Purification and characterization of a novel thermostable esterase from *Thermus sp.* NCCB 100425<sup>T</sup>

*Thermus sp.* NCCB 100425<sup>T</sup>den yeni bir ısıya dayanıklı esterazın saflaştırılması ve karakterizasyonu

**Objective:** The purpose of the present study was to purify and characterize an esterase from a thermophilic bacterium *Thermus sp.* NCCB 100425<sup>T</sup> and to check its suitability for industrial applications.

**Methods:** *Thermus sp.* NCCB 100425<sup>T</sup> esterase was purified by using ammonium sulphate precipitation and hydrophobic interaction chromatography and then characterized biocchemically.

**Results:** The purity of the enzyme was observed as a single band on native- and SDS- PAGE. In the presence of *p*-nitrophenyl acetate (*p*NPA) as a substrate, the optimum pH and temperature of the enzyme were found to be 7.5 and 60°C, respectively. *K<sub>m</sub>* and *V<sub>max</sub>* values are calculated as 18.32 mM and 96.15 U/mg protein, respectively, with *p*NPA. pH stability was investigated in the range of pH 4.0-9.0 at 4°C and 60°C. After 7 days incubation, activity of pure enzyme was retained 85-90% for all pH at 4°C and 59±5.2% for pH 8.0 at 60°C. It was determined that approximately 80% of enzyme activity was retained between 30-60°C after 7 days incubation. In the presence of 10% ethanol and DMSO, the enzyme activity was retained 96±2.7% and 78±2.5%, respectively. Additionally, it was detected that some metal ions affect the enzyme activity at different ratios.

**Conclusion:** It is clear that *Thermus sp.* NCCB 100425<sup>T</sup> esterase might have advantages for industrial and/or clinical applications in terms of especially its high thermal- and pH-stability.

**Key Words:** Esterase, *Thermus sp.* NCCB 100425<sup>T</sup>, Thermophilic, Hydrophobic Interaction Chromatography, Purification

**Correspondence Address**

Dr. Melike Yıldırım Akatın
Karadeniz Technical University, Faculty of Science, Department of Chemistry, 61080 Trabzon, Türkiye
Phone: +90 462 3777653
E-mail: melikey80@yahoo.com

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**Introduction**

Esterases are the enzymes catalyzing the hydrolysis and formation of ester bonds and they are widely distributed in animals, plants and microorganisms. Among esterases, carboxyl ester hydrolases (EC 3.1.1.1) hydrolyze esters of short chain carboxylic acids (≤12) and triacylglycerol hydrolases or lipases (EC 3.1.1.3) display maximum activity towards insoluble long chain (≥12) acylglycerides [1]. In organic media, they catalyze reactions such as esterification, inter-esterification and trans-esterification [2]. Therefore, they have become two of the most widely used enzymes in organic synthesis and various industrial applications such as detergent industry, oleochemical industry, pulp and paper industry [3,4,5]. Also, the modification of triglycerides for fat and oil industry, synthesis of flavor esters for food industry, resolution of racemic mixtures used for the synthesis of fine chemicals for the pharmaceutical industry can be performed with esterases [6]. It was reported that the carboxylesterase produced by *Bacillus subtilis* has been used in the synthesis of naproxen as a nonsteroidal anti-inflammatory drug [3] and 2-arylpropanionic acids with high enantioselectivity [7]. An esterase cloned from *Thermomyces lanuginosus* exhibited high enantioselectivity (E=95) in the kinetic resolution of 2-carboxyethyl-3-cyano-5-methylhexanoic acid ethyl ester into (3S)-2-carboxyethyl-3-cyano-5-methylhexanoic acid, a valuable chiral intermediate for Pregabaline [8], which is a lipophilic derivative of 4-aminobutyric acid and has been developed to a new blockbuster drug for the treatment of several central nervous system disorders [9].

In spite of esterases have been isolated from various species, microbial esterases are attractive because the cost to grow and maintain them is less and they are easy to manipulate. But, the major reasons of limiting industrial usage of known esterases are their limited thermostability, mainly at high temperatures; pH stability; and instability in the organic solvent in operating industrial conditions. For example, organic solvents are often needed to solubilize substrates and products. If the reaction can be performed at higher temperature, there is no need to add (and remove at the end) organic solvents, therefore the reaction can be performed at lower cost in an environmentally friendly process [10]. From this point of view, the exploration for new microbial enzyme sources is vital for the advancement of new thermostable and organic solvent resistant enzymes and their applications [11].

The global market for enzymes (carbohydrases, proteases, lipases, and others) has showed a great increase in the last years. It was USD 4,411.6 million in 2013 and is expected to increase to USD 7,652.0 million by 2020, growing at a CAGR of 8.3% from 2014 to 2020 [12]. Because providing novel enzymes to the market is very important, we purified and biochemically characterized a novel esterase from a thermophilic strain *Thermus sp.* NCCB 100425T. Thus, this study can be considered as a step to obtain an industrially suitable esterase having excellent features.

**Materials and Methods**

**Chemicals**

Substrates (*p*-nitrophenyl acetate, *p*-nitrophenyl butyrate, *p*-nitrophenyl laurate and *p*-nitrophenyl palmitate), Bovine Serum Albumin (BSA), sodium dodecyl sulphate (SDS), N,N,N',N'-tetramethyl ethylenediamine (TEMED) and Phenyl-Sepharose Fast Flow were purchased from Sigma Chem. Co. (St. Louis, MO, USA). Chloride salts of metal ions (Na+, Li+, Mg2+, Ni2+, Mn2+, Zn2+, Cu2+, Co2+ and Ca2+) and solvents were purchased from Merck A.G. (Darmstadt, Germany). All chemicals were analytical and microbiological grade.

**Bacterial strain and crude enzyme extraction**

*Thermus sp.* NCCB 100425T bacterial strain isolated from a geothermal source in Buharkent/Aydin in Turkey was provided from Department of Biology, Karadeniz Technical University (Trabzon). Strain was grown aerobically at 65°C in Nutrient-Broth (NB) medium for overnight. Grown cells were removed from NB medium by centrifugation at +4°C, at 17136xg for 10 min (Rotina 35 R, Hettich Zentrifugen). Then, cells were suspended in 20 mM Tris-HCl (pH 8.0) buffer and 50 μL lysozyme (10 mg/mL) was added. After incubation at 37°C for 30 min., cells were disrupted by sonication (Bandelin-Sonopuls, Germany) in an ice bath (80% amplitude, 1.0 cycle for 5 min). Finally, cell proteins were removed by centrifugation at +4°C, at 17136xg for 10 min and supernatant was used as crude enzyme extract.

**Purification procedure**

Esterase enzyme was purified from crude enzyme extract by two steps; ammonium sulphate precipitation and hydrophobic interaction chromatography. Precipitation procedure was applied as reported previously [13]. The crude enzyme extract was slowly precipitated with ammonium sulphate (20% (w/v) concentration) in an ice bath. After 1 hour incubation, precipitated protein was removed from suspension by centrifugation at +4°C, 29068xg for 30 min (Hermle Z366 Centrifuge). Then, proteins were resuspended in 20 mM Tris-HCl (pH 8.0) buffer. Enzyme solution was ultrafiltrated by using 10,000 kDa cut-off centrifugal filter device (Millipore Amicon, USA). Hydrophobic interaction chromatography (HIC) was applied as described previously [14,15]. Solid ammonium sulphate was slowly added to the enzyme solution to a final concentration of 1.0 M. Phenyl-Sepharose Fast Flow (Sigma Chem. Co., USA) was loaded onto column (diameter 1.5 cm and height 30 cm) and column was equilibrated with 20 mM Tris-HCl (pH 8.0) buffer including 1.0 M ammonium sulphate. After enzyme solution was loaded column, column was washed with the same buffer. Proteins were eluted reverse gradient of ammonium sulphate (from 1.0 M to 0 M) in 20 mM Tris-HCl (pH 8.0) buffer at a flow...
at 650 nm were utilized to prepare calibration curve and protein concentration was calculated via calibration curve. Activity assay and substrate specificity

Esterase activity was separately determined by using a spectrophotometric assay with $p$-nitrophenyl acetate ($p$NPA), $p$-nitrophenyl butyrate ($p$NPB), $p$-nitrophenyl laurate ($p$NPL) and $p$-nitrophenyl palmitate ($p$NPP) as substrates [19,20,21]. For the enzyme assay, a substrate solution was prepared by mixing stock substrate solution (10 mM), ethanol and 50 mM phosphate buffer (pH 8) in ratio of 1:4:95 (v/v/v), respectively. To determine the enzymatic activity, 100 µL of the enzyme was added to 1400 µL of the substrate solution. After the incubation of the reaction mixture at 55°C for 15 min, the change in absorbance at 410 nm was monitored spectrophotometrically. The background hydrolysis of the substrate was deducted by using a reference sample of identical composition to the incubation mixture except for the enzyme. One unit of enzymatic activity was defined as the amount of protein releasing 1 µmol of $p$-nitrophenol per minute.

Effects of pH and temperature

The activity of Thermus sp. NCCB 100425T esterase as a function of pH was assayed at 50°C by using $p$-nitrophenyl acetate ($p$NPA) as a substrate and 50 mM buffer systems with overlapping values: Mcilvaine (pH 4.0-7.5) rate of 1.0 mL min$^{-1}$. Finally, the fractions with the highest esterase activity and the relatively lower protein contents were pooled and concentrated by using 10,000 kDa cut-off centrifugal filter device (Millipore Amicon, USA). For then specific activity were calculated.

Native-PAGE, activity staining and SDS-PAGE

Gel electrophoresis was performed in a Mini-protean Tetra System (Bio-Rad, CA, USA). Purification of esterase enzyme was monitored by Native- and SDS-PAGE. Native-PAGE was carried out by using 5% stacking gel and 10% separating gel under non-denaturing conditions [16]. Gels were run at 25 mA for 90 min at 4°C and then stained with Coomassie Brilliant Blue R-250.

For activity staining, a gel prepared as describe above was stained by incubating in 100 mL of 20 mM Tris-HCl buffer (pH 8.0) including 2% (v/v) $\beta$-naphthyl acetate (30 mM stock solution) at 55°C for 25 min. At last, 0.04% (w/v) Fast Blue B salt was added and esterase activity bands were observed [17].

SDS-PAGE was performed by using 12% separating gel and then stained with Coomassie Brilliant Blue R-250 [16].

Protein determination

Protein concentration was determined according to Lowry method by using bovine serum albumin (BSA, Sigma Chem. Co., USA) as a standard [18]. Absorbance values at 650 nm were utilized to prepare calibration curve and protein concentration was calculated via calibration curve.

Activity assay and substrate specificity

Esterase activity was separately determined by using a spectrophotometric assay with $p$-nitrophenyl acetate ($p$NPA), $p$-nitrophenyl butyrate ($p$NPB), $p$-nitrophenyl laurate ($p$NPL) and $p$-nitrophenyl palmitate ($p$NPP) as substrates [19,20,21]. For the enzyme assay, a substrate solution was prepared by mixing stock substrate solution (10 mM), ethanol and 50 mM phosphate buffer (pH 8) in ratio of 1:4:95 (v/v/v), respectively. To determine the enzymatic activity, 100 µL of the enzyme was added to 1400 µL of the substrate solution. After the incubation of the reaction mixture at 55°C for 15 min, the change in absorbance at 410 nm was monitored spectrophotometrically. The background hydrolysis of the substrate was deducted by using a reference sample of identical composition to the incubation mixture except for the enzyme. One unit of enzymatic activity was defined as the amount of protein releasing 1 µmol of $p$-nitrophenol per minute.

Effects of pH and temperature

The activity of Thermus sp. NCCB 100425T esterase as a function of pH was assayed at 50°C by using $p$-nitrophenyl acetate ($p$NPA) as a substrate and 50 mM buffer systems with overlapping values: Mcilvaine (pH 4.0-7.5)

Table 1. Purification steps of Thermus sp. NCCB 100425T esterase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Fraction volume (mL)</th>
<th>Protein (mg/mL)</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Activity (U/mL)</th>
<th>Specific activity (U/mg protein)</th>
<th>Yield (%)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>65</td>
<td>10.8</td>
<td>702</td>
<td>286.8</td>
<td>4.4</td>
<td>0.41</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitation (20%)</td>
<td>5</td>
<td>1.5</td>
<td>7.5</td>
<td>13.2</td>
<td>2.6</td>
<td>1.76</td>
<td>4.6</td>
<td>4.3</td>
</tr>
<tr>
<td>Phenyl-Sepharose FF</td>
<td>0.5</td>
<td>0.15</td>
<td>0.075</td>
<td>2.15</td>
<td>4.2</td>
<td>28.67</td>
<td>0.75</td>
<td>69.9</td>
</tr>
</tbody>
</table>
and Tris-HCl (pH 7.5-9.0). The activity was expressed as percent relative activity with respect to maximum activity, which was considered as 100% [22].

The optimum temperature of the enzyme was determined at optimum pH value by measuring the activity at different temperatures in the range of 20-90°C with 10°C increments by using pNPA as a substrate. The activity was expressed as percent relative activity in relation to the temperature optimum, which was considered as 100% [22].

**Enzyme kinetics**

Enzyme kinetic parameters of *Thermus sp. NCCB 100425* esterase were obtained by measuring the rate of p-nitrophenyl acetate (pNPA) hydrolysis at various substrate concentrations (5-400 μM) in the standard reaction mixture (at 60°C in 50 mM Tris-HCl buffer, pH 7.5). The Michaelis-Menten constant (K_m) and maximum velocity (V_max) were determined from the Lineweaver-Burk plot using the Microsoft Excel software.

**pH- and thermal- stability**

To observe pH-stability of purified *Thermus sp. NCCB 100425* esterase, two trails were made separately at 4°C and 60°C. The purified enzyme was incubated at various pH values Mcilvaine (pH 4.0-7.0) and Tris-HCl (pH 8.0-9.0) for 7 days. At the end of the storage period, the activity was assayed under standard reaction conditions. The percentage residual enzyme activity was calculated by a statistical analysis. A value of p<0.05 was taken as statistically significant.

**Results**

**Enzyme purification**

In this study, *Thermus sp. NCCB 100425* esterase was purified in successive steps including ammonium sulphate precipitation and hydrophobic interaction chromatography (HIC) (Figure 1). The enzyme activity and total protein concentration were determined for all fractions collected. The fractions with the highest esterase activity and the relatively lower protein contents (27-30) were pooled and concentrated.

After the purification procedure, the total protein, total activity, specific activity, yield and purification fold of each fraction were determined (Table 1). The enzyme purified with ammonium sulphate precipitation and HIC showed the recovery of 4.6% and 0.75% respectively. The corresponding purification folds were seen to be 4.3 and 69.9. In addition, specific activity was seen to increase from 1.76 to 28.67 U/mg protein.

**Native-PAGE, activity staining and SDS- PAGE**

A single protein band observed in both native and SDS-PAGE indicated that the esterase enzyme was successfully purified from *Thermus sp. NCCB 100425*. Also, activity staining indicated that the enzyme was purified successfully as an active esterase (Figure 2).

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-nitrophenyl acetate (pNPA)</td>
<td>100</td>
</tr>
<tr>
<td>p-nitrophenyl butyrate (pNPB)</td>
<td>79.7</td>
</tr>
<tr>
<td>p-nitrophenyl laurate (pNPL)</td>
<td>16.5</td>
</tr>
<tr>
<td>p-nitrophenyl palmitate (pNPPP)</td>
<td>11.4</td>
</tr>
</tbody>
</table>

To investigate the effect of metal ions on the enzyme activity, Na⁺, Li⁺, Mg²⁺, Mn²⁺, Zn²⁺, Ca²⁺, Co²⁺ and Cu²⁺ were directly added to the standard reaction mixture in a final concentration of 1 mM and 10 mM, separately. Enzyme activity determined in the absence of metal ion was defined as 100% [26].

The effect of some organic solvents on the enzyme activity was studied by adding methanol, ethanol, isopropanol, acetonitrile, acetone and dimethylsulphoxide (DMSO) to the standard reaction mixture in the final concentration of 10% and 30%. Enzyme activity determined in the absence of organic solvent was defined as 100% [26].

**Statistical analysis**

All experiments were carried out in triplicates. Data are represented by the mean±standard deviation. Statistical significance was calculated using oneway analysis of variance and Duncan’s test. A value of p<0.05 was taken as statistically significant.
with 10°C increment. Data showed that highest activity was at 60°C (Figure 3b). It was observed that the enzyme retained 70% and 90% of its maximum activity at 50°C and 70°C, respectively.

**Substrate specificity**

Substrate specificity of the purified esterase was tested on various p-nitrophenyl esters: p-nitrophenyl acetate (pNPA), p-nitrophenyl butyrate (pNPB), p-nitrophenyl laurate (pNPL) and p-nitrophenyl palmitate (pNPP). Results showed that the enzyme was more active to hydrolyze short-chain carboxylic acids esters (Table 2).

**Effect of pH and temperature**

Effect of pH on the purified *Thermus sp. NCCB 100425T* esterase was analyzed at 50°C between pH values of 4.0 and 9.0 using 50 mM buffer systems with overlapping values; Mcilvaine (pH 4.0-7.5) and Tris-HCl (pH 7.5-9.0) and p-nitrophenyl acetate (pNPA) was used as a substrate. As shown in Figure 3a, maximal relative esterase activity was measured at pH 7.5.

Effect of temperature on the enzyme activity was investigated at different temperatures between 20°C to 90°C with 10°C increment. Data showed that highest activity was at 60°C (Figure 3b). It was observed that the enzyme retained 70% and 90% of its maximum activity at 50°C and 70°C, respectively.

**Enzyme kinetics**

Kinetic parameters of esterase purified from *Thermus sp. NCCB 100425T* were determined by using p-nitrophenyl acetate (pNPA) as substrate. $K_m$ and $V_{max}$ values were calculated from the Lineweaver–Burk plot as 18.32 mM and 96.15 U/mg protein, respectively (Figure 4).

**pH- and thermal- stability**

pH stability of esterase from *Thermus sp. NCCB 100425T* was analyzed by incubating the purified enzyme at 4°C and 60°C in the buffers having different pH values for up to 7 days. After incubation, the esterase activity assay was performed under standard reaction conditions. The pure enzyme was quite stable all pH value at 4°C. After 7 days

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**Figure 3.** (a) pH-activity profile of *Thermus sp. NCCB 100425T* esterase. The activity of the purified esterase as a function of pH was assayed at 50°C by using pNPA as a substrate and over a pH range from 4.0 to 9.0 using 50 mM Mcilvaine (pH 4.0-7.5) and Tris-HCl (pH 7.5-9.0). 100% activity corresponds to 50.25 U/mg protein. (b) Temperature-activity profile of *Thermus sp. NCCB 100425T* esterase. The optimum temperature was determined at pH 7.5 by measuring the activity at different temperatures in the range of 20-90°C with 10°C increments by using pNPA as a substrate. 100% activity corresponds to 70.84 U/mg protein.

**Figure 4.** Lineweaver-Burk plot of purified *Thermus sp. NCCB 100425T* esterase.
incubation, the enzyme was retained approximately 90% of its original activity for all pH values (Figure 5a). After 7 days incubation in different pH at 60°C, the enzyme was retained nearly 19±4.5%, 49±5.0% and 59±5.2% of its activity at pH 4.0, 6.0 and 8.0, respectively (Figure 5b). Thermal stability of Thermus sp. NCCB 100425T esterase was determined by pre-incubating for 1, 2 and 7 days at different temperature between 30-90°C (Figure 6). It was observed that enzyme was highly stable at 30-60°C and conserved nearly 90% and 95% of its activity at 30 and 40°C after 7 days incubation, respectively. On the other hand, the enzyme pre-incubated at 80 and 90°C for 7 days lost its activity completely.

Effect of some metal ions on the enzyme activity

To study the effect of metal ion on activity of Thermus sp. NCCB 100425T esterase, Na+, Li+, Mg2+, Mn2+, Zn2+, Ca2+, Co2+, Cu2+ and Ni2+ ions were added separately at 1 mM and 10 mM final concentration (Table 3). Na+, Li+, Mg2+, Mn2+, Zn2+, Ca2+ and Co2+ ions did not have significant effect on esterase activity at 1 mM final concentration. Ni2+ and Cu2+ ions inhibited the enzyme 25±2.8% and 83±0.9% at 10 mM concentration.

Effect of some organic solvents on the enzyme activity

To investigate the effect of some organic solvents on the esterase activity, methanol, ethanol, isopropanol, acetonitrile, acetone and dimethylsulphoxide (DMSO) were added into the reaction mixture in the final concentration of 10% and 30%. The esterase activity were inhibited approximately 50% at the 10% final concentration of methanol, isopropanol and acetone (Table 4). There was 4±2.7% and 22±2.5% inhibition of esterase activity in presence of 10% ethanol and DMSO, respectively. There was no esterase activity in presence of methanol, isopropanol, acetonitrile and acetone at the 30% final concentration. Ethanol and DMSO also inhibited the original esterase activity by 25±3.1% and 57±3.5%, respectively at the 30% final concentration.
In this study, aimed to obtain a novel esterase having features above animal feed, and in bio-conversion. For these reasons, we included: thermophilic nature; tolerance to a varied range of pH, metal ions and organic solvents; stability of enzyme activity over a range of temperature and pH; and other special characteristics of enzymes are exploited for their utility in bio-industries such as food, leather, textiles, and other harsh reaction conditions. Such enzymes have proven activity over a range of temperature and pH; and other harsh reaction conditions. Such enzymes have proven their utility in bio-industries such as food, leather, textiles, animal feed, and in bio-conversion. For these reasons, we aimed to obtain a novel esterase having features above mentioned.

In this study, Thermus sp. NCCB 100425T esterase was successfully purified with ammonium sulphate precipitation and hydrophobic interaction chromatography (HIC). Then, detailed biochemical characterization of the enzyme was carried out in terms of substrate specificities, thermal activation and stability, pH optimum and stability. In addition, the effects of some metal ions and organic solvents on esterase activity were studied.

The esterase was purified 69.9 fold with a specific activity of 28.67 U/mg protein. Various purification folds by using different chromatographic systems were previously reported about esterase purification such as 73.0 fold from Streptococcus thermophilus [13,27,28]. The purified esterase had maximal relative activity to- wards NCCB 100425T esterase and 36.2 fold esterase I and 37.0 fold esterase II from Sparassis crispa, 52.0 fold from Melanocarpus albomyces, 36.2 fold esterase I and 37.0 fold esterase II from Streptococcus thermophilus [13,27,28].

The purified esterase had maximal relative activity towards p-nitrophenyl acetate (pNPA, 2 carbons) and p-nitrophenyl butyrate (pNPB, 4 carbons), respectively, whereas it could slightly cleavage ester bonds of p-nitrophenyl laurate (pNPL, 12 carbons) and p-nitrophenyl palmitate (pNPp, 16 carbons). This confirms the assumption that the enzyme is an esterase rather than a lipase. Similarly, Pseudomonas citronellolis ATCC 13674 esterase hydrolytic activity decreased as carbon chain length increased where it exhibited higher substrate specificity for pNPB [29]. A thermostable esterase from Bacillus licheniformis was active on short chain fatty acids esters containing 2 to 8 carbon atoms. The activity was very low with longer chain length substrates (C10 to C18) [30].

Effect of pH on the purified Thermus sp. NCCB 100425T esterase was analyzed at 50°C between pH values of 4.0 and 9.0. Maximal relative esterase activity was measured at pH 7.5. Similar results were earlier reported that Thermus thermophilus HB27 esterase and Geobacillus sp. TF17 esterase had maximal activity at pH 8.0 and pH 7.5 in presence of p-nitrophenyl dodecanoate and pNPB, respectively [31,32].

Effect of temperature on the enzyme activity was investigated at different temperatures between 20°C to 90°C and the highest activity was measured at 60°C. Similar results were also reported for esterases of Streptococcus thermophilus and Bacillus licheniformis S-86 [28,33].

Kₘ and V_max values of esterase purified from Thermus sp. NCCB 100425T were calculated from the Lineweaver–Burk plot as 18.32 mM and 96.15 U/mg protein, respectively, by using pNPA as substrate. It was previously reported that Bacillus subtilis DR8806 esterase acting on pNPA had Kₘ of 4.2 mM and V_max of 151 μmol min⁻¹ mg⁻¹ respectively [34]. The Kₘ values of enzymes range widely, but for most industrially used enzymes they lie in the range of 10⁻¹ to 10⁻³ M when acting on biotechnologically important substrates, under normal reaction conditions. Kₘ is a measure of the affinity of an enzyme for a particular substrate, a low Kₘ value representing a high affinity and a high Kₘ a low affinity [35]. A high affinity for a substrate may be important especially some applications where the substrate is limiting. Because, low Kₘ means that low concentration of the substrate is enough to run

**Discussion**

Because industrial processes proceed often under extreme conditions, the broad application of enzymes in industry is hindered by the very limited repertoire of available enzymes, their low activity and stability, and high costs. The special characteristics of enzymes are exploited for their commercial interest and industrial applications, which include: thermophilic nature; tolerance to a varied range of pH, metal ions and organic solvents; stability of enzyme activity over a range of temperature and pH; and other harsh reaction conditions. Such enzymes have proven their utility in bio-industries such as food, leather, textiles, animal feed, and in bio-conversion. For these reasons, we aimed to obtain a novel esterase having features above mentioned.

**Table 3. Effect of metal ions on activity of Thermus sp. NCCB 100425T esterase. 100% activity corresponds to 82.63 U/mg protein**

<table>
<thead>
<tr>
<th>Metal Ion</th>
<th>1 mM Final</th>
<th>10 mM Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>90±2.3</td>
<td>89±2.5</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>95±1.5</td>
<td>97±1.4</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>102±3.2</td>
<td>17±0.9</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>96±1.2</td>
<td>94±1.2</td>
</tr>
<tr>
<td>Na⁺</td>
<td>99±1.6</td>
<td>89±1.0</td>
</tr>
<tr>
<td>Li⁺</td>
<td>95±0.9</td>
<td>94±2.0</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>94±2.1</td>
<td>95±2.1</td>
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<tr>
<td>Zn²⁺</td>
<td>97±1.8</td>
<td>95±1.6</td>
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<tr>
<td>Ni²⁺</td>
<td>87±3.1</td>
<td>75±2.8</td>
</tr>
</tbody>
</table>

**Table 4. Effect of some organic solvents on the activity of Thermus sp. NCCB 100425T esterase. 100% activity corresponds to 82.63 U/mg protein**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>10% Final</th>
<th>30% Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Methanol</td>
<td>54±3.2</td>
<td>0</td>
</tr>
<tr>
<td>Ethanol</td>
<td>96±2.7</td>
<td>75±3.1</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>49±2.2</td>
<td>0</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>25±3.4</td>
<td>0</td>
</tr>
<tr>
<td>Acetone</td>
<td>49±2.8</td>
<td>0</td>
</tr>
<tr>
<td>DMSO</td>
<td>78±2.5</td>
<td>43±3.5</td>
</tr>
</tbody>
</table>
the reaction at half of its maximum speed. When the pH stability of the enzyme was examined, it was seen that the pure enzyme was quite stable both at 4°C and 60°C. It was previously reported that esterase activity of Geobacillus caldoxylosilyticus TK4 phosphotriesterase was quite stable at all pH values between 3.0 and 9.0 and retained over 90% of its original activity after 2 days incubation at 50°C [19]. Biocatalysts are usually less stable under operational conditions compared to chemical catalysts. Although it sometimes is beneficial to adapt industrial processes to mild and environmentally benign conditions favored by the enzyme, the use of more extreme conditions is often desirable. Both esterases and lipases have rarely been utilized in industrial processes due to their low stability under operational process conditions [36]. For this reason, pH-stability of Thermus sp. NCCB 100425T may be an advantage in industrial applications.

Thermal stability of Thermus sp. NCCB 100425T esterase was determined by pre-incubating for 1, 2 and 7 days at different temperature between 30-90°C. The result showed that Thermus sp. NCCB 100425T esterase is more stable than esterases from B. licheniformis (50% at 64°C for 1 h), Bacillus sp. (50% at 65°C for 10 h), and Thermotoga maritima (50% at 80°C for 30 min) [30,37,38].

Effect of some metal ions on the activity of Thermus sp. NCCB 100425T esterase was examined. Ni²⁺ and Cu²⁺ ions inhibited the enzyme 25±2.8% and 83±0.9% at 10 mM concentration. These results are consistent with the literature. It was reported that G. caldoxylosilyticus TK4 PHP, Picrophilus torridus and Geobacillus sp. HBB-4 esterases were also inhibited by Cu²⁺ [19,26,39].

Organic solvents can be advantageous in various industrial enzymatic processes; e.g. the reaction media used in biocatalytic esterification and trans-esterification contains less than 1% water. The use of organic solvents can increase the solubility of non-polar substrates, increase the thermal stability of enzymes, decrease water-dependent side reactions, or eliminate microbial contamination [40]. For these reasons, the effect of some organic solvents on the esterase activity was also investigated. The esterase activity was inhibited in different ratios in different solvents. It was previously reported that 18% and 24% inhibitions was observed on the activity of Kluyveromyces marxianus CBS 15532 esterase in presence of methanol and ethanol at the 15% final concentration [14]. L. pyformae esterase activity was severely inhibited by acetonitrile [20]. It was also reported that Geobacillus sp. HBB-4 esterase was inhibited by 50% with acetone, acetonitrile and ethanol [39]. Methanol in the 10% final concentration caused 21% inhibition of G. caldoxylosilyticus TK4 esterase [19].

**Conclusion**

In conclusion, the present study reports the purification and characterization of an esterase for the first time from Thermus sp. NCCB 100425T. The enzyme had high specificity towards p-nitrophenyl acetate. The resistance of esterase to some metal ions and organic solvents, along with the high pH- and thermal- stability, can make it a good candidate for applications in some industrial processes.

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**Conflict of Interest**

There are no conflicts of interest among the authors.

**References**


