Appl Microbiol Biotechnol
DOI 10.1007/s00253-009-2079-4

MINI-REVIEW

Molecular cloning of fungal xylanases: an overview

Sibtain Ahmed · Saba Riaz · Amer Jamil

Received: 13 November 2008 / Revised: 5 June 2009 / Accepted: 5 June 2009
© Springer-Verlag 2009

Abstract Xylanases have received great attention in the development of environment-friendly technologies in the paper and pulp industry. Their use could greatly improve the overall lignocellulosic materials for the generation of liquid fuels and chemicals. Fungi are widely used as xylanase producers and are generally considered as more potent producers of xylanases than bacteria and yeasts. Large-scale production of xylanases is facilitated with the advent of genetic engineering. Recent breakthroughs in genomics have helped to overcome the problems such as limited enzyme availability, substrate scope, and operational stability. Genes encoding xylanases have been cloned in homologous and heterologous hosts with the objectives of overproducing the enzyme and altering its properties to suit commercial applications. Owing to the industrial importance of xylanases, a significant number of studies are reported on cloning and expression of the enzymes during the last few years. We, therefore, have reviewed recent knowledge regarding cloning of fungal xylanase genes into various hosts for heterologous production. This will bring an insight into the current status of cloning and expression of the fungal xylanases for industrial applications.

Keywords Fungal xylanases · Xylanase gene cloning · Xylanase expression

S. Ahmed · S. Riaz · A. Jamil (✉)
Molecular Biochemistry Laboratory,
Department of Chemistry and Biochemistry,
University of Agriculture,
Faisalabad 38040, Pakistan
e-mail: amerjamil@yahoo.com

Introduction

Cellulose, hemicellulose, and lignin are the major components of plant cell walls, with cellulose being the most abundant component followed by hemicelluloses (Han et al. 2003; Khandeparker and Numan 2008). Plant biomass comprises on average 23% lignin, 40% cellulose, and 33% hemicellulose by dry weight (Sa-Pereira et al. 2003). Biomass is an alternative natural source for chemical and feedstock production (Kulkarni et al. 1999; Kumar et al. 2008). Annually, 830 Gt of renewable plant biomass is formed consisting mainly of cellulose and hemicelluloses (Rauscher et al. 2006).

Hemicellulose is composed of xylan as a major component (Nair et al. 2008) that constitutes about 20–40% of total plant biomass (Ninawe et al. 2008) and accounts for approximately one third of all renewable organic carbon on earth (Prade 1996; Ning et al. 2008). Xylan is a heteroglycan having a backbone made up of β-1,4-linked D-xylopyranose residues with substitutions of L-arabinofuranose, D-glucuronic acid, and 4-*O*-methyl-D-glucuronic acid at 2′ and 3′ positions (Wakiyama et al. 2008). It represents an immense resource of biopolymers for practical applications accounting for 25–30% of the dry biomass of woody tissues of dicots and lignified tissues of monocots and occurs up to 50% in some tissues of cereal grains (Moure et al. 2006). Xylan has a high potential for conversion to useful end products. Complete conversion of the hemicellulose requires the action of several main-chain- and side-chain-cleaving enzymes: endoxylanase (endo-1,4-β-xylanase, E.C.3.2.1.8), β-xylosidase (xylan 1,4-β-xylosidase, E.C.3.2.1.37), α-glucuronidase (α-glucosiduronase, E.C.3.2.1.139), α-arabinofuranosidase (α-L-arabinofuranosidase, E.C.3.2.1.55), and acetylxylan esterase (E.C. 3.1.1.72; Choi et al. 2000). Endo-β-1,4-xylanases catalyze the hydrolysis of the

backbone of xylan to produce xylooligosaccharides, which in turn can be converted to xylose by β-xylosidase (Zhang et al. 2007).

Based on amino acid sequence homologies and hydrophobic cluster analysis, xylanases have been grouped mainly into two families of glycosyl hydrolases: family F or GH10 and family G or GH11 (Jeffries 1996; Zhou et al. 2008). However, other glycoside hydrolase families, 5, 7, 8, and 43, have also been found to contain distinct catalytic domains with a demonstrated endo-1,4-β-xylanase activity (Collins et al. 2005). Xylanases of family G are of low molecular mass with p*I* 8–9.5 compared to the family F xylanases that are of high molecular mass with lower p*I* values (Buchert et al. 1994). The optimum pH for xylan hydrolysis is around 5 for most fungal xylanases, whereas pH optima of bacterial xylanases are generally slightly higher (Yu et al. 1987). The xylanases and cellulases together with pectinases account for about 20% of the world enzyme market (Polizeli et al. 2005).

Nature is abound with bacteria and fungi that can produce cell wall-degrading enzymes to solubilize the complex components to simple molecules for completing the carbon cycle. Most of the microorganisms isolated from soil/waste/composting waste material are capable of producing a spectrum of cell wall-degrading enzymes (Badhan et al. 2007). Filamentous fungi have been used for more than 50 years in the production of industrial enzymes (Dalboge 1997). They are widely used as xylanase producers and are generally considered as more potent xylanase producers than bacteria and yeasts (Polizeli et al. 2005; Pedersen et al. 2007) that secrete much higher amounts of xylanolytic enzymes into the medium than bacteria or yeast (Bergquist et al. 2002; Fang et al. 2008). Species of fungi genera that are known to produce xylanases include *Aspergillus*, *Disporotrichum*, *Penicillium*, *Neurospora*, *Fusarium*, *Chaetomium*, *Trichoderma*, etc. (Kulkarni et al. 1999; Mach and Zeilinger 2003; Saleem et al. 2008). However, xylanases are produced mainly by *Aspergillus* and *Trichoderma* on the industrial scale (Fengxia et al. 2008).

Cloning of fungal xylanase genes

Introduction of recombinant DNA technology has resulted in the selection of xylanolytic enzymes that are more suitable for industrial applications (Sunna and Antranikian 1997; Beg et al. 2001). The main challenges for recombinant DNA technology in xylan bioconversion are: production of xylanolytic systems free of cellulolytic enzymes and the improvement of fermentation characteristics of the industrially important organisms by introducing genes for xylanase and xylosidase (Biely 1985).

It is difficult to obtain a pure form of a particular enzyme from a fungal preparation. Recombinant DNA technology can be applied with more success for this purpose (Korona et al. 2006). Genes encoding xylanases have been cloned in homologous and heterologous hosts with the objectives of overproducing the enzyme and altering its properties to suit commercial applications (Baba et al. 1994; Jamil et al. 2005). A number of genes of industrially important enzymes have been cloned and expressed for enhanced production of enzymes, improvement in substrate utilization, and other commercially useful properties (Chand and Mishra 2003).

Cloning in *Escherichia coli*

Escherichia coli has been widely used for cloning of genes and for heterologous expression of recombinant proteins including xylanases from fungi (Table 1). This is mainly due to the ease of DNA cloning, wide choice of cloning vectors, overproduction of recombinant enzymes, and in many cases, secretion of heterologous proteins from *E. coli* into the culture medium to avoid the difficulties associated with purification of the protein from their natural hosts. *E. coli* expression systems have long been used for the production of recombinant proteins either intracellularly or extracellularly (Baneyx 1999; Mergulhaoa et al. 2005; Sorensen and Mortensen 2005). The main limitation of using *E. coli* as expression host is that not every protein is secreted efficiently. Overexpression of recombinant enzymes in *E. coli* allows the engineering of the enzymes for the study of structure–function relationship by site-directed mutagenesis, as well as improvement of the enzyme properties using directed evolution technology (Alcalde et al. 2006; Kaur and Sharma 2006).

Gene isolation

The cloning of genes depends on the construction of DNA libraries followed by expression of the genes. Isolation and expression of the fungal genes in *E. coli* is mainly done by construction of cDNA libraries. The cDNA is synthesized from RNA isolated from xylanase-induced cultures of fungi, as xylanases are induced and expressed in the presence of certain carbon sources, while repressed in the presence of others (Ilmen et al. 1996, 1997; Ahmed et al. 2005; Saadia et al. 2008). It is cloned mainly in plasmids or bacteriophage lambda vectors and transformed in *E. coli*. The cDNA libraries are screened for the isolation of xylanase genes by different methods such as radiolabeled probe or antibody screening or by detecting specific activity of the corresponding enzyme. The probes for the screening of the libraries are generated by polymerase chain reaction

Table 1 Cloning of different fungal xylanase genes in *E. coli*

Source organism	Gene	Vector	Host	Molecular mass of the recombinant enzyme (kDa)	Characteristics of the recombinant enzyme	Reference
Aspergillus cf. *niger* BCC14405	*xyn*	pGEM-T Easy	*E. coli* DH5α	20.1		Krisana et al. 2005
Aspergillus oryzae KBN 616	*xynF3*	pNAN-d	*E. coli* DH5α			Kimura et al. 2002
Aspergillus usamii E001	*xynII*	pET-28a(+)	*E. coli* BL21-CodonPlus (DE3)-RIL	20	pH4.6, temperature=50°C Enzyme activity=49.6 U mg^{-1} Stability 1 h at 40°C	Zhou et al. 2008
Aureobasidium pullulans Y-2311-1	*xynA*	pCRII λ ZapII pBluescript	*E. coli* INVαF′, SURE, XL-Blue			Li and Ljungdahl 1994
Claviceps purpurea	*cpxyl1* *cpxl2*	pUC19 and pBluescriptII SK(−)	*E. coli*	21.5 33.8		Giesbert et al. 1998
Cochliobolus sativus	xyl2	Lambda ZAP	*E. coli* SOLR	25		Emami and Hack 2001
Cochliobolus sativus	xyl1	Lambda ZAP	*E. coli*			Emami and Hack 1998
Helminthosporium turcicum H-2	*htxyl2*	pBluescript SK(+)	*E. coli* DH5α			Degefu et al. 2004
Neocallimastix frontails	*xyn11A* *xyn11B*	pET-21a	*E. coli*			Huang et al. 2005
Neocallimastix patriciarum	*xynA*	pBTac2	*E. coli*			Xue et al. 1995
Neocallimastix patriciarum	*xyns20*	pTriptx2-S20	*E. coli*			Liu et al. 2008
Penicillium purpurogenum	*xynA*	pATH-3	*E. coli*			Chavez et al. 2001
Penicillium purpurogenum	*xynB*	pATH3	*E. coli* RR1	19.2		Diaz et al. 1997
Penicillium sp. 40	*xynA*	pUC119	*E. coli* DH5α	20.7		Kimura et al. 2000
Pichia stipitis NRRL Y-11543	*xynA*	pUC19	*E. coli* DH5αF′	43	K_m=1.4 mg mL^{-1} V_{max}=0.8 μmol mL^{-1} pH5.5, temperature 45°C Stability 30–40°C	Basaran et al. 2001
Thermomyces lanuginosus DSM 5826	*xynA*	pBluescript II SK(−)	*E. coli* SURE	24–26		Schlacher et al. 1996
Trichoderma harzianum E-58	*xyn2*	pUC18, FLAG	*E. coli* DH10B			Ahmed et al. 2007
Trichoderma reesei Rut C-30	*xyn2*	pET-28a	*E. coli* BL21 (DE3)	24	K_m=0.114 mg mL^{-1}, k_{cat}=106 s^{-1} pH5.0, temperature=50°C Stability >70% after 30 min at 60°C	Jun et al. 2008
Trichoderma reesei PC-3-7	*xyn3*	pT7Blue-T	*E. coli* JM109	33.1		Ogasawara et al. 2006

(PCR) amplification of a partial gene sequence with the help of degenerate primers commonly generated on the basis of N-terminal sequence and highly conserved region of the corresponding enzyme. Xylanase genes from *Penicillium purpurogenum* (Diaz et al. 1997) and *Aureobasidium pullulans* (Li and Ljungdahl 1994) were isolated by screening of the libraries with biotin-labeled probes. A xylanase gene was isolated from *Cochliobolus sativus* by screening a cDNA library with ^{32}P-labeled probe (Emami and Hack 2001). Schlacher et al. (1996) isolated a xylanase gene from *Thermomyces lanuginosus* by screening with rabbit antixylanase antiserum. A more direct way to isolate xylanase genes is by PCR and reverse transcription polymerase chain reaction (RT-PCR). A full-length endo-1-4-β-xylanase gene was amplified from *Aspergillus* cf. *niger* by RT-PCR (Krisana et al. 2005). The forward primer was designed from the nucleotide sequence of an endoxylanase B from *Aspergillus* species, and the reverse primer from oligo(dT) flanked region. Zhou et al. (2008) isolated a part of the *xynII* gene from *Aspergillus usamii* by RT-PCR and employed 5′-rapid amplification of cDNA ends approach to obtain the full-length gene.

The *xynA* gene from *A. pullulans* and *T. lanuginosus* encoded the xylanases with 221 and 225 amino acids, respectively. The endo-1,4-β-xylanase gene from *Aspergillus niger* also corresponded to a protein with 225 amino acids. The *xynB* gene from *P. purpurogenum* encoded 208 amino acid residues for the enzyme. The calculated molecular mass of the mature enzyme was 19.3 kDa that was lower than the value estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis (~23 kDa) might be due to glycosylation of the mature enzyme. The cDNA sequence obtained from *C. sativus* was predicted to code for the Xyl2 polypeptide with a molecular mass of 25 kDa.

Noncoding regions of xylanase genes

The genomic libraries are used to isolate full-length gene and to find out the number and positions of introns within the genes. Analysis of the 5′ noncoding regions of the genes also help in unraveling the mechanism of induction of xylanase biosynthesis by cellulosic and xylanosic substrates. Kimura et al. (2000) isolated a *xynA* gene from the genomic library of *Penicillium* sp. 40 constructed in pBluescript II KS(+). The library was screened with a digoxigenin-11-dUTP-labeled probe. The gene appeared to be 721 bp long interrupted by a single intron of 58 bp. The open reading frame (ORF) of the gene was predicted to encode 221 amino acid residues. Analysis of the noncoding region of the gene revealed a TATAAA sequence at 89 bp upstream from the translation initiation site. Sequences similar to the binding sites for transcriptional factors XlnR (transcriptional activator/regulator for xylanolytic enzymes) and CreA (catabolite repressor protein) were also identified. Ogasawara et al. (2006) characterized a xylanase gene, *xyn3*, isolated from genomic libraries of *Trichoderma reesei* constructed in *E. coli* XL1-Blue MRA (P2). They found three introns in the gene. The 5′ noncoding region of the gene contained the TATA box, CCAAT box, and binding sequences for ACEI, ACEII, and CREI. The 3′ end noncoding region had the AATAAA polyadenylation site. Characterization of the noncoding regions of the gene and deletion analysis of the *xyn3* promoter helped to investigate the mechanism of xylanase induction and repression under different carbon sources. Comparison of the cDNA and genomic DNA clones for *xynA* gene from *A. pullulans* revealed that a single intron of 59 bp was present in the gene (Li and Ljungdahl 1994). The *xynA* gene from *T. lanuginosus* was interrupted by a single 106-bp long intron (Schlacher et al. 1996). The 5′ noncoding region of the gene also had conserved TATAAA and CCAAT sequences 89 and 202 bp, respectively, upstream of the translation initiation codon. The *xynII* gene from *A. usamii* also contained one short intron (Zhou et al. 2008).

Detection of xylanase activity

The xylanase activity in *E. coli* cells transformed with the xylanase genes is detected on agar plates by the Congo red method of Teather and Wood (1982). Halos around the extracts from transformed cells showed xylanase activity. Alternatively, xylan covalently linked with remazol brilliant blue (RBB-xylan) was used to detect the xylanase activity in the transformed cells (Basaran et al. 2001). Xylanase activity is also detected by Western blot analysis using antibodies raised against the xylanase (Diaz et al. 1997).

Expression in *E. coli*

cDNA clones of the fungal xylanases are expressed in *E. coli* as intron processing is absent in the host. In many cases, the recombinant xylanases expressed in *E. coli* accumulate in the cytoplasm or periplasm (Schlacher et al. 1996), although extracellular activity has also been reported (Karlsson et al. 1998; Ebanks et al. 2000). Level of gene expression depends on the efficiency of transcription that is a function of the promoter sequence (Youderian et al. 1982). Several expression vectors and host strains are available for heterologous gene expression in *E. coli*. Eukaryotic genes are usually not expressed in *E. coli* due to the lack of a functional promoter. Basaran et al. (2001) expressed a β-xylanase from *Pichia stipitis* under its own promoter in *E. coli*, although the enzyme activity was significantly lower (4 U mg^{-1}) compared to the activity from the parent strain (30 U mg^{-1}). pET expression vector systems are among the most effective means of expression

of recombinant proteins in *E. coli*. Cloned genes are expressed under the control of a bacteriophage T7 promoter which remains silent until the expression of a chromosomal copy of T7 RNA polymerase gene in *E. coli* is induced (Studier and Moffatt 1986). Zhou et al. (2008) cloned the *xynII* cDNA of *A. usamii* into the pET-28a(+) expression vector and transformed into *E. coli* BL21-Codon plus (DE3) RIL. They obtained maximum activity of 49.4 U mg^{-1} from cellular extracts of *E. coli* BL21-Codon plus (DE3) RIL harboring pET 28a::*xynII* after induction with isopropyl β-D-1-thiogalactopyranoside (IPTG). The recombinant protein also had His-tag for further purification. A β-xylanase gene, *xyn2*, from *T. reesei* was also expressed in *E. coli* using the pET expression vector (Jun et al. 2008) with expression level up to 650 U mg^{-1}. The *tac* promoter is also used in some vectors for enhanced expression. An RT-PCR amplified *xynIII* gene from *T. reesei* was cloned in the pAG9-3 vector under *tac* promoter and expressed in *E. coli* JM109 with IPTG induction (Ogasawara et al. 2006). The enzyme activity detected in the cellular extract was very low (26 mU mL^{-1} medium). However, when the inclusion bodies were refolded in 8 m urea solution, the XynIII activity raised to about 500 times higher (13.2 U mL^{-1} medium) compared to the soluble supernatant. The enzyme activity may be enhanced by modification of the corresponding genes. Xue et al. (1995) isolated a *xynA* gene from anaerobic fungus *Neocallimastix patriciarum* and modified it for high-level expression in *E. coli*, accounting for approximately 25% of the cellular proteins.

Recombinant xylanases

E. coli is generally used as a cloning host for fungal xylanases due to lower expression levels that may be due to the fact that enzyme activity is mostly associated with the cells rather than secretion in the culture medium. Absence of post-translational modifications may also be a reason for the low levels of activity of the resulting xylanases (Wong et al. 1988). Lack of glycosylation in *E. coli* is an important factor that accounts for lower affinity of the enzyme with substrate and decrease in stability of the enzyme. The recombinant nonglycosylated XynA expressed from *E. coli* showed three-fold lower affinity for the substrate and tenfold lower V_{max} compared to the glycosylated enzyme isolated from *P. stipitis* (Basaran et al. 2001). The recombinant enzyme was also less stable than the native xylanase at the same temperature. The recombinant Xyn2 expressed in *E. coli*, however, had improved thermostability and was active over a wider range of pH compared to the native enzyme from *T. reesei* (Jun et al. 2008). The apparent K_m and k_{cat} for the recombinant Xyn2 were also higher than the native enzyme.

Suitable fermentation strategies need to be developed for the use of *E. coli* as a production host of xylanases at the industrial level. Although several yeast and fungi are used as expression hosts for fungal xylanase gene expression, *E. coli* will continue to be used as a cloning host, since it provides a convenient way for elucidation of primary structure of the enzymes that may lead to the improvements in the enzyme characteristics by point mutations and by protein engineering approach.

Cloning in yeast

Cloning in *Saccharomyces cerevisiae*

Several fungal xylanase genes have been cloned and expressed in *Saccharomyces cerevisiae* (Table 2). It is an attractive host for the expression of heterologous proteins (Das and Shultz 1987) including β-xylanases (Romanos et al. 1992). It provides an efficient post-translational processing such as glycosylation, proper folding of the proteins, proteolysis, etc. (Innis et al. 1985; Sa-Pereira et al. 2003). Another advantage of *S. cerevisiae* as a host is that it secretes only a few proteins; therefore, purification of the expressed proteins is easier (Das and Shultz 1987).

Xylanase genes for expression in S. cerevisiae

The expression of fungal xylanase genes in *S. cerevisiae* is achieved mainly by cloning cDNA obtained by RT-PCR. Presence of introns in the genes hinders their heterologous expression in yeast due to lack of proper splicing of introns (Moreau et al. 1992). Li and Ljungdahl (1996) compared the xylanase activity of *S. cerevisiae* clones expressing the gene *xynA* from *A. pullulans*. Significantly higher level of xylanase expression was found from the yeast transformed with the *xynA* gene without intron (26.2 U mL^{-1}) compared to the expression from the gene with intron (16.7 U mL^{-1}). Chavez et al. (2002) made the first successful attempt to express the *xynA* gene isolated from the genomic DNA library of *P. purpurogenum* in *S. cerevisiae*. The *xynA* gene, including its eight introns, was integrated into the yeast genome and spliced correctly. The recombinant clone was expressed and secreted in culture supernatant of *S. cerevisiae* under transcriptional control with glucose acting as repressor and xylose or xylan as inducers. Presence of binding site for XlnR (xylanolytic activator protein) and three binding sites for CreA (catablite repressor protein) upstream of the start codon might be responsible for this control (van Peij et al. 1998). The 5′ noncoding region of other fungal xylanase genes has also been studied and similar sites were found (Ito et al. 1992; Ohta et al. 2001). The *xyn1* gene from *Cryptococcus flavus*, when expressed in yeast, was induced in the presence of xylose and repressed in the presence of glucose (Parachin et al. 2009).

Table 2 Cloning of different fungal xylanase genes in yeast

Source organism	Gene	Vector	Host	Molecular mass of the recombinant enzyme (kDa)	Characteristics of the recombinant enzyme (pH, temperature, K_m, V_{max}) [secretion level, enzyme activity] {stability}	Reference
Aspergillus niger	*anxA*	pPIC9K	*P. pastoris* GS115	20	(5, 50, 4.8 mg mL^{-1}) k_{cat}=123.2 s^{-1} [175 U mg^{-1}] {At pH3.0–8.0}	Liu et al. 2006
Aspergillus niger	*xylA*	pHIL-D2	*P. pastoris* GS115	30	(3.5, 50, 12.6 mg mL^{-1}) k_{cat}=150 s^{-1} {90% after 3 h at 50°C}	Berrin et al. 2000
Aspergillus niger BCC14405	*xylB*	pPICZαA	*P. pastoris* KM71	21	(5.0, 55°C) [3,676 U mL^{-1}]	Ruanglek et al. 2007
Aspergillus niger CGMCC1067	*xynB*	pGAPZαA	*P. pastoris* X33	21, 30, 35	(5.0, 50) [62 IU mL^{-1}] {95% at 37–41°C}	Deng et al. 2006
Aspergillus niger IBT-90	*xyn6*	pPICZB	*P. pastoris* GS115	20	(pH3.5) [180 mg L^{-1} with α-MF signal, 220 mg L^{-1} with own signal]	Korona et al. 2006
	xynB	pPICZαA		17.5	(pH5.0) [140 mg L^{-1} with α-MF signal, 150 mg L^{-1} with own signal]	
Aspergillus sulphureus	Endo-β-1, 4-xylanase	pGAPZαA	*P. pastoris* X33		(2.4–3.4, 50) [120 U mL^{-1}] {>70% at 80°C for 30 min}	Cao et al. 2007
Aspergillus terreus BCC129	*xyn10*	pPICZαA	*P. pastoris* KM71	33	(5.0, 60, 4.8 mg mL^{-1}, 757 μmol min^{-1} mg^{-1}) [238.5 mg L^{-1}] {pH4–10 at 40°C, 4 h; 90% at 50°C, 30 min}	Chantasingh et al. 2006
Aureobasidium pullulans ATCC20524	*xynII*	pPIC3.5	*P. pastoris* GS115	39	[36 mg L^{-1}]	Tanaka et al. 2006
Aureobasidium pullulans	*xyl6*	pPIC3.5 pHILS1 pPIC9	*P. pastoris* GS115	24	(2.5, 40) [178 mg L^{-1}]	Tanaka et al. 2004
Cryptovalsa mangrovei BCC7197	Xylanase 10	pPICZα A (Invitrogen)	*P. pastoris* KM71	33		Boonyapakron et al. 2005
Lentinula edodes CS-2	*xyn11A*	pGAPZαA	*P. pastoris* GS115	29.5	(4.5, 50, 1.5 mg mL^{-1}, 2.1 mmol min^{-1} mg^{-1})	Lee et al. 2005
Magnaporthe grisea CP987	*xyl6*	pPicH	*P. pastoris*	47	[75–100 mg L^{-1}]	Wu et al. 2006
Neocallimastix frontalis	*Xyn11B*	pPK9K	*P. pastoris* KM71			Tsai and Huang 2008
Penicillium citrinum	*xynA*	pPIC3.5	*P. pastoris* GS115			Tanaka et al. 2005

Penicillium citrinum FERM P-15944	*xynB*	pPIC9	*P. pastoris* GS115	32.6	[5.74 U mL^{-1}]	Wakiyama et al. 2008
Plectosphaerella cucumerina HB174	*xynZG*	pHBM905B	*P. pastoris* GS115	19	(6.0, 40, 2.06 mg mL^{-1}, 0.49 mmol min^{-1} mg^{-1}) [362 U mL^{-1}]	Zhang et al. 2007
Scytalidium acidophilum ATCC 26774	*xyl1*	pPICZαA	*P. pastoris* X-33	25	(3.2, 56, 14.8 mg mL^{-1}, 270.4 μmol min^{-1} mg^{-1})	Balaa et al. 2006
Thermobifida fusca NTU22	*xynA*	pPICZα A	*P. pastoris* KM71H	36	(7.0, 70) [324.2 U mL^{-1}] {70% at 70°C for 3 h}	Cheng et al. 2005
Thermomonospora fusca	Xylanase A (*TfxA*)	pPIC9K	*P. pastoris* GS115	31	(6.0, 60, 2.45 mg mL^{-1}) k_{cat}=139 s^{-1} [117.3 U mg^{-1}] {Stability over wide pH range 5–9}	Sun et al. 2007
Thermomyces lanuginosus IOC-4145	*xynA*	pPIC9	*P. pastoris* GS115	26.9	(75°C) [148 mg mL^{-1}, 40.2 U mL^{-1}] {60% at 70°C after 80 min}	Damaso et al. 2003
Trichoderma reesei	*xyn2*	pRDH12	*P. stipitis* TJ26		[136.7 nkat mL^{-1}]	Den Haan & van Zyl 2003
Aspergillus kawachii	*xynC*	pRDH16	*P. stipitis* TJ26		[171.8 nkat mL^{-1}]	Den Haan & van Zyl 2003
Aspergillus kawachii IFO4308	*xynA*	pG3	*Saccharomyces cerevisiae* DBY747			Ito et al. 1992
Aspergillus kawachii IFO4308	*xyn3*	Multicopy episomal plasmid	*Saccharomyces cerevisiae*		(3.0, 60) [300 nkat mL^{-1}] {30–50°C}	Crous et al. 1995
Aspergillus nidulans G191	*xlnA* *xlnB*	Yeplac181	*Saccharomyces cerevisiae* OL1			Perez-Gonzalez et al. 1996
Aspergillus niger ATCC 90196	*xyn4* *xyn5*	pDLG1	*Saccharomyces cerevisiae* Y294		(4, 60) (4, 60)	Luttig et al. 1997
Aureobasidium pullulans	*xynA*	pYES2	*Saccharomyces cerevisiae* INSC1	25, 27	[28.6 U mL^{-1}]	Li and Ljungdahl 1996
Aureobasidium pullulans var. *melanigenum* ATCC 20524	*xyn1*	pYES2	*Saccharomyces cerevisiae* INVSc1			Ohta et al. 2001
Cryptococcus flavus I-11	*Cfxyn1*	Yep351PGK	*Saccharomyces cerevisiae* MFL	21.2	(3.0, 50) [2.5 U mL^{-1}] {80% at pH2.0 and 44% at pH6.0; 70% at 50°C}	Parachin et al. 2009
Penicillium purpurogenum ATCC MYA-38	*xynA*	pYEplac181	*Saccharomyces cerevisiae* YM335::RY171			Chavez et al. 2002
Trichoderma reesei QM 9414	*xyn1* *xyn2*	pTL2M-2	*Schizosaccharomyces pombe*			Okada et al. 1999
Trichoderma reesei QM6a	*xyn2*	pDLGI	*Saccharomyces*		(4, 60)	La Grange et al.

Table 2 (continued)

Source organism	Gene	Vector	Host	Molecular mass of the recombinant enzyme (kDa)	Characteristics of the recombinant enzyme (pH, temperature, K_m, V_{max}) [secretion level, enzyme activity] {stability}	Reference
			cerevisiae Y294		[1,200 nkat mL^{-1} with *ADH2* promoter; 160 nkat mL^{-1} with *PGK1* promoter] {79% at 50°C after 60 min}	1996
Trichoderma reesei QM6a	*xyn2*	*URA3*-based multicopy shuttle vectors	*Saccharomyces cerevisiae* Y294		[1,577 nkat mL^{-1} xylanase only]	La Grange et al. 2001
Aspergillus niger ATCC 90196 (coexpression)	*xlnD* (xylosidase)				[860 nkat mL^{-1} xylanase activity when coexpressed]	

Promoters in S. cerevisiae

The xylanase genes are generally expressed in *S. cerevisiae* under glycolytic promoters, such as alcohol dehydrogenase I and II (*ADHI* and *ADHII*) and phosphoglycerate kinase (*PGK*), that are the most powerful promoters of *S. cerevisiae* (Romanos et al. 1992). *ADHII* is an inducible promoter and is almost undetectable when the yeast is grown on sugars like glucose or galactose, but derepressed to significantly higher levels of xylanase expression when it is grown on nonfermentable sugars at concentrations lower than 1% (La Grange et al. 1996). Inducible expression of xylanase genes under *ADHII* promoter was also found by Luttig et al. (1997). La Grange et al. (2001) coexpressed the xylosidase gene (*xlnD*) from *A. niger* and *xyn2* gene from *T. reesei* under the *ADHII* promoter in *S. cerevisiae*. La Grange et al. (2000) also coexpressed a *Bacillus pumilus* β-xylosidase gene with the *T. reesei* β-xylanase gene in the yeast under the same promoter. The *PGK1* is a strong constitutive promoter that can give a high level of expression constituting 4% to 10% of the total soluble proteins (Hitzeman et al. 1982). This promoter is also used for xylanase gene expression in the yeast (Crous et al. 1995; Parachin et al. 2009). La Grange et al. (1996), however, obtained significantly less xylanase activity with the *PGK1* promoter (160 nkat mL^{-1}) compared to the enzyme activity under the *ADHII* promoter (1,200 nkat mL^{-1}). The promoters of the galactose-regulated genes (*GAL*) are among the most powerful tightly regulated promoters of *S. cerevisiae* (Romanos et al. 1992) and have also been used in xylanase gene expression (Li and Ljungdahl 1996). Another yeast promoter, glyceraldehyde-3-phosphate dehydrogenase (*GPD*), is also employed for the expression of the xylanase genes in *S. cerevisiae* (Ito et al. 1992). The actin gene promoter with constitutive expression has also been reported by Perez-Gonzalez et al. (1996) for the expression of xylanase genes from *A. nidulans* in *S. cerevisiae*.

Terminators of transcription

Effective termination of transcription is also needed for maximal gene expression. The *ADH2* and *PGK1* terminators are among the most commonly used termination signals in the expression cassettes expressing xylanase genes in *S. cerevisiae* (Luttig et al. 1997; La Grange et al. 2000; Parachin et al. 2009).

Secretion of xylanases from S. cerevisiae

Efficient secretion of the enzymes is necessary for achieving maximal expression from the yeast cells and for correct folding and stability of the proteins. Most of the

secreted yeast proteins have hydrophobic short signal peptides at the N-terminal regions of protein precursors that are cleaved off by specific peptidases during the secretion process (Li and Ljungdahl 1996). The leader peptides translocate the proteins to the endoplasmic reticulum from where these are mostly glycosylated. β-Xylanase (La Grange et al. 1996) and XynA (Li and Ljungdahl 1996) secreted from *S. cerevisiae* were hyper-glycosylated compared to the enzymes secreted from their parent hosts. Li and Ljungdahl (1996) compared the efficiency of a native XynA signal peptide from *A. pullulans* with the two most commonly used signal peptides (invertase and α-factor) from *S. cerevisiae*. They found similar levels of xylanase-specific mRNA in Northern blot analysis; however, the XynA signal peptide was found more capable of translocating xylanase out of the yeast cells compared to the *S. cerevisiae* signals.

Detection of xylanase activity

LEU2 or *URA3* are generally used as selectable markers for the *S. cerevisiae* transformations. The yeast strains transformed with the plasmids containing the complementing functional genes have been found to grow on uracil-deficient (La Grange et al. 1996; Perez-Gonzalez et al. 1996; Luttig et al. 1997) or leucine-deficient (Chavez et al. 2002; Parachin et al. 2009) medium. The *FUR1* gene of *S. cerevisiae* encoding uracil phosphoribosyl transferase is disrupted; hence, uridine-5-phosphate cannot be synthesized. Such recombinant strains are transformed with the yeast vectors containing the *URA3* gene for uridine-5-phosphate. The recombinant clones growing on the uracil-deficient or leucine-deficient plates are tested for xylanase activity by plate assay either by using oat spelt xylan or 4-*O*-methyl-D-glucurono-D-xylan-RBB-xylan as substrate. The positive transformants show clear halos around the colonies.

Although secretion levels of xylanases from other yeasts are found higher than from *S. cerevisiae* (Muller et al. 1998), it can be used as a producer of xylanases at the industrial level due to some attributes. It cannot degrade cellulose or other polymers in wood, thus β-xylanases produced from the recombinant strains are free from contaminating cellulases, important for use in paper industry (La Grange et al. 1996). *S. cerevisiae* can be grown on inexpensive culture media to produce β-xylanases, thus economical for industrial production. The pH and temperature optima for the recombinant β-xylanases produced by *S. cerevisiae* are also found suitable for their use in many industries.

Cloning in *Pichia pastoris*

Pichia pastoris is able to perform many eukaryotic post-translational modifications and, thus, is considered as an excellent host system for heterologous protein expression (Cheng et al. 2005). Heterologous gene expression by *P. pastoris* is a useful alternative when scaling up to industrial process (Berrin et al. 2000) and has several advantages over *S. cerevisiae* as a host for heterologous expression because of high secretion efficiency, high cell densities attained in inexpensive culture media, and the relative ease of scale up of industrial process (Cregg 1999). It is a particularly attractive expression host due to the availability of strong and regulatory promoters that are involved in methanol metabolism (Gellissen 2000; Tsai and Huang 2008).

Promoters in P. pastoris

P. pastoris is a methylotrophic yeast capable of metabolizing methanol. The enzymes involved in methanol metabolism are present at substantial levels only when the cells are grown on methanol (Cereghino and Cregg 2000). Alcohol oxidase (AOX) is the enzyme that catalyzes the first step in the methanol utilization pathway (Egli et al. 1980). It is encoded by two genes in *P. pastoris*: *AOX1* and *AOX2*. The *AOX1* gene accounts for the majority of AOX activity in the cell (Cregg et al. 1989). *AOX1*, therefore, is a promoter of choice by most researchers for the expression of xylanase genes in *P. pastoris* as it gives high levels of xylanase expression under methanol induction. Ruanglek et al. (2007) could achieve an enzyme activity of 3,676 U mL^{-1} for the gene product of *xylB* from *A. niger* when expressed under *AOX1* in *P. pastoris*. In fact, this is the highest expression of any recombinant xylanase expressed from *P. pastoris* reported so far. Similarly, Cheng et al. (2005) and Chantasingh et al. (2006) also attained high xylanase activity (342.2 U mL^{-1} and 238.5 mg mL^{-1}, respectively) under this promoter (67-fold and four-fold higher recombinant xylanase activity, respectively, compared to the native fungal xylanases). However, this promoter may not be suitable under certain industrial applications. For instance, it is not appropriate to use methanol for the induction of genes in the food industry due to its toxicity. Moreover, it may be a potential fire hazard in large-scale fermentations. A constitutive *GAP* (glyceraldehyde-3-phosphate dehydrogenase) gene promoter has been employed successfully for the xylanase gene expression in *P. pastoris* by some workers (Lee et al. 2005; Deng et al. 2006; Cao et al. 2007). Deng et al. (2006) obtained 50-fold increase in XynB expression under this promoter compared to the expression of the enzyme by the native species *A. niger*.

Vectors for P. pastoris

The expression vectors for xylanase gene expression in *P. pastoris* are designed as *E. coli*/*P. pastoris* shuttle vectors

having origin of replication and selection markers for propagation in both of the hosts (Cereghino and Cregg 2000). A shuttle vector pPICZαA is the vector of choice by most of the workers and contains tightly regulated *AOX1* promoter and the *S. cerevisiae* α-factor secretion signal (Ruanglek et al. 2007). The xylanase genes under *GAP* promoter are expressed in the pGAPZαA vector (Deng et al. 2006). Another vector, pPIC9, has promoter and terminator of *AOX1* gene, α-M signal peptide sequence (see also the "Secretion of xylanases from *P. pastoris*" section) and *HIS4* selectable marker (Wakiyama et al. 2008). Wu et al. (2006) incorporated His_6tag in the pPICH vector to facilitate purification of the Xyl6 by one-column chromatography.

Transformation and detection of xylanase activity

The vectors with cloned xylanase genes are mostly transformed into host strains by electroporation, although spheroplast and polyethylene glycol (PEG)-mediated transformations are also reported (Berrin et al. 2000; Zhang et al. 2007). The *P. pastoris* strain GS115 (*his4*) has a mutated *HIS4* gene that allows for the selection of the corresponding expression vectors (pPIC and pHIL series). Most of the *P. pastoris* strains have Mut^+ (methanol utilization) phenotype; however, His^+Mut^s (methanol utilization slow) phenotype transformants have also been used for xylanase expression (Berrin et al. 2000; Liu et al. 2006; Sun et al. 2007). Other *P. pastoris* strains for xylanase gene expression are KM71, KM71H, and X33 that accommodate pPICZαA and pGAPαA vectors with zeocin-resistant gene as selection marker. The integration of the xylanase gene into the genome of *P. pastoris* is determined by PCR. The positive transformants for xylanase activity are tested on agar plates containing RBB-xylan or Congo red staining (Boonyapakron et al. 2005; Damaso et al. 2003; Lee et al. 2005; Zhang et al. 2007).

Secretion of xylanases from P. pastoris

Secretion of heterologous proteins to culture medium serves as a substantial step in their purification and further utilization. Secretion level of the endogenous proteins is very low in *P. pastoris*. Therefore, secretion signals are added to the N terminus of the heterologous proteins expressed in the yeast. Alpha mating factor (α-MF) signal peptide from *S. cerevisiae* is the most widely used secretion signal for xylanase expression from *P. pastoris*. It consists of a 19-amino acid (pre)sequence followed by 66-residue (pro)sequence (Kurjan and Herskowitz 1982). Three N-linked glycosylation sites and a Kex2 endopeptidase processing site are present on the (pro)sequence. Other *S. cerevisiae* secretion signals such as acid phosphatase (*PHO1*; Tanaka et al. 2004) and sucrose-proton symporter 2 (*SUC2*; Berrin et al. 2000) have also been used efficiently for the secretion of xylanases in the yeast. Native leader sequences of fungal xylanases also deliver high secretion levels of the enzymes from *P. pastoris*. The activities of Xyn6 and XynB from *A. niger* secreted from *P. pastoris* were 73% and 89% lower with α-MF signal peptide compared to their native secretion signals, respectively (Korona et al. 2006). The amount of recombinant Xyn1 secreted into the culture medium of *P. pastoris* under the native signal was found comparable to that secreted with α-MF signal peptide and two-fold higher than that secreted by *PHO1* signal peptide (Tanaka et al. 2004). The use of native secretion signals allow for correct processing of the proteins because the accordance of the ORF of mature proteins and foreign leader peptides is not required, if native signals are used (Korona et al. 2006).

Characteristics of the recombinant xylanases

The recombinant xylanases expressed from *P. pastoris* generally exhibit similar or even better characteristics compared to the enzymes produced from native species. It serves as an excellent mesophilic host for the expression of thermophilic xylanases. A thermostable xylanase from *T. lanuginosus* showed similar thermostability when expressed in *P. pastoris* (Damaso et al. 2003). Similarly, the recombinant xylanases expressed by Cheng et al. (2005) exhibited high optimal temperature and thermostability. The recombinant xylanase from *Thermomonospora fusca* also showed higher optimal temperature; however, it was less stable than the native enzyme (Sun et al. 2007). Many of the recombinant xylanases had optimal pH in acidic range (Table 2). The acidophilic stability of the enzymes is of significance for their use in animal feeds that must survive transit through the stomach having low pH (<3.0). The recombinant xylanase expressed by Liu et al. (2006) not only showed high activity at low pH, but was also found stable over a broad pH range compared to the native xylanase. Specific activity and kinetic parameters of the recombinant xylanases expressed in *P. pastoris* also closely resembled their native counterparts (Balaa et al. 2006; Berrin et al. 2000; Liu et al. 2006). *P. pastoris*, therefore, has been proven to provide much higher expression of xylanases with comparable or better characteristics favorable for their use in industry.

Cloning in *Pichia stipitis*

P. stipitis has also been reported as a cloning and expression host for xylanase genes from various fungi. Indigenously, *P. stipitis* produces low levels of xylanase activity. However, the xylanase genes from *T. reesei* and *Aspergillus*

kawachii showed high levels of expression when expressed in *P. stipitis* under the transcriptional control of inducible *P. stipitis* xylose reductase gene (*XYL1*) promoter (Den Haan and van Zyl 2003). Coexpression of the xylanase genes with β-xylosidase gene from *A. niger* enhanced the xylanolytic activity to a significantly high level.

Cloning in fungi

Filamentous fungi are attractive hosts for protein expression because of their natural ability to secrete large amounts of proteins into the medium. Fungi that dominate the market as expression hosts are *A. niger*, *Aspergillus oryzae*, and *T. reesei*. Most of the xylanase genes have been expressed in fungi under homologous system.

Cloning in *Aspergillus*

Currently, there are only a few reports on heterologous production of fungal xylanases in *Aspergillus* (Table 3).

Cloning in Aspergillus oryzae

The xylanase genes from *A. oryzae* have been cloned and expressed mainly to control the browning of soy sauce (Kitamoto et al. 1998; Kimura et al. 2002). The *xynF1* gene was overexpressed under a strong *A. oryzae TEF1* gene promoter that gives high expression levels even in the presence of glucose (Kitamoto et al. 1999). Another *A. oryzae* promoter, P-No 8142, is reported to be a strong promoter that is also activated in the presence of glucose. The *xynF3* (Kimura et al. 2002) and *xynG2* (Kimura et al. 1998) genes were expressed under this promoter and the products were secreted with the help of the native signals. XynF1, XynF3, and XynG2 homologously expressed in *A. oryzae* had characteristics similar to other fungal xylanase. The characteristics of the recombinant xylanases, together with high secretion levels, make the expression system attractive for the industrial production of the enzymes. Promoters of the genes *xynF1* and *xynF3* were analyzed by Kimura et al. (2002). The *xynF1* gene was induced in the presence of xylan and repressed in the presence of glucose, whereas no such induction/repression mechanism was found for *xynF3*. Sequence analysis of the 5′ noncoding region of the *xynF1* gene revealed XlnR and CreA consensus binding sites that might be responsible for the induction/repression of the gene. No such sequence could be found for the *xynF3* gene. Similar binding sites were also found in the 5′ noncoding region of the *xynG2* gene (Kimura et al. 1998). Introduction of multiple copies (64) of the *xynF1* gene promoter in the promoter region of the xylanase gene in *A. oryzae* titrated the transacting regulatory proteins that led to repression in the xylanase activity (Kitamoto et al. 1998).

Cloning in A. niger

A. niger is an attractive organism for homologous and heterologous expression of genes due to its capacity to produce and secrete large amounts of proteins (up to 30 g L^{-1}) in the culture medium (Hessing et al. 1994). The *xyn2* and *eglI* genes of *T. reesei* were successfully expressed in *A. niger* D15 under the transcriptional control of the glyceraldehyde-6-phosphate dehydrogenase promoter from *A. niger* and *glaA* terminator of *Aspergillus awamori* (Rose and van Zyl 2002). The Xyn2 enzyme showed the highest activity at pH5–6 and temperature 50–60°C and retained more than 75% of its activity after 3 h of incubation at 50°C. Levasseur et al. (2005) cloned *xynB* cDNA of *A. niger* under the control of the strong and constitutive glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) gene promoter from *Aspergillus nidulans*. A signal sequence and prosequence were present in the cDNA for secretion of the gene. The recombinant plasmids were transformed into *A. niger* D15 for homologous expression. The uridine prototype transformants ($pyrG^+$) were selected on selective medium. Maximum xylanase activity of 625 U mL^{-1} was detected on day 6 by the best transformant. Conclusively, the homologous gene expression in *A. niger* was found to be highly efficient with 6 days incubation time, compatible with fast and large-scale production of enzymes. The kinetic parameters of the recombinant xylanase also favor their subsequent use in industrial processes. The *xynB* gene from *Phanerochaete chrysosporium* yielded the highest (4.5-fold) xylanase activity compared to the expression of the *xynA* and *xynC* genes in *A. niger* (Decelle et al. 2004). Resistance to high temperatures, stability over a wide pH range, and relatively higher substrate specificity of the XynB makes it a potential candidate for use in the biobleaching process.

Cloning in Aspergillus nidulans and Aspergillus awamori

A. nidulans is also used as a host for the heterologous expression of xylanase genes. The xylanase genes were introduced into *A. nidulans* G191 by transformation with the *Neurospora crassa pyr4* gene as a primary selection marker. The *cgxA* gene from *C. gracile* (Yoshino et al. 1995) and *xynG1* from *A. oryzae* (Kimura et al. 1998), when expressed under their own promoters in *A. nidulans*, showed similar induction/repression in the presence of xylan and glucose as discussed above for *xynF1* gene expressed in *A. oryzae* (Kimura et al. 2002). The *cgxB* gene appeared to be expressed constitutively under its own promoter in *A. nidulans*. Xylanase activity (up to 40-fold) was enhanced by the introduction of multiple copies of

Table 3 Cloning of different fungal xylanase genes in fungi

Source organism	Gene	Vector	Host	Molecular mass of the recombinant enzyme (kDa)	Characteristics of the recombinant enzyme (pH, temperature, K_m, V_{max}) [secretion level, enzyme activity] {stability}	Reference
Aspergillus oryzae KBN 616	*xynG2*	pNAN8142	*Aspergillus oryzae* KBN616-ND1	21	(6.0, 58°C, 7.1 mg mL^{-1}, 123 µmol min^{-1} mg^{-1})	Kimura et al. 1998
Aspergillus oryzae KBN 616	*xynF3*	pNAN8142	*Aspergillus oryzae* KBN616-ND1	32	(5.0, 58°C, 6.5 mg mL^{-1}, 435 µmol min^{-1} mL^{-1})	Kimura et al. 2002
Aspergillus oryzae KBN 616	*xynF1*	pXPR64 (pUC118 based)	*Aspergillus oryzae* KBN616-39			Kitamoto et al. 1998
Aspergillus oryzae KBN 616	*xynF1*	pTFXF200 (pUC19 based)	*Aspergillus oryzae* KBN616-39	35	(5.0, 60) [180 mg L^{-1}]	Kitamoto et al. 1999
Aspergillus oryzae KBN 616	*xynGI*	pDJB1	*Aspergillus nidulans* G191			Kimura et al. 1998
Aspergillus awamori ATCC11358	*exlA*	pAW14S	*Aspergillus awamori*			Hessing et al. 1994
Aspergillus niger BRFM281	*xynB*	pAN52.3	*Aspergillus niger* D15#26	23	(5.5, 50, 7.1 mg mL^{-1}, 3,881 U mg^{-1}) [900 mg L^{-1}]	Levasseur et al. 2005
Aspergillus niger biAI	*xlnD*	pUC18 (pXDEI) pGW635	*Aspergillus nidulans* G191			Perez-Gonzalez et al. 1998
Chaetomium gracile IFO6568	*cgxA* *cgxB*	pDJB1	*Aspergillus nidulans* G191			Yoshino et al. 1995
Phanerochaete chrysosporium RP78	*xynA* *xynB*	ANEp2	*Aspergillus niger* N593	52 32	(4.5, 70, 3.42 mg mL^{-1}) (4.5, 60, 9.96 mg mL^{-1})	Decelle et al. 2004
	xynC			50	(4.5, 70, 3.71)	
Acrophialophora nainiana	*xyn6*	pHEN11 exp (pUC19-based)	*Trichoderma reesei* Rut C-30	19	172 mg L^{-1} (secretion level)	Salles et al. 2007
Chaetomium thermophilum CBS730.95	*Ctxyn11A*	pUC19	*Trichoderma reesei* ALK04468	27	(6, 70°C) [148 ukat/mL, 9.2 mg mL^{-1}] {>90% at 80°C, pH5–6}	Mantyla et al. 2007
	Ctxyn11B			23	(6, 70°C) [57.7 µkat mL^{-1}] {<40% at 80°C}	
	Ctxyn11C			22	[1.4 7 µkat mL^{-1}]	
Humicola grisea var. *thermoidea*	*xyn2*	pHEN	*Trichoderma reesei* HEP1		(6.5, 70°C) [500 mg L^{-1}, 12,700 nkat mL^{-1}]	de Faria et al. 2002
Orpinomyces sp. PC-2	*xynA*	pT3C	*Trichoderma reesei* Rut C-30	28	150 mg L^{-1} (secretion level) 1,250–1,700 s^{-1} (k_{cat})	Li et al. 2007
Trichoderma reesei	*xln2*	pBluescript, pUC19	*Trichoderma reesei*			Saarelainen et al. 1993
ALK02721					[3,700 nkat mL^{-1}]	
ALK02221					[3,800 nkat mL^{-1}]	
VTT-D-79125					[10,000 nkat mL^{-1}]	
Cochliobolus carbonum	*xyl2* *xyl3*	pXLB37-2 pHYG2	*Cochliobolus carbonum* XYL mutant strain			Apel-Birkhold and Walton 1996
Cochliobolus carbonum	*xyl1*	pCC167	*Cochliobolus carbonum* XYL mutant strain	20.8		Apel et al. 1993

β-1,4-xylanase A gene, *exlA*, in *A. awamori* (Hessing et al. 1994). The properties of the recombinant xylanase were found comparable to the wild-type enzyme. These results indicate that the *exlA* promoter may be useful for high-level regulated gene expression in *Aspergillus* species.

Cloning in *Trichoderma*

T. reesei (anamorph *Hypocrea jecorina*) has been used as a host for the production of many homologous and heterologous proteins (Mantyla et al. 1998; Nevalainen et al. 2005). It has been singled out as the most promising organism for the production of enzymes to convert lignocellulosic material into simple sugars (Li et al. 2007). In general, higher production levels have been obtained when the expressed genes were from organisms taxonomically related to the host. This was also true for the expression of xylanases in *T. reesei* (Mantyla et al. 2007).

Attempts have been made by researchers to develop *T. reesei* strains with hyperexpression of xylanases. The transformations in *T. reesei* are mostly done by microparticle bombardment or with the help of PEG. The positive transformants are selected on the basis of acetamidase (*amdS*) selection marker or hygromycin B resistance. A *xln2* gene from *T. reesei* was expressed in the fungus under its own promoter (Saarelainen et al. 1993). The gene was targeted to the *cbhI* locus in the host strain resulting to the CBHI^{-} phenotype; however, the results demonstrated that the integration was not required for enhanced expression under the control of the *xln2* promoter. The gene was expressed in three different strains with respect to enzyme production: high cellulase (VTT-D-79125), high endogenous xylanase (ALKO 2721), and low protease (ALKO 2221). A twofold to fourfold increase in the xylanase activity was observed in the transformants. Maximum expression of the *xln2* gene was found in the host strain with high endogenous xylanase activity. Most of the xylanase genes from other fungi are expressed in *T. reesei* under the control of a strong *cel7A* (cellobiohydrolase I, *cbhI*) promoter. Genes for thermotolerant xylanases can easily be expressed in heterologous fungal hosts like *T. reesei*. A *xyn2* gene (cDNA) from *Humicola grisea* was cloned under the *cbhI* promoter and secretion signal and overexpressed in *T. reesei* (de Faria et al. 2002). Maximum activity was found with avicel as a substrate that might have activated the *cbhI* promoter. Avicel–lactose promoted higher xylanase activity than avicel–cellulose as a carbon source. Genomic copies of three xylanase genes, *Ctxyn11A*, *Ctxyn11B*, and *Ctxyn11C*, from a thermotolerant fungus, *Chaetomium thermophilum*, were expressed under *cel7A* (*cbhI*) promoter and terminator in low-protease mutant strains of *T. reesei* with deleted endogenous endoglucanase I, endoglucanase 2, and cellobiohydrolase 1 (*cel7A*) genes (Mantyla et al. 2007). The amount of recombinant *Ctxyn11A* was significantly higher than the other two xylanases. The xylanase activity for CtXyn11A, CtXyn11B, and CtXyn11C in the culture supernatants of the recombinant strains were about 260-fold, 100-fold, and 2.5-fold higher, respectively, than the host strain. CtXynA was stable at high temperature and neutral pH, the conditions desirable for pulp-bleaching applications. A xylanase gene, *xyn6*, from another thermotolerant fungus *Acrophialophora nainiana* was successfully expressed in *T. reesei* Rut C-30 under *cbh1* promoter and secretion signal (Salles et al. 2007). A xylanase gene, *xynA*, from an anaerobic fungus *Orpinomyces* sp. was expressed in *H. jecorina* under the *cel7A* promoter and terminator (Li et al. 2007). The enzyme was secreted into the culture broth with *cel5A* signal of *H. jecorina*. The original AT-rich *xynA* gene could not be expressed in the host until it was enriched with higher guanine–cytosine content. The recombinant enzyme showed high specific activities against xylans of birch wood, wheat, and oat spelt with highest activity against wheat insoluble xylan (1,710 s^{-1}), whereas the hydrolysis of corn fiber was negligible. The expression of xylanases from anaerobic fungi will have industrial significance as the specific activities of the hydrolytic enzymes from anaerobic fungi are much higher than their aerobic counterparts (Chen et al. 1997), but these fungi cannot be used as industrial enzyme manufacturing hosts due to anaerobic growth requirements and low secretion levels.

Concluding remarks and future prospects

The market demand of xylanases has been significantly increased during the last few decades. The practical application of the enzymes cannot be achieved unless they are available in sufficient quantity. The native production of the enzymes cannot meet the demand due to low yields and incompatibility of the standard industrial fermentation processes with the conditions required for the growth of many microorganisms. Therefore, heterologous expression is the main tool for the production of xylanases at the industrial level.

E. coli does not give efficient expression of xylanases. However, it has been found as a good cloning host for fungal xylanase genes and will continue to be used for the detailed study of the xylanase gene structure and for the improvement of the enzymes by protein engineering. *S. cerevisiae* secretes high amount of xylanases in the culture medium. Since it has already been established as an industrial microorganism, it can be used conveniently for the industrial production of xylanases at low cost. Moreover, the absence of contaminating cellulases makes it an attractive host of the production of xylanases for the paper

industry. *P. pastoris* has emerged as an excellent host for the commercial production of xylanases due to very high expression under its own promoters. However, one of the promoters has limitation for use at a large scale due to health and fire hazards of methanol. Filamentous fungi have also been found as efficient producers of xylanases both by heterologous and homologous gene expression. Their own promoters express the enzymes with high yields.

Optimal temperature, pH stability, and kinetics of xylanases play important roles for their effective utilization. The recombinant xylanases produced by the yeast and fungal strains have been shown to possess equivalent or even better properties than the native enzymes. Thermostable enzymes are important in many industrial applications; however, thermophilic microorganisms cannot be propogated at a large scale due to extreme fermentation conditions. *P. pastoris* and *T. reesei* expressed the genes for thermostable xylanases with high secretion levels. Similarly, the xylanase genes from anaerobic microorganisms are expressed successfully in such hosts that can be employed in the fermentation industry.

There is a possibility of exploring new fungal hosts capable of producing recombinant xylanases. Further technical advancements in the improvement and development of fungal expression systems by genetic engineering approach will help in the hyperexpression of heterologous xylanases for their production and use at the industrial level.

Acknowledgement The authors highly acknowledge the Higher Education Commission, Government of Pakistan for providing the grant to conduct research work on molecular cloning and expression of fungal xylanases.

References

Ahmed S, Aslam N, Latif F, Rajoka MI, Jamil A (2005) Molecular cloning of cellulase genes from *Trichoderma harzianum*. In: Atta-ur-Rehman, Choudhary, Khan (eds) Frontiers in natural product chemistry, vol 1. Bentham Science, The Netherlands, pp 73–75

Ahmed S, Jabeen A, Jamil A (2007) Xylanase from *Trichoderma harzianum*: enzyme characterization and gene isolation. J Chem Soc Pak 29:176–182

Alcalde M, Ferrer M, Plou FJ, Ballesteros A (2006) Environmental biocatalysts: from remediation with enzymes to novel green processes. Trends Biotechnol 24:281–287

Apel P, Panaccione D, Holden F, Walton J (1993) Cloning and targeted gene disruption of XYL1 a β-1,4-xylanase gene from the maize pathogen *Cochliobolus carbonum*. Mol Plant–Microb Interact 6:467–473

Apel-Birkhold P, Walton J (1996) Cloning, disruption and expression of two endo-β-1,4-xylanase genes, *XYL2* and *XYL3*, from *Cochliobolus carbonum*. Appl Environ Microbiol 62:4129–4135

Baba T, Shinke R, Nanmori T (1994) Identification and characterization of clustered genes for thermostable xylan-degrading enzymes, β-xylosidase and xylanase of *Bacillus stearothermophilus* 21. Appl Environ Microbiol 60:2252–2258

Badhan AK, Chadha BS, Kaur J, Saini HS, Bhat MK (2007) Production of multiple xylanolytic and cellulolytic enzymes by thermophilic fungus *Myceliophthora* sp. IMI 387099. Bioresour Technol 98:504–510

Balaa AB, Wouters J, Dogne S, Rossini C, Schaus J, Depiereux E, Vandenhaute J, Housen I (2006) Identification, cloning, and expression of *Scytalidium acidophilum XYLI* gene encoding for acidophilic xylanase. Biosci Biotechnol Biochem 70:269–272

Baneyx F (1999) Recombinant protein expression in *Escherichia coli*. Curr Opin Biotechnol 10:411–421

Basaran P, Hang YD, Basaran N, Worobo RW (2001) Cloning and heterologous expression of xylanase from *Pichia stipitis* in *Escherichia coli*. J Appl Microbiol 90:248–255

Beg QK, Kapoor M, Mahajan L, Hoondal GS (2001) Microbial xylanases and their industrial applications: a review. Appl Microbiol Biotechnol 56:326–338

Bergquist P, Teo'O V, Gibb M (2002) Expression of xylanase enzymes from thermophilic microorganisms in fungal host. Extremophiles 6:177–184

Berrin JG, Wiliamson G, Puigserver A, Chaix JC, McLauchlan WR, Juge N (2000) High level production of recombinant fungal endo-β-1,4-xylanase in the methylotrophic yeast *Pichia pastoris*. Protein Expr Purif 19:179–187

Biely P (1985) Microbial xylanolytic systems. Trends Biotechnol 3:286–290

Boonyapakron K, Pootanakit K, Chantasingh D, Kirtikara K, Eurwilaichitr L (2005) Cloning and expression of xylanase 10 from *Cryptovalsa mangrovei* (BCC7197) in *Pichia pastoris*. DNA Seq 16:372–378

Buchert J, Tenkanen M, Kantelinen A, Viikari L (1994) Application of xylanases in the pulp and paper industry. Bioresour Technol 50:65–72

Cao Y, Qiao J, Li Y, Lu W (2007) De novo synthesis, constitutive expression of *Aspergillus sulphureus* beta-xylanase gene in *Pichia pastoris* and partial enzymic characterization. Appl Microbiol Biotechnol 76:579–585

Cereghino JL, Cregg JM (2000) Heterologous protein expression in methylotrophic yeast *Pichia pastoris*. FEMS Microbiol Rev 24:45–66

Chand S, Mishra P (2003) Research and application of microbial enzymes-India's contribution. Adv Biochem Eng Biotechnol 85:95–124

Chantasingh DK, Champreda PV, Kanokratana P, Eurwilaichitr L (2006) Cloning, expression and characterization of a xylanase 10 from *Aspergillus terrus* (BCC129) in *Pichia pastoris*. Protein Expr Purif 46:143–149

Chavez R, Fierro F, Gordillo F, Martín JF, Eyzaguirre J (2001) Electrophoretic karyotype of the filamentous fungus *Penicillium purpurogenum* and chromosomal location of several xylanolytic genes. FEMS Microbiol Lett 205:379–383

Chavez R, Navarro C, Calderon I, Peorano A, Bull P, Eyzaguirre J (2002) Secretion of endoxylanase A from *Penicillium purpurogenum* by *Saccharomyces cerevisia*e transformed with genomic fungal DNA. FEMS Microbiol Lett 212:237–241

Chen H, Li XL, Ljungdahl LG (1997) Sequencing of a 1,3-1,4-beta-D-glucanase (lichenase) from the anaerobic fungus Orpinomyces strain PC-2: properties of the enzyme expressed in *Escherichia coli* and evidence that the gene has a bacterial origin. J Bacteriol 179:6028–6034

Cheng YF, Yang CH, Liu WH (2005) Cloning and expression of *Thermoibifida* xylanase gene in the methylotrophic yeast *Pichia pastoris*. Enzyme Microb Technol 37:541–546

Choi ID, Kim HY, Choi YJ (2000) Gene cloning and characterization of α-glucuronidase of *Bacillus stearothermophilus* no. 263. Biosci Biotechnol Biochem 64:2530–2537

Collins T, Gerday C, Feller G (2005) Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol Rev 29:3–23

Cregg JM (1999) Expression in methylotrophic yeast *Pichia pastoris*. In: Fernandez JM, Hoeffler JP (eds) Gene expression systems. Academic, New York, pp 157–191

Cregg JM, Madden KR, Barringer KJ, Thill GP, Stillman CA (1989) Functional characterization of the two alcohol oxidase genes from the yeast *Pichia pastoris*. Mol Cell Biol 9:1316–1323

Crous JM, Pretorius IS, van Zyl WH (1995) Cloning and expression of an *Aspergillus kawachii* endo-1,4-β-xylanase gene in *Saccharomyces cerevisiae*. Curr Genet 28:467–473

Dalboge H (1997) Expression cloning of fungal enzyme genes; a novel approach for efficient isolation of enzyme genes of industrial relevance. FEMS Microbiol Rev 21:29–42

Damaso MCT, Almeida MS, Kurtenbach E, Martins OB, Pereira N, Andrade CMMMC, Albano RM (2003) Optimized expression of a thermostable xylanase from *Thermomyces lanuginosus* in *Pichia pastoris*. Appl Environ Microbiol 69:6064–6072

Das RC, Shultz JL (1987) Secretion of heterologous proteins from *Saccharomyces cerevisiae*. Biotechnol Prog 3:43–48

de Faria FP, Teo VSJ, Bergquist PL, Azevedo MO, Nevalainen KM (2002) Expression and processing of major xylanase (XYN2) from the thermophilic fungus *Humicola grisea* var. *thermoidea* in *Trichoderma reesei*. Lett Appl Microbiol 34:119–123

Decelle B, Tsang A, Storms RK (2004) Cloning, functional expression and characterization of three *Phanerochaete chyrsosporium* endo-1,4-β-xylanases. Curr Genet 46:166–175

Degefu Y, Lohtander K, Paulin L (2004) Expression patterns and phylogenetic analysis of two xylanase genes (*htxyl1* and *htxyl2*) from *Helminthosporium turcicum*, the cause of northern leaf blight of maize. Biochimie 86:83–90

Den Haan R, van Zyl WH (2003) Enhanced xylan degradation and utilization by *Pichia stipitis* overproducing fungal xylanolytic enzymes. Enzyme Microb Technol 33:620–628

Deng P, Li D, Cao Y, Lu W, Wang C (2006) Cloning of a gene encoding an acidophilic endo-β-1,4-xylanase obtained from *Aspergillus niger* CGMCC1067 and constitutive expression in *Pichia pastoris*. Enzyme Microb Technol 39:1096–1102

Diaz R, Sapag A, Peirano A, Steiner J, Eyzaguirre J (1997) Cloning, sequencing and expression of the cDNA of endoxylanase B from *Penicillium purpurogenum*. Gene 187:247–251

Ebanks R, Dupont M, Shareck F, Morosoli R, Kluepfel D, Dupont C (2000) Development of an *Escherichia coli* expression system and thermostability screening assay for libraries of mutant xylanase. J Ind Microbiol Biotech 25:310–314

Egli T, van Dijken JP, Veenhuis M, Harder W, Fiechter A (1980) Methanol metabolism in yeast: regulation of the synthesis of catabolic enzymes. Arch Microbiol 124:115–121

Emami K, Hack E (1998) A xylanase gene from *Cochliobolus sativus* (*Bipolaris sorokiniana*). In: Duveiller E, Dubin HJ, Reeves J, McNab A (eds) Helminthsosprium blights of wheat: spot blotch and tan spot. CIMMYT, Mexico, pp 314–321

Emami K, Hack E (2001) Characterization of a xylanase gene from *Cochliobolus sativus* and its expression. Mycol Res 105:352–359

Fang H-Y, Chang S-M, Lan C-H, Fang TJ (2008) Purification and characterization of a xylanase from *Aspergillus carneus* M34 and its potential use in photoprotectant preparation. Process Biochem 43:49–55

Fengxia L, Mei L, Zhaoxin L, Xiaomei B, Haizhen Z, Yi W (2008) Purification and characterization of xylanase from *Aspergillus ficuum* AF-98. Bioresour Technol 99:5938–5941

Gellissen G (2000) Heterologous protein production in methylotrophic yeasts. Appl Microbiol Biotechnol 54:741–750

Giesbert S, Lepping HB, Tenberge KB, Tudzynski P (1998) The xylanolytic system of *Claviceps purpurea*: cytological evidence for secretion of xylanases in infected rye tissue and molecular characterization of two xylanase genes. Biochem Cell Biol 88:1020–1030

Han SO, Yukawa H, Inui M, Doi RH (2003) Regulation of expression of cellulosomal cellulase and hemicellulase genes in *Clostridium cellulovorans*. J Bacteriol 185:6067–6075

Hessing JG, Rotterdam CV, Verbake JM, Roza M, Maat J, Gorcom RFV, Hondel CAVD (1994) Isolation and characterization of a 1,4-β-endoxylanase gene of *A. awamori*. Curr Genet 26:228–232

Hitzeman RA, Hagie FE, Hayflick JS, Chen CY, Seeburg PH, Derynck R (1982) The primary structure of the *Saccharomyces cerevisiae* gene for 3-phosphoglycerate kinase. Nucleic Acids Res 10:7791–7808

Huang YH, Huang CT, Hseu RS (2005) Effects of dockerin domains on *Neocallimastix frontalis* xylanases. FEMS Microbiol Lett 243:455–460

Ilmen M, Thrane C, Penttila M (1996) The glucose repressor gene *cre1* of *Trichoderma*: isolation and expression of a full-length and a truncated mutant form. Mol Gen Genet 251:451–460

Ilmen M, Saloheimo A, Onnela ML, Pentilla ME (1997) Regulation of cellulase gene expression in the filamentous fungus *T. reesei*. Appl Environ Microbiol 63:1298–1306

Innis MA, Holland MJ, McCabe PC, Cole GE, Wittman VP, Tal R, Watt KWK, Gelfand DH, Holland JP, Meade JH (1985) Expression, glycosylation, and secretion of an *Aspergillus* glucoamylase by *Saccharomyces cerevisiae*. Science 228:21–26

Ito K, Ikemasu T, Ishikawa T (1992) Cloning and sequencing of the *xynA* gene encoding xylanase A of *Aspergillus kawachii*. Biosci Biotechnol Biochem 56:906–912

Jamil A, Naim S, Ahmed S, Ashraf M (2005) Production of industrially important enzymes using molecular approaches; cellulases and xylanases. In: Thangadurai D, Pullaiah T, Balatti PA (eds) Genetic resources and biotechnology II, volume two. Regency, New Delhi

Jeffries T (1996) Biochemistry and genetics of microbial xylanases. Curr Opin Biotechnol 7:337–342

Jun H, Bing Y, Zhang K, Ding X, Daiwen C (2008) Expression of a *Trichoderma reesei* β-xylanase gene in *Escherichia coli* and activity of the enzyme on fiber-bound substrates. Protein Expr Purif 67:1–6

Karlsson EN, Dahlberg L, Torto N, Gorton L, Holst O (1998) Enzymatic specificity and hydrolysis pattern of the catalytic domain of the xylanase *xyn*1 from *Rhodothermus marinus*. J Biotechnol 60:23–35

Kaur J, Sharma R (2006) Directed evolution: an approach to engineer enzymes. Crit Rev Biotechnol 26:165–199

Khandeparker R, Numan MT (2008) Bifunctional xylanases and their potential use in biotechnology. J Ind Microbiol Biotech 35:635–644

Kimura T, Kitamoto N, Kito Y, Karita S, Sakka K, Ohmiya K (1998) Molecular cloning of xylanase gene *xynG1* from *Aspergillus oryzae* KBN 616, a Shoyu Koji mold, and analysis of its expression. J Ferment Bioeng 85:10–16

Kimura T, Ito J, Kawano A, Makino T, Kondo H, Karita S, Sakka K, Ohmiya K (2000) Purification, characterization, and molecular cloning of acidophilic xylanase from *Penicillium* sp. 40. Biosci Biotechnol Biochem 64:1230–1237

Kimura T, Suzuki H, Furuhashi H, Aburatani T, Morimoto K, Sakka K, Ohmiya K (2002) Molecular cloning, characterization and expression analysis of the *xynF*3 gene from *Aspergillus oryzae*. Biosci Biotechnol Biochem 66:285–292

Kitamoto N, Yoshino S, Ito M, Kimura T, Ohmiya K, Tukagoshi N (1998) Repression of the expression of genes encoding xylanolytic enzymes in *Aspergillus oryzae* by introduction of multiple copies of the *xyn*F1 promoter. Appl Microbiol Biotechnol 50:558–563

Kitamoto N, Yoshino S, Ohmiya K, Tsukagoshi N (1999) Purification and characterization of the overexpressed *Aspergillus oryzae* xylanase, xynF1. Biosci Biotechnol Biochem 63:1791–1794

Korona B, Korona D, Bielecki S (2006) Efficient expression and secretion of two co-produced xylanases from *Aspergillus niger* in *Pichia pastoris* directed by their native signal peptides and the *Saccharomyces cerevisiae* α-mating factor. Enzyme Microb Technol 39:683–689

Krisana A, Rutchadaporn S, Jarupan G, Lily E, Sutipa T, Kanyawim K (2005) Endo β-1,4-xylanase B from *Aspergillus* cf. *niger* BCC14405 isolated in Thailand: purification, characterization and gene isolation. J Biochem Mol Biol 38:17–23

Kulkarni N, Shendye A, Rao M (1999) Molecular and biotechnological aspects of xylanases. FEMS Microbiol Rev 23:411–456

Kumar R, Singh S, Sing OV (2008) Bioconversion of lignocellulosic biomass: biochemical and molecular prospectives. J Ind Microbiol Biotech 33:377–391

Kurjan J, Herskowitz I (1982) Structure of a yeast pheromone gene (MF alpha): a putative alpha-factor precursor contains four tandem copies of mature alpha-factor. Cell 30:933–943

La Grange DC, Pretorius IS, van Zyl WH (1996) Expression of a *Trichoderma reesei* β-xylanase gene (*xyn2*) in *Saccharomyces cerevisiae*. Appl Environ Microbiol 62:1036–1044

La Grange DC, Claeyssens M, Pretorius IS, van Zyl WH (2000) Co-expression of the *Bacillus pumilus* β-xylosidase (*xynB*) gene with the *Trichoderma reesei* β-xylosidase 2 (*xyn2*) gene in yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 54:195–200

La Grange DC, Pretorius IS, Claeyssens M, van Zyl WH (2001) Degradation of xylan to D-xylose by recombinant *Saccharomyces cerevisiae* coexpressing the *Aspergillus niger* beta-xylosidase (*xlnD*) and the *Trichoderma reesei* xylanase II (*xyn2*) genes. Appl Environ Microbiol 67:5512–5519

Lee CC, Wong DW, Robertson GH (2005) Cloning and characterization of the *xyn11*A gene from *Lentinula edodes*. Protein J 24:21–26

Levasseur A, Asther M, Record E (2005) Overproduction and characterization of xylanase B of *Aspergillus niger*. Can J Microbiol 51:177–183

Li XL, Ljungdahl LG (1994) Cloning, sequencing and regulation of a xylanase gene from *Aureobasidium pullulans* Y-2311-1. Appl Environ Microbiol 60:3160–3166

Li XL, Ljungdahl LG (1996) Expression of *Aureobasidium pullulans xynA* in, and secretion of the xylanase from, *Saccharomyces cerevisiae*. Appl Environ Microbiol 62:209–213

Li XL, Skory CD, Ximenes EA, Jordan DB, Dien BS, Hughes SR, Cotta MA (2007) Expression of an AT-rich xylanase gene from the anaerobic fungus *Orpinomyces* sp. strain PC-2 in and secretion of the heterologous enzyme by *Hypocrea jecorina*. Appl Microbiol Biotechnol 74:1264–1275

Liu MQ, Wang XY, Sun JY (2006) Expression of recombinant *Aspergillus niger* xylanase A in *Pichia pastoris* and its action on xylan. Protein Expr Purif 48:292–299

Liu JR, Chung-Hang D, Xin Z, Jason T, Kuo-Joan C, Cheng-Kang P (2008) Cloning of a rumen fungal xylanase gene and purification of the recombinant enzyme via artificial oil bodies. Appl Microbiol Biotechnol 79:225–233

Luttig M, Pretorius IS, van Zyl WH (1997) Cloning of two β-xylanase encoding genes from *Aspergillus niger* and their expression in *Saccharomyces cerevisiae*. Biotechnol Lett 19:411–415

Mach RL, Zeilinger S (2003) Regulation of gene expression in industrial fungi: *Trichoderma*. Appl Microbiol Biotechnol 60:515–522

Mantyla A, Paloheimo M, Suominen P (1998) Industrial mutants and recombinants strains of *Trichoderma reesei*. In: Harman GE, Kubicek CP (eds) *Trichoderma* and *Gliocladium*, vol 2. Taylor and Francis, London, pp 291–309

Mantyla A, Paloheimo M, Hakola S, Lindberg E, Leskinen S, Kallio J, Vehmaanpera J, Lantoo R, Suominen P (2007) Production in *Trichoderma reesei* of three xylanases from *Chaetomium thermophile*: a recombinant thermoxylanase for bleaching of kraft pulp. Appl Microbiol Biotechnol 76:377–386

Mergulhaoa FJM, Summersb DK, Monteiro GA (2005) Recombinant protein secretion in *Escherichia coli*. Biotechnol Adv 23:177–202

Moreau A, Durand S, Morosoli R (1992) Secretion of a *Cryptococcus albidus* xylanase in *Saccharomyces cerevisiae*. Gene 116:109–113

Moure A, Gullón P, Domínguez H, Parajo JC (2006) Advances in the manufacture, purification and applications of xylo-oligosaccharides as food additives and utraceuticals. Process Biochem 41:1913–1923

Muller S, Sandal T, Kamp-Hansen P, Dalboge H (1998) Comparison of expression systems in the yeasts *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Klyveromyces lactis*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Cloning of two novel promoters from *Yarrowia lipolytica*. Yeast 14:1267–1283

Nair SG, Sindhu R, Shashidhar S (2008) Purification and biochemical characterization of two xylanases from *Aspergillus sydowii* SBS 45. Appl Biochem Biotechnol 149:229–243

Nevalainen KMH, Teo VSJ, Bergquist PL (2005) Heterologous protein expression in filamentous fungi. Trends Biotechnol 23:468–474

Ninawe S, Kapoor M, Kuhad RC (2008) Purification and characterization of extracellular xylanase from *Streptomyces cyaneus* SN32. Bioresour Technol 99:1252–1258

Ning L, Yang P, Wang Y, Luo H, Meng K, Wu N, Fan Y, Yao B (2008) Cloning, expression, and characterization of protease-resistant xylanase from *Streptomyces fradiae* var. k11. J Microbiol Biotechnol 18:410–416

Ogasawara W, Shida Y, Furukawa T, Shimada R, Nakagawa S, Kawamura M, Yagyu T, Kosuge A, Xu J, Nogawa M, Okada H, Morikawa Y (2006) Cloning, functional expression and promoter analysis of xylanase III gene from *Trichoderma reesei*. Appl Microbiol Biotechnol 72:995–1003

Ohta K, Moriyama S, Tanaka H, Shige T, Akimoto H (2001) Purification and characterization of an acidophilic xylanase from *Aureobasidium pullulans* var. *melanigenum* and sequence analysis of the encoding gene. J Biosci Bioeng 92:262–270

Okada H, Wakamatsu M, Takano Y, Nogawa M, Morikawa Y (1999) Expression of two *Trichoderma reesei* xylanases in the fission yeast *Schizosaccaromyces pombe*. J Biosci Bioeng 88:563–566

Parachin NS, Siqueira S, de Faria FP, Torres FAG, de Moraes LMP (2009) Xylanases from *Cryptococcus flavus* isolate I-11: enzymatic profile, isolation and heterologous expression of *CfXYN1* in *Saccharomyces cerevisiae*. J Mol Catal B Enzym 59:52–57

Pedersen M, Lauritzen HK, Frisvad JC, Meyer AS (2007) Identification of thermostable beta-xylosidase activities produced by *Aspergillus brasiliensis* and *Aspergillus niger*. Biotechnol Lett 29:743–748

Perez-Gonzalez AJ, De Graff LH, Visser J, Ranmon D (1996) Molecular cloning and expression in *Saccharomyces cerevisiae* of two *Aspergillus nidulans* xylanase genes. Appl Environ Microbiol 62:2179–2182

Perez-Gonzalez JA, van Peij NNME, Bezoen A, MacCabe AP, Ramon D, Graff LHD (1998) Molecular cloning and transcriptional regulation of the *Aspergillus nidulans xlnD* gene encoding a β-xylosidase. Appl Environ Microbiol 64:1412–1419

Polizeli ML, Rizzatti AC, Monti R, Terenzi HF, Jorge JA, Amorim DS (2005) Xylanases from fungi: properties and industrial applications. Minireview. Appl Microbiol Biotechnol 67:577–591

Prade RA (1996) Xylanases: from biology to biotechnology. Biotechnol Genet Eng Rev 13:101–131

Rauscher R, Würleitner E, Wacenovsky C, Aro N, Stricker AR, Zeilinger S, Kubicek CP, Penttilä M, Mach RL (2006) Tran-

scriptional regulation of *xyn1*, encoding xylanase I, in *Hypocrea jecorina*. Eukaryot Cell 3:447–456

Romanos MA, Scorer CA, Clare JJ (1992) Foreign gene expression in yeast: a review. Yeast 8:423–488

Rose SH, van Zyl WH (2002) Constitutive expression of the *Trichoderma reesei* beta-1,4-xylanase gene (*xyn2*) and the beta-1,4-endoglucanase gene (*egl*) in *Aspergillus niger* in molasses and defined glucose media. Appl Microbiol Biotechnol 58:461–468

Ruanglek V, Sriprang R, Ratanaphan N, Tirawongsaroj P, Chantasigh D, Tanapongpipat S, Pootanakit K, Eurwilaichitr L (2007) Cloning expression, characterization and high cell density production of recombinant endo 1,4-β-xylanases from *Aspergillus niger* in *Pichias pastoris*. Enzyme Microb Technol 41:19–25

Saadia M, Ahmed S, Jamil A (2008) Isolation and cloning of *cre*1 gene from a filamentous fungus *Trichoderma harzianum*. Pak J Bot 40:421–426

Saarelainen R, Paloheimo M, Fagerstrom R, Suominen PL, Nevalainen KMH (1993) Cloning, sequencing and enhanced expression of the *Trichoderma reesei* endoxylanse II (p*I* 9) gene *xln*2. Mol Gen Genet 241:497–503

Saleem F, Ahmed S, Jamil A (2008) Isolation of a xylan degrading gene from genomic DNA library of a thermophilic fungus *Chaetomium thermophile* ATCC 28076. Pak J Bot 40:1225–1230

Salles BC, Teo VSJ, Gibbs MD, Bergquist PL, Filho EXF, Ximenes EA, Nevalainen KM (2007) Identification of two novel xylanase-encoding genes (*xyn5* and *xyn6*) from *Acrophialophra nainiana* and hetrologous expression of *xyn*6 in *Trichoderma reesei*. Biotechnol Lett 29:1195–1201

Sa-Pereira P, Paveia H, Costa-Ferreira M, Aires-Barros MR (2003) A new look at xylanases: an overview of purification strategies. Mol Biotechnol 24:257–281

Schlacher A, Holzmann K, Hayn M, Steiner W, Schwab H (1996) Cloning and characterization of the gene for the thermostable xylanase XynA from *Thermomyces lanuginosus*. J Biotechnol 49:211–218

Sorensen HP, Mortensen KK (2005) Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. J Biotechnol 115:113–128

Studier FW, Moffatt BA (1986) Use of bacteriphage T7 RNA polymerase to direct selective high level expression of cloned genes. J Mol Biol 189:113–130

Sun YJ, Liu MQ, Wang XY, Qian LC, Gu SH (2007) Expression of recombinant *Thermomonospora fusca* xylanase A in *Pichia pastoris* and xylooligosachharides released from xylan by it. Food Chem 104:1055–1064

Sunna A, Antranikian G (1997) Xylanolytic enzymes from fungi and bacteria. Crit Rev Biotechnol 17:39–67

Tanaka H, Okuno T, Moriyama S, Muguruma M, Ohta K (2004) Acidophilic xylanase from *Aureobasidium pullulans*: efficient expression and secretion in *Pichia pastoris* and mutational analysis. J Biosci Bioeng 98:338–343

Tanaka H, Nakamura T, Ohta K (2005) Purification and properties of an extracellular xylanase from *Penicillium citrinum* and characterization of the encoding gene. J Biosci Bioeng 6:623–630

Tanaka H, Muguruma M, Ohta K (2006) Purification and properties of a family-10 xylanase from *Aureobasidium pullulans* ATCC 20524 and characterization of the encoding gene. Appl Microbiol Biotechnol 70:202–211

Teather RM, Wood PJ (1982) Use of Congo red polysaccharides interactions in enumeration and characterization of cellulolytic bacteria form the bovine rumen. Appl Environ Microbiol 43:777–780

Tsai CT, Huang CT (2008) Overexpression of the *Neocallimastix frontalis* xylanase gene in the methylotrophic yeasts *Pichia pastoris* and *Pichia methanolica*. Enzyme Microb Technol 42:459–465

van Peij NN, Visser J, De Graaff LH (1998) Isolation and analysis of *xlnR*, encoding a transcriptional activator co-ordinating xylanolytic expression in *Aspergillus niger*. Mol Microbiol 27:131–142

Wakiyama M, Tanaka H, Yoshihara K, Hayashi S, Ohta K (2008) Purification and properties of family-10 endo-1,4-β-xylanase from *Penicillium citrinum* and structural organization of encoding gene. J Biosci Bioeng 105:367–374

Wong KKY, Tan LUL, Saddler JN (1988) Multiplicity of β-1,4-xylanase in microorganisms: functions and applications. Microbiol Rev 52:305–317

Wu SC, Halley JE, Luttig C, Fernekes LM, Sanchez GG, Dravill AG, Albersheim P (2006) Identification of an endo-β-1,4-D-xylanase from Magnaporhte grisea by gene knockout analysis, purification, and heterologous expression. Appl Environ Microbiol 72:986–993

Xue GP, Denman SE, Glassop D, Johnson JS, Dierens LM, Gobius KS, Aylward JH (1995) Modification of a xylanase cDNA isolated from an anaerobic fungus *Neocallimastix patriciarum* for high-level expression in *Escherichia coli*. J Biotechnol 38:269–277

Yoshino S, Oishi M, Moriyama R, Kato M, Tsukagoshi N (1995) Two family G xylanase genes from *Chaetomium gracile* and their expression in *Aspergillus nidulans*. Curr Genet 29:73–80

Youderian P, Bouvier S, Susskind MM (1982) Sequence determinants of promoter activity. Cell 30:843–853

Yu EKC, Tan LUL, Chan MK-H, Deschatelets L, Saddler JN (1987) Production of thermostable xylanase by a thermophilic fungus, *Thermoascus aurantiacus*. Enzyme Microb Technol 9:16–24

Zhang GM, Huang J, Huang GR, Ma LX, Zhang XE (2007) Molecular cloning and expression of a new xylanase gene from *Plectosphaerella cucmerina*. Appl Microbiol Biotechnol 74:339–346

Zhou C, Bai J, Deng S, Wang J, Zhu J, Wu M, Wang W (2008) Cloning of a xylanase gene from *Aspergillus usamii* and its expression in *Escherichia coli*. Bioresour Technol 99:831–838