Ann Microbiol (2014) 64:561–569
DOI 10.1007/s13213-013-0689-7

ORIGINAL ARTICLE

Enhancing expression level of an acidophilic β-mannanase in *Pichia pastoris* by double vector system

Cun-Duo Tang · Jing Guo · Jian-Fang Li · Xi-Huan Wei · Die Hu · Shu-Juan Gao · Xin Yin · Min-Chen Wu

Received: 24 January 2013 / Accepted: 29 June 2013 / Published online: 20 July 2013

Abstract An acidophilic β-mannanase-encoding gene (*Auman5A*) from *Aspergillus usamii* YL-01-78 was amplified and inserted into pPIC9K and pPICZαA vectors. The resulting recombinant vector, pPIC9K-*Auman5A*, was transformed into *Pichia pastoris* GS115. One strain having the highest recombinant β-mannanase activity of 54.6 U/ml, labeled GSKM4-8, was chosen from the first-batch *P. pastoris* transformants. Then, the pPICZαA-*Auman5A* was transformed into GSKM4-8 again. From the second-batch transformants, one strain (GSKZαM4-2) with the highest β-mannanase activity of 78.1 U/ml was obtained, and used to optimize expression conditions. As GSKZαM4-2 was induced under the optimized conditions (initial pH value 6.5, induction period 120 h, methanol concentration 1.5 %, and induction temperature 32 °C), β-mannanase activity reached 162.8 U/ml. Protein and carbohydrate assays showed that the β-mannanase, a glycoprotein with an apparent molecular weight of 49.8 kDa and a carbohydrate content of 21.3 %, was extracellularly expressed. It displayed the maximum activity at pH 3.0 and 70 °C, and was stable at a pH range of 3.0–7.0 and at 60 °C. Its activity was not significantly affected by metal ions tested and EDTA, but inhibited by Ag^{+} and Hg^{2+}. Its most favorable substrate was locust bean gum, followed by konjac flour and guar gum. The K_m and V_{max} towards locust bean gum were 1.36 mg/ml and 415.8 U/mg, respectively. These results suggested that the β-mannanase can be expressed with higher level and possesses superior enzymatic properties, making it a good candidate in industrial processes.

Keywords *Aspergillus usamii* · β-Mannanase · *Pichia pastoris* · Double vector system · Expression optimization

C.D. Tang and J. Guo, the two first authors, contributed equally to this work.

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

J. Guo · S.-J. Gao
School of Pharmaceutical Science, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu 214122, People's Republic of China

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

M.-C. Wu (✉)
Wuxi Medical School, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu 214122, People's Republic of China
e-mail: biowmc@126.com

Introduction

β-Mannanases (β-1,4-D-mannan mannohydrolases, EC 3.2.1.78) can catalyze the cleavage of internal β-1,4-D-mannosidic linkages in mannan and heteromannan, the major hemicellulosic components in cell walls of plants and some algae, as well as in some types of plant seeds (Dhawan and Kaur 2007). Recently, β-mannanases have attracted much attention owing to their industrial potential applications, such as bleaching pulps, depolymerizing anti-nutritional factors in feedstuffs, extracting oils from leguminous seeds, and the production of mannooligosaccharides (van Zyl et al. 2010). Similar to other glycoside hydrolases (GHs), various β-mannanases with the corresponding properties have played important roles in simplifying the industrial processes, in improving the quality of products, and in reducing the environmental pollution caused by using the chemicals (Luo et al. 2009). Based on their remarkable differences in physicochemical properties, amino acid sequences, and hydrophobic clusters, almost all β-mannanases hitherto reported have been classified into GH families 5, 26, and 113 (http://www.cazy.org/fam/acc_GH.html). Analysis

of their three-dimensional (3-D) structures demonstrated that β-mannanases in three GH families all belong to GH clan-A (Zhang et al. 2008).

β-Mannanases are widely present in various organisms, such as bacteria, actinomycetes, fungi, plants, and molluscs. To date, much research has been performed on exploiting novel β-mannanases with superior properties, expressing β-mannanase-encoding genes in heterologous cells such as methylotrophic yeast *P. pastoris*, or enhancing β-mannanase catalytic activities by mutating enzyme-producing strains and optimizing fermentation/expression conditions, with the aim of producing β-mannanases on an industrial scale (Wu et al. 2011a; Li et al. 2012; Roth et al. 2009). However, the commercialization and broad applications of β-mannanases are still limited by their low catalytic activities and expensive production costs. Therefore, it is necessary to increase their catalytic activities and/or production yields to satisfy the increasing needs for β-mannanases. It is known that many factors can influence the expression levels of heterologous proteins in *Pichia pastoris*, such as promotor strength, mRNA secondary structure, codon usage, and post-translational modification and secretion (Zhang et al. 2011). Moreover, *P. pastoris* transformants with multiple copies of heterologous genes could lead to high expression levels of proteins (Sreekrishna et al. 1997).

In our previous studies, the filamentous fungus of *Aspergillus usamii* YL-01-78 was isolated from the soil in China and used for the β-mannanase production by solid-state fermentation (Li et al. 2006b). Subsequently, an acidophilic β-mannanase (abbreviated to AuMan5A) was purified from the cultivated koji of *A. usamii*, and its enzymatic properties were characterized (Li et al. 2006a). In this work, an AuMan5A-encoding gene (*Auman5A*) was cloned, and efficiently expressed in *P. pastoris* GS115 by a double vector system. Meanwhile, the expression conditions of *P. pastoris* GSKZαM4-2 were optimized by using both the 'one-factor-at-a-time' and 'orthogonal design' methods (Ma et al. 2011). In addition, the expressed recombinant AuMan5A (reAuMan5A) was purified to homogeneity, and its enzymatic properties were characterized, suggesting its potential applications in industries. To our knowledge, this is the first report on enhancing expression level of an acidophilic β-mannanase in *P. pastoris* by a double vector system.

Materials and methods

Strains, vectors and media

Aspergillus usamii YL-01-78 was used as a donor of the *Auman5A*, which was cultured at 32 °C in the medium comprising (g/l): tryptone 10, yeast extract 5, dextrose 10, and locust bean gum 5 (Sigma, St. Louis, MO, USA); pH 6.0. *Escherichia coli* JM109 and pUCm-T vector (Sangon, Shanghai, China) were used for gene cloning and DNA sequencing, while *E. coli* DH5α and both pPIC9K and pPICZαA vectors (Invitrogen, San Diego, CA, USA) were used for construction of two recombinant expression vectors. *E. coli* JM109 and DH5α were cultured at 37 °C in Luria-Bertani medium (Sambrook and Russell 2001). *P. pastoris* GS115 was cultured at 30 °C in yeast extract peptone dextrose (YPD) medium, and its transformants were cultured and induced in the following media: minimal dextrose (MD), geneticin G418- or Zeocin-containing YPD, buffered glycerol-complex (BMGY), and buffered methanol-complex (BMMY), which were prepared according to the manual of Multi-Copy Pichia Expression Kit (Invitrogen).

Cloning of an AuMan5A-encoding gene

Based on both the complete genomic DNA sequence of the *Auman5A* (GenBank accession no. HQ839639) and determined N-terminal 15 amino acid residues (NH_2-SFASTS GLQFTIDGE) of the AuMan5A purified from *A. usamii* (Li et al. 2006a), a pair of specific PCR primers was designed. The forward and reverse primers, synthesized by Sangon (China), were M-F: 5′-GAATTCTCCTTCGCCAGCACCTCC-3′ with an *Eco*RI site (underlined) and M-R: 5′-GCGGCCGCTT AGGCACTATCAATAGC-3′ with a *Not*I site (underlined), respectively.

An Oligo dT-Adaptor Primer, 5′-GTTTTCCCAGTCAC GAC-Oligo dT-3′ provided by RNA PCR Kit (TaKaRa, Dalian, China), was applied for reverse transcription of the first-strand cDNA from the total RNA which was extracted from *A. usamii* using the RNA Extraction Kit (Sangon) (Wu et al. 2011b). Using the resulting first-strand cDNA as the template, the first-round PCR was carried out with primers M-F and M13 Primer M4 (5′-GTTTTCCCAGTCACGAC-3′, identical to the Oligo dT-Adaptor Primer except Oligo dT) with the following conditions: an initial denaturation at 94 °C for 2 min; 30 cycles of at 94 °C for 30 s, 53 °C for 30 s, 72 °C for 75 s; and an extra elongation at 72 °C for 10 min. Then, the first-round PCR products were agarose gel-purified, respectively, and subjected to the second-round PCR with primers M-F and M-R (nested PCR) under the same conditions as the first-round PCR, except at an annealing temperature of 56 °C. The amplified target PCR product was purified using the EZ-10 Spin Column DNA Gel Extraction Kit (BBI, Markham, Canada), inserted into pUCm-T vector and transformed into *E. coli* JM109. The recombinant T-vector, designated pUCm-T-*Auman5A*, was confirmed by restriction enzyme analysis and DNA sequencing.

Construction of two recombinant expression vectors

The *Auman5A* was excised from the pUCm-T-*Auman5A* by digestion with *Eco*RI and *Not*I, and then inserted into the pPIC9K vector (containing a Kanamycin-resistant gene) and into the pPICZαA vector (containing a Zeocin-resistant gene) digested with the same enzymes, followed by transforming them into *E. coli* DH5α. Two recombinant expression vectors, designated pPIC9K-*Auman5A* and pPICZαA-*Auman5A*, respectively (Fig. 1), were confirmed by DNA sequencing.

Expression by double vector system

First, the resulting pPIC9K-*Auman5A* was linearized with *Sal*I, and transformed into *P. pastoris* GS115 by electroporation on a Gene Pulser apparatus (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instruction. The first-batch transformants, uniformly called *P. pastoris* GSKM strains, were spread on a MD plate, and then successively inoculated on geneticin G418-containing YPD plates at increasing concentrations of 1.0, 2.0, and 4.0 mg/ml for screening multiple copies of the *Auman5A*. Expression of the *Auman5A* in *P. pastoris* was carried out according to the manual of the Multi-Copy Pichia Expression Kit (Invitrogen). In brief, each single colony of *P. pastoris* GSKM strains, cultured on YPD plates containing different concentrations of G418, was inoculated into 30 ml BMGY medium in a 250-ml flask, and cultured at 30 °C at 220 rpm until the OD_{600} reached 2–4. Then, the yeast cells were collected by centrifugation, resuspended in 30 ml BMMY medium, and induced for expression of the *Auman5A* at 30 °C for 96 h by adding methanol to a final concentration of 0.5 % (v/v) at 24-h intervals. From those GSKM strains tested, one strain having the highest reAuMan5A activity, labeled GSKM4-8, was selected and used for the subsequent transformation.

Secondly, the selected GSKM4-8 was transformed again with pPICZαA-*Auman5A* linearized with *Sac*I. The second-batch transformants, called *P. pastoris* GSKZαM strains, were successively inoculated on Zeocin-containing YPD plates at increasing concentrations of 100, 200, and 400 μg/ml for screening multiple copies of the *Auman5A*. Through flask expression test, one strain having the highest reAuMan5A activity, labeled as GSKZαM4-2, was obtained, and used to optimize the expression conditions. *P. pastoris* GS115 transformed with pPIC9K or pPICZαA vector was used as the negative control, labeled *P. pastoris* GSKC or GSZαC.

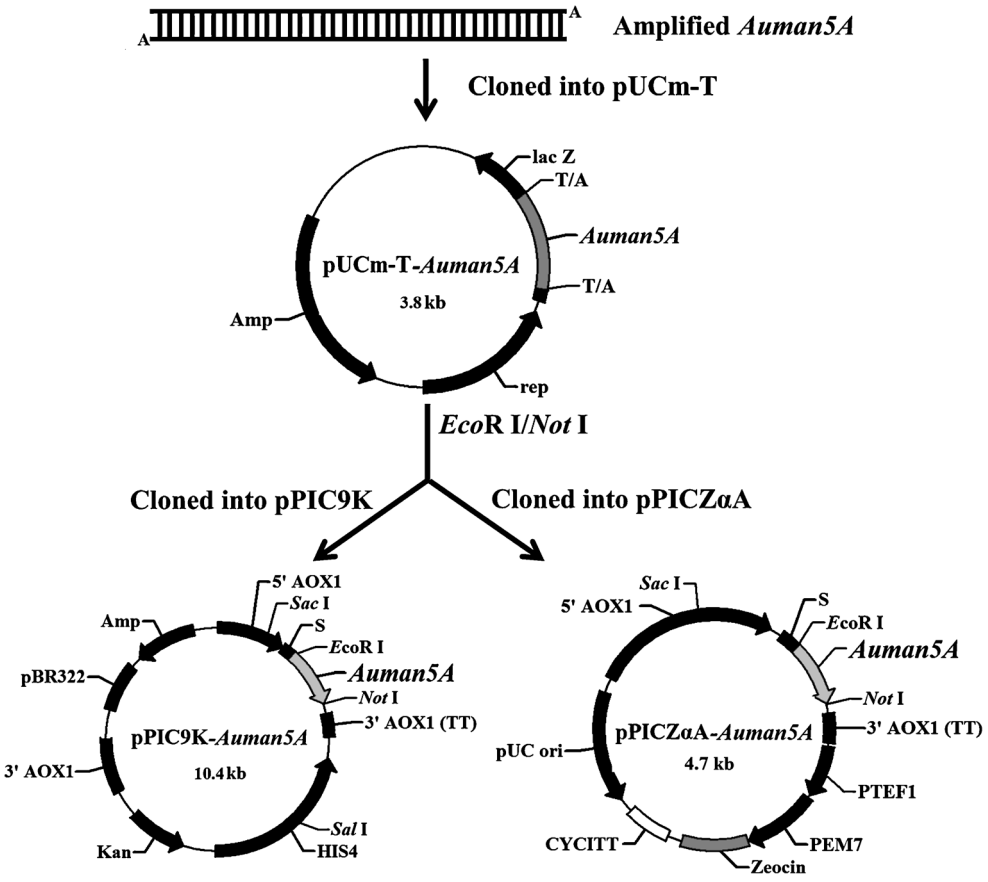

Fig. 1 Diagramatic representation for construction of the recombinant expression vectors, pPIC9K-*Auman5A* and pPICZαA-*Auman5A*

Optimization of the expression conditions

Four major factors (initial pH value, methanol concentration, induction temperature, and induction period) for the reAuMan5A expression by GSKZαM4-2 strain were first optimized using the 'one-factor-at-a-time' method which keeps all other factors at constant levels except the one being studied (Li et al. 2011). Based on the results obtained above, the mutual or synergistic effect of four factors was further investigated using the 'orthogonal design' method (Ma et al. 2011). In this work, the $L_9(3^4)$ of 'orthogonal design' (four factors, each at three levels) was arranged to optimize the expression conditions, by which just 9 experiments were done. If the 'full factorial design' method had been applied to examine the mutual effect of four factors (each at three levels), a total of 64 experiments would have had to have been performed.

Purification of the expressed reAuMan5A

After GSKZαM4-2 was induced under the optimized conditions, the cultured medium was centrifuged at 8,000 rpm to remove yeast cells. A total of 20 ml supernatant was brought to 75 % saturation by adding solid ammonium sulfate, and left overnight. The resulting precipitate was collected, dissolved in 4.0 ml 20 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0), and dialyzed against the same buffer. The dialyzed solution was concentrated to 1.0 ml by ultrafiltration using a 10-kDa cut-off membrane (Millipore, Billerica, MA, USA), and then loaded onto a Sephadex G-75 column (ϕ1.6×80 cm; Amersham Pharmacia Biotech, Uppsala, Sweden), followed by elution with the same buffer at a flow rate of 0.3 ml/min. Aliquots of 1.5 ml eluent containing only the reAuMan5A were pooled and concentrated for further studies. All purification procedures were performed at 4 °C.

Enzyme activity and substrate specificity assays

β-Mannanase activity was assayed by incubating 0.1 ml of suitable diluted enzyme solution with 2.4 ml of 0.5 % (w/v) locust bean gum (Sigma) in 50 mM Na_2HPO_4–citric acid buffer (pH 3.5) at 60 °C for 10 min. The released reducing sugars were measured with the 3,5-dinitrosalicylic acid (DNS) method as described previously (Li et al. 2012), using D-mannose (Sigma) as the standard. One unit (U) of β-mannanase activity was defined as the amount of enzyme liberating 1 μmol of reducing sugar equivalent per min under the standard assay conditions. Substrate specificity of the reAuMan5A was assayed by measuring the enzyme activities towards different substrates under the standard assay conditions.

Protein and carbohydrate assays

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 12.5 % gel using the method of Laemmli (1970). The isolated proteins were visualized by staining with Coomassie Brilliant Blue R-250 (Sigma), and their apparent molecular weights were estimated using the Quantity One software based on the standard protein markers. Protein concentration was determined with the BCA-200 Protein Assay Kit (Pierce, Rockford, IL, USA), using bovine serum albumin as the standard.

Carbohydrate or sugar content of the purified reAuMan5A was determined with the phenol-sulfuric acid method (Dubois et al. 1956), using D-mannose as the standard. N-deglycosylation assay was performed as follows: the purified reAuMan5A solution was heated at 100 °C for 10 min, and cooled to room temperature, followed by incubating it with an endoglycosidase H (New England Biolabs, Ipswich, MA, USA) at 37 °C for 1.0 h.

pH optimum and stability of the reAuMan5A

The pH optimum of the reAuMan5A was determined under the standard assay conditions, except 0.5 % (w/v) locust bean gum in 50 mM Na_2HPO_4–citric acid buffer at a pH range of 2.5–7.0. To evaluate its pH stability, aliquots of reAuMan5A were incubated at 40 °C and at varied pH values from 2.5 to 7.5 for 1.0 h, and then the residual enzyme activity was measured under the standard assay conditions. In this work, the pH stability was defined as a pH range, over which the residual reAuMan5A activity was more than 85 % of its original activity.

Temperature optimum and stability of the reAuMan5A

The temperature optimum of the reAuMan5A was determined, at pH optimum, at temperatures ranging from 50 to 80 °C. To estimate its thermostability, aliquots of reAuMan5A were incubated at pH 3.5 and at various temperatures (45–75 °C) for 1.0 h, and then the residual enzyme activity was measured under the standard assay conditions. Here, the thermostability was defined as a temperature, at or below which the residual reAuMan5A activity retained over 85 % of its original activity.

Effects of metal ions and EDTA on the reAuMan5A

To estimate its resistance to metal ions and EDTA, aliquots of reAuMan5A were incubated with various metal ions and EDTA at a final concentration of 5.0 mM in 20 mM Na_2HPO_4–citric acid buffer (pH 3.5) at 40 °C for 1.0 h, and then the residual enzyme activity was measured under

the standard assay conditions. The enzyme solution without any additive was used as the control.

Kinetic parameters of the reAuMan5A

Hydrolyzing reaction rate (U/mg) of the reAuMan5A was determined under the standard assay conditions, except locust bean gum 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 reAuMan5A conforms to the Michaelis–Menten equation. The kinetic parameters, K_m and V_{max}, of the reAuMan5A were graphically determined from the Lineweaver–Burk plotting.

Statistics analysis

All data were obtained from three independent experiments and expressed as the mean ± standard deviation (SD). Statistics comparison was carried out by Student's *t* test using a computerized statistical package. The levels of statistical significance were defined as $P<0.05$ and $P<0.01$, respectively.

Results and discussion

Cloning of an AuMan5A-encoding gene

Analytical results of the total RNA extracted from *A. usamii* YL-01-78 demonstrated that the ratio of OD_{260} to OD_{280} was 1.95, and that the 18S and 28S rRNA bands, characterized as eukaryotic cells, were specific on formaldehyde denatured agarose gel electrophoresis (data not shown), indicating that the isolated *A. usamii* total RNA has high purity and was not decomposed (Wu et al. 2003). Using the first-strand cDNA transcribed reversely from the total RNA as the template, a c.1.25-kb clear band and several faint bands were amplified by the first-round PCR. Then, each band was purified, and subjected to the second-round PCR. As a result, a c.1.05-kb specific band was amplified only using a 1.25-kb band as the template, and inserted into pUCm-T vector, followed by DNA sequencing. The sequencing results were adopted as the inserted gene (*Auman5A*) sequences of three randomly picked clones were identical to one another, otherwise the PCR amplification and DNA sequencing were redone.

Analysis of the *Auman5A* and AuMan5A sequences

Sequence alignment using the DNAMAN 6.0 software verified that the sequence of the cloned *Auman5A* was identified as the AuMan5A-encoding complete genomic DNA, except: a 5′-flanking regulatory region, 5′- and 3′-untranslated regions, two short introns, and a signal peptide-encoding sequence. The N-terminal 15 amino acid residue sequence deduced from 5′-end of the *Auman5A* was in agreement with that determined by amino acid sequencing (Li et al. 2006a). The *Auman5A* was exactly 1,038 bp in length, which encodes a 345-aa AuMan5A with a theoretical molecular weight of 37,614 Da and a calculated isoelectric point (pI) of 4.09. The calculated pI was similar to the determined one (pI 4.2), by IEF-PAGE analysis, of the native AuMan5A purified from *A. usamii* YL-01-78 (Li et al. 2006a). Multiple sequence alignment using the ClustalW program (http://www.ebi.ac.uk/ClustalW) showed that the AuMan5A amino acid sequence also contains seven functional residues that were strictly conserved among GH family 5 members: two catalytic residues (acid/base, Glu168; nucleophile, Glu276) and five active site residues (Arg52, Asn167, His241, Tyr243, and Trp306) (Chen et al. 2007).

Screening of the *P. pastoris* transformants

Pichia pastoris transformant that can resist a higher concentration of G418 or Zeocin might have multiple copies of integration of the heterologous gene into the *P. pastoris* genome, which could potentially lead to a high expression level of the heterologous protein as explained in the manual of Multi-Copy Pichia Expression Kit (Invitrogen). However, the expression level is not directly proportional to the concentration of G418 or Zeocin (Li et al. 2012; Tan et al. 2011). For those reasons, from the first-batch transformants, we picked out 10 strains resistant to 1.0, 2.0, and 4.0 mg/ml of G418, labeled *P. pastoris* GSKM1-1 to GSKM1-10, GSKM2-1 to GSKM2-10, and GSKM4-1 to GSKM4-10, respectively, for the flask expression test using the standard protocol (Invitrogen). After induction by methanol for 96 h, the cultured supernatants of 30 strains were used for β-mannanase activity assay. As a result, one strain (GSKM4-8) expressing the highest reAuMan5A activity of 54.6 U/ml was chosen (Table 1), and used as the host strain for transformation of pPICZαA-*Auman5A*. From the second-batch transformants, a total of 30 strains resistant to 100, 200, and 400 μg/ml of Zeocin, labeled *P. pastoris* GSKZαM1-1 to GSKZαM1-10, GSKZαM2-1 to GSKZαM2-10, and GSKZαM4-1 to GSKZαM4-10, respectively, were picked out for the flask expression test. The GSKZαM4-2 having the highest reAuMan5A activity of 78.1 U/ml was obtained (Table 1), and used for optimization of the expression conditions. No β-mannanase activity of the negative control, *P. pastoris* GSKC or GSZαC, was detected under the same expression conditions.

Optimization of the expression conditions

In this work, expression conditions of the GSKZαM4-2 were tested using the 'one-factor-at-a- time' method. First, the strain wasinoculated into BMMY media with initial pH values ranging from 5.0 to 8.0, and induced by adding methanol to a final

Table 1 Screening of the *P. pastoris* transformants for multiple copies of the *Auman5A*

G418 (mg/ml)	Representative strain	Enzyme activity (U/ml)[a]	Zeocin (μg/ml)	Representative strains	Enzyme activity (U/ml)[a]
1.0	GSKM1-2	36.7±1.3	100	GSKZαM1-3	63.1±1.1[b]
	GSKM1-5	39.0±1.6		GSKZαM1-6	60.5±0.8[b]
	GSKM1-7	34.9±1.3		GSKZαM1-7	65.2±1.4[b]
2.0	GSKM2-1	40.2±1.2[c]	200	GSKZαM2-1	69.4±1.6[b]
	GSKM2-3	49.0±1.3[b]		GSKZαM2-4	70.8±1.1[b]
	GSKM2-6	37.6±1.1		GSKZαM2-9	72.3±1.3[b]
4.0	GSKM4-3	49.7±1.4[b]	400	**GSKZαM4-2**	**78.1±1.2[b]**
	GSKM4-5	39.2±1.6[c]		GSKZαM4-5	73.4±1.5[b]
	GSKM4-8	**54.6±1.4[b]**		GSKZαM4-7	71.8±1.6[b]

Strains showing the highest activity for G418 or Zeocin are highlighted in bold

[a] All data were obtained from three independent experiments and expressed as the mean±SD

[b] The statistical comparison of β-mannanase activities between the GSKM1-2 and other strains displayed significant differences at $P<0.01$

[c] The statistical comparison displayed significant differences at $P<0.05$

concentration of 0.5 % at 24-h intervals at 30 °C for 96 h. The highest reAuMan5A activity reached 99.2 U/ml at initial pH 7.0 (Fig. 2a). Second, the strain was inoculated into BMMY media (pH 7.0) and induced by adding methanol concentrations ranging from 0.5 to 3.0 % (v/v). The maximum reAuMan5A activity was up to 112.5 U/ml by adding 1.5 % of methanol (Fig. 2b). It was reported that the over-high methanol concentration is toxic to *P. pastoris*; in contrast, a too low methanol concentration effectively induces heterologous protein expression (Katakura et al. 1998). Finally, the GSKZαM4-2 was induced in a BMMY medium (pH 7.0) by adding 1.5 % of methanol at 24-h intervals until 144 h at three temperatures of 28, 30, and 32 °C, respectively. As shown in Fig. 3, the reAuMan5A activity reached 139.9 U/ml at 30 °C for 120 h, and either increased slowly or decreased gradually from 120 to 144 h at different temperatures. As a result, the expression conditions tested primarily were as follows: initial pH value of 7.0, methanol concentration of 1.5 %, induction temperatures of 30 °C, and induction period of 120 h.

Based on the results obtained by the single-factor test, the $L_9(3^4)$ of 'orthogonal design' was applied to investigate the mutual effect of the four major factors (Table 2). The experimental and analytical results are listed in Table 3 and 4, indicating that four major factors had different effects on the reAuMan5A expression. According to *R* values of various factors, the order affecting the enzyme expression level was as follows: initial pH value (C) > induction period (D) > methanol concentration (A) > induction temperature (B). Corresponding to the maximal K (or k) value of each factor, the optimal combination of factors was $C_1D_3A_2B_3$, that is to say, the optimized expression conditions: initial pH value of 6.5, induction period of 120 h, methanol concentration of 1.5 %, and induction temperature of 32 °C. As the GSKZαM4-2 was induced under the optimized conditions, the maximum reAuMan5A activity reached 162.8 U/ml, being 2.08 times as high as that (78.1 U/ml) expressed using the standard protocol (Invitrogen), and 2.98-fold higher than that (54.6 U/ml) expressed by GSKM4-8. Compared with the results reported previously, the catalytic activity of the reAuMan5A expressed by GSKZαM4-2 under the optimized conditions was much higher than those of the recombinant *Aspergillus sulphureus*, *Phialophora* sp. P13, and *Aspergillus fumigates* β-mannanases, respectively (Chen et al. 2007; Zhao et al. 2010; Duruksu et al. 2009).

Purification and deglycosylation of the reAuMan5A

The *P. pastoris* expression system has many advantages, one of which is that the purities of expressed recombinant proteins or enzymes are very high according to the description of Multi-Copy Pichia Expression Kit (Invitrogen), which can greatly facilitate or simplify the purification procedures and decrease the industrial production costs. It was reported that the purity of the recombinant *A. sulphureus* β-mannanase expressed in *P. pastoris* X-33 was 97 % (Chen et al. 2007). In this work, the amount of the reAuMan5A expressed in the cultured supernatant of GSKZαM4-2 accounted for more than 85 % of all proteins measured by band-scanning (Fig. 4, lane 1). So, the reAuMan5A was purified to homogeneity only by a simple combination of ammonium sulfate precipitation, ultrafiltration, and Sephadex G-75 gel filtration (Fig. 4, lane 2). The specific activity of the purified reAuMan5A, towards locust bean gum under the standard assay conditions was 341.3 U/mg, which was similar to those of the recombinant β-mannanases from *Aspergillus niger* LW-1 (Li et al. 2012) and *A. niger* BK01 (Bien-Cuong et al. 2009), respectively.

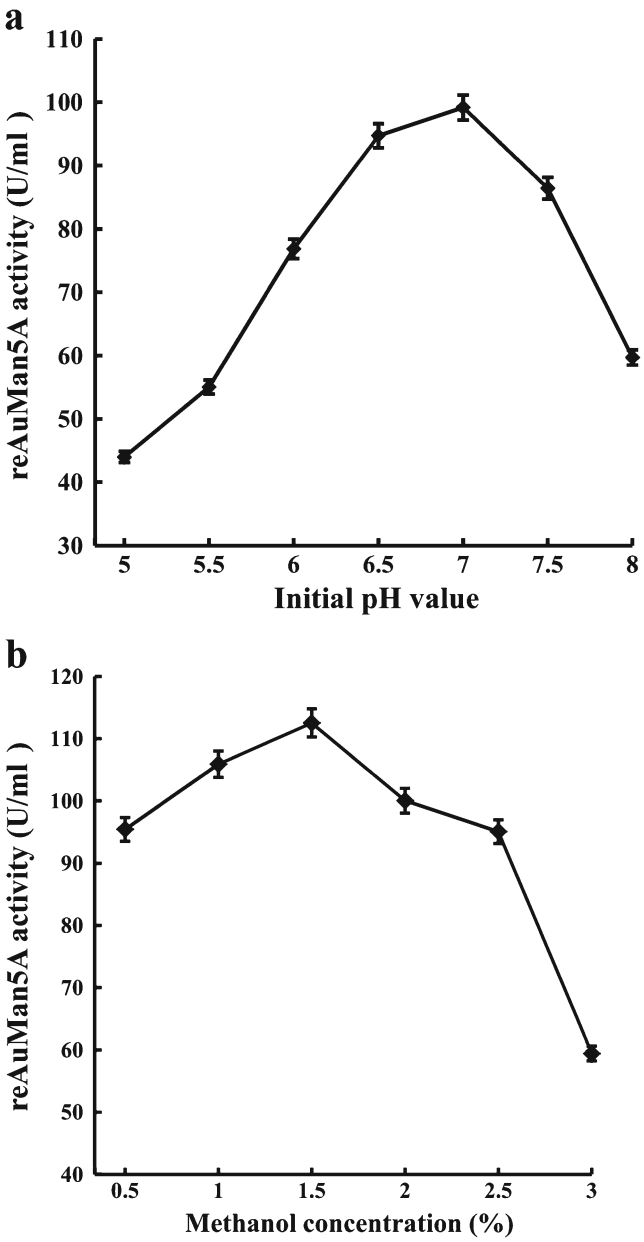

Fig. 2 Effects of initial pH value and methanol concentration on expression level of the reAuMan5A. **a** The GSKZαM4-2 was inoculated into BMMY media with initial pH values ranging from 5.0 to 8.0, and induced using the standard protocol. The statistical comparison between pH 7.0 and original pH 6.0 designated by standard protocol was significant at $P<0.01$. **b** Inoculated into BMMY media (pH 7.0), the GSKZαM4-2 was induced by adding methanol concentrations from 0.5 to 3.0 %. The statistical comparison between the optimized and original methanol concentrations was significant at $P<0.05$

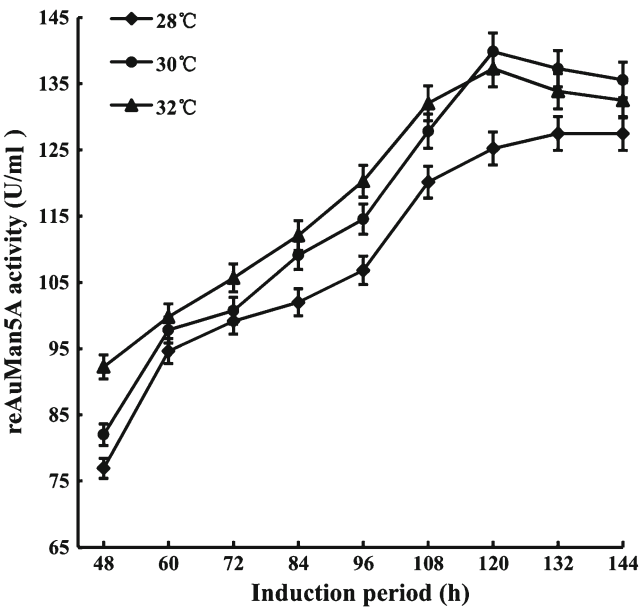

Fig. 3 Effects of induction temperature and period on expression level of the reAuMan5A. The GSKZαM4-2 was induced in a BMMY medium (pH 7.0) by adding methanol of 1.5 % at 24-h intervals until 144 h at three temperatures of 28, 30 and 32 °C, respectively

SDS-PAGE analysis of the purified reAuMan5A displayed one single protein band with an apparent molecular weight of 49.8 kDa (Fig. 4, lane 2), much larger than its theoretical one (37,614 Da). *Pichia pastoris* enables some post-translational modifications of a protein, including the assembly of disulfide bridge, exclusion of signal peptide, and N- and/or O-glycosylation, etc. Analysis by using the NetNGlyc program 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) displayed that there are two putative N-glycosylation sites (N156-S157-S158 and N225-F226-T227) in the AuMan5A sequence. To verify whether the difference between apparent and theoretical molecular weights was due to N-glycosylation, the N-deglycosylation assay was performed. As a result, one protein band of about 38.0 kDa was observed on SDS-PAGE (Fig. 4, lane 3). Simultaneously, carbohydrate content of the purified reAuMan5A was determined to be 21.3 %. Those experimental results verified that the reAuMan5A is an N-glycosylated protein. The glycosylation of a protein is one of the most common post-translational modifications that occurrs in eukaryotic cells. The carbohydrate moiety of a glycoprotein plays a key role in preventing a protein from proteolytic degradation and thermal denaturation (Srimathi

Table 2 Factors and level arranged by the $L_9(3^4)$ of 'orthogonal design'

Level [b]	Factors [a]			
	Methanol concentration (A)	Induction temperature (B)	Initial pH value (C)	Induction period (D)
1	1.0 %	28 °C	6.5	96 h
2	1.5 %	30 °C	7.0	108 h
3	2.0 %	32 °C	7.5	120 h

[a] *A*, *B*, *C*, and *D* represent four factors of methanol concentration, induction temperature, initial pH value, and induction period, respectively

[b] *1*, *2*, and *3* represent three levels of each factor, respectively

Table 3 The array and experimental results by the $L_9(3^4)$ of 'orthogonal design'

Number	A	B	C	D	reAuMan5A activity (U/ml)
1	1	1	1	1	71.12
2	1	2	2	2	62.78
3	1	3	3	3	40.82
4	2	1	2	3	131.04
5	2	2	3	1	35.85
6	2	3	1	2	132.92
7	3	1	3	2	20.41
8	3	2	1	3	138.33
9	3	3	2	1	70.90

The arrays of levels (*1*, *2*, and *3*) in columns (*A*, *B*, *C*, and *D*) were arranged.

Each of 9 experiments was conducted in triplicate

and Jayaraman 2005; Shental-Bechor and Levy 2008). Therefore, the glycosylation may be an advantage for the large-scale production and industrial utilization of the reAuMan5A.

Enzymatic properties of the reAuMan5A

The reAuMan5A showed higher catalytic activities at a pH range of 2.5–4.5, among which the maximum activity was at pH 3.0. Incubated at 40 °C for 1.0 h at varied pH values, the reAuMan5A was highly stable at a broad pH range of 3.0–7.0. The temperature optimum of the reAuMan5A was 70 °C (measured at pH 3.0). Incubated at pH 3.5 and at various temperatures for 1.0 h, the reAuMan5A activity retained more than 85 % of its original activity at 60 °C or below, but declined rapidly over 60 °C and only retained 36.8 % of its original activity at 75 °C.

Table 4 The analytical results by the $L_9(3^4)$ of 'orthogonal design'

Level	A	B	C	D
K_1=total level 1	174.72	222.57	342.37	177.87
K_2=total level 2	299.81	236.96	264.71	216.10
K_3=total level 3	229.64	244.64	97.09	310.20
$k_1=K_1/3$	58.24	74.12	114.13	59.29
$k_2=K_2/3$	99.93	78.99	88.24	72.03
$k_3=K_3/3$	76.55	81.55	32.37	103.40
$R=k_{max}-k_{min}$	41.69	7.43	81.76	44.11
Affecting order	C > D > A > B			
Optimal combination	$C_1D_3A_2B_3$			

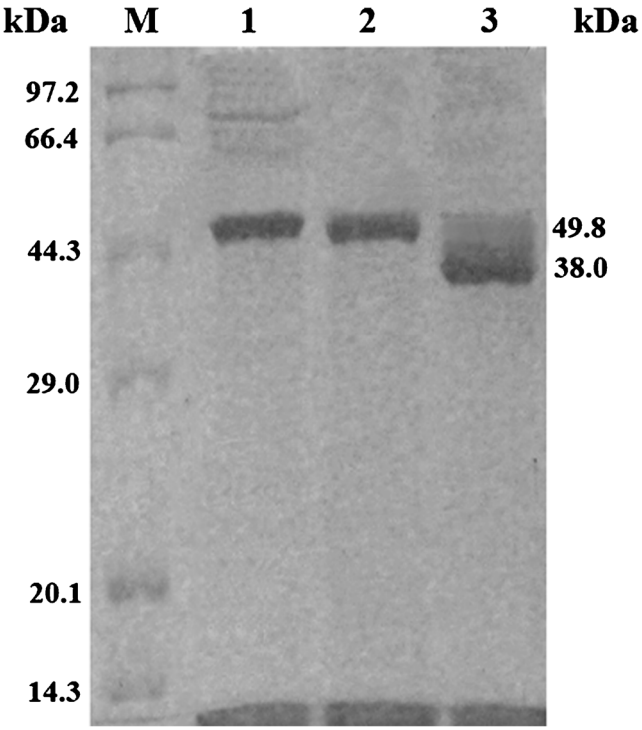

Fig. 4 SDS-PAGE analysis of the expressed reAuMan5A. *Lane M* protein marker. *Lane 1* the cultured supernatant of the GSKZαM4-2. *Lane 2* the purified reAuMan5A. *Lane 3* the N-deglycosylated reAuMan5A

The reAuMan5A activity was not significantly affected by Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Al^{3+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, Ba^{2+}, Pb^{2+}, Mn^{2+}, and EDTA, but strongly inhibited by Ag^{+} and Hg^{2+} with only 36.5 and 28.4 % of its original activity, respectively. The reAuMan5A showed the highest activity towards locust bean gum (100 % of relative activity), but lower activities towards konjac flour (53.8 %) and guar gum (31.6 %). No activity was detected towards birchwood xylan, soluble starch, or carboxyl methyl cellulose. The kinetic parameters, K_m and V_{max}, of the reAuMan5A towards locust bean gum were 1.36 mg/ml and 415.8 U/mg, respectively.

Conclusion

This work led to high-level expression of the *Auman5A* in *P. pastoris* GS115 by a double vector system and by optimization of the expression conditions, and characterized enzymatic properties of the purified reAuMan5A. The GSKZαM4-2 expressing the maximum reAuMan5A activity of 78.1 U/ml was obtained through two batches of transformation with pPIC9K-*Auman5A* and pPICZαA-*Auman5A*, respectively. As the GSKZαM4-2 was induced under the optimized conditions, the reAuMan5A activity reached 162.8 U/ml. The

reAuMan5A displayed superior enzymatic properties, such as high specific activity, good temperature and pH stabilities, and strong resistance to the chemicals, which making it a good candidate in industrial processes, especially in food and feedstuff industries. Considering the increasing needs for β-mannanases, we are going to require an industrial scale production of the reAuMan5A with high activity and low costs.

Acknowledgments This work was financially supported by the National Nature Science Foundation of China (No. 31271811), Doctoral Research Funds of Jiangnan University (No. JUDCF11011) and Postgraduate Innovation Training Project of Jiangsu (No. CXZZ11_0480). We are grateful to Prof. Xianzhang Wu (School of Biotechnology, Jiangnan University) for providing technical assistance.

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