J Ind Microbiol Biotechnol (2013) 40:75–83
DOI 10.1007/s10295-012-1201-2

BIOTECHNOLOGY METHODS

Expression of a family 10 xylanase gene from *Aspergillus usamii* E001 in *Pichia pastoris* and characterization of the recombinant enzyme

Jun-Qing Wang · Xin Yin · Min-Chen Wu · Hui-Min Zhang · Shu-Juan Gao · Jing-Tu Wei · Cun-Duo Tang · Jian-Fang Li

Received: 29 May 2012 / Accepted: 18 September 2012 / Published online: 5 October 2012

Abstract A cDNA gene (*Auxyn10A*), which encodes a mesophilic family 10 xylanase from *Aspergillus usamii* E001 (abbreviated to AuXyn10A), was amplified and inserted into the *Xho*I and *Not*I sites of pPIC9K^{M} vector constructed from a parent pPIC9K. The recombinant expression vector, designated pPIC9K^{M}-*Auxyn10A*, was transformed into *Pichia pastoris* GS115. All *P. pastoris* transformants were spread on a MD plate, and then inoculated on geneticin G418-containing YPD plates for screening multiple copies of integration of the *Auxyn10A*. One transformant expressing the highest recombinant AuXyn10A (reAuXyn10A) activity of 368.6 U/ml, numbered as *P. pastoris* GSX10A4-14, was selected by flask expression test. SDS-PAGE assay demonstrated that the reAuXyn10A was extracellularly expressed with an apparent M.W. of 39.8 kDa. The purified reAuXyn10A displayed the maximum activity at pH 5.5 and 50 °C. It was highly stable at a broad pH range of 4.5–8.5, and at a temperature of 45 °C. Its activity was not significantly affected by EDTA and several metal ions except Mn^{2+}, which caused a strong inhibition. The K_m and V_{max}, towards birchwood xylan at pH 5.5 and 50 °C, were 2.25 mg/ml and 6,267 U/mg, respectively. TLC analysis verified that the AuXyn10A is an endo-β-1,4-D-xylanase, which yielded a major product of xylotriose and a small amount of xylose, xylotetraose, and xylopentose from birchwood xylan, but no xylobiose.

Keywords *Aspergillus usamii* · Family 10 xylanase · pPIC9K^{M} vector · *Pichia pastoris* · Heterologous expression

J.-Q. Wang · M.-C. Wu
Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, Jiangnan University, 1800 Lihu Road, Wuxi 214122, Jiangsu, People's Republic of China

J.-Q. Wang · X. Yin · C.-D. Tang
Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, 1800 Lihu Road, Wuxi 214122, Jiangsu, People's Republic of China

M.-C. Wu (✉) · S.-J. Gao · J.-T. Wei
School of Medicine and Pharmaceutics, Jiangnan University, 1800 Lihu Road, Wuxi 214122, Jiangsu, People's Republic of China
e-mail: biowmc@126.com

H.-M. Zhang · J.-F. Li
School of Food Science and Technology, Jiangnan University, 1800 Lihu Road, Wuxi 214122, Jiangsu, People's Republic of China

Introduction

Xylan, one of the major structural constituents of plant cell walls, is both covalently and non-covalently attached to cellulose, lignin, pectin, and other polysaccharides to maintain cell wall integrity [8, 22]. Endo-β-1,4-D-xylanase (EC 3.2.1.8), commonly abbreviated to xylanase, randomly catalyzes the internal β-1,4-D-xylosidic bonds of xylan or heteroxylan backbone to produce xylooligosaccharides [1]. Based on their remarkable differences in amino acid sequences and hydrophobic clusters, almost all xylanases reported hitherto have been classified into glycoside hydrolase (GH) families 5, 7, 8, 10, 11, 16, 43, and 62 (http://www.cazy.org/fam/acc_GH.html), whereas most xylanases belong to GH families 10 and 11 [7]. More recently, xylanases from GH family 30 have also been reported [19]. Compared with GH family 11 counterparts, GH family 10 xylanases present higher molecular weights (> 30 kDa),

lower substrate specificities, and acidic isoelectric points (p*I*s). Family 10 xylanases also have catalytic activities towards certain cellulosic substrates, such as aryl cellobioside [2]. In addition, the three-dimensional (3D) structures of GH family 10 xylanases are very similar to those of GH family 5 enzymes, all consisting principally of the $(\beta/\alpha)_8$ barrel fold, which has been likened to a "salad bowl" [12].

Up to now, various xylanases have been isolated and characterized from saprophytic microorganisms, plants and rumen microbiota, among which the filamentous fungi were considered to have great potentials for the production of xylanases [6, 23]. Unfortunately, the commercialization of xylanases was still limited by their low catalytic activities and expensive production costs. Due to those reasons, many xylanase genes from organisms, especially from fungi, such as *Aspergillus niger* [24], *A. usamii* [29], *A. terreus* [4], and *Fusarium oxysporum* [21], have been cloned, characterized, modified, and expressed in heterologous cells. Some expressed recombinant xylanases have higher catalytic activities and superior properties [24, 31]. The *Pichia pastoris* eukaryotic expression system was a favorite one for expression of heterologous proteins owing to its many advantages, such as protein processing, folding, and post-translational modification and secretion. In addition, *P. pastoris* could be cultured and induced in inexpensive media, and the expression levels and purities of recombinant proteins were high [13].

In our previous studies, four genes encoding GH family 11 xylanases, i.e., AuXyn11A, B (named as XynI, II in previous references), C and D were cloned from *A. usamii* E001. The AuXyn11B gene (*Auxyn11B*) has also been expressed in *E. coli* [30] and *P. pastoris* [31]. More recently, a novel GH family 10 xylanase (AuXyn10A) was purified from the same strain. Then, the full-length cDNA and complete DNA sequences of the AuXyn10A gene were obtained and characterized [26]. In this work, we reported the expression of the *Auxyn10A* in *P. pastoris*, the purification of the reAuXyn10A, and its characterization. Most importantly, by means of a pPIC9K^M vector initially constructed in our lab, the expressed reAuXyn10A retained a native N-terminus of the AuXyn10A. The purified reAuXyn10A displayed the high specific activity, broad pH stability, and strong resistance to most metal ions tested and EDTA, making it a potential candidate for applications in industrial processes, especially in the production of xylooligosaccharides. TLC analysis verified that the AuXyn10A is an endoxylanase, which produced a major product of xylotriose and a small amount of xylose, xylotetraose, and xylopentose from xylan, but no xylobiose. To our knowledge, this is the first report on the expression of the *Auxyn10A* in *P. pastoris* by means of a pPIC9K^M vector and subsequent characterization of the recombinant enzyme.

Materials and methods

Strains, vectors, and culture media

Aspergillus usamii E001, isolated from the soil in China [9] and deposited in the Center of Industrial Culture Collection of China (accession number: CICC2239), was used for total RNA extraction. *E. coli* JM109 and pUCm-T vector (Sangon, Shanghai, China) were used for gene cloning and DNA sequencing. *E. coli* DH5α and pPIC9K^M vector constructed from a parent pPIC9K (Invitrogen, Carlsbad, CA, USA) were used for construction of the recombinant expression vector. The *Auxyn10A* encoding the *A. usamii* family 10 xylanase was expressed in *P. pastoris* GS115 (Invitrogen). *E. coli* JM109 and DH5α were cultured at 37 °C in a LB medium containing 1.0 % tryptone, 0.5 % yeast extract and 1.0 % NaCl, pH 7.2. *P. pastoris* was cultured at 30 °C in following media that were prepared according to the manual of Multi-Copy Pichia Expression Kit (Invitrogen, Carlsbad, CA, USA): minimal dextrose (MD), yeast extract peptone dextrose (YPD), buffered glycerol-complex (BMGY), and buffered methanol-complex (BMMY).

Total RNA extraction

Aspergillus usamii E001 was cultured in a xylan-containing medium, containing 1.0 % tryptone, 0.5 % yeast extract, 1.0 % glucose, and 0.5 % birchwood xylan, pH 6.0, at 30 °C on a rotary incubator with 220 rpm. After 36-h cultivation, the mycelia were collected by filtration and thoroughly washed with sterile SE buffer (150 mM NaCl, 100 mM EDTA, pH 8.0). The total RNA was extracted using the RNA Extraction Kit (Sangon, China) according to the method as reported previously [29].

Construction of the pPIC9K^M vector

An oligonucleotide, 5′-CTCGAGAAAAGA-3′ with an *Xho*I site (underlined), was added to the 5′-end of the *Auxyn10A*, and inserted into the *Xho*I and *Not*I sites of pPIC9K vector. The modified *Auxyn10A* encoded a preAuXyn10A with a single protease Kex2 signal cleavage site between Leu-Glu-Lys-Arg and mature peptide, where it can be exclusively cleaved, retaining the native N-terminus of AuXyn10A. Liu et al. [17] also reported that a salivary plasminogen activator from vampire bat was expressed in *P. pastoris* with native N-terminus by means of the above-mentioned design. Unfortunately, there are two *Xho*I sites (in the kanamycin-resistant gene *kan*^{r} and nearby the multiple cloning sites, respectively) on pPIC9K vector, making construction of the recombinant vector very difficult and haphazard. In this study, an *Xho*I site (CTCGAG) in the

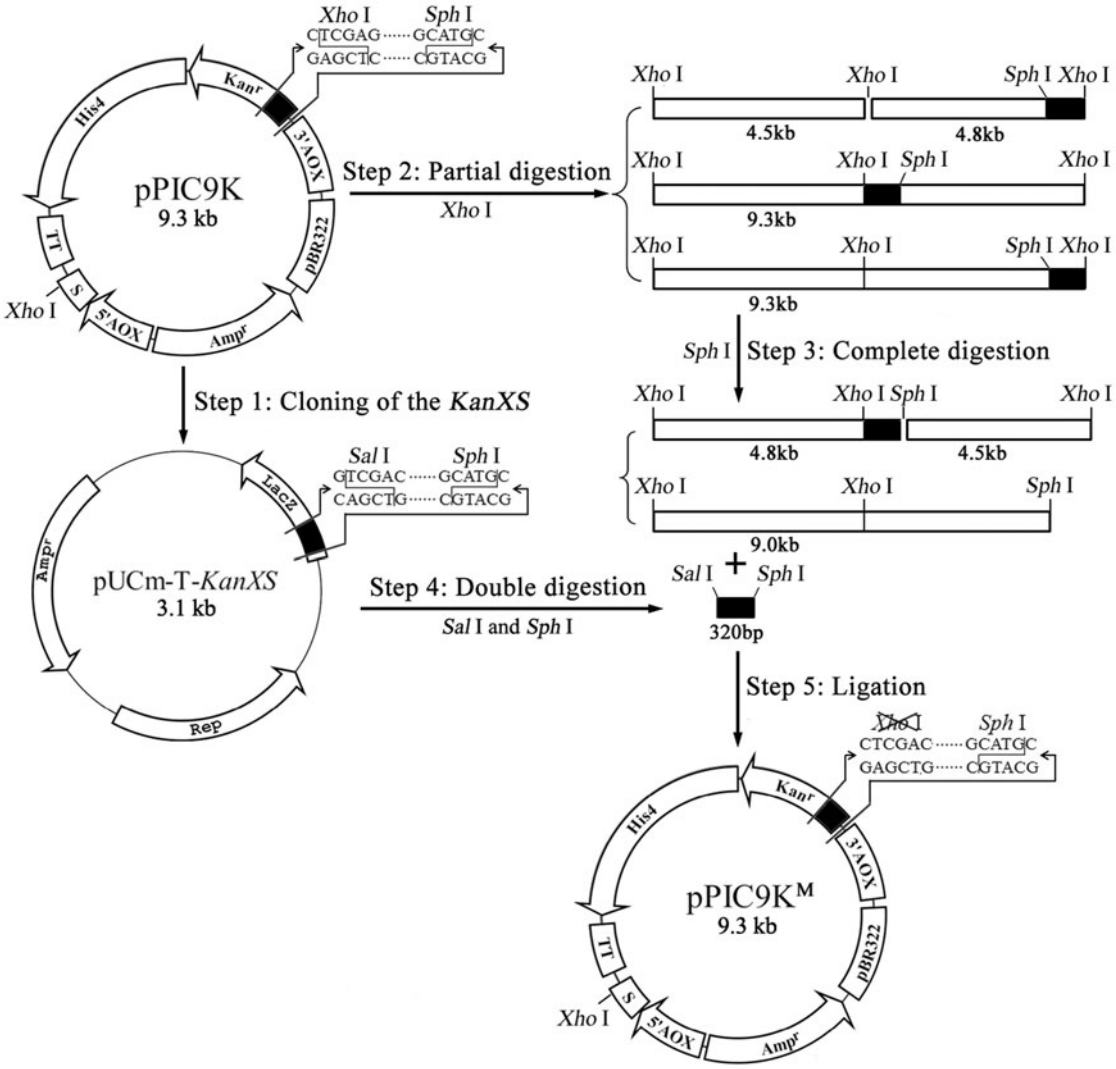

Fig. 1 A constructing flowchart of the pPIC9K^{M} from a parent pPIC9K vector

kan^{r} was mutated to CTCGAC by five steps (Fig. 1). The detailed manipulation procedures were as follows:

Using pPIC9K as the template, a DNA fragment (*kanXS*) in the *kan*^{r} between *Xho*I and *Sph*I sites was amplified, where an *Xho*I site was replaced by a *Sal*I site, using primers KanF (5′-GTCGACCAAGACGTTTCCC-3′ with a *Sal*I site, underlined) and KanR (5′-GCATGCGCAAGGAGATGGC-3′ with a *Sph*I site, underlined), and cloned into pUCm-T vector (pUCm-T-*kanXS*). Meanwhile, the pPIC9K vector was first partially digested with *Xho*I. The longest linearized DNA fragment (9.3 kb) was agarose gel-purified, and subjected to complete digestion with *Sph*I. A longer digested DNA fragment (9.0 kb) was purified, and ligated with a *kanXS* excised from pUCm-T-*kanXS* with *Sal*I and *Sph*I owing to *Xho*I and *Sal*I being isoschizomers, followed by transforming it into *E. coli* DH5α. The modified pPIC9K vector with only one *Xho*I site nearby the multiple cloning sites, named pPIC9K^{M} vector, was confirmed by restriction enzyme analysis with *Xho*I, and its kanamycin- and geneticin G418-resistant activities were tested in *E. coli* and *P. pastoris*, respectively.

Cloning of the cDNA gene encoding the AuXyn10A

A pair of specific PCR primers was designed based on the complete DNA sequence of the *Auxyn10A* (GenBank accession: HQ709245) and determined N-terminal sequence of 15 residues (QASVSIDTKFKAHGK) of the native AuXyn10A purified from cultivated koji of *A. usamii* E001 [26]. Both forward and reverse primers, synthesized by Sangon (China), were XynCF: 5′-CTCGAGAAAAGACAGGCTTCAGTGAGTATTGA-3′ containing an oligonucleotide 5′-CTCGAGAAAAGA-3′ with an *Xho*I site (underlined) and XynCR: 5′-GCGGCCGCCTAGAGAGCATTTGCGATAG-3′ with a *Not*I site (underlined).

An Oligo dT-Adaptor Primer, 5′-GTTTTCCCAGTCACGAC(dT_{18})-3′ provided by RNA PCR Kit (TaKaRa, Dalian, China), was used for reverse transcription of the first-strand cDNA from the total RNA. Using the resulting first-strand cDNA as the template, the first-round PCR was carried out using primers XynCF and M13 Primer M4 (identical to Oligo dT-Adaptor Primer except Oligo dT) with the following conditions: a denaturation at 94 °C for 2 min; 30 cycles of amplification at 94 °C for 30 s, 53 °C for 30 s, 72 °C for 75 s; and an elongation at 72 °C for 10 min. Then, the first-round PCR product was subjected to the second-round PCR using primers XynCF and XynCR (nested PCR) under the same conditions except with an annealing temperature of 55 °C. The amplified target band was purified using the EZ-10 Spin Column DNA Gel Extraction Kit (BBI, Markham, Canada), inserted into pUCm-T (pUCm-T-*Auxyn10A*), and confirmed by restriction enzyme analysis and DNA sequencing.

Construction and transformation of the recombinant vector

The *Auxyn10A* was excised from pUCm-T-*Auxyn10A* with *Xho*I and *Not*I, agarose gel-purified, and then inserted into the *Xho*I and *Not*I sites of pPIC9K^{M} vector, followed by transforming it into *E. coli* DH5α. The proper recombinant expression vector containing the *Auxyn10A*, designated pPIC9K^{M}-*Auxyn10A*, was confirmed by DNA sequencing. Then, the resulting pPIC9K^{M}-*Auxyn10A* was linearized with *Sal*I, and transformed into *P. pastoris* GS115 by electroporation on a Gene Pulser Apparatus (Bio-Rad, USA) according to the manufacturer's instruction.

Screening and expression of *P. pastoris* transformants

All *P. pastoris* transformants were first spread on an MD plate and then successively inoculated on G418-containing YPD plates at increasing concentrations of 1.0, 2.0, and 4.0 mg/ml for screening multiple *Auxyn10A* copies. Expression of the *Auxyn10A* in *P. pastoris* GS115 was performed according to the instruction of Multi-Copy Pichia Expression Kit (Invitrogen) with slight modification [14]. Each single colony of the transformants was inoculated into 30 ml of BMGY medium and cultured at 30 °C with 220 rpm until the OD_{600} reached 2–4. The cells were then collected by centrifugation, resuspended in 30 ml of BMMY medium, and induced for expression of the *Auxyn10A* by adding methanol to a final concentration of 2.0 % (v/v) at 24-h intervals at 30 °C for 96 h. One transformant expressing the highest reAuXyn10A activity, numbered as *P. pastoris* GSX10A4-14, was selected and used for further studies.

Purification of the expressed reAuXyn10A

After the GSX10A4-14 was induced by methanol for 96 h, a total of 30 ml of cultured supernatant was brought to 75 % saturation by adding solid ammonium sulfate and left overnight. The resulting precipitate was harvested by centrifugation, dissolved in 3.0 ml of 50 mM sodium phosphate buffer (pH 7.0), and dialyzed against the same buffer. The dialyzed solution was concentrated to 1.0 ml by ultrafiltration at 8,000 rpm using a 10-kDa cut-off membrane (Millipore, Billerica, MA, USA), then loaded onto a Sephadex G-75 column (1.6 × 80 cm), followed by elution with the same buffer (pH 7.0) at a flow rate of 0.3 ml/min. Aliquots of 3.0 ml eluent only containing the reAuXyn10A were pooled and concentrated. All purification procedures were performed at 4 °C.

Enzyme activity and protein assays

Xylanase activity was assayed using the previously described method [28] with appropriate modification. In brief, 100 µl of suitably diluted enzyme solution with 2.4 ml of 0.5 % (w/v) birchwood xylan (Sigma, St. Louis, MO, USA) in 50 mM Na_2HPO_4–citric acid buffer (pH 5.5) was incubated at 50 °C for 15 min. The released reducing sugars were measured with the 3,5-dinitrosalicylic acid (DNS) method [20], using D-xylose as the standard. One unit of xylanase activity was defined as the amount of enzyme liberating 1 µmol of reducing sugar equivalent per minute under the assay conditions.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a 12.5 % gel using the method of Laemmli [11], and the isolated proteins were visualized by staining with Coomassie brilliant blue R-250 (Sigma, USA). Protein concentration was determined by the Bradford method [3] using bovine serum albumin as the standard. The prediction of *N*- and *O*-linked glycosylation sites were performed using the NetNGlyc program 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc program 3.1 (http://www.cbs.dtu.dk/services/NetOGlyc/), respectively.

pH optimum and stability

The pH optimum of the purified reAuXyn10A was assayed under the standard xylanase activity assay conditions, except at a pH range of 3.5–7.5. To estimate its pH stability, aliquots of reAuXyn10A were preincubated at 30 °C for 1.0 h at varied pH values (Na_2HPO_4–citric acid buffer: pH 3.5–7.5; Tris–HCl buffer: pH 8.0–9.0). The residual enzyme activities were measured under the standard assay conditions (at pH 5.5 and 50 °C for 15 min). Here, the pH stability was defined as a pH range, at which the residual reAuXyn10A activities were more than 90 % of its original activity.

Temperature optimum and stability

The optimal temperature for the reAuXyn10A activity was determined, at pH optimum, at various temperatures ranging from 30 to 60 °C. To evaluate its thermostability, aliquots of reAuXyn10A were preincubated at 45, 50, and 55 °C, respectively, for 10, 20, 40, 60, 80, and 100 min. The residual enzyme activities were measured under the standard assay conditions. The thermostability in this work was defined as a temperature at which the residual reAuXyn10A activities retained over 90 % of its original activity within 1.0 h.

Effects of metal ions and EDTA

To estimate the effects of metal ions and EDTA on the reAuXyn11A activity, an array of metal ions and EDTA were added to the enzyme reaction system, respectively, at a final concentration of 5.0 mM in 20 mM Na_2HPO_4–citric acid buffer (pH 5.5) at 30 °C for 1.0 h. The residual reAuXyn11A activities were measured under the standard assay conditions. The reaction system without adding any additive was used as the control.

Kinetic parameter assay

Hydrolyzing reaction rates (U/mg) of the reAuXyn10A were measured under the standard assay conditions (at pH 5.5 and 50 °C for 15 min), except birchwood xylan concentrations ranging from 1.0 to 10 mg/ml. The reaction rate versus the substrate concentration was plotted to confirm whether the hydrolyzing mode of the reAuXyn10A conforms to the Michaelis–Menten equation. The K_m and V_{max} values of the reAuXyn10A were graphically determined from the Lineweaver–Burk plotting.

Thin-layer chromatography (TLC) assay

The purified reAuXyn10A (5.0 U/ml reaction solution) was mixed with 1.0 % (w/v) of birchwood xylan in 50 mM Na_2HPO_4–citric acid buffer (pH 5.5), and incubated at 37 °C for 4, 6, 8, 10, and 12 h, respectively. Hydrolytic products were separated on the high silica gel plate GF254 (Haiyang, Qingdao, China) developed in a solvent system consisting of *n*-butyl alcohol, glacial acetic acid, and water (2:1:1, by vol). The isolated products were visualized by spraying with coloration solution comprising aniline, diphenylamine, 85 % phosphoric acid, and acetone (1:1:5:50, by vol), followed by heating at 105 °C for 5 min. Xylose and xylooligosaccharides (xylobiose, xylotriose, xylotetraose, and xylopentose) purchased from Megazyme (Bray, Ireland) were used as standards.

Results and discussion

Construction of the pPIC9K^M vector

An about 320-bp DNA fragment, *kanXS*, was amplified using primers KanF and KanR. The DNA sequencing result verified that an *Xho*I site was replaced with a *Sal*I site (Fig. 1, step 1). An about 9.0-kb DNA fragment was obtained from pPIC9K vector by a partial digestion with *Xho*I, then a complete digestion with *Sph*I (steps 2 and 3). The pPIC9K^M vector with only one *Xho*I site was constructed by ligating the 9.0-kb fragment with the *kanXS* excised from pUCm-T-*kanXS* with *Sal*I and *Sph*I (steps 4 and 5), and confirmed by restriction enzyme analysis with *Xho*I. The kanamycin- and geneticin G418-resistant activities of pPIC9K^M in *E. coli* and *P. pastoris*, respectively, were not affected by site-directed mutagenesis (G → C) in the *kan*^{r} of pPIC9K vector.

Cloning of the cDNA gene encoding the AuXyn10A

Analytical results of the total RNA extracted from *A. usamii* E001 demonstrated that the ratio of OD_{260} to OD_{280} was 1.97, and that the 18S and 28S rRNA bands, characterized as eukaryotes, were specific on formaldehyde denatured agarose gel electrophoresis (data not shown), indicating that the isolated total RNA has high purity and is not decomposed [27].

Using the reversely transcribed first-strand cDNA as the template, an about 1.1-kb band and several faint bands were amplified by the first-round PCR using primers XynCF and M13 Primer M4. Each band was agarose gel-purified and subjected to the second-round PCR using primers XynCF and XynCR. As a result, an about 900-bp specific band was amplified only using the 1.1-kb band as the template. The sequencing results were adopted as the inserted cDNA gene sequences of three picked clones were identical to one another, or else the experiment was redone. The homology alignment verified that the sequence of the cDNA gene (*Auxyn10A*) is entirely identical to that of the complete DNA (GenBank accession: HQ709245), except a 5′-flanking regulatory region, 5′- and 3′-untranslated regions, a signal peptide sequence, and nine short introns [26]. The *Auxyn10A* is exactly 929 bp in length (containing an *Xho*I site followed by AAAAGA and a *Not*I site), encoding a 302-aa AuXyn10A with native N-terminus by means of a single protease Kex2 signal cleavage site. Its theoretical M.W. of 32,731 Da is in good agreement with the determined one (33.0 kDa) of the native AuXyn10A purified from *A. usamii* E001 as reported previously [26].

Screening and expression of *P. pastoris* transformants

Pichia pastoris transformants that could resist higher concentrations of G418 might have multiple copies of integration of heterologous genes into the *P. pastoris* genome, which could potentially lead to higher expression levels of heterologous proteins [25]. However, the expression level was not directly proportional to the concentration of G418 as elucidated in the manual of Multi-Copy Pichia Expression Kit (Invitrogen). For those reasons, we respectively picked out 20 transformants resistant to 1.0, 2.0 and 4.0 mg/ml of geneticin G418, numbered as *P. pastoris* GSX10A1-1 to GSX10A1-20, GSX10A2-1 to GSX10A2-20 and GSX10A4-1 to GSX10A4-20, for flask expression tests. *P. pastoris* transformed with pPIC9K^{M} vector, numbered as *P. pastoris* GSC, was used as the negative control. After 96-h induction by adding 2.0 % (v/v) methanol at 24-h intervals, the cultured supernatants of 60 transformants were harvested for xylanase activity and recombinant protein assays, respectively. Among all transformants tested, one strain expressing the maximum xylanase activity of 368.6 U/ml, numbered as *P. pastoris* GSX10A4-14, was selected and used for subsequent studies. No xylanase activity was detected in the cultured supernatant of *P. pastoris* GSC under the same expression conditions.

Purification of the expressed reAuXyn10A

The reAuXyn10A expressed by GSX10A4-14 was purified to homogeneity by ammonium sulfate precipitation, ultrafiltration, and Sephadex G-75 gel filtration. The specific activity of the reAuXyn10A, towards birchwood xylan at pH 5.5 and 50 °C, was 5,448 U/mg, which was higher than those (2,540, 2,279 and 1,047 U/mg, respectively) of the native AuXyn11A, 11B, and 10A purified from the cultivated koji of *A. usamii* E001 [26, 28]. SDS-PAGE analysis of the purified reAuXyn10A displayed a single protein band with an apparent M.W. of 39.8 kDa (Fig. 2, lane 3), which is larger than the theoretical one of the AuXyn10A. *P. pastoris* enables some post-translational modifications, such as assembly of disulfide bond, exclusion of signal peptide, and *N*- and/or *O*-glycosylation of mature peptide. Analytical results demonstrated that there is no *N*-linked glycosylation site, but there are *O*-linked glycosylation sites in the AuXyn10A sequence. Simultaneously, the carbohydrate content of the purified reAuXyn10A was determined to be 3.12 %. Those results verified that the increased M.W. of the reAuXyn10A expressed in *P. pastoris* GS115 may be resulted from *O*-linked glycosylation.

The yeast *P. pastoris* expression system has many advantages, one of which is that the purities of the expressed recombinant proteins are very high according to the description of Multi-Copy Pichia Expression Kit (Invitrogen),

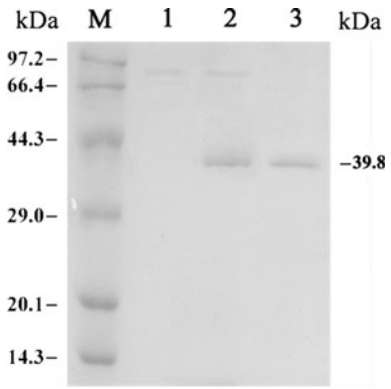

Fig. 2 SDS-PAGE analysis of the cultured supernatants of *P. pastoris* transformants (GSC and GSX10A4-14) and the purified reAuXyn10A. *Lanes*: *M* protein marker, *1* the cultured supernatant of the GSC, *2* the cultured supernatant of the GSX10A4-14, *3* the reAuXyn10A purified by a combination of ammonium sulfate precipitation, ultrafiltration and Sephadex G-75 gel filtration

which can greatly facilitate or simplify the purification procedures, and decrease the industrial production and application costs. Purification of the xylanase from *Thermomyces lanuginosus* 195 was performed by a combination of ultrafiltration, anion exchange chromatography and gel filtration, resulting in a 9.3-fold purification with a specific activity of 6,182 U/mg and a recovery yield of 7.6 % [10]. Ammonium sulfate fraction and gel filtration chromatography were applied for purification of the recombinant *A. niger* xylanase expressed in *P. pastoris*, resulting in a 4.1-fold increase in specific activity (717.5 U/mg) [16]. Chen et al. [5] reported that the purity of the recombinant *A. sulphureus* β-mannanase expressed in *P. pastoris* X-33 was 97 %. In our work, the purity of the reAuXyn10A expressed by GSX10A4-14 was more than 85 % (Fig. 2, lane 2).

N-terminus sequencing of the reAuXyn10A

The N-terminal sequence of the reAuXyn10A was assayed on a 470A automatic sequencer obtained from Applied Biosystems (Foster City, CA, USA). The sequence of N-terminal 10 amino acid residues was QASVSIDTKF, which is entirely identical to that of the native AuXyn10A purified from *A. usamii* E001 [26], demonstrating that there was no any additional peptide segment, such as EAEAYVEF, EAYVEF or YVEF, at the N-terminus of the reAuXyn10A expressed in *P. pastoris* by means of the pPIC9K^{M} vector.

Characterization of the purified reAuXyn10A

The purified reAuXyn10A exhibited higher catalytic activities over a pH range of 5.0–6.0, among which the maximum enzyme activity was at pH 5.5 (measured at 50 °C for 15 min). Preincubated at 30 °C for 1.0 h at varied pH

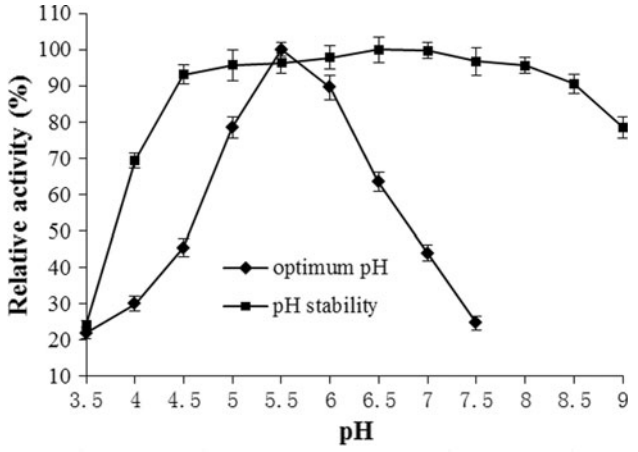

Fig. 3 Effect of pH value on the catalytic activity and stability of the reAuXyn10A. The pH optimum of the purified reAuXyn10A was assayed under the standard xylanase activity assay conditions, except 0.5 % (w/v) birchwood xylan in 50 mM Na_2HPO_4–citric acid buffer (pH 3.5–7.5), respectively. The pH stability of the reAuXyn10A was estimated by preincubating it at 30 °C for 1.0 h at varied pH values (pH 3.5–9.0), respectively, and the residual enzyme activities were measured under the standard assay conditions

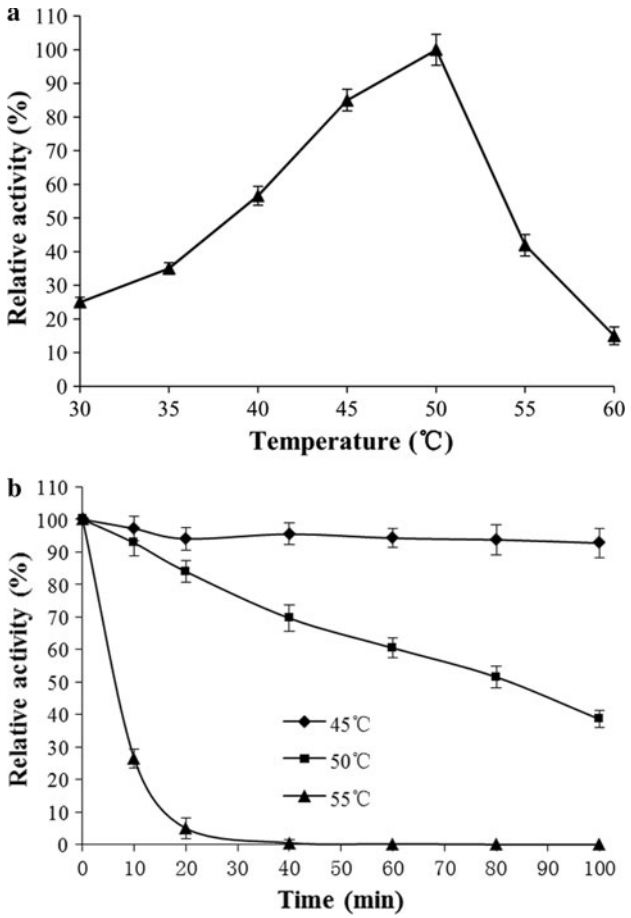

Fig. 4 Effect of temperature on the catalytic activity and stability of the reAuXyn10A. **a** The optimal temperature for the reAuXyn10A activity was determined, at pH 5.5, at various temperatures ranging from 30 to 60 °C. **b** To evaluate its thermostability, aliquots of reAuXyn10A were preincubated at 45, 50, and 55 °C, respectively, for 10–100 min, and the residual enzyme activities were measured under the standard assay conditions

values (3.5–9.0), the reAuXyn10A was highly stable over a broad pH range of 4.5–8.5, retaining more than 90 % of its original activity (Fig. 3). The optimal temperature for the reAuXyn10A activity (measured at pH 5.5 for 15 min) was 50 °C (Fig. 4a). The reAuXyn10A was highly stable (more than 90 % of its original activity) when preincubated at 45 °C for 100 min, and retained 83.2 % of its activity at 50 °C for 20 min, but entirely lost its activity at 55 °C for 40 min (Fig. 4b).

The reAuXyn10A activity was not affected (100 ± 5 %) by Mg^{2+} (103.8 %), Fe^{2+} (104.3 %), Al^{3+} (96.5 %) or Ba^{2+} (101.9 %), and slightly affected (100 ± 20 %) by Fe^{3+} (91.4 %), Zn^{2+} (109.2 %), Ca^{2+} (91.4 %), Sn^{2+} (89.2 %), Co^{2+} (83.7 %), Li^{+} (87.1 %), Cu^{2+} (82.4 %) or EDTA (84.8 %), but strongly inhibited by Mn^{2+} (23.2 %). The kinetic parameters, K_m and V_{max} values, of the purified reAuXyn10A towards birchwood xylan at pH 5.5 and 50 °C were 2.25 mg/ml and 6,267 U/mg, respectively.

All enzymatic properties of the reAuXyn10A characterized above are similar to those of the native AuXyn10A purified from the cultivated koji of the same strain (*A. usamii* E001), and partially in agreement with those of the xylanases from *A. usamii* E001 (AuXyn11A and 11B) [28] and *A. ficuum* AF-98, respectively [18].

TLC analysis of the xylooligosaccharides

TLC analysis of the hydrolytic products (xylose and xylooligosaccharides) from birchwood xylan revealed that the purified reAuXyn10A produced xylotriose (X3) as the major product, and a small amount of xylose (X1), xylotetraose (X4), and xylopentose (X5) (Fig. 5). Surprisingly, no trace of xylobiose (X2) could be detected in the hydrolytic products, on which, to our knowledge, this was a first report. The hydrolytic products from birchwood xylan hydrolyzed by the recombinant family 11 xylanase from *Bacillus licheniformis* (reBlxA) were xylose (X1) to xylopentose (X5), among which xylotriose (X3) was the major product [15].

Conclusions

The AuXyn10A-encoding cDNA gene (*Auxyn10A*) from *A. usamii* E001 was expressed in *P. pastoris* by means of the $pPIC9K^M$ vector initially constructed in our lab, retaining the native N-terminus of AuXyn10A. The reAuXyn10A displayed the high specific activity, broad pH stability and strong resistance to metal ions and EDTA. TLC analysis

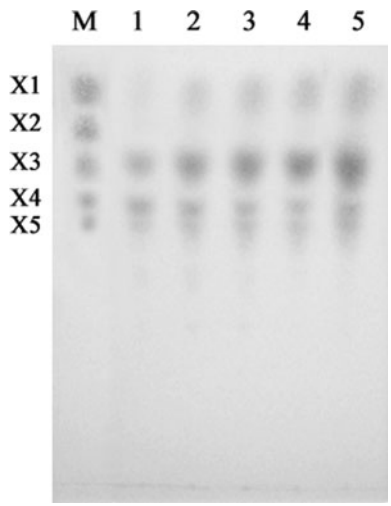

Fig. 5 TLC analyses of the hydrolytic products (xylose and xylooligosaccharides) from birchwood xylan. *Lanes*: *M* the standard xylose (X1) and xylooligosaccharides (X2–X5), *1–5* the hydrolytic products catalyzed by reAuXyn10A at pH 5.5 and 37 °C for 4, 6, 8, 10 and 12 h, respectively

verified that the AuXyn10A is an endo-β-1,4-D-xylanase, which produced a major product of xylotriose but no xylobiose. The superior enzymatic properties made the reAuXyn10A a good candidate in various industrial processes, especially in the production of functional xylooligosaccharides. Considering the low thermostability of the AuXyn10A and the availability of the primary and 3D structures of many thermostable xylanases, we will next focus our interests on improving the AuXyn10A thermostability by genetic engineering, such as by increasing the number of salt bridges, the addition of thermostabilizing domains, and the introduction of disulfide bridges, particularly at the N- or C-terminus or in the α-helix region.

Acknowledgments This work was financially supported by the National Nature Science Foundation of China (No. 31101229) and the Fundamental Research Funds for the Central Universities of China (No. JUDCF11032). The authors are grateful to Prof. Xianzhang Wu (School of Biotechnology, Jiangnan University) for providing technical assistance.

References

1. Berrin JG, Juge N (2008) Factors affecting xylanase functionality in the degradation of arabinoxylans. Biotechnol Lett 30:1139–1150
2. Biely P, Vršanská M, Tenkanen M, Kluepfel D (1997) Endo-β-1,4-xylanase families: differences in catalytic properties. J Biotechnol 57:151–166
3. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72:248–254
4. Chantasingh D, Pootanakit K, Champreda V, Kanokratana P, Eurwilaichitr L (2006) Cloning, expression, and characterization of a xylanase 10 from *Aspergillus terreus* (BCC129) in *Pichia pastoris*. Protein Express Purif 46:143–149
5. Chen XL, Cao YH, Ding YH, Lu WQ, Li DF (2007) Cloning, functional expression and characterization of *Aspergillus sulphureus* β-mannanase in *Pichia pastoris*. J Biotechnol 128:452–461
6. Chen XZ, Xu SQ, Zhu MS, Cui LS, Zhu H, Liang YX, Zhang ZM (2010) Site-directed mutagenesis of an *Aspergillus niger* xylanase B and its expression, purification and enzymatic characterization in *Pichia pastoris*. Process Biochem 45:75–80
7. Collins T, Gerday C, Fellar G (2005) Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol Rev 29:3–23
8. Coughlan MP, Hazlewood GP (1993) β-1,4-D-Xylan-degrading enzyme systems: biochemistry, molecular biology and applications. Biotechnol Appl Biochem 17:259–289
9. Fu DD, Xie H, Wu MC (2005) Solid fermentation for xylanase production by *Aspergillus usamii*. Food Ferment Ind 31:50–53
10. Gaffney M, Carberry S, Doyle S, Murphy R (2009) Purification and characterization of a xylanase from *Thermomyces lanuginosus* and its functional expression by *Pichia pastoris*. Enzyme Microb Technol 45:348–354
11. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
12. Larson SB, Day J, Barba de la Rosa AP, Keen NT, McPherson A (2003) First crystallographic structure of a xylanase from glycoside hydrolase family 5: implications for catalysis. Biochemistry 42:8411–8422
13. Li JF, Zhao SG, Tang CD, Wang JQ, Wu MC (2012) Cloning and functional expression of an acidophilic β-mannanase gene (*Anman5A*) from *Aspergillus niger* LW-1 in *Pichia pastoris*. J Agric Food Chem 60:765–773
14. Li JF, Tang CD, Shi HL, Wu MC (2011) Cloning and optimized expression of a neutral endoglucanase gene (*ncel5A*) from *Volvariella volvacea* WX32 in *Pichia pastoris*. J Biosci Bioeng 111:537–540
15. Liu MQ, Liu GF (2008) Expression of recombinant *Bacillus licheniformis* xylanase A in *Pichia pastoris* and xylooligosaccharides released from xylans by it. Protein Express Purif 57:101–107
16. Liu MQ, Weng XY, Sun JY (2006) Expression of recombinant *Aspergillus niger* xylanase A in *Pichia pastoris* and its action on xylan. Protein Expres Purif 48:292–299
17. Liu Y, Su C, Song XS, Tang YL, Bao ZH (2009) Construction of *Pichia pastoris* strain expressing salivary plasminogen activator from vampire bat (*Desmodus rotundus*). Chin J Biotech 25:566–574
18. Lu FX, Lu M, Lu ZX, Bie XM, Zhao HZ, Wang Y (2008) Purification and characterization of xylanase from *Aspergillus ficuum* AF-98. Biores Technol 99:5938–5941
19. Luo HY, Yang J, Li J, Shi PJ, Huang HQ, Bai YG, Fan YL, Yao B (2010) Molecular cloning and characterization of the novel acidic xylanase XYLD from *Bispora* sp. MEY-1 that is homologous to family 30 glycosyl hydrolases. Appl Microbiol Biotechnol 86:1829–1839
20. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:246–248
21. Moukouli M, Topakas E, Christakopoulos P (2011) Cloning and optimized expression of a GH-11 xylanase from *Fusarium oxysporum* in *Pichia pastoris*. New Biotechnol 28:369–374
22. Pastor FIJ, Gallardo O, Sanz-Aparicio J, Diaz P (2007) Xylanases: molecular properties and applications. In: Polaina J, MacCabe AP (eds) Industrial Enzymes. Springer, Berlin Heidelberg New York, pp 65–82
23. Polizeli MLTM, Rizzatti ACS, Monti R, Terenzi HF, Jorge JA, Amorim DS (2005) Xylanases from fungi: properties and industrial applications. Appl Microbiol Biotechnol 67:577–591
24. 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-xylanase from *Aspergillus niger* in *Pichia pastoris*. Enzyme Microb Technol 41:19–25

25. Tan ZB, Li JF, Wu MC, Tang CD, Zhang HM, Wang JQ (2011) High-level heterologous expression of an alkaline lipase gene from *Penicillium cyclopium* PG37 in *Pichia pastoris*. World J Microbiol Biotechnol 27:2767–2774
26. Wang JQ, Zhang HM, Wu MC, Tang CD (2011) Cloning and sequence analysis of a novel xylanase gene, *Auxyn10A*, from *Aspergillus usamii*. Biotechnol Lett 33:1029–1038
27. Wu MC, Qian ZK, Jiang PH, Min TS, Sun CR, Huang WD (2003) Cloning of an alkaline lipase gene from *Penicillium cyclopium* and its expression in *Escherichia coli*. Lipids 38:191–199
28. Wu MC, Fu DD, Zhu J, Xia MF (2005) Purification and some properties of two xylanases from *Aspergillus usamii*. J Food Sci Biotechnol 24:29–33
29. Wu MC, Wang JQ, Zhang HM, Tang CD, Gao JH, Tan ZB (2011) Cloning and sequence analysis of an acidophilic xylanase (XynI) gene from *Aspergillus usamii* E001. World J Microbiol Biotechnol 27:831–839
30. Zhou CY, Bai JY, Deng SS, Wang J, Zhu J, Wu MC, Wang W (2008) Cloning of a xylanase gene from *Aspergillus usamii* and its expression in *Escherichia coli*. Biores Technol 99:831–838
31. Zhou CY, Wang YT, Wu MC, Wang W, Li DF (2009) Heterologous expression of xylanase II from *Aspergillus usamii* in *Pichia pastoris*. Food Technol Biotechnol 47:90–95