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RESEARCH ARTICLE

Partial purification and characterization of lipase from *Geobacillus stearothermophilus* AH22

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Abstract

The lipase was partially purified by ion exchange chromatography and gel filtration column chromatography, and was characterized from *Geobacillus stearothermophilus* AH22 strain. The lipase was purified 18.3-folds with 19.7% recovery. The lipase activity was determined by using *p*-nitrophenyl esters (C_2–C_{12}) as substrates. The K_m values of the enzyme for these substrates were found as 0.16, 0.02, 0.19 and 0.55 mM, respectively, while V_{max} values were 0.52, 1.03, 0.72 and 0.15 U mg^{-1}. The enzyme showed maximum activity at 50 °C and between pH 8.0 and 9.0. The enzyme was found to be quite stable at pH range of 4.0–10.0, and thermal stability between 50 and 60 °C. It was found that the best inhibitory effect of the enzyme activity was of Hg^{2+}. The inhibitory effect as orlistat, catechin, propyl paraben, *p*-coumaric acid, 3,4-dihydroxy hydro-cinnamic acid was examined. These results suggest that *G. stearothermophilus* AH22 lipase presents very suitable properties for industrial applications.

Keywords

Characterization, *Geobacillus stearothermophilus* AH22, lipase, partial purification

History

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Introduction

Lipases are triaclyglycerol acylhydrolases (EC 3.1.1.3) that catalyze the hydrolysis of triaclyglycerol to diacylglycerols, monoacylglycerols, fatty acids and glycerol at the oil–water interface. They often express other activities such as phospholipase, isophospholipase, cholesterol esterase, cutinase, amidase and other esterase type of activities[1]. The number of available lipases has increased since the 1980s and used as industrial biocatalysts because of their properties such as bio-degradability, high specificity and high catalytic efficiency. Some unique properties of lipase such as their specificity, temperature, pH dependency, activity in organic solvents and nontoxic nature lead to their major contribution in the food processing industries[2]. Lipases are ubiquitous in nature, including plants, animals and microorganisms. Especially, lipases of microbial origin, which are used in food, diary, cosmetic, detergent and leather industries, are particularly attractive because of their tremendous catalytic potential. Due to the wide range of applications, lipases are accepted as one of the most important industrial enzymes[3]. Some important lipase-producing bacterial genera are *Bacillus*, *Pseudomonas* and *Burkholderia* and fungal genera include *Aspergillum*, *Penicillium*, *Rhizopus*, *Candida*[4,5]. Microbial lipases have gained special industrial importance due to their ability toward extremes of temperature, pH and organic solvents and chemo-, regio- and enantioselectivity. Therefore, researches on lipases are concentrated particularly on structural characterization, disclosure of mechanism of action, kinetics, sequencing and cloning of lipase genes, and general characterization of performance[6,7].

In this study, a thermostable lipase was partially purified from *Geobacillus stearothermophilus* AH22 strain, a new isolated thermophilic bacterium, and characterized in the presence of different substrates, cations, some inhibitors and surfactants.

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Materials and methods

Chemicals

4-Nitrophenyl laurate, 4-nitrophenyl butyrate, 4-nitrophenyl acetate, 4-nitrophenyl caprylate, DEAE-Cellulose, molecular weight marker, bovine serum albumin, Coomassie Brilliant Blue R-250, acrylamide and *N*,*N*′-methylenebisacrylamide were purchased from Sigma-Aldrich (St. Louis, MO).

Lipase production in this study, *G. stearothermophilus* AH22 strain, isolated from Erzurum Ilıca hot springs by Adiguzel et al.[8] was used as the enzyme source.

Culture and growth conditions

An overnight growth of the isolated *G. stearothermophilus* AH22 strain was inoculated into Luria-Bertani medium (NaCl 0.5%, yeast extract 0.5% and Tryptone 1.0%; pH 7.2) contained in conical flask. The flask was incubated at 55 °C with shaking (150 rpm) for 16 h. After the centrifugation (10 000 *g* for 10 min at 4 °C), bacterial cells were decomposed with sound waves by sonicator in Tris–HCI buffer (pH 7.5). After sonication, the cell culture was centrifugated (10 000 *g* for 20 min at 4 °C) and supernatant was assayed for lipolytic activity as described below. Supernatant was used for crude enzyme source.

Enzyme assay

Lipase activity was also estimated using a spectrophotometric assay[9] with *p*-nitrophenyl esters as a substrate. The absorbance of *p*-nitrophenol released was measured at 405 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol *p*-nitrophenol per min under standard assay conditions.

Partial purification of lipase

The crude enzyme (100 mL) was incubated to determine suitable incubation temperature and time at various temperatures ranging from 50 to 90 °C for 15 and 30 min in a water bath. The denatured proteins were removed by centrifugation (13 000 rpm, 30 min, 4 °C). The supernatant (95 mL) was loaded onto a DEAE-Cellulose column (30 cm × 1.5 cm) (Sigma Chemicals) previously equilibrated with the 50 mM Tris–HCl buffer (pH 8.0). The column was washed thoroughly with the initial buffer, and the elution was performed with a linear gradient of 0–1.0 M NaCl in the equilibrated at flow rate of 30 mL/h. Fractions showing lipase activity were pooled, and dialyzed by using Amicon Ultra centrifugal filter unit (MWCO: 10 kDa) (Sigma-Aldrich, St. Louis, MO) with 50 mM phospate buffer (pH 7.5). The dialyzed protein solution was then applied to Sephadex G-150 column (60 cm × 3 cm) (medium grade; Pharmacia, Uppsala, Sweden) chromatography for further purification. The column was eluted 50 mM phospate buffer (pH 7.5), and 5 mL fractions were collected at a flow rate of 0.5 mL/min. Fractions showing lipase activity were combined. The combined fractions were loaded following by G-150 column again, and the above-mentioned process was repeated exactly by the G-25 column. The fractions obtained in each step of gel filtration chromatography was concentrated by Amicon Ultra centrifugal filter unit (MWCO: 10 kDa). Furthermore, the specific activity of the purified enzyme was compared with that of crude enzyme and purification fold was calculated after each purification step.

Protein determination assay

Protein concentration was measured by using bovine serum albumin as standard method of Lowry et al.[10].

Polyacrylamide gel electrophoresis and activity staining

SDS–polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Laemmli[11] using a 12% cross-linked polyacrylamide gel and a mini-vertical apparatus (Bio-RAD, Herts, UK). The protein bands were stained with Coomassie brilliant blue R-250 (Sigma). Relative molecular weight of the lipase was estimated by comparison with molecular mass standard markers in the range 250.0–10.0 kDa (Fermentas, ThermoFisher Scientific (Waltham, MA)).

PAGE of purified lipase under nondenaturing conditions was also carried out using 10% separating gel at 4 °C. For activity staining, the gel containing nondenaturing protein was placed into a petri dish containing Solution C (Solution A: 20 mg of 1-naphtylacetate dissolved in 5 mL of acetone completed to 50 mL with 0.1 M Tris–HCI buffer pH 7.5; Solution B: 50 mg Fast Red TR, 0.5 g Triton X-100 completed to 50 mL 0.1 M Tris–HCI buffer pH 7.5; Solution C: equal volumes of Solutions A and B added to each other) and, incubated for 10 min at room temperature. The appearance of a Brown color indicates the hydrolysis of α-naphtylacetate into 1-naphthol and formation of complex with Fast-Red[12]. After activity staning, the protein bands on the gel were stained with Coomassie brilliant blue R-250 and, activity stained protein band was compared with other bands.

Effect of pH on enzyme activity and stability

To investigate the optimal pH, lipase activity was assayed at various pH from 4.0 to 12.0 in the following buffers: 50 mM sodium acetate buffer (pH 4.0–5.0), 50 mM MOPS buffer (pH 6.0–7.0), 50 mM Tris–HCl buffer (pH 8.0–9.0) and 50 mM glycine–NaOH buffer (pH 10.0–12.0). pH stability in the range of 4.0–10.0 was examined by incubating the enzyme solution for 30 days at +4 °C with different buffers, and then determined the residual activity.

Effect of temperature on enzyme activity and stability

The enzyme activity was measured in the range of 10–80 °C using the standard activity assay procedure at related temperature. Thermostability of the lipase was investigated by measuring the residual activity after incubating the enzyme solution at 40–90 °C at various times from 15 min to 15 days in 50 mM Tris–HCl buffer (pH 8.0).

Effect of protein concentration on enzyme activity

Lipase activity was assayed in the range of 0.5–100 μg/mL protein concentration using *p*-nitrophenyl (NP)-butyrate as substrate to determine the maximum lipase activity at optimal pH and temperature observed.

Determination of Michaelis–Menten constant and maximum reaction velocity

Initial rate measurements with partial purified lipase at final concentration of 30 μg/mL, at constant temperature 25 °C and pH 8.0 with increasing substrate concentration of *p*-NP esters (0.01–1.0 mM), were performed to determine the kinetic parameters such as maximum reaction rate (V_{max}) and Michaelis–Menten constant (K_m). The *p*-NP esters between C2 and C12 were determined using *p*-NP-acetate (*p*-NPA), *p*-NP-butyrate (*p*-NPB), *p*-NP-octanoate (*p*-NPO) and *p*-NP-laurate (*p*-NPL) as the synthetic substrate and by using the spectrophotometric assay[9]. The kinetic parameters were estimated from the Lineweaver–Burk equation plot.

Effect of metal ions on lipase activity

Various metal ions [Ba^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, Ni^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+}, Fe^{2+}, Cu^{2+} and the metal-chelating agent ethylenediaminetetraacetic acid (EDTA)] at final concentrations of 0.1, 0.5 and 1.0 mM were added to the enzyme in 50 mM Tris–HCl buffer, pH 8 and the solution was preincubated at room temperature for 5 min and then assayed for lipase activity. The relative activity of the enzyme was calculated by comparing with enzyme incubated under similar condition without metal ions.

Effect of inhibitors and surfactants on lipase activity

The effect of inhibitors on lipase activity was determined using β-mercaptoethanol, PMSF, orlistat, catechin, catechol, propyl paraben, *p*-coumaric acid and 3,4-dihydroxy hydro-cinnamic acid at various final concentrations. The enzyme/inhibitor mixture was then taken to assay the lipase activity. Relative activity was calculated and enzyme solution without inhibitor was used to compare as reference. Similarly, the effect of surfactants (Triton X-100, Triton X-114, Tween 80 and SDS) at the final concentration of 0.1%, 0.5% and 1.0% on the lipase was investigated and the remaining enzyme activity was examined by the standard assay method. Relative activity was calculated by comparing with control (enzyme incubated without surfactants).

Results and discussion

An intracellular lipase, *G. stearothermophilus* AH22 isolated from Erzurum Ilıca hot springs by Adiguzel et al.[8] was purified partial and characterized in this study.

Table 1. Flowsheet of procedure used to partial purification of lipase from *G. stearothermophilus* AH22.

	Volume (mL)	Protein (mg/mL)	Total protein (mg)	Activity (U/mL)	Total activity (U)	Specific activity (U/mg protein)	Purification fold	Yield (%)
Crude extract	100	9.32	932	2.01	201	0.216	1	100
Heat treatment (30 min at 70 °C)	95	6.50	617.5	1.8	171	0.28	1.3	85
DEAE-Cellulose	30	2.60	78	3.15	94.5	1.21	5.6	47
Sephadex G-150	13	2.32	30.2	5.38	69.9	2.31	10.7	34.7
Sephadex G-150 2nd loading	12.5	1.37	17.1	4.1	51.3	2.99	13.9	25.5
Sephadex G-25	11	0.91	10	3.60	39.6	3.96	18.3	19.7

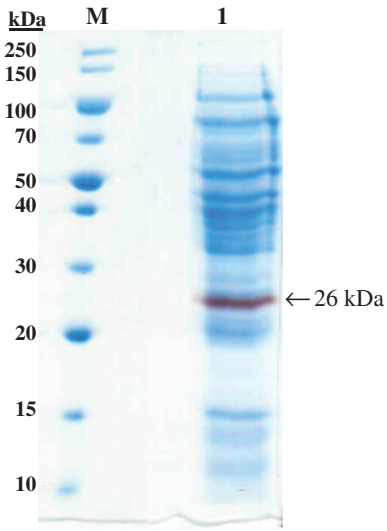


Figure 1. Staining SDS–PAGE (12%) gel with Coomassie brilliant blue R-250 after activity staining. The lanes are as follows: M, marker proteins with relative molecular masses indicated on the left; Lane 1, crude extract of *G. stearothermophilus* AH22 lipase.

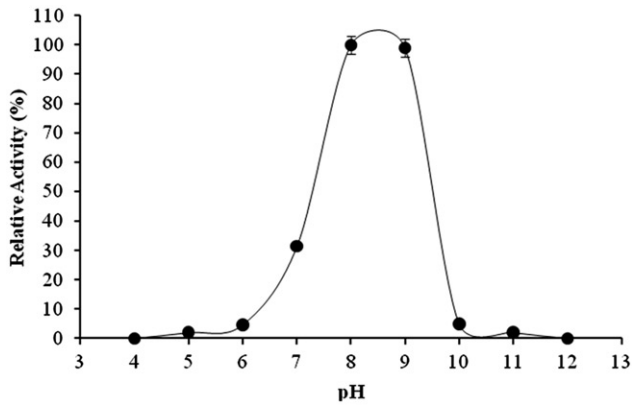


Figure 3. Optimum pH for activity of AH22 lipase.

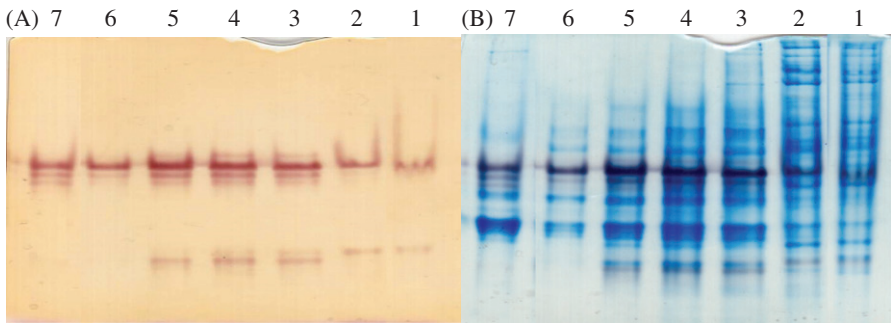


Figure 2. Nondenaturing PAGE pattern of partial purified lipase. The partial purified protein was electrophoresed on 10% (w/v) polyacrylamide gel under nonreducing conditions. (A) Activity staining gel. (B) Staining gel with Coomassie brilliant blue R-250 after activity staining. Lane 1: crude extract of *G. stearothermophilus* AH22 lipase; Lane 2: heat treatment (30 min at 70 °C); Lane 3: pooled fractions from DEAE-Cellulose chromatography; Lane 4: pooled fractions from Sephadex G-150 chromatography; Lane 5: pooled fractions from 2nd Sephadex G-150 chromatography; Lane 6:

The lipase from *G. stearothermophilus* AH22 strain was subjected to heat precipitate (70 °C, 30 min), DEAE-Cellulose sephadex G-150 and sephadex G-25 gel permeation chromatography (Table 1) in sequence resulting in its partial purification to 19.7% yield by the factor of 18.3-fold. The fold purification and yields were comparable to those reported previously; for example, *Bacillus stearothermophilus* MC 7 lipase has been purified to 10.2% yield with 19.25-fold and a specific activity of about 3.96 U mg^{-1} purification by ultrafiltration, Sephadex G-200 and DEAE-cellulose chromatography[13]. Masomian et al. have reported 15.6-fold purification and 19.7% yield in the case of *Aneurinibacillus thermoaerophilus* HZ lipase by steps of Q Sepharose and Sephadex G 75 chromatography[14]. Generally, the yield in lipase purification procedure is comparatively low – between 2% and 20%[13]. The responsibility of aggregation-related problems for the low yield of purification was speculated by the other authors[15]. The molecular mass of the protein showing lipase activity was estimated about 26 kDa, based on SDS–PAGE (Figure 1). It was reported that molecular mass of some lipases from *Aspergillus carneus*, *Thermomyces lanuginosus and Pseudomonas stutzeri* LC2-8 were as 27, 33 and 32 kDa, respectively[16–18]. Native PAGE (10% w/v) was subjected to confirm partially purity of AH22 lipase and then, acitivity staining was also performed in order to further verify the existence of lipase activity (Figure 2). After activity staining of native PAGE, the resulting dark brown bands showed presence of the lipase. Attempts to further purify lipase from this complex using Octyl–Sepharose hydrophobic interaction chromatography have been unsuccessful (data not shown).

The optimal pH for the lipase activity was obtained at pH 8–9 (Figure 3). It was observed that the enzyme had no activity at acidic pHs and substrate was self-hydrolysis at very high pHs.

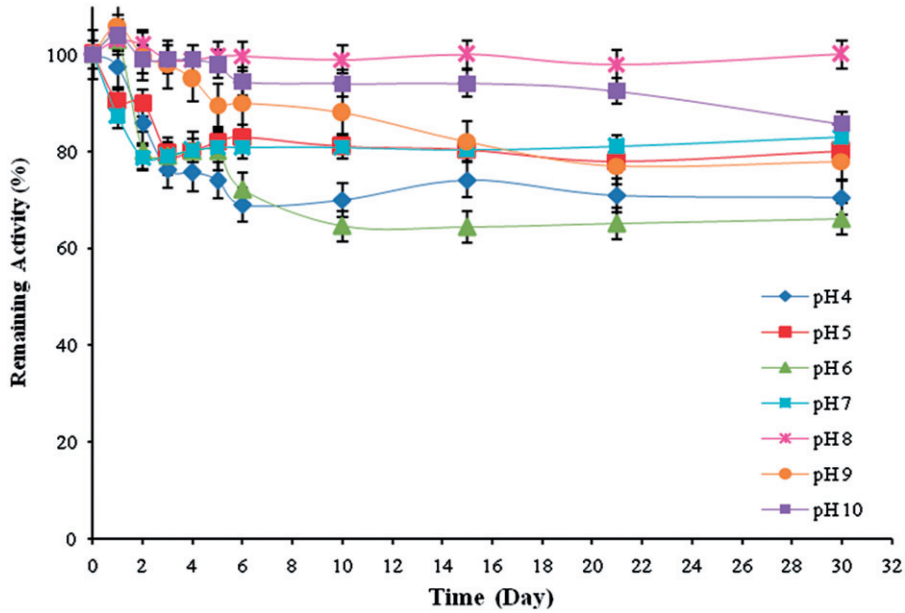


Figure 4. Effect of pH on thermostability of AH22 lipase.

Similar optimum pH activity profiles were observed in a few lipases from *Bacillus thermoleovorans* ID-1, *Bacillus cereus* C71, *P. stutzeri* LC2-8, *Pseudomonas aeruginosa*, *P. stutzeri* PS59[18–22]. The pH stability profile of the enzyme has also shown in Figure 4. After incubation at +4 °C for 30 days, more than 70% of the original activity could be retained at pH 7.0–10.0 but the original activity decreased slightly at pH 4.0–6.0. However, AH22 showed lower stability toward slightly acidic range than alkaline pH. Similar stability profiles were reported in a few lipases from *Penicillium* sp. DS-39 (DSM 23773) and *Microbacterium luteolum*[23,24]. This high stability can make the lipase applicable at especially alkaline pH conditions for the use in laundry and household detergents. These properties suggest that the lipase from *G. stearothermophilus* AH22 might be a novel pH stable lipase.

To determine effect of the temperature on AH22 lipase activity was assayed over a range from 10 to 80 °C (Figure 5). Partially purified AH22 lipase had an optimal temperature of 50 °C, which is similar or very close to other lipases reported from *Mortieralla alliacea*, *Staphylococcus aureus* and *B. stearothermophilus* P1[25–27]. The enzymatic activity decreased significantly at temperatures below 20 °C and above 70 °C. The enzyme was also highly active in a broad temperature range (20–70 °C). At 30, 40, 60 and 70 °C, it retained 66%, 82%, 75% and 49% of its maximum activity, respectively. It lost about 80% of the activity due to denaturation at 80 °C. While studying the thermal stability profile, AH22 lipase was found to be stable with remaining activity about 100% at 40 and 50 °C for 10 days (Figure 6). At room temperature, activity was maintained about 60% at the end of 15 days. At 60 °C, the enzyme activity was retained for 30 h, while remaining activity reduced to 20% at the end of 48 h (Figure 7). For higher temperatures, remaining activity decreased to lower than 35% at 70 °C, and it was lost completely at 80 and 90 °C owing to heat-denaturation of the enzyme occurred after 15 min of incubation (Figure 8). These results showed that the enzyme activity was significantly stable at room temperature and 40–60 °C, with a residual activity greater than 65% along 24 h. In addition, thermal properties of AH22 lipase with thermodynamic parameters (Table 2) indicate heat-inactivation of the enzyme. The activation energy of AH22 lipase was calculated to be 129.52 kJ/mol by using Arrhenius equation.

The kinetic analysis of the partially purified AH22 lipase was performed on the various *p*-nitrophenyl esters as substrate under

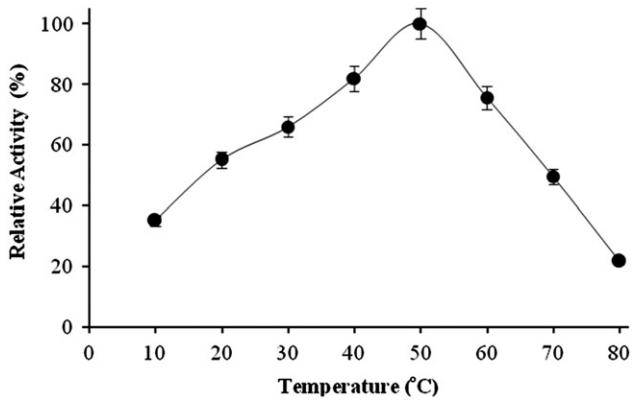


Figure 5. Optimal temperature for AH22 lipase.

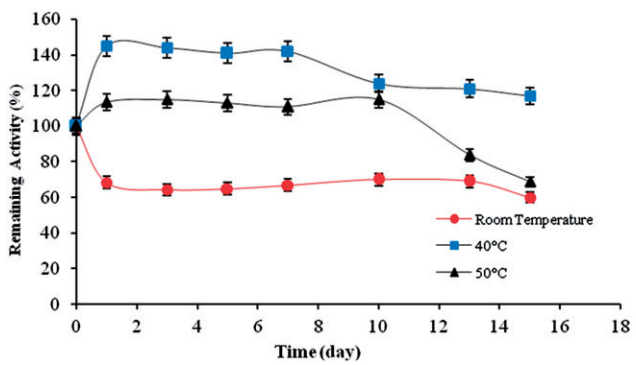


Figure 6. Effect of temperature on thermostability of AH22 lipase at room temperature, 40 and 50 °C.

optimal conditions using a Lineweaver–Burk equation plot corroborating the Michaelis–Menten behavior of the enzyme. K_m and V_{max} values were determined to be 0.16 mM and 0.52 U mg^{-1} for *p*-NPA, 0.02 mM and 1.03 U mg^{-1} for *p*-NPB, 0.19 mM and 0.72 U mg^{-1} for *p*-NPO and 0.55 mM and 0.15 U mg^{-1} for *p*-NPL. These results showed that the K_m values were lower than that of many lipases for various substrates from different resources, such as *Bacillus* sp. (0.5 mM *p*-NPL), *Aureobasidium pullulans* HN2.3 (0.608 mM *p*-NPL), *Geoacillus*

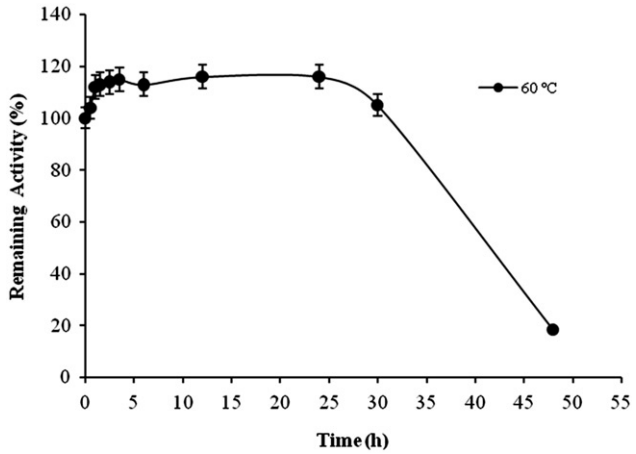


Figure 7. Effect of temperature on thermostability of AH22 lipase at 60 °C.

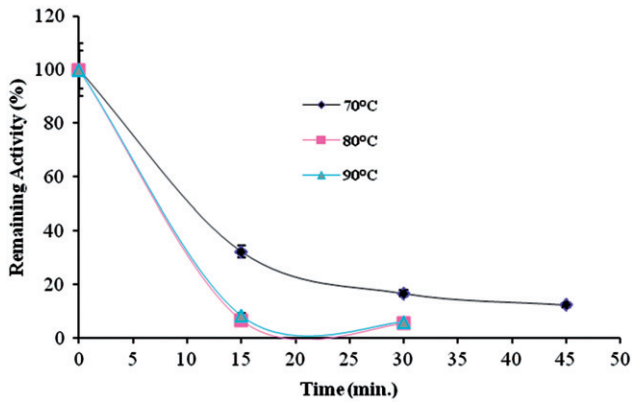


Figure 8. Effect of temperature on thermostability of AH22 lipase at 70, 80 and 90 °C.

sp. SBS-4S (3.8 mM *p*-NPA)[28–30]. It was reported that the K_m values of most industrial enzymes varied in the range of 10^{-1}–10^{-5} M when acting on biotechnologically important substrates[31]. The K_m values of AH22 lipase for each substrate are included in this range.

The activity of the partially purified AH22 lipase in the presence of metal ions is shown in Table 3. Among the metal ions studied, Mn^{2+}, Ca^{2+}, Mg^{2+}, Co^{2+}, Ba^{2+}, Fe^{2+}, Ni^{2+} and especially Cu^{2+} stimulated or stabilized the enzyme activity, whereas Hg^{2+} and Zn^{2+} inhibited the AH22 lipase. Similarly, lipases from *A. pullulans* HN2.3, *Penicillium* sp. DS-39 (DSM 23773), *M. luteolum* and *Saccharomyces cerevisiae* were inhibited by Hg^{2+} and Zn^{2+} cations[23,24,29,32]. The inhibition by mercuric ions may indicate the importance of thiol-containing amino acid residues in the enzyme function[33]. It has been also reported that the activity of lipases, isolated from various sources, was stimulated by some cations as *P. aeruginosa* PseA lipase (Ca^{2+}, Mg^{2+} and Fe^{2+}) and *P. aeruginosa* LX1 lipase (Ca^{2+}, Mg^{2+} and Ba^{2+})[34,35]. Cofactors are generally not required for lipase activity, but divalent cations like calcium often stimulate enzyme activity. This effect has been suggested to be due to the formation of long-chain fatty acid calcium salts[36,37]. Table 4 also shows the effect of different metal ions and EDTA on the activity of the partially purified AH22 lipase. The presence of the chelating agent EDTA did not inhibited but activated the enzyme activity, demonstrating that the partially purified enzyme was not metalloenzyme[38].

Surfactants relieve the access of substrate to the enzyme by stabilizing the interfacial area where catalytic reaction of lipase takes place[39]. It was interesting to note that the relative lipase activity decreased in the presence of increasing concentration of Triton X-100, Triton X-114, Tween-80 and SDS. In contrast, *P. stutzeri* LC2-8 lipase was stimulated by X-100 and activated slightly by Tween 20 and Tween-80[18]. Furthermore, many lipases from different sources, such as *Pseudomonas* sp., *Pseudomonas gessardii*, *Geoacillus* sp. SBS-4S and *Bacillus sphaericus* MTCC 7542 were inhibited by Triton X-100, Triton X-114, Tween-80 or SDS like AH22 lipase[30,40–42]. However, the effect of β-mercaptoethanol (a thiol reducing agent), PMSF (a serine protease inhibitor) and some chemicals such as orlistat, catechol, catechin, propylparaben, *p*-coumaric acid and 3,4-dihydroxy hydro-cinnamic acid on AH22 lipase activity is shown in Table 4. β-Mercaptoethanol did not affect lipase activity in high amount. Contrarily, PMSF and the other chemicals had significant inhibitory effect on AH22 lipase. This inhibitor effect of PMSF suggests that this lipase belongs to the class of serine hydrolases. It was shown that lipases from *Burkholderia multivorans* V2, *Nomuraea rileyi* MJ and *A. thermoaerophilus* HZ were also inhibited by PMSF[14,43,44]. It was observed that even very low concentrations of the other chemicals especially catechin (0.05 mM, 59% relative activity) and orlistat (2 µg/mL, 68% relative activity) inhibited considerably lipase (Table 4). Otherwise, IC_{50} values for orlistat, catechin, propyl paraben, *p*-coumaric acid, 3,4- dihydroxy hydro-cinnamic acid were calculated as 4.2 µg/mL, 0.06 mM, 0.5 mM, 1.3 mM and 1.7 mM, respectively.

This article describes the partial purification and characterization of a thermophilic lipase from *G. stearothermophilus* AH22 strain. The data available for the AH22 lipase are similar to the previous lipases. The lipase, thermostable and pH stable, may be applied to treat lipid-rich industrial effluents treatments or to synthesize useful chemical compounds. In addition, studies on *G. stearothermophilus* AH22 strain as well as their lipases may

Table 2. Thermodynamic parameters for thermal inactivation of *G. stearothermophilus* AH22 lipase.

Temparature (K)	k (s^{-1})	$\Delta G\#$ ($J\,mol^{-1}$)	$\Delta H\#$ ($J\,mol^{-1}$)	$\Delta S\#$ ($J\,mol^{-1}\,K^{-1}$)
298	2.00×10^{-7}	295 589	127 043	−566
313	2.00×10^{-7}	310 595	126 918	−587
323	5.00×10^{-7}	323 063	126 835	−608
333	2.00×10^{-6}	336 988	126 752	−631
343	5.00×10^{-4}	362 937	126 669	−689
353	2.00×10^{-4}	370 914	126 586	−692
363	4.00×10^{-4}	383 597	126 502	−708

#Standard condition is 25 °C and 1 atm for physicochemical parameters.

Table 3. Effect of various metal ions and EDTA on the lipase activity.

	Relative activity (%)		
Metal ions	0.1 mM	0.5 mM	1 mM
Control	100	100	100
Mn^{2+}	135	136	98
Ca^{2+}	105	119	91,5
Cu^{2+}	158	121	125
Ba^{2+}	112	124	153
Co^{2+}	145	127	152
Zn^{2+}	72.7	62.2	99
Fe^{2+}	122	103	96.8
Ni^{2+}	105	124	83.5
Mg^{2+}	104.5	120.7	93.8
Hg^{2+}	56	27.8	29.1
EDTA	105	119	106

Table 4. Effect of some surfactants and inhibitors on the lipase activity.

Surfactants and inhibitors	Concentration	Relative activity (%)	IC_{50}	Surfactants and inhibitors	Concentration	Relative activity (%)	IC_{50}
Control Triton X-114 (%)	–	100		Control PMSF (mM)	–	100	
	0.1	67			0.5	70	
	0.5	47	0.42		1.0	63	1.6
	1.0	23			2.0	43	
Triton X-100 (%)	0.1	85		Catechol (mM)	1.0	80	
	0.5	57	0.62		5.0	62	8.5
	1.0	37			10.0	48	
Tween-80 (%)	0.1	65		Catechin (mM)	0.05	59	
	0.5	46	0.40		0.1	31	0.06
	1.0	25			0.25	24	
SDS (%)	0.1	31		Propyl paraben (mM)	0.5	52	
	0.5	20	0.04		1.0	42	0.5
	1.0	12			2.0	29	
β-Mercaptoethan ol (mM)	0.1	114		*p*-Coumaric acid (mM)	0.5	64	
	0.5	107	–		1.0	53	1.4
	1.0	106			2.0	38	
Orlistat (μg/mL)	2.0	68		3,4-Dihydroxy hydro-cinnamic acid (mM)	1.0	80	
	4.0	51	4.2		2.5	43	1.7
	6.0	46			5.0	23	

lead to further understanding on the evolution of the thermophilic bacteria.

Declaration of interest

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