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ORIGINAL ARTICLE

High-level expression of *Thermomyces dupontii* thermo-alkaline lipase in *Pichia pastoris* under the control of different promoters

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Abstract

In this study, 15 methanol-inducible and 9 constitutive promoters were used to drive the expression of *Thermomyces dupontii* lipase (TDL) in *Pichia pastoris*. Of the 15 methanol-inducible promoters, formaldehyde dehydrogenase promoter (P_{FLD1}) showed the highest efficiency in driving lipase production, followed by alcohol oxidase 1 (P_{AOX1}) and dihydroxyacetone synthase (P_{DAS1}) promoters. The maximum lipase activity of transformants with P_{FLD1}, P_{AOX1} and P_{DAS1} promoters in 5-l bioreactor was 27,076, 24,159 and 22,342 U/ml, respectively. For the nine constitutive promoters, glycosyl phosphatidyl inositol-anchored protein promoter (P_{GCW14}) produced the highest amount of lipases in a medium containing glucose or glycerol as the only carbon source, followed by mitochondrial alcohol dehydrogenase isozyme (P_{0472}) and glyceraldehyde-3-phosphate dehydrogenase (P_{GAP}) promoters. The maximum lipase yields in 5-l bioreactors under the control of P_{GCW14}, P_{0472} and P_{GAP} promoters were 17,353, 15,046 and 14,276 U/ml, respectively. The result of this study not only identifies a few highly efficient promoters for the heterologous expression of TDL in *P. pastoris*, but also casts some insight into the optimization of protein production in heterologous systems.

Keywords Lipase · *Pichia pastoris* · Promoter · *Thermomyces dupontii*

Introduction

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) can be defined as a class of hydrolase. They can hydrolyze long-chain acylglycrols in aqueous medium. Meanwhile, they catalyze synthesis of esters and fine chemicals through direct esterification and transesterification in no-aqueous medium. Nowadays, lipases are widely used in industries of feed additives, detergent, food processing, fine chemicals, and biodiesel production (Jaeger and Eggert 2002; Singh et al. 2016).

The lipase from *Thermomyces dupontii* (formerly classified as *Talaromyces thermophilus*) is a thermo-active and alkaline enzyme (named TDL). Previous studies revealed TDL with great potential values in many 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, the high transesterification activity of TDL is very useful for its application in biodiesel production (Romdhane et al. 2013). Furthermore, TDL can be used as a biocatalyst for the synthesis of chiral intermediate of Pregablin through kinetic resolution of 2-carboxyethyl-3-cyano-5-methylhexanoic acid ethyl ester (CNDE) (Ding et al. 2018). Although TDL shows a potential value for commercial application, the low production of TDL and secretion of TDL with other hydrolytic enzymes by *T. dupontii* are the main hurdles limiting its further application (Romdhane et al. 2012). Heterologous expression of TDL in *P. pastoris* is an effective method

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to overcome these hurdles. Previous study demonstrated that the production of TDL in *P. pastoris* was 260% higher than that of *T. dupontii* at flask shake culture. Furthermore, SDS–PAGE showed that the recombinant TDL was almost free from contaminating proteins, since the recombinant strain secretes few native proteins, which facilitates downstream processing (Zhang et al. 2015).

Promoter plays an important role in the expression of heterologous lipases in *P. pastoris*. Generally, the promoters for the expression of lipases in *P. pastoris* include methanol-inducible and constitutive promoters. Until now, the most commonly used methanol-inducible and constitutive promoters are alcohol oxidase 1 promoter (P_{AOX1}) and glyceraldehyde-3-phosphate dehydrogenase promoter (P_{GAP}), respectively. P_{AOX1} is extensively employed for the expression of lipases in *P. pastoris* (Borrelli and Trono 2015). Compared to P_{AOX1}, P_{GAP} is used to drive some lipase expression (Wang et al. 2012a, b). Besides P_{AOX1} and P_{GAP}, few promoters are employed for the expression of lipase in *P. pastoris*. In this study, TDL is expressed in *P. pastoris* under the control of 15 methanol-inducible and 9 constitutive promoters. The results of this study will provide some suitable promoters for heterologous expression of TDL and lay a foundation for further improvement of this lipase in *P. pastoris*.

Materials and methods

Strains, plasmids and reagents

The *Escherichia coli* strain Top 10 was conserved in our laboratory and used to propagate plasmids. *P. pastoris* X33 was purchased from Invitrogen (Carlsbad, CA, USA) and used as host for the expression of TDL. The vector pPICZαA was also purchased from Invitrogen. DNA polymerase (PrimeSTAR^{TMHS}), restriction enzymes (EcoRI, NotI and SacI), infusion cloning kit and T_4-DNA ligase were purchased from Takara Biotechnology (Dalian, China). The gene of TDL (GenBank: JF414585.1) without signal sequence was optimized according to the preference of *P. pastoris* and synthesized by the Genewiz (Suzhou, China). Sodium hydroxide was purchased from Merck (Darmstadt, Germany).

Medium

Media for *E. coli* Top 10 and *P. pastoris* X33 included LBZ (LB with 25 μg/ml zeocin), YPD (yeast extract peptone dextrose medium), YPDZ (yeast extract peptone dextrose medium with 100 μg/ml zeocin), BMGY (buffered glycerol complex medium), BMMY (buffered methanol complex medium) and BMDY (buffered dextrose complex medium). LBZ, YPDZ, BMGY, BMDY and BMMY were prepared according to the protocol provided by Invitrogen (https://www.thermofisher.com).

Promoter cloning and construction expression vectors

Based on the results of previous studies (De Schutter et al. 2009; Xu et al. 2018), 15 methanol-inducible and 9 constitutive promoters were selected for heterologous expression of TDL. The optimized gene (*tdl-opt*) without the predicted signal sequence was digested by EcoRI and NotI, and ligated into pPICZαA to form pPICZαA–*tdl-opt*, for the expression of TDL under promoter P_{AOX1}. The following 23 expression vectors with different promoters were constructed based on the pPICZαA–*tdl-opt*. The 3.6-kb fragment of pPICZαA–*tdl-opt* without promoter P_{AOX1} was amplified from pPICZαA–*tdl-opt* using the specific primers. And then, the 23 different promoters were ligated to 3.5-kb fragment by in-fusion cloning to form 23 expression vectors with different promoters. All the expression vectors contained the *saccharomyces cerevisiae* α-factor secretion signal downstream of different promoters. The details of these 24 promoters are listed in Table 1. The primers used in this study are listed in supplementary Table 1.

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

The recombinant *E. coli* Top 10 containing different expression plasmids were grown in LBZ at 37 °C and 220 rpm for 18 h. The expression plasmids were isolated and purified using TIANprep Midi Plasmid Kit (TIANGEN, Beijing, China). The expression vectors with different promoters were linearized with SacI and the concentration of SacI-linearized plasmids were analyzed by NanoDrop 2000 (Thermo Scientific). 80 ng SacI-linearized plasmids were transformed into *P. pastoris* X33 competent cell. The transformants were plated and screened on YPDZ plates. The detailed protocol for the selection of recombinant strains is provided in supplementary material. The gene copy numbers of recombinant strain with different promoters were detected by Quantitative real-time PCR (qPCR). The single-copied recombinant strains were isolated for shake flask fermentation.

Shake flask cultures

For the expression of TDL under methanol-inducible promoters, the single-copied recombinant strains were inoculated into 10 ml BMGY in a 150-ml flask 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 250-ml flask, incubated at 30 °C and 200 rpm. 0.75% (v/v) methanol

Table 1 List of selected promoters for the heterologous expression of TDL

Promoters	Corresponding genes	Locus ID
P_{AOX1}	Alcohol oxidase 1	PAS_chr4_0821
P_{AOX2}	Alcohol oxidase 2	PAS_chr4_0152
P_{DAS1}	Dihydroxyacetone synthase 1	FJ752551.1
P_{DAS2}	Dihydroxyacetone synthase 2	FJ752552.1
P_{FLD1}	Formaldehyde dehydrogenase	AF066054.1
P_{TPI}	Triose phosphate isomerase	PAS_chr3_0951
P_{CAT}	Catalase A	PAS_chr2-2_0131
P_{FDH}	$NAD^{(+)}$-dependent formate dehydrogenase	PAS_chr3_0932
P_{FBP}	Fructose 1,6-bisphosphatase	PAS_chr3_0868
P_{FBA2}	Fructose 1,6-bisphosphate aldolase	PAS_chr1-1_0319
P_{0547}	Peroxiredoxin	PAS_chr1-4_0547
P_{0043}	Mitochondrial aldehyde dehydrogenase	PAS_chr4_0043
P_{FGH}	Non-essential intracellular esterase	PAS_chr3_0867
P_{0374}	Hypothetical protein	PAS_chr3_0374
P_{0099}	Mitochondrial NAD^+ transporter	PAS_chr3_0099
P_{GAP}	Glyceraldehyde 3-phosphate dehydrogenase	PAS_chr2-1_0437
P_{GCW14}	Glycosyl phosphatidyl inositol-anchored protein	PAS_chr1-4_0586
P_{0472}	Mitochondrial alcohol dehydrogenase isozyme	PAS_chr2-1_0472
P_{0769}	Pyruvate kinase	PAS_chr2-1_0769
P_{0188}	Pyruvate decarboxylase isozymes	PAS_chr3_0188
P_{0090}	Polyamine transport protein specific for spermine	PAS_chr1-4_0090
P_{0065}	Plasma membrane multidrug transporter of the major facilitator superfamily	PAS_chr2-2_0065
P_{0016}	2-Deoxyglucose-6-phosphate phosphatase	PAS_chr2-1_0016
P_{TEF}	Translation elongation factor 1	M10992.1

was added to the culture at every 24 h, and 0.5 ml culture was harvested every 24 h for lipase and biomass concentration assay. The recombinant strains showing higher lipase activity were isolated for high cell density fermentation.

For the expression of TDL under constitutive promoters, the single-copied recombinant strains were also inoculated into 10 ml YPD in a 150-ml flask and cultured overnight at 30 °C and 200 rpm. 1 ml of overnight culture was transferred to 250-ml flask containing 50 ml BMGY or BMDY, and incubated at 30 °C and 200 rpm for 72 h. 1 ml culture was harvested every 24 h for lipase and biomass concentration assay. The recombinant strains with higher lipase activity were isolated for high cell density fermentation.

High cell density fermentation

High cell density fermentation was carried out in 5-l bioreactor. The detailed protocol of high cell density fermentation is provided in supplementary material. For the expression of TDL under methanol-inducible promoters, the recombinant strains were cultivated for about 23 h in batch on glucose medium. When the glucose was exhausted, it was then changed to methanol induction phase. For the expression of TDL under constitutive promoters, the recombinant strains were cultivated on basal salt medium and using glucose or glycerol as sole carbon source. The enzyme activity, total protein concentration and dry cell weight (DCW) were monitored throughout the 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 determined according to the previous method (Wang et al. 2013). The qPCR experiments were performed according to the previous method (Sha et al. 2013). The glyceraldehyde 3-phosphate dehydrogenase gene (GAP) was selected as the reference gene.

Results

Construction of expression vectors with different promoters

15 methanol-inducible and 9 constitutive promoters were selected for the heterologous expression of TDL in *P. pastoris* (Table 1). These 15 methanol-inducible promoters included P_{AOX1}, P_{FLD1} and P_{DAS1}, etc. The nine constitutive promoters included P_{GAP}, P_{GCW14}, mitochondrial alcohol dehydrogenase isozyme promoter (P_{0472}), etc. Those methanol-inducible and constitutive promoters were cloned from *P. pastoris* X33, which were about 800 bp upstream of the start codon ATG. The process for construction of different expression vectors is depicted in Fig. 1.

Heterologous expression of TDL under methanol-inducible promoters

Those 15 expression vectors with different methanol-inducible promoters were linearized and transformed to *P. pastoris* X33. The recombinant strain with classic promoter P_{AOX1} was used as control. Due to a small amount (80 ng) of linearized plasmids were transformed into *P. pastoris* X33, only two to four clones were isolated for each promoter. The mean relative lipase activity of recombinant strains with different methanol-inducible promoters are mostly distributed from 4.8 to 120.3% compared to the recombinant strain with promoter P_{AOX1} (Fig. 2). Meanwhile, the gene copy numbers of recombinant strains with different methanol-inducible promoters were determined by qPCR. The results of qPCR showed that these recombinant strains were single-copied clone. The lipase activities of recombinant strain using P_{AOX1} (named X33–P_{AOX1}–*tdl*), P_{DAS1} (named X33–P_{DAS1}–*tdl*) and P_{FLD1} (named X33–P_{FLD1}–*tdl*) were much higher than other promoters (Fig. 2). So X33–P_{AOX1}–*tdl*, X33–P_{DAS1}–*tdl* and X33–P_{FLD1}–*tdl* were isolated for shake flask fermentation. After 120 h methanol induction, the maximum lipase activity of X33–P_{AOX1}–*tdl*, X33–P_{DAS1}–*tdl* and X33–P_{FLD1}–*tdl* were 542, 602 and 585 U/ml, respectively (supplementary Fig. 1). The OD_{600} values of these three recombinant strains were similar during the process of cultivation.

Furthermore, X33–P_{AOX1}–*tdl*, X33–P_{DAS1}–*tdl* and X33–P_{FLD1}–*tdl* were cultivated in 5-l bioreactor. The lipase activity, total protein concentration and DCW of these three recombinant strains are shown in Fig. 3. X33–P_{FLD1}–*tdl* also exhibited the highest activity among these three recombinants in 5-l bioreactor. The maximum lipase activity of X33–P_{FLD1}–*tdl* reached 27,076 U/ml after 168 h induction, which was 1.11- and 1.21-fold higher than those of the recombinant strains X33–P_{AOX1}–*tdl* and X33–P_{DAS1}–*tdl*, respectively (Fig. 3a). The maximum total protein concentration of X33–P_{AOX1}–*tdl*, X33–P_{DAS1}–*tdl* and X33–P_{FLD1}–*tdl* were 3.65, 3.35, and 4.63 g/l, respectively (Fig. 3b). The maximum DCW of X33–P_{FLD1}–*tdl*, X33–P_{AOX1}–*tdl* and X33–P_{DAS1}–*tdl* were 147, 146 and 141 g/l, respectively (Fig. 3c).

The heterologous expression of TDL under constitutive promoters

Those nine constitutive expression vectors were transformed to *P. pastoris* X33 by electro-transformation. The recombinant strains were cultured in YPD medium and lipase activity was determined after 36 h cultivation at 30 °C and 200 rpm. The recombinant strain with P_{GCW14} showed the highest lipase activity, followed by P_{0472} and P_{GAP} transformant (Fig. 4). Meanwhile, the results of qPCR revealed that these recombinant strains were single-copied clone. The P_{GCW14}, P_{0472} and P_{GAP} transformant (named X33–P_{GAP}–*tdl*, X33–P_{GCW14}–*tdl* and X33–P_{0472}–*tdl*) were selected for shake flask fermentation. These three recombinant strains were cultured in BMGY or BMDY medium for 72 h at 30 °C and 200 rpm. The maximum lipase activity of X33–P_{GAP}–*tdl*, X33–P_{GCW14}–*tdl* and X33–P_{0472}–*tdl* in BMGY medium were 241, 251 and 265 U/ml, respectively. The maximum lipase activities of these three recombinant strains in BMDY medium were 261, 273 and 286 U/ml, respectively (supplementary Fig. 2).

The expression level of TDL under P_{GAP}, P_{GCW14} and P_{0472} was further evaluated in 5-l bioreactor. As seen in Fig. 5, the maximum lipase activity and total protein concentration produced by X33–P_{GCW14}–*tdl* in medium using glucose as sole carbon source reached 16,569 U/ml and 2.71 g/l, respectively. Compared with X33–P_{GAP}–*tdl*, the maximum lipase activity and total protein concentration were increased by 18.8 and 24.3%, respectively. Meanwhile, the maximum lipase activity and total protein concentration of TDL driven by P_{GCW14} were 1.18- and 1.23-folds higher than that by P_{0472}. The DCW of X33–P_{GAP}–*tdl*, X33–P_{GCW14}–*tdl* and X33–P_{0472}–*tdl* were almost same during the high cell density fermentation (Fig. 5c). The lipase activity, total protein concentration and DCW of these three recombinant strains in medium using glycerol as sole carbon source are depicted in Fig. 5. The maximum lipase activity of X33–P_{GCW14}–*tdl* was 17,353 U/ml, which was 115.3 and 121.5% to that of X33–P_{0472}–*tdl* and X33–P_{GAP}–*tdl*,

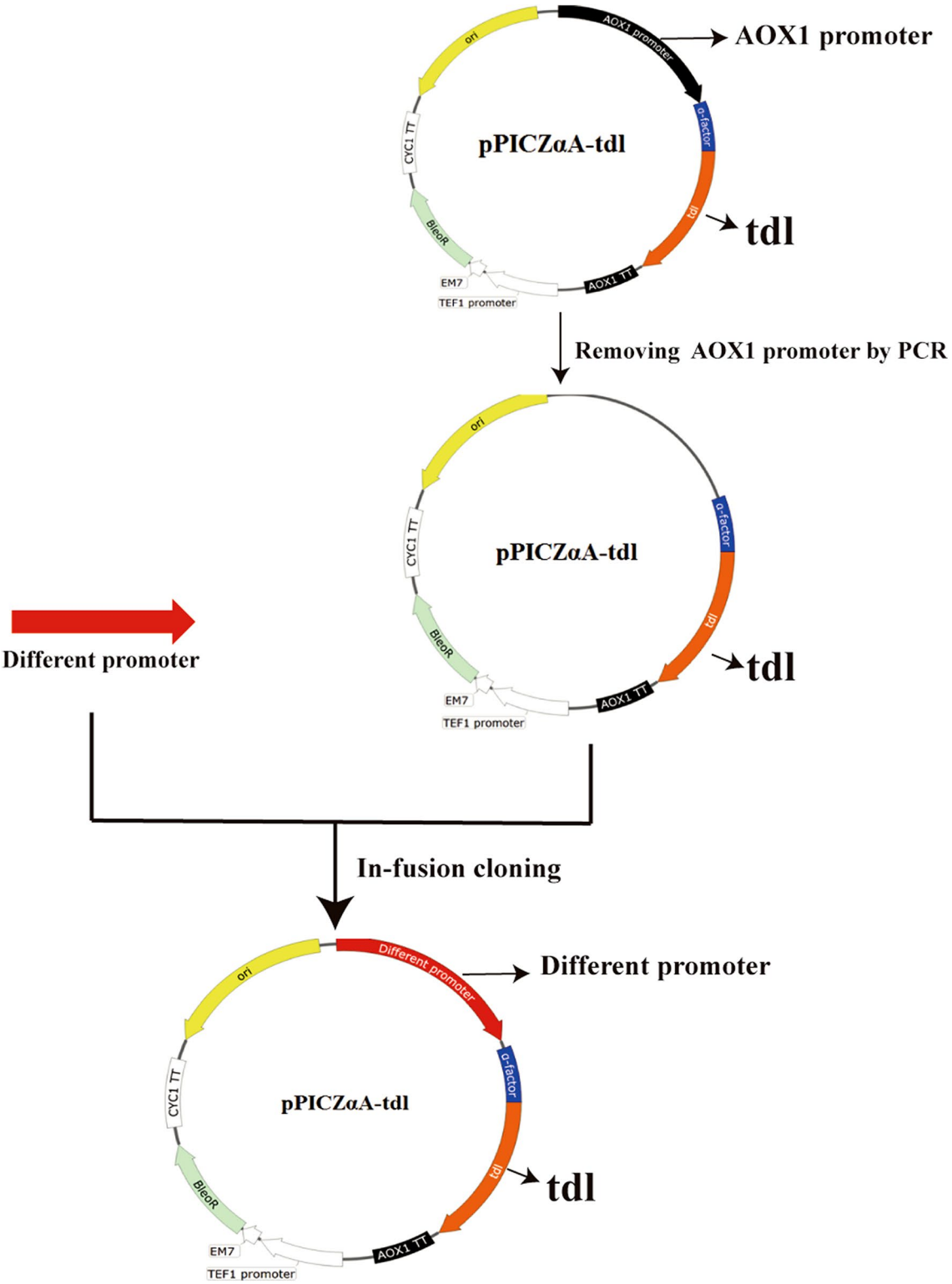

Fig. 1 Construction of the recombinant vectors containing different promoters

respectively (Fig. 5a). The maximum total protein concentration of X33–P_{GCW14}–*tdl* reached 3.12 g/l at 120 h, which was 120.4 and 126.3% to that of X33–P_{0472}–*tdl* and X33–P_{GAP}–*tdl*, respectively (Fig. 5b). The maximum DCW of X33–P_{GAP}–*tdl*, X33–P_{GCW14}–*tdl* and X33–P_{0472}–*tdl* was 148, 145 and 147 g/l, respectively (Fig. 5c).

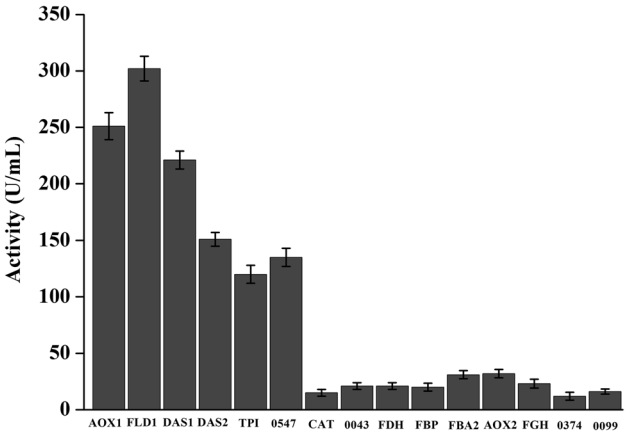

Fig. 2 Lipase activities of the transformant representatives controlled by different promoters

Discussion

As a thermo-active and alkaline lipase, TDL shows a great potential value in many industrial applications. The production of TDL from the wild-type strain *T. dupontii* is less than satisfactory, which limits its further applications. For commercial exploitation of TDL, it is crucial to achieve high yield of this protein. Previous study demonstrated that heterologous expression of TDL in *P. pastoris* was an effective method to improve the production of TDL (Zhang et al. 2015). As one of the most commonly used expression systems, *P. pastoris* has been used extensively for heterologous protein production. *P. pastoris* has many advantages such as powerful secretion ability, ease of genetic manipulation, and mature fermentation process (Ahmad et al. 2014; Li et al. 2017).

Promoter is a key factor for heterologous lipase production in *P. pastoris* (Vogl and Glieder 2013; Arruda et al. 2016). Until now, P_{AOX1} is the most commonly used promoter for the efficient expression of heterologous lipase. Besides P_{AOX1}, there are few methanol promoters which have been used to drive the expression of lipase. In this study, TDL was expressed under the control of 15 methanol promoters. To circumvent gene copy number on the production of TDL, only single-copied clone was selected for study. X33–P_{FLD1}–*tdl* showed the highest lipase activity at flask shake (602 U/ml) and 5-l fermentor (27,076 U/ml) among all the 15 promoter transformants, followed by X33–P_{AOX1}–*tdl* and X33–P_{DAS1}–*tdl*. Alcohol oxidase 1 (AOX1), formaldehyde dehydrogenase (FLD1) and dihydroxyacetone synthase (DAS) are key enzymes of the methanol utilization pathway. These genes are strongly induced when methanol is only carbon source (Yurimoto et al. 2011). The lipase activity from P_{FLD1} was 1.06-fold higher than P_{AOX1} which agreed with previous research

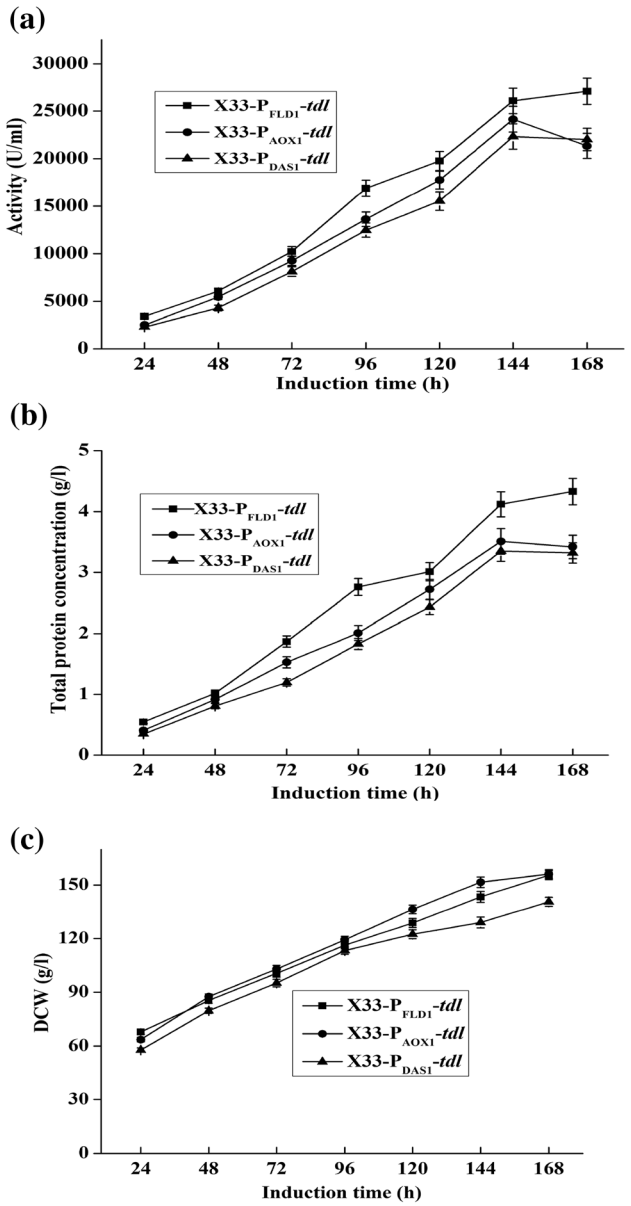

Fig. 3 Lipase activity (**a**), total protein concentration (**b**) and DCW (**c**) of X33–P_{FLD1}–*tdl*, X33–P_{AOX1}–*tdl* and X33–P_{DAS1}–*tdl* in 5-l bioreactor. The lipase activity was detected by pH-stat method (pH 9.5 and 60 °C) using olive oil as substrate. Total protein concentration was detected by Bradford method. DCW was obtained by centrifuging 10 ml samples in a pre-weighted 20 ml centrifuge tube and discarding supernatant, and then allowing the pellet to constant weight at 100 °C. Measurements are the average value ± standard error from independently duplicate fermentations

that the expression level of *Yarrowia lipolytica* lipase 2 under control of P_{FLD1} was comparable with P_{AOX1} (Wang et al. 2012a, b). Meanwhile, the production of TDL under P_{DAS1} was 22,342 U/ml, which was 90.9% of P_{AOX1}. These results indicate that P_{FLD1} and P_{DAS1} are alternative methanol-inducible promoters to P_{AOX1} for the TDL production in *P. pastoris*.

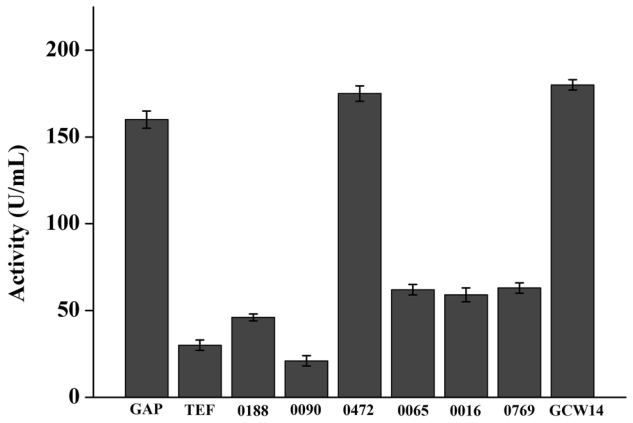

Fig. 4 Lipase activities of different constitutive promoter transformants during initial isolation

Expression under the control of constitutive promoters is also an effective method to improve the production of TDL. Compared to methanol-inducible promoters, constitutive promoters have several advantages such as omits the use of methanol as inducer, simplifies cultivation efforts, and shortens fermentation time (Vogl and Glieder 2013). In the present study, nine constitutive promoters were chosen for the expression of TDL based on literature data. These nine promoters were tested on glycerol and glucose as carbon sources. P_{GCW14} transformant showed the highest lipase activity among all test promoters, followed by P_{0472} and P_{GAP} transformant, when glucose is only carbon source. The change to glycerol as only carbon source, P_{GCW14} transformant also exhibited the highest lipase activity. These results are similar to previous studies, the expression level of EGFP under control of P_{GCW14} was ten and five times higher than P_{GAP} when carbon sources were glucose and glycerol, respectively (Liang et al. 2013). Previous study showed that the maximum amylase activity from P_{0472} recombinant strain was 1.25-fold higher than P_{GAP} strain during fed batch cultivation at 5-l bioreactor (Xu et al. 2018). These results suggest that P_{GCW14} and P_{0472} are an attractive alternative to P_{GAP} for the expression of TDL in *P. pastoris*.

The production of TDL under the control of other promoters was lower than above methanol-inducible (P_{FLD1}, P_{DAS1} and P_{AOX1}) and constitutive promoters (P_{GAP}, P_{GCW14} and P_{0472}), which means they are not the ideal promoters for the heterologous expression of TDL. However, these different promoters will become a toolbox in our further research. The effects of co-expression of chaperones on the production of TDL in *P. pastoris* will be investigated in our further study. Different chaperones will be expressed under the control of these promoters. The results of this study demonstrated that P_{FLD1}, P_{DAS1}, P_{GCW14} and P_{0472} were an attractive alternative to classic promoter P_{AOX1} and P_{GAP} for the expression of TDL in *P. pastoris*. These

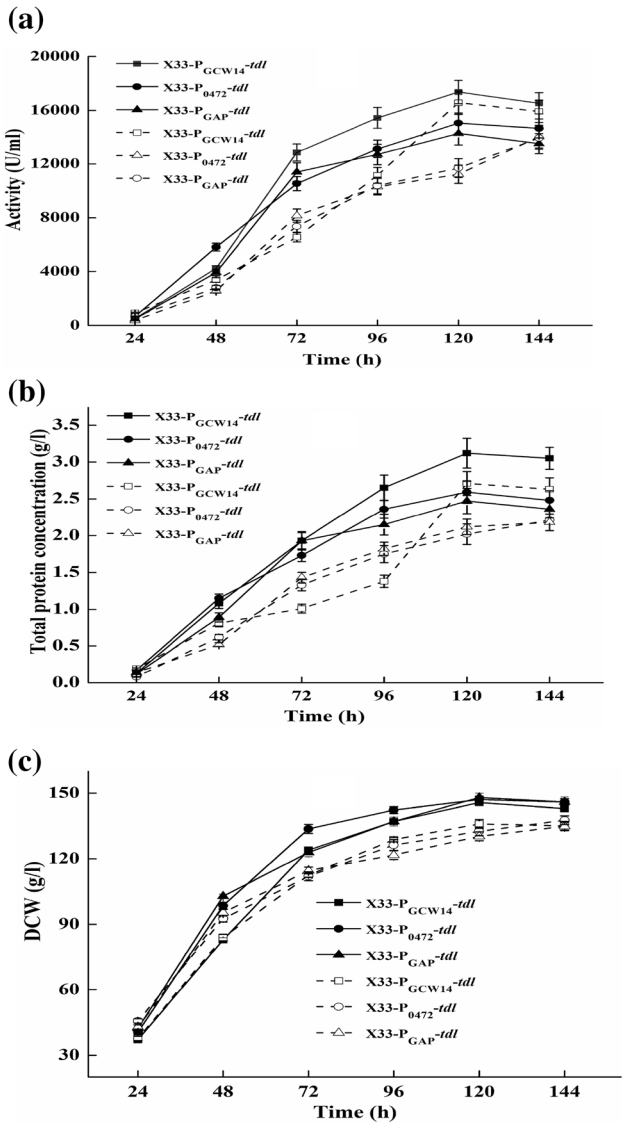

Fig. 5 Lipase activity (**a**), total protein concentration (**b**) and DCW (**c**) of X33–P_{GCW14}–*tdl*, X33–P_{0472}–*tdl* and X33–P_{GAP}–*tdl* in 5-l bioreactor. Solid line represents glycerol as sole carbon source and dashed line represents the glucose as sole carbon source. Measurements are the average value ± standard error from independently duplicate fermentations

24 promoters will lay a foundation for our further study and also give some clues for the heterologous expression of recombinant protein in *P. pastoris*.

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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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