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High-Level Secretory Production of Recombinant E2-Spy Antigen Protein via Combined Strategy in *Pichia pastoris*

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Abstract: E2-Spy (abbreviated as ES) plays a vital role as a component in the Bacterial-Like Particles (BLPs) vaccine against classical swine fever virus (CSFV). This vaccine demonstrates remarkable immunoprotection, highlighting the importance of augmenting ES production in the development of CSFV subunit vaccines. In this study, a *Pichia pastoris* strain capable of high-yield secretory production of ES was developed through signal peptide engineering, gene dosage optimization and co-expression of molecular chaperones. Initially, a hybrid signal peptide cSP3 was engineered, leading to a 3.38-fold increase in ES production when compared to the control strain 1-α-ES. Subsequently, cSP3 was evaluated for its expression efficiency alongside different commonly used signal peptides under multicopy conditions. SDS-PAGE analysis revealed that 2-αd14-ES exhibited the highest ES production, displaying a 4.38-fold increase in comparison to 1-α-ES. Afterwards, SSA1, YDJ1, BIP, LHS1, and their combinations were integrated into 2-αd14-ES, resulting in a 1.92-fold rise in ES production compared to 2-αd14-ES (equivalent to a 6.18-fold increase compared to 1-α-ES). The final yield of ES was evaluated as 168.3 mg/L through comparison with serially diluted BSA protein bands.

Keywords: E2-Spy; hybrid signal peptide cSP3; gene dosage optimization; molecular chaperone

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1. Introduction

Classical swine fever (CSF) is a highly contagious disease in pigs, primarily caused by the classical swine fever virus (CSFV). The E2 protein is the main protective antigen [1,2]. In our previous study, a truncated form of E2 (E2-Spy, abbreviated as ES) was expressed in a secreted manner in *P. pastoris*. It was then displayed on the surface of Gram-positive enhancer matrix (GEM) particles using the SpyTag/SpyCatcher system. The resulting complex was referred to as Bacterial-Like Particles (BLPs). Animal experiments demonstrated that BLPs exhibited significantly enhanced immunogenicity compared to non-particulate ES, indicating great potential for application [3]. Hence, enhancing the production yield of recombinant ES antigens is presently a critical concern that necessitates attention.

Signal peptide optimization is a highly effective strategy for enhancing the production of target proteins. The nSB signal peptide demonstrated a threefold increase in CalB production compared to the commonly utilized α-factor signal peptide [4], while the α-factor Δ57-70 (abbreviated as αd14) signal peptide resulted in a 1.59-fold increase in HRP production [5]. A typical secretory signal peptide comprises a pre-region and a pro-region, where the pre-region dictates the translocation pathway of the precursor protein into the endoplasmic reticulum (ER), and the pro-region mediates receptor-dependent packaging into ER-derived COPII transport vesicles [6,7]. The α-factor signal peptide guides precursor proteins into the ER through the post-translational translocation pathway [7,8]. In this process, the precursor protein may undergo folding and the formation of complex structures

before entering the ER [9]. Due to the narrow diameter of the translocation channel in the ER membrane, only proteins with an α-helical conformation or containing simple secondary structures can pass through [9]. ES possesses a typical disulfide bond structure. The six cysteine residues at the N-terminus form three pairs of nested disulfide bonds (Cys^{4}–Cys^{48}, Cys^{103}–Cys^{167}, Cys^{129}–Cys^{139}), resulting in a tightly folded conformation [1,10]. This conformation is unfavorable for ES to enter ER for subsequent processing.

The alternative translocation pathway is co-translational translocation. Nascent polypeptide chains enter the ER in a "linear" form and fold into their native conformations within the organelle [11]. This mode of protein translocation, where synthesis and transmembrane transport occur simultaneously, prevents premature folding of the precursor protein and facilitates the secretion of the target protein [12–14]. Literature reports have demonstrated that both the chicken lysozyme signal peptide (pre-cSIG) [15] and the *Saccharomyces cerevisiae* Oligosaccharyltransferase 1 signal peptide (pre-Ost1) [16] can guide precursor proteins into the ER through the co-translational translocation pathway. The use of the hybrid signal peptide pre-Ost1-pro-α-factor resulted in a 20-fold increase in the yield of tetrameric red fluorescent protein E2-Crimson in the fermentation broth, and a 10-fold elevation in the production of lipase BTL2 [7]. In our previous study, the intracellular yield of ES was increased 17.87-fold (as determined by Western blot) using the cSIG signal peptide. Unfortunately, ES was not secreted into the culture medium. These observations confirm that precursor proteins prone to spontaneous folding exhibit higher efficiency in transmembrane transport through the co-translational translocation pathway compared to the post-translational translocation pathway.

Enhancing protein secretion through optimization of gene dosage and co-expression of molecular chaperones presents a viable strategy. Intracellular expression often exhibits a strong linear correlation between the yield of the target protein and the gene copy number. For example, a strain with eight gene copies led to an 18.06-fold increase in the yield of HBsAg [17], while a strain with fourteen gene copies resulted in a more than 30-fold increase in the yield of tetanus toxin fragment C [18]. In the context of secretory expression of recombinant proteins, increasing gene dosage typically results in an initial increase in protein production followed by a decline, and the gene copy number associated with the strain showing the highest production is termed the optimal copy number [19]. It is hypothesized that the overexpression of precursor proteins may consume a substantial amount of cellular resources, such as molecular chaperones, and impose stress on the yeast cells [20]. Co-expressing molecular chaperones can mitigate cellular stress and restore homeostasis. For instance, PDI is responsible for the isomerization of disulfide bonds during peptide chain folding and also functions independently as a molecular chaperone to aid protein folding [21]. The co-expression of Hsp70s (SSA1, BIP), Hsp40s (YDJ1), and NEFs (SNL1, LHS1) may enhance the translocation competence of the recombinant protein [22]. Additionally, SSO1 facilitates the secretion of target proteins from the Golgi to the extracellular space [23].

In this study, a three-step approach was employed to enhance the production of ES (as depicted in Figure 1). The approach was validated through SDS-PAGE and semi-quantitative analysis since protein quantification methods, such as indirect ELISA, were not available. Initially, various signal peptides were fused to the N-terminus of ES, and their pro-region sequences were combined with pre-cSIG to generate hybrid signal peptides capable of effectively directing the secretory expression of ES. The performance of the highly efficient hybrid signal peptide was also compared with other well-known signal peptides. Subsequently, the yields of different signal peptide-ES combinations were evaluated under varied copy number conditions to determine the optimal combination of signal peptide and copy number. Finally, molecular chaperones were co-expressed to enhance the folding and transportation of ES. This strategy is expected to provide valuable insights for future research on recombinant envelope glycoprotein expression.

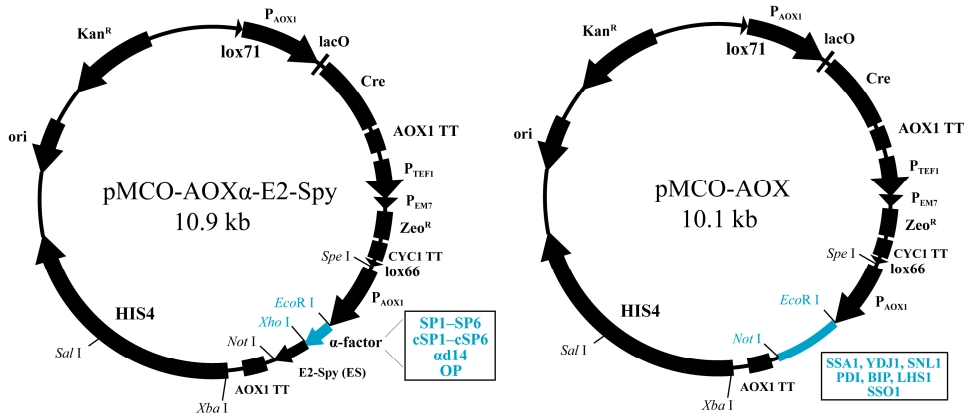

Figure 1. Schematic presentation of the optimized production of ES. The expression elements and partial multiple cloning sites were labeled.

2. Materials and Methods

2.1. Strains, Plasmids, and Culture Medium

P. pastoris strain GS115, *E. coli* strain DH5α were purchased from Invitrogen (Carlsbad, CA, USA). Plasmids pMCO-AOXα and pMCO-AOXα-E2-Spy were stocked in this lab. *E. coli* strains were cultured in LB medium (1% (*w*/*v*) NaCl, 1% (*w*/*v*) tryptone and 0.5% (*w*/*v*) yeast extract, pH 7.0). YPD medium (2% (*w*/*v*) peptone, 2% (*w*/*v*) dextrose, 1% (*w*/*v*) yeast extract) was used in *P. pastoris* culture, while YPDZ plates (YPD plus 100 μg/mL Zeocin) were used for the selection of positive *P. pastoris* transformants. The *P. pastoris* transformants were cultured in BMGY medium (2% (*w*/*v*) peptone, 1.34% (*w*/*v*) yeast nitrogen base, 1% (*w*/*v*) yeast extract, 1% (*w*/*v*) glycerol), and were transferred to BMMY medium (2% (*w*/*v*) peptone, 1.34% (*w*/*v*) yeast nitrogen base, 1% (*w*/*v*) yeast extract, 1% (*w*/*v*) methanol) for inducing expression of the target protein. All reagents were purchased from Sangon Biotech (Shanghai, China).

2.2. Construction of Recombinant Plasmids with Various Signal Peptides or Molecular Chaperones

The signal peptides used in this study, as detailed in Table 1, underwent codon optimization and were subsequently synthesized in vitro and subcloned into pMCO-AOXα-E2-Spy via *Eco*R I and *Xho* I, as depicted in Figure 1. The pro-region of these signal peptides (highlighted in blue in Table 1) has been analyzed and predicted as previously described [24]. The resulting plasmids were labeled as pMCO-αd14-ES, pMCO-OP-ES, and so forth. The primers shown in Supplementary Table S1 were utilized to amplify the molecular chaperone genes, which were integrated into pMCO-AOX via *Eco*R I and *Not* I, as depicted in Figure 1. The resulting plasmids, expressing intracellular molecular chaperones, were denoted as pMCO-SSA1, pMCO-YDJ1, and others.

Table 1. Signal peptides used in this study.

Abbreviated Name	Full Name	Sequence	Plasmid Name	Yeast Strain Name
α	α-factor	MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGVSLEKR	pMCO-α-ES	1-α-ES
αd14	α-factor Δ57-70	MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSASIAAKEEGVSLEKR	pMCO-αd14-ES	1-αd14-ES
OP	pre-Ost1-pro-α-factor	MRQVWFSWIVGLFLCFFNVSSAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGVSLEKR	pMCO-OP-ES	1-OP-ES
SP1	nSB	MKLLSLTGVAGVLATCVAATPLVKR	pMCO-SP1-ES	1-SP1-ES
SP2	254570357	MKLSTNLILAIAAASAVVSAAPVAPAEEAANHLHKR	pMCO-SP2-ES	1-SP2-ES
SP3	254565023	MINLNSFLILTVTLLSPALALPKNVLEEQQAKDDLAKR	pMCO-SP3-ES	1-SP3-ES
SP4	254572688	MKSQLIFMALASLVASAPLEHQQQHHKHEKR	pMCO-SP4-ES	1-SP4-ES
SP5	254570078	MKISALTACAVTLAGLAIAAPAPKPEDCTTTVQKRHQHKR	pMCO-SP5-ES	1-SP5-ES
SP6	254573224	MQLQYLAVLCALLLNVQSKNVVDFSRFGDAKISPDDTDLESRERKR	pMCO-SP6-ES	1-SP6-ES
cSP1	pre-cSIG-pro-SP1	MRSLLILVLCFLPLAALGKVATPLVKR	pMCO-cSP1-ES	1-cSP1-ES
cSP2	pre-cSIG-pro-SP2	MRSLLILVLCFLPLAALGKVAPVAPAEEAANHLHKR	pMCO-cSP2-ES	1-cSP2-ES
cSP3	pre-cSIG-pro-SP3	MRSLLILVLCFLPLAALGKVLPKNVLEEQQAKDDLAKR	pMCO-cSP3-ES	1-cSP3-ES
cSP4	pre-cSIG-pro-SP4	MRSLLILVLCFLPLAALGKVAPLEHQQQHHKHEKR	pMCO-cSP4-ES	1-cSP4-ES
cSP5	pre-cSIG-pro-SP5	MRSLLILVLCFLPLAALGKVAPAPKPEDCTTTVQKRHQHKR	pMCO-cSP5-ES	1-cSP5-ES
cSP6	pre-cSIG-pro-SP6	MRSLLILVLCFLPLAALGKVKNVVDFSRFGDAKISPDDTDLESRERKR	pMCO-cSP6-ES	1-cSP6-ES

SP1 to SP6 were predicted to contain pro-regions via in-silico analysis [4,24]. Pre-region of cSIG was colored in green and pro-regions of various signal peptides were colored in blue.

2.3. Generation of Yeast Recombinants and Shake Flask Culture

The plasmids for secretory expression of ES were linearized by *Sal* I and then transformed into *P. pastoris* GS115 by electroporation, and the resultant transformants harboring 1-copy target gene were designated as 1-αd14-ES, 1-OP-ES, etc. The plasmid pMCO-AOXα was also transformed into GS115 for generating a negative control (designated as NC). Positive transformants were selected on YPDZ plates and then cultured in 50 mL of BMGY liquid medium at 29 °C until the yeast cells reached the stationary phase. Afterward, the yeast cells were collected and resuspended in 50 mL of BMMY liquid medium. Additional 500 µL of methanol were supplemented every 24 h. After 96 h of induction, the supernatants of the culture medium were harvested by centrifugation.

2.4. Deglycosylation and Semi-Quantitative Analysis

The supernatant from the *P. pastoris* culture medium was directly treated by Endo H_f (New England Biolabs, Beverly, MA, USA) at 37 °C for 12 h, and then applied onto an SDS-PAGE gel stained with Coomassie Brilliant Blue. Subsequently, the gray intensity value of each positive band was measured using ImageJ 1.48v (National Institutes of Health, Bethesda, MD, USA).

2.5. RNA Extraction and Quantitative Real-Time PCR Analysis

The total RNA was extracted from the induced transformants using the Yeast Total RNA Isolation Kit (Sangon Biotech, Shanghai, China). The concentration of total RNA was quantified using the NanoDrop Spectrophotometer (ND-1000, Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized from 1 µg of RNA using the HiScript III RT SuperMix for qPCR Kit (Vazyme, Nanjing, China), and genomic DNA was simultaneously removed following the manufacturer's instructions. To compare the transcription levels of the target gene in different strains, real-time quantitative PCR (qPCR) was performed with the ChamQ Universal SYBR qPCR Master Mix kit (Vazyme, Nanjing, China). The mRNA levels of the target gene were analyzed using the $2^{-\Delta\Delta CT}$ method [25], and the mRNA level was normalized using the GAPDH (PAS_chr2-1_0437) gene as the endogenous control (housekeeping gene). The qPCR primers used for mRNA quantification are shown in Supplementary Table S1.

2.6. Construction of the Tandem Multicopy Expression Plasmids and Yeast Transformants

Plasmid pMCO-αd14-ES was digested via *Spe* I and *Xba* I to generate a 2.7 kb expression cassette. Simultaneously, pMCO-αd14-ES was digested by *Xba* I. These two fragments were then ligated using T4 ligase to form the plasmid pMCO-2-αd14-ES, which harbors two copies of the target genes. Similarly, plasmids like pMCO-4-αd14-ES were constructed using the same method. Subsequently, these plasmids were transformed into *P. pastoris* GS115 via electroporation, and the resulting transformants were designated as 2-αd14-ES, 4-αd14-ES, and so on. The copy numbers of the target genes in the multicopy transformants were confirmed using qPCR and the $2^{-\Delta\Delta CT}$ method, as described by Li et al. [26].

2.7. Obliteration of Resistance toward Zeocin for P. pastoris Transformants

Strain 2-αd14-ES was cultured in BMMY medium for 24 h to induce the expression of Cre recombinase, which promotes the genetic rearrangement of lox71-P_{AOX1}-lacO-Cre-AOX1TT-P_{TEF1}-P_{EM7}-Zeo^R-CYC1TT-lox66 fragment, as illustrated in Figure 1. The 24 h BMMY cultures were streaked onto YPD plates, and single colonies were then transferred to YPD and YPDZ plates and incubated at 28 °C overnight to isolate Zeocin-sensitive strains for the generation of electrocompetent cells.

2.8. Generation of Multicopy Recombinants Co-Expressing Molecular Chaperones

The plasmids containing various molecular chaperones, as listed in Supplementary Table S1, were linearized and subsequently introduced into Zeocin-sensitive 2-αd14-ES

electrocompetent cells. After confirming the production of the target protein, the expression cassettes of the identified positive molecular chaperones were integrated, leading to plasmids carrying either 2 or 4 expression cassettes of positive molecular chaperones. These resulting plasmids were then introduced into 2-αd14-ES cells, and the production of the target protein was evaluated. To assess the yield of the target protein in the optimized yeast strain, the protein bands were compared with serially diluted BSA.

2.9. Statistical Analysis

The statistical differences were assessed by one-way ANOVA and the *t*-test, with the statistical significance set at $p < 0.05$. The experiments were performed at least three times; and all the data are expressed as mean ± SD.

3. Results

3.1. Signal Peptide Optimization of ES

In the previous study, the recombinant ES protein exhibited smeared bands ranging from 35 to 70 kDa. However, these bands were found unsuitable for semi-quantitative analysis (Supplementary Figure S1). To address this issue, the ES protein was deglycosylated by treatment with Endo Hf, resulting in a distinct band at approximately 25 kDa and a minor band at 24 kDa. This observation suggests that the ES expressed in *P. pastoris* may undergo heterogeneous glycosylation modification, leading to differential deglycosylation efficiency. In this study, the culture supernatants were treated with Endo H_f prior to SDS-PAGE analysis without any concentration.

In order to identify potential pro-regions that can function in conjunction with the pre-cSIG, SP1 to SP6 were inserted between the AOX1 promoter and ES, and the expression of the target gene was evaluated. As illustrated in Figure 2, ES was detected in strains 1-SP1-ES to 1-SP6-ES, with consistent transcription levels of ES observed across these strains. Although the yields of ES with these signal peptides were lower than that of 1-α-ES, it is speculated that these signal peptides all contain functional pro-region sequences.

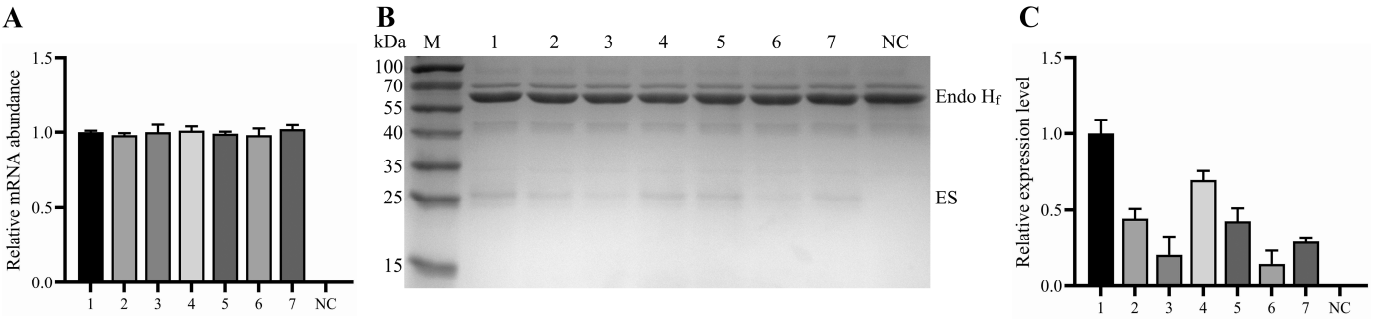

Figure 2. Expression analysis of ES with signal peptides SP1 to SP6. (**A**) Relative transcription level analysis. (**B**) SDS-PAGE analysis. (**C**) Semi-quantitative analysis. Lane 1, 1-α-ES; Lanes 2–7, 1-SP1-ES to 1-SP6-ES. NC: negative control. Equal volumes of each deglycosylated supernatant sample (20 μL) were loaded onto each lane of the SDS-PAGE gel. To facilitate comparison, the mRNA levels and ES expression levels were comparatively presented using relative quantification methods (normalized to the control strain 1-α-ES, Lane 1).

Following this, the pre-cSIG was fused with the pro-region of SP1 to SP6, the resultant signal peptides were designated as cSP1 to cSP6, respectively. Subsequently, the expression levels of ES were evaluated. As shown in Figure 3, ES was detected in strains 1-cSP2-ES to 1-cSP6-ES, with consistent transcription levels of ES observed across these strains. The use of cSP3 notably enhanced the expression of ES, resulting in a 3.38-fold increase in yield. In contrast, the yields of ES with other signal peptides were lower than the control, prompting their exclusion from further investigations.

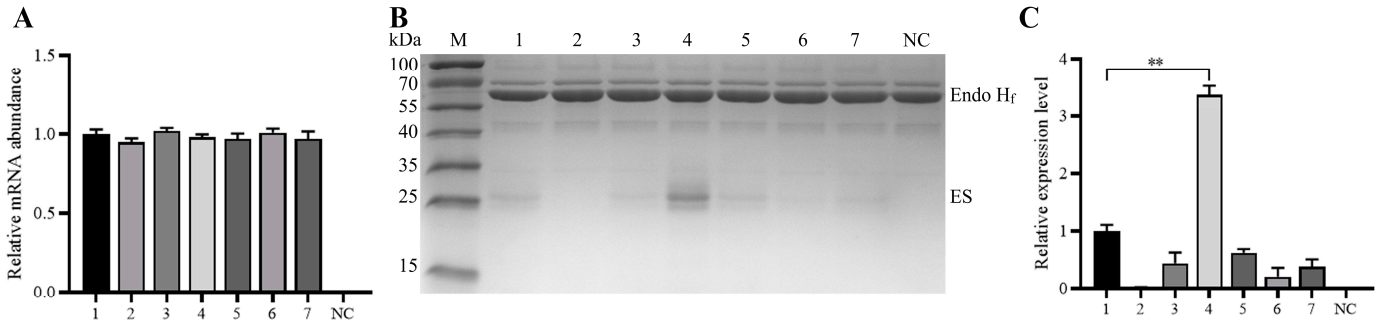

Figure 3. Expression analysis of ES with signal peptides cSP1 to cSP6. (**A**) Relative transcription level analysis. (**B**) SDS-PAGE analysis. (**C**) Semi-quantitative analysis. Lane 1, 1-α-ES; Lanes 2–7, 1-cSP1-ES to 1-cSP6-ES. NC: negative control. Equal volumes of each deglycosylated supernatant sample (20 μL) were loaded onto each lane of the SDS-PAGE gel. To facilitate comparison, the mRNA levels and ES expression levels were comparatively presented using relative quantification methods (normalized to the control strain 1-α-ES, Lane 1). ** $p < 0.01$.

3.2. Comparison of cSP3 with Other Commonly Used Signal Peptides

The signal peptides αd14 and OP, targeting the post-translational and co-translational translocation pathways, are commonly utilized in recombinant expression assays. They were employed to compare the expression efficiency of cSP3. Figure 4 illustrated that the transcription levels of ES were consistent across these strains, and the expression efficiency of OP is the lowest. The ES yield in strain 1-cSP3-ES significantly surpassed that of other strains, exhibiting a 3.75-fold increase compared to 1-α-ES and a 1.61-fold increase compared to 1-αd14-ES. However, it is uncertain whether the expression efficiency of cSP3 can exceed that of other signal peptides under multicopy conditions. Therefore, in vitro construction of multicopy plasmids containing different signal peptides was conducted.

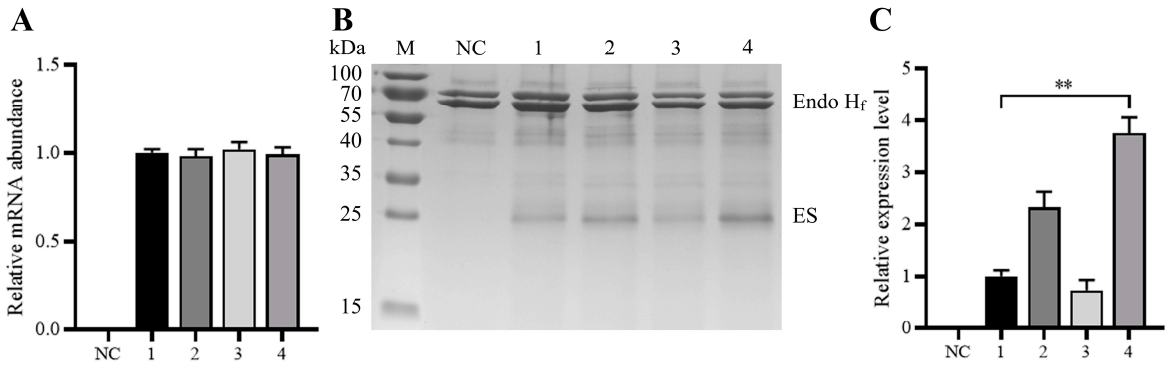

Figure 4. Comparison of cSP3 with other commonly used signal peptides. (**A**) Relative transcription level analysis. (**B**) SDS-PAGE analysis. (**C**) Semi-quantitative analysis. Lane 1, 1-α-ES; Lane 2, 1-αd14-ES; Lane 3, 1-OP-ES; Lane 4, 1-cSP3-ES. NC: negative control. Equal volumes of each deglycosylated supernatant sample (20 μL) were loaded onto each lane of the SDS-PAGE gel. To facilitate comparison, the mRNA levels and ES expression levels were comparatively presented using relative quantification methods (normalized to the control strain 1-α-ES, Lane 1). ** $p < 0.01$.

3.3. Construction of the Tandem Multicopy Expression Plasmids

The isocaudamer method, as previously described [27], was employed to generate multicopy plasmids for α-ES, αd14-ES, OP-ES, and cSP3-ES, as illustrated in Figure S2A. To validate the proper integration of expression cassettes, the recombinant multicopy plasmids were subjected to digestion with *Spe* I and *Xba* I. Subsequent gel electrophoresis analysis revealed a gradual increase in molecular weight related to the inserted expression cassettes, while the molecular weight of the vector frame remained approximately 8.5 kb

(as indicated by the arrow in Figure S2B). This result confirmed the precise construction of the multicopy plasmids.

3.4. Generation of Multicopy Transformants and Expression Identification

The linearized multicopy plasmids were transformed into *P. pastoris* GS115 and subjected to shake flask culture. The relative transcription levels of ES in the multicopy yeast strains were confirmed using qPCR and the $2^{-\Delta\Delta CT}$ method (demonstrated in Figures 5A and 6A). Interestingly, strain 2-cSP3-ES exhibited lower ES production compared to the other 2-copy strains, while the ES production of 2-α-ES and 2-OP-ES showed improvement (Figure 5B,C). Notably, the ES production of 2-αd14-ES was the highest, which was 4.38 times that of 1-α-ES.

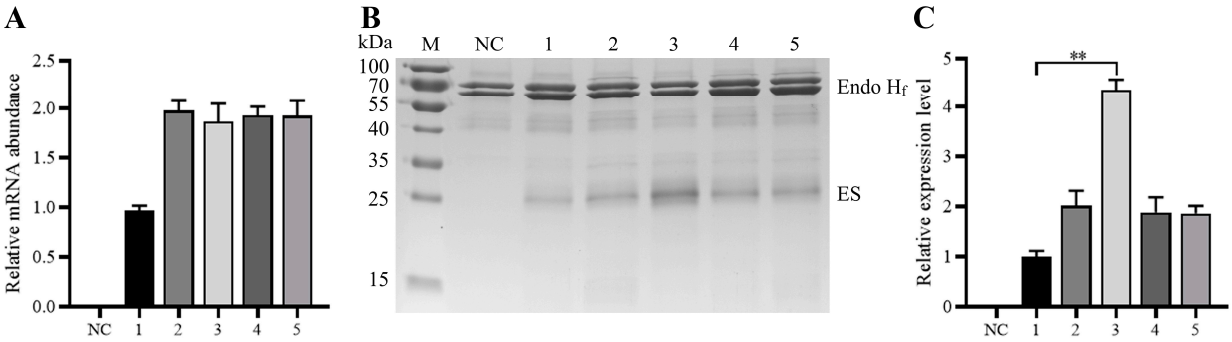

Figure 5. Comparison of ES production for 2-copy strains. (**A**) Relative transcription level analysis. (**B**) SDS-PAGE analysis. (**C**) Semi-quantitative analysis. Lane 1, 1-α-ES; Lane 2, 2-α-ES; Lane 3, 2-αd14-ES; Lane 4, 2-OP-ES; Lane 5, 2-cSP3-ES. NC: negative control. Equal volumes of each deglycosylated supernatant sample (20 µL) were loaded onto each lane of the SDS-PAGE gel. To facilitate comparison, the mRNA levels and ES expression levels were comparatively presented using relative quantification methods (normalized to the control strain 1-α-ES, Lane 1). ** $p < 0.01$.

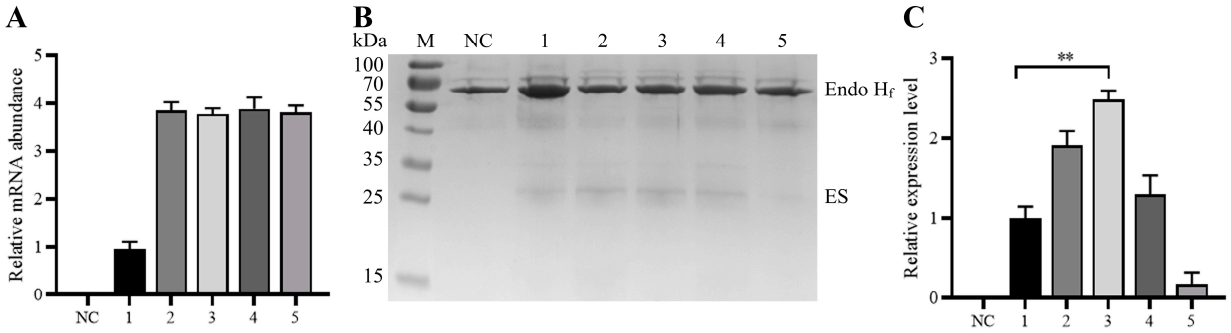

Figure 6. Comparison of ES production for 4-copy strains. (**A**) Relative transcription level analysis. (**B**) SDS-PAGE analysis. (**C**) Semi-quantitative analysis. Lane 1, 1-α-ES; Lane 2, 4-α-ES; Lane 3, 4-αd14-ES; Lane 4, 4-OP-ES; Lane 5, 4-cSP3-ES. NC: negative control. Equal volumes of each deglycosylated supernatant sample (20 µL) were loaded onto each lane of the SDS-PAGE gel. To facilitate comparison, the mRNA levels and ES expression levels were comparatively presented using relative quantification methods (normalized to the control strain 1-α-ES, Lane 1). ** $p < 0.01$.

In strains with a target gene copy number of 4, the yield of 4-cSP3-ES decreased to an undetectable level, whereas 4-αd14-ES exhibited the highest ES yield (Figure 6B,C). Additionally, the yield of 4-OP-ES decreased to 1.26-fold relative to 1-α-ES. Overall, the yield of ES in 2-αd14-ES was the highest among all strains, being 4.38-fold higher than the control strain 1-α-ES. Consequently, 2-αd14-ES was selected for molecular chaperone co-expression experiments.

3.5. Obliteration of Resistance toward Zeocin for P. pastoris Transformants

The pMCO plasmid and its derivatives contain specific expression elements that allow for the expression of the Cre recombinase under methanol induction conditions. Cre recombinase can induce genetic recombination, thereby obliterating the antibiotic resistance gene cassette located between lox71 and lox66. The single colonies of 2-αd14-ES in BMMY medium were isolated, and their sensitivity to Zeocin was assessed. Four Zeocin-sensitive colonies were randomly chosen and designated as 2-αd14-ES-S#5, 2-αd14-ES-S#11, 2-αd14-ES-S#17 and 2-αd14-ES-S#22, respectively. The transcription and expression levels of ES in the Zeocin-sensitive yeast strain were verified to be consistent with strain 2-αd14-ES, as demonstrated by qPCR and SDS-PAGE analyses (Figure 7). Consequently, the strain 2-αd14-ES-S#5 was selected for preparing competent cells.

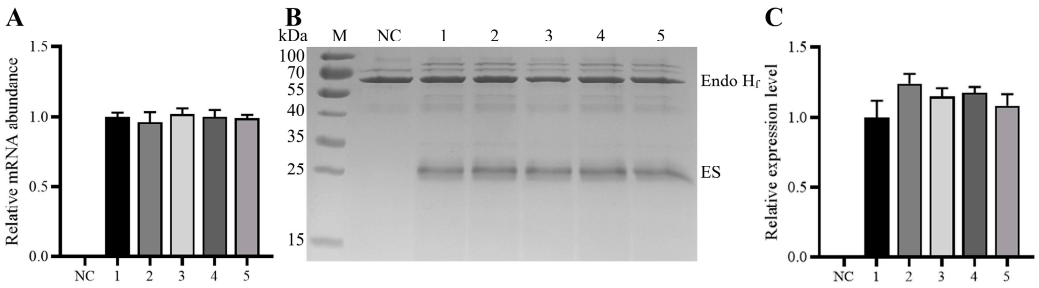

Figure 7. Validation of ES production for Zeocin-sensitive strains. (**A**) Relative transcription level analysis. (**B**) SDS-PAGE analysis. (**C**) Semi-quantitative analysis. Lane 1, 2-αd14-ES; Lane 2, 2-αd14-ES-S#5; Lane 3, 2-αd14-ES-S#11; Lane 4, 2-αd14-ES-S#17; Lane 5, 2-αd14-ES-S#22. NC: negative control. Equal volumes of each deglycosylated supernatant sample (20 μL) were loaded onto each lane of the SDS-PAGE gel. To facilitate comparison, the mRNA levels and ES expression levels were comparatively presented using relative quantification methods (normalized to the control strain 2-αd14-ES, Lane 1).

3.6. Co-Expression of a Single Molecular Chaperone in Multicopy Yeast Strains

Three cytoplasmic proteins (SSA1, YDJ1, SNL1), three lumenal ER proteins (PDI, BIP, LHS1), and one plasma membrane t-SNARE protein (SSO1) were selected. The expression plasmids corresponding to these molecular chaperones were integrated into the genome of the 2-αd14-ES-S#5 strain, resulting in yeast strains capable of co-expressing specific molecular chaperones. The mRNA levels of ES were primarily examined to ensure that changes in ES production in recombinant yeast strains were caused by the introduction of specific molecular chaperones, as depicted in Figure 8. The co-expression of SSA1, YDJ1, BIP, and LHS1 led to a significant increase in ES production compared to the control strain 2-αd14-ES. Notably, the ES yield of 2-αd14-ES-LHS1 was 1.34 times higher than that of 2-αd14-ES. The increase in ES production for 2-αd14-ES-SSA1, 2-αd14-ES-YDJ1, and 2-αd14-ES-BIP ranged from 1.11 to 1.29 times. In contrast, the co-expression of SNL1, PDI, and SSO1 led to a decrease in ES production compared to the control strain 2-αd14-ES, although these yields were still higher than that of the initial strain 1-α-ES.

3.7. Co-Expression of Molecular Chaperone Combinations in Multicopy Yeast Strains

To investigate the potential improvement in ES production by co-expression of SSA1, YDJ1, BIP, and LHS1, we examined the impact of co-expressing different combinations of these molecular chaperones: SSA1-YDJ1, BIP-LHS1, and SSA1-YDJ1-BIP-LHS1. As depicted in Figure 9, the mRNA levels of ES were confirmed to be consistent among strains co-expressing molecular chaperone combinations. Co-expression of SSA1-YDJ1 and BIP-LHS1 led to a 1.28-fold and 1.51-fold increase in ES production, respectively, compared to 2-αd14-ES (equivalent to a 4.11-fold and 4.86-fold increase compared to 1-α-ES). Further validation was performed to assess the effects of co-expressing SSA1-YDJ1-BIP-LHS1 on ES production. The results indicated a significant 1.92-fold increase in the ES yield of the

2-αd14-ES-SSA1-YDJ1-BIP-LHS1 strain compared to 2-αd14-ES, representing a 6.18-fold increase compared to 1-α-ES. Compared to a series of diluted BSA bands, the final ES yield of the 2-αd14-ES-SSA1-YDJ1-BIP-LHS1 strain reached 168.3 mg/L (Figure 10).

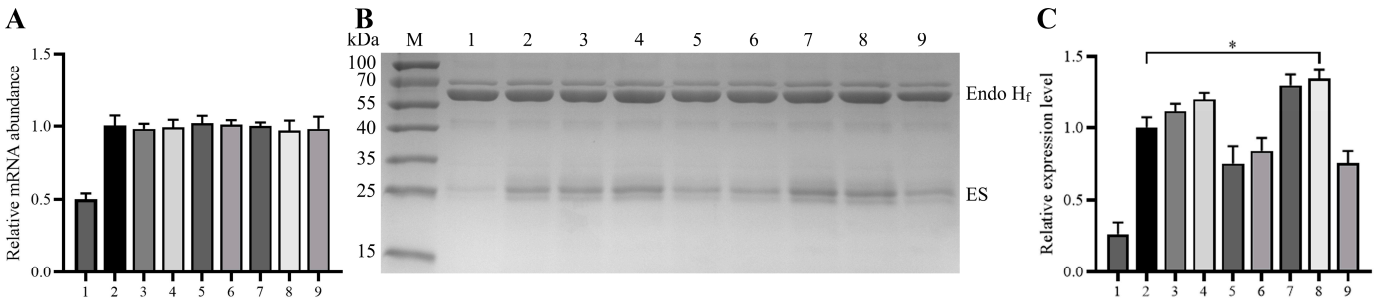

Figure 8. Validation of ES production for the strains co-expressing specific molecular chaperones. (**A**) Relative transcription level analysis. (**B**) SDS-PAGE analysis. (**C**) Semi-quantitative analysis. Lane 1, 1-α-ES; Lane 2, 2-αd14-ES; Lane 3, 2-αd14-ES-SSA1; Lane 4, 2-αd14-ES-YDJ1; Lane 5, 2-αd14-ES-SNL1; Lane 6, 2-αd14-ES-PDI; Lane 7, 2-αd14-ES-BIP; Lane 8, 2-αd14-ES-LHS1; Lane 9, 2-αd14-ES-SSO1. Equal volumes of each deglycosylated supernatant sample (20 μL) were loaded onto each lane of the SDS-PAGE gel. To facilitate comparison, the mRNA levels and ES expression levels were comparatively presented using relative quantification methods (normalized to the control strain 2-αd14-ES, Lane 2). * $p < 0.05$.

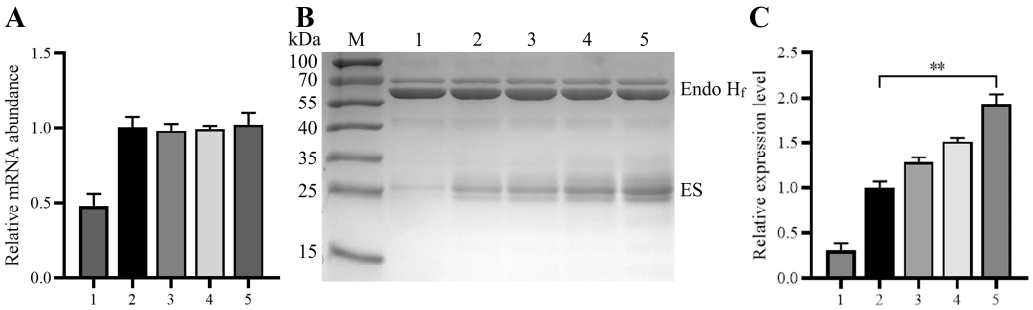

Figure 9. Validation of ES production for the strains co-expressing specific molecular chaperone combinations. (**A**) Relative transcription level analysis. (**B**) SDS-PAGE analysis. (**C**) Semi-quantitative analysis. Lane 1, 1-α-ES; Lane 2, 2-αd14-ES; Lane 3, 2-αd14-ES-SSA1-YDJ1; Lane 4, 2-αd14-ES-BIP-LHS1; Lane 5, 2-αd14-ES-SSA1-YDJ1-BIP-LHS1. Equal volumes of each deglycosylated supernatant sample (20 μL) were loaded onto each lane of the SDS-PAGE gel. To facilitate comparison, the mRNA levels and ES expression levels were comparatively presented using relative quantification methods (normalized to the control strain 2-αd14-ES, Lane 2). ** $p < 0.01$.

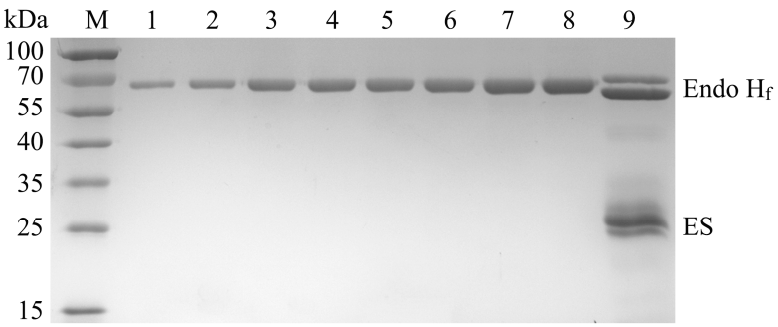

Figure 10. Final quantification of ES production. Lanes 1–8, 25, 50, 75, 100, 125, 150, 175, 200 mg/L BSA. Lane 9, deglycosylated ES producted by 2-αd14-ES-SSA1-YDJ1-BIP-LHS1 strain. Equal volumes of each sample (20 μL) were loaded onto each lane of the SDS-PAGE gel.

4. Discussion

The study aims to increase the yield of recombinant antigen ES. Both constitutive and induced expression pathways were considered during the experimental planning stages. Strangely, unlike the initial strain 1-α-ES in this study, yeast transformants expressing ES under the constitutive promoter P_{GAP} could not grow normally on YPDZ plates. This was supported by a 90% reduction in the quantity of positive transformants, and the limited number of positive transformants did not exhibit growth following the transfer to liquid YPDZ medium. In the constitutive expression strategy, cell growth and target protein expression occur simultaneously, so the presence of nested disulfide bonds in ES may cause a cellular stress response of yeast cells when overexpressed. Therefore, opting for the utilization of the inducible promoter P_{AOX1}, instead of constitutive promoters like P_{GAP} and P_{PMA1} [28], for controlling the expression of ES is a preferable option. This approach will allow yeast cells to attain a higher density under non-induced conditions before commencing the production of the target protein, ultimately resulting in a higher yield.

The cSP3 peptide exhibited superior performance in the initial selection of leader peptides but demonstrated decreased efficiency as the expression cassette copy number was increased. Explaining this phenomenon poses a particularly challenging issue. The review conducted by Delic et al. compared the differences in the secretion pathways among various yeast hosts and highlighted the lack of available information on the essentiality of the co-translational translocation pathway in *P. pastoris* [20]. Additionally, other studies have only mentioned that four proteins may utilize both co- and post-translational translocation pathways in *P. pastoris* [29], and that utilizing the pre-Ost1-pro-α-factor signal peptide can significantly enhance the production of target proteins through the co-translational translocation pathway targeting [7,30–33]. However, these successful cases have not confirmed that knocking out key components of the signal recognition particle (SRP) further reduces the yield of the target protein, thus failing to provide conclusive evidence of the effectiveness of the co-translational translocation pathway. Therefore, we propose several hypotheses to explain the observed phenomenon in this study.

First, the limited abundance of co-translational translocation-associated proteins within *P. pastoris* cells may lead to a scarcity or inefficient cycling of the SRP-related proteins when the target protein is overexpressed, resulting in decreased secretion efficiency. The second potential explanation could be that the co-translational translocation pathway of *P. pastoris* is not as efficient as in other eukaryotic hosts. *Yarrowia lipolytica* yeast exhibits a more developed co-translational translocation mechanism, indicated by a strong tendency for the nascent polypeptide to translocate to the ER lumen co-translationally. This was evidenced by approximately 75% of the translocation pores being co-fractionated with ribosomes [34], a much higher percentage compared to only 30% in *S. cerevisiae* [35]. In *Y. lipolytica* yeast, the inactivation of the two genes encoding the 7S RNA component of the SRP is lethal [36], while the deletion of the SRP54 and SEC65 genes results in a markedly slow growth rate [37]. Additionally, it has been demonstrated that the components of the SRP-dependent targeting are not indispensable for *S. cerevisiae* cell survival. Although the absence of any elements of SRP ribonucleoprotein led to slower growth and impaired protein translocation, the cells remained viable [38].

The third potential explanation for the observed phenomena may lie in the inadequacy of the current experimental design, wherein this study fails to investigate the influence of terminators on protein yield. Previous research on terminators has mainly concentrated on comparing termination strengths and examining the impact of secondary structures on termination efficiency [39,40]. The AOX1 terminator is generally recognized as the most widely used and strongest terminator in *P. pastoris*, commonly employed in recombinant expression experiments [39]. However, a recent study uncovered a phenomenon of SRP pre-recruitment [41]. Experimental evidence, along with previous studies, led to the conclusion that the terminators of PMP1 and PMP2 facilitated binding with the SRP even before the emergence of the secretion signal, indicating that SRPs can be pre-recruited independently of the signal [42]. It is important to note that the 3′UTR is not sufficient

for SRP-mediated targeting, as binding of the leader peptide to the SRP54 hydrophobic groove is important during the co-translational translocation process [41]. Furthermore, another study found that the combined use of a multicopy plasmid, Ost1-pro-MFα1 signal peptide, and PMP1 terminator increased the secretion level of CelA by 5-fold [30]. This data not only underscores the effectiveness of SRP pre-recruitment but also demonstrates that the use of multicopy and the co-translational translocation signal peptide Ost1 can synergistically enhance protein yield.

The engineering of strains with molecular chaperones often entails a trial-and-error approach that is highly specific to the considered protein [43]. The identification of expression bottlenecks for different target proteins is associated with changes in the protein yield and the functions of molecular chaperones that enhance yields [44]. Thus, methods that can universally enhance the production of target proteins hold significant promise and are highly relevant for reference. Staudacher et al. demonstrated a substantial increase in the expression of the target protein by overexpressing factors associated with the closed-loop conformation, a structure that improves stability and translation initiation rates. Furthermore, the co-expression of eIF4E, eIF4A, eIF4G, and PAB1 under the control of the GAP promoter resulted in a 2.5-fold increase in the target protein yield, concurrently enhancing global translational activity [45]. Moreover, a push-and-pull strategy has recently been utilized to improve the secretory expression of heterologous proteins [22]. Concurrently expressing Hsp70s, Hsp40s, and nucleotide exchange factors (NEFs) enhanced both the translocation competence and targeting of the recombinant protein to the ER membrane, facilitating protein transportation into the ER and subsequent folding in the ER lumen. Using this strategy, the yield of Fab increased by 4.77-fold, scFv by 2.71-fold, and carboxylesterase by 2.75-fold. It is important to note that the expression strengths of different molecular chaperones vary. Hsp70s and Hsp40s utilized very strong constitutive promoters like P_{GAP}, P_{GAPn}, and P_{MDH3}, while NEFs used a medium strong promoter, P_{POR1}. Earlier studies have found cellular abundances of approximately 336,941 for BIP, 2420 for SIL1, and 139 for LHS1 [46,47]. This suggests that uniformly regulating the expression of different molecular chaperones using the AOX1 promoter in this study may not be entirely appropriate. This could be an explanation for the decrease in ES production observed with the co-expression of SNL1, PDI, and SSO1.

In future research, would be valuable to investigate specific strategies aimed at enhancing the ES production in more effective ways. For instance, integrating plasmids into the yeast genome at specific sites, such as the nontranscribed intergenic spacer (NTS) in the rDNA repeat locus [48], may lead to higher transcription levels of ES proteins, as the *P. pastoris* genome contains around 16 copies of the rDNA repeat region [49]. Furthermore, the optimization of expression elements warrants further exploration. Prielhofer et al. have documented a promoter (P_{GTH1}) that enables the separate regulation of cell growth and heterologous protein expression by varying glucose concentrations in the culture medium, offering potential advantages for sustaining protein production under low oxygen and low nutrient conditions [50]. Utilizing mutated variants of the AOX1 promoter [51] to regulate the expression of molecular chaperones may offer advantages, as well as considering the use of the PMP1 terminator [30] to pre-recruit the SRPs in *P. pastoris*. It is also important to explore the use of additional molecular chaperones (e.g., eIF4G, PAB1) to increase the ES yield. Finally, further experimentation on the strategies proposed in this study is crucial to improving the expression of antigen proteins for various enveloped viruses, such as dengue virus [52] and hepatitis C virus [53], both of which have limited yields of the protective antigen E protein in *P. pastoris*. These studies will significantly advance the research and implementation of subunit vaccines for flaviviruses.

5. Conclusions

A hybrid signal peptide, cSP3, was constructed and employed to improve the production of ES, demonstrating superior expression efficiency compared to other signal peptides. Strain 2-αd14-ES showed the highest yield under multiple copy conditions, exhibiting

a 4.38-fold increase compared to the initial strain 1-α-ES. Furthermore, co-expression of molecular chaperones further increased the production of ES to 6.18-fold compared to 1-α-ES. Following multiple optimization steps, the final yield of ES reached 168.3 mg/L.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10020099/s1, Table S1: Primers used in this study; Figure S1: SDS-PAGE analysis of the deglycosylation for ES; Figure S2: Construction and identification of the multicopy plasmids.

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