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ORIGINAL PAPER

[illegible] *Pichia pastoris*

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[illegible] To obtain the protein e pression of a h brid lanase in east, the gene encoding it as modi ed according to the codon bias of *Pichia pastoris* and e pressed e tracellularl in this east as an active lanase, MBt , e hibited a molecular mass of appro imatel 35 kDa on SDS PAGE. The pH behavior of MBt in terms of both activit and stabilit as similar to that of Bt , original gene product in *Escherichia coli*, hile a certain difference as observed in optimal temperature for activit and in thermal stabilit . HPLC anal sis revealed the lan in heat could be h drol ed b MBt and the major h drol sis product as lotriose. These results sho ed codon usage pla ed a ke role in regulating the e pression of the h brid lanase in *P. pastoris* and the recombinant h brid lanase, MBt , produced b *P. pastoris* could be potentiall useful in feed industr .

[illegible] H brid lanase · Gene modi cation · *Pichia pastoris* · E pression · Feed industr

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X lan is the major constituent of hemicelluloses and is the second abundant rene able resource after cellulose ith a high potential for degradation to useful end products. The main chain of this heterogenous pol saccharide consists of β-1, 4-linked D- lop ranoside residues hich can be substituted ith acet l, arabinos l, and glucuronos l side chains depending on the botanical origin. Several en mes are involved in the h drol sis of lan pol mer; the most important one is the *endo-β*-(1, 4)- lanase (EC 3.2.1.8), hich has been used commerciall in the paper, food, and feed industries (Li et al. 2000). In animal feeds, supplementation ith e ogenous lanases can reduce the viscosit of intestinal contents and improve nutrient digestibilit in domestic animal fed on diets containing heat (Steenfeldt et al. 1998; He et al. 1998).

Catal tic and biochemical properties of man ild t pe and recombinant lanases have been studied (Karlsson et al. 1998; Katapodis et al. 2003; Chantasingh et al. 2006; Berrin et al. 2000), but little is kno n about catal tic and h drol tic properties of constructed h brid lanase hose parents are famil 11 lanases. In our laborator , a gene encoding a h brid lanase as constructed b substituting the 31 *N*-terminal amino acid residues of the *Thermomonospora fusca* lanase A (Tf A) for the corresponding region of 22 amino acid residues of the *Bacillus subtilis* lanase A (Bs A). E pressing the construct gene, *btx*, in *Escherichia coli* BL21 resulted in a recombinant h brid lanase, Bt , ith e cellent thermostabilit (Weng and Sun, 2005). To produce this h brid lanase in large scale for commercial use, the industrial east, *Pichia pastoris,* as selected to be the host. Ho ever, it is dif cult to obtain its protein e pression in *P. pastoris* probabl due to rare codons and potential mRNA instabilit or pol aden lation motifs represented b ve or more A/T or G/C repeats in *btx*. In this stud , *btx* as modi ed according to the codon usage bias of *P. pastoris* (Sreekrishna et al. 1997; Sinclair and Cho 2002) and e pressed e tracellularl in this east as an active lanase, MBt .

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Strains and plasmids

In this stud , the *E. coli TOP 10F'* cells ere used for DNA manipulation hile *P. pastoris* strain GS115 (*his4*) as used for protein e pression. Vector pPICZαA, carr ing the Zeocin resistance gene for selection of both *E. coli* and *P. pastoris* transformants, as used for e tracellular production of the recombinant protein. The t o strains and the plasmid listed above ere contained in Eas Select™ *Pichia* E pression Kit, production of Invitrogen (San Diego, CA). pGEM -T Eas vector used for DNA cloning as obtained from Promega (Madison, WI).

Reagents

T4 DNA ligase and PCR kit ere obtained from Promega; pfu DNA pol merase kit and restrict en mes ere from MBI Fermentas (Burlington, CA). Oligonucleotides (Table 1) ere s nthesi ed b Shangon (Shanghai, China). Ni-NTA agarose resin as from Qiagen (Hilden, German). Birch lan and dinitrosalic lic acid (DNS) ere purchased from Sigma (St. louis, MO). Wheat bran-insoluble lan as kindl provided b Dr. Chen (Southern Yangt e Universit). The standard looligosaccharides (X2 X6) ere from Mega me (Wicklo , Ireland). X lose (X) as from Merck (Darmstadt, German).

Gene modi cation

A fragment (mbt 02) designed to replace the middle fragment of the original gene, *btx*, for removing ve or more A/

o idase 1 (*AOX1*) locus, and transformed into *P. pastoris* stain GS115 ith the electroporation method as described in the manual for Eas Select™ *Pichia* E pression Kit of Invitrogen. The transformants ere screened on YPDS + Zeocin™ plates (1% east e tract, 2% peptone, 2% de trose, 1 M sorbitol, 2% agar, 100 μg/ml Zeocin™) and the Zeocin™-resistant *Pichia* colonies ere replica-plated onto MDH and MMH plates (1.34% east nitrogen base, 0.4 mg/l biotin, 2% agar, 40 mg/l histidine and 2% de trose or 0.5% methanol, respectivel) to determine their methanol-utili ing phenot pes. The Mut^+ phenot pe gre normall on both MMH and MDH pates hereas the Mut^- phenot pe gre ver slo l on MMH plates. The Mut^+ transformants ere selected and incubated at 30 C in a shaking incubator (250 rpm) in 5 ml BMGY (1% east e tract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 0.4 mg/l biotin, 1% gl cerol) for 20 h. After the cultures reached an $OD_{600} = 4$, the east cells ere harvested b centrifugation (2,000*g,* 5 min) and re-suspended in 50 ml BMMY (the same as BMGY but ith 0.5% methanol instead of gl cerol) to induce the e pression of the h brid lanase, MBt , in a shaking ask. Ever 24 h, 100% methanol as added into the culture to a nal 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 as centrifuged and the amount of MBt in the supernatant as estimated b activit measurement assa s.

Production, puri cation and SDS PAGE anal sis of MBt

The scale up e pression as performed in 2-l shake ask containing 500 ml BMMY medium. The culture supernatant collected at the optimal inducing time point as free e-dried and applied for Ni-NTA af nit chromatograph according to the manufacturer's instruction (Qiagen). Aliquots of the puri cation product and the culture supernatant ere subjected to sodium dodec l sulfate pol - acr lamide gel electrophoresis (SDS PAGE), using the culture supernatant of GS115 ith pPICZαA transformed in it as control. The protein concentration of the recombinant en me as measured b the d e-binding assa method of Bradford ith the bovine serum albumin (BSA) as the standard (Bradford 1976).

X lanase activit assa

The lanase activit as measured ith 1% birch ood (/v) as substrate at 50 C in McIlvaine's buffer (pH 5.0) (Baile et al. 1992). Reducing sugars freed b en matic h drol sis ere quanti ed b the dinitrosalic lic acid (DNS) (Miller et al. 1960). One unit of lanase activit as de ned as the amount of en me that produced 1 μmol of lose equivalent per minute.

pH optimum and stabilit

The effect of pH on lanase activit as measured over a range of pH 3.0 7.0 (McIlvaine's buffer s stem) and 8.0 9.0 (0.2 M gl cine, 0.2 M NaOH buffer s stem) at 60 C. The pH stabilit of the en me as determined b incubating the lanase in various pH buffers at 25 C for 1 h. The residual activit as estimated follo ing the procedure described above.

Temperature optimum and stabilit

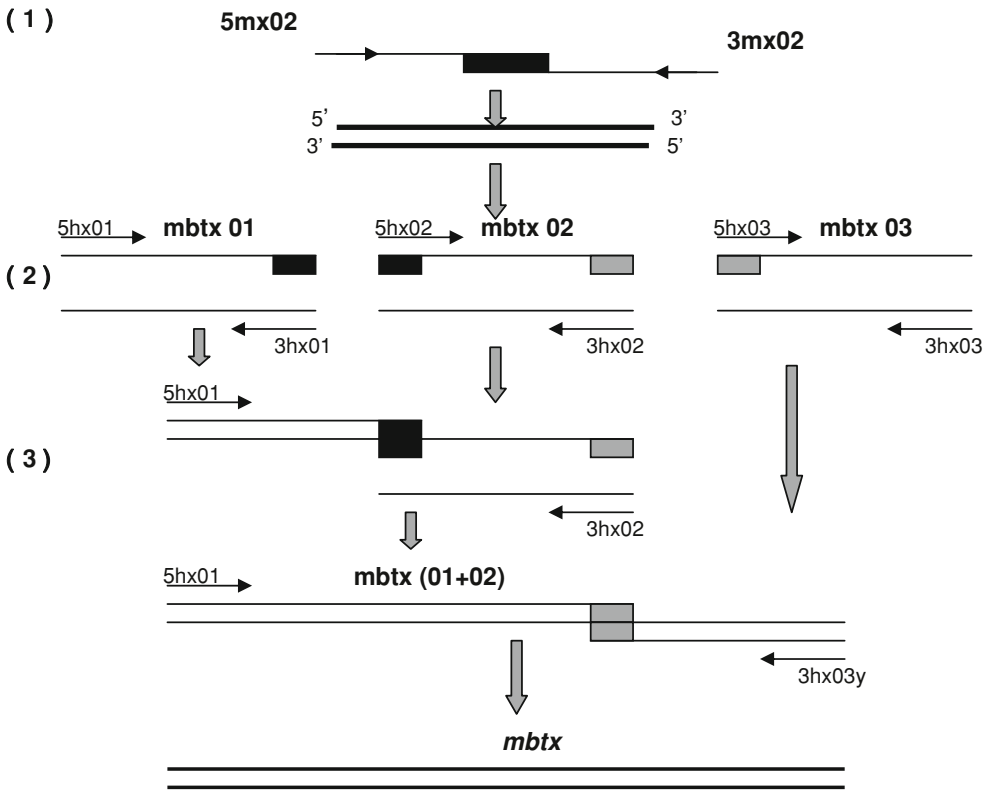
The effect of temperature on the en me activit as estimated at optimal pH at temperature ranging from 30 to 90 C. The thermo stabilit of lanase as determined b pre-incubating the en me in the absence of substrate at different temperature for 2 min, respectivel , then cooling on ice for 5 min before residual lanase activit measurements.

H drol sis products of bran insoluble lan b MBt

X lose (X) and standard looligosaccharides (X2 X6) ere resolved in pure ater. Samples of sugar (X X6) ere anal ed b HPLC separatel ith Sugar-PakTM1 column (300 mm × 6.5 mm; Waters, Milford, MA), pure ater as mobile phase (0.5 ml/min) and injection volumes of 20 μl. The areas of sugar peaks ere screened and calculated using a Waters 2,401 refractive inde detector, and the standard concentration curves of looligosaccharides (X X6) ere obtained according to the correlation of peak area and concentration. The 16 mg/ml bran insoluble lan solution in McIlvaine's buffer (pH 6.0) as h dro- l ed b MBt at 40 C ith constant shaking. In the reaction mi ture, the substrate as e cessive. The h dro- l tic products in this s stem for 20 h ere anal ed under same HPLC conditions and quanti ed according to standard curves.

Gene modi cation

Sequencing result sho ed *mbtx* as identi ed ith the theoretical design. Thirt -eight codons ere replaced to t for the preference of *P. pastoris* cells, hile the deduced amino acid sequence as not changed (Fig. 2).



Process of the gene modi cation Oligonucleotides are sho n as the line ith *arrowhead*. Overlapping sequences are represented ith *solid shading* or *lightly stippled*. Reagents used in PCR ere from pfu DNA Pol merase kit; The reaction s stem as in 50 µl; The PCR protocol used in step (1) as as follo s: 94 C for 2 min, 94 C for 50 s, 70 C for 50 s, 72 C for 1 min, nall cooling at 4 C. Then the PCR product as gel eluted and ampli ed, mean hile, mbt 01 and mbt 03 ere also ampli ed from *btx*. All the ampli cation conditions in step (2) involved 10 c cles of 94 C 50 s, 60 C −0.4 C per c cle for 50 s, 72 C for 1 min and 30 c cles of 94 C 50 s, 56 C −0.2 C per c cle for 50 s, 72 C for 1 min, nall cooling at 4 C. In step (3) mbt 01, mbt 02 and mbt 03 ere spliced one b one to form a modi ed gene *mbtx* b the technique of SOE b PCR. The PCR protocols in this step included 10 c cles of 94 C for 50 s, 70 C − 0.4 C per c cle for 50 s, 72 C for 1 min and 30 c cles of 94 C 50 s, 66 C −0.2 C per c cle 50 s, 72 C 1 min and nall remained at 4 C

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

A total of 15 transformants of pPICZαA-*mbtx* ere ana- l ed for their e pression performance and e pression time courses. Almost same levels of lanase activities ere detected in culture supernatants of the 15 transforments gro n on BMMY medium in shaken culture. The highest en me activit (4 0.12 U/ml) as reached after 60 h 0.5% methon induction.

Production, puri cation and SDS PAGE anal sis of MBt

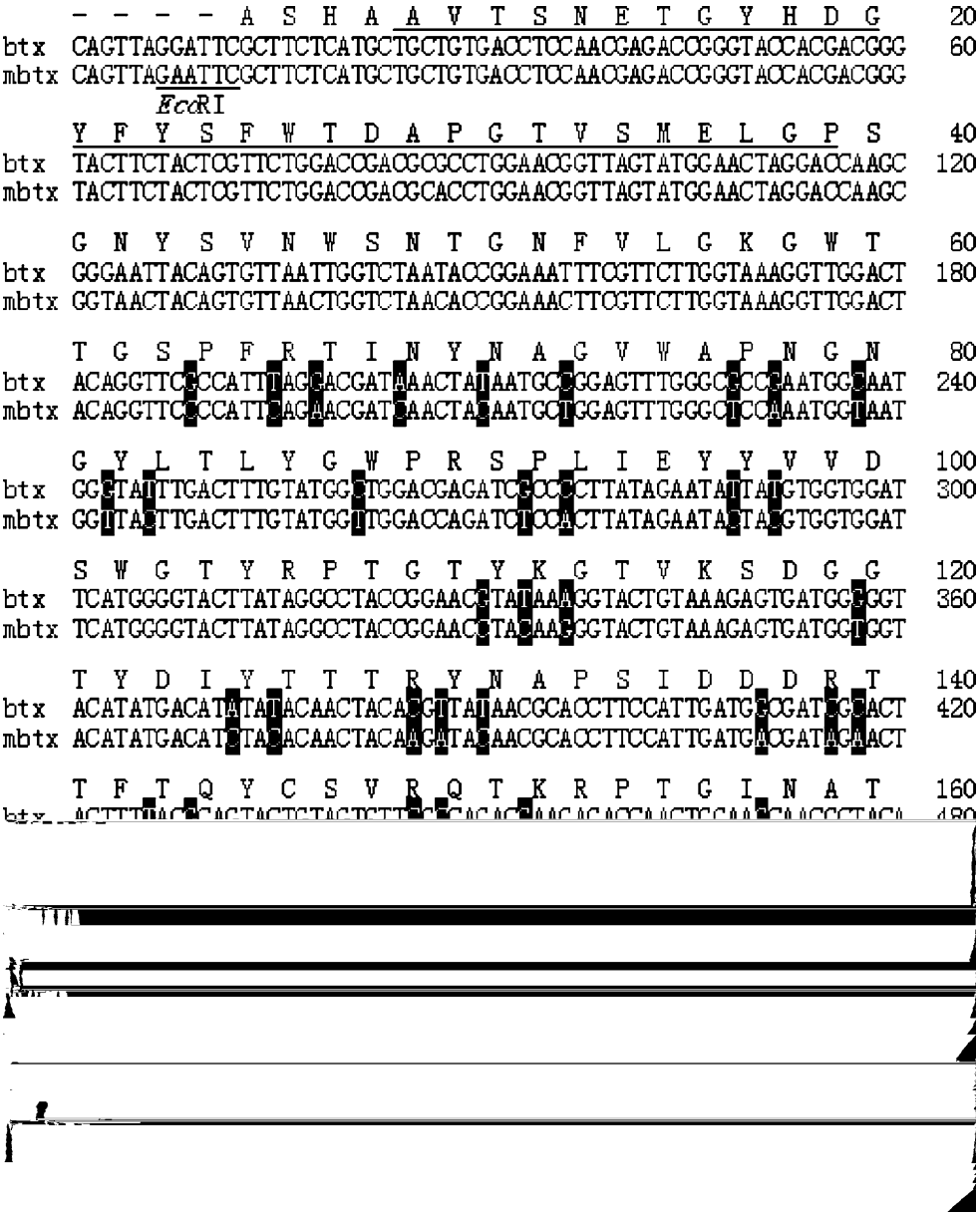
The ield of the puri ed 6His-tagged MBt as 1.8 mg in 500 ml of culture ith 1,338 U total activit . Anal sis of SDS PAGE sho ed MBt ran at a speci c protein band about 35 kDa, hich is consistent ith the theoretical molecular mass 34.4 kDa (Fig. 3), calculated on the basis of the deduced amino acid sequence.

Effects of pH and temperature

The effects of pH and temperature on MBt ere com- pared ith Bt . As sho n in Fig. 4a, MBt and Bt have almost the identical activit curves ith the optimum pH at 7.0 from 3.0 to 7.0. Ho ever, the activit of MBt has a dramatic improvement from 8.0 to 9.0, hich as not seen on Bt . Figure 4b indicated the t o en mes ere all stable over a ide pH range (4.0 10.0). Figure 4c revealed the optimum temperature rang (40 50 C) of MBt as lo er than that of Bt (50 60 C). Temperature-stabilit stud indicated that MBt as not thermostable as Bt . As sho n in Fig. 4d, MBt lost its activit drasticall above 60 C, hile Bt kept more than 90% activit belo 80 C.

H drol sis products of bran insoluble lan b MBt

The h drol sis products of heat-bran insoluble lan including lose(X) and looligosaccharides (X2 X6). X lotriose(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

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 drol ed b MBt .

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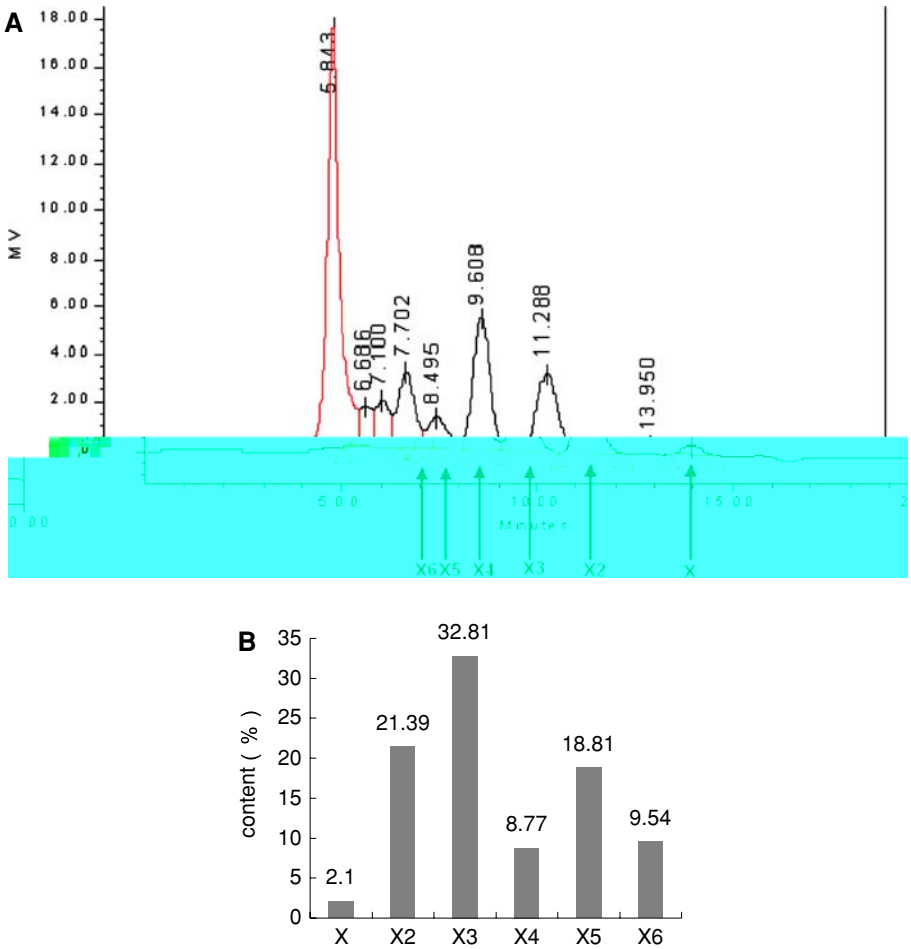
In these ears, the meth lotrophic east *Pichia pastoris* has developed into a highl successful s stem for production of a ide range of heterologous proteins. This s stem permits high-densit fermentation, tightl regulated e pression, and ef cient secretion of recombinant proteins. These attributes make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

Ho ever, the amount of the desired gene product in the shaken culture as relative lo , hich ere likel due to impossibilit of maintaining methanol concentrations ithin the narro range required for promoter induction; on the other hand, further addition of methanol during cultivation in shaken culture as not possible for the risk of incurring in cell accumulation of methanol that could result in c toto ic effects (Guarna et al. 1997). Unfavorable dissolved o gen concentration as a consequence of the impossibilit to control o gen suppl in shaken culture could be another reason of lo production of MBt (Lee et al. 2003a). Lo cell concentration reached in shaken



HPLC pro les and contents; , of h drol sis products of heat-bran insoluble lan b MBt after 20 h. The positions of lose (X), lobiose (X2), lotriose (X3), lotetraose (X4), lopentaose (X5), lohe aose (X6) are sho n

arabino lan in cell alls of cereal grains. The main h drol sis product released from heat to bran insoluble lan b MBt as different from reTf A, the recombinant Tf A e pressed b *P. pastoris* (Sun et al. 2007), hich implies the h drol tic properties of MBt ma relate to its catal tic domains inherited from Bs A.

In conclusion, codon usage pla ed a ke role in e pression of the h brid lanase in *P. pastoris* and the recombinant h brid lanase, MBt , produced b *P. pastoris* could be potentiall applied in feed industr .

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