



## Original Research Article

High-efficiency patatin expression strategies in *Komagataella phaffii* (*Pichia pastoris*): Expression cassette toolbox and regulation of protein secretionYue Huang<sup>a</sup>, Shao Jia<sup>a</sup>, Ying Lin<sup>a</sup>, Jiali Wang<sup>a</sup>, Luyuan Nong<sup>a</sup>, Lei Ye<sup>b, \*\*</sup>, Shuli Liang<sup>a,\*</sup><sup>a</sup> Guangdong Key Laboratory of Fermentation and Enzyme Engineering, School of Biology and Biological Engineering, South China University of Technology, Guangzhou, 510006, China<sup>b</sup> Institute of Food Safety and Nutrition, College of Life Science and Technology, Jinan University, Guangzhou, 510632, China

## ARTICLE INFO

## ABSTRACT

**Keywords:**  
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 $CAT1$  promoter  
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Patatin, a prominent food protein derived from potatoes, is renowned for its exceptional nutritional value. Patatin has been characterized for its diverse physiological attributes, including esterase activity, antioxidative properties, cholesterol-lowering effects, and high lysine content, alongside notable physicochemical traits such as foaming, emulsification, and gelation capabilities. Conventional methods for patatin extraction are fraught with inefficiencies, elevated costs, and detrimental impacts on protein structural and functional integrity. Herein, we leveraged an optimized strategy integrating an expression cassette toolbox and regulation of protein secretion to harness *Komagataella phaffii* as the expression host and achieved an expression level of 3.2 g per litre (g/L) in a 5-Litre bioreactor, which is the highest yield of patatin production using engineered bacteria and funguses that has been reported thus far. In this study, we innovatively refined the endogenous promoter  $P_{CAT1}$ , and its efficacy in driving heterologous protein expression under methanol induction surpassed that of the conventional  $AOX1$  promoter. Furthermore, crucial nodes for patatin heterologous expression in yeast were identified, substantially curtailing the production costs associated with patatin synthesis.

## 1. Introduction

Protein is the foundation of human growth and development. The recommended daily intake of protein for healthy adults is approximately 0.8–0.9 g/kg (body weight)/day. For adolescents, heavy labourers, fitness enthusiasts, athletes, etc., a more adequate protein intake of approximately 1.2–1.6 g/kg (body weight)/day is often required [1]. As people's living standards improve, various meat products rich in high-quality protein are favoured. However, according to statistics, 5%–15 % of total global greenhouse gas emissions are directly attributed to the production of animal-derived foods [2]. Therefore, research on nonanimal protein is highly important. Plant protein is a promising substitute for meat protein, and many plant proteins have been proven to have high nutritional value [3]. Here, we introduce a high-quality plant protein, patatin, a glycoprotein derived from potatoes. Potatoes are perennial herbaceous plants of the nightshade family and are very rich in nutritional value and inexpensive, making them a common food. With the development of the potato processing industry, research on the

nutritional and functional properties of potato proteins and their production and purification methods has continued to expand. Potato protein is composed of 19 amino acids, of which essential amino acids account for 47.9 % of the total amino acids, which is similar to egg white protein (49.7 %). According to their molecular weight and functional characteristics, potato proteins can be divided into high-molecular-weight proteins, patatins, and protease inhibitors. Patatins are the storage proteins of potato tubers and account for more than 40 % of the total protein content in potato tubers [4].

According to previous reports, patatin has many favourable functional properties, including solubility, gel-forming ability, foaming ability, and emulsifying ability, among other physical properties [5]. Additionally, patatin has many biological activities, such as esterase activity [6], antioxidant activity [7], and renin inhibitory activity [8]. Patatin has also been demonstrated to have the ability to lower cholesterol and regulate blood pressure [9]. As a good source of lysine, patatin is considered a suitable substitute for wheat [10]. Patatins isolated from *S. tuberosum* have exhibited antidiabetic effects in the model

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organism zebrafish, suggesting that patatin may act through its influence on lipid metabolism [11]. Peptide fragments produced by the gastric proteolysis of patatin have been shown to possess anti-inflammatory properties [12]. Overall, patatin, with its small molecular weight (40–45 kDa) and many excellent properties, is highly suitable as a protein source for food or as a safe food additive.

Currently, the production of patatin and other potato proteins in industry mainly involves the separation, extraction, and purification of these proteins from the byproducts, potato fruit juice, of the potato starch industry [6]. Although some studies have reported enzymatic methods for isolating patatin [13], these methods are considered time-consuming, laborious, inefficient, and costly. In addition, inappropriate operations during the production process may lead to the destruction of the original functional activity of patatin [14]. Owing to these drawbacks, the use of the *Komagataella phaffii* (*Pichia pastoris*) expression system is considered an excellent strategy for patatin production. The *K. phaffii* expression system is currently a very popular eukaryotic expression system for proteins and other products and has been used to express more than 6000 types of exogenous proteins, such as enzymes, antigens, membrane proteins, antimicrobial peptides, antibodies, and regulatory proteins [15]. The yield of some exogenous proteins in *K. phaffii* fermentation broth can even exceed 10 g/L [16]. Compared with other host expression systems, the *K. phaffii* system has many advantages [17], including powerful methanol-inducible promoter *AOX1*, simple operation, simple and controllable cell culture conditions, the ability to achieve high-density fermentation [18], the ability to perform post-translational modification of exogenous proteins [19], and the ability to secrete exogenous proteins into the extracellular space, simplifying the process of separation and purification [20]. Currently, Gelley et al. successfully produced patatin from *K. phaffii* for the first time and initially confirmed the feasibility of patatin expression in the fungus [21]. Dai et al. further demonstrated the excellent gel-forming properties of yeast-produced patatin [22]. In this study, patatin-B2 [21], which exhibits outstanding functional characteristics in various aspects, was selected from several patatins for protein expression in *K. phaffii*, and the yield of patatin was improved by screening for efficient elements from the expression cassette toolbox and globally regulating protein expression.

## 2. Materials and methods

### 2.1. Strains, reagents, plasmids, and culture conditions

*Komagataella phaffii* GS115 and *E. coli* top10 were used as the expression hosts for recombinant patatin-B2 production and plasmid construction. *E. coli* top10 was cultured in Luria broth (LB) at 37 °C, which contained 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl. Minimal dextrose medium (MD) containing 20 g/L glucose and 13.4 g/L yeast nitrogen base without amino acids was used. Yeast extract peptone dextrose medium (YPD) containing 20 g/L peptone, 10 g/L yeast extract, and 20 g/L glucose was used. Methanol-complex medium (BMMY) supplemented with 20 g/L peptone, 10 g/L yeast extract, 13.4 g/L yeast nitrogen base without amino acids, 10 % (v/v) 1 mol/L phosphate-buffered saline (PBS) (pH 6.0), and 1 % (v/v) methanol was used. The composition of BMGY is roughly the same as that of BMMY, where 1 % (v/v) methanol is replaced by 1 % (v/v) glycerol. All media were supplemented with 2 % (w/v) agar, and solid media was obtained. The plasmids and strains mentioned in this article are in our laboratory's collection. Unless otherwise specified, chemicals and reagents were purchased from Sangon Biotech (Shanghai, P. R. China). Restriction enzymes were obtained from Thermo Fisher Scientific (Waltham, MA, U.S.A.).

### 2.2. Construction of plasmids and yeast transformation

The relevant information concerning the primers, plasmids, and

bacterial strains mentioned in this article can be found in Tables S1 and S2. The amino acid sequence of patatin-B2 (accession number: P15477) was optimized according to the codon preference of *K. phaffii*, avoiding commonly used restriction endonuclease cleavage sites. The final sequence was synthesized by Sangon Biotech (Shanghai, China). During the synthesis of the patatin-B2 DNA, an EcoR I site was added to the 5' end of the gene, and a 6 × HIS tag followed by a NotI site was sequentially added to the 3' end. First, both patatin-B2 and the pHKA plasmid were digested with EcoR I and NotI at 37 °C. Subsequently, the digested B2 fragment and pHKA were ligated using T4 DNA ligase at 16 °C to obtain pHKA-B2 (Fig. 1b). The pHKA-B2 transformed into *E. coli* top10. The transformed cells were selected on LB agar plates containing 50 mg/L kanamycin, and the correct colonies were screened and extracted for plasmid preparation. The plasmids were sequenced by Sangon Biotech (Shanghai, China), and the correctly sequenced plasmids were linearized and transformed into *K. phaffii* GS115. Positive clones were screened and identified on MD plates. The transformation processes of the other plasmids were similar, except that different selection media were used during screening. The detailed transformation methods can be found in previous studies [23]. All plasmids in this study were constructed by restriction endonucleases and T4 DNA ligase or by Gibson assembly.

Multiple gene strains were constructed via a zeocin resistance marker recycling vector, pZACH, which was constructed in our previous work and employs the Cre/loxP recombination system. The zeocin resistance marker could be excised by induction of the Cre protein, allowing for the repeated use of the plasmid (Fig. S1). This system was employed to construct multicopy strains and overexpress relevant genes to achieve global regulation of protein expression. Specific operational details can be found in our previously published papers [24].

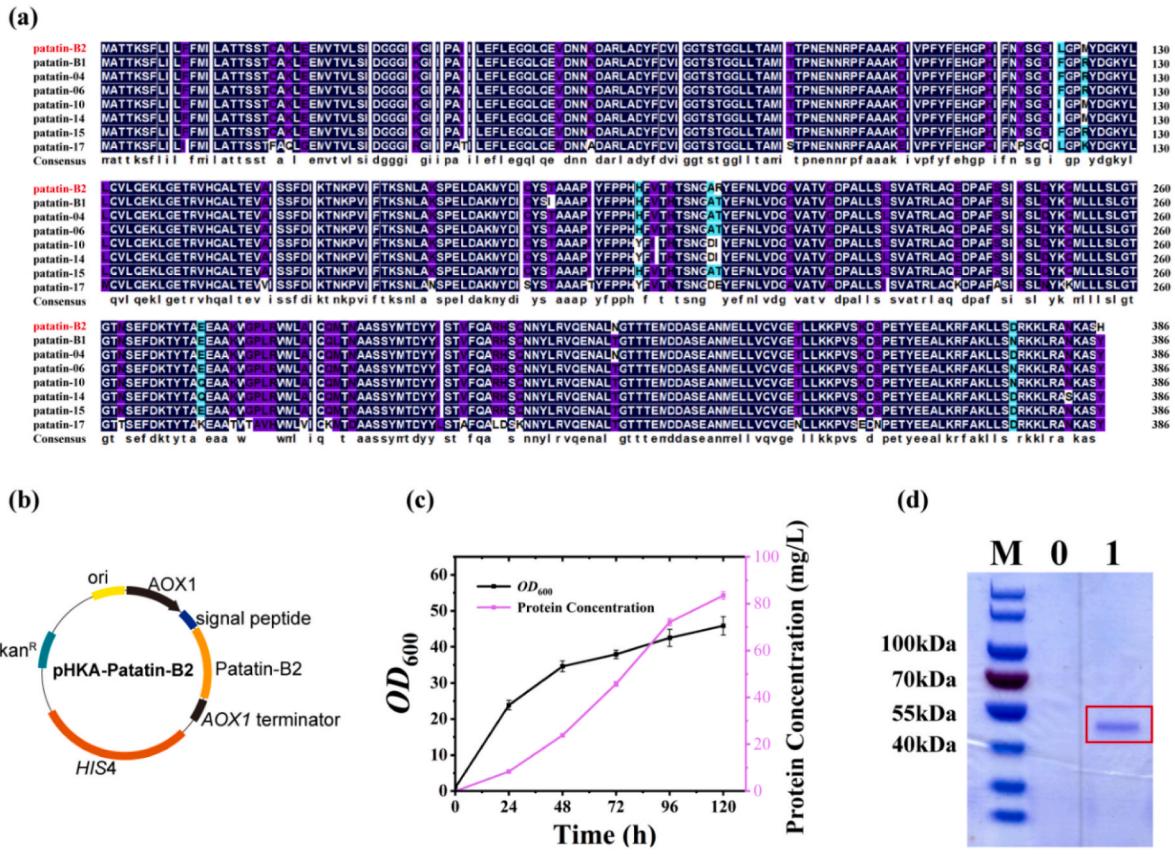
### 2.3. Quantification of the gene copy number of the recombinant protein patatin-B2 in yeast via real-time PCR

The actual copy number of the multicopy strain was determined through real-time quantitative PCR (qPCR). The strains were cultured in YPD for 24 h, and genomic DNA was extracted from the yeast cells via the Dr. GenTLE™ High Recovery Kit (Takara, Shiga, Japan). Genomic DNA served as the amplification template, with GAPDH as the reference gene. Copy number determination was performed via a QuantStudio™ 1 Real-Time PCR instrument (Thermo Fisher Scientific). Data processing and calculation of the final copy numbers followed procedures described in previous methods [25].

### 2.4. Shaking flask culture and fed-batch fermentation

The positive transformants successfully identified via tablet screening were inoculated into BMGY media and cultured at 30 °C and 250 rpm for approximately 20 h. Each strain was represented by five individual colonies selected as parallel controls. The culture with an initial OD<sub>600</sub> of 1.0 was subsequently transferred into 25 mL of BMMY medium and continuously cultured at 30 °C and 250 rpm for 120 h. During fermentation, 250 µL of the culture was removed from the medium every 24 h to monitor the growth status and measure the data. Additionally, 1 % methanol was added to the medium as a carbon source. After fermentation was complete, the fermentation broth was centrifuged at 12,000 rpm for 2 min to retain the supernatant.

Fed-batch fermentation was carried out in a 5 L bioreactor. The strain was inoculated in YPD medium and cultured overnight at 30 °C. Fresh seed cultures (8 %, v/v) were inoculated into 2 L of basal salt medium (BSM) containing 27 mL/L 85 % H<sub>3</sub>PO<sub>4</sub>, 40 g/L glycerol, 18 g/L K<sub>2</sub>SO<sub>4</sub>, 14.9 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O, 4.13 g/L KOH, and 0.93 g/L CaSO<sub>4</sub>. Additionally, 4.35 mL/L of PTM1 solution was added as a supplement to trace elements to support optimal cell growth. The fermentation process was divided into three stages. The first stage involved cell growth, with oxygen levels maintained above 30 % by controlling the agitation rate. The



**Fig. 1.** Expression of patatin-B2 with pPICHKA in *K. phaffii*. (a) Alignment of different patatin gene sequences. (b) Structure of the pPICHKA-patatin-B2 plasmid. (c) Fermentation time and yield curves. (d) SDS-PAGE of fermentation supernatant; 0: empty plasmid strain as a blank control; 1: patatin-expressing strain.

second stage was the glycerol feeding phase (with 12 mL/L PTM1 solution), in which the temperature was maintained at 30 °C and the oxygen level was above 30 %. Ammonium hydroxide was added during the first two stages to maintain the fermentation pH at 5.5. The third stage was the induction phase, which was induced by methanol (with 12 mL/L PTM1 solution). The cultivation temperature was controlled at 25 °C, the pH was adjusted to 6.0. During the initial phase of methanol induction (approximately within the first 6 h), this period is characterized as the adaptation phase to the change in carbon source, during which yeast needs to synthesize enzymes related to methanol metabolism. As a result, methanol consumption is slower during this stage. The initial flow rate is set to 2 mL of methanol per litre of fermentation broth per hour. A flow rate that is too fast can lead to methanol accumulation, causing methanol toxicity in the strain. Subsequently, as the biomass grows, the methanol flow rate is gradually increased while maintaining the dissolved oxygen level between 15 % and 25 %. The methanol feed rate should not exceed 10 mL per litre of fermentation broth per hour at its fastest. In the later stages of induction, as the number of aging cells increases, the yeast's utilization rate of methanol slows down, and it is appropriate to reduce the feed rate accordingly. The detailed fed-batch fermentation methods can be found in our previous study [26].

## 2.5. Bradford assay and SDS-PAGE

The protein concentration was determined using the Bradford assay. First, a standard curve was generated by preparing different concentrations of bovine serum albumin (BSA) standard solutions, adding 20 µL of each solution to a 96-well plate, and mixing with Bradford reagent. After incubation at 30 °C for 5 min, the absorbance at 595 nm ( $OD_{595}$ ) was measured using a microplate reader. The slope, intercept, and  $R^2$

value of the standard curve were calculated based on the  $OD_{595}$  values of the BSA standards at different concentrations. To determine the protein concentration of the samples, 20 µL of properly diluted purified samples were added to a 96-well plate, mixed with Bradford reagent, and incubated at 30 °C for 5 min. The  $OD_{595}$  value of the blank control was subtracted from the  $OD_{595}$  value of the samples, and the resulting value was used to calculate the actual protein concentration using the standard curve.

Protein size and purity were characterized by SDS-PAGE. The gel used for SDS-PAGE consisted of a stacking gel with 4 % acrylamide and a separating gel with 12 % acrylamide. The samples were mixed with 5 × protein loading buffer and boiled for 8 min before electrophoresis. The gel was run at 60 V until the bromophenol blue marker reached the boundary between the stacking and separating gels, and then the voltage was increased to 120 V until the electrophoresis was completed. After electrophoresis, the gel was stained with a solution containing 0.25 % Coomassie brilliant blue (R-250), 50 % ethanol, and 10 % acetic acid at high temperature for 2 min and then repeatedly destained in a destaining solution containing 10 % acetic acid and 5 % ethanol until the protein bands were visible on the gel.

## 2.6. Protein purification

Protein purification was conducted using a Ni-NTA 6FF Pre-Packed Gravity Column (Sangon Biotech, Shanghai, P. R. China). Following fermentation, the culture was centrifuged to obtain the supernatant. The supernatant was then filtered through a 0.22 µm membrane to eliminate residual cells and small particles. For the initial purification steps, the clear supernatant was loaded onto a gravity column. Approximately 10 mL of the supernatant was loaded, followed by washing with 10 mL of

purification buffer ( $\text{pH} = 8.0$ , Tris-HCl solution containing 500 mM/L NaCl). Subsequently, purification buffers containing 60 mM, 120 mM, and 150 mM imidazole were used to elute contaminating proteins, with 10 mL of each solution applied. Finally, patatin expressed with a 6 × HIS tag was eluted using a purification buffer containing 400 mM imidazole, with a total of 20 mL applied in three separate portions (5 mL, 5 mL, and 10 mL).

### 2.7. Esterase activity assay

The esterase activity was determined via a UV spectrophotometer. Using *p*-nitrophenyl caprylate as the substrate, esterase activity was assessed by hydrolysing *p*-nitrophenyl caprylate to release *p*-nitrophenol. The absorbance at 405 nm after the reaction was used to quantify the enzyme activity. The *p*-nitrophenyl caprylate substrate was prepared at a concentration of 0.025 mol/L, with the addition of 2.5 mL/L Triton X-100. After dissolution, the mixture was homogenized at high speed for 5 min and stored in the dark. Solutions of *p*-nitrophenol at different concentrations were prepared to create a standard curve for enzyme activity calculation. One unit of enzyme activity (U) was defined as the amount of enzyme that decomposes the substrate to produce 1  $\mu\text{mol}$  of *p*-nitrophenol per minute. The hydrolysis reaction was conducted at 37 °C. First, 900  $\mu\text{L}$  of pure water and 50  $\mu\text{L}$  of substrate solution were added to a 2 mL centrifuge tube. After thorough mixing, the mixture was preheated for 3 min. Subsequently, 50  $\mu\text{L}$  of enzyme mixture was added, and the reaction proceeded for 5 min. After 5 min, the reaction was rapidly terminated by centrifugation at 4 °C and 12,000 rpm. Finally, the absorbance at 405 nm was measured using a spectrophotometer.

### 2.8. Antioxidant activity

Patatin possesses various soluble, exposed amino acids with free radical scavenging capabilities, such as methionine, tryptophan, tyrosine, phenylalanine, cysteine, and histidine, indicating its antioxidative properties, as reported previously [27]. In this study, the antioxidant activity of recombinant patatin-B2 from *K. phaffii* was determined using the DPPH assay with a kit obtained from Cominbio (Suzhou, China). The specific experimental procedures can be found in the kit manual available at [www.cominbio.com](http://www.cominbio.com). DPPH, a stable free radical, is soluble in polar solvents such as methanol and ethanol, with maximum absorption at 515 nm. The decolorization reaction occurs when antioxidants are added to the DPPH solution, allowing changes in absorbance to characterize the antioxidative capacity of the substance. The formula for calculating DPPH radical scavenging activity is as follows:

$$\text{DPPH radical scavenging activity (\%)} = \frac{(A_b - A_m)}{A_b} \times 100\% \quad (1)$$

Here,  $A_b$  represents the absorbance of the blank control at 515 nm, and  $A_m$  represents the absorbance at 515 nm of the test sample after the reaction.

## 3. Results

### 3.1. Expression of patatin-B2 in *K. phaffii*

The potato genome contains 10–18 copies of patatin genes. Patatin coding regions are highly homologous, with sequence homology exceeding 90 %. Some patatins even presented only a 2 % difference in amino acid sequence (Fig. 1a). Different clusters of patatin genes are activated at different stages of potato growth, with the highest production of patatin in potato tubers [28]. Compared with other homologues, patatin-B2 has been proven to have superior biological properties and high commercial value and potential. The amino acid sequence of patatin-B2 can be downloaded from the NCBI database (accession number: P15477). The signal peptide of patatin-B2 was

removed, and the remaining sequence was codon optimized according to the preferred codons of *K. phaffii*. This optimization aimed to increase the expression of patatin-B2 in *K. phaffii* [29] and avoid common restriction enzyme recognition sites for ease of molecular cloning experiments. The final optimized sequence was synthesized by Sangon Biotech (Shanghai, China). The patatin-B2 gene was subsequently cloned and inserted into the pPICHKA plasmid, which is an expression vector previously constructed and preserved in our laboratory [30] (Fig. 1b). The pPICHKA plasmid contains the *AOX1* promoter,  $\alpha$ -factor signal peptide, and *His4* complementation gene marker. The pPICHKA-patatin-B2 plasmid was electrotransformed into *K. phaffii* GS115, yielding the GS115-pPICHKA-patatin-B2 strain. The GS115-pPICHKA-patatin-B2 strain was fermented in 25 mL of BMGY for 120 h (with 5 mL of BMGY used for seed culture). The fermentation results, as depicted in the figure, revealed protein bands at approximately 45 kDa on the SDS-PAGE gel, which was consistent with the theoretical position (Fig. 1d). This confirmed the successful expression of codon-optimized patatin-B2 in GS115, which reached a level of 83.5 mg/L by the end of the 120-h period (Fig. 1c).

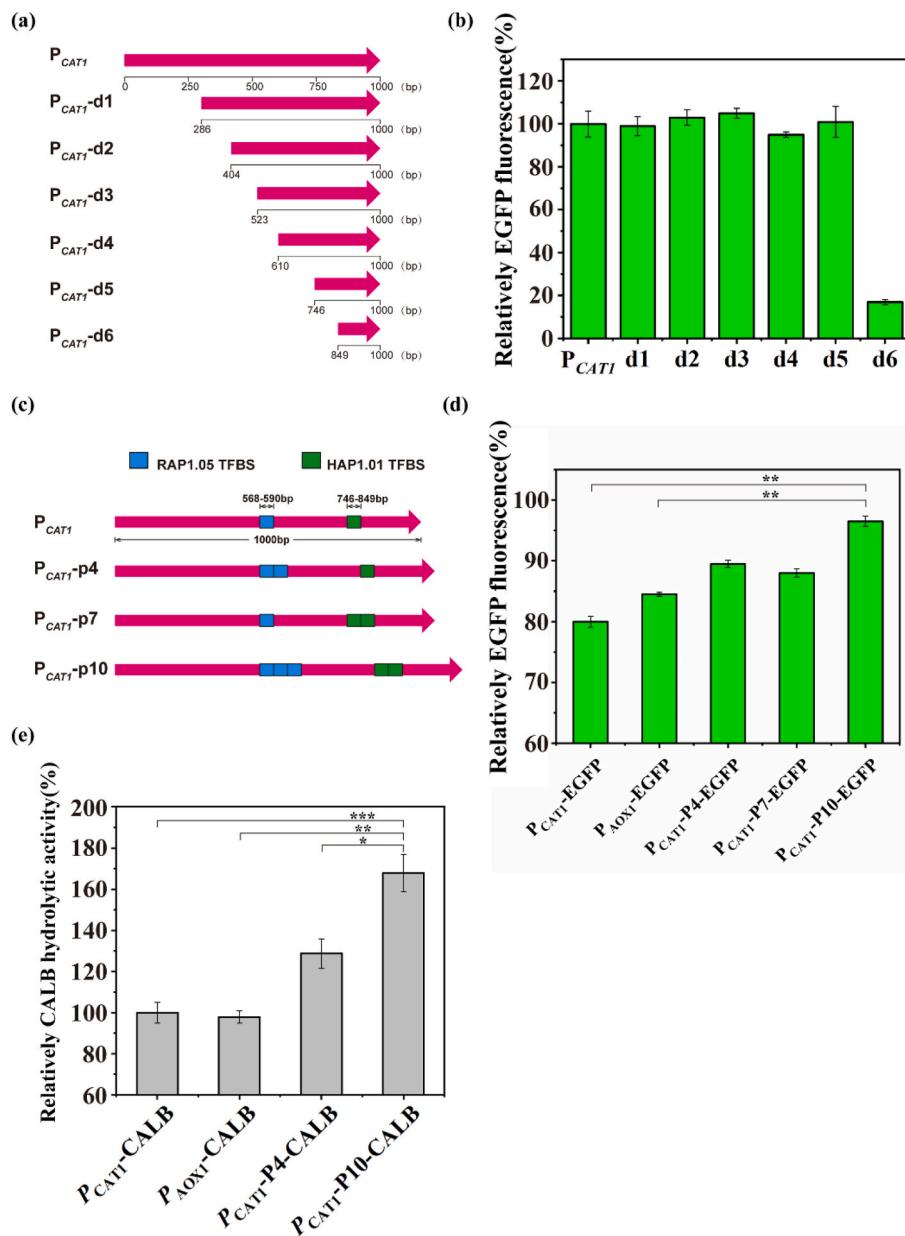
### 3.2. Amelioration of the *CAT1* promoter

Promoters are used for the initiation of gene transcription, and the expression strength of different promoters varies. In terms of protein expression, promoters with high expression strength have attracted significant attention. In our previous study, we used MatInspector from the Genomatix Suite to predict transcription factor-binding sites (TFBSs) of the catalase promoter *P<sub>CAT1</sub>* in *K. phaffii*. By repeating binding sites for the RAP1.05 transcription factor within *P<sub>CAT1</sub>*, the *P<sub>CAT1</sub>-P4* variant significantly increased the production of the EGFP fluorescent protein and the lipase CALB compared with the original *P<sub>CAT1</sub>* and *P<sub>AOX1</sub>* promoters [31]. In this study, leveraging these findings, a fragment deletion approach was used to investigate the impact of varying degrees of truncation on the expression strength of *P<sub>CAT1</sub>* (Fig. 2a). The results revealed that the d1–d5 variants did not significantly change the expression of EGFP and were essentially consistent with the wild type. However, the expression strength of the d6 variant dramatically decreased, indicating that the position of the 746–849 bp fragment of *P<sub>CAT1</sub>* is extremely important (Fig. 2b), and this region includes a HAP1.01 transcription factor-binding site. Therefore, we designed the *P<sub>CAT1</sub>-P7* variant and the *P<sub>CAT1</sub>-P10* variant. The *P<sub>CAT1</sub>-P7* variant repeats the HAP1.01 transcription factor-binding site within this region. For the *P<sub>CAT1</sub>-P10* variant, the RAP1.05 site was repeated twice, and the HAP1.01 site was repeated once (Fig. 2c). Three variants, *P<sub>CAT1</sub>-P4*, *P<sub>CAT1</sub>-P7*, and *P<sub>CAT1</sub>-P10*, were individually employed to secrete the EGFP fluorescent protein and amylase CALB. All three variants presented significantly higher expression levels than did the original *P<sub>CAT1</sub>*, with *P<sub>CAT1</sub>-P10* demonstrating the highest expression (Fig. 2de), indicating a synergistic effect of the repeats at the two sites.

### 3.3. Optimizing expression with the expression cassette toolbox

The expression of a gene requires a complete expression cassette, and for secretory expression, the cassette comprises three components: a promoter, a signal peptide, and a terminator sequence. As mentioned in the previous section, we obtained strains with a production yield of 83.5 mg/L in a 25 mL culture. However, this production level is relatively low. To address this, we introduce the concept of a cassette toolbox, which uses different expression cassette components to replace the existing *AOX1* promoter,  $\alpha$ -factor signal peptide, and *AOX1* terminator. This study aimed to explore the optimal combination of expression cassette components for patatin-B2. Our cassette toolbox comprises different promoters, signal peptides, and terminators. These three components enhance expression, including those generated in this study and those reported in other studies (Fig. 3a).

First, we optimized the promoter of the existing expression cassette.

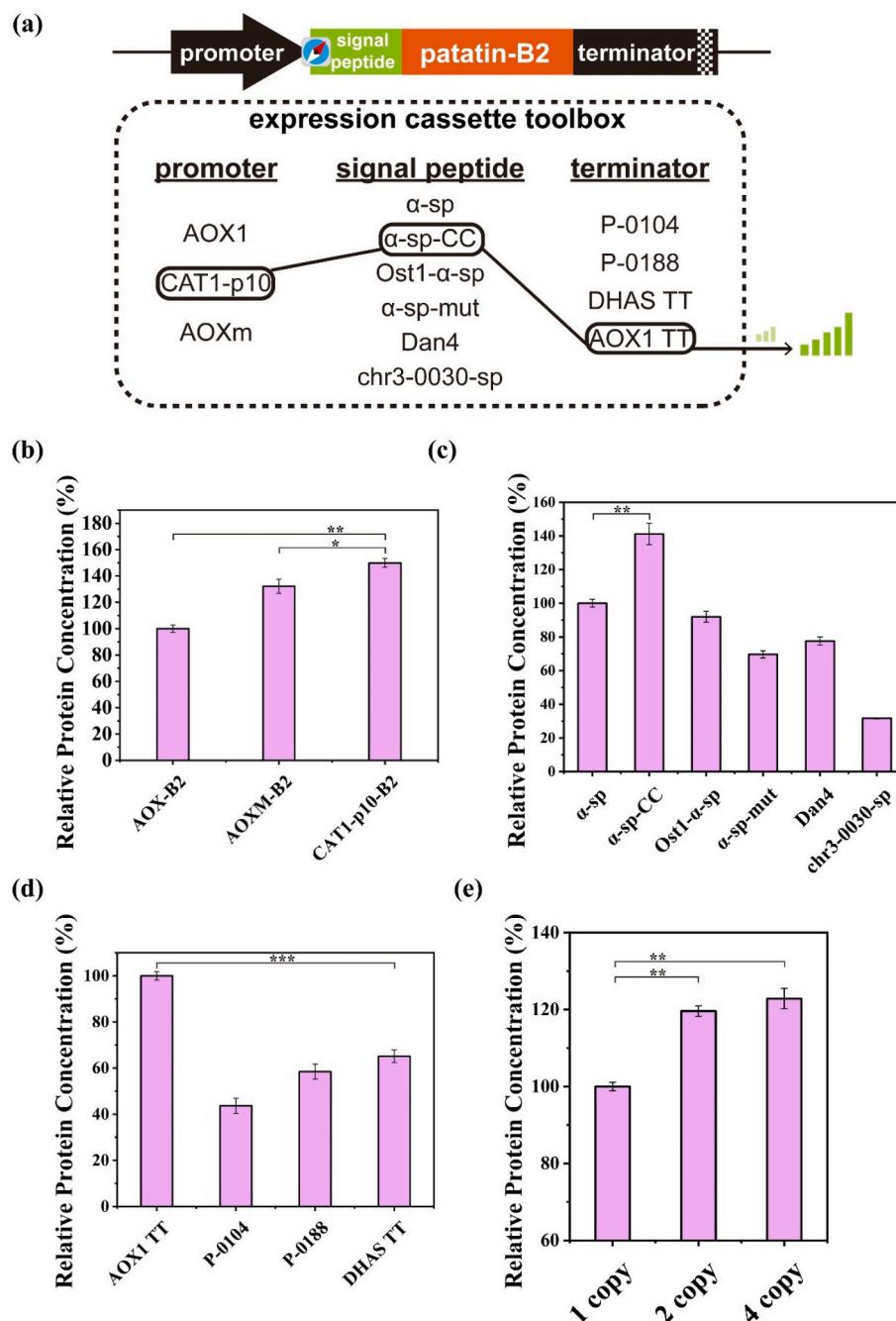


**Fig. 2.** Improvement of the *CAT1* promoter. (a) Truncated variants of the *CAT1* promoter of different lengths. (b) Differences in the expression of EGFP in the truncated *CAT1* promoter. (c) Construction of p7 and p10 variants by repeating the transcription factor-binding sites in the *CAT1* promoter. (d) Differences in the expression of EGFP in the p4, p7, and p10 variants of the *CAT1* promoter in 120 h of methanol induction. (e) Differences in the expression of the lipase CALB in the p4 and p10 variants of the *CAT1* promoter, wild-type *CAT1*, and *AOX1* promoter in 120 h of methanol induction. Statistical significance determined by Student's *t*-test is indicated with an asterisk (\**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001).

Previous studies have confirmed that the p10 variant of the *CAT1* promoter has a relatively high expression level and is superior to both the traditional *AOX1* promoter and the wild-type *CAT1* promoter. Additionally, the previously obtained *AOXM* promoter, which was optimized from the traditional *AOX1* promoter in our earlier research, was also included as a candidate promoter. In our previous study,  $P_{AOXM}$  was confirmed to have stronger protein expression capabilities than  $P_{AOX1}$  [30]. Consequently, using  $P_{CAT1}\text{-p10}$  and  $P_{AOXM}$  to drive the expression of patatin-B2, we compared their differences with those of  $P_{AOX1}$ -driven expression. The results demonstrated that the expression intensities of both promoters in the cassette toolbox surpassed those of the original  $P_{AOX1}$  promoter. Specifically,  $P_{CAT1}\text{-p10}$  presented the highest expression strength, with a 45 % increase compared with that of  $P_{AOX1}$  (Fig. 3b).

The next step is the optimization of the signal peptide. The

expression of secretory proteins in *K. phaffii* requires the involvement of a signal peptide. After translation, the nascent polypeptide chains are guided by the signal peptide to enter the endoplasmic reticulum and Golgi apparatus for folding. Finally, mature proteins are secreted into the extracellular space through vesicles [32,33]. The most commonly used signal peptide in the *K. phaffii* recombinant protein expression system is the  $\alpha$ -mating factor prepro leader sequence derived from *Saccharomyces cerevisiae*, referred to as the  $\alpha$ -signal peptide [34]. We then investigated the impact of different signal peptides from the cassette toolbox on the expression strength of patatin-B2. The candidate signal peptides included  $\alpha$ -signal peptide-CC, a variant derived from the wild-type  $\alpha$ -signal peptide through codon context (CC) optimization by Ahn et al. [35], which can produce more protein in the protein synthesis pathway than the wild-type  $\alpha$ -signal peptide. The Ost1- $\alpha$ -signal peptide, a hybrid created by J. Barrero et al. by combining the signal peptide Ost1



**Fig. 3.** Optimization of expression with the expression cassette toolbox. (a) Components contained in the expression cassette toolbox used in this study. (b) Effects of different promoters on the expression level of Patatin. (c) Effects of different signal peptides on the expression level of Patatin. (d) Effects of different terminators on the expression level of Patatin. (e) Effects of different copy numbers on the expression level of Patatin. All data are derived from samples taken at 120 h post methanol induction in shake flasks. Statistical significance determined by Student's *t*-test is indicated with an asterisk (\**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001).

from *Saccharomyces cerevisiae* with the α-signal peptide, significantly promotes the secretion of some structurally complex proteins [36]. α-signal peptide-mut, a variant with improved protein expression capacity, was obtained by Aza et al. [37] through point mutations on the wild-type α-signal peptide, including the combination of the A9D, A20T, L42S, and D83E mutations. Additionally, some endogenous signal peptides of *K. phaffii* reported in other studies, such as Dan4 [38] and the chr3-0030-signal peptide [39], were included, as they were reported to be more favourable for the expression of certain proteins than the α-signal peptide was. Based on the results of previous experiments and under the premise of selecting the *P<sub>CAT1</sub>-p10* promoter as the expression cassette promoter, the five aforementioned signal peptides, along with

the wild-type α-signal peptide, were individually involved in the expression of patatin-B2 to test the differences among them. As shown in Fig. 3c, under the selected *P<sub>CAT1</sub>-p10* promoter, the α-signal peptide CC increased the production of patatin-B2 by 42 % compared with that of the wild-type α-signal peptide, whereas the other signal peptides resulted in varying degrees of reduction in production.

Finally, the impact of different terminators from the cassette toolbox on protein expression was investigated. The terminator controls transcription termination and can determine mRNA stability and half-life, thereby influencing protein expression [40]. The candidate terminators in this study included our two previously discovered endogenous terminators from *K. phaffii*, *P-0104* and *P-0188*. These terminators have

demonstrated better performance than the *AOX1 TT* when involved in the expression of certain proteins (data not presented). Additionally, Ramakrishnan et al. reported that the terminator of dihydroxyacetone synthase in *K. phaffii* (*DHAS TT*) performed better than *AOX1 TT* when the lipase CALB was expressed [41]. Based on previous studies, we tested three candidate terminators and *AOX1 TT* under the premise of selecting the *P<sub>CAT1</sub>-p10* promoter and  $\alpha$ -signal peptide-CC. The results, as shown in the figure, indicate that, compared with *AOX1 TT*, all three candidate terminators individually reduced the expression of patatin-B2 by 30–60 %. These findings suggest that among these promoters, *AOX1 TT* is the optimal choice for the expression of patatin-B2 (Fig. 3d).

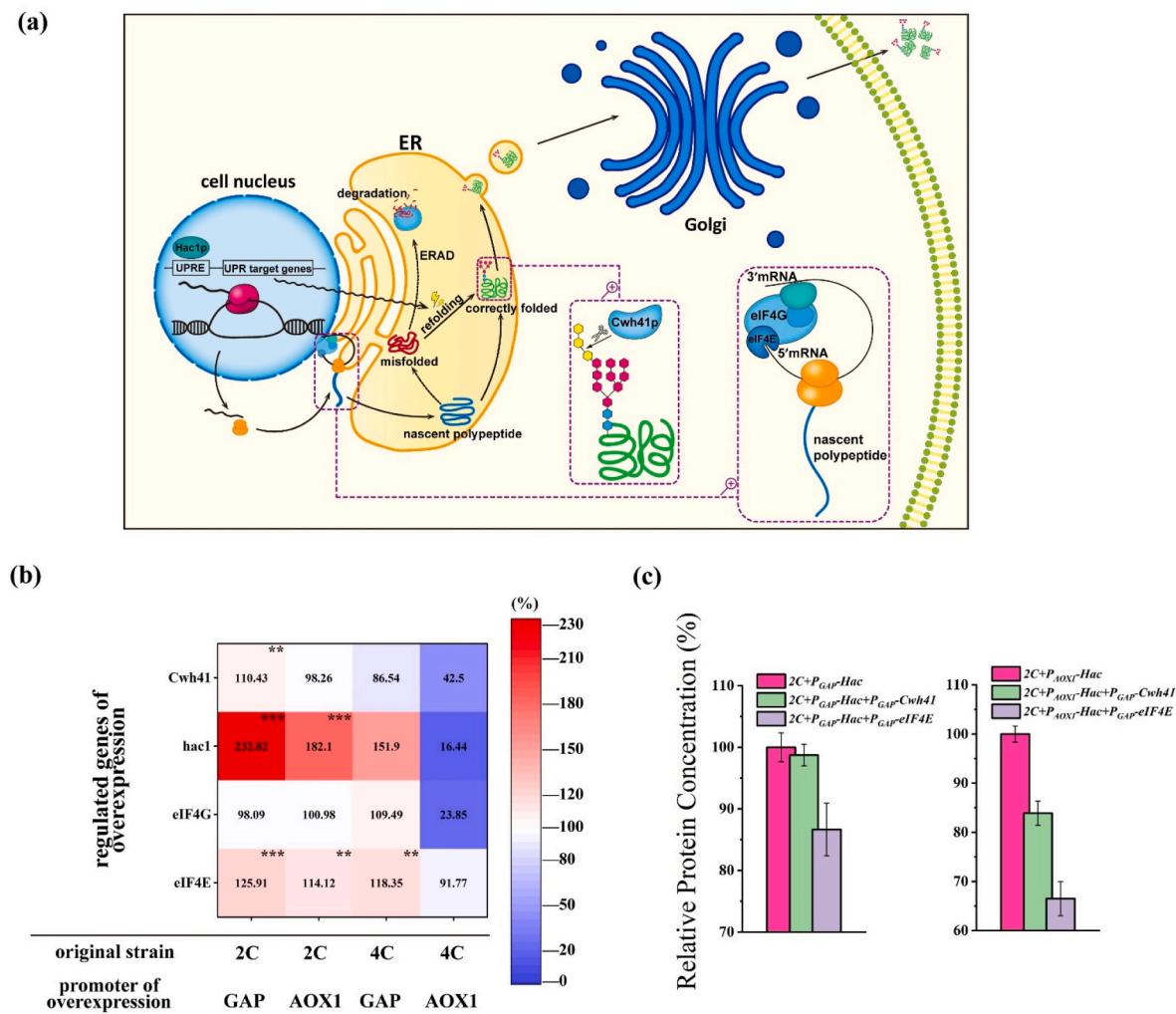
#### 3.4. Optimizing expression by increasing gene dosage

An increase in gene dosage is a commonly employed strategy to increase protein expression levels [42,43]. Specifically, this involves the construction of multicopy strains. Based on the aforementioned results, we selected the *P<sub>CAT1</sub>-p10* promoter,  $\alpha$ -factor-CC signal peptide ( $\alpha$ -CC), and *AOX1 TT* terminator as the optimal components for expressing

patatin-B2. The strain was designated GS115-*PCAT1-p10- $\alpha$ CC*. Building upon this combination, we further constructed a two-copy strain (GS115-*P<sub>CAT1</sub>-p10- $\alpha$ CC-2C) and a four-copy strain (GS115-*P<sub>CAT1</sub>-p10- $\alpha$ CC-4C). Shake flask culture experiments were conducted, and qPCR analysis was employed to determine the relationships among the actual copy numbers in the one-copy, two-copy, and four-copy strains. The results from quantitative PCR demonstrated that the actual copy numbers in the single-copy, two-copy, and four-copy strains were essentially consistent with the theoretical values. Notably, in comparison with the single-copy strain, the two-copy strain presented a 19.61 % increase in patatin expression, whereas the four-copy strain presented a 22.86 % increase. However, after statistical analysis, no significant difference in yield was observed between the two-copy and four-copy strains. (Fig. 3e).**

#### 3.5. Optimizing expression by global regulation of protein secretion

The process of protein expression in yeast encompasses several pivotal steps, namely, transcription, translation, folding, and secretion,



**Fig. 4.** Optimize expression with global regulation of protein secretion. (a) Regulation of protein expression in *Komagataella phaffii* by Hac1p, eIF4G, eIF4E, and Cwh41p. Hac1p activates the UPR pathway, allowing misfolded or incorrectly folded proteins to be refolded into their correct form. Polypeptides that do not achieve correct folding are degraded via the ERAD pathway. eIF4G: The eIF complex is an important regulatory protein in the translation process, with eIF4G serving as a scaffold protein in the eIF complex, stabilizing its structure. eIF4E binds to the 5' cap of mRNA, promoting the formation of a closed-loop structure between the eIF complex and mRNA, enhancing mRNA stability and regulating the initiation of translation. (b) Relationships between the overexpression of regulated genes (rg) at varying intensities and the expression levels of patatin in two-copy (2C) and four-copy (4C) strains. (c) Overexpression of two regulated genes and their impact on the expression level of patatin. All data are derived from samples taken at 120 h post methanol induction in shake flasks. Statistical significance determined by Student's *t*-test is indicated with an asterisk (\**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001).

each of which is meticulously regulated by numerous critical factors. Consequently, optimization of the entire protein synthesis pathway to augment the production of heterologous proteins can be achieved through the overexpression or knockout of key regulatory genes. In this study, we modulated the entire expression pathway of patatin-B2 by overexpressing the *Hac1*, *eIF4G*, *eIF4E*, and *Cwh41* genes (Fig. 4a). The *Hac1* gene serves as a transcription factor for target genes in the unfolded protein response (UPR) pathway. As the transcription and translation of the target gene intensify, a substantial accumulation of nascent polypeptide chains occurs within the endoplasmic reticulum (ER), exceeding the capacity of molecular chaperones to aid in proper folding. This leads to misfolding or a lack of folding of many peptide chains, inducing ER stress and drastically diminishing protein synthesis efficiency [44,45]. Therefore, overexpression of the *Hac1* gene enhances the UPR pathway, facilitating proper polypeptide folding and increasing protein expression levels [46,47]. The eIF complex plays an important role in the translation process, with each of its monomeric proteins having different functions to ensure the smooth progress of translation. *eIF4G* acts as a scaffold protein to stabilize the eIF complex. *eIF4E* binds to the 5' end of mRNA, promoting the formation of a "closed-loop" structure in the mRNA during translation, thereby increasing its stability. The genes *eIF4G* and *eIF4E* have been validated to effectively amplify protein secretion levels upon overexpression [48]. *Cwh41p*, a glucosidase, catalyses the initial step of N-glycan trimming in protein N-glycosylation and initiates the progression of folding. The overexpression of the *Cwh41* gene has been demonstrated to increase protein glycosylation in *Saccharomyces cerevisiae*, increasing the capacity for glycoprotein production [49]. Given the high homology of *Cwh41* within the *Komagataella phaffii* genome, this research focused on the overexpression of the native *Cwh41* gene in *Komagataella phaffii*.

In summary, we selected four expression-enhancing factors from the perspectives of translation, folding, and glycosylation to comprehensively optimize the expression of patatin-B2 in *K. phaffii*. Using the GS115-*P<sub>CAT1</sub>-p10-αCC-B2* strains with either two or four copies as the starting point, we first overexpressed individual expression-enhancing factors to investigate their impact on the patatin-B2 yield. Concurrently, we used strong *P<sub>AOX1</sub>* and weaker *P<sub>GAP</sub>* promoters for overexpression to examine the influence of overexpression intensity on patatin-B2 production. As shown in Fig. 4b, all four molecular chaperones increased the patatin-B2 yield to varying extents. Notably, the two-copy strain significantly outperformed the four-copy strain. Furthermore, under equivalent copy numbers, using the weaker promoter for overexpression yielded more pronounced enhancements, potentially attributed to the perturbation of protein synthesis homeostasis by excessive overexpression. The strain overexpressing the *Hac1* gene from the two-copy configuration presented the most substantial increase in yield, namely, 232.82 % compared with that of its nonoverexpressing counterpart. The overexpression of *Cwh41p* led to a yield increase only in the two-copy strain with the weaker promoter. The overexpression of *eIF4G* resulted in an approximately 9 % increase only in the four-copy strain with the weaker promoter. In the two-copy strain with the weaker promoter, overexpressing *eIF4E* elevated the yield by 25.91 %. Importantly, we observed a pronounced reduction in yield in the four-copy strain when the strong promoter was overexpressed. This decline may be attributed to the disruptive effects of excessive factors overexpression on the protein expression pathway or the burden imposed on the cell by excessive mRNA.

Based on the results of overexpressing individual genes, we further investigated the combined impact of overexpressing two genes simultaneously on the yield of patatin-B2. Using the two copies of the GS115-*P<sub>CAT1</sub>-p10-αCC-B2* strain and the overexpression of *Hac1p* as the starting point (with both strong and weak promoters), we subsequently individually overexpressed *Cwh41p* and *eIF4E* with a weaker promoter. As depicted in the figures, the concurrent overexpression of both genes not only failed to increase the patatin-B2 yield but also led to a variable degree of reduction. These findings suggest that the combinations of

overexpression genes selected did not have synergistic effects, and the specific reasons warrant further investigation. (Fig. 4c).

### 3.6. Fed-batch fermentation

Through optimization of the expression cassette based on the cassette toolbox concept and attempts at global regulation of the protein synthesis pathway, we ultimately identified the optimal strain for the expression of patatin-B2. The expression cassette comprised the *P<sub>CAT1</sub>-p10* promoter, the α-CC signal peptide, and the *AOX1* terminator. Additionally, *Hac1p* was overexpressed using the *P<sub>GAP</sub>* promoter in the two-copy strain, resulting in a strain designated GS115-*P<sub>CAT1</sub>-p10-αCC-B2-2C::P<sub>GAP</sub>-Hac1*. To further investigate the feasibility of high-density fermentation for patatin-B2 production, the strain was fermented in a 5 L bioreactor with methanol induction for 96 h. Fig. 5a shows the theoretical band of patatin-B2 at approximately 45 kDa. The yield of patatin-B2 increased progressively with increasing methanol concentration. After fermentation, 3.2 g of purified patatin-B2 was obtained per litre of culture broth. LC-MS identification of the theoretical bands from the SDS-PAGE gel blocks revealed a sequence coverage of 83 %, further confirming the successful expression of recombinant patatin-B2 (Fig. 5b). The SDS-PAGE of the purification process can be seen in Fig. 5c, and the growth curve of the strains during the fermentation process can be seen in Fig. 5d.

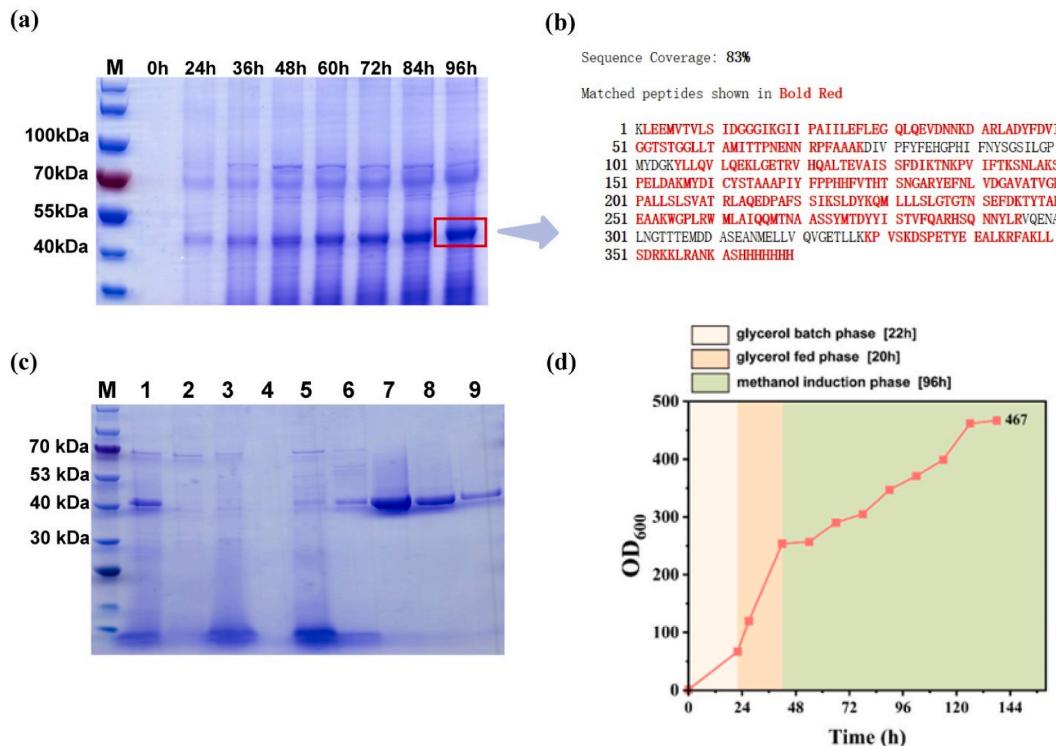
### 3.7. Esterase activity and antioxidant activity

Numerous studies have reported the esterase activity of patatin-B2, which exhibits hydrolytic activity towards various substrates [22]. Several examples have demonstrated the applicability of patatin-B2 in food processing, such as facilitating the release of short-chain fatty acids during cheese ripening to enhance flavour [50]. In this study, the esterase activity of patatin-B2 was verified by the use of *p*-nitrophenyl butyrate as a substrate. Upon calculation, the esterase activity in the fermentation supernatant reached 2.86 U/mg, whereas in the purified protein sample, the esterase activity was 3.46 U/mg.

Patatin-B2 possesses antioxidative properties and contains a plethora of amino acids capable of scavenging free radicals. Seo et al. demonstrated that native patatin-B2 exhibits substantial DPPH radical scavenging activity [51], with its clearance activity equivalent to that of ferulic acid ( $IC_{50} = 27.2 \mu M$ ), a potent antioxidant. Here, the DPPH assay was conducted using a kit procured from Cominbio (Suzhou, China) to investigate the antioxidative activity of patatin-B2, with a DPPH solution concentration of 0.169 mM/L. The results revealed that the DPPH radical scavenging rate of the fermentation supernatant of patatin-B2 reached 18.5 % after 5 min of reaction (the impact generated by the BSM medium was subtracted). Additionally, the purified protein sample (4.5 g/L) had a DPPH radical scavenging rate of 32.5 %. As a control, the DPPH radical scavenging rate of the purified xylanase sample (normalized to the same concentration) was merely 7.5 %, underscoring the robust antioxidative activity inherent to patatin-B2. Furthermore, through comparison, the recombinant patatin-B2 produced by *Komagataella phaffii* in this study exhibited superior antioxidant activity to that of patatin obtained through natural extraction methods [52].

## 4. Discussion and conclusions

In recent years, potato protein has garnered significant attention because of its rich nutritional profile and diverse physiological properties, positioning it as a promising candidate for functional food protein applications. However, inefficient and costly extraction methods have hindered the production of potato protein. *K. phaffii* has emerged as a powerful recombinant protein expression system, successfully expressing a wide variety of proteins. Although there are numerous hosts available for protein expression, *K. phaffii* offers several distinctive



**Fig. 5.** Fed-batch fermentation of recombinant patatin-B2. (a) SDS-PAGE analysis of supernatant samples from fed-batch fermentation at different time points (diluted 4-fold). (b) LC-MS determination of the sequence alignment of the target protein. (c) SDS-PAGE of the purification process, lanes numbered 1–9 represent the following: 1. Diluted fermentation supernatant. 2. Flow-through of the supernatant. 3. Flow-through of the purification buffer (washing away impurities). 4–6. Flow-through of the purification buffer with 60 mM, 120 mM, and 150 mM imidazole (eluting contaminating proteins). 7–9. Flow-through of the purification buffer with 400 mM imidazole (eluting patatin in three separate steps). (d) Growth curve of the strains during the fermentation process.

advantages. For instance, *E. coli*, a commonly used host, has relatively low protein secretion efficiency, often requiring cell lysis and resulting in limited protein modification. Additionally, it is prone to forming inclusion bodies and producing endotoxins [53,54]. Mammalian cells, while capable of providing better post-translational modifications, come with high costs and typically lower yields, making them less suitable for industrial-scale production [55]. While *Saccharomyces cerevisiae* was utilized as a protein expression tool before *K. phaffii*, its expression efficiency is generally lower than that of *K. phaffii* [56], which is why *K. phaffii* is increasingly chosen as the preferred expression host. In this study, we employed a combinatorial approach using the expression cassette toolbox and regulation of protein expression to achieve efficient production of patatin-B2 in *K. phaffii*, achieving a yield of 3.2 g/L, which is the highest reported level of engineered microbial fermentation for patatin production to date. Notably, we innovatively modified the endogenous promoter *P<sub>CAT1</sub>* in *K. phaffii* to obtain a variant, designated *P<sub>CAT1</sub>-p10*, that exhibited superior activity compared with the *P<sub>AOX1</sub>* promoter, thereby providing a more potent tool for future studies of *K. phaffii* protein expression. Additionally, we identified for the first time that solitary overexpression of the glucosidase Cwh41p, UPR pathway target gene transcription factor Hac1p, and translation process regulatory protein eIF4G or eIF4E enhances patatin-B2 production at a specific overexpression intensity. However, this advantage is negated when two genes that regulate the expression of proteins are overexpressed at the same time, which may due to the excessive modification of the genome lead to the disorder of protein expression pathway, and the specific reasons are worthy of further investigation. Previous studies have reported that recombinant patatin produced from yeast has superior bioactivity and improved stability compared with its native counterpart [14]. This study presents an advanced methodological and conceptual framework to produce recombinant patatin, positioning the optimized *K. phaffii* protein expression system as one of the most promising and

cost-effective approaches for patatin production currently available that can also be implemented for the adhibition of other protein expression optimization.

#### CRediT authorship contribution statement

**Yue Huang:** Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Shao Jia:** Methodology, Investigation, Conceptualization. **Ying Lin:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition. **Jiali Wang:** Methodology, Conceptualization. **Luyuan Nong:** Methodology, Investigation, Data curation, Conceptualization. **Lei Ye:** Resources, Methodology, Funding acquisition. **Shuli Liang:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition.

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#### 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.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.synbi.2025.01.005>.

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