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Improvement of the alkali stability of *Penicillium cyclopium* lipase by error-prone PCR

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ABSTRACT

Background: Lipases are extensively exploited in lots of industrial fields; cold-adapted lipases with alkali-resistance are especially desired in detergent industry. *Penicillium cyclopium* lipase I (PCL) might be suitable for applications of detergent industry due to its high catalytic efficiency at low temperature and relatively good alkali stability. In this study, to better meet the requirements, the alkali stability of PCL was further improved via directed evolution with error-prone PCR.
Results: The mutant PCL (N157F) with an improved alkali stability was selected based on a high-throughput activity assay. After incubating at pH 11.0 for 120 min, N157F retained 70% of its initial activity, which was 23% higher than that of wild type PCL. Combined with the three-dimensional structure analysis, N157F exhibited an improved alkali stability under the high pH condition due to the interactions of hydrophilicity and β-strand propensity.
Conclusions: This work provided the theoretical foundation and preliminary data for improving alkali stability of PCL to meet the industrial requirements, which is also beneficial to improving alkali-tolerance ability of other industrial enzymes via molecular modification.
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1. Introduction

Lipases can hydrolyze triacylglycerol to produce diacylglycerols, monoacylglycerols, and glycerol by releasing free fatty acids [1]. Thus they are extensively applied in various industrial fields [2,3,4,5,6,7,8,9,10]. As is known, the detergent industry is the primary consumer of lipases, both in terms of volume and value [11], approximately 1000 tonnes of lipases is added to 13 billion tonnes of detergents annually [12]. The applications of lipases in household detergents can improve their ability to remove stubborn oil or grease stains, making them more eco-friendly by reducing or replacing the synthetic detergents [13]. In laundry, the aqueous solution is usually alkaline [14], ideally, lipases added in detergents should be both active and stable in alkaline solution, especially under harsh conditions (pH 8.0–11.0) [15]. Compared with the detergents without cold-adapted enzymes, alkaline detergents supplemented with the cold-adapted lipases could exhibit higher catalytic efficiency at low temperatures, which was beneficial to environmental protection and energy saving [16]. Therefore, lipases with high catalytic activities at low temperatures and outstanding alkali tolerance are crucial for alkaline detergent industries.

Lipases are reported in a large variety of animals, plants, bacteria, yeast, and fungi [17]. In contrast to the plant or animal lipases, lipases produced by microbial sources are suitable for industrial applications as a result of the cost-effective extracellular production by large-scale fermentation [18]. Recently, lots of lipases from microbial sources, such as *Bacillus subtilis* [19], *Pseudomonas* species [20], *Yarrowia lipolytica* [21], *Penicillium expansum* [22], *Aspergillus niger* [23], *Bacillus licheniformis* [24], and *Candida rugosa* [25], have been expressed and characterized. Among them, a lipase isolated from *Penicillium cyclopium* (PCL) demonstrated its pH stability in an expansive range of 8.0 to 10.0 under 30°C, suggesting that it was cold-adapted and alkali-stable. These unique characteristics of PCL made it suitable for laundry, which is generally performed in alkaline environment and low temperature [26]. However, to better satisfy the potential application of PCL in laundry detergents, its alkali-tolerance should be further improved to meet the requirements.

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Currently, numerous protein engineering methods have been developed to improve various lipases' properties including activity (in aqueous solution or organic solvents) [27], thermostability [28], substrate specificity [29], and enantioselectivity [30]. However, limited studies for enhancing the characteristics of PCL have been reported, leading to few theoretical foundation and preliminary data for elucidating the relationship between structure and function at high alkali condition from the mutants generated from site-directed DNA mutagenesis. Alternatively, directed evolution can effectively create the desired mutants for investigating the functional amino acid residues of an enzyme without its detailed structural information [31, 32,33,34]. Therefore, directed evolution has demonstrated its potential for amending the functions of the enzymes via creating and screening the target mutants exhibiting appropriate catalytic behavior.

Based on the aforementioned reasons, a gene mutation library of PCL gene was established via error-prone PCR technology. Thereafter mutant PCL with enhanced alkali resistance was selected to further determine the enzymatic properties and kinetic parameters. Subsequently, enzymatic properties in association with its tertiary structure were investigated to interpret the alkaline-stable mechanism from molecular level and provide molecular basis for understanding the relationship between structure and function in the high alkaline tolerant lipases.

2. Materials and methods

2.1. Chemicals and enzymes

The artificial substrates *p*-nitrophenyl caprylate (pNPC, C8), *p*-nitrophenyl laurate (pNPL, C12), and *p*-nitrophenyl palmitate (pNPP, C16) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All enzymes and genetic manipulation kits used for molecular operation were produced by TaKaRa Bio (TaKaRa, Dalian, China). Reagents and chemicals mentioned in this study were of at least analytical grade.

2.2. Strains, plasmid, and media

pET-22b(+) (Novagen, San Diego, USA) was used for cloning the mutant PCL genes. *Escherichia coli* DH5α cells were used to maintain and propagate plasmids, but *E. coli* BL21 (DE3) cells were utilized for recombinant protein expression. Plasmid pET-*mpcl*, containing the codon-optimized *pclm* gene was synthesized by BGI (Shenzhen, China). All cells were cultivated at 37°C in Luria-Bertani (LB) medium containing appropriate ampicillin (100 μg/mL).

2.3. Creation of the mutant library

Error-prone PCR was performed using pET-*mpcl* as the template with forward primer (5′-cgc*ggatcc*ggcaaccgcagacgcagcag-3′) and reverse primer (5′-ccc*aagctt*actcagataaccgcaacctgccgtc-3′) containing the restriction sites of *Bam*HI and *Hin*dIII (underlined), respectively. The reaction mixture (100 μL) contained 1 × PCR buffer, 0.2 mM dATP, 0.2 mM dGTP, 1 mM dTTP, 1 mM dCTP, 10 μM of forward primer, 10 μM of reverse primer, 10 ng of template DNA, 5 mM $MgCl_2$, 0.25 mM $MnCl_2$, and 5 U Taq DNA polymerase. The resulting product was digested using both *Bam*HI and *Hin*dIII, thereafter integrated to *Bam*HI–*Hin*dIII-linearized pET22b(+), the obtained plasmids were named as pET-*mpclms* and transformed into the competent cells of *E. coli* BL21 Gold (DE3) using electroporation for recombinant proteins expression. Subsequently, the transformants, harboring the mutant PCL library, were incubated at 37°C for 12 h after spreading on LB agar plates supplemented with ampicillin (100 μg/mL).

2.4. Screening of the mutant library

After cultivation, the colonies were placed in the 96-well plates for incubation at 37°C. After the cells grew to $OD_{600} = 0.5$, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to the culture to induce the overexpression of mutant PCLs and wild type PCL (WT) with a final concentration of 0.5 mM at 16°C for 20 h. The supernatant was transferred to replica 96-well plates after being centrifugally harvested at 5000*g* for 5 min at 4°C. Lipase activity of one plate was directly measured at 25°C, but the activity of the second plate containing 50 mM glycine–NaOH buffer (pH 11.0) was measured at 25°C after incubation at 25°C for 30 min. The original activity and the residual activity of mutant PCLs and WT after being treated in 50 mM glycine–NaOH buffer (pH 11.0) were determined using the method described by Sigurgísladóttir et al. [35] with modification. This reaction was performed at 25°C for 10 min using *p*-nitrophenyl laurate as substrate in the buffer (pH 8.0). Due to the enzymatic hydrolysis of *p*-nitrophenyl laurate, the released *p*-nitrophenol was quantified by accurately monitoring its absorbance at the wavelength of 405 nm. Generally, one unit of lipase was defined as the quantity of lipase liberating 1 μM of *p*-nitrophenol from the substrate of *p*-nitrophenyl laurate per min. The positive mutants with enhanced alkali resistance were selected based on the ratio of the residual activity to the original activity of each construct.

2.5. Purification of PCLs

E. coli BL21 (DE3) cells carrying pET-*N157F* and pET-*mpcl* were cultivated at 37°C in 5 mL of LB medium for 12 h. Subsequently, 1 mL of the precultures was subinoculated into 100 mL of LB medium with ampicillin (100 μg/mL) and cultivated at 37°C until OD reached 0.5. Then the cultures were induced with IPTG (1 mM) at 16°C for 20 h to express N157F and WT. To purify N157F and WT tagged with 6 × His, the cultures were centrifugally collected and disrupted by sonication. Then the resulting lysates were purified using a nickel–nitrilotriacetic acid (Ni–NTA) agarose gel column after centrifugation at 20,000*g* at 4°C for 10 min. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the purity and apparent molecular mass of target proteins. Bradford method was conducted to measure the protein concentration using bovine serum albumin for calibration [36].

2.6. Assay of PCL activity

The activity of lipases was measured as stated by the Chinese Industrial Standard (GB-T 23535-2009). One milliliter of N157F or WT solution was mixed with the substrate solution composed of 4 mL of emulsified olive oil. 1.0% (w/v) polyvinyl alcohol and olive oil were emulsified at a volume ratio of 3:1 using a homogenizer (AIK, Shanghai, China) and 5 mL of glycine–NaOH buffer (50 mM, pH 10.0). These reactions were terminated by adding 15 mL of anhydrous ethanol after incubation for 10 min at 25°C. The released fatty acids were estimated by titrating 50 mM NaOH solution using phenolphthalein as an indicator. One micromole of fatty acid released per minute was defined as 1 unit.

2.7. Characteristics of PCLs

The optimal temperatures of N157F and WT were investigated under temperatures ranging from 15 to 40°C in sodium glycine–NaOH buffer (50 mM, pH 10.0). In order to conclude the optimal pH, N157F and WT were estimated in sodium glycine–NaOH buffer (50 mM, pH 9.0–11.0) at 25°C. The maximum activities of N157F and WT at the optimal temperature or pH were used as 100%, respectively.

The thermostability of N157F and WT was determined by incubating at temperatures of 30°C, 35°C, and 40°C in the absence of substrate for

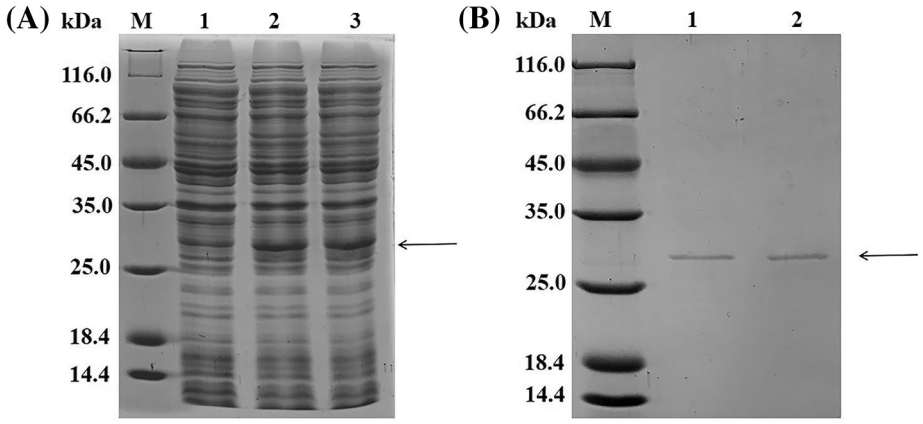

Fig. 1. SDS-PAGE evaluation of the purification of N157F and WT. (A) N157F and WT produced in *E. coli* cells. Lane 1: supernatant of BL21/pET22b(+) culture; Lane 2: supernatant of BL21/pET-*mpcl* culture; Lane 3: culture supernatant of BL21/pET-*N157F*; Lane M: protein ladder. (B) The evaluation of purified N157F and WT using SDS-PAGE. Lane 1: WT; Lane 2: N157F; Lane M: protein ladder.

30, 60, 90, and 120 min in sodium phosphate buffer (50 mM, pH 8.0). Similarly, their stability of pH was studied by incubation at different pH (9.0, 10.0, and 11.0) and 30°C without substrate for 40, 80, and 120 min in 50 mM glycine–NaOH buffer. Their residual activities were measured according to the standard method after cooling in ice bath for 10 min. The initial activities of N157F and WT without any treatment were used as 100%, respectively.

2.8. Characterization of the kinetic parameters

The kinetic parameters of N157F and WT were calculated as mentioned in the section of "Screening of the mutant library", using the substrates pNPC, pNPL, and pNPP in different concentrations (0.5–9.0 mM) at 25°C and pH 8.0. The Michaelis–Menten constants were calculated based on the Lineweaver–Burk plot.

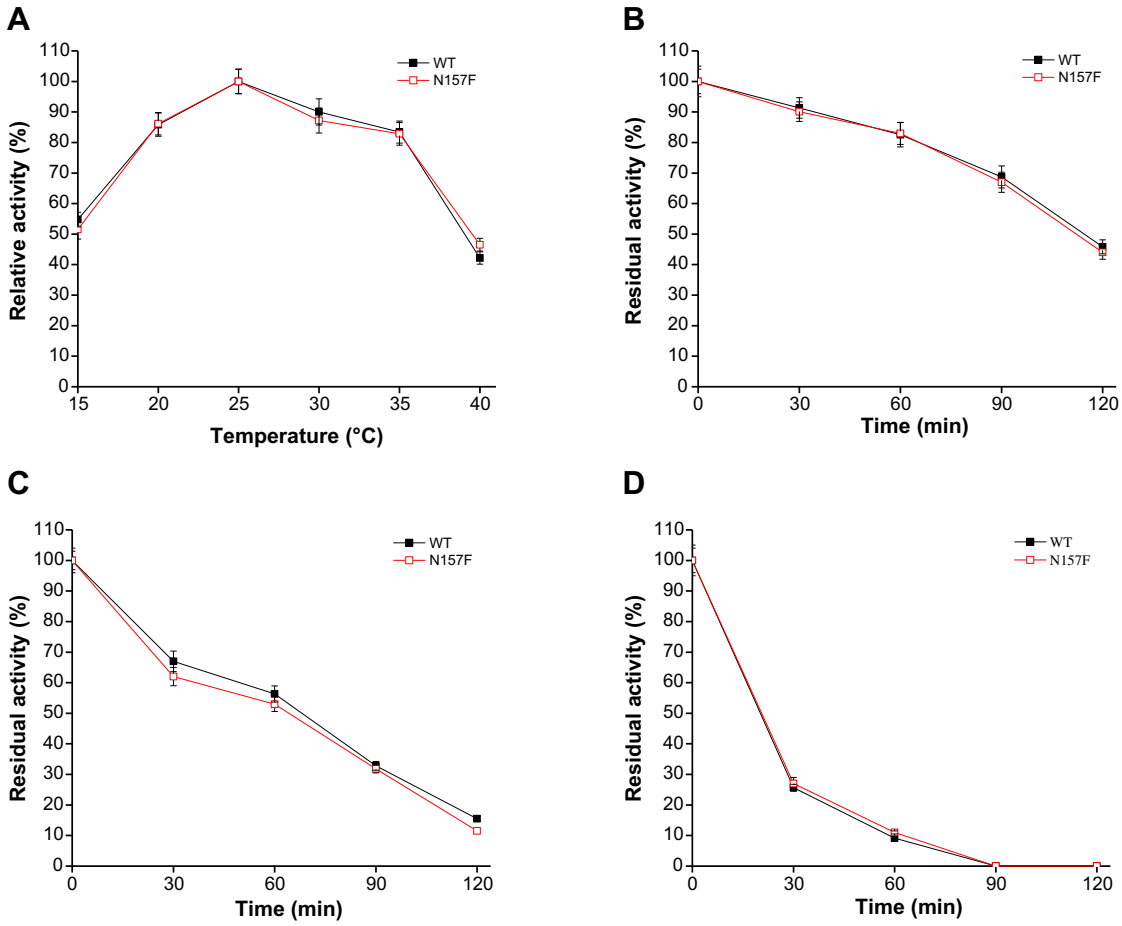

Fig. 2. The optimal temperature and stability of N157F and WT. (A) The optimum temperatures of N157F and WT were determined over a temperature range of 15–40°C at pH 10.0. While their thermostabilities were determined by monitoring the residual activity of N157F and WT after incubating at (B) 30°C, (C) 35°C, and (D) 40°C and pH 8.0 for various time without substrate. The values are expressed as means ± SD of three independent experiments.

2.9. Modeling studies

Both of the tertiary structural models of N157F and WT were built using PyMOL molecular graphic system and Swiss-Model (http://swissmodel.expasy.org/) with the template PEL (PDB code: 3G7N). Differences in the mutation positions of these models were compared in PyMOL by analyzing the interaction possibilities and clash problems.

3. Results and discussion

3.1. Screening of mutant library

After sequencing 10 mutants selected randomly, an average mutation rate of 0.2% was achieved by optimizing the error-prone PCR conditions. The mutant PCLs were screened by measuring their residual hydrolytic activity after incubation under alkaline conditions. Nearly 5100 clones in the library were screened to distinguish the mutants with enhanced alkali tolerance, and about 1700 transformants expressed functional lipases. Among them, three transformants which showed a higher alkali tolerance than the parent strain were used for a second screening, but no mutant with further enhanced alkali tolerance was obtained. Then plasmids were isolated from these three positive transformants and then the mutations were verified by sequencing. These three mutants showed nucleotide alteration at the same position (AAC → TTC), leading to the replacement of one amino acid residue (N157F). Because of the only one mutation in the mutant N157F, it could have direct correlation with the enhancement of alkali stability. Therefore, the mutant N157F was chosen for further characterization.

3.2. Purification of N157F and WT

The activities of N157F and WT were measured with the supernatant lysate after cell disruption by sonication. No detectable lipase activity was found in the lysate supernatant of cells containing pET-22b(+). It was found that mutant N157F and WT demonstrated the same migration pattern on SDS-PAGE (Fig. 1A, lanes 2–3) with an approximate molecular weight of 32 kDa, but no analogous band was observed in the negative control lane (Fig. 1A, lane 1). Subsequently, N157F and WT were purified with the Ni–NTA column using chromatography, and the purified N157F and WT were indicated as a single band on the gel with a molecular weight around 32 kDa, which was identical in size (Fig. 1B, lanes 1–2) and could be used for the functional analysis. Additionally, the specific activities of the purified N157F and WT were determined to be 1086 U/mg and 1177 U/mg, indicating that the enzymatic activity was not affected by the introduced mutation.

3.3. Enzymatic properties of N157F and WT

Similar to WT, N157F indicated an optimum temperature of 25°C (Fig. 2A). As demonstrated in Fig. 2B, Fig. 2C and Fig. 2D, N157F exhibited similar tendency of thermostability to those of WT at 30°C, 35°C, and 40°C, suggesting that there was no noteworthy difference between N157F and WT in the thermostability.

WT and N157F both exhibited over 50% activity in the pH range from 9.0 to 11.0 with an optimum pH at 10.0 (Fig. 3A). As demonstrated in Fig. 3B, Fig. 3C and Fig. 3D, 54%, 61%, and 47% of WT initial activity were

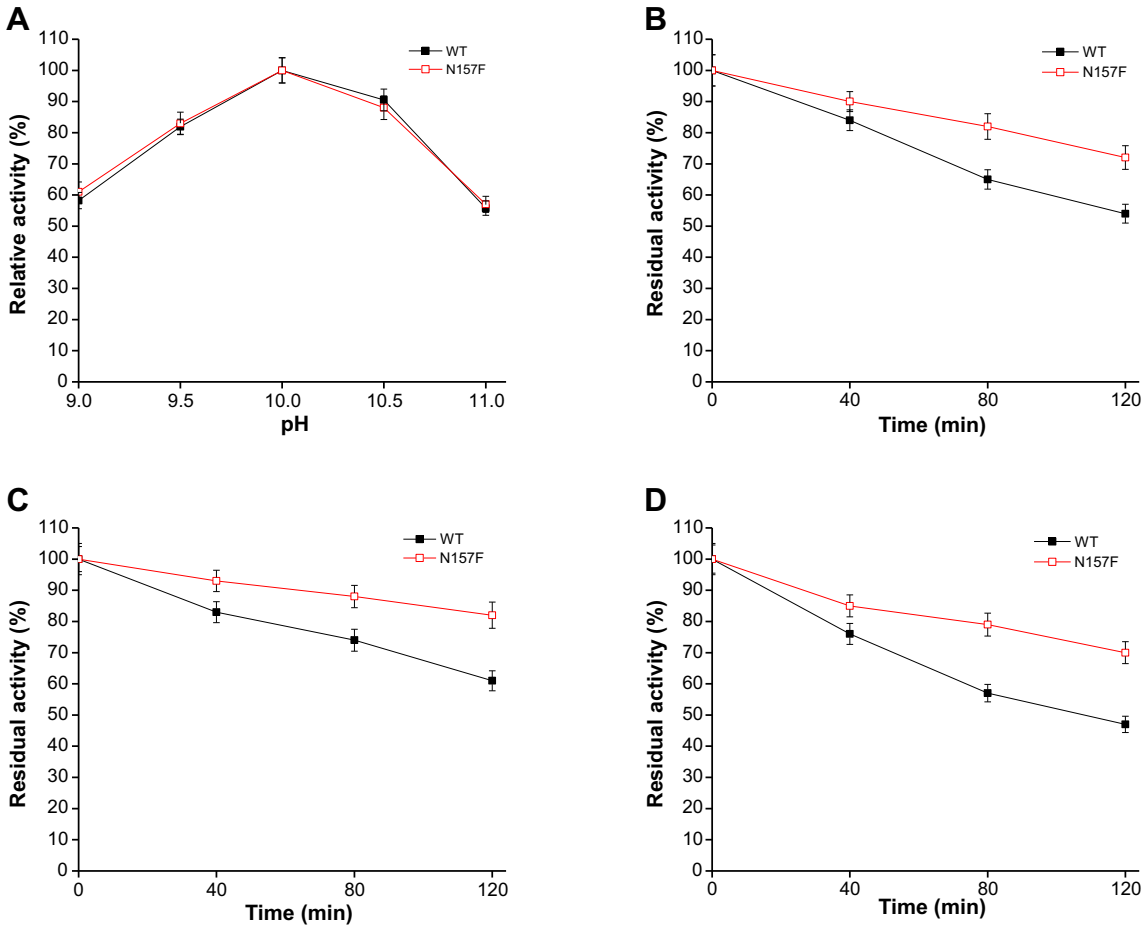

Fig. 3. The optimal pH and pH stability of N157F and WT. (A) The activity of N157F and WT was estimated at different pH starting from 9.0 to 11.0 at 25°C. The residual activity of N157F and WT was estimated after incubating at (B) pH 9.0, (C) pH 10.0, (D) pH 11.0 at 30°C for various time without substrate to determine the pH stability. The values are indicated as means ± SD of three independent experiments.

Table 1
The kinetic parameters of N157F and WT at 30°C under pH 8.0.

Lipases	*p*-Nitrophenyl caprylate (C8)			*p*-Nitrophenyl laurate (C12)			*p*-Nitrophenyl palmitate (C16)		
	k_{cat}[a] (s^{-1})	K_m[a] (mM)	k_{cat}/K_m (s^{-1}·mM)	k_{cat}[a] (s^{-1})	K_m[a] (mM)	k_{cat}/K_m (s^{-1}·mM)	k_{cat}[a] (s^{-1})	K_m[a] (mM)	k_{cat}/K_m (s^{-1}·mM)
WT	0.65 ± 0.028	2.54 ± 0.081	0.26 ± 0.0062	0.93 ± 0.039	1.77 ± 0.062	0.53 ± 0.019	0.44 ± 0.020	3.23 ± 0.120	0.14 ± 0.0064
N157F	0.66 ± 0.032	2.62 ± 0.092	0.25 ± 0.0075	0.87 ± 0.044	1.75 ± 0.070	0.50 ± 0.019	0.48 ± 0.015	3.27 ± 0.168	0.15 ± 0.0051

[a] Data are presented in the form of average ± standard deviation (n = 3).

detected after incubating at pH 9.0, 10.0, and 11.0 for 120 min at 30°C. However, N157F maintained 72%, 82%, and 70% of its initial activity, respectively, indicating that the mutation improved the alkali stability.

So far, lots of studies have been performed to improve the activity [37], thermostability [38], substrate specificity [39], and enantioselectivity [40] of lipases using protein engineering to enlarge their applications as biocatalysts in various industries, and the relationship between the structures and functions of mutants has been elucidated. However, limited studies were performed to enhance the alkali stability of lipase, resulting in little information on molecular mechanisms of alkaline tolerance. Typically, lipases used in detergent are required to be active and stable under alkaline environments [15]. To this end, a cold-adapted and alkali-stable lipase would be more beneficial to use in detergent industry due to economic and environmental benefits [41]. Nevertheless, the practical applications of lipases are somehow limited because of their relatively lower stabilities and catalytic activities particularly under low temperatures and extreme pH conditions. In this study, PCL, a cold-adapted lipase most active at 25°C and thermostable below 30°C, was modified by directed evolution to enhance its alkali stability, providing useful information to further understand the alkaline adaptation strategies.

3.4. Kinetic parameters of the N157F and WT

Kinetic parameters of N157F and WT on C8, C12, and C16 were measured at 30°C and pH 8.0, respectively (Table 1). K_m, k_{cat}, and k_{cat}/K_m values of N157F towards these three substrates with different chain lengths were not significantly altered when compared to WT, indicating that the mutation did not affect its overall integrity despite the significant variation in the alkali stability.

3.5. Analysis of the tertiary structure

The three-dimensional structures of WT and N157F were built using the crystal structure of lipase from *P. expansum* (PEL) as the template due to their 99% homology in amino acid sequence. As shown in Fig. 4, PCL has a modified α/β hydrolase fold containing seven β-strands situated in a parallel β-sheet which is surrounded by α-helices. The active center is identified to be formed by Ser132, Asp188, and His241. The residue at position 157 is far away from the active site in its three-dimensional structure, demonstrating no direct interactions with the catalytic site.

Residue Asn157 or Phe157 in the interior of WT or N157F is situated at the fourth residue of β5 strand. Asn157 residue containing three hydrogen bonds plays a role in a hydrogen bonding network: Asn157 ↔ Ala128, Asn157 ↔ Gly130, and Asn157 ↔ Asn181 (Fig. 5A), but Phe157 consisting of two hydrogen bonds takes part in a hydrogen bonding network: Phe157 ↔ Ala128 and Phe157 ↔ Gly130 (Fig. 5B). Although the structure stability might be moderately reduced by the destroyed hydrogen bond, the substitution of Asn with Phe at position 157 takes the place of a hydrophobic side chain using a hydrophilic group, leading to the increment of alkali stability for N157F due to the benefit of enhancing the structure stability. Additionally, it suggested that most energy was exhausted by destroying the interaction of the residues situated in the α-helix or β-strand during protein unfolding. Compared with Asn, Phe is highly apt to generate the β-strand, which is conducive to stabilize the β5 strand in N157F, leading to

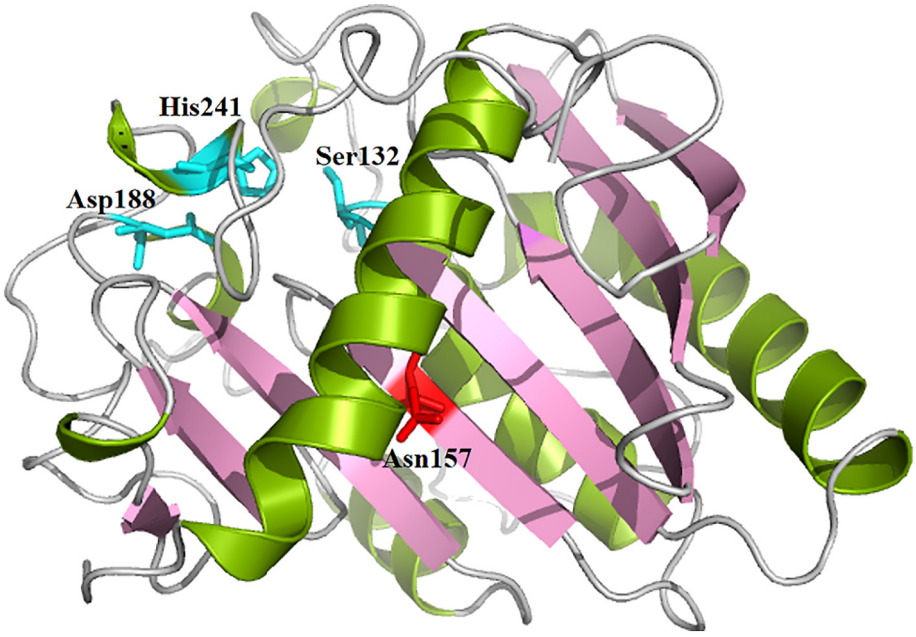

Fig. 4. The modeled PCL tertiary structure. Ser132, Asp188, and His241 which formed the catalytic center are labeled in blue. The α-helices are presented in green and β-sheets are colored in purple. The mutated position 157 is marked in red.

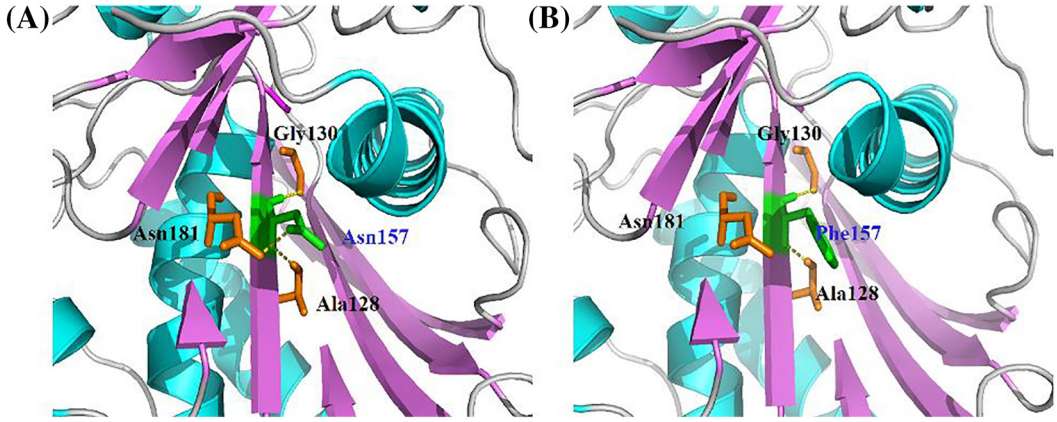

Fig. 5. Stereo view of the residuals (Asn157 or Phe157) and their surroundings. The residue at position 157 and residues closed to position 157 are labeled in green and orange, respectively. The networks of hydrogen-bonds closed to Asn157 and Phe157 are labeled with yellow dotted lines, respectively. The α-helices are colored in blue and the β-sheets are represented in purple.

the enhanced rigidity to improve the comprehensive stability under extremely alkaline conditions.

4. Conclusion

In summary, a novel cold-adapted lipase (N157F) with enhanced alkali stability was constructed by error-prone PCR technology. The tertiary structure evaluation indicated that these interactions of hydrophilicity and β-strand propensity improved the alkali stability of N157F under high pH condition. The present study is beneficial to understanding the relationship between structure and function in PCL mutant tolerant to high pH. It also provides the theoretical foundation and preliminary information on improving alkali stability in PCL to meet the industrial requirements via protein engineering. Moreover, N157F demonstrated great potential for applications in detergent industry as a novel cold-adapted and alkali-stable lipase.

Conflict of Interest Statement

There are no conflicts to declare.

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