

Pichia pastoris

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To obtain the protein expression of a hybrid lanase 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 lanase, MBt. We inhibited a molecular mass of approximately 35 kDa on SDS-PAGE. The pH behavior of MBt in terms of both activity and stability was similar to that of Bt, 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 lan in heat could be hydrolyzed by MBt and the major hydrolysis product was lotriose. These results showed codon usage played a key role in regulating the expression of the hybrid lanase in *P. pastoris* and the recombinant hybrid lanase, MBt, produced by *P. pastoris* could be potentially useful in feed industry.

Hybrid lanase · Gene modification ·
Pichia pastoris · Expression · Feed industry

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 heterogeneous

polysaccharide consists of β -1, 4-linked D-glucopyranoside residues which can be substituted with acetate, arabinose, 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 animals fed on diets containing heat (Steenfeldt et al. 1998; He et al. 1998).

Catalytic and biochemical properties of mammalian 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 (TfA) for the corresponding region of 22 amino acid residues of the *Bacillus subtilis* xylanase A (BsA). Expressing the construct gene, *btx*, in *Escherichia coli* BL21 resulted in a recombinant hybrid xylanase, Bt, 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 one 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 Cho 2002) and expressed extracellularly in this yeast as an active xylanase, MBt.

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Strains and plasmids

In this study, the *E. coli* TOP10F' 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 Eas Select™ *Pichia* Expression Kit, production of Invitrogen (San Diego, CA). pGEMT-T Eas 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 restriction enzymes were from MBI Fermentas (Burlington, CA). Oligonucleotides (Table 1) were synthesized by Shanon (Shanghai, China). Ni-NTA agarose resin was from Qiagen (Hilden, Germany). Birch lan and dinitrosalic acid (DNS) were purchased from Sigma (St. Louis, MO). Wheat bran-insoluble lan was kindly provided by Dr. Chen (Southern Yangtze University). The standard monooligosaccharides (X2-X6) were from Megazyme (Wicklow, Ireland). Xylose (X) was from Merck (Darmstadt, Germany).

Gene modification

A fragment (mbt02) designed to replace the middle fragment of the original gene, *btx*, for removing one or more A/

oxidase 1 (*AOX1*) locus, and transformed into *P. pastoris* strain GS115 with the electroporation method as described in the manual for Eas Select™ *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 plates 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 OD₆₀₀ = 4, the yeast cells were harvested by centrifugation (2,000g, 5 min) and resuspended in 50 ml BMMY (the same as BMGY but with 0.5% methanol instead of glycerol) to induce the expression of the hybrid lanase, MBt, 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 MBt in the supernatant was estimated by activity measurement assays.

Production, purification and SDS PAGE analysis of MBt

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 (1/v) as substrate at 50 °C in McIlvaine's buffer (pH 5.0) (Baile 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 glucose 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 thermostability 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 MBt

Xylose (X) and standard oligosaccharides (X2–X6) were resolved in pure water. Samples of sugar (X–X6) were analyzed by HPLC separately with Sugar-Pak™1 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 2401 refractive index detector, and the standard concentration curves of oligosaccharides (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 MBt 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.

Gene modification

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

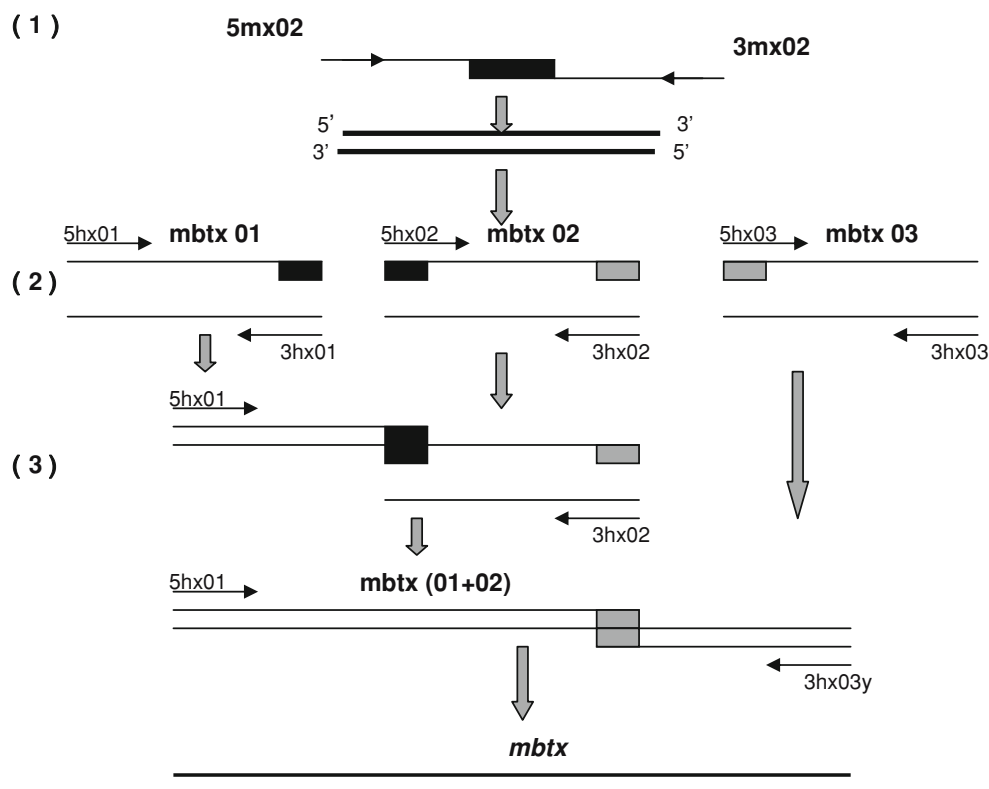


Fig. 1 Process of the gene modification. Oligonucleotides are shown as the line with arrowhead. Overlapping sequences are represented with solid shading or lightly stippled. Reagents used in PCR are from pfu DNA Polymerase kit; The reaction system is in 50 μ l; The PCR protocol used in step (1) is as follows: 94 $^{\circ}$ C for 2 min, 94 $^{\circ}$ C for 50 s, 70 $^{\circ}$ C for 50 s, 72 $^{\circ}$ C for 1 min, natural cooling at 4 $^{\circ}$ C. Then the PCR product is gel eluted and amplified, meanwhile, mbt 01 and mbt 03 are also amplified from btx. All the amplification

conditions in step (2) involved 10 cycles of 94 $^{\circ}$ C 50 s, 60 $^{\circ}$ C –0.4 $^{\circ}$ C per cycle for 50 s, 72 $^{\circ}$ C for 1 min and 30 cycles of 94 $^{\circ}$ C 50 s, 56 $^{\circ}$ C –0.2 $^{\circ}$ C per cycle for 50 s, 72 $^{\circ}$ C for 1 min, natural cooling at 4 $^{\circ}$ C. In step (3) mbt 01, mbt 02 and mbt 03 are 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 $^{\circ}$ C for 50 s, 70 $^{\circ}$ C –0.4 $^{\circ}$ C per cycle for 50 s, 72 $^{\circ}$ C for 1 min and 30 cycles of 94 $^{\circ}$ C 50 s, 66 $^{\circ}$ C –0.2 $^{\circ}$ C per cycle 50 s, 72 $^{\circ}$ C 1 min and natural remained at 4 $^{\circ}$ C.

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

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

Production, purification and SDS PAGE analysis of MBt

The yield of the purified 6His-tagged MBt was 1.8 mg in 500 ml of culture with 1,338 U total activity. Analysis of SDS PAGE showed MBt 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 MBt were compared with Bt. As shown in Fig. 4a, MBt and Bt have almost the identical activity curves with the optimum pH at 7.0 from 3.0 to 7.0. However, the activity of MBt has a dramatic improvement from 8.0 to 9.0, which was not seen on Bt. Figure 4b indicated the two enzymes were all stable over a wide pH range (4.0–10.0). Figure 4c revealed the optimum temperature range (40–50 $^{\circ}$ C) of MBt was lower than that of Bt (50–60 $^{\circ}$ C). Temperature-stability studies indicated that MBt was not thermostable as Bt. As shown in Fig. 4d, MBt lost its activity drastically above 60 $^{\circ}$ C, while Bt kept more than 90% activity below 80 $^{\circ}$ C.

Hydrolysis products of bran insoluble alanine MBt

The hydrolysis products of heat-bran insoluble alanine including fucose(X) and oligosaccharides (X2–X6). X3 (trifucose(X3)) is the major products. After 20 h

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

	- - - - A S H A A V T S N E T G Y H D G	20
<i>btx</i>	CAGTTAGGATTTCGCTTCTCATGCTGCTGTGACCTCCAAACGAGACCGGGTAACACGACGGG	60
<i>mbtx</i>	CAGTTAGAATTTCGCTTCTCATGCTGCTGTGACCTCCAAACGAGACCGGGTAACACGACGGG	
	<u>Y F Y S F W T D A P G T V S M E L G P S</u>	40
<i>btx</i>	TACTTCTACTCGTTCTGACGACGCGCCTGGAACGGTTAGTATGGAAC TAGGACCAAGC	120
<i>mbtx</i>	TACTTCTACTCGTTCTGACGACGCGCACCTGGAACGGTTAGTATGGAAC TAGGACCAAGC	
	G N Y S V N W S N T G N F V L G K G W T	60
<i>btx</i>	GGGAATTACAGTGTTAATTGGTCTAATACCGGAAATTTGTTCTTGGTAAAGGTTGGACT	180
<i>mbtx</i>	GGTAAC TACAGTGTTAATCTGGTCTAATACCGGAAACTTCTGTTCTTGGTAAAGGTTGGACT	
	T G S P F R T I N Y N A G V W A P N G N	80
<i>btx</i>	ACAGGTTCCCATTTAGCAACGATAACTATAATGCCGGAGTTTGGGCGCCCAATGGCAAT	240
<i>mbtx</i>	ACAGGTTCCCATTTAGCAACGATAACTATAATGCTGGAGTTTGGGCGCCCAATGGCAAT	
	G Y L T L Y G W P R S P L I E Y Y V V D	100
<i>btx</i>	GGTAATTGACTTTTGATGGGTGGACGAGATCCCGCTTATAGAATAATATGTGGTGGAT	300
<i>mbtx</i>	GGTAATTGACTTTTGATGGGTGGACGAGATCCCGCTTATAGAATAATATGTGGTGGAT	
	S W G T Y R P T G T Y K G T V K S D G G	120
<i>btx</i>	TCATGGGTAAGTTATAGGCTACCGGAACGTATAAAGGTACTGTAAAGAGTGATGGTGGT	360
<i>mbtx</i>	TCATGGGTAAGTTATAGGCTACCGGAACGTATAAAGGTACTGTAAAGAGTGATGGTGGT	
	T Y D I Y T T T R Y N A P S I D D D R T	140
<i>btx</i>	ACATATGACATATATACAAC TACAAGTTATAACGCACTTCCATTGATGACGATGCAACT	420
<i>mbtx</i>	ACATATGACATATATACAAC TACAAGTTATAACGCACTTCCATTGATGACGATGCAACT	
	T F T Q Y C S V R Q T K R P T G I N A T	160
<i>btx</i>	ACTTTTACCACTACTTCTAGTCTTCTGACACGAAACACACCAACTGCAACCAACCTTAC	480
<i>mbtx</i>	ACTTTTACCACTACTTCTAGTCTTCTGACACGAAACACACCAACTGCAACCAACCTTAC	

incubation, lotriose accounted for 32.81% of total h drol sis products and its concentration as 1.3 mg/ml (Fig. 5). In this process, about 29.5% heat-bran insoluble lan as h drolz ed b MBt .

Conclusions

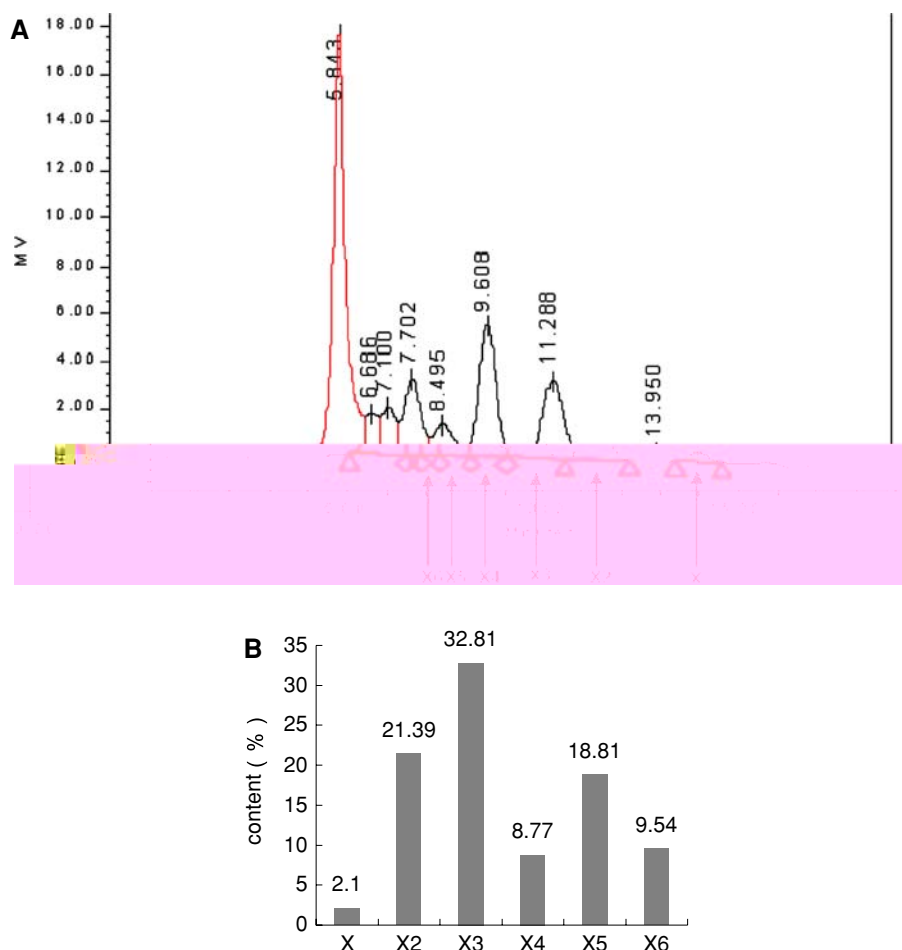
In these ears, the meth lotrophic east *Pichia pastoris* has developed into a highl successful s stem for production of a ide range of heterologous proteins. This s stem permits high-densit fermentation, tightl regulated e pression, and ef cient secretion of recombinant proteins. These attributes make it meet the industrial demands of interest proteins (Cereghion and Cregg 2000). Despite the success

of the *P. pastoris* s stem, opportunities e ist to develop a larger range of proteins that can be e pressed in the s stem (Romanos 1995). Codon usage could pla a ke role in regulating gene e pression and in the production of large quantities of high-qualit heterologous protein (Eckart and Bussineau 1996). Even the regional optimi ation of the 5' end of the coding region or the removal of onl particularl rare codons throughout the gene has sho n to have a signi cant impact on heterologous protein production (Outchkourov et al. 2002; Li et al. 2008; Hu et al. 2006; Trinh et al. 2004). In this stud , the successful e pression of the modi ed gene in *P. pastoris* con rmed the codon optimi ation to ards the s non mous codon usage bias of *P. pastoris* had a positive impact on e pression levels (Sinclair and Cho 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 MBt (Lee et al. [2003a](#)). Low cell concentration reached in shaken

HPLC profiles and contents of hydrolysis products of heat-bran insoluble lan b MBt after 20 h. The positions of glucose (X), lobiose (X2), lotriose (X3), lotetraose (X4), lopentaose (X5), lohe aose (X6) are shown



arabino lan in cell walls of cereal grains. The main hydrolysis product released from heat to bran insoluble lan b MBt as different from reTf A, the recombinant Tf A expressed by *P. pastoris* (Sun et al. 2007), which implies the hydrolytic properties of MBt may relate to its catalytic domains inherited from Bs A.

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

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