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Altering the Regioselectivity of T1 lipase from *Geobacillus zalihae* toward sn-3 acylglycerol by a rational design approach

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Abstract: The regioselectivity characteristic of lipases facilitate a wide range of novel molecules unit construction and fat modifications. Lipases can be categorized as sn-1,3, sn-2, and random regiospecificity. *Geobacillus zalihae* T1 lipase catalyzes the hydrolysis of the sn-1,3 acylglycerol chain. The T1 lipase structural analysis shows that oxyanion hole F16 and its lid domain undergoes structural rearrangement upon activation. To study the structure of these parts and function, site-directed mutagenesis performed by substitution of the lid domain residues F180G/F181S and the oxyanion hole residue F16W. The novel lipase (3M) has switched the regioselectivity from sn-1,3 to only sn-3. The (3M) shifted the optimum pH to 10, altered selectivity toward p- nitrophenyl ester selectivity to C14-C18, and maintained similar catalytic efficiency of 518.4×10-6 (s-1 /mM). The secondary structure of 3M lipase has contained 15.8% and 26.3% of α-helix and β-sheet, respectively, with a predicted Tm value of 67.8 °C. In silico analysis was conducted to reveal the role of F180G/F181S/F16W mutations at the atomic level in blocking the binding of the sn-1 acylglycerol chain and orientating the substrate to bond to the sn-3 acylglycerol which resulted in switching the T1 lipase regioselectivity.

Keywords: T1 lipase; sn-3 regioselectivity; kinetic study; MD simulation; Molecular docking.

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Supplementary figures and tables

Table S1: Characteristic of lipase variants.

	Optimum Temperature (°C)	pH optimum	Melting Temperature (T_m, °C)
wt-T1	70	9	73.5±0.05
F180G/F181S	70	9	66.19±0.06
F180G/F181S/F16D	70	9	67.29 ±0.16
F180G/F181S/F16Y	70	9	73.3±0.3
F180G/F181S/F16L	70	9	65.85±0.1
F180G/F181S/F16N	70	9	65.81±0.1
F180G/F181S/F16H	70	10	66.47±0.3
F180G/F181S/F16V	60	9	70.45±0.2
F180G/F181S/F16W (3M)	70	10	67.85±0.06
F180G/F181S/F16I	70	10	65.38±0.2
F180G/F181S/F16S	70	10	68.09±0.1
F180G/F181S/F16C	70	10	68.82±0.2
F180G/F181S/F16G	70	9	65.12±0.2

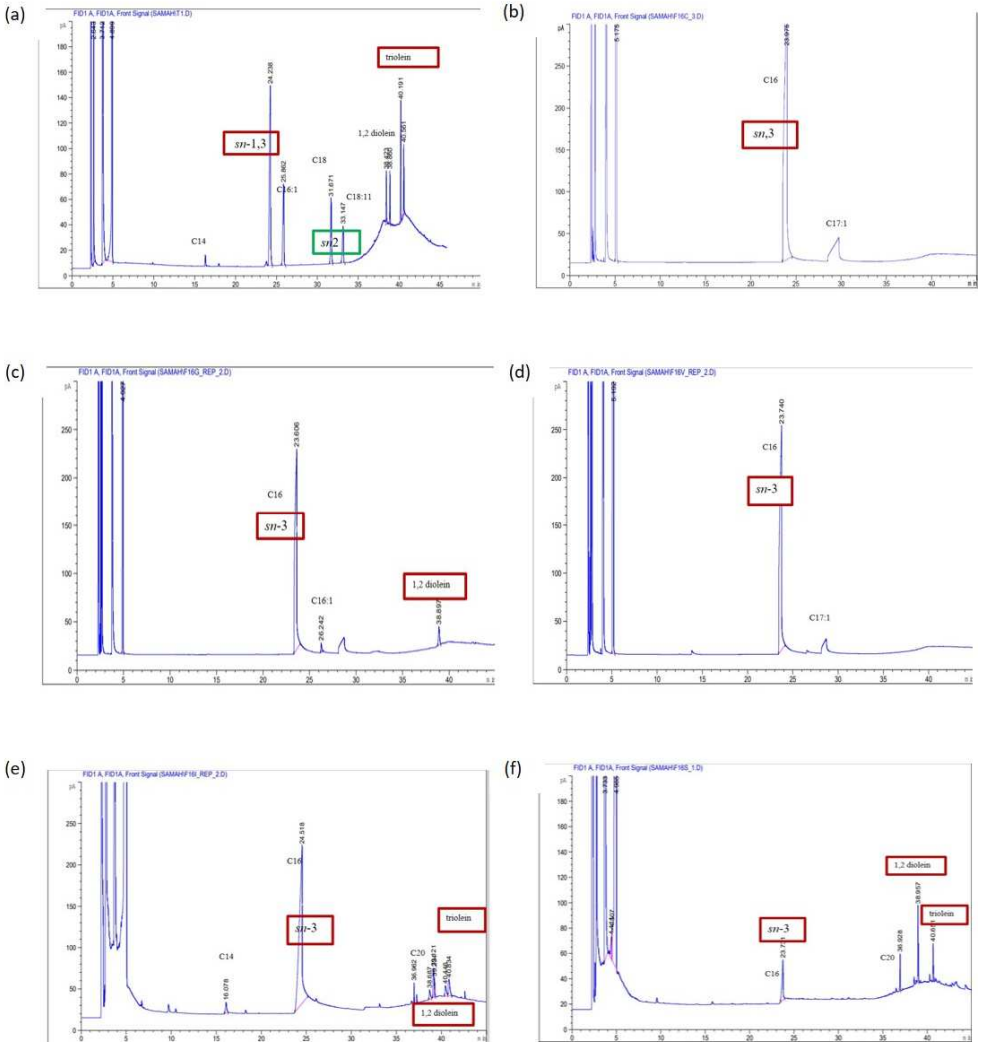

Figure S1: Gas chromatography flame ionization detector (GC FID) analysis for *sn*-3 modified regiospecific variants. (a) wt-T1 (b)F16C (c)F16G (d)F16V (e)F16I (f) F16S respectively. The acylglycerol position highlighted with red box.

Table S2: Purification table of wt-T1 and its 3M variant lipases.

Lipase	Purification step	Volume mL	Total protein (mg)	Total activity (U)	Specific Activity (U/mg)	Yield (%)	Purification fold
wt-T1	Crude	20	92.2	9690	105.1	100	1
	Affinity	10	17.4	5311	305.2	54.8	3
3M	Crude	20	58	2220	38.3	100	1
	Affinity	10	17	1968	115.8	88.6	3

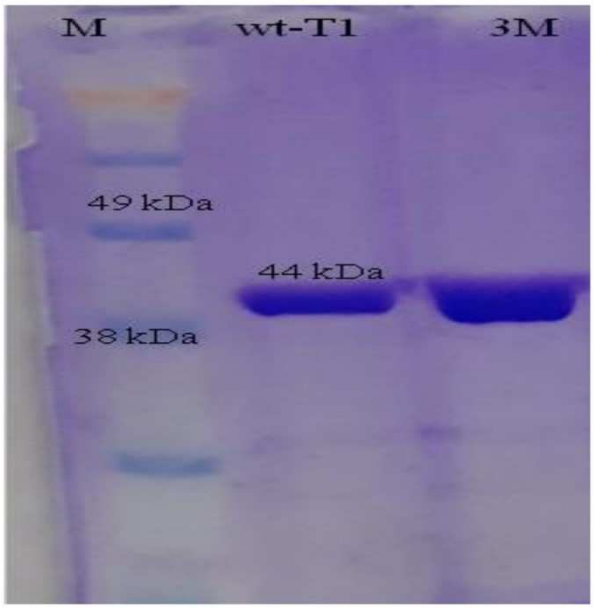

Figure S2: Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified wt-T1 and 3M variant lipases.

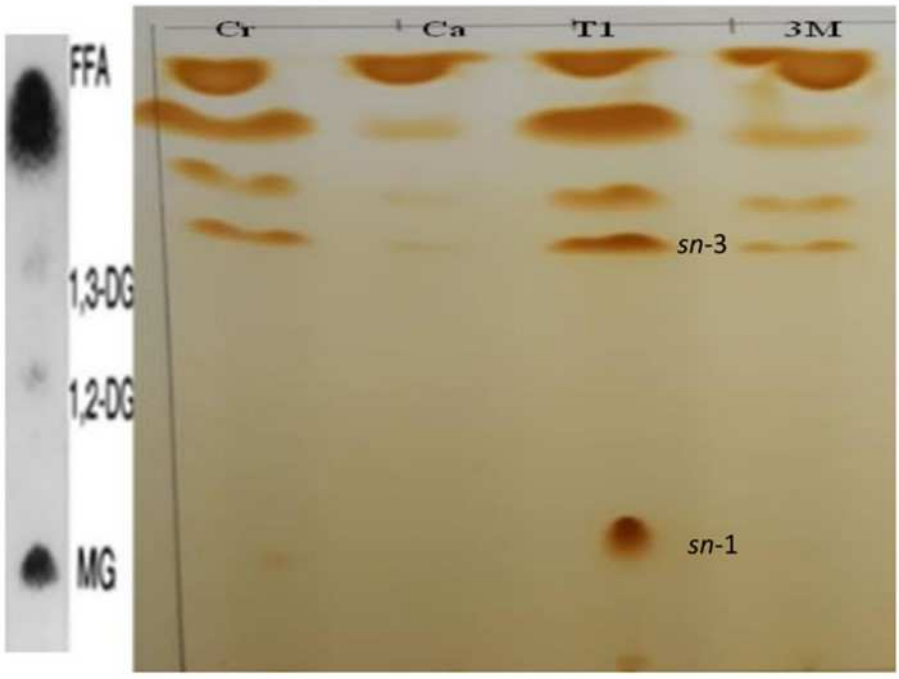

Figure S3: Thin-layer chromatography (TLC) sheet of purified wt-T1 and 3M variant lipases: Cr= *Candida rugosa* lipase, Ca= *Candida antarctica* lipase, T1= wt-T1 lipase, 3M= 3M variant lipase, respectively.

Table S3. Biochemical characterization of wt-T1 and its variant 3M

Lipase	T_{Opt}[1](°C)	pH_{opt}	T_m[2]	Energy changes[3] (kcal/mol)	Dissociation constant (pM)
wt-T1	70	9	73.5	-7.2360	47742780.0
3M	70	10	67.8	-5.6440	19516978.0

[1]Optimum temperatures and optimum pH of wt-T1 and 3M variant were determined using *p*NP-C12 as substrate. [2]The melting temperature of wt-T1 and 3M lipases. [3]Energy changes were calculated using YASARA software .YASARA definition of binding energy is more positive energies indicate stronger binding, and negative energies mean no binding.

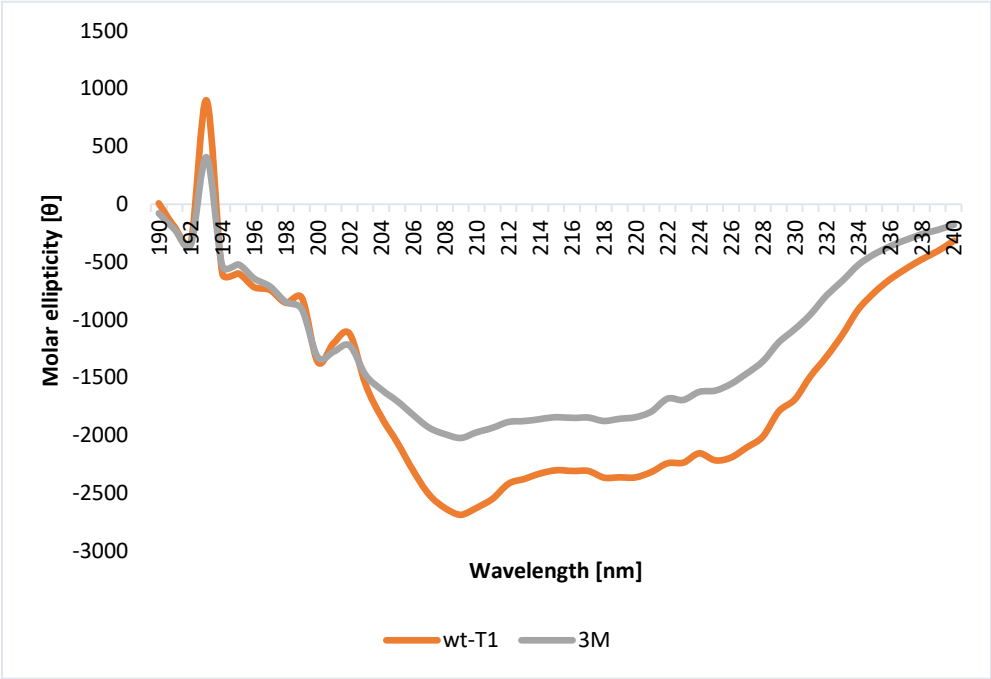

Figure S4: Circular dichroism (CD) spectra analysis of wt-T1 and 3M lipases at 190 to 240 nm. All scans were performed at temperature of 20 °C. Analysis of CD

spectral reference based on Yang [58], showing α-helix (190–220), beta sheet (194–204), turn (199–240) and random coil (190–193).

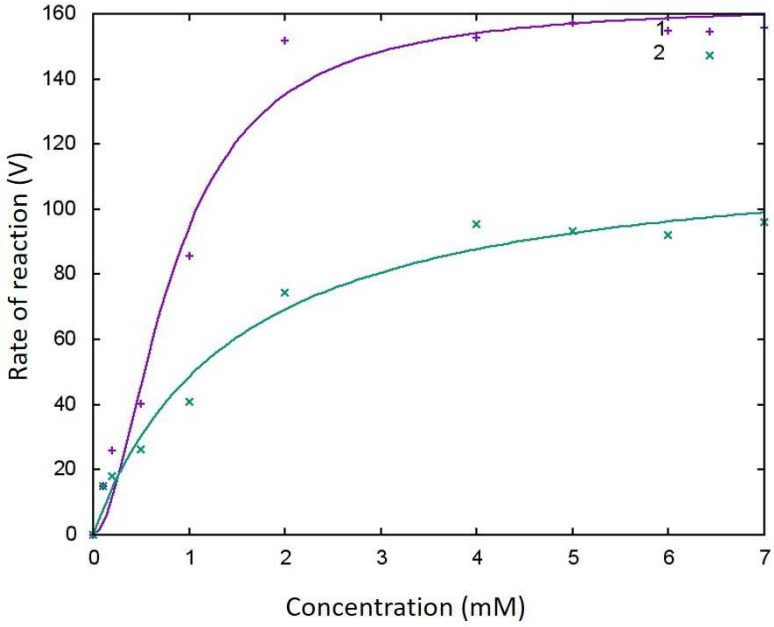

Figure S5. The change in the initial rate of the reaction at different concentrations of *p*NP-12 for wt-T1 (purple line) and 3M (green line) are shown. The figure is generated from online tool http://www.ic50.tk/kmvmax.html.

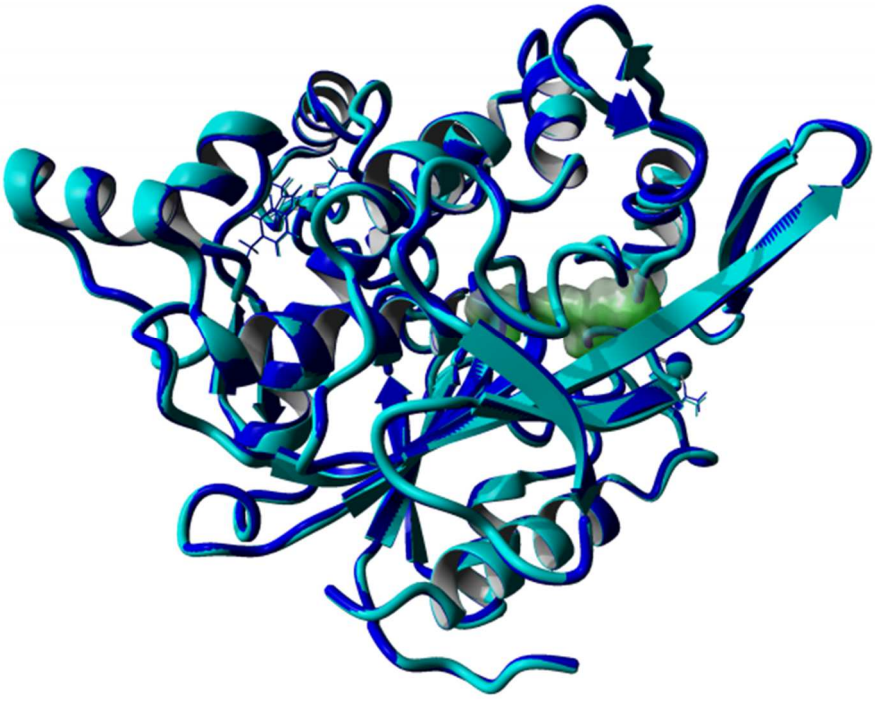

Figure S6: Superimposition of open conformation wt-T1 (blue) and 3M variant (cyan) structures. The green surface residues represent the catalytic triad.

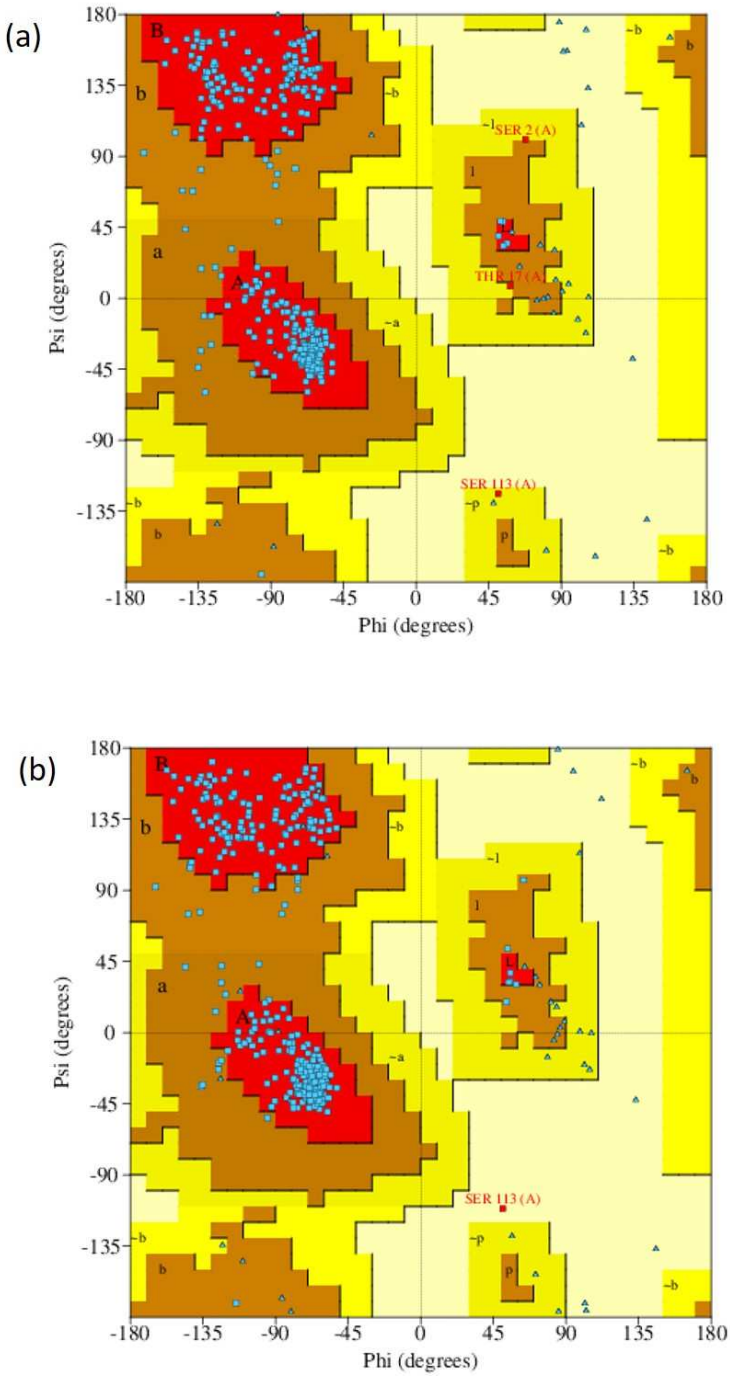

Figure S7. Ramachandran plot of wt-T1 (a) and 3M (b) lipases. The images generated using PDBsum (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html)

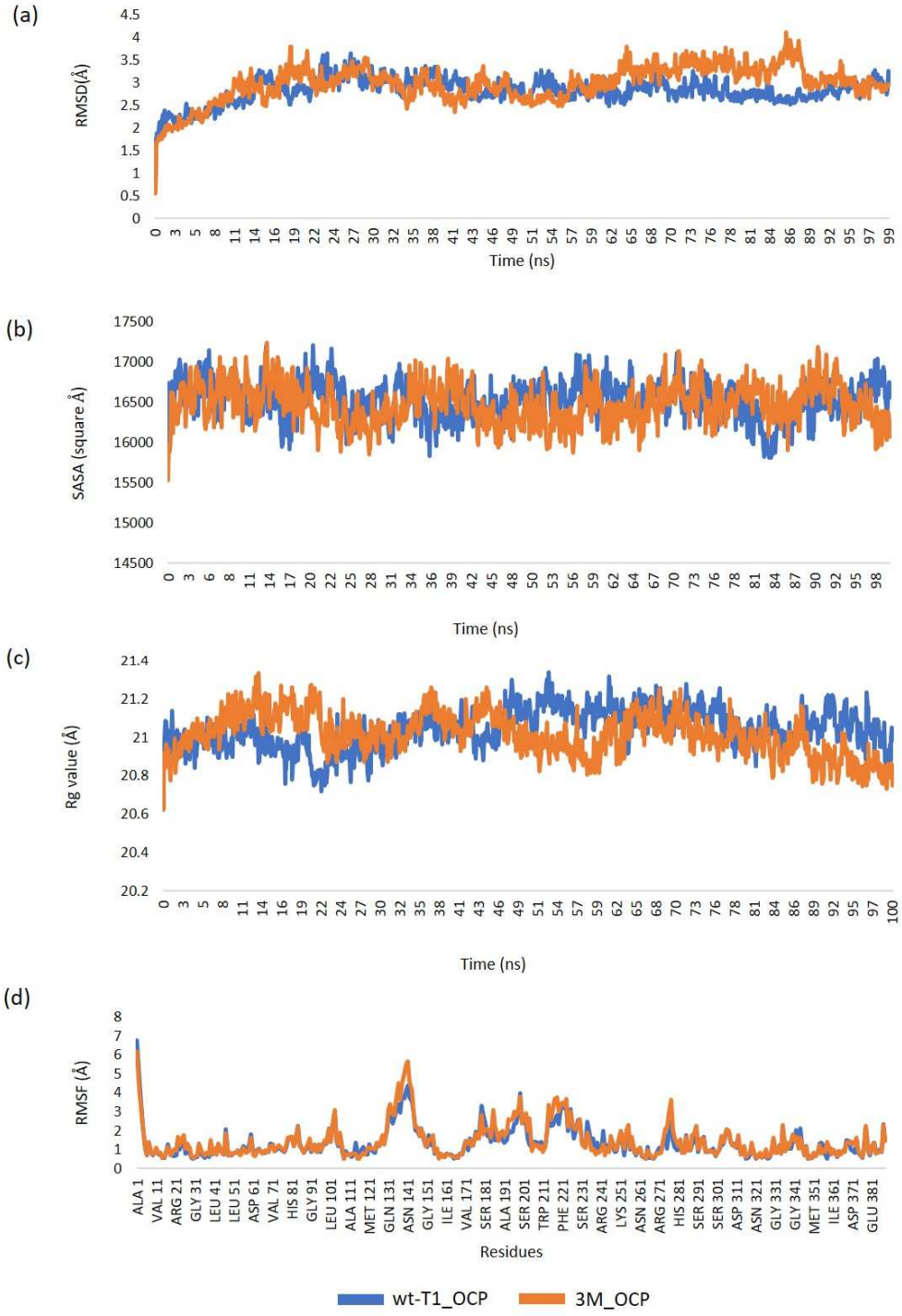

Figure S8. Molecular dynamics simulation analyses of lipases-complexed with the substrate (OCP). (a) Root Mean Square Deviation (RMSD). (b) Accessible surface area (SASA). (c) Radius of gyration. (d) Root means square fluctuation (RMSF).

Table S4. Residues with higher RMSF value peak and their corresponding location on wt-T1 lipase structure.

Residue	Lipase	RMSF (Å) range	Structural part

Ala1-Ala5 (Peak 1)	wt-T1	1-6.7	N-terminal
	3M	1-6.1	
pro95- Arg106 (peak 2)	wt-T1	1.1-1.8	Coil
	3M	1.2-1.9	
Ser130-Asn141 (Peak 3)	wt-T1	1.9-4.3	α5-helix
	3M	2-5.6	
Val171-Lys 185 (Peak 4)	wt-T1	1.4-2.5	α6-helix
	3M	1.4-3	
Val193-Tyr 199 (Peak 5)	wt-T1	2-3.9	coil
	3M	2.6-3.8	
Arg214-His223 (Peak 6)	wt-T1	2.7-3.2	Coil and α7-helix
	3M	3.3-3.7	
Tyr273-Leu277 (Peak 7)	wt-T1	1.3-2.4	turn
	3M	1.3-3.6	

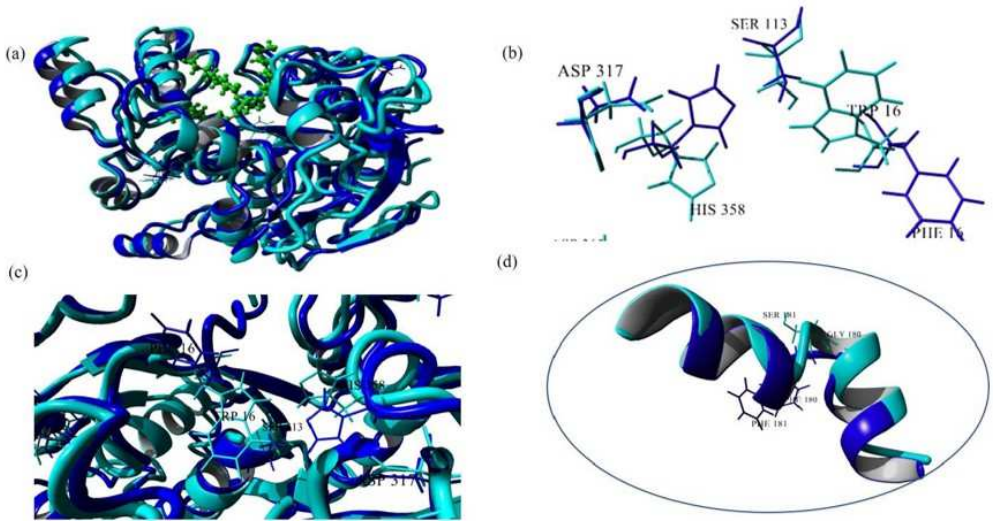

Figure S9. Superimposition of wt-T1 and 3M variant structure complexed with the substrate (OCP). (a) Global structure of wt-T1 and *sn*-3-regiospecific variant structure (b) Close up of the catalytic site residues (Asp317, His358, and Ser113). (c) The ribbon of binding site residues (d) Close up of the lid structure of lipases. The wt-T1 coloured in blue and 3M coloured in cyan.