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# Enhancing activity and thermostability of Lipase A from *Serratia marcescens* by site-directed mutagenesis

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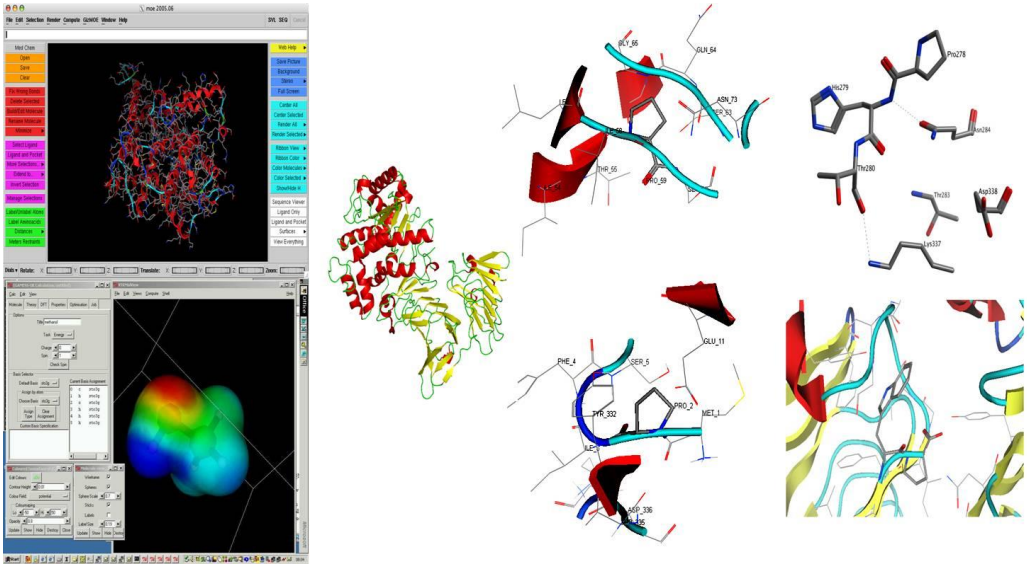
## Graphical abstract



## Highlights

- We examine the effect of site-direct mutagenesis on the thermal stability and activity of SML.
- Four mutant lipases of SML were constructed by site-direct mutagenesis
- The thermal stability and activity of SML (wild type and mutants) was compared.
- Using protein modeling program and creating mutation, can enhance lipase activity and/or thermostability of SML

## Abstract

Lipases as significant biocatalysts had been widely employed to catalyze various chemical reactions such as ester hydrolysis, ester synthesis, and transesterification. Improving the activity and thermostability of enzymes is desirable for industrial applications. The lipase of *Serratia marcescens* belonging to family I.3 lipase has a very important pharmaceutical application in production of chiral precursors. In the present study, to achieve improved lipase activity and thermostability, using computational predictions of protein, four mutant lipases of SML (MutG2P, MutG59P, Mut H279K and Mut L613W A614P) were constructed by site-directed mutagenesis . The recombinant mutant proteins were over-expressed in *E. coli* and purified by affinity chromatography on the Ni-NTA system. Circular dichroism spectroscopy, differential scanning calorimetry and kinetic parameters (Km and kcat) were determined. Our results have shown that the secondary structure of all lipases was approximately similar to one another. The MutG2P and MutG59P were more stable than wild type by approximately 2.3 and 2.9 in $T_{1/2}$, respectively. The catalytic efficiency (kcat/Km) of MutH279K was enhanced by 2-fold as compared with the wild type ($p<0.05$). These results indicate that using protein modeling program and creating mutation, can enhance lipase activity and/or thermostability of SML and it also could be used for improving other properties of enzyme to the desired requirements as well as further mutations.


**Key words**: Family I.3 Lipase, Site-directed mutagenesis, *Serratia marcescens*, Differential scanning calorimetry , CD spectra

# 1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are serine hydrolases that catalyze both the hydrolysis and the synthesis reactions [1]. Moreover, microbial lipases do not require cofactors and possess broad substrate specificity, hence are widely used in a variety of industrial applications such as the synthesis of fine chemicals and optically active pharmaceuticals [2]. Lipases have been widely produced by microorganisms, mainly bacterial, and are commercially important due to their diversity in catalytic activity, high yield and low cost production, as well as the relative ease of genetic manipulation [3, 4]. Bacterial lipases based on the difference in amino acid sequence and biochemical properties classified into eight different families (I-VIII). Family I, being the largest group and is further divided into seven subfamilies (I.1-I.7) with the first three subfamilies (I.1-I.3) including of gram-negative bacteria true lipases [5, 6]. Subfamily I.1 and I.2 lipases share relatively high amino-acid sequence similarity, in-contrast subfamily I.3 lipases possess low amino-acid sequence similarity (<20%) to either family I.1 or family I.2 lipases [7]. In the other hand, those have higher molecular size than lipases from subfamilies I.1 and I.2 and both the lack of Cys residue [8]. Family I.3 lipases, which are represented by lipases from *Serratia marcescens* (SML) and *Pseudomonas fluorescens*, are separated from other lipases not only by their amino acid sequences but also by their secretion mechanisms and biological properties [9]. The SML is composed of 613-614 amino acid residues with a molecular weight between 64-65kDa [10, 11]. The lipase A from *Serratia marcescens* has been the subject of research for more than 30 years, as it is used for production the large- scale kinetic resolution of racemic 3-(4-methoxyphenyl) glycidic acid methyl ester which it is a chiral precursor for diltiazem synthesis, a calcium-channel blocker and coronary vasodilator [12] . In recent years, many efforts have been made to improve lipase activity, and in particular, to enhance lipase stability. Various strategies to protect lipases from inactivation and to increase the operational

stability and activity of lipases have been developed, including the use of chemical modification of structure, protein engineering, medium engineering, etc [13]. Many factors are that influence enzyme stability, which of all potentially deactivating factors, temperature is the best-studied [14]. In order to use lipases as biocatalysts in industrial applications, it is desirable improving properties such as the thermostability and activity, that prolongs product shelf half -life, increase energy efficiency, and save costs hence, there is great interest to generate enzymes with desired properties [15-17]. Nevertheless to achieve the above objective, requires the use of the modern methods of genetic engineering combined with an increasing knowledge of the structure and function of lipases [18, 19]. Thermostable lipases are more attention for industrial processes because the reactions might be performed at elevated temperatures therefore should be the structure of enzyme in harsh condition is maintained , furthermore includes other advantages such as increased solubility of lipid substrates in water, faster reaction and reduced possible risk of contamination [20, 21]. To improve one or more properties such as the thermostability and the activity of the enzyme ,widely used strategy include hydrogen bonding, the formation of disulfide bridges, hydrophobic or aromatic interactions, ion pairing, etc [22]. Therefore, to achieve these goals a suitable and efficient method must choose. For this purpose, there are two major and principally different routes: (i) the first, structure-based rational design, in here requires to structural information and knowledge about three-dimensional (3D) of protein. Using this method can predict and improved the activity and stability of enzyme, for example, the site-directed mutation (SDM) technique here is used [23-26] . The second, (ii) random or “irrational” mutagenesis design, relying on entirely random mutagenesis techniques by various protocols for generating large variant libraries of genes, followed by screening or selection for improved variants for specific properties of an enzyme, such as utilizing the gene (DNA) shuffling and error-prone PCR (epPCR) technique. In here for directed evolution usually does not require prior knowledge of the structure of the enzyme (protein) [27-32]. The fact that the rational protein

design methods through computational models have been developed and it have provided a new valuable tool for improving enzyme properties to the desired requirements [33]. This method has experienced important with varying degrees of success in recent years, with considerable achievements in the design of novel enzymes ([34-36]. Structural features of an enzyme can affect the thermostability as well as plays a crucial role in promoting thermostability. For example, thermostability is associated with large numbers of electrostatic interactions, a large number and tight metal-binding sites, high packing density and core hydrophobicity. Thus, a rational approach ( e.g. site-directed mutagenesis) can be used in the presence of empirical testing to enzyme engineering as well as optimizing the activity and thermal properties of an enzyme [37].Understanding the structural basis of altered properties of proteins due to changes, provides useful insight in designing proteins with improved catalytic activity or thermostability. We describe here progress in improving the stability and activity of SML by rational site-directed mutagenesis (SDM) based on homology modeling and molecular dynamics (MD) simulations as well as experimentally testing. In this study, four mutant proteins of SML were designed and constructed, and was analyzed enzyme activity and thermostability.

## 2. Materials and methods

### *2.1. Bacterial strains, plasmids, enzymes, materials and culture conditions*

Marine *Serratia marcescens* (GenBank No. GQ471957), which is deposited into the Microorganisms collection of the Microbial Technology and Products (MTP) Research Center of Iran (Collection No. UTMS 2342), was utilized for experiments. *Escherichia coli XL1* -Blue [recA1 *endA1 gyrA96 thi-1 hsdR17 supE44 [F' proAB lacI$^q$ZΔ15 Tn10 (Tet$^r$)*] (Novagen-USA) and *Escherichia coli BL21* (DE3) [*F$^-$ ompT hsdSB (rB$^-$ mB $^-$) gal dcm (lcIts857 ind1 Sam7 nin5 lacUV5-T7 gene1*] (Novagen-USA) were employed as host strains for the gene manipulation and protein expression, respectively. The plasmids used for the cloning and protein expression was

pUC19 Amp$^r$ and pET-28a (+) Kan$^r$ (Novagen-USA). All the restriction enzymes were from Thermo Scientific (USA), all the kits were purchased from Qiagen (USA)/ Roche (Germany). All other chemicals and reagents used in the study were of good analytica grade, obtained from Merck (Germany)/Sigma Aldrich (USA). The *S. marcescens* was grown in a nutrient broth (0.3% beef extract, 0.5% peptone and 0.5% NaCl) at 30 ◦C. LB medium (1% peptone, 0.5% yeast extract, 1% NaCl) or solid medium contained bacto-agar (1.5%) were used for culture and growth of *E.coli* at 37 ◦C. Ampicillin (100μg/ml) and Kanamycin (50μg/ml) were added to medium for recombinant *E.coli* when needed. Isopropyl β-1-D-thiogalactopyranoside (IPTG) was used at indicated time point for induction of lipase production when needed. The cell growth was determined by measuring the optical density at 600 nm using a spectrophotometer.

### *2.2. Construction of wild-type expression plasmid*

The genomic DNA of *S.marcescens* was extracted by the bacteria genomic DNA extraction kit (Roche Germany). The SML gene *(lipA)* (accession no. KF372589) of *S. marcescens* was amplified using Pwo DNA polymerase (Roch) according to the procedures recommended by the Supplier in combination of primer 1 (5′-GCCCAT**ATG**GGCATCTTTAGCTATAAGG-3′), primer 2 (5′- TAGGATCC**TTA**GGCCAACACCACCTGATC -3′) where, the underlined bases represent the *Nde*I site for primer 1, and *Bam*HI site for primer 2. The ATG codon for the initiation of the translation and the sequence complementary, TAA, to the termination codon are shown in bold type. The PCR products were digested with with *Nde*I and *Bam*HI , purified, and ligated into the *Nde*I and *Bam*HI sites of pET-28a(+) vector and was then transformed into XL-1 Blue cells for cloning and verification through sequencing. The verified pET28a-LipA was transformed into *E. coli* BL21 (DE3) for overexpresssion of the lipA gene.

### *2.3. Homology model*

#### *2.3.1. Computational design of mutants*

In order to identify potential mutations that would lead to enhanced catalytic activity and thermostability, studied SML structure was modelled using the homology model module of MOE 2012.10 based on the existing crystal structures of SML (PDB code 2QUA). Identification of putative mutations was performed based on two approaches: comparison of SML structure with *Pseudomonas* lipase (PML) (PDB code 2Z8X) and direct investigation on SML structure itself. In order to evaluate catalytic activity of SML, the obtained SML model was superimposed on PML structure and calcium-binding sites were compared in the two enzymes. Hence, the MutH279K mutant was constructed. Direct investigation on 3D structure of SML was performed using Protein Design Module of MOE 2012.10 [38] which includes a rotamer exploration of the proposed mutations and a molecular dynamics simulation of the obtained mutants in order to identify putative sites. In which it was used to calculate dihedral angles ψ against φ of amino acid residues in protein structure, where changing residues would result in higher thermostability. Therefore, three mutants, MutG2P, MutG59P and pMutL613W-A614P were constructed.

### *2.4. Site-directed mutagenesis*

Site- directed mutagenesis (SDM) was carried out using splicing by the overlap extension method (SOE) [39] to create the four mutants of SML. Plasmid of pET28a-LipA was used as template for mutagenesis that involved the application of primers as described in Table 1. The 1845 bp amplicons obtained were cloned into pET-28a (+). Expression plasmids derived from pET-28a (+), which carry different SML mutant genes, were named pMutG2P, pMutG59P, pMutH279K and pMutL613W-A614P, and were transformed into *E. coli* XL1-Blue. The nucleotide sequence of the mutated genes was verified by DNA sequencing. *E. coli* BL21 (DE3)

cells were transformed with the constructs then transformants were selected on Luria-Bertani medium plates supplemented with kanamycin (50μg/ml) for expression.

*2.5. Overexpression and purification of recombinant enzymes*

For Overexpression , single colonies of each clone *E.coli* BL (DE3) harboring the pET28a-LipA and mutant plasmids were inoculated in 5 ml of LB broth containing 50μg/ml kanamycin and grown at 37 $^{o}$ C overnight with shaking. The resulting cultures were used to inoculate 200 ml of the same medium until OD600 reached spanning 0.7-0.8, isopropyl-L-D-thiogalactopyranoside (IPTG) was added to a final concentration 1 mM, and the cells were cultivated further at 35 °C for 12 hours. Cells were harvested by centrifugation at (5000×g, 10 min, 4 $^{o}$ C) and washed twice with 50mM Tris-HCl, pH 8.0 solution. The cell pellet was then resuspended in buffer A (6 M Guanidine Hydrochloride, 20mM $NaH_2PO_4$, 500 mM NaCl, pH 7.8) and the cells were disrupted by gentle sonication treatment, cooled on ice, for six cycles, 10 s and 30 s intervals. The supernatant was obtained by centrifugation at (15000 × g, 20 min, 4 $^{o}$C). The eight ml of the supernatant fraction containing soluble recombinant lipase was mixed with 1.5 ml of nickel affinity resin (Ni-NTA, Invitrogen) previously equilibrated with buffer B (8 M Urea , 20 mM Sodium Phosphate pH 7.8 , 500 mM NaCl), incubated for 1 h at 22 $^{o}$ C and applied to an empty column. The column was washed several times with buffer B (pH 6). Finally, recombinant proteins were refolded and eluted with buffer C containing 100 mM Tris- HCl, pH 8.0, 250 mM NaCl, 20 mM $CaCl_2$, and 250 mM imidazole. The fractions containing protein solution were pooled and several times was dialyzed using dialysis tubing (12 kDa cut-off ) for overnight at 4 °C against 50 mM Tris- HCl (pH 8.0) buffer containing 150 mM NaCl, 10mM $CaCl_2$. The protein eluted from the column was used for biochemical characterization. The protein concentration was determined according to [40] using bovine serum albumin (BSA) as a standard and absorbance was recorded at 605 nm. Finally, proteins were used for biochemical characterization.

*2.6. Enzyme assay*

The residual and relative enzyme activities were determined by a spectrophotometric method at 410nm in 1-cm path length cells with a double-beam using *p*-nitrophenyl laurate (p-$NPC_{12}$) as the substrate. Assay mixtures as follows: One part of Solution A , *p*-nitrophenyl laurate (in isopropanol ) 5mM (to a final concentration (0.5mM) as substrate , and nine part of Solution B, 50mM Tris –HCl (pH 8.00) buffer , containing 5mM $CaCl_2$ ,0.1% (w/v) gum arabic 0.2% (w/v) sodium deoxycholate 0.4% (v/v) Triton X-100 . Therefore, 950 µl of the substrate solution was mixed with 50 µl of the enzyme solution (20-60 µg) to make a final volume 1ml. The activity assay was done at indicated temperature for 5 minutes. The reaction was terminated by addition of 25 µl of 0.5 M EDTA solution. The molar absorptivity of *p*-nitrophenol in same buffer was experimentally determined as 14200 $M^{-1}$ $cm^{-1}$. The background hydrolysis of the substrate was deducted by using a reference sample of identical composition to the incubation mixture. One unit of enzymatic activity was defined as the amount of enzyme that liberated one µmol of *p*-nitrophenol per min under standard assay conditions [11]. The total enzyme activity was defined in U and specific activity was defined as U/mg of protein**.** The experiments were performed triplet and the processed data were given as the means ±SD of triplicate measurements from three independent determinations for each enzyme.

*2.7. Gel electrophoresis*

The expression level of rSML (wild type and mutated lipases) in the cells , molecular mass and the purity of the proteins were analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to [41] followed by staining with Coomassie brilliant blue R-250 and destined with methanol/acetic-acid/water (3:1:6 v/v/v).

*2.8. Effects of temperature and pH on lipase activity and stability*

Effects of temperature and pH on lipase activity wild type and mutated proteins were measured by the spectrophotometric assay at 410 nm using *p*-nitrophenyl laurate (p-$NPC_{12}$) as the

substrate. The optimal temperature for activity was determined under various temperatures (30-50 $^{o}$C) at the optimum pH value using a circulation water bath and the relative activities (%) were measured. The thermal stability of SMLs , wild –type and mutants, was tested by pre-incubating the enzyme sample at various temperatures ranging from 25 to 80 $^{o}$C with 5 $^{o}$C increments for 1 h in optimal pH buffer. Immediately after chilling the samples on ice, the residual activity (%) at the optimum temperature for each enzyme was determined under the standard reaction conditions. The optimal pH for the purified lipases was evaluated by incubating the enzyme–substrate at various pH from 4 to 12 in following buffers: 50mM sodium acetate buffer, 50mM Tris– HCl buffer, and 50mM glycine/NaOH buffer. Enzyme reactions at each pH value were carried out at optimum temperature and their relative activities (%) were measured. In order to determine the stability for enzymes at pH values of 4.0 and 12.0, a pre-incubation was performed at each pH value at room temperature for 24 h. The residual activity (%) was measured using the standard assay as described previously.

*2.9. Substrate specificity*

The substrate specificity was determined by the spectrophotometric assay, by incubating the purified lipases with four the *p* NP-derived ester in various lengths (*p* NP acetate, $C_2$; *p* NP butyrate, $C_4$; as short- chain fatty acid esters, *p* NP laurate, $C_{12}$; as medium- chain fatty acid ester; *p* NP palmitate, $C_{16}$ as long- chain fatty acid ester) as substrate and measuring the amount of *p* NP released.

*2.10. Kinetic Parameters*

The kinetic parameters were calculated using the spectrophotometric activity assay with varied concentrations of $pNPC_{12}$ (0.05–2.0 mM) as substrate of the lipase. The kinetic parameters, affinity constant (Km), maximal velocity (Vmax), and the turnover number (kcat) values, were calculated from the experimental data using Sigma plot 12 software. The parameter Catalytic efficiency was also obtained by using the equation kcat/Km.

*2.11. Statistical analysis of data*

The data reported in all figures and tables are an average of triplicate observations and were subjected to one-way analysis of variance (ANOVA) and compared by F-test using the STATISTICA 20 (Stat Soft, Inc., USA) software package. The final values was presented as mean ± standard deviation. Differences were considered to be significant when $P<0.05$.

*2.12. Circular dichroism (CD)*

The circular dichroism (CD) spectrum was determined on Aviv circular dichroism spectrometer model 215 (Aviv Instruments, NJ, and USA). The far-UV (260-200 nm) CD spectrum was measured in 50mM Tris-HCl (pH 8.0) containing 0.15 M NaCl and 10 mM $CaCl_2$ at 25 $^{o}$ C. The protein concentration and optical path length were 0.2 mg $ml^{-1}$ and 0.1 cm, respectively .The buffer 50mM Tris-HCl (pH 8.0) with 0.15 M NaCl and 10 mM $CaCl_2$ was used as blank and its spectrum was subtracted to CD spectrum of protein. The results are expressed as molar ellipcity $[\theta]_\lambda$(deg $cm^2$ $dmol^{-1}$) by using a mean amino acid residue weight (MWR) of 110. The CD unit used is the mean residue ellipticity for CD spectrum in the far-UV range. The ellipticities are calculated from the following relations, $[\theta]_\lambda Far = (\theta m \times 100\ MWR)/(c.l.n)$ where $\theta m$ is the measured ellipticity in degrees wavelength $\lambda$, $c$ is the protein concentration in mole per liter, $l$ is the light path length of the cell in centimeter and $n$ is the number of residues.

*2.13. Differential scanning calorimetry (DSC)*

Thermal analysis (thermograms) of samples was analyzed using a Differential Scanning Calorimeter (DSC- 823 e, Mettler Toledo, Switzerland). Approximately 4-5 mg of Freeze-dried protein using a small spatula were weighed in an aluminium pan (Mettler, ME-27331, Mettler-Toledo Switzerland) and 10µl of 50mM Tris-HCl (pH 8.0) containing 0.15 M NaCl and 10 mM $CaCl_2$ was added and hermetically sealed. The DSC analyzer was calibrated using an empty sealed aluminium pan as reference. DSC scans were performed in the range between 20 and 95 °C, at a scan rate of 60 °C/h in a nitrogen atmosphere. Thermograms, denaturation temperatures

(Td), were determined automatically using Origin Software (STARe Thermal analysis version 3.1 software; Mettler Toledo, Switzerland) at which the denaturation peak in the thermogram is a maximum.

# 3. Results and discussion

## *3.1. Rational design of mutations and production of Mutants*

Three-dimensional model structure of *S. marcescens* lipase A belonging to family I.3 lipase with 614 amino acid residues (and high similarity to SML 2QUA.pdb) shows that the polypeptide chain is folded into two domains (N-C). The N-catalytic domain contains the active site residues Ser207, Asp256, and His314 is composed of residues from 1 to 320, and C-terminal β-sandwich domain is formed by residues 321 to 614 [6, 11, 12]. It was shown earlier from structural and biochemical/biophysical studies on family I.3 lipases that the N-catalytic domain both are nearly identical with each other, except for the structures of two lids, termed lid 1 and lid 2, and the $Ca^{2+}$-binding sites [5]. The lid is a surface loop, covering the active-site of lipases and its conformation is changed upon interfacial activation, therefore providing the interaction between the active- site serine residue and the substrate [9, 42]. The N-catalytic domain of SML possesses calcium-binding sites Ca1 and Ca2, while PML has one additional site indicated as Ca1–Ca3. As the Ca3 site is missing in the SML structure, it may be mentioned that all three $Ca2^{+}$-binding sites in the N-catalytic domain of family I.3 lipase are not conserved [5, 42, 43]. The Ca1 site of SML is related to the lid, which is an α-helical polypeptide chain overing the active site, and which is anchored in its position by the $Ca2^{+}$ ion. This ion is coordinated by Asp153 (monodentate) and Asp157 (bidentate), the carbonyl oxygen atoms of Thr118 and Ser144, and the side chain of Gln120 the coordination sphere is completed by a water molecule. [12]. All the residues participating in Ca-binding site are strongly conserved in lipases of family I.3 with the

same residue numbers. Mutation at this site prevents the formation of the Ca1 site and almost fully abolishes the enzymatic activity as for PML [43, 44]. The $Ca^{+2}$ ion of the Ca2 site is heptacoordinated by the side chains of Glu254 (monodentate), Asp276 (bidentate) and Asn285, main chain oxygen atoms of Asn284, and two water molecules [12]. Residues adjacent to calcium in the Ca3 site of PML are shown in Fig. 1A, as P277, K278, E279, T282, D283, K336, and D337. Equivalent residues of SML (obtained by structural superimposition, Fig.2B) are P278, H279, T280, T283, N284, K337 and D338. Comparison of residues composition of SML with PML in this region, as well as their orientation shows that H279 of SML to be the most prominent difference between these two regions. In one of the crystallized structures of SML (2QUB.A), it could be observed that in absence of a calcium ion, K337 side chain interacts with the backbone of H279. In conclusion, as there is one Lysine residue instead of this Histidine in PML, and as this residue seemed to be the most important differing part in the structure, hence, it was hypothesized that a mutation of H279K may lead to the formation of a calcium-binding site structure in SML. In fact, the Ca3 site may play an important role in increasing activity and /or thermal stability of family I.3 lipases .It has been shown that in a family I.3 lipase from *Pseudomonas sp*. MIS38 (PML), by removing the Ca3 site through the mutation of Asp337 to Ala (D337A-PML), the stabilization was decreased .It has been shown that a family I.3 lipase from *Pseudomonas sp*. MIS38 (PML) by removing the Ca3 site through the mutation of Asp337 to Ala (D337A-PML), the stabilization was decreased [5] as well as a lipase from *pseudomonas fragi* has been shown that mutations in the lid region affected in chain length specificity, activity and thermostability of the enzyme [45]. It has been proposed that the lids of family I.3 lipases to interact with the substrate and open conformation must be stabilized by the $Ca^{2+}$ ion bound and in the absence of this $Ca^{2+}$ ion, the lid structure in the open conformation may be unstable [42]. Thus $Ca^{2+}$ ion is essential for lipase activity and the conformational change of calcium-stimulated lipases associated with ca- binding sites that includes a change in their secondary structures as well an

increase in α-helix and β-sheet contents [46]. Several studies showed that family I.3 lipases requires $Ca^{2+}$ ion for activity , $Ca^{2+}$ -dependent activity , and do not a functional conformation in the absence of the $Ca^{2+}$ ion [5, 9, 42, 43, 46].Further exploring possibilities to stabilize the protein, other mutations were investigated using the Protein Design module of MOE program, using SML model . Mutation of glycine to to proline was systematically explored in the whole protein. Stability of a protein can be increased by selected amino acid substitutions that decrease the configurational entropy of unfolding [47].Generally proline residues is the only naturally occurring amino acid in which the side chain is connected to the backbone nitrogen, forming a five-membered pyrrolidine ring. Proline residues exhibit special dihedral angles of φ against ψ of amino acid residues in the protein backbone [48]. The proline residues due to the pyrrolidine ring, has the lowest conformational entropy than any other residues hence it can decrease the conformational entropy of the unfolded structures, thus stabilizes the protein. The pyrrolidine ring can decrease the conformational entropy of the unfolded structures, therefore proline has the lowest conformational entropy than any other residues [47, 49]. In contrast glycine, in the unfolded state, is the residue with the highest conformational entropy [50]. Thus, changing glycine to proline may result into increase in the stability of proteins by decreasing the entropic difference between the unfolded and the folded state. The effect of all glycine to proline substitutions was checked, and finally G2 and G59 mutations were predicted to result in best stability. Location of these glycine residues and modelled mutations are represented in Fig. 2A, B, C, D, where, in case of P59, potential new interactions may happen (e.g. with L150 and S60). Substitution of Gly residues with Pro increases the free energy difference and local rigidity and influences the stability of the enzyme. Mutational studies on various proteins have been reported by proline substitution as an important factor involved in increased thermostability [51-58]. Since the C-terminal of family I.3 lipase is suggested to play an important role in the enzyme stability [59], inducing a mutation at this site was also considered. As shown in Fig. 2 E,F, L613 and

A614 residues are the last residues of the sequence and it was hypothesized that mutation of these residues to more voluminous W613 and P614 would result into less conformational freedom, as well as potential for other interactions of the tryptophan side chain. According to a study that was carried out on a lipase has been reported that the tryptophan residues affected the thermal stability [60].

### *3.2. Cloning, expression and purification of SML and its mutants*

The wild-type lipase A and its four mutants were cloned into the pET-28a (+) vector under the control of the T7 promoter for expression of protein. The performance of the mutation and the absence of PCR-generated random mutations were confirmed by DNA sequencing. Sequence analysis of the cloned fragments revealed one major open reading frame of 1845 bps, which encodes a polypeptide of 614 residues. The sequence of wild-type lipase A from *S. marcescens* in this study has been submitted to GenBank and is available under accession number AGT95802. The recombinant lipases were overexpressed in *E. coli* BL21 (DE3) cells transformed with PET-28a (+) which places the lipase gene under control of a T7 promoter and induced with IPTG. The inclusion bodies were solubilized and purified in the presence of 6M Gn-HCl or 8 M urea as well as refolded in the presence of the $Ca^{+2}$ ions. The proteins, with a histidine tag in N-terminal region of the protein, were purified by one-step purification using metal-affinity chromatography on Ni-NTA resin column. The recombinant lipases attached to Ni-NTA resin column was eluted with imidazole. To avoid any interferences, remaining imidazole from the elution was removed by dialysis against 50mM Tris-HCl. After protein purification, the recombinant lipases were confirmed by a single band on an SDS-PAGE gel with an apparent molecular mass of 65 kDa stained with Coomassie Brilliant Blue (Fig. 3) this molecular mass of *S. marcescens* lipase is in agreement with previous reports [10, 61, 62].

### *3.3. Biochemical characterization*

#### *3.3.1. Effect of temperature and pH on enzyme activity and stability*

The effect of temperature on the activity of the SML and its mutants was analyzed at 30-50 $^{o}$C. The enzymes exhibited high activities at temperatures range of 37–45°C, and displayed their optimal activity at 42◦C (Fig. 4a). Therefore, no tangible changes in optimum temperature of mutants were observed compared with wild type. The identical results for optimum temperature also reported for *S. marcescens* Sr41 8000 lipase [63] and *S. marcescens* ECU1010 lipase [64]. To evaluate the improvement in thermostability, the residual activity of wild type and the mutants after pre-incubation for 1h over a temperature range from 25◦C to 80◦C was measured. As shown in Fig. 4b. All enzymes retained above 100 % activity at temperatures below 35°C, but the stability of the enzymes decreased when the temperature was over 50°C, however the two mutants, MutG2P and MutG59P, displayed favorable effects on thermostability and retained activity higher than that of wild type. The thermo stability /tolerance of any of the lipases from *Serratia* species as well as mutation on the thermal stability have not yet been reported so far. The lipases from *S. marcescens* Sr41 8000 [63] and *S. marcescens* ECU1010 [64] are two of the reported examples of SML that without showing the details reported the lipase (wild type) retained 60-70% activity after being incubated at 50°C for 1 h. The effect of pH on the catalytic activity of mutants and wild type recombinant lipases was investigated in the pH range 4.0–12.0 (Fig. 4c). All enzymes were displayed highest activity at pH values between 7.0 and 9.0. The optimum pH of wild type and mutants was pH 8.0 at 42°C and no distinct changes were found in the mutants. The pH- stability profile in the form of residual activity was presented in Fig. 4d, as that showed the highest residual activity all enzymes was observed in the pH 9.0 value. This is somewhat similar to that reported for lipases from *S. marcescens* optimum pH (pH 7.5-8.0) and pH stability (pH 6.0-8.0) [65], and *Serratia marcescens* Sr41 8000 optimum pH: 8.0 and pH stability (pH 6.0-8.0) [63], and *S. marcescens* ECU1010 optimum pH: 8.0 and pH stability (pH

6.0-9.0) [64], and *S. marcescens* SM6 optimum pH: 8.0 and pH stability (pH 6.0-10) [66]. Therefore both the wild type lipase and mutants display the similar profiles in pH stability and were very stable at alkaline pH and decreased activities at acidic pH values. In addition an increase in stability was observed with the increase in pH buffers this is particularly was shown in the MutH279K compared with wild type and other mutants. The relative increase in pH stability of the MutH279K may be associated with basic property of lysine as similar results have been obtained on some other lipase [67].

### *3.4. Substrate specificity*

The substrate specificity of enzymes, were evaluated towards several *p*-nitrophenyl esters of different chain lengths having alkyl chain lengths of $C_2$, $C_4$, $C_{12}$, and $C_{16}$, by spectrophotometrically under the assay conditions. As shown in Fig.5, the highest hydrolytic activity was obtained with $C_4$ and $C_{12}$ *p*-nitrophenyl esters, with a remarkable preference of toward *p*-nitrophenyl laurate ($C_{12}$) whereas with reduce was the activities toward $C_2$ and $C_{16}$ esters, indicating a clear preference of the enzyme for medium acyl chain lengths. As it has been reported a lipase from *S. marcescens* Sr41 8000 [63] showed the highest activity on substrates with short-chain length ($C_4$–$C_8$) rather than the long chain length. On the other hand the lipases from *S. marcescens* ES-2 (44, 50) [62, 68], exhibited maximum activity towards substrates with medium-chain length ($C_8$–$C_{12}$).

### *3.5. Kinetic parameters*

Kinetic parameters of *S. marcescens* lipase (wild type and mutants) were determined by measuring rates of hydrolysis of different concentrations of $pNPC_{12}$ as substrate using a spectrophotometric activity assay. The kinetic parameters such as, affinity constant (Km), maximal velocity (Vmax), turnover number (kcat), and catalytic efficiency (kcat/Km) were determined for five enzymes. The results are summarized in Table 2. Kinetic data was plotted and shown in Fig.6. The MutH279K displayed a affinity constant (Km) of 0.36 mM and

maximal velocity (Vmax) of 1207 μmol/min/mg that compared to the wild type , exhibited significant increase of lipase activity (P<0.05). The catalytic efficiency (kcat/Km ) for MutH279K was twofold greater than that the wild type enzyme, showing that substitution His279 by Lys and formation of additional calcium binding sites might lead to an increase in the enzyme activity. Therefore, it can serve as a good candidate for the engineering of enzyme. The *K*m value of Mut L613WA614P was 0.63mM, which was higher than to that of wild type enzyme (0.39 mM) showing this mutation decreased the enzyme affinity for the substrate, however its maximal velocity (Vmax) and turnover number (kcat) was slightly higher in compared with wild type.The affinity constant (Km), maximal velocity (Vmax), turnover number (kcat), and catalytic efficiency (kcat/Km) of the thermostable mutants, the MutG2P and MutG59P, was almost similar as that of the wild type enzyme and no significant differences was observed (p>0.05) therefore, in spite of the increase of MutG2P and MutG59P thermal stability, but not observed important change in the kinetic parameters. The effects of mutagenesis on activity of different microbial lipases were investigated by site-directed mutagenesis using substitution different amino acids around or far from the active site. The results reveal that substitution the amino acid residues, affects enzyme catalysis activity [45, 67, 69-74].

### *3.6. CD spectra*

To inspect whether the secondary structure of SML is changed by the mutations , the far- CD spectrum of the wild type and mutant lipases , MutG2P , MutG59P , Mut H279K and Mut L613WA614, were measured in 50 mM Tris- HCI (pH 8.0) containing 10 mM $CaCl_2$, at 25°C as previously described. The far-UV CD spectrum (below 250 nm) reflects the secondary structure of the protein [75]. Results indicated the far-UV CD spectrum of mutant proteins were similar to those of the wild type protein (Fig. 7), suggesting that the overall

secondary structures of several SML mutants were not significantly changed by mutations. A similar spectral without change has been observed for a family I.3 lipase from *Pseudomonas sp*. MIS38 (PML) where five single and two double mutations were created [69].

### *3.7. Differential scanning calorimetry (DSC)*

Differential scanning calorimetry (DSC) was performed on SMLs to evaluate their thermal stability. The lipases were submitted to gradual temperature increase from the initial temperature of 25 °C to the final temperature of 95 °C at a scan rate of 60 °C/h. The denaturation process was characterized by determining the midpoint of denaturation temperature (Td), during which half of the protein molecules were in a denatured state. Based on these results the peak denaturation temperature (Td) of the enzymes were 53.02°C for wild type, 55.29°C for MutG2P, 55.88°C MutG59P, 52.82 °C for Mut H279K and 53.96°C for Mut L613WA614P as are shown in Fig. 8. As observed, Td both MutG2P and MutG59P respectively 2.3 and 2.8 °C were higher than to that of wild type as was expected. Thermal denaturation of H279K was similar to those of the wild type. However, DSC thermogram of Mut L613WA614P was 0.9 °C higher when compared with that of the wild type but it was less important than to that of MutG2P and MutG59P.According to computational analysis of the enzyme structure it was expected that the thermostability of the Mut L613WA614P would be higher, but based on results the increased thermostability of Mut L613WA614P is not significant comparing to that of MutG2P and/or MutG59P. Until now, limited information is available regarding the effect of site-directed amino acid substitutions on the thermostability of SML. Other lipases , *H. lanuginosa* lipase, has also been reported by substitution G225P, the temperature stability was increased about 2 $^{o}$C measured by DSC [76].

## 4. Conclusion

In conclusion, we explored the thermal stability and activity of SML by site-directed mutagenesis. Hence three mutants of SML (MutG2P, MutG59 and Mut L613W A614P) with improved thermal stability were successfully obtained by proline substitution mutagenesis. Especially, mutations MutG2P and MutG59 increased thermal stability without distinct loss of specific activity and catalytic efficiency. This shows the importance of proline residues for the thermal stability of SML and makes SML potentially more useful in industrial processes. As described above, proline stabilizes a protein by decreasing the backbone conformational entropy of the unfolded state. The MOE predicts the stability of proline mutations due to calculating thermodynamic quantities (entropic and enthalpic contributions*)* in protein stability modulation. Therefore, the preceding glycine residues at the site where proline is replaced can be candidate mutation sites for improving thermal stability of SML. However, this remains replacing another amino acid by proline using MOE program and three-dimensional structure analysis of SML to the greater thermal stability. On the other hand, one mutant obtained by the substitution of histidine 279 for lysine, H279K, caused an increase in lipase activity compared with wild type, and shows the important role of His 279 in SML in lipase activity. Our starting hypothesis was a probable formation of one additional calcium-binding site in SML via this substitution. However, it should be noted that the aspartate residue (D283) observed in the original calcium binding site of PML is also involved in the interaction with the calcium ion. The corresponding residue is N284 in SML, which lacks one oxygen atom compared with D283, and may lead to a potentially weaker interaction. With regard to the experimental results, it may be suggested that combining the successful mutations G2P, G59P, and H279K could result in both higher stability and activity in SML. Since these residues are located relatively far from each other, and since the mutations to proline had not adversely affected the enzyme's activity, a simultaneous increase in activity and stability could be achieved. Overall, in our study, hypothesized stabilizing mutations to proline residue were more successful in the N-terminal region of the enzyme. Interestingly, the third

successful mutation is also located in the N-terminal domain of the enzyme's structure. This is suggestive of a real potential for this domain as a target for increasing the enzyme's activity and stability. The reported mutations are novel in SML, and show the effectiveness of rationally designed point mutations as a possible way for improvement activity or thermal stability of SML for potential industrial applications.

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## Figure legends

**Fig. 1.** Comparison of residues surrounding calcium ion in PML with equivalent residues in SML. A: Calcium binding site in PML. Residues are shown that are located in 5.0 Å distance from calcium. B: Equivalent residues in SML model. Backbone atoms that were not involved in interactions have been hidden for better visualization. Dots indicate putative hydrogen bonds.

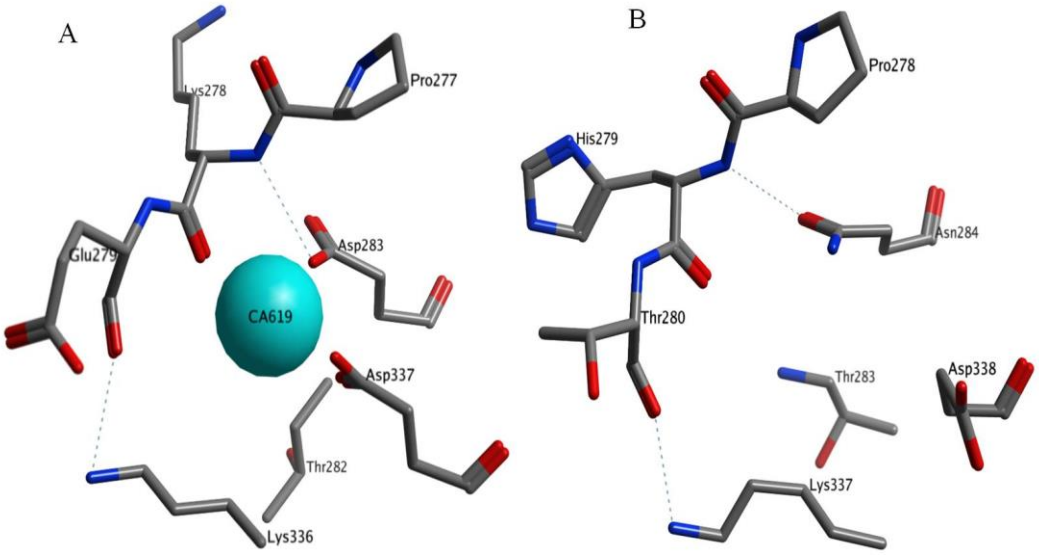
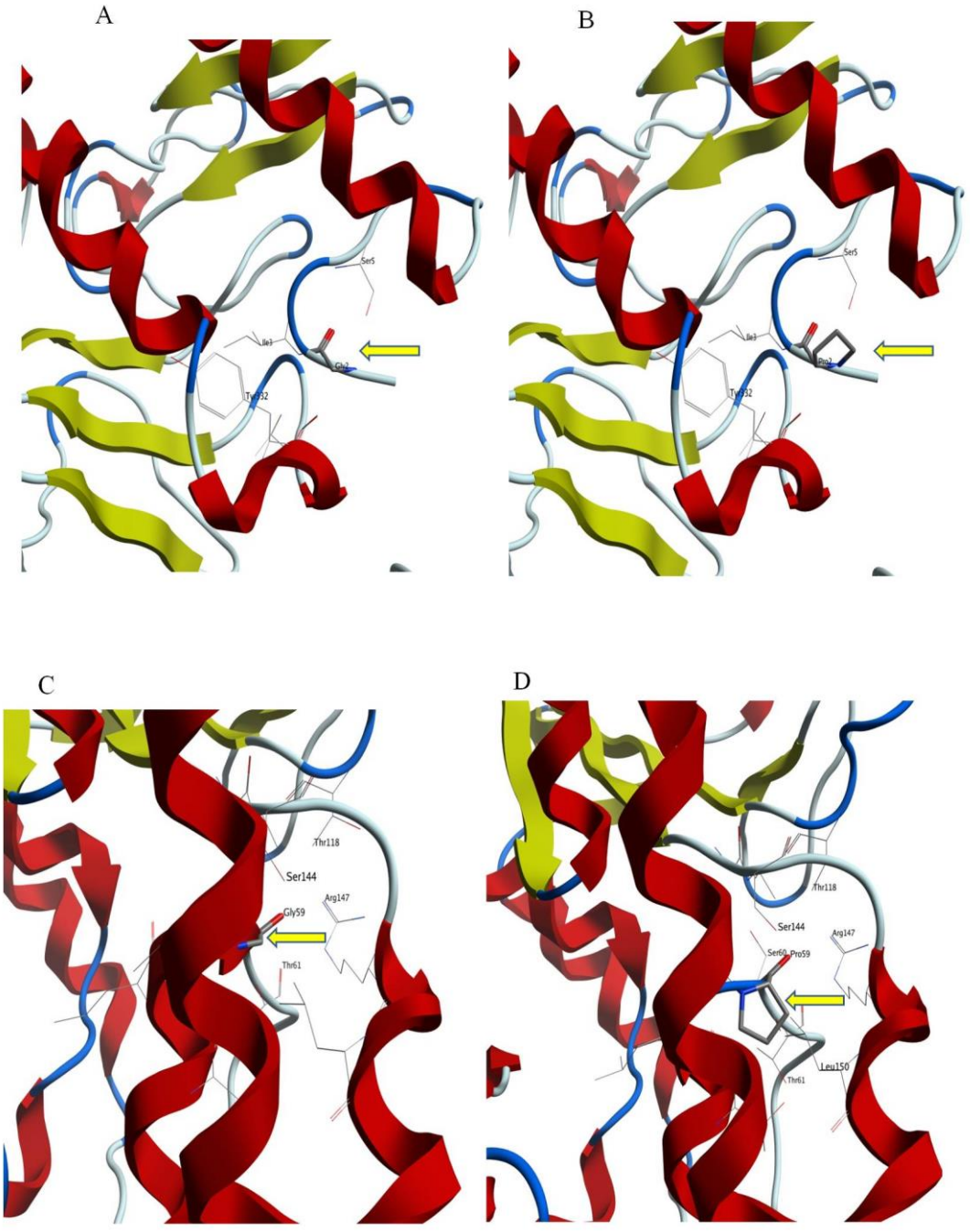


**Fig.2**. Glycine 2 location in SML (A) and potential mutation to proline 2 (B). Glycine 59 location in SML (C) and potential mutation to proline 59 (D). Location and adjacent residues to L613 and A614 (E) as well as the effect of mutating these residues to W613 and P614 (F).



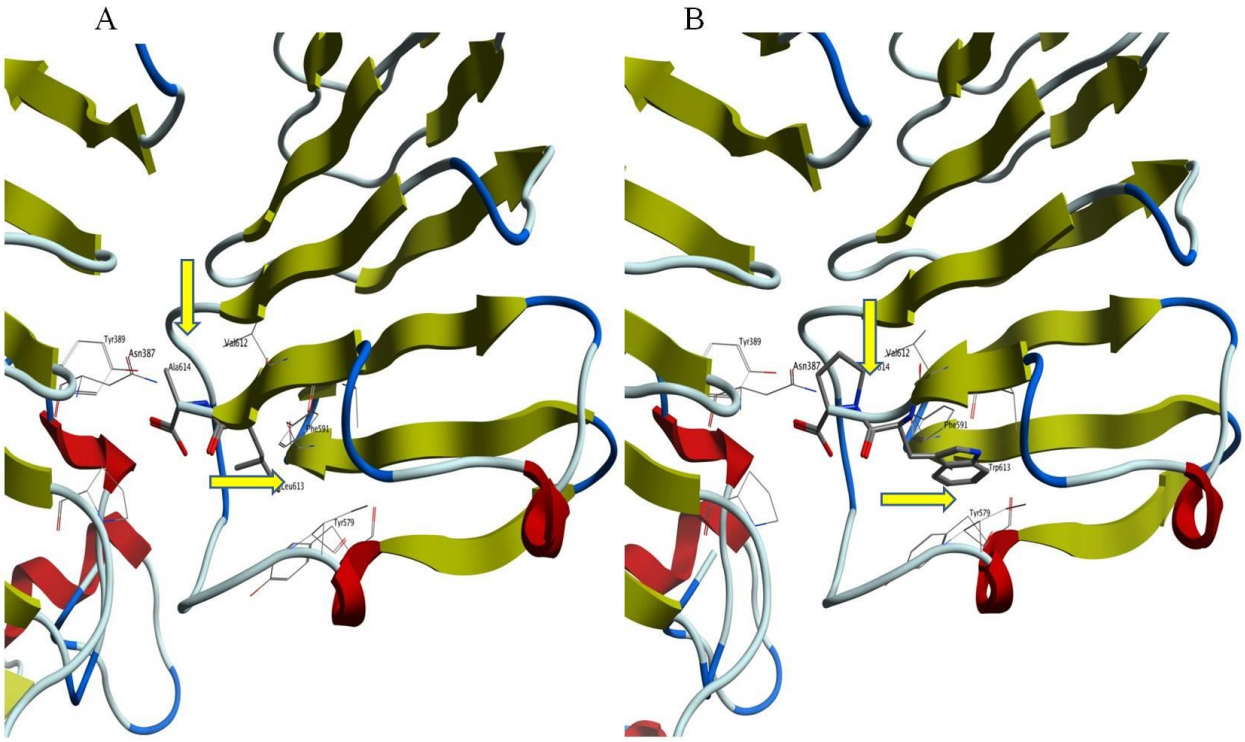


**Fig.3**. SDS-PAGE (12% gel) analysis of overexpressed proteins. The SML-WT (wild type) and mutants were overproduced in the *E. coli* BL21 (DE3) cells transformed with plasmid PET-28a (+) derivatives carrying the *lip*A gene as described in Materials and Methods. The gel was stained by Coomassie brilliant blue R-250. Lane M, molecular weight marker (kilo Daltons, kDa) ; lane 1, cell lysates without induction (control) ; lane 2, 3 ,4 ,5 and 6 precipitate of cell lysate (Inclusion Body) SML-WT , MutG2P, MutG59P, Mut H279K and Mut L613W A614P respectively, induced by 1mM IPTG; lane 8,9,10,11 and 12 , purified SML-WT , MutG2P, MutG59P, Mut H279K and Mut L613W A614P respectively. Recombinant proteins are indicated by arrows which are approximately 65kD.

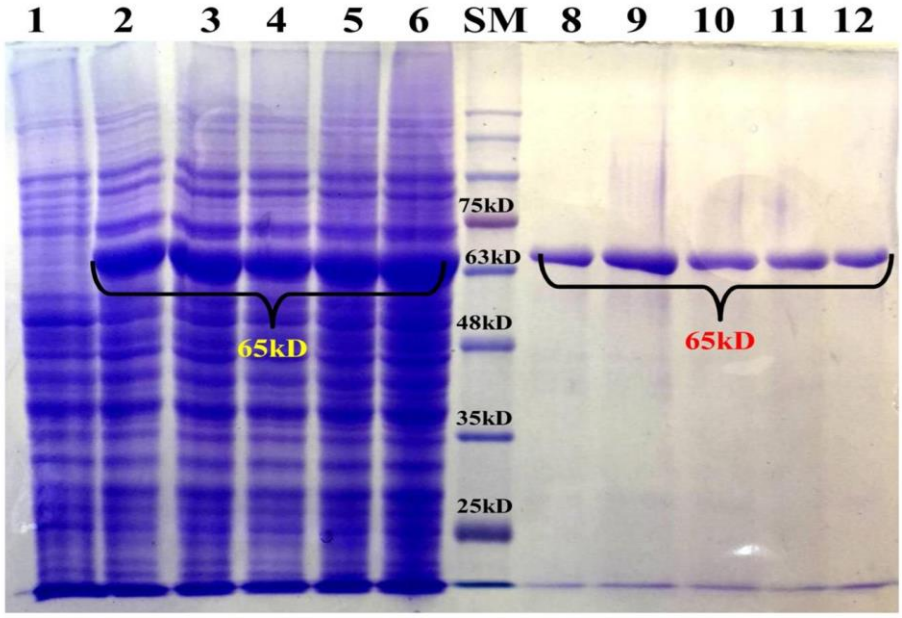


**Fig. 4**. Effect of temperature and pH on enzyme activity and thermostability of SML (wild type and mutants). The temperature optimum (a) and stability (b) profiles of SML (wild type and mutants). The reaction mixture in in 50 mM Tris-HCl buffer (pH 8.0) was incubated at various temperatures (30-50 $^{o}$ C) and the relative activity was measured using $pNPC_{12}$ as substarte at 410 nm. For temperature stability, the purified enzymes were incubated in 50 mM Tris-HCl buffer, pH 8.0, at temperatures of 25–80 $^{o}$ C with 5 $^{o}$C increments for 60 min and residual activity was measured under assay conditions and compared with unincubated. Date presented were the average values of three independent experiments and the error bars indicated standard deviations. The optimal pH (c) was determined at various pH from 4.0 to 12.0 in the presence of 10 mM $CaCl_2$ by using $pNPC_{12}$ as substrate according to standard assay method. The pH stability (d) was analyzed after preincubating enzymes for 24 h at 25◦C in various buffers at various pH levels (4.0–12.0). Residual activity was measured under assay condition and compared with unincubated. Date presented were the average values of three independent experiments and the error bars indicated standard deviations.

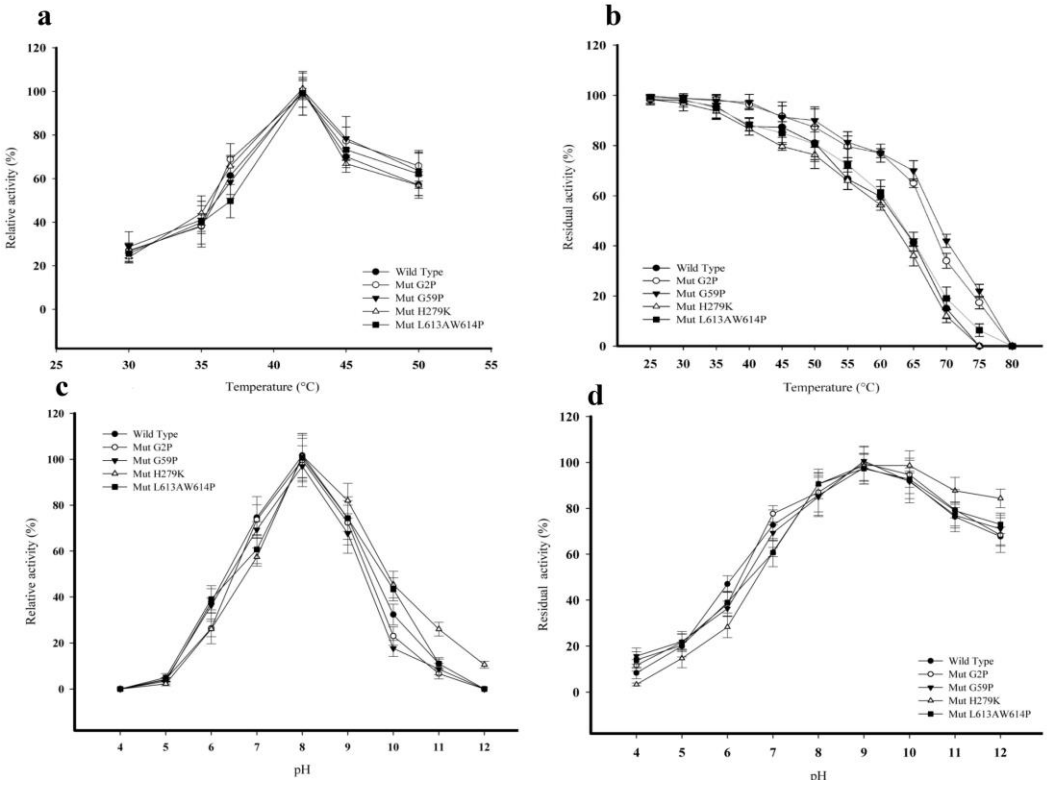


**Fig. 5.** Relative activities of the lipases on various *p*-NP esters. Lipase activities are expressed as the percentage of that of *p*-nitrophenyl laurate ($C_{12}$), which was taken as 100%. Date presented were the average values of three independent experiments and the error bars indicated standard deviations.

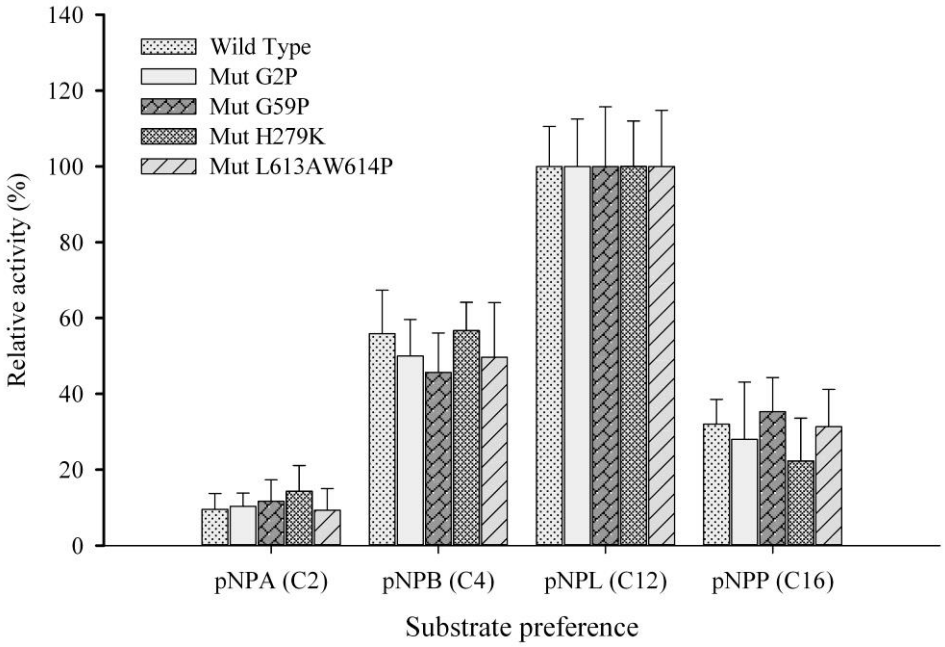


**Fig. 6.** Michaelis-Menten and Lineweaver-Burk plots of *S. marcescens* recombinant lipase wild type (a) and lipase mutants, MutG2P (b), MutG59P (c), Mut H279K (d) and Mut L613WA614P (e) . Kinetic data were measured using *p*- nitrophenyl laurate ($C_{12}$) as substrates at a series of concentrations. Kinetic analyses were determined in 50 mM Tris HCl buffer (pH 8.0) in the presence of 10 mM $CaCl_2$ at 42 ºC. Date presented were the average values of three independent experiments and the error bars indicated standard deviations.

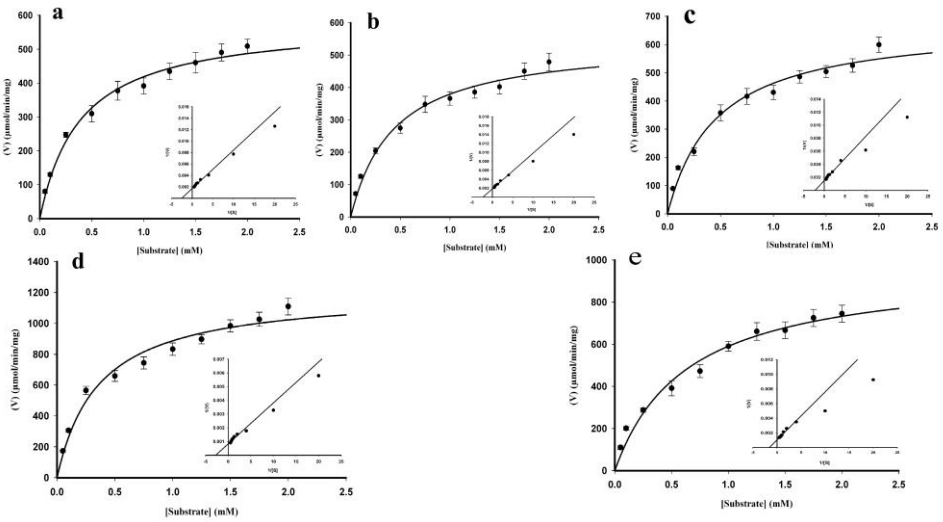


**Fig. 7**. The CD spectra of SML (wild type and mutants). The far-UV CD spectra were measured in 50 mM Tris- HCI (pH 8.0) containing 10 mM $CaCl_2$, at 25°C as described in Materials and Methods Section. The far-UV CD spectra of wild type (Triangle up), MutG2P (Square filled), MutG59P (Square) , Mut H279K (Diamond ) and Mut L613W A614P (Circle) are shown.

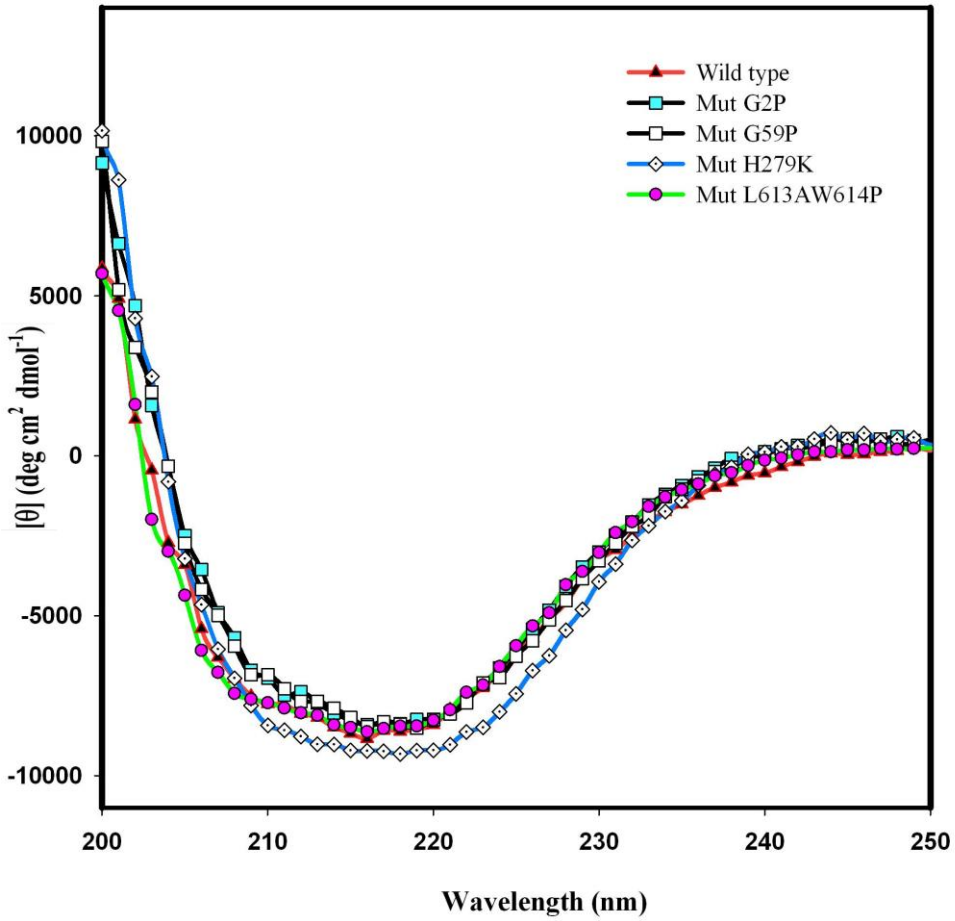


**Fig. 8**. Differential scanning calorimetry (DSC) thermograms representing the five proteins. The midpoint of the transition of thermal denaturation curve , at which 50% of the protein is denatured ($T_{1/2}$), of the Wild type, MutG2P, MutG59P, Mut H279K and Mut L613WA614P were obtained, 53.02°C, 55.29°C, 55.88°C, 52.82 °C and 53.96°C, respectively.

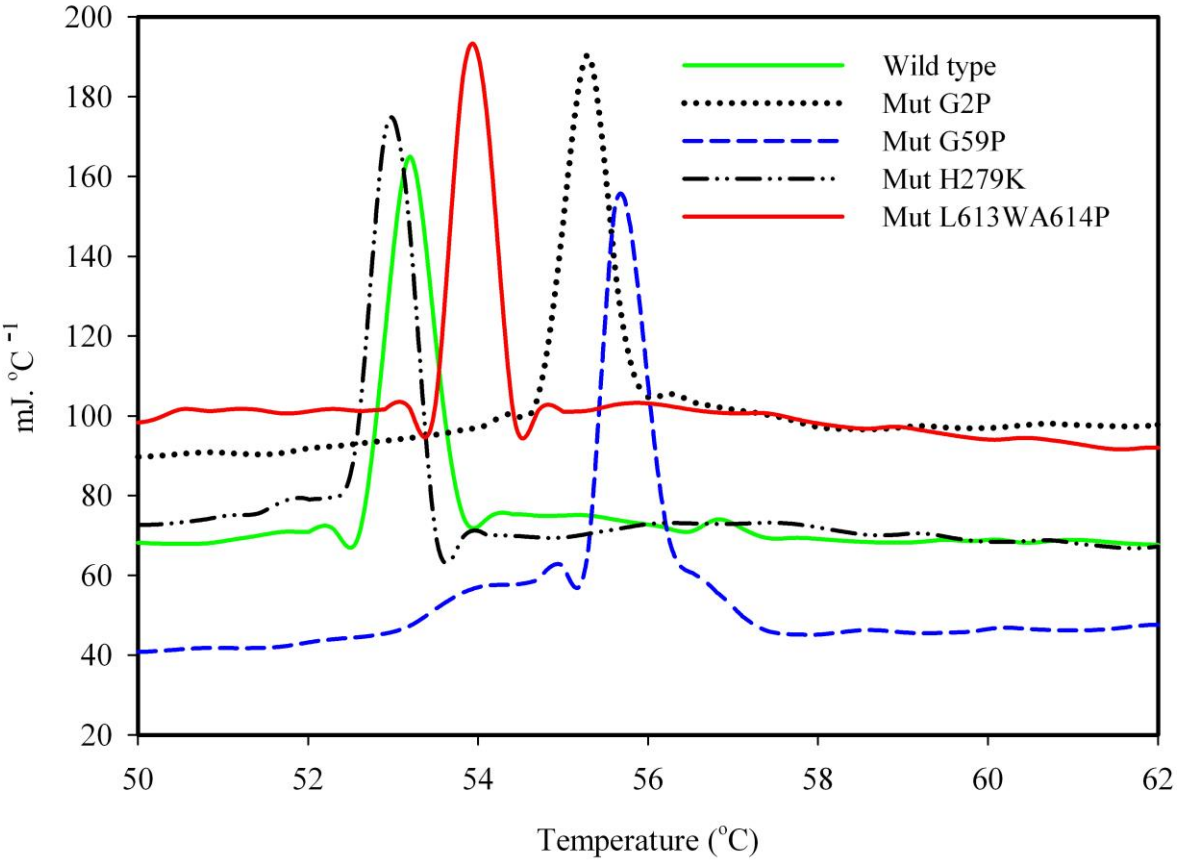


Table 1. Names of the mutants and oligonucleotides used for site-directed mutagenesis

| Mutation sites | Name of the mutants<br>Base change (codon) | Oligonucleotide primres* |
|---|---|---|
| G2P | MutG2P (single mutant)<br>→GGC CCG | [a]F:5′-GCCATATG**CCG**ATCTTTAGCTATAAGGATC-3′ |
| G59P | MutG59P (single mutant)<br>→GGC CCG | F:5′-GGCGCTCATC**CCG**AGCACCCAGTC-3′<br>R: 5′-GACTGGGTGCT**CGG**GATGAGCGCC-3′ |
| H279K | MutH279K (single mutant)<br>→ *CAT* *AAA* | F:5′-CGATGCTCCG**AAA**ACCTCCGCCACC-3<br>R:5′-GGTGGCGGAGGT**TTT**CGGAGCATCG-3′ |
| L613WA614P | L613WA614P (double mutant)<br>TTG→ TGG GCC→ CCG | [b]R:5′-TAGGATCCTTA**CGGCCA**CACCACCTGATC-3′ |

*Bolded sequences represent the designated mutation for the target amino acid residue .The underline indicates [a]the *Nde*I site and [b]*Bam*HI site. F: forward & R: reverse

Table.2. Kinetic parameters of the lip A *Serratia marcescens* wild type and mutants

| Lipase | Specific Activity ($Umg^{-1}$) | $V_{max}$ (μmol/min/mg) | $K_m$ (mM) | $K_{cat}$ ($s^{-1}$) | $K_{cat}/K_m$ ( $s^{-1}$ $mM^{-1}$) |
|---|---|---|---|---|---|
| **WT** | 309.6 ±41.7 | 598.4± 52.6 | 0.39± 0.12 | 0.65 | 1.66 |
| **MutG2P** | 276.7±29.1 | 544.7± 65.2 | 0.43±0.13 | 0.59 | 1.37 |
| **MutG59P** | 351.2± 46.8 | 675.7± 93.4 | 0.45±0.18 | 0.73 | 1.62 |
| **MutH279K** | 662.3± 75.4 | 1207.2± 145.8 | 0.36±0.16 | 1.30 | 3.61 |
| **MutL613WA614P** | 398.7± 53.5 | 964.9±161.7 | 0.63±0.21 | 1.01 | 1.59 |

Enzyme assays were performed in 10 mM $CaCl_2$ with 50 mM Tris HCl buffer (pH 8.0) at using pNPL(C12) as the substrate. Results are mean ± SD (triplicate for each sample)