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Characterization of a New Thermostable Carboxylesterase from *Aneurinibacillus* sp. PDF24

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Abstract

In this study, esterase of *Aneurinibacillus* sp. PDF24 strain, a thermophilic bacteria, was purified to homogenity (5.25 fold purification) by column chromotography, and characterized. The molecular weight of *Aneurinibacillus* sp. PDF24 esterase was determined about 40 kDa. The maximum activity of the purified esterase was analyzed at 55°C, pH 8.5. The esterase was found to be stable at 40ºC, 50ºC and 60ºC for 1 hour. K_m and V_{max} values for p-nitrophenyl butyrate were determined as 0.120 mM and 3164.8 U/mg, respectively. Considering K_m values in the literature, *Aneurinibacillus* sp. PDF24 esterase was found to have a good K_m value compared to other esterases. In the presence of 1 mM and 5 mM metal salts of Mg^{2+}, Li^{+}, Ca^{2+}, K^{+}, no significant change occured in enzyme activity. The activity of *Aneurinibacillus* sp PDF24 esterase was found to be stable also in the presence of ethanol, DMSO, EDTA, DTT and ß-mercaptoethanol. The data obtained suggest that the enzyme is a serine esterase, not a metalloprotein, and that disulfide bonds are not required to maintain enzyme conformation, and therefore, depending on its features, this esterase may be a suitable candidate for industrial applications.

Keywords: *Aneurinibacillus*, characterization, esterase, thermophilic

1. INTRODUCTION

Lipolytic enzymes are the most significant group of biocatalysts for biotechnological implementations. Hydrolases are a class of enzymes that displays a wide range of substrate specificity. Lipases (EC 3.1.1.3, triacylglycerol hydrolases) and esterases (EC 3.1.1.1, carboxyester hydrolases) which are two main groups of hydrolases are important biocatalysts with high industrial applications [1]. They catalyze the hydrolysis of ester bonds and are divided into some subclasses depending on the ester bonds they hydrolyze [2]. The three-dimensional structure of both groups of enzymes are characterized by the folding of the

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characteristic α/β-hydrolase [3]. They are common in animals, plants and microorganisms. [4]. Esterases and lipases are found everywhere in nature and are produced by various plants, animals and microorganisms. Bacterial and fungal enzymes help in the classification of enzymes in organic chemistry and biotechnological applications [5]. Importance of scientific and biotechnological enzyme studies and the importance of microorganisms in obtaining enzymes are increasing day by day due to the increase in usage areas of enzymatic products in the industrial area and the importance of economic value. Esterases are used in processing the skin and post and in the removal of industrial wastewater by benefiting from the ability of esterases to cleave lipids [6]. The most important commercial area for hydrolytic esterases is detergents used in domestic and industrial washing machines and domestic dishwashers. The detergency of such detergents is at the highest level, all detergents contain similar ingredients and are based on similar detergent mechanisms. [7]. In particular, recent studies on fats have made biological processes more important than chemical processes. The most important reason for this is the formation of by-products in chemical processes and these by-products are high in number and also high temperature, pressure, pH etc. It requires exceptional circumstances. Furthermore, biotechnological methods are more economical than chemical methods. In recent years, esterases derived from thermophilic microorganisms have come to the forefront in industry. Thermophilic bacteria are organisms that are adapted to live in extreme temperature conditions, and the thermophilic enzymes are recently used in biotechnological and industrial fields. The stability of thermophilic enzymes against pH changes and high temperatures is the reason why these enzymes are preferred in industrial areas. Thermophilic enzymes are stable and active even at a temperature higher than the optimum growth temperature of microorganisms. These high temperatures significantly reduces the risk of contamination can occur during the reaction. Because most of the contaminating bacteria in the biological cycles are mesophilic. In the light of this information, in this study, a novel esterase was identified and purified from *Aneurinibacillus* sp. PDF24 strain and characterized to determine its suitability and potency for industrial processes or other applications in the field.

2. MATERIALS AND METHODS

2.1. Substrats and chemicals

p-nitrophenyl butyrate, p-nitrophenyl myristate, p-nitrophenyl deconoate, p-nitrophenyl laurate, p-nitrophenyl octanoate, p-nitrophenyl palmitate were purchased from Sigma (St. Louis, MO, USA). Protein ladder was purchased from NEB (Ipswich, MA) and all other chemicals were purchased from Merck AG (Darmstadt, Germany) and Sigma (St. Louis, MO, USA).

2.2. Bacterial strains and growth conditions

Aneurinibacillus sp. PDF 24 was isolated from Dikili Nebiler Hotspring in Turkey and identified as *Aneurinibacillus* sp. according to its morphological, physiologial, and biochemical properties and 16S rDNA sequences previously [8]. *Escherichia coli* JM101 was used as negative control of carboxylesterase activity because it does not have carboxylesterase. *Aneurinibacillus* sp. PDF 24 strain was grown at 50°C at pH 7.5 in Degryse medium and allowed to grow overnight.

2.3. Determination of carboxylesterase activity

Aneurinibacillus sp. PDF 24 and *Escherichia coli* JM101 were grown on Tributyrin agar plate. Plates were prepared by adding 1% tributyrin in Leura Bertani (LB) Broth. After inoculation, plates were incubated at 50°C for three days. Clear zone formation around the colonies indicated esterase activity.

2.4. Enzyme production

Overnight culture of *Aneurinibacillus* sp. PDF24 were inoculated into Degryse medium with an optical density (OD_{600}) of 0.1 and incubated at 50°C for 24 h with a shaking rate of 150 rpm. The cells were harvested by centrifugation at 10,000 rpm for 5 min at 4°C and pellet was resuspended

in 50 mM Tris-HCl (pH 8.0) buffer, sonicated with Sartorius Labsonic M at 70% amplitude for 0.6 min at 5 min. To remove cell debris, the cell-free extract was centrifuged at 14,800 rpm for 15 min at 4°C and assayed for carboxylestrase activity.

2.5. Purification of carboxylesterase

A crude extract of *Aneurinibacillus* sp. PDF24 esterase was heated at 55°C for 15 min and precipitated proteins were removed by centrifugation at 14,800 rpm for 15 min at 4°C. After heat shock, ammonium sulfate precipitation was carried out between 20-80% $(NH_4)_2SO_4$ saturation. Resulting precipitates were dissolved in 50 mM Tris-HCl (pH 8.0) buffer and dialyzed overnight against the same buffer. The samples that have esterase activity were collected and loaded onto a Q sepharose Fast Flow column (50 x 1.5 cm) equilibrated with 20 mM Tris-HCl (pH 8.0). Elution was carried out with 500 mL gradient of NaCl (0.02–0.5 M) in the same buffer at a 1 mL/min flow rate. The fractions that have carboxylesterase activity were collected. The combined fractions was concentrated by centrifugation at 3,500 g for 15 min at 4°C using concentrator. The enzyme was then passed through a hydrophobic interaction column chromatography, Phenyl Sepharose 6 Fast Flow (20 x 0.75 cm). The column was equilibrated with 100 mL of 20 mM Tris-HCl (pH 8.0.) buffer including 1.3 M ammonium sulfate. Proteins were eluted with the gradient of ammonium sulfate from 1.3 M to 0 M and then 50 mL of 10% ethylene glycol was passed through the column with a flow rate of 0.5 mL/min. The amount of proteins in each fractions were determined by measurements at 280 nm wavelength in each purification step. In all eluted samples, the carboxylesterase activity was determined spectrophotometrically and fractions with the highest activity were selected. Protein concentration was determined by the Bradford method [9]. The purity and molecular mass were confirmed on SDS-PAGE (12% separating and 4.0% stacking) according to the method of Maniatis et al. [10].

2.6. Spectrophotometric carboxylesterase activity assay

The substrate (pNPB) solution for the determination of activity was prepared by mixing ethanol and 50 mM Tris–HCl (pH 8.0) to a final composition of 1:4:95 (v/v/v) of substrates dissolved in acetonitrile-isopropanol (1:1) /ethanol/buffer (50 mM Tris-HCl, pH 8,5), respectively.

Substrate solution (0.9 mL) and 50 mM Tris-HCl (pH 8.5) buffer (0.27 mL) were added to the enzyme (0.03 mL) and incubated at 55°C for 20 min [11]. After incubation, the enzyme activity was measured by monitoring the absorbance at 410 nm. One enzymatic unit was defined as 1 µmol p-nitrophenol formed in 1 minute from p-nitrophenyl butyrate (pNPB) at pH 8.5 and 55ºC. The molar absorption coefficient of p-nitrophenol at 410 nm was calculated as $1.457x10^5 cm^2 mol^{-1}$. All conditions that the reactions were carried out (reaction temperature, reaction pH, amount of enzyme) were rearranged as the enzymes optimum working conditions are determined.

2.7. Characterization of enzyme

2.7.1. Effects of temperature on activity and stability

Reactions, for determining the effects of temperatures on carboxylesterase activities were carried out at different temperatures ranging from 25ºC to 90ºC at pH 8.5 using pNPB as the substrate. Results were expressed as relative activity (%). For the stability of carboxylesterase, enzyme was incubated in 50 mM Tris-HCl (pH 8.5) buffer, at 40ºC, 50ºC, 60ºC, 65ºC, 70ºC and 75ºC for 20, 40 and 60 minutes. The residual activity (%) after incubation was calculated by comparison with untreated enzyme.

2.7.2. Effects of pH on activity and stability

Optimum pH of enzyme was determined by incubating enzyme in the range of pH from 5.0 to 10.0 at 55°C. pNPB was used as the substrate. The following buffers (50 mM) were used; sodium acetate (pH 5.0-5.5), potasium phosphate (pH

6.0), Tris-HCl (pH 7.0-8.0-8.5-9.0) and CAPS (pH 10.0). Results were expressed as relative activity (%).

To determine the stability of the enzyme at pH values of 5.0-10.0, a pre-incubation was performed at room temperature and 55°C for 60 min at each pH value. The residual enzyme activity was determined under the standard assay conditions.

2.7.3. Substrate specifity

To determine the substrate specificity of the esterase, p-nitrophenyl butyrate (4C), p-nitrophenyl caprylate (8C), p-nitrophenyl caprate (10C), p-nitrophenyl laurate (12C) p-nitrophenyl myristate (14C) and p-nitrophenyl palmitate (16C) were used. The relative activity (%) was determined under the standard assay conditions.

2.7.4. Effect of metal ions

Mg^{2+}, Li^{+}, Ca^{2+}, K^{+}, Zn^{2+}, and Co^{2+} were performed with chloride salts of metal ions. The enzyme was incubated with 1 mM metal ions for 15 minutes and 5 mM metal ions for 60 minutes at 55°C. To examine the effect of metal ions on the activity of carboxylesterase, incubated samples were assayed at 55ºC and pH 8.5 with pNPB as the substrate. The carboxylesterase activity of the enzyme without metal ions was defined as the 100% level. The residiul activity (%) was assayed spectrophotometrically.

2.7.5. Effect of detergents, potential inhibitors or organic solvents on the enzyme activity

To determine the effects of detergents, potential inhibitors or organic solvents such as 1% (v/v) of dimethyl sulfoxide (DMSO), β-Mercaptoethanol, ethanol and isopropanol, 0.1% of (v/v) Triton X-100, sodium dodecyl sulfate (SDS) and Tween 20, and 5 mM of EDTA, dithiothreitol (DTT), phenylmenthane sulfonyl fluoride (PMSF) were used. Carboxylesterase activity were assayed by the same procedure for 1 hour in 50 mM Tris-HCl (pH 8.5) buffer using pNPB as the substrate. The carboxylesterase activity of the enzyme without organic solvents, detergents and potantial inhibitors were defined as the 100% level. The residual activity (%) was assayed spectrophotometrically.

2.7.6. Kinetic parameters

The carboxylesterase activity was measured using various concentrations (25-1000 μM) of pNPB as substrate. The values of Michaelis-Menten kinetic parameters, V_{max} and K_m of the enzyme were calculated using a Lineweaver–Burk plot.

3. RESULTS AND DISCUSSION

3.1. Determination of carboxylesterase activity

Aneurinibacillus sp. PDF24 strain was shown to have esterase activity. *E.coli* JM101 strain that have no esterase activity and *Staphylococcus aureus* that have esterase activity were used as a positive and negative controls, respectively. After 3 days of incubation, the formation of clear zones around bacteria indicated the carboxylesterase activity (Figure 1).

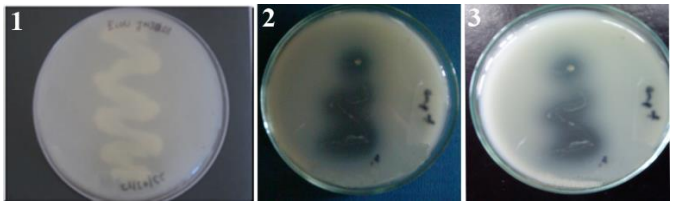

Figure 1 The esterase activity of the PDF24 bacterium on the Degryse-Tributrin agar. 1-*E.coli* JM101 esterase negative, 2-*Aneurinibacillus* sp. PDF24 esterase positive, 3-*Staphylococcus aureus* positive

3.2. Purification of the carboxylesterase

Aneurinibacillus sp. PDF24 carboxylesterase was expressed and purified by heat-shock, ammonium sulphate precipitation, ion exchange and hydrophobic interaction column chromatography. Protein concentrations and specific activities were determined after each purification steps. Enzyme purities were checked by SDS-PAGE analysis (Figure 2). Purifcation yields of enzymes after purifcation steps were shown in Table 1.

Table 1 Summary of purification steps

Purification step	Total volume (mL)	[Protein] (mg/mL)	Total protein (mg)	Total activity (U)	Spesific activity (U/mg)	Yield	Purification (fold)
Crude extract	17.0	1.9	32.5	116.9	3.6	100	1.0
Heat treatment	11.0	1.9	20.9	98.2	4,7	84.0	1.3
Ammonium sulfate precipitation	5.0	3.7	18.5	94.3	5.1	80.7	1.4
Ion exchange (Q-Sepharose)	4.1	0.7	2.9	45.8	15.7	39.2	4.4
Hydrophobic interaction	0.4	2.6	1.0	19.3	18.9	16.6	5.2

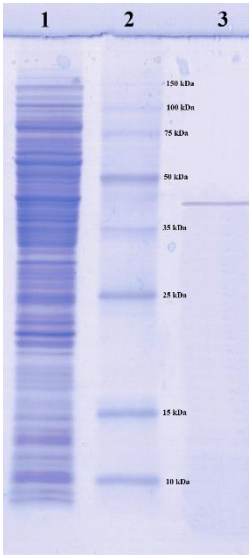

Figure 2 SDS-PAGE analysis of purified *Aneurinibacillus* sp. PDF24 esterase. Lane 1: Crude extract Lane 2: Moleculer weight marker Lane 3: Purified PDF24 esterase

3.3. Characterization of the carboxylesterase

3.3.1. Optimum pH and temperature

The optimum temperature for carboxylesterase was observed to be 55ºC and reactions were carried out at this temperature in subsequent experiments (Figure 3a)

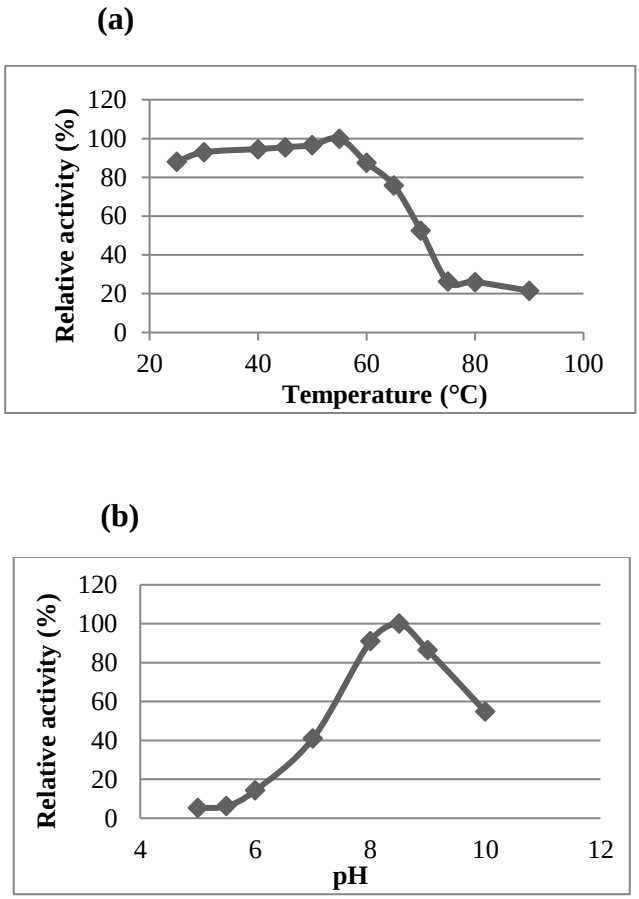

Figure 3 Effect of (a) temperature and (b) pH on activity of the purified PDF24

In literature, the optimum temperature of *Geobacillus thermodenitrificans* EstGtA2 and *Geobacillus* sp. DF20 esterases are reported to have 50°C [12, 13], *Geobacillus* sp. Est1, Est2 and Est3 esterases are reported to have 65°C [14] temperature optima. The optimum temperature of *Geobacillus thermoleovorans* YN EstA is reported as 60-65ºC [15]. When most of the esterases in the literature are reviewed, the optimum temperatures of the enzymes vary between 50-65ºC. The optimum temperature of *Aneurinibacillus* sp. PDF24 esterase is also in these ranges. However, there are also enzymes that operate at higher temperatures than these. For example, the optimum temperatures of *Thermoanaerobacterium thermosaccharolyticum* ThLip1 and ThLip2 are reported as 80ºC and 75ºC respectively [16].

The effect of pH on esterase activity was examined by a series reaction performed in

buffers at pH 5.0-10.0. As shown in Figure 3b, optimum pH of *Aneurinibacillus* sp. PDF24 esterase was observed to be pH 8.5. Most of the previously studied esterases have been reported to operate at an optimum pH between 7.5 and 9.5 with a few exceptions, for example, *Thermoanaerobacterium thermosaccharolyticum* ThLip1 and ThLip2 have optimum pHs of 6.5 [16]. *Geobacillus thermodenitrificans* EstGtA2 esterase is reported to have a 8.0 optimum pH [12]. Optimum pH of *Geobacillus stearothermophilus* Est55 is in the range of 8.0-9.0. The optimum pH of *Geobacillus* sp. Est1 and Est3 is 9.5 [14] and the optimum pH of *Thermus* sp. NCCB 100425T is 7.5 [17]. Carboxylesterases in the literature generally have high optimum pHs. *Aneurinibacillus* sp. PDF24 esterase works better in alkaline environment than acidic environment and is compatible with other esterases in the literature.

3.3.2. pH and Temperature Stability

The enzyme was incubated at 40ºC, 50ºC, 60ºC, 65ºC, 70ºC and 75ºC for 1 hour to determine the effect of temperature on the stability of *Aneurinibacillus* sp PDF 24 esterase. At the end of incubation, there was no decrease in carboxylesterase activity at 40ºC, 50ºC and 60ºC. It was found that after 1 hour incubation at 65ºC, it retained 59% of its activity, 5% at 70ºC and 2% at 75ºC. (Figure 4a)

Comparing *Aneurinibacillus* sp PDF24 esterase to other similar esterases, *Geobacillus* sp Est1, Est2 and Est3 esterases retain 90% of their activities at 55-65°C for 1 hour [14], while *Geobacillus thermodenitrificans* EstGtA2 esterase retains 90% of the relevant activity at 55-60°C, however, only 15% at 75°C, and 83% at 65°C [12].

To determine the pH stability of the *Aneurinibacillus* sp PDF24 esterase, the pure enzyme was incubated at different pHs. There was no significant change in enzyme activity after 1.5 hours incubation at room temperature (data not shown). But, activity decreased to 90% at pH 5.0 and 97% at pH 6.0-10.0 after incubation at 55ºC for 1.5 hours. (Figure 4b)

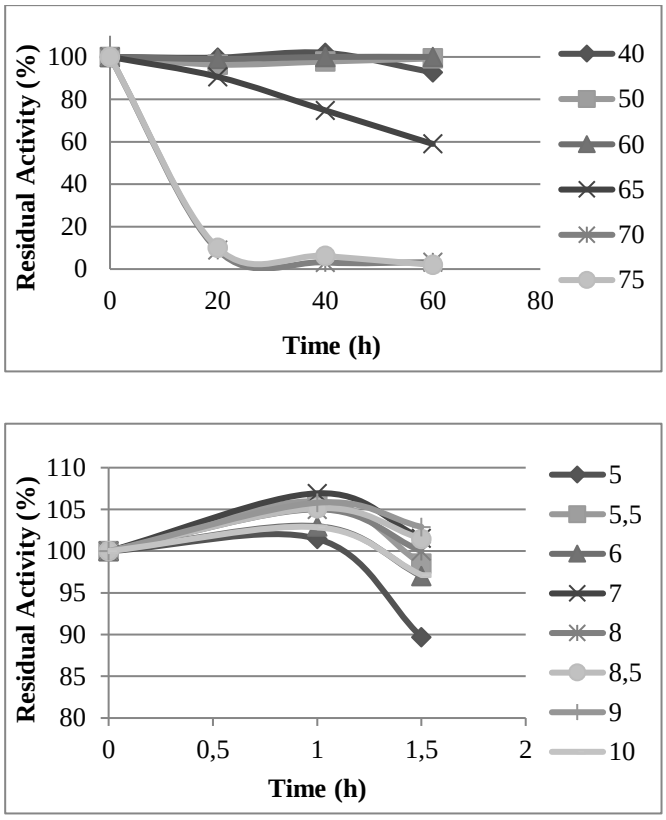

Figure 4 (a) Thermal stability of the purified PDF24 (b) pH stability of the purified PDF24 at 55 °C

The pH stability of *Geobacillus thermodenitrificans* EstGtA2 esterase at 50ºC was found to be 62%, and 36% at pH 6.0, and 11.0, respectively [12]. As a result, *Aneurinibacillus* sp. PDF24 esterase was found to have better pH stability than other esterases.

3.3.3. Substrate Specifity

The rates of hydrolysis of p-nitrophenyl acylates with chain length of C4, C8, C10, C12, C14, and C16 were determined for *Aneurinibacillus* sp. PDF24 esterase. The results demonstrated that *Aneurinibacillus* sp. PDF24 esterase has the maximal activity toward C4, while decreasing activities for C8, C10, C12, C14, and C16. *Aneurinibacillus* sp. PDF24 esterase prefers substrates with short-chain fatty acids instead of long-chain fatty acids (Table 2). The substrate specificity of esterases can vary, as the side chains of residues in the substrate binding pocket form steric hindrance. Esters with acyl chain lengths longer than C4 bind more difficult to the substrate

binding site because of this steric hindrance [18]. Similarly, *Thermus* sp. NCCB 100425T, *Geobacillus* sp. DF20 and *Bacillus* sp. 4 [17, 13, 19] also showed a strong preference for the Hydrolysis of pNPB.

Table 2 Substrate specificity of the purified PDF24

Substrates	Relative activity (%)
***p*-NPB (C4)**	100
***p*-NPO (C8)**	74.5
***p*-NPD (C10)**	54.2
***p*-NPL (C12)**	28.9
***p*-NPM (C14)**	6.0
***p*-NPP (C16)**	1.8

3.3.4. Effect of Metal ions

For determining the effects of metal ions on esterase activity; chloride salts of Mg^{2+}, Li^{+}, Ca^{2+}, K^{+}, Zn^{2+}, and Co^{2+} were used. It was observed that in the presence of 1 mM metal salts of Mg^{2+}, Li^{+}, Ca^{2+}, K^{+}, no significant change occurs in activity, however Zn^{2+} decreased activity to 50% and Co^{2+} to 95%. In 1 hour incubation of enzyme with these salts (5 mM) Mg^{2+}, Li^{+}, Ca^{2+}, K^{+} showed still no significant change in activity, but Zn^{2+} decreased activity to 32% and Co^{2+} metal salt to 90%. (Figure 5)

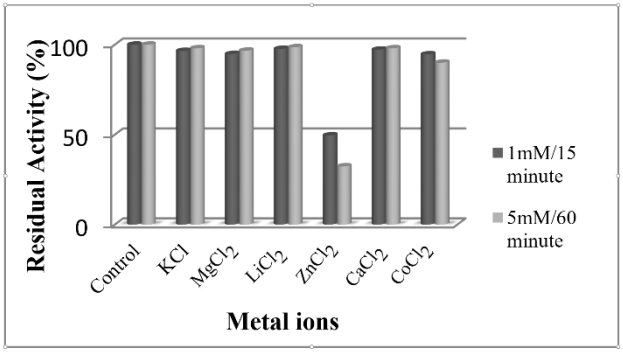

Figure 5 Effect of metal ions on PDF 24 activity

Metal ions have important roles in ensuring enzyme activity and structure by linking amino acids with negative charge in specific areas. *Geobacillus thermoleovorans* YN EstA esterase activity decreased to 17% with 1 mM Zn^{2+} but activity increased to 170% and 152% after incubations with Mg^{2+} and Ca^{+}, respectively [15]. The inhibition of esterase activity in the presence of Zn^{+2} has been reported in this study is in accordance with *Aneurinibacillus* sp. PDF24 esterase inhibition with Zn^{+2}. 1 mM Ca^{2+}, Zn^{2+}, K^{+} increased activity of *Geobacillus* sp Est1 esterase to 110%, 111%, 101% and 1 mM Cu^{2+}, Mg^{2+}, Li^{+} salts decreased activity to 88%, 92%, 89%, respectively [12]. 1 mM salts of Ca^{2+}, Zn^{2+}, K^{+}, Cu^{2+}, Mg^{2+}, Li^{+} increased *Geobacillus* sp Est2 esterase activity to 121%, 104%, and decreased activity to 98%, 81%, 95%, respectively [14]. Effect of metal ions on *Geobacillus* sp. Est3 esterase activity was determined and it was found that, Ca^{2+} and Zn^{2+} salts increase the activity of the enzyme to 103%, 115%, respectively. K^{+}, Cu^{2+}, Mg^{2+}, Li^{+} salts decrease the activity to 99%, 83%, 97% and 93%, respectively [14]. Considering all these datas, Ca^{2+} improves esterase activities in all these studies. Some esterases have a Ca^{2+} binding motif that increases enzyme activity and thermostability [20]. When the effect of Ca^{2+} on the activity of *Aneurinibacillus* sp. PDF24 esterase was examined, it was seen that the enzyme did not show Ca^{2+} dependent activation, which indicates that the enzyme does not have a Ca^{2+} binding site. Also the other metal ions that are used in this study (e.g Mg^{2+}, Zn^{2+}, K^{+}), don`t improve the activity of PDF24 esterase as they improve the activities of other enzymes as mentioned above.

3.3.5. Effect of detergents, organic solvents and potential inhibitors on the enzyme activity

The effects of detergent and organic solvents on the activity of *Aneurinibacillus* sp PDF4 esterase were investigated (Table 3). After treating the enzyme with these chemicals, the remaining activities at the end of 15, 30 and 60 minute incubations were calculated. The greatest inhibition was observed in the presence of SDS (an ionic detergent), which reduced *Aneurinibacillus* sp. PDF24 esterase activity to

7% after 1 hour incubation. Similarly, it was observed that SDS also inhibited Est1, Est2 and Est3 esterases of *Geobacillus* sp. [14]. Non-ionic detergents such as Triton X-100 and Tween 20 were found to reduce *Aneurinibacillus* sp. PDF24 esterase activity to 70% and 75%, respectively. Similarly, Triton X-100 and Tween 20 were also found to decrease the activity of *Geobacillus* sp. Est1, Est2 and Est3 esterases [14]. Most of the esterases and lipases contain a serine amino acid at their central active site [21]. The effect of PMSF, a serine inhibitor to *Aneurinibacillus* sp. PDF24 esterase, was found to reduce the activity of the enzyme to 10% after 1 hour incubation as expected for esterases with a serine in their active site. This data demonstrates that the enzyme is a serine esterase. *Geobacillus stearothermophilus* Est55 and Est30 esterase activity was reduced to 0% [22] and *Geobacillus* sp. Est1, Est2 and Est3 esterase activity were reduced to 45%, 58% and 55% respectively with PMSF as expected for esterases with a serine in their active site [14].

Organic solvents, which disrupt the hydrophobic interactions between the nonpolar side chains of amino acids, alter the natural structure of proteins. But activity of *Aneurinibacillus* sp. PDF24 esterase was found to be stable in the presence of organic solvents such as ethanol, DMSO and isopropanol, reducing agents that reduce disulfide bonds such as DTT and ß-Mercaptoethanol and in the presence of EDTA (a metal chelating agent). Studies on DTT and ß-mercaptoethanol, which are known as inhibitors of thiol groups, show that sulfhydryl groups do not have an important role in the active site of *Aneurinibacillus* sp. PDF24 esterase and suggesting that disulfide bonds are not obligatory to maintain the enzyme conformation [23]. As EDTA did not inhibit the enzyme, it can be suggested that this enzyme is not a metalloprotein [20]. In the literature, DMSO, EDTA and DTT were found to increase the activity of *Geobacillus* sp. Est1, Est2 and Est3 esterases [14]. Organic solvent such as isopropanol was found to reduce activity to 88% [14].

Table 3 Effect of inhibitors on PDF 24 activity

		Residual Activity (%)			
Inhibitors	**Concentration**	**0 min**	**15 min**	**30 min**	**60 min**
Control	0	100	100	100	100
DMSO	% 1 (v/v)	102	97	95	93
Ethanol	% 1 (v/v)	102	92	92	92
Isopropanol	% 1 (v/v)	101	92	92	88
β-Mercaptoethanol	% 1 (v/v)	104	102	101	100
EDTA	5 mM	98	95	94	92
PMSF	5 mM	50	5	11	10
DTT	5 mM	97	97	96	98
Triton X-100	%0,1 (v/v)	82	67	65	70
Tween 20	%0,1 (v/v)	83	75	70	75
SDS	%0,1 (v/v)	6	4	8	7

3.3.6. Kinetic Parameters

In determination of the kinetic parameters of *Aneurinibacillus* sp. PDF4 esterase, the substrate-activity graph was plotted in the presence of pNPB as the substrate and the enzyme was found to fit the simple Michaelis-Menten kinetics. The values of K_m and V_{max} were 0.120 mM and 3164.8 U/mg, respectively. K_m of esterases in the industrial applications was reported to be between 0.01 mM and 100 mM [24]. In the literature, K_m of *Geobacillus* sp. Est1 was found to be 0.095 mM [14], K_m of *Thermoanaerobacterium thermosaccharolyticum* esterase was 3.337 mM [16], K_m of *Thermus* sp. NCCB 100425T esterase

was 18.32 mM [17], K_m of *Geobacillus* sp. DF20 esterase was 0.12 mM [13], K_m of *Geobacillus* sp. Est2 was 0.24 mM [14], K_m of *Geobacillus* sp. Est3 esterase was 0.17 mM [15], K_m of *Bacillus circulans* esterase was found to be 0.24 mM [25] Considering K_m values in the literature, *Aneurinibacillus* sp. PDF24 esterase was found to have a compatable K_m value compared to other esterases in the literature.

As a conclusion, in this study, a thermophilic esterase from the strain of *Aneurinibacillus* sp. PDF24 was purified and characterized. According to the results of SDS-PAGE, molecular weight of *Aneurinibacillus* sp. PDF24 esterase was determined to be 40 kDa. *Aneurinibacillus* sp. PDF24 esterase has an optimum pH of 8.5 and an optimum temperature of 55°C. Kinetic parameters of pNPB as the substrate of the enzyme were examined and K_m value was determined as 0.120 mM and V_{max} value was 3164.8 U/mg for this substrate. After incubation of enzyme for 1.5 hours at 55°C, the activity of the enzyme was decreased to 90% at pH 5.0, 97% at pH 6.0 and pH 10.0. In the presence of 1 mM $MgCl_2$, LiCl, $CaCl_2$, KCl, enzyme activity did not change significantly after 15 minutes incubation. It was observed that $ZnCl_2$ metal salt reduced activity to 50% and $CoCl_2$ metal salt reduced to 95%. The effects of detergents, organic solvents and other inhibitors on the activity of *Aneurinibacillus* sp. PDF24 esterase were investigated. The activity of *Aneurinibacillus* sp. PDF24 esterase was reduced down to 10% by PMSF (a serine inhibitor), down to 70% by Triton X-100, and down to 75% of Tween 20. SDS significantly reduced the activity. It was determined that activity remained stable in the presence of ß-Mercaptoethanol, DMSO, ethanol and EDTA, and activity decreased to 88% in isopropanol. Considering the obtained data, the biochemical features of the enzyme suggest the possible importance of the enzyme for industrial applications.

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