

# 米根霉脂肪酶基因 *pro-ROL* 和 *m-ROL* 在毕赤酵母中的密码子优化、表达和酶学性质的比较分析

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**摘要:** 米根霉 *Rhizopus oryzae* 脂肪酶不仅在众多工业领域中具有良好的应用价值, 其典型的分子内伴侣结构 (Intramolecular chaperon) 也是研究蛋白质翻译后加工和成熟的理想材料。以 *R. oryzae* HU3005 为材料, 克隆了其脂肪酶前体基因 (*pro-ROL*) 和成熟脂肪酶基因 (*m-ROL*), 并实现了其在巴斯德毕赤酵母 *Pichia pastoris* GS115 中的分泌表达。酶学性质比较分析表明: *m-ROL* 对中等链长底物 (C10 和 C12) 具有更高的水解活性, 而 *pro-ROL* 更倾向于短链底物 (C4), 且在 pH 8.0 时活性最高。再者, *pro-ROL* 具有比 *m-ROL* 更好的温度稳定性。推测 *m-ROL* 和 *pro-ROL* 脂肪酶酶学性质的差异可能是由前序列对脂肪酶的折叠和修饰的影响而导致。为提高 *m-ROL* 的表达水平, 采用重叠延伸 PCR 技术将基因中 8 个低频密码子替换为高频密码子。在摇瓶条件下, 发酵 72 h 后, 经密码子优化后的 *m-ROL* 酶活和蛋白质含量分别达到 132.7 U/mL 和 50.4 U/mL, 而初始 *m-ROL* 和 *pro-ROL* 酶活和蛋白质含量分别仅为 28.7 U/mL 和 14.4 mg/L、29.6 U/mL 和 14.1 mg/L。

**关键词:** 米根霉脂肪酶, *m-ROL*, *pro-ROL*, 重叠延伸 PCR, 毕赤酵母

## Codon optimization, expression and enzymatic comparison of *Rhizopus oryzae* lipases *pro-ROL* and *m-ROL* in *Pichia pastoris*

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**Abstract:** *Rhizopus oryzae* lipase (*ROL*) is not only a biocatalyst used in a broad range of biotechnological fields, but also a model to investigate the function of intramolecular chaperone in the post-translational processing of lipase. In this study, we cloned and expressed the mature lipase gene (*m-ROL*) containing the pre-sequence (*pro-ROL*) of *R. oryzae* HU3005 in *Pichia*

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*pastoris* GS115 and characterized their enzymatic activities. m-ROL exhibited higher hydrolysis activity towards middle-chain substrates (C<sub>10</sub> and C<sub>12</sub>) at pH 9.0, whereas pro-ROL preferred short-chain substrates (C<sub>4</sub>) and displayed maximal activity at pH 8.0. Moreover, pro-ROL possessed better thermal stability than m-ROL. This enzymatic discrepancy between m-ROL and p-ROL may be due to the pre-sequence that affects the folding and conformation of the mature lipase domain. To improve the expression level of *m-ROL* in *P. pastoris*, overlap extension PCR was conducted to substitute eight less-frequently used codons of *m-ROL* with frequently used codons. After methanol-induced expression for 72 h, the activity and protein content of the codon optimized m-ROL reached 132.7 U/mL and 50.4 mg/L, while the activity of the parental m-ROL and pro-ROL are 28.7 U/mL and 14.4 mg/L, 29.6 U/mL and 14.1 mg/L, respectively.

**Keywords:** *Rhizopus oryzae* lipases, *m-ROL*, *pro-ROL*, overlap extension PCR, *Pichia pastoris*

## Introduction

*Rhizopus oryzae* lipases (EC 3.1.1.3) have been widely utilized in hydrolysis of triglycerides, synthesis of aromatic esters and biofuel, and kinetic resolutions of prochiral compounds<sup>[1-3]</sup>. Typically, *Rhizopus oryzae* lipase (ROL) consists of three parts that includes a signaling peptide responsible for the translocation and secretion of ROL, pre-sequence, and mature lipase (m-ROL) domain<sup>[4]</sup>. The pre-sequence is an intramolecular chaperone involved in the correct folding and efficient secretion of m-ROL. Although intramolecular chaperones have been found in several protein families<sup>[5-6]</sup>, to our knowledge, *Rhizopus* lipase is the only member of the lipase family to possess intramolecular chaperone-like pre-sequences. It remains unknown how the pre-sequence contributes to folding, post-translational processing and maturation of *Rhizopus* lipase.

Currently, *Rhizopus* lipase has been successfully expressed in *Escherichia coli*, *Saccharomyces cerevisiae* and *Pichia pastoris*. In *E. coli*, pro-ROL and m-ROL exists as insoluble inclusion bodies. To obtain their active forms, a complex refolding process was needed<sup>[4,7]</sup>. m-ROL exhibited no enzymatic activity when expressed in *S. cerevisiae*, suggesting the pre-sequence is necessary for correct protein folding of m-ROL<sup>[8-9]</sup>. However, m-ROL displayed enzymatic activity when expressed in *Pichia*<sup>[10-11]</sup>. It means that without the help of pre-sequence the mature domain of ROL can form the active conformation in *P. pastoris* by itself. But questions such as whether pro-ROL would actively express in *P. pastoris* and whether pro-ROL and m-ROL were enzymatically identical characteristics still need to be clarified.

*P. pastoris* expression system is an easy and efficient system suitable for high-density fermentation, and now broadly used to produce recombinant heterologous proteins<sup>[12-14]</sup>. Like most organisms, *Pichia* displays a non-random pattern of synonymous codon usage and showed general bias towards a subset of codons. This bias can affect the heterogenous expression efficiency of genes in *Pichia*. Codon optimization by substituting rare codons with frequently used ones has been established as an efficient measure to enhance the expression level<sup>[15-17]</sup>.

In this study, we cloned and expressed *R. oryzae* lipase genes, *pro-ROL* and *m-ROL*, in *P. pastoris*. To improve its expression levels, we conducted overlap extension PCR to optimize the codons of *m-ROL* gene. We further compared the enzymatic activity of pro-ROL and m-ROL to assess the function of the pre-sequence on the maturation of ROL, and to acquire parameters for potential biotechnological applications.

## 1 Materials and methods

### 1.1 Reagents, media and strains

All molecular biological reagents were purchased from TaKaRa, Dalian. p-Nitrophenyl acetate (pNPA, C<sub>2</sub>), p-Nitrophenyl butyrate (pNPB, C<sub>4</sub>), p-Nitrophenyl caprate (pNPC, C<sub>10</sub>), p-Nitrophenyl laurate (pNPL, C<sub>12</sub>), and p-Nitrophenyl palmitate (pNPP, C<sub>16</sub>) were purchased from Sigma.

*R. oryzae* strains HU3005 was obtained from the China Centre of Industrial Culture Collection (CICC) with deposition number CICC3005. *E. coli* strain DH10B was cultured at 37 °C in Luria-Bertani (LB) medium unless otherwise noted. Ampicillin (100 mg/L) and kanamycin (50 mg/L) were added when necessary.

## 1.2 Lipase genes cloning

*R. oryzae* pre-mature lipase gene *pro-ROL* was amplified using the primer pairs ROL1 (5'-CTGAATTCTGTTCTGTTTCTGGTAAATC-3', *EcoR* I site) and ROLA1 (5'-CTGCGGCCGCTTACAAACAGCTTCCTCGT-3', *Not* I site), and mature lipase gene *m-ROL* was amplified with the primer pairs MROL2 (5'-CTGAATTCTCTGATGGTGGTAAGTTG-3', *EcoR* I site) and MROLA2 (5'-CTGCGGCCGCTTACAAACAGCTTCCTCGT-3', *Not* I site) (Fig. 1).

## 1.3 RNA extraction

Total RNA from *R. oryzae* was extracted by Trizol reagent (Gibcol) according to the manufacturer's protocol. The first strand cDNA was synthesis by using the RevertAid First Strand cDNA Synthesis Kit (Fermentas). PCR was carried out in a 50  $\mu$ L reaction containing 200 mmol/L dNTPs, 0.1 mmol/L primers,

1.5 mmol/L  $MgCl_2$ , and 1 U of *pfu* DNA polymerase (TaKaRa). The PCR conditions is as followed: denaturation at 94  $^{\circ}C$  for 4 min, 28 cycles of 94  $^{\circ}C$  for 30 s, 56  $^{\circ}C$  for 50 s and 72  $^{\circ}C$  for 1 min, and final elongation at 72  $^{\circ}C$  for 6 min. The PCR product was cloned into the pMD18-T simple vector (TaKaRa), and then sequenced by Sangon Ltd., Shanghai. The sequence of *R. oryzae* lipase gene was deposited into GenBank with the accession number GQ502721.

## 1.4 Codon optimization

Overlap extension PCR amplifications were conducted to substitute eight less-frequently used codons with the frequently used codons (Table 1). Three fragments which overlap each other were amplified with the primers containing the mutant nucleotides (Table 2), and then assembled into the full-length *m-ROL* gene by a second PCR using 5'- and 3'-end primers (Fig. 2).

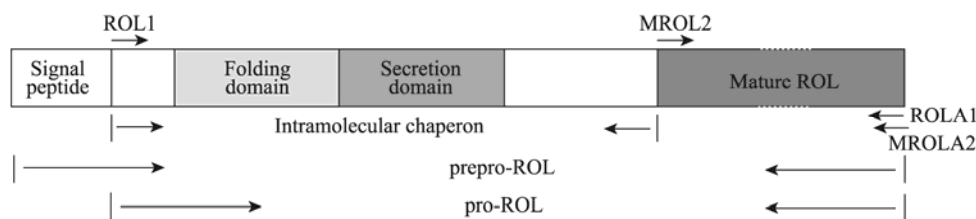


Fig. 1 Primary structure of *R. oryzae* lipase ROL.

Table 1 The optimized codons of *R. oryzae* lipase gene *m-ROL* and their usage in *Pichia*

Site in <i>m-ROL</i>	Original codon	Codon usage in <i>Pichia</i> /1 000	Optimized codon	Codon usage in <i>Pichia</i> /1 000	Amino acid coded
474	CTC	7.6	CTG	15.5	Leu
501	CCC	6.5	CCA	17.5	Pro
513	AGC	7.3	AGT	12.2	Ser
732	TGC	4.4	TGT	8.3	Cys
744	ATC	19.2	ATT	31.0	Ile
756	ACC	14.0	ACT	23.7	Thr
762	CTC	7.6	CTT	16.1	Leu
801	AGC	8.3	AGT	12.2	Ser

**Table 2** The primers used for codon optimization of *ROL* and the size of PCR products.

Primers	Sequence (5'–3')	PCR product
S1	CTGAATTCTCTGATGGTGGTAAGGTTG, <i>Eco</i> R I site	Fragment F1 (~500 bp)
A1	CTT <b>TGG</b> AGACAATCTTGGTTCACGTTGGTA <b>CAG</b>	
S2	CAAGATTGTCT <b>CCA</b> AAGAATTG <b>AGT</b> ATC	Fragment F2 (~270 bp)
A2	GAAAGGAAC <b>AAT</b> AGAGTTACT <b>ACA</b> ATC	
S3	CT <b>ATT</b> GTTCC <b>TTC</b> <b>ACT</b> TCT <b>CTT</b> CT	Fragment F3 (71 bp)
A3	CTGCGGCCGCTTACAAACA <b>ACT</b> TC, <i>Not</i> I site	

Square framed are the optimized codons.

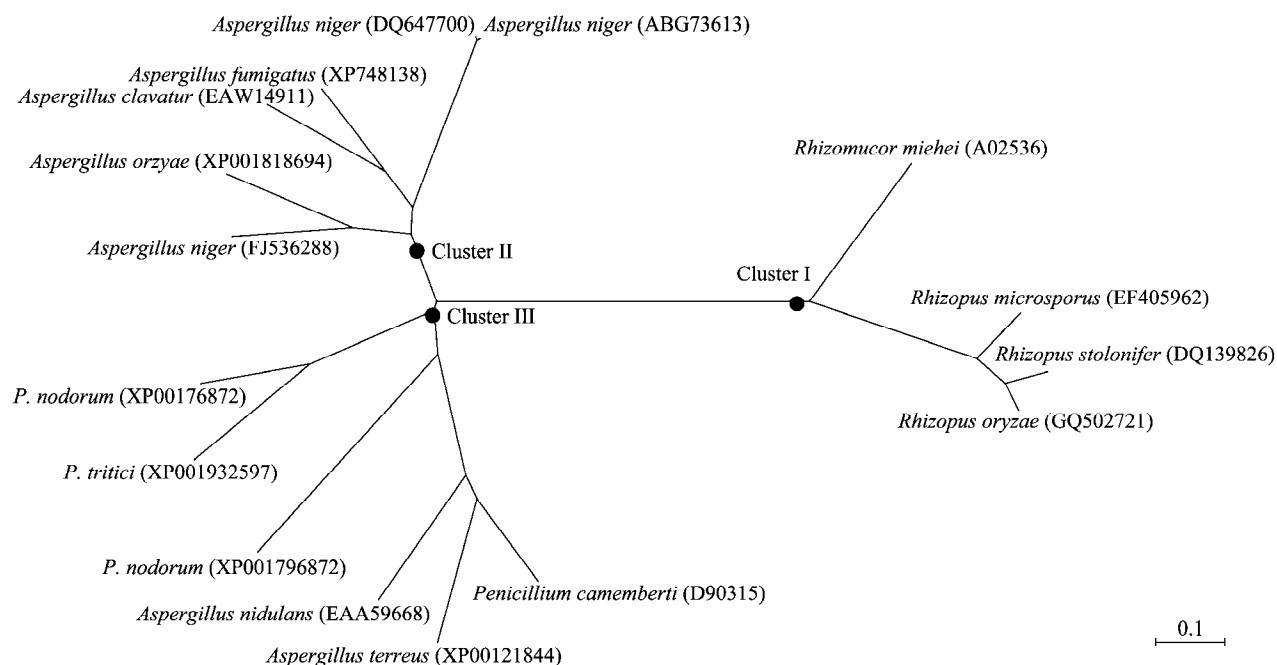


Fig. 2 Phylogeny tree of *R. oryzae* lipase (*ROL*) and the related species. Bracketed are the GenBank accession numbers. *P. nodorum*: *Phaeosphaeria nodorum*; *P. tritici*: *Pyrenophora tritici*.

### 1.5 Plasmid construction, transformation, and recombinants selection

PCR product of *m-ROL* and *pro-ROL* were digested with *Eco* R I and *Not* I, and then inserted into vector pPIC9K (Invitrogen) to obtain plasmids pPIC9K-*m-ROL* and pPIC9K-*pro-ROL*, with a fusion expression of  $\alpha$ -factor. *Sac* I was used to linearize the plasmid pPIC9K-*m-ROL* and pPIC9K-*pro-ROL* for the single crossover with *P. pastoris* genome to generate the methanol-utilized phenotype ( $Mut^+$ ). About 6  $\mu$ g of linearized DNA was mixed with 80  $\mu$ L of yeast

competent cells, and transformed into *P. pastoris* GS115 cells by electroporation conducted on Gene Pulser (Bio-rad) according to the manufacturer's suggestion for *S. cerevisiae*. Positive clones were initially selected by MD medium (1.34% yeast nitrogen base,  $4 \times 10^{-5}$ % biotin, 2% dextrose) plates and then checked by colony PCR.

The lipase activity of the recombinants was initially checked by BMMY agar plate containing olive oil and rhodamine B. Positive clones displayed orange fluorescent halos visible upon UV irradiation<sup>[18]</sup>.

## 1.6 Fermentation

A single colony of recombinant bacteria was selected and inoculated into 50 mL BMGY (1% yeast extract, 2% peptone, 100 mmol/L potassium phosphate buffer with pH 6.0, 1.34% yeast nitrogen base,  $4 \times 10^{-5}$ % biotin, 1% glycerol) medium, and grown at 28 °C in a shaking incubator (250 r/min) until the culture reached an  $OD_{600}$  of 3.0. The cells were harvested, and a portion was transferred into 50 mL BMMY (1% yeast extract, 2% peptone, 100 mmol/L potassium phosphate buffer pH 6.0, 1.34% yeast nitrogen base,  $4 \times 10^{-5}$ % biotin, and 0.5% methanol) medium to obtain a cell suspension with  $OD_{600}=1.0$ . The cells were grown for another 5 d and expression of lipase was induced by methanol at a final concentration of 0.5%. Lipase activity of the supernatant of fermentation broth was checked at intervals. For every type of recombinant, three colonies were randomly selected for fermentation.

## 1.7 Lipase activity and characterization

Lipase activity was quantified at pH 7.5 by free fatty acid titration with 50 mmol/L NaOH after incubation in a thermostated vessel for 10 min. The assay mixture consisted of 5 mL 50 mmol/L Tris-HCl buffer, 50 mmol/L NaCl, 4 mL emulsified olive oil and 1 mL enzyme solution. One unit (U) of the activity was defined as the amount of enzyme liberating 1 micromole of fatty acid per min at 40 °C. Properties of lipase such as its thermal stability and optimal pH were determined as previously reported<sup>[13-14]</sup>. All experiments were carried out in triplicate. Protein content of the fermentation broth was determined by the Bradford method<sup>[25]</sup>.

## 1.8 Phylogenetic analysis

Phylogenetic analysis was performed by molecular evolutionary genetics analysis (MEGA3.1) software, which was also used to produce a phylogenetic dendrogram reflecting the evolutionary relationship between the cloned genes and other reference strains by the neighbor-joining method according to the Kimura 2-parameter model<sup>[19]</sup>.

## 2 Results

### 2.1 Cloning of *R. oryzae* lipase genes

*R. oryzae* premature lipase gene *pro-ROL* and

mature lipase gene *m-ROL* were amplified by RT-PCR. The ORFs of *pro-ROL* and *m-ROL* genes were 1 101 bp and 810 bp, respectively. Phylogenetic analysis based on the similarity of amino acid sequences between *R. oryzae* and the related reference lipases revealed three distinct clades (I to III). Clade I consisted of lipases from *Rhizopus* and *Rhizomucor*. Cloned *R. oryzae* lipase exhibited a 95% amino acid similarity with *R. stolonifer* (Fig. 2).

### 2.2 Codon optimization and expression in *P. pastoris*

Bias codon usages between different microorganisms are one of the main factors that restrict gene expression in heterogeneous host. To achieve a high level expression of *m-ROL* in *Pichia*, the less frequently used codons consisting of eight amino acids were substituted with the frequently used ones by overlap extension PCR (Fig. 3). After transformation, yeast recombinants carrying codon-optimized *m-ROL*, *pro-ROL* and original *m-ROL* were acquired. Both *m-ROL* and *pro-ROL* showed lipase activity on plates. However, the enzymatic activity of the recombinants carrying codon-optimized *m-ROL* (optimized-*ROL*) is significantly higher than the non-optimized *m-ROL* and *pro-ROL* (Fig. 4A). After methanol-inducible expression in *Pichia*, the molecular weight of the enzymes *m-ROL* and *pro-ROL* determined by PAGE were 30 kDa and 35 kDa, respectively (Fig. 4B). The expression level of codon-optimized *m-ROL* is higher than the original *m-ROL* and *pro-ROL*. After 72 h of fermentation, the enzymatic activity (Fig. 5A) and protein content (Fig. 5B) of the codon-optimized *m-ROL* reached 132.7 U/mL and 50.4 mg/L, while the activity of the parental *m-ROL* and *pro-ROL* are 28.7 U/mL and 14.4 mg/L, 29.6 U/mL and 14.1 mg/L, respectively.

### 2.3 Characterization of *pro-ROL* and *m-ROL*

We determined the optimal substrate, pH and temperature of *pro-ROL* and *m-ROL* (Fig. 6). pNP esters with different carbon chain lengths were selected as substrates. *pro-ROL* and *m-ROL* showed differential preferences to these substrates. *pro-ROL* preferred short- and middle-chain substrates ( $C_4$  and  $C_{10}$ ), while *m-ROL* preferred middle-chain substrates with optimal activity observed using  $C_{10}$  substrates (Fig. 6A).

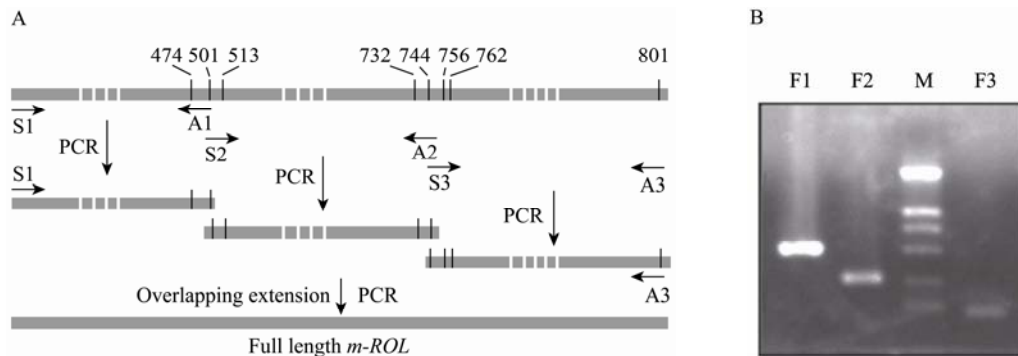


Fig. 3 Flow chart of codon optimization of *m-ROL* by overlap extension PCR (A), and the gel picture of the three fragments (B), F1 (500 bp), F2 (270 bp) and F3 (71 bp). M: DNA marker (2 000, 1 000, 750, 500, 250, 100 bp)

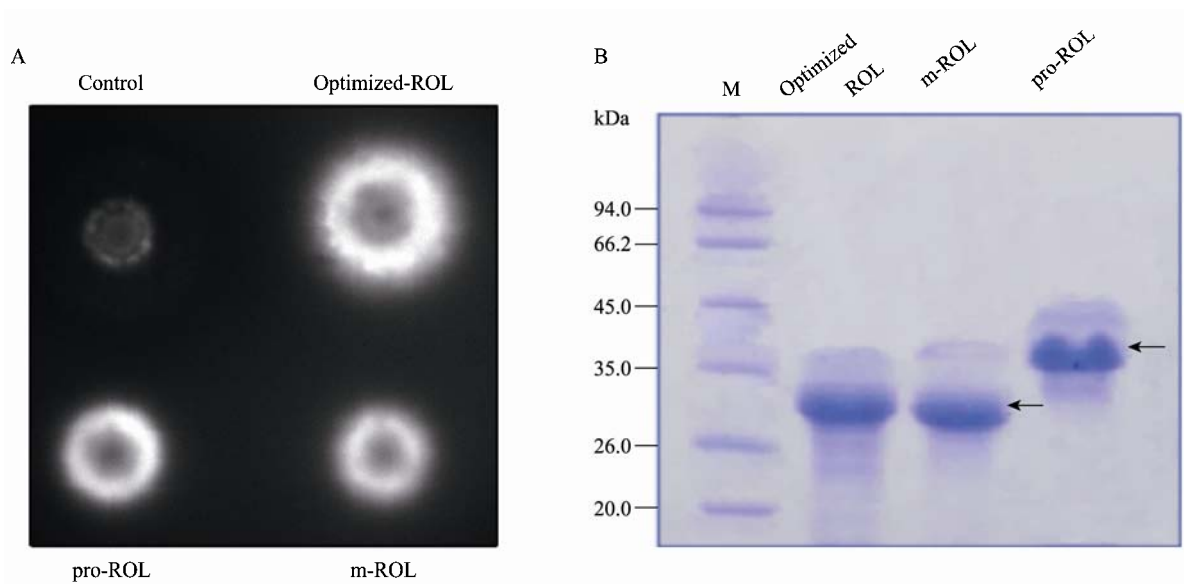


Fig. 4 Expression of codon-optimized ROL (optimized ROL), *pro-ROL* and original *m-ROL* in *P. pastoris*. (A) The morphology of different recombinants on the plate under the UV light. (B) SDS-PAGE of supernatant product of recombinant lipase.

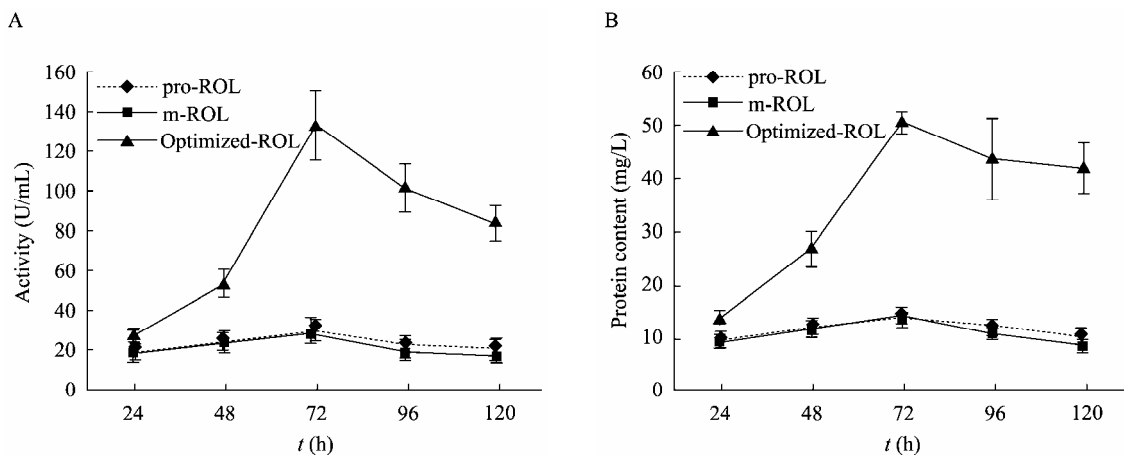


Fig. 5 Time course of the production of lipases by recombinants carrying *pro-ROL*, *m-ROL* and the codon-optimized *m-ROL* (optimized-ROL). (A) and (B) directed out lipase activity and protein content of the fermentation broth, respectively.

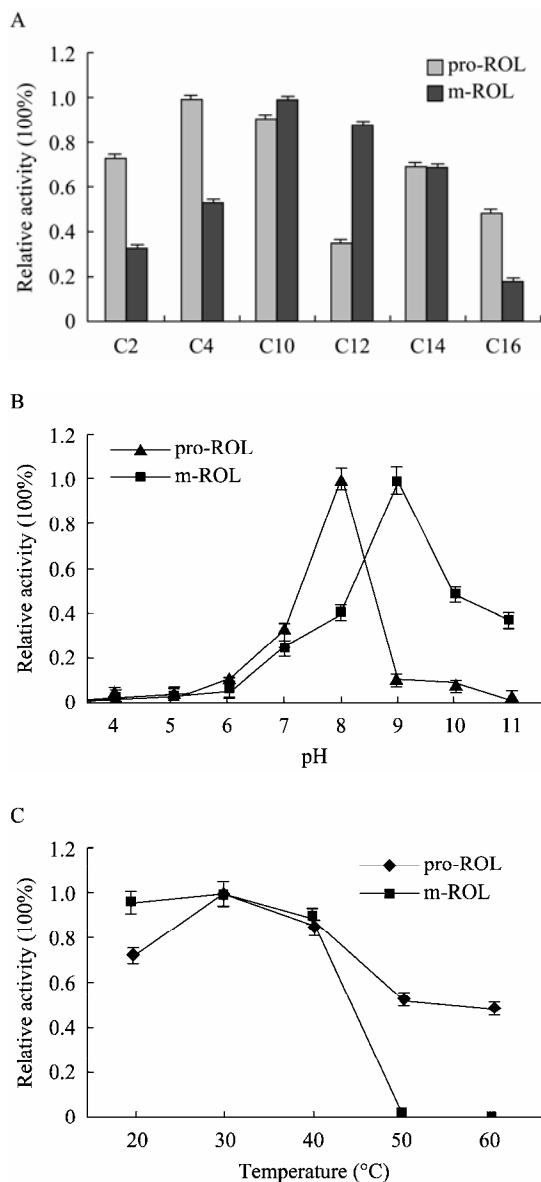


Fig. 6 Enzymatic characterization of pro-ROL and m-ROL. (A) Substrate specificity of pro-ROL and m-ROL towards ester of nitrophenol. (B) and (C) Effects of temperature and pH on lipase activity.

The optimal pH was determined by incubating lipases in reaction buffer with different pH values. As shown in Fig. 6B, both pro-ROL and m-ROL preferred alkaline environments. Unlike pro-ROL, which has an optimal pH value 8.0, the optimal pH value of m-ROL was 9.0. The suitable pH range, determined as having >60% remaining activity, of m-ROL is 8.5–9.5, while the suitable pH range of pro-ROL is from 7.5 to 8.5.

Enzymatic activity significantly decreased when the lipases were not maintained at their optimal pH.

To determine the optimal reaction temperature and thermal stability, lipase activities were assessed under different temperatures (20 °C–60 °C) and calculated as percentages of the maximal activity. As shown in Fig. 6C, the optimal temperatures for both pro-ROL and m-ROL were 30 °C. At 50 °C, enzymatic activity was not detected for m-ROL, whereas pro-ROL had 51% activity. Additionally, pro-ROL was more thermally stable than m-ROL.

### 3 Discussion

Pre-sequence plays an important role on the folding, post-translational processing and secretion of *R. oryzae* lipase<sup>[4,7,9]</sup>. Previous reports showed lack of enzymatic activity when m-ROL was expressed in *S. cerevisiae*<sup>[8]</sup>. This suggested that the lack of the pre-sequence in m-ROL may have inhibited formation of its active conformation. In our study, we actively expressed pro-ROL and m-ROL in *P. pastoris*. The enzymatic characteristics of pro-ROL in *Pichia* are different than those in *S. cerevisiae*. In *Pichia*, pro-ROL prefers short and middle-length carbon chains (C4–C10), while in *S. cerevisiae*, pro-ROL favors middle-chain substrates<sup>[8]</sup>. It is possible that in *P. pastoris*, post-translational modification may modulate the function of the pre-sequence and facilitate correct protein folding of m-ROL. Moreover, intramolecular chaperones may also affect enzymatic properties by mediating the folding and conformational changes of the mature protein domain<sup>[20]</sup>. In this study, pro-ROL was more thermally stable than m-ROL. Similar phenomenon was observed by Beer and his colleague. In their study, they found that in contrast to m-ROL, pro-ROL and prepro-ROL had considerably higher thermostability<sup>[7]</sup>. This suggests that the pre-sequence domain may also protect the m-ROL region from dramatic conformational change when exposed to high temperatures.

A special characteristic of lipase is the interfacial activation. Generally, the active site (catalytic triad) is completely buried beneath a short amphiphilic helical segment, which prevents substrate access. When the enzyme contact with the hydrophobic substrates, it

will go through a series structural rearrangement, and the enzyme changes from a closed to a open conformation that make the substrates enter into the activity site<sup>[21]</sup>. Although there were no report on the effect of pre-sequence on the substrate selectivity of lipase before, but it is possible that this pre-sequence of pro-ROL can spatially retard the conformational change of mature ROL domain. When the long-chain substrates contacted with enzymes, although the enzymes have conformational change, the open size of enzyme is still not enough to permit the long-chain substrates enter into the active site. On the contrary, this half-open conformation will be more accessible to the shorter chain substrate (C4). As observed in this study, the favorable substrate of pro-ROL and m-ROL are C4, while for m-ROL is C10 (Fig. 6A).

Codon-usage frequency is a major impediment to the efficient expression of foreign gene in heterologous host. In ROL gene, four codons 155Leu (CTC), 171Ser (AGC), 254Leu (CTC) and 267Ser (AGC), which have the lowest usage frequency, are the bottleneck of gene expression in *P. pastoris*. Our study shows that codon optimization by overlap extension PCR to substitute less frequently used codons with frequently used ones could significantly improve the expression level of ROL. Compared to other methods such as chemical gene synthesis, overlap extension PCR is simple and effective. The initial enzymatic activity achieved based on the flask fermentation method could reach 132.7 U/mL. Although our product yield is not as high as a previous study<sup>[22]</sup> using a large-scale bioreactor (644.0 U/mL), it is still significantly better than other reported results<sup>[23-24]</sup>. We believe that lipase production and enzymatic activity from recombinants carrying optimized ROL gene may be improved under a batch-induced mode with further optimization in pH, methanol concentration and aeration during the fermentation process.

In this study, we actively expressed pre-mature (*pro-ROL*) and mature lipase gene (*m-ROL*) of *R. oryzae* in *P. pastoris*. The differences in enzymatic characteristics between these two forms of lipase may be caused by the intramolecular chaperone-like pre-sequence in pro-ROL, which could affect the conformation of m-ROL domain. Codon optimization by overlap extension PCR has effectively improved the

expression level by 4.6-fold. This newly codon-optimized ROL gene may be useful for future large-scale production of *R. oryzae* lipase. Further characterization of pro-ROL and m-ROL may offer a valuable tool in industrial applications.

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