High-level expression of *Thermomyces dupontii* thermophilic lipase in *Pichia pastoris* via combined strategies

Jianrong Wang¹ · Zongze Wu¹ · Tianyu Zhang¹ · Yonghua Wang² · Bo Yang¹

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Abstract

In this study, a combined strategy was used to improve the production of *Thermomyces dupontii* lipase (TDL) in *Pichia pastoris*. First, the native gene of TDL was optimized based on the codon usage of *P. pastoris*, ligated to pPICZαA and transformed in *P. pastoris* X33. A recombinant strain designated X33-T23 with the highest activity (1020 U/mL in shake flasks) amongst 216 recombinant colonies was selected for further investigations. To further increase the production of TDL, nine different secretion helper factor genes were transformed in the recombinant strain, X33-T23. The recombinant strain co-expression with the gene encoding protein disulfide isomerase, designated X33-T23-PDI, exhibited the highest activity in shake flasks (1760 U/mL) and in 5 L bioreactor (57521 U/mL) which were 1.67- and 1.46-fold higher, respectively, than for strain X33-T23. Additionally, the optimization of the inducers (temperature and pH) for the recombinant strain X33-T23-PDI in 5 L bioreactor produced, as expected, much higher lipase activity (81203 U/mL). The results of this study will provide an effective method to produce TDL and give some clues on how to improve production of heterologous proteins in *P. pastoris*.

Keywords Codon optimization · *Pichia pastoris* · Secretion helper factor · Thermophilic lipase · *Thermomyces dupontii*

Introduction

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are interfacial enzymes which hydrolyze ester linkages of triacylglycerols in aqueous medium. Meanwhile, they also catalyze transesterification, esterification, and inter-esterification reactions in non-aqueous medium (Jaeger and Eggert 2002; Singh et al. 2016). As versatile biocatalysts, lipases are widely used in many industries such as agriculture, food, oil modification, biodiesel production, fine chemicals, etc. (Borrelli and Trono 2015).

The lipase from *Thermomyces dupontii* (formerly classified as *Talaromyces thermophiles*) is a thermophilic enzyme (named TDL) which exerts its maximal activity at 60 °C and retains more than 50% residual activity after treatment at 70 °C for 1 h (Romdhane et al. 2010). Previous studies found that TDL with potential values in several industrial applications. TDL presented good stability in alkaline pH and different surfactants, which is helpful for its application as additive in detergents (Romdhane et al. 2010). Meanwhile, TDL exerts excellent transesterification activity, which is useful for its application in oil modification and biodiesel production (Romdhane et al. 2011). Furthermore, TDL shows its potential for preparation of chiral pharmaceutical intermediate and chemicals (Romdhane et al. 2013; Ding et al. 2018). Although TDL shows potential value for industrial application, the production of TDL from *T. dupontii* is very low. So it is crucial to achieve high yield of this protein.

Heterologous expression is an effective strategy to improve the production of TDL. Previous studies found that the production of TDL in *P. pastoris* was 156 U/mL, which was 2.6-fold higher than *T. dupontii* (60 U/mL) at flask shake culture (Romdhane et al. 2010; Zhang et al. 2015). The methylotrophic yeast *P. pastoris*, currently reclassified as *Komagataella phaffii*, has been used extensively and is one

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✉ Bo Yang
yangbo@scut.edu.cn

¹ School of Bioscience and Bioengineering, South China University of Technology, Guangzhou 510006, People's Republic of China

² School of Food Science and Engineering, South China University of Technology, Guangzhou 510640, People's Republic of China

of the most commonly used expression systems for heterologous protein production. *P. pastoris* has several advantages over other expression systems such as high expression level, powerful secretion ability, mature fermentation process, etc. (Ahmad et al. 2014). Previous studies found that several factors affect the production of heterologous protein in *P. pastoris*, including the gene copy numbers, codon optimization, protein folding in the endoplasmic reticulum (ER), unfolded protein response (UPR), protein translocation from the ER to the Golgi apparatus, and cultivation conditions (Gu et al. 2015; Li et al. 2016, 2017; Yang and Zhang 2018). Optimization of these factors is an effective method to improve the production of heterologous protein in *P. pastoris*. Previous studies demonstrated that the maximum activity of *Candida rugosa* lipase Lip1 reached 13,490 U/mL by combination of codon optimization and co-expression of chaperons (Li et al. 2016). Co-expression of two or three chaperones could improve the production of glucose oxidase in *P. pastoris* and the maximum activity of the recombinant strain S17 (co-expressed with *GCN4*, *CNE1* and *SEC53*) was 5.11-fold higher than the control strain (Gu et al. 2015). In our previous study, the production of *Aspergillus usamii* endo- β -1,4-xylanase was improved by 181% (from 20,937 to 58,729 U/mL) by combination of codon optimization, co-expression with *Vitreoscilla hemoglobin* and cultivation optimization (Wang et al. 2016).

In this study, a combined strategy was used to improve the expression of TDL in *P. pastoris*. First, the gene of TDL was optimized, ligated to pPICZ α A, and transformed to *P. pastoris* X33 to isolate recombinant strain with multiple copy integration. Then, we studied the effects of different secretion helper factors on the production of TDL. Finally, the induction temperature and pH were optimized in 5 L bioreactor. The results present here will offer an effective method to improve the production of recombinant TDL and provide an important basis for further applications of TDL in industrial applications.

Materials and methods

Strains, plasmids, and reagents

The *E. coli* strain Top 10 and *P. pastoris* X33 are routinely conserved in our laboratory. Zeocin and pPICZ α A, pGAPZA and pPIC9K were from Invitrogen (Carlsbad, CA, USA). DNA polymerase (PrimeSTARTM HS), restriction enzymes (EcoRI, NotI, and SacI), T₄-DNA ligase and in-fusion cloning kit were from Takara Biotechnology (Dalian, China). The codon optimization gene without signal sequence (*tdl-opt*) and the oligonucleotides were synthesized by the GENEWIZ (Suzhou, China). Sodium hydroxide was purchased from Merck (Darmstadt, Germany).

Medium

Media for *E. coli* include LBZ (LB with 25 μ g/mL zeocin) and LBK (LB with 30 μ g/mL kanamycin). Media for *P. pastoris* include YPDG (yeast extract peptone dextrose medium with 0.5 mg/mL G418 sulfate), YPDZ (yeast extract peptone dextrose medium with 100 μ g/mL zeocin), BMGY (buffered glycerol complex medium), BMMY (buffered methanol complex medium). LBZ, LBK, YPDG, YPDZ, BMGY, and BMMY were prepared according to the protocol provided by Invitrogen (<https://www.thermofisher.com>).

Gene optimization

The gene of TDL without signal sequence (GenBank: JF414585.1) was optimized according to the preferred codons of *P. pastoris* (OPTIMWIZ, GENEWIZ, Suzhou, China). The amino acid sequence of TDL was not changed during the optimization process.

Construction of expression vectors

The optimized gene (*tdl-opt*) was digested by EcoRI and NotI and then ligated into pPICZ α A to form pPICZ α A-*tdl-opt*. The sequence of pPICZ α A-*tdl-opt* was confirmed by DNA sequencing. The process for construction of different secretion helper factor expression vectors is shown in supplemental Fig. 1. The 2.7-kb fragment of pGAPZA without zeocin was amplified from pGAPZA. The gene of kanamycin was cloned from pPIC9K and ligated to 2.7 kb fragment by in-fusion cloning to form pGAPZAK. The genes of different secretion helper factors were cloned from the chromosomal DNA of *P. pastoris* X33 and ligated into pGAPZAK to form different molecular chaperone expression vectors. All primers used in this study are listed in supplemental Table 1.

Transformation of *P. pastoris* and isolation of recombinant clones

The expression vector pPICZ α A-*tdl-opt* was linearized and transformed into *P. pastoris* X33. Transformants were plated on YPDZ plates loaded with different zeocin content (from 100 to 1000 μ g/mL). The method for screening transformants was the same as in our previously described method (Wang et al. 2016). The detailed protocol of selection of recombinant strains is provided in supplemental material.

The recombinant strains with higher activities were inoculated into 10 mL BMGY in 150 mL flasks and incubated at 30 °C and 200 rpm for 24 h. Then the recombinant cells were harvested by centrifugation, re-suspended in BMMY,

and transferred to 50 mL BMMY (OD_{600} is 1.0) in a 500-mL flask, which also incubated at 30 °C and 200 rpm. 0.75% (v/v) methanol was added to the culture at every 24 h, and 0.5 mL culture was harvested every 24 h for lipase activity assay. Quantitative PCR (qPCR) was used to analyze the gene copy number of *tdl-opt* in the recombinant strain X33-T23 which showed highest lipase activity. Meanwhile, the recombinant strain X33-T23 was isolated for the next step.

Determination of gene copy number

The qPCR experiments were performed according to the previous method (Sha et al. 2013a, b). The glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) was selected as the reference gene. Specific primers used to amplify *GAPDH* and *PAOXI* were designed by Primer Express software. The qPCR reactions were performed on CFX Connect™ Real-Time System (Bio-Rad) using SYBR® Green Real-time PCR Master Mix (Toyobo). The detailed protocol of qPCR experiments is provided in supplemental material. The copy number of *tdl-opt* in the recombinant strain X33-T23 was analyzed using the Pfaffl method (Pfaffl 2001; Sha et al. 2013a, b).

Co-expression with secretion helper factors

The recombinant strain with the highest lipase activity was used as host and transformed with linearized secretion helper factor expression vectors. Positive transformants were plated on YPDG plates. The method for screening transformants was the same as described in supplemental material. The clones with higher activity were cultivated in shake flask and 5 L bioreactor.

High cell density fermentation

High cell density fermentation was carried out in 5 L bioreactor. The cultivation conditions and medium composition were the same as the previously described (Wang et al. 2013). The detailed protocol of high cell density fermentation is provided in supplemental material. The enzyme activity, total protein, and the cell density (dry cell weight) were monitored throughout the fermentation.

Optimization of induction pH and temperature in 5 L bioreactor

To further improve the production of recombinant TDL in *P. pastoris*, the induction pH and temperature were optimized in 5 L bioreactor. During glycerol fed-batch phase, the condition was maintained at pH 5.0, 30 °C. During methanol feed-phase, the temperature was set in the range of 24–30 °C, and the pH was controlled at from 4.0 to 7.0,

respectively. The enzyme activity, total protein concentration, and DCW were monitored at 24-h interval.

Large-scale production of TDL in 50 L bioreactor

The method for high cell density fermentation in 50 L bioreactor was the same as described above except the induction pH and temperature were changed to 6.0 and 27 °C. The enzyme activity, total protein concentration, and DCW were monitored at 24-h interval during the process of fermentation.

Detection methods

The lipase activity was detected by the pH-stat (Metrohm, Herisau, Switzerland) method using olive oil as substrate according to the previous method (Wang et al. 2013). The pH and temperature for lipase activity determination were set at 9.5 and 60 °C, respectively. The amount of enzyme that liberates 1 µmol fatty acid per minute was defined as one unit (U) of the activity. The concentration of total protein was detected by Bradford method using BSA as standard. DCW was obtained by centrifuging 10-mL samples in a pre-weighed centrifuge tube at 8000×g for 10 min and discarding supernatant and then drying the pellet at 100 °C. The cell-specific activity (Q_p) and mean cell-specific productivity (q_p) were according to the previous method (Gu et al. 2015).

Results and discussion

Codon optimization

Codon usage is a key factor that affects heterologous protein production in *P. pastoris*. In this study, the sequence of native TDL gene was analyzed first by codon optimization software. It was observed that the codon usage between *T. dupontii* and *P. pastoris* was quite different. Some amino acid residues were encoded by codons present in the native gene, which were rarely used in *P. pastoris* such as CGG (Arg), CCG (Pro), CTC (Leu), and TCG (Ser), etc. Consequently, the native TDL gene without signal sequence was optimized according to the codon usage of *P. pastoris* (Coghlan and Wolfe. 2000; Zhao et al. 2000). The GC content was adjusted from 55.4- to 50.2%, which is close to the appropriate range for optimal expression in yeast (Marín et al. 2003). Totally, 183 nucleotides were optimized in native TDL gene, and the optimized gene (*tdl-opt*) showed 77.8% identity to the native gene (supplemental Fig. 2).

Transformation and isolation

The expression vector pPICZαA-*tdl-opt* was linearized and transformed into *P. pastoris* X33. A total of 216 recombinant colonies were screened according the method described in supplemental material. Three transformants named X33-T23, X33-T105, and X33-T163 with higher lipase activity were selected for shaking flask fermentation. The recombinant strain X33-T23 with the highest activity (1020 U/mL) after 96 h of methanol induction was followed by X33-T163 (982 U/mL) and X33-T105 (960 U/mL). Meanwhile, the gene copy numbers of X33-T23 were determined by qPCR. The results of qPCR revealed that the gene copy number of X33-T23 was three copies. The recombinant strain X33-T23 was selected for further study.

Effects of secretion helper factors on TDL production

Co-expression of secretion helper factors is an effective method to improve the production of heterologous protein in *P. pastoris*. Generally, the protein secretion pathway in *P. pastoris* involves protein translocation across the ER membrane, protein folding in ER, transport from ER to Golgi, etc (Delic et al. 2013). Secretion helper factors involved in protein secretion can be divided into several modules such as protein folding, stress responses, translocation, endoplasmic reticulum-associated protein degradation (ERAD), vesicular trafficking, etc (Idiris et al. 2010). In this study, nine secretion helper factors from different modules were chosen to investigate their effects on the production of TDL (Table 1). The recombinant strain X33-T23 was used as host and transformed with these 9 secretion helper factors genes. For initial screening, at least 96 recombinant colonies of each single secretion helper factor co-expression were selected and analyzed. The transformants with higher activity were isolated for shake flask fermentation. The mean relative lipase activities of recombinant strains' co-expression with

different single secretion helper factor were mostly distributed from 99 to 157% compared to the recombinant strain X33-T23 (Fig. 1). The *PDI* transformant exhibited the highest activity (1760 U/mL), followed by *KAR2* (1568 U/mL), *HAC1* (1512 U/mL), and *ERO1* transformant (1400 U/mL). The *PDI*, *KAR2*, *ERO1*, and *HAC1* transformants (named X33-T23-*PDI*, X33-T23-*KAR2*, X33-T23-*ERO1*, and X33-T23-*HAC1*) were further analyzed in 5 L bioreactor, and X33-T23 was used as control.

The lipase activity, protein concentration of TDL in supernatant, and DCW of recombinant strains in 5 L bioreactor are presented in Table 2. The maximum lipase activity of X33-T23-*PDI* in 5 L bioreactor reached 57,521 U/mL after 168 h of induction. Compared with the shaking flasks procedure, a 32.7-fold increase in the volumetric lipase activity was obtained (from 1760 to 57,521 U/mL). For high cell density fermentation, the fermentation parameters (such as temperature, pH, dissolved oxygen, etc.) of bioreactor are easier to control than shaking flasks, which is helpful for the growth of recombinant strain and production of recombinant protein. The DCW and protein concentration of TDL in culture supernatant of X33-T23-*PDI* in 5 L bioreactor reached 157 and 6.99 g/L, respectively. As seen from supplemental Fig. 3, at shaking flasks level, the DCW and protein concentration of TDL in culture supernatant were only 15 and 0.21 g/L, respectively. Compared with fed-batch fermentation, the relatively scarce supply of 0.75% methanol every 24 h may be the reason for relatively low productivity in shake flasks.

In this study, the highest DCW of recombinant strain X33-T23-*PDI* reached 157 g/L. We speculate that there may be two reasons for this. First, co-expression of the molecular

Table 1 Secretion helper factor used in this study

Secretion helper factor	Locus ID ^a	Module
<i>PDI</i>	CAC33588.1	Protein folding
<i>HAC1</i>	XP_002490039.1	Stress responses
<i>KAR2</i>	XP_002491027.1	Protein folding
<i>ERO1</i>	XP_002489645.1	Protein folding
<i>CNE1</i>	XP_002491218.1	Protein folding
<i>YDJ1</i>	XP_002492708.1	Vesicular trafficking
<i>UBC1</i>	XP_002493859.1	ERAD
<i>HRD1</i>	XP_002493560.1	ERAD
<i>SSA1</i>	XP_002492443.1	Vesicular trafficking

^aThe locus ID is from National center for biotechnology information

Fig. 1 Effects of different secretion helper factor on the production of TDL at shake flask fermentation. The abbreviation CK is the recombinant strain X33-T23. *ERO1*, *CNE1*, *PDI*, *YDJ1*, *HAC1*, *HRD1*, *SSA1*, *UBC1*, or *KAR2* represents the recombinant strain X33-T23 co-expression with corresponding gene. Measurements are the average value $\pm$ standard error from independent triplicate fermentations

Table 2 Comparison of TDL productivity obtained by co-expression of single helper factor in *P. pastoris*

Help factor	Activity (U/mL)	Protein concentration of TDL in supernatant (g/L)	DCW (g/L)	TDL product yield (g/g _{DCW})	Mean Qp (U/g _{DCW} /h)	Mean qp (mg/g _{DCW} /h)
CK	39,479 ± 1488 ^a	4.50 ± 0.18 ^a	120.33 ± 0.82 ^a	0.0375	1952	0.222
<i>PDI</i>	57,521 ± 1154 ^b	6.99 ± 0.16 ^b	157.33 ± 1.21 ^b	0.0444	2180	0.265
<i>KAR2</i>	54,280 ± 1611 ^c	6.26 ± 0.10 ^c	151.00 ± 1.41 ^c	0.0414	2139	0.246
<i>HAC1</i>	54,234 ± 1395 ^c	6.38 ± 0.10 ^c	155.50 ± 1.05 ^d	0.0410	2082	0.245
<i>ERO1</i>	48,156 ± 1103 ^d	5.68 ± 0.09 ^d	136.50 ± 1.05 ^e	0.0416	2107	0.248

Data are mean ± standard deviation of results from independent duplicate fermentations. Values with different letters (a–e) show significant differences ($p < 0.05$) between samples for each analysis

chaperones could enhance the growth rate of recombinant strain X33-T23 which is similar to previous studies. Li et al. found that co-expression of *KAR2* and *ERO1* improved the wet cell weight of recombinant strain from 396 to 452 g/L (Li et al. 2016). Gu et al. also reported that the DCW of recombinant strain was improved by co-expression with molecular chaperone genes (Gu et al. 2015). Second, the long cultivation time (23 h glycerol and 168 h methanol induction fed-batch phase) may be the other reason for higher DCW.

X33-T23-*PDI* also exhibited the highest lipase activity in 5 L bioreactor (Table 2). Pdip, belonging to the protein folding module, plays an important role in formation and rearrangement of disulfide bonds in newly folding proteins (Delic et al. 2014). TDL was found to have three disulfide bonds by SWISS-MODEL analysis. Therefore, overexpression of *PDI* may help in correct formation of disulfide bonds during the process of TDL folding in ER, which results in improving the production of recombinant TDL. This result was similar with previous studies. The production of recombinant lipase r27RCL and lipase MAS1 in *P. pastoris* was improved by co-expression with *PDI* (Sha et al. 2013a, b; Lan et al. 2016). As a multifunctional molecular chaperone, Kar2p is a member of the heat shock protein 70 kDa (Hsp70) family, which is involved in translocation, folding, ERAD, and UPR (Yu et al. 2015). Previous studies found that overexpression of *KAR2* shows different effects on the production of heterologous protein in *P. pastoris*. For instance, the secretions of 2F5 Fab fragment and A33 single-chain antibody fragment were enhanced 1.5- and threefold by overexpression of *KAR2* (Gasser et al. 2007; Damasceno et al. 2007). However, co-expression with *KAR2* decreased expression of CALB in *P. pastoris* (Samuel et al. 2013). In this study, co-expression with *KAR2* increased TDL activity from 39,479 to 54,280 U/mL (Table 2). The mean Qp increased from 1952 to 2139 U/g_{DCW}/h, and the mean qp increased from 0.222 to 0.246 mg/g_{DCW}/h. Generally, high-level expression of heterologous protein in *P. pastoris* is prone to produce unfolded protein in ER, which could lead to the UPR and

affect the production of recombinant protein. Hac1p is a transcription factor of the UPR pathway, which could be used to improve the secretion of heterologous protein in *P. pastoris* (Guerfal et al. 2010). As shown in Table 2, co-expression with *HAC1* resulted in an increase in lipase activity (from 39,479 to 54,234 U/mL) and protein concentration of TDL in supernatant (from 4.50 to 6.38 g/L). The mean Qp and qp were 2082 U/g_{DCW}/h and 0.245 mg/g_{DCW}/h, respectively. As a flavoenzyme, Ero1p is a specific oxidant of Pdip which directly interacts with Pdip. As seen from Table 2, compared with the strain X33-T23, the mean Qp increased from 1952 to 2107 U/g_{DCW}/h, and the mean qp increased from 0.222 to 0.248 mg/g_{DCW}/h. Overexpression of *ERO1* led to a significant improvement of HAS/GH fusion protein in *P. pastoris* (Wu et al. 2014).

Compared to the above chaperones, Ssa1p, Ydj1p, Cne1p, Ubc1p, and Hrd1p had little influence on the production of TDL. Ssa1p, as a cytosolic chaperone, is responsible for protein trafficking from ribosome to the ER. Ydj1p belongs to hsp40 protein family which shows synergy with Ssa1p for protein translocation. In previous study, overexpression of *SSA1* and *YDJ1* could improve the production of CALB and GOD in *P. pastoris* (Samuel et al. 2013; Gu et al. 2015). In this study, co-expression of *SSA1* or *YDJ1* had little effect on the production of TDL (Fig. 1). Cne1p is a membrane-bound lectin-chaperone in ER which plays an important role in calnexin cycle. Klabunde et al. reported a twofold increase of fungal phytase in a *CNE* co-expression *Hansenula polymorpha* strain (Klabunde et al. 2007). Gu and his coworker found that the activity of GOD increased from 322.9 to 779.3 U/mL by overexpression of *CNE* in *P. pastoris* (Gu et al. 2015). Nevertheless, as shown in Fig. 1, the lipase activity of recombinant strain X33-T23-*CNE1* showed only 1.05-fold of the control strain. Hrd1p and Ubc1p are important members of the ERAD which help degrading misfolded proteins in the ER. In this study, when *HRD1* or *UBC1* was co-expressed with TDL, the lipase activities of these recombinant strains were almost the same as the control (Fig. 1).

Fig. 2 Influence of induction temperatures on the lipase activity (a) and protein concentration of TDL in supernatant (b), and DCW (c) in a 5-L bioreactor. Measurements are the average value $\pm$ standard error from independent duplicate fermentations

Optimization of induction temperature and pH in 5 L bioreactor

Temperature is an important factor affecting the recombinant protein production in *P. pastoris* during fermentation process. The effect of induction temperature on the expression of TDL in X33-T23-*PDI* was investigated in a 5-L bioreactor and the induction temperatures were set in the range from 24 to 30 °C. As shown in Fig. 2, lower induction temperature was beneficial for production of TDL. The maximum lipase activity at 30 °C reached 57,012 U/

Fig. 3 Influence of induction pH on the lipase activity (a) and protein concentration of TDL in supernatant (b), and DCW (c) in a 5-L bioreactor. Measurements are the average value $\pm$ standard error from independent duplicate fermentations

mL (Fig. 2a). Changing the temperature to 27 and 24 °C resulted in a 1.19-fold (68,301 U/mL) and 1.30-fold (74,241 U/mL) respective increase in the maximum lipase activity (Fig. 2a). As seen from Fig. 2b, the maximum protein concentration of TDL in culture supernatant at 27 and 24 °C was 8.40 and 9.31 g/L showing 1.2- and 1.33-fold higher values than at 30 °C, respectively (7.01 g/L). These results agreed with previous studies in that lower induction temperature has a positive effect for protein production in

Fig. 4 Large-scale production of TDL in 50 L bioreactor. **a** Lipase activity, protein concentration of TDL in supernatant and DCW during fed-batch fermentation in a 50-L bioreactor; **b** SDS-PAGE of supernatant from different induction time (*M* marker, lane 1–7 represented supernatant from 24 to 168 h); **c** SDS-PAGE of TDL after EndoHf treatment (*M* marker, lane 1: supernatant from 48 h, lane 2 and 3: de-glycosylated TDL and Endo Hf)

P. pastoris. At lower induction temperatures, *P. pastoris* has been proposed to reduce cell death and increase cell viability, which could improve the production of heterologous proteins (Li et al. 2001). Meanwhile, the activity of AOX1 could be improved largely at lower induction temperatures, then strengthen the expression of downstream target gene (Wang et al. 2009). Lowering induction temperature can contribute to relatively high ATP content and total adenine nucleotide level, which increases enzyme production (Chuang et al. 2005). Furthermore, at lower temperatures, the folding stress of *P. pastoris* is decreased which leads to efficient heterologous protein secretion (Dragosits et al. 2009). Zhong and co-workers reported that the folding efficiency of the G3-pro-rhIL10 in the ER at 20 °C was much higher than 30 °C (Zhong et al. 2014). Although higher production of TDL could be obtained below 27 °C, temperatures below 27 °C are not usually acceptable for industrial production. Therefore, the induction temperature was set to 27 °C for TDL production.

Induction pH is also an important factor that affects the cell growth and protein production. In order to evaluate the effects of induction pH on cell growth and TDL production, the recombinant strain X33-T23-*PDI* was cultivated at 27 °C

and the pH was set from 4.0 to 7.0. As shown in Fig. 3a, the maximum lipase activity was 81,203 U/mL at pH 6.0, which was 1.39-, 1.19-, and 1.03-fold higher than at pH 4, 5, and 7, respectively. The highest protein concentration of TDL in supernatant (10.43 g/L) was also obtained at pH 6.0 (Fig. 3b). There were no significant differences on cell growth under different induction pH (Fig. 3c). These results were consistent with previous report showing that optimization of fermentation pH can improve the production of heterologous proteins in *P. pastoris* (Lan et al. 2016).

Large-scale production of TDL in 50 L bioreactor

The recombinant strain X33-T23-*PDI* was further cultivated in a 50-L bioreactor under optimized induction conditions (27 °C and pH 6.0). As shown in Fig. 4a, the maximum lipase activity and protein concentration of TDL in culture supernatant produced by X33-T23-*PDI* reached 81,062 U/mL and 10.23 g/L, respectively. SDS-PAGE showed that there were two forms of recombinant TDL with molecular masses about 38 and 30 kDa (Fig. 4b). TDL was found to have one N-glycosylation site (N⁵⁵VTC) by N-glycosylation site analysis (<http://www.cbs.dtu.dk/>

services/NetNGlyc/). As shown in Fig. 4c, After EndoH_f treatment, there was only a single band about 30 kDa, suggesting that recombinant TDL is a glycoprotein. The recombinant protein TDL accounted for approximately 75% of the total protein in culture supernatant which was estimated by the software quantity one (Bio-Rad). Compared to *T. dupontii*, *P. pastoris* secreted few native proteins which facilitate downstream processing (Fig. 4b).

Conclusions

In this study, we combined codon optimization, co-expression of secretion helper factor genes, and induction temperature and pH optimization as a whole to improve the production of TDL in *P. pastoris*. To our knowledge, this is the first report to improve the production of TDL in *P. pastoris* by this strategy. The result of this study not only identifies an effective strategy for the heterologous expression of TDL in *P. pastoris*, but also casts some insight into the optimization of protein production in the heterologous systems.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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