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ORIGINAL ARTICLE

Study the effect of F17S mutation on the chimeric *Bacillus thermocatenulatus* lipase

Seyed Hossein Khaleghinejad [a], Gholamreza Motalleb [a,*], Ali Asghar Karkhane [b,*], Saeed Aminzadeh [b], Bagher Yakhchali [b]

[a] *Department of Biology, Faculty of Sciences, University of Zabol, Zabol, Iran*
[b] *Department of Industrial and Environmental Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran*

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Cloning;
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Abstract Lipases (*triacylglycerol acylhydrolase*, EC 3.1.1.3) are one of the highest value commercial enzymes as they have potential applications in biotechnology for detergents, food, pharmaceuticals, leather, textiles, cosmetics, and paper industries; and are currently receiving considerable attention because of their potential applications in biotechnology. *Bacillus thermocatenulatus* Lipase 2 (BTL2) is one of the most important research targets, because of its potential industrial applications. In this study, the effect of substitution Phe17 with Ser in mutated BTL2 lipase, which conserved pentapeptide (^{112}Ala-His-Ser-Gln-Gly^{116}) was replaced with similar sequences (^{207}Gly-Glu-Ser-Ala-Gly^{211}) of *Candida rugosa* lipase (CLR) at the nucleophilic elbow region. Docking results confirmed the mutated lipase to be better than the chimeric lipase. So, cloning was conducted, and the mutated and chimeric btl2 genes were expressed in *Escherichia coli*, and then the enzymes were purified by anion exchange chromatography. The mutation increased lipase lipolytic activity against most of the applied substrates, with the exception of tributyrin when compared with chimeric lipase. Further, the mutated lipase exhibited higher activity than the chimeric lipase at all temperatures. Optimum pH of the mutated lipase was obtained at pH 9.5, which was more than the chimeric one. Enzyme activity of the mutated lipase in the presence of organic solvents, detergents, and metal ions was also improved than the chimeric lipase.

1. Introduction

Lipases can catalyze both hydrolysis and synthesis of esters formed from glycerol, and long-chain fatty acids in water–lipid interfaces [32]. Microbial lipases are more useful than lipases derived from plants, or animals, because microbial lipases are the most suitable for industrial applications due to their

* Corresponding authors at: Department of Biology, Faculty of Science, University of Zabol, PO Box 98615-53, Zabol, Iran (G. Motalleb), Department of Industrial and Environmental Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran (A.A. Karkhane).
E-mail addresses: rezamotalleb@gmail.com, reza.motaleb@uoz.ac.ir (G. Motalleb), karkhane@nigeb.ac.ir (A.A. Karkhane).

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low price, simple production, susceptibility to expression in host microorganisms, and diverse specificity [1]. Bacteria are susceptible to genetic modifications (site-directed mutagenesis, directed evolution), and this feature helps to improve the properties of their lipases [10]. Lipases usually show exquisite chemoselectivity, regioselectivity, and stereoselectivity. Lipases are available in plenty, because most of them can be produced in high yields from microbial organisms, viz., fungi and bacteria [19]. Lipases are practical in the food industry [14,7] medical, detergents, textile industries [24,17] biodiesel, and chiral compounds [2,13]. So, lipases are now extensively studied for their potential industrial applications [11]. Extremozymes are premier to the traditional biocatalysts, because these proteins have unique properties, and show suitable activity, even at 100 °C and in the presence of organic solvents, and detergents [27]. Many lipases are moderately stable at high temperature, strict pHs that can reduce their efficiency. However, this could be solved by using lipases from thermophilic microorganisms, whose stability in extreme conditions has been developed by nature [12]. The thermophile *Bacillus thermocatenulatus* produces two lipases (BTL1 and BTL2) [29]. While the molecular weight of the majority lipase from *Bacillus* is between 16 and 22 kDa, molecular weight of BTL2 lipase is 43 kDa. BTL2 showed high stability at medium temperatures (50 °C), alkaline pH (9.0–11.0), and organic solvents (2-propanol, acetone and methanol) [28]. Crystal structure of the BTL2 in an open formation with two molecules of Triton detergent that are currently in the active site has already been reported [5]. In the closed form of L1 lipase it was shown that catalytic serine is in tight side-chain packing with some residues of the active site (His113, Phe17, Ile320, Thr270, and Met326) resulting in the stabilization of the serine loop, and lipase thermostability. Catalytic machinery of BTL2 includes the catalytic triad (Ser114, His359, and Asp318) and the oxyanion hole, also Phe17 has a fundamental site in the oxyanion hole formation in the BTL2 open conformation [5,20]. In this study, to create more space for substrates, Phe17 is replaced with Ser in chimeric BTL2 lipase, in which the already conserved pentapeptide (^{112}Ala-His-Ser-Gln-Gly^{116}) had been replaced completely with similar sequences (^{207}Gly-Glu-Ser-Ala-Gly^{211}) of *Candida rugosa* lipase (CLR) at the nucleophilic elbow region. Firstly, the effect of this substitution on the structure and function of chimeric lipase was investigated by bioinformatics studies. Then, the F17S mutation was induced by site directed mutagenesis, and the enzyme was expressed in *E. coli*. The pure enzyme was characterized, and its physicochemical properties were compared to those of the chimeric lipase.

2. Materials and methods

The recombinant plasmid *pKHT.E*, which contained the chimeric *B. thermocatenulatus* lipase gene, was used for generating mutated *btl2* gene (GenBank accession No. X95309). The plasmids *pGEM*-5zf(-) and pET-26b (+) (Novagen, USA) were used for cloning and expression in *E. coli* DH5α, and playsS (Invitrogen, USA), respectively. MODELLER v9.10 [9], and VMD 1.9 [18] Software were used for Homology modeling, and analysis of the protein structure. DE-52 cellulose column (Maidstone, England) was provided from Whatman (Maidstone, England). All the ligand molecules were purchased from Sigma (St. Louis, USA). All other chemicals were purchased from Merck (Darmstadt, Germany).

2.1. Homology Modeling and 3D-Structure Analysis

Homology modeling for the mutated lipases was carried out as a template, with MODELLER v9.10 (http://www.salilab.org/modeller/) [9], by using opened form of BTL2 lipase (2W22) [20]. The MODELLER generated structure of the mutated lipase was further evaluated by Ramachandran plot generated by Procheck [22], Errat plot [8], Q mean server [3] and ProSA-web score [31]. The Root Mean Square Deviation (RMSD) values were also calculated by superimposing all Cα atoms of the mutated lipase to the corresponding Cα atoms of the chimeric lipase [18].

2.2. Site-directed mutagenesis and cloning of mutated btl2 gene

The pKHT.E plasmid containing chimeric *btl2* gene (previous work) was used as a template for generating mutated *btl2* gene using site-directed mutagenesis, as described previously [16]. The mutated *btl2* gene was generated by PCR using the F17S forward (5′- GATGGCCATGGCGGCATCCC CACGCGCCAATGATGCACCCATCGTGCTTCTCCATG GGTCCACAGGATGGGGGCGAGAG-3′, *Mlu* NI site and point mutation are underlined) and F17S reverse (5′-TTGAGCTCATCATC CCTTCATTAAGGC -3′, *Sac* I site) primers. The PCR product was cloned into pGEM-5zf plasmid, and leading to the formation of pGKKM.17S plasmid, which then was transformed into the *E. coli* DH5α. The resulting transformants were selected on LB plates supplemented with ampicillin (100 mg/ml). To confirm the mutations recombinant, the pGKKM.17S plasmid DNA was sequenced using universal M13 forward, and reverses primers (Macrogen Inc., Seoul, South Korea). The pGKKM.17S plasmid was digested with *Mlu* NI and *Sac*I enzymes, then the resulting (1188 bp) was ligated into the corresponding restriction sites of the expression vector pET-26b(+)to pKKM.17S plasmid that was constructed. Subsequently, the pKKM.17S plasmid was transformed into *E. coli* pLysS. *B. thermocatenulatus* lipase gene was also cloned, and expressed in *E. coli* pLysS that was used as chimeric lipase to compare with the mutated *B. thermocatenulatus* lipase.

2.3. Expression and purification of mutated lipase

The mutated and chimeric plasmids were transformed into *E. coli* pLysS. The transformants were grown (overnight) at 37 °C in LB liquid medium supplemented with kanamycin (30 mg/ml). The cultures were transferred into fresh LB liquid media at 1:20 (v/v). Protein expression was induced at 37 °C and 150 rpm, by the addition of 0.5 mM IPTG, when the cell density (OD600) reached 0.6. After induction for 18 h, cells were collected by centrifugation. The cell pellets were resuspended in 25 ml of 100 mM Tris base buffer (pH 8.5). PMSF was added to a final concentration of 1 mM, and then cells were disrupted by ultrasonication on ice for six sets of one-minute pulses (100 mHz) with 15-s intervals. The cells lysate extracts were centrifuged (9000×*g*, 10 min, 4 °C). Then supernatant and precipitate were collected, and analyzed by

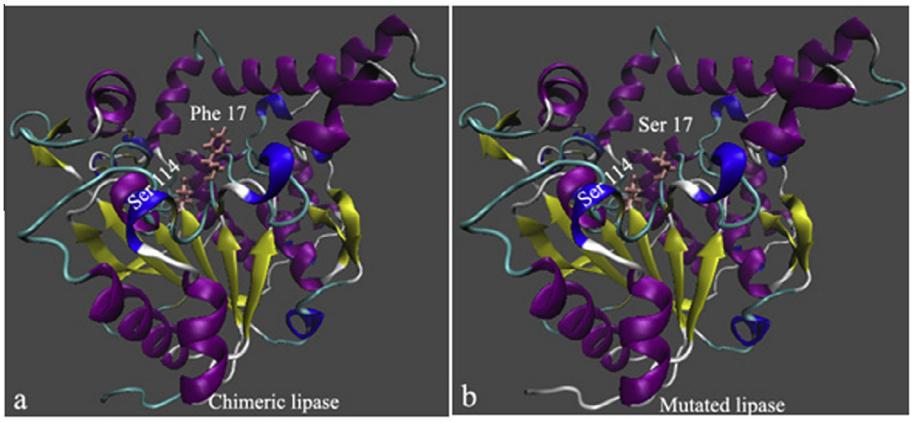

Figure 1 Optimized 3D structure of the opened form of chimeric lipase (a), and mutated lipase (b) that was generated by MODELLER v9.10, using 2W22 as the template. Visualization was performed using VMD software.

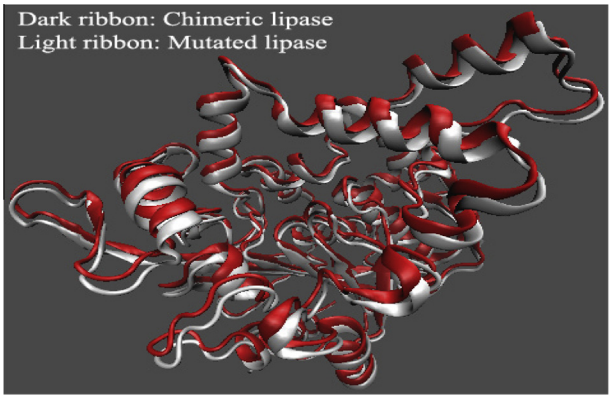

Figure 2 Superposition of Chimeric lipase (Dark ribbon) and Mutated lipase (Light ribbon), the picture was taken by VMD software.

SDS–PAGE. Inclusion bodies of the mutated and chimeric lipases were rearranged; and then they were purified with ion-exchange chromatography [21]. Subsequently, the outputs of the column were collected and visualized using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) [26]. Protein bands were stained by Coomassie Brilliant Blue R-250. Expression and purification were confirmed by Western blot.

2.4. Protein assay

The protein concentrations of mutated and chimeric lipases were measured by the Bradford method [4].

2.5. Lipase assay and substrate specificity

Lipase activity was determined by triacylglycerol substrates in pH-STAT (Metrohm Ltd., Herisau, Switzerland) at 55 °C (pH 9.5). For substrate specificity, 2 mg/ml of various substrate, including tributyrin (C4), tricaprylin (C8), tricaprin (C10), trilaurin (C12), trimyristin (C14), tripalmitin (C16), and olive oil (C18) were emulsified in distilled water containing Arabic gum

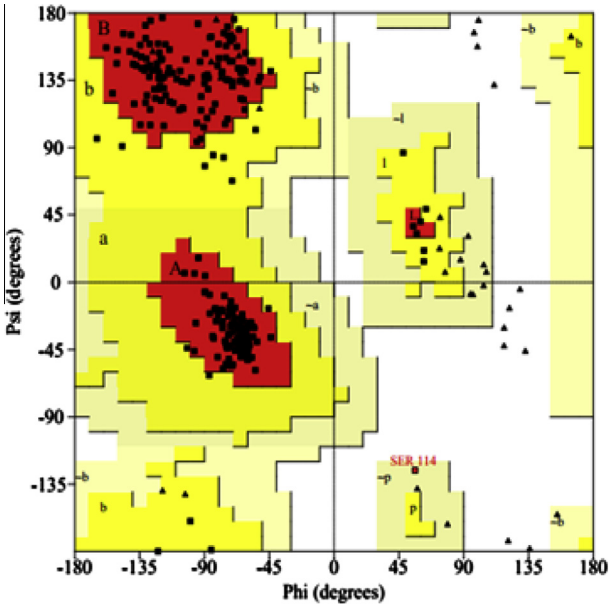

Figure 3 Ramachandran plot of mutated lipase, Glycine residues are shown with triangle, and all other residues are shown with a square.

(20 mg/ml) using ultrasonic treatment (Hielscher GmbH, Teltow, Germany) for 5 min at maximum power [16].

2.6. Effect of temperature on lipase activity

The effect of temperature on the enzyme activity was determined at 45, 50, 55, 60, and 65 °C (pH 9.5) with tricaprylin as substrate. Enzyme thermostability was tested by preincubation of the enzyme at different times (15, 30, 45, and 60 min) at 60 °C and 50 mM glycine buffer (pH 9.5) [21].

2.7. Effect of pH on lipase activity

pH profile was measured with tricaprylin as substrate at pH range of 8.0–10.0 at 55 °C [21].

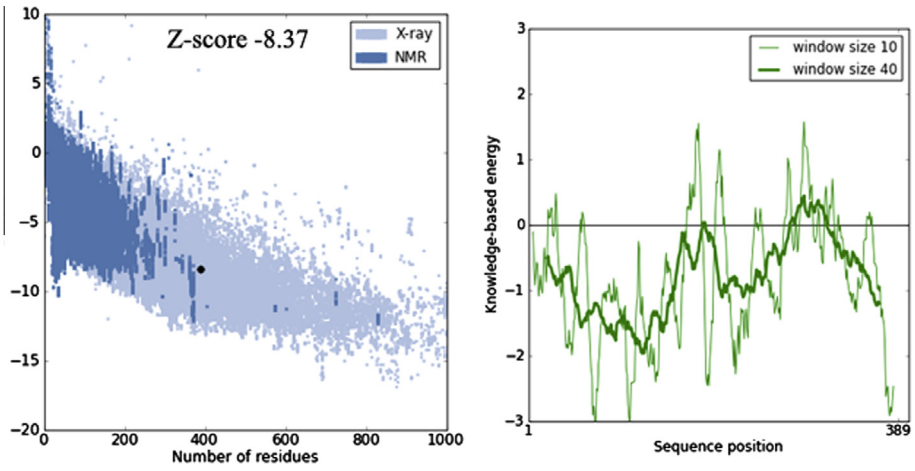

Figure 4 Z-Score and the energy profile for the mutated lipase.

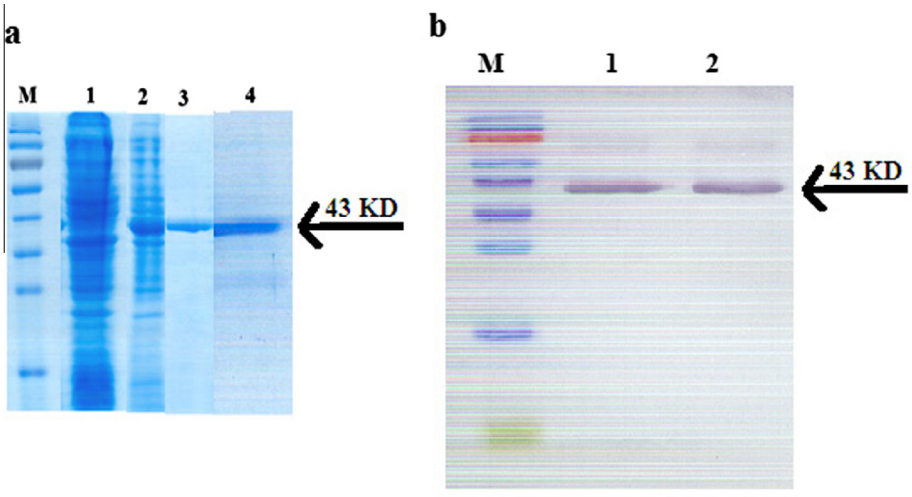

Figure 5 (a) SDS–PAGE analysis of the chimeric and mutated lipase. Lane M, protein size marker; lane 1, Crude cell extract before induction with IPTG; lanes 2, Crude cell extract after induction with IPTG; lanes 3 and 4, purified mutated, and chimeric lipases respectively, (b) Western blot analysis of the chimeric and mutated lipase. Lane M, protein size marker; lanes 1, and 2 purified chimeric and mutated lipases, respectively.

2.8. Metal ions, organic solvents and detergents on lipase activity

To examine the effects of different metal ions on lipase activity, the enzymes were incubated at room temperature for 60 min in the presence of 1-mM various metal ions ($MgCl_2$, KCl, $CaCl_2$, $FeCl_2$, $ZnSO_4$, $CuSO_4$), and EDTA. The effect of organic solvents on the lipase activity was determined after the enzymes were incubated in 30% methanol, acetone, N-heptane, N-hexane, and chloroform for 60 min. The effect of various detergents (SDS, TWEEN 20, TWEEN 40, TWEEN 85, and Triton x-100) on the lipase activity was checked after incubation, the enzymes in 1% concentration for 60 min. Eventually, residual lipase activity was measured by pH-STAT in pH 9.5 and 55 °C using tricaprylin as substrate [21].

2.9. Statistical analysis

One-way analysis of variance (ANOVA) was used to compare differences between values. P values < 0.05 were considered significant.

3. Results

3.1. Homology modeling and structure evaluation

Tertiary structures of mutated and chimeric lipases were predicted using MODELLER software (Fig. 1). The MODELLER-generated structure was evaluated by Ramachandran plot, which was generated by Procheck [22], ProSA-web [31], and root-mean-square deviation (RMSD) [18]. An RMSD of 0.125 Å was found between the model (mutated lipase) and the template (chimeric lipase) structures. This result shows that the mutation causes no change in the overall structure of the mutated lipase (Fig. 2). Ramachandran plot of the mutated lipase revealed that 94.5% of the amino acid residues were present in the most favored region, 5.2% in addition to allowed region, and 0.3 % in the generously allowed region (Fig. 3). Z-Score of −8.37 and the energy profile for mutated lipase were determined by ProSA-web (Fig. 4). The quality of the proposed model for a mutated lipase was confirmed by this observation.

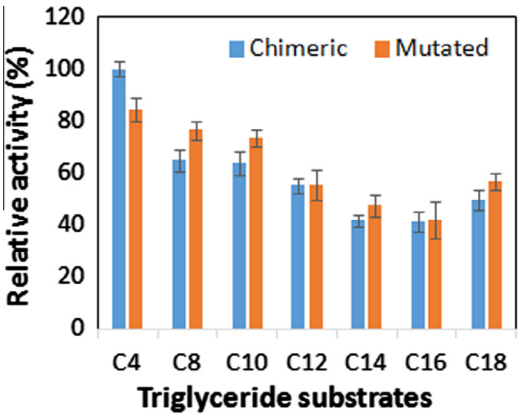

Figure 6 Substrate specificity of the chimeric and mutated lipases.

3.2. Cloning, expression, and purification

The F17S mutation was exerted in chimera *B. thermocatenulatus* lipase using Site-directed mutagenesis method. Then, the chimeric lipase (previous work) and mutated lipase genes were cloned in a cloning vector (*pGEM*-5zf). The presence of the F17S mutation in the gene was confirmed by DNA sequencing. The chimeric and mutated lipases were expressed in *E. coli* pLysS. Then, both lipases were purified by ion exchange chromatography. Single bands of mutated and chimeric lipases with an apparent molecular mass of about 43 kDa were detected by SDS–PAGE electrophoresis (Fig. 5a). Purified lipases were checked with western blot (Fig. 5b).

3.3. Substrate specificity

The enzyme specificity of the mutated lipase was compared to the chimeric lipase by a broad range of the lipase substrates of varying acyl chain lengths from C_4 to C_{18}. The results showed that the relative activity of mutated lipase was higher than chimeric lipase for all substrates with exception of tributyrin (C4) substrate. The difference in activity was more significant for tricaprylin (C8) and tricaprin (C10) (Fig. 6).

3.4. Thermal activity and stability

Optimum temperature was obtained at 60 °C (Fig. 7a). The relative activity of mutated lipase was improved compared with chimeric at all temperatures. Enzyme thermostability was measured and the results showed that enzyme activity of mutated lipase was higher than chimeric lipase, but after 30 min activity of mutated lipase decreased more compared to chimeric lipase (Fig. 7b).

3.5. Effect of pH, metal ions, organic solvents and detergents on lipase activity

The effects of pH, metal ions, organic solvents, and detergents on the enzyme activity are shown in Fig. 8. The relative activity of both enzymes was improved by increasing pH toward alkaline pH. The maximum activity was observed at pH 9.5. However, the relative activity of mutated lipase was improved at alkaline pH.

4. Discussion

Schmidt-Dannert et al. [30] have previously reported that BTL2 lipase has maximum activity at pH between 7.5 and 9 [30]. Generally, metal ions are especially important in thermostable enzymes. Hence, these have a structural role with bind to the surface of the molecule [6]. The present study showed that the enzyme activity of both lipases was slightly inhibited by Ca^{2+} and Fe^{2+}. Arnaldo et al., have been also reported similar results of the effects of 1 mM metal ions, such as, of Zn^{2+},Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Na^{+}, and Ca^{2+} on lipase activity, especially decreased activity to 33 and 74% by Fe^{2+} and Ca^{2+}, respectively [30]. Inhibition of the lipase activity in the presence of Fe^{3+} has also been reported by Higaki and Pooreydy [15,23]. The relative activity of the mutated lipase decreased much less in the presence of 5 mM EDTA compared to the chimeric lipase. The effects of organic solvents (30% v/v), and detergents (1% w/v) on the enzyme activity of mutated and chimeric lipases were also investigated. The results showed that with the exception of hexane, the effect of other organic solvents on mutated lipase improved compared to chimeric lipase. Dinh et al., reported that metha-

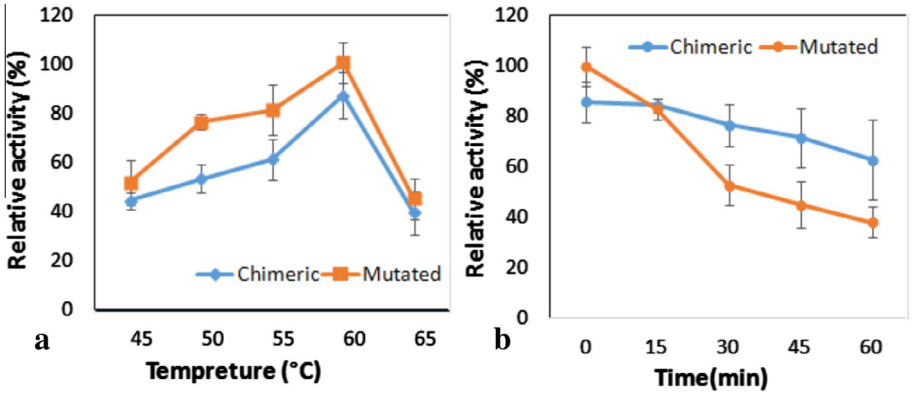

Figure 7 The effect of temperature on mutated and chimeric lipase: (a) temperature profile of mutated and chimeric lipases, (b) thermostability of mutated and chimeric lipases after incubation at 60 °C. pH, rather than chimeric lipase.

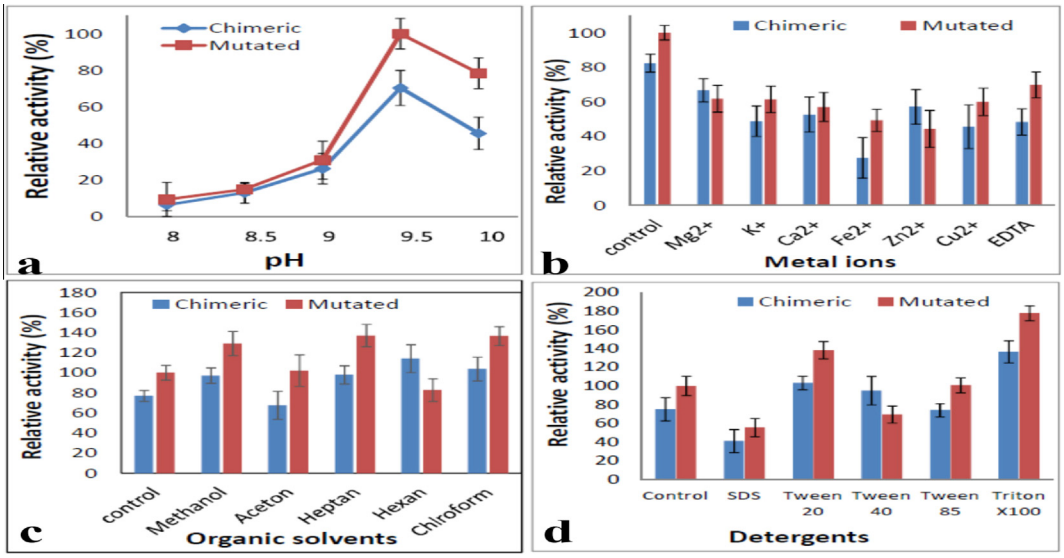

Figure 8 The Effects of pH (a), metal ions and EDTA (b), organic solvents (c), and detergents (d) on the mutated and chimeric lipases.

nol and acetone indicated an inhibitory effect on the BTL2 lipase activity [25]. The results showed that TWEEN 20, TWEEN 85, and Triton x-100 enhanced, and SDS extremely decreased the catalytic activities of both enzymes. However, effect of TWEEN 20 and Triton x-100 was highly significant in increasing the relative activity of mutated lipase. Quinn et al., reported that the addition of 1% (w/v) of Triton x-100 and TWEEN 80 increased the BTL2 lipase activity by around 30% and 6%, respectively [25].

5. Conclusions

Phe-17 plays an essential role at the active site to achieve substrate and oxyanion hole formation. Substitution of conserved phenylalanine 17 (Phe-17) residue with small residues (Ala, Ser, etc.) simplifies oxyanion hole formation, and declines steric hindrance in the enzyme active site.

Ethical approval

Animals or human samples were not contained in the article by any of the authors.

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