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Comparison of two codon optimization strategies enhancing recombinant *Sus scrofa* lysozyme production in *Pichia pastoris*

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Abstract

Lysozyme has played an important role in animal feed additive industry, food additive industry and biological engineering. For improving expression efficiency of recombinant lysozyme from *Sus scrofa*, two genes respectively designed by the most used codon optimization strategies, "one amino acid one codon" and "codon randomization", were synthesized and expressed in *Pichia pastoris* X-33. At shaking flask level, *Sus scrofa* lysozyme (SSL) under two conditions had a highest activity of 153.33±10.41 and 538.33±15.18 U/mL after a 5 days induction of 1% methanol, with secreted protein concentration 80.03±1.94 and 239.60±4.16 mg/L, respectively. Compared with the original SSL gene, the expression of optimized SSL gene by the second strategy showed a 2.6 fold higher level, while the first method had no obvious improvement in production. In total secreted protein, the proportions of recombinant SSL encoded by the original gene, first method optimized gene and the second-strategy optimized one were 75.06±0.25%, 74.56±0.14% and 79.00±0.14%, respectively, with the same molecular weight about 18 kDa, optimum acidity pH 6.0 and optimum temperature 35°C.

Key words: Lysozyme, *Sus scrofa*, Codon optimization, One amino acid one codon, Codon randomization, *Pichia pastoris.*

Introduction

With the high frequency of food safety incidents, antibiotics in animal feed have caught a good many attentions. Therefore, many countries have prohibited the use of them. To replace antibiotics in animal feed, the substitute must be safe and effective. As a natural alkaline protein, lysozyme plays an important role in animal immune system because it could break down the β-1,4-glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine which are important compositions of the peptidoglycan in microbial cell walls (1). Considering the important role of pig (*Sus scrofa*) in livestock breeding and that of *Sus scrofa* lysozyme (SSL) in the phyletic evolution of lysozymes, SSL has aroused a flurry of interest (2-4). However, there has been no report about the production of SSL on an industrial scale.

In order to produce lysozyme on an industrial scale, the heterologous expression of different lysozymes has been attempted in a number of host organisms, including *Escherichia coli (E. coli)* (5-7), *Bacillus strains* (8), *Lactococcus lactis* (9), *Saccharomyces cerevisiae* (10, 11), *Pichia pastoris (P. pastoris)* (12), rice grain (13, 14) and transgenic goats (15). However, the expression level of this recombinant protein is too low for industrial application. Currently, *P. pastoris* is one of the most effective and versatile systems for the expression of heterologous proteins. Its success is due to its powerful methanol-inducible alcohol oxidase 1 (AOX1) promoter, capacity to perform post-translational modifications and pathways leading to recombinant products secretion (16). With the wide application of *P. pastoris*, numerous strategies enhancing protein expression level have also been developed, including the introduction of a high efficient transcriptional promoter (17), intercalation of an appropriate signal peptide in expression vector (18), the high gene copy strategy (19) and the optimization of fermentation (20, 21). However, these optimization procedures did not universally result in high protein production for every recombinant event as expected (22) and their final actual effect depended on each individual case.

Codon optimization is considered to be a promising technique for enhancing protein expression. Difference of codon usage between the native gene and expression host has been shown to have a significant impact on the expression of recombinant protein (23). An optimization of codon regions according to the codon bias of the host usually results in a significant increase of target protein secretion (23-25). There are two most common used codon optimization strategies in *E. coli,* namely "one amino acid one codon" and "codon randomization" (26). However, the former one has now seldom been reported in *P. pastoris*. In this study, SSL gene was optimized by these two strategies and expressed in X-33, and the expression levels were compared.

Materials and methods

Strains, plasmids, and growth conditions

E. coli JM109 (from our laboratory) was used for maintenance and manipulation of plasmids. *P. pastoris* X-33 (Invitrogen) was used as a host strain for secretion of SSL. The *E. coli* cells were grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0) at 37°C, and Zeocin (25 μg/mL) was

added, if necessary. The *P. pastoris* X-33 cells were grown in YPD medium (1% yeast extract, 2% tryptone, 2% glucose) at 28-30°C. The expression vector was pPICZαA.

Synthesis of the genes and construction of the expression vectors

The SSL mRNA-complete cds (NCBI-ID: 1174173) was synthesized for control with signal peptides removing. Meanwhile, the amino acid (AA) sequence of SSL (GenBank accession no. AAA86644.1) was backtranslated by "codon randomization" strategy and "one amino acid one codon". In "one amino acid one codon" method, the preferred codons found in the entire genome of *P. pastoris* were assigned to each amino acid (Table 1). The second strategy used for codon optimization consisted on randomly assigning a triplet for each amino acid using Table 1, with a probability based on the weight of each codon within the set encoding a given amino acid (26). By this algorithm, an optimized sequence was designed using the GeMS software package. The three genes were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). The synthesized products (firstly stored in vector pPICZA) were obtained by PCR using primers in Table 2. Then they were digested by *Kpn* I/*Not* I for ligating into pPICZαA, and the recombinant vectors were transformated into *E. coli* JM109. The numbered colony was picked by a pipette tip which was then dabbed into the master mix (25 μL of 5 pmol/L primer1, 5 pmol/L of primer 2 (Table 2), 2.5 μL 10 × PCR buffer and 0.5 μL dNTPs). The PCR reaction was initiated at 95°C for 4 min, followed by 30 amplification cycles (94°C, 30 s; 52°C, 30 s; 72°C, 30 s) and terminated with a 5 min extension at 72°C. Positive control with synthesized products as templates and negative control with distilled water as template were also carried out. Five microliter PCR product was loaded onto 1.0% agarose gel and separated at 110 V using the Bio-Rad electrophoresis system (Bio-Rad, America). PCR products were ligated into the pMD18-T vector, and transformed into *E. coli* JM109 cells by heat-pulse transformation. The antibiotic resistant transformants were selected for sequencing by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). The identified recombinant vectors were termed pPICZαA/SSL/Ori, pPICZαA/SSL/Opt/O and pPICZαA/SSL/Opt/R, respectively.

Table 1. Synonymous codon usage frequency of *P. pastoris.*

Amino acid	Codon	Frequency [a]	Amino acid	Codon	Frequency
Gly	**GG**G	5.76	Asn	**AA**T	25.07
	A	19.06		**C**	**26.67**
	T [b]	**25.52** [b]			
	C	8.06	Ile	**AT**A	11.14
				T	**31.14**
Glu	**GA**G	29.03		C	19.43
	A	**37.43**			
			Thr	**AC**G	6.04
Asp	**GAT**	**35.66**		A	13.75
	C	25.87		**T**	**22.39**
				C	14.45
Val	**GT**G	12.28			
	A	9.89	Cys	**TGT**	**7.70**
	T	**26.91**		C	4.38
	C	14.88			
			Tyr	**TA**T	15.99
Ala	**GC**G	3.86		**C**	**18.12**
	A	15.10			
	T	**28.92**	Leu	**TTG**	**31.51**
	C	16.58		A	15.56
				CTG	14.94
Arg	**AG**G	6.63		A	10.74
	A	**20.1**		T	15.85
	CGG	1.94		C	7.63
	A	4.18			
	T	6.94	Phe	**TTT**	**24.14**
	C	2.15		C	20.60
Ser	AGT	12.55	Gln	**CA**G	16.27
	C	7.64		**A**	**25.45**
	TCG	7.36			
	A	15.18	His	**CAT**	**11.81**
	T	**24.39**		C	9.07
	C	16.53			
			Pro	**CC**G	3.94
Lys	**AAG**	**33.8**		**A**	**18.94**
	A	29.93		T	15.77
				C	6.80

a. The frequency of synonymous codon is shown in times per 1000 codons, and synonymous codon frequency was compiled from 137 complete protein coding genes (81301 codons) of *P. pastoris.*

b. The most preferred synonymous codons and the frequency are indicated in bold.

Transformation into P. pastoris and positive recombinants selection

The plasmids pPICZαA/SSL/Ori, pPICZαA/SSL/Opt/O and pPICZαA/SSL/Opt/R were linearized with *Sac* I (NEB), and transformed into *P. pastoris* by electroporation using a Gene Pulser (Bio-Rad) apparatus, according to the manufacturer's instructions. The transformants were grown on YPDS-Z medium (1% yeast extract, 2% tryptone, 2% glucose, 1% sorbitol, 100 μg/mL Zeocin, 2% agar). Following a course of denaturation at 98°C for 10 minutes, all of the targets were identified with colony polymerase chain reaction, in which two other primers (RG-primers, in Table 2) were used. After the identification of colony PCR, the genomes of positive ones were extracted and used as templates for another PCR reaction identification, with the same primers used in the former step. Finally, the positive clones were confirmed via sequencing again by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China).

Assay of SSL activity

The SSL activity was assayed using turbidimetry method according to Minagawa's method (27) with a little modification. A suspension of *M. lysodeikticus* (0.2 mg/mL in 50 mM phosphate buffer, pH 6.2) was mixed with the samples to a final volume of 1.2 mL. The reaction was allowed to proceed at 25°C and the

Table 2. Primers used for PCR identification in this study.

Primers	Sequences
SSL-Ori-F	5′-GGGGTACCAAGGTTTACGATAGATGTGA-3′
SSL-Ori-2R	5′-AAGGAAAAAAGCGGCCGCCAACTTACAACCTCTAATGT-3′
SSL-Opt-O-F	5′-GGTACCAAGGTATACGACCGATGTGAATTCGC-3′
SSL-Opt-O-R	5′-GCGGCCGCTAATTTACAGCCTCTAATATATTGTGA-3′
SSL-Opt-R-F	5′-GGGGTACCAAGGTCTATGATCGGTGCGA-3′
SSL-Opt-R-R	5′-AAGGAAAAAAGCGGCCGCCCAGTTTGCAACCCCGAATG-3′
RG-primer-F	5′-TACTATTGCCAGCATTGCTGC-3′
RG-primer-R	5′-GCAAATGGCATTCTGACATCC-3′

absorbance at 450 nm was measured after 30 s and 4.5 min. A unit (U) of lysozyme activity was defined as the amount of sample causing a decrease in absorbance of 0.001 per min. All of the experiments were performed three times and the results were shown as mean value ± standard deviation.

The optimum acidity and temperature

The recombinant SSL was firstly purified by affinity chromatography His-trap 1mL according to its procedure. Simply, the broth of fermentation was centrifugeted and filtered by 0.45 μm membranes. 30 mL samples were loaded onto the equilibrated column by flow rate of 0.5 mL/min. After washing with 20 column volume wash buffer (20 mM sodium phosphate, 0.5 M NaCl, 100 mM imidazole, pH 7.4), 10 column volume elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4) was used for one-step procedure, with flow rate of 1 mL/min. Then the optimum pH of three recombinant SSL was measured with *M. lysodeikticus* in different buffers of various pH (3.5–9.0) at 25°C. The optimum temperature of the enzyme was measured with *M. lysodeikticus* in phosphate buffer of pH 6.0 at various temperatures (15–80°C). The pH stability of the enzyme was measured from residual activities after incubation in the buffer with various pH at optimum temperature for 30 min, and thermal stability was measured in buffers with optimum pH at various temperatures for 30 min. The residual activities of the enzyme were measured as mentioned before. The buffers used were: 0.1 M sodium acetate buffer (pH 3.5–5.5), 0.1 M sodium phosphate buffer (pH 6.0–8.0) and 0.1 M Tris–HCl buffer (pH 8.5–9.0). All of the experiments were performed in triplicates with a standard deviation of below 10%.

Screening of transformants for high-level expression of SSL

The transformants obtained were screened for their secretion ability of SSL with induction of methanol. The colonies were firstly cultured in 500 mL shaking flask containing 100 mL BMGY medium (1% yeast extract, 2% tryptone, 1% glycerol, 1.34% YNB, 4×10^{-5}% biotin, and 100 mmol/L potassium phosphate, pH 6.0) with shaking at 28°C and 225 rpm to reach OD_{600} 2-6. Then the cells were harvested by centrifugation and resuspended into 500 mL shaking flasks containing 50 mL BMMY medium (1% yeast extract, 2% tryptone, 1% methanol, 1.34% YNB, 4×10^{-5}% biotin, and 100 mmol/L potassium phosphate, pH 6.0). The induction expressions of the recombinant proteins were operated at 30°C of 250 rpm for 7 days, with methanol concentration 1% of total fermentation volume (V/V) and the enzyme activities of culture supernatants were assayed every 12 hours (h) during induction. The secreted proteins were assayed everyday by the enzyme activity assay and analyzed using SDS-PAGE. The protein concentration and proportion were assayed by Bradford method (28) and JD801 Gel imaging and analysis system (Jiangsu, China), respectively. At last, the clone with the highest SSL activity was selected for further studies.

Results

Synthesis of the original and optimized genes and construction of expression vectors

In comparison with online analysis (http://www.kazusa.or.jp/codon) of synonymous codon usage frequency of *P. pastoris* (Table 1), some amino acid residues in SSL gene were encoded by codons that are rarely represented in the host, namely CTC (Leu), TCG/AGC (Ser), GCG (Ala), CGG/C and AGG (Arg), all of which were less than 15% of usage percentage. Moreover, more than half of these rare codons usually exist in clusters of two to six consecutive codons, resulting in a much lower expression in *P. pastoris*. Thus, it was surmised that codon optimization of the SSL gene might result in an increase in protein expression.

Among the three genes, *Kpn* I and *Not* I sites were added in 5′ and 3′ end, respectively. Totally, the original SSL gene has a 44.36% G + C content, but the genes optimized by two methods have a 37.43% and 42.05% content, respectively. The synthetic complete genes

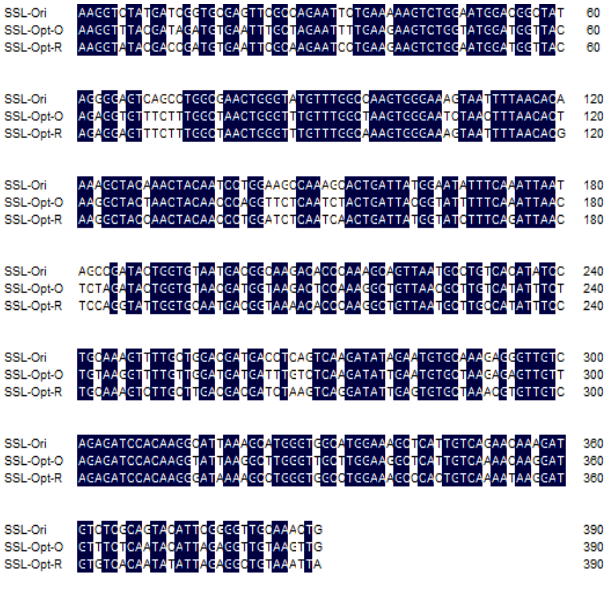

Figure 1. Alignment of nucleotide of the optimized and original SSL genes. Characters with shadow are the same nucleotides, and others are different ones.

(Fig. 1) were obtained by PCR and introduced into the expression vector pPICZαA where target DNA fragments were cloned into frame with the propeptide of the *Saccharomyces cerevisiae* mating α factor.
Colony PCR screening of the positive recombinants was performed using primers mentioned in Table 2. From sequence analysis, these DNA fragments in the recombinant vectors were in consistence with our designed ones and at the right position in an open reading frame coding for 130 amino acids.

Screening of transformants at shaking flask level

The identified recombinant vectors were then electroporated into the competent cell of *P. pastoris* X-33. Via colony PCR identification and genome PCR (Data were not shown), followed by sequencing, recombinnts of original SSL gene and optimized SSL genes were obtained. The SSL activity of the best recombinants of the original, "one amino acid one codon" optimized and "codon randomization" optimized SSL genes were 178.33±7.64, 153.33±10.41and 538.33±15.18 U/mL according to turbidimetry method at flask level (Fig. 2), respectively. In all three cases, fermentation was terminated at 144 h, and SSL activity was determined every 12 h. As shown in this work, the highest SSL activity level appears at 120 h after induction, and the targeted protein was approximately 75.06±0.25% , 74.56±0.14% and 79.00±0.14% of the total secreted protein (analyzed in Fig. 3 by JD801 Gel imaging and analysis system (Jiangsu, China)), respectively. The ability of *P. pastoris* to perform post-translational modifications including glycosylation and disulfide bond formation makes that target protein was the main part of secreted product, unlike in *E. coli*, from which the majority of protein is expressed in inclusion bodies (29).

SDS-PAGE of recombinant SSL

The SDS-PAGE of aliquots of the culture supernatant showed the recombinant protein stained with Coomassie brilliant blue (Fig. 3) was a size of 18 kDa assayed by JD801 Gel imaging and analysis system (Jiangsu, China). The value was about 4 kDa larger than 14.4 kDa, the calculated molecular weight of the nonglycosylated SSL. The expressed target product in *P. pastoris* accounted for 79.00±0.14% of the total secreted protein in the best recombinant, which was beneficial for latter purification.

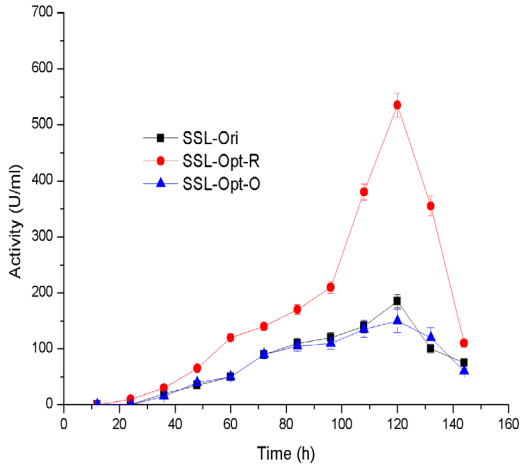

Figure 2. Fermentation and secretion curve of recombinant SSL from yeast. The recombinants were cultured at 30°C, 250 rpm, with an induction of 1% methanol. The culture supernatants were obtained by centrifugation with 12000×g for 5 minutes. Error bars indicate standard deviations.

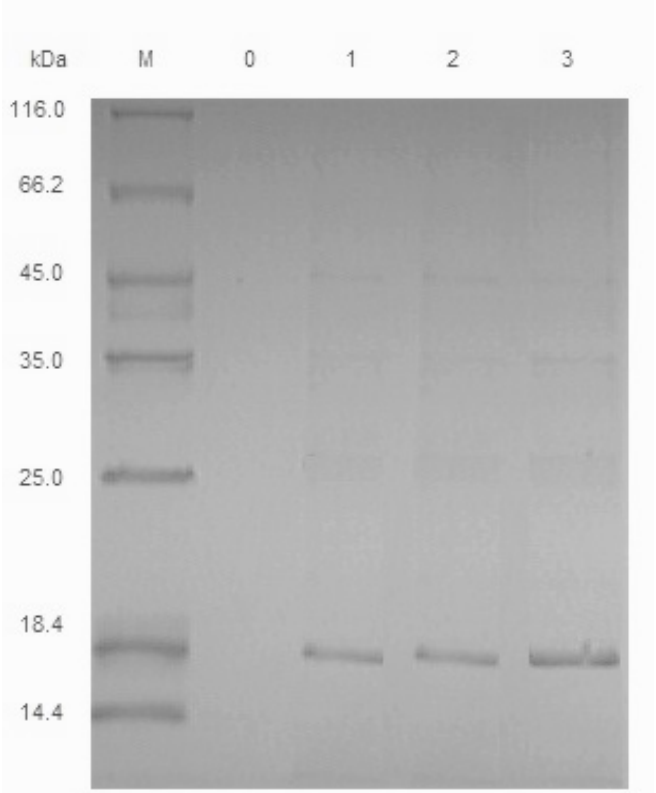

Figure 3. Coomassie brilliant blue R-250 (CBB)-stained SDS-PAGE gel. 15 microliters of culture supernatants, after centrifugation by 12000×g for 5 minutes, of different recombinants were loaded. Lane M, 0, 1, 2, 3 refers to Marker, X-33, recombinant of original SSL gene, recombinant of "one amino acid one codon"strategy optimized SSL gene and recombinant of "codon randomization"strategy optimized SSL gene, respectively.

Characterization of recombinant SSL

The protein concentration in fermentation supernatant of the best recombinants of the original, "one amino acid one codon" optimized and "codon randomization" optimized SSL genes were 89.97±2.90, 80.03±1.94 and 239.60±4.16 mg/L, respectively. As shown in Table 3, the corresponding specific activity of this recombinant enzyme were 2640.07±19.86, 2567.96±121.27 and 2845.38±124.19 U/mg, and the results were confirmed by the purified enzyme. With *Micrococcus lysodeikticus* used as substrate, the optimal acidity and temperature for recombinant SSL expressed in *P. pastoris* were approximately pH 6.0 and 35°C (Fig. 4 and 5), respectively. They were basically the same as that of natural SSL (2). After incubation at 50°C, 60°C, 70°C and 80°C for 30 min, the residual activity was 73.33%, 52.54%, 10.00% and 0.00% of corresponding initial activity (Fig. 4), respectively. The enzyme was stable between pH 5.0 and 6.5, retaining more than 60% of the highest activity. Moreover, it retained no less than 90% of the original activity after treatment at pH 4 or 9 for 30 min (Fig. 5). In addition, there was a trough at pH 7.0 to 8.0, indicating that electrostatic charge may not be the only factor affecting the bacteriolysis of this lysozyme, which was similar to that of egg white lysozyme (30).

Table 3. Comparison of the expression levels of original and optimized SSL genes.

Gene	Secreted protein (mg/L)	SSL activity (U/mL)	SSL in secreted protein (%)	Specific activity (U/mg)
SSL-Ori	89.97±2.90	178.33±7.64	75.06±0.25	2640.07±19.86
SSL-Opt-O	80.03±1.94^{**a}	153.33±10.41^{*b}	74.56±0.14*	2567.96±121.27
SSL-Opt-R	239.60±4.16**	538.33±15.18**	79.00±0.14**	2845.38±124.19*

a, $^{**}P$<0.01 vs. Control; b, $^{**}P$<0.05 vs. Control.

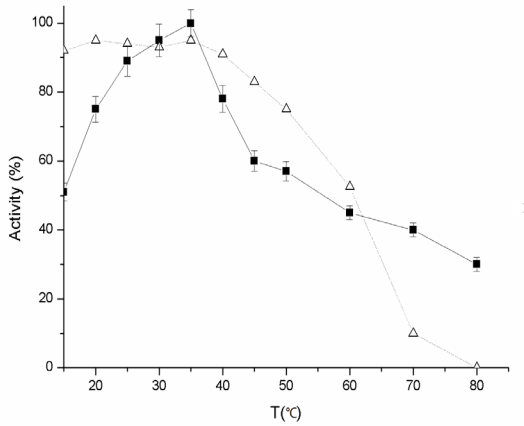

Figure 4. Effects of temperature on enzyme activity. Empty triangle, temperature stability; filled square, temperature optimum. Activity is shown as % activity and maximum activity was taken as 100%. Error bars indicate standard deviations.

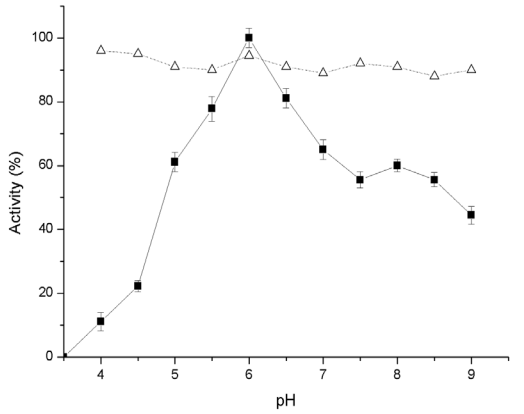

Figure 5. Effects of pH on enzyme activity. Empty triangle, pH stability; filled square, pH optimum. Activity is shown as % activity and maximum activity was taken as 100%. Error bars indicate standard deviations.

The consistence of characteristics among all recombinant SSL and the natural one suggests production of SSL on an industrial scale by fermentation be feasible and promising.

Discussion

Compared with the native enzyme gene expression in *P. pastoris* (in Table 3), the secreted SSL concentration of "codon randomization" optimized gene was approximately 239.60±4.16 mg/L, and the expression showed a 2.6 fold higher level (P<0.01). However, the first strategy had no obvious improvement in the expression level of SSL, even had a little decrease (81 mg/L, P<0.05).

As reported, rare codons can interrupt mRNA stability and reduce its translation rate (26), but most frequently used codons are able to prevent the potential depletion of tRNAs encoding the same amino acid (23). In addition, adjusting of the G + C may also contribute to the improvement of protein expression (24). In this study, when G + C content decreased from 44.36% to 42.05% ("codon randomization" strategy), a 2.6 higher expression level was obtained; however, when G + C content decreased to 37.43% ("one amino acid one codon" method), the expression level had a little decrease. As a matter of fact, the decrease of G + C content means an increase of A + T proportion and chances of A + T-rich stretches (e.g., 166-179 sites in Fig. 1), which is harmful for protein expression by causing fortuitous polyadenylation (31). These results were similar to that in *E. Coli* (26), suggesting that "codon randomization" strategy used in this study may be a better way than "one amino acid-one codon" method in codon optimization. It can be concluded that, in a successful codon optimization in *P. pastoris*, only taking codon preference into consideration may be not enough, and there are still some other factors (e.g., eliminating A + T-rich stretches) involved.

It was found that some extracellular activity of SSL secreted from the yeast cells decreased in the late period of fermentation after 120 h of induction (Fig. 2), probably due to proteolysis of endogenous proteases secreted by host *P. Pastoris* (23). Therefore, it is necessary to study the effect of protease inhibitors such as serine protease inhibitor, phenylmethylsulfonyl fluoride (31) and cation chelators on the production of recombinant SSL. Moreover, protease-deficient expression host may be a good candidate for further study.

The deviation in molecular weight of the recombinant protein from natural ones in SDS-PAGE might result from the basic amino acid residues of His-tag which might retard the mobility of the fusion protein bands (32). Moreover, many reports referred this deviation phenomenon to N-glycosylation of recombinant proteins, and recent studies have shown the key importance of attached carbohydrate chains by N-glycosylation and their vital role in the stability of glycoproteins (33). However, there are no potential glycosylation sites in the amino acid sequence of SSL, but two sites Asn-Ser-Thr-Asn-Asn and Asn-Thr-Thr are found in the signal peptide α-factor used in this study, according to the basis of the consensus sequence of protein glycosylation (34). Considering the molecular weight of α-factor, its imperfect removal from recombinant SSL when SSL was secreted to extracellular space was possible, with or without glycosylation. Furthermore, the optimum pH and temperature of recombinant SSL obtained by three genes were all 6.0 and 35°C (Fig. 4 and 5), respectively. They were basically the same as that of the natural one, and there were no obvious improvement in acid-resistance and thermostability, suggesting that no glycosyla-

tion seem to have occurred.

In this study, the effect of gene codon on various type of SSL was basically the same, except for the expression level, as shown in Figure 3, and the results were similar to that on β-1,3-1,4-glucanase (23). As a matter of fact, all three enzymes were investigated in the work, but for the same characters among the three enzymes, only one (original SSL) of them was shown in the Figure 4 and 5.

In summary, "codon randomization" strategy may be a better way than "one amino acid one codon" method in SSL gene expression, and some other factors (e.g., eliminating of A + T-rich stretches and avoiding of introduction of cryptic splice site.) besides codon preference should be also considered in a successful codon optimization. Given that the similar result was obtained in *E.coli* (26), "codon randomization" strategy seemed to be a better choice when an optimization is to be carried out, although the mechanism is still unknown (35). Take the same characters of enzymes encoded by different codon into consideration, codon optimization will certainly play an important role in their production in the future. Furthermore, to construct and choose new protease-deficient strain like SMD1168H as an alternative host might alleviate the problem of possible extracellular degradation of the secreted protein.

Acknowledgements

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