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Improved extracellular endo-1,4-*β*-mannosidase activity of recombinant *Pichia pastoris* by optimizing signal peptide

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Abstract: In order to improve the extracellular endo-1,4-*β*-mannosidase (MAN) activity of recombinant *Pichia pastoris*, optimization of signal peptides was investigated. At first, five potential signal peptides (W1, MF4I, INU1A, αpre, HFBI) were chosen to be analyzed by SignalP 4.0, among which W1 was designed. Then, the widely used signal peptide *α*-factor in expression vector pGAPZαA was replaced by those five signal peptides to reconstruct five new expression vectors. MAN activity was assayed after expression vectors were transformed into *Pichia pastoris*. The data show that the relative efficiencies of W1, MF4I, INU1A, αpre, and HFBI signal peptides are 23.5%, 203.5%, 0, 79.7%, and 120.3% compared with *α*-factor, respectively. The further gene copy number determination by the quantitative real-time PCR reveals that the MAN activities mediated by *α*-factor from 1 to 6 gene copy number levels are 12.95, 43.33, 126.63, 173.53, 103.23 and 88.63 U/mL, while those mediated by MF4I are 79.22, 133.89, 260.14, 347.5, 206.15 and 181.89 U/mL, respectively. The maximum MAN activity reached 347.5 U/mL with 4 gene copies mediated by MF4I. These results indicate that replacing the signal peptide *α*-factor with MF4I and increasing MAN gene copies to a proper number can greatly improve the secretory expression of MAN.

Key words: endo-1,4-*β*-mannosidase; *Pichia pastoris* (*P.pastoris*); signal peptide; optimization

1 Introduction

Endo-*β*-mannanase (EC 3.2.1.78) is an endohydrolase degrading hemicellulose applied in food processing industry, feed production, and second generation biofuels production. *Pichia pastoris* (*P.pastoris*) is a highly effective and versatile yeast for the expression of heterologous proteins. This yeast secretes only low levels of endogenous proteins, and secreted heterologous protein constitutes the vast majority of total protein in the medium. So, the efficient secretion of an interested protein into the culture broth is an important measure to improve the extracellular endo-1,4-*β*-mannosidase activity of recombinant *P. pastoris*, which contributes to fewer steps of the next purification and lower cost for industrial production than before. However, the secretion of recombinant proteins is usually mediated by an N-terminal secretory signal peptide, of which the *S. cerevisiae α*-factor prepro-signal (*α*-factor) is the most widely and successfully used one [1]. Many other secretory signals are also used in *P. pastoris* expression systems, including inulinase signal peptides [2], *Phaseolus vulgaris* phytohaemagglutinin (PHA-E) [3], acid phosphatase [4], viral preprotoxin [5], MF4I signal peptides [6], human serum albumin [7], and the HFBI from *Trichoderma reesei* [8]. Signal peptides differ widely in the secretory efficiencies of recombinant proteins, thus it is necessary to identify multiple available secretory signal peptides and find the optimum signal peptide for a protein.

In the previous study, the endo-1,4-*β*-mannosidase man26A from *Aspergillus niger* CBS 513.88 was successfully expressed in *P. pastoris* using constitutive expression vector pGAPZαA [9]. In this work, to further improve the extracellular activity of endo-1,4-*β*-mannosidase in *P. pastoris*, *α*-factor was replaced by other five potential signals peptides (W1, MF4I, INU1A, αpre, and HFBI), and the characteristics of these potential signal peptides was investigated briefly.

2 Materials and methods

2.1 Strains and growth conditions

Escherichia coli DH5α cells were cultivated in low salt LB medium (1% tryptone, 0.5% NaCl, and 0.5%

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yeast extract). *P. pastoris* strain X33 (Invitrogen) was used as a host and cultivated in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose). The transformed cells were selected on YPDSZ plates (1% yeast extract, 2% peptone, 2% dextrose, 1 mol/L sorbitol, 100 μg/mL Zeocin and 2% agar) and YPDSKT plates (1% yeast extract, 2% peptone, 2% dextrose, 1 mol/L sorbitol, 5% konjaku flour, 0.02% Trypan Blue and 2% agar).

The protein sequence of W1 was designed according to the signal design rules [10−11], the protein sequences of *α*-factor, MF4I, INU1A, and HFBI were obtained from the GenBank. The *α*-factor signal peptide consists of a pre-region and a pro-region. The protein sequence of αpre was the pre-region of *α*-factor signal, consisting of 19 amino acids. The signal peptides sequences and primers used in this work are summarised in Tables 1 and 2, respectively.

2.2 Prediction of signal peptides

To predict the probability and secretory efficiencies

Table 1 Signal peptide sequence

Name	Signal peptide sequence
α-factor	MRFPSIFIAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIA AKEEGVSLE
W1	MRRRAIPLLLLLLLLLLLGSSALA
MF4I	MAIPRFPSIFIAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLEEAEAE AEPKFINTTIASIAAKEEGVSLE
INU1A	MKLAYSLLLPLAGVSASVINYKR
αpre	MRFPSIFTAVLFAASSALA
HFBI	MKFFAIAALFAAAAVA

Table 2 Primers

Name	Oligonucleotides
Fz	GGGGTACCGCTTCTAATCAAACTTTGTCTTATGGTAAT
Rz	CCGCGCTTCGAAATAGTTGTTCAATTGATTG
Fw	GACTACTTCGAAACGATGAGAAGAAGAGCCATTCCTTTGTTGTTGTTGTTGTTGCTTTT
Rw	GGGGTACCGGCAAGGGCGGAGGAACCCAACAAAAGCAAAAGCAACAACAACAACAAC
Fm1	GACTACTTCGAAACGATGGCTATTCCAAGATTCCCATCTATCTTCATCGCTGTCTTGTT
Rm1	GTTGACAGGAGCAGCCAAGGCAGAAGAAGCAGCGAACAAGACAGCGATGAAGATAGATG
Fm2	CTGCCTTGGCTGCTCCTGTCAACACTACTACCGAGGATGAAACTGCTCAAATCCCTGCT
Rm2	GTCACCCTCCAGGTCAGAGTAACCGATGACAGCCTCAGCAGGGATTTGAGCAGTTTCAT
Fm3	GTTACTCTGACCTGGAGGGTGACTTCGACGTCGCTGTCTTGCCATTCTCTAACTCCACC
Rm3	CAGCTTCAGCTTCAGCCTCCTCCAACAAACCGTTGTTGGTGGAGTTAGAGAATGGCAAG
Fm4	GGAGGCTGAAGCTGAAGCTGAACCTAAATTCATCAACACTACTATCGCTTCTATCGCTG
Rm4	GGGGTACCCTCGAGGGAAACACCCTCCTCCTTAGCAGCGATAGAAGCGATAGTAGTGT
Fn	GACTACTTCGAAACGATGAAGTTGGCTTATAGTTTGCTTTTGCCTTTGGCTGGAG
Rn	GGGGTACCTCTCTTGTAATTGATAACAGATGCAGAAACTCCAGCCAAAGGCAAAAGC
Fp	GACTACTTCGAAACGATGAGATTCCCATCTATCTTCATCGCTGTCTTGTTCGCTGCTTC
Rp	GGGGTACCAGCCAAGGCAGAAGAAGCAGCGAACAAGACAGCGATGAAGATAGATG
Fh	GACTACTTCGAAACGATGAAGTTTTTCGCCATTGCCGCCTTGTTTG
Rh	GGGGTACCAGCGACGGCGGCGGCGGCAAACAAGGCGGCAATGGCGAAAAACT
GAP-1	ATGTCTTGGTGTCCTCGTCC
GAP-2	GGTCTTTTGAGTGGCGGTC
RTMAN-1	ATGGTACAAGGGTTTCTACACTGA
RTMAN-2	TGATTTGAGCAGCGATAGCAT
RTGAP-1	TTGTCGGTGTCAACGAGGAG
RTGAP-2	GGTCTTTTGAGTGGCGGTC

of signal peptides for the endo-1,4-*β*-mannosidase in *P. pastoris*, the protein sequences of *α*-factor, W1, MF4I, INU1A, αpre and HFBI were fused to reporter proteins MAN in frame. The fusion proteins sequences were examined by the SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP-4.0/) [12].

2.3 Construction of vectors

The pGAPZA-MAN gene was amplified from plasmid pGAPZA-α-MAN constructed in the previous study [9] using the Fz/Rz primers (Table 2). Subsequently, the DNA sequences of potential signal peptides W1, INU1A, αpre and HFBI were synthesized by using primers Fw/Rw, Fn/Rn, Fp/Rp and Fh/Rh primers, respectively (Table 2). MF4I was synthesized by successive PCR using Fm1/Rm1, Fm2/Rm2, Fm3/Rm3 and Fm4/Rm4 primers with 1−2 ng inner primers and 10−20 ng external primers. Those PCR external primers contain suitable restriction cleavage sites of BspT104I and KpnI for cloning. After digestion with these restriction enzymes, all of the PCR products were ligated by T4 DNA ligase into the pGAPZA-MAN gene with no signal sequence, resulting in vectors pGAPZA-W1-MAN, pGAPZA-MF4I- MAN, pGAPZA-INU1A-MAN, pGAPZA-αpre-MAN and pGAPZA-HFBI-MAN. All of the five plasmids were transformed into *E. coli* DH5α and confirmed by colony PCR at first, and screened transformants were chosen for the DNA sequencing.

2.4 Clone library construction and strains screening

Those five kinds of plasmids and existing pGAPZA-α-MAN plasmid were linearised with Bgl Ⅱ, and transformed into *P. pastoris* X33 competent cells using the electroporation method according to the manufacturer's protocol. The transformed cells were selected on YPDSZ plates and incubated at 30 °C for 48 h.

And 360 strains for each signal peptide were screened on YPDSKT by hydrolysis holes method [13]. A total of 30 strains with different sizes of hydrolysis holes were selected from 360 strains and inoculated respectively into 5 mL YPD medium in 50 mL Erlenmeyer flask at 30 °C (250 r/min) for 24 h. Then, 50 μL of cultures was inoculated into 10 mL YPD medium in a 50 mL Erlenmeyer flask at 30 °C (250 r/min). After 36 h, the extracellular activity of MAN was determined.

2.5 Assay of extracellular MAN activity

Because the secreted heterologous protein constitutes the most part of total protein in the medium, and our previous researches have proven that the activity of endo-1,4-*β*-mannosidase was positively correlated with the expression of MAN and there was a few MAN detected in *P. pastoris* cell [14]. We just assay the endo-1,4-*β*-mannosidase in extracellular fluid to measure the secretory expression level of MAN at the same time. Extracellular MAN activity of each 30 transformants was determined respectively using the 3,5-dinitrosalicylic acid (DNS) method [15] after 36 h culture. 1 mL of culture was harvested by centrifugation at 7378 x g for 1 min at room temperature. Then, the culture supernatant was appropriately diluted as enzyme sample for assay. The reaction was started by mixing 0.1 mL of appropriately diluted enzyme sample with 0.9 mL of 5.56 g/L locust bean gum (G0753, Sigma, USA) in 0.1 mmol/L citrate phosphate buffer (pH 5.0). After incubation at 40 °C for 5 min, the reaction was stopped by the addition of 1 mL of DNS reagent. The reaction solution was boiled for 10 min and the absorbance was measured at 540 nm. One unit of endo-1,4-*β*-mannosidase activity was defined as the amount of enzyme releasing 1 μmol of mannose equivalents per minute. All experiments were done in triplicate [16].

2.6 Extraction of genomic DNA and MAN gene copy number identification

Those strains with different MAN activity levels were selected for the extraction of genomic DNA using the yeast DNA iso-Kit (Omega, USA). MAN gene copy number identification was performed by quantitative real-time PCR, which makes it possible to compare the secretory efficiencies of *α*-factor and MF4I signal peptide, ruling out any influence on the secretion of reporter proteins due to copy number. Quantitative real-time PCR was performed using SYBR green real-time PCR Master Mix (TaKaRa, Japan) in an MYIQ Real-time PCR System (Bio-rad, USA) with RTMAN-1/RTMAN-2 and RTGAP-1/RTGAP-2 as primers (Table 2). The copy number of reporter gene MAN was analyzed using the plasmid double standard curve method. The double standard curves of the reporter gene MAN and the GAP fragment were generated with plasmids pGEM-MAN and PMD19-GAP. The GAP fragment, consisting of a portion of the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene and the following genomic sequence, was used as the reference sequence because there is only a single copy in the *P. pastoris* genome [17]. GAP gene was amplified using two primers GAP-1/GAP-2 (Table 2) and inserted into PMD19-T vector (named T-GAP). The plasmid pGEM-T carrying MAN gene (named T-MAN) was constructed in Ref. [14]. T-GAP and T-MAN were used as templates to establish double standard curves with two pairs of primers RTMAN-1/RTMAN-2 and RTGAP-1/RTGAP-2. The mean Ct values were plotted against the log10 of the initial plasmid copy number, and the double standard curves were generated using a linear regression of the plotted points. The copy number of the MAN gene in

each transformant was calculated using the Ct value of the *P. pastoris* genomic DNA and the standard curve. The copy number was determined using the following equation:

$$N_{MAN}=N_{MAN,standard}/N_{GAP} \quad (1)$$

where N_{MAN} is reporter gene MAN copy number; $N_{MAN,standard}$ is the reporter gene MAN copy number determined from standard curve; N_{GAP} is the GAP fragment copy number determined from standard curve.

3 Results

3.1 Prediction of signal peptides

SignalP 4.0 was applied to calculating a discrimination score (D-score) for a particular amino acid sequence to predict whether it might act as a signal peptide. Sequences with a D-score>0.7 have a high probability of being a signal peptide. As listed in Table 3, the D-scores of all peptides except INU1A (0.443) are above 0.45, indicating a basic probability of the sequence being a signal peptide. The D-scores of W1, αpre and HFBI sequences were below 0.7, indicating the low efficiencies of secreting MAN; while the D-score of MF4I was the highest, 0.900.

In conclusion, these prediction results suggest that INU1A might not be a signal peptide for secreting MAN, and the transformants with W1, αpre and HFBI sequences might not be so efficient in secreting MAN. The MF4I with the highest D-score might be the most efficient signal peptide.

Table 3 Analysis results of signal sequences by software SignalP 4.0

Construct	Signal peptide sequence	Prediction result	
		D-score	Cleavage site
α-factor-MAN	MRFPSIFTAVLFAASSALA ↓APVNTTTEDETAQIPAE AVIGYSDLEGDFDVAVLP FSNSTNNGLLFINTTIASI AAKEEGVSLE-ASNQTLS	0.884	*AA 19-20
W1-MAN	MRRRAIPLLLLLLLLLLL GSSALA↓-ASNQTLS	0.565	*AA 24-25
MF4I-MAN	MAIPRFPSIFIAVLFAASS ALA↓APVNTTTEDETAQI PAEAVIGYSDLEGDFDVA VLPFSNSTNNGLLEEAEA EAEPKFINTTIASIAAKEE GVSLE-ASNQTLS	0.900	*AA 22-23
INU1A-MAN	MKLAYSLLLPLAGVSA SVINYKR-ASNQTLS	0.443	—
αpre-MAN	MRFPSIFTAVLFAASSALA ↓-ASNQTLS	0.453	*AA 19-20
HFBI-MAN	MKFFAIAALFAAAAVA-A SNQTLS	0.638	* AA 4-5

Note: Signal peptide prediction of the fusion proteins was performed. *AA means amino acid.

3.2 Construction of vectors

The pGAPZA-MAN gene was amplified from plasmid pGAPZA-α-MAN (Fig. 1). All five signal sequences were assembled correctly by one-step PCR (Fig. 2). Five new plasmids were confirmed by colony PCR (Fig. 3) and DNA sequencing. All the five signal sequences were accurately ligated into the pGAPZA-MAN gene.

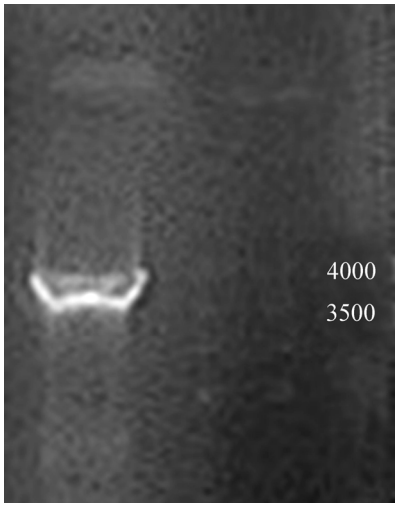

Fig. 1 pGAPZA-MAN gene amplified from plasmid pGAPZA-α-MAN

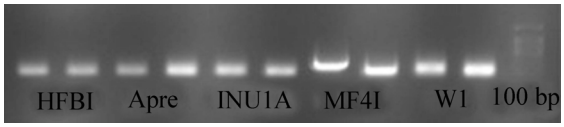

Fig. 2 Identification of five signal sequences synthesized by PCR

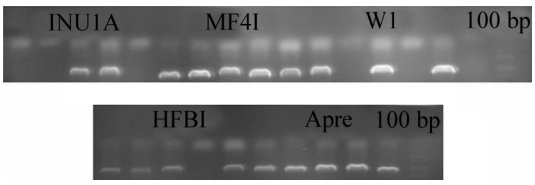

Fig. 3 Five thansformants of each new plasmids confirmed by colony PCR

3.3 Clone library construction and strains screening

Six kinds of plasmids including the existing pGAPZA-α-MAN plasmid were linearised with BglII (Fig. 4).

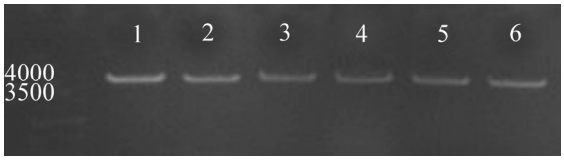

Fig. 4 Six kinds of plasmid linearised with BglII

Strains of each signal peptide were screened on YPDSKT by hydrolysis holes method (Fig. 5).

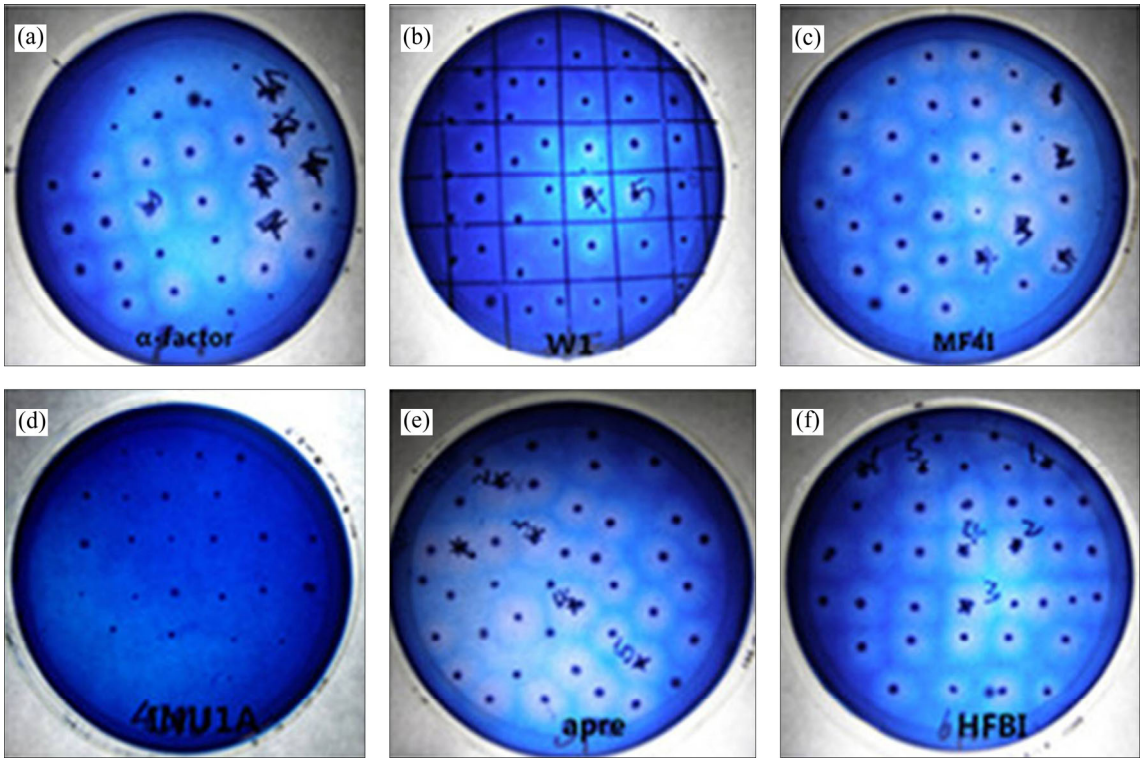

Fig. 5 Hydrolysis holes of different transformants cvith α factor (a), w1 (b), MF4I (c), INU1A (d), apre (e) and HFBI (f) as signal peptides on YPDSKT

3.4 Assay of extracellular MAN activity

The results of MAN activity assay are listed in Table 4. Relative efficiency was the ratio calculated by dividing MAN activity of five other transformants by that of α-factor transformants. INU1A can't mediate the secretion of MAN. The MF4I is the most efficient among six signal sequences.

Table 4 Results of MAN activity assay

Name	Highest MAN activity/(U·mL^{-1})	Mean MAN activity/(U·mL^{-1})	Relative efficiency/%
α-factor	173.5	83.3	100.0
W1	23.4	19.6	23.5
MF4I	347.5	169.5	203.5
INU1A	0	0	0
αpre	97.0	66.4	79.7
HFBI	208.1	100.2	120.3

3.5 Measurement of MAN gene copy number

MF4I signal peptide seems to be more efficient than other signal peptides. Thus, ten transformants with pGAPZA-MF4I-MAN expression vectors were chosen for the determination of MAN gene copy number and compared with positive controls (pGAPZA-α-MAN). The standard curves of the GAP fragment and the MAN gene were obtained, and the correlation coefficients were 1.000 and 0.998. The melting curve of the GAP fragment and the MAN gene displayed single peak value, suggesting that the data was believable ((Figs. 6(a), (b) and (c)). MAN gene copy numbers of different transformants with pGAPZA-α-MAN or pGAPZA-MF4I-MAN vector were measured respectively, which were normalised with the reference GAP fragment.

The relativities between the copy numbers of the MAN genes and MAN activity in transformants with MF4I and α-factor respectively are shown in Fig. 7. The MAN activities of α-factor transformants from 1 to 6 gene copy number levels were 12.95, 43.33, 126.63, 173.53, 103.23 and 88.63 U/mL, while those of MF4I transformants were 79.22, 133.89, 260.14, 347.5, 206.15 and 181.89 U/mL. These results indicated that the number of MAN gene copies in these transformants ranged from 1 to 6. The activity of endo-1,4-β-mannosidase increased with the increase of MAN gene copy number from 1 to 4 and decreased at 5 and 6 copies, which may be due to the negative feedback regulation caused by the overexpression pressure [18]. The max MAN activity reached 347.5 U/mL with 4 gene copies.

The MAN activities improved by MF4I from 1 to 6 MAN gene copies were 66.30, 90.56, 133.37, 173.97, 102.92 and 102.26 U/mL; while the relative efficiencies reached 512.0%, 209.0%, 105.3%, 100.2%, 99.7% and 105.2% of that mediated by α-factor. ((Figs. 8(a) and (b)).

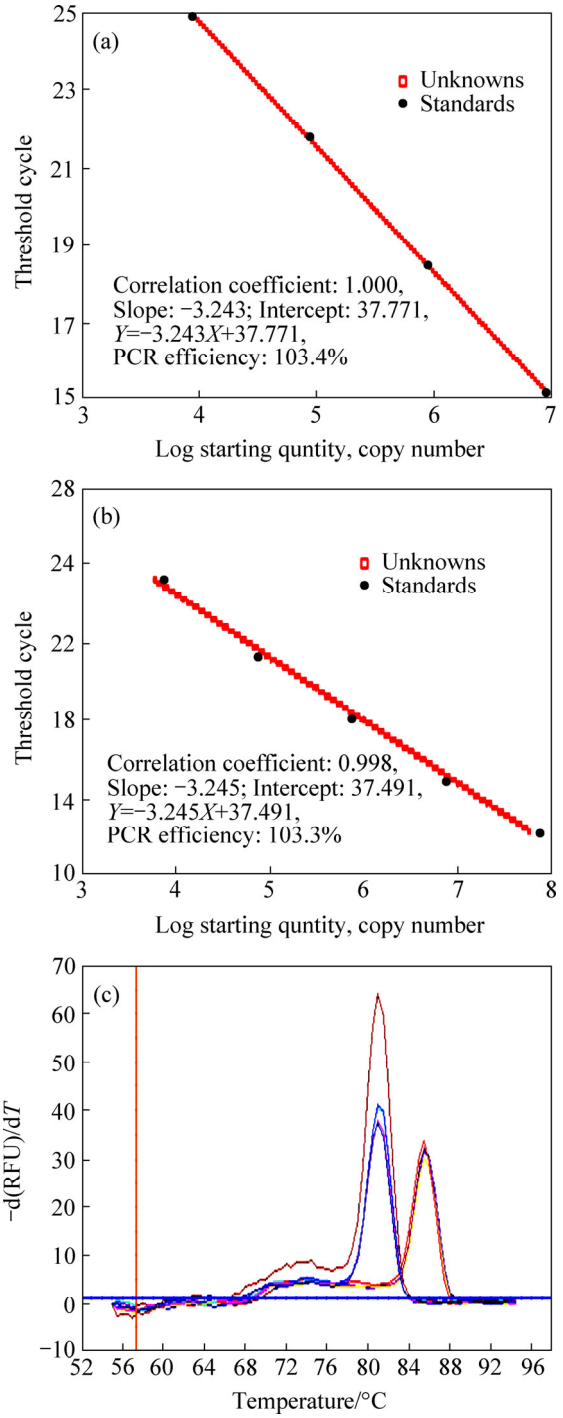

Fig. 6 Standard curves and melting curve of GAP fragment and MAN gene: (a) Standard curve of GAP fragment; (b) Standard curve of MAN gene; (c) Melting curve of GAP fragment and MAN gene

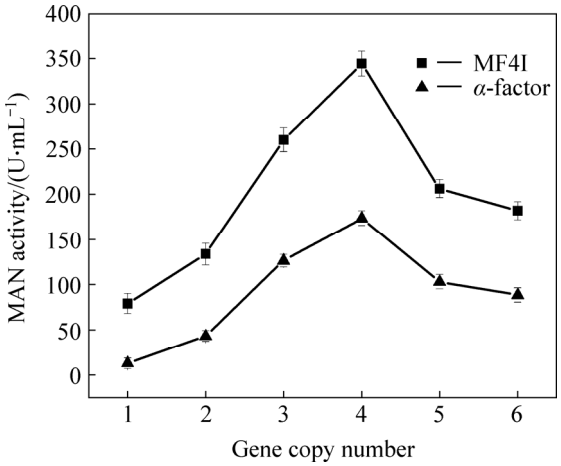

Fig. 7 Correlation between copy numbers of MAN genes and MAN activity in transformants with MF4I and α-factor respectively

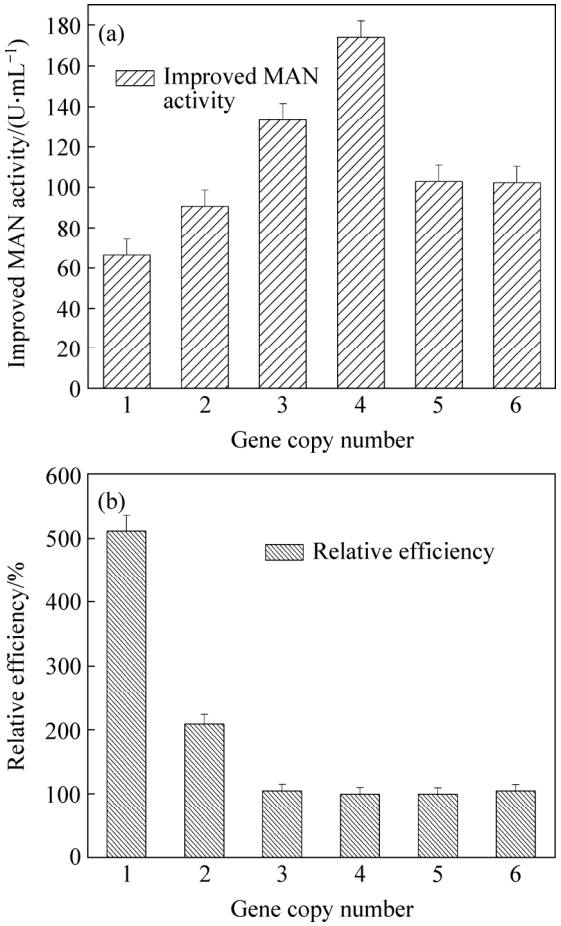

Fig. 8 Improved MAN activity (a) and relative efficiency (b) at different gene copy number levels

4 Discussion

Compared with positive control (α-factor), the relative efficiencies of MF4I and HFBI signal peptides for secreting MAN increased up to 203.5% and 120.3%; while those of W1, INU1A, and αpre decreased down to 23.5%, 0, and 79.7%, respectively. The results support that previous prediction of six signal peptides is basically right except the HFBI with lower D-score but higher MAN activity compared with α-factor, which also indicates that SignalP 4.0 is useful for the prediction of signal peptides mediating the expression of endo-1, 4-β-mannosidase.

By replacing the signal peptide of *P. pastoris* expression system to MF4I, the max MAN activity reached 347.5 U/mL with 4 gene copies, which is 2-fold of that mediated by *α*-factor. Thus, choosing an efficient signal peptide and improving heterologous gene copies to a proper number at the same time would be able to improve the extracellular expression of MAN in *P. pastoris*.

According to a predicted structure model of the *α*-factor built by Geoff, the *α*-factor signal peptide has a pre-region containing mainly an alpha helix and a pro-region consisting of a large loop region framed by two interacting helices (Fig. 9) [19]. The chosen αpre signal was just the pre-region of *α*-factor, which is believed to be important for interaction with the signal recognition particle, subsequent translocation into the ER, and folding of the nascent protein [20]. Absence of the entire pro-region (αpre) reduced the MAN secretion by 21.3%. This result suggests that the pro-region played an important role in secretion efficiency and deserved more attention and further modification. These pro-region helices sandwich a loop region needing sufficient freedom in order to interact with transacting factors for the secretory expression. Therefore, any mutations that increase the flexibility of this region may favor interactions and thus secretion. While the MF4I was reconstructed from *α*-factor by inserting a 10-amino acid sequence (EEAEAEAEPK) in the third helix of the pro-region at 64 sites, which increased the flexibility of the pro-region and thus the secretion of MAN. Besides, it is clear that increasing the overall hydrophobicity of the signal peptide just like W1 with insertion of an 11-amino acid sequence rich in hydrophobic amino acids (Leucine acid) reduced the secretion efficiency of the MAN reporter by 76.5%, suggesting that polar and charged residues play a positive role in secretion. Thus, optimizing sequence structure of signal peptide like MF4I also has great potential to improve the secretory expression of MAN and the extracellular MAN activity of *Pichia pastoris*.

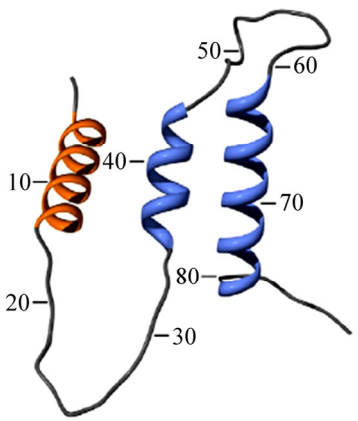

Fig. 9 Predicted structure of *α*-factor signal (First helix is pre-region, whereas following two helices are pro-region)

Furthermore, signal peptides differ widely in their ability to secrete heterologous proteins, so it is of crucial importance to have several secretion signal peptides to choose. Future research should focus on the construction of signal peptide libraries, the prediction and modification of their structure, or even the rational design of a new signal sequence that is more efficient to secrete heterologous proteins in *P. pastoris*. By taking these optimizing measures, the extracellular activity of endo-1,4-*β*-mannosidase and other heterologous proteins produced by recombinant *Pichia pastoris* may have great potential to be greatly improved.

5 Conclusions

1) SignalP 4.0 is useful for the prediction of signal peptide mediating the expression of endo-1,4-*β*-mannosidase.

2) The mean MAN activity mediated by positive control (*α*-factor) is 83.3 U/mL, and those mediated by other five signal peptides (W1, MF4I, INU1A, αpre, HFBI) are 19.6, 169.5, 0, 66.4 and 100.2 U/mL, respectively. INU1A can't mediate the secretion of MAN. The MF4I is the most efficient among these signal sequences.

3) The activity of endo-1,4-*β*-mannosidase increases with the increase of MAN gene copy number from 1 to 4 and decreases at 5 and 6 copies. The MAN activities of *α*-factor transformants from 1 to 6 gene copy number levels are 12.95, 43.33, 126.63, 173.53, 103.23 and 88.63 U/mL; while those of MF4I transformants are 79.22, 133.89, 260.14, 347.5, 206.15 and 181.89 U/mL. The max MAN activity reached 347.5 U/mL with 4 gene copies. Optimizing the signal peptide of *P. pastoris* expression system and improving MAN gene copies to a proper number can greatly improve the extracellular activity of MAN.

4) The polar and charged residues in signal peptides sequence play a positive role in secretion. Optimizing sequence structure of signal peptide also has great potential to improve the secretory expression of MAN and the extracellular MAN activity of *Pichia pastoris*.

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