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Enhanced production of *Thermomyces lanuginosus* lipase in *Pichia pastoris* via genetic and fermentation strategies

Zhonggang Fang · Li Xu · Dujie Pan · Liangcheng Jiao · Ziming Liu · Yunjun Yan

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Abstract This study attempted to enhance the expression level of *Thermomyces lanuginosus* lipase (TLL) in *Pichia pastoris* using a series of strategies. The *tll* gene was first inserted into the expression vector pPIC9 K and transformed into *P. pastoris* strain GS115. The maximum hydrolytic activity of TLL reached 4,350 U/mL under the optimal culture conditions of a 500 mL shaking flask containing 20 mL culture medium with the addition of 1.2 % (*w/v*) methanol, cultivation for 144 h at pH 7.0 and 27 °C. To further increase the TLL expression and copy number, strains containing two plasmids were obtained by sequential electroporation into GS115/9k-TLL #3 with a second vector, either pGAPZαA-TLL, pFZα-TLL, or pPICZαA-TLL. The maximum activity of the resultant strains GS115/9KTLL-ZαATLL #40, GS115/9KTLL-FZαATLL #46 and GS115/9KTLL-GAPTLL #45 was 6,600 U/mL, 6,000 U/mL and 4,800 U/mL, respectively. The *tll* copy number in these strains, as assessed by real-time quantitative PCR, was demonstrated to be seven, five, and three, respectively, versus two copies in GS115/9k-TLL #3. When a co-feeding strategy of sorbitol/methanol was adopted in a 3-L fermenter, the maximum TLL activity of GS115/9k-TLL #3 increased to 27,000 U/mL after 130 h of fed-batch fermentation, whereas, the maximum TLL activity was 19,500 U/mL after 145 h incubation when methanol was used as the sole carbon source.

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Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are a class of hydrolase that catalyze both the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids at the interface between aqueous and non-aqueous media [2, 5, 20, 27, 33]. These kinds of interfacial enzymes have been widely applied in many industries, including the detergent, food, flavor, pharmaceutical, fine chemicals, biodiesel production, biosensor and bioremediation industries [2, 14, 16, 27, 29]. However, their poor stability and high production costs of the enzymes should be improved for industrial biocatalysis [3]. Over the past 20 years, such bottlenecks were circumvented either by enzyme immobilization [10, 15, 21, 23, 24, 38, 39], directed evolution [26, 45], chemical modification [12, 15], or protein engineering strategies [3]. Moreover, utilization of thermostable lipases with unusual features from mesophiles and thermophiles has attracted increasing attention in recent years [13, 28, 44]. As a noticeable thermostable enzyme, the lipase from *Thermomyces lanuginosus* (TLL) (formerly called *Humicola lanuginosa*) was first commercialized as lipolase™ in 1994 by Novo Nordisk. Currently, TLL is used as an industrial biocatalyst in both soluble and immobilized forms in many different industrial areas including the modification of oils and fats, biodiesel production, organic chemistry, environmental applications, detergents, and the cosmetic industry [13, 26]. All of these practical applications require large quantities of enzymes. As a result, there is an urgent need to achieve high levels of lipase expression in commonly used industrial strains.

Pichia pastoris is a widely used system for heterologous protein expression [6, 8, 9, 17, 40]. The popularity of this expression system is due to several advantages: the simplicity of the techniques needed for its molecular genetic manipulation; its capability to perform eukaryotic post-translational modifications; and the alcohol oxidase 1 (*AOX1*) promoter being tightly regulated by methanol. Additionally, *P. pastoris* can grow to high cell density in a simple mineral salts medium and it secretes only a few endogenous proteins in a bioreactor, which simplifies product recovery and purification [7, 17]. The factors that significantly affect the high-level expression of heterologous proteins in *P. pastoris* include promoter strength, copy number of the expression cassette, mode of chromosomal integration of the expression cassette, secretion signal, and growth conditions [29, 30]. One of the common strategies to increase the expression level of a specific gene is to screen and select a strong promoter. Many microbial lipases have been expressed in *P. pastoris* under different promoters, of which the pAOX1-regulated systems are the most common [8]. *Rhizopus chinensis* lipase [41], *T. lanuginosus* lipase [44], *R. oryzae* lipase [25] and *Yarrowia lipolytica* lipase LIP2 (YlLIP2) [34] were expressed under the formaldehyde dehydrogenase promoter (pFLD1). Wang et al. [35] expressed YlLIP2, and Zhao et al. [43] expressed *Candida rugosa* lipase in *P. pastoris* using the glyceraldehyde-3-phosphate promoter (pGAP). However, there are fewer examples of lipases expressed under different promoters in one strain. In addition, in the *P. pastoris* expression system, increasing the copy number of the expression cassette often has the positive effect of improving the amount of target protein expression [40]. Until now, multicopy strains were often generated using an expression vector with an antibiotic resistance marker to select for multiple genomic integration events using high drug concentrations; in vitro construction of vectors with multicopy expression cassettes; or sequential electroporation with a second expression vector containing a different antibiotic marker [36, 46].

The carbon source, which is another important fermentation parameter, has changed from glycerol to a combination of multi-carbon substrates with methanol. This combination strategy could overcome the repression of the *AOX1* promoter caused by glycerol and increase the production of the recombinant target protein [31, 42]. Although glycerol is a common carbon source [42], it represses the *AOX1* promoter, which may lower the specific production of recombinant proteins. The use of a less-repressing carbon source may result in higher specific production rates, improving overall productivity and eliminating the need for tight control of residual substrate levels [31]. Among these, sorbitol is a widely accepted non-repressive carbon source for *P. pastoris* [32].

In this study, TLL was heterologously expressed in *P. pastoris* strain GS115 using sequential electroporation with two different expression vectors containing different promoters and antibiotic markers. The TLL expression level was investigated in shaking flasks and a 3-L bioreactor. Moreover, two different feeding strategies, sole methanol addition and methanol/sorbitol co-feeding, were compared in the induction phase of fed-batch fermentation. Finally, the effect of lipase gene copy number on the high-yield expression of recombinant strains was investigated using real-time PCR.

Materials and methods

Strains, plasmids, and media

Escherichia coli TOP10 (Invitrogen, Carlsbad, CA, USA) and the pMD18-T simple vector (Takara, Japan) were used for DNA manipulation, gene cloning and sequencing. The recombinants were screened at 37 °C with Luria–Bertani (LB) medium plates (0.5 % [*w/v*] yeast extract, 1 % [*w/v*] tryptone, and 2 % [*w/v*] agar) containing 100 μg/mL ampicillin, 50 μg/mL kanamycin or low Luria–Bertani (LLB) medium plates (its composition is similar to LB, but with the addition of 0.5 % [*w/v*] sodium chloride) containing 25 μg/mL Zeocin. *P. pastoris* strain GS115 (his4) and vectors pPIC9K, pPICZαA, and pGAPZαA were purchased from Invitrogen Co (Invitrogen, USA). The vector pFZα was constructed and preserved in our laboratory [34]. We substituted the *FLD1* promoter for the *AOX1* promoter in the vector pPICZαA. *P. pastoris* strain GS115 and recombinant GS115 were grown in either YPD medium (1 % [*w/v*] yeast extract, 2 % [*w/v*] dextrose, 2 % [*w/v*] tryptone) or BMGY/BMMY medium (1 % [*w/v*] yeast extract, 2 % [*w/v*] dextrose, 1.34 % [*w/v*] yeast nitrogen base with no amino acids [YNB], 4×10^{-5} % [*w/v*] biotin, 100 mM potassium buffer, 1 % [*w/v*] glycerol or 0.5 % [*v/v*] methanol). MD medium plates (1.34 % [*w/v*] YNB, 4×10^{-5} % [*w/v*] biotin, 2 % [*w/v*] dextrose, and 2 % [*w/v*] agar) or YPDS medium plates (YPD medium containing 1 mol/L D-sorbitol and 100 μg/mL Zeocin) were used to screen yeast recombinants.

Vector construction and *P. pastoris* transformation

The mature *tll* gene (GenBank Accession No. AF054513) without the 66 bp signal peptide sequence was amplified by PCR using the primer pair tll-kF/tll-R (which incorporated *Sna*BI/*Not*I restriction sites) and plasmid pUC57-TLL as a template. The PCR products and plasmid pPIC9 K were digested with *Sna*BI and *Not*I, then the *tll* gene and vector pPIC9 K were ligated after purification using a gel

Table 1 Primers used for plasmid construction and real-time quantitative PCR analysis

Name	Sequence (5′–3′)	Annotation
tll-kF	AGCTTACGTAGAGGTCTCGCAGGATCTGTTTAA	*Sna*BI site (underlined)
tll-F	CCGCTCGAGAAAAGAGAGGTCTCGCAGGATCTGTTTAA	*Xho*I site (underlined)
tll-R	ATTTGCGGCCGCCTAAAGACATGTCCCAATTAACCC	*Not*I site (underlined)
qtll-F	GACATAGCTTGGGTGGTGC	qPCR for *tll*
qtll-R	AAAGCCCTGTTTCCGACT	qPCR for *tll*
qGAP-F	CGGTGTTTTCACCACTTTGGA	qPCR for *gapdh*
qGAP-R	CAACGAACATTGGAGCATCCT	qPCR for *gapdh*

extraction kit. Finally, the resulting product, pPIC9 K-TLL, was transformed into *E. coli* TOP10, and positive recombinant strains were screened via PCR. Similarly, the recombinant plasmids pGAPZαA-TLL, pFZα-TLL, and pPICZαA-TLL were constructed and positive clones were screened. PrimerSTAR™ HS DNA Polymerase, DNA ligation kit ver. 2.0 and restriction endonucleases were purchased from TaKaRa Biotechnology Co. (Dalian, China). All the aforementioned recombinant plasmids were confirmed by DNA sequencing by Sunny Biotechnology Co. (Shanghai, China), and all primers used in this study are listed in Table 1.

The plasmids pPIC9K-TLL and pPIC9K were linearized by *Pme*I and transformed into *P. pastoris* GS115 by electroporation with a Gene Pulser apparatus (Bio-Rad, Hercules, CA, USA). According to the manufacturer's protocol, the parameters were 1,500 V, 200 Ω, 25 μF, using a 0.2 cm cuvette (Invitrogen). Transformants were selected on MD plates after incubation for 2–3 days at 28 °C, then further screened using YPD plates containing various concentrations of G418 (1.0–4.0 mg/mL).

The constructed recombinant vectors, pGAPZαA-TLL, pFZα-TLL and pPICZαA-TLL, were re-electroporated into GS115/pPIC9K-TLL #3 after linearization with *Bln*I, *Sph*I and *Pme*I, respectively. Positive recombinant transformants were identified using YPDS plates (100 μg/mL Zeocin) after incubation at 28 °C for 2–3 days.

Recombinants screening and shaking flask culture

Transformants with high G418 resistance were plated onto BMMY-rhodamine B-olive oil (BRBO) medium plates containing 0.008 % (*w/v*) rhodamine B and 1 % (*v/v*) emulsified olive oil. About 2 days later, white colonies grew on the BRBO plates. Subsequently, 200 μL of methanol was added to the culture dish every day, and 30 colonies with large, clear transparent circles were picked and inoculated into a 500-mL Erlenmeyer flask containing 25 mL of BMGY medium. These selected recombinant cells were harvested by centrifugation and transferred into 25 mL of BMMY medium after incubation at 28 °C for 24 h. In addition, methanol was added to the culture every 24 h and maintained at a final concentration of 1.2 % (*v/v*) to induce lipase expression.

Fermentation cultivation

The fermentation inoculum of *P. pastoris* was prepared by cultivating the cells at 28 °C with shaking at 220 rpm for 18–20 h in a 500 mL shaking flask containing 120 mL of YPD medium. Then, 10 % (*v/v*) of the culture was inoculated into a 3-L fermenter (BIOTECH-3BG-7000A, Baoxing Co. Shanghai, China) with 1.2 L of FM22 medium, which contained (g/L): KH_2PO_4, 42.9; $(NH_4)_2SO_4$, 5; $CaSO_4{\cdot}2H_2O$, 1.0; K_2SO_4, 14.3; $MgSO_4{\cdot}7H_2O$, 11.7; glycerol, 40; and 2.5 mL/L *Pichia* trace minerals 4 (PTM4) solution. The PTM4 solution was composed of (g/L): $CuSO_4{\cdot}5H_2O$, 2.0; NaI, 0.08; $MnSO_4{\cdot}H_2O$, 3.0; $Na_2MoO_4{\cdot}2H_2O$, 0.2; H_3BO_3, 0.02; $CaSO_4{\cdot}2H_2O$, 0.5; $CoCl_2$, 0.5; $ZnCl_2$, 7; $FeSO_4{\cdot}7H_2O$, 22; biotin, 0.2; and 1 mL/L concentrated H_2SO_4 [32]. The cultivation parameters were as follows: the pH was adjusted to 5.0 by adding 25 % ammonium hydroxide, while the dissolved oxygen (DO) level was maintained at over 20 % of air saturation by controlling the air flow rate (2–6 L/min) and the stirring speed (300–900 rpm) at 30 °C. The fermentation process of recombinant *P. pastoris* comprised three phases. In phase 1, the cells were incubated at 30 °C and pH 5.0 until the glycerol in the medium was consumed; a sudden increase in the DO value indicated the end of this phase. Phase 2 (the glycerol fed-batch phase) was initiated by feeding medium containing 50 % (*w/v*) glycerol and 2 mL/L PTM4 solution. Cells substantially grew during this phase, and an antifoaming agent (Dowfax DF103, USA) was pumped into control the production of foam. When the OD_{600} reached ca. 150, glycerol feeding was terminated and a carbon-source starvation period of 30 min was followed to exhaust the glycerol. Meanwhile, the pH was adjusted to 5.5, and the temperature was reduced to 27 °C. Phase 3 was the methanol induction phase. Pure methanol or mixtures of methanol and sorbitol (100 % methanol: 50 % sorbitol in a 1:1 ratio, *v/v*) containing 2 mL/L PTM4 solution was fed to start the induction. The feed frequency was adjusted once every 2–3 h, and the agitation rate and airflow rate were controlled to maintain the DO level at 20–50 %. Samples were taken at regular intervals

and analyzed for biomass, lipase activity, total protein concentration, and other experiments. Each condition was measured in triplicate, and each experiment was investigated three times. The results are shown as the mean ± standard deviation (SD) from three independent experiments.

Optimization of TLL cultivation in shaking flasks

To further improve the lipase productivity and achieve the maximal bioactivity, the effects of methanol induction time, various pH values, culture medium volume, inoculum size, methanol concentration, and induction temperature on lipase activity were investigated in shaking flask experiments.

Based on the initial cultivation conditions (pH 6.5, culture medium volume 50 mL, 4 % inoculum size, 1 % methanol, and 28 °C induction temperature) in shaking flasks, the parameters including initial pH (pH 5.5–8.0), initial culture medium volume (20–60 mL in 500-mL shaking flask), temperature (22, 25, 27 and 30 °C), the inoculum size (from 1 to 5 %) and methanol concentration (from 0.8 to 1.8 %) were investigated to evaluate their effects on TLL productivity and other parameters. Each condition was measured in triplicate, and each experiment was investigated three times. The results are shown as the mean ± SD from three independent experiments.

Biomass analysis and optical density (OD)

Biomass was expressed as wet cell weight (WCW, g/L), and was measured by centrifuging 10 mL samples in a preweighed centrifuge tube at 8,000 *g* at 4 °C for 10 min, and the supernatant was refrigerated for subsequent determinations. The precipitates were subsequently washed twice with distilled water and measured in triplicate. The fermentation broth samples were diluted with distilled water to measure the OD at 600 nm.

Lipase activity assay and total protein concentration

The lipase activity of supernatants was determined by the alkali titration method, using olive oil as substrate [24]. The reaction was conducted in a mixture of 5 mL of 50 mM Tris–HCl (pH 8.0), 4 mL of emulsion of olive oil [25 % (*v*/*v*) olive oil emulsified with 2 % (*w*/*v*) polyvinyl alcohol solution] and 1 mL of proper dilute enzyme solution at 60 °C for 10 min in a shaking water bath. Finally, a 15 mL mixture of acetone/ethanol (1:1, *v*/*v*) was added to terminate the reaction. The amount of liberated fatty acids was measured by titration with 50 mM NaOH using phenolphthalein as an indicator. One unit (U) of lipase activity was defined as the amount of lipase necessary to liberate 1 μmol per min of fatty acids from the olive oil. The total

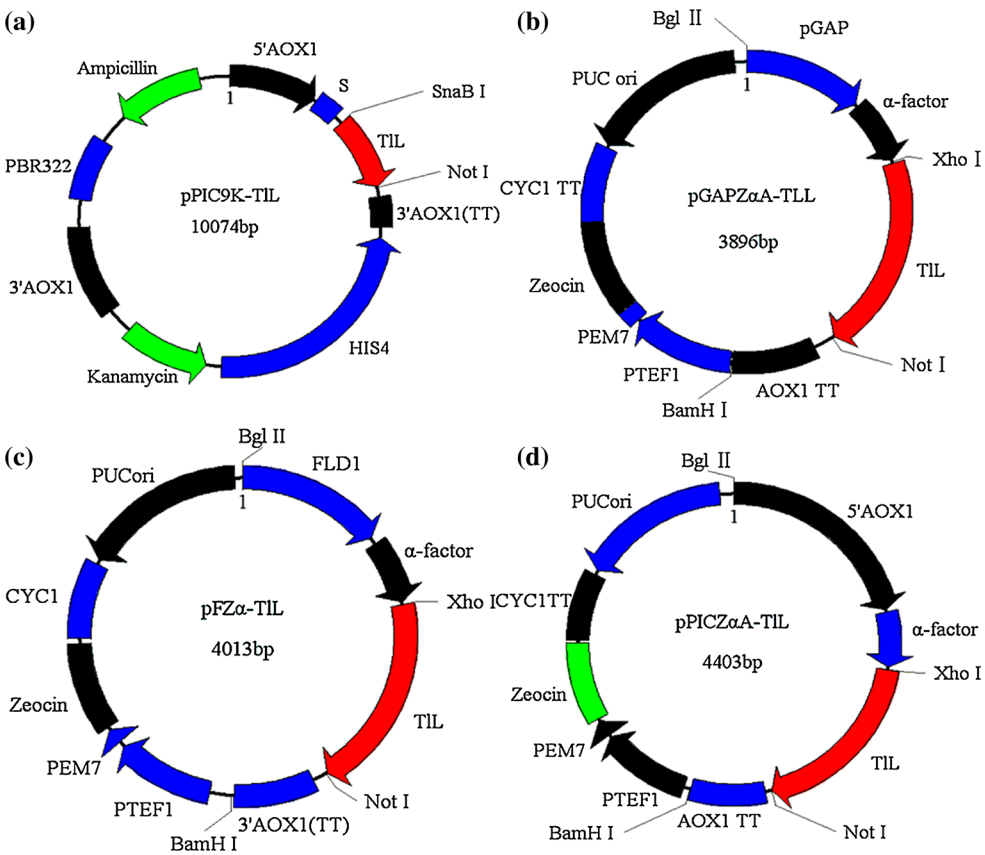

Fig. 1 The schematic diagram of recombinant plasmid pPIC9 K-TLL **a**, pGAPZαA-TLL **b**, pFZα-TLL **c**, and pPICZαA-TLL **d**

protein concentration was determined by the method of Bradford, using BSA as a standard [4]. Each condition was measured in triplicate, and each experiment was investigated three times. The results are shown as the mean ± SD from three independent experiments.

SDS-PAGE analysis

The recombinant lipase in the supernatant was analyzed by SDS-PAGE, which was conducted using a 6 % stacking gel and a 12 % separating gel on a vertical mini gel apparatus (Bio-Rad, USA), as described by Laemmli [18]. Protein molecular weight marker was purchased from Fermentas (Burlington, Canada). Samples were mixed equally with 2× loading buffer and heated at 100 °C for 8 min before electrophoresis. Proteins were stained with Coomassie Brilliant Blue R-250 (Amresco, Solon, OH, USA).

Determination of gene copy number by quantitative PCR

Absolute quantification was used to determine the copy numbers of the target gene using SYBR green. Primer design was performed using Primer Express v3.0 software (Applied Biosystems, CA, USA), and all primers used for qPCR are listed in Table 1. According to the product manual, PCR was performed in a 10 μL reaction mixture containing 5 μL of 2× SuperReal PreMix Plus (TIANGEN BIOTECH Co., Beijing, China), 0.3 μL of each 10 μM primer, 1 μL of 50× ROX Reference Dye, and 1 μL of template. The amplification reaction was initiated with a 15 min step at 95 °C, followed by 40 cycles of 10 s at 95 °C, 20 s at 55 °C and 30 s at 72 °C. After 40 cycles, the specificity of the amplification was analyzed by the melting curve. Real-time PCR was accomplished using an ABI 7900HT system with SDS v2.4 Software (Applied Biosystems). The GAPDH gene of *P. pastoris* was set as the reference gene. A tenfold series dilution (10^{-2}–10^{-8}) of linearized plasmid, including cloned target and reference genes, was utilized as templates to establish the standard curves. Genomic DNA of *P. pastoris* was used as the template in real-time PCR analysis. The PCR efficiency was calculated from the slope of each standard curve by the following equation [19]: $E = 10^{-1/\text{slope}} - 1$. As per the method of Abad et al. [1], data were collected and the copy number of the target gene was analyzed in different recombinant strains.

Results

Construction of expression vectors and screening of positive *P. pastoris* clones

The plasmid pPIC9K-TLL (Fig. 1a) was linearized by *Pme*I and inserted at the *AOX1* locus to create Mut$^+$ (Methanol utilization plus) transformants. After screening with high concentrations of G418, the *P. pastoris* colonies were transferred to BRBO medium plates to further investigate lipase expression. During the 72 h methanol induction, there were clear halos around the colonies of the transformants, whereas no halos were observed around the control cells. Thirty colonies with large-sized halos were picked and inoculated into 500 mL shaking flasks. After induction for 144 h by methanol, the TLL lipase activity reached its highest value. The positive clone, termed GS115/9K-TLL #3, with the maximum activity of 4,350 U/mL, was chosen for all further experiments.

The expression vectors pGAPZαA-TLL, pFZα-TLL, and pPICZαA-TLL (Fig. 1b–d) were linearized and transformed into GS115/9K-TLL #3 via a second round of electroporation. The transformants were screened by a high concentration of Zeocin (500 μg/mL) on YPDS plates. After reselecting several circles, the lipase productivity and bioactivity of these positive recombinants were investigated quantitatively in shaking flasks. As shown in Fig. 2, the maximum activities of the selected recombinants GS115/9KTLL-GAPTLL

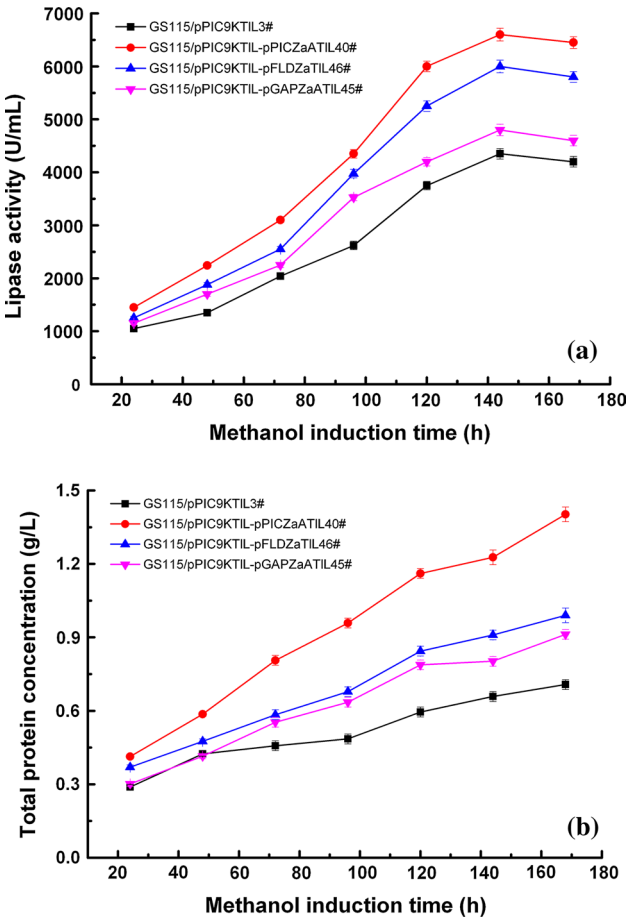

Fig. 2 The cultivation properties of the recombinant strains in shaking flasks. **a** Time course of lipase activity; **b** Time course of total protein concentration. Data are presented as the mean ± SD of triplicate determinations

#45, GS115/9KTLL-FZαTLL #46, and GS115/9KTLL-ZαATLL #40 reached 4,800, 6,000, and 6,600 U/mL, respectively. The maximum total protein concentrations for these three recombinants, GS115/9KTLL-GAPTLL #45, GS115/9KTLL-FZαTLL #46, and GS115/9KTLL-ZαATLL #40, were 0.912, 0.990, and 1.402 g/L, respectively.

Optimization of the *tll* recombinant strain in shaking flask

As shown in Fig. 3a, the maximum lipase activity in the supernatant from GS115/9K-TLL reached 1,960 U/mL after methanol induction for 144 h, after which the lipase activity started falling immediately. Ultimately,

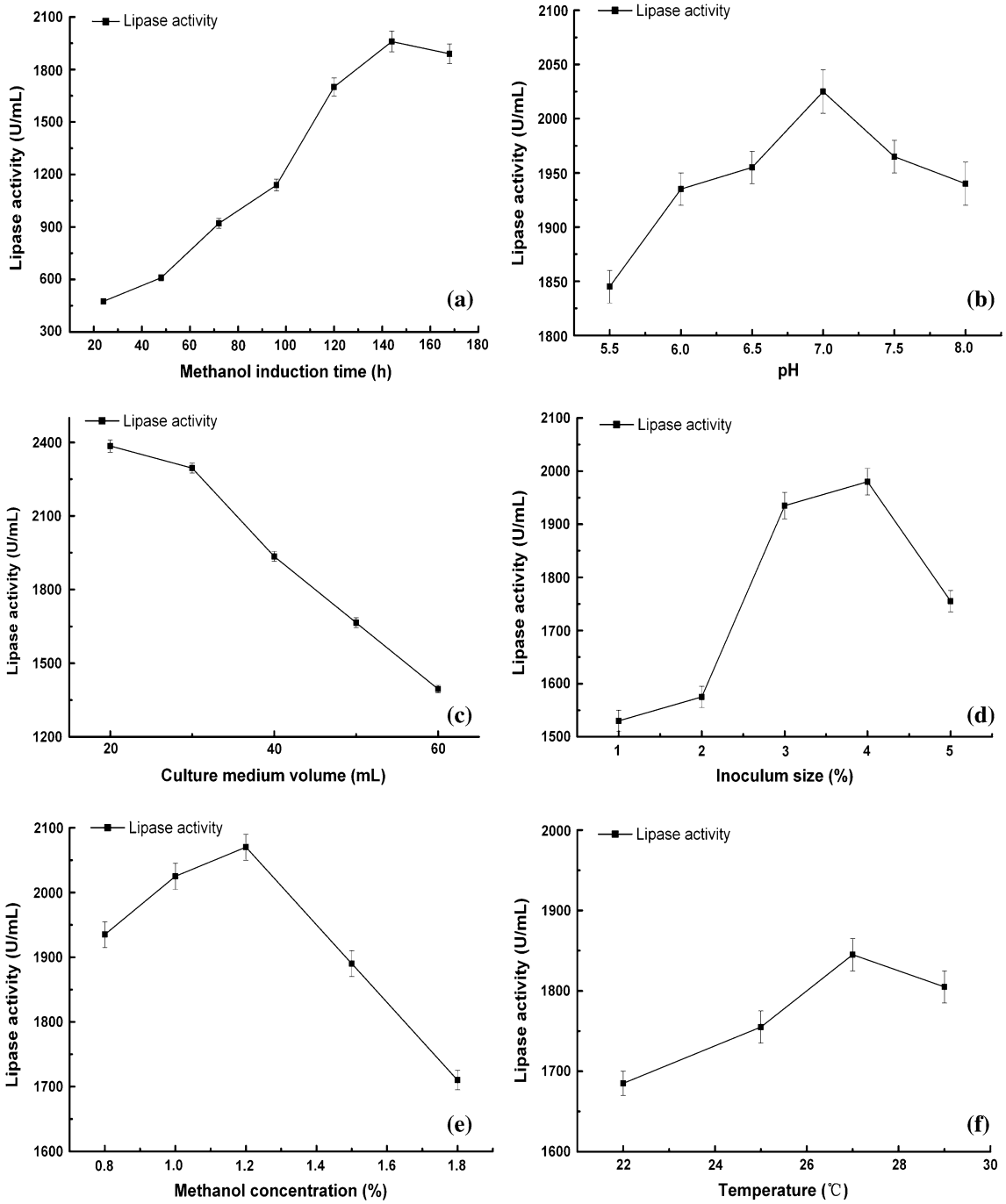

Fig. 3 Optimization of TLL cultivation in shaking flasks. **a** Time course of lipase activity; **b** Effects of variation of initial pH on lipase activity; **c** Effects of different initial culture medium volumes in the 500 mL shaking flask; **d** Relationship between lipase activity and inoculum size; **e** Effects of different methanol concentrations on the production of TLL; **f** Effects of variation in the induction temperature on lipase activity. All values are mean ± SD from three independent experiments

the maximum TLL lipase activity was obtained with a 4 % inoculum size and the optimal culture condition was achieved by the daily addition of 1.2 % (*v/v*) methanol to a 500 mL shaking flask containing 20 mL culture medium for 144 h at pH 7.0 and 27 °C.

Gene copy numbers of the target gene *tll* in recombinant strains

RT-qPCR assays were performed to precisely determine the *tll* gene copy number in the genomes of the integrants. Fig. 4 shows that the standard curves of the target gene, *tll*, and the reference gene, *gapdh,* were linear in the tested range, and the amplification efficiencies were 93.78 and 95.36 %, respectively. Both matched the requirements of the qPCR assay. A single-peak melting curve suggested that the PCR products were singlets (data not shown). The average Ct value of *tll* and *gapdh* were calculated based on triplicate experiments. As shown in Table 2, the copy numbers of the *tll* gene in the different recombinant strains, GS115/9K-TLL #3, GS115/9KTLL-ZαATLL #40, GS115/9KTLL-FZαTLL #46, and GS115/9KTLL-GAPTLL #45, were two, seven, five and three, respectively.

Fed-batch fermentation studies

To obtain a high cell biomass and TLL productivities, fed-batch studies were conducted in 3-L fermenters using recombinant strain GS115/9K-TLL #3. Induction of TLL expression by the *AOX1* promoter was controlled using methanol as the sole carbon source (Table 3). The maximum TLL lipase activity and maximal WCW reached 19,500 U/mL and 425 g/L, respectively, and the maximal OD_{600} reached 420 after 145 h of methanol induction.

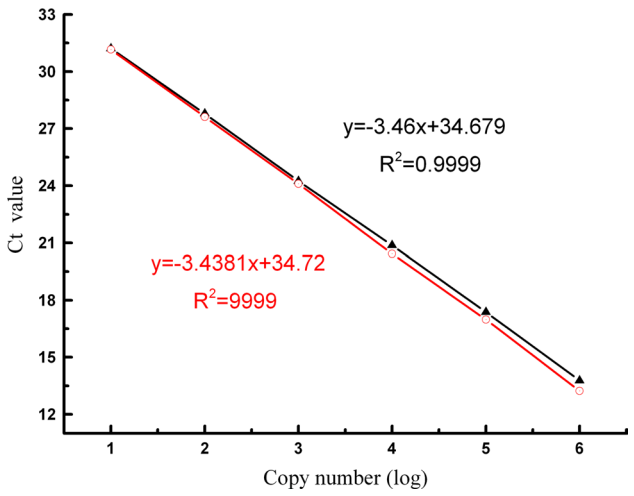

Fig. 4 The standard curves of the target gene *tll* (*open circle*) and the reference gene *gapdh* (*filled triangle*)

Additionally, the total protein concentration in the supernatant achieved a maximum value of 3.28 g/L (Fig. 5a). Meanwhile, the effect of co-feeding with sorbitol and methanol on the recombinant strains was compared with that using a sole carbon source. As shown in Fig. 5b, the maximum TLL lipase activity, WCW, and total protein concentration in the supernatant were 27,000 U/mL, 513, and 4.02 g/L, respectively, after 130 h of sorbitol/methanol fed-batch fermentation. The maximal OD_{600} reached 530. The lipase bioactivity, productivity, and cell growth rate were all improved by the co-feeding strategy. Furthermore, the lipase activity, total protein concentration and OD_{600} were approximately 6.20-, 5.69- and 12.62-fold higher than those obtained in shaking flasks.

SDS-PAGE electrophoresis

Equal volumes of supernatants from shaking flask cultivation and 3-L fermenter fed-batch cultures were run on 12 % SDS-PAGE, and the target lipase TLL migrated as a 35 kDa band, as expected (Fig. 6). As shown in Fig. 6a, there were few contaminating proteins present in the supernatant from shaking flask cultivation. The TLL target band obtained from the sorbitol/methanol fed-batch culture was much more intense than that of other bands, which is indicative of increased TLL production from the co-feeding strategy (Fig. 6b).

Discussion

The *tll* gene, which encodes a thermostable industrial enzyme, was cloned from the thermophilic fungus *T. lanuginosus* and expressed in *P. pastoris*. The activity of the purified lipase was 1,328 U/mL [44]. Immobilized TLL has been widely applied in many fields, such as in detergents, biodiesel production, organic chemistry, the modification of oils and fats, and environmental recovery [13].

Table 2 Copy numbers of the *tll* gene detected by real-time PCR using SYBR Green

Strain	Ct value		Copy number
	gapdh	*tll*	*tll*
GS115/9 K-TLL 3#	11.964 ± 0.465	10.830 ± 0.063	2
GS115/9 KTLL-ZαATLL 40#	13.709 ± 0.224	11.028 ± 0.115	7
GS115/9 KTLL-FZαTLL 46#	14.098 ± 0.039	11.767 ± 0.137	5
GS115/9 KTLL-GAPTLL 45#	12.451 ± 0.235	10.767 ± 0.147	3

Values are means ± SDs from three independent experiments

Table 3 The yield and growth characteristics of different recombinant strains

Scale	Recombinant strains	Lipase activity (U/mL)	Total protein concentration (g/L)	WCW (g/L)	OD_{600}	Feeding methanol
500 mL shaking flask	GS115/9K-TLL 3#	4,350	0.707	70	42	
	GS115/9KTLL-GAPZαATLL 45#	4,800	0.912	70	40	
	GS115/9KTLL-FZαTLL 46#	6,000	0.990	72	43	
	GS115/9KTLL-ZαATLL 40#	6,600	1.402	74	45	
3-L fermenter	GS115/9K-TLL 3#	19,500	3.279	425	420	Methanol fed-batch
		27,000	4.024	513	530	Sorbitol/methanol fed-batch

Values are means ± SDs from three independent experiments

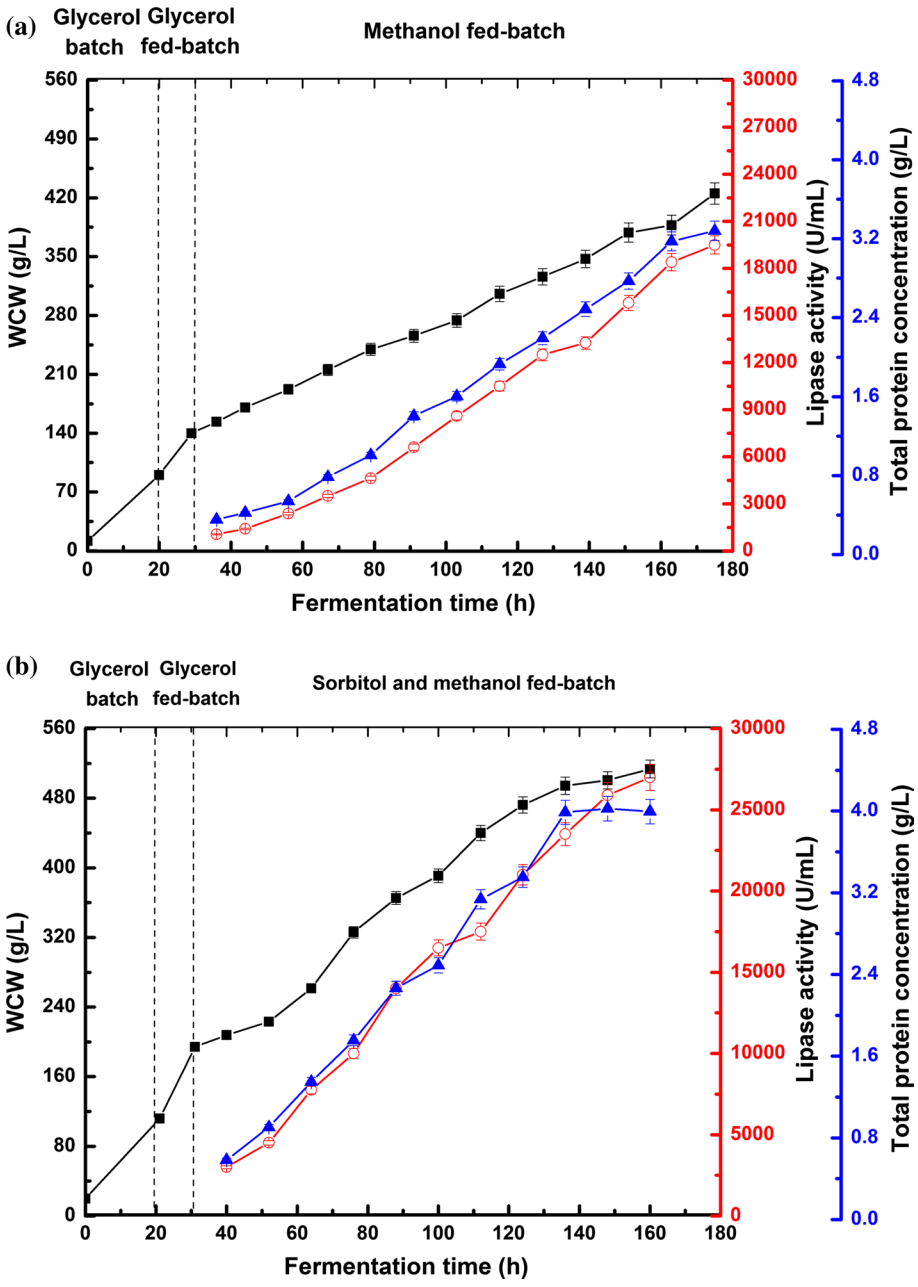

Fig. 5 Time course of lipase activity (*open circle*), biomass (*filled square*), and total protein concentration (*filled triangle*) during fed-batch fermentation in the 3-L fermenter with a 1.2 L modified FM22 medium. **a** Methanol was used as the sole carbon source and inductor; **b** Sorbitol and methanol were used as the co-feeding substrates. All values are mean ± SD from three independent experiments

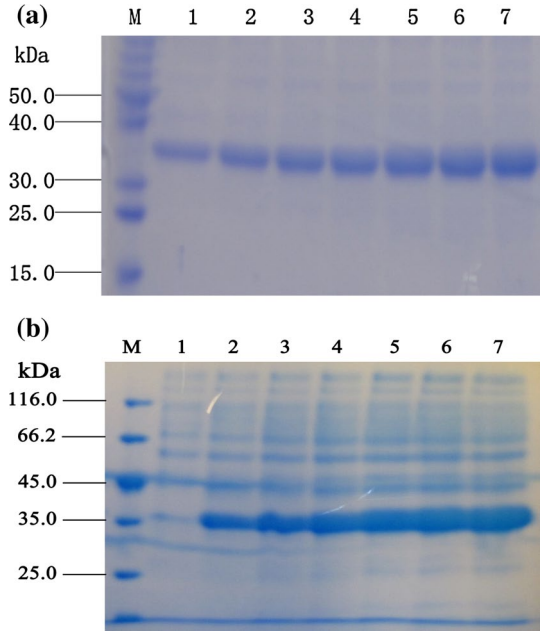

Fig. 6 Time course of SDS-PAGE analysis of culture supernatants during the cultivation. **a** Shaking flask cultivation. Lane M, low molecular weight marker (Fermentas, Burlington, Canada); lanes 1–7, 15 μL of culture supernatants after 24, 48, 72, 96, 120, 144, and 168 h of incubation, respectively. **b** Sorbitol/methanol fed-batch cultivation in a 3-L fermenter. Lane M, low molecular weight marker; lanes 1–7, 10 μL of culture supernatants in a 3-L fermenter after 31, 40, 52, 64, 76, 88 or 100 h of cultivation

TLL displayed on the surface of *P. pastoris*, another form of immobilization, was investigated, and its activity was only 257.8 U/g dry cells in shaking flasks [39]. Therefore, the productivity of TLL should be further improved to meet increasing industrial requirements. However, the strategy of using dual- or multiple-promoters as an improved, effective method to increase target protein yields was not utilized to efficiently express TLL [11, 37].

In this context, TLL was expressed under the control of pAOX1 and the recombinant strain, GS115/9 K-TLL #3, with the maximum activity of 4,350 U/mL was obtained and selected as host cells for a second round of electroporation with pGAP expression vectors (pFLD1 or pAOX1) containing a *tll* gene expression cassette to enhance TLL expression. As expected, the lipase activity of recombinant strains GS115/9KTLL-GAPTLL #45, GS115/9KTLL-FZαTLL #46, and GS115/9KTLL-ZαATLL #40 were 1.10-, 1.38-, and 1.52-fold higher, respectively, than that of GS115/9K-TLL #3, which is in accordance with a previous report [37]. More interestingly, the results of the high activity, recombinant GS115 strains with multiple lipase gene copies demonstrate again the positive correlation between lipase activity and lipase gene dosage. In *P. pastoris*, increased expression cassette copy number has been reported to increase the yield of a number of recombinant proteins [36]. Thus, sequential electroporation of plasmids with different promoters and antibiotic resistance markers into *P. pastoris* is an efficient method to increase target gene copy numbers and expression levels.

Cultivation is essential for secreted proteins because yield correlates largely with cell density. With the aim of reaching an ideal bioactivity at high cell densities under optimal culture parameters in fermenters, the cultivation conditions were optimized in shaking flasks. The culture medium volume and methanol concentration were the major factors, and the lipase activity increased 2.2 times compared with that of the same recombinant strain under the initial culture conditions. From the results of the aforementioned shaking flask studies, we concluded that modifications should be made to obtain optimum aeration (dissolved oxygen), induction temperature, pH, inoculum size, and methanol concentration to obtain maximum cell growth and lipase activity, according to the specific situation of each recombinant strain.

Furthermore, the co-feeding of sorbitol and methanol was adopted to induce TLL expression at the high cell densities in fermenters, instead of using methanol as the sole carbon source. Compared with methanol fed-batch fermentation, the highest lipase activity, WCW, total protein concentration, and OD_{600} obtained from the sorbitol/methanol fed-batch strategy were increased by 38.5, 20.7, 22.6, and 26 %, respectively. More importantly, the addition of sorbitol as a co-substrate shortened the fermentation time needed to achieve an activity of 19,500 U/mL from 112 to 49 h because sorbitol eliminates the long lag phase of the cells [6]. This is a major advantage in the industrial process. The activity, WCW, total protein concentration, and OD_{600} of the recombinant strains were 6.2-, 5.69-, 7.33- and 12.62-fold higher following a fed-batch strategy compared to shaking flasks. The hydrolytic activity of TLL in our study is higher than the previously reported value of 1,328 U/mL [44]. These results demonstrate that co-feeding of sorbitol and methanol is a good choice to reduce oxygen consumption [22] and protease production, and enhances the target protein yield as a result of the elimination of the lactic acid build-up [6].

In conclusion, we used sequential electroporation with two different expression vectors containing different promoter and antibiotic markers to express the microbial lipase *tll* gene in *P. pastoris*. The investigation of the *tll* gene copy number showed that the foreign gene dosage in *P. pastoris* had a great influence on the protein expression level. On the basis of molecular up-stream operations, the cultivation conditions of the TLL recombinant strains were optimized in shaking flasks, and the high-density cultivation of TLL was further explored in a 3-L fermenter. The maximum lipase activity reached 27,000 U/mL in a 3-L fermenter using a sorbitol/methanol fed-batch strategy, which was

13.8-fold higher than that of the original strain. Therefore, the strategy of combined up-stream molecular operation and down-stream fermentation processing is an efficient method for extracellular lipase production that is compatible with industrial and commercial needs, which offers a promising approach for the enhancement of biomass and heterologous protein production in less fermentation time for industrial applications.

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