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Increasing gene dosage and chaperones co-expression facilitate the efficient dextranase expression in *Pichia pastoris*

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ABSTRACT

Dextranase (DEX) is an important hydrolase in the food industry, which can degrade the dextran into low-molecular-weight polysaccharides. However, its commercial applications are limited by ineffective heterologous recombination system. Here, we build an efficient *Pichia pastoris* system for DEX following two different engineering strategies. First, through increasing the gene dosage, the DEX activity increased from 40.13 U/mL to 121.02 U/mL. The up-regulation of the endoplasmic reticulum stress gene *ERO1* was detected by the qPCR. Since chaperones are involved in the protein folding and modification, co-expression of chaperones was applied to relieve the endoplasmic reticulum stress and facilitated further increase in DEX activity to 164.78 U/mL. Then, high cell density cultivation of *P. pastoris* was performed in a 5 L fermenter (cell dry weight reached 105.37 g/L), resulting in the DEX activity to 619.78 U/mL. Finally, under the conditions of 25 °C, pH 5.0, 5% sorbitol as co-substrates, and a feed rate of 7.2 mL/h, a DEX yield of 3257.23 U/mL was obtained through 5-L fed-batch fermentation. DCFH-DA staining showed that the reactive oxygen species level decreased by 68.83% due to sorbitol co-feeding. Our study thus represents an important step for building a robust *P. pastoris* expression system for recombinant DEX production.

1. Introduction

DEX is an important hydrolase in the food industry, which can degrade the dextran into low-molecular-weight polysaccharides (Juntarachot, Kantachote, Peerajan, Sirilun, & Chaiyasut, 2020). DEXs are usually classified into endo- and exo-DEXs according to the mode of action (Yang et al., 2018). The endo-DEX from fungi hydrolyzes dextran into isomaltose, isomaltotriose, and a small amount of D-glucose (Khalikova, Susi, & Korpela, 2005), whereas the hydrolysis of dextran by bacterial endo-DEX mainly produces oligo-oligosaccharides. Exo-DEX hydrolysates vary according to the class of enzymes, the main hydrolysis products are glucose, isomaltose, isomaltotriose, and cyclodextrin. Currently, commercialized dextranases mainly derive from fungi (Roca et al., 1996). However, the production of DEX poses challenges as the complex metabolism in the wild-type strain could lead to unwanted secondary metabolites and byproducts such as roquefortine C (Perrone & Susca, 2017) and echinocandin A (Dwibedi et al., 2023), making downstream purification processes complex and labor consuming. On the contrary, heterologous expression of enzymes using engineered strains can effectively reduce the secondary metabolites, thus bringing convenience to subsequent isolation and purification (Pan, Yang, Wu, Yang, & Fang, 2022). Heterologous expression of DEX has been intensively investigated; however, the expression of these engineered strains is limited by the low level of enzyme activity. For example, Zhao et al. expressed *Chaetomium gracile* derived DEX in *Bacillus subtilis*, after optimizing the fermentation conditions, the DEX activity increased from 2.625 to 64.34 U/mL (Zhao, Wang, Wei, Li, & Liu, 2021). Roca et al. achieved the highest reported heterologous expression of DEX by expressing the enzyme from *Penicillium minioluteum* in *P. pastoris*. The enzyme activity reached 98 U/mL in shake flasks and 2500 U/mL in a 5-L fermenter (Roca et al., 1996).

To further enhance the production of DEX, it is necessary to construct

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a more robust heterologous expression system. *P. pastoris* has developed rapidly in industrial enzyme applications in recent years attributed to its high-density fermentation potential, well post-modification ability, and excellent protein secretion capacity (Yang & Zhang, 2018). It has been reported that *P. pastoris* was used to express DEX (Martínez et al., 2021; Roca et al., 1996), but its expression level needs to be further improved for large-scale production. There are various strategies for achieving high protein expression in *P. pastoris*. Promoter (Yadav, Ranjan, McHunu, Le Roes-Hill, & Kudanga, 2021) and signal peptide (Duan et al., 2019) engineering are common methods and have been proven to be effective. In addition, increasing the copy number of exogenous genes can also enhance protein expression (Sallada, Harkins, & Berger, 2019; Shen et al., 2020). However, this approach could burden the host, leading to a large number of unfolded proteins remaining in the endoplasmic reticulum (ER) and adopting an unfolded conformation, which triggers the unfolded protein response (UPR) (Fauzee, Taniguchi, Ishiwata-Kimata, Takagi, & Kimata, 2020). In addition, the unfolded proteins could also trigger the ER-assisted protein degradation (ERAD) to maintain ER homeostasis (Zahrl, Mattanovich, & Gasser, 2018). introducing the concept of metabolic engineering in the protein expression process to co-express molecular chaperones involved in the protein secretion pathway (As shown in Fig. 1) is an effective and reasonable method to improve protein folding and increase protein secretion. In addition, it is reported that sorbitol has been applied in the production of many proteins as a non-repressing carbon source (Berrios et al., 2017) in *P. pastoris*. Co-feeding sorbitol in the induction phase exhibits several advantages, including reducing heat formation and alleviating toxic metabolites accumulation (Ergün, Berrios, Binay, & Fickers, 2021). Thus, sorbitol co-feeding was also investigated in this study to achieve a higher DEX production under the induction of multi-carbon substrate.

In this study, the efficient expression of DEX in *P. pastoris* was achieved by augmenting the DEX gene dosage. It was found that DEX producing strains were accompanied with elevated ER stress. Thus, co-expressing molecular chaperones was introduced. The effect of molecular chaperones co-expression under the regulation of constitutive promoter GAP or inducible promoter AOX1 in strains with different DEX copy numbers was studied. In this way, the molecular chaperones for the efficient expression of DEX in *P. pastoris* was comprehensively investigated. In the end, 5-L scale-up fermentation with the combination of sorbitol co-feeding was applied to achieve a higher DEX production.

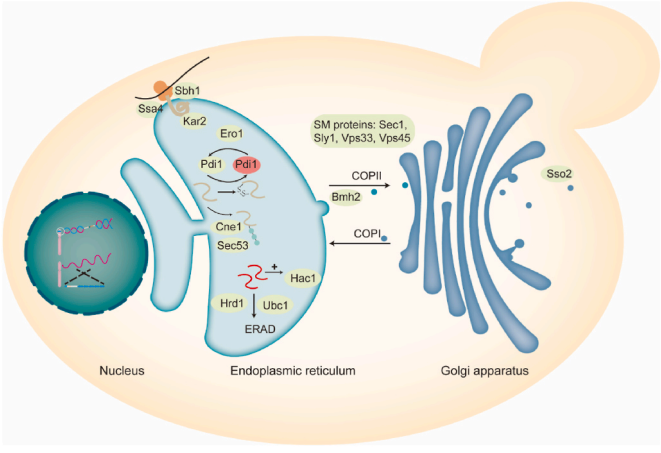

Fig. 1. Schematic diagram of protein secretion in *P. pastoris*. The newly synthesized proteins need to pass the ER membrane and fold into a proper conformation. Correctly folded proteins are transported to the Golgi apparatus through COP II vesicles and finally achieve secretory expression.

2. Material and methods

2.1. Strain construction

The DEX gene from *Penicillium minioluteum* was deposited in our laboratory. A plasmid containing multi-DEX was constructed as shown in Fig. 2a. The amplified fragment was digested with *Bgl* II and *Sal* I, then ligated with *Bam*H I and *Sal* I digested pPIC9K through T4 DNA Ligase (TaKaRa) to get the 2-copy plasmid pPIC9K-2DEX. The same is for the subsequent 3-copy and 4-copy plasmid construction. The strains, plasmids and primers used were listed in Table 1 and Table S1.

The chaperones were amplified from the genomic DNA of *P. pastoris* GS115 using primers listed in Table S1. Plasmid pPICZA and pGAPZA was digested with *Eco*R I-*Not* I and *Eco*R I-*Sal* I respectively. After digestion, the plasmids and chaperone genes were gel purified using the FastPure Gel DNA Extraction Mini Kit (Vazyme), then ligated through ClonExpress II One Step Cloning Kit (Vazyme). Ligation products were transformed into *E. coli* JM109 for plasmid amplification.

2.2. Transformation of P. pastoris

E. coli JM109 harboring recombinant plasmids was incubated on LB medium at 37 °C for 12 h, then the strains were collected and the plasmids were extracted using FastPure EndoFree Plasmid Maxi Kit (Vazyme). The pPIC9K plasmid was linearized with *Sal* I; pPICZA was linearized with *Sac* I, and pGAPZA was linearized using *Bln* I. Then linearized plasmids are gel purified using FastPure Gel DNA Extraction Mini Kit (Vazyme), and DNA concentration was measured using the "Nucleic Acid" mode of NanoDrop (Thermo scientific). 1 μg linearized plasmid was added into the competent *P. pastoris* cells. The transformation of *P. pastoris* was accomplished by the electroporation method (Becker & Guarente, 1991) using Gene Pulser Xcell (Bio-Rad) with instrument settings of 2.0 kV. 1 M sorbitol was added after an electric shock, then recovered at 30 °C for 2 h and spread on minimal dextrose (MD) plates. Plates were grown at 30 °C for 2–3 days.

2.3. Strain cultivation

Luria–Bertani (LB) medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L sodium chloride) containing 25 μg/mL zeocin or 100 μg/mL ampicillin was used for the cultivation of *E.coli* JM109 transformants. The selection of *P. pastoris* transformants was performed on YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) containing 100 μg/mL zeocin or MD medium (13.4 g/L Yeast Nitrogen Base, 0.4 mg/L biotin, 20 g/L glucose), 20 g/L agar was added in all solid medium. The recombinant *P. pastoris* strains were cultivated on YPD liquid medium at 30 °C overnight, and then sub-inoculated into 20 mL buffered glycerol-complex medium (BMGY) (10 g/L yeast extract, 20 g/L tryptone, 13.4 g/L YNB, 11.8 g/L KH_2PO_4, 4.0 g/L $K_2HPO_4{\cdot}3H_2O$, 10 g/L glycerin, 4×10^{-4} g/L biotin) 30 °C for 24 h. The cells were collected in a 50 mL centrifuge tube and centrifuged at 8000 rpm for 5 min. The fermentation broth was poured off and the cells were washed by adding 20 mL of distilled water. The cells were then centrifuged again, harvested, and transferred to 50 mL buffered methanol-complex medium (BMMY) (10 g/L yeast extract, 20 g/L tryptone, 13.4 g/L YNB, 11.8 g/L KH_2PO_4, 4.0 g/L $K_2HPO_4{\cdot}3H_2O$, 10 g/L methanol, 4×10^{-4} g/L biotin) for the production of DEX. Basal salts medium (BSM) (26.7 ml/L 85% phosphoric acid, 0.93 g/L calcium sulfate, 18.2 g/L potassium sulfate, 14.9 g/L magnesium sulfate-$7H_2O$, 4.13 g/L potassium hydroxide, 40 g/L glycerol) was used for the fed-batch high cell density fermentation (as provided in section 2.7).

2.4. Copy number determination by quantitative PCR (qPCR)

Quantitative PCR was performed on the Bio-Rad CFX Connect Real-Time System with Bio-Rad CFX Manager 3.1 version. The PowerUp™

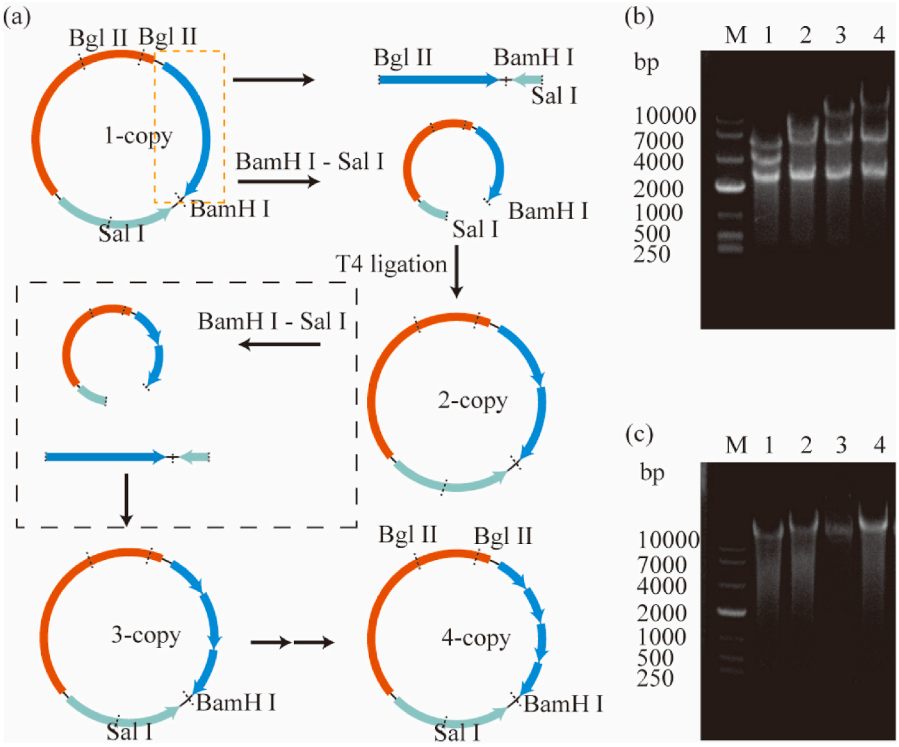

Fig. 2. Multi-copy DEX plasmid construction *in vitro*. (a) Schematic diagram of the multi-copy plasmid construction process. (b) *Bgl* II and *Bam*H I enzyme digestion. (c) *Sal* I digestion. The marker is marked as M. The number of lanes indicates plasmids with different copy numbers. 1-copy (lane 1), 2-copy (lane 2), 3-copy (lane 3), 4-copy (lane 4), 5-copy (lane 5), 6-copy (lane 6).

Table 1
Strains and plasmids used in this study.

Strains and plasmids	Source
P. pastoris GS115	Laboratory preservation
E. coli JM109	Laboratory preservation
pPIC9K	Laboratory preservation
pGAPZA	Laboratory preservation
pPICZA	Laboratory preservation
pPIC9K-DEX	This study
pPIC9K-2DEX	This study
pPIC9K-3DEX	This study
pPIC9K-7DEX	This study

SYBR™ Green (ThermoFisher) was used as a marker. qPCR was conducted in a mixture comprising 0.425 μL primer with a concentration of 5 μM, 8.5 μL of 2 x SYBR Green Mix, 2.5 μL of RNase-free H_2O, 1.25 μL of genomic DNA template (Water used as template was control). The reaction initiates with a 2 min step at 50 °C followed by 95 °C for 10 min, then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Melting curve analysis was performed to exclude unspecific amplification. All determinations were carried out in triplicate. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as the reference. The establishment of a standard curve was accomplished through the linearization of plasmid harboring *DEX* or *GAPDH* gene followed by the qPCR determination. The calculation of gene copy number was according to the method described previously (Jiao, Zhou, Su, Xu, & Yan, 2018). The *DEX* gene copy number in the *P. pastoris* genome was determined with the following equation: *DEX* gene copy number = (*DEX* gene copy number calculated by standard curve)/(*GPADH* fragment copy number calculated by standard curve). The *P. pastoris* genome extraction protocol was based on the method established by Lõoke et al. (Lõoke, Kristjuhan, & Kristjuhan, 2011). *P. pastoris* colony was picked from the plates, resuspended in 100 μL of lysis solution (100 μL 200 mM, LiOAc 1% SDS) and incubated at 70 °C for 15 min. Add 300 μL of 96% ethanol to precipitate genomic DNA and centrifuge at 12,000 rpm for 5 min. The precipitated DNA is dissolved in 100 μL TE solution (10 mM Tris-Hcl, 1 mM EDTA, pH 8.0).

2.5. Induction and measurement of UPR

To simulate the UPR in *P. pastoris*, a tunicamycin (5 μg/mL) method was performed (Graf et al., 2008). *P. pastoris* was cultivated in 10 mL YPD overnight, and inoculated into 10 mL BMMY to reach the exponential growth phase (OD_{600} at around 6). Then, tunicamycin was added, and strain was incubated for 1 h to induce UPR. After this, 1 ml of cells was collected and added into 1.5 ml of RNase-free centrifuge tube, centrifuged at 10000 rpm at 4 °C for 1 min. The Spin Column Yeast Total RNA Purification Kit (Sangon Biotech) was used to extract RNA from the collected cell. Reverse transcription was performed using the All-In-One 5X RT MasterMix (Applied Biological Materials Inc). The qPCR analysis of target gene expression level was performed as described in 2.4. The primers used were reported by (Dragosits, Mattanovich, & Gasser, 2011).

2.6. Enzyme activity measurement

Dextran T-2000 was used as the substrate of DEX. As the method described by Miller (Miller, 1959), the reducing sugar released from the dextran T2000 can react with 3,5-dinitrosalicylic acid reagent. One unit of DEX activity was defined as the amount of enzyme that catalyzing the liberation of the equivalent of 1 μmol of glucose in 1 min from dextran T-2000.

2.7. Fed-batch high cell density fermentation

Fermentation was carried out in a 5-L scale fermenter and the protocol was performed according to *Pichia* Fermentation Process Guidelines Overview (Invitrogen, 2002). Single colonies were picked from the

MD plate and cultivated in flaks containing 10% of the initial fermentation volume at 30 °C, 220 rpm until OD = 2–6. Set the temperature to 30 °C, adjust the pH of the BSM to 5.0 with 28% ammonium hydroxide, agitation and aeration to operating conditions (maximum rpm and 1.0 vvm air). At this phase (phase I), the DO will continue to decrease because the growth of the strains will reduce the DO. Continue to culture the strains until the glycerol is completely consumed (18–24 h). This is indicated by an increase in the DO to 100%. Then, in phase II (glycerol fed-batch phase), a 50% w/v glycerol feed at a rate of 36.3 ml/h was performed. Then, a 2 h carbon-source starvation was carried out when the wet cell weight reached 250 g/L. In phase III (methanol induction phase), initiate the induction by starting a 100% methanol feed with a rate of 7.2 ml/h and the fermentation temperature was maintained at 25 °C for the DEX induction. Sorbitol was added at a concentration of 50 g/L in the methanol fed-batch phase (Azadi, Mahboubi, Naghdi, Solaimanian, & Mortazavi, 2017). *P. pastoris* would consume oxygen as it grows, reducing the dissolved oxygen content. In the phase III (methanol induction phase), methanol was added as an inducer and carbon source. Therefore, it is important to maintain the DO concentration at a certain level (>20%) to ensure the growth of *P. pastoris* on methanol because oxygen is required for the first step of methanol catabolism. The dissolved oxygen was kept higher than 20% through the monitoring of the methanol feed rate and agitation rate (the agitation rate was maintained at the maximum value of 900 rpm in this study).

3. Results

3.1. Construction of multicopy DEX in vitro

As depicted in Fig. 2a, plasmids with different DEX gene copy numbers were successfully constructed. All plasmids were digested with *Bgl* II and *Bam*H I to ensure the correct expression cassette (Fig. 2b), and *Sal* I single digestion was also carried out to ensure the correct length of the constructed plasmids (Fig. 2c).

When integrating heterologous genes into the genome, multiple integration events are possible in *P. pastoris*, with a probability of around 1% (Romanos, Scorer, Sreekrishna, & Clare, 1998). Besides, many articles report gene integration instability in *P. pastoris* (Zhu et al., 2009). Therefore, the multi-copy *in vitro* construction results are not consistent with the transformation results. Thus, it is necessary to ensure the DEX gene copy number after transformation into *P. pastoris*. The qPCR was performed to identify the copy number of DEX. As shown in Table 2, the 1-copy, 2-copy, and 3-copy were consistent with the *in vitro* construction as excepted, but the 4-copy turns out to be 7-copy.

3.2. The expression of DEX in strains with different DEX gene copy number

To assess the effect of copy number on DEX activity and the growth of *P. pastoris*, plasmids with different copy numbers of DEX were transformed into *P. pastoris*. The dry cell weight (DCW) and DEX activity time curve of different DEX gene copy number strains were measured. Compared with the control strain GS115, strains with different DEX gene copy numbers showed little difference in growth, and the DCW curves showed that the dry cell weight was still increasing at 168 h (Fig. S1). However, there were obvious differences in enzyme activity (Fig. 3a), and they all reached the maximum activity at 144h. Among them, the activity of the 1-copy and 2-copy strains was not significantly different, and their maximum activities were 40.13 U/mL and 39.06 U/mL, respectively. As the copy number continued to increase, secreted activity increased steadily. The activity of 7-copy strain showed the most significant increase with the DEX activity of 121.02 U/mL.

Table 2
The copy number of the DEX gene was determined by qPCR and the GAPDH gene was used as an internal reference.

Recombinant strain	Ct value		Copy number
	GAPDH	DEX	
1	16.39 ± 0.13	16.96 ± 0.03	1.41
2	15.24 ± 0.03	15.26 ± 0.04	2.01
3	15.82 ± 0.09	15.16 ± 0.09	3.22
4	15.6 ± 0.04	13.81 ± 0.19	6.93
5	15.21 ± 0.05	15.39 ± 0.05	2.31
6	16.62 ± 0.04	15.97 ± 0.05	3.25

To detect the difference in DEX protein expression level of different copy number strains, the supernatant of 144 h fermentation broth was taken for SDS-PAGE analysis. As shown in Fig. 3b, The DEX was successfully expressed in *P. pastoris* with a molecular weight of 71 kDa (Fig. 3b). The protein production of 1 and 2 copy strains are equivalent, as the copy number increases, the expression level increases accordingly, and the 7-copy strain reaches the highest. These results indicate that the efficient expression of DEX in *P. pastoris* by constructing multicopy strains is effective and could significantly enhance the DEX expression.

3.3. Detection of UPR-related genes

It is reported that the overexpression of heterologous proteins could induce the UPR (Lin et al., 2013). To verify the upregulation of the UPR. *HAC1*, *PDI1*, *ERO1* were selected as target genes to detect their expression levels during methanol induction. Compared with the control strain *P. pastoris* GS115, it was found that there were no notable alterations in the expression level of both *HAC1* and *PDI1*. However, the expression of the *ERO1* was significantly elevated in GS115-DEX and GS115-7DEX strains, respectively (Fig. 4). The up-regulated expression level of *ERO1* is related to the heavy secret loads of cells (Tu and Weissman 2004), thus in the follow-up experiments, the focus will be on engineering the secretion pathway to overcome the secretion bottleneck.

3.4. Inducible co-expression of molecular chaperones in low and high DEX copy strains

The aforementioned results showed that increasing the copy number could effectively improve the secretory expression level of DEX. However, large amounts of protein usually disrupt ER homeostasis (Walter & Ron, 2011). To enhance the expression of DEX in *P. pastoris*, molecular chaperones involved in protein translocation, folding and degradation were co-expressed with AOX1 promoter. Considering that gene dosage of DEX also affect the enhancing effect of molecular chaperone co-expression (Sallada et al., 2019), the plasmid pPICZA containing different molecular chaperones were transformed into *P. pastoris* GS115-DEX/GS115-7DEX which harboring 1-copy/7-copy of DEX, respectively. As shown in Fig. 5a and c, in the 1-copy group, results showed that only Ubc1 and Hrd1 got a slight increase of 9.38% and 12.73%. While in the 7-copy group, the co-expression of Ubc1 was significantly more effective than that of the 1-copy group and resulted in an increment of 36.16%. In addition, the Hac1 also showed a 28.69% enhancement of DEX secretory expression. There was no significant differences in the DCW of the 1-copy group, but all of the 7-copy group showed a decrease in DCW (Fig. 5d), with the highest degree of decrease in DCW caused by co-expression of Ubc1 at 27.12%. However, the DCW of Hac1 overexpressing strain, which also served to elevate the enzyme expression level, did not change significantly.

3.5. Constitutive co-expression of molecular chaperones in low and high DEX copy strains

The use of the same promoter for molecular chaperones and heterologous genes has been reported to cause a titration effect of transcription factors (TF). The presence of multiple targets for the same TF

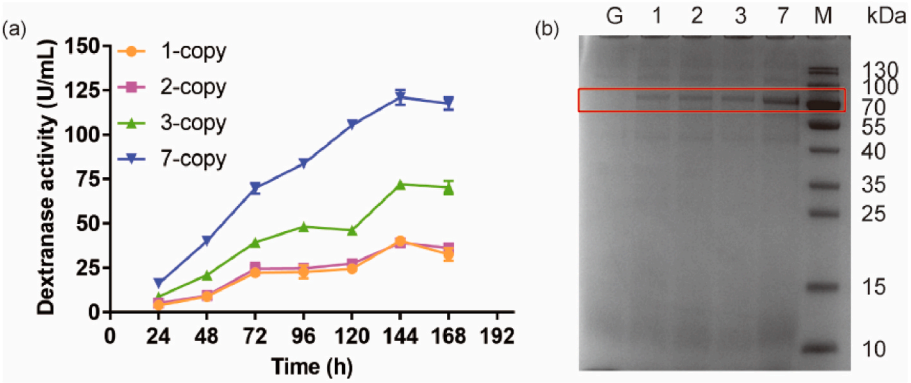

Fig. 3. The increment in copy number improves the production of DEX. (a) The time curve of DEX activity with different copy numbers. The DEX activity was 40.13 U/mL (GS115-DEX), 39.06 U/mL (GS115-2DEX), 72.02 U/mL (GS115-3DEX), 121.02 U/mL (GS115-7DEX). (b) SDS-PAGE at 144 h. M: Marker, G: *P. pastoris* GS115, 1: GS115-DEX, 2: GS115-2DEX, 3: GS115-3DEX, 4: GS115-7DEX.

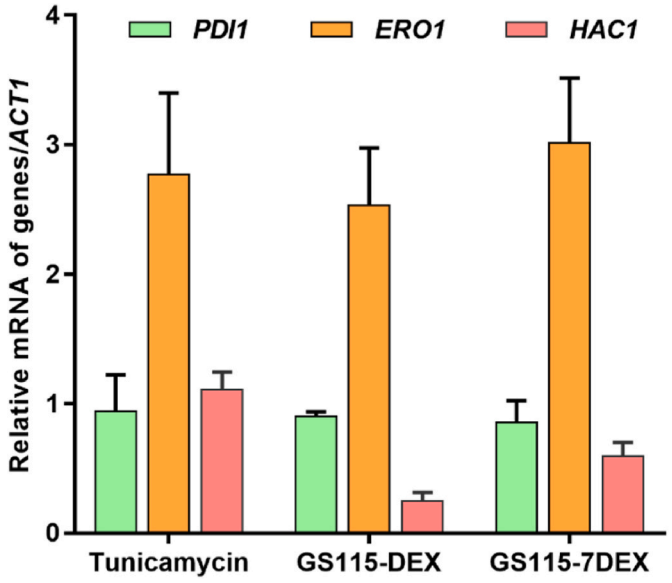

Fig. 4. The qPCR results of *PDI1*, *ERO1*, and *HAC1* compared with *P. pastoris* GS115. Tunicamycin treatment was the positive control group. No change in the expression of *PDI1* gene was observed, and *HAC1* was downregulated in both GS115-DEX and GS115-7DEX. All three groups showed an increase in ERO1 gene expression level.

could result in a competition that decreases the transcription of heterologous genes (Huang, Gao, Zhou, Zhang, & Cai, 2017). To avoid the titration effect, experiments were conducted on molecular chaperone co-expression under the regulation of the constitutive promoter GAP.

Similar to the co-expression results under the control of AOX1 promoter, the co-expression effect was apparently higher in the 7-copy group than in the 1-copy group, but the molecular chaperones that exerted positive effects were different. In the 1-copy group, the expression of molecular chaperones showed slight improvement on DEX activity, only Hac1 and Hrd1 overexpression demonstrated a 12.13% and 11.48% improvement respectively (Fig. 6a). In the 7-copy group, Hac1 and Hrd1 overexpression decreased the DEX activity of 7.42% and 9.72%. In addition, overexpression of Sly1, Sbh1, and Ssa4 showed 23.66%, 20.60%, and 30.36% elevation, respectively (Fig. 6c). No significant difference in DCW was observed in the 1- and 7-copy group (Fig. 6b and d), which was different from the inducible co-expression of molecular chaperones.

3.6. Fed-batch high cell density fermentation in 5-L fermenter

In the above work, the strain GS115-7DEX co-expression with Ubc1 under the control of AOX1 promoter exhibited the highest activity of 164.78 U/mL. In this work, fed-batch high cell density fermentation was applied to explore the growth and DEX expression ability of this strain. The DCW and activity continued to increase with the initiation of methanol induction. Maximum activity of 619.78 U/mL was achieved with a DCW of 105.37 g/L (Fig. 7a). The DEX activity in the 5-L

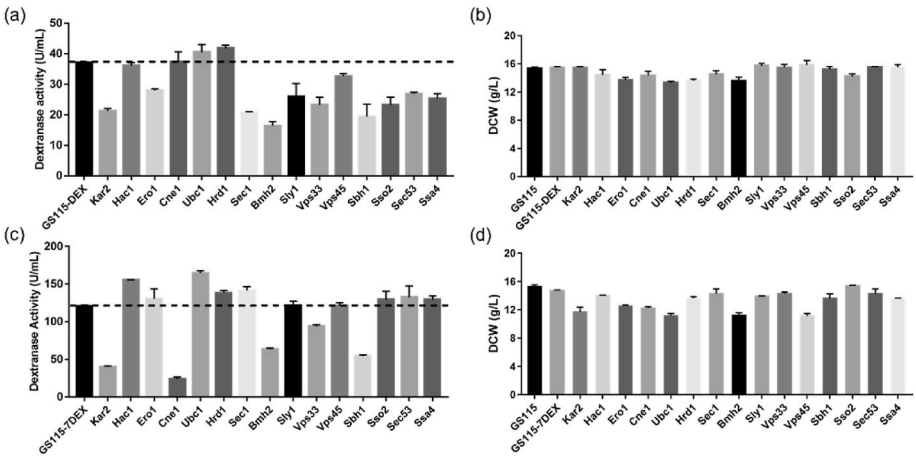

Fig. 5. Inducible co-expression of molecular chaperones in strains harboring 1 or 7 copies of DEX. The 144 h DEX activity of *P. pastoris* harboring 1-copy (a) and 7-copy (c) DEX gene. The 144 h DCW of 1-copy (b) showed slight differences. (d) The 144 h DCW of 7-copy exhibited a remarkable decrease.

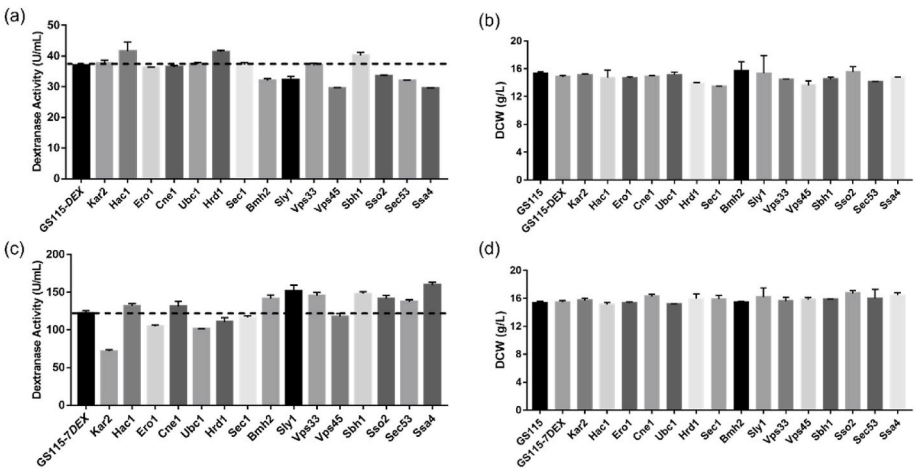

Fig. 6. Constitutive co-expression of molecular chaperones in strains harboring 1 or 7 copies of DEX. The 144 h DEX activity of *P. pastoris* harboring 1-copy (a) and 7-copy (c) DEX gene. The 144 h DCW of 1-copy (b) and 7-copy (d). Constitutive co-expressing molecular chaperone did not affect the growth of *P. pastoris*.

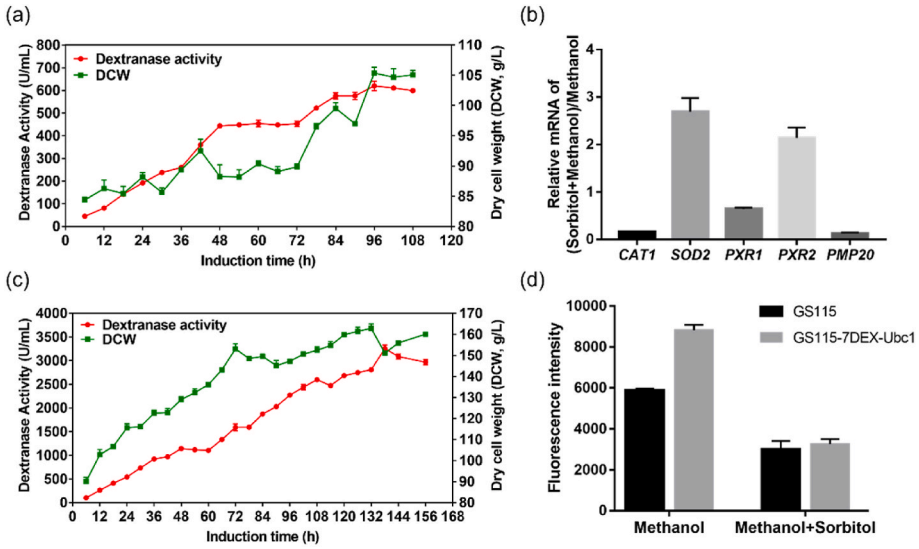

Fig. 7. 5-L scale-up fermentation of *P. pastoris* GS115-7DEX inducible co-expression with Ubc1. (a) DEX activity through methanol induction exhibited 619.78 U/mL at 96 h. (b) The expression of SOD2 and PXR2 exhibited 2.69- and 2.14- fold increments respectively during sorbitol co-feeding. (c) Sorbitol co-feeding resulted in a 5.26-fold improvement and a DEX activity of 3257.23 U/mL at 138 h was obtained. (d) The intracellular ROS of GS115-7DEX-Ubc1 decreased by 68.83% when sorbitol co-feeding was introduced.

fermenter is 3.76 times than that of the shake flask fermentation. Moreover, it takes 96 h to reach the highest activity, which is 48 h earlier than the shake flask fermentation.

It is reported that the co-feeding strategy could considerably enhance the production of recombinant proteins (Liu et al., 2019). As a non-repressing carbon source, the sorbitol co-feeding strategy was tested to increase the expression of DEX. By co-feeding sorbitol in the concentration of 50 g/L during methanol induction. The DEX production was significantly improved, and the maximum activity was 3257.23 U/mL (Fig. 7c), which was 5.25-fold of that in the methanol induction and 20.4-fold than the shake flask fermentation. It is worth noting that the DCW maintained increase in the late fermentation stage. Under the sorbitol co-feeding strategy, DCW at 138 h was 151.05 g/L, which is 1.43-fold than inducing expression using methanol alone. The DCW increased by 67% during the entire induction period of the sorbitol co-feeding, which exceeded 24% than that of the methanol feeding alone.

Methanol is oxidized to formaldehyde in *P. pastoris* and releases the ROS, thus leading to the elevation of ROS levels. High concentrations of ROS were toxic to the strains (Innokentev & Kanki, 2021). This study found that the promotion of sorbitol on the growth of *P. pastoris* was associated with a decrease in ROS. The expression of genes related to antioxidant (*CAT1*, *SOD2*, *PXR1*, *PXR2* and *PMP20*) was determined by qPCR. It was found that the *SOD2* gene encoding superoxide dismutase and *PXR2* gene encoding mitochondrial peroxidase were upregulated (Fig. 7b). In addition, DCFH-DA staining showed a 68.83% decrease in intracellular ROS when sorbitol was co-supplemented (Fig. 7d).

4. Discussion

In this study, a recombinant *P. pastoris* GS115 harboring 7 copies of DEX was constructed. It was found that DEX-expressing strains showed significant ER stress. To relieve this stress, a series of chaperone proteins were co-expressed. In addition, the scale-up fermentation in a 5-L fermenter was conducted to pave the way for the industrialization of DEX expression with *P. pastoris*.

Through the construction of plasmids with different copies of DEX, *P. pastoris* with 1-copy, 2-copy, 3-copy and 7-copy of DEX was constructed. *In vitro* multi-copy construction does not require tedious screening work but results in the consequences of oversized plasmids. Previous studies have shown that when the copy number is less than 6, the yeast would have a stable copy number, but once the copy number exceeds 6, it would become unstable (Zhu et al., 2009). Therefore, the 7 copies of DEX was attributed to the occurrence of multiple integrations,

and the oversized plasmid which makes this integration unstable. In fact, the construction of subsequent 5 or 6-copy plasmids was also tried (Fig. S2). However, it was found that copy number was inconsistent with the *in vitro* construction results, only a very low copy number strains were obtained (Table 2). This indicates that the integration efficiency would decrease with the increase of plasmid length when multi-copy construction is performed *in vitro*, which is also consistent with previous research (Lin-Cereghino et al., 2008). In addition, the DEX activity was improved significantly with the increase of gene copy number. This is consistent with previous reports that increasing the copy number is an effective way to improve the expression of heterologous proteins in *P. pastoris* (Shen et al., 2020; Shu et al., 2016). When the heterologous gene was in a low copy number (1 or 2), it seems that the enhancement of heterologous protein expression is not effective (He et al., 2020; Shen, Wu, Wang, Naranmandura, & Chen, 2012). The same results are also shown in this study, the yield of DEX does not increase until the number of copies continues to increase (greater than 3).

Overexpression of heterologous protein often saturates the secretory pathway, hence triggering the UPR (Huang et al., 2017). So, the expression level of genes relevant to the UPR was examined. The expression level of *ERO1* in strains expressing heterologous proteins and tunicamycin treatment was found to be higher than the control strain. The elevated *ERO1* expression level was associated with increased ROS due to excessive ER secretion burden (Harding et al., 2003). To overcome this bottleneck, the co-expression of proteins related to the secretory pathway was investigated.

This study gave a comprehensive understanding on the effect of molecular chaperones co-expression. It was found that the co-expression of molecular chaperones was more effective when the DEX gene was in 7 copies. Similar results have been reported that molecular chaperones co-expression is more effective in *P. pastoris* encountering higher folding stress (Huang et al., 2020). In addition to this, the effect of chaperones co-expression also varies with the control of different promoters. Protein secretion initiates with the crossing of the ER membrane. In this process, the Sec complex catalyzes the translocation of proteins into the ER (Delic et al., 2013). Constitutive co-expression of the Sbh1 which is the subunit of Sec61, increased the expression of DEX by 20.60%. The cytoplasmic Hsp70 protein Ssa4 plays important roles in protein quality control (Andersson et al., 2021) and the transport of target nascent proteins to the ER membrane (Gasser et al., 2007). The constitutive co-expressing of Ssa4 resulted in a 30.36% enhancement of DEX production. The proteins which help heterologous proteins to fold, form disulfide bonds and glycosylation: Cne1, Ero1 and Sec53 display negligible effects on DEX production. These results suggest that it is possible that the protein enters the ER, rather than folding in the ER, which becomes the bottleneck of secretion in *P. pastoris*. The UPR which regulates the ER homeostasis in *P. pastoris* was activated by a transcription factor Hac1. In this work, inducible co-expression of Hac1 leads to the improvement of DEX by 28.69% while continuous co-expression of Hac1 shows a slight effect (7.42%). Continuous expression of Hac1 consistently activates UPR, which may not be necessary to increase heterologous protein expression (Guerfal et al., 2010). To reduce the load pressure of ER, inducible co-expression of ubiquitin-conjugating enzyme Ubc1 which is responsible for the degradation of misfolded proteins (Hwang and Qi 2018), was performed and an obvious improvement on DEX activity was obtained. Proteins correctly folded in the ER need to be transported into the Golgi apparatus through vesicles and eventually secreted out of the cell. Thus, the constitutive expression of SM protein Sly1, which participates in the fusion of the vesicle at the membrane (Kosodo et al., 2002), resulted in an improvement of 23.66% on DEX production. Proteins related to the vesicular trafficking, Bmh2 and Sso2 were co-expressed in this study. It is worth noting that when co-expressed with AOX1 promoter, Bmh2 decreased its activity by 47.58%, while Sso2 showed a slightly increase (7.1%). However, when constitutive co-expression was performed, both showed an increase of more than 10% (15.48% and 15.57% respectively). The difference may be attributed to the titration effect (Huang et al., 2017).

Furthermore, efficient expression of DEX in *P. pastoris* with BSM via high cell density fermentation was achieved. Compared with methanol, a drastic change was observed when employing the sorbitol co-feeding strategy. The DCW was 1.43-fold than the methanol feed alone. By enhancing the expression of ROS resistance genes, sorbitol co-feeding resulted in a significant decrease of intracellular ROS (68.83%), which alleviated the damage caused by ROS and led to the enhancement of cell viability and DCW. In addition, sorbitol co-feeding in the induction phase could reduce the consumption of oxygen (Azadi et al., 2017) and high dissolved oxygen is reported to be beneficial to the expression of heterologous proteins (Jia et al., 2018).

In conclusion, an engineered *P. pastoris* with high DEX production was successfully constructed in this work. This study suggested that increasing gene dosage could improve the high DEX expression in *P. pastoris*. In addition, the production of DEX was further enhanced by the comprehensive study on chaperones co-expression. This study also demonstrates, for the first time, that chaperones co-expression is more effective in DEX high-copy strains. On this basis, a 5-L scale-up fermentation was further carried out and the DEX activity of 3257.23 U/mL with a DCW of 151.05 g/L was achieved. It is believed that strategies applied in DEX high level expression could also facilitate the application of *P. pastoris* in the production of other proteins.

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CRediT authorship contribution statement

Chao Liu: Experiments, Methodology, Writing – original draft. **Jin-Song Gong:** Supervision, Writing – review & editing. **Chang Su:** Formal analysis, Software. **Heng Li:** Formal analysis. **Jiufu Qin:** Supervision, Methodology, Writing – review & editing. **Zheng-Hong Xu:** Project administration, Writing – review & editing. **Jin-Song Shi:** Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.114753.

References

Azadi, S., Mahboubi, A., Naghdi, N., Solaimanian, R., & Mortazavi, S. A. (2017). Evaluation of sorbitol-methanol Co-feeding strategy on production of recombinant human growth hormone in *Pichia pastoris*. *Iranian Journal of Pharmaceutical Research, 16*(4), 1555–1564.

Becker, D. M., & Guarente, L. (1991). High-efficiency transformation of yeast by electroporation. *Methods in Enzymology, 194*, 182–187.

Berrios, J., Flores, M. O., Díaz-Barrera, A., Altamirano, C., Martínez, I., & Cabrera, Z. (2017). A comparative study of glycerol and sorbitol as co-substrates in methanol-induced cultures of *Pichia pastoris*: Temperature effect and scale-up simulation. *Journal of Industrial Microbiology & Biotechnology, 44*(3), 407–411.

Dragosits, M., Mattanovich, D., & Gasser, B. (2011). Induction and measurement of UPR and osmotic stress in the yeast *Pichia pastoris*. *Methods in Enzymology, 489*, 165–188.
Duan, G., Ding, L., Wei, D., Zhou, H., Chu, J., Zhang, S., et al. (2019). Screening endogenous signal peptides and protein folding factors to promote the secretory expression of heterologous proteins in *Pichia pastoris*. *Journal of Biotechnology, 306*, 193–202.
Dwibedi, V., Rath, S. K., Jain, S., Martínez-Argueta, N., Prakash, R., Saxena, S., et al. (2023). Key insights into secondary metabolites from various *Chaetomium* species. *Applied Microbiology and Biotechnology, 107*(4), 1077–1093.
Ergün, B. G., Berrios, J., Binay, B., & Fickers, P. (2021). Recombinant protein production in *Pichia pastoris*: From transcriptionally redesigned strains to bioprocess optimization and metabolic modelling. *FEMS Yeast Research, 21*(7), foab057.
Fauzee, Y., Taniguchi, N., Ishiwata-Kimata, Y., Takagi, H., & Kimata, Y. (2020). The unfolded protein response in *Pichia pastoris* without external stressing stimuli. *FEMS Yeast Research, 20*(7), Article foaa053.
Graf, A., Gasser, B., Dragosits, M., Sauer, M., Leparc, G. G., Tüchler, T., et al. (2008). Novel insights into the unfolded protein response using *Pichia pastoris* specific DNA microarrays. *BMC Genomics, 9*, 390.
Harding, H. P., Zhang, Y., Zeng, H., Novoa, I., Lu, P. D., Calfon, M., et al. (2003). An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Molecular Cell, 11*(3), 619–633.
He, H., Wu, S., Mei, M., Ning, J., Li, C., Ma, L., et al. (2020). A combinational strategy for effective heterologous production of functional human lysozyme in *Pichia pastoris*. *Frontiers in Bioengineering and Biotechnology, 8*, 118.
Huang, J., Zhao, Q., Chen, L., Zhang, C., Bu, W., Zhang, X., & Yang, Z. (2020). Improved production of recombinant Rhizomucor miehei lipase by coexpressing protein folding chaperones in *Pichia pastoris*, which triggered ER stress. *Bioengineered, 11*(1), 375–385.
Huang, M., Gao, Y., Zhou, X., Zhang, Y., & Cai, M. (2017). Regulating unfolded protein response activator HAC1p for production of thermostable raw-starch hydrolyzing α-amylase in *Pichia pastoris*. *Bioprocess and Biosystems Engineering, 40*(3), 341–350.
Innokentev, A., & Kanki, T. (2021). Mitophagy in yeast: Molecular mechanism and regulation. *Cells, 10*(12), 3569.
Invitrogen. (2002). *Pichia fermentation process guidelines*. Retrieved from https://tools.thermofisher.cn/content/sfs/manuals/pichiaferm_prot.pdf/. (Accessed 6 January 2022).
Jia, L., Gao, M., Yan, J., Chen, S., Sun, J., Hua, Q., et al. (2018). Evaluation of the sub-optimal induction strategies for heterologous proteins production by *Pichia pastoris* Mut(+)/Mut(S) strains and related transcriptional and metabolic analysis. *World Journal of Microbiology and Biotechnology, 34*(12), 180.
Jiao, L., Zhou, Q., Su, Z., Xu, L., & Yan, Y. (2018). High-level extracellular production of *Rhizopus oryzae* lipase in *Pichia pastoris* via a strategy combining optimization of gene-copy number with co-expression of ERAD-related proteins. *Protein Expression and Purification, 147*, 1–12.
Juntarachot, N., Kantachote, D., Peerajan, S., Sirilun, S., & Chaiyasut, C. (2020). Optimization of fungal dextranase production and its antibiofilm activity, encapsulation and stability in toothpaste. *Molecules, 25*(20), 4784.
Khalikova, E., Susi, P., & Korpela, T. (2005). Microbial dextran-hydrolyzing enzymes: fundamentals and applications. *Microbiology and Molecular Biology Reviews, 69*(2), 306–325.
Lin, X. Q., Liang, S. L., Han, S. Y., Zheng, S. P., Ye, Y. R., & Lin, Y. (2013). Quantitative iTRAQ LC-MS/MS proteomics reveals the cellular response to heterologous protein overexpression and the regulation of HAC1 in *Pichia pastoris*. *Journal of Proteomic, 91*, 58–72.
Lin-Cereghino, J., Hashimoto, M. D., Moy, A., Castelo, J., Orazem, C. C., Kuo, P., et al. (2008). Direct selection of *Pichia pastoris* expression strains using new G418 resistance vectors. *Yeast, 25*(4), 293–299.
Liu, W. C., Inwood, S., Gong, T., Sharma, A., Yu, L. Y., & Zhu, P. (2019). Fed-batch high-cell-density fermentation strategies for *Pichia pastoris* growth and production. *Critical Reviews in Biotechnology, 39*(2), 258–271.
Lõoke, M., Kristjuhan, K., & Kristjuhan, A. (2011). Extraction of genomic DNA from yeasts for PCR-based applications. *Biotechniques, 50*(5), 325–328.
Martínez, D., Menéndez, C., Chacón, O., Fuentes, A. D., Borges, D., Sobrino, A., et al. (2021). Removal of bacterial dextran in sugarcane juice by *Talaromyces minioluteus* dextranase expressed constitutively in *Pichia pastoris*. *Journal of Biotechnology, 333*, 10–20.
Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry, 31*(3), 426–428.
Pan, Y., Yang, J., Wu, J., Yang, L., & Fang, H. (2022). Current advances of *Pichia pastoris* as cell factories for production of recombinant proteins. *Frontiers in Microbiology, 13*, Article 1059777.
Perrone, G., & Susca, A. (2017). *Penicillium* species and their associated mycotoxins. *Methods in Molecular Biology, 1542*, 107–119.
Roca, H., Garcia, B., Rodriguez, E., Mateu, D., Coroas, L., Cremata, J., et al. (1996). Cloning of the *Penicillium minioluteum* gene encoding dextranase and its expression in *Pichia pastoris*. *Yeast, 12*(12), 1187–1200.
Romanos, M., Scorer, C., Sreekrishna, K., & Clare, J. (1998). The generation of multicopy recombinant strains. *Methods in Molecular Biology, 103*, 55–72.
Sallada, N. D., Harkins, L. E., & Berger, B. W. (2019). Effect of gene copy number and chaperone coexpression on recombinant hydrophobin HFBI biosurfactant production in *Pichia pastoris*. *Biotechnology and Bioengineering, 116*(8), 2029–2040.
Shen, Q., Wu, M., Wang, H. B., Naranmandura, H., & Chen, S. Q. (2012). The effect of gene copy number and co-expression of chaperone on production of albumin fusion proteins in *Pichia pastoris*. *Applied Microbiology and Biotechnology, 96*(3), 763–772.
Shen, Q., Yu, Z., Lv, P. J., Li, Q., Zou, S. P., Xiong, N., et al. (2020). Engineering a *Pichia pastoris* nitrilase whole cell catalyst through the increased nitrilase gene copy number and co-expressing of ER oxidoreductin 1. *Applied Microbiology and Biotechnology, 104*(6), 2489–2500.
Shu, M., Shen, W., Yang, S., Wang, X., Wang, F., Wang, Y., et al. (2016). High-level expression and characterization of a novel serine protease in *Pichia pastoris* by multi-copy integration. *Enzyme and Microbial Technology, 92*, 56–66.
Walter, P., & Ron, D. (2011). The unfolded protein response: From stress pathway to homeostatic regulation. *Science, 334*(6059), 1081–1086.
Yadav, D., Ranjan, B., McHunu, N., Le Roes-Hill, M., & Kudanga, T. (2021). Enhancing the expression of recombinant small laccase in *Pichia pastoris* by a double promoter system and application in antibiotics degradation. *Folia Microbiologica, 66*(6), 917–930.
Yang, Z., & Zhang, Z. (2018). Engineering strategies for enhanced production of protein and bio-products in *Pichia pastoris*: A review. *Biotechnology Advances, 36*(1), 182–195.
Zahrl, R. J., Mattanovich, D., & Gasser, B. (2018). The impact of ERAD on recombinant protein secretion in *Pichia pastoris* (*syn Komagataella* spp.). *Microbiology (Reading), 164*(4), 453–463.
Zhao, J., Wang, L., Wei, X., Li, K., & Liu, J. (2021). Food-grade expression and characterization of a dextranase from *Chaetomium gracile* suitable for sugarcane juice clarification. *Chemistry and Biodiversity, 18*(1), Article e2000797.
Zhu, T., Guo, M., Sun, C., Qian, J., Zhuang, Y., Chu, J., et al. (2009). A systematical investigation on the genetic stability of multi-copy *Pichia pastoris* strains. *Biotechnology Letters, 31*(5), 679–684.