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-acetylglicosamine 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 has been shown to have a significant impact on the expression of recombinant products 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 \textit{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.

\textbf{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 was translated 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 \textit{Kpn I/Not I} for ligating into pPICZαA, and the recombinant vectors were transformed into \textit{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 \textit{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.

\textbf{Transformation into \textit{P. pastoris} and positive recombinants selection}

The plasmids pPICZαA/SSL/Ori, pPICZαA/SSL/Opt/O and pPICZαA/SSL/Opt/R were linearized with \textit{Sac I} (NEB), and transformed into \textit{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).

\textbf{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 \textit{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 optical density was read at 600 nm.
Table 2. Primers used for PCR identification in this study.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSL-Ori-F</td>
<td>5′-GGGGTACCAAGGTCTATGATCGGTGCGA-3′</td>
</tr>
<tr>
<td>SSL-Ori-2R</td>
<td>5′-AAAGAAAAAAGCCGGCCGAACCTACACGCTCATATGT-3′</td>
</tr>
<tr>
<td>SSL-Opt-O-F</td>
<td>5′-GGTACCAAGGTATACGACCGATGTGAATTCGC-3′</td>
</tr>
<tr>
<td>SSL-Opt-O-R</td>
<td>5′-GGGGTACCAAGGTTTACGATAGATGTGA-3′</td>
</tr>
<tr>
<td>SSL-Opt-R-F</td>
<td>5′-GCGGCCGCTAATTTACAGCTCTAATATATTGTGA-3′</td>
</tr>
<tr>
<td>SSL-Opt-R-R</td>
<td>5′-AAGGAAAAAAGCGGCCGCCAGTTTGCGACCCCAATGT-3′</td>
</tr>
<tr>
<td>RG-primer-F</td>
<td>5′-TACTATTTGCCAGATTTGCCTG-3′</td>
</tr>
<tr>
<td>RG-primer-R</td>
<td>5′-GCAAAATGGCATTCTGACATCC-3′</td>
</tr>
</tbody>
</table>

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

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^{-3} % 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^{-3} % 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

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 centrifuged 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%.
kDa, the calculated molecular weight of the nonglycosylated SSL. The expressed target product in \textit{P. pastoris} accounted for 79.00±0.14% of the total secreted protein in the best recombinant, which was beneficial for latter purification.

**Screening of transformants at shaking flask level**

The identified recombinant vectors were then electroporated into the competent cell of \textit{P. pastoris} X-33. Via colony PCR identification and genome PCR (Data were not shown), followed by sequencing, recombinants 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±10.41 and 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 \textit{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 \textit{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

![Figure 2](image2.png)

**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](image3.png)

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Secreted protein (mg/L)</th>
<th>SSL activity (U/mL)</th>
<th>SSL in secreted protein (%)</th>
<th>Specific activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSL-Ori</td>
<td>89.97±2.90</td>
<td>178.33±7.64</td>
<td>75.06±0.25</td>
<td>2640.07±19.86</td>
</tr>
<tr>
<td>SSL-Opt-O</td>
<td>80.03±1.94**</td>
<td>153.33±10.41*</td>
<td>74.56±0.14</td>
<td>2567.96±121.27</td>
</tr>
<tr>
<td>SSL-Opt-R</td>
<td>239.60±4.16**</td>
<td>538.33±15.18**</td>
<td>79.00±0.14**</td>
<td>2845.38±124.19*</td>
</tr>
</tbody>
</table>

a, **P<0.01 vs. Control; b, **P<0.05 vs. Control.

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 secreed 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 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 also be 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.

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