

Research Article

Characterization of the Recombinant Thermostable Lipase (Pf2001) from *Pyrococcus furiosus*: Effects of Thioredoxin Fusion Tag and Triton X-100

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In this work, the lipase from *Pyrococcus furiosus* encoded by ORF PF2001 was expressed with a fusion protein (thioredoxin) in *Escherichia coli*. The purified enzymes with the thioredoxin tag (TRX-PF2001Δ60) and without the thioredoxin tag (PF2001Δ60) were characterized, and various influences of Triton X-100 were determined. The optimal temperature for both enzymes was 80°C. Although the thioredoxin presence did not influence the optimum temperature, the TRX-PF2001Δ60 presented specific activity twice lower than the enzyme PF2001Δ60. The enzyme PF2001Δ60 was assayed using MUF-acetate, MUF-heptanoate, and MUF-palmitate. MUF-heptanoate was the preferred substrate of this enzyme. The chelators EDTA and EGTA increased the enzyme activity by 97 and 70%, respectively. The surfactant Triton X-100 reduced the enzyme activity by 50% and lowered the optimum temperature to 60°C. However, the thermostability of the enzyme PF2001Δ60 was enhanced with Triton X-100.

1. Introduction

One of the main limitations for the use of enzymes in industrial processes is their low stability under operational conditions (e.g., high temperatures, organic solvents, and extremes of pH). Enzymes from thermophilic and hyperthermophilic microorganisms are inherently more stable towards a variety of enzyme denaturants and thus represent promising alternatives for the development of industrial biocatalytic processes [1]. In addition to the potential biotechnological applications of these enzymes, the structural requirements that underlie their superior thermodynamic stability are of great interest to researchers [2].

Carboxylesterases (E.C. 3.1.1.1) and lipases (E.C. 3.1.1.3) are enzymes that catalyze the hydrolysis of ester bonds involving a carboxylic acid of variable chain length, that is,

from C2 to C18 or more. In organic media, these two classes of enzymes also possess the ability to catalyze several other types of biotransformations such as esterification, transesterification, alcoholysis, aminolysis, and acidolysis [3]. These unique properties have increased the biotechnological interest of enzymes for a number of important industrial biotransformations, either as hydrolytic or as synthetic catalysts [4]. For instance, they are used by organic chemists for chiral molecule preparation by optical resolution from racemic mixtures; sugar modification and flavor ester synthesis for the food industry; biodiesel production and waste treatment [3–9]. Despite their significant biotechnological potential, the use of more resistant enzymes (extremozymes) could open up more possibilities for lipases and esterases in industrial processes.

A number of lipases and esterases from extremophilic organisms have been cloned in *E. coli*, but in general their expression levels and purification yields are low. This effect could be caused by the hydrophobic nature of lipases that causes folding problems. In a previous work, Almeida et al. [10] cloned the gene PF2001 from *P. furiosus* fused to a thioredoxin tag (TRX-PF2001Δ60) in *E. coli*. The enzyme was characterized as an esterase according to its substrate preference to MUF-Hep ($C < 10$). This enzyme was immobilized on microporous polypropylene at low ionic strength, showing the hyperactivation phenomenon [11]. Recently, the same enzyme was immobilized on more hydrophobic supports showing, once more, the hyperactivation phenomenon. The immobilization on hydrophobic supports under low ionic strength and hyperactivation is a characteristic of lipases suggesting that PF2001 enzyme is a lipase and not an esterase [12].

In this work, we investigated the effects of the TRX tag on the lipase activity of the purified enzyme. To this end, the purification of the recombinant *P. furiosus* lipase (TRX-PF2001Δ60) was carried out, and the thioredoxin tag was removed. The purified enzyme with and without the thioredoxin tag (PF2001Δ60) was then characterized. Furthermore, the influence of Triton X-100 (a surfactant commonly used in esterase/lipase assays) on the optimum temperature and thermostability was investigated.

2. Materials and Methods

2.1. Enzyme and Chemicals. Calf intestine enterokinase was purchased from Roche (Nutley, NJ, USA); Isopropyl- β -D-thiogalactopyranoside (IPTG), 4-methylumbelliferyl-acetate (MUF-Ace), 4-methylumbelliferyl-heptanoate (MUF-Hep), and 4-methylumbelliferyl-palmitate (MUF-Pal) were purchased from Sigma (Sigma, Chemicals, USA.). All other chemicals were of the highest reagent grade commercially available.

2.2. Expression. The *E. coli* strain BL21 (DE3) pLysS containing the plasmid with the ORF PF2001 and the thioredoxin tag, obtained by Almeida et al. [10], was grown in LB medium (0.5% yeast extract, 1% tryptone, and 0.5% NaCl) containing ampicillin (100 μ g/mL) and chloramphenicol (12.5 μ g/mL). After incubation with shaking at 35°C until $A_{600\text{nm}}$ reached 0.3, the induction was carried out by adding IPTG at a final concentration of 0.5 mM and incubation for 3 h at 35°C. Cells were collected by centrifugation, resuspended in sodium phosphate buffer (50 mM, pH 7.0) and disrupted by sonication. The lysed cells were centrifuged and the clarified supernatant was used for affinity purification.

2.3. Purification. A Hitrap Chelating resin (bed volume 1 mL) (Amersham Biosciences/GE Healthcare) was washed with 10 mL of sterile water and charged with Ni^{2+} using 5 mL of a 100 mM NiSO_4 solution. After a second wash with 10 mL of sterile water, the charged resin was equilibrated with 10 mL of sodium phosphate buffer (50 mM, pH 7.0). Eight milliliters of the clarified supernatant of the cell lysate

was applied directly to the column. A washing step with 100 mM imidazole in equilibration buffer was done in order to elute nonspecific ligands. Elution of the TRX-PF2001Δ60 was carried out with 150 mM imidazole in the equilibration buffer. Fractions of 1 mL were collected at a flow rate of 0.2 mL/min and monitored by their absorbance at 280 nm.

2.4. Enterokinase Hydrolysis. Twenty-five micrograms of purified enzyme were dialyzed against Tris-Cl buffer (50 mM, pH 7.5). The dialyzed enzyme was treated with 0.6 μ g of enterokinase at 4°C for 18 h to remove the TRX tag. The digestion mixture was analyzed by SDS-PAGE and zymography.

2.5. Electrophoresis and Zymogram Analysis. Nonreducing gel electrophoresis (SDS-PAGE) was carried out with a 10% separating gel on a vertical slab minigel apparatus (Bio-Rad) at 120 V for 1 h [13].

After the run, the gels were soaked for 30 min in 2.5% Triton X-100 at room temperature, briefly washed in 50 mM sodium phosphate buffer, pH 7.0, and covered by a solution of 100 μ M MUF-Hep in ethylene glycol monomethylether. Soon after, the bands became visible under UV illumination [14]. Following zymogram analysis, the gels were silver-stained, and the protein bands were visualized.

2.6. Enzymatic Activity Assay. Enzyme assays were carried out using 4-methylumbelliferone (MUF) derivatives as substrates (MUF-Ace, MUF-Hep and MUF-Pal) in a Varian Cary Eclipse spectrofluorimeter, as described elsewhere [10, 14]. The reaction mixture contained 0.6 mL of 50 mM sodium phosphate buffer pH 7.0 containing 0.1% gum arabic and 2.4 μ L of MUF-derivative substrate stock solution (25 mM in ethylene glycol monomethylether). The enzymes TRX-PF2001Δ60 and PF2001Δ60 (0.5 μ g) were added to start the reaction. Enzymatic activity was determined at 70°C by measuring the increase of fluorescence emission ($\lambda_{\text{ex}} = 323$ nm and $\lambda_{\text{em}} = 448$ nm) due to the release of MUF. All rates were measured during the linear part of the progress curve. A calibration curve was constructed with 4-methylumbelliferone (MUF). One unit of activity was defined as the amount of enzyme required to release 1 μ mol of MUF per minute under the conditions described above. Protein concentration was determined by the Bradford method [15]. All tests were carried out 3 times in triplicate.

2.7. Temperature and pH Effects. For optimum temperature determination, the reaction was assayed using MUF-Hep as substrate at 50, 60, 70, and 80°C with 50 mM phosphate buffer pH 7.0.

For optimum pH determination, the reactions were performed at 70°C with 100 mM BIS-TRIS-propane buffer at pH 6, 7, 8, and 9.

2.8. Thermostability Analysis. The enzymes TRX-PF2001Δ60 and PF2001Δ60, in sodium phosphate buffer (50 mM pH 7.0) containing 0.1% gum arabic and 0.4% Triton X-100, were preincubated for different times at 55, 75, and 95°C.

Tubes were removed periodically and assayed for lipase activity using MUF-Hep at 70°C. The Triton X-100 influence on thermostability was determined for PF2001Δ60 at 70°C with and without 0.4% Triton X-100.

2.9. Substrate Preference and Influence of Ions and Inhibitors.

The substrate preferences of TRX-PF2001Δ60 and PF2001Δ60 were determined using 100 μM of the MUF-Ace, MUF-Hep, and MUF-Pal substrates. The effects of metal ions on lipase PF2001Δ60 activity were assessed by measuring the residual activity after preincubation in phosphate buffer 50 mM with 10 mM of metal ion at 70°C and pH 7.0 for 10 min. The metal ions used were KCl, MgCl₂, FeCl₂, and CaCl₂. To determine the effects of enzyme inhibitors, the PF2001Δ60 enzyme was pre-incubated as above, and then the substrate was added in order to measure lipase activity. The inhibitors and concentrations used were sodium dodecyl sulfate (SDS) 1 mM, dithiothreitol (DTT) 1 mM, ethylene diamine tetraacetic acid (EDTA) 1 and 10 mM, and ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) 1 and 10 mM.

2.10. Data Analyses. Lipase activity was analyzed using MINITAB v.14 package. The values expressed were based on the average of triplicate experiments. Experimental errors were between 5 and 10%, and averages were compared using *t*-test ($P < 0.05$).

3. Results and Discussion

In a previous work, we reported the identification and cloning of a novel lipase from *Pyrococcus furiosus* encoded by the ORF PF2001 [10]. The enzyme was cloned and expressed without the first 60 nucleotides (a hypothetical signal peptide). A thioredoxin tag was fused to the amino terminal domain of the enzyme. The enzyme in a crude extract from *E. coli* presented the best activity at 60°C (pH 7.0). This temperature is 40°C lower than the optimal *P. furiosus* growth temperature. In order to understand the reasons for this large difference, and to validate the fusion of the TRX tag as a method for expressing the extremophilic lipases, we performed in this work the purification of the enzyme and its characterization before and after thioredoxin tag removal.

3.1. Purification and Enterokinase Hydrolysis. The TRX-PF2001Δ60 purification results are summarized in Table 1. The enzyme was completely purified with a yield of 89.8% (Figure 1, lane 3). A purification of 6.6-fold was achieved. The specific activity of the purified enzyme was 8.9 U/mg using MUF-Hep as the substrate.

The purified enzyme was cleaved with enterokinase, and the hydrolyzed product showed two protein bands (about 48.8 and 26.5 kDa, representative of TRX-PF2001Δ60 and PF2001Δ60, resp.) in the SDS-PAGE analysis (lane 4, Figure 1(a)). Both proteins rendered a positive signal in the zymogram analysis. Therefore, the PF2001Δ60 preparation also contained some of TRX-PF2001Δ60. However, densitometry analyses show that less than 10% of the enzyme

remains in the TRX-PF2001Δ60 form (Figure 1(a)). Furthermore, the zymogram (Figure 1(b)) is not a quantitative analysis. So the influence of this form of protein is minimal, within experimental error.

The purified enzyme was stable at -20°C for several months (data not shown).

3.2. Effects of Thioredoxin Tag

3.2.1. Optimal Temperature. The activities of TRX-PF2001Δ60 and PF2001Δ60 increased linearly from 50 to 70°C. At 80°C, the measured activities were higher than at 70°C, but the slope of the curve over this range was less steep than between 50 and 70°C. Among the temperatures tested, the temperature at which both enzymes show maximum activity was 80°C. This temperature was considered as the optimal temperature for both enzymes (Figure 2(a)).

At 50°C, TRX-PF2001Δ60 and PF2001Δ60 exhibited the same level of activity (3 U/mg), but the difference between them increases with temperature. At 80°C, the enzyme without the thioredoxin tag is twice as active as the enzyme with the thioredoxin tag.

Thioredoxin is a peptide of 11.7 kDa, responsible for 24% of the total molecular weight of the recombinant fusion protein. Although this tag is considered a thermostable protein [16], Pedoni et al. [17] demonstrated that 90% of the enzyme is denatured at 90°C. These facts may explain the reason that the lipase with and without thioredoxin tag has the same optimal temperature, but with TRX-PF2001Δ60 presenting lower activity at higher temperatures. We believe that the TRX-tag does not modify the lipase structure, but it diminishes the quantity of active enzyme.

3.2.2. Optimal pH. The enzymes TRX-PF2001Δ60 and PF2001Δ60 showed optimum activity in pH 7.0. The enzyme with and without the thioredoxin tag demonstrated 73 and 90% of its maximum activity, respectively, at pH 8.0 (Figure 2(b)). The optimal pH for lipases and esterases according to Petersen is determined by the electric charge distribution on the active site surface [18]. These results suggest that the thioredoxin tag is sufficiently distant from the active site to cause any effect on the charge distribution.

3.2.3. Thermostability. The resistance to heating in the presence of Triton X-100 was investigated at the temperatures of 55, 75, and 95°C and showed that the enzyme is endowed with high thermostability. Around 100% activity was recovered after 6 h pre-incubation at 55 and 75°C. However, less than 50% activity was retained after 10 min at 95°C. No differences were observed between PF2001Δ60 and TRX-PF2001Δ60 (Figure 3).

3.3. Substrate Preference and Effects of Ions and Inhibitors. Among the different substrates tested, the enzyme was more active towards MUF-Hep. The activities towards MUF-Ace and MUF-Pal were, respectively, equivalent to 20.8 and 6.4% of the activity towards MUF-Hep.

TABLE 1: Summary of the purification of the recombinant TRX-PF2001Δ60 from *P. furiosus*.

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Soluble Ext.	16.9	22.5	1.34	100	—
Ni-NTA	2.28	20.4	8.9	89.8	6.63

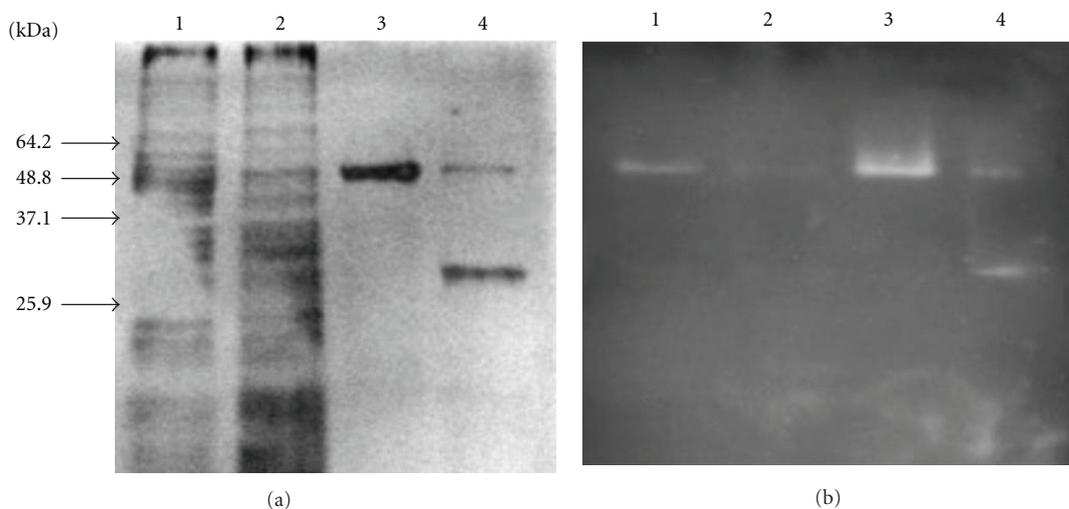


FIGURE 1: SDS-PAGE (a) and zymography (b), on a 15% polyacrylamide gel, of expressed and purified protein encoded by the PF2001Δ60 gene. (1) soluble extract; (2) flow-through; (3) recombinant lipase eluted with 150 mM imidazole; (4) lipase treated with enterokinase to remove the thioredoxin tag.

TABLE 2: Effects of metal ions, chemicals, and detergents on PF2001Δ60 lipase activity. The enzyme (0.5 μg) was incubated at 70°C in 50 mM sodium phosphate buffer (pH 7.0) in the presence of the tested agent using MUF-Hep as substrate. After 10 min, the mixture was assayed for residual activity using the standard lipase assay. The activity of the control was considered 100%.

Chemical	Concentration (mM)	Relative activity (%)
KCl	10.0	107.5
MgCl ₂	10.0	77.9
CaCl ₂	10.0	54.8
SDS	1.0	37.0
DTT	1.0	94.6
Triton X-100	6.4	47.0
	1.0	97.8
EDTA	10.0	195.7
	1.0	103.4
EGTA	10.0	169.0
PMSF	1.0	0.0

In order to define the ion and inhibitor effects on the PF2001Δ60, enzyme the activity assays were performed in the presence of the above-mentioned substrates.

The influence of mono- and divalent metal ions was studied at a concentration of 10 mM. K⁺ did not affect the activity of the enzyme significantly, but significant activity loss was observed with Mg²⁺ and Ca²⁺ (20 and 45%, resp.). This suggests that PF2001Δ60 enzyme is not a metalloenzyme. This was reinforced by a significantly higher activity of the enzyme

in the presence of 10 mM of metal chelators: EDTA (activity increased by 96%) and EGTA (69%) (Table 2).

PF2001Δ60 retained full activity in the presence of the disulfide bond reducing agent DTT. The enzymatic stability against DTT suggests that the Cys166 and Cys167 are not disulfide bonded, or that this covalent bond does not affect the protein structure.

In the presence of the detergents: SDS (1mM) and Triton X-100 (0.4%–0.6 mM), the activity decreased by 63% and 53%, respectively. Similar inhibitory effects on microbial lipases and esterases activity have been observed by other groups [19–22].

Because Triton X-100 has traditionally been used in a concentration of 0.4% as an emulsifier to measure lipase and esterase activities, its effects were further investigated.

3.4. Effects of Triton X-100 on Optimal Temperature and Thermostability. Figure 4(a) shows that the presence of Triton X-100 in the reaction mixture drastically modifies the temperature dependence profile of the enzyme. Using 0.4% Triton X-100, the optimal temperature of the enzyme was shifted from 80°C to 60°C. This data explains the results described in our previous report [10] that the optimal reaction temperature for this enzyme was 60°C. In those experiments, the enzyme activity was measured in a reaction mixture containing 0.4% of Triton X-100 because this nonionic surfactant is frequently used to help dissolve lipids in water.

The Triton X-100 cloud point temperature is probably one of the main reasons for shift in optimal temperature of the enzyme. At 60°C, most non-ionic surfactants in aqueous

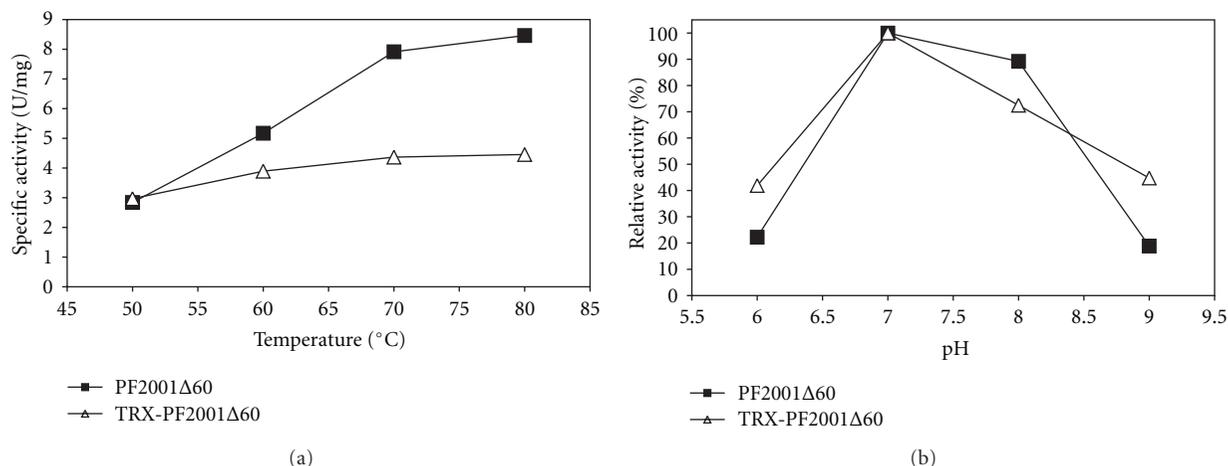


FIGURE 2: (a) Effects of temperature on lipase activity. The purified lipases, TRX-PF2001Δ60 and PF2001Δ60, were assayed at temperatures ranging from 50 to 80°C, in 50 mM phosphate buffer, pH 7.0. (b) Effects of pH on lipase activity. The purified lipases, TRX-PF2001Δ60 and PF2001Δ60, were assayed at pH ranging from 5.0 to 9.0, in 100 mM BIS-TRIS-propane buffer at 70°C. All assays were carried out without Triton X-100.

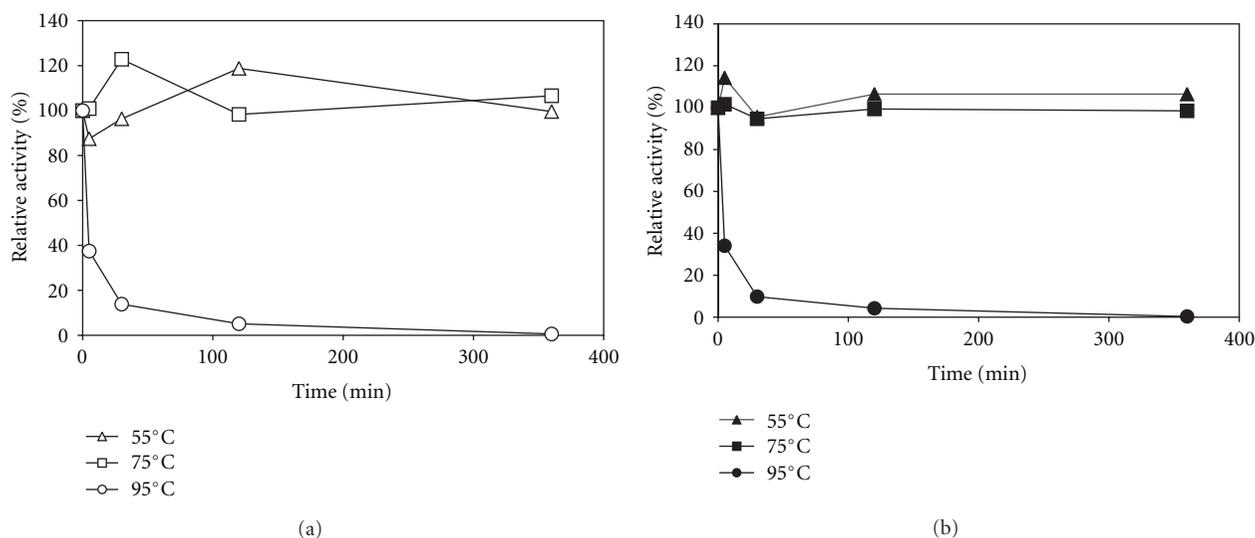


FIGURE 3: Thermal stability of the purified enzyme, with (a) and without (b) the thioredoxin tag. The enzyme samples in phosphate buffer (50 mM pH 7.0) containing 0.1% gum arabic and 0.4% Triton X-100 were incubated at the indicated temperatures for 5 min, 30 min, 2, and 6 h. The residual enzyme activity was assayed at 70°C, pH 7.0, using the substrate MUF-Hep.

solutions form micelles and become turbid when heated. Above this temperature, the micellar solution separates into a surfactant-rich phase, in which the surfactant concentration is close to the critical micellar concentration.

Although the presence of Triton X-100 affects the activity of the enzyme at higher temperatures, the thermostability was higher when the enzyme was incubated with the detergent. At 70°C, 89% of enzyme activity was recovered after 85 min of incubation in the presence of Triton, but only 40% was recovered when Triton X-100 was not used in the incubation mixture (Figure 4(b)). Studies using this enzyme immobilized on Butyl and Octadecyl-Sepabeads also showed an increase in enzyme thermostability in the presence of Triton X-100 [12]. We believe that interactions between the

Triton X-100 molecules and the enzyme provided a more rigid external backbone for the lipase molecules. Also, the effect of higher temperatures in breaking the interactions that were responsible for the proper globular, catalytic active structure, became less prominent, thus increasing the thermal stability of the immobilized lipase.

In spite of its high optimal temperature and thermostability, it is curious to observe the rapid denaturation of PF2001Δ60 at 95°C with or without Triton X-100, a temperature close to the optimal growth temperature of *P. furiosus*. Similar effects were observed when other enzymes were heterologously expressed in *E. coli*; where the enzymes appeared to be significantly less thermostable and barostable than in their natural hosts [23]. These results suggest that

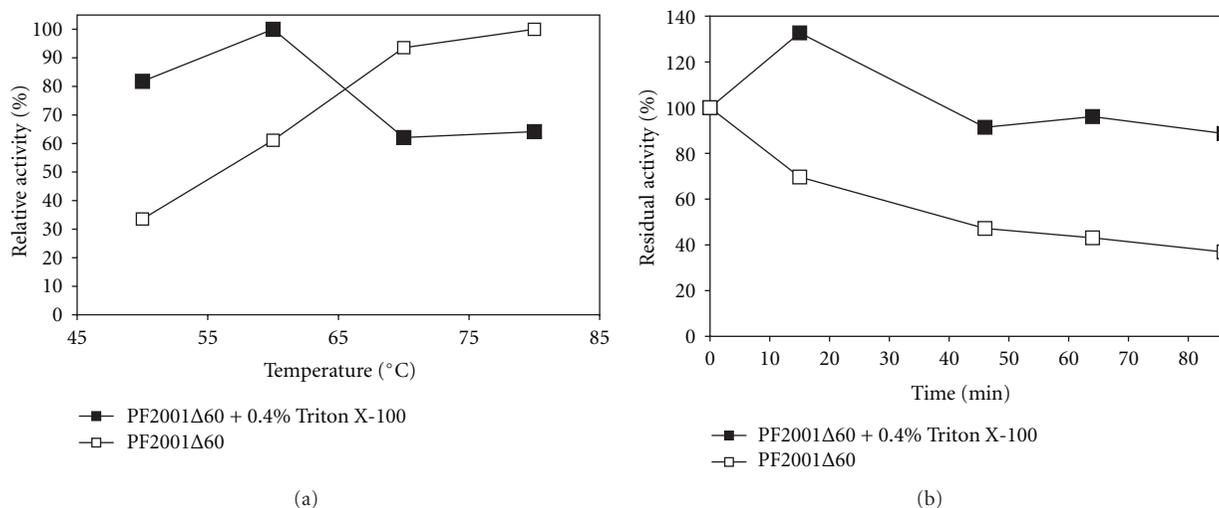


FIGURE 4: (a) Effect of Triton X-100 on optimal temperature. The lipase PF2001Δ60 was assayed at temperatures ranging from 50 to 80°C, in 50 mM phosphate buffer, pH 7.0, in the presence and absence of Triton X-100. (b) Effects of Triton X-100 on thermal stability of the PF2001Δ60. The enzyme samples in phosphate buffer (50 mM pH 7.0) containing 0.1% gum arabic were incubated at 70°C for 85 min and in the same conditions with 0.4% Triton X-100. The residual enzyme activity was assayed at 70°C, pH 7.0, using the substrate MUF-Hep.

PF2001Δ60 might be stabilized in part by association with other cellular components.

4. Conclusions

The purified recombinant lipase exhibits its highest activity at 80°C—one of the highest temperatures described for a lipase with medium chain length substrate preference. Furthermore, the TRX tag did not influence the optimal pH and optimal temperature of the enzyme; however, the temperature profile was influenced by the TRX tag, reducing its specific activity at 80°C by 50%.

In addition, we show here that Triton X-100, a commonly used detergent in lipase essays, influenced the enzymatic performance; it shifted the optimal temperature to 60°C, diminished the enzyme activity by 50, and stabilized the enzyme towards the temperature.

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