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Analysis of a thermostable lipase from CTG-clade yeast: Molecular expression, characterization and structure prediction

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ABSTRACT

Aims: This study was aimed to express *Meyerozyma guilliermondii* strain RT lipase using *Komagataella phaffii* X-33 expression system and its biochemical characterization and analyse the predicted structure of the product.
Methodology and results: *Meyerozyma guilliermondii* strain RT obtained from the previous study was used as the source of RT lipase gene. Extracellular *M. guilliermondii* strain RT lipase expression has significantly been improved up to 56 U/mg at 24 h cultivation in Yeast extract-Peptone-Dextrose (YPD) medium containing (in w/v): 1% yeast extract, 2% peptone, 2% dextrose with 0.5% v/v methanol induction. Characterization of RT lipase showed optimum activity at 45 °C and pH 9. It exhibited stability in the alkaline pH range (8 to 10) and retained 50% of its residual activity at 30 °C for 30 min. Substrate specificity analysis revealed that it preferred short to medium-chain triacylglycerols (C2-C12) with the highest activity towards caprylic acid (C8). Pairwise alignment revealed three substitutions (S2L, S92L and S193L) present in non-CTG-clade hosts (*K. phaffii*). Homology modelling (YASARA) was used to predict the structures of RT lipase [wild type (wt) and recombinant (rc)]. Mutational analysis of the structures showed the differences in loops that might attribute to the reduction of the optimum temperature from 75 °C (wt) to 45 °C (rc).
Conclusion, significance and impact of study: RT lipase was successfully overexpressed extracellularly using *K. phaffii* expression system with 91.8-fold higher specific activity than the native host. The conceptual advances on the importance of codon optimization before expressing a protein from a CTG-clade species in a non-CTG-clade yeast have been highlighted and the effect of the rare codon usage in recombinant protein characteristics has been evident.

Keywords: Characterization, extracellular lipase, *M. guilliermondii*, stability, structure prediction

INTRODUCTION

Lipases (EC 3.1.1.3) are ubiquitous carboxylic ester hydrolases that catalyze the hydrolysis and synthesis of acylglycerols in the presence of water (Sarkar *et al.*, 2012). High global demand for lipase such as from China, Brazil and India enhances the market scope beyond the estimated target (Chandra *et al.*, 2020). Lipases can be obtained from various sources encompassing plants, animals and microorganisms. Microbial lipases are preferred over plant and animal lipases due to their wide range of advantages for industrial applications. Generally, lipases of microbial origins have a significant impact in the biotechnology industry since they can be easily manipulated and have high yield production, rapid growth in cultivation using inexpensive media, as well as a variety of catalytic activities (Hasan *et al.*, 2006). In the detergent industry, there is an elevated demand for microbial lipases as an alternative to chlorine bleach, which causes environmental pollution (Hauthal, 2016). Alkaline lipases have high activity and stability in various parameters, including temperature, pH, metal ions,

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surfactants and oxidants (Wang *et al.*, 1995). Lipases decomposed fatty material, which makes the enzyme capable of removing fatty stains such as fats, butter, salad oil, sauces and the tough stains on collars and cuffs (Hasan *et al.*, 2010). In addition, lipases are widely used in the food industry, especially for dairy products for the hydrolysis of milk fat and to enhance the flavour of cheese by modifying the length of fatty acids (Balcão and Malcata, 1998). Recently, lipases in their powdered forms are more common and dominate the microbial lipase market because of their stability and ease of handling and packaging (Roohi *et al.*, 2017).

The yeast, *M. guilliermondii* (previously known as *Pichia guilliermondii*) has been receiving attention recently because of its potential applications, ranging from biodiesel degradation (Ganapathy *et al.*, 2019) to promising feed lipase using cheese whey (Knob *et al.*, 2020). It can be extensively isolated from various sources, including fruits, arthropods and human blood (Corte *et al.*, 2015) and phylogenomic analysis of its genome locates it as a CTG clade yeast (Santos *et al.*, 2011). *Meyerozyma guilliermondii* strain RT, previously isolated from rotten tomato was found to exhibit thermostable lipase activity when assayed at 75 °C (Oslan *et al*., 2012). The full sequence of RT lipase of this strain showed 100% similarity to a hypothetical protein from *M. guilliermondii* ATCC6260. The lipase shared a common ancestor with the bacterial thermostable lipase family, *Thermomyces lanuginosus* and 55% similarity to triacylglycerol lipase of *Candida albicans*. The highest intracellular RT lipase expression was obtained at 0.61 U/mg, which is far below industrial expectations (Mahyon *et al*., 2018).

There were a few studies reported on the use of *K. phaffii* (previously known as *Pichia pastoris*) to express lipase from CTG-clade yeast (*Candida rugosa*) (Chang *et al.*, 2011; Kuo *et al.*, 2015). Generally, lipases from the wild-type host are produced at a low level. Therefore, molecular cloning and expression of the recombinant lipase in another expression host are useful for its overproduction. Being a commercial yeast expression system, *K. phaffii* has been widely used to express recombinant lipases from various sources (Cortesini *et al*., 2020). It offers many advantages and outperforms other expression systems as they are easy to handle, less complex, suitable for genetic analysis and produces haploid and diploid cells (Ahmad *et al*., 2014). Thus, cloning and expression of the CTG-clade lipase in *K. phaffii* expression system could facilitate research into the biochemical and structural properties of the enzyme. In addition, the structural study of CTG-clade lipase has not been comprehensively explored to understand its function at the molecular level. Considering the great potential and novelty of the RT lipase, this study sought to highlight the improved expression of RT lipase from a CTG-clade yeast using *K. phaffii* secretory expression system, characterize its biochemical properties and predict its 3D structure.

MATERIALS AND METHODS

Strains and plasmids

Meyerozyma guilliermondii strain RT was obtained from a previous study (Oslan *et al*., 2012). Cultivation was carried out in YPD plate containing (in w/v): 1% yeast extract (Oxoid, UK), 2% peptone (Oxoid, UK), 2% dextrose (Chemiz, MY) and 2% agar (BD, USA) at 30 °C for 3 days. A single colony was then inoculated into 10 mL YPD broth (same composition as YPD agar without agar) and incubated at 30 °C, 200 rpm for 24 h. The pPICZαB expression plasmid and *K. phaffii* strain X-33 (wild-type) were purchased from Invitrogen, USA. *Escherichia coli* TOP10 was used for subcloning and plasmid propagation.

Construction of recombinant plasmid

Isolation of the total RNA from *M. guilliermondii* strain RT was performed using Presto Mini. The extracted RNA (250 ng/µL) was reverse-transcribed using *TransScript*® II One-Step gDNA Removal and cDNA Synthesis SuperMix kit (Transgen, Beijing). For this purpose, 1 µL of RNA template was mixed with 1 µL of transcript enzyme mix, 1 µL of gDNA remover, 1 µL of random primer and 10 µL of the reaction mix. The reaction was carried out at 42 °C for 30 min, followed by 85 °C for 5 sec to stop the reaction. The PCR product was amplified by using PCR master mix (2× PCR Taq MasterMix, USA) and primers flanked with *Pml*I and *Xba*I restriction sites at the 5' and 3' termini as follows (the underlined letters represent the RE recognition sequences): forward *Pml*I: 5'-GATCTTCACGTGATGCTGCAAGTC-3', reverse *Xba*I: 5'-CCTCTAGAGGAAATCCATGTTTAT-3'. The reaction was performed at 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 10 min. The amplified product was purified using the Geneaid PCR Cleanup Kit (Taiwan). Following this, the PCR product and pPICZαB vector was digested with *Pml*I and *Xba*I (Thermo Fisher, USA) and the gel-purified DNA was then ligated using T4 DNA ligase (Thermo Fisher, USA). For ligation, RT lipase (insert) and pPICZαB (vector) at a ratio of 1:6 (vector: insert) were added to the ligation mixture, which consists of 2 µL ligation buffer (ThermoFisher Scientific, USA), 2 µL 50% PEG 4000 solution (ThermoFisher Scientific, USA), 1 µL (5 U/µL) T4 DNA ligase (ThermoFisher Scientific, USA) and distilled water up to a final volume of 20 µL. The mixture was incubated for 14-16 h at 4 °C. Next, the ligated plasmid pPICZαB/RT was transformed into *E. coli* TOP10 cloning host *via* heat shock transformation method described by Sambrook *et al*. (1989). A single colony of the transformant was picked and cultured into 10 mL Luria-Bertani (LB) broth (BD, USA) supplemented with 25 µg/mL zeocin at 37 °C for 18 h with an agitation speed of 200 rpm. PureYield™ Plasmid Miniprep System (Promega, USA) was used to extract the recombinant plasmid from 16-18 h bacterial culture. Then, the recombinant plasmid was validated by performing double digestion, PCR and sequencing analyses.

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Transformation and extracellular expression of RT lipase by *Komagataella phaffi* X-33

The validated recombinant plasmid (pPICZαB/RT) was linearized using *Sac*I (Thermo Fisher, USA) before electroporation (Godiska *et al*., 2009). The transformants were spread onto YPDS-Zeocin agar (similar to YPD agar but with 1 M D-sorbitol and 100 µg/mL zeocin). The plates were incubated at 30 °C for 3 days for the transformants to grow. Then, a few colonies were selected for colony PCR to confirm the positive transformants. Colony PCR was performed using the AOX priming site in pPICZαB plasmid. Another amplification was done to further confirm the positive clones using the forward primer (AOX5fwd: 5'-GAGATCTAACATCCAAAGACG-3') and the insert primer (FRTlipase: 5'-ATGCTGCAAGTCGGTAGA-3') as the reverse primer to determine that RT lipase was successfully cloned into the *K. phaffi* X-33 expression system.

Determination of lipase activity and protein concentration

A single colony of the transformant was inoculated into 10 mL YPD broth and incubated overnight at 30 °C and 200 rpm. Then, 1 mL of the culture was transferred into 100 mL YPTG medium in 500 mL conical flask and shaken for 24 h. The culture was harvested, and the pellet was transferred into 100 mL YPTM medium, supplemented with 0.5% (v/v) methanol. The cells were harvested when the final OD_{600nm}=15 and supernatant was collected for enzymatic assay.

Lipase activity was assayed at 45 °C based on the method by Kwon and Rhee (1986). Briefly, olive oil substrate (Bertolli, Italy) was prepared by emulsification with 50 mM Tris-HCl buffer (pH 9) at a 1:1 ratio (v/v) supplemented with 20 µL of 20 mM $CaCl_2$. Absorbance at 715 nm was measured using a UV-Visible spectrophotometer (Amersham Biosciences, UK). The oleic acid standard curve was constructed. One unit (U) of lipase activity was defined as the rate of fatty acid formation (µmole per minute) at 45 °C. The protein concentration of the sample was determined at 595 nm *via* Bradford assay (1976) using bovine serum albumin (BSA) (Sigma, USA) as the standard.

Effect of temperature on crude lipase activity and stability

The effect of temperature on recombinant RT lipase was assessed at temperatures ranging from 30-65 °C for 30 min in 50 mM Tris-HCl buffer (pH 9). For the measurement of thermostability, RT lipase was incubated at 30-45 °C at an increasing temperature of 5 °C for 30 min. Lipase assay was performed every 5 min interval at 45 °C for 30 min and the relative activity was determined.

Effect of pH on crude RT lipase activity and stability

The effect of pH on the activity of recombinant RT lipase was evaluated at pH 4-12. The respective buffers with different pH were used: 50 mM acetate buffer (pH 4-6), 50 mM sodium phosphate buffer (pH 6-8), 50 mM Tris-HCl buffer (pH 8-9) and 50 mM glycine-NaOH (pH 9-11). For pH stability, 0.02 mg/mL (2 mL) enzyme was incubated at 25 °C for 30 min at various pH ranging from pH 4 to pH 12. A lipase assay was performed, and the relative activity was determined.

Substrate specificity analysis

The substrate specificity of RT lipase was investigated using natural oils and synthetic substrates. For natural oil, palm (MPOB, MY), rice bran (Pure Harvest, MY), corn (Masola, MY), soybean (Masola, MY), sunflower (Masola, MY), coconut (Parachuta, MY) and olive oils (Bertolli, Italy) were used as the substrates. They were firstly emulsified with 50 mM Tris-HCl buffer (pH 9) at a 1:1 ratio (v/v) before being used as substrates for lipase activity assay at 45 °C for 30 min.

For synthetic substrates, *p*-nitrophenol ester was used in the assay based on the method by Becker *et al*. (1997). Triacetin (C2), tributyrin (C4), tricaproin (C6), tricaprylin (C10), triolein (C12), trimyristin (C14) and tripalmitin (C16) were used. The reaction was carried out in 1 mL assay reaction mixture consisting of Tris-HCl buffer (950 µL, 50 mM, pH 9), *p*NP (25 µL, 10 mM in isopropanol) and 25 µL crude enzyme solution. The reaction was carried out at 45 °C for 10 min and the absorbance was measured at 410 nm. One unit (U) of lipase activity was defined as the rate of 1 µmole of fatty acids liberated per minute.

Sequential and structure analyses of wild-type and recombinant RT lipase

Protein structure prediction was performed by subjecting the wild-type (wt) (XP_001482631.1) and recombinant (rc) (EDK41553.2.) RT amino acid sequence for homology modelling. Yet Another Scientific Artificial Reality Application (YASARA) (http://www.yasara.org/) was used to predict the structures. Then, the predicted protein models were evaluated by PROCHECK (https://servicesn.mbi.ucla.edu/PROCHECK/), ERRAT (https://servicesn.mbi.ucla.edu/ERRAT/) and Verify3D (https://servicesn.mbi.ucla.edu/Verify3D/). The active site identification was made by structural alignment of the predicted RT lipase structures with the top template from all of the servers. Next, triacylglycerol lipase from *Burkholderia glumae* (PDB ID: 1TAH) was used to construct the pairwise alignment with the RT lipase (wt). The alignment was made using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The superimposition of the wt and rc RT lipases models was performed using Mustang from YASARA.

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RESULTS

Cloning and expression of RT lipase gene

RT lipase gene from *M. guilliermondii* strain RT was cloned into *K. phaffii* expression system using a strong and tightly regulated *AOX1* promoter. The lipase gene was correctly fused with the α-factor secretion signal and polyhistidine tag (His_6) in the pPICZαB expression vector (Figure S1). The positive clones of recombinant pPICZαB/RT in *K. phaffii* X-33 were successfully verified *via* PCR using AOX5fwd and FRTlipase primers with an expected single band size of 1.5 kb (Figure S2). RT lipase was successfully expressed in a shake flask using a secretory *K. phaffii* X-33 expression system using 0.5% (v/v) methanol induction.

Effect of temperature on recombinant RT lipase activity and stability

To study the effect of temperature on recombinant RT lipase, various assays were performed over a range of temperatures for 30 min. The highest activity of recombinant RT lipase was observed at 45 °C (Figure 1a), which was comparable to the intracellular crude enzymes reported by Mahyon *et al*. (2018). It is noteworthy that the thermostability of the enzyme was reduced from 75 °C (wild-type; wt) to 45 °C. Recombinant RT lipase was most stable at 30 °C by retaining nearly 50% of its residual activity for 15 min of incubation (Figure 1b). At 45 °C, the lipase started to lose its activity for about 80% after only 5 min of incubation.

Effect of pH on lipase activity and stability

Recombinant RT lipase was found to be highly sensitive to pH change and the highest activity was observed in alkaline pH, which is pH 9. The enzyme showed more than 50% of its optimum activity over pH range of 8-10 and had an optimum pH at pH 9 with 50 mM Tris-HCl buffer (Figure 1c). Its activity decreased and almost completely inactivated above pH 9 and at pH below pH 6. Moreover, RT lipase showed high pH stability (>50% activity) at the pH ranging from 7-9 at room temperature (25 °C) (Figure 1d) based on 30-min incubation at various pH ranging from pH 4 to pH 12 before being assayed in 50 mM Tris-HCl buffer (pH 9).

Substrate specificity towards natural oils

The catalytic effect of RT lipase on natural oils was investigated. The relative activity of the lipase was compared to the olive oil assay (Figure 1e). The most effective hydrolysis reaction of RT lipase was observed towards coconut oil with a relative activity of >50% above olive oil. The results showed that RT lipase specifically preferred natural oil that contains medium fatty acid chain length such as coconut oil (C12:0). RT lipase showed moderate hydrolytic activity towards long-chain unsaturated natural oils such as corn oil (C18:2), soybean oil (C18:2) and sunflower oil (C18:2) with relative activities of 195, 177 and 136%, respectively when olive oil (C18:1) was used as a control (100%). RT lipase assayed with rice bran oil which is mainly composed of monounsaturated and polyunsaturated fatty acids (C18:1; C18:2) showed a relative activity of 60%.

Substrate specificity toward *p*-nitrophenol esters

Similar to natural oil, the recombinant RT lipase appeared to prefer short to medium fatty acids (C4-C12) when the synthetic substrate was used (Figure 1f). The enzyme showed the highest activity toward octanoic acid (C8). Butyric acid (C4) was also effectively hydrolyzed by the crude recombinant RT lipase.

In silico structural studies of RT lipase

Primary structural analysis

The active site is responsible for the biocatalytic activity to predict the protein function by a structure-based approach. It is suggested that the active sites of the proteins are more conserved than the overall fold. The catalytic triad of lipases usually consists of a serine nucleophile, a histidine base and an aspartic acid (Skolnick and Fetrow, 2000).

The search of the template for structure prediction showed that RT lipase was structurally closer to bacterial lipases instead of any yeast lipases. These proteins were lipases from *B. glumae* (PDB ID: 1TAH), *P. aeruginosa* (PDB ID: 1EX9) and *B. cepacia* (PDB ID: 2NW6) (Table 1). Therefore, the analysis of the RT lipase structure was pursued by using the bacterial lipases template. The lower E-value indicated higher significance with RT lipase; therefore, lipase from *B. glumae* (PDB ID: 1TAH) was used for the identification of the active site in RT lipase.

The position of the catalytic triad of *B. glumae* lipase was identified as Ser126, Asp302 and His324. Figure 3 shows the pairwise sequence alignment of the RT lipase wt and *B. glumae* lipase, where the catalytic triad of RT lipase was determined accordingly. The active sites for RT lipase were identified as Ser175, Asp289 and His311 (Figure 2).

Structures of both wt and rc RT lipase were built, having lower identities than their respective templates. An identity with more than 50% produces models with features that are correctly predicted (Tramontano, 1998). Both sequences only shared 29% identity with the templates, resulting in less accurate models. The final model structures were predicted by YASARA. Both predicted models are shown in Figure 4 and the folding of the structures is different from one another.

Validation of the predicted structures was done using PROCHECK, ERRAT and Verify3D. The predicted structures from YASARA were labelled as wt (wild type) and rc (recombinant). Ramachandran plot analysis (PROCHECK) showed both wt and rc had 86.8% and 87.2% of the amino acids present in the most favoured

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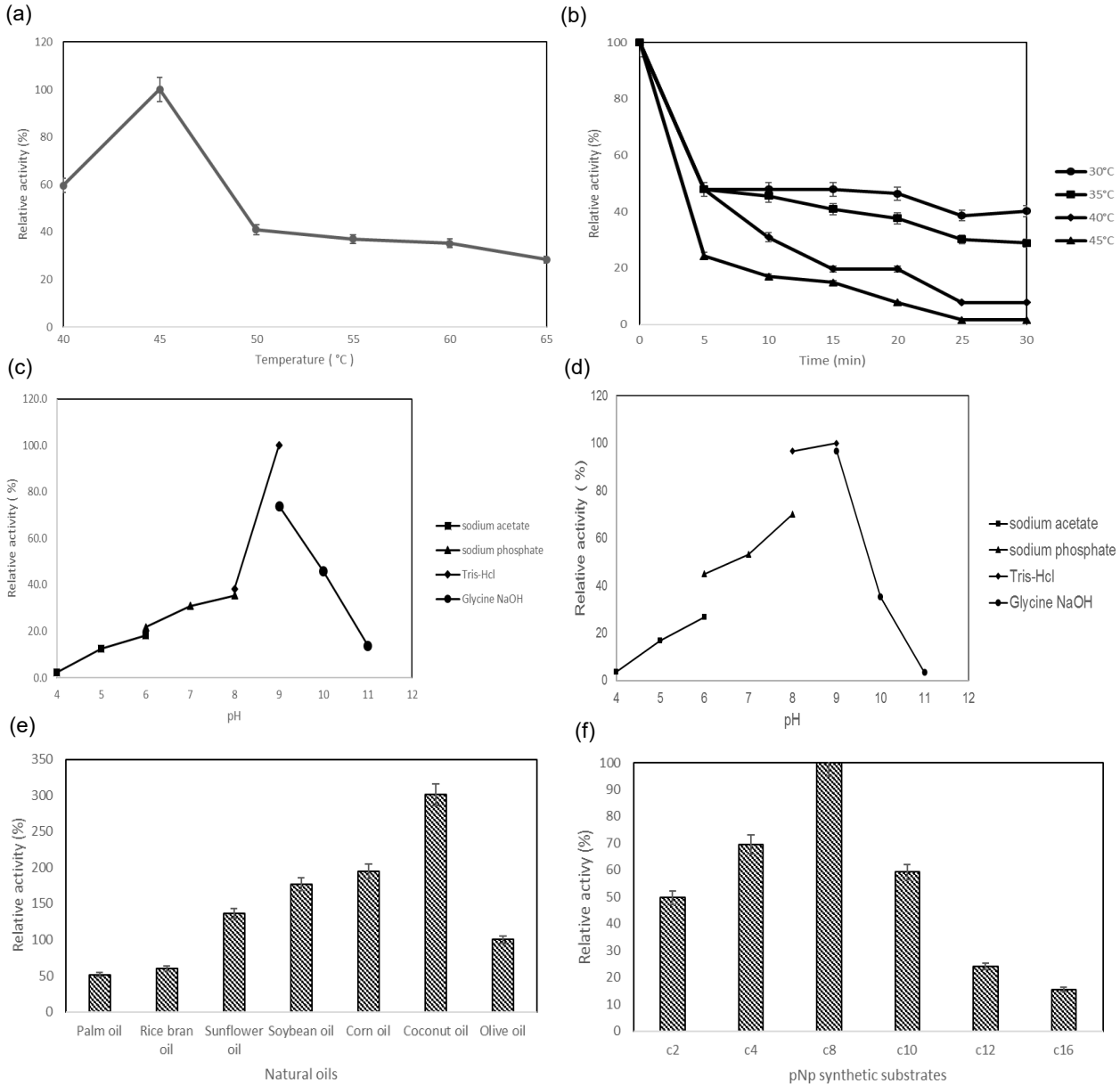

Figure 1: Characterization of recombinant RT lipase. (a) The temperature profile of crude RT lipase. RT lipase was assayed with olive oil as substrate in temperatures ranging from 30 °C to 65 °C at pH 9 for 30 min. (b) Thermostability profile of RT lipase. RT lipase was incubated at different temperatures in 50mM Tris-HCl pH 9 and the activity was assayed at a respective time interval. (c) pH profile of RT lipase. RT lipase was assayed at various pH ranges from pH 4 to pH 11 for 30 min. (d) pH stability of RT lipase. RT lipase was incubated at room temperature 25 °C for 30 min at various pH ranging from pH 4 to pH 11 and assayed on olive oil at pH 9 for 30 min. (e) Substrate specificity towards natural oils. RT lipase was assayed with a different type of natural oils as a substrate for 30 min. (f) Specificity towards *p*-nitrophenol esters. RT lipase was assayed with a different type of pNp synthetic substrates for 10 min.

Table 1: Lists of proteins that were used for multiple sequence alignment and their catalytic triad position.

PDB ID	Template	Similarity (%)	E-value
1TAH	*Burkholderia glumae*	29	2e-054
1EX9	*Pseudomonas aeruginosa*	29	5e-053
2NW6	*Burkholderia cepacian*	29	8e-053

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RTlipase_WT      MSQVGRRRLGNWLTYSGFQRLYSSSTKELVITDKFSTLRPEYKSPRYPIVLCHGFSGFDK   60
RTlipase_RC      MLQVGRRRLGNWLTYSGFQRLYSSSTKELVITDKFSTLRPEYKSPRYPIVLCHGFSGFDK   60
                 * ************************************************************

RTlipase_WT      LAFPKLTDVLNQQKKLESAVEATVDKLSSPLVSLDYWMGIREALERIGSTVLIAKVPAFG   120
RTlipase_RC      LAFPKLTDVLNQQKKLESAVEATVDKLSSPLVLLDYWMGIREALERIGSTVLIAKVPAFG   120
                 ******************************** ************************

RTlipase_WT      TIKERAESLNEFIERQCKQLRKKESKASIYDTGSADSRSFKHINERIKVNLVAHSMGGLD   180
RTlipase_RC      TIKERAESLNEFIERQCKQLRKKESKASIYDTGSADSRSFKHINERIKVNLVAHSMGGLD   180
                 ************************************************************

RTlipase_WT      CRYMISKLEHKSFEIVSLTTISTPHHGSECADFVVQLINQFPALKAVCPKSIPELTTSHM   240
RTlipase_RC      CRYMISKLEHKLFEIVSLTTISTPHHGSECADFVVQLINQFPALKAVCPKSIPELTTSHM   240
                 ********** ***************************************************

RTlipase_WT      RQFNKEVLDDPDVSYLSYGASFDPHWFNIFKITSDIMKTRIKSENHKNDGLVSVESAKWG   300
RTlipase_RC      RQFNKEVLDDPDVSYLSYGASFDPHWFNIFKITSDIMKTRIKSENHKNDGLVSVESAKWG   300
                 ************************************************************

RTlipase_WT      HYLGTLDQVDHLDLINWTNKARTMVDKAMFAHDPKFNAIALYLDVADNLYKHGF 354
RTlipase_RC      HYLGTLDQVDHLDLINWTNKARTMVDKAMFAHDPKFNAIALYLDVADNLYKHGF 354
                 ******************************************************
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Figure 2: Pairwise sequence alignment of wt and rc RT lipase sequences. The red boxes from the alignment sequence indicate the position of mutation at S2L, S92L and S193L.

```
RT_lipase                ----MSQVGRRRLGNWLT----YSGFQRLYSSSTKELVITDKFSTLRPEYKSPRYPIVLC     52
Burkolderia_glumae_lipase MVRSMRSRVAARAVAWALAVMPLAGAAGLT-------MAASPAAVAADTYAATRYPVILV     53
                             * .    *   *       :*   *        : :.  :.    * : ***::*

RT_lipase                HGFSGFDKLAFPKLTDVLNQQKKLESAVEATVDKLSSPLVSLDYWMGIREALERIGSTVL     112
Burkolderia_glumae_lipase HGLAGTDKFA----------------------------NVVDYWYGIQSDLQSHGAKVY     84
                         **::* **:*                               :*** **:. *:  *:.*

RT_lipase                IAKVPAFGTIKERA---ESLNEFIERQCKQLRKKESKASIYDTGSADSRSFKHINERIKV     169
Burkolderia_glumae_lipase VANLSGFQSDDGPNGRGEQLLAYV----------------------KQVLAATGATKV     120
                         :*:: .* : .      *.*  ::                        :..   .   **

RT_lipase                NLVAHSMGGLDCRYMISKLEHKSFEIVSLTTISTPHHGSECADFVVQLINQFPALKAVCP     229
Burkolderia_glumae_lipase NLIGHSQGGLTSRYVAAVAP---QLVASVTTIGTPHRGSEFADFVQDVLKTDPTGLSSTV     177
                         **:.** *** .**: :       :.*:***.***:*** **** ::::  *:  :

RT_lipase                -K---------------------SIPELTTSHMRQFN----KEVLDDPDVS--------     254
Burkolderia_glumae_lipase IAAFVNVFGTLVSSSHNTDQDALAALRTLTTAQTATYNRNFPSAGLGAPGSCQTGAATET     237
                                          ::  ***::   :*    .  *. *. .

RT_lipase                -------YLSYGA-SFDP-----------------------HWFNIFKITSDIMKTR     280
Burkolderia_glumae_lipase VGGSQHLLYSWGGTAIQPTSTVLGVTGATDTSTGTLDVANVTDPSTLALLA-TG---AVM     293
                                *:*. :::*                          : ::  *.    .

RT_lipase                IKSENHKNDGLVSVESAKWGHYLGTLDQVDHLDLINWTNKARTMVDKAMFAHDPKFNAIA     340
Burkolderia_glumae_lipase INRASGQNDGLVSRCSSLFGQVISTSYHWNHLDEINQLLGVRGAN-----AEDPV----A     344
                         *:  . :******  *: :*: :.*  : :*** **    .*       *.**     *

RT_lipase                LYLDVADNLYKHGF   354
Burkolderia_glumae_lipase VIRTHVNRLKLQGV   358
                         :    .:.*  :*.
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Figure 3: Pairwise sequence alignment of RT lipase (wt) sequence with *B. glumae* lipase sequence. The yellow box is the active sites, the green box is the binding sites and the red box is the calcium-binding sites.

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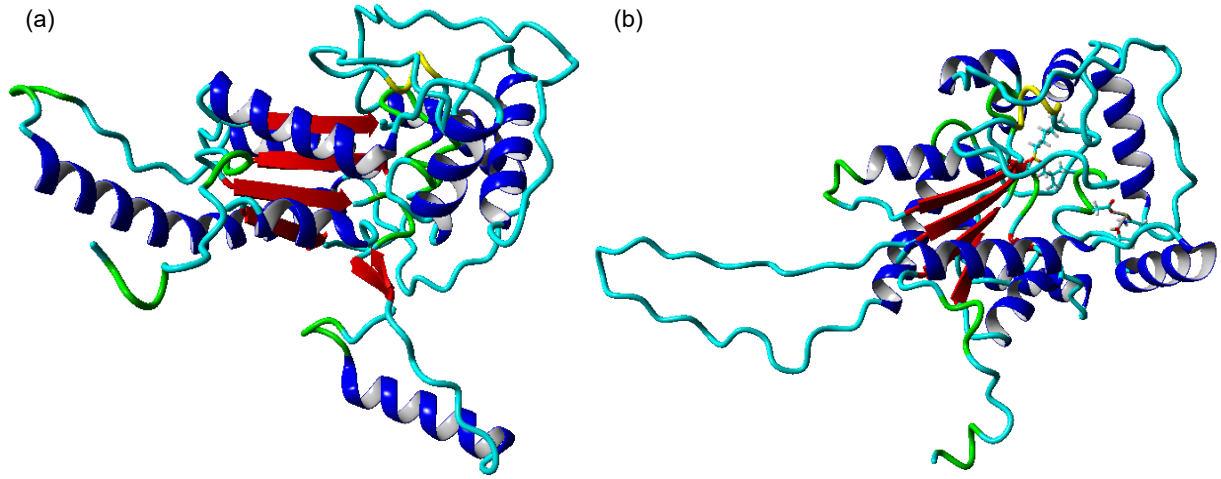

Figure 4: Structure prediction of RT lipase using YASARA. (a) wt (wild-type) and (b) rc (recombinant).

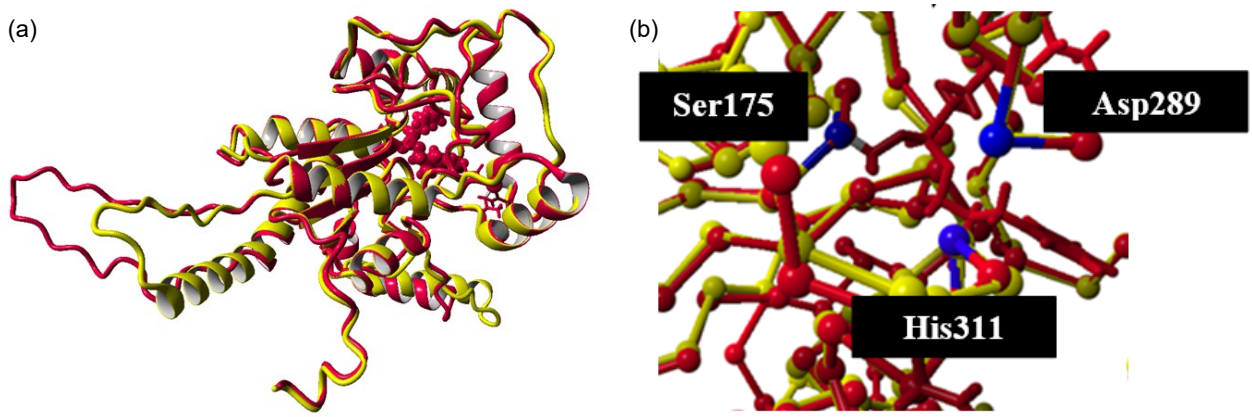

Figure 5: Superimposition of wt with rc RT lipases. (a) Yellow colour: wt (wild type); Red colour: rc (recombinant). Both of the structure was superimposed by using Mustang, YASARA server. (b) The blue residues are the position of the catalytic triad in RT lipase structure.

region. These parameters of protein structure demonstrated that the predicted models displayed were of sufficiently good quality, stable and adequate for further analysis. The overall quality factor ERRAT value for both of the structures were 95.0% (wt) and 92.3% (rc), thus concluding the quality of the structures within 3.5 Å. Both structures failed the Verify3D validation with scores of only 58.33% and 45.06% for wt and rc RT lipases. This verification measures the compatibility of the model built with its amino acid sequence by assigning the location based on the amino acid class.

Superimposition between both wt and rc RT lipases was performed using Mustang YASARA to examine the possible effects of three mutation sites (S2L, S92L and S193L) on the structural properties of rc RT lipase. In structure modelling, the superimposition of protein structures is a common strategy to translate and align the structural domains (Wu and Wu, 2010). Based on the analysis, the root-mean-square-deviation (RMSD) value was 0.682 Å over 283 aligned residues with 97.53% sequence identity. The RMSD value indicated the average distance between the atoms between superimposed proteins. As shown in Figure 5, the alterations in the protein loop were observed, which could be attributed to the decreased thermostability in rc RT lipase since several factors have been reported to affect the thermostable property of a protein, including shortening of loops and characteristics of the polar amino acids in the protein (Khan *et al*., 2017; Zhou *et al*., 2020).

DISCUSSION

The objective of this study was to increase the expression of RT lipase. Strikingly, the highest activity obtained was 56 U/mg, being 91.8-fold higher than the intracellular lipase (0.61 U/mg) in the native host (*M. guilliermondii* strain RT). Previously, this yeast from a rotten tomato (Selangor, Malaysia) was identified as *Pichia* sp. strain RT (later changed to *M. guilliermondii* strain RT) and found to have thermostable lipase (RT lipase). The

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morphological features of the yeast were studied under SEM (Scanning Electron Microscopy) and TEM (Transmission Electron Microscopy). A full sequence of the RT lipase was identified, and it shows that the lipase is 100% percent identical to the hypothetical protein PGUG_05651 (*M. guilliermondii* ATCC 6260).

In this study, the optimum temperature for RT lipase was 45 °C. As compared to the previous study by Mahyon *et al*. (2018), the wt lipase was relatively thermostable and retained nearly up to 50% of its activity when incubated for 30 min at 75 °C. These results have led to two possible hypotheses: (1) intracellular localization of lipase helps to maintain its thermostability or (2) mutations occurred at CTG positions (CUG codons) in recombinant RT lipase resulting in an adverse effect on the optimum temperature. Recombinant RT lipase activity was optimum in alkaline pH. Other lipases such as the thermostable lipase from *Geobacillus* sp. strain ARM (Masomian *et al.*, 2013) and extracellular lipase from *Pseudomonas aeruginosa* JCM5962(T) (Sachan *et al.*, 2018) were also reported to function optimally at alkaline pH. Lipase from the CTG-clade *C. albicans* (CaLIP10) was also reported to exhibit optimum activity at pH 8 (Lan *et al.*, 2011).

For substrate specificity, RT lipase was less selective towards saturated palm oil (C16:0) as the residual activity was only 50% as compared to other natural oils. Our data suggested that RT lipase had high selectivity towards natural oils consisting of a medium-length fatty acid chain (MLFA) since coconut oil contains mostly saturated linoleic acid. Another lipase produced by CTG-clade yeast, *C. rugosa* was reported to preferably synthesize the short-chain fatty acids (SCFA; C4 and C6) from its substrates, contributing to the sharp, tangy flavour in the food (Öztürk, 2001). Its preference towards MCFA was similar to *C. rugosa* lipase (Nguyen *et al*., 2018). However, long-chain fatty acids (LCFA) synthetic substrates were less preferable to RT lipase. Our data suggested that RT lipase favored the synthetic MCFA as compared to other substrates. Lipases from fungi and yeast are usually exploited as a source in cheese manufacturing. These lipases have been chosen due to the short-chain and medium-chain substrates selectivity, which contributes to the cheese flavouring during the ripening process (Jooyandeh *et al.*, 2009).

From *in vitro* study of RT lipase activity, the optimum temperature for wt and rc RT lipases had shifted from 75 °C to 45 °C. Based on the BLAST results, the amino acid sequence for wt was 100% similar to XP_001482631.1, while rc was 99% similar to EDK41553.2, where both were hypothetical proteins of PGUG_05651 (*M. guilliermondii* ATCC 6260). These two sequences shared identical nucleotide sequences but had distinct amino acid sequences where serine was changed to leucine, which could contribute to the change in optimum temperature of RT lipase after being cloned into *K. phaffii*. In addition, those two sequences in the Genbank were deposited based on the direct translation (non-CTG-clade) (EDK41553.2) and CTG-clade yeast ambiguity (XP_001482631.1).

Pairwise sequence alignment of both RT lipase sequences showed three mutation sites in rc RT lipase, which were S2L, S92L and S193L, as compared to its native wt (Figure 2). Generally, these CUG codons encode leucine. But in CTG clade yeast, the CUG codons were instead translated as serine (Butler *et al*., 2009). Some of the lipase-producing CTG yeasts are *C. rugosa* (Matsumae *et al*., 1993), *C. albicans* (Lan *et al*., 2011) and *Candida parapsilosis* (Tóth *et al*., 2015). Serine and leucine exhibited different polar properties, which might contribute to the conformational change in rc RT lipase. Therefore, further investigation on their sequences was performed to examine the possible structural alteration in rc RT lipase.

From the structural study, the data explained the weakness of the predicted structures where they had less than 30% sequence identity with the template. Thus, the predicted models became technically more problematic and produced less accurate results. Nevertheless, the models can still be used in some cases to run experiments directed to understand the function of the protein, as suggested by Tramontano (1998).

CONCLUSION

RT lipase was extracellularly expressed using *K. phaffi* system with 91.8-fold higher production than the wild type (*M. guilliermondii* strain RT). The characterization was conducted and showed that the thermostability of the recombinant enzyme had dropped from 75 °C to 45 °C. Based on the structural analysis, differences in the protein loop could be observed between the wild-type and recombinant protein structures, which are suggested to be the factor for the thermostability reduction. Further studies such as multiple cloning constructs can be performed to improve the expression of RT lipase when transformed into *K. phaffii* expression system. RT lipase can be purified for better characterization and crystallization to improve RT lipase structure and to understand its biochemical properties. This study provided insights into the importance of codon optimization before expressing a protein from a CTG-clade species in a non-CTG-clade organism.

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SUPPLEMENTARY INFORMATION

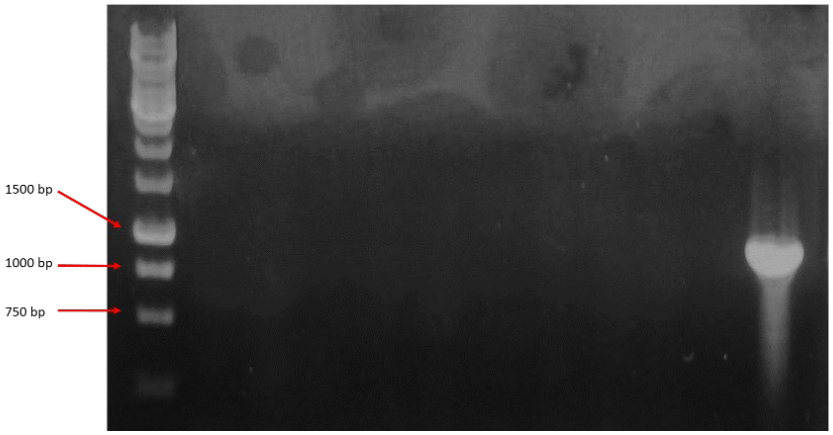

Figure S1: Construction of recombinant plasmids. Analysis of recombinant RT lipase. PCR product of recombinant plasmid pPICZαB/RT.

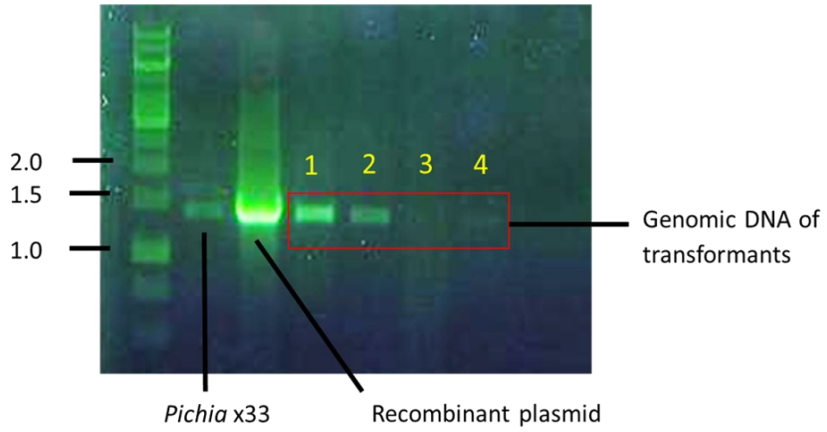

Figure S2: Gel electrophoresis of PCR product from *K. phaffii* transformants. PCR amlification by using (AOX5fwd) and (FRlipase).

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