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Increasing Hydrolytic Activity of Lipase on Palm Oil by PCR-Based Random Mutagenesis

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ABSTRACT

Random mutagenesis technique is a powerful technique capable of producing enzymes with desired biocatalytic activity. This study aims to obtain a mutant lipase with improved hydrolytic activity on palm oil substrate using random mutagenesis technique. Random mutagenesis by error-prone PCR was used to generate mutant lipases. A total of 1101 mutants were obtained, out of which two mutants, Lip M14.25, and Lip M14.57, showed an increased relative hydrolytic activity. Lip M14.25 and Lip M14.57 demonstrated a 14% and 16% increased activity respectively. A comparison of the mutants' hydrolytic activities using p-nitrophenyl esters showed a significantly high preference for p-nitrophenyl palmitate. Furthermore, the mutant, Lip M14.25 showed its highest activity at pH 5, and Lip M14.57 exhibited a 10 ^{o}C decrease in optimum temperature. The two mutants' protein modelling showed the substitution of N44S/S202N on M14.25 and F154L/S265C on M14.57 lipase, which caused changes in conformation and active site residue distance of the lipase. The study found two mutants of lipase, M14.25 and M14.57, which showed improved hydrolytic activity on palm oil substrate.

Keywords: biotransformation, enzyme, error-prone PCR, *Geobacillus stearothermophilus*, lipid.

INTRODUCTION

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are hydrolase enzymes that act on carboxylic ester bonds, catalyse the hydrolysis of triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. Lipases can also perform the reverse reaction of esterification, interesterification, alcoholysis, and acidolysis reactions (Sharma & Kanwar 2014). They are widely used as catalysts in the food, detergent, pharmaceutical, leather, textile, cosmetics, paper, and biodiesel industries. Humans, animals, plants, and microorganisms can produce lipase (Melani *et al.* 2020). However, microbial lipases are the most widely used in inindustrial activities due to simple of production, low price, various substrate specificities,

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and uncomplicated of genetic modifications. Lipases from genera *Pseudomonas, Burkholderia, Bacillus, Candida*, and *Staphylococcus* have been reported potential for industries (Gurung *et al.* 2013).

One of genetic modification methods used to increase an enzyme's catalytic activity is random mutagenesis (error-prone PCR). Main principles of this method are mutation and selection. Mutation is made by changing the condition of PCR that can increase the mutation rate during DNA amplification. Mutation can be achieved by adding $MnCl_2$, an unbalanced amount of dNTP, increasing the concentration of $MgCl_2$, increasing the concentration of DNA polymerases, and using low-fidelity DNA polymerases (Cadwell & Joyce 1992). Recent studies have reported that error-prone PCR can be used to create enzymes with desirable characteristics, such as enhancing enzyme stability under acidic conditions, enhancing stability at high temperatures, enhancing stability in organic solvents, and changing enzyme-substrate specificities (Liebeton *et al.* 2000; Khurana *et al.* 2010; Liu *et al.* 2012; Dror *et al.* 2014).

In this work, we applied a method of random mutagenesis (error-prone PCR) to obtain lipase mutants of *Geobacillus stearothermophilus* T1.2RQ with higher hydrolytic activity on palm oil substrate. The lipase used in this research has 90% homology with thermostable lipase *G. stearothermophilus* L1. This lipase comes from I.5 lipase family, which is generally active and stable at high temperatures, so it is a potential candidate for industries (Apituley 2012). Palm oil is a vegetable oil widely produced and used in Indonesia and a potential substrate in the oleochemical industry (Yuliansyah *et al.* 2009; Abdelmoez & Mustafa 2014). The substrate selectivity of T1.2RQ and mutant lipase to p-nitrophenyl palmitate and palm oil were investigated comprehensively. Crude extract enzyme of T1.2RQ lipase and potential mutants were characterized and analyzed for their protein structures to determine their potential in industrial applications.

MATERIALS AND METHODS

Material

Mutation of *G. stearothermophilus* T1.2RQ lipase gene (PT Wilmar Benih Indonesia, Indonesia) was performed using GeneMorph II Random Mutagenesis Kit (Agilent, USA). pET-28a plasmid (Novagen, USA) was used for the expression vector in *Echerichia coli* BL21 (DE3) (TaKaRa, Japan). Restriction enzymes EcoRI, NotI, and T4 DNA ligase were purchased from New England Biolabs (USA), and the p-nitrophenyl substrates were purchased from Sigma-Aldrich (USA).

Random Mutagenesis

Random mutagenesis was done using GeneMorph II Random Mutagenesis Kit. The PCR (gradient) reaction was carried out in Thermo Scientific thermal cycler at 95 °C for 2 min, 95 °C for 30 sec, 58 °C for 1 min, and 72 °C for 75 sec with a final extension 72 °C for 10 min followed by 30 cycles. The PCR product was cloned in pET28a vector after double digestion with EcoRI and NotI, and plasmid recombinants were transformed into *Echerichia coli* BL21 (DE3) cells via heat shock as a mutagenic library.

Screening of Potential Mutants

The expression of recombinant proteins performed in 96-well plates follows the Böttcher and Bornscheuer (2006) methods. E. coli BL21 (DE3) cells harbouring recombinant pET-28a plasmid were grown in a microplate overnight at 37°C in

200 µL LB media with 50 µg/mL kanamycin antibiotic. The next day, 1% (v/v) overnight mutant cultures were inoculated into 200 µL LB media contain 50 µg/mL kanamycin and incubated for 4 hours at 37 °C, induction of lipase expression by 0.5 mM IPTG at 30 °C for 16 hours. Cells were harvested and lysis using lysis buffer (50 mM Tris-HCl buffer [pH 8], 43% glycerol, and 0.05% Triton X-100) at 50 °C for 3 hours. The crude extract enzymes were harvested with a centrifugation speed of 1300 ×g at 4 °C for 30 min.

Lipase activity was determined using a colorimetric test based on p-nitrophenyl palmitate (pNPP) hydrolysis. Enzyme activity was measured based on yellow color resulted from the release of p-nitrophenyl (pNP) on absorbance at 405 nm using a microplate reader (Park *et al.* 2018). Two microliters of crude lipase were added to 196 µL assay mixture (188 µL Tris-HCl buffer [pH 8] 0.1 M and 8 µL ethanol absolute), and subsequently 2 µL 8 mM pNPP (30 mg pNPP dissolved in 10 mL isopropanol). The assay mixture containing crude lipase was incubated for 5 min at 35 °C and measured at 405 nm in a microplate reader spectrophotometer. One enzyme activity unit is defined as the number of enzymes that hydrolyze 1 µmol p-nitrophenyl/min. Mutants with higher lipolytic activity than lipase control were selected for further study.

Production of Recombinant Enzyme

Lipase production of potential mutants obtained from the first selection was done in 50 mL LB media with 50 µg/mL kanamycin antibiotic. Lipase expression was induced with 0.5 mM IPTG at an optical density (OD) of ~ 0.5-0.6 at 600 nm. Cells were harvested and disrupted with lysis buffer (50 mM Tris-HCl buffer [pH 8], 43% glycerol, and 0.05% Triton X-100) and incubated at 50 °C for 3 hours. The crude extract was harvested by centrifugation at a speed of 10,000 ×g at 4 °C for 10 min. Molecular weight of crude extract enzyme was analyzed using denaturing 12% SDS-PAGE gel (Laemmli 1970).

Hydrolytic Activity on Palm Oil

Hydrolytic activitity of lipase mutants on triglyceride were measured based on free fatty acids released during hydrolysis of palm oil in pH-stat (902 Titrando, Metrohm). Fifty milliliters substrates (1% (v/v) palm oil, 10% (v/v) gum arabic stock solution [20 mM NaCl, 5 mM CaCl, and 0.1% (w/v) gum arabic], and distilled water) were put into reaction with 5 µL crude extract enzyme at 50 °C and titrated with 10 mM NaOH. One lipase unit is defined as the amount of enzyme that releases 1 µmol of fatty acids per minute (Kim *et al.* 2000). Protein concentration was determined by bicinchoninic acid (BCA) kit (Novagen, Germany) method.

Biochemical Characterization

Substrates specificity of lipase T1.2RQ and mutants were determined using pNP ester with various chain lengths: pNP-butyrate (C4), pNP-caproate (C6), pNP-caprylate (C8), pNP-lauric (C12), pNP-palmitate (C16), and pNP-stearate (C18). The effect of temperature on mutants lipase activity was measured by reacting mutant lipases at various temperatures (30 - 60 °C) to determine optimum temperature of lipase T1.2RQ and mutants. The activity of lipase T1.2RQ and mutants at acidic pH was determined using sodium acetate pH 5.0 at 35 °C substrate pNP-lauric (C12). The effect of organic solvent was also measured by incubation for 0, 60, 120, and 180 min in 50% (v/v) methanol solvent.

Amino Acid and 3D Structure of Protein Analysis

Mutations in base sequences were confirmed by sequencing potential mutant plasmid using T7 promoter and T7 terminators primers (Macrogen, South Korea). The base sequence determined using AB® Genetic Analyzer 3130 (Applied Biosystem, USA), and analysis of base mutations using Geneious software 11.0.5. The protein model structure was acquired as PDB using I-TASSER and visualized by visual molecular dynamics (VMD) 1.9.3 (Humphrey *et al.* 1996; Yang & Zhang 2015).

RESULTS AND DISCUSSION

Screening and Recombinant Protein Expression

Random mutagenesis (error-prone PCR) was chosen for creating variants of T1.2RQ lipase with higher hydrolytic activity in palm oil substrate compared to the control (T1.2RQ). A total of 1,101 transformants showing a clear zone in tributyrin medium, transformants were transferred to 96-well plates, cultivated and used as a master plate. Recombinant proteins were produced on 96-well plates and screened using a p-nitrophenyl palmitate substrate. The second screening of potential mutants was carried out with hydrolytic activity lipase on the palm oil substrate. Two potential mutants were found with increased hydrolytic activity on the palm oil. These variants, M14.25 and M14.57, had 14% and 16% higher relative activity compared to the control, respectively (Table 1).

Table 1 Relative activity of control lipase, M14.25, and M14.57 to palm oil

Lipase	Relative activity (%)
Control	100 ± 6.71
M14.25	114 ± 5.54
M14.57	116 ± 6.01

**Deviation standar n= 3.*

Protein expression was achieved with induction using 0.5 mM IPTG. Crude extract enzymes were harvested by breaking the cells using lysis buffer and incubation at 50 °C. Molecular weight of the control and mutant lipase proteins were checked on 12% SDS-PAGE electrophoresis. Single bands of control and mutated lipase were similar at ~43 kDa (Figure 1).

Biochemical Characterization

Mutants lipases' substrate specificity were compared with controls using p-nitrophenyl ester with various fatty acid lengths from C4 to C18. The results showed that both mutant and control lipases had maximum activity on pNP-caprylate (C8). Lipases M14.25 and M14.57 showed 24% and 16% higher relative activity on pNP-butyrate (C4) and 11% and 12% on pNP-palmitate (C16) substrates than control respectively. Moreover, M14.57 lipases also showed a 13% higher relative activity on pNP-lauric (C12) compared to control (Figure 2). This change in substrate specificity probably led to an increase in the hydrolytic activity of lipases M14.25 and M14.57 on palm oil substrate containing 43.5% palmitic fatty acid (C16) (SambanthamurthI 2000). *G. stearothermophilus* T1.2RQ lipase is an alkaline lipase that will work maximum in basic pH but begins to lose its activity at acidic pH (Apituley 2012). However, the mutant lipase, M14.25, showed an 18% increase in relative activity at acidic pH 5 compared to the control (Figure 3).

Methanol is short-chain alcohol widely used in biodiesel production, but methanol in high concentrations has also been reported to inhibit lipase activity when used in alcoholysis reactions (Lotti *et al.* 2014). The stability of control and mutants

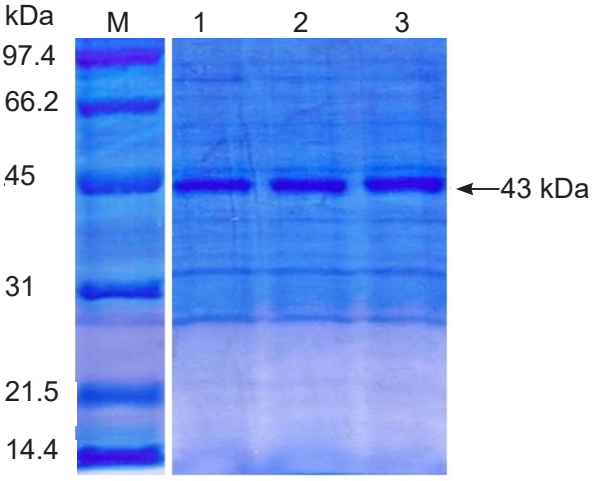

Figure 1 SDS-PAGE analysis of control and mutant crude extract enzyme. Lane M: protein molecular weight marker; lanes 1-3: protein sample from control, M14.25, and M14.57.

3D Protein Homology Modelling Analysis

A 3D homology model of mutant lipase was made based on crystal structure of G. stearothermophilus P1 lipase (code PDB IJ13) (Tyndall *et al.* 2002). Amino acid substitutions in M14.25 lipase are located on the protein surface, N44S located in the helix α1 region, and S202N in the loop region lipase lid. Lipase M14.57 underwent F154L amino acid substitution in the loop region adjacent to β6, while S266C substitution was located in the strand β7 region (Figure 6). Based on protein modeller, these two mutants have different secondary structures in some parts of the protein. The α-helix structure

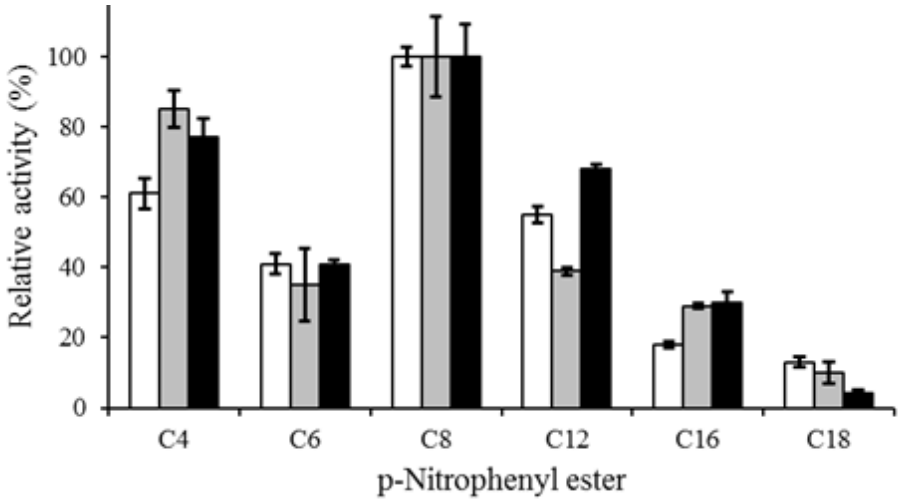

Figure 2 Substrate specificity of control (white), M14.25 (grey), and M14.57 (black) crude extract enzyme on p-nitrophenyl butyrate (C4), p-nitrophenyl caproate (C6), p-nitrophenyl caprylate (C8), p-nitrophenyl lauric (C12), p-nitrophenyl palmitate (C16), and p-nitrophenol stearate (C18).

lipases in organic solvents were tested by incubation for 0-180 minutes in 50% methanol. Based on test results, there was no differential effect between control and mutant lipases (Figure 4). The effect of temperature on lipase activity shows that control lipase has an optimum temperature at 50 °C, M14.25 at 50 °C, and M14.57 decrease at 40°C (Figure 5).

formed by the folding of amino acids at 305-308 transformed to primary structure, whereas primary structure of amino acids at 232-235 and 289-293 formed the α-helix structure.

The substitution of N44S on M14.25 caused a conformational alteration and loss of one hydrogen bond between Trp40 and Arg373. Whereas, the substitution of the amino acid S202N located in the make up lid area caused the formation

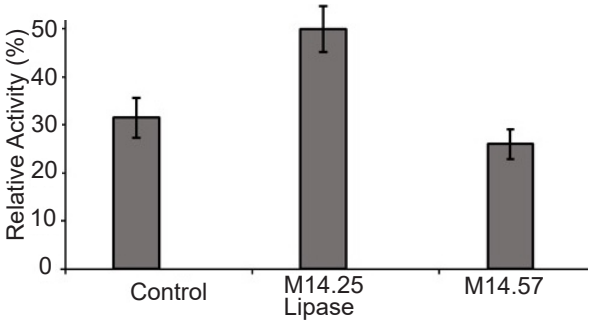

Figure 3 The effect of acidic condition (pH 5) on enzime activities

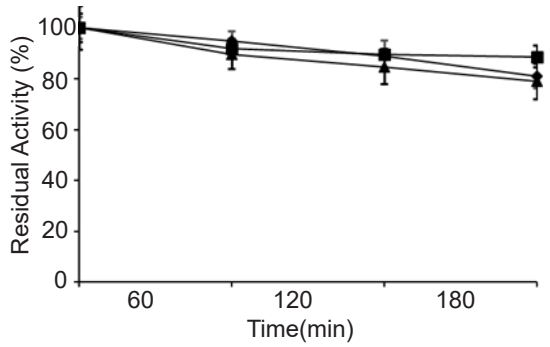

Figure 4 Control (●), M14.25 (■), and M14.57 (▲) crude extract lipases activity after incubation at different times in 50% methanol.

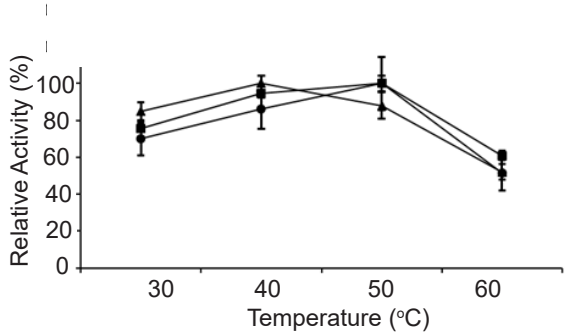

Figure 5 Effect of temperature on the control (●), M14.25 (■), and M14.57 (▲) crude extract lipases activity at 30 °C, 40 °C, 50 °C, and 60 °C.

of hydrogen bonds between Asp205 and Arg214 and conformational alteration (Figure 7). Amino acid substitution in the lid lipase region has been widely reported to induce increased activity and change lipase substrate specificity (Karkhane *et al.* 2009; Shih and Pan, 2011; Tang *et al.* 2019). These two substitutions N44S/S202N even caused alteration conformation and distance between Asp317 and His358 on the catalytic triad (Figure 9).

The substitution of amino acid F154L on M14.57 affected amino acid residues' conformation and forms a new hydrogen bond between Arg153 and His261. Based on a conformational change, This substitution affected catalytic triad Ser113, where the substitution of S266C was a factor that affected the conformation of Glu270, whose position affected the conformation of catalytic triad Asp317 (Figure 8). This conformational change caused loss of hydrogen bond between Asp317 and His358 residues (Figure 9). Previous studies show that substitution of amino acid N355K locates close with catalytic residue Asp317 cause a decrease in mutant lipases' optimum temperature by 10 °C, mutations in this region also affect the substrate specificity of the lipase mutant (Sharma *et al.* 2013).

Based on the analysis of active site residual, the distance of hydrogen bonds between His358 and Asp317 in the control lipase are 2,75 Å and 2,00 Å, while in M14.25 lipase, the hydrogen bonds distance between His358 and Asp317 changed to 2,72 Å and 2,07 Å. M14.57 lipase shows more significant distance change, the distance between His358 and Asp317 are 4,44 Å and 4,03 Å (Figure 9). This distance change causes loss of hydrogen bonds in those residues. The hydrogen bond is known to make a beneficial contribution to protein stability because it involves hydroxyl groups, which tend to increase protein stability (Nick Pace *et al.* 2014). A recent study reported in mutant lipases that increase its optimum temperature, there is a new hydrogen bond form on the active site residue (Sani *et al.* 2017). The loss of hydrogen bonds on F154L/S266C lipase is probably the cause of optimum temperature to 40 °C (Figure 6).

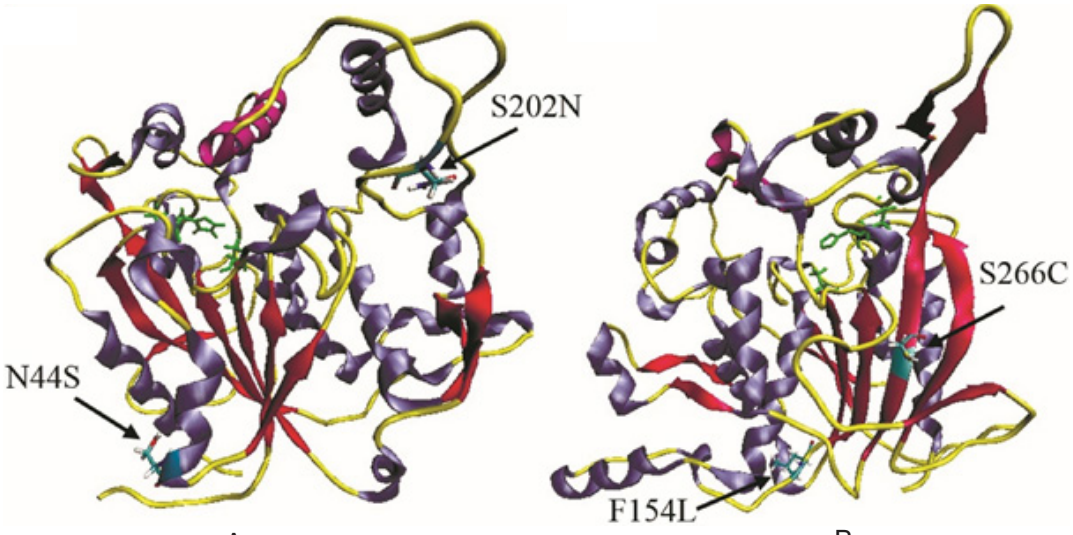

Figure 6 3D structural model of the mutant lipase protein. The catalytic triad (Ser113, Asp317, and His358) in green, lipase lid (residue 176-192) in magenta, amino acid substitutions (A) M14.25 (B) M14.57 in cyan.

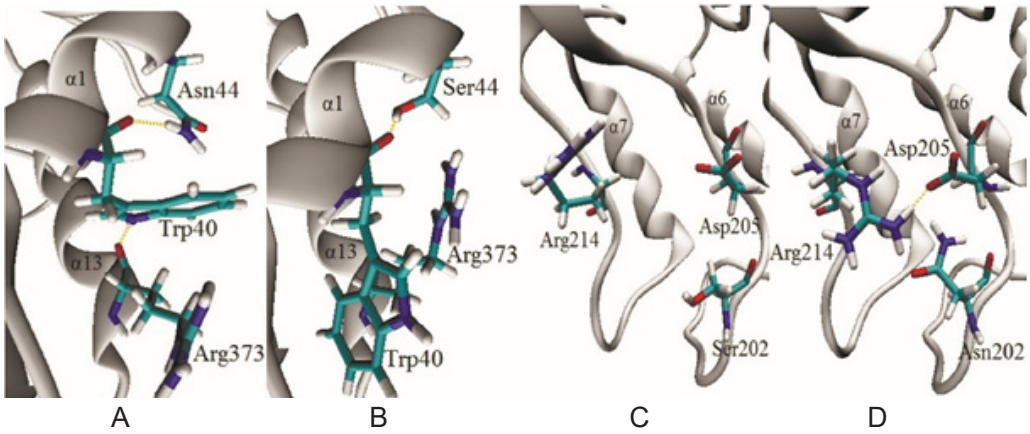

Figure 7 Close-up of structural mutation site in M14.25 lipase with hydrogen bonds. (A-B) N44S amino acid substitution; (C-D) S202N amino acid substitution.

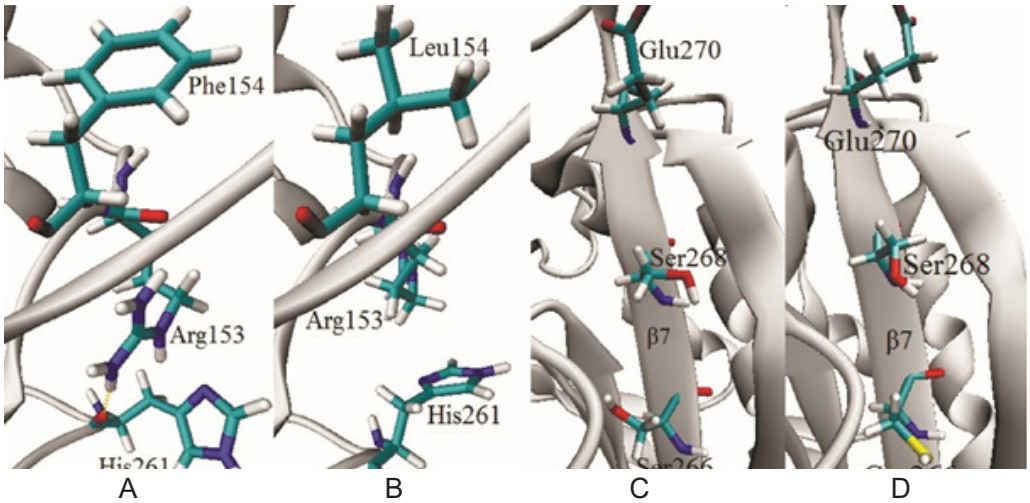

Figure 8 Close up of structure mutation site in M14.57 lipases with hydrogen bonds. (A-B) F154L amino acid substitution; (C-D) S266C amino acid substitution.

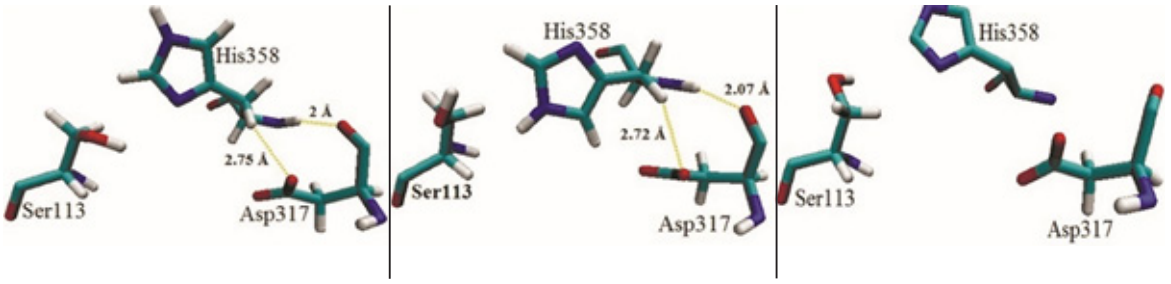

Figure 9 Close up of the residual structure of active site lipases with hydrogen bonds. (A) control; (B) M14.25; (C) M14.57.

CONCLUSION

We found two mutants of lipase which generated from random mutagenesis (error-prone PCR), lipase M14.25 and M14.57, that showed 14% and 16% enhanced hydrolytic activity on palm oil substrate respectively and showed a significantly high preference for p-nitrophenyl palmitate. Moreover, lipase M14.25 showed an increase in hydrolytic activity at pH 5, and lipase M14.57 showed a decrease in optimum temperature. The two mutants' protein modelling showed the substitution of N44S/S202N on M14.25 and F154L/S265C on M14.57 lipase.

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