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Original Research Article

Combined strategies for improving the heterologous expression of a novel xylanase from *Fusarium oxysporum* Fo47 in *Pichia pastoris*

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ABSTRACT

Xylanase, an enzyme capable of hydrolyzing non-starch polysaccharides found in grain structures like wheat, has been found to improve the organizational structure of dough and thus increase its volume. In our past work, one promising xylanase FXYL derived from *Fusarium oxysporum* Fo47 and first expressed 779.64 U/mL activity in *P. pastoris*. It has shown significant potential in improving the quality of whole wheat bread, making it become a candidate for development as a new flour improver. After optimization of expression elements and gene dose, the xylanase activity of FXYL strain carrying three-copies reached 4240.92 U/mL in *P. pastoris*. In addition, 12 factors associated with the three stages of protein expression pathway were co-expressed individually in order in three-copies strain, and the translation factor Pab1 co-expression increased FXYL activity to 8893.53 U/mL. Nevertheless, combining the most effective or synergistic factors from three stages did not exhibit better results than co-expressing them alone. To further evaluate the industrial potential, the xylanase activity and protein concentration reached 81184.51 U/mL and 11.8 g/L in a 5 L fed-batch fermenter. These engineering strategies improved the expression of xylanase FXYL by more than 104-fold, providing valuable insights for the cost-effective industrial application of FXYL in the baking field.

1. Introduction

Xylanases are hydrolytic enzymes which randomly cleave the β-1,4-glucoside bond in the xylan skeleton and display varying physico-chemical characteristics, substrate specificities, hydrolytic activities (yields, rates and products) [1,2]. Many xylanases has exhibited excellent market potential in various industries, including food baking, paper pulping, and animal feed [3]. In the baking field, xylanase is supposed to substitute chemical additives when used at optimum levels for it enhances the organizational structure of fermented dough, making the flour products more fluffy and improving its sensory properties [4]. Thus, there is an increasing trend in baking industry towards the application of xylanases in bread or dough production.

In our past work, xylanase FXYL (GenBank: EWZ46984.1) derived from *F. oxysporum* Fo47 with an optimum pH of 5.0 and a temperature of 45 °C were found to be suitable for the acidic environment of dough fermentation [5]. When it was applied to whole wheat bread, the loaf volume was increased by 13.06%, and hardness was reduced by 32.20%. Compared with other xylanases applied in bread baking from *Streptomyces* sp. [6], *Aspergillus niger* [7] and *Plectosphaerella cucumerina* [8], FXYL showed outstanding performance on improving bread quality and is expected to develop into a new type of flour improver. However, FXYL production with as low as 779.64 U/mL at present could not meet the requirements of industrial level and commercialization demand, apparently.

P. pastoris is known as one of the most important workhorse to produce heterologous proteins in industry [9]. It has successfully expressed more than 5000 proteins and over 70 protein products have been launched into the market [10]. With the rapid advancements of its genetic manipulation, many strategies encompassing the optimization of promoters as well as signal peptides [11,12], enhancement of gene dosage [13,14] could be simple and easy to be employed to augment enzymes production capacity. Boer *et al.* revealed that growth rate of the GS115-Cel7A^{GAP} strain with GAP (glyceraldehyde-3-phosphatedehydrogenase) promoter on glycerol was significantly lower than that of GS115-Cel7A^{AOX1} driven by the AOX1 (alcohol oxidase) promoter when *Trichoderma reesei* cellobiohydrolase Cel7A were heterologous expression in *P. pastoris* in a fermenter [15]. Paifer *et al.* reported that

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ɑ-amylase secretion increased from 500 REU/mL to 1600 REU/mL when fused with the *SUC2* gene signal sequence from *Saccharomyces cerevisiae* compared with its own signal sequence in *P. pastoris* [16]. In addition to the above, gene dosage optimization with multimerization approach in vitro [17] or CRISPR/Cas9 mediated genome editing [18] were complemented to achieve the protein high-yield secretory production. Nevertheless, it may surpass the original capacity of the ER and obstruct the normal secretion when the gene dosage is increased [19]. In this case, co-expression of transcription factors or molecular chaperones are recognized as promising approach to solve the bottlenecks, for example, co-expression of HAC1 (one of unfolded protein response transcription factors) and PDI (protein disulfide isomerase) has been proven to enhance the secretion of heterodimeric Fab fragments by 1.3- and 1.9-fold, respectively [20].

In this study, the expression elements including promoters and signal peptides as well as gene dose for high-yield secretory production of xylanase FXYL in *P. pastoris* were optimized step by step. Subsequently, 12 factors related to protein translation, ER folding processing and ER-Golgi vesicle trafficking were employed individually in order in multi-copy strains. In addition, the optimal factors from different stages of expression pathway are further recombined in multi-copy strains and their synergistic effect was investigated for the first time. After all optimizations were completed, the final engineered strain was evaluated for xylanase FXYL production performance and the three-phase fed-batch fermentation in a 5-L fermenter was implemented. The result demonstrated high-density fermentation in fed-batch bioreactors enabled the secretory production of FXYL at high levels and makes FXYL promising for commercial applications, especially for baking industry.

2. Materials and methods

2.1. Strains, reagents and culture medium

Escherichia coli TOP10 (Invitrogen) was used for plasmid construction and amplification. *P. pastoris* X33 (Invitrogen) was used as the host strain to express xylanase and other protein. The *P. pastoris* recombinant strain F1/pPICZɑA-*FXYL* was constructed and preserved by our laboratory for the expression of xylanase FXYL [5]. The restriction endonucleases and zeocin antibiotic were bought from Thermo Fisher Scientific (Shanghai, China). Hygromycin B antibiotic was purchased from Macklin (Shanghai, China). DNA Ligation Kit and TB Green® Premix Ex Taq™ II were purchased from TaKaRa Biotechnology Co. (Dalian, China). Beechwood xylan was purchased from Sigma-Aldrich (St. Louis, MO, United States). *E. coli* recombinants were grown at 37 °C in Luria-Bertani (LB) or low salt Luria-Bertani (LBL) medium, and zeocin or hygromycin B was appropriately added. *P. pastoris* recombinants were cultivated in yeast extract peptone dextrose (YPD) medium with appropriate antibiotics at 30 °C. BMGY medium (1% yeast extract, 2% peptone, 1.34% YNB, 0.1 mM sodium phosphate buffer pH 6.0, and 1% glycerol) and BMMY medium (1% yeast extract, 2% peptone, 1.34% YNB, 0.1 mM sodium phosphate buffer pH 6.0, and 1% methanol) were used as seed solution and fermentation medium, respectively. Basal salts medium (BSM) (1.49% $MgSO_4{\cdot}7H_2O$, 0.094% $CaSO_4$, 1.82% K_2SO_4, 0.413% KOH, 2.67% H_3PO_4, 4% glycerol, and 0.435% PTM1) and the trace metal solution PTM1 (0.6% $CuSO_4{\cdot}5H_2O$, 0.008% NaI, 2% $ZnCl_2$, 0.3% $MnSO_4{\cdot}H_2O$, 0.02% $Na_2MoO_4{\cdot}2H_2O$, 0.002% H_3BO_3, 0.05% $CoCl_2$, 0.02% Biotin, and 6.5% $FeSO_4{\cdot}7H_2O$) were used in 5-L fed-batch fermentation.

2.2. Construction of recombinant plasmids

The strains and plasmids used or constructed in this work are listed in Table S1, primers are listed in Table S2. To optimize the expression elements of FXYL, pre-Ost1 signal peptide from *S. cerevisiae* was used to replace the pre-α signal peptide in the pPICZɑA-*FXYL* (primers preOst1-F and preOst1-R). On this basis, *AOXm* mutant promoter was used to replace the *AOX1* promoter (primers AOXm-F and AOXm-R). Here, the gene of *His4* was inserted into the plasmid to make it as an integration site in the yeast genome (primers His4-F and His4-R). Then the plasmids PHZOA-*FXYL* and PHZOAm-*FXYL* were constructed. The single-copy plasmid PHZOAm-*FXYL* was digested by isocaudamer *Bgl* II and *Bam*H I to release the FXYL expression cassette. Next, the FXYL expression cassette was ligated into the PHZOAm-*FXYL* at *Bam*H I site to obtain new recombinant plasmid PHZOAm-2*FXYL* which carrying two copies of FXYL gene. This procedure was repeated to obtained plasmids PHZOAm-3*FXYL* and PHZOAm-4*FXYL*. Subsequently, for plasmid pGAPZA, the antibiotic zeocin was replaced by hygromycin B (primers HygR-F/R) and *His4* by *Int12* integration site (primers Int12-F/R) to obtained the plasmid pGAPHI. The yeast protein expression pathway related genes *eIF4A*, *eIF4E*, *eIF4G*, *Pab1*, *Rli1*, *HAC1*, *FHL1*, *PDI1* were amplified using primers pairs eIF4A-F/R, eIF4E-F/R, eIF4G-F/R, Pab1-F/R, Rli1-F/R, HAC1-F/R, FHL1-F/R, PDI1-F/R with the genomic DNA of *P. pastoris* as the template, respectively. With the *S. cerevisiae* genomic DNA as the template, transcription/vesicle factors genes *MSN4*, *GLO3*, *GCS1* were amplified by primers pairs, MSN4-F/R, GLO3-F/R, GCS1-F/R. *P180* (N-terminal) is stored in our laboratory and constructed into pGAPHI using primers P180–F/R. Through homologous recombination, above factors genes could be inserted into the pGAPHI vector, and obtained 12 recombinant plasmids pGAPHI-*eIF4A*, pGAPHI-*eIF4E*, pGAPHI-*eIF4G*, pGAPHI-*Pab1*, pGAPHI-*Rli1*, pGAPHI-*HAC1*, pGAPHI-*P180*, pGAPHI-*PDI1*, pGAPHI-*FHL1*, pGAPHI-*MSN4*, pGAPHI-*GLO3*, pGAPHI-*GCS1*. Then, the factors gene expression cassettes that may have synergistic effects or the best effect in different stages of expression pathway, were combined in pGAPHI vector for co-expression. The factors related to the heterologous protein expression pathway in yeast applied in this study are listed in Table 1.

Table 1
The factors related to protein expression pathway applied in this study.

Type	Name	Origin	Characteristic	GenBank	Reference
Translation factors	eIF4Ap	*P. pastoris*	DEAD-box RNA helicase	CCA39568.1	[21]
	eIF4Ep	*P. pastoris*	mRNA cap binding protein	CCA39169.1	[21]
	eIF4Gp	*P. pastoris*	Scaffold protein	CCA36511.1	[21]
	Pab1p	*P. pastoris*	Interacting with the 3′poly(A) tail of the mRNA	CCA38887.1	[21]
	Rli1p	*P. pastoris*	Multifunctional ABC-family protein	CCA38888.1	[21]
Transcription factors	HAC1p	*P. pastoris*	Transcription factor of UPR-responsive	AOA66916.1	[22]
	P180p (N-terminal)	Canis	A membrane-bound protein that mediates mRNA anchoring to ER	X87224.1	[23,24]
	FHL1p	*P. pastoris*	Activator of ribosome biosynthesis processing	CAY71926.1	[25]
	MSN4p	*S. cerevisiae*	A zinc finger protein that regulates various stress responses, including HSR	WNV94252.1	[26]
Chaperone	PDI1p	*P. pastoris*	Protein disulfide isomerases	AOA70013.1	[27]
Vesicle trafficking factors	GLO3p	*S. cerevisiae*	ADP-ribosylation factor GTP activating proteins in the process of COPI-coated vesicle formation	WNV72558.1	[28]
	GCS1p	*S. cerevisiae*		WNF19576.1	[28]

2.3. Construction of recombinant strains

The constructed recombinant plasmids were linearized and electrically transformed into *P. pastoris* X33. The recombinant strains integrating plasmids PHZOA-*FXYL* and PHZOAm-*FXYL* were referred to as strain F2 and F3, respectively. After that, the plasmids carrying different FXYL gene copy was linearized and integrated the genome of F3, resulted in recombinant strains 2F3, 3F3 and 4F3 containing two to four copies were obtained, respectively. Expression element optimization and multi-copy recombinant strains were screened using a YPDZ plate supplemented with antibiotics zeocin (100 μg/mL). The recombinant strains with the best copy number were generally used as chassis cells for co-expressing secretory pathway-related factors, and the co-expressed recombinant strains were screened using YPDH plates supplemented with hygromycin B antibiotic (750 μg/mL). In addition, to ensure the presence of the expression plasmid in the chassis cells, the spot-plate operation is also performed at the same location in the YPDZ plates when constructing co-expression recombinant strains. After being cultivated at 30 °C for approximately 3 days, colony PCR was performed to identify the positive transformants by primers FXYL-F and FXYL-R.

2.4. Shake flask cultures

Recombinant strains expressing xylanase FXYL were successfully constructed and identified, then positive strains were selected and inoculated into 10 mL BMGY medium, cultured about 24 h at 30 °C and 250 rpm. After the expression strain accumulated to a certain growth density in BMGY, it was transferred to 25 mL BMMY medium with $OD_{600} = 1$. The cultures were incubated in BMMY at 30 °C at 250 rpm for 5 days, 1% methanol was added daily, and 1 mL of fermentation broth was sampled to measure the growth density and enzyme activity.

2.5. Fed-batch fermentation

Fed-batch fermentation was performed in a 5-L fermenter according to the reference protocol in the "Pichia Fermentation Process Guidelines" (Invitrogen). The single activated strain was inoculated into 10 mL YPD medium at 30 °C with shaking at 250 rpm for 24 h. Next, 4% of the seed solution was transferred to 160 mL YPD medium and cultured under the same conditions for about 20 h. Eventually, 8% of the seed solution was inoculated into BSM in a 5-L fermenter. Fed-batch fermentation mainly includes three phases: glycerol batch fermentation, glycerol feed culture and methanol feed culture. The initial culture temperature was set at 30 °C, and the glycerol in BSM was depleted at the end of the first phase. Glycerol feed culture was started by feeding 50% (w/v) glycerol containing 1.2% (v/v) PTM1 at a flow rate of 20–48 g/h, and it was stopped until the OD_{600} was increased to 200–300. Afterwards, the cells were maintained in a hungry state for about 1 h and the culture was transitioned to the methanol feed phase. The temperature was adjusted to 25 °C and methanol solution containing 1.2% (v/v) PTM1 was fed at a speed of 5–20 g/h. Samples of fermentation solution were taken every 12 h for determination of OD_{600}, enzyme activity and protein concentration. During the fermentation process, the pH was maintained at 5.5 by adding ammonia solution (25%, v/v). The dissolved oxygen (DO) in the fermenter was maintained at about 20%–30% by adjusting the stirring speed (1200 rpm) and the airflow rate (4 L/min).

2.6. Xylanase activity determination

Xylanase activity was determined via 3,5-dinitrosalicylic acid (DNS) colorimetric method. The fact that xylanase can hydrolyze xylan (from Beechwood) to produce reducing sugar and develop color after co-heating with DNS [29]. Briefly, 10 μL of properly diluted enzyme solution was mixed with 90 μL PBS buffer (pH 5.0), then added 100 μL 1% xylan substrate. After reaction at 45 °C for 10 min, 300 μL DNS was immediately added and then boiled the solution for 5 min to terminate the reaction. When cooled to room temperature, the absorbance was measured at 540 nm utilizing a microplate reader (Gene Com. Ltd., Hong Kong, China). According to the tested solution absorbance, the total reducing sugar concentration in the sample can be calculated from the xylose standard curve, and then the xylanase activity was determined. Under the above determination conditions, the amount of enzyme required to generate 1 μmoL reducing sugar by hydrolyzing substrate per minute is defined as an enzyme activity unit (U/mL).

3. Results and discussion

3.1. Optimization of gene expression elements for FXYL expression

The predominant promoter and signal peptide (SP) combination for protein expression in *P. pastoris* is typically P_{AOX1} and the α-mating factor (α-MF) prepro-leader from *S. cerevisiae*. However, it cannot be definitively asserted as the optimal expression pairing for all proteins [30–32]. Hartner et al. created a promoter library by deleting and replicating putative transcription factor binding sites within the P_{AOX1} sequence [33]. P_{AOXm}, an enhanced variant, results from deleting the −777 to −721 regions of P_{AOX1} and contributing a copy of the *cis*-acting elements from the P_{AOX1}'s −230 to −190 regions. This modification augments the expression of foreign proteins in *P. pastoris*. Except for the promoter, efficient protein secretion in *P. pastoris* necessitates the incorporation of an appropriate secretion signal at the N-terminal of the target protein. It has been reported that by replacing the pre-region of the α-MF with the pre-Ost1 signal sequence (SP_{Ost1} from *S. cerevisiae*), the lipase BTL2 production was increased 3-fold [34]. Consequently, substituting P_{AOXm} and $SP_{pre\text{-}Ost1}$ may be a preferable choice for xylanase expression elements in *P. pastoris*.

Utilizing SWISS-MODEL online server (https://swissmodel.expasy.org/) according to the xylanase from *Fusarium proliferatum* (PDB: A0A365MZL8.1.A) conducted homology modeling for FXYL (Fig. 1A). Structural analyses revealed its possession of a family 11 glycoside hydrolase domain spanning amino acids 25 to 198, classifying it within the GH11 family. The SDS-PAGE analysis of FXYL expression in strain F1 showed that its molecular weight was slightly larger than the theoretical 31.6 kDa (Fig. 1B). NetNGlyc prediction disclosed two N-glycosylation sites in FXYL, suggesting varying glycosylation degrees during expression in *P. pastoris*, leading to distinct target zones. After treatment with the deglycosylated enzyme PNGase F, only a single destination band remains (Fig. 1B). The elements of the recombinant protein gene expression cassette, including promoters, secreted signal peptides, target genes, and terminators, play crucial roles. We focused on optimizing these elements based on the successful expression of FXYL in *P. pastoris* (Fig. 1C). Employing fusion PCR, the pre of the Ost1 signal peptide was fused with the pro of α-signal peptide to create a novel signal peptide for FXYL secretion. This modification led to the construction of the recombinant strain F2, exhibiting a FXYL activity of 2131.1 U/mL, a 21.8% improvement over F1. Further optimization involved replacing the original AOX1 promoter with a mutant AOXm promoter in strain F3. Following signal peptide and promoter optimization, FXYL activity in F3 reached 2360.28 U/mL, which increased by 34.9% (Fig. 1D). The combination optimization did not prominently impact strain growth compared to *P. pastoris* X33 without the integrated target gene. While these results indicated that optimizing gene expression cassette elements enhanced FXYL expression in *P. pastoris* without affecting the strains growth significantly, additional measures are suggested to further improve expression yields.

3.2. Regulation of gene copy number for FXYL expression

Fluorescent quantitative PCR (qPCR) assays were employed to precisely determine the copy number of xylanase gene *FXYL* in the recombinant strain F3 genome after optimization of expression elements,

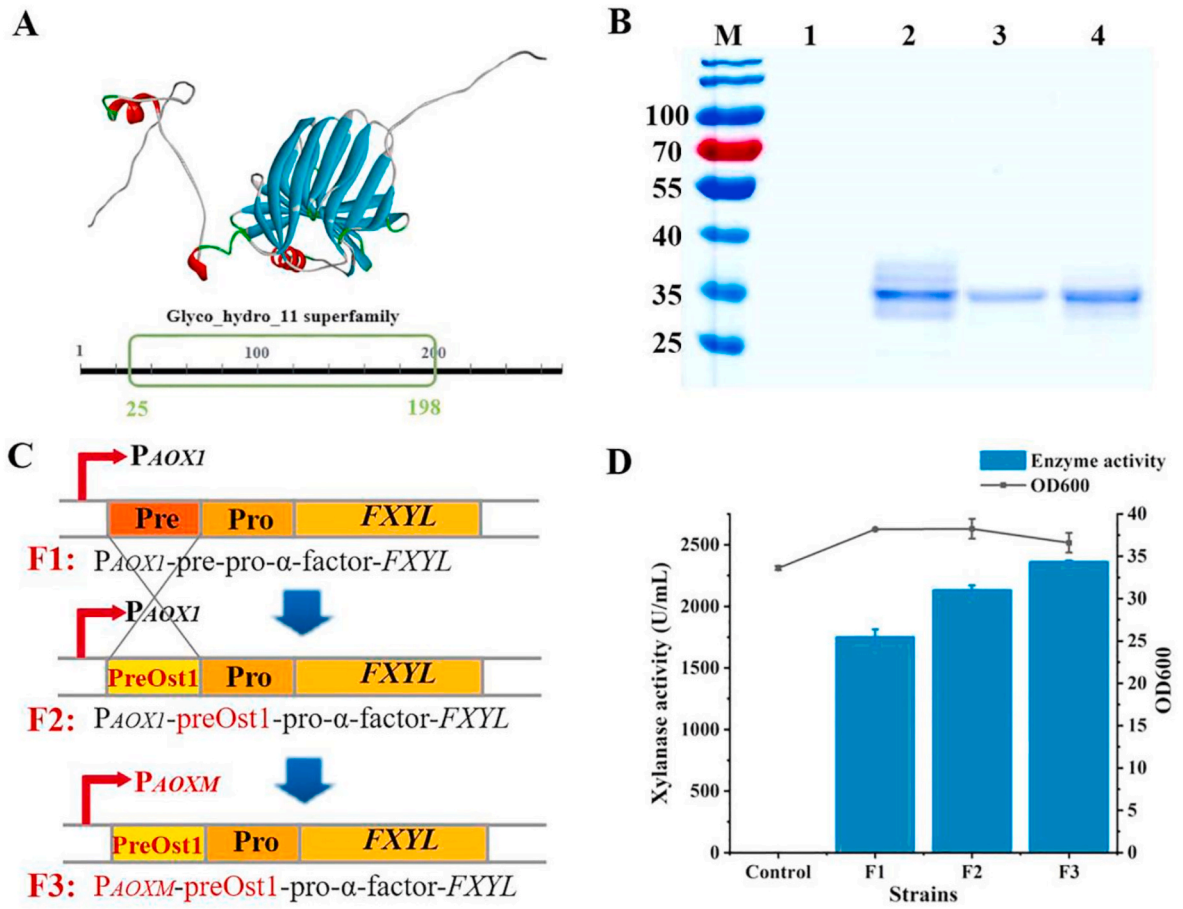

Fig. 1. Three-dimensional structure, SDS-PAGE, expression elements optimization and fermentation assay of FXYL. (A) 3D structure and conserved domains analysis of the FXYL amino acid sequence. (B) SDS-PAGE before and after glycosylation of FXYL with F1 at 120 h. Lane M, Protein marker; Lane 1, X33/pPICZαA; Lane 2, F1/pPICZαA-*FXYL* fermentation broth supernatant; Lane 3–4, The sample of Lane 1 and Lane 2 were treated with PNGase F, respectively. (C) Expression cassette elements optimization process; F1, F2 and F3 were recombinant strains in which plasmid pPICZαA-*FXYL*, PHZOA-*FXYL* and PHZOAm-*FXYL* were inserted into *P. pastoris* X33, respectively. (D) The FXYL activity and growth curve of *P. pastoris* recombinant strains F1, F2 and F3 after induced fermentation for 120 h. Control, *P. pastoris* X33 strain integrated with pPICZαA, as negative control.

and strain F3 was identified as a single copy strain (Table S3). In instances where the expression of the recombinant protein is modest in single-copy strains, augmenting the gene dosage stands out as an effective strategy to significantly enhance protein expression level. Utilizing an in vitro multimerization approach, we constructed a single expression vector containing multiple gene expression cassettes. Subsequently, *P. pastoris* strains were transformed to screen the positive transformants that successfully inserted into the multi-expression cassette plasmid. The copy number of FXYL gene in the transformants genome was determined by qPCR (Table S3), multi-copy engineered strains 2F3 (two copies of FXYL), 3F3 (three copies of FXYL), and 4F3 (four copies of FXYL) were obtained (Fig. 2A).

It is noteworthy that, after 120 h of methanol-induced culture, the growth density of multi-copy strains exhibited a decreasing trend, particularly in the case of the four-copies. This trend suggests that the increased gene dosage imposes growth pressure on the strains. Nevertheless, within certain limits, the expansion of gene copy numbers has a positive effect on FXYL expression (Fig. 2B). Notably, the enzyme activity of the two-copies strain 2F3 increased by 59.1% compared with the F3, reaching its peak at the three-copies strain 3F3 with an enzyme activity of 4240.92 U/mL. The SDS-PAGE analysis illustrated this phenomenon (Fig. 2C). However, upon reaching four copies, the enzyme activity began to decline, dropping by 17.8% compared to three copies. This decline may be attributed to enzyme activity reaching a plateau, where further increases in copy number no longer contribute to improved protein expression and may even have negative effects. Higher copy numbers can lead to decreased cell growth activity and the release of cell lysates, including proteases, resulting in lower enzyme activity. This phenomenon mirrors observations in the secretion and expression of α-galactosidase (GalA) in *P. pastoris* [35]. When the copy number exceeded four copies, the expression level of GalA gene diminishes, and a linear relationship between the expression level and the copy number was no longer evident. The protein expression in *P. pastoris* is not unlimited, reaching an upper limit where protein expression levels stabilize or decrease with further increases in gene dose [36]. Perhaps that is because the physiology of high copy strain was affected, and thus affected its metabolism and oxidative stress associated with protein folding [37]. We conclude that certain factors restrict the expression and secretion of recombinant proteins in *P. pastoris*, possibly including the ability of the ER to fold and process proteins, the limitation of the membrane to accommodate proteins, and the degradation of extracellular proteases. Consequently, it is imperative to take appropriate measures to overcome potential limiting factors, and further improve the expression of FXYL in *P. pastoris*.

3.3. Co-expression of protein expression pathway related factors for FXYL expression

Insufficient translation ability and secretion transport flux pose limitations on the effective synthesis and secretion of recombinant

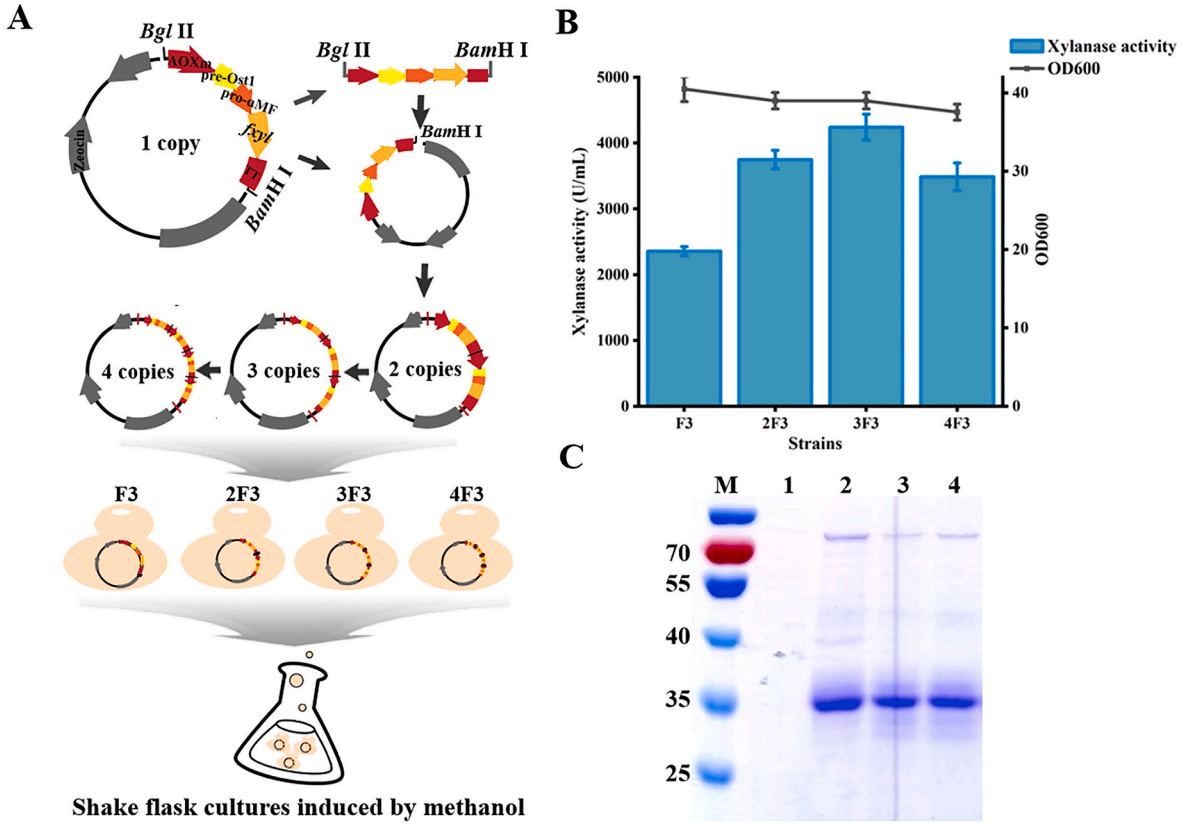

Fig. 2. Construction and fermentation assay of FXYL multi-copy recombinant strain. (A) Multi-copy recombinant strain construction process. F3 was a single copy strain in which the plasmid PHZOAm-*FXYL* was inserted into *P. pastoris* X33; 2F3, 3F3 and 4F3 were the two-, three- and four-copies of *FXYL*, respectively. (B) The FXYL activity and growth density of F3, 2F3, 3F3 and 4F3 after induced by methanol for 120 h. (C) Optimization of gene copy number by SDS-PAGE analysis. Lane M, Protein marker; Lane 1, X33/pPICZαA; Lane 2, 3F3/PHZOAm-3*FXYL*; Lane 3, 2F3/PHZOAm-2*FXYL*; Lane 4, F3/PHZOAm-*FXYL*.

proteins, thereby impeding the efficient expression of such proteins in yeast [21,38]. The expression pathways of recombinant proteins in *P. pastoris* mainly include the synthesis of new peptide chains after translation, protein modification and folding in the ER, vesicle trafficking of the ER to Golgi, and further transport from the Golgi to cell membrane, culminating in secretion into the extracellular environment (Fig. 3). On the basis of recombinant strain 3F3, 12 factors related to protein translation, ER folding processing and ER-Golgi vesicle trafficking were co-expressed individually, which were supposed to solve the protein expression bottlenecks to some degree (Table 1).

3.3.1. *Translation factors*

The yeast translation mechanism plays a pivotal role in the heterologous proteins production, and co-expression of translation factors can further improve the translation efficiency, ensuring the synthesis of a large number of new peptide chains. Using strain 3F3 with three copies as chassis cells, we expressed critical translation factors such as eIF4A, eIF4E, eIF4G, Pab1 and Rli1 (highlighted in orange in Fig. 3), respectively. Recombinant strains 3F3-eIF4A, 3F3-eIF4E, 3F3-eIF4G, 3F3-Pab1 and 3F3-Rli1 were obtained. As shown in Fig. 3A, the co-expression of the five translation factors (eIF4A, eIF4E, eIF4G, Pab1, Rli1) all promoted the expression of FXYL to varying degrees, increasing by 43.5%, 2.4%, 20.4%, 109.75% and 2.7%, respectively. Notably, the co-expression of Pab1 had the most remarkable effect on the FXYL activity, reaching 8893.53 U/mL. This result can be attributed to the fact that Pab1 is a poly(A)-binding protein so that can bind the 3′-poly(A) tail of mRNA. It determines whether the mRNA can be effectively exported to the nucleus, as well as mRNA metabolism during translation initiation and termination [39,40]. Consequently, Pab1 increases the total amount of mRNA that can participate in the translation process and is of paramount importance in promoting the translation synthesis of recombinant proteins. However, it is noteworthy that despite Pab1 having the most significant effect among the five translation factors, the growth density of the strain 3F3-Pab1 after 120 h of fermentation was slightly lower than that of other strains during the same period (Fig. 3A). This indicates that Pab1 co-expression exerts a substantial effect on cells, not only enhancing FXYL productivity but also restricting cell growth. Moreover, the promotion of translation mechanisms and protein expression may be greater than the growth disturbance brought by it. The inconsistencies between cell growth and recombinant protein activity also appeared in the enhancement of specific vHH production through co-expression of translation factors [21].

3.3.2. *ER-associated transcription factors and chaperones*

Under the guidance of the signal peptide, the nascent polypeptide chain is translocated through a channel into the ER lumen for modification, processing, and folding. Intriguingly, the ER incorporates quality control mechanisms. As the gene dose increases, faulty or unfolded proteins in the ER accumulate excessively, leading to the accumulation of intracellular reactive oxygen species (ROS). In this case, the ER activates the unfolded protein response (UPR) which regulates protein synthesis, folding, and degradation, maintaining protein homeostasis in the ER [41]. Under stress conditions, the heat shock response (HSR) is up-regulated, leading to the production of heat shock protein (HSP) that assist in the folding of nascent chains [42]. Specific transcription factors in cells can activate or inhibit these stress responses, promoting the proper folding and transport of proteins. Herein, five yeast endogenous chaperone/transcription factors (highlighted in blue in Fig. 3) were selected for co-expression in *P. pastoris*: HAC1, P180, PDI1, FHL1, and MSN4. Fig. 3B indicates that, apart from the chaperone PDI, the

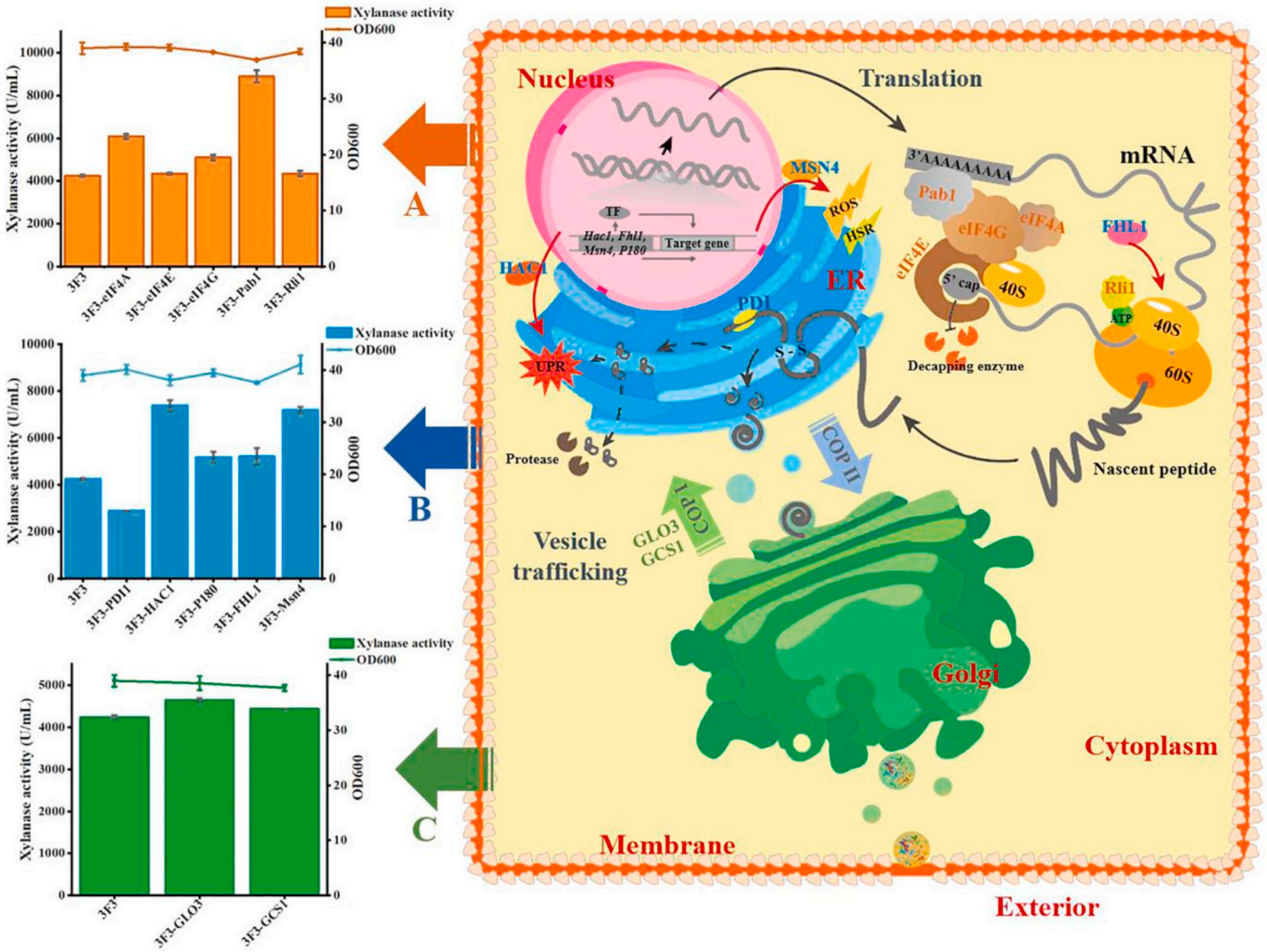

Fig. 3. Overview of the protein expression pathway related factors applied in this work and explored their secretion-promoting effects in *P. pastoris*. (A) Translation factors (eIF4A, eIF4E, eIF4G, Pab1, Rli1) were co-expressed in three-copies strain 3F3, and FXYL activity of the recombinant strain after induced by methanol for 120 h. (B) ER-associated transcription factors (FHL1, MSN4, HAC1, P180) and chaperone (PDI1) were co-expressed in three-copies strain 3F3, and FXYL activity of the recombinant strain after induced by methanol for 120 h. (C) Vesicle trafficking factors (GLO3, GCS1) were co-expressed in three-copies strain 3F3, and FXYL activity of the recombinant strain after induced by methanol for 120 h.

remaining factors all have certain effect. The transcription factors HAC1 and MSN4 exhibited the most significant impact, with the FXYL activity of the recombinant strain increasing by 73.8% and 69.4% to 7371 U/mL and 7183.4 U/mL, respectively. HAC1 is a commonly utilized effector transcription factor. It can activate the UPR signaling pathway and then regulate downstream target genes of UPR, enhancing protein processing and transport capabilities [43–45]. In contrast, MSN4 is a zinc finger protein derived from *S. cerevisiae*. Despite the specific regulatory mechanism of MSN4 on heterologous protein expression is not entirely clear, studies have shown that MSN4 can regulate various stress responses during ER processing, such as HSR [26,46]. This may increase the protein folding flux of the ER, thereby promoting protein expression. In addition, P180 and FHL1 increased by 21.9% and 22.8%, respectively, resulting in FXYL activities of 5170.5 U/mL and 5208.9 U/mL. However, FXYL activity decreased by 32.1% after the co-expression of PDI. Further analysis revealed that only one cysteine (Cys) existed in the 270 amino acids of FXYL, and it is located near the N-terminus of the peptide chain, which also leads to the inability to form intra-chain disulfide bonds. Nevertheless, as a disulfide isomerase, co-expression of PDI1 may cause inter-chain folding disordered and reduce the enzyme activity.

3.3.3. *Vesicle trafficking related factors*

The nascent peptide chains that have been modified and properly folded in the ER are transported to the Golgi lumen via vesicles for further processing and packaging. Eventually, they are secreted out of the cell through vesicles fusion with the plasma membrane. Interestingly, proteins can undergo bidirectional transport between the ER and Golgi, with reverse and forward traffic mediated by the vesicle coat protein complex (COP) I and II, respectively [47]. Herein, we co-expressed ADP-ribosylation factor GTP activating proteins, GLO3 and GCS1 (highlighted in green in Fig. 3), resulting in the construction of recombinant strains 3F3-GLO3 and 3F3-GCS1. These two factors are involved in COPI vesicles formation and can promote the reverse traffic of Golgi to ER. As shown in Fig. 3C, compared with the original strain 3F3, the FXYL activity in the recombinant strain 3F3-GLO3 and 3F3-GCS1 slightly improved by 9.6% and 4.6%, respectively, reaching 4648 U/mL and 4435.2 U/mL. It is generally known that secreted proteins are primarily involved in the forward transport of COPII vesicles in the ER and Golgi. We speculated that the increase of gene dose also causes an expansion in the forward transport flux to some extent. Therefore, the co-expression of reverse transport related factors may alleviate and balance the pressure in forward transport. On the other hand, it also indicates that the balance between vesicle bidirectional

transport is very important to maintain the efficiency of protein secretion. This opinion was also reflected in the study of Bao et al., who further increased α-amylase secretion by maintaining the balance of bidirectional vesicular transport [28].

3.3.4. Combined co-expression of protein expression pathway related factors

As mentioned, earlier, the co-expression of relevant factors in the secretion process can have either positive or negative effects on FXYL expression to varying degrees. The three pathways involved, namely translation, folding modification in the ER, and vesicular traffic of the ER-Golgi, and one link to another for protein secretion. In addition to single co-expression, Zhang et al. also found that there may act synergistically between different factors to promote protein expression [48]. In that way, it is a feasible strategy to connect the three pathways through combination expression, or to concentrate on enhancing the flux of one of them. As a consequence, by means of concatenating expression cassettes of different factors, we tried to combine factors with the best effects in different pathways or with potential synergistic effects in same pathway. These expression cassettes were then inserted into the three-copies strain for co-expression (Fig. 4A). Undoubtedly, the best factors (Pab1, HAC1, GLO3) in the above three processes were co-expressed on the same vector in series, resulting in the construction of strain 3F3-Z1. Furthermore, in translation mechanisms, the closed-loop conformation is generally believed to reinforce mRNA stability and recycling, controlling translation efficiency. The closed loop is mainly composed of Pab1 (binding to the 3′poly (A) tail of mRNA), eIF4G (coordinating bridging mechanism) and eIF4E (interacting with the 5′cap of mRNA) [49] (Fig. 3). Pab1 can also activate another translation initiation factor, eIF4A, to enhance its ATPase and helicase activities [50]. Recombinant strains 3F3-Z2 (eIF4A, Pab1) and 3F3-Z3 (eIF4E, eIF4G, Pab1) were constructed by co-expressing synergistic factor combinations. It has been reported that the combined co-expression of MSN4 and HAC1 may have a positive effect on the secretion of recombinant protein [26], so we simultaneously constructed strain 3F3-Z4 (MSN4, HAC1).

Later, four recombinant strains (3F3-Z1, 3F3-Z2, 3F3-Z3, 3F3-Z4) were screened for double antibiotics, and the secretion activity of FXYL was analyzed by methanol-induced culture, as shown in Fig. 4A. Surprisingly, after the combined co-expression of related factors, the growth of the recombinant strain was not as restricted as co-expression alone (Fig. 4B), and there was no significant difference compared to the strain 3F3. Strain 3F3-Z3 demonstrated prominent FXYL secretion activity, reaching 5338.1 U/mL, which was augmented by 26.5% compared with 3F3 (Fig. 4C). This also suggests that closed-loop structures can indeed stimulate translation efficiency to a certain extent. The other three combinations showed no secretory effect, and the combined co-expression of related factors weakened the secretory effect of these factors co-expressed separately. Compared with strain 3F3-Pab1, FXYL activity of 3F3-Z1, 3F3-Z2 and 3F3-Z3 decreased by 59.3%, 68.7% and 40.0%, respectively. On the one hand, we suspect that the combined expression of cassettes may not fully unleash the combination

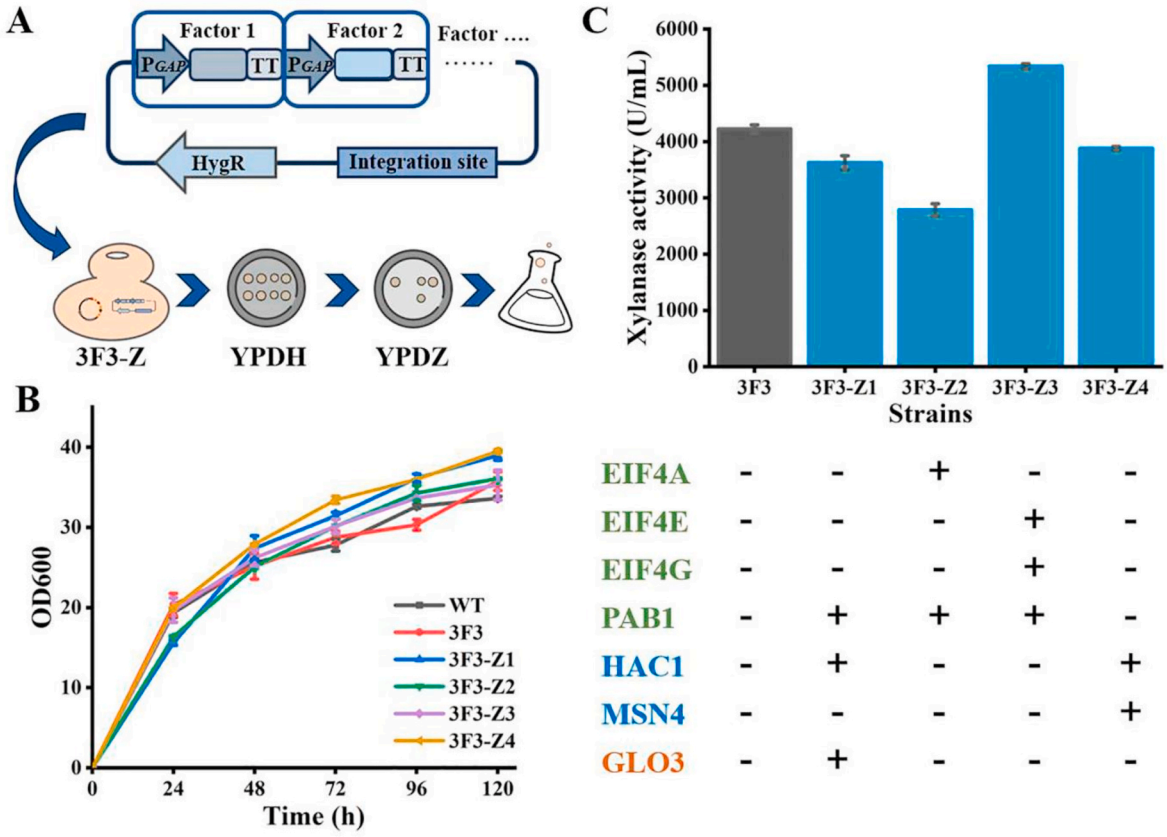

Fig. 4. Combined co-expression of effective protein expression pathway related factors and FXYL expression assay of recombinant strains. (A) The process of combination co-expression by series expression cassette method. The screening marker of the co-expression plasmid was hygromycin B (HgyR), and the promoter of each factor was P_{GAP}.The plasmid was inserted into the three-copy strain (screening marker was Zeocin), and the recombinant strain was obtained by double-layer resistance screening. (B) The growth curve of recombinant strains 3F3, 3F3-Z1, 3F3-Z2, 3F3-Z3 and 3F3-Z4. WT, *P. pastoris* X33 strain integrated with pPICZαA, as negative control. (C) The FXYL activity of combined co-expression recombinant strains after induced fermentation for 120 h. The factor compositions of each combination have been shown.

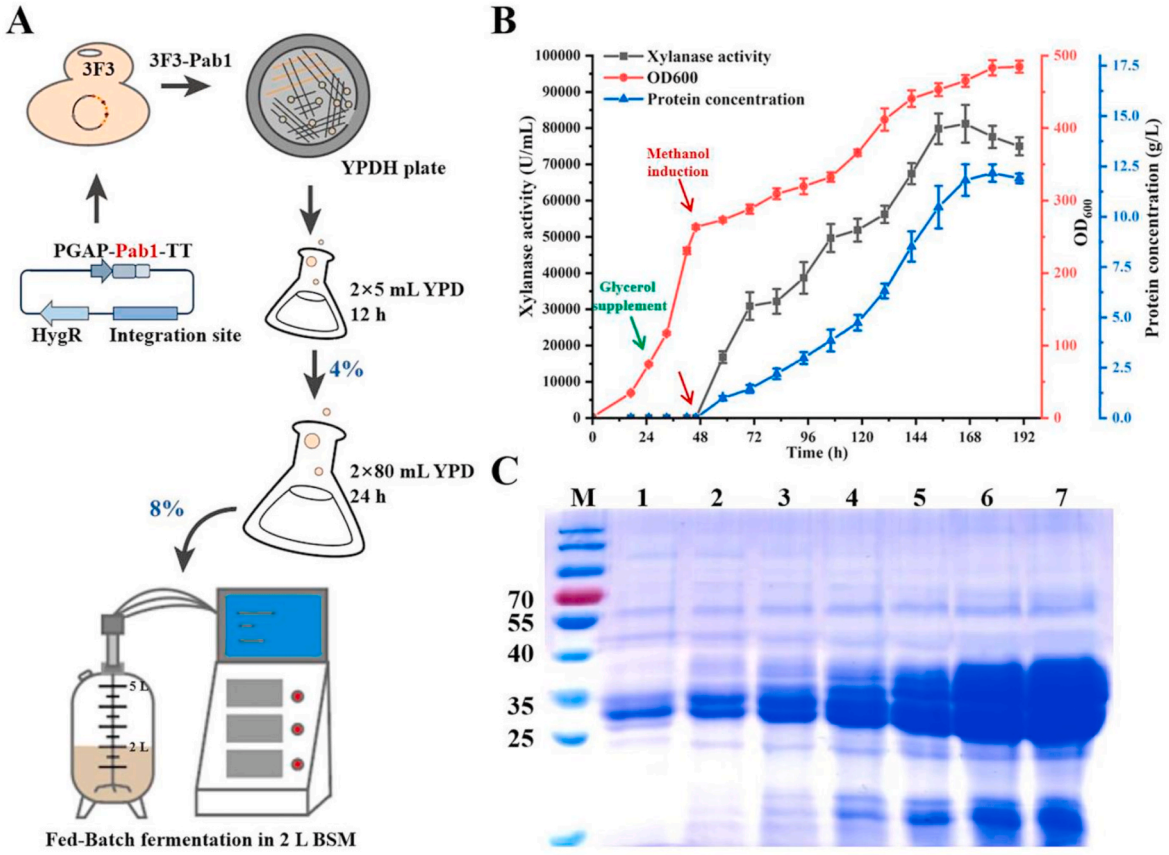

Fig. 5. Production of FXYL from the recombinant strain 3F3-Pab1 via high-density cultivation in 5-L fermenter. (A) The scheme for fed-batch fermentation operation process. (B) FXYL production from the recombinant strain 3F3-Pab1 in 5 L fermenter for 190 h. Glycerol was fed at 25 h and methanol induced culture at 46 h. Fermentation was terminated at 144 h after methanol feeding. (C) The SDS-PAGE analysis of FXYL production from the recombinant strain 3F3-Pab1 under different methanol induction time. Lane M, Protein marker; Lane 1–7: Fermentation supernatant (twice diluted) after methanol induction for 12, 24, 48, 72, 96, 120, 144 h.

advantages, but instead limit the single effect of related factors. On the other hand, it also reveals that the best factors combination of factors may not exhibit the promotion effect of win-win co-operation, and perhaps the random combination of several factors can achieve unexpected effects, which needs further research.

3.4. Production of FXYL in a 5 L fermenter

In an effort to evaluate the industrial production value of FXYL recombinant strains and considering that the combined co-expression of effective factors did not show a better impression than the single, strain 3F3-Pab1 was selected for high-density culture in 5 L fermenter. As shown in Fig. 5A, the freshly activated engineered strain was selected and cultured in YPD to prepare primary as well as secondary seed solution, then transferred into 2 L BSM medium. The BSM medium contains 40 g/L glycerol to sustain rapid growth of the strain until the target gene is expressed. It can be seen in Fig. 5B, glycerol in BSM was exhausted at 25 h, entering the glycerol supplement culture stage, with 46 h OD_{600} reaching 263.5 and starting methanol-induced culture. The target gene is regulated by methanol-induced P_{AOXm} promoter, and the engineered strain began to produce FXYL after methanol supplementation. At the initial stage of methanol feeding, the methanol feed flow rate was controlled to 5–10 g/h, and the starved cells began to adapt and utilize methanol. After fed for 24 h, xylanase activity and protein concentration enhanced significantly. With the increase of the growth density, the methanol flow rate gradually increased to 15–20 g/h. When methanol was fed for 120 h, xylanase enzyme activity was the highest, reaching 81184.51 U/mL, and protein concentration was 11.8 g/L (Fig. 5B). Fermentation was terminated after methanol induction for 144 h, biomass reached an OD_{600} of 485, and FXYL accumulation in the fermenter tended to be stable, as displayed in Fig. 5C on SDS-PAGE.

4. Conclusions

In this study, we employed strategies including expression element optimization and gene copy number increase to reinforce FXYL secretion in *P. pastoris*. Utilizing the three-copies engineered strain as the chassis, we selected three links in the protein expression pathway and co-expressed the related factors in these links. In addition to PDI, all the factors in the three links demonstrated positive effects on FXYL activity. Furthermore, we tried to combine the prominent factors or the factors with potential synergistic effects in above three links, yet four combinations obtained could not transcend the effect of co-expressing them alone. Ultimately, the FXYL activity and protein concentration reached 81184.51 U/mL and 11.8 g/L after methanol induction for 120 h in a 5 L fed-batch fermenter. The FXYL activity was 104.13-fold that of the first report. This research not only enhances our understanding of xylanase expression in *P. pastoris* but also provides a xylanase with potential industrial application value, particularly in the food baking industry.

CRediT authorship contribution statement

Chun Liu: Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Yaping Zhang:** Conceptualization, Investigation, Formal analysis, Methodology, Writing – review & editing. **Chunting Ye:** Investigation, Data curation, Validation. **Fengguang Zhao:** Conceptualization, Formal analysis, Funding acquisition, Writing – review & editing. **Yian Chen:**

Conceptualization, Writing – original draft. **Shuangyan Han:** Conceptualization, Formal analysis, Funding acquisition, Supervision, Project administration, Writing – review & editing.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.03.012.

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