Appl Microbiol Biotechnol (2016) 100:707–717
DOI 10.1007/s00253-015-7056-5

Enhanced production of leech hyaluronidase by optimizing secretion and cultivation in *Pichia pastoris*

Zhen Kang[1,2,3] · **Na Zhang**[1,3] · **Yunfeng Zhang**[1,3]

Received: 29 May 2015 / Revised: 24 September 2015 / Accepted: 2 October 2015 / Published online: 17 October 2015

Abstract Leech hyaluronidase (LHAase) was recently cloned and successfully expressed in *Pichia pastoris*. To increase its secretory expression level, four signal peptides (nsB, YTP1, SCS3, and HKR1) and six amphipathic peptides (APs) were comparatively investigated. After substitution with nsB and fusion with AP2, the production of LHAase was significantly increased, from 8.42×10^5 to 1.24×10^6 U/ml. Compared with the parental LHAase, the variant AP2-LHAase showed a lower optimum pH (5.0), higher optimum temperature (50 °C), and a broader range of thermal stability (20–60 °C). To further promote fermentative production of the variant AP2-LHAase, the cultivation temperature was systematically optimized according to cell viability and alcohol oxidase activity. Eventually, through a combination of N-terminal engineering and optimization of cultivation, the production of LHAase was improved to 1.68×10^6 U/ml, with a high productivity of 1.87×10^4 U/ml/h.

Keywords Leech hyaluronidase · *Pichia pastoris* · N-terminal engineering · Signal peptides · Amphipathic peptides · Variable temperature control strategy

Introduction

Hyaluronidases (HAases) are a large family of glycoside hydrolases that predominantly degrade hyaluronan (HA) (El-Safory et al. 2010). HAases are commonly classified into three groups according to their different substrate specificities and hydrolysis products: hyaluronate lyases (EC 4.2.2.1, e.g., *Streptococcus* hyaluronate lyase), hyaluronate 4-glycanohydrolases (EC 3.2.1.35, e.g., bovine testicular hyaluronidase (BTH)), and hyaluronate 3-glycanohydrolases (EC 3.2.1.36, e.g., leech hyaluronidase (LHAase)) (Stern et al. 2006). Extracted BTH has been commercialized and its hydrolysis mechanism studied extensively. Although commercial BTH has been used in clinical medicine as a spreading factor, it suffers from a high content of other proteins, low purity, and strong immunogenicity, which all increase risks during medical use (Holubova et al. 2014). Consequently, preparation and application of recombinant HAases without any potential risk of animal cross-infection will become a standard method in the future. Additionally, because of a wide range of potential applications in food, medicine, and cosmetics, production of low-molecular weight HA oligosaccharides has attracted much attention (Stern and Jedrzejas 2006). Although many physical and chemical degradation methods (Stern et al. 2007) and de novo chemical synthesis methods (Boltje et al. 2009) have been developed and applied for preparing HA oligosaccharides, many disadvantages (such as high polydispersity of the degradation mixtures, low transformation efficiency towards specific HA oligosaccharides, and time-consuming and expensive manufacturing processes) restrict

Electronic supplementary material The online version of this article (doi:10.1007/s00253-015-7056-5) contains supplementary material, which is available to authorized users.

✉ Zhen Kang
zkang@jiangnan.edu.cn

[1] The Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China

[2] Synergetic Innovation Center of Food Safety and Nutrition, 1800 Lihu Road, Wuxi, Jiangsu 214122, China

[3] School of Biotechnology, Jiangnan University, Wuxi 214122, China

their widespread practical applications (Ferguson et al. 2011). In comparison, enzymatic production of narrow-spectrum HA oligosaccharides in mild conditions is a much more promising and attractive approach. In this regard, achievement of high-level expression of recombinant HAases with desirable enzymatic properties is of great importance.

Pichia pastoris (current accepted name is *Komagataella pastoris*), a methylotrophic yeast, is widely used for heterologous protein production because of its fast growth on minimal media, powerful secretion ability, and absence of production of intrinsic endotoxins or viral DNAs (Cereghino et al. 2002; Gellissen 2000; Macauley-Patrick et al. 2005; Vogl and Glieder 2013). Many pharmaceutical proteins and industrial enzymes, including hepatitis B surface antigen (Lünsdorf et al. 2011; Vanz et al. 2012), insulin precursor (Gurramkonda et al. 2010), single-chain variable fragment (Eldin et al. 1997), and trypsin (Macouzet et al. 2005), have been functionally expressed in *P. pastoris*. Impressively high levels of heterologous protein expression have been achieved in *P. pastoris* using the P_{AOX1}-promotor, for example 22 g/l of hydroxynitrile lyase (intracellular) and 15 g/l gelatin (secreted), respectively (Hasslacher et al. 1997; Werten et al. 1999). Although the bee hyaluronidase encoding gene has been cloned and successfully overexpressed in *P. pastoris*, the expression level was relatively low (Reitinger et al. 2008). Recently, we identified the first LHAase-encoding gene by applying the random amplification of cDNA ends polymerase chain reaction (RACE-PCR) approach and successfully realized its high-level production in *P. pastoris* (8.42×10^5 U/ml) (Jin et al. 2014). Compared with the two other families of HAases, LHAase shows higher substrate specificity (Linker et al. 1957). More recently, by modulating the concentration of LHAase and controlling the hydrolysis time, we achieved large-scale production of specific molar-mass-defined HA oligomers (30,000, 10,000, and 4000 Da) with high efficiency (Yuan et al. 2015), suggesting the applicability of this LHAase for the preparation of specific HA oligosaccharides on a commercial scale.

Although the alpha-factor mating signal sequence from *Saccharomyces cerevisiae* has been used frequently for secretory expression of heterologous proteins in *P. pastoris*, it does not always lead to high-level secretion. Several review papers have recognized that engineering of signal peptides should be a potential tool to improve the secretory expression of interesting enzymes (Celik and Calik 2012; Damasceno et al. 2012; Gasser et al. 2013). Recently, the production of *Candida antarctica* lipase B was significantly increased by using its native signal peptide (nsB) in *P. pastoris*, suggesting this peptide might be a better choice than the alpha-factor mating signal sequence (Vadhana et al. 2013). Moreover, amphipathic peptides with special structures are also considered promising tools for modification of enzyme properties (Gao et al. 2010). On fusion with amphipathic peptides, the specific activity of *Pseudomonas aeruginosa* lipoxygenase was significantly enhanced (Lu et al. 2013). In addition, it has been discovered that culture conditions (especially temperature) also play important roles in heterologous protein expression in *P. pastoris* (Hong et al. 2002; Woo et al. 2004). Here, to improve recombinant LHAase production in *P. pastoris*, four signal peptides and six amphipathic peptides were fused to the N-terminus of the LHAase and comparatively investigated. By combining the best modifications with incubation process optimization, the production of recombinant AP2-LHAase was increased from 8.42×10^5 to 1.68×10^6 U/ml.

Materials and methods

Strains and plasmids

All strains, plasmids, and oligonucleotides used in this study are listed in Tables 1 and 2. *Escherichia coli* JM109 was used for construction of recombinant plasmids. *P. pastoris* GS115 was used as the expression host for recombinant LHAase production. The plasmid pPIC9K (Invitrogen, Carlsbad, USA) was used as the expression vector and *S. cerevisiae* W303-1A genomic DNA was used for amplification of signal peptides. The plasmid pET-22b (+)-*SAPs-LOX* (Lu et al. 2013) was used for cloning amphipathic peptides.

The signal peptide encoding genes *YTP1*, *HKR1*, and *SCS3* (Supplementary Table S1) were amplified from *S. cerevisiae* W303-1A genomic DNA with designed oligonucleotides (Table 1) and ligated into plasmid pPIC9K-*LHAase* to substitute for the alpha-factor mating signal sequence after digestion with endonucleases *Eco*RI and *Bam*HI. The nsB signal peptide (25 amino acids) was fused at the N-terminus of the *LHAase* gene by three-step extension PCR. Then, the product *nsB-LHAase* was digested with *Not*I and *Bam*HI and ligated into pPIC9K. DNA encoding six different amphipathic peptides (Supplementary Table S1) was cloned from pET-22b(+)-*SAPs-LOX* and the sequences individually inserted downstream of the nsB signal peptide after digestion with endonucleases *Bam*HI and *Eco*RI.

Medium composition

YPD medium contained (per liter) 20 g peptone, 10 g yeast extract, 20 g glucose, and 20 g agar and was used for seed growth. MD medium (per liter: 20 g glucose, 4×10^{-4} g biotin, 13.4 g yeast nitrogen base without amino acids [YNB]) was used for growing transformants of *P. pastoris* and screening positive colonies. BMGY medium (per liter: 20 g peptone, 10 g yeast extract, 3 g K_2HPO_4, 11.8 g KH_2PO_4, 1 ml pure glycerol, 13.4 g YNB, 4×10^{-4} g biotin) and BMMY medium (the same as BMGY except 1 % methanol was substituted for the glycerol) were used for expression studies. BSM medium

Table 1 Oligonucleotides used in this study

Oligonucleotides	Sequence (5′-3′)
nsB-Lhyal-F1	ACTTGCGTTGCAGCCACTCCTTTGGTGAAGCGTCACCACCACCACCACCACATGAAAGA
Lhyal-R	CGCGGATCCGCGGCCGCTTACTTTTTGCACGCTTCAACATTT
nsB-F2	TACTCTCTCTGACCGGTGTGGCTGGTGTGCTTGCGACTTGCGTTGCAGCCACTCCTTTG
nsB-F3	CGCGGATCCAAACGATGAAGCTACTCTCTCTGACCGGTGTGGCTG
SCSC-F	CGCGGATCCAAACGATGTCTAGCAAATGGTTTAA
SCS3-R	CCGGAATTCCCCTTTCTTTACCAACATAGC
YTP1-F	CGCGGATCCAAACGATGACAGCAGCTAATAAGAA
YTP-R	CCGGAATTCAGATTTCGACCCAGCCTGCACAATT
HKR1-F	CGCGGATCCAAACGATGGTCTCATTGAAAATAAA
HKR1-R	CCGGAATTCTTGCGTTGTCGTCGTCACACTAATA
AP1-F	ACTTGCGTTGCAGCCACTCCTTTGGTGAAGCGTGTGAATTACGGTAACGGCGT
AP1-R	CCGGAATTCGCGACGGCCGATGCTA
AP2-F	ACTTGCGTTGCAGCCACTCCTTTGGTGAAGCGTGCAGAAGCAGAAGCGAAAGC
AP2-R	CCGGAATTCTTTAGCCTTAGCTTCCGCCTC
AP3-F	ACTTGCGTTGCAGCCACTCCTTTGGTGAAGCGTGATTGGCTGAAGGCTTTTTA
AP3-R	CCGGAATTCGAACGCTTCCTTCAGTTTTTC
AP5-F	ACTTGCGTTGCAGCCACTCCTTTGGTGAAGCGTGATTGGCTGAAAGCGTTCTA
AP5-R	CCGGAATTCGAAGGCTTCCTTCAGTTTTTCCG
AP6-F	ACTTGCGTTGCAGCCACTCCTTTGGTGAAGCGTGATTGGCTGAAGGCATTCTA
AP6-R	CCGGAATTCAAAAGCCTCTTTCAGTTTTTCTGCC
AOX1-F	GACTGGTTCCAATTGACAAGC
AOX1-R	TCCTACAGTCTTACGGTAAACGG
*Lyl*RT-F	CGAAAGGGTTGTGGTCATTTGTTG
*Lyl*RT-R	CGTTGCTGTCTCAGGTGTCTTATC
GAPDH-F	CGCTTCTGGTAACATCATTCC
GAPDH-R	ACGGTCAAGTCAACAACGG

Restriction endonuclease digestion sites were underlined

(per liter: 40 g pure glycerol, 18 g K_2SO_4, 7.28 g $MgSO_4$, 4.13 g KOH, 0.93 g $CaSO_4$, 27 ml 85 % H_3PO_4, and 4.4 ml particular trace metal solution [PTM1]) was used for fed-batch fermentation. PTM1 contained (per liter) 6 g $CuSO_4{\cdot}5H_2O$, 0.09 g KI, 3 g $MnSO_4$, 0.02 g H_3BO_3, 0.2 g $MoNa_2O_4{\cdot}H_2O$, 0.5 g $CoCl_2$, 20 g $ZnCl_2$, 65 g $FeSO_4{\cdot}7H_2O$, 0.2 g biotin, and 5.0 ml H_2SO_4.

Transformation and screening of recombinant strains

After digestion with endonuclease *Sal*I, 5 μg linearized plasmids was mixed with 80 μl competent cells and then transferred to pre-cooled electroporation cuvettes (0.2 cm). Transformation was performed with a Bio-Rad electroporator (1500 V, 200 Ω, 25 μF). Once electrotransformation was finished, all the cells were resuspended in 1 mol/l sorbitol, plated onto MD plates, and incubated at 30 °C for 3–4 days. Then, all the colonies were transferred to YPD plates containing G418 (gradually increased from 1 to 4 g/l) for gradient screening of the multi-copy integrated transformants. The screened recombinant strains were further confirmed by PCR with the primers 5′AOX1 and 3′AOX1. All recombinant *P. pastoris* strains are described in Table 2.

Determination of gene copy number of recombinant LHyal by real-time PCR

The genomic DNA of strains screened on 4 g/l G418 was prepared for absolute quantification using the TIANamp Yeast DNA Kit (Tiangen Biotech, Beijing, China). *GAPDH* (PAS_chr2-1_0437) was chosen as the reference gene and is present on chromosome 2 of *P. pastoris* as a single copy. The plasmids pPIC9K-*LHyal* (Jin et al. 2014) and pMD19-T-*GAPDH* (with insertion of the *GAPDH gene* into the T-vector pMD19-T, Takara Bio, Dalian, China) were used to establish standard curves, respectively, for the amount of plasmid measured using a Qubit® 2.0 fluorometer (Invitrogen Life Technologies, Carlsbad, USA). Real-time PCR primers were designed using Beacon Designer 7 software (Table 1). The PCR mixture were prepared using SYBR® Premix Ex *Taq*™ II (Tli RNaseH Plus) (Takara Bio, Dalian, China), and amplification was performed using a LightCycler® 480

Table 2 Strains and plasmids used in this study

Strains and plasmids	Genotype and characteristics	Reference
Strains		
Escherichia coli JM109	*e14*$^-$(*mcrA*), *endA1*, *recA1*, *hsdR17*(*rk*$^-$, *mk*$^+$), (*lac-proAB*) *lacIqZM15*, *relA1*	Invitrogen, Carlsbad, USA
S. cerevisiae W303-1A	*MATa ade2-1*, *his3-11*, *15*, *leu2-3*, *112 trp1-1*, *can1-100*, *ura3-1*	Invitrogen, Carlsbad, USA
P. pastoris GS115	*his4*, *Mut*$^+$, *His*$^-$ (*aox1*$^+$, *aox2*$^+$)	Invitrogen, Carlsbad, USA
P. pastoris GS115-9 K-*Lhyal*	GS115 integrated pPIC9K-*Lhyal*	Jin et al. (2014)
P. pastoris GS115-*nsB-Lhyal*	GS115 integrated pPIC9K-*nsB-Lhyal*	This study
P. pastoris GS115-*SCS3-Lhyal*	GS115 integrated pPIC9K-*SCS3-Lhyal*	This study
P. pastoris GS115-*YTP1-Lhyal*	GS115 integrated pPIC9K-*YTP1-Lhyal*	This study
P. pastoris GS115-*HKR1-Lhyal*	GS115 integrated pPIC9K-*HKR1-Lhyal*	This study
P. pastoris GS115-*nsB-AP1-Lhyal*	GS115 integrated pPIC9K-*nsB-AP1-Lhyal*	This study
P. pastoris GS115-*nsB-AP2-Lhyal*	GS115 integrated pPIC9K-*nsB-AP2-Lhyal*	This study
P. pastoris GS115-*nsB-AP3-Lhyal*	GS115 integrated pPIC9K-*nsB-AP3-HAase*	This study
P. pastoris GS115-nsB-AP4-Lhyal	GS115 integrated pPIC9K-*nsB-AP4-HAase*	This study
P. pastoris GS115-*nsB-AP5-Lhyal*	GS115 integrated pPIC9K-*nsB-AP5-HAase*	This study
P. pastoris GS115-*nsB-AP6-Lhyal*	GS115 integrated pPIC9K-*nsB-AP6-HAase*	This study
Plasmids		
pET-22b (+)-*SAPs-LOX*	*Amp*^{r}	Lu et al. (2013)
pMD19-T	*Amp*^{r}	(Takara Bio, Dalian, China)
pMD19-T-*GAPDH*	*Amp*^{r}	This study
pPIC9K	*HIS4 Amp*^{r} and *Kan*^{r}	Invitrogen, Carlsbad, USA
pPIC9K-*LHyal*	*HIS4 Amp*^{r} and *Kan*^{r}	Jin et al. (2014)
pPIC9K-*nsB-LHyal*	*HIS4 Amp*^{r} and *Kan*^{r}	This study
pPIC9K-*SCS3-LHyal*	*HIS4 Ampr* and *Kan*^{r}	This study
pPIC9K-*YTP1-LHyal*	*HIS4 Amp*^{r} and *Kan*^{r}	This study
pPIC9K-*HKR1-LHyal*	*HIS4 Amp*^{r} and *Kan*^{r}	This study
pPIC9K-*nsB-AP1-LHyal*	*HIS4 Amp*^{r} and *Kan*^{r}	This study
pPIC9K-*nsB-AP2-LHyal*	*HIS4 Amp*^{r} and *Kan*^{r}	This study
pPIC9K-*nsB-AP3-LHyal*	*HIS4 Amp*^{r} and *Kan*^{r}	This study
pPIC9K-*nsB-AP4-LHyal*	*HIS4 Amp*^{r} and *Kan*^{r}	This study
pPIC9K-*nsB-AP5-LHyal*	*HIS4 Amp*^{r} and *Kan*^{r}	This study
pPIC9K-*nsB-AP6-LHyal*	*HIS4 Amp*^{r} and *Kan*^{r}	This study

instrument (Roche Diagnostics Ltd., Rotkreuz, Switzerland). For each sample, reactions were performed in triplicate. Standard curves were generated by a linear regression of the mean Ct values plotted against $\log^{10}$ of gradient diluted template. The copy number of the target gene was determined by the following equation (Abad et al. 2010)

$$\text{Copy number}_{\text{Lhyal GENE}} \frac{\text{Copy quantity}_{\text{LHyal GENE}}}{\text{Copy quantity}_{\text{GAPDH GENE}}}$$

Cultivation of HAase-producing strains

Shaken-flask cultivations were performed in 250-ml Erlenmeyer flasks containing 50 ml BMGY medium. A 2 % (*v*/*v*) inoculum from a preculture grown in YPD medium for 24 h was used. When cells reached middle log phase (OD_{600} = 15), they were harvested by centrifugation at 4 °C and resuspended in 50 ml induction medium (BMMY). Induction was carried out at 25 °C for 96 h. The cultures were fed with pure methanol to a final concentration of 10 g/l every 24 h. At different times, samples were centrifuged at 2100×*g* at 4 °C for 5 min. The supernatant was stored at −80 °C until further analysis. Optical density values were determined at 600 nm using a spectrophotometer (Biospe-1601; Shimadzu Co., Kyoto, Japan).

Fed-batch fermentation was carried out in a 3-l fermenter (LiFlus GM Biotron, Gyeonggi-Do, Republic of Korea) containing 800 ml BSM medium. A 10 % (*v*/*v*) inoculum was applied and the temperature was maintained at 30 °C. The

pH was maintained at 5.5 by addition of 25 % ammonium hydroxide and 85 % phosphoric acid. During glycerol batch phase growth, the dissolved oxygen (DO) level was maintained above 30 % of air saturation by cascaded control of the agitator speed at 500–1000 r/min and the aeration rate (2.5 l/min). During the glycerol fed-batch phase, 50 % (*w*/*v*) glycerol containing 12 ml/l PTM1 solution was pumped into the fermenter according to a predetermined protocol (Jin et al. 2014). After glycerol was exhausted, pure methanol containing 12 ml/l PTM1 solution was added to cultures to maintain a constant methanol concentration of 18 g/l (Ling et al. 2012). During the methanol fed-batch phase, the methanol feed rate was controlled with a methanol online sensor (FC-2000, SuperInfo. Tech. Co. Ltd., Shanghai, China). In experiments to test the effect of induction temperature on production of LHAase, three temperatures were used (30, 25, and 22 °C). In experiments involving a two-stage temperature strategy, the temperature was maintained at 25 °C until 60 h from addition methanol and then changed to 22 °C.

Measurement of cell concentration, cell viability, and alcohol oxidase (AOX) activity

Ten-milliliter cultures were centrifuged at 8000×*g* for 10 min. Then the cell pellets were dried to a constant weight at 105 °C for determination of dry cell weight (DCW, g/l). Cell viability was measured by the methylene blue dye exclusion technique, as described previously (Sinha et al. 2005). One unit of enzymatic activity of AOX was defined as the amount of enzyme producing 1 μmol/l of H_2O_2 per minute, as described previously (Suye et al. 1990).

HAase activity assay

One unit of enzymatic activity was defined as equal to the reducing power of glucuronic acid (glucose equivalents in micrograms) liberated per hour from HA at 38 °C and pH 5.5 (Jin et al. 2014). The specific activity was defined as units of enzyme per milligram of protein. HAase activity was quantified by measuring the amount of reducing sugar liberated from hyaluronic acid using the 3,5-dinitrosalicylic acid (DNS) colorimetric spectrophotometric method (Ghose 1987).

Purification and SDS-PAGE analysis of HAases

Cultures were centrifugated at 8000×*g* for 30 min at 4 °C and the supernatants were applied for SDS-PAGE analysis. LHAases, which were His-tagged, were purified using Ni-NTA agarose chromatography (Qiagen, Hilden, Germany). Samples were loaded onto an equilibrated column and washed with a stepwise gradient of imidazole (20–100 mM) in phosphate buffer (pH 6.0). Then, the target protein was eluted from the column with 500 mM imidazole and desalted by dialysis, as reported previously (Jin et al. 2014).

For SDS-PAGE analysis, the culture samples were centrifuged at 8000×*g* at 4 °C for 10 min. After thermal denaturation, 10 μl culture supernatant was separated on a 12 % gel. After electrophoresis on a vertical mini-gel apparatus at 150 V for 1 h, the gel was stained with 1 % Coomassie Brilliant Blue (R-250) (Sigma-Aldrich, St. Louis, USA) and destained with a methanol/water/acetic acid mixture (5:4:1) for 1 h. Protein concentrations were measured by the Bradford method (Bradford 1976) using bovine serum albumin as the standard.

Results

Improvement of LHAase secretory expression in *P. pastoris* with the nsB signal peptide

Signal peptide engineering is a feasible approach to enhance extracellular secretion of heterologous proteins (Zhang et al. 2013). Here, four different signal peptides, HKR1 YTP1, SCS3, and nsB (Sahara et al. 2007) (Supplementary Table S1), were investigated in comparison with the alpha-factor mating signal sequence. Although similar biomass and copy numbers of genes were obtained respectively (Fig. 1a, 1b and Supplementary Fig. S1a), application of the nsB signal peptide resulted in the highest extracellular LHAase activity (7.96×10^4 U/ml), which was 126 % higher than that with the alpha-factor mating signal sequence (Fig. 1b). To confirm and evaluate the secretory capacity of nsB, fed-batch fermentation was carried out in a 3-l fermenter. Methanol was maintained at about 18 g/l throughout the fermentation. At 90 h, the activity of LHAase was 1.03×10^6 U/ml and 1.14×10^4 U/ml/h (Fig. 1c), while the biomass accumulated to 187.7 g/l (cell dry weight). Consistently, SDS-PAGE analysis results showed that, compared with the alpha-factor mating signal sequence, nsB resulted in a thicker band of LHAase (Fig. 1d). Consequently, nsB was applied to improve the production of LHAase in further studies.

Fusion with amphipathic peptides to improve specific activity

Recently, some amphipathic peptides with special structures (such as β-sheet) have been applied for production of active proteins and enhancement of enzyme thermal stability (Wang et al. 2008; Zhang et al. 1993; Zhou et al. 2012). To further improve the expression level and to optimize the enzymatic properties of LHAase at the molecular level, six amphipathic peptides (APs) (Supplementary Table S1) were, respectively, inserted downstream of the nsB signal peptide and comparatively investigated. As expected, significant differences were observed between the strains with different APs (Fig. 2a),

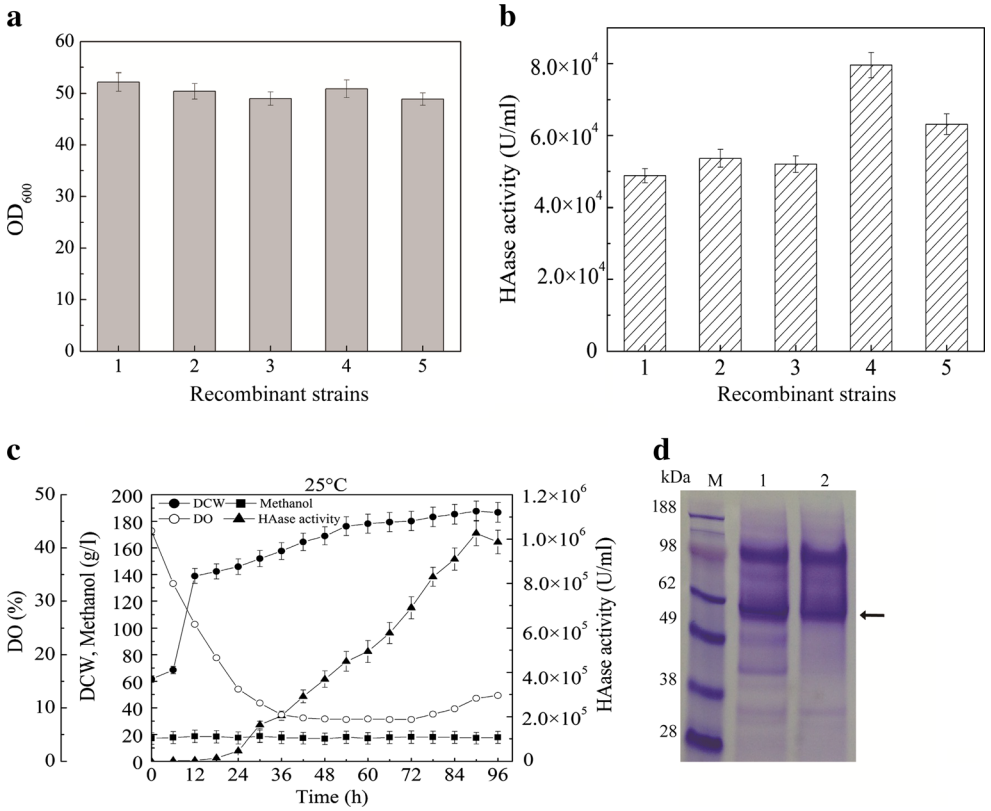

Fig. 1 Effects of different signal peptides on the production of HAase in recombinant *P. pastoris*. **a** The value of OD_{600} in shaken-flask cultures. The value for each strain was monitored throughout the fermentation course and is shown at 96 h when the extracellular enzyme activity reached its maximum. Samples 1–5 were GS115-*HKR1-LHyal*, GS115-*YTP1-LHyal*, GS115-*SCS3-LHyal*, GS115-*nsB-LHyal*, and GS115-*alpha-factor-LHyal*, respectively. **b** Extracellular HAase activity in shaken-flask cultures. **c** Fed-batch fermentation of the recombinant strain GS115-*nsB-LHyal* (25 °C); time course during the methanol fed-batch phase. **d** SDS-PAGE analysis of the extracellular HAase. *Lane M*, protein marker; *lanes 1 and 2* show supernatants of cultures of cells expressing GS115-*nsB-LHyal* and GS115-*alpha-factor-LHyal*, respectively. Each value is the mean of three independent experiments

although the copy numbers of each gene were similar (Supplementary Fig. S1b). Only the recombinant AP2-LHAase showed higher HAase activity (9.69×10^4 U/ml) than the parental LHAase secreted by the nsB signal peptide, indicating that insertion of other peptides might negatively affect the function of LHAase. The culture supernatants of AP2-LHAase and LHAase were also investigated by SDS-PAGE assay. Interestingly, no obvious differences were observed (Fig. 2b), suggesting that the higher activity of AP2-LHAase might be attributable to an increased specific activity. Thus, AP2-LHAase was purified, characterized, and compared with the parental LHAase. As expected, AP2-LHAase showed higher specific activity and catalytic efficiency (k_{cat}/K_m) than the parental LHAase (Table 3). Peptide AP2 may make the structure around the active site more flexible (Yang et al. 2013). More interestingly, we found that fusion with AP2 generated a lower optimum pH (5.0), higher optimum temperature (50 °C), and a wider range of thermal stability (20–60 °C) than were observed for the parental enzyme. These results indicate that the recombinant AP2-LHAase could have wide applications in enzymatic production of hyaluronan oligosaccharides as lower pH and higher temperature are beneficial in avoiding bacterial contamination.

Optimization of the induction temperature to increase LHAase production

In most cases, temperature plays a vital role in cell growth and heterologous protein expression in *P. pastoris* (Wang et al. 2009). Thus, the effect of temperature on the production of AP2-LHAase was investigated and optimized. Cells were initially cultivated at 30 °C, then the inducing temperature was 30, 25, or 22 °C, respectively. As shown in Fig. 2c, although no obvious changes were observed in cell growth, there was a remarkable influence of the inducing temperature on LHAase expression. Incubation at 22 °C produced the highest activity of AP2-LHAase (1.22×10^5 U/ml) (Fig. 2d), which was approximately 126 and 342 % higher than that at 25 and 30 °C, respectively. Subsequently, fed-batch fermentation was carried out at different temperatures to confirm these results.

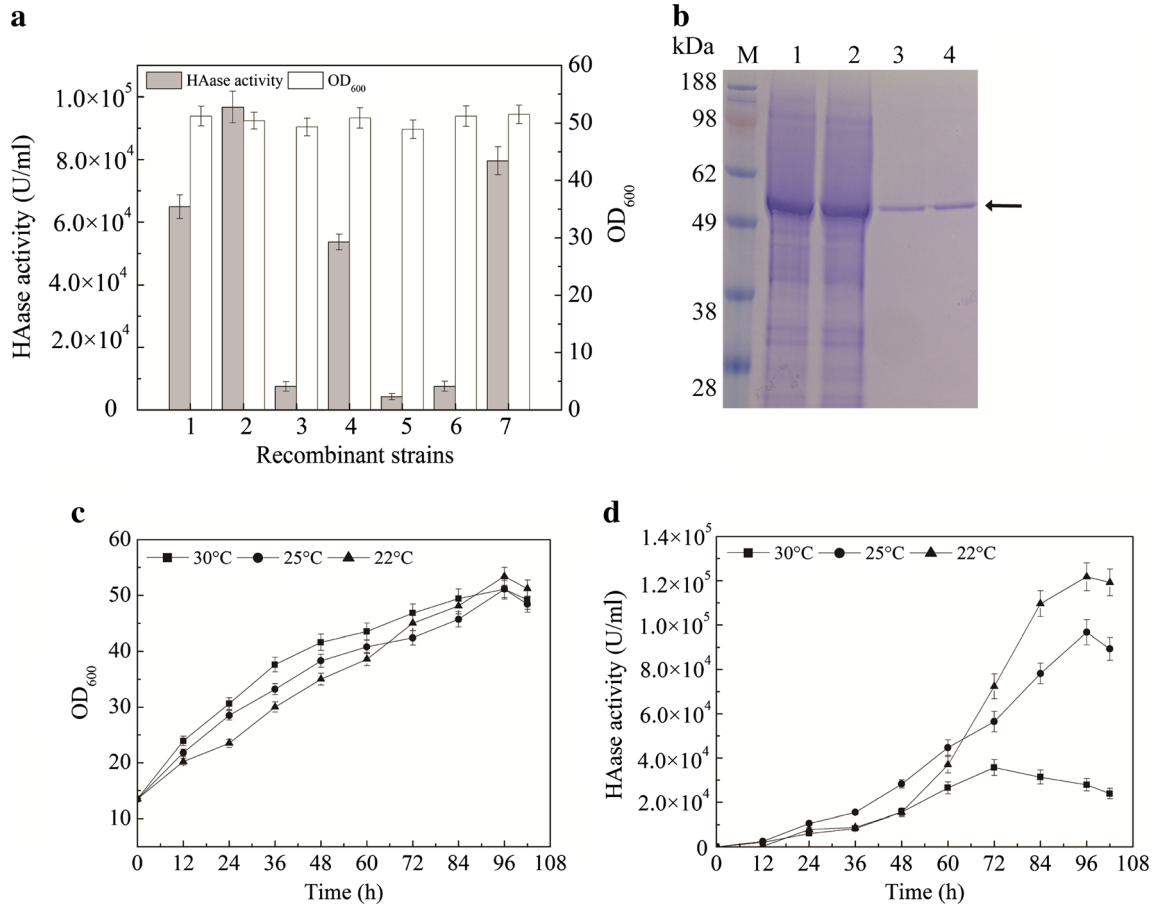

Fig. 2 Production of HAase with different amphipathic peptides and induction temperatures. **a** Extracellular HAase activity and cell biomass (25 °C). Samples 1–7 were the variants GS115-*AP1-LHyal*, GS115-*AP2-LHyal*, GS115-*AP3-LHyal*, GS115-*AP4-LHyal*, GS115-*AP5-LHyal*, GS115-*AP6-LHyal*, and GS115-*alpha-factor-LHyal*, respectively. **b** SDS-PAGE analysis of extracellular and purified HAases. *Lane M*, protein marker; *lanes 1 and 2*, the culture supernatants of cells expressing *LHyal* and *AP2-LHyal*, respectively; *lanes 3 and 4*, purified HAase and AP2-HAase, respectively. **c** Effect of induction temperature on cell growth. **d** Effect of induction temperature on HAase expression in shaken-flask cultures. Each value is the mean of three independent experiments

Biomass accumulated to about 190 g/l after 90 h at each temperature, but a higher activity of AP2-LHAase (1.48×10^6 U/ml, 1.49×10^4 U/ml/h) (Fig. 3a) was observed at 22 °C, which was 119 and 307 % higher than that at 25 °C (Fig. 3b) and 30 °C (Fig. 3c), respectively. SDS-PAGE analysis (Fig. 3d) of the culture supernatants confirmed that lower temperature resulted in production of more AP2-LHAase.

Table 3 Enzymatic properties of hyaluronidase with amphipathic peptide 2

Parameters	nsB-LHAase	nsB-AP2-LHAase
Specific activity (U/mg)	1.23×10^6	1.44×10^6
Optimum pH	6.5	5.0
pH Stability	3.0–8.0	3.0–8.0
Optimum temperature (°C)	45	50
Thermal stability (°C)	20–55	20–60
K_m (g/l)	1.73 ± 0.16	1.56 ± 0.13
k_{cat} (1/min)	879 ± 65	1312 ± 139
k_{cat}/K_m (g/min/l)	508 ± 37	841 ± 70

Low induction temperature maintained higher AOX activity and cell viability

In the methanol utilization plus phenotype *P. pastoris* (Mut$^+$), the intracellular AOX activity is commonly used as an indicator of the expression level of interesting enzymes because induction of the enzyme of interest is under the control of the P_{AOX1}-promotor. Thus, we also determined the AOX activities in cells at different temperatures. Lower temperatures resulted in higher AOX activities throughout the whole cultivation process (Fig. 4a); methanol was nearly depleted by the strain at each temperature and a similar dry cell weight was observed in each temperature condition (Fig. 3a–3c). Because of the vital role of cell viability in the production of target

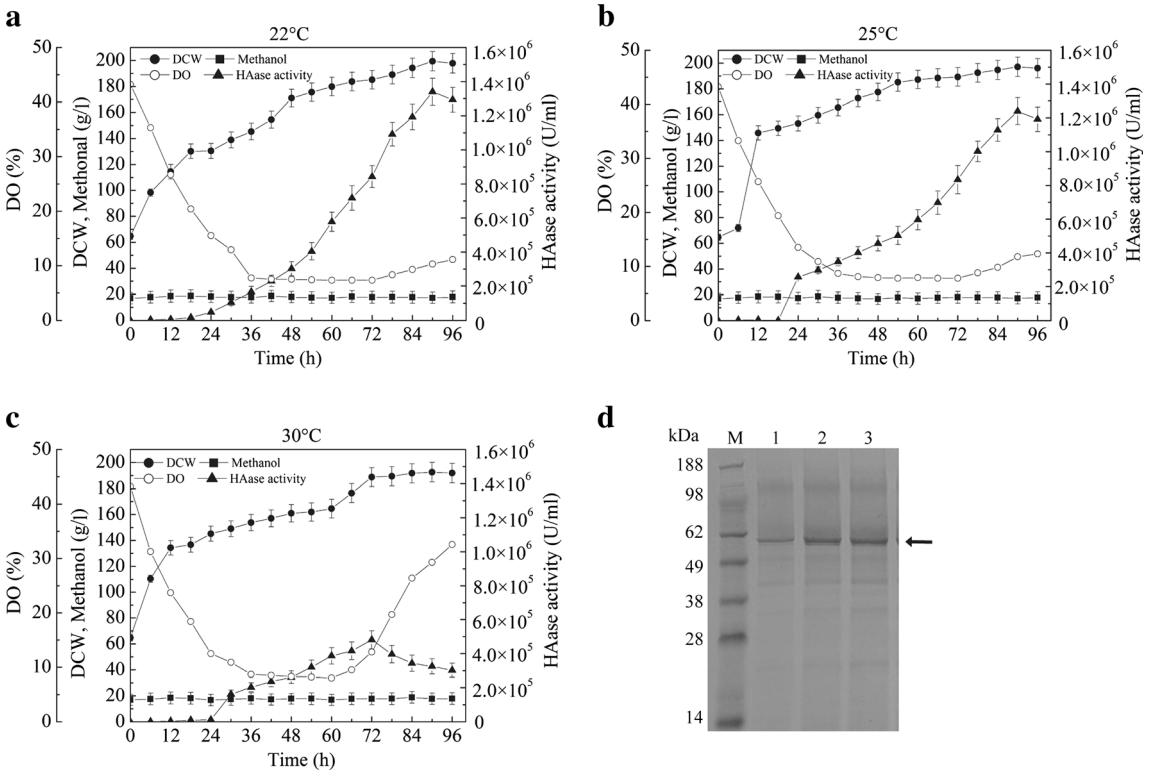

Fig. 3 Fed-batch fermentation of the recombinant strain GS115-*nsB-AP2-LHyal*. **a** 22 °C; **b** 25 °C; **c** 30 °C (different inducing temperature during the methanol fed-batch phase). **d** SDS-PAGE analysis of the extracellular HAase at different induction temperatures. *Lane M*, protein marker; *lanes 1, 2, and 3* show the culture supernatants at 30, 25, and 22 °C, respectively. Each value is the mean of three independent experiments

proteins (Jahic et al. 2003), the cell viabilities at different temperatures were also analyzed. Consistent with the AOX activities, higher cell viability was observed at lower temperature throughout the fermentation process (Fig. 4b), indicating that higher activity of HAase at lower temperature should be ascribed to higher cell viability.

Production of LHAase with a variable induction temperature strategy

Although higher production of LHAase was obtained at 22 °C, a higher cell growth rate and enzyme accumulation rate were observed during the methanol fed-batch phase (Fig. 3). In consideration of both cell growth and cell viability, a two-stage temperature control strategy was applied to further improve the secretory expression of AP2-LHAase. Specifically, the temperature was controlled at 25 °C until 60 h from addition methanol then changed to 22 °C until the end of fermentation. As shown in Fig. 5, AOX activity increased until reaching a stable, high level of 1750 U/ml. At the same time, no obvious decline in cell viability was observed through the fermentation, which indirectly suggests the success of the two-stage temperature control strategy.

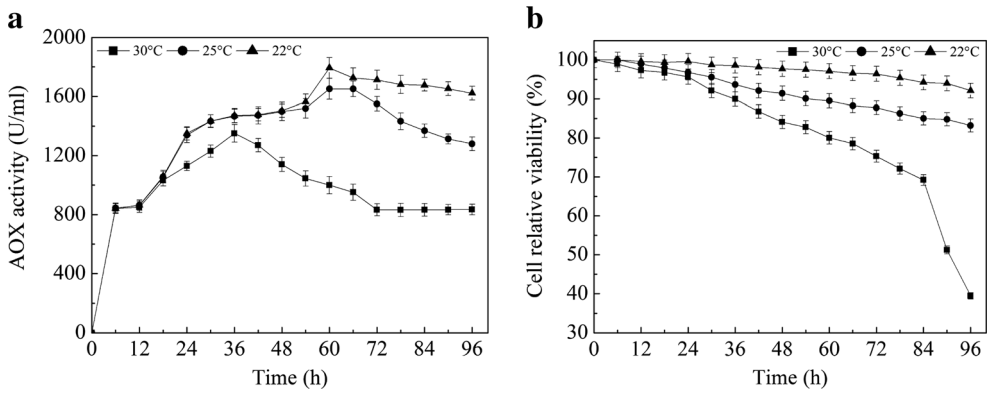

Fig. 4 Effect of induction temperature on **a** AOX activity and **b** cell viability

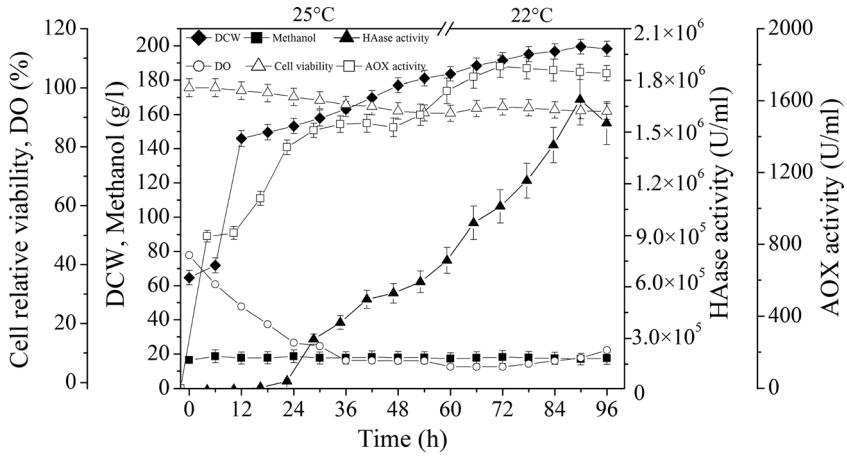

Fig. 5 Fed-batch fermentation with a variable induction temperature approach. The time course shown is during the methanol fed-batch phase. At 60 h, the culture temperature was changed from 25 to 22 °C. Each value is the mean of three independent experiments

More directly, it was found that the activity of HAase was increased to 1.68 × 10^6 U/ml (Fig. 5) with a high productivity of 1.87 × 10^4 U/ml/h (Table 4).

Discussion

Although the alpha-factor mating signal sequence has commonly been used for heterologous protein expression in *P. pastoris*, it is not always optimal (Cregg et al. 2000; Liang et al. 2013; Vogl and Glieder 2013). In addition, the Glu-Ala repeats in the alpha-factor mating signal sequence usually cause incomplete processing, which results in a redundant fragment at the N-terminus of the proteins of interest (Kozlov and Yagudin 2008; Ling et al. 2012). Thus, many other signal peptides have been investigated and applied in *P. pastoris* expression systems (Liang et al. 2013). Here, four different commonly used signal peptides were comparatively investigated. The results demonstrate that, compared with the alpha-factor mating signal sequence, the nsB signal peptide results in higher secretory expression of LHAase. In contrast, the other tested signal peptides exhibited a lower secretory capacity than the alpha-factor mating signal sequence, which further confirms that the nsB signal peptide can be a good choice for production of heterologous protein in *P. pastoris* (Vadhana et al. 2013). To further improve the secretory capacity and promote wide applications of the recombinant protein, directed evolution of the nsB signal peptide is advisable for production of individual enzymes (Kang et al. 2015) because of its short length (25 amino acids).

Increasingly, more data suggest that the N-terminal sequence of a protein (including the signal peptide) plays critical roles in determining its expression level and enzymatic properties (Kang et al. 2014; Ling et al. 2013). Here, our results showed that fusion with the amphipathic peptide 2 of only 16 amino acids increased the specific activity and also improved the thermal stability of the recombinant LHAase (Table 3). Similarly, Lu et al. (2013) significantly increased the specific activity of lipoxygenase by fusion with amphipathic peptides. In addition, Zhou et al. (2012) successfully drove soluble proteins into active aggregates by fusion with short amphipathic surfactant-like peptides, demonstrating that amphipathic peptides with specific structures can be applied for protein expression and engineering.

In addition to molecular engineering, optimization of cultivation parameters is always practical and effective for enhancing enzyme production. In particular, temperature, as a critical factor, is usually optimized for expression of heterologous proteins in *P. pastoris*. Our results here demonstrate that a lower cultivation temperature resulted in a higher activity of HAase. It has been reported that lower induction temperature led to a higher rate of correctly folded protein (Gao et al.

Table 4 Hyaluronidase production in different expression hosts

Source of HAase	Expression host	Culture time (h)	HAase activity (U/ml)	Productivity (U/ml/h)	Reference
Human	Insect cell	240	1.50 × 10^{-1}	6.25 × 10^{-4}	Hofinger et al. (2007)
Human	*E. coli*	4	1.00 × 10^{-3}	2.50 × 10^{-4}	Hofinger et al. (2007)
Bacillus. sp	*Bacillus* sp. A50	18	1.50 × 10^4	8.33 × 10^2	Guo et al. (2014)
Bee	*P. pastoris*	72	1.01	1.40 × 10^{-2}	Reitinger et al. (2008)
Leech	*P. pastoris*	96	8.42 × 10^5	8.77 × 10^3	Jin et al. (2014)
Leech	*P. pastoris*	90	1.68 × 10^6	1.87 × 10^4	This study

2015). Moreover, our results demonstrate that lower temperature resulted in higher cell viability, which might be ascribed to smooth methanol induction and a lower cell death rate at low temperature (Jahic et al. 2003). In conclusion, through a combination of N-terminal engineering and optimization of the cultivation process, the activity of LHAase was significantly increased, reaching 1.68×10^6 U/ml. To the best of our knowledge, this is the highest value reported to date.

Acknowledgments This work was financially supported by a grant from the Key Technologies R & D Program of Jiangsu Province, China (BE2014607); National High Technology Research and Development Program of China (863 Program, 2012AA022005); Program for Changjiang Scholars and Innovative Research Team in University (no. IRT1135); the National Science Foundation for Post-doctoral Scientists of China (2013M540414); the Jiangsu Planned Projects for Postdoctoral Research Funds (1301010B); and the 111 Project.

Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests.

References

Abad S, Kitz K, Hormann A, Schreiner U, Hartner FS, Glieder A (2010) Real-time PCR-based determination of gene copy numbers in *Pichia pastoris*. Biotechnol J 5:413–420

Boltje TJ, Buskas T, Boons GJ (2009) Opportunities and challenges in synthetic oligosaccharide and glycoconjugate research. Nat Chem 1: 611–622

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Celik E, Calik P (2012) Production of recombinant proteins by yeast cells. Biotechnol Adv 30:1108–1118

Cereghino GP, Cereghino JL, Ilgen C, Cregg JM (2002) Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. Curr Opin Biotechnol 13:329–332

Cregg JM, Cereghino JL, Shi J, Higgins DR (2000) Recombinant protein expression in *Pichia pastoris*. Mol Biotechnol 16:23–52

Damasceno LM, Huang CJ, Batt CA (2012) Protein secretion in *Pichia pastoris* and advances in protein production. Appl Microbiol Biotechnol 93:31–39

El-Safory NS, Fazary AE, Lee CK (2010) Hyaluronidases, a group of glycosidases: current and future perspectives. Carbohydr Polym 81: 165–181

Eldin P, Pauza ME, Hieda Y, Lin GF, Murtaugh MP, Pentel PR, Pennell CA (1997) High-level secretion of two antibody single chain Fv fragments by *Pichia pastoris*. J Immunol Methods 201(1):67–75

Ferguson EL, Roberts JL, Moseley R, Griffiths PC, Thomas DW (2011) Evaluation of the physical and biological properties of hyaluronan and hyaluronan fragments. Int J Pharm 420:84–92

Gao MJ, Zhan XB, Gao P, Zhang X, Dong SJ, Li Z, Shi ZP, Lin CC (2015) Improving performance and operational stability of porcine interferon-α production by *Pichia pastoris* with combinational induction strategy of low temperature and methanol/sorbitol co-feeding. Appl Biochem Biotechnol 176:493–504

Gao Y, Zhao F, Wang Q, Zhang Y, Xu B (2010) Small peptide nanofibers as the matrices of molecular hydrogels for mimicking enzymes and enhancing the activity of enzymes. Chem Soc Rev 39:3425–3433

Gasser B, Prielhofer R, Marx H, Maurer M, Nocon J, Steiger M, Puxbaum V, Sauer M, Mattanovich D (2013) *Pichia pastoris*: protein production host and model organism for biomedical research. Future Microbiol 8:191–208

Gellissen G (2000) Heterologous protein production in methylotrophic yeasts. Appl Microbiol Biotechnol 54:741–750

Ghose T (1987) Measurement of cellulase activities. Pure Appl Chem 59: 257–268

Guo X, Shi Y, Sheng J, Wang F (2014) A novel hyaluronidase produced by *Bacillus* sp. A50. PLoS One 9:e94156

Gurramkonda C, Polez S, Skoko N, Adnan A, Gäbel T, Chugh D, Swaminathan S, Khanna N, Tisminetzky S, Rinas U (2010) Application of simple fed-batch technique to high-level secretory production of insulin precursor using *Pichia pastoris* with subsequent purification and conversion to human insulin. Microb Cell Factories 9:31

Hasslacher M, Schall M, Hayn M, Bona R, Rumbold K, Lückl J, Griengl H, Kohlwein SD, Schwab H (1997) High-level intracellular expression of hydroxynitrile lyase from the tropical rubber tree *Hevea brasiliensis* in microbial hosts. Protein Expr Purif 11:61–71

Hofinger ES, Spickenreither M, Oschmann J, Bernhardt G, Rudolph R, Buschauer A (2007) Recombinant human hyaluronidase Hyal-1: insect cells versus *Escherichia coli* as expression system and identification of low molecular weight inhibitors. Glycobiology 17:444–453

Holubova L, Korecka L, Podzimek S, Moravcova V, Rotkova J, Ehlova T, Velebny V, Bilkova Z (2014) Enhanced multiparametric hyaluronan degradation for production of molar-mass-defined fragments. Carbohydr Polym 112:271–276

Hong F, Meinander NQ, Jönsson LJ (2002) Fermentation strategies for improved heterologous expression of laccase in *Pichia pastoris*. Biotechnol Bioeng 79:438–449

Jahic M, Wallberg F, Bollok M, Garcia P, Enfors SO (2003) Temperature limited fed-batch technique for control of proteolysis in *Pichia pastoris* bioreactor cultures. Microb Cell Factories 2:6

Jin P, Kang Z, Zhang N, Du G, Chen J (2014) High-yield novel leech hyaluronidase to expedite the preparation of specific hyaluronan oligomers. Sci Rep 4:4471

Kang Z, Yang S, Du G, Chen J (2014) Molecular engineering of secretory machinery components for high-level secretion of proteins in *Bacillus* species. J Ind Microbiol Biotechnol 41:1599–1607

Kang Z, Zhang J, Jin P, Yang S (2015) Directed evolution combined with synthetic biology strategies expedite semi-rational engineering of genes and genomes. Bioengineered 6:136–140

Kozlov DG, Yagudin TA (2008) Antibody fragments may be incorrectly processed in the yeast *Pichia pastoris*. Biotechnol Lett 30:1661–1663

Liang S, Li C, Ye Y, Lin Y (2013) Endogenous signal peptides efficiently mediate the secretion of recombinant proteins in *Pichia pastoris*. Biotechnol Lett 35:97–105

Ling Z, Ma T, Li J, Du G, Kang Z, Chen J (2012) Functional expression of trypsin from *Streptomyces griseus* by *Pichia pastoris*. J Ind Microbiol Biotechnol 39:1651–1662

Ling Z, Liu Y, Teng S, Kang Z, Zhang J, Chen J, Du G (2013) Rational design of a novel propeptide for improving active production of *Streptomyces griseus* trypsin in *Pichia pastoris*. Appl Environ Microbiol 79:3851–3855

Linker A, Hoffman P, Meyer K (1957) The hyaluronidase of the leech: an endoglucuronidase. Nature 180:810–811

Lu X, Liu S, Zhang D, Zhou X, Wang M, Liu Y, Wu J, Du G, Chen J (2013) Enhanced thermal stability and specific activity of *Pseudomonas aeruginosa* lipoxygenase by fusing with self-

assembling amphipathic peptides. Appl Microbiol Biotechnol 97: 9419–9427

Lünsdorf H, Gurramkonda C, Adnan A, Khanna N, Rinas U (2011) Virus-like particle production with yeast: ultrastructural and immunocytochemical insights into *Pichia pastoris* producing high levels of the hepatitis B surface antigen. Microb Cell Factories 10:48

Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM (2005) Heterologous protein production using the *Pichia pastoris* expression system. Yeast 22:249–270

Macouzet M, Simpson BK, Lee BH (2005) Expression of a cold-adapted fish trypsin in *Pichia pastoris*. FEMS Yeast Res 5:851–857

Reitinger S, Boroviak T, Laschober GT, Fehrer C, Müllegger J, Lindner H, Lepperdinger G (2008) High-yield recombinant expression of the extremophile enzyme, bee hyaluronidase in *Pichia pastoris*. Protein Expr Purif 57:226–233

Sahara T, Gouda T, Tochigi Y, Ohgiya S (2007) A highly efficient secretory signal peptide and a protein expression system using the peptide thereof. Eur Pat: EP1790661

Sinha J, Plantz BA, Inan M, Meagher MM (2005) Causes of proteolytic degradation of secreted recombinant proteins produced in methylotrophic yeast *Pichia pastoris*: case study with recombinant ovine interferon-tau. Biotechnol Bioeng 89:102–112

Stern R, Asari AA, Sugahara KN (2006) Hyaluronan fragments: an information-rich system. Eur J Cell Biol 85:699–715

Stern R, Jedrzejas MJ (2006) Hyaluronidases: their genomics, structures, and mechanisms of action. Chem Rev 106:818–839

Stern R, Kogan G, Jedrzejas MJ, Soltes L (2007) The many ways to cleave hyaluronan. Biotechnol Adv 25:537–557

Suye S, Ogawa A, Yokoyama S, Obayashi A (1990) Screening and identification of *Candida methanosorbosa* as alcohol oxidase-producing methanol using yeast. Agri Biol Chem 54:1297–1298

Vadhana AK, Samuel P, Berin RM, Krishna J, Kamatchi K, Meenakshisundaram S (2013) Improved secretion of *Candida antarctica* lipase B with its native signal peptide in *Pichia pastoris*. Enzym Microb Technol 52:177–183

Vanz AL, Lünsdorf H, Adnan A, Nimtz M, Gurramkonda C, Khanna N, Rinas U (2012) Physiological response of *Pichia pastoris* GS115 to methanol-induced high level production of the hepatitis B surface antigen: catabolic adaptation, stress responses, and autophagic processes. Microb Cell Factories 11:103

Vogl T, Glieder A (2013) Regulation of *Pichia pastoris* promoters and its consequences for protein production. New Biotechnol 30:385–404

Wang Q, Yang Z, Gao Y, Ge W, Wang L, Xu B (2008) Enzymatic hydrogelation to immobilize an enzyme for high activity and stability. Soft Matter 4:550

Wang Y, Wang Z, Xu Q, Du G, Hua Z, Liu L, Li J, Chen J (2009) Lowering induction temperature for enhanced production of polygalacturonate lyase in recombinant *Pichia pastoris*. Process Biochem 44:949–954

Werten MW, van den Bosch TJ, Wind RD, Mooibroek H, de Wolf FA (1999) High-yield secretion of recombinant gelatins by *Pichia pastoris*. Yeast 15:1087–1096

Woo JH, Liu YY, Stavrou S, Neville DM (2004) Increasing secretion of a bivalent anti-T-cell immunotoxin by *Pichia pastoris*. Appl Environ Microbiol 70:3370–3376

Yang H, Lu X, Liu L, Li J, Shin HD, Chen RR, Du G, Chen J (2013) Fusion of an oligopeptide to the N terminus of an alkaline α-amylase from *Alkalimonas amylolytica* simultaneously improves the enzyme's catalytic efficiency, thermal stability, and resistance to oxidation. Appl Environ Microbiol 79:3049–3058

Yuan P, Lv M, Jin P, Wang M, Du G, Chen J, Kang Z (2015) Enzymatic production of specifically distributed hyaluronanoligosaccharides. Carbohydr Polym 129:194–200

Zhang J, Kang Z, Ling Z, Cao W, Liu L, Wang M, Du G, Chen J (2013) High-level extracellular production of alkaline polygalacturonate lyase in *Bacillus subtilis* with optimized regulatory elements. Bioresour Technol 146:543–548

Zhang S, Holmes T, Lockshin C, Rich A (1993) Spontaneous assembly of a self-complementary oligopeptide to form a stable macroscopic membrane. Proc Natl Acad Sci U S A 90:3334–3338

Zhou B, Xing L, Wu W, Zhang XE, Lin Z (2012) Small surfactant-like peptides can drive soluble proteins into active aggregates. Microb Cell Factories 11:10