Chapter

Xylanase and Its Industrial Applications

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Abstract

Lignocellulosic biomass is a renewable raw material. Industrial interest with new technology has grown to take advantage of this raw material. Different microbial enzymes are treated with biomass to produce the desired products under ideal industrial conditions. Xylanases are the key enzymes that degrade the xylosidic linkages in the xylan backbone of the biomass, and commercial enzymes are categorized into different glycoside hydrolase families. Thermophilic microorganisms are an excellent source of thermostable enzymes that can tolerate the extreme conditions of industrial processing. Thermostability of xylanases from thermophilic microorganisms has given the importance for a specific activity at elevated temperatures and distinction due to biochemical properties, structure, and mode of action. Optimized xylanases can be produced through genetic engineering: a novel xylanase is isolated from an extreme environment and then genetically modified to improve suitability for industrial contexts. Recombinant protein techniques have made it possible to engineer and express thermostable xylanases in bacteria, yeasts, and filamentous fungi. We will discuss the biotechnological potential of xylanases from thermophilic microorganism and the ways they are being optimized and expressed for industrial applications.

Keywords: xylanases, thermophilic xylanases, genetic engineering, applications

1. Introduction

Increasing energy costs and environmental concerns have pushed the global demand for sustainable renewable fuels. The impacts of plant biomass gain particular interest due to their availability as the most abundant raw material worldwide and certainly play an important role because of its significant role as a renewable source of energy [1]. Plant biomass refers to lignocellulosic biomass and its growing demand for the more effective utilization appears to evolve as one of the most important area with great industrial interest. Lignocellulose is composed of the structural polymers of cellulose, hemicellulose, pectin, and lignin. Cellulose is the most abundant polysaccharide in nature followed by hemicelluloses. Xylan is a major structural component of hemicullose and makes up 20–40% of total plant biomass [1].

Various hydrolyzing enzymes are required to degrade lignocellulosic biomass. Carbohydrate-active enzymes, the second largest group of industrial enzymes are sourced from animals, plants, and microorganisms. The hydrolytic enzymes make up nearly 75% of the total extent of industrial enzymes worldwide [2]. Efficient

degradation of plant biomass remains a major challenge that requires the availability of enzymes for the hydrolysis of lignocellulosic biomass. Biomass can be efficiently degraded by combining multiple enzymes that hydrolyze complex polysaccharides into fermentable sugars under ideal industrial conditions. These enzymes must be active during a wide range of conditions, such as high temperature and pH [3]. Xylanases are a crucial group of depolymerizing enzymes used for the hydrolysis of the xylan that is a major component of hemicellulose. There is a critical need for thermophilic xylanases operating more efficiently at higher temperatures than current commercially available ones. Xylanase producing thermophilic microorganisms are of prime importance and well-suited for industrial applications. Therefore, there is growing interest in exploring thermophilic microorganisms from extreme environment for biotechnological applications toward biomass degradation. Because of their biotechnological characteristics, xylanases are most often produced from microorganisms for commercial applications. Recently, there has been much industrial interest in xylanases for biofuel production, chemical and pharmaceutical industries, wood pulp bioleaching, papermaking, the manufacture of food and beverages, and animal nutrition. Let us look briefly at the genesis and some promising industrial uses of xylanases.

2. Structure of xylan and xylanases

Lignocellulosic biomass is a potential raw material and its growing demand for the more effective utilization appears to evolve as one of the most important technologies with great industrial interest. Hemicellulose is a complex of polymeric carbohydrates including xylan, xyloglucan (heteropolymer of D-xylose and D-glucose), glucomannan (heteropolymer of D-glucose and D-mannose), galactoglucomannan (heteropolymer of D-galactose, D-glucose and D-mannose) and arabinogalactan (hetero-polymer of D-galactose and arabinose) [4]. Xylan is a major structural polysaccharide found in the cell walls of plants, in which they may constitute more than 30% of the dry weight [5].

The structure of xylan holds a backbone of β-1,4-linked xylopyranosyl residues with an equatorial configuration and a diversity of substituted groups such as arabinose, acetyl, glucuronic acids, ferulic acid, and p-coumaric acid [6]. However, the structure of xylan is variable, ranging from linear 1,4-β-linked polyxylose sugars other than D-xylose, but the main chain of xylan is analogous to that of cellulose, composed of D-xylose instead of D-glucose. Moreover, xylan interacts with lignin and polysaccharides through covalent and non-covalent linkages, respectively. Different types of covalent linkages are responsible to interconnect xylan and lignin in the secondary cell wall [7]. These covalent interactions include glycosidic linkages between xylopyranosyl and p-coumaric acid and ester linkages between arabinofuranosyl residues and p-coumaric acid or ferulic acid. Feruloyl residues in arabinoxylans are known to be bridging units between xylan and lignin. The distribution pattern of the substituents on xylan affects a series of functional properties, including their solubility, interactions with other polymeric cell wall substances, degradability by enzymes, and solution behavior [8]. In addition, a relationship between the chemical structure of xylan results in a certain degree of complexity of xylan-containing materials, that may have several different xylan polymers of related structures but differ by more or less important features [8].

Xylanases are a crucial group of depolymerizing enzymes used for the hydrolysis of the xylan that is a major component of hemicellulose. Enzymatic depolymerization of hemicellulose to monomer sugars needs the synergistic action of multiple enzymes. These enzymes include endo-xylanase (endo-1,4-β-xylanase),

β-xylosidase (xylan-1,4-β-xylosidase), α-glucuronidase (α-glucosiduronase), α-arabinofuranosidase (α-L-arabinofuranosidase) and acetylxylan esterase. Among them endo-xylanases and β-xylosidases are the two key enzymes responsible for the hydrolysis of xylan. Endo-xylanases randomly cleaves the xylan backbone into xylo-oligosaccharides, while β-xylosidases further hydrolyze the xylo-oligosaccharides from non-reducing end into xylose monomers. The removal of side groups is catalyzed by α-D-glucuronidases, α-L-arabinofuranosidases, ferulic acid esterases, acetylxylan esterases and p-coumaric acid esterases [9, 10].

3. Classification of xylanases

Initially xylanases were classified into two groups, those with low molecular weight (less than 30 kDa) and basic isoelectric points (pI), secondly those with high molecular weight (greater than 30 kDa) and acidic pI. However, this classification system was unable to classify most of the recently discovered xylanases [11]. Afterward, another classification system was introduced that were based on the comparisons of primary structure of the catalytic domains and these enzymes were grouped into families based on related sequences. This classification system now considered the standard means for the classification of enzymes including xylanases. In addition, this classification system gave an extra edge that classifies the glycosidases in general [11]. The most extensive group of enzymes is "Glycoside hydrolases" that refers to catalyze the glycosidic bond cleavage between carbohydrates or between carbohydrate and non-carbohydrate moiety. In glycoside hydrolases (GH) families, some family protein folds are more conserved than their amino acid sequences, and these families are further grouped into clans. Presently, 14 different clans have been proposed (GH-A to GH-N), with most clans encompassing two or more than two families [11].

According to the information provided in the Carbohydrate-Active Enzymes Database (CAZy), xylanases have been classified into 13 families, however only the GH10 (formerly F) and GH11 families (formerly G) with exclusive activities for endo-β-xylanase in them. The difference between these two families based on sequence, different catalytic properties, substrate specificity, three-dimensional structure and mechanism of action [11, 12]. Besides the GH family 10 and 11, xylanases activity are also found in families of GH5, GH7, GH8, GH16, GH26, GH43, GH52 and GH62 [11, 13]. For the reason that some bifunctional enzymes are containing two catalytic domains, for example xylanases having domain of family GH10 or GH11 and it contains a domain of glycosidase as well. Among the other families, GH8 xylanases act solely on xylan whereas GH5, GH7, and GH43 xylanases also show activities as endo-glucanases, licheninases or arabino-furanosidases. Therefore, the enzymes with xylanase activity are solely not only confined to families GH10 and GH11 but also expanded to include other families like GH5, GH7, GH8, GH16, GH43, GH52, GH62 [11].

Xylanases belongs to GH families 10 and 11, which hydrolyze glycosidic bonds by acid base-assisted catalysis through a double displacement mechanism leading to retention of anomeric configuration at the cleavage site [14]. The xylanases from GH family 10 belongs to clan GH-A and the crystal structures display an $(\alpha/\beta)_8$ barrel fold or "salad bowl" shape with extended loops creating a catalytic cleft that contains at least four to seven xylose-binding subsites [15]. The catalytic site contains two glutamate residues, one acting as a nucleophile and the other as an acid/base catalyst. Catalytic amino acids and enzymatic mechanism are conserved, presenting a domain for catalysis of 250–450 amino acids. From the biochemical point of view, most of them have high molecular weight though there are reports of

low molecular weight enzymes [16]. The values of their pI are generally alkaline (8.0–9.5), however, some also have acid values and all of them sustain the same three-dimensional structure. Most of the substrate binding subsites are highly conserved in xylanases, but the affinity differences between these subsites significantly affect their mode of action, as well as substrate and product preferences [17]. As heat stability has great concern in commercial usages of xylanases. For this purpose, a number of studies analyzed the crystal structures of thermostable xylanases. Intra- and intermolecular interactions in structural topography such as disulfide bond and hydrogen bond, compact the overall fold and stabilized N and C terminal end, fusion with CBM (carbohydrate-binding motif) and lower B-factor have been proposed to bestow the enzyme for increased heat stability [18].

The xylanases from the GH11 family belongs to clan GHC. It displays exclusive substrate specificity toward xylose containing substrates and a preference for insoluble polymeric substrates. The structure of GH11 is highly homologous and contains a single major α-helix and two extended pleated β-sheets which form a jelly-roll fold [19]. The structural features include a compact globular structure and a thumb-like structure as an 11-residue long loop that connects β-strands β8 and β7, and a long cleft that spans the entire molecule and contains the active site [20]. The catalytic machinery is composed of two glutamate residues, acting as a nucleophile and an acid/base catalyst, located in the middle of the long cleft [19]. Moreover, catalytic amino acids and enzymatic mechanism of GH11 are conserved and presenting domains for catalysis of 180–200 amino acids that fold into β-sheet conformation curved on itself.

GH family	Fold	Clan	Mechanism	Nucleophile/ proton donor	Xylanase Activity	PDB No.*
GH5	(β/α)8	CH-B	Retaining	Glu/Glu	Endo-β-1,4-xylanase (EC 3.2.1.8), Arabinoxylan-specific endo-β-1,4-xylanase (EC 3.2.1.-)	2Y8K 5G56 4U3A
GH7	β-jelly roll	CH-B	Retaining	Glu/Glu	Endo-β-1,4-glucanase (EC 3.2.1.4), Endo-β-1,3–1,4-glucanase (EC 3.2.1.73)	1EG1 3OVW
GH8	(α/α)8	CH-M	Inverting	Asp/Glu	Endo-1,4-β-xylanase (EC 3.2.1.8)	1H13 1XW2
GH10	(β/α)8	CH-A	Retaining	Glu/Glu	Endo-1,4-β-xylanase (EC 3.2.1.8), Endo-1,3-β-xylanase (EC 3.2.1.32)	4QCE 1NQ6 1 W32
GH11	β-jelly roll	CH-A	Retaining	Glu/Glu	Endo-1,4-β-xylanase (EC 3.2.1.8), Endo-1,3-β-xylanase (EC 3.2.1.32)	3WP3 1YNA 1XNK
GH30	(β /α) 8	GH-A	Retaining	Glu/Glu	Endo-β-1,4-xylanase (EC 3.2.1.8), Endo-β-1,4-xylanase (EC 3.2.1.136), β-xylosidase (EC 3.2.1.37)	4FMV 4FMV
GH43	5-fold β-propeller	GH-F	Inverting	Asp/Glu	Xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37)	5GLN 2EXJ

PDB, Protein data base number.

Table 1.
Characteristics of different glycoside hydrolase family containing enzymes with a demonstrated xylanase activity.

GH 5 is the largest glycoside hydrolase family with varying activities including endo-1,4-β-xylanase. It hydrolyzes the β-1,4 xylan chain at a specific site directed by the position of an α-1,2-linked glucuronate moiety. The structural analysis XynA (of the family 5 xylanase) showed that, the catalytic domain displayed a common $(\beta/\alpha)_8$ barrel fold [21]; whereas, the β-barrels aligned well with those of another family 5 enzyme. The α-helices and loops were different, showing variances in the positioning, length and orientation. The xylanases belongs to family GH8 are classified in clan CH-M also contains endo-1,4-β-xylanase along with other glycoside hydrolase enzymes. It has also the aptitude to hydrolyze the β-1,4 xylan chain and exhibits the $(\alpha/\alpha)_6$ barrel structure formed by six inner and six outer α helices [22]. Similarly, the GH26 are the member of the clan CH-A and exhibits the (β/α)8 structure. This family contains different glycoside hydrolase enzymes including β-1,3-xylanase, capable of hydrolyzing β-1,3-xylan. Activity, mechanisms and the structure of other member of glycoside hydrolase enzymes are listed in **Table 1**.

4. Sources/genesis of xylanases

Demystifying definitional issue for common understanding, the xylanases are enzymes commonly found in microorganisms, marine algae, protozoans, snails, crustaceans, insects, seeds, plants, and other natural sources [23]. Recently, there has been much industrial interest in xylanases for wood pulp bioleaching, paper-making, the manufacture of food and beverages, animal nutrition, and bioethanol production. Because of their biotechnological characteristics, xylanases are most often produced from microorganisms for commercial applications.

Nature is replete with myriad microorganisms producing enzymatic complexes that degrade cellulose and hemicellulose releasing sugars, used for attainment of products with high economical value [24]. Microbial xylanases are of prime importance in industrial application. Most of commercial enzymes are accrued from mesophilic microorganisms. The thermostable enzymes from thermophilic micro-organisms can better meet the need of high temperatures in the industrial processes for preparing end products. Of course there is a growing interest for multiple studies in exploring the importance of enzymes producing thermophilic microorganisms in relationship with biotechnological application. The microorganisms being extremophilic in nature can survive and thrive in extreme environments on account of which thermo-stability is provided to industrial processes. Biological sources including bacteria, fungi and yeasts have been reported as xylanase producing organisms in a natural process.

4.1 Bacterial source

Xylanase producing thermophilic bacteria are found in variegated environments and the recent one was isolated in Tunisian hot springs. Various thermophilic *Bacillus* strains isolated and the identification of *Bacillus* strains was based on the phenotypic characteristics of *Bacillus* genus and phylogenetic analysis of the 16S rDNA sequence. Activity tests of these *Bacillus* strains confirmed the xylanase producing strains [25]. A thermophilic anaerobic bacteria *Caldicoprobacter algeriensis* TH7C1(T), isolated from the hydrothermal hot spring has been reported as extracellular thermostable xylanase (XYN35) producing organism [26]. The isolation of thermophilic gram positive strain Rxl, a member of genus *Thermoanaerobacterium* with xylan degrading ability was reported from hot springs in Baoshan of Yunnan Province, China. The successful cultivation of the bacterium was made through utilization of xylan, starch and wide range of monosaccharide and

polysaccharides [27]. Others like xylanase genes, was cloned from bacterial strain *Planococcus* sp., SL4, was isolated from the sediment of Soda Lake Dabusu of high alkalinity nature [28].

4.2 Fungal source

In comparison with the bacteria, the filamentous fungi have been in use as most potent industrial enzyme producers for the last five decades. Filamentous fungi are exuberant producers of xylanolytic enzymes in medium being used for the purpose. The genomes of lignocellulolytic fungi like for example *Trichoderma reesei*, *Aspergillus niger*, and *Myceliophthora thermophila* are producing diversity of enzymes that breakdown the complex cell wall components [29–31]. *M. thermophila* known as a powerful cellulolytic organism was used in new advanced technologies for industrial enzyme production, like the biomass-derived fuels. Due to its peculiarities *M. thermophila* distinguishes from rest of xylanase producers such *A. niger* and *T. reesei*. It is best source of gene encoding extracellular thermophilic xylanases. It has presence of a relatively high number of (glucurono) arabinoxylan degrading enzymes. It has lignocellulolytic enzymes that synthesize a complete set of enzymes necessary for the breakdown of cellulose. On the sequence analysis and the genome of the *M. thermophilia* has revealed a large repertoire of genes responsible for the production of thermostable lignocellulolytic enzymes such as carbohydrate-active enzymes, proteases, oxidoreductases, lipases and xylanase [30, 32, 33]. *M. thermophila* has 9110 genes, organized in 7 chromosomes, sequenced and annotated and also consists of 250 genes encode carbohydrate-active enzymes of which 180 are potential glycoside hydrolases. Thirteen out of 180 genes has ability to encode xylanases [34, 35].

Other thermophilic fungus like genus *Humicola* a nonpathogenic and nontoxic fungus also produces a wide range of hemicellulases and cellulases. Thermophilic *Humicola insolens* Y1 is an excellent producer of xylanolytic enzymes, including the thermophilic xylanases from family GH10 and GH11 [36]. More to the list is the *Thermoascus aurantiacus* another potential fungus with the ability to produce thermostable cellulases and xylanase, reported from Aravali forest area of University of New Delhi [37]. The fungus was able to produce antioxidant compounds as byproduct of its inoculum preparation process, which could be used for exploiting crop residues for biofuel production.

4.3 Xylanases from archaeal domain

In the field of biotechnology, the thermophilic micro-organisms from archeal domain have been reported/isolated with ability to express enzymes that can tolerate high temperatures (80–115°C), extreme pH, and high salt concentration [38]. These thermophilic enzymes with attribute of hydrolyzing lignocellulosic biomass were characterized, cloned and expressed in various hosts. *Sulfolobus solfataricus* is thermoacidophile that can live in acidic volcanic hot springs and grows optimally up to 87°C and pH 2–4. It produces enzymes with carbohydrate depolymerizing activities, such as endoglucanases and xylanases, as well as β-glucosidases/xylosidases involved in the degradation of plant-derived complex polysaccharides [39]. The genome of *S. solfataricus* has been sequenced, and three open reading frames (sso1354, sso1949, and sso2534) coding for putative extracellular endo-glucanases have been identified. These enzymes belong to a GH12 of glycoside hydrolases family and member of clan C [40]. Sources of microbial xylanases with demonstrated xylanase activity are listed in **Table 2**.

Sources	Gene	Substrate	Xylanase activity	References
Humicola insolens	Xyn11B	Beechwood Xylan	382.0 U/mg	[36]
Streptomyces sp.	XynA	Beechwood Xylan	250.69 U/mg	[41]
Streptomyces sp.	—	Birchwood Xylan	5098.28 U/mg	[42]
Schizophyllum commune	XynA	Beechwood Xylan	5768 U/mg	[43]
Streptomyces sp.	XynBS27	Oat spelt Xylan	3272.0 U/mg	[44]
Aspergillus niger	XAn11	Birchwood Xylan	909.4 U/mg	[45]
Aspergillus. niger	XAn11	Birchwood Xylan	415.1 U/mg	[45]
Planococcus sp.	XynSL4	Birchwood Xylan	244.7 U/mg	[28]
Acrophialophora nainiana	Xyn6	Oat spelt xylan	172 mg/L	[46]
Trichoderma reesei	Xyn2	Birchwood Xylan	1600 U/mg	[47]
Myceliophthora thermophila	MYCTH_56237	RBB-Xylan*	1533.7 U/mg	[48]
Myceliophthora thermophila	MYCTH_49824	RBB-Xylan*	1412.5 U/mg	[48]
Thermothelomyces thermophila	MYCTH_39555	Birchwood Xylan	105.42 U/mg	[49]

**RBB-Xylan, Remazol brilliant blue-Xylan.*

Table 2.
Sources of microbial xylanases with demonstrated activity.

5. Expression systems for xylanases

To acquire a pure form of a particular enzyme from a given source is challenging. Also it is inconvenient to have cultivation of bacteria or fungi for large scale protein production that often leads to many interfering enzymes. It might need multiple purification steps to get the intended enzymes purified from a pool of proteins which in turn will increase the cost. Therefore, recombinant DNA technology is recommendable for application with success prospects for desired object [50]. Recombinant DNA technology allows large scale expression of enzymes in both homologous and heterologous protein expression. The genes of enzymes with industrial importance were reportedly cloned and expressed in expression hosts in order to enhance specific enzymes production plus improvement in substrate utilization, and other commercially useful properties. Likewise, genes encoding thermophilic xylanases from different sources have been cloned with the objectives of overproduction of the xylanases and changing its properties to suit commercial applications [9].

5.1 Bacterial expression system

Escherichia coli are the most promising host for cloning and expression of heterologous recombinant proteins. Success of this platform as a recombinant expression host mainly due to the ease of is attributed toward some factors such as wide choice of cloning vectors, rapid growth, inexpensive media and simple techniques required for transformation, secretion of heterologous proteins into the culture medium and avoid the difficulties associated with purification of the recombinant protein [9]. *E. coli* expression systems been used for recombinant proteins production both intracellularly and extracellularly. In spite of the many advantages of using *E. coli* as expression host, there are certain limitations such as upon gene over expression, recombinant protein aggregates to form inclusion bodies in the cytoplasm. In order

to reduce the inclusion bodies, several strategies could be used such as regulation of the protein synthesis rate, co-expression of chaperone genes and empowering the secretion of proteins into the periplasm. However, the control rate of protein synthesis can be achieved by altering the promoter to regulate the level of expression, fusing the target gene to another gene, and adjusting the growth conditions, such as pH and temperature of the medium [9].

Although previously it has been reported that the expression of the xylanase genes usually cannot be functionally expressed in *E. coli* due to some factors including the repetitive appearance of rare codons and the requirement for specific post translational modifications such as disulfide bond formation and glycosylation [51] and also it require N-glycosylation whereas *E. coli* can only perform simple O-glycosylation [52]. However, recently recombinant xylanase of family GH10 (XYN) from *Thermoanaerobacterium thermosaccharolyticum* (DSM 571) was successfully overexpressed in *E. coli* (strain BL21) [53]. Similarly, another xylanase gene (xyn10B) encoding the endo-xylanase from *Thermotoga thermarum*, was successfully cloned and expressed in *E. coli* (strain BL21) and exhibits the thermostability at high temperature [54]. These finding indicated that *E. coli* might be an effective and suitable host for the expression of xylanases. Furthermore, xylanase (gene xynA) from thermophilic fungus *Thermomyces lanuginosus* exhibits the activity endo-xylanase of GF11 and the expression of optimized sequence of xynA in *E. coli* was found to be a high level. However, the recombinant XynA was mainly found in inclusion bodies, and only a small proportion was soluble and active [55]. For this purpose, a strategy was exposed to overcome inclusion-body formation, an expression plasmid named pHsh exhibit a synthetic heat-shock (Hsh) promoter, in which gene expression is regulated by an alternative sigma factor (σ32). pHsh derivative was constructed by fusing a signal peptide to xynA2 gene, eases to export the recombinant protein to periplasm and xylanase was successfully produced in a soluble form [56].

5.2 Fungal expression system

Filamentous fungus is the promising organism for protein expression and its production by fermentation has a long history in industrial area. Even developed other expression systems for recombinant protein expression, fungal expression system also considered an appropriate candidate for the expression [9]. Natural capability of fungal expression system to secrete large amounts of proteins into the medium gave an advantage to this expression system. Furthermore, it has feasibility for functional expression of other xylanases from remote sources by using of native xylanase expressing machinery [7, 9]. Most of the xylanase genes have been expressed in fungi under homologous expression system and frequently used fungus as expression hosts are *T. reesei*, *A. niger* and *Aspergillus oryzae* [57]. *T. reesei* system relies on the integration of the transforming DNA into the fungal genome, which results in excellent stability of transformants. The vectors construct provided a variety of N and C-terminal modifications that facilitate gene product processing and purification. In *T. reesei*, the most frequent choice of a promoter used for recombinant gene expression is the *cbh1* (cellobiohydrolase 1) gene encoding the cellulose. For high level expression of recombinant proteins in *T. reesei* is to use a variety of strong promoters that simultaneously transcribe the target gene, instead of one promoter with multiple copies of genes, which might be leads to the depletion of specific transcription factors for the promoter. Recently *T. reesei* strain expressed the recombinant bacterial xylanase XynB under the promoters of *egl2* (endoglucanase 2), *xyn2* and *cbh2*. Promoter of the *T. reesei xyn2* gene encodes the endo-1,4-β-xylanase II (Xyn2). Gene expression cassettes with the *xyn2* and *cbh2* promoters were introduced simultaneously into a *T. reesei* strain (EC-21), which

produced XynB under the egl2 promoter [58]. Expression of the *xynB* gene under all three different promoters resulted in improvements of the enzyme [58]. Two novel genes of family GH11 xylanases *xyn5* and *xyn6*, isolated from the thermophilic filamentous fungus *Acrophialophora nainiana,* were successfully expressed in an industrially-exploited fungal host *T.* reesei [46]. Moreover, the beneficial aspect of this fungus use for recombinant gene expression is the secretion of proteins into the growth medium and consequent gene products achieved comparatively straightforward. However, degradation of recombinant gene products also occurs due to the secreted acidic proteases into the cultivation medium [58].

5.3 Yeast expression system

Yeasts considered as excellent and attractive host for the expression of heterologous proteins and offer many advantages over the other established expression systems especially in protein maturation [59]. The methylotrophic yeast *Pichia pastoris* is an established protein expression host for the production of industrial enzymes. It can be grown to very high cell densities, produces high titer of recombinant proteins and ability to secrete proteins into fermentation media thus it is a very useful expression host, especially when scaling up to industrial process [60]. *P. pastoris* can be expressed intercellularly and provides extra benefits over the other expression systems such as ability to perform eukaryotic post-translational modifications, glycosylation, proper folding of the proteins [61]. Moreover, the most significant feature the of this expression host are due to the availability of strong and regulatory promoter of alcohol oxidases AOX1, involved in the methanol utilization pathway which provided exceptionally high levels of heterologous recombinant protein [62]. Because of all such features, the expression of xylanase genes in *P. pastoris* preferred mostly and provides high yield of recombinant xylanases expression under methanol induction. The enzyme activity of xylanase was reported 3676 U mL^{-1} for the gene product of xylB from *A. niger*, when expressed under AOX1 in *P. pastoris*. In fact, this is one of the highest expressions of recombinant xylanase expressed from *P. pastoris* reported [63]. Similarly, Cheng et al. [61] and Chantasingh et al. [64] also attained high xylanase activity (342.2 U mL^{-1} and 238.5 mg mL^{-1}, respectively) under this promoter (67-fold and 4-fold) higher recombinant xylanase activity, compared to the native fungal xylanases. The gene coding xylanase (*xynS14*) from a thermophilic xylan degrading actinomycetes *Actinomadura* S14, were expressed in both *E. coli* and *P. pastoris* [65]. The specific activity of purified recombinant xylanase from *P. pastoris* transformants was approximately 2.4-fold higher than that of purified recombinant xylanase from *E. coli* transformants, suggesting that *P. pastoris* is a better host for expression of recombinant XynS14. Although both recombinant XynS14 showed approximately the same basic properties, such as substrate specificity, optimal pH and temperature, stability for pH and temperature, and effects of EDTA and metal ions, whereas XynS14 (*P. pastoris*) showed higher specific activity and kinetic values (V_{max} and K_{cat}) than XynS14 (*E. coli*) [65]. These finding suggested the glycosyl chains present in XynS14 (*P. pastoris*) stabilized the enzyme and the enzymes were folded properly in *P. pastoris*. Cloning and expression of another xylanase gene belongs to family GH11 from *T. fusca* NTU22, also reported higher yield and thermostability than the original strain [61].

6. Xylanases: genetic engineering and optimization

In most of the cases xylanases need to undergo some genetic modifications in order to enhance expression level, enzymes activity and that might have some

influence on substrate specificity and stability to high temperature and pH. The gene encoding cellulolytic and xylanolytic enzymes are usually regulated by a repressor/inducer system in fungi. Xylanolytic transcriptional regulators have been reported in thermophilic fungi. The strong promoter MtPpdc (pyruvate decarboxylase) recently used for the overexpression of xylanases from *M. thermophila* ATCC42464 (MtXyr1). The extracellular xylanase activity of the recombinant was reported higher as compared to the wild type indicating the MtXyr1 is a positive regulatory factor for xylanase gene expression and its feasibility of improving xylanase production by overexpressing Mtxyr1 in *M. thermophila* represented an effective approach to increase total xylanase productivity [66]. Similarly, a new N-glycosylated site was created in the coding sequence by amino acid replacements for the expression of endoxylanase in *T. reesei* M2C38. For this purpose, amino acid Asn at position 131 associated with Thr/Ser at position 133 was inserted that resemble a conserved feature for family GH11 xylanases. The new created N-glycosylation site Asn-Xaa-Thr/Ser displayed 40% enhanced protein expression in comparison with wild type [67]. The downregulation of Cre1 plays an important role in enhancing enzyme production and also the silencing of CRE1 improves cellulase and xylanase expression. In *T. reesei* and other fungi, the key regulator of CCR (Carbon catabolite repression) is the Cys_2His_2-type transcription factor CRE1. The role of CRE1 in *M. thermophilia* was verified through RNAi and suggested the feasibility of improving cellulase production by modifying the expression of regulators in thermophilic fungi [68].

In order to fulfill the demands of industrial requirements, gene mining and protein engineering are applied to develop thermostable xylanases. Although some of thermophilic xylanases were produced from thermophiles but their lower expression levels and specific activities making them unable to be applied efficiently. The higher specific activity with enhanced thermostability of xylanases is therefore needed through genetic engineering. Recently the thermostability of mesophilic xylanase (AuXyn10A from *Aspergillus usamii* E001), was improved through elucidation of some local structures affecting the thermostability of mesophilic xylanases in corresponding to thermophilic *Thermoascus aurantiacus*. The temperature optimum of the mutant was 10°C higher than that of AuXyn10A [69]. The thermostability and alkalophilicity of another endo-xylanase from *T. reesei* was improved by replacement of amino acids at different positions and the replacement of NH_2 terminal amino acid sequence of *Thermomonospora fusca* along with the addition of some extra amino acids selected from N-terminus of *Clostridium acetobutylicum* xynB. All these strategies increased the thermophilicity and alkalophicility of the enzyme from 55 to 75°C and pH 7.5 to 9.0 respectively [70]. Similarly, the thermostability of mutant xylanase (Xyn10A_ASPNG) was improved by 17.4°C [71].

7. Factors affecting xylanase activity

7.1 Substrates

The enzymatic degradation reaction of insoluble polysaccharides had great importance. However, glycoside hydrolase enzymes often inaccessible to the active site of the appropriate enzymes, such polysaccharides relatively inefficiently. In order to overcome these problems, many of the glycoside hydrolases that utilize insoluble substrates are modular, comprising catalytic modules appended to one or more non-catalytic carbohydrate-binding modules (CBMs) [72]. Two xylanase genes belongs to family GH11 (xynC81 and xynC83) from a thermophilic strain

Achaetomium sp. Xz-8, with substantial xylanase activity. Substrate specificity and the hydrolysis analysis of purified recombinant revealed that XynC81 and XynC83 were moderate on beechwood xylan (67 and 69%, respectively) and birchwood xylan (45 and 52%, respectively), and weak on barley β-glucan (18 and 14%, respectively). Only XynC81 had detectable activity against insoluble wheat arabinoxylan (22%). Although enzymatic properties of XynC81 and XynC83 were similar but XynC81 with CBM 1 had activity against insoluble wheat arabinoxylan (22%), whereas XynC83 had not. This result further implied the importance of CBM in enzyme activity toward insoluble substrate. As all of the GH11 xylanases characterized so far, about 25% of them carry at least one CBM [73]. Unlike the serine/threonine/asparagine-rich linker sequence found in other fungal xylanases, the XynC81 linker sequence is extremely glycine-rich. Therefore, it could be predicted that CBMs take part in the action of cellulolytic enzymes toward insoluble substrates [74].

Moreover, some xylanases bears CBMs specific for cellulose, which probably assist indirectly localization of xylanase to the xylan substrate, since it is in close association with cellulose. The number of characterized fungal xylanases harboring CBM1 module is relatively reduced. It includes xylanases from *Penicillium funiculosum* XynB [75, 76], *Neocallimastix patriciarum* XynS20 [77], *Lentinula edodes* Xyn11A [78] and *Phanerochaete chrysosporium* XynB/XynB-1 [79]. Recently, a Xyl-11 from *Podospora anserina* harboring a C-terminal CBM1 efficiently supplemented the industrial cocktail produced by *T. reesei* by improving significantly the release of reducing sugars upon hydrolysis of wheat straw [80].

7.2 Metals and chemicals

The metal ion and chemical reagents had been proved to be one of the critical factors which affected the enzyme activity of xylanases. The effect of metal ions and chemical reagents on the xylanase activities has been determined on various metal ions (Na^+, K^+, Ca^{2+}, Li^+, Co^{2+}, Cr^{3+}, Ni^{2+}, Cu^{2+}, Mg^{2+}, Fe^{3+}, Zn^{2+}, Pb^{2+}, and Ag^+) and chemical reagents (SDS, β-mercaptoethanol, ethanol, Triton X-100, and EDTA) at the standard condition. Ca^{2+}, Pb^{2+}, K^+, Mn^{2+}, EDTA, β-ME, Cu^{2+} and Triton X-100 were reported to enhance enzyme activity by 6.4–29.9% [81], while Fe^{3+}, Cd^{2+}, Hg^{2+}, and Ba^{2+} completely suppressed the xylanase activity. Besides this, the enzyme had certain ability to resist the Fe^{2+}, Mg^{2+}, Ag^{2+}, SDS, ethanol and SDS. Xylanase activity was not inhibited by chelating reagents such as EDTA and EGTA. Moreover, it is predicted that Ca^{2+} and Mg^{2+} ions enhance the enzyme activity by stabilizing the enzyme–substrate complex. In contrast, EDTA is a chelating agent and it removes ions from the enzymes, thus inhibits the enzyme activity [7]. More detailed studies are needed in order to understand the mechanistic effect of metal ions on enzyme activity. Similarly, a xylanase activity isolated from *Planococcus* sp. *SL4* was enhanced by Ca^{2+} and β-mercaptoethanol. K^+, Cr^{3+}, Li^+, and Na^+ showed little or no effect on xylanase activity. Ag^+, Cu^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+}, Fe^{3+}, and Cr^{3+} resulted in an almost complete loss of activity [28].

7.3 Temperature and pH

As thermophilic enzymes are preferred over the mesophilic enzymes complements because of high temperatures, which had a great influence on many factors such as decreases contamination risk and viscosity of substrate [82]. In a study carried out with the effect of xylanase activity from *A. niger* DFR-5 on different temperature (between 20 and 60°C). The xylanase activity was increased with increase in temperature with maximum activity of at 40°C. On further increase in

Species	Strain	pH	Temperature	References
Caldicoprobacter algeriensis	TH7C1(T)	11.0	70°C	[26]
Microcella alkaliphila	JAM-AC0309	8.0	65°C	[86]
Planococcus sp.	SL4	7.0	70°C	[28]
Geobacillus sp.	WBI	7.0	65°C	[87]
Caldicellulosiruptor sp.	F32	6.6	75°C	[88]
Actinomadura sp.	Cpt20	10.0	80 °C	[89]
Thermofilum pendens	Tpbgl	3.5	95°C	[90]
Sulfolobus solfataricus	MT4	7.0	90°C	[91]
Thermococcus zilligii	AN1	6.0	75°C	[92]
Penicillium occitanis	Pol6	3.0	65°C	[93]
Saccharopolyspora pathumthaniensis	S582	6.5	70°C	[94]
Paecilomyces thermophila	J18	7.0	75°C	[95]
Bispora sp.	MEY-1	4.5	85°C	[96]
Malbranchea cinnamomea	S168	6.5	80 °C	[97]
Penicillium oxalicum	B3–11(2)	5.0	50 °C	[98]

Table 3.
List of xylanase producing microbial species/strains with demonstrated pH and temperature.

temperature, the enzyme activity declined gradually and at 60°C, enzyme exhibited 35.5% of the maximum activity [83]. Similarly, the temperature effect of purified xylanase (xynZF-2) from *A. niger*, was evaluated [84]. The optimum temperature recombinant enzyme was 40°C and the enzyme activity was observed relatively stable on the temperature below 40°C, however enzyme activity decreased rapidly with rise of temperature (above than 40°C). The activity became completely lost on incubation at 50°C for 15 min [84].

Evaluation of the pH stability is a vital part of any enzyme characterization before it can be exploited commercially. Similarly effect of pH had great influence on the xylanase activity. The effect of pH on extracellular xylanase from *A. niger* DFR-5 was evaluated [83]. That suggested the formation of an improper ionic form of the xylanase and/or substrate between pH 4.0 and 5.0 and between pH 5.0 and 6.5. Because the activity of pre-incubated xylanase at pH >6.5 or pH <4.0, was full not recovered at pH 5.0 again. The decline activity at pH above than 6.5 and pH below than 4.0 resulted from irreversible enzyme inactivation [83]. Many researchers have reported the pH stability of xylanase but without interpretation. Xylanase isolated from *Planococcus* sp. SL4 was highly active and stable over the neutral and alkaline pH range from 6 to 11, with maximum activity at pH 7 and more than 60% activity at pH 11 [28]. Xylanase purified from *Arthrobacter* sp. MTCC 5214 was found stable in a narrow pH range of 7.0–8.0 [85]. List of xylanase producing microbial species/strains with demonstrated pH and temperature are given in **Table 3**.

8. Application of xylanases

Because of their biotechnological characteristics, xylanases are most often produced from microorganisms for commercial applications. The environmental

hostility and lethality to most of living organisms due to extreme hot environments is obvious but such factors are counter-productive in the presence of specialized microorganisms. These microorganisms from harsh environments can tolerate high or low temperatures, extremes pH and high concentrations of salts. Based on the unique stability of their enzymes at high temperature, extremes of pH they are expected to be a powerful tool in industrial biotransformation processes that run at harsh conditions. Enzymes derived from extremophiles have great importance to the local enzymes because they can perform industrial processes even under harsh conditions, under which conventional proteins are completely denatured. The extremophile research, the high demands of the biotech industries for tailor-made novel biocatalysts, and the rapid development of new techniques such as genomics, proteomics, metabolomics, directed evolution and gene shuffling will stimulate the development of new industrial processes on the basis of biocatalysts from extremophiles.

Enzymatic hydrolysis of xylan has become attractive due to its biotechnological applications in the food, animal feed, waste treatment, ethanol production, textile, and pulp and paper industries [11]. Xylanases gained its importance for the saccharification process in degradation of lignocellulosic biomass, because xylanases assist the hydrolysis of xylan and ultimately leads to the hydrolysis of hemicellulose and cellulose, to obtain industrially important products [99]. In addition, biofuel production from lignocellulosic biomass has great concern in industrial sectors worldwide. However, biofuel production is still a bottleneck, because the initial conversion of biomass into sugars requires multiple enzymes (including xylanases) with high activity and excellent enzyme properties. Therefore, recent development in biotechnological applications xylanases toward the biomass conversion is on way [100]. Moreover, highly stable enzymes, active under high temperature and wide range of pH, displayed several advantages over the enzymes from mesophilic or neutrophilic origin. For example, highly active xylanases under extreme conditions reduce the contamination risk due to fast reaction rate. In second generation ethanol industries, mild pretreatment of biomass requires to reduce the total costs and sustain the high contents of hemicellulose. However, high dosage of other enzymes is still needed for the complete conversion of hemicellulose. Therefore, the biotechnological application of xylanases especially thermophilic xylanases and its interaction with other enzymes such as cellulases, xylosidases, arabinofuranosidase seems to be an effective in saccharification process.

The use of xylanases, as accessory enzymes, has shown to improve the hydrolysis of xylan and cellulose, and it has contributed to the reduction of enzyme dosage, but it seems to be a substrate dependent reaction [101]. Recently, less severe pretreatments of lignocellulosic biomass are gaining popularity, thus, hemicellulase characterization and studies regarding synergism of cellulases and xylanases, can contribute to reducing pretreatment severities and enhancing glucose and xylose release [102]. Xylanases are very important in bioconversion of xylan into value added products, such as xylitol. Xylitol is used in soft drinks, candies, ice cream, chewing gum and various pharmaceutical products. Xylitol have key role in sweeten food products and used as a natural sweetener in toothpaste. Xylanases have great importance in the bio-bleaching of wood pulp and in the bioprocessing of textiles.

Xylanases are very useful in manufacturing of animal feed. Xylanases reduce the viscosity and enhance the absorption rate by degrading the starch polysaccharides in rich fiber and barley based feeds. Pre-treatment of agricultural silage and grain feed employed with xylanases increases the nutritional value and improves the feed digestion in ruminants. Similarly, feed supplementation combined with xylanases for broiler diets, not only increases growth performance like weight gain but also helpful in the yield production of poultry products.

Conclusively, the xylanases have potential applications in a wide range of industrial processes, covering all sectors of industrial enzymes markets. Arguably, these are attractive for research studies and deeper investigation with aims to explore dividends and utility of these useful products in industrial context in relation to industrial enzymes producing organisms.

9. Summary

Microbial xylanases are gaining importance in industrial applications. The thermophilic microorganisms are of interest in the field of biotechnology because of their ability to express enzymes that tolerate high temperature and pH. The thermophilic bacteria, and an archaeal species, there are also filamentous fungi that grow in diverse environments and produce xylanase. New recombinant DNA technology can be used to express xylanases in both homologous and heterologous host organisms. Compared to *E. coli, P. pastoris* considered the most suitable and excellent host for the heterologous proteins expression including xylanases. Xylanases generally must be genetically modified in order to increase the expression level with improved xylanase activity, but these alterations can change substrate specificity and stability at high temperatures and pH. Many xylanases have xylan-binding domains that can affect the thermostability of the enzymes and their ability to bind insoluble substrates. In conclusion, the lignocellulosic biomass and enzyme-producing microorganisms are compelling subjects for research studies aiming to explore the growing importance of xylanases in various industrial products.

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Conflict of interest

The authors declare no conflict of interest.

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