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ORIGINAL PAPER

Modification of a gene encoding hybrid xylanase and its expression in *Pichia pastoris*

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Abstract To obtain the protein expression of a hybrid xylanase in yeast, the gene encoding it was modified according to the codon bias of *Pichia pastoris* and expressed extracellularly in this yeast as an active xylanase, MBtx, exhibited a molecular mass of approximately 35 kDa on SDS–PAGE. The pH behavior of MBtx in terms of both activity and stability was similar to that of Btx, original gene product in *Escherichia coli*, while a certain difference was observed in optimal temperature for activity and in thermal stability. HPLC analysis revealed the xylan in wheat could be hydrolyzed by MBtx and the major hydrolysis product was xylotriose. These results showed codon usage played a key role in regulating the expression of the hybrid xylanase in *P. pastoris* and the recombinant hybrid xylanase, MBtx, produced by *P. pastoris* could be potentially useful in feed industry.

Keywords Hybrid xylanase · Gene modification · *Pichia pastoris* · Expression · Feed industry

Introduction

Xylan is the major constituent of hemicelluloses and is the second abundant renewable resource after cellulose with a high potential for degradation to useful end products. The main chain of this heterogenous polysaccharide consists of β-1, 4-linked D-xylopyranoside residues which can be substituted with acetyl, arabinosyl, and glucuronosyl side chains depending on the botanical origin. Several enzymes are involved in the hydrolysis of xylan polymer; the most important one is the *endo*-β-(1, 4)-xylanase (EC 3.2.1.8), which has been used commercially in the paper, food, and feed industries (Li et al. 2000). In animal feeds, supplementation with exogenous xylanases can reduce the viscosity of intestinal contents and improve nutrient digestibility in domestic animal fed on diets containing wheat (Steenfeldt et al. 1998; Hew et al. 1998).

Catalytic and biochemical properties of many wild type and recombinant xylanases have been studied (Karlsson et al. 1998; Katapodis et al. 2003; Chantasingh et al. 2006; Berrin et al. 2000), but little is known about catalytic and hydrolytic properties of constructed hybrid xylanase whose parents are family 11 xylanases. In our laboratory, a gene encoding a hybrid xylanase was constructed by substituting the 31 *N*-terminal amino acid residues of the *Thermomonospora fusca* xylanase A (TfxA) for the corresponding region of 22 amino acid residues of the *Bacillus subtilis* xylanase A (BsxA). Expressing the construct gene, *btx*, in *Escherichia coli* BL21 resulted in a recombinant hybrid xylanase, Btx, with excellent thermostability (Weng and Sun, 2005). To produce this hybrid xylanase in large scale for commercial use, the industrial yeast, *Pichia pastoris,* was selected to be the host. However, it is difficult to obtain its protein expression in *P. pastoris* probably due to rare codons and potential mRNA instability or polyadenylation motifs represented by five or more A/T or G/C repeats in *btx*. In this study, *btx* was modified according to the codon usage bias of *P. pastoris* (Sreekrishna et al. 1997; Sinclair and Choy 2002) and expressed extracellularly in this yeast as an active xylanase, MBtx.

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Materials and methods

Strains and plasmids

In this study, the *E. coli TOP 10F'* cells were used for DNA manipulation while *P. pastoris* strain GS115 (*his4*) was used for protein expression. Vector pPICZαA, carrying the Zeocin resistance gene for selection of both *E. coli* and *P. pastoris* transformants, was used for extracellular production of the recombinant protein. The two strains and the plasmid listed above were contained in EasySelect™ *Pichia* Expression Kit, production of Invitrogen (San Diego, CA). pGEM®-T Easy vector used for DNA cloning was obtained from Promega (Madison, WI).

Reagents

T4 DNA ligase and PCR kit were obtained from Promega; pfu DNA polymerase kit and restrict enzymes were from MBI Fermentas (Burlington, CA). Oligonucleotides (Table 1) were synthesized by Shangon (Shanghai, China). Ni-NTA agarose resin was from Qiagen (Hilden, Germany). Birch xylan and dinitrosalicylic acid (DNS) were purchased from Sigma (St. louis, MO). Wheat bran-insoluble xylan was kindly provided by Dr. Chen (Southern Yangtze University). The standard xylooligosaccharides (X2–X6) were from Megazyme (Wicklow, Ireland). Xylose (X) was from Merck (Darmstadt, Germany).

Gene modification

A fragment (mbtx02) designed to replace the middle fragment of the original gene, *btx*, for removing five or more A/T or G/C repeats and replacing the codons with an abundance of <10% with more frequently occurring ones was generated through the overlap extension of two long oligonucleotides 5mbtx02 and 3mbtx02 with overlapping sequence in the 3′ or 5′ end. The flanking fragments mbtx01 and mbtx03 were amplified from *btx* previously cloned into *E. coli* expression vector pET-30(a) (Weng and Sun, 2005), and introduced restriction enzyme sites *Eco*RI at 5′ end of mbtx01 and *Xba*I at 3′ end of mbtx03. In their other ends, they have overlapping sequences with mbtx02. Then these three fragments were spliced one by one by the technique of splicing by overlap extension by the polymerase chain reaction (SOE by PCR) (Warrens et al. 1997) (Fig. 1). The resulted modified gene, *mbtx*, was subcloned into the pGEM®-T Easy vector, and subjected to DNA sequencing to confirm that no errors were generated during the PCR.

Construction of expression plasmid

The *mbtx* fragment digested from pGEM®-T Easy vector was inserted into pPICZαA vector at *Eco*RI and *Xba*I sites to fuse in frame to the α-factor secretion signal with c-myc epitope and polyhistidine (6His) tag formed in the *C*-terminus. The construct pPICZαA-mbtx was transformed into competent *E. coli* TOP*10F'* cells which were plated on Low Salt LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl; 1.6% agar in plates, pH 7.5) containing 25 ug/ml Zeocin™ plates. The positive colonies were then grown to prepare DNA for transformation.

Transformation of *P. pastoris* and selection of secreting colonies

The pPICZαA-*mbtx* plasmid was linearized with *Sac*I for integration into the *P. pastoris* genome at the alcohol

Table 1 Oligonucleotides used in the process of the gene modification

Name	Sequence and restriction sites[a]
5hx01	5-TAG^AATTC (*Eco*RI) GCCTCTCATGCTGCT-3
3hx01	5-TTCTGAATGGGGAACCTGTAGTC-3
5hx02	5-ACAGGCTCCCCATTCAGAACTATCAA-3
3hx02	5-GTTGTACCTGGTGGTTGTGTAGATGTC-3
5hx03	5-ACATCTACACAACTACAAGATACAACGCACC-3
3hx03	5-TCTAGATGCCACACTGTTACGTTAGAACTTC-3
3hx03y	5-TCTAG^A (*Xba*I) TGCCACACTGTTACGTTAG-3
5mx02	5-ACAGGTTCCCCATTCAGAACGATCAACTACAATGCTGGAGTTTGGGCTC CAAATGGTAATGGTTACTTGACTTTGTATGCTTGGACCAGATCTCCACTTA TCGAATACTACGTGGTGGAT-3
3mx02	5-GTTGTATCTTGTAGTTGTGTAGATGTCATATGTACCACCATCACTCTTT ACAGTACCCTTGATGGTTCCGGTAGGTCTGTAAGTACCCCATGAATCCA CCACGTAGTATTCGA-3

[a] The sequences in same color shadings are the overlapping sequences

oxidase 1 (*AOX1*) locus, and transformed into *P. pastoris* stain GS115 with the electroporation method as described in the manual for EasySelect™ *Pichia* Expression Kit of Invitrogen. The transformants were screened on YPDS + Zeocin™ plates (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar, 100 μg/ml Zeocin™) and the Zeocin™-resistant *Pichia* colonies were replica-plated onto MDH and MMH plates (1.34% yeast nitrogen base, 0.4 mg/l biotin, 2% agar, 40 mg/l histidine and 2% dextrose or 0.5% methanol, respectively) to determine their methanol-utilizing phenotypes. The Mut^+ phenotype grew normally on both MMH and MDH pates whereas the Mut^- phenotype grew very slowly on MMH plates. The Mut^+ transformants were selected and incubated at 30°C in a shaking incubator (250 rpm) in 5 ml BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 0.4 mg/l biotin, 1% glycerol) for 20 h. After the cultures reached an $OD_{600} = 4$, the yeast cells were harvested by centrifugation (2,000*g,* 5 min) and re-suspended in 50 ml BMMY (the same as BMGY but with 0.5% methanol instead of glycerol) to induce the expression of the hybrid xylanase, MBtx, in a shaking flask. Every 24 h, 100% methanol was added into the culture to a final concentration of 0.5% to maintain induction. At each of the times (0, 6, 12, 24, 36, 48, 60, 72, 84, and 96 h) 1 ml of the culture was centrifuged and the amount of MBtx in the supernatant was estimated by activity measurement assays.

Production, purification and SDS–PAGE analysis of MBtx

The scale up expression was performed in 2-l shake flask containing 500 ml BMMY medium. The culture supernatant collected at the optimal inducing time point was freeze-dried and applied for Ni-NTA affinity chromatography according to the manufacturer's instruction (Qiagen). Aliquots of the purification product and the culture supernatant were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), using the culture supernatant of GS115 with pPICZαA transformed in it as control. The protein concentration of the recombinant enzyme was measured by the dye-binding assay method of Bradford with the bovine serum albumin (BSA) as the standard (Bradford 1976).

Xylanase activity assay

The xylanase activity was measured with 1% birch wood (w/v) as substrate at 50°C in McIlvaine's buffer (pH 5.0) (Bailey et al. 1992). Reducing sugars freed by enzymatic hydrolysis were quantified by the dinitrosalicylic acid (DNS) (Miller et al. 1960). One unit of xylanase activity was defined as the amount of enzyme that produced 1 μmol of xylose equivalent per minute.

pH optimum and stability

The effect of pH on xylanase activity was measured over a range of pH 3.0–7.0 (McIlvaine's buffer system) and 8.0–9.0 (0.2 M glycine, 0.2 M NaOH buffer system) at 60°C. The pH stability of the enzyme was determined by incubating the xylanase in various pH buffers at 25°C for 1 h. The residual activity was estimated following the procedure described above.

Temperature optimum and stability

The effect of temperature on the enzyme activity was estimated at optimal pH at temperature ranging from 30 to 90°C. The thermo stability of xylanase was determined by pre-incubating the enzyme in the absence of substrate at different temperature for 2 min, respectively, then cooling on ice for 5 min before residual xylanase activity measurements.

Hydrolysis products of bran insoluble xylan by MBtx

Xylose (X) and standard xylooligosaccharides (X2–X6) were resolved in pure water. Samples of sugar (X–X6) were analyzed by HPLC separately with Sugar-PakTM1 column (300 mm × 6.5 mm; Waters, Milford, MA), pure water as mobile phase (0.5 ml/min) and injection volumes of 20 μl. The areas of sugar peaks were screened and calculated using a Waters 2,401 refractive index detector, and the standard concentration curves of xylooligosaccharides (X–X6) were obtained according to the correlation of peak area and concentration. The 16 mg/ml bran insoluble xylan solution in McIlvaine's buffer (pH 6.0) was hydrolyzed by MBtx at 40°C with constant shaking. In the reaction mixture, the substrate was excessive. The hydrolytic products in this system for 20 h were analyzed under same HPLC conditions and quantified according to standard curves.

Results

Gene modification

Sequencing result showed *mbtx* was identified with the theoretical design. Thirty-eight codons were replaced to fit for the preference of *P. pastoris* cells, while the deduced amino acid sequence was not changed (Fig. 2).

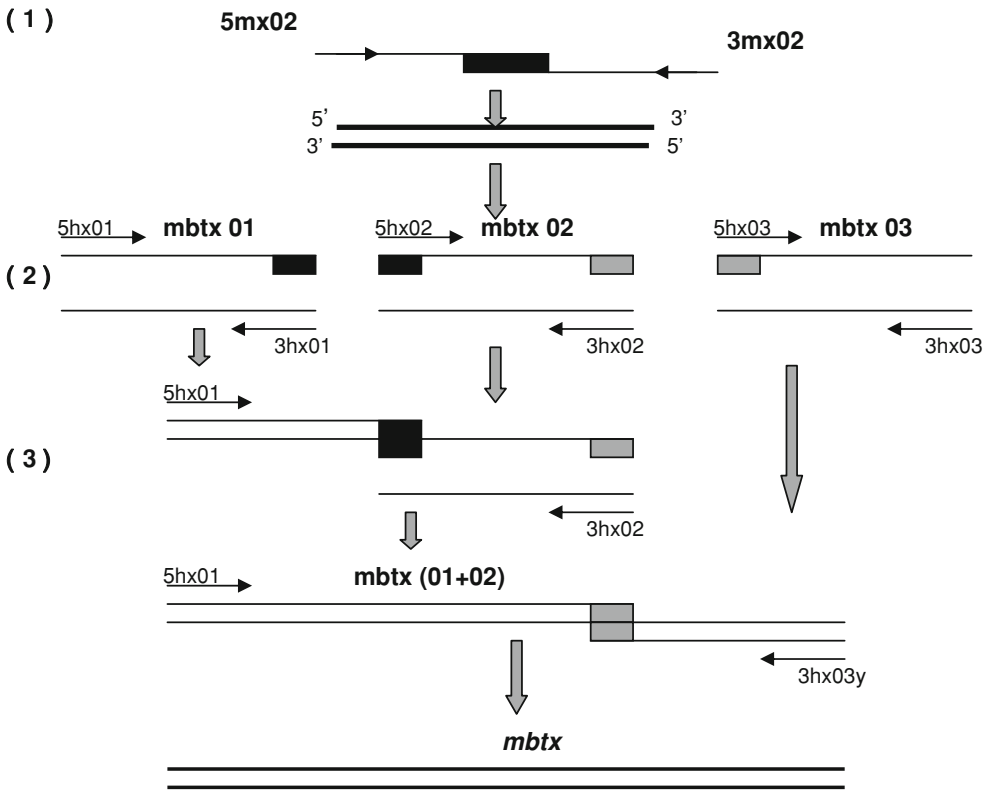

Fig. 1 Process of the gene modification Oligonucleotides are shown as the line with *arrowhead*. Overlapping sequences are represented with *solid shading* or *lightly stippled*. Reagents used in PCR were from pfu DNA Polymerase kit; The reaction system was in 50 μl; The PCR protocol used in step (1) was as follows: 94°C for 2 min, 94°C for 50 s, 70°C for 50 s, 72°C for 1 min, finally cooling at 4°C. Then the PCR product was gel eluted and amplified, meanwhile, mbtx01 and mbtx03 were also amplified from *btx*. All the amplification conditions in step (2) involved 10 cycles of 94°C 50 s, 60°C −0.4°C per cycle for 50 s, 72°C for 1 min and 30 cycles of 94°C 50 s, 56°C −0.2°C per cycle for 50 s, 72°C for 1 min, finally cooling at 4°C. In step (3) mbtx01, mbtx02 and mbtx03 were spliced one by one to form a modified gene *mbtx* by the technique of SOE by PCR. The PCR protocols in this step included 10 cycles of 94°C for 50 s, 70°C −0.4°C per cycle for 50 s, 72°C for 1 min and 30 cycles of 94°C 50 s, 66°C −0.2°C per cycle 50 s, 72°C 1 min and finally remained at 4°C

Construction of expression plasmid, transformation of *P. pastoris* and selection of secreting colonies

A total of 15 transformants of pPICZαA-*mbtx* were analyzed for their expression performance and expression time courses. Almost same levels of xylanase activities were detected in culture supernatants of the 15 transforments grown on BMMY medium in shaken culture. The highest enzyme activity (4 ± 0.12 U/ml) was reached after 60 h 0.5% methon induction.

Production, purification and SDS–PAGE analysis of MBtx

The yield of the purified 6His-tagged MBtx was 1.8 mg in 500 ml of culture with 1,338 U total activity. Analysis of SDS–PAGE showed MBtx ran at a specific protein band about 35 kDa, which is consistent with the theoretical molecular mass 34.4 kDa (Fig. 3), calculated on the basis of the deduced amino acid sequence.

Effects of pH and temperature

The effects of pH and temperature on MBtx were compared with Btx. As shown in Fig. 4a, MBtx and Btx have almost the identical activity curves with the optimum pH at 7.0 from 3.0 to 7.0. However, the activity of MBtx has a dramatic improvement from 8.0 to 9.0, which was not seen on Btx. Figure 4b indicated the two enzymes were all stable over a wide pH range (4.0–10.0). Figure 4c revealed the optimum temperature rang (40–50°C) of MBtx was lower than that of Btx (50–60°C). Temperature-stability study indicated that MBtx was not thermostable as Btx. As shown in Fig. 4d, MBtx lost its activity drastically above 60°C, while Btx kept more than 90% activity below 80°C.

Hydrolysis products of bran insoluble xylan by MBtx

The hydrolysis products of wheat-bran insoluble xylan including xylose(X) and xylooligosaccharides (X2–X6). Xylotriose(X3) is the major products. After 20 h

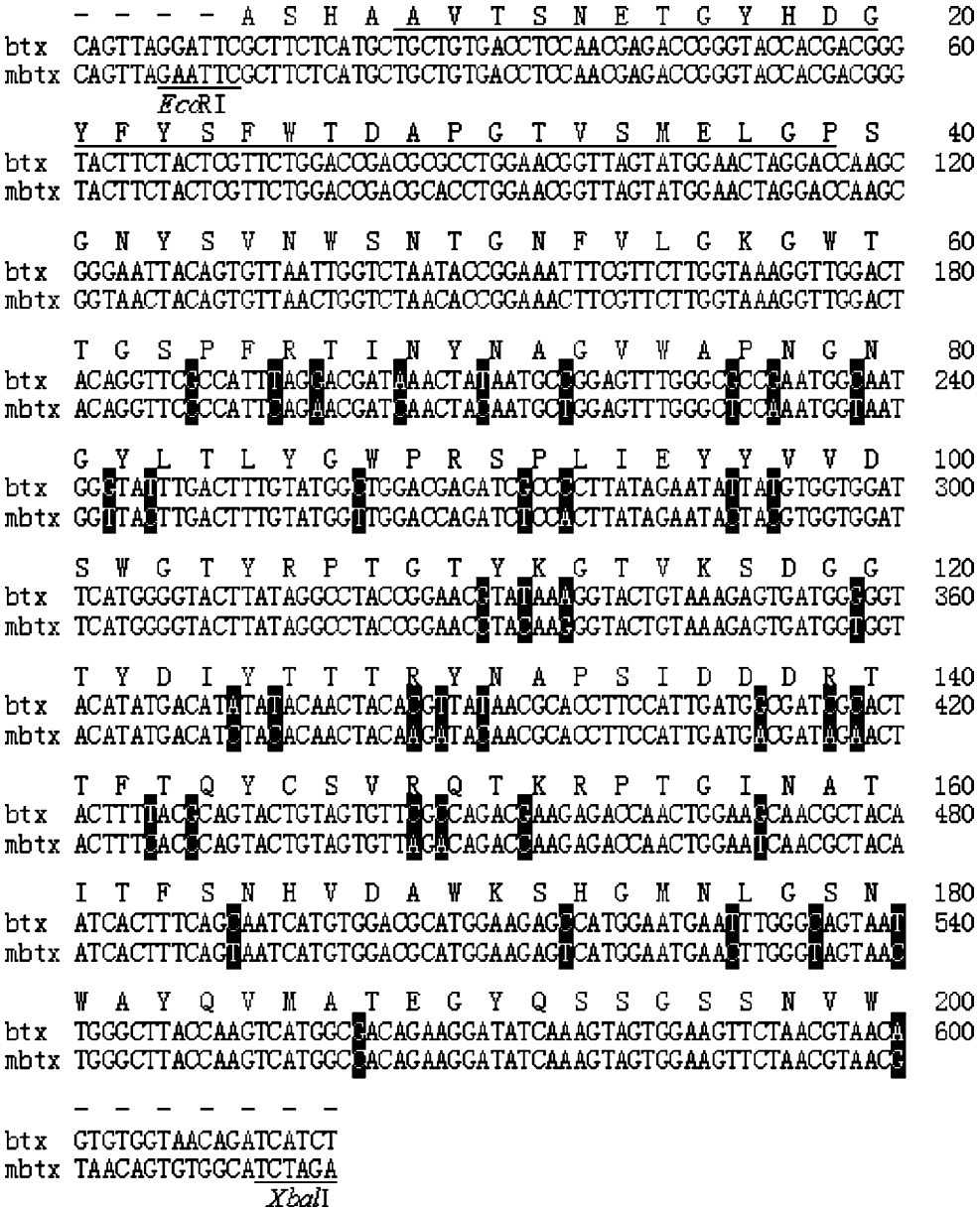

Fig. 2 Nucleotide sequences of *btx* and *mbtx* and their deduced amino acid sequence. *Note* Amino acids underlined are from *N* terminus of *Thermomonospora fusca* xylanase. The bases in panes are the codons that be replaced

incubation, xylotriose accounted for 32.81% of total hydrolysis products and its concentration was 1.3 mg/ml (Fig. 5). In this process, about 29.5% wheat-bran insoluble xylan was hydrolyzed by MBtx.

Discussion

In these years, the methylotrophic yeast *Pichia pastoris* has developed into a highly successful system for production of a wide range of heterologous proteins. This system permits high-density fermentation, tightly regulated expression, and efficient secretion of recombinant proteins. These attributes make it meet the industrial demands of interest proteins (Cereghion and Cregg 2000). Despite the success of the *P. pastoris* system, opportunities exist to develop a larger range of proteins that can be expressed in the system (Romanos 1995). Codon usage could play a key role in regulating gene expression and in the production of large quantities of high-quality heterologous protein (Eckart and Bussineau 1996). Even the regional optimization of the 5′ end of the coding region or the removal of only particularly rare codons throughout the gene has shown to have a significant impact on heterologous protein production (Outchkourov et al. 2002; Li et al. 2008; Hu et al. 2006; Trinh et al. 2004). In this study, the successful expression of the modified gene in *P. pastoris* confirmed the codon optimization towards the synonymous codon usage bias of *P. pastoris* had a positive impact on expression levels (Sinclair and Choy 2002).

However, the amount of the desired gene product in the shaken culture was relative low, which were likely due to impossibility of maintaining methanol concentrations within the narrow range required for promoter induction; on the other hand, further addition of methanol during cultivation in shaken culture was not possible for the risk of incurring in cell accumulation of methanol that could result in cytotoxic effects (Guarna et al. 1997). Unfavorable dissolved oxygen concentration as a consequence of the impossibility to control oxygen supply in shaken culture could be another reason of low production of MBtx (Lee et al. 2003a). Low cell concentration reached in shaken culture was also observed in this study. Therefore, high density fermentation could be an efficient method to improve the production of MBtx by *P. pastoris*, with the fermentation protocols to be further studied and optimized (Lee et al. 2003b; Files et al. 2001).

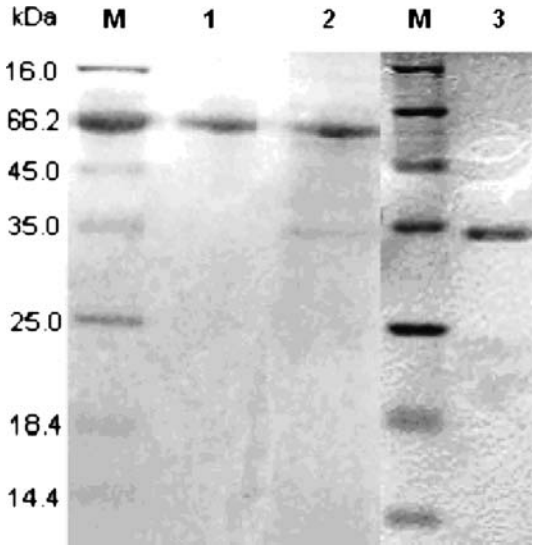

Fig. 3 SDS–PAGE analysis of MBtx *line 1* is the control (GS115 with pPICZαA transformed in it) *Line 2* is MBtx in culture supernatant of secreting colons induced by 1% methanol for 60 h. *Line 3* is purified MBtx. *M* is protein mark

In addition, MBtx is not thermal stable as Btx. Post-translation modification, disulphide isomerization or phosphorylation readily performed by *P.pastoris* cells probably are causes of the reduced thermo stability. Some researches have reported extensive *N*-glycosylation in *P. pastoris* could reduce the thermal stability of enzymes (Tull et al. 2001; Gemmill and Trimble 1999). However, compared with other recombinant xylanases that expressed in *P. pastoris* in our laboratory (Sun et al. 2005; Sun et al. 2007), MBtx could remain more residue activity in high temperature feed pelleting process. Furthermore, the optimum pH and temperature of MBtx are so close to the physiological conditions of animal small intestine that it would have excellent hydrolysis performance in small intestine.

HPLC analysis of wheat-bran insoluble xylan hydrolyzed by MBtx revealed that MBtx can hydrolyze the

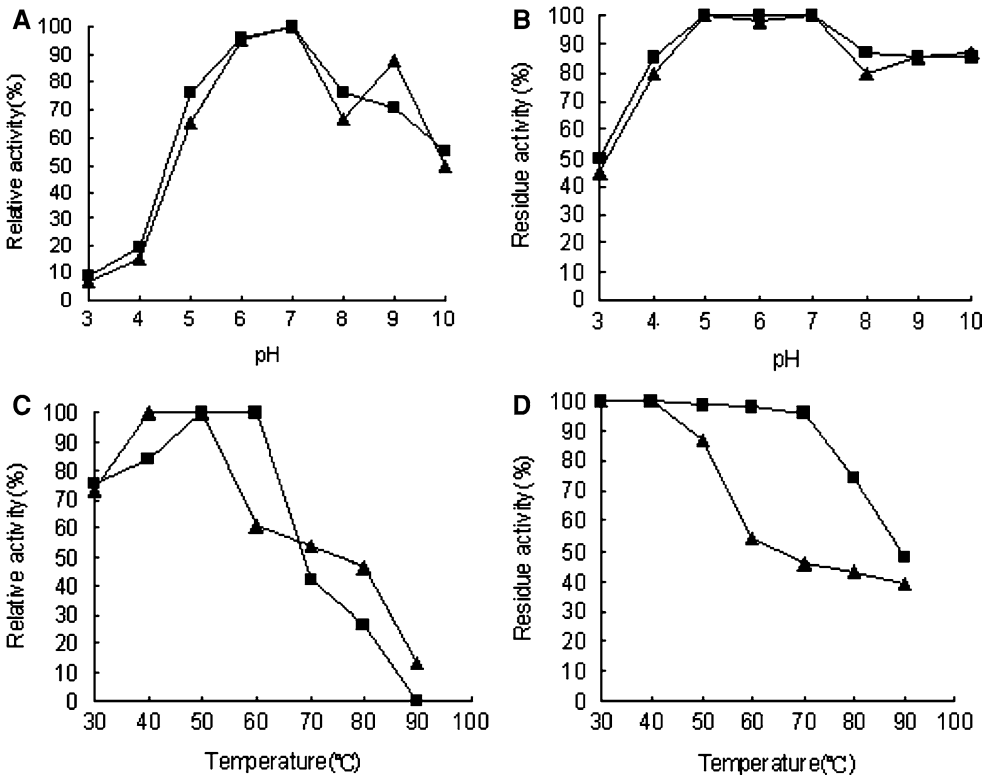

Fig. 4 Effect of pH and temperature on the xylanase activity of MBtx (—▲—) and Btx (—■—) **a** pH optimum; **b** pH stability; **c** temperature optimum; **d** thermostability. *Note* The activity assays were performed as described under "Materials and methods" using birch wood xylan as substrate. The highest xylanase activity was taken as 100%

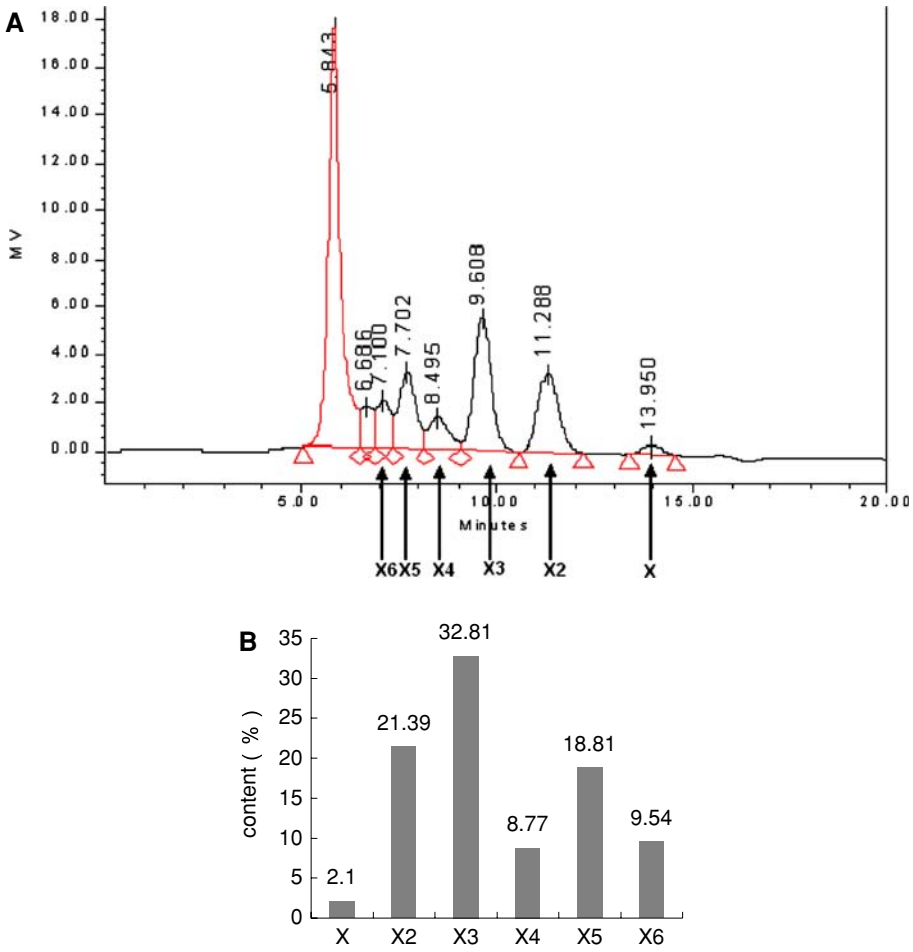

Fig. 5 HPLC profiles **a** and contents; **b** of hydrolysis products of wheat-bran insoluble xylan by MBtx after 20 h. The positions of xylose (X), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), xylohexaose (X6) are shown

arabinoxylan in cell walls of cereal grains. The main hydrolysis product released from wheat to bran insoluble xylan by MBtx was different from reTfxA, the recombinant TfxA expressed by *P. pastoris* (Sun et al. 2007), which implies the hydrolytic properties of MBtx may relate to its catalytic domains inherited from BsxA.

In conclusion, codon usage played a key role in expression of the hybrid xylanase in *P. pastoris* and the recombinant hybrid xylanase, MBtx, produced by *P. pastoris* could be potentially applied in feed industry.

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References

Bailey MJ, Biely P, Poutanen K (1992) Interlaboratory testing of methods for assay of xylanase activity. J Biotechnol 32:257–270. doi:10.1016/0168-1656(92)90074-J

Berrin JG, Williamson G, Puigserver A, Chaix JC, McLauchlan WR, Juge N (2000) High-level production of recombinant fungal endo-b-1, 4-xylanase in the methylotrophic yeast pichia pastoris. Protein Expr Purif 19:179–187. doi:10.1006/prep.2000.1229

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. doi:10.1016/0003-2697(76)90527-3

Cereghion JL, Cregg JM (2000) Heterologous protein expression in the methylotrophic yeast Pichia pastoris. FEMS Microbiol Rev 24:45–66. doi:10.1111/j.1574-6976.2000.tb00532.x

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 Expr Purif 46:143–149. doi:10.1016/j.pep.2005.09.013

Eckart MR, Bussineau CM (1996) Quality and authenticity of heterologous proteins synthesized in yeast. Curr Opin Biotechnol 7:525–530. doi:10.1016/S0958-1669(96)80056-5

Files D, Ogawa M, Scaman CH, Baldwin SA (2001) A *Pichia pastoris* fermentation process for producing high-levels of recombinant human cystatin-C. Enzyme Microb Technol 29:335–340. doi:10.1016/S0141-0229(01)00395-7

Gemmill TR, Trimble RB (1999) Overview of *N*- and *O*-linked oligosaccharide structures found in various yeast species. Biochim Biophys Acta 1426:227–237

Guarna MM, Lesnicky GJ, Tam BM, Robinson J, Radziminsky CZ, Hasenwinkle D, Boraston A, Jeris E, MacGiliray RTA, Turner RFB, Kilburn DG (1997) On-line monitoring and control of methanol concentration in shake flask cultures of *Pichia*

pastoris. Biotechnol Bioeng 56:279–286. doi:10.1002/(SICI)1097-0290(19971105)56:3<279::AID-BIT5>3.0.CO;2-G

Hew LI, Ravindran V, Mollah Y, Bryden WL (1998) Influence of exogenous xylanase supplementation on apparent metabolisable energy and amino acid digestibility in wheat for broiler chickens. Anim Feed Sci Technol 75:83–92. doi:10.1016/S0377-8401(98)00206-5

Hu S, Li L, Qiao J, Guo Y, Cheng L, Liu J (2006) Codon optimization, expression, and characterization of an internalizing anti-ErbB2 single-chain antibody in *Pichia pastoris*. Protein Expr Purif 47:249–257. doi:10.1016/j.pep.2005.11.014

Karlsson EN, Dahlberg L, Torto N, Gorton L, Holst O (1998) Enzymatic specificity and hydrolysis pattern of the catalytic domain of the xylanase Xyn1 from *Rhodothermus marinus*. J Biotechnol 60:23–35. doi:10.1016/S0168-1656(97)00178-8

Katapodis P, Vrsanska M, Kekos D, Nerinckx W, Biely P, Claeyssens M, Macris BJ, Christakopoulos P (2003) Biochemical and catalytic properties of an endoxylanase purified from the culture filtrate of Sporotrichum thermophile. Carbohydr Res 338:1881–1890. doi:10.1016/S0008-6215(03)00291-X

Lee CY, Lee SJ, Jung KH, Katoh S, Lee EK (2003a) High dissolved oxygen tension enhances heterologous protein expression by recombinant Pichia pastoris. Process Biochem 38:1147–1154. doi:10.1016/S0032-9592(02)00280-7

Lee CY, Nakano A, Shiomi N, Lee EK, Katoh S (2003b) Effects of substrate feed rates on heterologous protein expression by *Pichia pastoris* in DO-stat fed-batch fermentation. Enzyme Microb Technol 33:358–365. doi:10.1016/S0141-0229(03)00146-7

Li K, Azadi P, Collins R, Tolan J, Kim JS, Eriksson KEL (2000) Relationships between activities of xylanases and xylan structures. Enzyme Microb Technol 27:89–94. doi:10.1016/S0141-0229(00)00190-3

Li Z, Hong G, Wu Z, Hu B, Xu J, Li L (2008) Optimization of the expression of hepatitis B virus e gene in *Pichia pastoris* and immunological characterization of the product. J Biotechnol 138:1–8. doi:10.1016/j.jbiotec.2008.07.1989

Miller GL, Blum R, Glennon WE, Burton AL (1960) Measurement of carboxymethylcellulase activity. Anal Biochem 2:127–132. doi:10.1016/0003-2697(60)90004-X

Outchkourov NS, Stiekema WJ, Jongsma MA (2002) Optimization of the expression of equistatin in *Pichia pastoris*. Protein Expr Purif 24:18–24. doi:10.1006/prep.2001.1523

Romanos M (1995) Advances in the use of *Pichia pastoris* for high-level gene expression. Curr Opin Biotechnol 6:527–533. doi:10.1016/0958-1669(95)80087-5

Sinclair G, Choy FYM (2002) Synonymous codon usage bias and the expression of human glucocerebrosidase in the methylotrophic yeast, *Pichia pastoris*. Protein Expr Purif 26:96–105. doi:10.1016/S1046-5928(02)00526-0

Sreekrishna K, Brankamp RG, Kropp KE, Blankenship DT, Tsayn JT, Smith PL, Wierschke JD, Subramaniam A, Birkenberger LA (1997) Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast Pichia pastorid. Gene 190:55–62

Steenfeldt S, Hammershoj M, Mullertz A, Jensen JF (1998) Enzyme supplementation of wheat-based diets for broilers. Anim Feed Sci Technol 75:27–43. doi:10.1016/S0377-8401(98)00189-8

Sun JY, Liu MQ, Xu YL, Xu ZR, Pan L, Gao H (2005) Improvement of the thermostability and catalytic activity of a mesophilic family 11 xylanase by *N*-terminus replacement. Protein Expr Purif 42:122–130. doi:10.1016/j.pep.2005.03.009

Sun JY, Liu MQ, Weng XY, Qian LC, Gu SH (2007) Expression of recombinant Thermomonospora fusca xylanase A in Pichia pastoris and xylooligosaccharides released from xylans by it. Food Chem 104:1055–1064. doi:10.1016/j.foodchem.2007.01.028

Trinh R, Gurbaxani B, Morrison SL, Seyfzadeh M (2004) Optimization of codon pair use within the (GGGGS)3 linker sequence results in enhanced protein expression. Mol Immunol 40:717–722. doi:10.1016/j.molimm.2003.08.006

Tull D, Gottschalk TE, Svendsen I, Kramhøft B, Phillipson BA, Bisgård-Frantzen H, Olsen O, Svensson B (2001) Extensive *N*-glycosylation reduces the thermal stability of a recombinant Alkalophilic *Bacillus* a-amylase produced in *Pichia pastoris*. Protein Expr Purif 21:13–23. doi:10.1006/prep.2000.1348

Warrens AN, Jones MD, Lechler RI (1997) Splicing by overlap extension by PCR using asymmetric amplification: an improved technique for the generation of hybrid proteins of immunological interest. Gene 186:29–35. doi:10.1016/S0378-1119(96)00674-9

Weng XY, Sun JY (2005) Construction, expression, and characterization of a thermostable xylanase. Curr Microbiol 51:188–192. doi:10.1007/s00284-005-4543-4