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RESEARCH ARTICLE

Exploring the impacts of terminal mutations on the thermostability of *Bacillus* sp. L2 lipase

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Abstract
Protein engineering has been widely used to improve enzyme properties and make them appropriate for use as industrial biocatalysts. To study the effect of mutation at the N- and C-terminal of lipase, two double mutants (A8V/S385E and A8P/S385E) were generated by site-directed mutagenesis of L2 lipase from *Bacillus* sp. (wt-L2). The simultaneous mutations in the A8V/S385E and A8P/S385E resulted in significant changes in the lipase's properties compared to the wild-type (wt-L2). The mutants demonstrated increased thermostability compared to the wild-type. The melting temperature (T_m) analysis using circular dichroism revealed higher T_m values of 84.5 °C for A8P/S385E and 75.1 °C for A8V/S385E. This indicates that the enzyme can withstand higher temperatures before denaturation, a desirable trait in various industrial processes. Secondary structure analysis indicated alterations in the lipase structure caused by the simultaneous mutations. In summary, the simultaneous mutation at the C- and N-terminals had a multifaceted impact on the lipase, influencing its optimal temperature, thermostability, and structural characteristics. These findings provide insights into how specific genetic modifications can be employed to tailor the enzyme for improved performance in industrial applications.

Keywords: lipase; thermostability; secondary structure; melting temperature; double mutation

INTRODUCTION

Lipases (triacylglycerol acyl hydrolases; EC 3.1.1.3) are serine hydrolases that catalyze various bioconversion reactions. Under aqueous conditions, they catalyze the carboxyl ester bond of triacylglycerol liberating fatty acids and glycerol (Chandra et al., 2020; R. Gupta et al., 2004). Their physiological function is to hydrolyze triglycerides into diacylglycerol, monoacylglycerol, glycerol, and carboxylate. Because of their enzymatic activities, they are widely applied in biological detergent, food, and pharmaceutical industries (Yao et al., 2021). Microbial lipases have been preferred as biocatalysts over their animal and plant counterparts because of their stability in extreme temperatures and organic solvents (Bharathi & Rajalakshmi, 2019). Examples of such lipases are *Bacillus* lipases that belong to the subfamily five of a family I (I.5). These lipases share a conserved pentapeptide (Ala-X-Ser-X-Gly) comprising a catalytic serine residue (Kovacic et al., 2019).

Lipases with high thermal stability are highly sought after as they can be used in high-temperature

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industrial processes (Contesini et al., 2020; Gupta & Khare, 2009). A thermostable lipase designated L2 (Wt-L2) lipase, produced by *Bacillus* sp. strain L2 was previously identified by Shariff et al. (2007). The recombinant L2 lipase exhibited enzymatic activity at 70 °C and was found to be stable in reaction systems with a pH range of 9 to 10. The presence of metals such as Ca^{2+}, K^{+}, Na^{+}, and Mn^{2+} increased the activity of L2 lipase by more than 100% (Abd Rahman et al., 2012; Shariff et al., 2011). Additionally, the atomic structure of L2 lipase has been determined to have a high resolution of 1.5 Å (Abd Rahman et al., 2012).

The structural and functional information of L2 lipase allowed a rational design approach to produce lipase mutants with different properties (Sani et al., 2018). In a previous study, Sani et al. (2018) identified amino acid 385 at the C-terminus of L2 lipase to be critical for stability and activity. Substitution of Serine at position 385 into Glutamic acid increased the optimal temperature for the enzymatic activity to 80 °C, a 10 °C increase from its wild-type counterpart (Sani et al., 2018). Another study by Bukhari et al. (2020) indicated that a single substitution of amino acid located on the N-terminal (A8V and A8P) increased the stability of lipase at high temperatures compared to their wild-type L2 (Wt-L2).

Hence in this study, the effects of residue substitutions in both N- and C-terminals on the stability and activity of the lipase were evaluated. Based on the previous investigations conducted by Bukhari et al. (2020) which investigated N-Terminal mutations (A8V and A8P), and Sani et al. (2018) which focused on the C-Terminal mutation (S385E), the combination of mutation sites on N- and C-terminals (A8V/S385E and A8P/S385E) was conducted to study their effects on stability and activity. This study combines experimental evaluations of thermal stability with computational docking analysis to comprehensively understand how double mutations affect the lipase's functionality and interactions at the molecular level. This study highlights the significant roles of mutations in stability and enzymatic activity. While rational design typically focuses on catalytic site modifications, these findings demonstrate that targeted substitutions in the N-terminal region of L2 lipase contribute to enhanced thermal stability and catalytic efficiency, suggesting its involvement in protein stability. Similarly, mutations at the C-terminal region may either improve thermostability and enzymatic function or, conversely, disrupt structural integrity due to the intrinsic flexibility of this region. Given that the C-terminal residues in some proteins play crucial roles in stability and function, as reported by Gudiukaite et al. (2014), which emphasize the structural and functional importance of C-terminal residues in enzyme behavior.

MATERIALS AND METHODS

Expression and purification of mutant lipases A8V/S385E and A8P/S385E

Based on the previous studies conducted by Bukhari et al. (2020) and Sani et al. (2018), two double mutants, namely, A8V/S385E and A8P/S385E were generated by site-directed mutagenesis using a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, California) according to the manufacturer's protocol. The plasmid pET32(B)+ harbouring recombinant L2 (wt-L2) lipase was used as the template. The plasmid harbouring the double mutant gene was transformed into E. coli BL21(DE3) expression host. The clone was inoculated in Luria-Bertani broth (LB) medium supplemented with 100 μg/mL ampicillin at 37 °C in an incubator shaking at 180 rpm until the culture reached A600 of 0.6. Protein expression was induced using 0.75 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) at 37 °C for 16 h. The cells were harvested by centrifugation at 11,200 × g for 20 min. Subsequently, the harvested cells were resuspended in 10 mL of lysis buffer (50 mM Tris-HCl, 500 mM NaCl, and 30 mM imidazole, pH 8), and lysed using a sonicator at 50 kHz for four cycles (15 s) with 30 seconds rest intervals. The lysate was clarified by centrifugation at 11,200 × g for 20 min. The supernatant was filtered through a 0.45-μm filter, and loaded onto 5 mL Ni-Sepharose HP beads equilibrated with lysis buffer, and subsequently washed with 30 mL of lysis buffer. The protein bound was eluted by linear gradient using an elution buffer containing 20 mM sodium phosphate, 500 mM NaCl, and 500 mM imidazole. The eluted fractions were analysed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and lipase assay.

Lipase assay and protein concentration determination

Protein concentrations were determined by Bradford assay using the Bradford reagent from VWR Life Science AMRESCO (USA). The standard curve of protein determination was prepared using Bovine Serum Albumin (BSA) concentration ranging from 0-1 mg/ml as the standard curve. The protein quantitation was measured by reading the mixture at an absorbance of 595 nm.

Kwon and Rhee's method was applied for lipase activity determination. The substrate solution was prepared by mixing olive oil (Bertolli, Cordoba,

Spain) and 50 mM Glycine-NaOH buffer pH 9.0 with a 1:1 volume ratio. The reaction mixture contained 10 µL enzyme, 990 µL buffer, 20 µL of 20 mM calcium chloride, and 2.5 mL of substrate solution was incubated at 70 °C for 30 minutes with 200 rpm shaking. Afterwards, 1 mL of 6N HCl was added to stop the reaction followed by 5 mL of isooctane. The reaction mixtures were left for 30 minutes at room temperature. The upper layer of the reaction was mixed with copper pyridine solution pH 6.1 (1 mL) and the mixture was let to rest for 2 hours prior to absorbance measurement at 715 nm. The activity was defined as one unit (U) is the amount of enzyme that catalyzes the reaction of 1 µmol of substrate per minute.

Determination of temperature profile and pH stability of lipase mutants

The enzymatic activity was conducted using the Kwon and Rhee (1986) colorimetric method at the broad temperature from 45 to 80 °C with 5 °C intervals using olive oil as substrate. The enzyme solution was assayed for 30 minutes with 200 rpm shaking. The optimal activity of each lipase was recorded at 100 %.

The half-life of lipases at 60 °C was studied by pre-incubating the lipase samples at 60 °C for 16 hours. The samples of enzyme solution were taken out every 4 hours and the remaining activity was assayed at optimum temperature. The untreated enzyme was recorded as a control (100%).

For pH optimum determination, the lipase was assayed at different pH ranging from pH 4 to pH 11 using different buffers (50 mM sodium acetate buffer, pH 4, and 5; 50 mM sodium phosphate, pH 6, and 7; 50 mM Tris-HCl, pH 8 and 9; 50 mM glycine-NaOH, pH 10 and 11; and 50 mM disodium phosphate, pH 12.). These buffers were emulsified with olive oil with a ratio of 1:1 separately. All the experiments were conducted in three replicates. The activity was measured according to the standard assay condition.

Circular dichroism analysis

The secondary structure and thermodynamic stability of double mutant A8V/S385E and A8P/S385E were determined using JASCO J-810 CD spectropolarimeter (JASCO, Japan). The purified lipase 0.1 mg/mL was equilibrated in 5 mM sodium phosphate buffer (pH 8.0). 300 µL of the sample was used in the 0.1-cm path length cuvette and was measured at 20 °C. The structural elements of lipase mutants were estimated based on the far-UV spectral (190 - 240 nm). The spectral analysis of protein was subtracted with the subsequent blank containing 5 mM sodium phosphate buffer (pH 8.0). The structural element analysis was determined using Spectra Manager™ Suite Software (JASCO, Japan).

The denaturation of the protein was investigated by collecting the complete spectra as a function of temperatures. The warming period was set from 20 to 100 °C with a heating rate of 1 °C/min at 222 nm of wavelength. The denaturation temperatures (T_m) were defined as the point at which 50 % of the protein sample denatured.

Homology modeling and docking analysis

Yet Artificial Reality Application (YASARA) software was applied for lipase structure prediction (Krieger and Vriend, 2015). The open structures of wt-L2 lipase and its mutants A8V/S385E and A8P/S385E were modeled using *Geobacillus thermocatenulatus* lipase (PDB ID: 2W22) as a template (96 % identity). The model structures were uploaded to SAVES v6.0 (https://saves.mbi.ucla.edu/) for structure evaluation using ERRAT, Verify3D, and Ramachandran plot (Colovos & Yeates, 1993; Laskowski et al., 1993).

The ligand p-nitrophenyl decanoate (pNP-C10) was retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The C10 p-nitrophenyl substrate was docked into the lipase structure to study the ability of the lipase to catalyze the substrate. The specific protein-ligand docking of lipase was investigated using the YASARA software package, using autodock VINA and AMBER03 force field. The simulation cell was prepared around the catalytic triad residues. The grid size was automatically determined by YASARA software package. The structures of the lipase and substrate were energy-minimized prior to the docking analysis. This docking study was performed in 25 runs. The docking results were ranked based on AutoDock VINA's scoring function, which estimates binding affinity in kcal/mol. Positive energies indicate stronger binding, and negative energies represents no binding. The visual analysis of structures and preparation of figures was carried out using YASARA and LigPlot+ software (Krieger and Vriend, 2015; Laskowski and Swindells, 2011).

RESULTS AND DISCUSSION

Temperature and pH profiles

All lipases were purified by one-step affinity chromatography using Ni-Sepharose purification resin (Supplementary Figure 1). The recombinant double mutants A8V/S385E and A8P/S385E were evaluated for their enzymatic activities at various temperatures ranging from 40 to 80 °C (Figure 1a). Recombinant wt-L2 lipase exhibited the highest

activity at 70 °C. Intriguingly, A8V/S385E and A8P/S385E displayed the optimum enzymatic activity at 50 °C. This is significantly lower than its wild-type and single mutant S385E counterparts that exhibited optimum enzymatic activities at 70 °C and 80 °C, respectively (Bukhari et al., 2020; Sani et al., 2018). This indicated that A8P/S385E and A8V/S385E lipases became less active at high temperatures when the double mutant was incorporated.

The half-life of macromolecules is outlined as the time required for the target protein concentration to decrease by 50% relative to the extent of the initial form of the molecules (Zhou, 2004). Protein half-life is a crucial aspect of proteostasis, which is involved in many cellular processes and thus determines protein functionality (Rahman & Sadygov, 2017). Measuring a protein's half-life is essential to know how long the specific protein can retain its function when exposed to a particular temperature. The longer the protein can retain its activity, the more valuable the protein is in industry. In this study, we found that A8P/S385E lipase had 50% remaining activity after 7 hours of incubation at 60 °C (Figure 1b). The recombinant wt-L2 and A8V-S385E lipases had 50 % remaining activity after 3 hours of incubation at 60 °C. The combination of these two mutations (A8P/S385E and A8V/S385E) was expected to increase in thermal stability more than its single mutants (A8P, A8V, and S385E), which previously reported (Bukhari et al., 2020; Sani et al., 2018). Contrary to expectations, the double mutations reduced the optimal temperature for activity. A similar antagonistic effect was reported in ELBn12 lipase, where the substitution of Lys173 to Ala and Gln177 to Ala in the double mutant K173A/Q177A resulted in lower stability compared to its single mutants (K173A and Q177A) when exposed to 60 °C (Farrokh et al., 2018). In industrial applications, half-life is an important characteristic as it indicates an enzyme's stability over time, directly affecting efficiency and the economic feasibility of long-duration operations. In contrast to the wild-type (WT), the mutant A8P/S385E lipase showed a longer half-life, maintaining 50% of its activity after 7 hours. For long-duration applications like bioconversion or continuous processing, where prolonged enzymatic activity is crucial for maintaining productivity and reducing enzyme replacement costs, the increased stability of A8P/S385E makes it more beneficial. With its extended half-life, this variant may be better suited for industrial processes requiring sustained operational stability. Purified A8V/S385E and A8P/S385E lipases were assayed at different pH (pH 4 to 11) using different buffer systems includes 50 mM Sodium acetate pH 4-5, 50 mM Sodium phosphate pH 6-7, 50 mM Tris-HCl pH 8-9, 50 mM Glycine-NaOH pH 10-11, and 50 mM disodium phosphate pH 12. Based on Figure 2, mutants A8P/S385E and A8V/S385E were stable at a pH range of pH 7 – pH 10. Meanwhile, wt-L2 lipase was stable within the range of pH 8 – pH 10. The amino acid substitution was found to have a shift preference towards pH, as reported by Guan et al. (2020), in a study involving mutants generated from recombinant lipase of *Pseudomonas fluorescens* lipase (PFL).

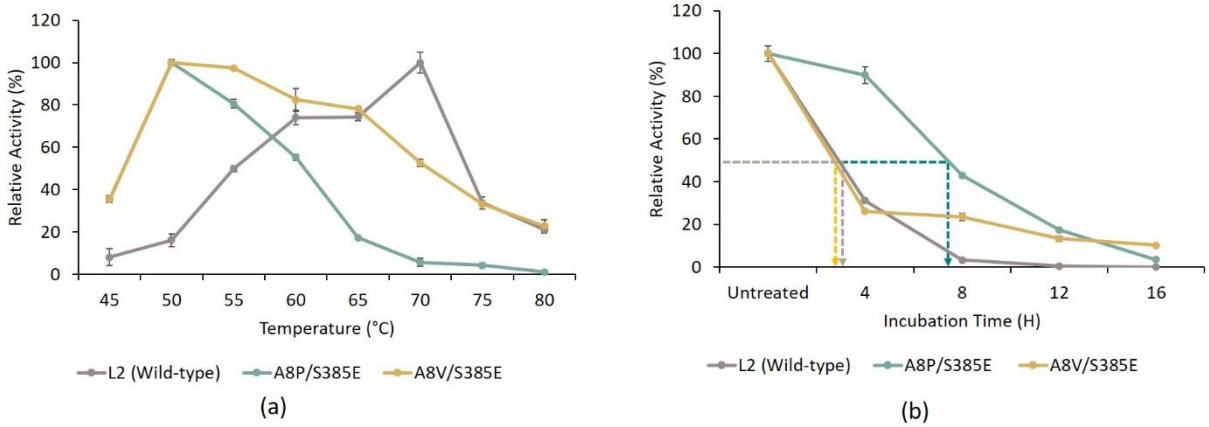

Figure 1. Effect of temperature on activity and stability of lipases. (a) Temperature profile of lipases. (b) Half-life study of lipases. The half-life of each lipase was remarked with the dotted line. The experiments were conducted in triplicate. The data are expressed as the mean ± standard deviation.

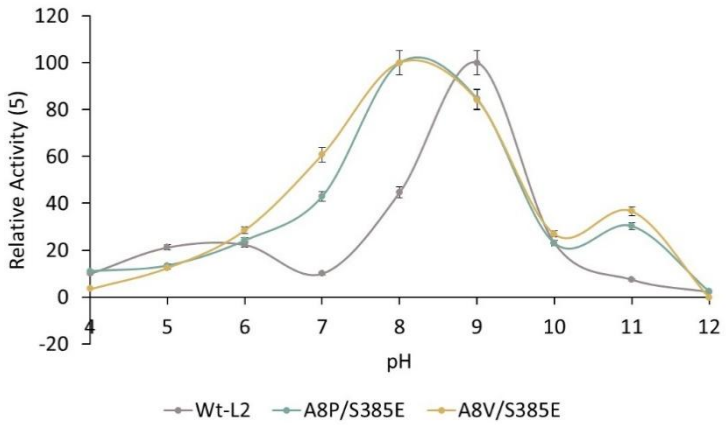

Figure 2. pH stability profile of the wt-L2 lipase and its variants. The buffer systems used in this experiment: 50 mM Sodium acetate (pH 4-5), 50 mM Sodium phosphate (pH 6-7), 50 mM Tris-HCl (pH 8-9), 50 mM Glycine-NaOH (pH 10-11), and 50 mM disodium phosphate (pH 12). The experiments were conducted in triplicate. The data are expressed as the mean ± standard deviation.

Circular dichroism analysis.

Circular dichroism (CD) spectroscopy is briefly defined as the different absorption of left-handed and right-handed circularly polarized light. It is a beneficial experimental method for the immediate determination of the secondary structure analysis and the folding properties of proteins (Greenfield, 2007). The secondary structure of a protein could be affected drastically even if only one amino acid changes in the primary sequences. The modification of the primary sequence might affect the formation of hydrogen bonds, affecting the secondary structure elements in protein. As reported, the substitution of amino acids was found to alter the secondary structure elements of lipase (Tian et al., 2021).

Based on data presented in Table 1, the mutant lipase A8V/S385E contains 41.8 % of α-helix, 10.5 % of β-pleated sheets, 16.3 % β-turn, and 31.4 % random structure, meanwhile, wt-L2 lipase structurally consists of 38.6 % of alpha helix, 2.2 % beta-pleated sheets, 23.6 % turn, and 35.6 % of the random structure. The results were significantly different from the secondary structure of mutant A8P/S385E, which possessed 18.0 % α-helix, 22.9 % ß-sheet, 30.6 % ß-turn, and 28.5 % random coil. The mutant A8P/S385E showed a decrease in α-helix percentage and an increase in the percentage of ß-sheet and ß-turn. The reduction in α-helix content in the A8P/S385E mutant indicates structural alterations that could affect enzyme flexibility. α-helices are commonly linked to preserving protein stability and structural integrity, particularly at elevated temperatures. A decrease in α-helix content might result in reduced structural stability, potentially make the enzyme less tolerant to temperature changes and lowering the optimal temperature. On the other hand, the increased in β-sheet and β-turn in A8P/S385E could lead to a compact structure, potentially enhanced the short-term stability while maintained conformational flexibility for effective catalysis at higher temperatures. The changes in secondary structure might explained the enhanced of enzyme stability for A8P/S385E mutant over time while having a lower optimal temperature for activity than the wild-type. The role of β-sheet content in enhancing structural rigidity while potentially reducing the flexibility needed for optimal enzymatic activity at higher temperatures has been previously discussed by Stapley & Doig (1997). Nonetheless, additional structural and dynamic investigations, like molecular dynamics simulations, may offer greater understanding of how these alterations affect enzyme activity.

Melting temperature is the temperature where the protein's folded conformation equals the unfolded conformation, also known as the temperature of heat denaturation (Sani et al., 2018). To investigate the effects of mutating residues at both the N- and C-terminals of the wt-L2 lipase on the stability of the lipase, the melting temperatures (T_m) of double mutants A8V/S385E and A8P/S385E were measured using Spectra Manager TM Suite Software (Japan Spectroscopic Company, Tokyo, Japan) (Figure 3). The comparison of previously characterized wt-L2 lipase and single mutants (A8P, A8V, and S385E) revealed that combination of mutation at the C- and N-terminal does affect the melting temperature of lipase (Table 2). Mutant A8V/S385E notably has a lower melting temperature than mutant S385E, however, it showed a high denaturation temperature compared to its single mutant A8V. Meanwhile, mutant A8P/S385E successfully retained its melting temperature compared to single mutant S385E and showed a high denaturation temperature compared to mutant A8P.

Table 1. Estimated secondary structure composition (%) of lipases.

Secondary structure	A8P/S385E	A8V/S385E
α-helix	18.0	41.8
β-sheet	22.9	10.5
Turn	30.6	16.3
Random	28.5	31.4

Table 2. Melting temperature analysis of lipase variants.

Lipase	A8P/S385E	A8V/S385E
Melting temperature (T_m) (°C)	84.5	75.1

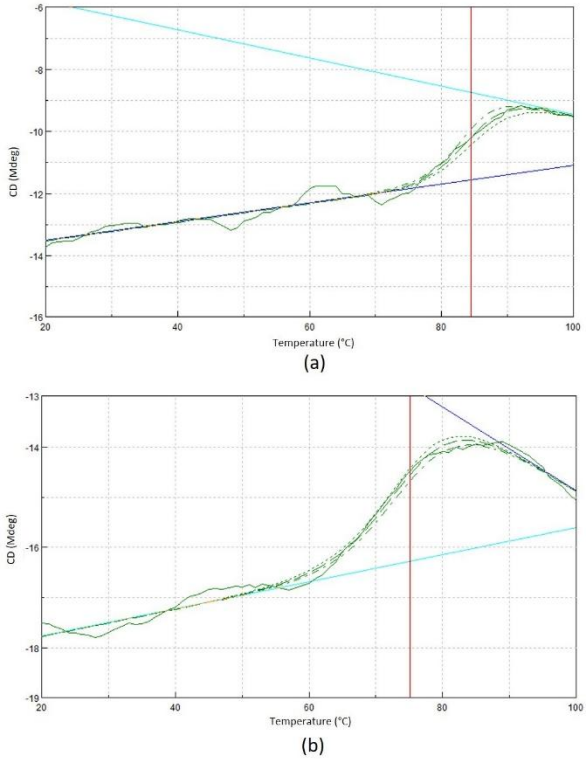

Figure 3. Melting temperature (T_m) profile of lipases. (a) A8P/S385E and (b) A8V/S385E. the melting temperature of lipases was evaluated using circular dichroism (CD) spectroscopy Spectropolarimeter J-810 (JASCO, Japan) from temperatures 20 to 100 °C.

All mutants showed a high denaturation temperature when compared with their wild-type L2. Another study by Zhang et al. (2022) reported that the cumulative five mutations of *Proteus mirabilis* lipase (PML), resulted in an increase in melting temperature by 10.6 °C of the wild-type. The enhancement of melting temperature was also achieved by mutant D25R of *Penicillium camembertii* (PCL) lipase and mutants L218P and P184C/M243C of *Pseudomonas fluorescens* lipase (PFL) (Guan et al., 2020; Liu et al., 2018). The melting temperature (T_m) analysis demonstrated that mutations at both the N- and C-terminals significantly influenced L2 lipase stability. While A8V/S385E exhibited a lower T_m than S385E, it retained a higher denaturation temperature compared to A8V, suggesting that its combined mutations introduced structural changes affecting stability differently than single substitutions. Similarly, A8P/S385E maintained its T_m relative to S385E but had a higher denaturation temperature than A8P, indicating that the proline substitution contributed to thermal resistance despite potential rigidity. Compared to previous study where strategic mutations generally enhanced thermostability (Zhang et al., 2022; Guan et al., 2020; Liu et al., 2018), our findings suggests that terminal modification on N- and C-terminals does not produce straightforward stabilizing effects. The observation in secondary structural changes, particularly the decrease in α-helix content and increase in β-sheet and β-turns, may have altered the enzyme's conformational flexibility, leading to differences in thermal stability and optimal activity temperature. These findings highlight the complex interplay between structural elements and thermostability, reinforcing the need for case-specific analysis in protein engineering.

The lid of L2 lipase is a flexible helix that covers the catalytic pocket and isolates the active site from solvent. The lid is formed by helix α6, which is part of an α/β hydrolase fold (Rahman et al., 2012). According to Ericsson et al. (2008), lipase exhibits interfacial activation, where a conformational change in the lid reg ion exposes the active site upon interaction with a lipid-water interface, enhancing substrate binding and catalysis. This structural flexibility facilitates high activity at low temperatures by reducing the energy barrier for substrate access. Despite this flexibility, the enzyme maintains a high melting temperature due to strong hydrophobic core interactions and stabilizing secondary structures makes the lipase more rigid, ensuring thermal stability.

Modeling and molecular docking analysis

Following the result of single substitution on N- and C-terminals, which generate more stable lipase, both mutations were combined to study their effects on the stability and activity of lipase. Mutants A8V, A8P, and S385E show increasing stability compared to their wild-type L2 lipase (Bukhari et al., 2020; Sani et al., 2018). Hence in this study, the combination of mutation sites on N- and C-terminals (A8V/S385E and A8P/S385E) was conducted to reveal their effects on lipase structure. The *in-silico* mutations were prepared using Yet Artificial Reality Application (YASARA) software. Since the wt-L2 structure (PDB ID: 4FDM) is a closed lid formation, the open structures of wt-L2 lipase and its mutants were predicted using the open lid formation *Geobacillus thermocatenulatus* lipase (PDB ID: 2W22) to ensure the proper binding simulation between the enzyme and the substrate. The predicted structures were validated using Ramachandran Plot, ERRAT, and Verify_3D. Table 3 shows the summary of the evaluation score, which concluded that all the models are within good quality models. Ramachandran plot delivers overall stereo-chemical quality, local, and residue-by-residue-reliability (Figure 4). It represents all possible protein structures in the dihedral angles of the polypeptide chain. None of the residues for all structures reside in the disallowed region. Ramachandran plot is important to determine the quality and any potential errors of protein model. The Ramachandran plots of wt-L2, A8P/S385E, and A8V/S385E models were generated using online server UCLA-DOE LAB - SAVES v6.1. The ERRAT analysis of all structures showed an overall quality value of 96 %.

All the structures were then superimposed to analyse their similarities and differences. There is no notable alteration when the modeled structures of A8P/S385E and A8V/S385E are superimposed with the wt-L2 modeled structure. The model structures of mutants A8P/S385E and A8V/S385E showed a root mean square deviation (RMSD) of 0.722 Å and 0.704 Å when superimposed with the wild-type L2 open structure model, respectively.

Table 3. Summary of the evaluation of the predicted structure.

Lipase structure models	**Ramachandran Plot**			**ERRAT score**	**Verify_3D (% of 3D-1D score)**
	Residues in most favoured regions (%)	Residues in additional allowed regions (%)	Residues in generously allowed regions (%)		
Wt-L2	91.8	7.3	0.9	96.6	94.1
A8P/S385E	92.1	7.3	0.6	96.3	91.8
A8V/S385E	92.1	7.6	0.3	96.8	92.5

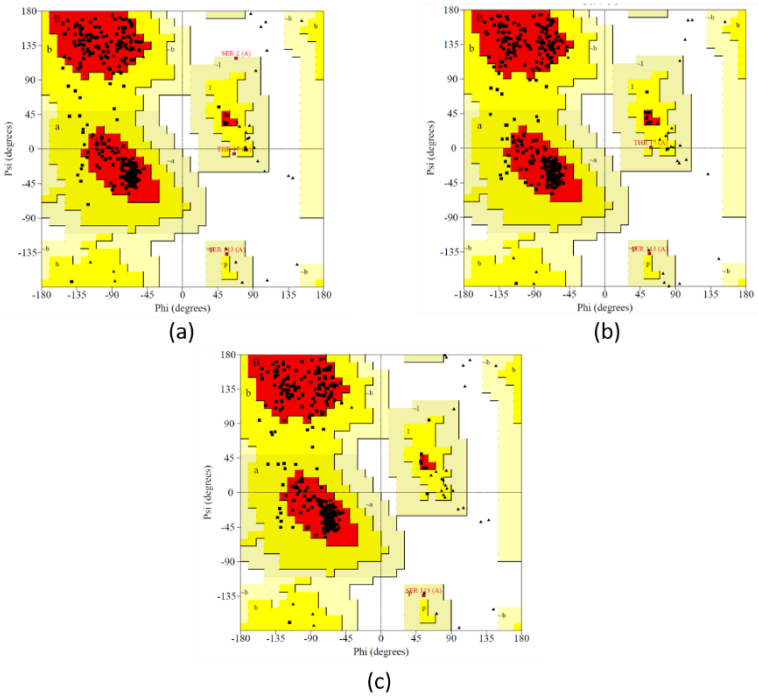

Figure 4. Ramachandran Plot of open structure wt-L2 and its mutants predicted using YASARA Software. (a) wt-L2 lipase (b) Mutant A8P/S385E (c) Mutant A8V/S385E.

To investigate the substrate binding of mutant lipase, docking analyses were conducted using YASARA software. The catalytic triad of wt-L2 lipase was reported to be Ser113, Asp317, and His358 (Abd Rahman et al., 2012). The specific docking analysis targeting the catalytic triad was performed by preparing the simulation cell around the catalytic triad to ensure the proper binding of the ligand. Based on the previous study, wt-L2 and single mutants preferred *p*Np-decanoate (C10) as substrate, hence, *p*Np-decanoate (C10) was chosen as the substrate for docking analysis. The three-dimensional model structure of wt-L2 and the mutants were docked with *p*Np-decanoate (C10) structure. The protein-ligand docking using YASARA showed a positive binding energy, which indicated stronger binding between a receptor and a ligand. YASARA scoring was defined as where positive energy means stronger binding and negative energy means no binding (Aamir et al., 2018). The protein-ligand docking showed an efficient, more robust, and stable binding of substrate with mutant lipases A8P/S385E and A8V/S385E with binding energy 5.98 and 4.96 kcal/mol, respectively. Meanwhile, the binding energy of wt-L2 is 4.58 kcal/mol. The contact residues involved in the binding of *p*Np-decanoate (C10) were visualized by Ligplot+ (Figure 5). Stronger binding energy is often associated with increased structural stability, however, it does not always correlate with optimal enzymatic activity at high temperatures. In some cases, stronger substrate binding reduces conformational flexibility, which is crucial for efficient catalysis at elevated temperatures. Additionally, excessive rigidity may limit substrate diffusion and product release, ultimately lowering overall catalytic efficiency (Almeida et al., 2024). This aligns with our findings, where despite increased stability, the mutants exhibited variations in optimal activity temperature, emphasizing the complex relationship between binding energy, structural dynamics, and enzyme performance.

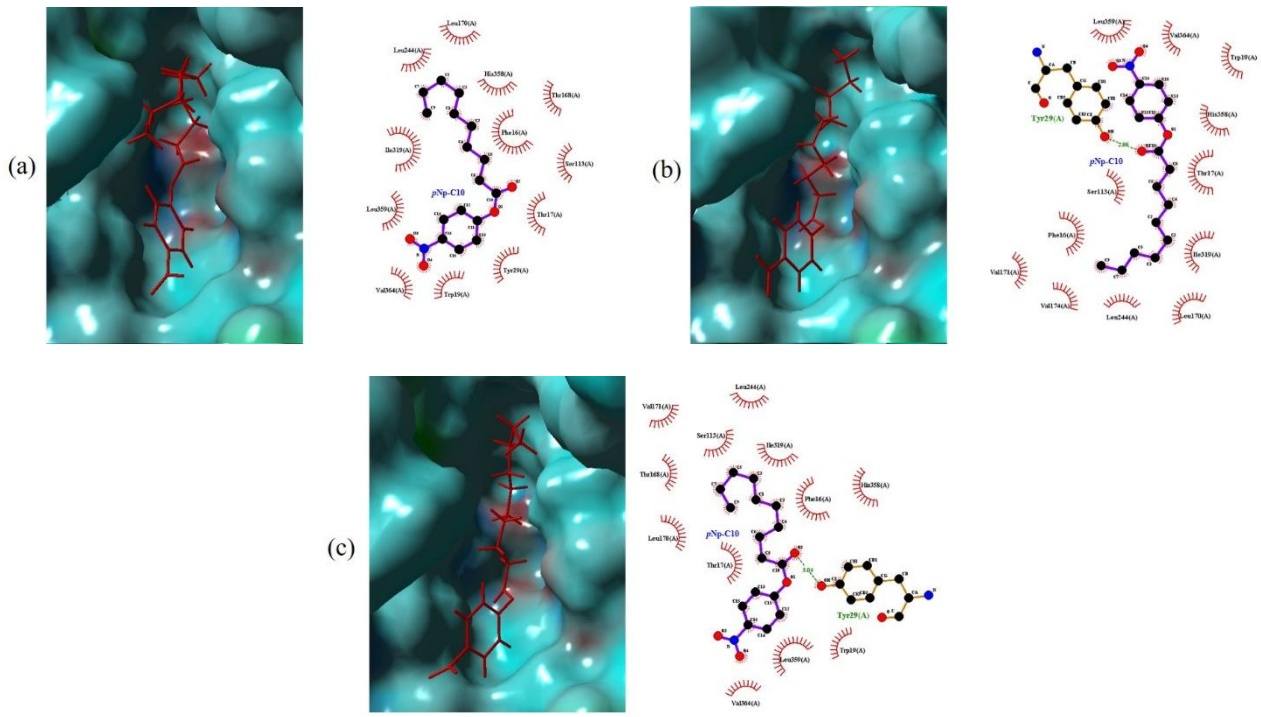

Figure 5. Molecular surface representation of lipases docked with *p*Np-C10 (The red stick represents ligand) and visual of Ligplot+ analysis (The dotted green line represents the hydrogen bonds, and the red spokes are representing the hydrophobic interactions. Ligands are coloured in purple. C, N, and O atoms are represented in black, blue, and red spheres, respectively. (a) wt-L2 lipase (b) A8P/S385E (c) A8V/S385E. The visuals were generated using YASARA and Ligplot+ (Krieger & Vriend, 2015; Laskowski & Swindells, 2011)

CONCLUSION

The simultaneous mutations introduced in L2 lipase significantly affected its activity and stability. The catalytic activity of the double mutants (A8P/S385E and A8V/S385E) shifted to 50 °C, which is 20 °C lower than the wild-type (wt-L2). Additionally, the mutations led to an increase in melting temperature and changes in secondary structure elements, as observed using circular dichroism spectropolarimetry. While studies on double mutations in proteins remain limited compared to single mutations, our findings highlight their significant impact on enzyme properties. Further research on double mutations is essential to enhance our understanding of their effects on protein stability and function. These insights could contribute to the rational design of enzymes with customized properties, optimizing their stability and activity for specific industrial and research applications. A more detailed comparison of the binding activities between the wild-type and mutant forms, particularly through RMSD values and stability analysis, would provide deeper insights into structural variations and their effects on enzymatic activity. Future studies should integrate these computational analyses to better correlate structural dynamics with functional performance, further refining the rational design of thermostable enzymes.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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