Turkish Journal of Chemistry

http://journals.tubitak.gov.tr/chem/

Research Article

Turk J Chem
(2014) 38: 538 – 546

doi:10.3906/kim-1308-23

Partial purification and biochemical characterization of an extremely thermo- and pH-stable esterase with great substrate affinity

Esra ÖZBEK, Yakup KOLCUOĞLU, Leyla KONAK, Ahmet ÇOLAK*, Fulya ÖZ
Department of Chemistry, Karadeniz Technical University, Trabzon, Turkey

Received: 07.08.2013 • **Accepted:** 30.11.2013 • **Published Online:** 11.06.2014 • **Printed:** 10.07.2014

Abstract: An esterase from a thermophilic bacterium, *Geobacillus* sp. DF20, was partially purified. Final purification factor was found to be 64.5-fold using Q-Sepharose ion exchange column chromatography. Native polyacrylamide gel electrophoresis indicated the presence of a single active esterase. The substrate specificity of this esterase was high for *p*-nitrophenyl butyrate (*p*NPB) substrate. The optimum pH and temperature for the enzyme activity were 7.0 and 50 °C, respectively. The pH and heat stability profiles show that this enzyme is more stable under neutral conditions at 50 °C. K_m and V_{max} values for this esterase acting on *p*NPB were 0.12 mM and 54.6 U/mg protein, respectively. Presence of 10% (v/v) acetonitrile in the reaction medium indicated that purified enzyme was strongly inhibited. It was also detected that some metal ions affected enzyme activity at different rates. As a result, it was observed that esterase from *Geobacillus* sp. DF20 has extreme temperature and pH stabilities. Therefore, the stability and K_m value of the enzyme make this study interesting when compared with the literature.

Key words: Thermophilic, esterase, *Geobacillus* sp., purification, pH stability

1. Introduction

Lipolytic enzymes, including esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3), are among the most frequently used groups of biocatalysts in industry and are widely distributed in nature. Esterases are ubiquitous and usually prefer to hydrolyze the esters of short chain fatty acids, while lipase hydrolyzes triglycerides with long-chain acyl groups.[1] Microbial esterases have attracted considerable attention because of their wide substrate specificity, and excellent capabilities to carry out regio-, stereo-, and enantiospecific reactions.[2,3] Although esterases are responsible for hydrolysis reactions in the presence of water, they also catalyze several types of biotransformations in anhydrous solvents.[4] Compared with other enzymes, this distinctive biotechnological feature has attracted interest in these enzymes. They are valuable in the industrial fields in the production of food (including dairy products), pharmaceuticals, detergents, textiles, paper, animal food, leather, and cosmetics.[5,6] The common use of this enzyme in various sectors of industry is stimulating increasing interest in the discovery and characterization of new esterases.

Geobacillus is a genus of moderately thermophilic bacilli and these bacteria have been regarded as important sources of thermostable enzymes because of their structural and functional stability in extreme environments.[7] In spite of growing interest in thermophiles and their biocatalysts, only a few esterases have been characterized from thermophilic archaea and bacteria.[8] Additionally, the common limitation of industrial

*Correspondence: acolak@ktu.edu.tr

application of lipases and esterases is their limited thermostability at high temperatures and pH stability in operating industrial conditions. Therefore, finding novel microbial enzyme sources is of great importance in the development of new thermostable enzymes and applications.[9]

Here, we present detailed information about purification of the intracellular esterase from a thermophilic bacterium, *Geobacillus* sp. DF20, and its biochemical characterization in terms of pH and temperature optima, thermal and pH stabilities, and kinetic parameters. The effects of some metal ions and organic solvents on purified enzyme activity were also investigated.

2. Results and discussion

2.1. Enzyme purification

Purification of the esterase from the intracellular supernatant of *Geobacillus* sp. DF20 was achieved by ion exchange chromatography using Q-Sepharose fast flow gel. Protein content was determined spectrophotometrically at 280 nm and activity measurement described previously was performed for each fraction. Figure 1 indicates that 2 distinct regional fractions having good protein amounts and esterase activities were eluted and the highest degree of purity was attained in the fractions between 54 and 57. Therefore, esterase I (EI) activity was used for further studies. The enzyme was purified 64.5-fold with a specific activity of 42.07 U/mg protein (Table 1). The presence of 3 major protein bands with Coomassie staining and a single band with activity staining, on native PAGE, confirmed the partial purity of the isolated esterase (Figure 2). Similar purification folds for esterases had been previously reported such as 42.7-fold from a salt-tolerant *Bacillus* species isolated from the marine environment of the Sundarbans and 62.8-fold from *Rhodococcus* sp. LKE-028 (MTCC 5562).[10,11]

Table 1. Purification of the esterase from *Geobacillus* sp. DF20.

Fraction	Protein	Total	Specific activity	Yield	Purity
	(mg/mL)	activity (U)	(U/mg protein)	(%)	(fold)
Crude extract	6.2	27.9	0.65	100	1
Q-Sepharose fast flow	3	18.9	42.07	67	64.5

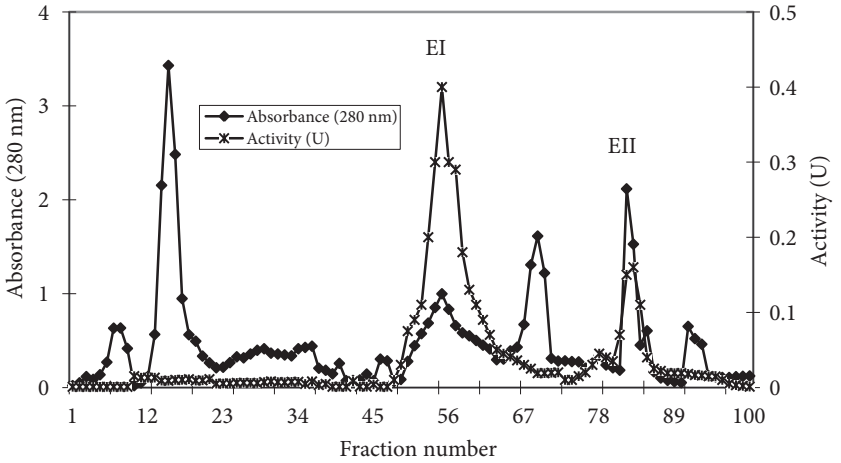

Figure 1. Purification of *Geobacillus* sp. DF20 esterase by Q-Sepharose fast flow anion exchange column.

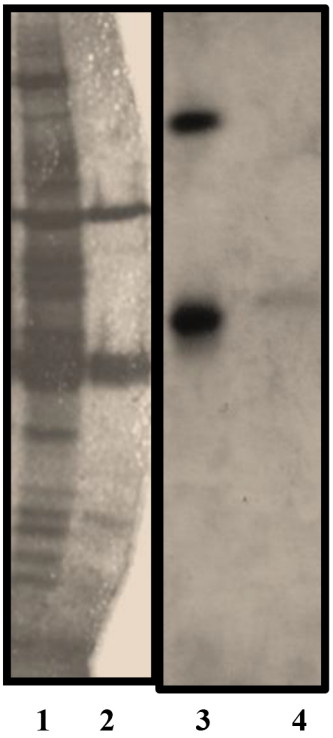

Figure 2. Native PAGE of purified esterase from of *Geobacillus* sp. DF20. With Coomassie staining Lane 1, crude enzyme extract; Lane 2, purified enzyme elution. With activity staining Lane 3, crude enzyme extract; Lane 4, purified enzyme elution.

2.2. Substrate specificity and enzyme kinetics

The substrate specificity of the esterase was tested in the presence of *p*-nitrophenyl esters with acyl chains of different lengths. The purified enzyme exhibited higher catalytic efficiency toward short acyl chain esters such as *p*NPA(C2) and *p*NPB (C4) and no significant esterase activity was observed for the substrates with longer chain lengths. The optimal substrate was determined to be *p*NPB and this preference of the enzyme suggested that it is a true esterase but not a lipase since esterases are known to hydrolyze short chain substrates and lipases are known to hydrolyze long chain ones. The reported esterases from *Bacillus subtilis* (RRL 1789),[12] *Vibrio* sp. GMD509,[13] *Geobacillus* sp. HBB-4,[14] and *Geobacillus thermodenitri?cans* T2[15] had also shown a strong preference for the hydrolysis of *p*NPB.

To determine kinetic parameters in the presence of *p*NPB substrate was studied with a substrate range of 0.005 to 0.5 mM and it was determined that the enzyme typically displayed a Michaelis–Menten kinetics pattern. The K_m and V_{max} values of the purified enzyme acting on *p*NPB were calculated as 0.12 mM and 54.6 U/mg protein, respectively, with a Lineweaver–Burk plot, under the standard reaction conditions described above. This Km value, which is lower than those of many known esterases in the literature, shows that the esterase has a great affinity for *p*NPB and it can be speculated that *Geobacillus* sp. DF20 esterase may be utilized in various biotechnological processes.

2.3. Effect of pH and temperature on enzyme activity

The effect of buffer conditions on *Geobacillus* sp. DF20 esterase activity was investigated at 50 °C by using *p*NPB with a pH range from 3.0 to 9.0. As shown in Figure 3A, the enzyme exhibited the highest activity at pH 7.0 and high activity (around 80% of the maximum) was still retained at pH 8.0. The neutral pH optimum was similar to those of some plant and microbial esterases from a thermoacidophilic archaeon, *Thermotoga maritima*, and *Chrysosporium lucknowense* C1[16–18] and pH 8.0 from *Streptococcus thermophilus* and *Sulfolobus solfataricus.*[19,20]

The effect of temperature on esterase activity was studied in the range of 30 to 80 °C at pH 7.0. The optimum temperature for the enzyme was 50 °C and at least 70% of maximum activity was retained between 30 and 70 °C, while 70% of maximum activity was lost at 80 °C (Figure 3B). The determined optimum temperature is the same as those reported for esterases from a chicken, deep-sea metagenomic library, the culinary medicinal mushroom *Sparassis crispa*, *Picrophilus torridus*, *Pseudomonas* sp. B11-1, and *Bacillus licheniformis.*[21–26]

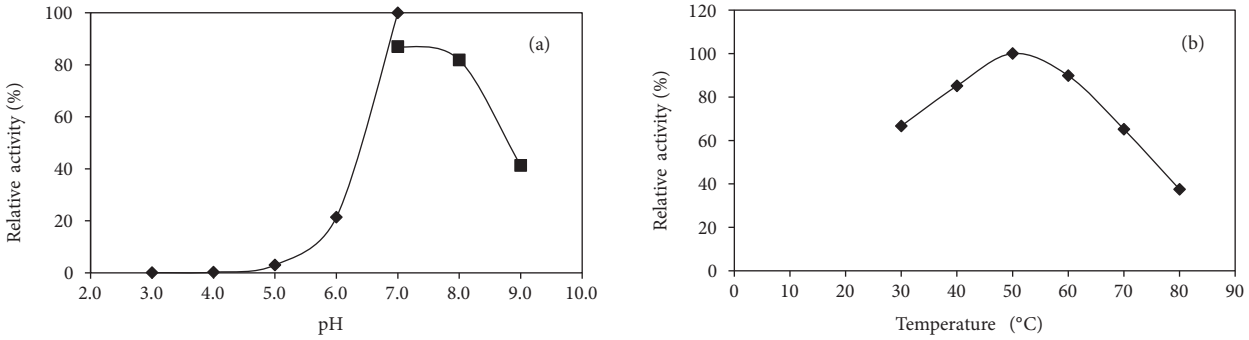

Figure 3. pH activity and optimum temperature profiles of *Geobacillus* sp. DF20 esterase. (A) Effect of pH on enzyme activity in 50 mM of different buffer systems: glycine-HCl (pH 3.0), sodium acetate (pH 4.0, 5.0), sodium phosphate (pH 6.0, 7.0), and Tris-HCl (pH 7.0–9.0). (B) The activity as a function of temperature was determined under standard reaction conditions in the range of 30–80 °C.

2.4. pH and thermal stability

The pH stability of the purified esterase was examined by incubating the enzyme solution for up to 5 days at 4 °C and 50 °C in buffer solutions having 2 different pH values (5.0 and 7.0). At the end of each storage period, the activities were assayed at incubation pH. As shown in the pH stability profile of the pure enzyme, the esterase conserved over 75% of its original activity at pH 5.0 after 3 days of incubation at 4 °C (Figure 4A). It is also shown in Figure 4B that *Geobacillus* sp. DF20 esterase was extremely stable at pH 5.0 and 50 °C for a day-long incubation period, and the activity was fully retained when the enzyme was incubated at pH 7.0 and 50 °C for 3 days. When compared with other esterases in the literature, *Anoxybacillus* sp. PDF1 conserved 90% of its original activity almost at room temperature for 30 min,[27] *Bacillus subtilis* (RRL 1789) esterase retained 45%–50% of its original activity up to 55 °C for 1 h,[12] and *Rhodococcus* sp. LKE-028 (MTCC 5562) lost 50% of its esterase activity after a 120-min incubation period at 70 °C.[11] It is clearly seen that *Geobacillus* sp. DF20 esterase displays high levels of activity under neutral conditions when incubated at both 4 and 50 °C. The stability of the enzyme in acidic and neutral pHs and at high temperatures suggests its usefulness in industrial applications.

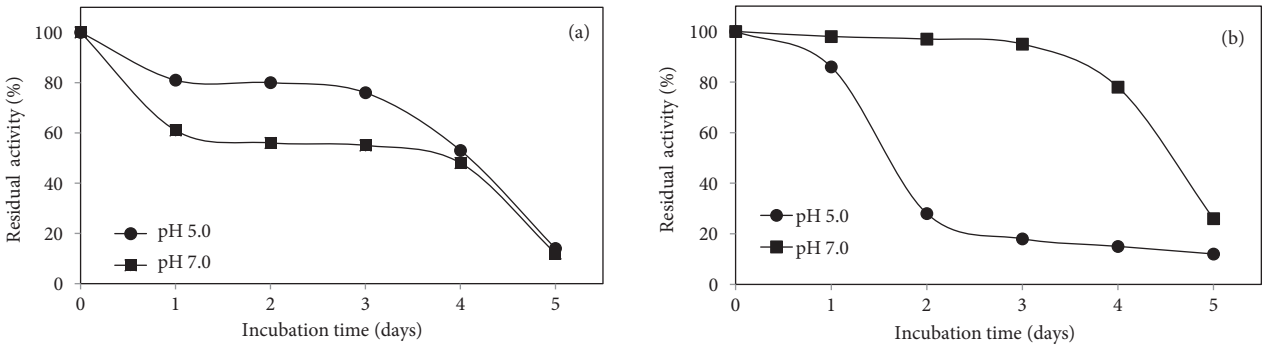

Figure 4. pH stability profiles of *Geobacillus* sp. DF20 esterase. For determining pH stability, the enzyme was incubated at 4 °C (A) and 50 °C (B) for 5 days.

The thermostability of *Geobacillus* sp. DF20 esterase was also determined by measuring the residual activity after incubation of the pure enzyme solution (in pH 7.0 elution buffer) at various temperatures (Figure 5). The activity was completely retained after incubation for 72 h at 50 °C. According to the temperature stability profile, upon 5 h of incubation at 70 °C, the activity sharply decreased. Comparable results were obtained with the esterase purified from *Anoxybacillus* sp. PDF1 maintained all of its original activity at 50 °C and lost all of its activity at 75 °C for 30 min.[27] For *Sporotrichum* thermophile esterase the rate of activity loss was at 60% at 50 °C and 100% at 60 °C after 6 h of incubation.[28] The results obtained from stability studies showed that the enzyme was highly thermostable in addition to its high pH stability. The correlation between thermostability of an enzyme in water and its resistance to denaturation in organic solvent has been reported earlier.[29] For this reason, thermostable enzymes are attractive to be used not only in aqueous media but also in organic media.[30] Esterases and lipases have not been utilized often in industrial processes due to their low stability under operational process conditions. Enzymes extracted from *Geobacillus* species have potential applications in biotechnological processes. Although it has been reported that *Geobacillus* species produced various thermostable enzymes including proteases, amylases, lipases, and pullulanase, there is little information on thermostable esterases from *Geobacilli.*[7]

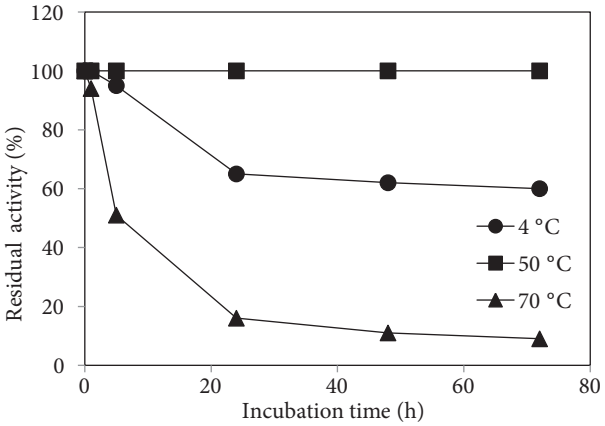

Figure 5. Thermal stability profile of *Geobacillus* sp. DF20 esterase. Thermostability of the enzyme was determined after incubating the reaction mixture at different temperatures for up to 72 h.

2.5. Effect of some metal ions and organic solvents on esterase activity

Various metal ions at 1 mM and 10 mM final concentrations in the reaction mixture were tested for their effects on *Geobacillus* sp. DF20 esterase activity (Table 2). While Ca^{2+} and Cu^{2+} reduced the esterase activity by about 35%, in the presence of other assayed metal ions it was determined that more than 80% of the enzyme activity was retained at 1 mM concentration. At 10 mM final concentrations, while Cu^{2+} inhibited esterase activity by nearly 50%, the esterase lost its activity completely in the presence of Zn^{2+}. Similar results for Zn^{2+} inhibition were also observed for *Acinetobacter baumannii* BD5 esterase.[31] It was reported that extremely thermostable *Picrophilus torridus* esterase was inhibited by Cu^{2+} and Co^{2+} while Na^{+} did not significantly affect the *Geobacillus* sp. DF20 esterase activity similar to *P. torridus* esterase.[24]

Table 2. Effect of various metal ions on the *Geobacillus* sp. DF20 esterase activity.

Metal ion	Residual activity (%)	
	1 mM	10 mM
Control (None)	100 ± 3	100 ± 3
Co^{2+}	97 ± 3	77 ± 3
Mn^{2+}	93 ± 3	77 ± 3
Na^{+}	93 ± 2	92 ± 2
Mg^{2+}	87 ± 2	91 ± 3
Li^{+}	86 ± 3	93 ± 3
Zn^{2+}	81 ± 2	1 ± 1
Ca^{2+}	68 ± 1	73 ± 2
Cu^{2+}	65 ± 1	57 ± 1

The catalytic efficiency of many enzymes is affected by organic solvents in different ways and organic solvent resistant enzymes can be useful in various industrial processes. The use of organic solvents in reaction media can increase the thermal stability of enzymes or eliminate microbial contaminations. In this study the effect of organic solvents on esterase activity was investigated by estimating residual activity of the purified enzyme under standard reaction conditions (Table 3). *Geobacillus* sp. DF20 esterase conserved approximately 75% of its original activity in the presence of methanol and ethanol. It was previously reported that *Kluyveromyces marxionus* CBS 1553 esterase had residual activity of 82% and 76% in the presence of methanol and ethanol, respectively.[32]

Table 3. Effect of organic solvents on the *Geobacillus* sp. DF20 esterase activity.

Organic solvent (10% final concentration)	Residual activity (%)
Control (None)	100 ± 3
Ethanol	77 ± 3
Methanol	72 ± 2
Isopropanol	56 ± 2
Acetonitrile	16 ± 1

In conclusion, this study describes the purification and characterization of an esterase from thermophilic *Geobacillus* sp. DF20. This enzyme is characterized in terms of substrate specificity, pH and temperature optima, organic solvent resistance, thermal and pH stability, and kinetic parameters. Biochemical characterization revealed several properties of *Geobacillus* sp. DF20 esterase and suggested that the enzyme may be used in suitable biotechnological applications.

3. Experimental

3.1. Chemicals

Geobacillus sp. DF20 was provided by the Department of Biology at Karadeniz Technical University, Trabzon (Turkey). All *p*-nitrophenyl esters used as substrates and Q-Sepharose fast flow were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of the reagent grade available and used as obtained.

3.2. Growth conditions and enzyme extraction

The bacterial strain *Geobacillus* sp. DF20 was grown in Erlenmeyer flasks (2 L) containing 500 mL of mineral medium[33] at 55 °C for 14 h on a rotary shaker (Barnstead/Lab-Line). Cells were harvested by centrifugation at 10,000 rpm and 4 °C for 10 min. The collected cells were then resuspended in 20 mM Tris-HCl buffer (pH 7.0) containing 10 mg/mL lysozyme and incubated at 37 °C for 30 min. The cell lysate was finally sonicated at 80% amplitude for 5 min and then centrifuged at 10,000 rpm for 10 min. The resulting supernatant was used for esterase purification.

3.3. Enzyme purification

The crude enzyme solution was applied to a Q-Sepharose fast flow anion exchange column (1.5 × 30 cm) equilibrated with 20 mM Tris-HCl (pH 7.0) buffer. Next, the unbound proteins were removed by washing the column with 150 mL of equilibration buffer. Proteins were eluted by using a linear gradient of NaCl solution from 0 to 0.6 M, in the same buffer, at the flow rate of 1 mL min^{-1} and 3 mL fractions were collected. The active fractions were concentrated and also partial purified with a centrifugal filter device (Ultracel Membrane 50,000 MWCO Millipore, Amicon, USA) and stored at –20 °C until used.

3.4. Native PAGE and activity staining

The purity of the eluted protein and the presence of an esterase were determined by native PAGE. Continuous nondenaturing PAGE was performed by using a 10% separating gel and 5% stacking gel.[34] Gels were run at 25 mA for 90 min at 4 °C. To monitor the migration and separation of the proteins, gel was stained with Coomassie Blue R-250. For activity staining, the native gel was incubated in 100 mL of 50 mM Tris-HCl buffer (pH 7.0) containing 2 mL of 30 mM β-naphthyl acetate (dissolved in acetone) at 50 °C for 15 min. Finally, 40 mg of Fast Blue B salt was added to the staining solution and the esterase activity was detected by a deep purple band on the gel.[35]

3.5. Enzyme activity and protein assay

Protein concentration was measured by the method of Lowry[36] using bovine serum albumin as a standard. Graphic interpolation on a calibration curve at 650 nm was used to obtain protein amounts.

Esterolytic activity was determined spectrophotometrically using *p*-nitrophenyl acetate (*p*NPA), *p*-nitrophenyl butyrate (*p*NPB), *p*-nitrophenyl laurate (*p*NPL), and *p*-nitrophenyl palmitate (*p*NPP) as substrates.[37–39] The substrate solution included stock substrate solution (10 mM), ethanol, and 20 mM Tris-HCl buffer (pH 7.0) in the ratio of 1:4:95 (v/v/v), respectively. Then 100 μL of enzyme solution was added to 1400 μL of the substrate solution and after the incubation of the reaction mixture at 50 °C for 20 min the

changes in absorbance at 405 nm were monitored. The nonenzymatic hydrolysis was subtracted by using a reference sample without enzyme. One unit of esterolytic activity was defined as the amount of enzyme producing 1 μmol of p-nitrophenol per minute.[37]

3.6. Characterization of the esterase

Functional properties of *Geobacillus* sp. DF20 esterase in different conditions were investigated by using the purified enzyme solution and all experiments were run under standard reaction conditions by using pNPB as substrate.

The pH profile of the enzyme for its esterolytic activity was assayed by using various buffers (all are 50 mM): glycine-HCl (pH 3.0), sodium acetate (pH 4.0 and 5.0), sodium phosphate (pH 6.0 and 7.0), and Tris-HCl (pH 7.0–9.0). To determine the optimum temperature of the enzyme, esterase activity was measured between 30 °C and 80 °C with 10 °C increments, at optimum pH value. The determined optimum pH and temperature values were used in the rest of the study.

To determine the substrate specificity, relative activities of the purified enzyme were investigated as described previously in the presence of different chain-length fatty acid esters. Values for K_m and V_{max} were calculated from the Lineweaver–Burk plot by estimating activities toward a series of pNPB concentrations (0.005–0.5 mM).

The effect of pH on the enzyme stability was studied with sodium acetate (pH 5.0) and Tris-HCl (pH 7.0) buffers by incubating the enzyme solution in both buffer solutions for up to 5 days, at 4 °C and 50 °C. Enzyme thermostability was determined after incubating enzyme solution in pH 7.0 buffer at different temperatures (4, 50, and 70 °C) for up to 72 h. The activities, after incubation, were assayed under standard reaction conditions and the percentage residual activity was calculated by comparison with unincubated enzyme activity.

To study the effect of metal ions on esterase activity, chloride salts of metals such as Na^+, Li^+, Mn^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, and Ca^{2+} were added to the reaction mixture in 2 different final concentrations (1 mM and 10 mM). The enzyme stability in organic solvents was assayed by diluting the enzyme sample in 10% (v/v) methanol, ethanol, isopropanol, and acetonitrile. Enzyme activity was treated in the same way but without any additive was defined as 100%. Residual activity was measured following the standard assay method with pNPB as substrate.

Acknowledgments

This work was supported by a research grant KTU-BAP. The authors would like to thank Prof Dr Ali Osman Beldüz, Prof Dr Sabriye Dülger Çanakçı, and their research groups for providing the bacterial strain. The authors also thank Asstist Prof Dr Elif Demirel (Department of English Language and Literature) for her proofreading.

References

1. Verger, R. *Trends Biotechnol.* **1997**, *15*, 32–38.
2. Bornscheuer, U. T. *FEMS Microbiol.* **2002**, *26*, 73–81.
3. Panda, T.; Gowrishankar, B. S. *Appl. Microbiol.* **2005**, *67*, 160–169.
4. Vieille, C.; Zeikus, G. J. *Microbiol. Mol. Biol.* **2001**, *65*, 1–43.
5. Hasan, F.; Shah, A. A.; Hameed, A. *Enzyme Microb. Technol.* **2006**, *39*, 235–251.

6. Jaeger, K. E.; Eggert, T. *Curr. Opin. Biotechnol.* **2002**, *13*, 390–397.
7. McMullan, G.; Christie, J. M.; Rahman, T. J.; Banat, I. M.; Ternan, N. G.; Marchant, R. *Biochem. Soc. Trans.* **2004**, *32*, 214–217.
8. Suzuki, Y.; Miyamoto, K.; Ohta, H. *FEMS Microbiol.* **2004**, *236*, 97–102.
9. Romdhanea, I. B. B.; Fendrib, A.; Gargourib, Y.; Gargouria, A.; Belghitha, H. *Bioche. Eng. J.* **2010**, *53*, 112–120.
10. Sana, B.; Ghosh, D.; Saha, M.; Mukherjee, J. *Process Biochem.* **2007**, *42*, 1571–1578.
11. Kumar, L.; Singh, B.; Adhikari, D.; Mukherjee, J.; Ghosh, D. *Process Biochem.* **2012**, *47*, 983–991.
12. Kaiser, P.; Raina, C.; Parshad, R.; Johri, S.; Verma, V.; Andrabi, K. I.; Qazi, G. N. *Protein Expres. Purif.* **2006**, *45*, 262–268.
13. Park, S. Y.; Kim, J. T.; Kang, S. G.; Woo, J. H.; Lee, J. H.; Choi, H. T.; Kim, S. J. *Appl. Microbiol. Biotechnol.* **2007**, *77*, 107–115.
14. Kubilay, M. Z.; Ateşlier, B. B.; Basbulbul, G.; Biyik, H. H. *J. Basic Microbiol.* **2006**, *46*, 400–409.
15. Yang, Z.; Zhang, Y.; Shen, T.; Xie, Y.; Mao, Y.; Ji, C. *J. Biosci. Bioeng.* **2012**, *115*, 1–5.
16. Kim, S.; Bok Lee, S. *Biosci. Biotechnol. Biochem.* **2004**, *68*, 2289–2298.
17. Levisson, M.; Van der Oost, J.; Kengen, S. W. M. *FEBS Journal.* **2007**, *274*, 2832–2842.
18. Kühnel, S.; Pouvreau, L.; Appeldoorn, M. M.; Hinz, S. W. A.; Schols, H. A.; Gruppen, H. *Enzyme Microb. Tech.* **2012**, *50*, 77–85.
19. Liu, S. Q.; Holland, R.; Crow, V. L. *Int. Dairy J.* **2001**, *11*, 27–35.
20. Chung, Y. M.; Park, C. B.; Lee, S. B. *Biotechnol. Bioprocess. Eng.* **2000**, *5*, 53–56.
21. Fendri, A.; Louati, H.; Sellami, M.; Gargouri, H.; Smichi, N.; Zarai, Z.; Aissa, I.; Miled, N.; Gargouri, Y. *Int. J. Biol. Macromol.* **2012**, *50*, 1238–1244.
22. Fu, C.; Hu, Y.; Xie, F.; Guo, H.; Ashforth, E. J.; Polyak, S. W.; Zhu, B.; Zhang, L. *Appl. Microbiol. Biotechnol.* **2011**, *90*, 961–970.
23. Chandrasekaran, G.; Kim, G. J.; Shin, H. J. *Food Chem.* **2011**, *124*, 1376–1381.
24. Hess, M.; Katzer, M.; Antranikian, G. *Extremophiles* **2008**, *12*, 351–364.
25. Alvarez-Macarie, E.; Augier-Magro, V.; Baratti, J. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 1865–1870.
26. Suzuki, T.; Nakayama, T.; Choo, D. W.; Hirano, Y.; Kurihara, T.; Nishino, T.; Esaki, N. *Protein Expr. Purif.* **2003**, *30*, 171–178.
27. Ay, F.; Karaoglu, H.; Inan, K.; Canakci, S.; Belduz, A. O. *Protein Expres. Purif.* **2011**, *80*, 74–79.
28. Vafiadi, C.; Topakas, E.; Biely, P.; Christakopoulos, P. *FEMS Microbiol. Lett.* **2009**, *296*, 178–184.
29. Owusu, R. K.; Cowan, D. A. *Enzyme Microb. Tech.* **1989**, *11*, 568–574.
30. Kademi, A.; Ait-Abdelkader, N.; Fakhreddine, L.; Baratti, J. C. *Enzyme Microb. Tech.* **1999**, *24*, 332–338.
31. Park, I. H.; Kim, S. H.; Lee, Y. S.; Lee, S. C.; Zhou, Y. *J. Microbio. Biotechnol.* **2009**, *19*, 128–135.
32. Monti, D.; Ferrandi, E. E.; Righi, M.; Romano, D.; Molinari, F. *J. Biotechnol.* **2008**, *133*, 65–72.
33. Degryse, N. G.; Pierard, A. *Arch. Microbiol.* **1978**, *117*, 189–196.
34. Laemmli, U. K. *Nature* **1970**, *227*, 680–685.
35. Karpushova, A.; Brümmer, F.; Barth, S.; Schmid, R. D. *Appl. Microbiol. Biotechnol.* **2005**, *67*, 59–69.
36. Lowry, O. H.; Rosebrogh, N. J.; Farr, A. L.; Randall, R. J. *Biol. Chem.* **1951**, *75*, 193–265.
37. Lee, D.; Koh, Y.; Kim, K.; Kim, B.; Choi, H. *FEMS Microbiol. Lett.* **1999**, *179*, 393–400.
38. Faiz, O.; Colak, A.; Saglam, N.; Canakcı, S.; Beldüz, A. O. *J. Biochem. Mol. Biol.* **2007**, *40*, 588–594.
39. Colak, A.; Camedan, Y., Faiz, Ö.; Sesli, E.; Kolcuoğlu,Y. *J. Food Biochem.* **2007**, *33*, 482–499.