Integrated Strategies for Enhancing the Expression of Chitosanase *Aq*CoA in the *Pichia Pastoris* by A Novel High-Copy Plasmid PMC-GAP

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Abstract

In our previous study, the chitosanase *Aq*CoA and its products chitooligosaccharides exhibited significant application in fungal disease protection. In this study, to enhance the expression of *Aq*CoA, we obtained various strains with multi-copy by a novel plasmid pMC-GAP with stable transformation ability in *Pichia pastoris* and built an integrated model combining the gene copy number, the chaperones and protein production of *Aq*CoA. In terms of gene dosage, the highest enzyme activity was 0.32 U/ml in the strain with four copies, which was 1.78-fold higher than that in strain with only one copy (0.18 U/ml). In addition, we found the chaperone like PDI, ERO1, HAC1, YDJ1, SSE1, SSA4 and SSO2 improved protein expression. Furthermore, the PDI/ERO1, SSA4/SSE1 and YDJ1/SSO2 pairs synergistically increased by 61%, 31% and 42% in expression levels of the strain GAP-1*Aq*CoA. Finally, we investigated the effect co-expression of gene copy and chaperones on protein expression. The maximum activity reached 2.319 U/ml by the strain with six chaperones intergrant plus sixteen copies, which was 13-fold higher than that by the control strain with only one copy (GAP-1AqCoA). Co-expression of gene dosage and chaperones significantly enhanced expression levels of *Aq*CoA, which presented a powerful tool to improve foreign protein expression.

Introduction

As the glycoside hydrolase (GH), chitosanase (EC3.2.1.132) catalyze the hydrolysis of the β-1,4-linked glycosidic bond in chitosan, forming the chitooligosaccharides (COSs) mixture (44). The COSs are commonly superior to chitosan polymers with the characteristics of water-soluble, less viscous and low molecular weight (25, 22). Furthermore, the COSs have many biological applications with its antimicrobial (54), anti-inflammatory (6), anti-oxidant (31), anti-photoaging (19), anti-tumor activities (35) and immuno-enhancer effects (33). In addition, the COSs are as significant immunity elicitors triggering plant resistance against fungal infection (52). Therefore, to facilitate the application of the chitosanase in production, establishment of the method used to maintain a high-level expression of protein is essential.

In the past few decades, the yeast *Pichia pastoris* has been widely used as host organism for heterologous protein production (8) with the advantages of rapid growth and carrying out the posttranslational modifications to most of proteins (42). However, yeast has limited ability to secrete proteins in its natural state. At present, many genetic methods utilized manipulated *Pichia pastoris* to obtain high yields of secreted proteins. In general, increasing the gene dosage to construct multi-copy strains is a common method (26). Furthermore, several high-throughput methods were established to screen many transformants with multi-copy integration. Scorer et al. (1994) found that a tight correlation was showed between the copy number and drug-resistance, and then proposed to utilize the high concentration of antibiotics to screen multi-copy strains (38). Moreover, multi-copy plasmids were constructed by the isocaudamer in vitro, like that Li et al (2009) created multiple *Pichia* clones carrying different copies of the gene expression cassette (23), however, the transformation into *P. pastoris* is inefficient as result of the constructed plasmids are very large by this way. In addition, Sunga et al. (2008) proposed the method of post-transformational vector amplifcation (PTVA), which mentioning that using

the antibiotic to generate the strains with different copies number (41). Strains that survived at higher antibiotic concentrations contained a higher number of intact copies of the gene of interest. In addition, the PTVA was widely employed in many studies, like that Zhu et al. (2009) adopted the method to obtained porcine insulin precursor with 52 copies (58), Rochelle et al. (2016) developed the liquid PTVA on the base of the PTVA (2). Except that, the PTVA also combined with other ways, such as Marx et al. (2009) established the method that combining the vector integration into the rDNA locus with the PTVA to produce strains with different gene copy number (27).

In the most cases, the copy number is determinant in promoting productivity of the recombinant protein; however, the improvement usually reaches a plateau with increasing gene dosage. Overexpression of heterologous proteins may bring about accumulation of misfolded protein (13), which triggering the endoplasmic reticulum (ER) stress and further activating the unfolded protein response (UPR) (49). Thus, overcoming the secretion block that caused by the ER stress may be crucial in enhancing foreign protein production. Recently, some measures, like overexpression of chaperones and foldase, were taken to overrule the obstacle of secretion. To improve the folding and secretion of proteins, the HAC1, as the unfolded-protein response (UPR) pathway regulator (57), was overexpression in the *P. pastoris*. For example, Li et al (2015) increased the yield of the phytase by 412% by the overexpressing HAC1 (21). However, the effect of Hac1p to heterologous protein expression needed to be evaluated on a case by case basis, like that Valkonen et al (2003) found that the HAC1 overexpression increased the α- amylase production by 70% but had no influence on the endoglucanase secretion (43). Furthermore, the chaperone PDI, involved in the disulfide bond formation in the ER, increased the capacity for processing proteins of ER in *P. pastoris* (11). Sha et al (2013) detected that the lipase r27RCL production in the strain with one copy chaperone gene PDI plus five copies was 2.74-fold higher than that in the control strain with only one copy (39). In addition, the Sso1 and Sso2, as the t-SNAREs protein that working at the targeting/fusion of the Golgi-derived secretory vesicles to the plasma membrane, were demonstrated to modulate the protein expression level (32). Ruohonen et al (1997) found the multiple copies of the SSO2 gene increased the secretion of invertase to the cell surface by 1.5 fold, which suggesting that the SSO gene exert the secretion-enhancing function in the process of the protein secretion (34).

In our previous work, the chitosanase *Aq*CoA from *Aquabacterium* sp. A7-Y was cloned and successfully expressed in *Escherichia coli* BL21 (DE3). Furthermore, the *Aq*CoA exhibited higher enzyme activity toward the chitosan and sodium carboxymethylcellulose (CMC) (47). However, the expression level of the *Aq*CoA is limited in *E.coli*, which cann't meet the application in the industrial production. Thus, in this study, we adopted expression system of *Pichia pastoris* to improve production of the *Aq*CoA. Furthermore, to elevate the yield of the *Aq*CoA, a novel high-copy yeast expression vector was constructed, which was based on the method for unmarked gene deletions proposed by Pan et al (2011) (30). In addition, the connection among gene copy number, the chaperones and production of *Aq*CoA was investigated.

Materials And Methods

Plasmid, strains, growth conditions

The plasmids used in this study are listed in Table S1. Standard cloning procedures were adopted in *E. coli* DH5α as described by Sambrook et al (36). Synthesis of oligonucleotides (listed in Table S2) and DNA sequencing were performed by Tsing Ke Biological Technology. *E. coli* DH5α cells used for DNA manipulations were cultivated in LB (1% peptone, 0.5% yeast extract, 1% NaCl) or low-salt LB medium (1% peptone, 0.5% yeast extract, 0.5% NaCl). Bacterial plasmid selection and maintenance were performed using 100 mg/l kanamycin or 25 mg/l zeocin. *P. pastoris* strains were cultured at 30℃ in YPD medium (1% yeast extract, 2% peptone, and 2% glucose). Transformants of *P. pastoris* were selected on YPDZ plates (1 % yeast extract, 2 % peptone, 2 % glucose, 2 % agar, and 100 mg/l zeocin) or BMGY (1 % yeast extract, 2 % peptone, 1% glycerol, 1.34 % YNB, 4×10^{-5}% biotin, 0.1M PBS and 2 % agar) or YPDZ-CMC plates (1 % yeast extract, 2 % peptone, 2 % glucose, 0.5% CMC, 2 % agar, and 100 mg/l zeocin) .

The construction of recombinant plasmid

First, seven multiple cloning sites (MCS) synthesized was integrated to the plasmid pUC52-kan. Next, some insertion elements, like His4 site, the cassette containing the AOX tanscription termination (AOX TT), the Zeo^r selectable marker (ZeocinR cassette) and lox66 sites, the gene *cre* (CRE) and promoter AOX1 (pAOX1), were amplificated by the primes in the table S2, and then PCR products were purified using the AxyPrep™ DNA Gel Extraction Kit (Axygen). At last, a new expression plasmid was constructed by inserting different insertion elements into the parent plasmid pUC52-Kan in turn. Detailed steps of plasmid construction were presented in the figure S1-S3.

The expression of the chitosanase *Aq*CoA in *Pichia pastoris*

The chitosanase *Aq*CoA gene of *Aquabacterium* sp. strain A7-Y, *Aq*coa [GenBank: MW 340224], was amplified from the vector pET29A-*Aq*coa (47) reported with the forward primer CCGCTCGAGAAAAGAGAGGCTGAAGCTGCCTGCGGCGGCGGCAGCGGCA and reverse primer CGGAATTCTCAGTGGTGGTGGTGGTGGTGGCGGCCTTTGCGGATCTGGTC.

Then, the PCR products of the chitosanase *Aq*CoA and the plasmid pMC-GAP were digested respectively with Xho I and EcoR I and then ligated to generate the plasmid pMC-GAP-*Aq*CoA by the T4 ligase. The transformation of *P. pastoris* with pMC-GAP-*Aq*CoA and the selection of transformants with pMC-GAP-*Aq*CoA were performed as described by Niu et al (29).

The transformants obtained were cultured overnight on the YPGZ plate at 30℃. Then single transformant selected was transferred to liquid mudium containing 15ml YPD and cultured at 30℃, 200rpm for 24 hours. After centrifugation, the cells harvested were transferred to 100 ml of BMGY medium and cultured at 30˚C, 220 rpm for four days. Lastly, crude enzyme was obtained by

centrifugation at 12,000×g at 4˚C for 10 min to remove yeast cells. The purification of recombinant protein and determination of content were executed as described by Li et al (24).

The enzymatic activity of the chitosanase *Aq*CoA

The activity of chitosanase *Aq*CoA toward 95% deacetylated chitosan was detected as presented by Wang et al (47). In addition, the activity of chitosanase *Aq*CoA against CMC was tested in this study. In brief, the transformants obtained were cultured overnight on the YPDZ and YPDZ-CMC plate, respectively. Then, YPGZ-CMC plates were stained with 0.1% Congo red solution at room temperature for 30 min, and decolorized with 1M NaCl for 10 min. The enzyme activity was determined by the size of the hydrolysis circle.

Immunodetection of protein expression levels

For western blotting, the crude enzymes from different strains with multi-copy were prepared. The expression level of strains with multi-copy was estimated by the methods described by Zhang et al (2006) (56). Briefly, the same volume supernatants were first separated by SDS-PAGE. Then, protein was transferred to nitrocellulose membranes, and nonspecific binding was blocked by incubating the membrane in solution A (10 mM Tris-HCl pH 8.0, 0.9% NaCl, 5% skim milk powder) overnight at 4 °C. The blocked membrane was then incubated with His antibody (10 mM Tris-HCl pH 7.4, 0.9% NaCl, 2% skim milk powder, 0.05% Tween-20, antibody 1:1000) for 2-3 h at room temperature. Goat anti-rabbit secondary antibody conjugated to horseradish peroxidase was used for the *Aq*CoA detection. Antibody-antigen complexes were visualized with ECL Advance Western Blotting Detection Kit. (Amersham Biosciences, Buchinghamshire, England)

Determination of the *Aq*coa copy number by quantitative PCR

The copy number of the *Aq*coa was studied according the method described by Li et al (2015) (21). In short, the GAPDH (48) gene with the forward primer GCTATCACTGTCGGTATTAACGG and reverse primer TGGGTAGAGTCGTACTTGAACAT was used as the reference sequence, the *Aq*coa was detected with the forward primer CACTTCTACGCGGCCTCTCA and reverse primer TGTTGAGCAGGTCGATGTAGG. The gene copy number of the *Aq*coa in the *P. pastoris* was the ratio of *Aq*coa gene copy number calculated by standard curve to the GPADH fragment copy number calculated by standard curve.

Result

The construction of the high-copy plasmid with stable transformation stability

According to the steps in the figure S1-S3, the recombined plasmids P1 and P2 were constructed successfully. However, in the process of verifying whether the plasmid P3 (figure S3) is successfully constructed, the target bands (3572 bp) between the two sites lox71 and lox66 in the plasmid P3 were not obtained (figure S4 (a)). Subsequently, the plasmid of the transformant 2 in the figure S4(a) was extracted and sequenced. The result showed that a new site lox72 replaced the sequence between the two sites lox71 and lox66 in the transformant 2 (figure S4 (b)), which suggested that leaky expression of AOX promoters in the *E. coli* drove the translation of the *cre* gene, resulting the recombination of sequence between lox71 and lox66 sites in the plasmid P3.

To avoid the leaky expression of AOX promoters in *E. coli* cell, the plasmid P4 was generated by inserting the operon lactose (*lac*O) gene between pAOX and CRE of the plasmid P3 (Fig. 1). Moreover, as shown in the Fig. 2a, the target bands (3572 bp) were amplified by PCR in the transformants 1 and 4. Meanwhile, the circular plasmid (8741 bp) extracted in the transformant 1 was consistent with the theoretical size (5827 bp) in nucleic acid gel (Fig. 2b), which suggested that the operon *lac*O controlled rigorously the expression of *cre* in *E. coli.*

To further verify the function of the plasmid P4, the plasmid P4 linearized by *Sal* I was transferred to *P. pastoris*. Then, five transformants selected were cultured and induced by methanol. After 24h, each strain was separately inoculated on two plates YPG and YPGZ for overnight culture at 30℃. As shown in the Fig. 2c, the colonies treated by methanol grew only on YPD plates, while they did not grow on the YPDZ containing bleomycin, which indicating that the resistance of the strain to bleomycin was eliminated after the inducement of methanol, the plasmid P4 was successfully constructed. In addition, to promote the expression of target genes, promoter GAP and secretory factor α-factor were inserted into the plasmid P4, meanwhile, the restriction sites *Xho I* and *Not* I between CRE cassette and ZeocinR cassette were deleted by overlapping extended PCR. At last, the plasmid pMC-GAP was constructed successfully based on the plasmid 4 (Fig. 3).

Heterologous expression of the chitosanase Aq**CoA in** P. pastoris

In our previous study, the chitosanase *Aq*CoA was heterologously expressed in *E. coli*. To improve the expression level of the *Aq*CoA and reduce the cost of postprocessing, the chitosanase *Aq*CoA was heterologously expressed in *P. pastoris*. Our previous study found the chitosanase *Aq*CoA was able to hydrolyze sodium carboxymethylcellulose (CMC), thus, the strain GAP-1*Aq*CoA was cultured on YPDZ plate containing CMC. As shown in Fig. 4(a), the hydrolytic circle was generated on the YPDZ plate containing CMC. Then, the activity of ten transformants selected in the liquid culture to 95% deacetylated chitosan was detected. As shown in the Fig. 4(b), compared to others, the transformant 3 showed the highest enzyme activity that reached to 0.18 U/ml (Fig. 4(b)).

Effect of different gene doses on expression of the AqCoA

To construct the plasmid pMC-GAP-2*Aq*CoA, the pMC-GAP-1*Aq*CoA was divided into two parts. One part was digested with two restriction enzymes Spe I and Xba I to produce a cassette with single copy (1 *Aq*CoA). The other part was cut with single enzyme Xba I and dephosphorized with alkaline phosphatase to prevent the vector from self-linking. The pMC-GAP-2*Aq*CoA was generated by linking the dephosphorization product with 1 *Aq*CoA cassette. The method was also applied in the construction of the plasmid pMC-GAP-4*Aq*CoA and pMC-GAP-8*Aq*CoA. The recombinant plasmids (pMC-GAP-2*Aq*CoA, pMC-GAP-4*Aq*CoA and pMC-GAP-8*Aq*CoA) were linearized and transferred into the *P. pastoris*. The transformants were selected according to the size of the hydrolysis circle to culture in vitro and activity detection. Similarly, the transformants containing pMC-GAP-3*Aq*CoA, pMC-GAP-5*Aq*CoA and pMC- GAP-5*Aq*CoA were obtained and named as GAP-3*Aq*CoA, GAP-5*Aq*CoA, GAP-7*Aq*CoA, respectively. As shown in the Fig. 4(c), activity of the *Aq*CoA against 95% deacetylated chitosan was gradually enhanced with increase of the *Aq*CoA cope number. Among them, the GAP-4*Aq*CoA exhibited the highest enzyme activity (0.32 U/ml). However, the enzyme activity began to decline as the copy number of the *Aq*CoA exceeded four.

Effects of different molecular chaperones on expression of the AqCoA

First, different molecular chaperones genes, such as PDI, ERO1, BIP, HAC1 and SSA1 that derived from *P. pastoris* and YJD1, SSA4, SSE1, SSO1 and SSO2 that originated from *S. cerevisiae*, were amplified by the primes in the table S4. Then, the amplified molecular chaperones genes were linked to the plasmid pMC-GAP linearized by *EcoR I* and *Not I* to form the recombinant plasmids. The localization and function of different molecular chaperones were shown in Table S3. The recombinant plasmids linearized were transferred to the strain GAP-1 *Aq*CoA or GAP-4 *Aq*CoA, respectively, the transformants obtained were named as GAP-1*Aq*CoA-PDI/ERO1/BIP/HAC1/SSA1/YJD1/SSA4/SSE1/SSO/SSO2 or GAP-4 *Aq*CoA-PDI/ERO1/BIP/HAC1/SSA1/YJD1/SSA4/SSE1/SSO/SSO2.

As shown in the Fig. 5, the molecular chaperones PDI, ERO1 and HAC1 from *P. pastoris* and the molecular chaperones YDJ1, SSA4, SSE1 and SSO2 from *S. cerevisiae* enhanced the expression of the *Aq*CoA. In the GAP-1*Aq*CoA, the enzyme of *Aq*CoA against 95% deacetylated chitosan was increased 31%, 16%, 17% and 21%, 15%, 24%, 11% by the molecular chaperone PDI, ERO1, HAC1, YDJ1, SSA4, SSE1 and SSO2, respectively. While the enzyme activity was separately enhanced 80%, 30%, 38%, 31%, 21%, 36% and 21% in the GAP-4*Aq*CoA, which indicating that the gene dose and molecular chaperones showed synergistic effects on the improvement of the *Aq*CoA expression. In addition, other molecular chaperones like BIP, SSA1 and SSO1 had no influence on the *Aq*CoA expression.

Co-expression of the molecular chaperones and copy numbers enhanced expression of the AqCoA

By screening molecular chaperones (PDI, ERO1, YJD1, SSA4, SSE1 and SSO2) that promoted the expression of the *Aq*CoA, the various expression boxes of combinations of two chaperones were established. The plasmids containing various expression boxes were constructed and named as pMC-

GAP-PDI-ERO1, pMC-GAP-SSA4-SSE1 and pMC-GAP-YDJ1-SSO2, respectively. Then, the recombinant plasmids linearized by *Nhe I* were transferred to GAP-1*Aq*CoA or GAP-4*Aq*CoA, respectively, the transformants obtained were named as GAP-1*Aq*CoA-PDI-ERO1, GAP-1*Aq*CoA-SSA4-SSE1, GAP-1*Aq*CoA-YDJ1-SSO2, GAP-4*Aq*CoA-PDI-ERO1, GAP-4*Aq*CoA-SSA4-SSE1 and GAP-4*Aq*CoA-YDJ1-SSO2, respectively. As shown in the Fig. 6(a) and 6(b), PDI/ERO1, SSA4/SSE1 and YDJ1/SSO2 pairs markedly improved the expression of the *Aq*CoA. Furthermore, in the strain GAP-1*Aq*CoA, enzyme activity was increased 61%, 31% and 42% by the expression boxes PDI-ERO1, SSA4-SSE1 and YDJ1-SSO2, respectively (Fig. 6(a)). While the enzyme activity was separately enhanced 110%, 53% and 71% in the GAP-4*Aq*CoA (Fig. 6(b)), which indicating that two integrated chaperones were capable of removing the secretory bottleneck more efficiently than the single chaperone.

To improve more efficiently the expression of the *Aq*CoA, the plasmid pMC-GAP-PDI-ERO1-SSA4-SSE1-YDJ1-SSO2 was constructed and transferred to the strain GAP-1*Aq*CoA or GAP-4*Aq*CoA or GAP-8*Aq*CoA, respectively. The transformants obtained were named as GAP-1*Aq*CoA – 6chaperone GAP-4*Aq*CoA – 6chaperone and GAP-8*Aq*CoA – 6chaperone, respectively. Then, based on the strain GAP-8*Aq*CoA-6chaperone, the transformants GAP-12*Aq*CoA-6chaperone, GAP-16*Aq*CoA-6chaperone, GAP-20*Aq*CoA-6chaperone and GAP-24*Aq*CoA-6chaperone were separately constructed. The result showed the synergistic effect between gene copy number and molecular chaperones in enhancing expression of the *Aq*CoA. As indicated in the Fig. 6(c), when the co-expression of six chaperones, the enzyme activity of *Aq*CoA enhanced gradually with the increase of the copy number of the *Aq*CoA, suggesting that combination of six chaperones synergistically enhanced the secretion levels of the recombinant protein. Moreover, the maximum enzyme activity reached 2.319 U/ml by the recombinant strain with six chaperones combination plus sixteen copies chitosanase gene *Aqcoa*, which was 13-fold higher than that for the control strain GAP-1*Aq*CoA with only one copy (Table 1). However, the activity gradually decreased as the copy number exceeded 16, indicating that molecular chaperones had certain limitation in relieving secretion bottlenecks of protein. Besides, the expression level of the *Aq*CoA was detected by western blot. The result exhibited that the expression level of the *Aq*CoA increased gradually with the increase of the copy number of the *Aq*CoA in the Fig. 6(d), the highest amount of protein attained 125.20 mg/l by the recombinant strain with six chaperones combination plus sixteen copies chitosanase gene *Aqcoa* (Table 1), which was consistent with the result in the Fig. 6(c).

Table 1
The maximum activity to 95% deacetylated chitosan obtained in strains with various copies and six chaperones

Strains	Maximum Activity(U/ml)	Production of *Aq*CoA (mg/l)	Improvement of activity (fold)
GAP-1*Aq*CoA	0.18 ± 0.005	9.71 ± 0.246	1.00
GAP-1*Aq*CoA-6chaperone	0.39 ± 0.011	21.05 ± 0.536	2.17
GAP-4*Aq*CoA-6chaperone	0.87 ± 0.024	46.95 ± 1.205	4.84
GAP-8*Aq*CoA-6chaperone	1.39 ± 0.038	75.01 ± 1.903	7.73
GAP-12*Aq*CoA-6chaperone	1.86 ± 0.050	100.38 ± 2.546	10.34
GAP-16*Aq*CoA-6chaperone	2.32 ± 0.063	125.20 ± 3.176	12.89
GAP-20*Aq*CoA-6chaperone	2.14 ± 0.058	115.49 ± 2.929	11.89
GAP-24*Aq*CoA-6chaperone	1.84 ± 0.050	99.30 ± 2.519	10.23

The copy number of target genes had a crucial effect on the production of recombinant protein, thus, the *Aqcoa* gene copy number in the genomes of the integrants was determined by the qPCR assays. As shown in Table 2, the *Aqcoa* gene copy numbers of strains GAP-1*Aq*CoA-6chaperone, GAP-4*Aq*CoA-6chaperone, GAP-8*Aq*CoA-6chaperone, GAP-12*Aq*CoA-6chaperone, GAP-16*Aq*CoA-6chaperone, GAP-20*Aq*CoA-6chaperone and GAP-24*Aq*CoA-6chaperone were 0.98, 2.77, 6.02, 9.79, 12.36, 15.47 and 18.02, respectively, which was not consistent with theoretical copy number. It was likely that the recombinant plasmid was not fully integrated into the yeast genome, which caused that the copy number of the gene had difference between the theoretical and measured values.

Table 2
The identification of copy numbers of the *Aq*CoA by different strains by qPCR

Strains	Copy numbers of *Aq*CoA
GAP-1*Aq*CoA-6chaperone	0.98 ± 0.10
GAP-4*Aq*CoA-6chaperone	2.77 ± 0.21
GAP-8*Aq*CoA-6chaperone	6.02 ± 0.15
GAP-12*Aq*CoA-6chaperone	9.79 ± 0.13
GAP-16*Aq*CoA-6chaperone	12.36 ± 0.15
GAP-20*Aq*CoA-6chaperone	15.47 ± 0.19
GAP-24*Aq*CoA-6chaperone	18.02 ± 0.25

Discussion

The pMC-GAP is a novel plasmid with rapid and convenient in construction of high-copy strain. At present, the method to build multi-copy strain mainly depended on the different concentration of antibiotics, which led to the increase of the cost in the experiment. Pan et al (2011) proposed the effective strategy to delete gene from genome, in which referred to that resistance genes on foreign plasmids integrated into *P. pastoris* genome via homologous recombination were at last deleted by the Cre recombinase (30). However, the application of this system in yeast expression has not been reported. Therefore, we rebuilt the plasmid P3 (figure S1-S3) and found the promoter AOX1 leaked expressing in the *E. coli* activated the translation of the Cre enzyme that led to recombination between the two lox sites (figure S4), which hindered the application of this system in yeast expression. Therefore, some strategies took to regulate the expression of the Cre protein. Some investigators mentioned that the promoter lac (P_{lac}) (45) of *E.coli* was admittedly a rather simple system in gene regulation (20) and strictly modulated by the inducer isopropyl β-D-thiogalactopyranoside (IPTG) (1). To prevent the leakage of the gene expression, the plasmid P3 was added the promoter lac (P_{lac}) and generated the plasmid P4 (Fig. 1). In addition, different promoters were successfully employed for the expression of recombinant proteins in *P. pastoris* (7). The alcohol oxidase (AOX1) promoter, strongly induced by addition of methanol, was extensively used for recombinant protein production in *P. pastoris* (9, 15). However, the methanol needed to be added continuously during the growth of the *P. pastoris*, which increased the risk of bacterial contamination. Nevertheless, the glyceraldehyde-3-phosphate dehydrogenase constitutive promoter (pGAP) has been turn into a wide alternative (4, 55), the promoter utilized other carbon sources like glucose, glycerol as a substrate (48, 16) and avoid the use of methanol in the industry, which possessed important advantageous in industrial production. Thus, to facilitate industrial production of the recombinant proteins in *P. pastoris*, we employed the pGAP and constructed the novel plasmid pMC-GAP (Fig. 3). Moreover, in this study, some strains containing different gene copy were obtained, indicating that the plasmid pMC-GAP is more efficient in generating multi-copy strains.

Increase the copy number of the *Aq*CoA could significantly enhanced the protein yield (Fig. 4c). However, it gradually reached the plateau in the increase of the protein production once gene dosage exceeded a certain limit, suggesting that it was not the linear relation between gene dosage and protein yield. Previous studies showed that over-expression of heterologous proteins in yeasts increased the burden of the folding capacity in the ER and activated the unfolded protein response (17), which limiting the secretory pathway and further reducing the production of the foreign protein. To alleviate the stress on the ER, a few chaperone genes were overexpressed. In the study, ten chaperone genes were employed to augment the recombinant protein expression Different chaperones exhibited different efficiencies towards the foreign protein expression. In among of them, up-regulating of PDI, ERO1, HAC1, YDJ1, SSE1, SSA4 and SSO2 enhanced the expression of the *Aq*CoA (Fig. 5). Pdip, as the protein disulfide isomerase, assisted in rearrangement of incorrect disulfide pairings by isomerase activity (50). Overexpression of PDI increased recombined protein production in the *P. pastoris* (40). Similarly, the overexpression of the flavoenzyme Ero1p that producing disulfide bonds for oxidative protein folding in the ER (10) improved Fab fragment secretion in *P. pastoris* (46). As the most prominent components regulating the UPR (3), the overexpression of the transcription factor Hac1p increased translation of the chaperones and further enhanced expression of heterologous protein (14). Ydj1p and Ssa1p were the cytoplasmic chaperones. Ssa1p directly interacted with Ydj1p, that facilitating to assist in the transportation of intermediate proteins to the ER (28). Increasing the expression of chaperone Ydj1p and Ssa1p improved production of foreign proteins (37). Ssa4 was also the cytosolic chaperone and responsible for the transport of target nascent proteins to the ER membrane (5, 12). Overexpression of Ssa4 increased protein expression level in *P. pastoris* (53). As the secretion helper factor, Sso2 acted as one of the soluble N-ethylmaleimide-sensitive factor attachment protein receptors and aided the fusion of secretory vesicles at the plasma membrane (34, 51). Overexpression of Sso2 also achieved the purpose of enhancing protein production (18). Consistent with the above reports, in our work, enhancing the expression level of PDI, ERO1, HAC1, YDJ1, SSE1, SSA4 and SSO2 had a positive impact on the expression of the *Aq*CoA.

At last, we investigated the effects of co-expression between gene dosages and different chaperones on the expression of the *Aq*CoA. In the strain containing one copy, the yield of the protein produced by the co-expression of six chaperones increased by 2.17 times than that of the control strain with single copy (Table 1) and was higher than that of the strain with the individual overexpression of one chaperone, indicating the potential synergistic effects of combined chaperones. Based on the strain with one copy plus six chaperones, the strains with various copy numbers were generated. Furthermore, the highest yield of the protein reached 125.20 mg/l by the strain containing six chaperones and 16 copies, which increased by 13 times than that of the control strain with one copy (Table 1), suggesting co-expression of six chaperones markedly improve secretion pathway of the multi-copy strains to facilitate the expression of the *Aq*CoA. Zhang et al (2006) proposed that some pairs of chaperones could enhance the secretion levels of the protein. However, overexpression of the chaperone pairs exclusively located in the cytoplasm or ER had a negative effect to the protein expression, in which secretion was lower than that mediated by single chaperone (56). In this study, to avoid the above case, the chaperones from the ER (PDI and ERO1) and the cytoplasm (YDJ1, SSA1, SSO2 and SSE4) were co-expressed, which prominently increased the

yield of recombinant protein. Therefore, integrated expression of chaperones and gene dosages presented a powerful tool to enhance foreign protein expression.

Conclusion

In this study, a novel plasmid pMC-GAP with stable transformation ability was constructed in *P. pastoris*. By adopting pMC-GAP, we constructed series of plasmids with different copy number and obtained corresponding transformants. In point of gene dosages, the highest enzyme activity got 0.32 U/ml in the strain with four copies, which was 1.78-fold higher than that of strain with a single copy. In addition, the chaperones like PDI, ERO1, HAC1, YDJ1, SSE1, SSA4 and SSO2 elevated level of protein expression, and PDI/ERO1, SSA4/SSE1 and YDJ1/SSO2 pairs synergistically increased protein expression. Lastly, the maximum enzyme activity reached 2.319 U/ml by the recombinant strain with six chaperones (PDI-ERO1-SSA4-SSE1-YDJ1-SSO2) intergrant plus sixteen copies chitosanase gene *Aqcoa*, that was 13-fold higher than that for the control strain (GAP-1*Aq*CoA). It presented an effective strategy that co-expression of gene copy and *molecular chaperone in improvement* of *foreign protein expression.*

Declarations

Author Contribution

YX Wang performed the experiments and wrote the manuscript. YQ Zhao provided valuable suggestions for improving the experimental method. X Luo helped to finish the experiments. ZK Li helped to design the experimental strategy. XF Ye performed the data analysis. Y Huang provided many helpful comments in writing the manuscript. D Ling helped to carry out the experiment. ZL Cui contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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Data Availability

All data generated or analyzed during this study are included in this paper and its supplementary information files.

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Competing Interests The authors declare no competing interests.

References

1. Ackers, G. K., Johnson, A. D. and Shea, M. A. (1982) Quantitative model for gene regulation by lambda phage repressor. P. Nati. Acad. Sci. **79,** 1129-1133.

2. Aw, R. and Polizzi, K. M. (2016) Liquid PTVA: a faster and cheaper alternative for generating multi-copy clones in *Pichia pastoris*. Microb. Cell Fact. **15,** 1-11.

3. Back, S. H., Schröder, M., Lee, K., Zhang, K. and Kaufman, R. J. (2005) ER stress signaling by regulated splicing: IRE1/HAC1/XBP1. Methods,**35,** 395-416.

4. Boer, H., Teeri, T. T. and Koivula, A. (2000) Characterization of *Trichoderma reesei* cellobiohydrolase Cel7A secreted from *Pichia pastoris* using two different promoters. Biotechnol. Bioeng.,**69,** 486-494.

5. Boorstein, W. and Craig, E. (1990) Structure and regulation of the SSA4 HSP70 gene

of *Saccharomyces cerevisiae*. J. Biol. Chem.,**265,** 18912-18921.

6. Chung, M. J., Park, J. K. and Park, Y. I. (2012) Anti-inflammatory effects of low-molecular weight chitosan oligosaccharides in IgE–antigen complex-stimulated RBL-2H3 cells and asthma model mice. Int. Immunopharmacol.,**12,** 453-459.

7. Cos, O., Ramón, R., Montesinos, J. L. and Valero, F. (2006) Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: a review. Microb. Cell Fact.,**5,** 1-20.

8. Cregg, J. M., Cereghino, J. L., Shi, J. and Higgins, D. R. (2000) Recombinant protein expression in *Pichia pastoris.* Mol. Biotechnol.,**16,** 23-52.

9. Cregg, J. M., Vedvick, T. S. and Raschke, W. C. (1993) Recent advances in the expression of foreign genes in *Pichia pastoris*. Bio/technology,**11,** 905-910.

10. Frand, A. R. and Kaiser, C. A. (1999) Ero1p oxidizes protein disulfide isomerase in a pathway for disulfide bond formation in the endoplasmic reticulum. Mol. Cell,**4,** 469-477.

11. Freedman, R. B. (1989) Protein disulfide isomerase: multiple roles in the modification of nascent secretory proteins. Cell,**57,** 1069-1072.

12. Gasser, B., Sauer, M., Maurer, M., Stadlmayr, G. and Mattanovich, D. (2007) Transcriptomics-based identification of novel factors enhancing heterologous protein secretion in yeasts. Appl. Environ. Microb.,**73,** 6499-6507.

13. Graf, A., Gasser, B., Dragosits, M., Sauer, M., Leparc, G. G., Tüchler, T., Kreil, D. P. and Mattanovich, D. (2008) Novel insights into the unfolded protein response using *Pichia pastoris* specific DNA microarrays. BMC genomics,**9,** 1-13.

14. Guerfal, M., Ryckaert, S., Jacobs, P. P., Ameloot, P., Van Craenenbroeck, K., Derycke, R. and Callewaert, N. (2010) The HAC1 gene from *Pichia pastoris*: characterization and effect of its overexpression on the production of secreted, surface displayed and membrane proteins. Microb. Cell Fact.,**9,** 1-12.

15. Hartner, F. S., Ruth, C., Langenegger, D., Johnson, S. N., Hyka, P., Lin-Cereghino, G. P., Lin-Cereghino, J., Kovar, K., Cregg, J. M. and Glieder, A. (2008) Promoter library designed for fine-tuned gene expression in *Pichia pastoris*. Nucleic Acids Res.,**36,** 76-76.

16. Heyland, J., Fu, J., Blank, L. M. and Schmid, A. (2011) Carbon metabolism limits

recombinant protein production in *Pichia pastoris*. Biotech. Bioeng.,**108,** 1942-1953.

17. Inan, M., Aryasomayajula, D., Sinha, J. and Meagher, M. M. (2006) Enhancement of protein secretion in *Pichia pastoris* by overexpression of protein disulfide isomerase. Biotech Bioeng.,**93,** 771-778.

18. Jiao, L., Zhou, Q., Su, Z. and Yan, Y. (2018) Efficient heterologous production of

Rhizopus oryzae lipase via optimization of multiple expression-related helper proteins. Int. J. Mol. Sci.,**19,** 3372-3375.

19. Kong, S.-Z., Li, D.-D., Luo, H., Li, W.-J., Huang, Y.-M., Li, J.-C., Hu, Z., Huang, N., Guo, M.-H. and Chen, Y. (2018) Anti-photoaging effects of chitosan oligosaccharide in ultraviolet-irradiated hairless mouse skin. Exp. Gerontol.,**103,** 27-34.

20. Kuhlman, T., Zhang, Z., Saier, M. H. and Hwa, T. (2007) Combinatorial transcriptional control of the lactose operon of *Escherichia coli*. P. Nati. Acad. Sci.,**104,** 6043-6048.

21. Li, C., Lin, Y., Zheng, X., Pang, N., Liao, X., Liu, X., Huang, Y. and Liang, S. (2015) Combined strategies for improving expression of Citrobacter amalonaticus phytase in *Pichia pastoris*. BMC Biotechnol.,**15,** 1-11.

22. Li, K., Xing, R., Liu, S. and Li, P. (2016) Advances in preparation, analysis and biological activities of single chitooligosaccharides. Carbohyd. Polym,**139,** 178-190.

23. Li, Y.-T., Li, M.-T., Fu, C.-H., Zhou, P.-P., Liu, J.-M. and Yu, L.-J. (2009) Improvement of arachidonic acid and eicosapentaenoic acid production by increasing the copy number of the genes encoding fatty acid desaturase and elongase into *Pichia pastoris*. Biotechnol. Lett.,**31,** 1011-1017.

24. Li, Z., Xia, C., Wang, Y., Li, X., Qiao, Y., Li, C., Zhou, J., Zhang, L., Ye, X. and Huang, Y. (2019) Identification of an endo-chitinase from *Corallococcus* sp. EGB and evaluation of its antifungal

properties. Int. J. Biol. Macromol.,**132,** 1235-1243.

25. Liang, S., Sun, Y. and Dai, X. (2018) A review of the preparation, analysis and biological functions of chitooligosaccharide. Int J Mol. Sci.,**19,** 2197-2205.

26. Macauley-Patrick, S., Fazenda, M. L., McNeil, B. and Harvey, L. M. (2005) Heterologous protein production using the *Pichia pastoris* expression system. Yeast,**22,** 249-270.

27. Marx, H., Mecklenbräuker, A., Gasser, B., Sauer, M. and Mattanovich, D. (2009) Directed gene copy number amplification in *Pichia pastoris* by vector integration into the ribosomal DNA locus. Fems Yeast Res.,**9,** 1260-1270.

28. Ngosuwan, J., Wang, N. M., Fung, K. L. and Chirico, W. J. (2003) Roles of cytosolic Hsp70 and Hsp40 molecular chaperones in post-translational translocation of presecretory proteins into the endoplasmic reticulum. J. Biol. Chem.,**278,** 7034-7042.

29. Niu, X., Liu, C.-C., Xiong, Y.-J., Yang, M.-M., Ma, F., Liu, Z.-H. and Yuan, S. (2016) The modes of action of ChiIII, a chitinase from mushroom *Coprinopsiscinerea*, shift with changes in the length of GlcNAc oligomers. J. Agr. Food Chem.,**64,** 6958-6968.

30. Pan, R., Zhang, J., Shen, W.-L., Tao, Z.-Q., Li, S.-P. and Yan, X. (2011) Sequential deletion of *Pichia pastoris* genes by a self-excisable cassette. FEMS Yeast Res.,**11,** 292-298.

31. Park, P.-J., Je, J.-Y. and Kim, S.-K. (2003) Free radical scavenging activity of chitooligosaccharides by electron spin resonance spectrometry. J. Agr. Food Chem.,**51,** 4624-4627.

32. Protopopov, V., Govindan, B., Novick, P. and Gerst, J. E. (1993) Homologs of the synaptobrevin/VAMP family of synaptic vesicle proteins function on the late secretory pathway in *S. cerevisiae*. Cell,**74,** 855-861.

33. Rahimnejad, S., Yuan, X., Wang, L., Lu, K., Song, K. and Zhang, C. (2018) Chitooligosaccharide supplementation in low-fish meal diets for Pacific white shrimp (*Litopenaeus vannamei*): Effects on growth, innate immunity, gut histology, and immune-related genes expression. Fish Shellfish Immun.,**80,** 405-415.

34. Ruohonen, L., Toikkanen, J., Tieaho, V., Outola, M., SODERLUND, H. and KERANEN, S. (1997) Enhancement of protein secretion in *Saccharomyces cerevisiae* by overproduction of Sso protein, a late-acting component of the secretory machinery. Yeast,**13,** 337-351.

35. Salah, R., Michaud, P., Mati, F., Harrat, Z., Lounici, H., Abdi, N., Drouiche, N. and Mameri, N. (2013) Anticancer activity of chemically prepared shrimp low molecular weight chitin evaluation with the human monocyte leukaemia cell line, THP-1. Int. J. Biol. Macromol.,**52,** 333-339.

36. Sambrook, J. and Russell, D. Molecular Cloning: A Laboratory Manual, 3rd edn 2001 Cold Spring Harbor. NY Cold Spring Harbor Laboratory Press.

37. Samuel, P., Vadhana, A. K. P., Kamatchi, R., Antony, A. and Meenakshisundaram,

S. (2013) Effect of molecular chaperones on the expression of *Candida antarctica* lipase B in *Pichia pastoris*. Microbiol. Res.,**168,** 615-620.

38. Scorer, C. A., Clare, J. J., McCombie, W. R., Romanos, M. A. and Sreekrishna, K. (1994) Rapid selection using G418 of high copy number transformants of *Pichia pastoris* for high–level foreign gene expression. Bio/technology,**12,** 181-184.

39. Sha, C., Yu, X.-W., Lin, N.-X., Zhang, M. and Xu, Y. (2013) Enhancement of lipase r27RCL production in *Pichia pastoris* by regulating gene dosage and co-expression with chaperone protein disulfide isomerase. Enzyme Microb. Tech.,**53,** 438-443.

40. Smith, J. D., Tang, B. C. and Robinson, A. S. (2004) Protein disulfide isomerase, but not binding protein, overexpression enhances secretion of a non-disulfide-bonded protein in yeast. Biotechnol Bioeng,**85,** 340-350.

41. Sunga, A. J., Tolstorukov, I. and Cregg, J. M. (2008) Posttransformational vector

amplification in the yeast *Pichia pastoris*. Fems Yeast Res.,**8,** 870-876.

42. Valero, F. (2012) Heterologous expression systems for lipases: a review. Lipases

and phospholipases, 161-178.

43. Valkonen, M., Penttilä, M. and Saloheimo, M. (2003) Effects of inactivation and constitutive expression of the unfolded-protein response pathway on protein production in the yeast *Saccharomyces cerevisiae*. Appl. Environ. Microb.,**69,** 2065-2072.

44. Viens, P., Lacombe-Harvey, M.-È. and Brzezinski, R. (2015) Chitosanases from family 46 of glycoside hydrolases: from proteins to phenotypes. Mar. Drugs,**13,** 6566-6587.

45. Vilar, J. M., Guet, C. C. and Leibler, S. (2003) Modeling network dynamics: the lac operon, a case study. J. Cell Biol.,**161,** 471-471.

46. Wang, J., Wu, Z., Zhang, T., Wang, Y. and Yang, B. (2019) High-level expression of *Thermomyces dupontii* thermophilic lipase in *Pichia pastoris* via combined strategies. 3 Biotech,**9,** 62-64.

47. Wang, Y., Li, D., Liu, M., Xia, C., Fan, Q., Li, X., Lan, Z., Shi, G., Dong, W. and

Li, Z. (2021) Preparation of active chitooligosaccharides with a novel chitosanase AqCoA and their application in fungal disease protection. J. Agr. Food Chem., 69, 3351-3361.

48. Waterham, H. R., Digan, M. E., Koutz, P. J., Lair, S. V. and Cregg, J. M. (1997) Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. Gene,**186,** 37-44.

49. Whyteside, G., Alcocer, M. J., Kumita, J. R., Dobson, C. M., Lazarou, M., Pleass, R. J. and Archer, D. B. (2011) Native-state stability determines the extent of degradation relative to secretion of protein variants from *Pichia pastoris*. PLoS One,**6,** 22692-22695.

50. Wilkinson, B. and Gilbert, H. F. (2004) Protein disulfide isomerase. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics,**1699,** 35-44.

51. Yamamoto, T., Yamamoto, D., Rokugawa, K., Yoshimura, K., Imura, Y., Yoshimura,

E. and Suzuki, M. (2018) Decreased aluminium tolerance in the growth of *Saccharomyces cerevisiae* with SSO2 gene disruption. Biometals,**31,** 203-215.

52. Ye, W., Munemasa, S., Shinya, T., Wu, W., Ma, T., Lu, J., Kinoshita, T., Kaku, H., Shibuya, N. and Murata, Y. (2020) Stomatal immunity against fungal invasion comprises not only chitin-induced stomatal closure but also chitosan-induced guard cell death. P. Nati. Acad. Sci.,**117,** 20932-20942.

53. Yu, X.-W., Sun, W.-H., Wang, Y.-Z. and Xu, Y. (2017) Identification of novel factors enhancing recombinant protein production in multi-copy *Komagataella phaffii* based on transcriptomic analysis of overexpression effects. Sci. Rep.,**7,** 1-12.

54. Yue, L., Li, J., Chen, W., Liu, X., Jiang, Q. and Xia, W. (2017) Geraniol grafted chitosan oligosaccharide as a potential antibacterial agent. Carbohyd. Polym.,**176,** 356-364.

55. Zhang, A.-L., Luo, J.-X., Zhang, T.-Y., Pan, Y.-W., Tan, Y.-H., Fu, C.-Y. and Tu, F.-z. (2009) Recent advances on the GAP promoter derived expression system of *Pichia pastoris*. Mol. Biol. Rep.,**36,** 1611-1619.

56. Zhang, W., Zhao, H. l., Xue, C., Xiong, X. h., Yao, X. q., Li, X. y., Chen, H. p.

and Liu, Z. m. (2006) Enhanced secretion of heterologous proteins in *Pichia pastoris* following overexpression of *Saccharomyces cerevisiae* chaperone proteins. Biotechnol. Progr.,**22,** 1090-1095.

57. Zhou, J., Liu, C. Y., Back, S. H., Clark, R. L., Peisach, D., Xu, Z. and Kaufman, R. J.

(2006) The crystal structure of human IRE1 luminal domain reveals a conserved dimerization interface required for activation of the unfolded protein response. P. Nati. Acad. Sci.,**103,** 14343-14348.

58. Zhu, T., Guo, M., Tang, Z., Zhang, M., Zhuang, Y., Chu, J. and Zhang, S. (2009) Efficient generation of multi-copy strains for optimizing secretory expression of porcine insulin precursor in yeast *Pichia pastoris*. J. Appl. Microbiol.,**107,** 954-963.

Supplementary Tables

Supplementary Tables S1-S4 are not available with this version.

Figures

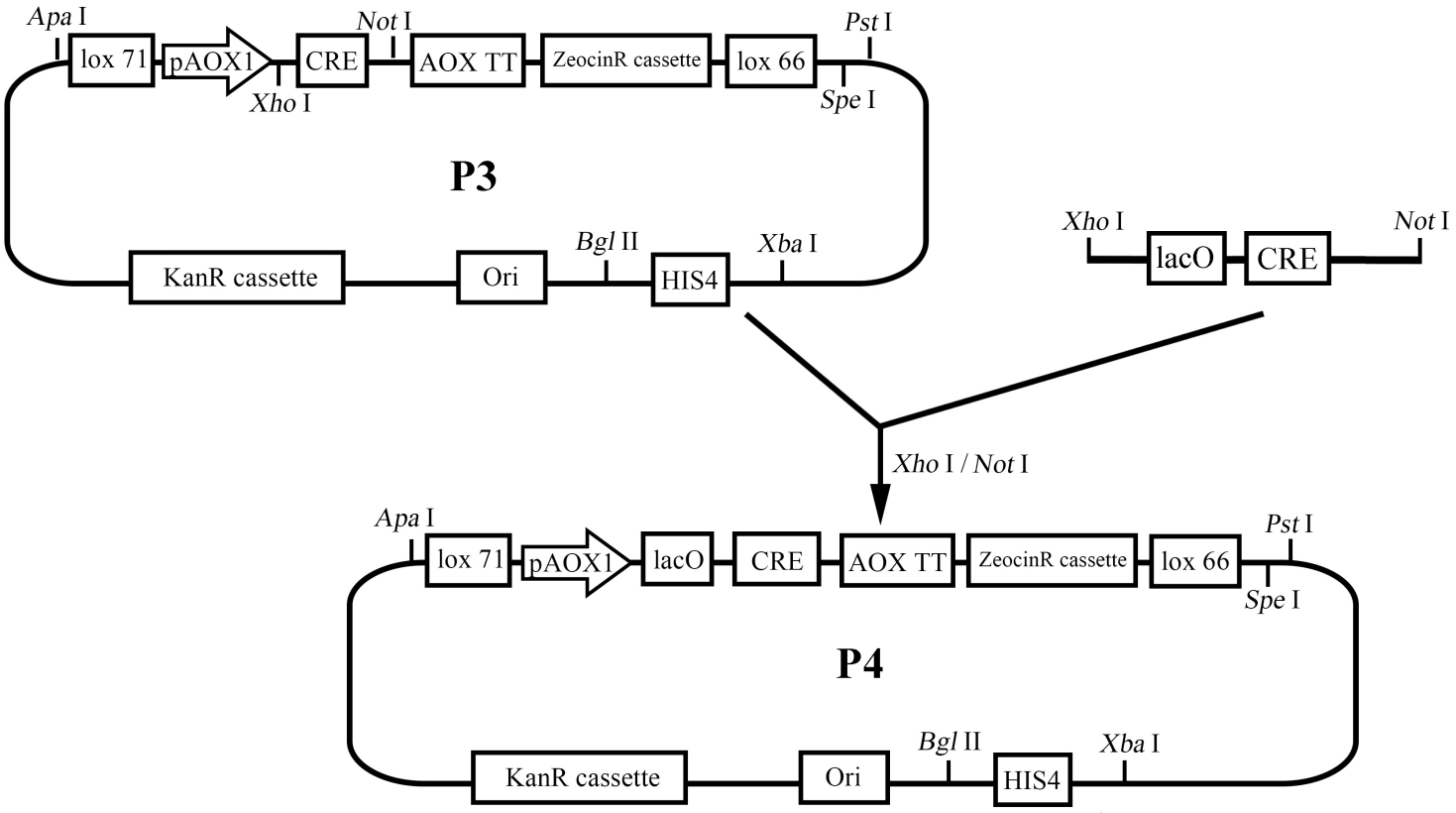

Figure 1

The addition of lactose operon (lacO) on the plasmid to prevent the leaked expression of AOX in E. coli. The cassette with the lacO and CRE was amplified. The cassette and the plasmid P3 were digested by Xho I and Not I and then ligated to generate the recombined plasmid by the T4 ligase. Finally, the plasmid P4 was generated by deleting the restriction site Xho I and Not I on the recombinant plasmid.

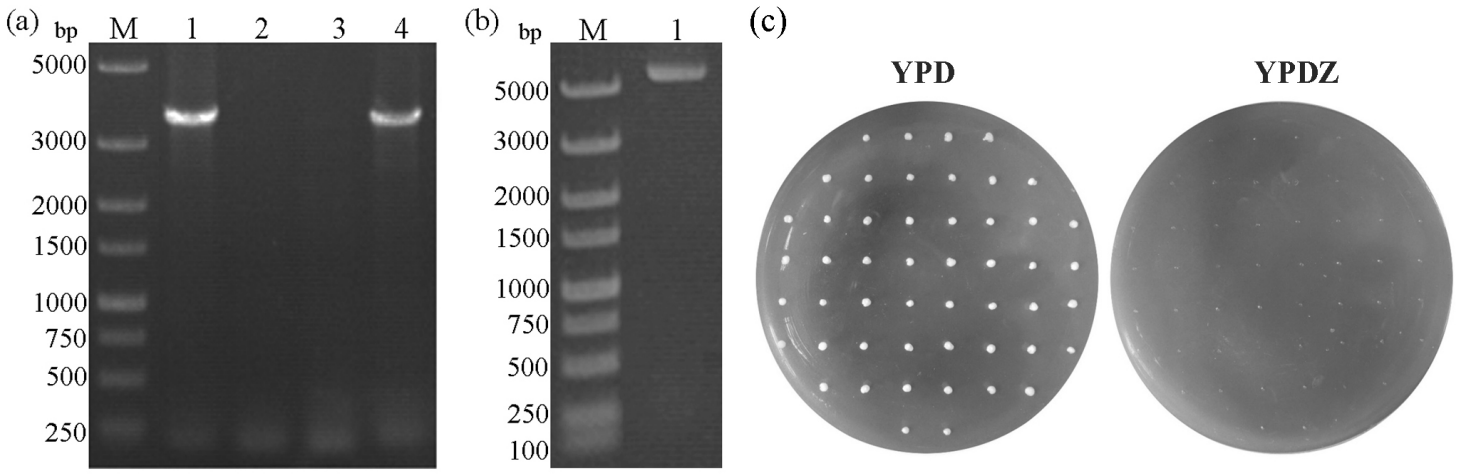

Figure 2

Confirmation of function in the plasmid P4. (a), Colony PCR validation of the target fragment located between site lox71 and lox66 in the E.coli transformants. The target bands were amplified in the transformants 1 and 4. (b), Plasmid was extracted from the E.coli transformant 1. The circular plasmid (8741 bp) in the transformant 1 is consistent with the theoretical size (5827 bp) in nucleic acid gel. (c) Growth of P. pastoris on the plate YPD and YPDZ after induction by methanol.

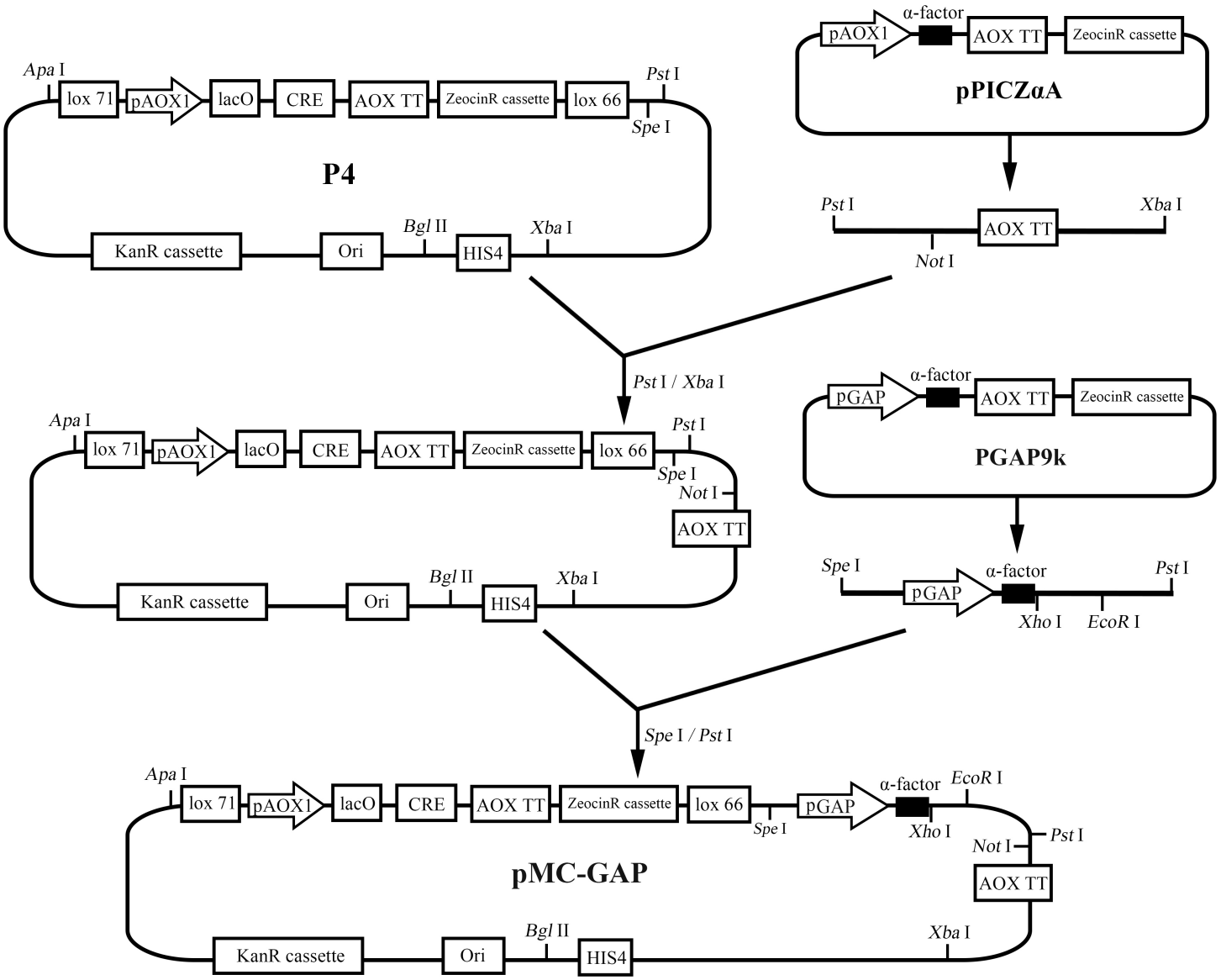

Figure 3

Construction of the plasmid pMC-GAP. The AOX tanscription termination codon (AOX TT) and the cassette with the promoter GAP and secretory factor α-factor were respectively amplified and ligated to the linearized plasmid P4 in turn to generate recombinant plasmid pMC-GAP.

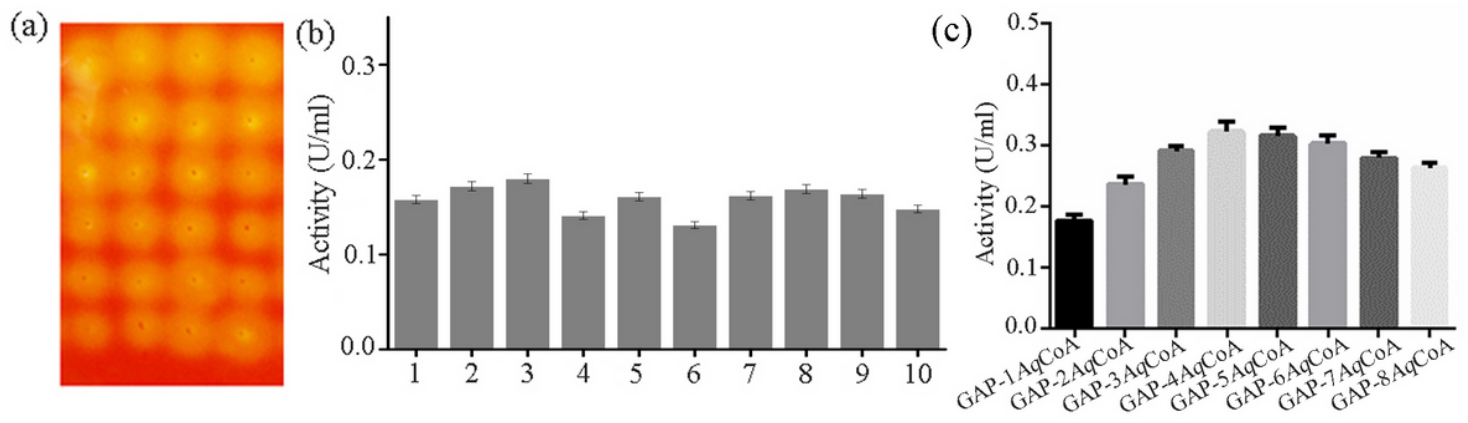

Figure 4

The activity assay of strain GS115 with one-copy or multi-copy. (a) Activity of the strain with single copy to the sodium carboxymethylcellulose (CMC) was detected in the plate YPDZ-CMC by the hydrolysis circle. (b) Activity determination to 95% deacetylated chitosan in ten transformants containing one copy. (c) Activity detection to 95% deacetylated chitosan in the strains with various copies.

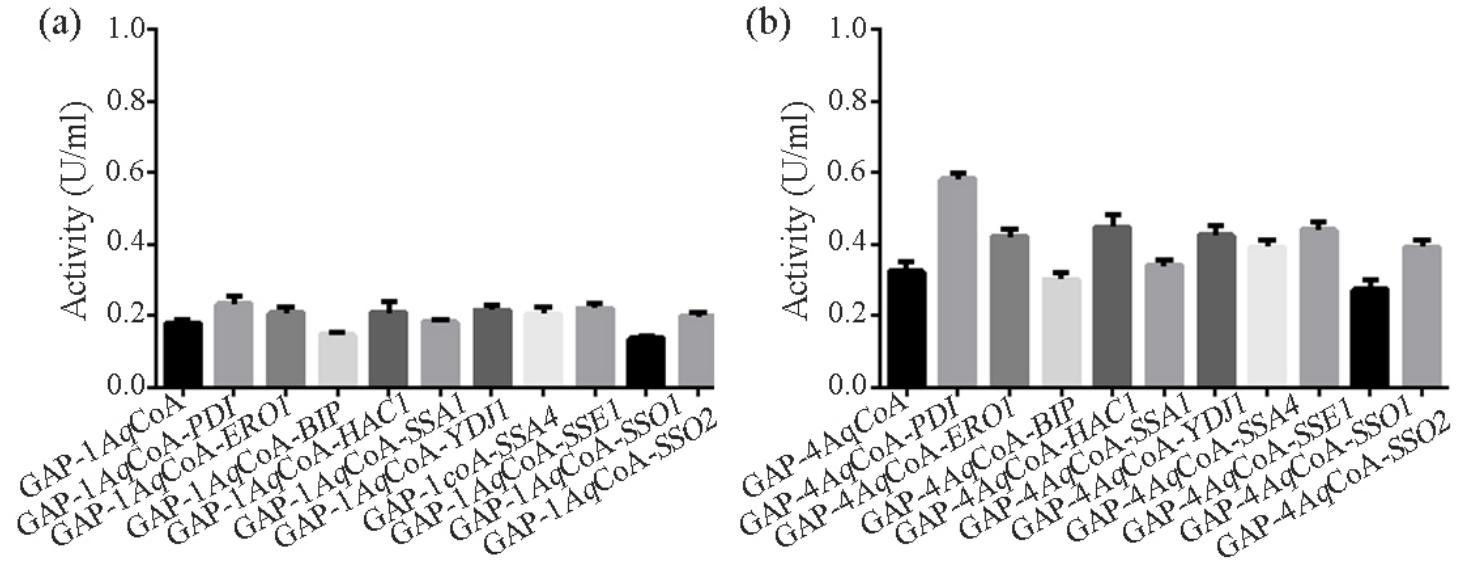

Figure 5

Effects of expression of different molecular chaperones on the hydrolysis activity toward 95% deacetylated chitosan in the strains with single-copy or four-copy. (a) Effects of expression of different molecular chaperones on the hydrolysis activity to 95% deacetylated chitosan in the strains with single copy. (b) Effects of expression of different molecular chaperones on the hydrolysis activity to 95% deacetylated chitosan in the strains with four copies.

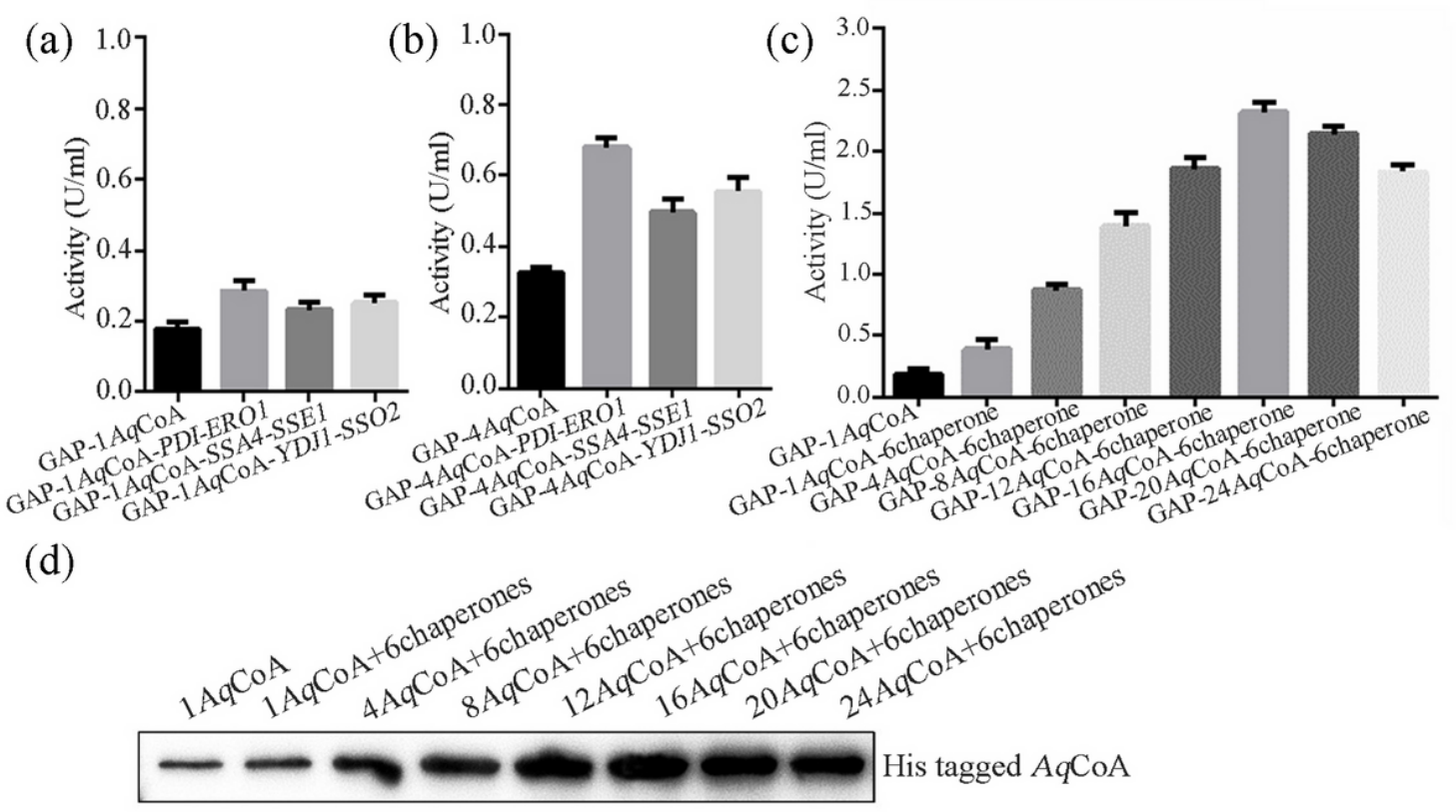

Figure 6

Effects of co-expression of different molecular chaperones on the hydrolysis activity to 95% deacetylated chitosan in the strains with multi-copy. (a) Effects of co-expression of pairs of chaperones on the hydrolysis activity to 95% deacetylated chitosan in the strains with single copy. (b) Effects of co-expression of pairs of chaperones on the hydrolysis activity to 95% deacetylated chitosan in the strains with four copies. (c) Effects of co-expression of six chaperones and gene copy number on the hydrolysis activity to 95% deacetylated chitosan. (d) Detection of protein expression level in the strains with various copies by western blot.

Supplementary Files

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