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Improved production of recombinant *Rhizomucor miehei* lipase by coexpressing protein folding chaperones in *Pichia pastoris*, which triggered ER stress

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ABSTRACT

Rhizomucor miehei lipase (RML) is a biocatalyst that widely used in laboratory and industrial. Previously, RML with a 70-amino acid propeptide (pRML) was cloned and expressed in *P. pastoris*. Recombinant strains with (strain containing 4-copy *prml*) and without ER stress (strain containing 2-copy *prml*) were obtained. However, the effective expression of pRML in *P. pastoris* by coexpressing ER-related elements in pRML-produced strain with or without ER stress has not been reported to date. In this study, an efficient way to produce functional pRML was explored in *P. pastoris*. The coexpression of protein folding chaperones, including *PDI* and *ERO1*, in different strains with or without ER stress, was investigated. *PDI* overexpression only increased pRML production in 4-copy strain from 705 U/mL to 1430 U/mL because it alleviated the protein folded stress, increased the protein concentration from 0.56 mg/mL to 0.65 mg/mL, and improved enzyme-specific activity from 1238 U/mg to 2186 U/mg. However, *PDI* coexpression could not improve pRML production in the 2-copy strain because it increased protein folded stress, while *ERO1* coexpression in the two strains all had a negative effect on pRML expression. We also investigated the effect of the propeptide on the substrate specificity and the condition for pRML enzyme powder preparation. Results showed that the relative activity exceeded 80% when the substrates C8–C10 were detected at 35°C and pH 6, and C8–C12 at 45°C and pH 8. The optimal enzyme powder preparation pH was 7, and the maximum recovery rate for pRML was 73.19%.

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Introduction

A fungal lipase from *Rhizomucor miehei* (*R. miehei* lipase; RML) is a widely used biocatalyst in the preparation of structured lipids, biodiesel, and enantiomeric separation of chiral drugs [1–3]. The synthesized intracellular RML contains a 70-amino acid propeptide (pRML), and the propeptide is removed after mature RML (mRML) is produced [4]. RML is a single-chain α/β type protein with the catalytic triad (Ser144, His257, Asp203), and the crystal structure with (PDB ID:6QP) and without propeptide (PDB ID: 1TIC) was reported [5,6]. Two commercial forms of RML (Palatase 2000 L in free form and Lipozyme RM IM in an immobilized form) can be purchased from Novozymes (Novo Nordisk A/S Corp, Hillerod, Denmark). Cost remains a key factor limiting the large-scale application of RML [7]. The increase in RML yield and the decrease in production cost are important goals [1].

P. pastoris expression system has many advantages when expressing heterologous proteins, such as simple genetic manipulation, capacity to perform post-translational modifications, mature industrial fermentation processes, and easy purification with extremely low secretion of endogenous proteins [8,9]. The protein expression level can significantly increase after conditions optimized [10–12]. In this protein expression system, as many as 350 kinds of foreign proteins are present, with a yield of more than 10 g/L [13]. And the highest reported value is ~18 g/L of *Trichoderma reesei* cellulase in cell-free broth [14,15]. The expression of RML in *P. pastoris* has also been studied in recent years. Strategies used

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Supplemental data for this article can be accessed here.

by different scholars to improve RML production include optimization of culture conditions [16], promoter selection [17], gene codons [18], and coexpression of isozymes [19]. However, the maximum extracellular enzyme activity is only 175 U/mL (shake flask fermentation), which cannot meet the requirements of industrial production [17]. In our previous study, RML was expressed in *P. pastors* X-33 with its propeptide [1]. Using the strategies of optimizing the signal peptide codon and lipase gene dosage, we obtained a 2-copy *prml* strain (2pRML-X33) that had the maximum extracellular enzyme activity (1200 U/mL) with extracellular pRML protein yield 0.46 mg/mL [1]. While extracellular pRML protein yield in 2-copy strain was not the maximum value, but 4-copy strain. The protein yield of a 4-copy strain (4pRML-X33) can reach 0.64 mg/mL, while its extracellular enzyme activity was only 713 U/mL [1]. The production of pRML protein production in a 4-copy strain is extremely high, but its enzyme activity cannot be simultaneously improved.

Many factors can affect the expression of heterologous proteins. Among numerous factors, host tolerance to foreign proteins is an important limiting step. Overexpressed protein can lead to protein fold pressure in host, and a high supply of protein folding-related elements was required for strain [20,21]. Therefore, the overexpressed protein cannot be successfully folded or unfolded and accumulated in the endoplasmic reticulum (ER), thereby leading to the steady state of ER imbalances [22,23]. The unfolded protein response (UPR) or ER stress is then triggered [22]. The overexpression of the protein fold-related elements can relieve ER stress on the host and improve the production of heterologous protein. Protein disulfide isomerase (PDI) and PDI oxidase (ER oxidoreductin 1, ERO1) are the enzymes related to protein folding. However, modifying the two elements in host can upregulate or decrease the production of heterologous proteins [24–26]. Overexpressing *PDI* increased the expression level of human immunodeficiency virus type I antibody fragments by 1.5- to twofold in *P. pastoris* [27], but decreased the expression level of the porcine insulin precursor in *P. pastoris* [26]. Overexpression with one copy of *ERO1* in strain carrying multicopies of the human serum albumin (HSA/GH) gene can increase the secretion of HSA/GH, but the excessive copies of *ERO1* decrease HSA/GH secretion [28]. Therefore, whether the overexpressed protein folding elements can further improve the extracellular enzyme activity of pRML should be further studied.

To analyze the reasons for not improving synchronously between pRML protein yield and extracellular enzyme activity in 4-copy strain, we compared *prml* mRNA level, enzyme-specific activity of pRML, and transcript level of the key genes (*HAC1, KAR2, PDI,* and *ERO1*) that participated in UPR between 4-copy strain and 2-copy strain [1,29]. The function of four UPR-related genes is shown in Table S1. The results showed that 1) *prml* mRNA level improved and enzyme specific activity decreased in 4-copy strain compared that in 2-copy strain; and 2) the mRNA levels of *HAC1, KAR2*, and *PDI* were significantly upregulated in 4-copy strain compared with that in the 2-copy strain [1,29]. These findings proved that protein folding pressure was generated in a 4-copy strain.

Now, no study has reported the high expression of pRML protein by engineering the protein folding system in the *P. pastoris* system for strains with different gene dosages and cellular physiologies. Thus, in this study, we aimed to increase pRML production by overexpressing protein folding-related elements (*PDI* and *ERO1*) in 2-copy and 4-copy strains and study the cellular physiologies changed in the overexpressed strains. Our work showed a correlation between the gene dosage and secretion of the pRML protein in *PDI*- or *ERO1*-overexpressed strains with or without ER stress. In addition, the effect of propeptide on enzyme properties and the preparation conditions of enzyme powder were also investigated.

Materials and methods

Overexpression of PDI or ERO1 in 2-copy and 4-copy strains

Recombinant strains containing two and four copies of the target *prml* gene were constructed

as described in the previous report [1]. The *PDI* and *ERO1* genes were cloned from the *P. pastoris* genome using primer pairs. *PDI* was amplified using the primer pair *PDI*-f (5′-CCG GAATTCATGCAATTCAACTGGGATAT-TAAA -3′, *Eco*R I site) and *PDI*-r (5′-ATAAGAATGCGGCCGCTTAAAGCTCGTCGT-GAGCG–3′, *Not* I site). *ERO1* was amplified using the primer pair *ERO1*-f (5′-CGGGATCCATGA GGATAGTAAGGAGCG-3′, *Bam*H I site) and *ERO1*-r (5′-ATAAGAATGCGGCCGCTTACAAG TCTACTCTATATGTGGT-3′, *Not* I site). The method for overexpressing *PDI* or *ERO1* in the 2-copy and 4-copy strains was described by Yang et al. (2016) [30], except that vector pPIC3.5K was used. The strains used in this study are listed in Table 1.

Strains culture and lipase activity

Each strain was flask cultured in BMGY/BMMY medium, and the target protein expression was induced as described previously [31]. Absolute methanol was added (final concentration: 1% v/v) for pRML expression. Cell density (estimated as OD_{600}) and lipase activity were evaluated using the NaOH titration method once per day [1]. There are three replicates for each experiment, and the average of them was used as the final value. The error bar was obtained by calculating the sample standard deviations of the three replicates.

Table 1. Strains used in this study.

Strains	Description
2pRML-X33 [1]	Two copies pRML expressed in *Pichia pastoris* X-33 using pPICMα A, named mα-2pRML-X33 in previous study[1].
2pRML-X33K	Intracellular overexpression vector pPIC3.5 K expressed in mα-2pRML-X33
2pRML-X33P	*PDI* was overexpressed in mα-2pRML-X33 used pPIC3.5 K
2pRML-X33E	*ERO1* was overexpressed in mα-2pRML-X33 used pPIC3.5 K
4pRML-X33 [1]	Four copies pRML expressed in *Pichia pastoris* X-33 using pPICMα A, named mα-4pRML-X33 in previous study[1].
4pRML-X33K	Intracellular overexpression vector pPIC3.5 K expressed in mα-4pRML-X33
4pRML-X33P	*PDI* was overexpressed in mα-4pRML-X33 used pPIC3.5 K
4pRML-X33E	*ERO1* was overexpressed in mα-4pRML-X33 used pPIC3.5 K
mα-1mRML-X33[1]	One copy mRML expressed in *Pichia pastoris* X-33 using pPICMα A [1]

2pRML-X33, 4pRML-X33, and mα-1mRML-X33 were constructed in the previous study [1], other strains constructed in this study.

RNA extraction and real-time quantitative PCR (RT-qPCR)

Each strain was cultured in BMGY/BMMY medium. The samples were obtained at 96 h for RNA extraction. TRIzol reagent (9108Q, TaKaRa Biotech Co., Beijing, China) was used to extract RNA [32]. TransScript Green Two-Step qRT-PCR SuperMix (AQ201-01, Transgen biotech Co., Beijing, China) was used to reverse RNA into cDNA. The transcript levels of *prml* and four UPR-related genes, namely, *HAC1*, *KAR2*, *PDI*, and *ERO1*, were analyzed by RT-qPCR. The primers of the abovementioned genes are listed in Tables S2.

The ABI StepOnePlus system was used for RT-qPCR. ABI PowerUp SYBR Green Master Mix (A25742, ABI, USA) was used as the fluorescent dye. The reference gene was the glyceraldehyde-3-phosphate dehydrogenase gene (*gap*) of *P. pastoris*. Reaction system was added according to the instructions of SYBR Green Master Mix and RT-qPCR condition was as follows: 95°C 5 min, 95°C, 10 s; 58°C, 15 s; 72°C, 15 s; 40 cycles. The relative gene expression was analyzed via the comparative crossing point (CP) method and presented as $2^{-\Delta\Delta C_P}$. Three replicates were set up in this experiment, and the data were averaged for the next analysis. The calculation of the error bar was referred to as M&M 2.2.

Target protein detection and enzyme characterization

Target lipase purification by Ni-NTA [29]. Bradford method was used to quantify the protein concentration, with bovine serum albumin as the standard [33].

The lipase hydrolysis activity was detected using NaOH titration [1]. Specific activity (U/mg), extracellular lipase concentration (mg/mL), extracellular lipase activity secretion efficiency (U/OD_{600}) and lipase protein secretion efficiency (mg/OD_{600}) were calculated according to Huang

et al. (2019) [1]. The calculation of the error bar was referred to as M&M 2.2.

Substrate specificity and position specificity

The substrate range was determined under standard conditions using pNP esters with acyl chains of various lengths, namely, pNP acetate (C2), pNP butyrate (C4), pNP caprylate (C8), pNP caprate (C10), pNP laurate (C12), pNP palmitate (C16), and pNP stearate (C18). Initial reaction velocities measured at various substrate concentrations were fitted to the Lineweaver–Burk transformation of the Michaelis–Menten equation. Kinetic analyses by curve fitting were performed with the fit linear program (OriginLab Corp., Northampton, Massachusetts, USA) [34]. The calculation of the error bar was referred to as M&M 2.2 from three replicates.

The position specificity of pRML detected by catalyzing the hydrolysis of soybean oil (triglycerides) and the hydrolyzate analyzed by thin-layer chromatography (TLC).

Enzyme powder preparation

Collecting the fermentation broth: After the strains were fermented at 28°C for 96 h, the supernatant was collected by centrifugation at 6000 rpm for 5 min at 4°C. About 40 mL of the supernatants was adjusted to different pH with 10 M NaOH, three repeats for each pH. The supernatants were placed at −20°C until the surface was frozen. 8 mL of the precooled acetone at −20°C was slowly added to the fermentation broth and then stirred and stood for 10 min. Then, repeat this step using 72 mL precooled acetone and stirred at −20°C for 2–3 h. The precipitate was collected by centrifugation at 8,000 rpm for 10 min at 4°C. Twofold higher precipitation volumes of the precooled acetone were added to the precipitate and slowly stirred, mixed, and centrifuged at 8,000 rpm for 10 min at 4°C. This step was repeated once. The precipitate was air-dried to obtain an enzyme powder. The calculation of average and error bar was referred to M&M 2.2 from three replicates.

Results and discussion

pRML yield increased by PDI overexpression in the 4-copy strain

Numerous studies on the *P. pastoris* expression system focused on optimizing the fermentation process, vector systems, and host strain to improve cell titer and cell-specific productivity [35]. The secretion production of the foreign proteins improved from milligrams to grams per liter after optimizing the above conditions. However, whether host- or protein-based factors limited cell-specific protein secretion levels should be examined [36]. Given the difficulty of optimizing the properties of target proteins by molecular engineering for effective secretion, studies on the improvement in cell-specific secretion titers have shifted their focus on host selection and engineering [36]. For example, engineering protein folding systems [8], the intracellular protein trafficking pathway [37] and minimization of post-secretory proteolytic degradation [38]. In our previous experiments, the strains with (4pRML-X33, 713 U/mL) and without (2pRML-X33, 1200 U/mL) ER stress were constructed by adding the propeptide, thereby optimizing the carrier signal peptide codon and gene dosage [1]. The accumulation of unfolded or misfolded proteins in ER triggered the UPR, which may be relieved by regulating protein folding-related elements, such as *PDI* and *ERO1* [39,40]. Thus, in the present study, *PDI* or *ERO1* overexpression was investigated in the 2- and 4-copy strains with or without ER stress.

The intracellular expression vector pPIC3.5K was used to overexpress *PDI* or *ERO1* in 2- and 4-copy strains, which were called 2pRML-X33P, 2pRML-X33E, 4pRML-X33P, and 4pRML-X33E (Table 1). The respective control strains were called 2pRML-X33K and 4pRML-X33K, containing only pPIC3.5K (Table 1). Each strain was flask-cultured, and daily measurements of the cell growth (OD_{600}) and lipase activity were conducted. The results are shown in Figure 1.

Compared with the 2-copy strain, 2pRML-X33K and *PDI*- or *ERO1*-overexpressed strain had no effect on cell growth and did not enhance extracellular enzyme activity (Figure 1a, b). However, the three strains delayed the time by 1 day to reach the

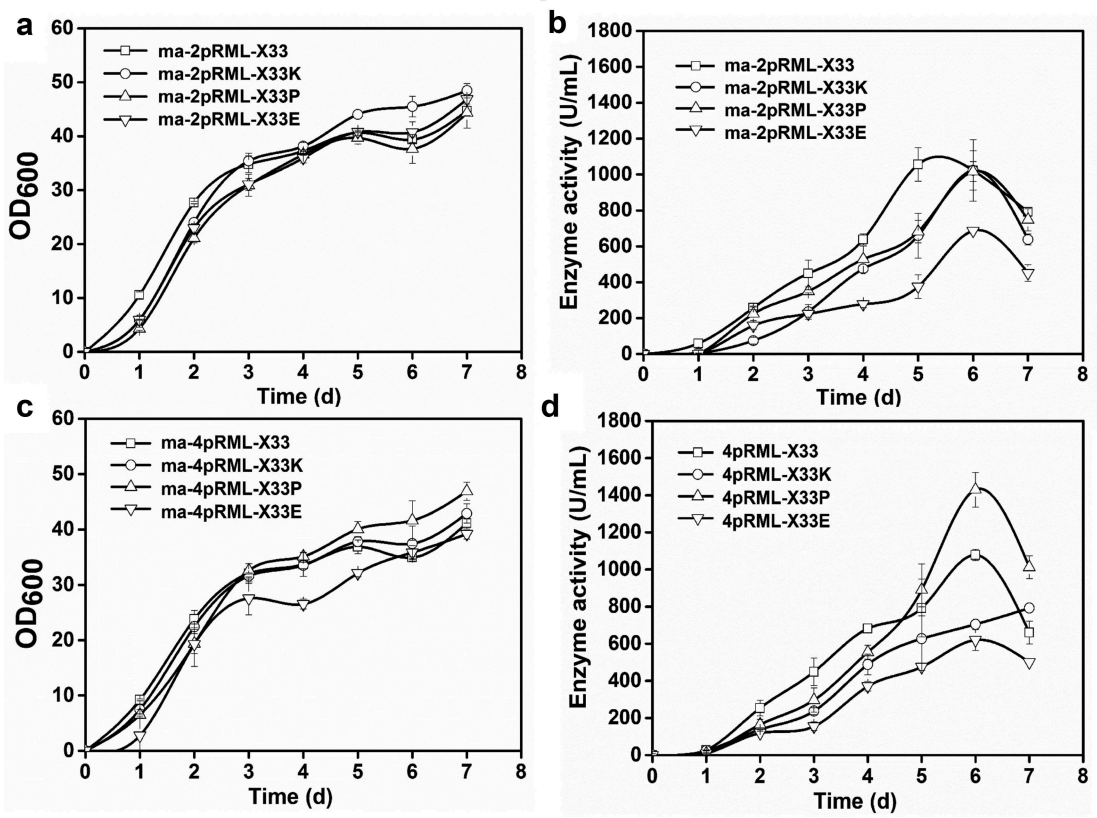

Figure 1. Cell growth and lipase activity of *PDI*- and *ERO1*-overexpressed strains during cultured in flask.
a: OD_{600} of 2-copy and its *PDI*- or *ERO1*-overexpressed strain. **b**: Extracellular lipase activity of 2-copy and *PDI*- or *ERO1*-overexpressed strains.**c**: OD_{600} of 4-copy and *PDI*- or *ERO1*-overexpressed strains. **d**: Extracellular lipase activity of 4-copy and *PDI*- or *ERO1*-overexpressed strains.
The OD_{600} and extracellular lipase activity of 2pRML-X33 and 4pRML-X33 were referred to the previous study [1].

maximum enzyme activity value compared with the 2-copy strain, which may be due to vector pPIC3.5K (Figure 1b). *PDI*-overexpressed strain presented maximum enzyme activities (1016 U/mL), which were similar to those in 2pRML-X33 (1023 U/mL) and 2pRML-X33K (1023 U/mL). Meanwhile, *ERO1*-overexpressed strain significantly reduced the extracellular enzyme activity to 686 U/mL, which was lower than that of 2pRML-X33 and 2pRML-X33K (Figure 1b).

Compared with the 4-copy strain, 4pRML-X33K and overexpressed *PDI* or *ERO1* gene did not significantly affect cell growth, and the culture time reached the maximum extracellular enzyme activity value was all on the 6th day (Figure 1c, d). The extracellular enzyme activity of 4pRML-X33K on the 6th day was 705 U/mL, which was decreased than that of the 4-copy strain (1078 U/mL, Figure 1d). The extracellular enzyme activity increased to 1430 U/mL after overexpressing *PDI* in 4-copy strain using the pPIC3.5K vector, which was 1.3- and 2.0-fold higher than 4pRML-X33 and 4pRML-X33K, respectively (Figure 1d). However, *ERO1*-expressed strain had the maximum extracellular enzyme activity of only 620 U/mL (Figure 1d). This result proved that overexpressed *ERO1* was harmful for pRML production in the 4-copy strain.

In the process of catalyzing the formation of disulfide bonds in protein molecules, PDI itself is reduced. ERO1 participates in oxidative protein folding through PDI. ERO1 reoxidizes PDI from the reduced state to the oxidation state [41]. But the process generates an amount of reactive oxygen species (hydrogen peroxide), which is harmful to host [42]. Overexpressed ERO1 may cause futile oxidation cycles, and this may be another reason for the low pRML production in *ERO1*-overexpressed

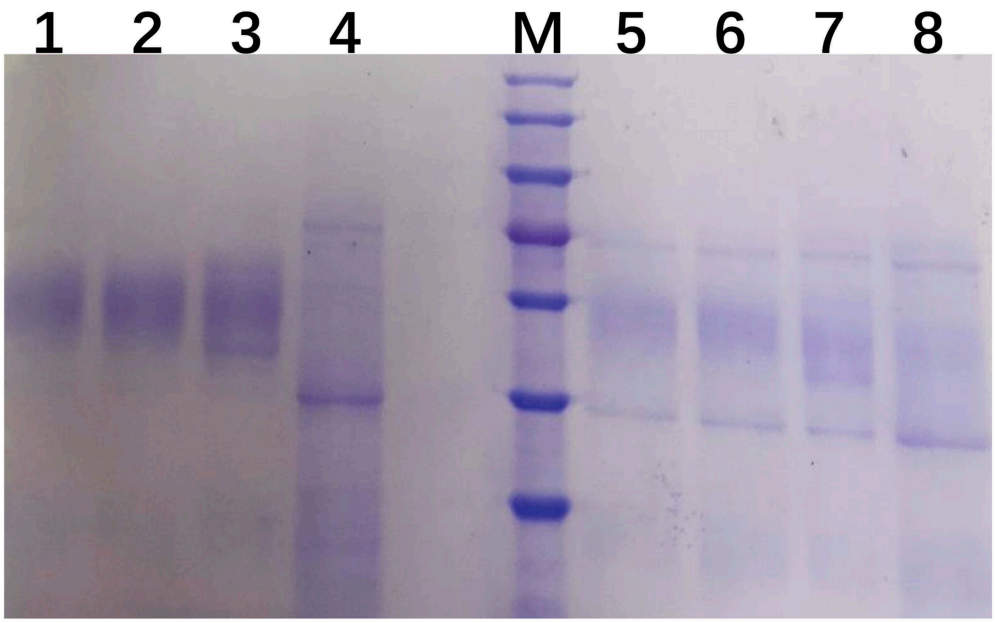

Figure 2. Extracellular protein expression in fermentation broth detected by SDS-PAGE.
Lane M: protein markers (180, 140, 100, 80, 60, 45 and 35 kDa). Lanes 1–4: recombinant strain 2pRML-X33, 2pRML-X33K, 2pRML-X33P and 2pRML-X33E. Lanes 5–8: recombinant strain 4pRML-X33, 4pRML-X33K, 4pRML-X33P and 4pRML-X33E. Target protein (45–80 kDa) increased in 2pRML-X33P and 4pRML-X33P, but decreased in 2pRML-X33E and 4pRML-X33E.

strains. Until now, the extracellular enzyme activity of pRML improved used different strategies in our study was shown in Table S3, and the 1430 U/mL was the maximum yield produced by *P. pastoris*.

Extracellular protein detection

The extracellular protein levels of the above-mentioned strains were detected by SDS-PAGE on the 6th day, and the result is shown in Figure 2. Each strain had the target pRML protein, with a broad molecular weight (MW) of 45–80 kDa. The broad MW was a result of glycosylation [1]. Overexpressed *PDI* in the 2- and 4-copy strains increased pRML protein secretion (Figure 2, lanes 3 and 7). By contrast, overexpressed *ERO1* in the 2- and 4-copy strains reduced pRML protein secretion (Figure 2, lanes 4 and 8).

Extracellular target protein produced by each strain was purified by Ni-NTA. The specific activity (U/mg), extracellular lipase concentration (mg/mL), extracellular lipase activity secretion efficiency (U/OD_{600}), and pRML protein secretion efficiency (mg/OD_{600}) were compared among different strains after the 6th day, and the result is shown in Table 2. Vector pPIC3.5K had no significant effect on the four parameters compared with the 2--copy strain. Overexpressed *PDI* in the 2-copy strain increased the pRML protein concentration to 0.66 mg/mL, and the pRML protein secretion efficiency increased to 0.017 mg/OD_{600}. By contrast, the pRML protein concentration and

Table 2. Parameters of lipase produced by different recombinant strains.

	Extracellular enzyme activity (U/mL)	OD_{600}	Specific activity (U/mg)	Extracellular protein concentration (mg/mL)	Extracellular lipase activity secretion efficiency (U/OD_{600})	pRML protein secretion efficiency (mg/OD_{600})
2pRML-X33	1023 ± 108	39 ± 0.5	2075 ± 51	0.50 ± 0.004	26.2 ± 0.67	0.013 ± 0.0014
2pRML-X33K	1023 ± 171	46 ± 1.9	1939 ± 32	0.53 ± 0.024	22.2 ± 0.51	0.012 ± 0.0008
2pRML-X33P	1016 ± 56	38 ± 2.7	1529 ± 105	0.66 ± 0.011	26.7 ± 0.40	0.017 ± 0.0012
2pRML-X33E	686 ± 0	41 ± 1.9	1716 ± 40	0.41 ± 0.030	16.7 ± 0.32	0.010 ± 0.0007
4pRML-X33	1078 ± 31	35 ± 0.2	1796 ± 35	0.59 ± 0.021	30.8 ± 0.71	0.017 ± 0.0017
4pRML-X33K	705 ± 0	37 ± 3.2	1238 ± 28	0.56 ± 0.017	20.3 ± 0.55	0.015 ± 0.0013
4pRML-X33P	1430 ± 93	42 ± 3.6	2186 ± 50	0.65 ± 0.027	34.1 ± 0.65	0.015 ± 0.0014
4pRML-X33E	620 ± 56	36 ± 1.3	1550 ± 26	0.40 ± 0.037	17.2 ± 0.33	0.011 ± 0.0015

pRML protein secretion efficiency of the control strain 2pRML-X33K were 0.53 mg/mL and 0.012 mg/OD_{600}, respectively. However, the specific activity of pRML secreted by the overexpressed *PDI* in 2-copy strain decreased from 1939 U/mg to 1529 U/mg, which may be caused by the misfolded lipase protein.

Compared with 4pRML-X33K, overexpressed *PDI* in the 4-copy strain did not affect the efficiency of pRML protein secretion (0.015 mg/OD_{600}), but the extracellular protein concentration increased to 0.65 mg/mL because of the addition in biomass. The specific activity of the enzyme also increased to 2186 U/mg from 1238 U/mg, which proved the correct protein fold in pRML. Therefore, the extracellular enzyme activity of 4pRML-X33P improved to 1430 U/mL because of the increased specific activity of pRML and extracellular lipase concentration.

Overexpressed *ERO1* in 2-copy strain caused the specific activity of pRML and the pRML protein secretion efficiency, respectively, decreased to 1716 U/mg and 0.010 mg/OD_{600}. Although overexpressed *ERO1* in 4-copy strain did not significantly affect pRML specific activity, its protein secretion efficiency dropped to 0.011 mg/OD_{600}. Therefore, the extracellular protein concentration, extracellular enzyme activity, and extracellular lipase activity secretion efficiency of 2pRML-X33E and 4pRML-X33E were all decreased.

ER stress analysis

The overexpressed protein caused protein fold pressure and produced the amount of unfolded or misfolded proteins [39]. Accumulation of unfolded or misfolded proteins in ER can trigger the UPR [40]. mRNA levels of marker genes (*HAC1, KAR2, PDI,* and *ERO1*) can be used for determining whether UPR is triggered [43]. The specific activity changed in pRML secreted by *PDI*- or *ERO1*-overexpressed strains may be caused by the conformation change of lipase.

To analyze the effects of overexpressed genes (*PDI* or *ERO1*) on the protein folding capacity of ER in different strains, we examined the transcript level change in *prml* and four UPR-related genes (i.e., *HAC1, KAR2, PDI*, and *ERO1*). The results are shown in Figure 3. Overexpressed *PDI* or *ERO1* in 2pRML-X33 upregulated the transcript level of *HAC1, KAR2* and *ERO1*/PDI (Figure 3a). This result proved that overexpressed *PDI* or *ERO1* in the 2-copy strain caused ER stress, which was produced by misfolded or unfolded proteins accumulated in the ER. So, the specific activity of lipase produced by 2pRML-X33P and 2pRML-X33E decreased to 1529 U/mL and 1716 U/mL (Table 2). Overexpressed *PDI* in the 2-copy strain did not affect the *prml* mRNA level, but *ERO1* overexpression significantly downregulated the *prml* mRNA level (Figure 3a). This may be another reason for 2pRML-X33E decreased its extracellular enzyme activity.

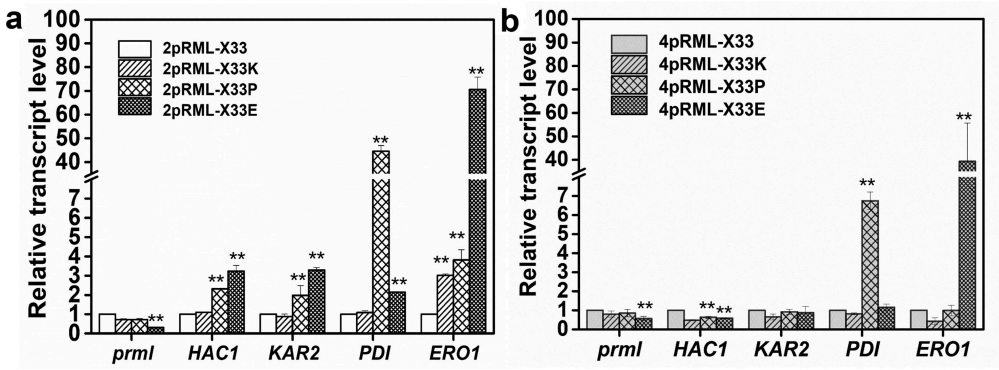

Figure 3. Transcription level analysis of *prml* and four UPR-related genes in different strains.

The skewness and kurtosis of the data were calculated by SPSS. For normally distributed data, Student's t-tests were used to evaluate its significance (p value), which represented by '*' or '**'. p Value ≤0.05 was marked '*' and p value ≤0.01 was marked '*'. p Value ≤0.05 and p value ≤0.01 were considered as significant difference and extremely significant difference, respectively. **a**: Transcription levels of *HAC1, KAR2, PDI*, and *ERO1* in 2-copy, *PDI*-overexpressed, and *ERO1*-overexpressed strains. **b**: Transcription levels of *HAC1, KAR2, PDI*, and *ERO1* in 4-copy, *PDI*-overexpressed, and *ERO1*-overexpressed strains.

Real-time PCR analysis was also conducted in the 4-copy strain overexpressed *PDI or ERO1*, and the result is shown in Figure 3b. Overexpressed *PDI* in 4-copy strain had no effect on the *prml* mRNA level but downregulated the transcript level of *HAC1* (Figure 3b). This result proved that ER stress was relieved in 4pRML-X33P, and the specific activity of lipase produced by 4pRML-X33P increased to 2182 U/mg. Although overexpressed *ERO1* downregulated *HAC1* mRNA level and relieved ER stress, it reduced *prml* expression (Figure 3b), thereby leading to low pRML production in 4pRML-X33E.

Propeptide had no effect on substrate specificity

pRML enzyme activity reached a maximum value of 1430 U/mL by overexpressing the protein folding-related element *PDI* in the 4-copy strain. This result exceeded that of the 2-copy strain (1023 U/mL) and was the highest value reported to date. The highly expressed pRML contained a 70-amino acid precursor peptide. Whether the precursor affects the substrate specificity of the enzyme should be answered in future work. Thus, we compared the substrate specificity between the lipase with (pRML) and without (mRML) propeptide using pNP (C4-C18) as a substrate. mRML was produced by mα-1mRML-X33, which was constructed in the previous study [1]. The results are shown in Figure 4. No effect on the substrate specificity between pRML and mRML was found. The substrate with relative activity exceeding 80% was C8-C10 when pRML and mRML were detected at 35°C with a pH of 6 (Figure 4a). While the substrate specificity was all changed to C8-C12 when the reaction temperature and pH were 45°C and pH 8 (Figure. 4b-d). The positional specificity of pRML to hydrolyze triglycerides was also been examined in this study (Figure S1). 1,2-DAG was detected in the hydrolysis product, but there was no 1,3-DAG, which proved that pRML still was a 1,3-position-specific lipase. Thus, the propeptide had no effect on the substrate specificity and position specificity of pRML. To

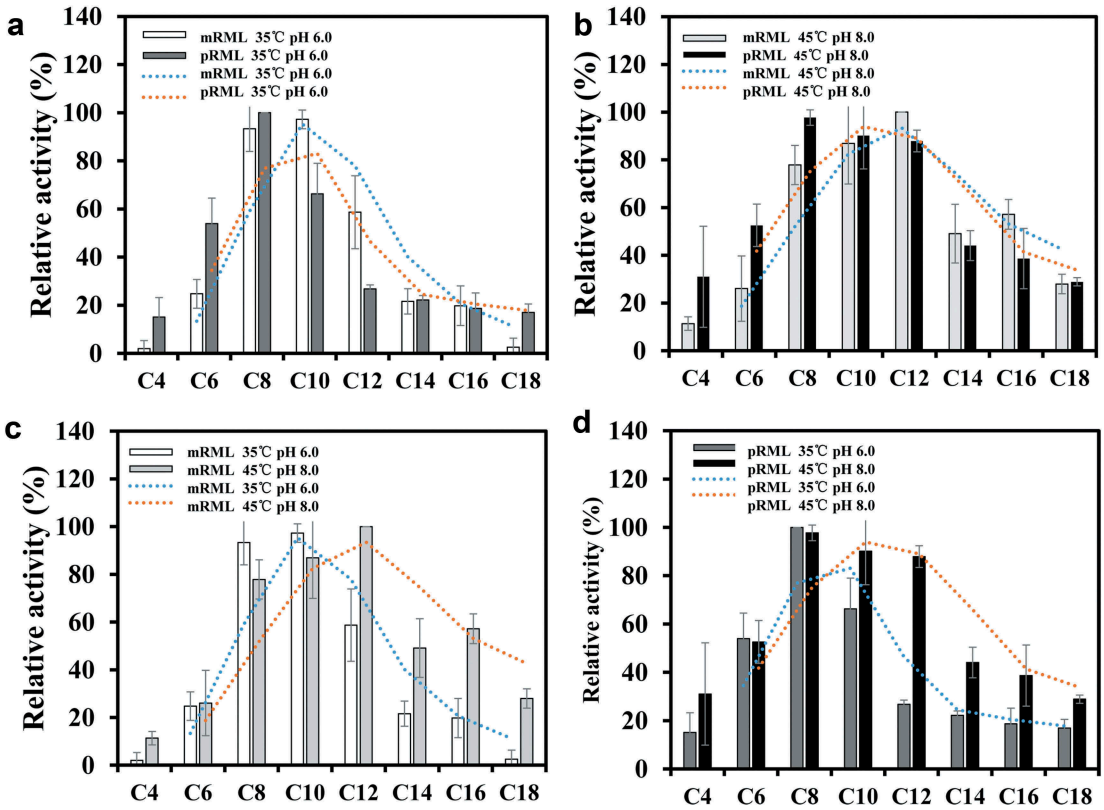

Figure 4. Comparison of substrate specificity between pRML and mRML.

a: Substrate specificity of pRML and mRML detected at 35°C and pH 6; **b**: Substrate specificity of pRML and mRML detected at 45°C and pH 8; **c**: Substrate specificity of mRML detected at 35°C with pH 6 and 45°C with pH 8; **d**: Substrate specificity of pRML detected at 35°C with pH 6 and 45°C with pH 8.

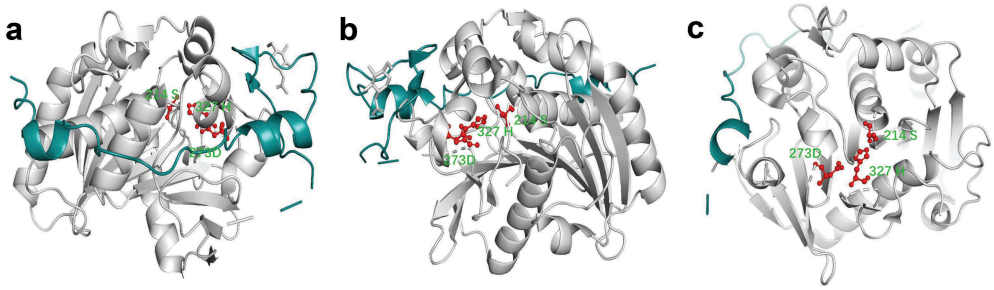

Figure 5. Location of propeptide and active site in 3D structure of pRML.
a: front; **b**: back; **c**: side. Blue represents a 70-amino acid propeptide, and red denotes the catalytic triad (Ser214-His327-Asp273).

this end, we investigated the location of the propeptide and the active center in pRML. pRML 3D structural model was constructed using SWISS-MODEL server (https://swissmodel.expasy.org), and a graphical figure of propeptide (1-70aa) and active site (Ser214-His327-Asp273) were marked using PyMOL Molecular Graphics System. The result is shown in Figure 5. Blue represents a 70-amino acid propeptide, and red denotes the catalytic triad (Ser214-His327-Asp273). From different perspectives (front, back, and side), the propeptide was encapsulated in the periphery of the protein and did not participate in the composition of the active center. Thus, the propeptide did not affect the substrate specificity and positional specificity of pRML.

Enzyme powder

Enzymes can be used directly in the form of fermentation broth, but fermentation broth is not conducive to storage and takes up considerable space. Enzyme powder is a good form for convenient storage and transportation. Therefore, after obtaining the high yield of the enzyme, we investigated the optimum pH to prepare the enzyme powder with acetone (Table 3). pRML from fermentation broth had different pH values ranging from 4 to 8. The recovery rate exceeded 50% when the fermentation broth pH was between 5 and 7. The highest recovery rate of pRML (73%) was obtained at pH 7. Under this condition, the specific activity reached 11.02 × 10^4 U per g (pRML), and 1.406 g of enzyme powder was harvested from 40 mL fermentation broth. However, the lowest specific activity (0.65 × 10^4 U per g) and recovery rate (3%) were obtained when the pH of the fermentation broth was adjusted to 8.

Conclusion

Our study showed a simple and direct way to optimize pRML secretion. Coexpressing *PDI* with *prml* is an attractive strategy for the development of efficient pRML expression systems. In this study, we improved pRML production to 1430 U/mL from the strain with the protein fold pressure strain by overexpressing *PDI*, and found that pRML production could not be improved in the strain without the protein fold pressure strain. Substrate specificity analysis found that propeptide had no effect on the substrate specificity and positional specificity of pRML, and the optimal enzyme powder preparation pH of pRML was 7, with the recovery rates reaching 73.19%.

Highlights

(1) Overexpressed PDI in 4pRML-X33 increased pRML production from 705 to 1430 U/mL.
(2) Propeptide had no effect on the substrate specificity of pRML compared with RML.

Table 3. Effect of the fermentation broth's pH on pRML enzyme power.

	pH 4.0	pH 5.0	pH 6.0	pH 7.0	pH 8.0
Specific activity (×10^4 U per g)	0.74 ± 0.032	9.05 ± 0.031	11.69 ± 0.034	11.02 ± 0.033	0.65 ± 0.032
Weight (g per 40 mL)	1.022 ± 0.0145	1.188 ± 0.0009	1.240 ± 0.0313	1.406 ± 0.0076	1.056 ± 0.0023
Recovery rate (%)	3.587 ± 0.103	50.77 ± 1.711	68.52 ± 3.707	73.19 ± 2.593	3.243 ± 0.127

(3) The maximum recovery rate for pRML was 73.19% when the pH of supernatant was 7.

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Disclosure Statement

No potential conflicts of interest were disclosed.

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