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**AN INSIGHT INTO THE ECO-FRIENDLY
VALORIZATION OF LIGNOCELLULOSIC
BIOMASS**

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VALORIZATION OF LIGNOCELLULOSIC
BIOMASS**

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*“Climbing the same mountain you're not getting higher you're
running after yourself, and you can't let go.*

*Hiding in that place you don't wanna be you push happiness so
far away, but it comes back to give you all that you've given
before to love you the way that you do, like a mirror.*

*Look in the air 'n catch that boomerang can't fall
anywhere else but in your own hand.”*

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Abstract

The transition from fossil-based economies to a circular bioeconomy is necessary to mitigate climate change by adopting more sustainable strategies. Replacing fossil resources and reducing greenhouse gas emissions is possible by biorefining renewable lignocellulosic biomass into biofuels and high-value products. In this context, thermophilic microorganisms have evolved to colonize extreme environments on Earth, and their thermozymes are regarded as powerful and eco-friendly tools in biorefinery to promote biomass valorization. They help maintain high operating temperatures that are associated with higher yields of lignocellulose transformation. Therefore, the research of new thermophilic enzymes that act on the carbohydrate matrix of biomass is needed to make the enzymatic process cost-competitive, reduce the enzyme load dosage, improve substrate and product solubility, and minimize the risk of microbial contaminations.

The first section of my doctoral project focuses on the characterization of two novel hemicellulolytic enzymes from the thermo-acidophilic bacterium *Alicyclobacillus mali* FL18, isolated from a hot spring of Pisciarelli, near Naples, Italy. The genome of *A. mali* FL18 was sequenced and annotated using the NCBI and RAST database [1]. Among several glycosyl hydrolases (GHs) retrieved from the genome, we selected a β -xylosidase (*Am* β Xyl) annotated as GH3 and an endo- β -1,4-xylanase (*Am*Xyn) annotated as GH10. The synthetic genes were cloned into pET-30 (a+) expression vectors and the two enzymes were produced recombinantly in *Escherichia coli* BL21 (DE3) RIL strain and subsequently biochemically characterized.

Am β Xyl showed a dimeric structure and an optimal catalytic activity at 80 °C and pH 5.6. It exhibited not only an excellent tolerance to organic solvents DMSO, ethanol, and methanol and monosaccharides xylose, arabinose, and glucose, but was also activated by 0.75 M xylose and up to 1.5 M by both arabinose and glucose. With the aim of developing a new enzymatic cocktail acting on hemicellulose, the recombinant *Am*Xyn was also biochemically characterized. Molecular exclusion chromatography coupled with light scattering showed a monomeric structure of the protein with a molecular weight of ~ 40 kDa. The enzyme displayed peculiar biochemical properties like an optimal hydrolytic activity at 80 °C and pH 6.6, a good thermostability at 60 °C, and a high catalytic efficiency on beechwood xylan, compared to other thermophilic

xylanases. *AmXyn* hydrolyzed exclusively xylan and not cellulose, that makes it very suitable in the paper and pulp industry for bio-bleaching process. The two enzymes from the same microorganism shared optimal activity at similar pH and temperature and a good stability at 60 °C and were used to develop a new cocktail for xylan hydrolysis. They exhibited a high degree of synergy in both sequential and simultaneous assays at 60 °C. According to TLC and HPAEC-PAD analyses, the combination of *AmXyn* and *AmβXyl* resulted in the complete degradation of xylan to xylose, with a conversion yield of 22.62 %. With the aim of valorizing lignocellulosic wastes as sources of fermentable sugars, *AmXyn* and *AmβXyl* were assayed on ultrasound-treated wheat bran (WB), showing a conversion yield into xylose of 91.56 %. The results obtained demonstrated the capability of these enzymes to degrade xylan substrates into fermentable sugars, important feedstocks in food, biofuel, and biochemical production.

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Chapter 1

Introduction

1.1 Lignocellulosic residues as raw material for biorefinery

The effects of climate change depend on the enhancement of greenhouse gas emissions and the utilization of fossil fuels, which still support 84 % of energy demand [1]. Promising alternative strategies that can promote the transition toward a circular economy include the utilization of lignocellulosic biomass [2], that can be exploited in biorefineries for food, biofuel, and biochemical production [2,3]. The first-generation biorefinery that exclusively utilizes biomass, particularly food crops, to produce biofuels [4] was succeeded by the second-generation biorefinery that relies on the production of value-added chemicals from industrial and domestic lignocellulosic wastes [5].

Lignocellulose is the main component of cell wall of plants, a complex structure composed of different layers: primary cell wall, secondary cell wall and middle lamella [6] (Figure 1). The secondary cell wall is formed only in some plants, and it is located between the primary cell wall and the plasma membrane only after the cell wall has stopped growing, resulting in increased rigidity [7]. A typical lignocellulosic biomass is composed of cellulose (40-60 %), hemicellulose (20-40 %), lignin (up to 25 %), as well as minor amounts of organic and inorganic constituents [8,9]. In general, cellulose and hemicellulose constitute the chief source of fermentable sugars, while lignin is devoid of sugar.

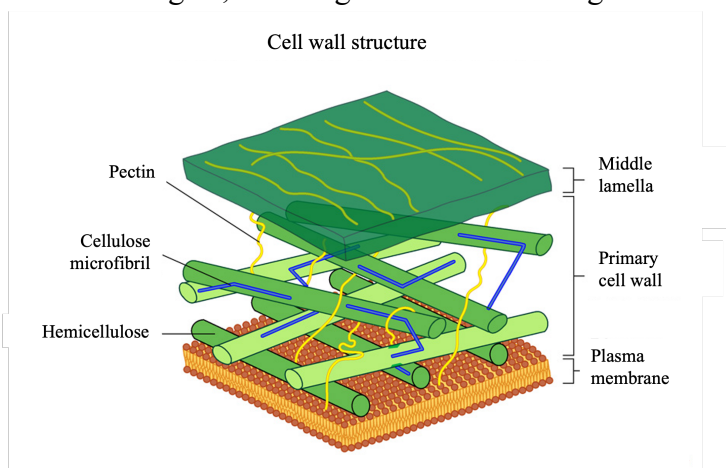


Figure 1. The structure and composition of the cell wall plants.

1.1.1 Cellulose

Cellulose, the most abundant biopolymer on Earth, is a linear polysaccharide composed of D-glucose subunits linked together by β -1,4-glycosidic bonds, which results in the formation of cellobiose, a glucose dimer (Figure 2).

Amorphous and crystalline regions are present in natural cellulose [10]. The latter are characterized by polymeric subunits bound by hydrogen bonds to form microfibrils that are insoluble in water and difficult to hydrolyze. Conversely, amorphous regions are less compact and easier to access [11]. Generally, hardwood biomass, such as mahogany, walnut, and birch, tends to have a higher cellulose content than softwood, like pine and larch [12].

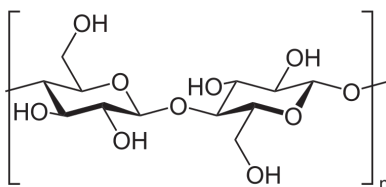


Figure 2. The smallest repeating unit, cellobiose, of the chemical structure of cellulose.

1.1.2 Hemicellulose

Hemicellulose is more structurally varied than cellulose and is more prevalent in agricultural biomass and softwood than in hardwood [12]. It consists of xylan, mannan, galactan, 4-O-methyl-glucuronoxylan, galactoglucomannan and arabinan polymers, including pentoses (C5) and hexoses (C6) sugars and organic acid substituents [9]. Among these, xylan and glucomannan are the most abundant polymers, while arabinan, mixed-linked glucan, and xyloglucan are less common [11]. They can also be covalently linked to lignin matrix to form lignin carbohydrate complexes (LCC), which increase the recalcitrance and rigidity of plant cell walls [13].

Xylan is the primary type of hemicellulose and accounts for around one-third of the total amount of renewable organic carbon in nature [14]. It consists of a main chain of β -1,4-linked xylosyl residues [15] with various substituents, such as arabinose, glucuronic acid, and acetyl [15] (Figure 3). Thus, the main sub-categories of xylan are homoxylan, arabinoxylan, glucuronoxylan, and arabinoglucuronoxylans (Figure 3).

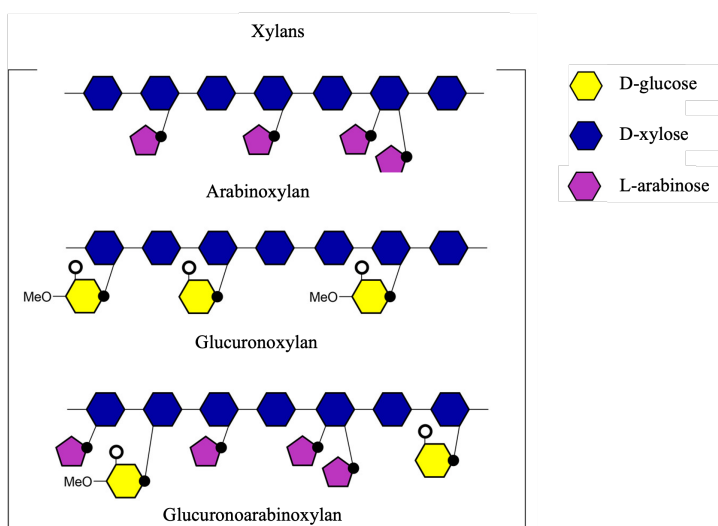


Figure 3. The main classes of xylan: arabinoxylan, glucuronoxylan, and arabinoglucuronoxylan.

1.1.3 Lignin

Lignin is the third major component of the plant cell wall. It consists of phenylpropane building blocks, including guaiacyl propanol, syringyl propanol, and *p*-hydroxyphenyl propanol, and it is also bound to side groups of hemicellulose and cellulose fibers [16–18]. Lignin content is higher in both hardwood and softwood biomass than in agricultural residues and herbaceous plants [12]. Softwood features a high percentage of guaiacyl propanol; hardwood has a shared composition of guaiacyl propanol and syringyl propanol. Herbaceous wood contains guaiacyl propanol and a smaller quantity of *p*-hydroxyphenyl propanol [2,19]. Based on the different composition and content of lignin units, the most suitable pretreatment is selected to achieve a high conversion rate into value-added products.

1.2 Bioconversion of lignocellulosic residues

Bioconversion of lignocellulosic biomass consists of three steps: i) pretreatment, ii) saccharification, and iii) fermentation [3,20].

Pretreatment is a key step in disrupting the stiff and reticulated structure of biomass to ensure saccharification and fermentation processes [21–23]. The optimal pretreatment involves delignifying biomass without altering the native lignin structure, consuming low energy, reducing cellulose crystallinity, avoiding

the production of enzyme inhibitors, and using environmentally friendly chemicals. Several techniques have been explored and can be classified into physical, chemical, and physico-chemical techniques [21,24].

Conventional chemical pretreatments are generally detrimental to the environment and the activity of value-added products. They are performed using acids like sulphuric acid, acetic acid, and phosphoric acid [25]; alkali catalysts such as potassium hydroxide, sodium hydroxide, ammonium hydroxide, and calcium hydroxide [26]; or organic solvents such as methanol, ethanol, ethylene glycol, and acetone [27]. Typically, higher temperatures ($> 200\text{ }^{\circ}\text{C}$) are employed with dilute acid (0.1 %) and lower temperatures ($< 50\text{ }^{\circ}\text{C}$) with concentrated acid (30-70 %) [28]; however, inhibitors are produced in both cases [21]. Although they increase enzyme accessibility to cellulose and hemicellulose, pretreatments with alkali and organic solvents also have several drawbacks. The first method demands longer reaction times and is ineffective on biomass with a high lignin content [29]. The latter is associated with high cost, volatility, flammability, and difficulty in the recovery [21].

Physical pretreatments include microwave irradiations which produce explosions within the treated material, resulting in swelling and fragmentation of lignocellulosic components [30]. Although these facilitate shorter enzymatic reaction times and higher end-product yields [31], they also necessitate high energy consumption and operational costs [21]. Other methods, such as ammonia fibre explosion [32], steam explosion [33], and hot water [34] have been carried out to expose carbohydrates for the downstream hydrolysis.

Ultrasound-assisted extraction (UAE) has emerged as an efficient, eco-friendly, and clean way to physically treat biomass in recent years, bypassing harsh chemical methods. Ultrasonic instruments emit mechanical-acoustic waves at frequencies ranging from 20 kHz to 500 MHz [35]. These generate radical reactions that break glycosidic bonds in the biomass [36] and cause the implosion of microbubbles at high pressure and temperature, which accelerate the reactions [35]. Ultrasonic frequencies of 20-50 kHz have been reported to increase the porosity of cellulose fibers and induce lignin separation from lignocellulosic matrix [37]. They can also be combined with new solvents, such as ionic liquids (ILs) (Figure 4) or deep eutectic solvents (DESs), to shorten the impulse time and make enzymatic attack easier. However, the recovery and recycling of these solvents have not been thoroughly studied [38].

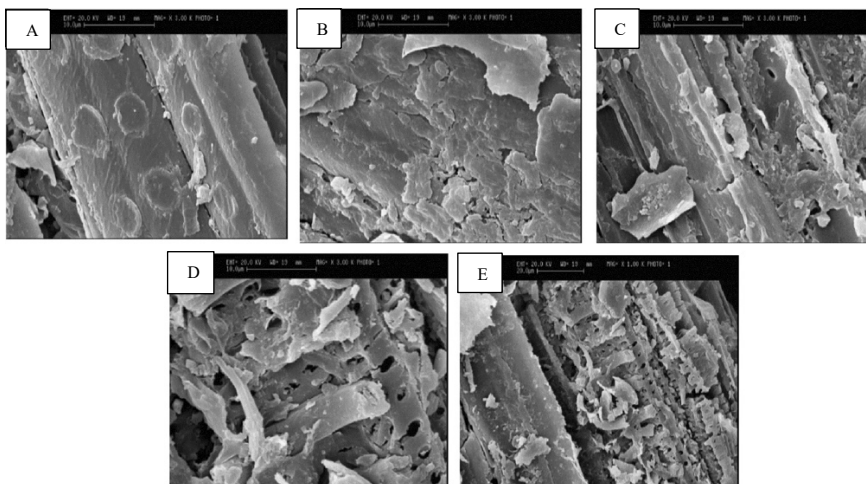


Figure 4. Scanning electron microscopy (SEM) of untreated wheat straw (A), IL-UAE pretreated at RT for 15 min (B), and 30 min (C), IL-UAE pretreated at 130 °C for 15 min (D), and 30 min (E).

After pretreatment there are four methods of bioconversion: i) SHF, in which saccharification and fermentation are performed separately; ii) SSF and SSFC, in which the two processes are carried out simultaneously (SSF refers to fermentation of hexoses only, SSFC of pentoses and hexoses); iii) CBP, in which a single organism is employed to produce the hydrolytic enzymes and perform fermentation [12].

1.2.1 Lignocellulose degrading enzymes: saccharification process

Once lignin has been removed and the cell walls have been affected by pretreatments, carbohydrates become more readily accessible for enzymatic saccharification to convert into C5 and C6 sugars [39]. The enzymes responsible for working on carbohydrates in plant biomass are known as carbohydrate-active enzymes (CAZymes). According to the CAZymes database (CAZy – www.cazy.org), they have been classified into 6 families based on amino acid sequence similarities: glycoside hydrolases (GHs), glycosyltransferases (GTs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), carbohydrate-binding modules (CBMs), and auxiliary activities (AAs) [39]. This thesis focuses on GHs, also known as glycosidases, which are currently grouped into 180 families [12] and are widely distributed in fungi, bacteria, and some archaea [40]. Notably, those from thermophilic microorganisms have gained major attention since they are active and stable at high operating temperatures [41]. GHs hydrolyze the

glycosidic bonds of two carbohydrates or a carbohydrate and another moiety. They are distinguished in exo- and endo- glycosidases depending on the position of the glycosidic bond and α - and β -glycosidases based on the stereospecificity of the substrate's anomeric carbon [42]. Lastly, the retaining or inverting action mechanisms are also used to classify them. Two amino acids are involved in these processes, one to act as a proton donor for the hydrolysis of glycosidic bonds, maintaining or reversing the anomeric atom configuration, and another to stabilize the charge [43,44].

1.2.2 Factors affecting enzymatic saccharification of lignocellulose

The conversion yield of lignocellulosic biomass into fermentable sugars can be affected by other factors besides the temperature, pH, and the choice of pretreatment method, which are essential for enzyme activity [39]. Hydrolysis time, enzyme loading, substrate concentration, and surfactants are also parameters that govern the effectiveness of enzymatic saccharification [45,46].

The minimization of hydrolysis time has been suggested to lower the overall cost of industrial processes and avoid the formation of inhibitory compounds such as acetic acid and furfural [39]. For industrial applications, higher substrate concentrations (in the range of 2.5-10 % w/v) are preferred to achieve a higher final concentration of fermentable sugars for downstream fermentation [47]. However, with high substrate loadings, the suspension is more viscous, and it becomes harder for the substrate to access the active site of enzymes [48]. Another issue related to the high substrate loading is enzymatic inhibition by end-products, which occurs after the release of sugars during saccharification [49,50]. A strategy to overcome this challenge could be to use of enzymes that are highly monosaccharide tolerant.

Moreover, low enzyme-loading rates are preferred to reduce bioconversion costs, which still pose a significant bottleneck to their practical deployment in the industrial field. The optimal enzyme loading is determined by the substrate's composition and the pretreatment method chosen [39]. Biomasses with a high lignin content may need a higher enzyme amount, as some of it can bind to the lignin surface [51,52]. In this regard, surfactants can prevent unproductive enzyme binding by attaching to lignin through hydrophobic interactions and competing with the free enzyme [53]. Therefore, the research of new enzymes tolerant to surfactants is also needed to lower the cost of many industrial processes.

1.2.3 Cellulose degrading enzymes

Cellulose degradation involves three different groups of GHs (Figure 5). The endo- β -1,4-glucanase (EC 3.2.1.74) randomly cleaves the internal β -1,4-linked glycosidic bonds and is distributed in 12 GH families (GH 5, 9, 12, 16, 17, 26, 44, 45, 48, 51, 74, 124) [44]. The cellobiohydrolase (EC 3.2.1.91), which hydrolyzes the reducing or non-reducing ends of polysaccharides, thus releasing cellobiose from chains, and is mainly distributed in GH 1, 3, 5, 6, 7, 9, 39, and 51 families [44]. Lastly, the β -glucosidase (EC 3.2.1.21) cleaves the non-reducing ends of cellobiose units into glucose and is distributed in GH 1, 2, 3, 5, 30, 39, and 116 families [44].

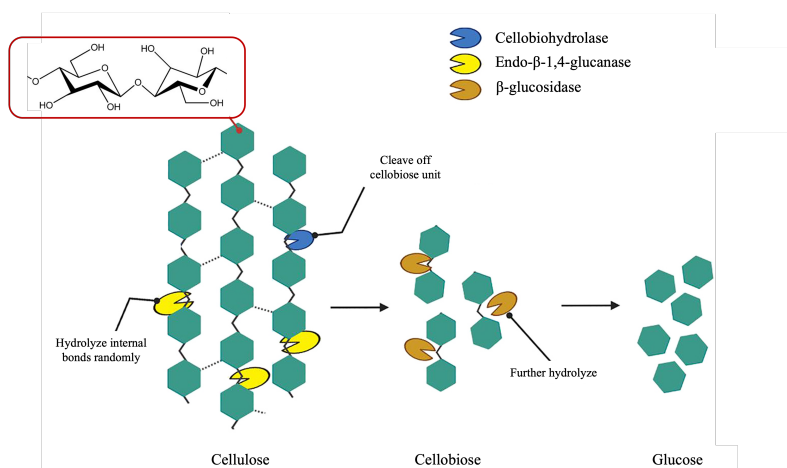


Figure 5. Schematic illustration of cellulose enzymatic degradation.

1.2.4 Hemicellulose (xylan) degrading enzymes

Hemicellulose is a complex and heterogeneous polysaccharide; thus, its degradation requires a full array of hydrolases with different substrate specificities. In this study only xylan-degrading enzymes are described, being more in line with the purpose of this thesis. Hydrolysis of xylan is mainly catalyzed by endo- β -1,4-xylanase (EC 3.2.1.8) and β -xylosidase (EC 3.2.1.37), which act to depolymerize xylan backbone into smaller xylo-oligosaccharides (XOS) and further convert them into xylose, respectively [44,54]. According to CAZy database xylanases are found in GH 5, 6, 8, 10, 11, 26, 30, 43, 51, 98 and 141 families and those mostly characterized belong to GH 10 and 11 families. β -xylosidases are found in GH 1, 2, 3, 5, 8, 10, 30, 39, 43, 51, 52, 54, 116 and 120 families. As shown in figure 7, to remove side chain substituents and decoration

and achieve the complete depolymerization of xylan, other additional enzymes are required, such as α -L-arabinofuranosidase (EC 3.2.1.55), acetyl xylan esterase (EC 3.1.1.72), α -D-glucuronidase (EC 3.2.1.139), and ferulic acid esterase (EC 3.1.1.73) [55].

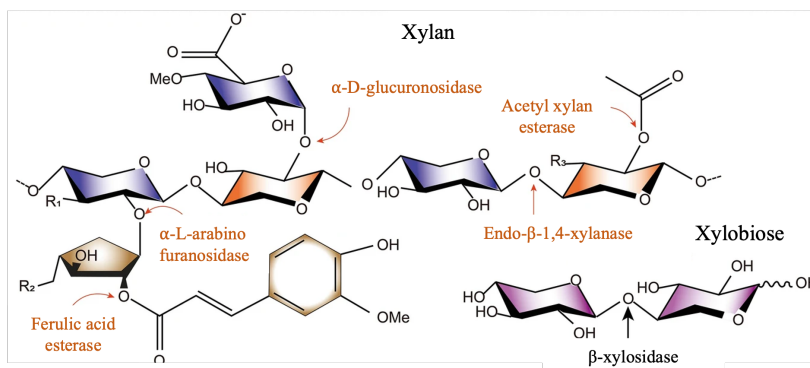


Figure 6. Hydrolases responsible for xylan degradation.

The focus of this thesis work was to examine thermophilic β -xylosidases belonging to GH 3 family and endo- β -1,4-xylanases belonging to GH 10 family in greater detail.

GH 3 is one of the largest GH families in the CAZy database, including 103 enzymes with β -xylosidase activity and various activities like β -glucosidase, β -glucosylceramidase, α -L-arabinofuranosidase, and β -N-acetylhexosaminidase [56]. Structural analyses revealed that most GH 3 members feature a single (β/α)8 TIM-barrel domain to which a second (α/β)-sandwich domain is connected via a short linker. Frequently, there is a fibronectin type III (FnIII) domain at the C-terminus with unknown function [57]. These enzymes hydrolyze the glycosidic bond by retaining reaction mechanism and display an aspartic acid residue as the nucleophile residue.

GH 10 enzymes display an (β/α)8-barrel structure and two glutamic acid residues involved in the retaining action mechanism [58]. However, they exhibit several structural variations that lead to highly flexible substrate specificities and different activities on glucan-based substrates, such as carboxymethyl cellulose (CMC) and lichenan, unlike the GH 11 family [59]. Moreover, the majority of GH10 enzymes are inefficient at cleaving short-chain XOS, unlike xylanases from other GH families [58].

1.2.5 The benefits of thermophilic GHs

Industrial biotechnology research on thermophilic and thermostable GHs has become a hot topic [60] as these enzymes can support current raw material pretreatment procedures and other conditions typical of some industrial processes such as paper and pulp bio-bleaching or animal feed and biochemical production [61,62].

Thermozymes come from thermophilic microorganisms, which grow in the temperature range of 50-80 °C, and extremophiles (above 80 °C), including archaea, bacteria, and some fungi from extreme environments such as volcanic area, hot springs, mud pots, and deep-sea hydrothermal vents [63–65]. These evolved adaptive and genomic mechanisms to thrive under harsh conditions, such as reduced genome size, shorter genes, a plasma membrane rich in saturated fatty acids that give it rigidity and strength, or thermostable proteins with a lower turnover [66–68].

Thermozymes have several benefits compared to mesophilic enzymes: i) promote the solubility of the substrate, thus accelerating the reaction rate, reducing the dosage of the enzymatic load, and improving the economic efficiency of the enzymatic process; ii) have a high tolerance and stability at high temperatures associated with a higher conversion rate of lignocellulose; iii) are more tolerant to denaturing agents, which are often used as additives during saccharification to attack the lignin of the pre-treated material and avoid unproductive enzymatic bonding; iv) lastly, reduce the risk of microbial contamination [44,54]. Therefore, they have received considerable attention for the efficient conversion of lignocellulosic residues into fermentable sugars and other industrial applications [69].

1.2.6 Discovery of new thermophilic GHs

The isolation and identification of lignocellulose-degrading microorganisms from extreme environments as well as the study of genomic DNA led to the discovery of new thermophilic GHs [70–72]. *Bacillus amyloliquefaciens*, *Pyrococcus*, *Thermotoga*, *Pseudothermotoga*, *Geobacillus*, *Alicyclobacillus*, and *Dictyoglomus* are among the microorganisms that produce GHs with high temperature activity [63,72–76]. Several enzyme formulations including xylanases, pectinases, laccases, and cellulases have been developed by Novozyme (Bagsvaerd, Denmark) or Megazyme (Bray, Ireland). To enrich the

industrial library of enzymatic cocktails, it is crucial to discover and characterize new GHs with properties of interest for the enhancement of lignocellulose.

Over the past few years, thermophilic bacteria belonging to the genus *Alicyclobacillus* have gained considerable attention as source of GHs that can withstand high temperatures, acidity as well as toxic metal compounds [72]. Several species of *Alicyclobacillus acidocaldarius*, *Alicyclobacillus vulcanalis*, *Alicyclobacillus hesperidum*, *Alicyclobacillus sendaiensis* have been isolated from hot soil or geothermal pool samples, with optimum growth temperature in the range from 40 to 60 °C [75,77–79].

In this regard, a new strain of *A. mali* FL18 was recently isolated from a hot mud pool with high level of arsenic in Pisciarelli, located within the volcanic zone of Campi Flegrei near Naples in Italy (Figure 7) [65]. It is an aerobic, Gram-positive, and thermo-acidophilic bacterium that grows at 55 °C and pH 5. Its genome comprises 48 contigs (all of them $\geq 1,000$ bp) with a total length of ~ 3 Mbp, an N50 value of 128,277 bp, and an average GC content of 61.5 % [65]. Interestingly, genomic annotation has revealed a high percentage of genes involved in processes related to amino acid and carbohydrate metabolism genomic [65]. There are 73 coding DNA sequences (CDSs) belonging to CAZymes, 32 GHs (Figure 8), 28 GTs, 11 CEs, and 2 AAs [72]. *A. mali* FL18 also possesses multiple genes that are involved in detoxifying metals and has a high tolerance to nickel, cobalt, mercury, and other metals [65].



Figure 7. The hot mud pool of Pisciarelli site

■ cellulases ■ hemicellulases ■ pectinases ■ amylases ■ hypothetical proteins

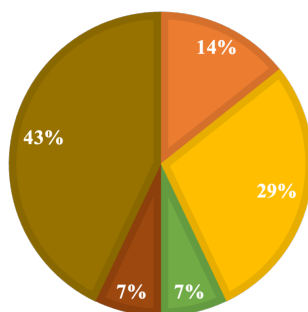


Figure 8. Percentage pie chart of *A. mali* FL18 predicted GHs

1.2.7 Value-added products from biomass saccharification

Oligosaccharides with prebiotic activity are value-added products that can be obtained from lignocellulosic residue saccharification [80]. They are a valuable alternative to classical synthetic compounds that are unstable and low yield [81]. Some examples of prebiotics are inulin, fructooligosaccharides, galactooligosaccharides, and XOS [82]. The latter have attracted the attention of food, pharmaceutical, cosmetic, and agriculture industries for their prebiotic, anticarcinogenic, antimicrobial, antioxidant, and immunomodulatory properties [83].

Lignocellulosic hydrolysates are also a promising and cheaper carbon source for microorganism fermentation, enabling the production of biofuel and other biomaterials [84]. Second-generation ethanol can be accomplished using glucose and cellobiose of byproduct hydrolysates as carbon sources for *Saccharomyces cerevisiae* yeast [85] or xylose and arabinose for *Scheffersomyces stipites* [86] or genetically modified *S. cerevisiae* [87]. Different microorganisms, including *Candida parapsilosis* and *Pichia stipitis* generate xylitol from xylose and arabinose [88]. This alcohol can find applications in food, odontological, and biomedical industries [89]. Other important biomaterials are organic acids, employed to produce pharmaceuticals, disinfectants, additives, or animal feed [90]. Among these, lactic acid, precursor of polylactic acid and lactate solvents [91,92], can be obtained from wheat straw hydrolysate by using *Bacillus coagulans* MA-13 [93] or sugarcane bagasse containing xylose by *Lactococcus lactis* IO-1 [94].

Agri-food wastes are also rich in polyphenols, which can be found both in soluble and insoluble-bound forms. Orange peels have been reported to contain 80 %

bound phenolics [95,96]. These molecules, released by enzymatic hydrolysis of the cell wall, can be converted into distinctive metabolites by the methylation, hydroxylation, carboxylation, and oxidation capabilities of microorganisms during fermentation [97]. Numerous studies have revealed that phenolic compounds like phenolic acids, anthocyanins, and flavonoids, possess antioxidant, antimicrobial, anticancer, and anti-inflammatory properties [98]. Their applications include cosmetic and cream formulations, pharmaceuticals to prevent hypertension, obesity, cardiovascular diseases, or diabetes, and food additives to prevent microbial contamination of food [99].

1.3 Aim of the work

Discovering new thermophilic enzymes that can work synergistically and tolerate certain inhibitory conditions in biorefinery is crucial in the development of new effective enzyme cocktails.

The purpose of this project focuses on the identification and biochemical characterization of carbohydrate-active enzymes from thermophilic microorganisms for the conversion of lignocellulosic biomass into fermentable sugars and bioactive compounds with beneficial effects.

The first part of this PhD thesis describes the biochemical characterization of new thermophilic xylanolytic enzymes from *A. mali* FL18 and their synergistic action in hemicellulose deconstruction (Chapter 2). The results are shown in the following manuscripts:

1. “*Alicyclobacillus mali* FL18 as a Novel Source of Glycosyl Hydrolases: Characterization of a New Thermophilic β -Xylosidase Tolerant to Monosaccharides” (<https://doi.org/10.3390/ijms232214310>).
2. “Identification and characterization of a novel endo-1,4- β -xylanase from *Alicyclobacillus mali* FL18 and its synergistic action with β -xylosidase in hemicellulose deconstruction” (manuscript submitted to International Journal of Biological Macromolecules).

The second part describes the use of commercial enzymatic cocktails to produce value-added compounds from different agri-food residues (Chapter 3). The results are reported in the manuscript in preparation:

1. “Antioxidant, antimicrobial, and antiproliferative properties of agri-food residues after enzyme-assisted extraction (EAE) using commercial enzymatic cocktails”.

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Chapter 2

Identification and biochemical characterization of new xylanolytic enzymes from the thermo-acidophilic bacterium *Alicyclobacillus mali* FL18

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

2.2 Identification and characterization of a novel endo-1,4- β -xylanase from
Alicyclobacillus mali FL18 and its synergistic action with β -xylosidase in
hemicellulose deconstruction

Summary

Saccharification is an effective alternative to chemical hydrolysis for bioconversion of lignocellulosic biomass, as it is less energy-intensive and more eco-friendly. In this context, thermozymes from thermophilic microorganisms have gained particular attention for their exceptional efficiency in breaking down lignocellulose into its constituent components. However, some challenges still need to be addressed to reduce enzyme inhibition by oligo and monosaccharides, non-sinergic enzyme mixture, or non-productive enzyme-lignin interactions in order to achieve high fermentable sugar yields and reduce the cost of the entire process. For promoting high-performing bioconversions, it is essential to utilize enzymes more tolerant and stable to organic solvents, monosaccharides, and other chemicals that can inhibit biocatalyst activity.

In the section 2.1 the manuscript “*Alicyclobacillus mali* FL18 as a Novel Source of Glycosyl Hydrolases: Characterization of a new thermophilic β -xylosidase tolerant to monosaccharides” is focused on the new β -xylosidase (*Am* β Xyl) from *Alicyclobacillus mali* FL18. The recombinant enzyme, purified to homogeneity by His-trap affinity and anionic exchange chromatography, showed a dimeric structure in solution. Its thermophilicity and thermotolerance, along with its capability of hydrolyzing xylooligosaccharides (XOS), suggested it could be useful in the saccharification process. In addition, *Am* β Xyl also exhibited the highest tolerance to organic solvents and monosaccharides discovered so far, making it very suitable for biorefinery.

In the section 2.2 the manuscript “Identification and characterization of a novel endo-1,4- β -xylanase from *Alicyclobacillus mali* FL18 and its synergistic action with β -xylosidase in hemicellulose deconstruction” is based on the biochemical characterization of a novel endo-1,4- β -xylanase (*Am*Xyn). The monomeric enzyme showed a peculiar thermophilicity and a high catalytic efficiency on beechwood xylan. It was found to be specific for xylan, with no activity on cellulose, unlike other xylanases belonging to the GH 10 family. Furthermore, synergistic action of *Am*Xyn and *Am* β Xyl was explored for the setting-up of a novel enzymatic cocktail for xylan degradation. The highest degree of synergy was obtained when the two enzymes were added sequentially on beechwood xylan. The cocktail developed for xylan was also employed for the hydrolysis of wheat bran residue, leading to a conversion yield into xylose of 91.56 %.

The results highlighted the potential application of *Am*Xyn and *Am* β Xyl as promising biocatalysts for the valorization of agricultural wastes.

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

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Keywords

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



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*

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 acetylxyylan 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].

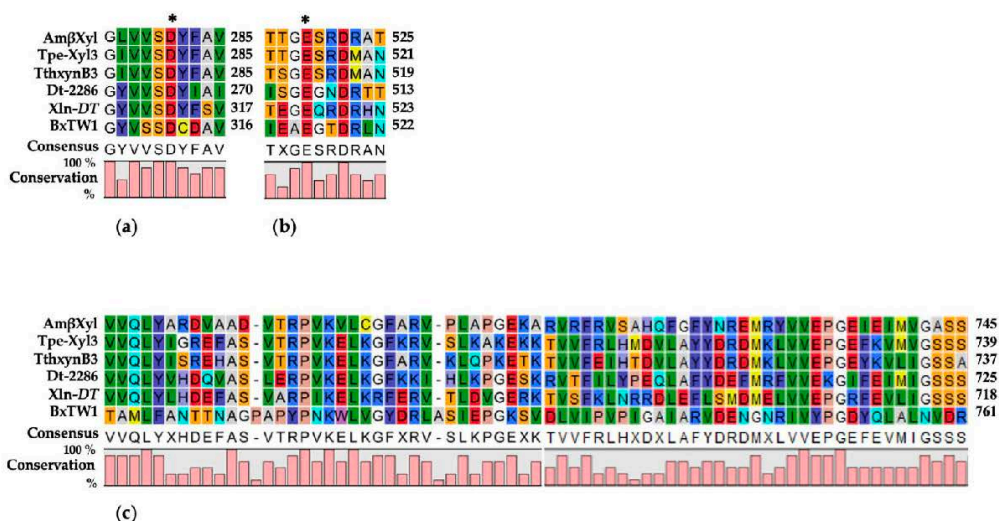


Figure 1. Sequence homology of β -xylosidases from different sources. The amino-acid sequences of *A. mali* FL18, *T. petrophila*, *P. thermarum*, *D. twigidum*, *D. thermophilum*, and *T. amestolkiae* were aligned to optimize the sequence similarity. (a,b) Amino-acid stretches surrounding the catalytic 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).

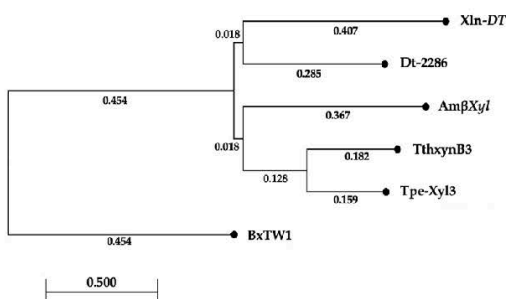


Figure 2. Phylogenetic analysis built on the basis of the multiple alignment of amino-acid sequences (numbers on nodes correspond to percentage bootstrap values for 200 replicates).

The tree showed that Am β Xyl 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.

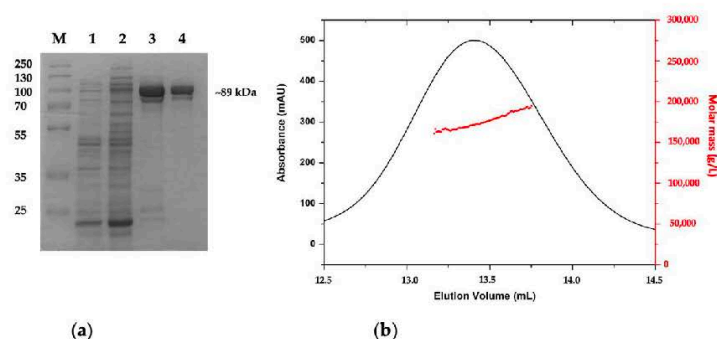


Figure 3. Analysis of the recombinant Am β Xyl expressed in *E. coli* BL21(DE)RIL/pET30a-Am β Xyl. (a) SDS-PAGE of the purification steps. M, molecular mass markers. (1) C.E. from non-induced cells; (2) C.E. from IPTG-induced cells; (3) A.C.; (4) A.E.C. (b) Analysis of purified Am β Xyl by gel filtration 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 thermotolaceus* 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).

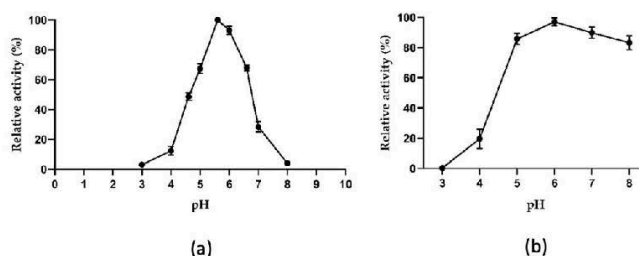


Figure 4. Effect of pH on the enzymatic activity of Am β Xyl. (a) The pH optimum was evaluated in buffers ranging from pH 3.0 to pH 8.0. (b) The pH stability was determined by incubating Am β Xyl in various buffers (from pH 3.0 to pH 8.0) for 16 h and assaying residual activity under optimal conditions.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

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].

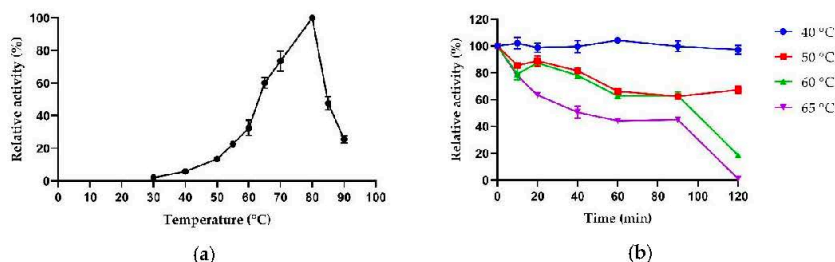


Figure 5. Effect of temperature on the enzymatic activity of AmβXyl. (a) The optimum temperature was determined by measuring enzyme activity in the range 30–90 °C. (b) Thermostability was determined by incubating the enzyme at 40 °C, 50 °C, 60 °C, and 65 °C for different times and then assaying for residual activity under optimal conditions. The activity of AmβXyl 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 AmβXyl. 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, AmβXyl 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 AmβXyl. 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 AmβXyl 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 AmβXyl a crucial enzyme to improve the efficiency of the saccharification process.

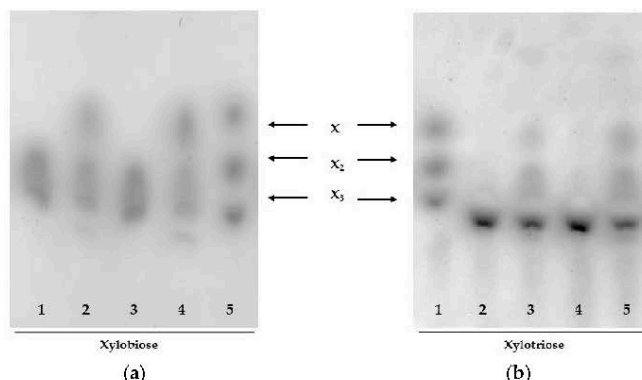


Figure 6. TLC analysis of xylobiose and xylotriose hydrolyzed by Am β Xyl. (a) Xylobiose hydrolysis: (1) control reaction without enzyme (1 min); (2) hydrolysis products (1 min); (3) control reaction without enzyme (30 min); (4) hydrolysis products (30 min); (5) standards XOS (X, X₂, X₃). (b) Xylotriose 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-Xyl13 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.

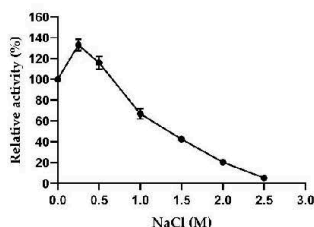


Figure 7. Effect of NaCl on Am β Xyl activity.

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].

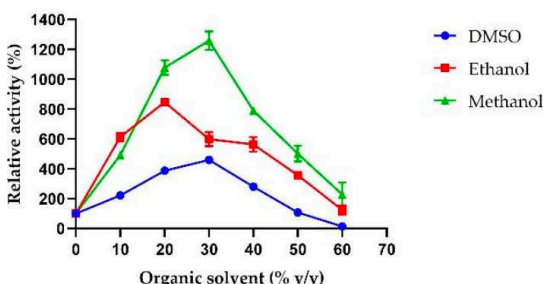


Figure 8. Effect of organic solvents on Am β Xyl activity. DMSO (circles), ethanol (squares), and methanol (triangles).

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).

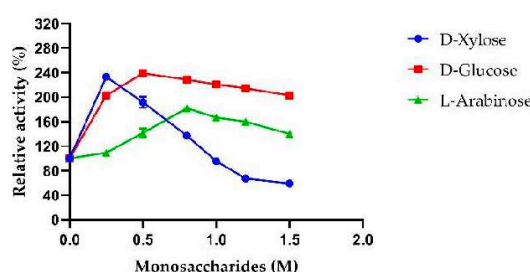


Figure 9. Effect of monosaccharides on Am β Xyl activity. D-xylose (circles), D-glucose (squares), and L-arabinose (triangles).

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 μ g/ μ L) and kanamycin (50 μ g/ μ L) (strain TopF'10) or chloramphenicol (33 μ g/ μ L) and kanamycin (50 μ g/ μ 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), *Pseudothermotoga 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 (Rh) 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 (pNP) (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 pNP 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 pNP 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 (X_2), and xylotriose (X_3) 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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2.2 Identification and characterization of a novel endo-1,4- β -xylanase from *Alicyclobacillus mali* FL18 and its synergistic action with β -xylosidase in hemicellulose deconstruction

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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 AmXyn, 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 mL mg⁻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 AmXyn and the previously characterized β -xylosidase Am β Xyl, deriving from the same microorganism, was assessed. The two enzymes had similar temperature and pH optima and showed the highest degree of synergy when AmXyn and Am β Xyl 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 AmXyn and Am β Xyl 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 nano-structured 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 *Alcalylobacillus 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 (AmXyn) 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 β Xyl) from the same microorganism was biochemically characterized, showing hydrolytic activity on xylobiose (X₂) and xylotriose (X₃) [38]. Therefore, a novel enzymatic mixture was set up by using the β -xylosidase (Am β Xyl) and the newly characterized endo-1,4- β -xylanase (AmXyn). 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, AmXyn, (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/pETAmXyn culture was grown to 0.5 OD_{600nm} in Luria-Bertani (LB) medium supplemented with kanamycin (50 μ g/mL) and chloramphenicol (33 μ g/mL) at 37 °C. AmXyn 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 $\times$ 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 $\times$ (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 μ g/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 $\times$ 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 $\times$ g for 40 min at 4 °C. The soluble fraction containing recombinant AmXyn 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 AmXyn 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 Barbara, 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 μ L, was preincubated at 80 °C for 5 min before the addition of 2 μ L of purified enzyme (0.25 μ g/ μ L). The reaction was carried out for 1 min at 80 °C and stopped on ice for 5 min. Then, 160 μ L 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 μ mol 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 AmXyn 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 (CuCl₂, ZnCl₂, LiCl, MgCl₂, CaCl₂, MnSO₄, and NiCl₂) and metal chelator EDTA on the xylanase activity of *AmXyn* 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, *AmXyn* 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 *AmXyn* 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 (pNP-β-xyl). All enzymatic measurements were performed in triplicate. The concentration of released pNP 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*)

AmβXyl from *A. mali* FL18 was expressed in recombinant strain BL21 (DE3)RIL/pET30a-*AmβXyl* 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 *AmXyn* and *AmβXyl* 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 *AmXyn* and 0.5 μg *AmβXyl* corresponding to 75 % – 25 %; b) 1.0 μg *AmXyn* and 1.0 μg *AmβXyl*, corresponding to 50 % – 50 %; c) 0.5 μg *AmXyn* and 1.5 μg *AmβXyl* 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 Y₁₊₂ indicates the μmol of reducing sugars released by enzymes when used together simultaneously or sequentially, Y₁ and Y₂ 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 *AmXyn* and *AmβXyl* were qualitatively assessed by thin layer chromatography (TLC) and high-performance

anion exchange chromatography equipped with pulsed amperometric detection (HPAEC-PAD). In detail, 75 % *AmXyn* (1.5 μg) and 25 % *AmβXyl* (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/ddH₂O (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 *AmXyn* (90 U/g biomass) and *AmβXyl* (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 *AmXyn*. 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 AmXyn 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 AmXyn was analyzed through size-exclusion chromatography coupled with a triple-angle light scattering QELS. This analysis showed a molecular weight of about 41 kDa $\pm$ 0.8 %, indicating that AmXyn 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 AmXyn 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 CbXyn10B 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 AmXyn 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.

AmXyn 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 AmXyn 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 AmXyn 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 AmXyn activity was evaluated (Table 2). The enzyme exhibited the ability to resist most of the metal ions tested, except for Zn^{2+} , 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 AmXyn activity, indicating that no metals are needed for the enzymatic reaction.

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

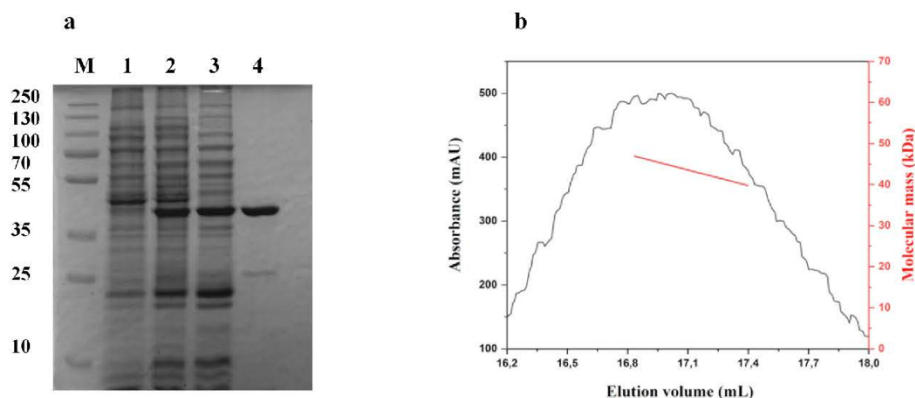


Fig. 1. Analysis of the recombinant AmXyn. a) SDS-PAGE of the purification steps. M) Protein Marker; 1) cellular extract from non-induced cells; 2) cellular extract from IPTG-induced cells; 3) crude extract of induced cells after 10 min at 60 °C; 4) AmXyn after affinity chromatography. b) Analysis of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

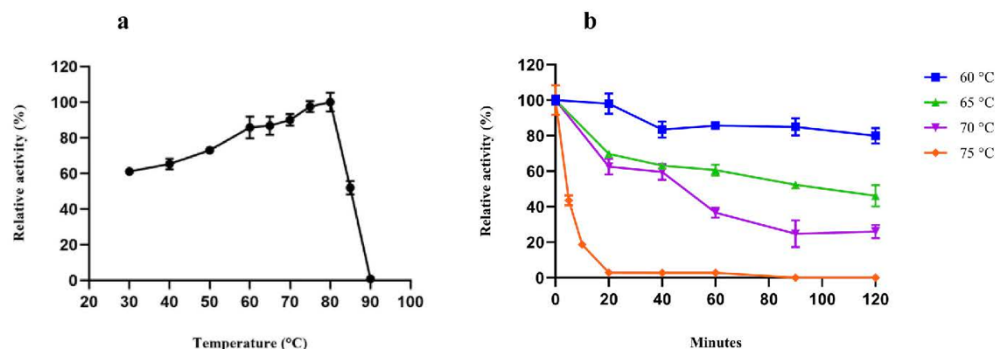


Fig. 2. Effect of temperature on enzymatic activity of *AmXyn*. a) Temperature optimum was determined by measuring enzymatic activity in the range 30–90 °C. b) Thermostability was evaluated by incubating the enzyme at 60, 65, 70, and 75 °C for different times and then assaying the residual activity under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

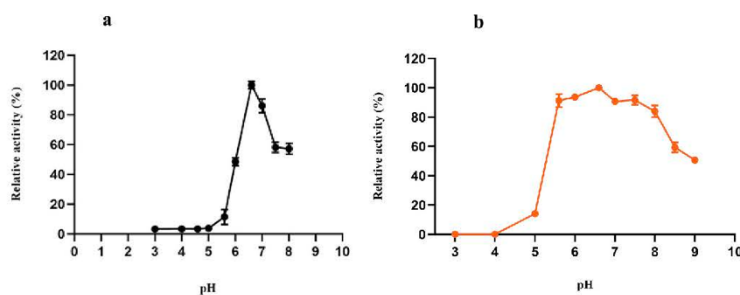


Fig. 3. Effect of pH on the enzymatic activity of *AmXyn*. a) pH optimum was evaluated in buffers ranging from pH 3.0 to pH 8.0. b) pH stability was determined by incubating *AmXyn* in various buffers (from pH 3.0 to pH 9.0) for 16 h and assaying residual activity under optimal conditions. The activity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

Table 2
Effect of metal ions and chemicals on *AmXyn* 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, *AmXyn* 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 *AmXyn* 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 micro-organism 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 *AmXyn* 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 *AmXyn* activity was also examined (Fig. 4). *AmXyn* 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].

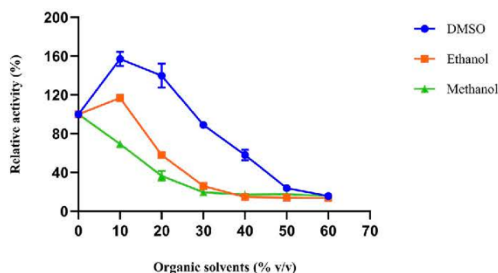


Fig. 4. Effect of organic solvents on *AmXyn* activity. DMSO (circles), ethanol (squares), and methanol (triangles).

Interestingly, *AmXyn* 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 *AmXyn* 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 (pNP- β -xyl) (10.30 ± 0.02 U/mg) (Table 3). It is worth mentioning that, in contrast with most GH10 xylanases [28], *AmXyn* 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 *AmXyn* on beechwood xylan were also compared with those of other characterized xylanases belonging to GH10 family. Interestingly, as shown in Table 4, *AmXyn* 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 *AmXyn* was corroborated by the already characterized *Am β Xyl* 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 % *AmXyn* - 25 % *Am β Xyl*; 50 % *AmXyn* - 50 % *Am β Xyl*; 25 % *AmXyn* - 75 % *Am β Xyl*), at different temperatures (60 °C and 80 °C), and reaction times

(1, 80, and 180 min). DNS assay results indicated that *Am β Xyl* 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 *AmXyn* and *Am β Xyl* (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 β Xyl* [76].

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* SFP2 which produces X_2 - X_4 but no xylose [79]. Liu et al. identified the hydrolysis products released from beechwood xylan by the xylanase rePBxA 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 *AmXyn* 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 *AmXyn* 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 *AmXyn*. 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 *AmXyn* and *Am β Xyl* 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, *AmXyn* and *Am β Xyl* were tested on physically pretreated WB. As shown in Table 5, the combined action of *AmXyn* and *Am β Xyl* 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 *AmXyn* 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 *AmXyn* are xylose and xylobiose. The latter, thanks to its prebiotic potential in increasing

Table 3
Substrate specificity of *AmXyn*. 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

Table 4
Comparison of specific activity and kinetic constants of *AmXyn* 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
<i>A. mali</i> FL18	<i>AmXyn</i>	5.29	486.11	91.89	440.00	This work
<i>A. acidocaldarius</i> ATCC27009	GH10-XA	1.81	98.20	54.30	152.00	[10]
<i>Streptomyces</i> SP7	XynST7	3.54	ND	27.98	ND	[53]
<i>Streptomyces</i> sp. B6	XynST10	10.40	149.00	14.30	84.79	[54]
<i>Thermotoga thermarum</i>	Xyn10B	1.80	520.00	289.00	192.00	[35]
<i>Caldicellulosigranor bescii</i>	CbXyn10B	1.90	378.4	199.2	497.00	[33]

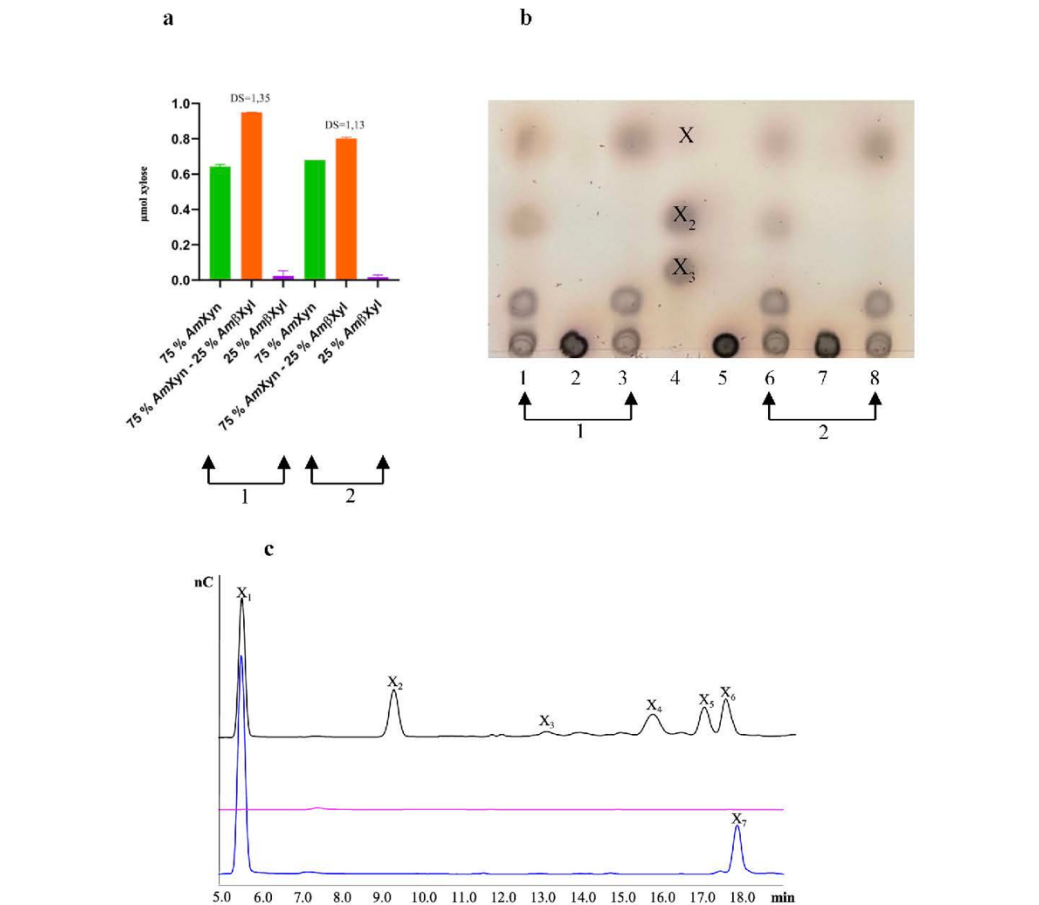


Fig. 5. Analysis of the hydrolytic activity of *AmXyn* and *AmβXyl*. a) Quantification of μmol xylose released by sequential (1) and simultaneous (2) addition of recombinant *AmXyn* and *AmβXyl* using 10 mg/mL beechwood xylan. Protein ratio was expressed as relative percentage. Xylose release was determined 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 *AmXyn* and *AmβXyl*: lane 1, hydrolysis products by *AmXyn*; lane 2, hydrolysis products by *AmβXyl*; lane 3, hydrolysis products by the combination of *AmXyn* and *AmβXyl*; lane 4, mixture of standards (X, X₂, X₃); lane 5, beechwood xylan; lane 6, hydrolysis products by *AmXyn*; lane 7, hydrolysis products by *AmβXyl*; lane 8, hydrolysis products by the combination of *AmXyn* and *AmβXyl*. c) HPAEC-PAD analysis of hydrolysis products from xylan. In black the chromatogram relative to *AmXyn* alone, in blue *AmXyn* and *AmβXyl* in sequential addition, in pink *AmβXyl* alone. Peak assignments: 1) xylose, 2) X₂, 3) X₃, 4) X₄, 5) X₅, 6) X₆, 7) X₇.

Table 5

HPAEC-PAD analysis of hydrolysis products from WB. The values represent the mean $\pm$ standard deviation of three measurements.

	UAE	UAE + AmXyn + AmβXyl
Arabinose	1.1 $\pm$ 0.05 g/L	3.48 $\pm$ 0.22 g/L
Glucose	3.62 $\pm$ 1.81 g/L	2.07 $\pm$ 1.22 g/L
Xylose	1.52 $\pm$ 0.07 g/L	20.71 $\pm$ 1.51 g/L

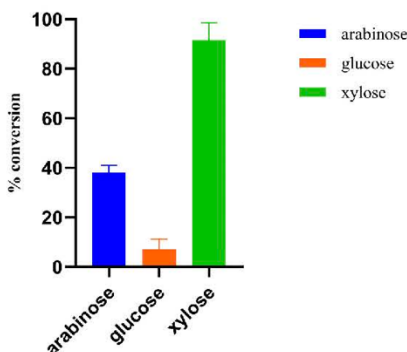


Fig. 6. Conversion percentage of arabinan, glucan, and xylan to monomers arabinose, glucose, and xylose. The values represent the mean $\pm$ standard deviation of three measurements.

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

Interestingly, AmXyn synergistically works with AmβXyl 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 AmXyn and AmβXyl 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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Chapter 3

Value-added products from agri-food wastes through enzyme technology



[REDACTED]

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Gaetano², Dominga Capasso², Emilia Pedone², Patrizia Contursi¹, Gabriella
Fiorentino¹, Danila Limauro^{1*}

[REDACTED]

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Keywords: agri-food waste, glycosyl hydrolases, lignocellulose, antioxidants,
bioactive compounds

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2.5. Assessment of antioxidant activity

SCGs, sunflower and citrus extracts

. Antioxidants react with DPPH, which is a stable free radical and converts it to α - α -diphenyl- β -picrylhydrazine. The degree of discoloration indicates the scavenging potential of the antioxidant. A 0.2 mM

A mixture of 25 of each enzymatic extract (4 225 DPPH solution was incubated at room temperature in the dark for 30 min, and the absorbance at 517 nm was measured using

The radical scavenging activity of the extracts was calculated as DPPH inhibition percentage using the following equation:

$$\% \text{ Inhibition} = [(A_0 - A_s)/A_0] \times 100$$

A_0 and A_s

$\pm$ SD

experiments each made in triplicate.

2.5.2 Free radical scavenging (ABTS) assay

The ABTS radical scavenging activity was measured

$$\% \text{ Inhibition} = [(A_0 - A_s)/A_0 \times 100]$$

A_0 and A_s

$\pm$ SD experiments each made in triplicate.

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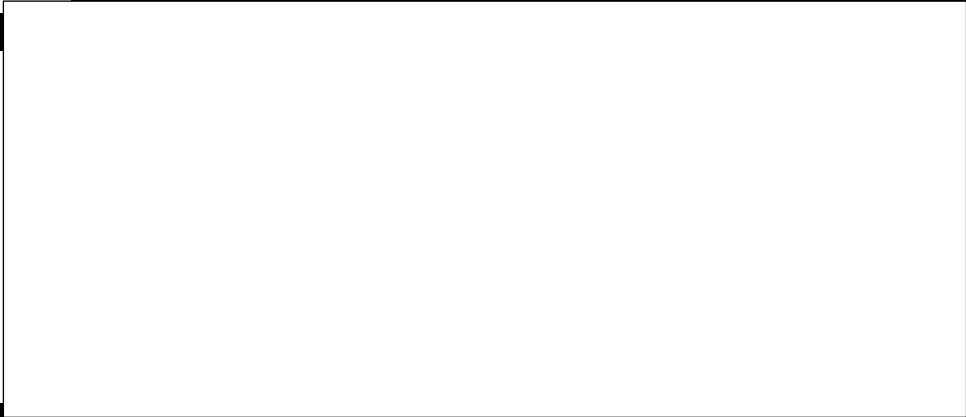
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Chapter 4

General conclusions

The shift to a bio-based society requires a greater utilization and valorization of lignocellulosic wastes as renewable biological resources to reduce dependence on fossil resources [1]. The bioconversion of biomass into valuable products, such as fermentable sugars, antioxidants, and biologically active compounds plays a key role for several sectors of the industry, including biofuel, pharmaceutical, cosmeceutical, bioplastic, and food [2]. In this context, enzymatic hydrolysis of lignocellulose is regarded as the most suitable alternative to harsh chemical treatments [3]. My PhD thesis focuses on the characterization of xylanolytic enzymes for the development of a novel cocktail for lignocellulose bioconversion, and the investigation of the potential industrial applications of various agri-food residues after EAE treatment with commercial enzyme mixtures.

In the first part of this work two putative novel glycoside hydrolases (GHs) were identified from the genome of *A. mali* FL18: *Am*βXyl and *Am*Xyn. These proteins were expressed in *E. coli*, purified, and biochemically characterized. The enzymes showed the optimal activity at the same temperature and pH, and were selected for a cocktail that would breakdown the hemicellulose backbone. Interestingly, the mixture efficiently hydrolyzed beechwood xylan to form mainly xylose, which can be used in a fermentation process to produce bioethanol, and xylobiose (X₂) which has a pronounced prebiotic activity, showing great applicability in both the food and pharmaceutical industries. The highest degree of synergy was obtained when *Am*Xyn and *Am*βXyl were added sequentially, highlighting that *Am*Xyn mainly operates during the initial phases of xylan hydrolysis releasing short XOS, that are further degraded into xylose by *Am*βXyl. During my internship at Vrije Universiteit of Brussels, in collaboration with Professors Eveline Peeters and David Cannella, the cocktail developed was also used to hydrolyze pre-treated wheat bran (WB) residue, leading to an excellent conversion of WB into xylose. This supports the application of these two enzymes as new biocatalysts in the saccharification process of agricultural wastes.



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List of communications

Salzano F., Aulitto M., Xu Y., Peeters E., Limauro D. Characterization of a novel thermophilic endo- β -1,4-xylanase from *Alicyclobacillus mali* FL18 and its heterologous expression in *Sulfolobus acidocaldarius*. **SIB congress 2023, University of Florence, Polo Didattico Morgagni, 07/09/2023-09/09/2023.**

Salzano F., Aulitto M., Fiorentino G., Contursi P., Limauro D., *Alicyclobacillus mali* FL18 as a novel source of Glycosyl Hydrolases: characterization of a new thermophilic β -xylosidase tolerant to monosaccharides. **Meeting Sezione SIB Campania 2022, University of Naples “Federico II”, 26/10/2022-26/10/2022.**

Salzano F., Aulitto M., Fiorentino G., Contursi P., Bartolucci S., Limauro D. Eco-friendly extraction of antioxidants from agri-food residues. **XVI FISV Congress, 3R: Research, Resilience, Reprise, University of Naples “Federico II”, 13/09/2022-15/09/2022.**

Salzano F., Aulitto M., Fiorentino G., Contursi P., Bartolucci S., Limauro D. *Alicyclobacillus mali* FL18 as a novel source of thermophilic glycosyl hydrolases: characterization of a new β - xylosidase. **Trends in Biotechnology: the SIB group perspectives, University of Naples Vanvitelli, 23/06/2022-24/06/2022.**

Gallo G., Aulitto M., Carbonaro M., **Salzano F.**, Limauro D., Contursi P., Fiorentino G. *Alicyclobacillus mali* FL18: a new polysaccharide waste-degrading extremophilic bacterium isolated from a hydrothermal hot- spring in Southern Italy. **FEBS 2021, The 45TH congress, Ljubjana, Slovenia, 02/07/2021-08/07/2021**

List of manuscripts

Salzano F., Aulitto M., Fiorentino G., Pedone E., Contursi P., Limauro D. *Alicyclobacillus mali* FL18 as a Novel Source of Glycosyl Hydrolases: Characterization of a New Thermophilic β -Xylosidase Tolerant to Monosaccharides. International Journal of Molecular Sciences. 2022 Nov 18. doi: <https://doi.org/10.3390/ijms232214310>.

Salzano F., Aulitto M., Fiorentino G., Cannella D., Peeters E., Limauro D. *A novel endo-1,4- β -xylanase from Alicyclobacillus mali FL18: biochemical*

characterization and its synergistic action with β -xylosidase in hemicellulose deconstruction. Manuscript submitted to International Journal of Biological Macromolecules.

Antioxidant, antimicrobial, and antiproliferative properties of agri-food residues after enzyme-assisted extraction (EAE) using commercial enzymatic cocktails. Manuscript in preparation.

Experience in foreign laboratory



Concerns

Invitation to host FLORA SALZANO
for a research stay at VUB

Date

Brussels, 30
January 2023

To whom it may concern,

I hereby formally invite Flora Salzano (PhD student at Department of Biology, University of Naples Federico II, Naples, Italy) for a research stay at the Research Group of Microbiology at the Vrije Universiteit Brussel (Brussels, Belgium) in the context of her PhD trajectory for the period 6/03/2023 – 6/09/2023.

The research project that Flora Salzano will carry out in my laboratory is entitled "Heterologous expression of xylosidase enzymes in the thermoacidophilic crenarchaeon *Sulfolobus acidocaldarius*". I commit to providing Flora with the necessary research environment and infrastructure for a successful execution of this project.

Thank you in advance for your kind attention.

Yours Sincerely,

Prof. Dr. Ir. Eveline Peeters

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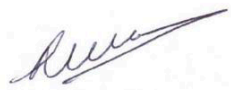
Concerns
Confirmation of research activities of
Flora Salzano at VUB

Date
Brussels, 6th September
2023

To whomever concerned,

I hereby confirm that Flora Salzano, who is enrolled as a PhD student at the University of Naples "Federico II", is currently spending a research stay in the context of a collaboration at the Vrije Universiteit Brussel and has performed research activities at VUB from 6th March 2023 until 6th September 2023. I am available in case more information is required regarding this statement.

Sincerely yours,



Prof. Dr. Ir. Eveline Peeters