found in hydrolysates of lignocellulosic material and are potent inhibitors of endo- β -1,4-xylanases and cellulases [7,40,41]. Therefore, xylobiose and xylotriose were incubated for different times with purified Am β Xyl and examined by TLC (Figure 6). A thermal effect on the migration of xylobiose (Figure 6a, lanes 1 and 3) and xylotriose (Figure 6b, lanes 2 and 4) was observed at the assay temperature; however, it did not influence XOS hydrolysis. Results showed that the enzyme hydrolyzed these short XOS into xylose (X), demonstrating its true xylosidase activity. Interestingly, the enzyme hydrolyzed X₂ in X (Figure 6a) and X₃ in X₂/X after only 1 min of incubation (Figure 6b). This capability of hydrolyzing XOS makes Am β Xyl a crucial enzyme to improve the efficiency of the saccharification process.

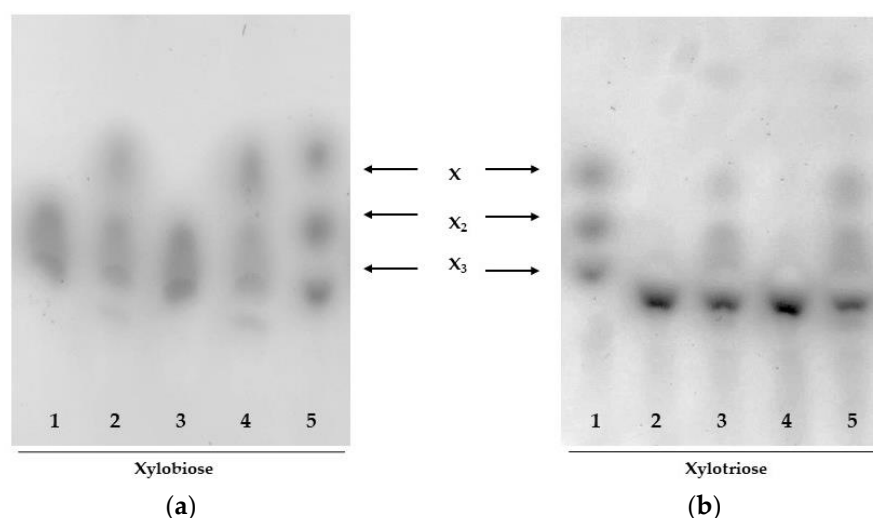


Figure 6. TLC analysis of xylobiose and xylotriose hydrolyzed by Am β Xyl. **(a)** Xylobiose hydrolysis: (1) control reaction without enzyme (1 min); (2) hydrolysis products (1 min); (3) control reaction without enzyme (30 min); (4) hydrolysis products (30 min); (5) standards XOS (X, X₂, X₃). **(b)** Xylotriose hydrolysis: (1) standards XOS (X, X₂, X₃); (2) control reaction without enzyme (1 min); (3) hydrolysis products (1 min); (4) control reaction without enzyme (30 min); (5) hydrolysis products (30 min).

2.3.3. Kinetic Parameters

The kinetic parameters of Am β Xyl were measured for both PNP- β -xyl and PNP- α -ara at optimal pH and temperature (Table 3).

Table 3. Comparison of kinetic parameters of Am β Xyl with those of some thermophilic β -xylosidases. ND: non detected.

Substrate	Enzyme	K _M (mM)	k _{cat} (s ⁻¹)	k _{cat} /K _M (mM ⁻¹ ·s ⁻¹)	Reference
PNP- β -xyl	Am β Xyl	0.52	1606.00	3088.46	This work
	TthxynB3	0.27	316.81	1173.40	[29]
	Dt-Xyl3	0.83	ND	ND	[28]
	Tpe-Xyl3	0.12	982.32	7808.00	[30]
	Dt-2286	0.12	651.59	5245.31	[27]
PNP- α -ara	Am β Xyl	10.56	2395.80	226.87	This work
	TthxynB3	0.21	106.23	505.90	[29]
	Dt-Xyl3	2.01	ND	ND	[28]
	Tpe-Xyl3	6.16	2201.64	357.89	[30]
	Dt-2286	0.50	1054.33	2077.35	[27]

Am β Xyl showed higher activity on PNP- β -xyl than PNP- β -ara with K_M, k_{cat}, and k_{cat}/K_M values of 0.52 mM, 1606.00 s⁻¹, 3088.46 mM⁻¹·s⁻¹ respectively, while kinetic parameters for Am β Xyl on PNP- α -ara were K_M = 10.56 mM, k_{cat} = 2395.80 s⁻¹, and k_{cat}/K_M = 226.87 mM⁻¹·s⁻¹.

As illustrated in Table 3, the comparison of kinetic parameters with other thermophilic GH3 β -xylosidases highlighted that Am β Xyl exhibited a lower catalytic efficiency for PNP- β -xyl (~2.5-fold) than TthxynB3 from *T. petrophila* but a higher catalytic efficiency for PNP- β -xyl (~2.5-fold) than TthxynB3 from *T. thermarum*. Regarding the enzymatic activity toward PNP- β -ara, only the catalytic efficiency of Dt-2286 from *D. turgidum* showed a 10-fold increase with respect to Am β Xyl; the other β -xylosidases reported in Table 3 showed similar catalytic efficiency.

2.3.4. Effect of Chemical Agents

Metal ions can be liberated during biomass degradation as a consequence of corrosion of the equipment used, leading to enzyme inhibition [42]. Recombinant Am β Xyl shows good tolerance to metals in accordance with the characteristics of *A. mali* FL18 strain, which is particularly tolerant to nickel, cobalt, mercury, and other metals, having been isolated from an arsenic-rich hot spring [21]. As reported in Table 4, metal ions and EDTA at the final concentrations of 1 and 5 mM did not inhibit the enzyme activity, which remained between 80% and 100%, except for Cu²⁺ at the concentration of 5 mM, which strongly inhibited Am β Xyl activity.

Table 4. Effect of metal ions and chemicals on Am β Xyl activity. ND: non detected.

Metal Ions or Chemical Agents	Relative Activity (%)		
	1 mM	5 mM	0.5%
None	100	100	100
CuCl ₂	32.10	ND	
ZnCl ₂	105.80	93.99	
LiCl	89.85	83.20	
MgCl ₂	91.64	77.81	
CaCl ₂	82.97	82.46	
MnSO ₄	78.13	59.59	
NiCl ₂	104.28	86.29	
EDTA	96.99	98.89	
SDS			1.30
Triton X-100			91.08
Tween 20			113.36

A similar effect of Cu²⁺ was observed in both enzymes Dt-2286 and Dt-Xyl3 from *D. turgidum* [27,28]. Cu²⁺ ion is known to catalyze the auto-oxidation of cysteine residues, leading to the formation of intra- and intermolecular disulfide bonds or sulfenic acid [28]. Indeed, eight cysteine residues are present in the Am β Xyl amino-acid sequence, and their oxidation state might influence the structure/function of the enzyme. Furthermore, at different concentrations of EDTA, no significant effect was observed, suggesting that this chelating agent did not affect enzymatic activity.

Regarding the effect of surfactants, Am β Xyl was only found to be very sensitive to the anionic detergent SDS (Table 4). Conversely, the nonionic detergents Tween-20 and Triton X-100 did not inhibit enzyme activity. These surfactants are often used during bioconversion of lignocellulosic biomass as they can bind to the residual lignin of biomass through hydrophobic interactions, thus preventing the adsorption of hydrolytic enzymes on lignin, and they are also able to protect enzymes from denaturing by heat [43,44].

Halotolerance is an interesting feature of xylanases and xylosidases for applications in the treatment of saline foods, such as soy sauce, which have a salt concentration between 0.5 and 2.5 M [45–47]. For this reason, Am β Xyl activity was evaluated with increasing NaCl concentrations. As shown in Figure 7, the enzyme activity gradually decreased with increasing NaCl concentrations, maintaining more than 100% activity at 0.5 M NaCl and approximately 50% activity in presence of 1 M NaCl, suggesting the possible use of Am β Xyl in saline food processing.

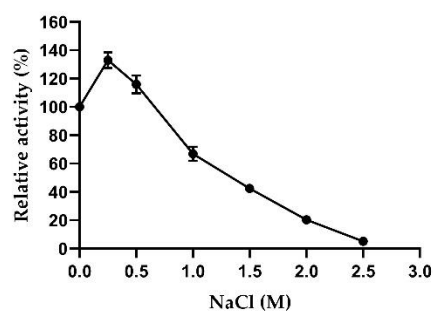


Figure 7. Effect of NaCl on Am β Xyl activity.

Lastly, organic solvents are often used during biomass saccharification to improve the solubilization of water-insoluble substrates and to overcome biomass recalcitrance to hydrolysis [48]. Therefore, the effect of DMSO, ethanol, and methanol on Am β Xyl activity at different concentrations was analyzed. As shown in Figure 8, Am β Xyl was active at high concentrations of solvents; enzymatic activity increased about fourfold in the presence of 30% DMSO, ninefold in the presence of 20% ethanol, and 12-fold in the presence of 30% methanol. Only in the presence of 60% DMSO was a slight reduction in activity compared with the control determined. This effect could be attributed to the presence of a higher percentage of hydrophobic amino acids in the thermostable enzymes compared to their mesophilic counterparts [49]. Indeed, Am β Xyl possesses 383 hydrophobic amino-acid residues (~46%) that might contribute to its high tolerance toward organic solvents. Organic solvent tolerance is not frequently reported in other thermophilic β -xylosidases; for example, TpeXyl3 from *T. petrophila* shows a slight increase in enzymatic activity in the presence of 15% of methanol while the activity is reduced by 50% in the presence of DMSO or methanol at the concentration of 30% [30].

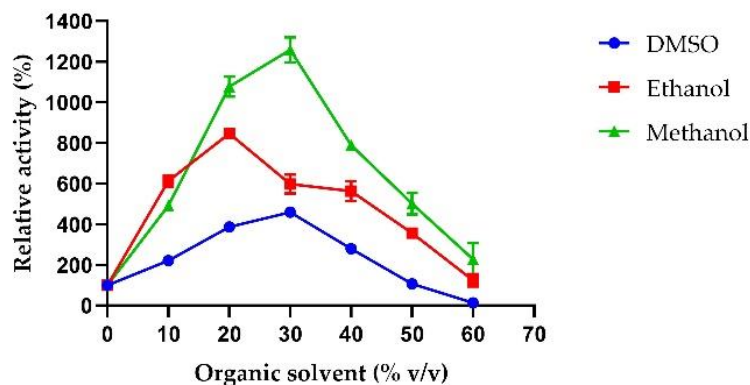


Figure 8. Effect of organic solvents on Am β Xyl activity. DMSO (circles), ethanol (squares), and methanol (triangles).

This result suggests that Am β Xyl is an enzyme that can efficiently work in the presence of an organic solvent that is required for the biotransformation of a poorly water-soluble substrate and that the tolerance of organic solvents makes Am β Xyl a suitable enzyme to be used to clarify alcoholic beverages such as wine or beer.

2.3.5. Sugar Tolerance of Am β Xyl

Xylose is a strong inhibitor of β -xylosidases; therefore, xylose-tolerant enzymes have great potential in biotechnological applications. In addition, the inhibition of enzymatic activity by glucose or arabinose of β -xylosidases is also a serious bottleneck in industrial biomass saccharification since these monosaccharides may accumulate to high concentrations reducing the enzymatic activity [7]. Hence, monosaccharide-tolerant β -xylosidases are of great interest; however, unfortunately, previous studies have underlined that most β -xylosidases are generally sensitive to the inhibition at low concentrations of these sugars.

β -Xylosidase from *Trichoderma harzianum* C completely loses activity at 2 mM xylose [50], while β -xylosidase from *H. insolens* is 50% inhibited by 29 mM xylose [51]. Among thermophilic GH3 β -xylosidases, the enzyme from *T. thermarum* loses 50% activity in the presence of 1 M xylose [29], while Dt-2286 from *D. turgidum* shows tolerance until 1 M arabinose and a strong decrease in enzymatic activity in the presence of both xylose and glucose by 0.5 M [27].

Therefore, the monosaccharide tolerance of Am β Xyl was investigated. Our results showed that Am β Xyl retained 100% of its activity in the presence of 1 M xylose and 60% in the presence of 1.5 M xylose. In addition, an increased activity of ~2.5-fold was observed in the presence of 0.25 M xylose (Figure 9). On the other hand, the presence of glucose and arabinose in a range of 0.5–1.5 M stimulated Am β Xyl activity; maximal increases in the enzymatic activity of ~1.8-fold in the presence of 0.8 M arabinose and ~2.4-fold in the presence of 0.5 M glucose were observed (Figure 9).

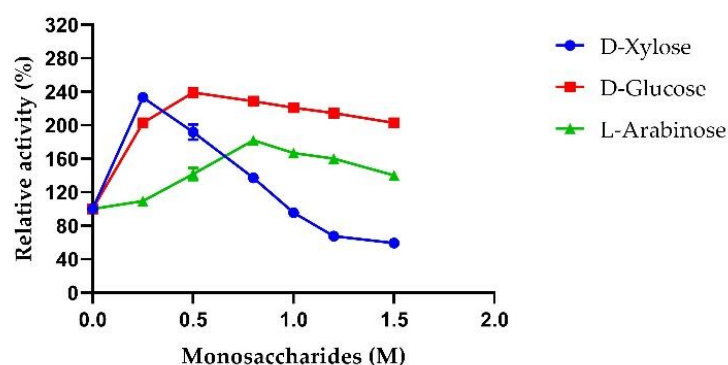


Figure 9. Effect of monosaccharides on Am β Xyl activity. D-xylose (circles), D-glucose (squares), and L-arabinose (triangles).

3. Materials and Methods

3.1. Gene Synthesis

E. coli strains were grown in solid or liquid LB medium at 37 °C containing tetracycline (15 $\mu\text{g}/\mu\text{L}$) and kanamycin (50 $\mu\text{g}/\mu\text{L}$) (strain TopF'10) or chloramphenicol (33 $\mu\text{g}/\mu\text{L}$) and kanamycin (50 $\mu\text{g}/\mu\text{L}$) (strain BL21 (DE3) RIL). Am β Xyl gene (locus tag IW967_11185) encoding the putative β -xylosidase from *A. mali* FL18 (GenBank Accession No. JADPKZ000000000) was synthesized by GenScript Biotech (Piscataway, NJ, USA), by optimizing the codon usage for the expression in *E. coli*. The optimized gene was cloned in pET-30a(+) vector (pET30a-Am β Xyl), by using *Nco*I and *Hind*III as restriction sites, with an in-frame His-tag at the N-terminus.

3.2. Sequence Analyses of Am β Xyl

The genome of *A. mali* FL18 was previously sequenced and annotated with NCBI and RAST [21]. Among several GHs retrieved in the genome, a β -xylosidase annotated as GH3 was selected. The protein sequence was analyzed by BlastP (www.Blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 15 May 2022) and CAZy (www.Cazy.org, accessed on 15 May 2022), and the possible presence of a signal sequence and N-glycosylation sites was analyzed using the tools SignalP 6.0 (<https://services.healthtech.dtu.dk/service.php?SignalP>, accessed on 15 May 2022) and NetNGlyc (<https://services.healthtech.dtu.dk/service.php?NetNGlyc-1.0>, accessed on 15 May 2022). The Pfam-(EMBL-EBI) (<http://pfam.xfam.org/search/sequence>, accessed on 15 May 2022) and I-TASSER (<http://zhanglab.cmb.med.umich.edu/I-TASSER/>, accessed on 15 May 2022) tools were used to analyze the structural model, the predicted catalytic sites, and the presence of conserved domains in Am β Xyl sequence. The amino-acid sequence was also aligned using the CLC Main Workbench 22.0.1 multiple sequence alignment tool with other GH3 β -xylosidases from *Thermotoga petrophila* (ABQ46867.1), *Pseudothromotoga thermarum* (AEH50242.1), *Dictyoglomus turgidum* (ACK42133.1), *D. thermophilum* (WP012548714.1), and *Talaromyces amestolkiae* (KP119719.1).

On the basis of the alignment, phylogenetic relationships were deduced using the neighbor-joining (NJ) method and the Jukes–Cantor protein distance measure. A total of 200 bootstrap replicates were used for evaluating the tree's topological structure.

3.3. Expression and Purification of Am β Xyl in *E. coli*

The *E. coli* BL21 (DE3) RIL strain was transformed with the vector pET30a-Am β Xyl to express the recombinant protein. The clone was grown in LB containing kanamycin 50 μ g/mL and chloramphenicol 33 μ g/mL at 37 °C (180 rpm/min in an orbital shaker) until the optical density at 600 nm was 0.5. Protein expression was induced by adding 0.5 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG) for 16 h at 37 °C. The bacterial culture (1L) was harvested by centrifugation (5000 \times *g*, 15 min, 4 °C); then, the pellet was resuspended in 30 mL of buffer A (20 mM imidazole, 100 mM NaCl, 50 mM sodium phosphate pH 7.5, and 1 mM DTT) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and underwent sonication (10 s of pulse on and 10 s of pulse off) for 10 min (Sonicator Heat System Ultrasonic, Inc., Farmingdale, NY, USA). The crude extract was centrifuged at 40,000 \times *g* for 30 min at 4 °C, to remove protein aggregates. The supernatant, containing Am β Xyl, was purified by a first step of affinity chromatography (HisTrapHP 1 mL column, GE Healthcare, Chicago, IL, USA), connected to an AKTA system and equilibrated in buffer A. The protein elution was carried out with a linear gradient of imidazole (0–500 mM), and all the fractions were pooled and dialyzed against 50 mM sodium phosphate pH 7.5 and 1 mM DTT at 4 °C for 16 h. Subsequently, the extract was further purified by anionic exchange chromatography on a HiTrap Q HP column (GE Healthcare, 5 mL). The column was equilibrated in buffer A (50 mM sodium phosphate and 1 mM DTT), and elution was performed through a linear gradient from 0 to 1 M NaCl. The fractions were dialyzed against 100 mM NaCl, 50 mM sodium phosphate pH 7.5, and 1 mM DTT, and then concentrated using an Amicon Ultrafiltration System (Millipore, Burlington, MA, USA) with a 10 kDa cutoff nitrocellulose membrane (Millipore) at room temperature and a maximum pressure of 75 MPa. The homogeneity of the protein was assessed by 12% SDS-PAGE stained with Coomassie brilliant blue R-250. Lastly, the purified protein concentration was estimated using the Bradford assay with bovine serum albumin as a standard.

3.4. Am β Xyl Biochemical Characterization

3.4.1. Quaternary Structure Determination

The native molecular weight of the purified Am β Xyl was analyzed using a size-exclusion chromatograph connected to a Mini DAWN Treos light scattering system (Wyatt Technology) equipped with a QELS (quasi-elastic light scattering) module mass value and hydrodynamic radius (*R*_h) measurements [52]. Briefly, 500 μ g of sample was loaded on an S200 column (16/60, GE Healthcare), equilibrated in 100 mM NaCl, 25 mM sodium phosphate pH 7.5, and 1 mM DTT. A constant flow rate of 0.5 mL/min was applied. Data were analyzed using Astra 5.3.4.14 software (Wyatt Technology, Santa Barbara, CA, USA).

3.4.2. β -Xylosidase Activity

The catalytic activity of the purified β -xylosidase was determined using *para*-nitrophenyl- β -D-xylopyranoside (PNP- β -xyl) as substrate. The reaction mixture containing 4 mM PNP- β -xyl, 50 mM sodium citrate (pH 5.6), and 1 mM DTT in a volume of 158 μ L was preincubated at 80 °C for 2 min before the addition of 2 μ L of purified enzyme (0.05 μ g/ μ L). The reaction was carried out for 1 min at 80 °C and stopped by adding 160 μ L of cold 0.5 M Na₂CO₃. All enzymatic reactions were carried out in triplicate into 1.5 mL tubes and then transferred to a 96-well microplate. The concentration of released *para*-nitrophenol (*p*NP) (ϵ_{mM} , 18.5 mM⁻¹·cm⁻¹) was immediately detected by measuring A_{405nm} with a microplate reader (Synergy H4 Biotek, Agilent, Santa Clara, CA, USA). One unit of β -xylosidase was defined as the amount of enzyme required to release 1 μ mol of *p*NP per min under assay conditions.

3.4.3. Substrate Specificity and Kinetic Parameters

The substrate specificity of Am β Xyl was tested firstly using the following substrates: *para*-nitrophenyl- β -D-xylopyranoside (PNP- β -xyl), *para*-nitrophenyl- α -L-arabinofuranoside (PNP- α -Ara) *para*-nitrophenyl- α -D-galactopyranoside (PNP- α -gal), *para*-nitrophenyl- β -D-galactopyranoside (PNP- β -gal), *para*-nitrophenyl- α -D-glucopyranoside (PNP- α -glu), *para*-nitrophenyl- β -D-glucopyranoside (PNP- β -glu), *para*-nitrophenyl- α -D-glucopyranoside (PNP- α -glu), beechwood xylan, and wheat arabinoxylan. All enzymatic measurements were performed in triplicate. The concentration of released *p*NP was determined by measuring $A_{405\text{nm}}$, while xylanase and arabinoxylanase activities were determined with the 3,5-dinitrosalicylic acid (DNS) method [53]. One unit of xylanase or arabinoxylanase activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar equivalent to xylose or arabinose from beechwood xylan or wheat arabinoxylan per minute under standard assay conditions.

Kinetic parameters, K_M , V_{max} , k_{cat} , and k_{cat}/K_M , were determined through the standard assay procedure utilizing PNP- β -xyl and PNP- α -ara as substrates at concentrations ranging from 0.1 to 8.0 mM using GraphPad 8.0 Prism software.

3.4.4. TLC Analysis

Xylose (X), xylobiose (X₂), and xylotriose (X₃) were purchased from Biosynth S.R.O. XOS were treated with purified Am β Xyl, and hydrolysis products were analyzed by thin-layer chromatography (TLC). The reaction mixture (50 μL) contained 10 mM XOS and 0.2U Am β Xyl in 50 mM sodium citrate (pH 5.6) and 1 mM DTT. Incubation times of 1 min, 30 min, and 2 h were tested at 80 $^{\circ}\text{C}$ and stopped by exposure to dry ice for 10 min. The reaction mixtures (12.5 μL) were loaded on silica gel 60 (F254, 0.25 mm) plates (Merck, Darmstadt, Germany) and separated using *n*-butanol/ethanol/*ddH*₂O (2:1:1 *v/v/v*) as the eluent. For the detection of sugars, the plates were soaked in a solution consisting of ethanol/sulfuric acid (90:10 *v/v*), followed by heating for few minutes at 120 $^{\circ}\text{C}$ in an oven. Mixture without enzyme was included in the analysis as control.

3.4.5. pH and Temperature Effects on Activity and Stability of the Enzyme

The optimal pH of recombinant Am β Xyl was evaluated by performing the β -xylosidase assay using PNP- β -xyl as a substrate, as reported above, in the pH range 3.0–8.0, at the temperature reported above.

To determine enzyme stability at different pH values, assays were carried out by incubating Am β Xyl in buffers ranging from pH 3.0 to 8.0 at 4 $^{\circ}\text{C}$ for 16 h. The residual β -xylosidase activity was measured under the standard conditions as previously reported.

The optimal temperature was determined by measuring the β -xylosidase activity at different temperatures ranging from 30 to 90 $^{\circ}\text{C}$. Thermal stability was determined through preincubation of the enzyme at different temperatures (40, 50, 60, 65, 70, and 80 $^{\circ}\text{C}$) in the reaction mixture for different times (10–120 min) without substrate. Aliquots of Am β Xyl were taken at regular time intervals to measure the residual activity under standard assay conditions. The activity of the enzyme assayed without preincubation was defined as 100%.

3.4.6. Effect of Chemicals and Monosaccharides on Enzyme Activity

The inhibition effect of metal ions and chelating agent EDTA on Am β Xyl activity was evaluated. The effect of different ions Cu^{2+} , Zn^{2+} , Li^{+} , Mg^{2+} , Ca^{2+} , Mn^{2+} , and Ni^{2+} , as well as the chemical agent EDTA, at final concentrations of 1 mM and 5 mM on enzymatic activity was determined.

Enzymatic activity was measured in the presence of nonionic (Tween-20 and Triton X-100) and ionic (SDS) detergents at a final concentration of 0.5% (*v/v*).

The β -xylosidase activity was also detected in the presence of different concentrations of D-xylose, D-glucose, L-arabinose (0.5–1.5 M), and NaCl (0.25–2.5 M) under standard assay conditions.

Lastly, Am β Xyl activity was tested at 60 °C and optimal pH with the addition of organic solvents (DMSO, ethanol, or methanol) at final concentrations of 10%, 20%, 30%, 40%, 50%, and 60% in the reaction mixture. The activity of the enzyme without organic solvents assayed at 60 °C was defined as 100%.

4. Conclusions

In this study, a thermophilic GH3 β -xylosidase (Am β Xyl) from the thermoacidophile bacterium *A. mali* FL18 was biochemically characterized. Am β Xyl displays a peculiar thermophilicity, thermoresistance, and stability in a wide range of pH. A common characteristic of several enzymes of the GH3 family is bifunctionality; in particular, Am β Xyl displays a good catalytic efficiency on both PNP- β -xyl and PNP- α -ara synthetic substrates. Furthermore, Am β Xyl is capable of hydrolyzing XOS, playing an important role in the saccharification process since it relieves the end-product inhibition of endoxylanase. Moreover, Am β Xyl has shown an excellent tolerance to organic solvents making it a suitable enzyme for several biotechnological applications. Interestingly, Am β Xyl is the first β -xylosidase reported to be not only resistant to end-product inhibition (i.e., xylose and arabinose) but also stimulated in the presence of high concentrations of several monosaccharides. Altogether, the data presented in this study are a good starting point both for the formulation of novel enzymatic mixtures and for the valorization of hemicellulosic materials in the context of a green economy.

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References

1. Chen, H.; Liu, J.; Chang, X.; Chen, D.; Xue, Y.; Liu, P.; Lin, H.; Han, S. A Review on the Pretreatment of Lignocellulose for High-Value Chemicals. *Fuel Process. Technol.* **2017**, *160*, 196–206. [[CrossRef](#)]
2. Ashokkumar, V.; Venkatkarthick, R.; Jayashree, S.; Chueter, S.; Dharmaraj, S.; Kumar, G.; Chen, W.H.; Ngamcharussrivichai, C. Recent Advances in Lignocellulosic Biomass for Biofuels and Value-Added Bioproducts—A Critical Review. *Bioresour. Technol.* **2022**, *344*, 126195. [[CrossRef](#)] [[PubMed](#)]
3. Ebringerová, A.; Hromádková, Z.; Heinze, T. Hemicellulose. *Adv. Polym. Sci.* **2005**, *186*, 1–67.
4. Juturu, V.; Wu, J.C. Microbial Xylanases: Engineering, Production and Industrial Applications. *Biotechnol. Adv.* **2012**, *30*, 1219–1227. [[CrossRef](#)]
5. Goldbeck, R.; Gonçalves, T.A.; Damásio, A.R.L.; Brenelli, L.B.; Wolf, L.D.; Paixão, D.A.A.; Rocha, G.J.M.; Squina, F.M. Effect of Hemicellulolytic Enzymes to Improve Sugarcane Bagasse Saccharification and Xylooligosaccharides Production. *J. Mol. Catal. B Enzym.* **2016**, *131*, 36–46. [[CrossRef](#)]
6. Bosetto, A.; Justo, P.I.; Zanardi, B.; Venzon, S.S.; Graciano, L.; dos Santos, E.L.; de Cássia Garcia Simão, R. Research Progress Concerning Fungal and Bacterial β -Xylosidases. *Appl. Biochem. Biotechnol.* **2016**, *178*, 766–795. [[CrossRef](#)]
7. Rohman, A.; Dijkstra, B.W.; Puspaningsih, N.N.T. β -Xylosidases: Structural Diversity, Catalytic Mechanism, and Inhibition by Monosaccharides. *Int. J. Mol. Sci.* **2019**, *20*, 5524. [[CrossRef](#)]
8. Aulitto, M.; Martinez-Alvarez, L.; Fiorentino, G.; Limauro, D.; Peng, X.; Contursi, P. A Comparative Analysis of *Weizmannia coagulans* Genomes Unravels the Genetic Potential for Biotechnological Applications. *Int. J. Mol. Sci.* **2022**, *23*, 3135. [[CrossRef](#)]
9. Aulitto, M.; Fusco, S.; Nickel, D.B.; Bartolucci, S.; Contursi, P.; Franzén, C.J. Seed Culture Pre-Adaptation of *Bacillus coagulans* MA-13 Improves Lactic Acid Production in Simultaneous Saccharification and Fermentation. *Biotechnol. Biofuels* **2019**, *12*, 45. [[CrossRef](#)]

10. Marcolongo, L.; la Cara, F.; del Monaco, G.; Paixão, S.M.; Alves, L.; Marques, I.P.; Ionata, E. A Novel β -Xylosidase from *Anoxybacillus* Sp. 3M towards an Improved Agro-Industrial Residues Saccharification. *Int. J. Biol. Macromol.* **2019**, *122*, 1224–1234. [[CrossRef](#)]
11. Ing, N.; Deng, K.; Chen, Y.; Aulitto, M.; Gin, J.W.; Pham, T.L.M.; Petzold, C.J.; Singer, S.W.; Bowen, B.; Sale, K.L.; et al. A Multiplexed Nanostructure-Initiator Mass Spectrometry (NIMS) Assay for Simultaneously Detecting Glycosyl Hydrolase and Lignin Modifying Enzyme Activities. *Sci. Rep.* **2021**, *11*, 11803. [[CrossRef](#)] [[PubMed](#)]
12. Saha, B.C. Hemicellulose Bioconversion. *J. Ind. Microbiol. Biotechnol.* **2003**, *30*, 279–291. [[CrossRef](#)] [[PubMed](#)]
13. Aulitto, M.; Fusco, F.A.; Fiorentino, G.; Bartolucci, S.; Contursi, P.; Limauro, D. A Thermophilic Enzymatic Cocktail for Galactomannans Degradation. *Enzym. Microb. Technol.* **2018**, *111*, 7–11. [[CrossRef](#)] [[PubMed](#)]
14. Aulitto, M.; Fusco, S.; Limauro, D.; Fiorentino, G.; Bartolucci, S.; Contursi, P. Galactomannan Degradation by Thermophilic Enzymes: A Hot Topic for Biotechnological Applications. *World J. Microbiol. Biotechnol.* **2019**, *35*, 32. [[CrossRef](#)]
15. Dalmaso, G.Z.L.; Ferreira, D.; Vermelho, A.B. Marine Extremophiles a Source of Hydrolases for Biotechnological Applications. *Mar. Drugs* **2015**, *13*, 1925–1965. [[CrossRef](#)] [[PubMed](#)]
16. Gallo, G.; Aulitto, M.; Contursi, P.; Limauro, D.; Bartolucci, S.; Fiorentino, G. Bioprospecting of Extremophilic Microorganisms to Address Environmental Pollution. *J. Vis. Exp.* **2021**, *178*, e63453. [[CrossRef](#)]
17. Aulitto, M.; Fusco, S.; Franzén, C.J.; Strazzulli, A.; Moracci, M.; Bartolucci, S.; Contursi, P. Draft Genome Sequence of *Bacillus coagulans* MA-13, a Thermophilic Lactic Acid Producer from Lignocellulose. *Microbiol. Resour. Announc.* **2019**, *8*, 13–15. [[CrossRef](#)]
18. Aulitto, M.; Tom, L.M.; Ceja-Navarro, J.A.; Simmons, B.A.; Singer, S.W. Whole-Genome Sequence of *Brevibacillus borstelensis* SDM, Isolated from a Sorghum-Adapted Microbial Community. *Microbiol. Resour. Announc.* **2020**, *9*, 15–16. [[CrossRef](#)]
19. Shao, W.; Xue, Y.; Wu, A.; Kataeva, I.; Pei, J.; Wu, H.; Wiegel, J. Characterization of a Novel β -Xylosidase, XylC, from *Thermoanaerobacterium saccharolyticum* JW/SL-YS485. *Appl. Environ. Microbiol.* **2011**, *77*, 719–726. [[CrossRef](#)]
20. Yan, Q.J.; Wang, L.; Jiang, Z.Q.; Yang, S.Q.; Zhu, H.F.; Li, L.T. A Xylose-Tolerant β -Xylosidase from *Paecilomyces thermophila*: Characterization and Its Co-Action with the Endogenous Xylanase. *Bioresour. Technol.* **2008**, *99*, 5402–5410. [[CrossRef](#)]
21. Aulitto, M.; Gallo, G.; Puopolo, R.; Mormone, A.; Limauro, D.; Contursi, P.; Piochi, M.; Bartolucci, S.; Fiorentino, G. Genomic Insight of *Alicyclobacillus mali* FL18 Isolated From an Arsenic-Rich Hot Spring. *Front. Microbiol.* **2021**, *12*, 639697. [[CrossRef](#)] [[PubMed](#)]
22. Aulitto, M.; Strazzulli, A.; Sansone, F.; Cozzolino, F.; Monti, M.; Moracci, M.; Fiorentino, G.; Limauro, D.; Bartolucci, S.; Contursi, P. Prebiotic Properties of *Bacillus coagulans* MA-13: Production of Galactoside Hydrolyzing Enzymes and Characterization of the Transglycosylation Properties of a GH42 β -Galactosidase. *Microb. Cell Fact.* **2021**, *20*, 71. [[CrossRef](#)] [[PubMed](#)]
23. Bertram, R.; Schlicht, M.; Mahr, K.; Nothaft, H.; Saier, M.H.; Titgemeyer, F. In Silico and Transcriptional Analysis of Carbohydrate Uptake Systems of *Streptomyces coelicolor* A3(2). *J. Bacteriol.* **2004**, *186*, 1362–1373. [[CrossRef](#)]
24. Tsujibo, H.; Takada, C.; Tsuji, A.; Kosaka, M.; Miyamoto, K.; Inamori, Y. Cloning, Sequencing, and Expression of the Gene Encoding an Intracellular β -D-Xylosidase from *Streptomyces thermoviolaceus* OPC-520. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 1824–1831. [[CrossRef](#)]
25. Pinphanichakarn, P.; Tangsakul, T.; Thongnumwon, T.; Talawanich, Y.; Thamchaipenet, A. Purification and Characterization of β -Xylosidase from *Streptomyces* Sp. CH7 and Its Gene Sequence Analysis. *World J. Microbiol. Biotechnol.* **2004**, *20*, 727–733. [[CrossRef](#)]
26. Eisenhuber, K.; Krennhuber, K.; Steinmüller, V.; Jäger, A. Comparison of Different Pre-Treatment Methods for Separating Hemicellulose from Straw during Lignocellulose Bioethanol Production. *Energy Procedia* **2013**, *40*, 172–181. [[CrossRef](#)]
27. Tong, X.; Qi, Z.; Zheng, D.; Pei, J.; Li, Q.; Zhao, L. High-Level Expression of a Novel Multifunctional GH3 Family β -Xylosidase/ α -Arabinosidase/ β -Glucosidase from *Dictyoglomus turgidum* in *Escherichia Coli*. *Bioorg. Chem.* **2021**, *111*, 104906. [[CrossRef](#)]
28. Li, Q.; Jiang, Y.; Tong, X.; Pei, J.; Xiao, W.; Wang, Z.; Zhao, L. Cloning and Characterization of the β -Xylosidase from *Dictyoglomus turgidum* for High Efficient Biotransformation of 10-Deacetyl-7-Xylosltaxol. *Bioorg. Chem.* **2020**, *94*, 103357. [[CrossRef](#)]
29. Shi, H.; Li, X.; Gu, H.; Zhang, Y.; Huang, Y.; Wang, L.; Wang, F. Biochemical Properties of a Novel Thermostable and Highly Xylose-Tolerant β -Xylosidase/ α -Arabinosidase from *Thermotoga thermarum*. *Biotechnol. Biofuels* **2013**, *6*, 27. [[CrossRef](#)]
30. Zhang, S.; Xie, J.; Zhao, L.; Pei, J.; Su, E.; Xiao, W.; Wang, Z. Cloning, Overexpression and Characterization of a Thermostable β -Xylosidase from *Thermotoga petrophila* and Cooperated Transformation of Ginsenoside Extract to Ginsenoside 20(S)-Rg3 with a β -Glucosidase. *Bioorg. Chem.* **2019**, *85*, 159–167. [[CrossRef](#)]
31. Graciano, L.; Corrêa, J.M.; Gandra, R.F.; Seixas, F.A.V.; Kadowaki, M.K.; Sampaio, S.C.; da Conceição Silva, J.L.; Osaku, C.A.; de Simão, R.C.G. The Cloning, Expression, Purification, Characterization and Modeled Structure of *Caulobacter crescentus* β -Xylosidase I. *World J. Microbiol. Biotechnol.* **2012**, *28*, 2879–2888. [[CrossRef](#)] [[PubMed](#)]
32. Fusco, F.A.; Fiorentino, G.; Pedone, E.; Contursi, P.; Bartolucci, S.; Limauro, D. Biochemical Characterization of a Novel Thermostable β -Glucosidase from *Dictyoglomus turgidum*. *Int. J. Biol. Macromol.* **2018**, *113*, 783–791. [[CrossRef](#)] [[PubMed](#)]
33. Teng, C.; Jia, H.; Yan, Q.; Zhou, P.; Jiang, Z. High-Level Expression of Extracellular Secretion of a β -Xylosidase Gene from *Paecilomyces thermophila* in *Escherichia Coli*. *Bioresour. Technol.* **2011**, *102*, 1822–1830. [[CrossRef](#)] [[PubMed](#)]
34. Shin, K.C.; Seo, M.J.; Oh, D.K. Characterization of β -Xylosidase from *Thermoanaerobacterium thermosaccharolyticum* and Its Application to the Production of Ginsenosides Rg1 and Rh1 from Notoginsenosides R1 and R2. *Biotechnol. Lett.* **2014**, *36*, 2275–2281. [[CrossRef](#)] [[PubMed](#)]

35. Zhang, S.; Wang, H.; Shi, P.; Xu, B.; Bai, Y.; Luo, H.; Yao, B. Cloning, Expression, and Characterization of a Thermostable β -Xylosidase from *Thermoacidophilic alicyclobacillus* Sp. A4. *Process. Biochem.* **2014**, *49*, 1422–1428. [[CrossRef](#)]
36. la Grange, D.C.; Pretorius, I.S.; Claeysens, M.; van Zyl, W.H. Degradation of Xylan to D-Xylose by Recombinant *Saccharomyces cerevisiae* Coexpressing the *Aspergillus niger* β -Xylosidase (XlnD) and the *Trichoderma Reesei* Xylanase II (Xyn2) Genes. *Appl. Environ. Microbiol.* **2001**, *67*, 5512–5519. [[CrossRef](#)] [[PubMed](#)]
37. Zhang, J.; Cui, T.; Li, X. Screening and Identification of an *Enterobacter ludwigii* Strain Expressing an Active β -Xylosidase. *Ann. Microbiol.* **2018**, *68*, 261–271. [[CrossRef](#)]
38. Sharma, M.; Soni, R.; Nazir, A.; Oberoi, H.S.; Chadha, B.S. Evaluation of Glycosyl Hydrolases in the Secretome of *Aspergillus fumigatus* and Saccharification of Alkali-Treated Rice Straw. *Appl. Biochem. Biotechnol.* **2011**, *163*, 577–591. [[CrossRef](#)]
39. Yang, X.; Shi, P.; Huang, H.; Luo, H.; Wang, Y.; Zhang, W.; Yao, B. Two Xylose-Tolerant GH43 Bifunctional β -Xylosidase/ α -Arabinosidases and One GH11 Xylanase from *Humicola insolens* and Their Synergy in the Degradation of Xylan. *Food Chem.* **2014**, *148*, 381–387. [[CrossRef](#)]
40. Fujii, T.; Yu, G.; Matsushika, A.; Kurita, A.; Yano, S.; Murakami, K.; Sawayama, S. Ethanol Production from Xylo-Oligosaccharides by Xylose-Fermenting *Saccharomyces cerevisiae* Expressing β -Xylosidase. *Biosci. Biotechnol. Biochem.* **2011**, *75*, 1140–1146. [[CrossRef](#)]
41. Kont, R.; Kurašin, M.; Teugjas, H.; Våljamäe, P. Strong Cellulase Inhibitors from the Hydrothermal Pretreatment of Wheat Straw. *Biotechnol. Biofuels* **2013**, *6*, 135. [[CrossRef](#)] [[PubMed](#)]
42. Turner, P.; Mamo, G.; Karlsson, E.N. Potential and Utilization of Thermophiles and Thermostable Enzymes in Biorefining. *Microb. Cell Fact.* **2007**, *6*, 9. [[CrossRef](#)]
43. Chen, Y.A.; Zhou, Y.; Liu, D.; Zhao, X.; Qin, Y. Evaluation of the Action of Tween 20 Non-Ionic Surfactant during Enzymatic Hydrolysis of Lignocellulose: Pretreatment, Hydrolysis Conditions and Lignin Structure. *Bioresour. Technol.* **2018**, *269*, 329–338. [[CrossRef](#)] [[PubMed](#)]
44. Zhou, Y.; Chen, H.; Qi, F.; Zhao, X.; Liu, D. Non-Ionic Surfactants Do Not Consistently Improve the Enzymatic Hydrolysis of Pure Cellulose. *Bioresour. Technol.* **2015**, *182*, 136–143. [[CrossRef](#)] [[PubMed](#)]
45. Li, N.; Han, X.; Xu, S.; Li, C.; Wei, X.; Liu, Y.; Zhang, R.; Tang, X.; Zhou, J.; Huang, Z. Glycoside Hydrolase Family 39 β -Xylosidase of *Sphingomonas* Showing Salt/Ethanol/Trypsin Tolerance, Low-PH/Low-Temperature Activity, and Transxylosylation Activity. *J. Agric. Food Chem.* **2018**, *66*, 9465–9472. [[CrossRef](#)] [[PubMed](#)]
46. Xu, B.; Dai, L.; Li, J.; Deng, M.; Miao, H.; Zhou, J.; Mu, Y.; Wu, Q.; Tang, X.; Yang, Y.; et al. Molecular and Biochemical Characterization of a Novel Xylanase from *Massilia* Sp. RBM26 Isolated from the Feces of *Rhinopithecus Bieti*. *J. Microbiol. Biotechnol.* **2015**, *26*, 9–19. [[CrossRef](#)]
47. Yegin, S. Single-Step Purification and Characterization of an Extreme Halophilic, Ethanol Tolerant and Acidophilic Xylanase from *Aureobasidium pullulans* NRRL Y-2311-1 with Application Potential in the Food Industry. *Food Chem.* **2017**, *221*, 67–75. [[CrossRef](#)]
48. Kucharska, K.; Rybarczyk, P.; Hołowacz, I.; Łukajtis, R.; Glinka, M.; Kamiński, M. Pretreatment of Lignocellulosic Materials as Substrates for Fermentation Processes. *Molecules* **2018**, *23*, 2937. [[CrossRef](#)]
49. Jain, I.; Kumar, V.; Satyanarayana, T. Applicability of Recombinant β -Xylosidase from the Extremely Thermophilic Bacterium *Geobacillus thermodenitrificans* in Synthesizing Alkylxylosides. *Bioresour. Technol.* **2014**, *170*, 462–469. [[CrossRef](#)]
50. de Ximenes, F.A.; de Paula Silveira, F.Q.; Filho, E.X.F. Production of β -Xylosidase Activity by *Trichoderma Harzianum* Strains. *Curr. Microbiol.* **1996**, *33*, 71–77. [[CrossRef](#)]
51. Xia, W.; Shi, P.; Xu, X.; Qian, L.; Cui, Y.; Xia, M.; Yao, B. High Level Expression of a Novel Family 3 Neutral β -Xylosidase from *Humicola insolens* Y1 with High Tolerance to D-Xylose. *PLoS ONE* **2015**, *10*, e0117578. [[CrossRef](#)] [[PubMed](#)]
52. Aulitto, M.; Fusco, S.; Fiorentino, G.; Limauro, D.; Pedone, E.; Bartolucci, S.; Contursi, P. *Thermus thermophilus* as Source of Thermostable Enzymes for Biotechnological Applications: Homologous Expression and Biochemical Characterization of an α -Galactosidase. *Microb. Cell Fact.* **2017**, *16*, 28. [[CrossRef](#)] [[PubMed](#)]
53. Miller, G.L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.* **1959**, *31*, 426–428. [[CrossRef](#)]