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Efficient secretion of lipase r27RCL in *Pichia pastoris* by enhancing the disulfide bond formation pathway in the endoplasmic reticulum

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Abstract The lipase r27RCL from *Rhizopus chinensis* CCTCC M201021 was heterologously expressed in *Pichia pastoris* GS115 by simultaneous co-expression with two secretion factors ERO1p and PDI involved in the endoplasmic reticulum (ER). Compared to the expression of the lipase alone (12,500 U/ml), co-expression with these two proteins resulted in the production of larger total quantities of enzymes. The largest increase was seen when the combined ERO1p/PDI system was co-expressed, resulting in approximately 30 % higher enzyme yields (16,200 U/ml) than in the absence of co-expressed secretion factors. The extracellular protein concentration of the recombinant strain Co XY RCL-5 reached 9.39 g/l in the 7-l fermentor. Simultaneously, the fermentation time was also shortened by about 8 h compared to that of the control. The substrate-specific consumption rate (Qs) and the product-specific production rate (Qp) were both investigated in this research. In conclusion, the space–time yield was improved by co-expression with ERO1p and PDI. This is a potential strategy for high level expression of other heterologous proteins in *P. pastoris*.

Keywords Recombinant lipase · *Pichia pastoris* GS115 · Chaperone · PDI · ERO1p

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Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are part of the family of hydrolases that hydrolyze triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. Lipases can catalyze esterification, interesterification, and transesterification reactions in nonaqueous media, which makes lipases the enzymes of choice for potential applications in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries [11].

In our previous study, the lipase gene *proRCL* was cloned from *Rhizopus chinensis* CCTCC M201021 and successfully expressed in *Pichia pastoris* GS115, which showed a high potential for industrial usage, including synthesis of eicosapentaenoic acid and docosahexaenoic acid, sorbitan oleate, ethyl esters, and biodiesel [28, 29, 32, 33, 35]. However, the cost of large quantities of lipase for use in industry is very high, which makes high level heterogenous production of lipase extremely important.

P. pastoris is one of the most promising host systems. However, overexpression of heterologous proteins in *P. pastoris* can lead to saturation or overloading of the secretory pathway and even trigger the unfolded protein response (UPR), a process which involves many secretion helpers, such as transcription factor *Hac1*, the protein folding factors *ERO1* and *PDI*, the translocation or other secretion helper factors *COG6*, *SSA4*, *SSE1*, *SSO2*, and so on [9]. In most cases, the rate-limiting step in the eukaryotic secretion pathway is the protein folding in the endoplasmic reticulum (ER) [24]. A proper balance between synthesis, maturation, and degradation in the ER is crucial for cell survival. The physiological roles and the molecular basis of the disulfide bond formation pathway were recently reviewed [23]. Two abundant ER resident proteins directly involved in these processes are endoplasmic

reticulum oxidoreduction 1 (ERO1p) and protein disulfide isomerase (PDI). Co-expression with PDI resulted in increased secretion levels or biological activity of the heterologous proteins in *P. pastoris* [16, 30]. However, in some cases, the increase of foreign proteins in *P. pastoris* could not solely be achieved by co-expression with chaperone PDI in the folding pathway [6]. In addition, co-overexpression with ERO1p and PDI has already been conducted in *Kluyveromyces lactis* and Chinese hamster ovary cells, but not reported in *P. pastoris* [17, 19].

In our previous research, we also observed that increasing the copy number of the lipase gene *proRCL* in *P. pastoris* resulted in a reduced secretion of the product r27RCL. UPR was probably activated in the recombinant strain with six copies of lipase gene by analysis of the transcription levels of relevant genes *ERO1* and *PDI* in the ER [25]. There are numerous instances in the literature wherein overexpression with ER chaperones or foldases has been used to increase secretion yields. To our knowledge, no study on the production of recombinant lipase in *P. pastoris* GS115 by co-expression with ERO1p and PDI has been conducted. Therefore, we investigated the effects of co-expression with these two ER-resident proteins on the lipase r27RCL secretion in *P. pastoris*.

Materials and methods

Enzymes and reagent

The following were used: restriction enzymes, T4 DNA ligase, polymerase chain reaction (PCR) reagent (TaKaRa Biotechnology (Dalian) Co., Ltd.), primers (SBS Gene Technology (Shanghai) Co., ltd.), gel extraction kit and plasmid mini kit I (OMEGA BIO-TEK), PCR purification kit (Bioflux), real-time quantitative PCR (RT-qPCR) reagent and material (Bio-Rad).

Strains, plasmids, and medium

P. pastoris GS115, plasmids pPIC9 K, pPIC3.5, and pPICZαA from Invitrogen BV were used as the host strain and the vectors, respectively. Recombinant plasmid pPIC9 K-*proRCL* was constructed before [35]. Yeast nutrient medium MD, YPD, BMGY, BMMY, YPD-G418, and YPD-Zeocin were prepared by means of the *P. pastoris* expression kit (*Pichia* Multi-Copy Expression Kit, version A, Invitrogen BV, the Netherlands).

Construction of expression plasmids

The *ERO1* gene (GenBank accession number FN392319) was amplified from chromosomal DNA of *P. pastoris* GS115 using the forward primer 5′-CGGATCCAT GAGGATAGTAAGGAGCG-3′ (*Bam*HI site underlined) and the reverse primer 5′-AGCGGCCGCTTACAAGTCT ACTCTATA TG-3′ (*Not*I site underlined). The *Bam*HI/*Not*I amplicon was cloned into a *Bam*HI/*Not*I-digested pPIC3.5 generating pPIC3.5-*ERO1* and placing the gene under the control of the *AOX1* promoter. The *PDI* gene (GenBank accession number AJ302014) was also amplified from chromosomal DNA of *P. pastoris* GS115 using the forward primer 5′-GGGTTCGAAACGATGCAATTCAAC TGGG-3′ (*Asu*II site underlined) and the reverse primer 5′-TTGCGGCCGCTTAAAGCTCGTCGTGAGCGTC-3′ (*Not*I site underlined). The *Asu*II/*Not*I amplicon was cloned into *Asu*II/*Not*I-digested pPICZαA resulting in pPICZ-*PDI* without the *Saccharomyces cerevisiae* α-mating factor secretion signal under the control of the *AOX1* promoter.

Transformation of *P. pastoris*

Plasmids of pPIC3.5-*ERO1*, pPICZ-*PDI*, and pPIC9 K-*proRCL* were separately linearized by *Sac*I or *Sal*I and transformed into corresponding *P. pastoris* competent cells by electroporation. The cells were pulsed in a 2-mm electroporation cuvette at 1.5 kV, 200 Ω, and 25 μF in a Gene Pulser electroporator (Bio-Rad) and 1 ml of ice-cold 1 M sorbitol was added immediately. Then transformed cells were spread on MD, YPD-Zeocin, or YPD-G418 agar plates. After about 2–5 days, the colonies were picked and the gene copy numbers in the recombinant strains were identified by RT-qPCR as established before [25].

Cultivation conditions in shake flasks

A single colony was transformed into 25 ml of BMGY and incubated for approximately 24 h at 30 °C until the OD_{600} of the culture reached 2–6. The cells were harvested by centrifugation and resuspended in 100 ml of BMMY, which was incubated at 250 rpm for 5 days at 30 °C. Methanol was added into the medium every 24 h to a final concentration of 1.0 % to maintain induction. Meanwhile, samples were taken every 24 h and centrifugated. The culture supernatants were checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE analysis of the expressed products

Denaturing SDS-PAGE was conducted in accordance with the method of laemmli [15]. The supernatant samples (20 μl) were resolved on 12 % SDS-PAGE under reducing conditions and visualized by Coomassie Blue R-250 staining.

Lipase activity determination

The lipase activity in the supernatant was assayed by the method developed previously [14]. One volume of a 1.08 mM solution of *p*NPP in 2-propanol was mixed just prior to use with nine volumes of 50 mM Tris–HCl buffer pH 8.0, containing 4 g/l Triton X-100 and 1 g/l arabic gum. The standard reaction was started by pre-equilibration of 2 ml of the above mixture at 40 °C and addition of 0.1 ml of enzyme solution at an appropriate dilution in 50 mM pH 8.0 Tris–HCl buffer. The variation of the absorbance of the assay at 410 nm against a blank without enzyme was monitored for 2–5 min using a UV–Vis spectrophotometer (UNICO UV-3102 PC, China). One enzyme unit was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute under the assay conditions. All the assays were conducted in triplicate and significant differences ($p < 0.05$) were measured.

Fermentor fermentation

The 7-l fermentation experiments were performed as reported previously [18]. The culture was incubated at 28 °C with a 2.8-l volume in a 7-l bioreactor (New Brunswick, BioFlo110, Edison, NJ, USA). The inocula were grown for 18 h at 28 °C in shake flasks at 250 rpm with BMGY medium. In the glycerol batch phase, 200 ml of inoculum was directly added into 2.6 l of a fermentation basal salts medium (40 g/l glycerol, 22.7 g/l H_3PO_4, 0.93 g/l $CaSO_4$, 18.2 g/l K_2SO_4, 14.9 g/l $MgSO_4{\cdot}7H_2O$, 4.13 g/l KOH, and 7.0 g/l K_2HPO_4) and trace solution (12 ml). Trace solution consisted of 6 g/l $CuSO_4{\cdot}5H_2O$, 0.08 g/l NaI, 3.0 g/l $MnSO_4{\cdot}H_2O$, 0.2 g/l $Na_2MoO_4{\cdot}2H_2O$, 0.02 g/l H_3BO_3, 0.5 g/l $CoCl_2$, 20 g/l $ZnCl_2$, 65 g/l $FeSO_4{\cdot}7H_2O$, 0.2 g/l biotin, and concentrated sulfuric acid (0.5 % v/v). The medium was sterilized by filtration. The pH of the medium was adjusted and controlled at 5.0 with the addition of 28 % (v/v) ammonium hydroxide. Dissolved oxygen (DO) concentration was constantly maintained between 20 and 60 % saturation and controlled in cascade mode. Aeration was constantly maintained at 1.0 vvm and pure oxygen was supplied as needed. The agitation rate was maintained between 300 and 700 rpm. The fermentation was conducted during the glycerol batch phase at 28 °C until all of the glycerol was consumed, which was indicated by a sharp increase in DO. The process then proceeded to a glycerol fed-batch phase at 28 °C, with feeding with 50 % (v/v) glycerol containing 1.2 % (v/v) trace solution at an average rate of 12.4 g/l/h. When needed, glycerol feeding rates were adjusted to control DO. After the desired biomasses were reached, the methanol fed-batch phase was initiated at 28 °C, during which the culture was supplied with 100 % (v/v) methanol containing 1.2 % (v/v) trace solution, and the methanol concentration was controlled at 0.1 % by an online methanol analyzer (FC2002, Super-Xinxi, Shanghai, China).

Cell concentration

Dry cell weight (DCW) of the cell suspension was determined by a previously reported method [18]. DCW of the cell suspension was determined by centrifugation of 5 ml cell broth in a pre-weighed centrifuge tube, followed by washing with distilled water and drying to constant weight at 80 °C in an oven.

Calculation of specific rates

The substrate specific consumption rate (Qs) and the product specific production rate (Qp) were determined by applying the methodology developed for this estimation [5].

Results

Construction of the recombinant strains with different gene copy numbers of lipase gene *proRCL* and chaperone genes *PDI* and *ERO1*

The plasmid pPICZ-*PDI* was linearized by *Sac*I and subsequently transformed into recombinant strain XY RCL-5, resulting in the strain PDI XY RCL-5. The ERO1p overexpressing strain ERO1 XY was transformed in *P. pastoris* GS115 with *Sac*I-linearized pPIC3.5-*ERO1* and selection was done in the MD plates. To construct an *ERO1*/*PDI* co-expressed strain ERO1 PDI XY, recombinant strain ERO1 XY was transformed with a *Sac*I-linearized plasmid pPICZ-*PDI* and selected in YPD-Zeocin plates. The chaperone-overexpressing strains ERO1 XY RCL-5, Co XY RCL-5, and Co XY RCL-6 were the products of transformation of recombinant strains ERO1 XY and ERO1 PDI XY with plasmid pPIC9 K-*proRCL* linearized by *Sal*I, which were selected in the YPD-G418 plates. The gene copy numbers of the lipase gene *proRCL* and the secretion helper genes *ERO1* and *PDI* in all these recombinant strains were identified by the RT-qRCR method developed before [25]. All these recombinant strains and the gene copy numbers are also listed in Table 1.

Effects of co-expression with PDI and ERO1p on the secretion of lipase r27RCL in shake flasks

Protein folding in the ER is a critical step both in mammals and yeast [10]. Proteins enter the ER as unfolded

Table 1 Recombinant strains with different copy numbers of secretion helper genes and lipase gene

Name of recombinant strains	Copy number of heterogenous gene *PDI*	Copy number of heterogenous gene *ERO1*	Copy number of heterogenous gene *proRCL*
XY RCL-5	0	0	5
XY RCL-6	0	0	6
PDI XY RCL-5	1	0	5
ERO1 XY RCL-5	0	1	5
Co XY RCL-5	1	1	5
Co XY RCL-6	1	1	6

polypeptide chains. Chaperones assist in the folding of the nascent polypeptide chain by redox reaction and isomerization, ensuring that the correct disulfide bonds are formed [4, 8, 23].

Firstly, we investigated the effects of co-expression with a single secretion factor on the lipase r27RCL production in XY RCL-5. As shown in Fig. 1a, within the set of strains expressing the lipase r27RCL, co-expression with PDI resulted in a small increase, but a small decrease in recombinant strain ERO1 XY RCL-5 compared with the control. Then, the dosage of the genes encoding ERO1p and PDI in the yeast *P. pastoris* was increased and utilized to evaluate the secretion of lipase r27RCL. As shown in Fig. 2a, the levels of the secreted lipase r27RCL in both recombinant strains with five copies of lipase gene were similar before 24 h. But after that, co-expression with ERO1p and PDI resulted in a striking increase in the amount of lipase r27RCL secreted by recombinant strain Co XY RCL-5 (Fig. 2a, b). The lipase enzyme activity of Co XY RCL-5 reached the maximum value of 370 U/ml at 96 h, which represented a more than 40 % increase compared to that of the control strain XY RCL-5 (Fig. 1a). The enzyme activity of the recombinant strain Co XY RCL-6 reached 390 U/ml, which represented a larger increase by approximately 56 % compared to that of the control strain XY RCL-6 (Fig. 2b).

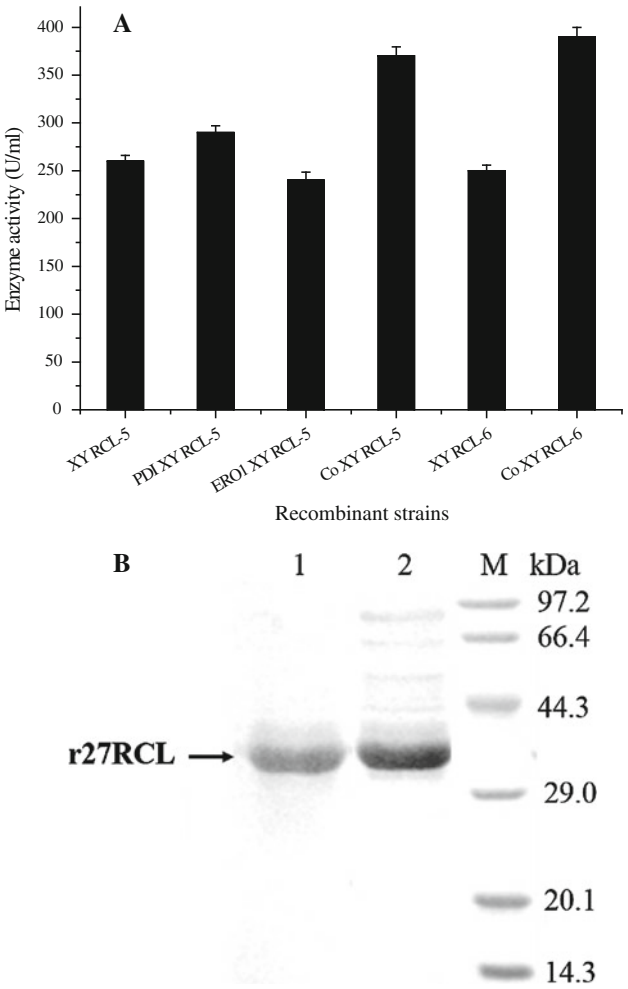

Fig. 1 Effects of co-expression with ERO1p and PDI on the lipase secretion in *P. pastoris* at 96 h in shake flasks. **a** Enzyme activity of recombinant strains by co-expression with ERO1p and PDI at 96 h in shake flasks. **b** SDS-PAGE of fermentation supernatant of recombinant strains XY RCL-5 and Co XY RCL-5 at 96 h in shake flasks. *1* XY RCL-5, *2* Co XY RCL-5, *M* protein marker

Fermentation of recombinant strains XY RCL-5 and Co XY RCL-5 in the 7-l fermentor

As shown in Fig. 3a, the growth rate of the recombinant strain Co XY RCL-5 was much higher than that of the control strain XY RCL-5 before 72 h, but it increased at a lower rate after 72 h. In accordance with the DCW, the enzyme activity of recombinant strain Co XY RCL-5 reached 16,200 U/ml at 80 h, a value nearly 30 % higher compared to the strain XY RCL-5 (Fig. 3b). Interestingly, the fermentation time of recombinant strain Co XY RCL-5 was apparently 8 h shorter by co-expression with these two chaperones. The protein concentration of the recombinant strain Co XY RCL-5 reached 9.39 g/l at 80 h, whereas the protein concentration of the control strain XY RCL-5 reached 8.06 g/l at 88 h (Fig. 3c). The specific activity of the fermentation supernatant of recombinant strain Co XY

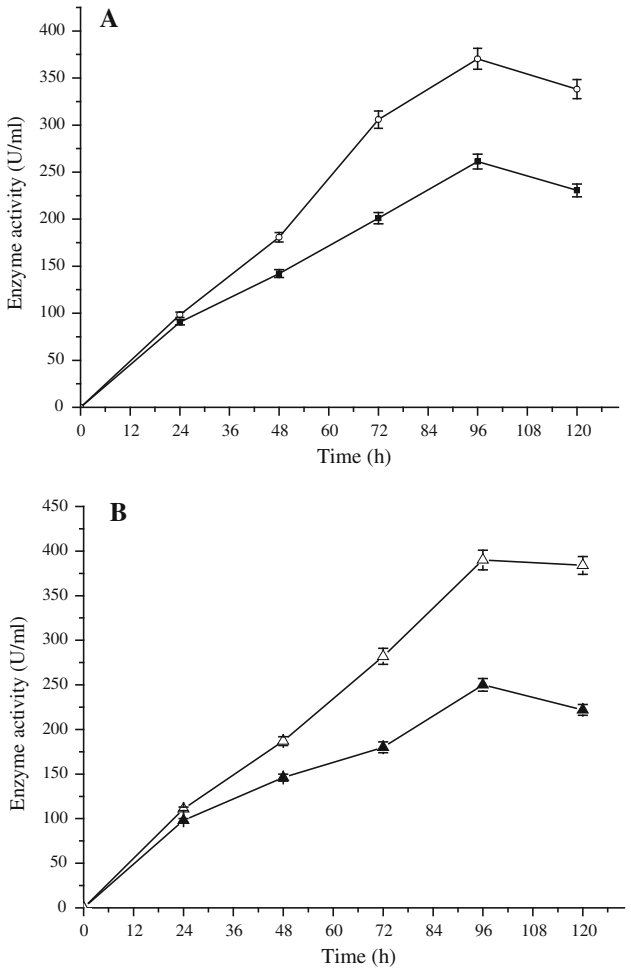

Fig. 2 Effects of co-expression with ERO1p and PDI simultaneously on the lipase secretion in *P. pastoris* in shake flasks. Recombinant strains with **a** five and **b** six copies of lipase gene. *Filled squares* XY RCL-5, *open circles* Co XY RCL-5, *filled triangles* XY RCL-6, *open triangles* Co XY RCL-6

RCL-5 is higher than that of the strain XY RCL-5 throughout the fermentation process (Fig. 3d). The SDS-PAGE of the supernatant of the recombinant strain Co XY RCL-5 is shown in Fig. 4.

In addition, as shown in Fig. 5a, the *Q*s of recombinant strain Co XY RCL-5 was much higher than that of control strain XY RCL-5 before 72 h; after that, the *Q*s of the strain Co XY RCL-5 rapidly declined. The highest *Q*s of the co-expression strain reached 0.072 $g_{methanol}/g_{DCW}/h$. Methanol was utilized as both the inducer and the carbon source during the fermentation process. The more methanol was consumed by the recombinant strain, the more product (lipase r27RCL) would probably be produced. As shown in Fig. 5b, the *Q*p of recombinant strain Co XY RCL-5 was maintained at a higher level and reached 1,226–1,442 $U/g_{DCW}/h$ during the period between 40 and 72 h.

Discussion

In our study, RT-qPCR (Pfaffl method) was selected to determine the copy number of the inserted genes and the method was efficient and easy to operate. Genomic DNA of GS115 strain and the housekeeping gene *GAPDH* are used as the external and the reference gene, respectively. The promoter of alcohol oxidase gene *AOX1* (the target gene *PAOX1*) exists in plasmids pPIC9 K-*proRCL*, pPIC3.5-*ERO1*, pPICZ-*PDI*, and *P. pastoris* genome simultaneously. The copy number of *PAOX1* minus one (the one existed in the genome of *P. pastoris* GS115) equals the copy numbers of the inserted heterogenous genes.

Previously, we have described the expression of lipase r27RCL in *P. pastoris* with different lipase gene copy numbers [25]. The highest enzyme activity of 12,500 U/ml was achieved by XY RCL-5 in the 7 l fermentor, but there was a decrease in the recombinant strain XY RCL-6. Overexpression of foreign proteins in *P. pastoris* will trigger UPR. The induction of UPR is disadvantageous to cell survival. An imbalance (called ER stress) between the load of unfolded proteins that enter the ER and the capacity of the cellular machinery that handles this load sets three main responses in motion: a reduction in the protein load that enters the ER, an increase in the capacity of the ER to handle unfolded proteins, and cell death is triggered [22]. In our previous research, we detected the higher transcription levels of the chaperone genes *PDI* and *ERO1* in the ER of the recombinant strain XY RCL-6, which suggested that UPR was probably triggered. Similar research has been conducted in the fed-batch cultivations to *Rhizopus oryzae* lipase (ROL) production [21]. We postulated that the bottleneck for secretion of lipase r27RCL in XY RCL-6 was attributed to the stress of the protein folding in the ER and UPR was probably activated because the transcription levels of genes *ERO1* and *PDI* were much higher than those in the lower copy recombinant strains. Thus we firstly investigated the effects of co-expression with a single secretion helper on the lipase r27RCL production in XY RCL-5.

In our study, co-expression with PDI or ERO1p had little effect on the lipase r27RCL production. Many researches have suggested that overexpression of PDI increased the yields of some heterologous proteins in *P. pastoris* [6, 16, 26]. PDI is an essential eukaryotic protein from the thioredoxin superfamily that catalyzes the oxidation, reduction, and isomerization of disulfide bonds. Interestingly, increasing the PDI levels also increased the secretion of β-glucosidase, which is not a disulfide-bonded protein [27]. In that case, PDI may act in a chaperone-like capacity or possibly create mixed disulfides with the β-glucosidase's single cysteine residue during the folding and assembly process. Co-expression with *ERO1* even

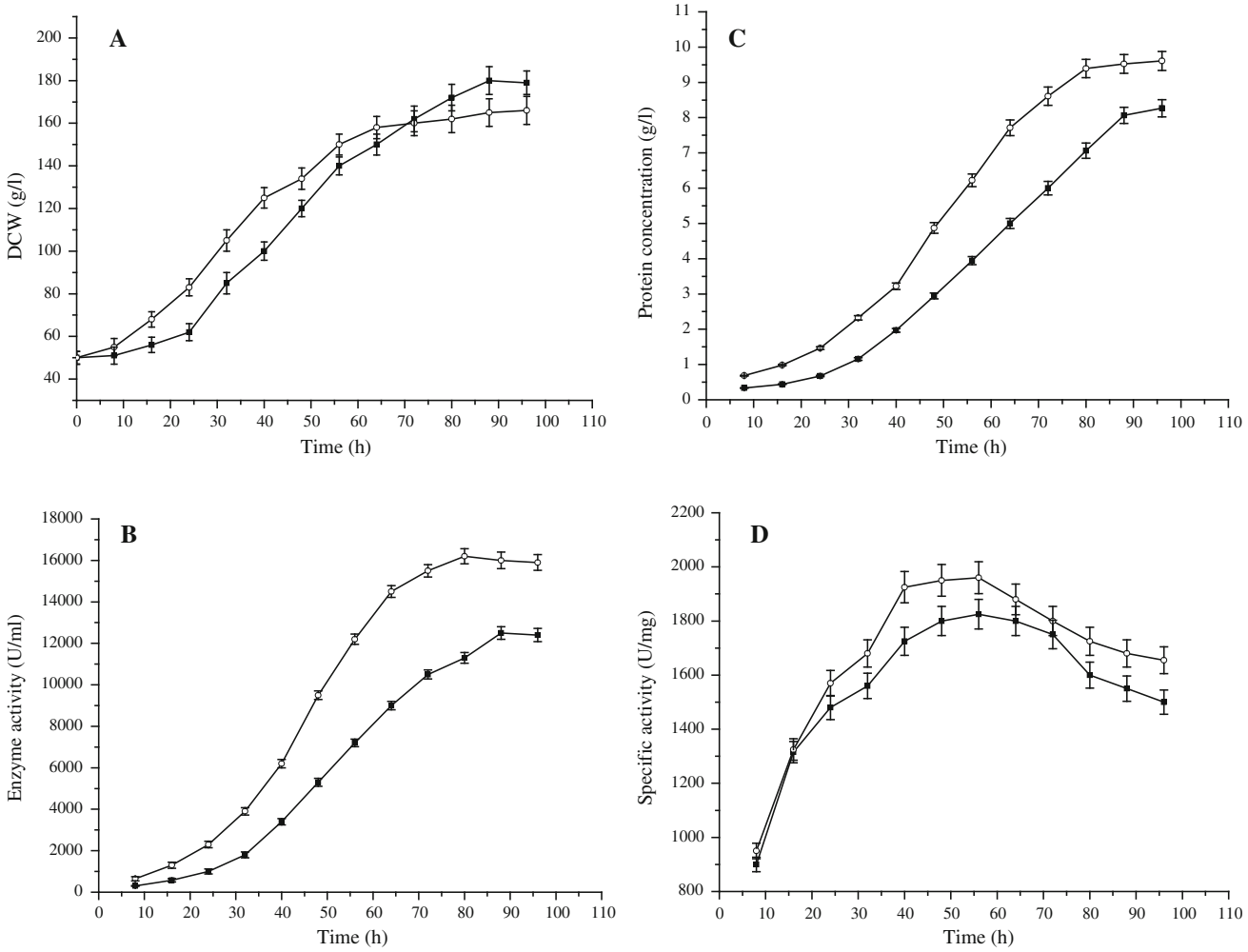

Fig. 3 Fermentation properties of the recombinant strains with or without the secretion helpers ERO1p and PDI in the 7-l fermentor. **a** Growth curves. **b** Enzyme activity. **c** Extracellular protein concentration. **d** Specific activity. *Filled squares* XY RCL-5, *open circles* Co XY RCL-5, *filled triangles* XY RCL-6, *open triangles* Co XY RCL-6

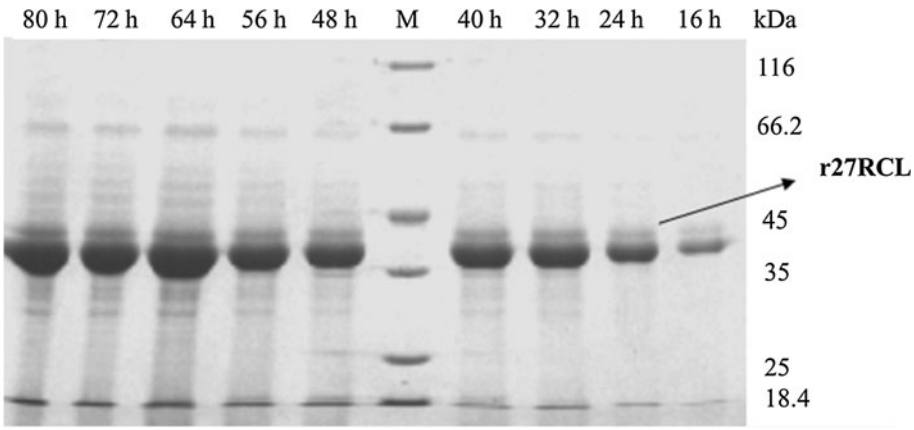

Fig. 4 SDS-PAGE of fermentation supernatant of recombinant strain Co XY RCL-5 in the 7 l fermentor

decreased the production of lipase r27RCL in our study, which suggests that ERO1p may not have a chaperone-like function such as that suggested for PDI in *P. pastoris*. Thus, whether the protein production could be improved by co-expression with chaperones is probably associated with the properties of the proteins expressed in *P. pastoris*.

Unfolded or misfolded proteins are prevented from leaving the ER by a proofreading mechanism of the protein

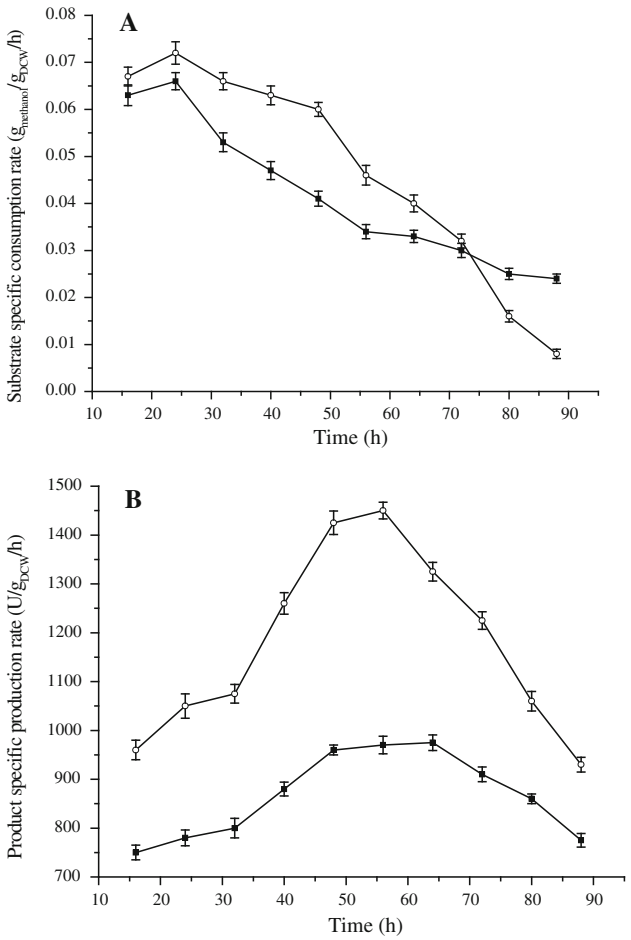

Fig. 5 Effects of recombinant strains with or without the secretion helpers ERO1p and PDI in the 7 l fermentor on the fermentation parameters. **a** Substrate-specific consumption rate. **b** Product-specific production rate. *Filled squares* XY RCL-5, *open circles* Co XY RCL-5, *filled triangles* XY RCL-6, *open triangles* Co XY RCL-6

quality control system [3]. Disulfide bond formation plays a crucial role in the folding of secretory proteins in the ER. The core pathway of the oxidative folding machinery includes two conserved proteins: ERO1p and PDI [4]. Secreted proteins enter the ER with reduced cysteines and leave it with oxidized cysteines. As shown in Fig. 6, the requirement for oxidative equivalents is fulfilled by ERO1 proteins, which transfer electrons from PDI to oxygen through a series of specific interchange reactions [31]. Thus we increased the dosage of the genes encoding ERO1p and PDI in the yeast *P. pastoris* and evaluated the secretion of the lipase r27RCL.

Since oxidizing equivalents flow directly from ERO1p to substrate proteins via PDI, the observed increase in lipase production suggested that the simultaneous co-expression with both secretion helpers accelerated the secretion of lipase r27RCL. In our previous research, the lipase r27RCL production had a decrease in the recombinant strain XY RCL-6, probably because of the UPR caused by the high-level gene copy numbers [25]. In this study, our results suggested that the protein folding stress in the recombinant strain XY RCL-6 may be relieved by co-expression with these two proteins present in the protein folding pathway. In addition, the protein production of both recombinant strains Co XY RCL-5 and Co XY RCL-6 was very similar, but there was an increase of oxygen consumption in Co XY RCL-6 in the 7-l fermentor and the concentration of methanol in the culture with recombinant strain Co XY RCL-6 could not be controlled easily owing to the high demand of oxygen (data not shown). Thus, Co XY RCL-5 was utilized to investigate the effects of co-expression with chaperones on the lipase production in *P. pastoris* in the 7-l fermentor.

The beneficial effects on the lipase secretion in the 7-l fermentor were all based on the yields and productivities of the lipase r27RCL in *P. pastoris*. All these data in the 7-l fermentor suggested that co-expression with ERO1p and PDI successfully increased both the enzyme activity and the total amount of proteins, and even improved the expression space–time yields, which was probably a new finding. The higher expression efficiency of recombinant strain Co XY RCL-5 was possibly due to the faster substrate utilization rate than that of the control strain XY RCL-5 (Fig. 5a). Protein folding in the ER is a crucial step both in mammals and yeast [10, 24]. Chaperones in the ER could assist in the folding of the nascent polypeptide chain. Numerous examples show that many overexpressed proteins in yeast accumulated in the ER [13, 37]. Different from those cases, in our study, the enzyme activity in the higher copy strains was extremely low in vivo and there was also no obvious band of lipase r27RCL by SDS-PAGE analysis, which may be due to the target protein degradation via the ER-assisted degradation (ERAD) [20]. Thus, the misfolded lipase r27RCL in the ER would probably be degraded quickly under the strict quality control system (QC).

ROL was functionally expressed in *P. pastoris* and the enzyme activity reached 1,334 U/ml, which was improved largely by the optimization of fermentation strategies in the mut^s phenotype, and the maximum specific productivity reached 268 $U/g_{DCW}/h$ [18]. In addition, the enzyme-specific production rates (Qp) were further increased to 244 $U/g_{DCW}/h$ by optimization of gene dosage in the mut^+ phenotype and 451.4 $U/g_{DCW}/h$ using mixed substrates in the mut^s phenotype, which were much lower than that by recombinant strain Co XY RCL-5 in our research [2, 5]. High-level expression in *P. pastoris* usually faces some potential bottlenecks, such as limitations in gene dosage, mRNA transcription, protein translation, translocation, or secretion, which could not be solved solely by the fermentation process control [7]. In recent years, more

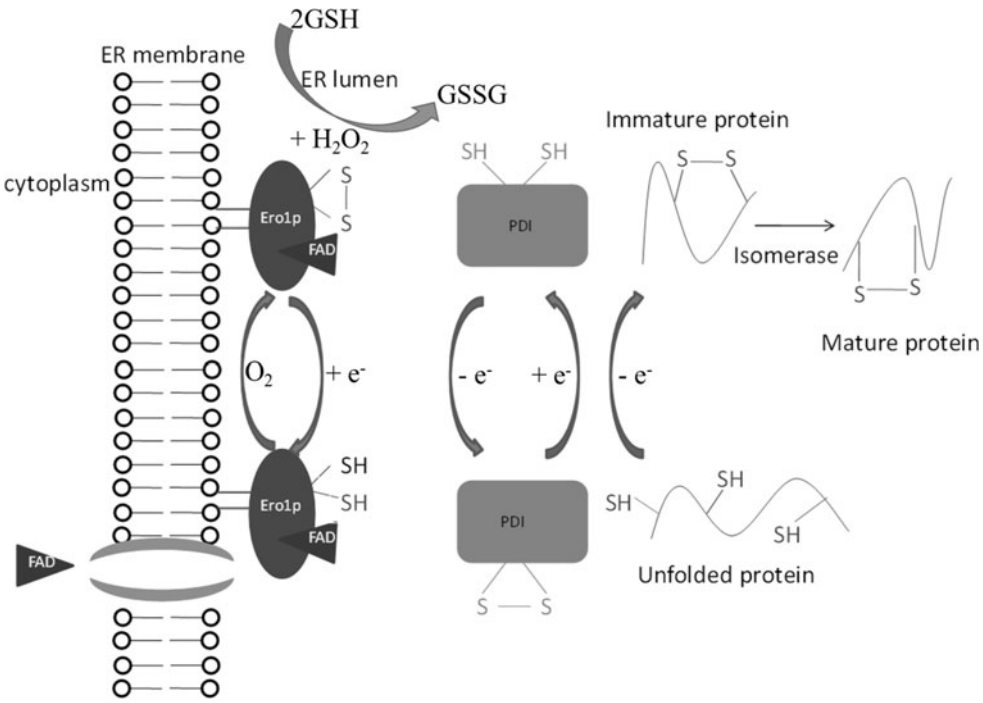

Fig. 6 Model of ERO1p-mediated oxidative folding in the ER

research has focused on the systematic engineering of the yeast strains for effective protein secretion [12]. The metabolism rate and the lipase production capacity of the recombinant strain Co XY RCL-5 were significantly enhanced by the synergies between ERO1p and PDI. The product yields achieved by recombinant strain Co XY RCL-5 and XY RCL-5 reached 30.8 and 20.2 mg product/g methanol, respectively. The fermentation time was also shorter with the recombinant strain Co XY RCL-5 and the yeast achieved the growth plateau more quickly. The enhancement of fermentation efficiency of recombinant strain Co XY RCL-5 very likely resulted from the altered metabolism and energy consumption pattern, e.g., the metabolic change of ATP (energy factor) and glutathione (the reducing agent) [1, 34, 36]. The analysis of the intracellular metabolism of recombinant strain Co XY RCL-5 is ongoing in our laboratory.

In conclusion, we have engineered a *P. pastoris* lipase r27RCL expression system capable of the secretion of over 9.39 g/l in the 7-l fermentor. The highest lipase enzyme activity reached 16,200 U/ml. The highest product-specific production rate (Qp) by the recombinant strain Co XY RCL-5 reached 1,442 U/g_{DCW}/h and the fermentation time was also shortened by 8 h. Thus, the space–time yield was mostly improved by co-expression with ERO1p and PDI in the ER. The strategy we developed should also be easily transferrable to the study and expression of other proteins in *P. pastoris*.

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