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# *Rhizopus chinensis* lipase: Gene cloning, expression in *Pichia pastoris* and properties

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ABSTRACT

Lipases are the most attractive enzymes for use in organic chemical processes. In our previous studies, a lipase from *Rhizopus chinensis* CCTCC M20102 was found to have very high ability of esterification of short-chain fatty acids with ethanol. In this study, we reported the cloning and expression of the lipase gene from *R. chinensis* in *Pichia pastoris* and characterization of the recombinant lipase. The lipase gene without its signal sequence were cloned downstream to the alpha-mating factor signal and expressed in *P. pastoris* GS115 under the control of *AOX1* promoter. In the induction phase, two bands of 37 kDa and 30 kDa proteins could be observed. The amino-terminal analysis showed that the 37-kDa protein was the mature lipase (30 kDa) attached with 27 amino acid of the carboxy-terminal part of the prosequence (r27RCL). The pH and temperature optimum of r27RCL and mRCL were pH 8.5 and 40 °C, and pH 8 and 35 °C, respectively. The stability, reaction kinetics and effects of metal ions and other reagents were also determined. The chain length specificity of r27RCL and mRCL showed highest activity toward *p*-nitrophenyl hexanoate or glyceryl tricaproate (C6) and *p*-nitrophenyl acetate or glyceryl triacetate (C2), respectively. This property is quite rare among lipases and gives this new lipase great potential for use in the field of biocatalysis.



## 1. Introduction

Lipases (triacylglycerol ester hydrolases EC 3.1.1.3) are well known hydrolases capable of hydrolyzing the ester bonds of water-insoluble substrates at the interface between substrate and water. Furthermore, contrary to many other enzymes, they show remarkable levels of activity and stability in non-aqueous environments, which facilitates the catalysis of several unnatural reactions such as esterification and transesterification. Because of these unique properties, lipases are the most attractive enzymes for use in organic chemical processes [1].

Lipase-mediated synthesis of aliphatic esters of longer chain substrates has shown their easy esterification abilities [2]. However, the synthesis of low molecular flavor ethyl esters from shorter chain substrate comparatively received less attention with no satisfaction as short fattyacids easily strip the essential water around enzymes to cause their deactivation or cause dead-end inhibition reacting with the serine residue at the active site of lipase [3]. In our previous studies, a fungal strain was isolated from leaven (mouldy grains) samples with very high ability of esterification of short-chain fatty acids with ethanol and was identified as *Rhizopus microsporus* var. *chinensis*, also named as *Rhizopus chinensis* [4]. In further studies two kinds of intracellular lipases were purified from this strain (RCL-lip1 and RCL-lip2), and RCL-lip2 showed a preference to hydrolyze short chain esters and its N-terminal sequence has a high homology with *Rhizopus oryzae* mature lipase [5].

*Rhizopus* species was mainly divided into three groups, including *R. microsporus*, *R. oryzae*, *Rhizopus stolonifer*, among which *R. oryzae* was investigated more widely. Many individually named isolates, such as *R. oryzae*, *Rhizopus arrhizus*, *Rhizopus delemar* and *Rhizopus javanicus* latterly recognized to be the same organism, in spite of some minor variations, were combined into *R. oryzae* [6]. *R. oryzae* lipase, *R. delemar* lipase and *R. javanicus* lipase have a substitution in the His134 and the Leu234 by an Asn and a Leu, respectively [7]. The lipase gene from *R. stolonifer* (Genebank No. DQ139862) was reported with 84% amino acid sequence identity to *R. oryzae* lipase. However, there is no report on the molecular characterization of lipase from *R. microsporus*.

The production of an active *Rhizopus* lipase has been performed in *Escherichia coli* [8], in *Saccharomyces cerevisiae* [9] and in *Pichia pastoris* [10,11]. Over the last few decades, the *P. pastoris* expression system has been used successfully for production of various recombinant heterologous proteins. This process offers several advantages, such as alcohol oxidase 1 (*AOX1*) gene promoter tightly regulated by methanol, easy growth to high cell densities, high levels of protein expression at the intra- or extra-cellular level, and the ability to perform eukaryotic protein modifications [12]. Several

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other microbial lipases have also been successfully expressed in *P. pastoris*, such as *Candida rugosa* lipase 1[13], *Candida parapsilosis* lipase/acyltransferase [14] and *Candida antarctica* lipase B [15].

In this study, we report the cloning and expression of the lipase gene from *R. chinensis* in *P. pastoris* and characterization of the recombinant lipase.

## 2. Materials and methods

### 2.1. Strains, plasmids and growth conditions

*R. chinensis* CCTCC M201021 producing lipase (RCL) was from our lab. *E. coli* DH5α (*sup*E44Δ*lac*U169(φ80 *lacZ*ΔM15)*hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1) has been used for DNA propagation. *P. pastoris* GS115 (*His*$^-$ *Mut*$^+$) and the pPIC9K expression vector are from Invitrogen BV (*Pichia* Multi-Copy Expression Kit, version A, Invitrogen BV, The Netherlands).

*R. chinensis* CCTCC M201021 was cultivated in the medium contained 10 g/L maltose, 60 g/L peptone, 2 g/L $K_2HPO4$, 0.5 g/L $MgSO_4{\cdot}7H_2O$ and 20 g/L olive oil, pH of the medium was adjusted to 5.5. *E. coli* was cultivated in Luria–Bertani medium. *P. pastoris* GS115 was grown and maintained in YPD medium (10 g/L yeast extract, 2 g/L peptone, 2 g/L dextrose). The YPD-G418 plates containing different concentrations (0.25–1.0 g/L) of geneticin (G418 sulfate, Invitrogen BV) were used for selection of *Pichia* transformants.

### 2.2. Cloning of rcl gene and construction of the lipase expression vector

The open reading form of the *rcl* gene was amplified directly from *R. chinensis* genomic DNA using a pair of primers, RCL-F1 (CCG**ATG**GTTTCATTCATTTCCATTTCTC) and RCL-R1 (*GC***TTA**CAAACAGCTTCCTTCGTT) according to the consensus sequence of lipases from other *Rhizopus* sp. (GenBank accession nos. AB013496, DQ139862, AF229435). The PCR fragment was DNA sequenced. Based on the sequence of *rcl* gene, the *rcl* gene without its own signal peptide (*prorcl*) was amplified using a pair of primers, RCL-F2 (ATC*CCTAGG*GTTCCTGTTGCTGGTCATAAAGGTTC) and RCL-R2 (CAGT*GCGGCCGC***TTA**CAAACAGCTTCCTTCGTT). The restriction sites *Avr*II and *Not*Iwere incorporated into the forward and reverse primer sequence, respectively. The PCR fragment was ligated into the respective sites of pPIC9K resulting in pPIC9K-proRCL under the control of the methanol inducible alcohol oxidase 1 promoter ($P_{AOX1}$) and fused in-frame with the α-factor secretion signal peptide of *S. cerevisiae*.

### 2.3. Transformation of P. pastoris and selection of His$^+$ multicopy recombinants

*P. pastoris* GS115 was transformed with 7 μg of *Bgl*II-linearized pPIC9K-proRCL vector by electroporation, and selection of *His*$^+$ transformants was done on minimal dextrose medium (MD, Invitrogen BV) plate. The screening of geneticin resistant was performed on solid YPD-G418 medium. The insertion and methanol metabolization was checked by PCR. The PCR amplifications were carried out according to Invitrogen's recommendations with genomic DNA and primers complementary to the 5′ and 3′ region of the *AOX1* gene. Lipase secretion was assayed at 28 °C on agar plate composed of minimal methanol medium (MM, Invitrogen BV) with 10 g/L olive oil and 1 mg/L fluorescent dye rhodamine B (MM-rhodamine) by the appearance of a fluorescent halo around colonies under UV light.

### 2.4. Expression of lipase in P. pastoris in shake flasks

The *P. pastoris His*$^+$ transformants were cultured in 25 mL of buffered glycerol-complex medium (BMGY, 10 g/L yeast extract, 20 g/L peptone, 100 mM potassium phosphate, pH 6.0, 13.4 g/L YNB, $4 \times 10^{-4}$ g/L biotin, 10 g/L glycerol) shaken at 28 °C and 250 rpm in 250 mL glass flasks. When cultures reached an $OD_{600}$ of about 6, the cells were centrifuged and resuspended in 20 mL of buffered methanol-complex medium (BMMY, 10 g/L yeast extract, 20 g/L peptone, 100 mM potassium phosphate, pH 6.0, 13.4 g/L YNB, $4 \times 10^{-4}$ g/L biotin, 5 g/L methanol) to an $OD_{600}$ of 1.0, shaken at 28 °C and 250 rpm in 100 mL glass flasks for 120 h. The cultures were supplemented with methanol (5 g/L) to induce the expression of lipase every 12 h. The culture was centrifuged, and the supernatant was collected for SDS-PAGE and lipase activity assay.

### 2.5. Expression of lipase in P. pastoris in bioreactor

Fermentation experiments were performed at 28 °C with 2.8 L volume in a 7-L bioreactor (New Brunswick, BioFlo 110, USA). The inoculums were grown for 18 h at 28 °C in shake flasks at 220 rpm with BMGY medium. In glycerol batch phase, 200 mL of inoculums was directly added into 2.6 L of a Fermentation Basal Salts Medium (40 g/L[1] 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, 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 $FeSO_4{\cdot}7H2O$, 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 maintained always above 30% saturation and controlled in cascade mode: Aeration was kept constant at 1.0 vvm and pure oxygen was supplied as needed, agitation rate kept between 300 rpm and 700 rpm.

The fermentation was operated in glycerol batch phase at 28 °C until all of the glycerol was consumed, which was indicated by a sharp increase in DO. Then Proceed to glycerol fed-batch phase at 28 °C, feeding with 50% (v/v) glycerol containing 1.2% (v/v) trace solution at the average rate of 12.4 g/(L h$^{-1}$) was performed until the desired biomasses reached ($OD_{600}$ = 90), and when needed, glycerol feeding rates were adjusted to control DO. After half an hour, start the methanol fed-batch phase at 20 °C, in which supplied with 100% (v/v) methanol containing 1.2% (v/v) trace solution and the methanol concentration was kept at about 2.5 g/L controlled by an on-line methanol analyzer (FC2002, Shanghai Super-xinxi, China). Biomass was determined by measuring the optical density (OD 600 nm). The correlation between $OD_{600}$ and dry cell weight (g/L) was a factor of about 3.

### 2.6. Lipase activity determination

Lipase activity was measured on emulsified *p*-nitrophenyl palmitate (pNPP) according to Kordel et al. [16]. One volume of a 1.08-mM solution of pNPP in 2-propanol was mixed just before used with 9 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 above mixture at 37 °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 at 410 nm of the assay against a blank without enzyme was monitored for 2–5 min using a UV–vis spectrophotometer (UNICO UV-3102 PC, China). The reaction rate was calculated from the slope of the curve absorbance versus time, using a molar extinction coefficient of 44,656 cm$^{-1}$ M$^{-1}$ for *p*-nitrophenol. One enzyme unit was defined

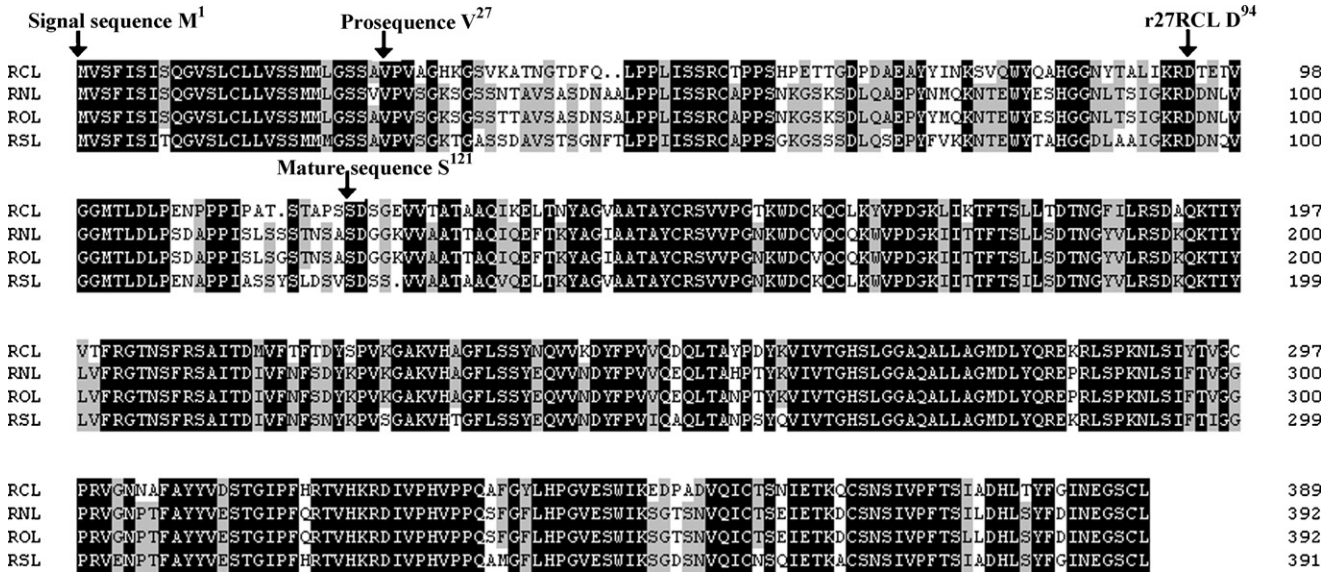


**Fig. 1.** Alignment of amino acid sequence from *Rhizopus* sp. lipase.

as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute under the assay conditions.

The enzymatic activities for hydrolyses of triglycerides were determined as follows. The reaction mixture that contained 100 μl of triglyceride and an appropriate amount of enzyme in 3 ml of 25 mM Tris–HCl buffer (pH 8.0) incubated at 37 °C at 250 rpm. The reaction was terminated by the addition of 10 ml of acetone–ethanol (1:1, v/v) and the liberated fatty acid was titrated with 10 mM NaOH with phenolphthalein as an indicator. One unit of enzymatic activity was defined as the amount of enzyme releasing 1 μmol of fatty acid per minute under the assay conditions.

All the assays were done in triplicate, and significant differences ($p < 0.05$) were measured.

### 2.7. Lipase purification

The 37-kDa and 30-kDa recombinant enzymes from the culture supernatant were purified after cultivation for 72 h and for 144 h, respectively. Cell free medium from the expression medium were concentrated and interchanged with 10 mM Tris–HCl buffer (pH 7.5) by ultrafiltration through a 10-kDa membrane (Millipore, USA). Then, the concentrated solution was loaded onto a SP-Sepharose column (Pharmacia, 1.6 cm × 20 cm) equilibrated with 20 mM Tris–HCl buffer (pH 7.5), eluted with 0–0.5 M NaCl in the same buffer. Fractions containing lipase activity were pooled, concentrated and chromatographed on a Phenyl-sepharose 6 FF (Pharmacia, 1.6 cm × 20 cm) equilibrated in 50 mM Tris–HCl buffer (pH 7.5) containing 1.6 M ammonium sulfate. Lipase was then eluted in an ammonium sulfate concentration gradient ranging from 1.6 to 0 M in 50 mM Tris–HCl buffer (pH 7.5), and 4 mL fractions were collected at a flow rate of 0.8 mL/min. Protein fractions were collected and assayed for protein concentration and lipase activity. Protein concentration was determined using Bradford assay. Bovine serum albumin (BSA) was used as a standard.

### 2.8. Endo-β-N-acetylglycosamidase H digestion

Samples were eventually deglycosylated using endo-β-*N*-acetylglycosaminidase H (*Endo* H, New England Biolabs) as described by the manufacturer's instructions.

### 2.9. N-terminal sequencing

Lipase was separated by SDS-PAGE and electroblotted onto a poly (vinylidene difluoride) membrane. The band was cut out and N-terminal Edman sequencing was performed on an Applied Biosystems 492cLC done by Shanghai GeneCore BioTechnologies Co., Ltd.

### 2.10. Effect of temperature and pH on enzyme stability and activity

Optimal pH was determined by examining the activity of the enzyme at 37 °C in the following buffers: 50 mM citrate (pH 5–6), 50 mM phosphate buffer (pH 7), 50 mM Tris–HCl (pH 8), and 50 mM glycine–NaOH (pH 9–10). Optimal temperature was determined by measuring the enzyme activity at optimal pH under various temperatures (20–60 °C). pH stability were determined by incubating lipase solution in buffers (50 mM, pH 5.0–10.0) for 1 h at 25 °C and analyzing the residual activity. Thermostability was determined by pre-incubating the purified enzyme for various intervals up to 3 h at temperatures ranging from 20 to 60 °C in optimal pH buffer and analyzing the residual activity.

### 2.11. Effect of metal ions and other reagents

Various additions such as metal ions (0.01 M each) and chemical modifiers (EDTA, β-Mercaptoethanol and PMSF, 0.1 M each) were added to the enzyme and incubated for 30 min at 40 °C followed by enzyme assay under standard conditions at 40 °C and pH 8.5. The sample without any additive/modifier was taken as control (100%). The reaction mix with respective additives but without enzyme served as control.

### 2.12. Chain length specificity and position specificity of recombinant lipases

The substrate specificity of the purified enzyme was analyzed using substrates of *p*-nitrophenyl-fatty acyl esters and triglycerides. Regioselectivity of triolein hydrolysis by lipase was carried out by adding 1 mL of triolein into 4 mL of enzyme solution at optimum temperature and 150 rpm for 30 min. Then the reaction mixture was

extracted by *iso*-propanol and *n*-hexane and analysised by HPLC [17].

### 2.13. Kinetic parameters

The Michaelis–Menten kinetic parameters $V_{max}$ and $K_m$ values of both enzymes were calculated using pNPP as substrate. Lineweaver-Burk plots were used to determine $V_{max}$ and $K_m$ parameters, assuming that the reactions followed a simple Michaelis–Menten kinetics.

## 3. Results

### 3.1. Cloning and sequence analysis of the gene encoding lipase

Based on the consensus sequence of lipases from other *Rhizopus* sp., about 1.2-kb fragment was amplified by PCR using *R. chinensis* CCTCC M201021 genomic DNA as template. Compared with the published sequence of *Rhizopus* sp. lipase, the sequence of *rcl* (GenBank accession no. EF405962) contained one complete open reading frame without intron, encoding a 389 amino acid protein including 26 amino acid signal sequence, 94 amino acid prosequence and 269 amino acid mature lipase sequence (Fig. 1). The enzyme was homologous to lipases from *Rhizopus niveus* lipase (RNL, 75.3% similarity, GenBank accession no. AB013496), *R. stolonifer* lipase (RSL, 73.8% similarity, GenBank accession no. DQ139862), and *R. oryzae* lipase (ROL, 75.6% similarity, GenBank accession no. AF229435). The X-ray analysis of *R. niveus* mature lipase showed that $S^{145}$-$H^{257}$-$D^{204}$ are the residues of the catalytic triad, and $Ser^{145}$ is involved in the oxyanion hole stabilizing the tetrahedral intermediates [18]. These residues are conserved in all homologous lipases, suggesting that the catalytic triad of *R. chinensis* mature lipase is composed of $S^{145}$-$H^{257}$-$D^{204}$, and that $S^{145}$ involved in the oxyanion hole.

### 3.2. Selection and expression of positive transformants

The linearized plasmids pPIC9K-proRCL by *Bgl* II were either integrated into the *P. pastoris* genome via a double crossover between the *AOX1* promoter and 3′ *AOX1* regions of the vector, which disrupted the wild-type *AOX1* gene to create *Mut*$^s$ (Methanol utilization slow) transformants or inserted at the *AOX1* locus to create *Mut*$^+$ (Methanol utilization plus) transformants. A total of fifty *P. pastoris* colonies resisting the highest G418 concentration of 0.75 g/L were randomly picked and checked for methanol utilization by PCR. Two kinds of transformants were discovered, *Mut*$^s$ GS115/pPIC9K-proRCL and *Mut*$^+$ GS115/pPIC9K-proRCL integrated with the pro- and mature sequence of *R. chinensis* lipase, both under the control of *AOX1* promoter induced by methanol. The transformants were transferred on MM-Rhodamine plates to check lipase expression level. After induction for 72 h by adding 0.3 mL of methanol onto the lid covering the plates, several colonies of each type that produced the largest fluorescent zone were chosen to express lipase in glass flasks. *P. pastoris* GS115/pPIC9K was used as negative control.

As shown in Fig. 2, *Mut*$^s$ transformant exibited higher activity than that of *Mut*$^+$ phenotype. After induction of 96 h by methanol the prolipase activity in the supernatant from *Mut*$^s$ GS115/pPIC9K-proRCL reached the maximum at 121 U/mL, which was 34 U/mL higher than that from *Mut*$^+$ phenotype, and the specific activities of *Mut*$^s$ and *Mut*$^+$ phenotype were 6050 U/(g DCW) and 3222 U/(g DCW), respectively.

The supernatant of culture loaded onto SDS-PAGE (Fig. 3) showed that the molecular weight of expressed lipase from *Mut*$^s$ GS115/pPIC9K-proRCL was 37 kDa at fermentation time from 24 h to 96 h and after that time two separated bands were observed at 37 kDa and 30 kDa, respectively. At the last stage, the 37-kDa band completely disappeared. Since the calculated molecular weight of proRCL was 39 kDa, we further purified these two forms of proteins for analysis of amino-terminal sequence, *Endo*-H digestion and comparison of properties studies.

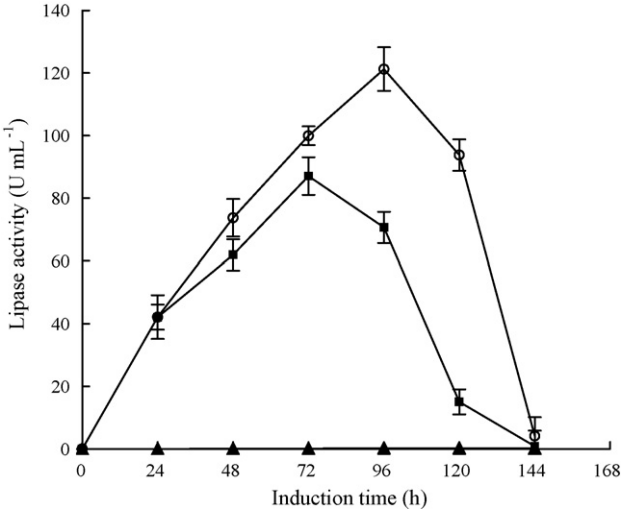


**Fig. 2.** Lipase activity from *Mut*$^s$ GS115/pPIC9K-proRCL (○) and *Mut*$^+$ GS115/pPIC9K-proRCL (■) induced by methanol. *Mut*$^s$ GS115/pPIC9K (▲) as a negative control.

### 3.3. Protein purification and N-terminal sequencing

The 37-kDa and 30-kDa recombinant enzymes from the culture supernatant were purified by a three-step purification protocol. A summary of the purification process is given in Table 1. The purified enzyme gave two bands on SDS-PAGE corresponding to 37 kDa (cultivation for 72 h) and 30 kDa (cultivation for 144 h), respectively (Fig. 4). The purification resulted in a significant increase in the specific activity of 22,625 and 3000 for 37-kDa and 30-kDa lipases, respectively. The N-terminal amino acid sequence of the 37-kDa protein was D-T-E-T-V-G-G, corresponding to 27 amino acids of C-terminal part of the prosequence. On the other hand, the N-terminal amino acid sequence of the 30-kDa protein was S-D-S-G-E-V, which was identical to the N-terminal part of the mature region of wild-type RCL-lip2 [5]. These results indicate that the 37-kDa protein was the mature region attached with 27 amino acids of the C-terminal part of the prosequence (r27ROL), while the 30-kDa protein was the mature lipase (mRCL) and probably the same as wild-type RCL-lip2.

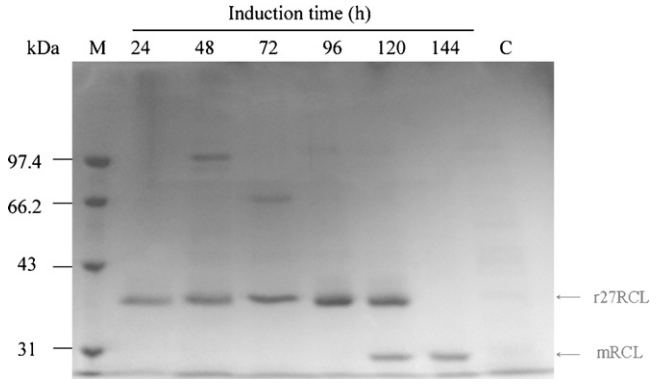


**Fig. 3.** SDS-PAGE analysis of the culture supernatants of *Mut*$^s$ GS115/pPIC9K-proRCL induced by methanol. C, supernatant of *Mut*$^s$ GS115/pPIC9K as negative control.

**Table 1**
Purification of recombinant lipase of r27RCL and mRCL.

| Purification step | Total activity (U) | | Total protein (mg) | | Specific activity (U/mg) | | Purification fold | | Yield (%) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | r27RCL | mRCL | r27RCL | mRCL | r27RCL | mRCL | r27RCL | mRCL | r27RCL | mRCL |
| Supernatant | 8458 | 322 | 85.00 | 16.00 | 99 | 20 | 1.0 | 1.0 | 100.0 | 100.0 |
| Ultrafiltration | 7858 | 308 | 2.20 | 0.50 | 3,572 | 616 | 35.9 | 30.6 | 92.9 | 93.5 |
| SP sephrose FF | 4680 | 116 | 0.40 | 0.08 | 11,700 | 1450 | 117.6 | 72.1 | 40.4 | 35.8 |
| Phenyl-sepharose 6 FF | 1810 | 30 | 0.08 | 0.01 | 22,625 | 3000 | 227.4 | 149.3 | 21.4 | 9.3 |

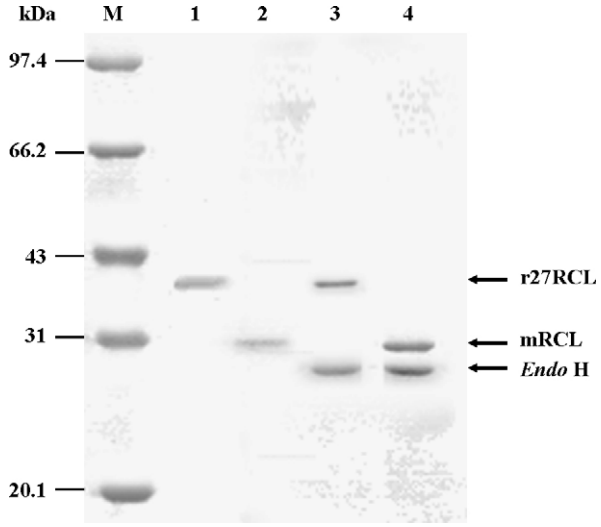


**Fig. 4.** SDS-PAGE analysis of r27RCL and mRCL treated with *Endo* H. Lane 1, purified r27RCL; Lane 2, purified mRCL; Lane 3, r27RCL treated with *Endo* H; Lane 4, mRCL treated with *Endo* H.

*Endo* H with molecular weight of about 28 kDa cleaves within the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins. The recombinant enzyme by treatment with *Endo* H showed that no differences in the molecular weight of the recombinant proteins could be observed before and after incubation with *Endo* H, suggesting that no *N*-glycosylation had occurred (Fig. 4).

### 3.4. Properties of r27RCL and mRCL produced by P. pastoris

The activity of r27RCL and mRCL was measured over a pH range of 5.0–10.0 and the profiles were similar (Fig. 5). r27RCL showed maximum activity at pH 8.5 and was stable over a narrow pH range of 7.5–8.5, and mRCL was most active at pH 8 and was stable over pH 7.5–8.0, which was similar to wild-type RCL-lip2 [5]. The effect of temperature on lipase activity was examined at various temperatures. As shown in Fig. 6, r27RCL and mRCL had the highest activity at 40 °C and 35 °C, respectively, while r27RCL was a little more thermostable than mRCL. The optimum temperature of wild-type RCL-lip2 was the same as that of mRCL, while its thermostability was similar with that of r27RCL [5]. The thermostability of *R. chinensis* lipase was higher than that of r28ROL [19] and rPro28RAL [20] from *R. oryzae*, which were unstable above 30 °C.

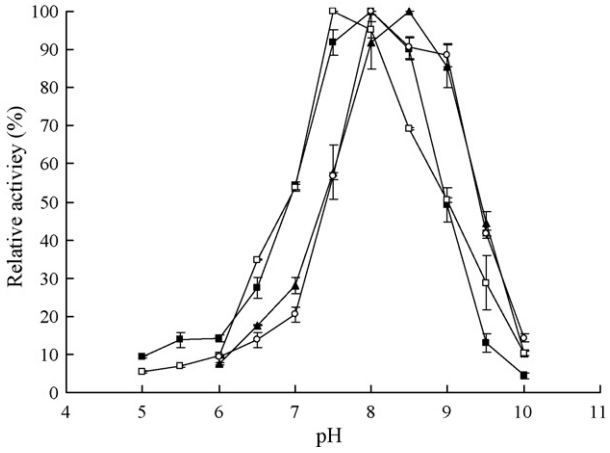


**Fig. 5.** Effect of pH on r27RCL stability (■), activity (▲) and mRCL stability (□), activity (○). Activity was determined under standard conditions at pH 5.0–10.0 and 37 °C.

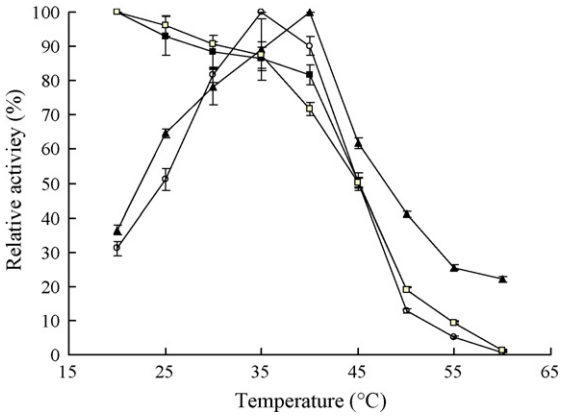


**Fig. 6.** Effect of temperature on r27RCL stability (■), activity (▲) and mRCL stability (□), activity (○). Activity was determined under standard conditions at 20–60 °C and pH 8.5 for r27RCL and pH 8.0 for mRCL.

Effects of metal ions, EDTA, β-Mercaptoethanol and PMSF on the recombinant lipase activities were studied (Table 2). Among the metal ions tested, only $Ca^{2+}$ increased lipase activities greatly by 16–24%, whereas $Mg^{2+}$ and $Na^{2+}$ had little effects on both enzyme activities. $Mn^{2+}$ and $K^{2+}$ showed middle effects on r27RCL, while had little effects on mRCL, and $Cu^{2+}$, $Zn^{2+}$ and $Hg^{2+}$ reduced both lipase activities significantly from 25% to 92%. Lipases belong to the class of serine hydrolases with the catalytic triad as

**Table 2**
Effects of different additives on r27RCL and mRCL activity.

| Reagents | Relative activity (%) | |
|---|---|---|
| | r27RCL | mRCL |
| Control | 100 | 100 |
| $K^+$ | 91.3 ± 1.3 | 97.4 ± 0.7 |
| $Na^+$ | 95.5 ± 1.5 | 96.3 ± 2.1 |
| $Mg^{2+}$ | 100.9 ± 0.4 | 102.5 ± 1.8 |
| $Ca^{2+}$ | 124.3 ± 0.6 | 116.4 ± 1.9 |
| $Cu^{2+}$ | 65.3 ± 1.4 | 74.8 ± 1.0 |
| $Zn^{2+}$ | 45.6 ± 1.4 | 41.7 ± 1.1 |
| $Mn^{2+}$ | 87.8 ± 1.7 | 95.7 ± 0.9 |
| $Hg^{2+}$ | 8.2 ± 1.1 | 14.2 ± 0.8 |
| EDTA | 89.9 ± 2.0 | 99.4 ± 1.1 |
| β-Mercaptoethanol | 99.6 ± 1.4 | 100.9 ± 0.4 |
| PMSF | 95.0 ± 1.2 | 93.2 ± 0.7 |

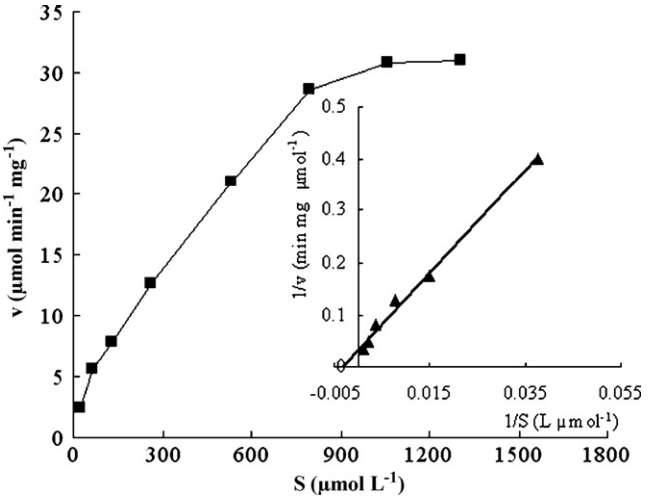


**Fig. 7.** Enzyme kinetic assay of the purified r27RCL. (Inset: the Lineweaver-Burk plot for the determination of kinetic parameters, $K_m$ and $V_{max}$). The enzymatic assay condition was at pH 8.5 and 40 °C, using various concentrations of pNPP as substrate.

S-H-D/E in this enzyme. Therefore, serine inhibitors are potential irreversible active-site lipase inhibitors, e.g. phenylmethylsulfonyl fluoride (PMSF), phenylboronic acid, diethyl-*p*-nitrophenyl phosphate. In contrast, PMSF had only slightly inhibitory effects on the enzyme activities at this reaction condition. Similar effects of serine inhibitors also reported on *Acinetobacter calcoaceticus* LP009 lipase [21] and *Rhizopus homothallicus* lipase [22]. β-Mercaptoethanol was found to have no effect on the lipase activities. Generally, lipases are not sulphydryl proteins; and thus in most lipases neither free –SH nor S–S bridges are important for their catalytic activity. This is also substantiated by the use of 2-mercaptoethanol, *p*-chloromercuric benzoate and iodoacetate, which have no detectable effect on lipase from *Staphylococcus aureus* 226 [23] and *A. calcoaceticus* LP009 [21]. Further, EDTA had little effect on the enzyme activity, which suggested that *R. chinensis* lipase does not require any metal cofactors, which is a typical characteristic of esterases/lipases [24–26].

The kinetic assay showed that $K_m$ and $V_{max}$ values for r27RCL (Fig. 7) were 0.304 mM and 30.76 μmol/(min mg$^{-1}$) and for mRCL were 0.345 mM and 0.822 μmol/(min mg$^{-1}$) from the Lineweaver-Burk plot, respectively.

### 3.5. Comparison of specificity of r27RCL and mRCL

A comparative study of chain length fatty acid specificity was studied by measuring the initial rate of hydrolysis of various fatty acid esters of *p*-nitrophenol and triglycerides by r27RCL and mRCL. As shown in Table 3, r27RCL preferred *p*-nitrophenyl hexanoate and glyceryl tricaproate (C6) whereas mRCL preferred short-chain ester (C2).

Substrate position specificity was analyzed by HPLC. Calibration curves of the ELSD detection data were established using knowing amounts of triolein, 1,3 diolein, 1,2(2,3) diolein, monoolein, and free fatty acid. Using these calibration curves, the quantitative decrease in triolein and the concomitant increase in oleic acid, diolein and monoolein were measured. After 30 min of triolein lipolysis by lipase, the molar concentration of 1,2(2,3) diolein was almost the same as that of 1,3 diolein. These results show that r27RCL and mRCL not only hydrolyze the ester bond of triacylglycerids in the sn-1,3 position, but also hydrolyze the ester bond of triacylglycerids in the sn-2 position, similarly to the result of wild-type RCL-lip2 [5] and *R. homothallicus* lipase described in the literature [27]. However,

**Table 3**
Chain length fatty acid specificity of the purified r27RCL and mRCL.

| Substrates | Activity (U/mg) | |
|---|---|---|
| | r27RCL | mRCL |
| (Triglycerides) | | |
| Glyceryl triacetate (C2) | 32 ± 2 | 42 ± 2 |
| Glyceryl tripropanoate (C3) | 40 ± 3 | 31 ± 1 |
| Glyceryl tricaproate (C6) | 89 ± 4 | 26 ± 2 |
| Glyceryl tricaprylate (C8) | 61 ± 3 | 23 ± 2 |
| Olive oil (C18) | 41 ± 2 | 22 ± 1 |
| (*p*-Nitrophenyl monoesters) | | |
| *p*-Nitrophenyl acetate (C2) | 95.8 ± 5.4 | 90.4 ± 1.2 |
| *p*-Nitrophenyl propionate (C3) | 114.2 ± 6.6 | 82.8 ± 0.6 |
| *p*-Nitrophenyl hexanoate (C6) | 223.3 ± 7.3 | 72.8 ± 0.7 |
| *p*-Nitrophenyl caprylate (C8) | 144.2 ± 5.2 | 61.6 ± 0.4 |
| *p*-Nitrophenyl laurate (C12) | 105.2 ± 3.1 | 31.2 ± 0.4 |
| *p*-Nitrophenyl palmitate (C16) | 100.8 ± 3.6 | 19.6 ± 0.2 |

*R. oryzae* lipase are 1,3-position specific in spite of high homology of *Rhizopus* lipases. Based on phylogenetic tree inferred from 18S rRNA sequences using the neighbour-joining approach, *R. chinensis* is much nearer to *R. homothallicus* than to *R. oryzae*.

### 3.6. Bioreactor batch cultivation

As shown in Fig. 8, in the methanol induction phase, the lipase activity in the supernatant increased up to 587 U/mL with 1.6 g protein per liter after 61 h of methanol induction. Reduced SDS-PAGE analysis of culture supernatant showed that degradation of r27RCL at lower induction temperature was completely inhibited (Data not shown). The percentage of recombinant lipase of the total protein was about 30–50% determined by scanning the area of each band on reduced SDS-PAGE gels, and then calculating with ImageMaster TotalLab software (Amersham Biosciences).

## 4. Discussion

Eukaryotic lipases, *Rhizopus* sp. lipases, are synthesized as pre-pro-proteins. In vivo expression and in vitro refolding experiments of *R. oryzae* lipase showed that the prosequence is necessary for the production of the active lipase [28]. Sayari et al. [29] assessed the important role of the N-terminal sequence from prolipase on its specific activity, regioselectivity, stereoselectivity and its binding to a lipidic interface through a structural explanation of the enhanced penetration capacity as well as the catalytic activity of prolipase. The lipase gene structure of *rcl* was similar to other *Rhizopus* sp. lipases genes, which is composed of

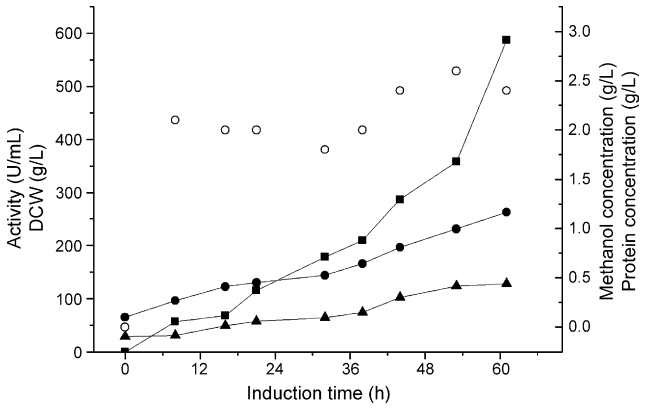


**Fig. 8.** r27RCL activity (■), protein concentration (●), methanol concentration (○) and dry cell weight (▲) in the bioreactor batch cultivation induced by methanol.

signal sequence, prosequence and mature lipase sequence without intron. Since the prosequence of the lipase might act as an intramolecular chaperone, we expressed the mature lipase in *cis* with prosequence in *P. pastors* extracellularly. As can be seen in Fig. 3, initially recombinant *P. pastors* produced one type of lipase with molecular mass of 37 kDa, and then along with the fermentation, the 37-kDa protein gradually degraded into a 30-kDa protein, and at the last stage completely converted into the 30-kDa protein. The N-terminal amino acid sequence analysis showed that the 37-kDa protein was cleaved by Kex2-like protease in *P. pastoris* at the recognition site Lys-Arg between -29 and -28 of the prosequence of RCL. The resulting lipase (r27RCL) consists of 27 amino acid prosequence and 269 amino acid mature lipase sequence. However, lipase from *R. oryzae, R. arrhizus* or *R. niveus* cleaved at the same Kex2p site (Lys-Arg) was found at amino acids -30 and -29 of the prosequence, which retained 28 amino acids of C-terminal part of the prosequence [20,30,31]. The one amino acids difference is probably due to gene variation during evolution. The N-terminal amino acid sequence of the 30-kDa lipase is identical to that of the mature region (mRCL) and the wild-type RCL-lip2 simultaneously, which suggests that mRCL expressed in *P. pastoris* might be the lip2 from *R. chinensis*. Researchers also found that the partial processed prolipase for *R. oryzae* (r28ROL) was gradually converted to mature lipase incubated at 0–6 °C for a few days [29,30]. Serine proteases present in the solution are likely to be responsible for this phenomenon, as the shift was strongly inhibited by phenylmethylsulfonyl fluoride (PMSF) [30].

The r27RCL activity from the methanol utilization plus phenotype, *Mut*$^+$ GS115/pPIC9K-proRCL was nearly one third percent lower than that from the methanol utilization slow phenotype, *Mut*$^s$ GS115/pPIC9K-proRCL. The *Mut*$^s$ phenotype, have a disruption in the *AOX1* gene. Since the cells must then rely on the weaker *AOX2* for methanol metabolism, a slower growing and slower methanol utilization strain is produced. Strains with *AOX* mutations are sometimes better producers of foreign proteins than wild-type strains [12]. Additionally, these strains do not require the large amounts of methanol routinely used for large-scale fermentations of *Mut*$^+$ strains.

Batch cultivations using a synthetic medium were investigated for further improvement of recombinant lipase production by *Mut*$^s$ GS115/pPIC9K-proRCL in a larger scale. Methanol concentration is a critical parameter in the cultivation of *Pichia* since the level of methanol affects both growth and the expression of the heterologous gene. Above certain concentrations (3.65 g/L), growth was substrate-inhibited by methanol [32]. In this research a methanol concentration of about 2.5 g/L was maintained during induction phase by avoiding buildup of methanol to cytotoxic levels and depletion of methanol to non-inducing levels. An on-line methanol monitoring and control system allows accurate control of methanol avoiding the periods of methanol exhaustion and build-up. Based on the gas–liquid phase equilibrium, a hollow silicone tube is submerged into the fermentor where methanol in the broth diffuses through the tubing, is picked up by a stream of air, and is carried to an external sensor, which detects methanol vapor that permeates from the broth across the silicone tube. The activity of r27RCL in the bioreactor was improved greatly to 587 U/mL. At lower induction temperature degradation of r27RCL was completely inhibited, which was probably caused by reduced proteolysis due to lower temperature and reduced proteolysis due to lower cell death and protease release to the medium [33]. The results obtained using expensive complex medium were 1600 U/mL for *R. niveus* lipase (mixed ProRNL and r28RNL) expressed in *S. cerevisiae* [34]. Takahashi et al. reported that the activity of *R. oryzae* Lipase (ProROL) by *S. cerevisiae* reached 2.88 U/mL (28.0 mg/L) after 120 h of cultivation in YPD medium [35]. The activity of *R. oryzae* lipase (r28ROL) expressed in *P. pastoris* obtained 140 U/mL after 92 h of cultivation in complex medium [20]. However, these values are not fully comparable since assay conditions were different. It is well known that the lipolytic activity test largely depends on a variety of factors, e.g. the substrate's interfacial area that is available for the enzyme, the type of substrate, as well as the equipment used [36]. The fermentation strategy needs to be further studied to improve the production of recombinant lipase and this work is in progress.

**Table 4**
Summary of properties of r27RCL, mRCL and wild-type RCL-lip2.

| Property | r27RCL | mRCL | Wild-type RCL-lip2[a] |
|---|---|---|---|
| Molecular weight (kDa) | 37 | 30 | 39 |
| Optimum pH | 8.5 | 8.0 | 8.5 |
| pH stability | 7.5–8.5 | 7.5–8.0 | 7.5–9.0 |
| Optimum temperature (°C) | 40 | 35 | 35 |
| Temperature stability (°C) | ≤40 | ≤35 | ≤40 |
| $K_m$ (mM) | | | |
| $V_{max}$ (μmol/(mL min$^{-1}$) | 0.304, 30.76 | 0.345, 0.822 | 0.578, 0.633 |
| Chain length specificity | C6 | C2 | C2 |
| Position specificity | No | No | No |
| N-terminal sequence | D-T-E-T-V-G-G | S-D-S-G-E-V | S-D-S-G-E-V-V |

[a] Data taken from Ref. [5].

Proteins secreted by *P. pastoris* are often glycosylated. Since the molecular mass of r27RCL but not mRCL was higher than the calculated molecular mass of 32 kDa and both proteins contain one common *N*-glycosylation sites of the sequence Asn-X-Ser/Thr, we investigated glycosylation of the recombinant enzymes by treatment with *Endo* H. However, the result showed that no *N*-glycosylation had occurred, which was also observed in lipase from *R. oryzae* (r28ROL) expressed in *S. cerevisiae* with a increased 3 kDa on SDS-PAGE compare with calculated molecular weight [19]. The increased molecular mass might be other kinds of post-translational modification of secreted proteins such as *O*-glycosylation, lipidation or phosphorylation [37].

The enzymatic properties of recombinant r27RCL and mRCL were characterized and summarized in Table 4. Compared with the wild-type RCL-lip2 [5], the pH optimum and stability, temperature optimum and thermostability were only a litter different and all of them showed no 1,3-position specific.

The prosequence not only plays an important role as an intramolecular chaperone to facilitate the correct folding of its mature portion, but also shows significant effects on its specific activity [9]. As shown in Table 3, the specific activity of r27RCL was generally higher than that of mRCL. r27RCL preferred middle-chain ester (C6) while mRCL preferred short-chain ester (C2), whose behavior was close to esterase. The chain length fatty acid specificity of r27RCL was a little different from lipases purified from *R. oryzae* and *R. homothallicus*, which showed specificity toward medium length fatty acid esters (C8) [22,38]. However, the specificity of mRCL favored short-chain ester was quite different from other *Rhizopus* sp. lipases. The interesting properties of the new lipase were correlated well with our previous studies that whole cell lipase from *R. chinensis* showed very high ability of esterification of short-chain fatty acids with ethanol [4]. This property is quite rare among lipases and gives this new lipase great potential for use in the field of biocatalysis. In addition, the activity of r27RCL in the bioreactor was improved greatly, and was five times higher than that in shake flask and was about 160 times higher than that of wild-type *R. chinensis* lipase [5], which makes *R. chinensis* lipase a highly interesting candidate for future practical applications.

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