

Determination and Characterization of Thermostable Esterolytic Activity from a Novel Thermophilic Bacterium *Anoxybacillus gonensis* A4

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A novel hot spring thermophile, *Anoxybacillus gonensis* A4 (*A. gonensis* A4) was investigated in terms of capability of tributyrin degradation and characterization of its thermostable esterase activity by the hydrolysis of *p*-nitrophenyl butyrate (PNPB). It was observed that *A. gonensis* A4 has an esterase with a molecular weight of 62 kDa. The extracellular crude preparation was characterized in terms of substrate specificity, pH and temperature optima and stability, kinetic parameters and inhibition/activation behaviour towards some chemicals and metal ions. Tributyrin agar assay showed that *A. gonensis* A4 secreted an esterase and V_{max} and K_m values of its activity were found to be 800 U/L and 176.5 μ M, respectively in the presence of PNPB substrate. The optimum temperature and pH, for *A. gonensis* A4 esterase was 60-80°C and 5.5, respectively. Although the enzyme activity was not significantly changed by incubating crude extract solution at 30-70°C for 1 h, the enzyme activity was fully lost at 80°C for same incubation period. The pH-stability profile showed that original crude esterase activity increased nearly 2-fold at pH 6.0. The effect of some chemicals on crude esterase activity indicated that *A. gonensis* A4 produce an esterase having serine residue in active site and -SH groups were essential for its activity.

Keywords: *Anoxybacillus*, Esterase, Lipase, Thermophile, Thermostability

Introduction

Esterases catalysing the cleavage and formation of ester bonds are known as α/β -hydrolases (EC 3.1.1.X) (Ollis *et al.*, 1992). They are widely distributed in animals, plants and microorganisms

(Bornscheuer, 2002). Among esterases true esterases (EC 3.1.1.1, carboxyl ester hydrolases) hydrolyze esters of short chain carboxylic acids (≤ 12) and lipases (EC 3.1.1.3, triacylglycerol hydrolases) display maximum activity towards insoluble long chain ($C \geq 12$) acylglycerides (Eggert *et al.*, 2002).

Microbial esterases and lipases are of considerable interest because of their potential application in biotechnology. The major reason of limiting industrial usage of known esterases is their limited thermostability, mainly at high temperatures, and pH stability in operating industrial conditions. Therefore the search for new microbial enzyme sources is important for the development of new thermostable enzymes and applications. The correlation between thermostability of an enzyme in water and its resistance to denaturation in organic solvent has been reported earlier (Owusu and Cowan, 1989). For this reason thermostable enzymes are attractive not only to be used in aqueous media but also in organic media (Kademi *et al.*, 1999). Thermostable enzymes are usually screened from thermophilic or hyperthermophilic organisms (Gowland *et al.*, 1987).

In organic media, lipases and esterases catalyse esterification, interesterification, alcoholysis or acidolysis reactions (Kawamoto *et al.*, 1987). The synthesis of flavor esters for food industry, modification of triglycerides for fat and oil industry, resolution of racemic mixtures used for the synthesis of fine chemicals for the pharmaceutical industry can be performed (Molinari *et al.*, 1996). The carboxylesterase originated from *Bacillus subtilis* has been used in the synthesis of naproxen as a non-steroidal anti-inflammatory drug (Quax and Broekhuizen, 1994) and 2-arylpropionic acids with high enantioselectivity (Azzolina *et al.*, 1995). Efficient kinetic resolutions were achieved in the synthesis of primary alcohols by esterase from *Bacillus coagulans* (Baumann *et al.*, 2000) and secondary alcohols from *Bacillus stearothermophilus* (Molinari *et al.*, 1996). The cloning, expression, purification and biochemical characterization of esterolytic enzymes from *Bacillus* species were reported (Kademi *et al.*, 2000a; Markossian *et al.*, 2000; Eggert *et al.*, 2002). Recently, diphenolases from *Anoxybacillus kestanbolensis* strains K1 and K4^T were studied (Yildirim *et al.*, 2005).

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Recently, a novel hot spring thermophile, *Anoxybacillus gonensis* A4, was isolated and characterized based on its biochemical, taxonomic and genetic properties (Belduz *et al.*, 2003). The present study was aimed at evaluating the ability of this thermophilic strain for degradation of *p*-nitrophenyl esters and characterization of its extracellular thermostable esterase activity. Many researches on screening and characterization of industrial extracellular and intracellular enzyme extract such as lipase, catecholase etc. from different organisms were reported (Lopes *et al.*, 2002; Yildirim *et al.*, 2005). Over the past few years, immobilization of enzymes and especially the whole cell immobilization has caused an increase in the usage of enzymes in industrial process. Whole cell immobilization not only eliminates expensive operations of enzyme purification steps, but also keeps the enzyme in its natural environment and causes the greater resistance to environmental changes and higher operational stability (Kierstan and Coughlan 1985). The data obtained from this study will light the way for the further investigation of immobilization of *A. gonensis* A4 for thermostable crude esterase activity.

Materials and Methods

Materials. Substrates were purchased from Sigma Chemical Co. (St. Louis) and other reagents were of analytical grade and used as obtained.

Screening of esterolytic enzyme producing thermophile. A novel thermophile *Anoxybacillus gonensis* A4 (*A. gonensis* A4) was screened for its esterolytic activity on tributyrin agar plate containing 1.5% tributyrin in Luria-Bertani (LB) solid medium at 60°C for three days. Further 4 days incubation at 4°C was done to bright clear zone on the plate (Suoniemi and Tynkynen 2002).

Culture media and growth conditions. The original Luria-Bertani (LB) liquid medium contained (g/L in distilled water) yeast extract 5.0, bactotryptone 10.0 and NaCl 5.0 at pH 7.5. LB medium was used to observe both activity and growth profiles of *A. gonensis* A4 at 60°C up to 24 h on a shaker operating at 200 rpm. LB medium supplemented with 0.3% carboxymethyl cellulose as an emulsifier and secondary carbon source was used only for activity profiles of the isolates at the same conditions. These profiles provided optimization of culture medium and growth conditions.

Preparation of extracellular crude extract. The extracellular enzyme extract was prepared by centrifugation of cells, grown in supplemented liquid medium for 15 h, at 4,000 rpm for 20 min. The pellet was discarded and the supernatant was used as crude extracellular enzyme extract for characterization and determination of the esterolytic activity (Nawani and Kaur, 2000).

Protein determination. Lowry method with bovine serine albumin as standard was used to determine the protein quantity of the enzyme extract. Graphic interpolation on a calibration curve at 650 nm was used to obtain values (Lowry *et al.*, 1951).

Electrophoresis. Electrophoresis was performed in 12% SDS-PAGE for extracellular crude extracts according to Musidlowska-Persson and Bornscheuer (Musidlowska-Persson and Bornscheuer, 2003) on a Hoeffer SE 600 Series Electrophoresis dual slab cell unit. Then, the gel was incubated in a solution containing 0.5% Triton X-100 in Tris buffer (0.1 M, adjusted to pH 7.5 with HCl) for 1 h renaturation. Renaturated gel was incubated for 30 min in a 1 : 1 mixture of solution A (8 mg α -naphthyl acetate dissolved in 3 mL acetone and added to 0.1 M Tris buffer which pH is 7.5) and B (20 mg Fast Red dissolved in 0.1 M Tris buffer which pH is 7.5) for activity staining.

Determination of esterolytic activity. Esterolytic activity was determined by using the spectrophotometric assay described by Lee *et al.*, (1999). For the preparation of stock substrate solution, *p*-nitrophenyl butyrate (PNPB) was dissolved in acetonitrile at a concentration of 10 mM. The substrate solution included stock substrate solution, ethanol and 50 mM phosphate buffer (pH 7.5) in ratio of 1 : 4 : 95 (v/v/v), respectively. To prepare the reaction mixture, 0.3 mL of the cell free supernatant was added to 0.9 mL of the substrate solution. After the incubation of the reaction mixture at 60°C for 15 min, the change in absorbance at 405 nm was monitored. The amount of released *p*-nitrophenol (*p*-NP) was determined for the esterolytic activity. The non enzymatic hydrolysis was subtracted by using a blank without enzyme. During this study, optima values obtained were adapted to standard enzymatic assays. One unit of enzyme activity was defined as 1 μ mol of *p*-NP formed per min under assay conditions.

Substrate specificity. The substrate specificity of crude enzyme extract was determined at standard reaction conditions by using PNPB in acetonitrile and *p*-nitrophenyl laurate (PNPL) in DMSO at 10 mM concentration of stock solution.

Optimum pH and pH stability. The effect of pH on the esterase activity was measured at 60°C by using 50 mM of following buffers; sodium acetate (pH 4.0-5.5) and potassium phosphate (pH 6.0-8.0) (Lee *et al.*, 1999). The same buffers with 1.0 pH value increments, and NaOH-Glycine buffer at pH 9.0 were used to determine the pH stability of the enzyme extract. The mixture of enzyme extract and the buffers given above (1 : 1) was incubated for 24 h at 4°C and then the residual activity was assayed by using 0.3 mL of this mixture at optimum conditions with PNPB as substrate (Yildirim *et al.*, 2005, Zhang *et al.*, 2005). The percentage residual esterase activity was calculated by comparison with unincubated enzyme.

Optimum temperature and thermostability. The temperature optimum assay was performed for different temperatures in the range of 30-90°C at the optimum pH value using a circulation water bath. The reaction mixtures were incubated for 15 min at various temperatures indicated above. Then, the relative activity was determined spectrophotometrically at 405 nm as rapidly as possible. In order to determine the thermal stability of the enzyme, the crude enzyme extract in test tubes were incubated at temperatures of 30-80°C with 10°C increments for 1 h, rapidly cooled and brought to 25°C. After the mixture reached to room temperature, the enzyme activity was assayed under the standard reaction conditions at optimum pH value (Özen *et al.*, 2004; Yildirim *et al.*, 2005).

Effect of substrate concentration on esterolytic activity. A substrate saturation curve was obtained by plotting the final substrate concentration in the range of 0-900 μM versus esterase activity in the presence of PNPB as substrate. The Michaelis-Menten constant (K_m) and the maximum velocity of the reaction (V_{max}) were calculated from Lineweaver-Burk plot (Lineweaver and Burk, 1934).

Effect of protein concentration on esterolytic activity. The hydrolysis of PNPB by crude enzyme extract was performed for different protein concentration ranging from 0.012 to 1.215 mg/mL. The activity was assayed under standard reaction condition at the optimum pH.

Effect of metal ions on esterolytic activity. The effect of various metal ions on the enzyme activity was tested by addition of 1 mM chloride salts Na^+ and K^+ for monovalent, Cu^{2+} , Ni^{2+} , Ca^{2+} , Mn^{2+} , Hg^{2+} , Zn^{2+} , Co^{2+} , Cd^{2+} for divalent and Cr^{3+} for trivalent ions. After addition of each metal ion solution on enzyme extract (1 : 1) and incubation of this mixture for 20 min at room temperature, the activity was assayed using 0.3 mL aliquot of metal ion:extract solution and PNPB as substrate. The residual activities were measured by comparison with standard assay mixture with no metal ion added and diluted enzyme extract in the ratio of 1 : 1, at optimum pH (Lee *et al.*, 1999).

Effect of some chemicals and chelating agent on esterolytic activity. The esterase activity was monitored in the presence of 2-mercaptoethanol and ethylenediamine tetraacetic acid (EDTA) at the concentration of 2, 2.5 and 5 mM, dithiothreitol (DTT) and *p*-methylphenyl sulfonyl fluoride (PMSF) at the concentration of 50, 100, 250 μM . The behaviour of the crude enzyme extract in the presence of these chemicals was assayed as indicated in the section of metal ion effect.

Statistical analysis of data. Analysis of variance of the data was performed with SPSS 10.0 for Windows (USA). The LSD multiple range test was employed to determine the statistical analysis. In all Figures and tables, data points represent mean of three determinations ($p \leq 0.05$).

Results and Discussion

Esterase secretion by *Anoxybacillus gonensis* A4 (*A. gonensis* A4) (Beldüz *et al.*, 2003) on tributyrin agar plate was qualitatively monitored by the formation of clear zone around the colonies after three days incubation at 60°C. A further incubation at 4°C for 4 days was performed in order to make the clear zone more visible (Fig. 1a). It can be easily extracted from the result of this qualitative test that *A. gonensis* A4 produce an extracellular enzyme responsible for the degradation of tributyrin. This method is not specific for esterase or lipase (Suoniemi and Tynkkyinen 2002; Hotta *et al.*, 2002) but shows that *A. gonensis* A4 can hydrolyze ester bounds with short chain.

SDS-PAGE gel electrophoresis on crude enzymes stained with a 1:1 mixture solution of 0.1 M α -naphthyl acetate and 0.1

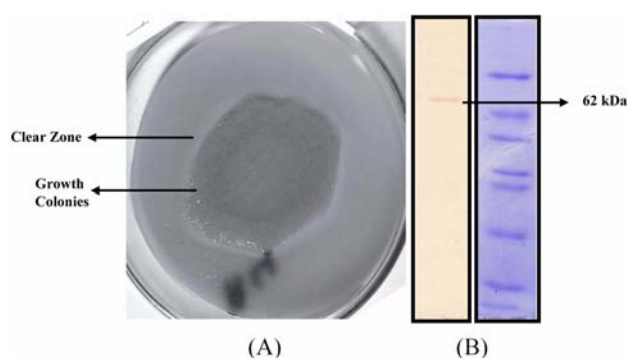


Fig. 1. (A) Esterolytic activity detection of *A. gonensis* A4. Determination of the extracellular esterolytic activity for *A. gonensis* A4 on tributyrin agar plate containing 1.5 % tributyrin in Luria-Bertani (LB) solid medium. (B) SDS-PAGE for *A. gonensis* A4 crude extracellular extract. Lane 1: Esterase on renaturated gel stained with α -naphthyl acetate and Fast Red, Lane 2: Molecular mass standard (albumin 66 kDa, ovalbumin 45 kDa, glyceraldehyde-3-phosphate dehydrogenase 36 kDa, carbonic anhydrase 29 kDa, trypsinogen 24 kDa, trypsin inhibitor 20.1 kDa, α -Lactalbumin 14.2 kDa, aprotinin 6.5 kDa).

M Fast Red solution from *A. gonensis* A4 indicated the presence of an esterase having molecular weight of approximately 62 kDa (Fig. 1b). It was reported that the molecular weights of *Bacillus circulans* esterase, *Vibrio harveyi* lipase and a carboxyl esterase from a hyperthermophilic archeon were found to be 95, 61 and 34 kDa, respectively (Kademi *et al.*, 2000a; Hotta *et al.*, 2002; Teo *et al.*, 2003).

It is known that composition of growth culture medium affects bacterial lipase/esterase production (Choi and Lee 2001; Lima *et al.*, 2003). While growing, the presence of an esterase activity of this isolate was checked by the hydrolysis of *p*-nitrophenylbutyrate (PNPB) to *p*-nitrophenol. Crude enzyme extracts prepared from each *A. gonensis* A4 grown in LB medium and LB medium supplemented with 0.3% carboxymethyl cellulose reached the activity maximum value after 18 and 15 h incubation period, respectively, in the presence of PNPB substrate. It was observed that maximum activity was higher in the presence of carboxymethyl cellulose in culture medium (Fig. 2). The effect of surfactants on the production of *Penicillium camembertii* Thom PG-3 lipase was reported (Tan *et al.*, 2004).

PNPB as a short chain nitrophenyl ester and *p*-nitrophenyl laurate (PNPL) as a long chain nitrophenyl ester were tested for substrate specificity of the crude esterase. In the presence of PNPB and PNPL, hydrolytic activities of crude extracts were measured as 347 and 48 U/L, respectively, at the end of 15 h bacterial growth period (Fig. 2). Substrate specificity clearly shows that the crude enzyme extract utilize both of these esters but possesses esterase activity since hydrolysis of PNPB was approximately 7-fold higher. This is in good agreement with the earlier reports (Kademi *et al.*, 1999, Teo *et al.*, 2003).

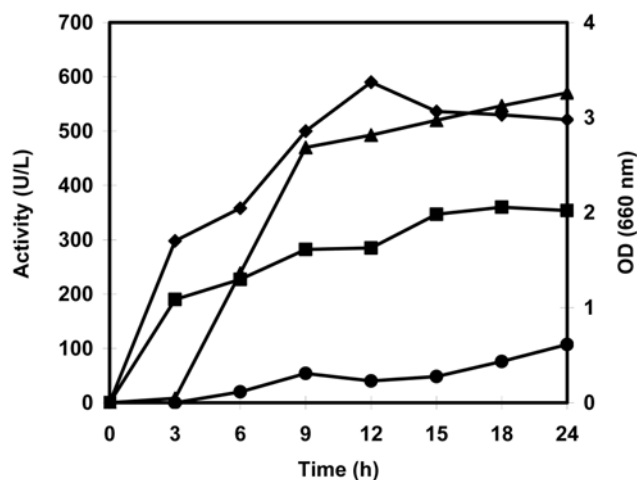


Fig. 2. Activity and growth profiles of *A. gonensis* A4. Activity profiles of *A. gonensis* A4 growth in LB medium in the presence of *p*-nitrophenyl laurate, *p*-nitrophenyl butyrate substrates and 0.3% carboxymethylcellulose supplemented LB medium in the presence of *p*-nitrophenyl butyrate substrate. Reaction mixture contained ethanol, pH 7.5 phosphate buffer and 1 mL of 10 mM of *p*-nitrophenyl butyrate in acetonitrile and *p*-nitrophenyl laurate in DMSO. Growth profile of *A. gonensis* A4 in LB medium.

The amount of protein (w/v) in the extracellular medium was determined to be 6.3 mg/mL. The effect of protein content on the PNPB esterase activity was investigated by varying protein concentration between 0.012 to 1.215 mg/mL. The enzyme activity gradually increased to 0.45 mg/mL protein content and remained constant at protein levels greater than 0.65 mg/mL. Therefore, crude enzyme extracts from *A. gonensis* A4 having 0.3 mg/mL protein content was used for the kinetic studies. Similar observations were reported for the crude depolymerases (Colak and Güner 2004; Çolak *et al.*, 2005).

The effect of pH on the *A. gonensis* A4 esterase activity was determined by using PNPB as a substrate with the pH buffers ranging from 4.0 to 8.0. pH optimum of this enzyme was observed at 5.5 (Fig 3). In addition, crude enzyme preparation showed a second peak at pH 7.5. This might indicate isoforms of the esterase or different enzymes hydrolyzing PNPB in the crude enzyme preparations. Similar results were reported earlier for polyphenol oxidases (Yildirim *et al.*, 2005; Çolak *et al.*, 2005). It is also reported that esterases have a pH optimum at approximately 6.0 while lipases show maximum activity around 8.0 (Fojan *et al.*, 2000). Further investigations were carried out at pH 5.5. The optimum pH values for extracellular esterases were found different among bacteria (Jung *et al.*, 2003).

The residual activity of the esterase from *A. gonensis* A4 was determined after 24 h of incubation at various pH values ranging from 4.0 to 9.0 (Fig. 3 inset). The pH-stability profiles showed that original crude esterase activity nearly 2-fold increased at pH 6.0. In addition, it appears that the esterase is

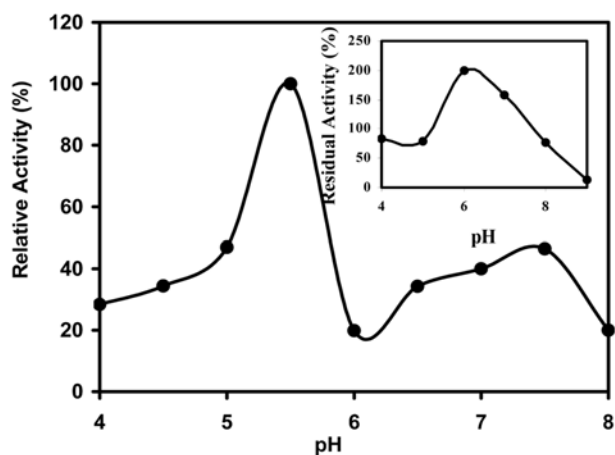


Fig. 3. Effect of pH on activity and pH stability of *A. gonensis* A4. Effect of pH on activity of *A. gonensis* A4 esterolytic activity in 50 mM acetate buffer (4.0-5.5), in phosphate buffer (6.0-8.0). The reaction mixture contained *p*-nitrophenyl butyrate and ethanol in the buffer indicated above. Inset: pH stability of the extracellular esterolytic activity of *A. gonensis* A4 residual activity after 24 h incubation at desired pH at 4°C. The activity was assayed under standard conditions, pH 5.5 acetate buffer at 60°C.

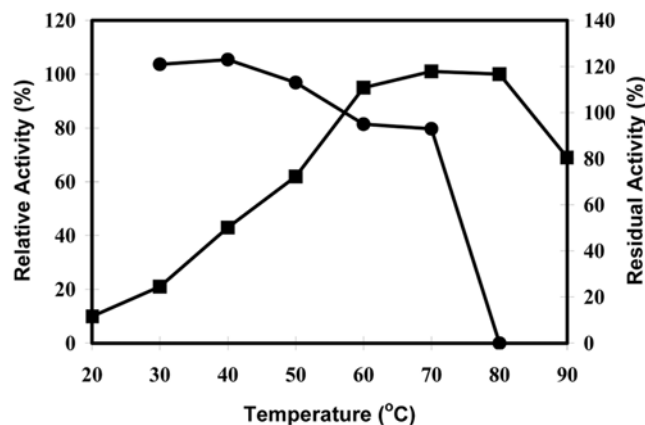


Fig. 4. Effect of temperature on activity and thermostability of *A. gonensis* A4. Temperature optimum and stability profiles of *A. gonensis* A4 esterase that hydrolyze *p*-nitrophenyl butyrate. The reaction mixture in pH 5.5 acetate buffer was incubated for different temperatures and the standard activity assay was performed at 405 nm. For temperature stability, the crude enzyme extract was incubated at temperatures of 30-80°C. The percentage residual activity was compared with unincubated enzyme.

quite stable at the pH range from 4.0 to 8.0. The stability of the enzyme in acidic and neutral pHs and at high temperatures suggests its usefulness in industrial applications. The pH stability profile of thermophilic *Bacillus* sp. esterase showed that enzyme retained almost its original activity when it was incubated at pH 4.0-8.0 up to 10 h at room temperature, but a decline of enzyme activity was observed at the end of 24 h incubation period (Burcu *et al.*, 2006).

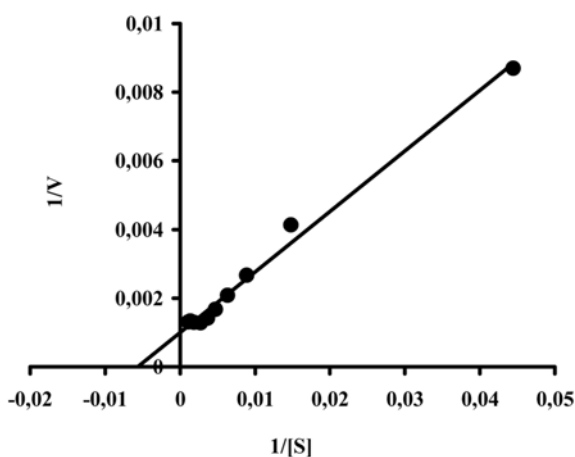


Fig. 5. Kinetic analysis of the reaction rates. Lineweaver-Burk plot of *A. gonensis* A4 esterase for the kinetic analysis of the reaction rates, at a series of concentrations for *p*-nitrophenyl butyrate.

Fig. 4 shows the thermal activity profile having a shoulder between 60-80°C for extracellular crude esterase from *A. gonensis* A4. It was observed that enzyme preparation has a higher esterase activity at higher temperatures above 50°C and this result was well consistent with the opinion which thermostable enzymes could be extracted from thermophilic microorganisms (Kademi *et al.*, 2000a; Markossian *et al.*, 2000). A thermostable esterase with an optimum temperature of 60 and 70-75°C was also reported for *Bacillus circulans* (Kademi *et al.*, 2000b) and *Bacillus thermoleovorans* ID-1 (Lee *et al.*, 1999), respectively.

The thermal stability profile of *A. gonensis* A4 esterase in the form of residual activity was presented in Fig. 4. The enzyme was stimulated by keeping at 30-50°C and almost retained its original activity at 60 and 70°C for 1 h incubation period. However, enzyme activity was fully lost at 80°C for the same incubation period. From both thermal activity and thermal stability profiles, it can be speculated that crude enzyme extract prepared from *A. gonensis* A4 can be used with any temperature from 60 to 70°C in a biotechnological applications. These characteristics are superior to those of other notable esterases or lipases from *Bacillus thermoleovorans* ID-1 lipase (Lee *et al.*, 1999) and *Bacillus* sp. esterase (Burcu

Table 1. Effect of some metal ions on *Anoxybacillus gonensis* A4 esterase activity. The esterase activity in the presence of a metal ion was compared with the control including no metal ion whose activity was taken as 100%

Metal Ion (1 mM)	Relative Activity (%)
Cu ²⁺	69 ± 3
Ni ²⁺	80 ± 3
Ca ²⁺	86 ± 4
Mn ²⁺	96 ± 4
Hg ²⁺	47 ± 2
Zn ²⁺	59 ± 4
Co ²⁺	65 ± 5
Cd ²⁺	86 ± 5
Cr ³⁺	53 ± 3
K ⁺	115 ± 5
Na ⁺	101 ± 5

et al., 2006). The thermostable esterases from soil thermophilic bacterial strains were also reported to be stable in the ratio of 98% at the end of 1 h incubation period at 70°C when compared with its original activity (Kademi *et al.*, 1999).

Substrate saturation curves for PNPB indicated that the *A. gonensis* A4 esterase follows simple Michaelis-Menten kinetics. The substrate saturation curve was obtained by interpolating the substrate concentrations against activity values. The reaction velocity increased up to approximately 360 μM PNPB and then reached a constant value. Michaelis-Menten constants (K_m) and maximum reaction velocities (V_{max}) were determined as 800 U/L and 176.5 μM, respectively, from a Lineweaver-Burk plot (Fig. 5). Similar K_m value (0.18 mM) was calculated for the carboxyl esterase Rv3487c from *Mycobacterium tuberculosis* against PNPB and V_{max} was found to be 300.5 ML⁻¹ s⁻¹ (Zhang *et al.*, 2005).

It is known that metal ions have an important role to maintain the enzyme in active and stable structure by binding to amino acid residues with negative charge in specific sites (Çolak *et al.*, 2005). The behaviour of *A. gonensis* A4 for some metal ions was examined by using chloride salts of each metals at a concentration of 1 mM (Table 1). In the presence of K⁺, the enhancement of esterase activity up to 115 ± 5% shows that this metal ion stimulates *A. gonensis* A4 esterase

Table 2. Effect of some reagents on *Anoxybacillus gonensis* A4 esterase activity. The esterase activity in the presence of a reagent was compared with the control which contained no reagent other than the compounds required for esterase assay. The control activity was taken as 100%

Concentration (μM)	Relative activity (%)		Concentration (mM)	Relative activity (%)	
	PMSF	DTT		2-ME ^a	Na-EDTA
50	94 ± 5	107 ± 5	2	127 ± 5	79 ± 4
100	90 ± 4	110 ± 5	2.5	130 ± 5	56 ± 3
250	85 ± 4	118 ± 4	5	153 ± 5	32 ± 3

^a2-Mercaptoethanol

activity. In addition, all tested divalent and trivalent metal ions inhibited enzyme activity in different ratio but not completely. The inhibition of esterase activity in the presence of EDTA (ethylenediamine tetraacetic acid) can be attributed to its metal chelating effect (Table 2). However, Dithiotreitol (DTT) and mercaptoethanol as reducing agent of disulfide bridges were also stimulated enzyme activity. It can be concluded from this result that -SH groups were essential for PNPB hydrolysis. The inhibition of enzyme activity with Hg^{2+} was also supported this result (Kademi *et al.*, 2000a). The little inhibition of esterase activity by PMSF may be attributed to the presence of serine residues responsible for the hydrolytic reaction in the active site since this inhibitor is known to interact selectively and irreversibly with the serine hydroxyl groups.

In conclusion, a thermophilic bacterium *A. gonensis* A4 isolated from hot springs in Turkey, secretes an extracellular hydrolase responsible for the degradation of some *p*-nitrophenyl esters. This extracellular crude preparation was investigated in terms of substrate specificity, pH and temperature optima, stability and kinetic parameters. It was found that *A. gonensis* A4 produced a good thermostable extracellular esterase and enzyme was highly stable at near neutral pH values. The stimulation of enzyme activity in the presence of DTT and Mercaptoethanol and inhibitor effect of Hg^{2+} shows that -SH groups in specific sites were required.

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