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Advances in Heterologous Expression of Xylanase (Postprint)

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Abstract

Abstract: Xylan is extremely abundant in nature and occurs in large quantities in crops and agricultural and forestry residues. As issues concerning energy and resources become increasingly prominent, research into the application of xylan has attracted growing attention. Xylanase is a class of hydrolases that can degrade xylan into xylooligosaccharides and xylose. In recent years, researchers have conducted extensive studies to achieve high-yield, high-activity expression of xylanase. This paper reviews the research progress on heterologous expression of xylanase.

Keywords: Xylan; Xylanase; Heterologous expression

Full Text

Preamble

Research Advances in Heterologous Expression of Xylanase

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Abstract

Xylan is abundantly present in crops and agricultural and forestry residues. With increasing emphasis on energy resource issues, research on xylan applications has attracted growing attention. Xylanase is a class of hydrolases that can degrade xylan into xylooligosaccharides and xylose. In recent years, researchers have conducted extensive studies to achieve high-yield, high-activity expression of xylanase. This review summarizes the research progress in heterologous expression of xylanase.

Keywords: xylan; xylanase; heterologous expression

Xylan is an extremely abundant polysaccharide in nature, second only to cellulose in content and constituting the main component of hemicellulose. It is widely distributed in plant cell walls [1]. Xylanase is the primary enzyme system capable of degrading xylan into xylose and xylooligosaccharides. Beyond the broad applications of its degradation products in food processing and pharmaceuticals, studies have reported that removal of hemicellulose can significantly enhance the release of cellulose and lignin, offering important implications for addressing energy resource utilization challenges. Xylanases are widely distributed in nature and can be obtained from animals, plants, and microorganisms, with bacterial and fungal xylanases being the most extensively studied and applied. However, natural microbial sources typically produce insufficient xylanase yields to meet industrial demands. Consequently, numerous researchers have cloned xylanase genes and expressed them in various expression systems to enhance production. This review summarizes the research advances in heterologous expression of xylanase.

1 Overview of Xylanase

Xylan, a component of plant hemicellulose found in cell walls, is the second most abundant polysaccharide in nature. Most xylan is a structurally complex, highly branched heteropolysaccharide containing various substituents. Therefore, complete xylan degradation requires not a single enzyme but an entire enzyme system.

1.1 Xylan-Degrading Enzyme System

The xylan-degrading enzyme system comprises -1,4-endoxylanase (endo-1,4- -D-xylanase), -L-arabinofuranosidase, -D-glucuronidase, acetyl xylan esterase, ferulic acid esterase, and -D-xylosidase. This enzyme series constitutes the broad definition of xylanase—the collective term for enzymes that can completely degrade xylan. In contrast, the narrow definition of xylanase refers only to -1,4-endoxylanase, which cleaves internal -1,4-xylosidic bonds in the main chain [2]. -1,4-endoxylanase (EC 3.2.1.8) is considered the key enzyme for xylan hydrolysis [3].

1.2 Xylanase Structure

Xylanases have relatively complex molecular structures. The typical quaternary structure contains a catalytic domain and several non-catalytic domains, such as cellulose-binding domains (which bind cellulose), xylan-binding domains (which bind xylan), thermostabilizing domains, linker sequences, and other domains of unknown function [4]. The spatial structures of different xylanases show significant variations, resulting in differences in their functional effects and target substrates. Some structures are relatively simple, while others require auxiliary non-catalytic domains for functions such as substrate binding or interaction with other xylanase system components.

The catalytic domain is an essential component of xylanase and the key functional domain responsible for catalyzing and degrading xylan. Most xylanases in nature contain a single catalytic domain in their spatial structure. However, *Ruminococcus flavefaciens* has been found to possess two catalytic domains located at the C-terminus and N-terminus of the protein, making such multi-catalytic domain xylanases quite rare [5].

The cellulose-binding domain primarily binds cellulose and is commonly found in cellulases. However, it also exists in xylanases with cellulose and hemicellulose hydrolysis functions, where it binds to cellulose connected to xylan substrates, playing a regulatory and anchoring role [5].

The thermostabilizing domain significantly contributes to enzyme thermal stability and is typically present in xylanases from thermophilic microorganisms. These domains generally contain abundant aromatic amino acids and exhibit high molecular folding. Linker sequences in xylanase structures connect various domains within the enzyme protein, thus possessing good flexibility and extensibility, though they vary considerably among different xylanases [7]. The solution structure of the Cbm10 cellulose-binding module from *Pseudomonas* xylanase A has been determined.

The xylan-binding domain functions to bind the xylan substrate and represents another type of functional domain. Research indicates that xylanase-substrate binding generally relies on electrostatic interactions on the macromolecular surface, with this domain playing a less significant role. However, in the xylanase produced by *Streptomyces olivaceoviridis* E-86, the xylan-binding domain plays a crucial role in xylan degradation. The intact xylanase can degrade both soluble and insoluble xylan, but deletion of the xylan-binding domain results in loss of activity against insoluble xylan [8].

1.3 Physicochemical Properties of Xylanase

Xylanases are ubiquitous in nature with numerous varieties, and their physicochemical properties vary considerably among different types. Bacterial xylanases generally contain a single subunit with molecular weights ranging from 8–145 kDa, whereas fungal xylanases have more complex structures, typically containing multiple subunits and thus exhibiting much larger molecular weights than bacterial xylanases [9]. Based on their optimal pH environments, xylanases are classified as acidic or alkaline. Acidic xylanases function at pH 4–7, are typically produced by bacteria, and have molecular weights below 30 kDa [10]. Alkaline xylanases operate at pH 8–10, are produced by both bacteria and fungi, and have molecular weights exceeding 30 kDa. The optimal temperature ranges also vary significantly among xylanases from different sources. Most thermostable xylanase-producing strains are bacteria, including *Bacillus* and *Streptomyces* species [11, 12]. Bacterial xylanases generally have broad temperature ranges around 40–60°C, while fungal xylanases are most stable around 50°C. The isoelectric points of xylanases from various sources range from 3–10, within which

they typically remain stable.

[Figure 1: see original paper]. The model 3-D structure of xylanase
Note: Image translated from Dal Rye Kim et al.

2 Heterologous Expression of Xylanase

Since Horikoshi et al. first reported xylanase genes from alkalophilic bacteria in 1973, numerous researchers have conducted extensive studies on xylanase genes, achieving their expression in multiple expression systems.

2.1.1 Heterologous Expression of Xylanase in *Escherichia coli*

The prokaryotic expression system using *E. coli* as host is the earliest and most extensively studied system, offering advantages such as clear genetic background, complete vector-receptor systems, simple cultivation, and short growth cycles. Consequently, increasing numbers of scientists have cloned xylanase genes from various microorganisms and achieved heterologous expression in *E. coli*. In 1995, researchers cloned multiple xylanase genes from *Aspergillus niger* [18]. The following year, Iefuji H et al. isolated and purified a xylanase-producing yeast *Cryptococcus* sp. S-2 [19]. By 2001, Beg et al. had isolated corresponding xylanases from over 20 bacterial species, 16 fungal species, 3 yeast species, and 8 actinomycete species, with successful expression in fungal or bacterial host systems [20]. In 2003, Xue et al. used PCR technology to clone the gene encoding a highly thermostable xylanase from *Thermotoga maritima* into the prokaryotic expression vector pET-20b, fusing it with a histidine tag for high-level expression in *E. coli* JM109(DE3) [21]. To improve cellulose utilization in feed, Wang et al. (2014) co-expressed xylanase and endoglucanase genes from *Bacillus subtilis* in *E. coli* [22]. In 2014, Liu cloned the xylanase gene *xylA* from *Bacillus agaradhaerens* from a genomic library and achieved heterologous expression in *E. coli*, characterizing the recombinant enzyme. By optimizing culture medium and induction conditions, the supernatant enzyme activity reached 3,729 U/mL, 2.3-fold higher than the original strain [23]. In 2016, He et al. synthesized the thermostable xylanase gene *xyn11Em* from family 11, cloned it into expression plasmid pET-28a(+), and transformed *E. coli* BL21(DE3) to construct the recombinant strain *E. coli* BL21/xyn11Em. The results demonstrated successful heterologous expression of the thermostable xylanase, which exhibited good thermal stability and industrial application potential [24]. In the same year, Wu fused the xylanase gene from *Bacillus pumilus* NJ-M2 with green fluorescent protein gene (*gfp*) for surface display on *E. coli* cells, creating a strain capable of displaying xylanase on its surface and providing a theoretical basis for industrial immobilization and fermentation applications [25]. In 2018, Hao et al. synthesized and codon-optimized a xylanase gene from *B. subtilis* for efficient secretory expression in *E. coli* BL21 and characterized the enzyme properties [26]. Also in 2018, Liu et al. heterologously expressed the bifunctional xylanase Xyn2038 from *Clostridium clariflavum* in *E. coli*, obtaining three recombinant enzymes and studying their enzymatic properties [27].

2.1.2 Heterologous Expression of Xylanase in *Bacillus subtilis*

Bacillus subtilis is another widely used expression system following *E. coli*. It is non-pathogenic, possesses a single-layer outer membrane, and can directly secrete many proteins into the culture medium. The complete genome sequencing of *B. subtilis* 168 has been jointly completed by eight countries (UK, France, Germany, Japan, Switzerland, etc.), with all 4,214,810 bases sequenced and publicly released. The published genetic map contains 2,379 genes, providing detailed background information for *B. subtilis* modification and engineering strain construction [28], and offering effective theoretical support for metabolic engineering-guided strain development [29-31].

In 2012, Cao et al. cloned an alkaline xylanase gene fragment (*xynA*) containing a signal peptide, inserted it into a previously constructed *E. coli*-*B. subtilis* shuttle vector, and transformed *B. subtilis* WB700 to obtain recombinant strain BXS-W, which was then induced to secrete large amounts of xylanase through various stress treatments [32]. In 2013, Verma et al. first achieved specific heterologous expression of a thermostable xylanase metagenome in *B. subtilis* [33]. In 2015, Huang et al. truncated a thermostable xylanase from *Thermoanaerobacterium aotearoense* SCUT27 and integrated the truncated gene into the *B. subtilis* genome, achieving enzyme activity four times higher than that in *E. coli* [34]. In 2016, Xiang et al. cloned an extremely thermostable xylanase gene *xynB*, fused it with a rokanycin promoter, constructed the fusion plasmid vector pXYNB2, and transformed it into *B. subtilis* Bs916 for heterologous secretory expression [35].

.1 Expression of xylanase in bacterial expression system

Xylanase	EC Number	Source	Protein Size	Optimal pH	Expression Vector	Promoter	Enzyme Activity
EC 3.2.1.8	*Bacillus subtilis*	43.0 kDa	NR	WB700	pET26b	T7/Lac	1,201.5 IU/mL
EC 3.2.1.8	*Aspergillus niger* XZ-3S	33.47 kDa	61.43 U/mg	NR	pET28a	T7/Lac	pelB
EC 3.2.1.8	*Bacillus pumilus* BYGS-20N	25.0 kDa	58.19 U/mg	NR	pGJ148	YnfF	NR
EC 3.2.1.8	*Bacillus* sp.	28.34 kDa	30.89 U/mL	WB700			

Note: NR indicates not reported in the literature

2.2 Heterologous Expression of Xylanase in Fungi

2.2.1 Heterologous Expression of Xylanase in *Pichia pastoris* The *Pichia pastoris* expression system offers genetic stability, post-translational protein processing, high expression efficiency, secreted products in culture medium, and suitability for high-density fermentation [36], making it a widely used system for efficient heterologous protein expression. Hundreds of foreign proteins have been successfully expressed at high levels in this system. Liu et al. [37] achieved high-level secretory expression of *Aspergillus niger* xylanase A gene in *P. pastoris*, reaching a specific activity of 175 U/mg, 1.9-fold higher than the parent strain. Under the regulation of the alcohol oxidase promoter AOX1, *Bacillus licheniformis* xylanase gene was expressed in *P. pastoris* for the first time, with a specific activity of 122.9 U/mg [38]. Zhang et al. expressed a thermophilic actinomycete xylanase gene in *P. pastoris*, achieving fermentation broth enzyme activity of 324.2 U/mL [39]. In 2000, Berrin et al. expressed *A. niger* xylanase gene in *P. pastoris*, with recombinant strain yield reaching 60 mg/L on synthetic medium, marking the first successful expression and effective secretion of an *A. niger* xylanase gene in this system [40]. In 2009, Han et al. cloned the xylanase XYN gene from *B. subtilis* B2 into pPIC9K vector, constructed the secretory expression vector pPIC9K-XYN, and transformed *P. pastoris* GS115 to obtain recombinant strain GS115-pPIC9K-XYN, achieving xylanase activity of 1,542.6 U/mL in shake-flask fermentation supernatant [41]. In 2014, Gao et al. codon-optimized the xylanase XynB-wild gene according to *Pichia* codon preferences, synthesized the optimized gene XynB-opt, and connected it to pPICZaA vector. After linearizing the recombinant plasmid and electroporating into *P. pastoris* CS115, an engineered strain capable of xylanase expression was obtained [42].

In 2015, Wu et al. used genomic DNA and cDNA from *Hypocrea orientalis* EU7-22 as templates to obtain the GH10 family xylanase III gene (*xynIII*) through PCR and chromosome walking, then constructed expression vectors for transformation into *P. pastoris*, obtaining high enzyme activity in fermentation broth after 168 h induction [42]. In 2016, Zhou et al. inserted the mature peptide gene of *A. niger* XZ-3S xylanase Xyn43A into expression vector pPIC9K. After linearizing the recombinant plasmid with Sal I, two *Pichia* strains GS115 and KM71 were electroporated to obtain recombinant strains GS115/Xyn43A (Mut+) and KM71/Xyn43A (Muts) [43]. In 2017, Han et al. transferred the thermophilic *Thermoascus* xylanase *xynA* into *P. pastoris* GS115 to obtain a xylanase with improved thermal stability compared to the original strain [44].

2.2.2 Heterologous Expression of Xylanase in *Saccharomyces cerevisiae* The *S. cerevisiae* expression system offers safety without toxin production, requiring no extensive host safety testing for its expression products, along with clear genetic background and easy manipulation. In brewing industry applications, *S. cerevisiae* cannot directly utilize xylan as a carbon source. Therefore, researchers screen for high-activity xylanases from other strains and

integrate them into the *S. cerevisiae* genome to obtain efficiently expressing strains [45, 46]. Li et al. [47] first reported the integration of *A. niger* T21 glucoamylase gene (glucoamylase; EC 3.2.1.3) and *A. niger* UV11 xylanase gene (endo-1,4- -D-xylan xylanohydrolase; EC 3.2.1.8) into the chromosome of industrial ethanol-producing yeast *S. cerevisiae* 2.346, obtaining an engineered strain capable of stably co-expressing both enzymes. De la Grange et al. [48] expressed *Trichoderma reesei* XYN2 gene in *S. cerevisiae* using different promoters ADH2 and PGK1, achieving enzyme activities of 1,200 nkat/mL and 160 nkat/mL, respectively. Nuyens et al. [49] expressed *Bacillus pumilus* xynA in *S. cerevisiae* using different cloning vectors, obtaining activities of 8.5 nkat/mL and 4.5 nkat/mL. In 2013, Zhang et al. cloned the *A. niger* xylanase gene *xynB* and transformed it into *S. cerevisiae* INVSC1 using lithium acetate transformation, obtaining two engineered yeast strains producing xylanase [50]. In 2017, Zhang et al. constructed xylanase-producing *S. cerevisiae* engineered strains using rDNA integration, achieving multicopy expression of the xylanase gene. Ten multicopy strains were obtained, with the highest enzyme activity reaching 308 U/mL [51].

2.2.3 Heterologous Expression of Xylanase in Filamentous Fungi Filamentous fungal expression systems have high potential for efficient protein secretion and can perform various correct post-translational modifications similar to higher eukaryotes. They are important for industrial enzyme production due to easy cultivation, rapid growth, suitability for large-scale culture, and mature fermentation technology [52]. Molecular chaperones participate in protein secretion in filamentous fungi, facilitating better protein modification. However, research on heterologous xylanase expression in filamentous fungi remains limited. Current studies typically employ strong promoters or increase gene copy numbers to enhance protein secretion and stability, though successful examples are not numerous. In 2007, Mantyla et al. [53] cloned three xylanase genes (Ctxyn11A, Ctxyn11B, and Ctxyn11C) from *Chaetomium thermophilum* CBS 730.95 and successfully expressed them in *T. reesei* using the *T. reesei* cel7A (cellobiohydrolase 1, cbh1) promoter. In 2013, Wang et al. [54] transformed a signal peptide-containing xylanase *xyn1* from *Stachybotrys* into *A. niger*, obtaining xylanase with specific activity approaching 392 U/mg.

.2 Expression of xylanase in fungal expression system

Xylanase	EC Number	Source	Protein Size	Optimal Temperature	Expression Vector	Promoter	Enzyme Activity
EC 3.2.1.8	*Aspergillus niger* XZ-3S	34.5 kDa	45°C	pPIC9k	*P. pastoris* GS115	- factor	145.24 U/mg

Xylanase	EC Number	Source	Protein Size	Optimal Temperature	Expression Vector	Promoter	Enzyme Activity
EC 3.2.1.8	*Thermotoga maritima*	40.0 kDa	60°C	pAo815	*P. pastoris* YS2	PHO5, -factor	8,000 U/mL
EC 3.2.1.8	*A. niger* strain IME-216	35.0 kDa	40°C	pPICZaA	*P. pastoris* GS115	AOX, -factor	90,000 U/mL
EC 3.2.1.8	*Trichoderma reesei*	21.0 kDa	60°C	pYES6/CT	*S. cerevisiae* INVSc	a-factor	298 U/mL
EC 3.2.1.8	*A. niger* MC062	23.0 kDa	55°C	pYES2	*S. cerevisiae* INVSC1	a-factor	7.59 U/mL

Note: NR indicates not reported in the literature

3 Expression of Xylanase in Plant Expression Systems

Plant expression systems offer higher safety compared to microbial systems. Producing xylanase in transgenic plants enables direct use as animal feed, with advantages including simple storage and transportation, large-scale production, and low cost.

In 1992, Laliberte [55] first transferred a yeast-derived xylanase gene into tobacco plants, demonstrating that introns in the gene could be spliced in tobacco, indicating transcriptional activity, though no further experiments were conducted. Subsequently, Herbers et al. [56, 57] achieved heterologous xylanase expression in tobacco, detecting enzyme activity in leaves and root exudates. In 2000, Hung et al. [58] fused the oleosin gene with a xylanase gene from aquatic organisms and transformed it into rapeseed, achieving xylanase expression. In the same year, researchers expressed specifically induced *xynA* in barley seeds, but the target protein was only detected in seeds, not in mature plants. In 2003, Kimura et al. [59] obtained the xylanase gene from *Clostridium thermocellum* and integrated it into the rice genome, detecting xylanase activity in both rice plants and seeds. In 2010, Huang et al. [60] introduced a hybrid xylanase gene *atx* into rice callus tissues, demonstrating transcription of the xylanase gene with variable expression levels among different rice plants. Dr. Yang [61] used tobacco and potato as bioreactors to express the high-specific-activity xylanase XYNB from *Streptomyces olivaceoviridis* A1 in both plants. The transgenic tobacco showed xylanase activity up to 170 IU/g fresh leaves—the highest reported level—with normal growth and stable inheritance. Xylanase activity was also

detected in potato leaves and tubers at 90 IU/g fresh leaves and 13 IU/g fresh tubers, respectively.

.1 Expression of xylanase in plant expression system

Xylanase	EC Number	Source	Protein Size	Optimal Temperature	Promoter	Signal Peptide	Enzyme Expression
EC 3.2.1.8	*Streptomyces olivaceoviridis*	21.0 kDa	23°C	35S promoter with AMV enhancer (constitutive)	Plant expression vector pCAMBIA1300	Proteinase inhibitor II signal peptide	90 IU/g fresh leaves, 13 IU/g fresh tubers
EC 3.2.1.8	*S. olivaceoviridis*	31.0 kDa	25°C	35S promoter (constitutive)	pCAMBIA1300	Patatin signal peptide	170 IU/g fresh leaves (23 IU/mg)
EC 3.2.1.8	*A. niger*	35S promoter (constitutive)					3.51 U/g fresh weight

Note: NR indicates not reported in the literature

Xylanase has found extensive applications in food, feed, paper, and pharmaceutical industries, prompting numerous studies on its large-scale production. Researchers have screened xylanase-producing strains and optimized cultivation to obtain strains with higher enzyme activity. However, naturally selected strains face limitations in mass production, including low yields, weak activity, labor-intensive screening, high costs, and time consumption. With advances in biotechnology, genetic engineering approaches using heterologous expression in eukaryotic or prokaryotic systems have been adopted to address these challenges. Although current heterologous expression studies still face issues—such as lack of protein modification organelles and low yields in bacterial systems, and problems with hyperglycosylation hindering secretion or interference from native proteins in fungal systems—these drawbacks do not overshadow the advantages. Heterologous expression of xylanase undoubtedly represents the future direction for large-scale production, and with continued research efforts, these existing problems will be progressively resolved.

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