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An esterase from *Thermus thermophilus* HB27 with hyper-thermoalkalophilic properties: Purification, characterisation and structural modelling

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ABSTRACT

A membrane-associated esterase (E34Tt) was detected in *Thermus thermophilus* HB27. The enzyme was purified to homogeneity in a three-step protocol. Detergent (CHAPS) above the CMC was found to be essential to solubilise the enzyme from cell membranes as well as for maintaining activity and stability.

By using mass fingerprinting, peptides were found to share identity with the YP_004875 protein, which was annotated as putative esterase in the genome analysis of *T. thermophilus* HB27, although experimental evidence was lacking. No homology was detected with any known lipase or esterase. However, a comparison with the high-scored sequences from a BLASTp search identified the consensus sequence for lipases/esterases between amino acids 157 and 161 (Gly-Cys-Ser^{159}-Ala-Gly). Further inhibition assays with E600 confirmed that Ser^{159} was involved in the catalytic mechanism.

The monomeric enzyme had a molecular mass of 34 kDa and exhibited esterase activity with preference for medium chain-length esters (C10). E34Tt was noticeable for its high thermal stability; the optimal reaction temperature was higher than 80 °C and the half-life of thermal inactivation at 85 °C was 135 min, which makes it even more thermostable than some hyperthermophilic esterases. These properties convert E34Tt into a very attractive enzyme for biotechnological purposes.

A theoretical structural model was constructed using as template a prolyl oligopeptidase from *Sus scrofa*, and a putative catalytic triad (Ser^{159}, Glu^{255} and His^{293}) with high similarity to the template was identified.

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1. Introduction

Enzymatic transformations are more specific and can be performed in milder conditions with a lower generation of by-products than chemical catalysis. As consequence, enzymes are gaining popularity in the current chemical industry as economically and environmentally friendly catalysts, and can be used to replace chemical steps with low environmental efficiency [1]. Biocatalysts can be implemented in a broad range of industrial processes, including detergent manufacturing, paper processing, agrochemicals, bioplastics food technology, and synthesis or modification of biologically active molecules for diagnostics and research [2]. Therefore, the demand for novel enzymes with better activity and stability properties increases exponentially [3].

Thermal stability is one of the most sought-after characteristics in new enzymes. In this regard, extreme thermophiles (microorganisms living at temperatures above 70 °C) are an interesting source of stable enzymes as, besides thermo-resistance, thermozymes frequently present an unusual resistance towards a number of chemical and physical denaturing agents [4], which make them suitable for harsh industrial conditions where conventional enzymes lose their function.

Amongst novel enzymes isolated from thermophilic microorganisms, hydrolases, and particularly lipases and esterases are experiencing an increasing demand. Lipases (EC 3.1.1.3) and esterases (EC 3.1.1.1) catalyse the cleavage of ester bounds in aqueous media and the reverse reaction in organic solvents. Both lipolytic enzymes have relevant applications in food, dairy, detergent, biofuel, and pharmaceutical industries. In particular, these enzymes are interesting due to their broad substrate specificity, high regio- and enantioselectivity, and ability to synthesise active chiral compounds [5,6].

Several lipase- and esterase-producing thermophiles have been studied (see reviews by Atomi [7], Egorova and Antranikian [8], Levisson et al. [9] or Salameh and Wiegel [10]). However, their biotechnological potential has not been extensively exploited, mainly due to the lack of knowledge about their production, purification systems, and catalytic properties.

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In relation with this, the thermophilic bacterium *Thermus thermophilus* HB27 is becoming a model organism for biotechnology and genetic engineering of extremophilic microorganisms [11]. The genus *Thermus*, with a temperature of growth ranging from 45 °C to 85 °C, belongs to one of the oldest branches of bacterial evolution and forms a phylum together with the genus *Deinococcus*. It most likely represents an evolutionary intermediate between today's Gram-positive and Gram-negative bacteria [12]. The genome sequences of two *T. thermophilus* strains, HB8 and HB27, are available [13]. Chromosomes are highly conserved with an identity of 94%, but variations are found, predominantly in cell envelope structures [14]. Several species of the genus *Thermus* are able to form, under certain environmental conditions, rotund bodies (also called multicellular bodies), i.e., a variable number of cells surrounded by a membrane formed by the fusion of the individual external membranes of each cell [15]. Castán et al. [16] demonstrated that the internal volume of the multicellular bodies constitutes a hypertrophic periplasmic space with a specific profile of soluble proteins (different from that of the cytoplasm and the membrane), which could be isolated by breaking the external membrane through repeated pipetting of cell suspensions.

These thermophiles produce a number of enzymes with considerable biotechnological interest such as proteases, phosphatases, catalases, several DNA processing enzymes, etc. [17,18]. However, little information is available on the lipolytic activities of *Thermus* species. In previous works the authors demonstrated the ability of several *Thermus* strains to produce enzymes active on lipolytic substrates [19].

T. thermophilus HB27 produces two extracellular enzymes with lipase/esterase activity and molecular weights of 34 and 62 kDa [20]. By zymography, the same enzymes were identified in the cytoplasm [21] and the periplasm of *T. thermophilus* HB27, although the 62 kDa-esterase was the predominant in the latter location [22]. Growth and esterase secretion was improved by modifying culture conditions such as temperature, mineral composition of the medium or reactor configuration [21,23–25]. However, purification was hampered by difficulties due to the low abundance of these enzymes and their tendency to aggregate [20].

Previous studies [26] and our own results [27] indicated that *T. thermophilus* produce additional cell-bound lipolytic activity as major fraction. Interestingly, the same 34 and 62 kDa esterases were associated to this fraction.

Taking advantage of that, in this paper we report on the analytical-scale purification of the 34-kDa protein (E34Tt) and characterisation for its biochemical and catalytic properties, including structural modelling. Wild-type E34Tt was remarkable for its elevated thermophilic and alkalophilic character. Tryptic digestion coupled to peptide mass fingerprinting of the enzyme was carried out, revealing identity with the putative esterase YP_004875 from *T. thermophilus* HB27.

2. Materials and methods

2.1. Materials

T. thermophilus HB27 was kindly supplied by Dr. Berenguer from the Universidad Autónoma de Madrid (Spain). *p*-Nitrophenyl esters, sodium cholate, Triton X-100, Coomassie Brilliant Blue R-250, Fast Red, α-naphthyl acetate, and CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) were from Sigma (St. Louis, MO, USA). PD-10 columns and Butyl-Sepharose CL-4B gel were from GE Healthcare (Sweden). Molecular weight markers for electrophoresis were obtained from Bio-Rad (Richmond, CA, USA). All other chemicals used were of the purest grade available.

2.2. Microorganisms and medium

Both, seed culture and cultivation in a 6-L bioreactor of *T. thermophilus* HB27, were made in MT-Burgas medium [23], containing per litre of spring thermal water (As Burgas, Galicia, Spain): tryptic case 8 g, yeast extract 4 g and NaCl 3 g. The final pH was adjusted to 7.5. The microorganisms were grown in this medium (MT-Burgas) during 24 h. Cells were harvested by centrifugation; vacuum dried and the pellets kept at −40 °C until they were used as inoculum.

2.3. Cultivation

Cultivation was carried out in a 6-L BIOFLO III bioreactor (New Brunswick Scientific, USA) filled up with 5 L of MT-Burgas medium under the following operation conditions: temperature 70 °C, stirring rate 70 rpm and air-flow rate of 3.5 L/min. The culture was started with a 2% (v/v) inoculum of a 24 h culture and maintained for 30 h until the culture was in the stationary phase.

2.4. Preparation of cell extracts and cell fractions

5-L of a 30 h-culture (0.5 g/L of biomass) were centrifuged at low speed (4500 × *g*, 10 min, 4 °C). In order to release the intercellular content of multicellular bodies (i.e., the periplasmic fraction) the procedure reported by Fuciños et al. [21] was followed; the recovered biomass was submitted to a freeze–thaw cycle (−40 °C/48 h), method that has been showed to be as effective as the pipetting protocol proposed by Castán et al. [16]. After thawing, the biomass was suspended in an aqueous solution of 1% (w/v) CHAPS in 50 mM sodium phosphate buffer (pH 7.0), obtaining a solution (500 mL) that constituted the crude extract.

2.5. Purification

All chromatographic steps were run on a Pharmacia FPLC Purification System at room temperature.

2.5.1. Precipitation steps

Thermal precipitation. The crude enzyme solution was subjected to thermal precipitation at 85 °C for 35 min obtaining a white rubbery low-density pellet that could not be completely removed by centrifugation at (35,000 × *g*, 30 min, 4 °C). A subsequent step of filtration through nylon membranes (0.2 μm) allowed its removal.

Ethanol/ether precipitation. Three volumes of an ice-cold mixture of ethanol and diethyl ether (1:1, v/v) were slowly added to the filtered solution under continuous stirring. The mixture was kept stirring for 30 min. The precipitate was collected by centrifugation (17,500 × *g*, 10 min, 4 °C) dissolved in sodium phosphate buffer (0.05 M, pH 7.0) and dialysed overnight at room temperature in the same buffer.

2.5.2. Chromatographic steps

Hydrophobic interaction chromatography. The chromatography was run on a Butyl-Sepharose CL-4B column (2.6 cm × 8.5 cm, volume 45 mL) equilibrated with 25 mM sodium phosphate buffer (pH 7.0). Elution was performed at a flow rate of 1.5 mL/min in two steps: (1) 1 mM sodium phosphate buffer (pH 7.0); (2) 1% (w/v) CHAPS in 1 mM sodium phosphate buffer (pH 7.0); in this case, after elution with 1 column volume, the flow was stopped and the column allowed to stand at room temperature for 2 h in order to promote weakening of the protein–ligand interactions.

Gel filtration chromatography. A HiPrep 16/60 Sephacryl S-100 HR column was equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl. The elution was performed at a flow rate of 0.5 mL/min and fractions of 1.5 mL were collected. The column was calibrated with aprotinin (6.5 kDa), cytochrome

C (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa) and blue dextran (2000 kDa).

2.6. Electrophoresis and zymogram analysis

SDS-polyacrylamide gel electrophoresis (SDS–PAGE) was performed with a 10% separating gel on a vertical slab mini gel apparatus (Model SE 250; Amersham Biosciences) at 40 mA for 2 h. The samples, diluted with electrophoresis sample buffer containing 4% (w/v) SDS, were boiled for 5 min. After migration, gels were stained for protein detection with Coomassie Blue Brilliant R-250 following standard procedures.

Detection of hydrolytic activity on the SDS–PAGE gels was carried out following procedures previously described [20]. Briefly, the gels were washed for 20 min at 65 °C in a solution of 20 mM Tris–HCl buffer (pH 8.0) containing 0.5% (w/v) Triton X-100. The esterase activity was detected with α-naphthyl acetate at 65 °C. After the reaction, the gels were briefly subjected to a cycle of staining/destaining with Coomassie Blue Brilliant R-250 in order to allow molecular weight determination. Broad range SDS–PAGE molecular weight standards from Bio-Rad were used. Proteins included were: myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lisozyme (14.4 kDa), and aprotinin (6.5 kDa).

2.7. N-terminal sequencing of the purified enzyme

The purified lipase was subjected to SDS–PAGE gel, blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) and stained with Coomassie Blue Brilliant R-250. The band of 34 kDa was excised from the membrane and used for N-terminal sequencing by the Servei de Proteòmica i Bioinformàtica (SePBio) of the Universitat Autònoma de Barcelona (Barcelona, Spain).

2.8. Peptide-mass fingerprinting of the purified enzyme

The 34-kDa protein isolated by SDS–PAGE was digested with trypsin and subjected to MALDI-TOF-MS analysis in the Centro de Genómica y Proteómica of the Universidad Complutense de Madrid (Madrid, Spain). For Peptide Mass Fingerprint analysis, the MASCOT search engine was used (http://www.matrixscience.com) [28].

2.9. In silico methods

Homologous sequences were identified through NCBI BLASTp searches (http://www.ncbi.nlm.nih.gov/blast/) [29]. The conserved domains were analysed with CD-Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) [30]. N-terminal sequence analysis of *T. thermophilus* HB27 enzyme was performed using the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) [31] and Phobius (http://phobius.cbr.su.se) [32]. The alignments amongst the amino-acid sequences were made using the Geneious Pro 4.5.5 software (Biomatters Ltd.). A three dimensional structure of E34Tt was modelled using the 3D-PSSM protein fold recognition server (http://www.sbg.bio.ic.ac.uk/servers/3dpssm) [33].

2.10. Analytical methods 44

Cell growth was monitored spectrophotometrically at 600 nm, and converted to dry weight from a standard curve.

Protein was measured using the detergent-compatible BCA protein assay (Pierce, Rockford, IL) in accordance with the manufacturer's instructions. Bovine serum albumin was used as standard.

The lipolytic activity was determined spectrophotometrically using 2.5 mM *p*-nitrophenyl dodecanoate as substrate [20], at pH 8, 65 °C and 10 min of reaction time. One activity unit was defined as the amount of enzyme that produced 1 μmol of *p*-nitrophenol per minute under standard assay conditions. The activities were expressed in U/mL. All measurements were carried out in triplicate.

2.11. Biochemical characterisation

The activity–pH dependence was determined at 65 °C using *p*-nitrophenyl dodecanoate as substrate. Buffers used were: 100 mM sodium citrate/citric acid (pH 3–5.5), 100 mM Tris/maleic (pH 6.1–8.0), Tris/HCl (pH 7.5–9.0), all containing 40 mM $CaCl_2$. The pH of the different buffer solutions was adjusted at 65 °C.

The activity–temperature dependence was determined in the range 40–85 °C in 100 mM Tris/HCl buffer (pH 8.5) containing 40 mM $CaCl_2$. The buffer solutions were adjusted at pH 8.5 at each assayed temperature.

Thermal inactivation was studied at 85 °C using samples of the pure enzyme solution diluted (100-fold) in 50 mM phosphate buffer (pH 7.0) and in 50 mM phosphate buffer (pH 7.0) containing 1% CHAPS. Aliquots (200 μL) were placed in PCR tubes that were closed and sealed with Teflon before introduced into a thermostatised water bath (Multi Temp II, GE Healthcare, Sweden). At regular times (0, 1, 3, 5, 7 and 10 h), aliquots were withdrawn, quickly cooled down on ice and centrifuged (14,000 × *g*, 15 s) in Mini Spin Plus (Eppendorf AG, Hamburg, Germany). The obtained supernatants were kept frozen at −40 °C until assaying the residual activity with the standard assay.

The substrate specificity was studied by following the hydrolysis of different *p*-nitrophenyl esters: decanoate (*p*NPC10), dodecanoate (*p*NPC12), tetradecanoate (*p*NPC14) and hexadecanoate (*p*NPC16), all at a final concentration of 2.5 mM in the assay. Activity was measured at 65 °C in 100 mM Tris/HCl buffer (pH 8.5) containing 40 mM $CaCl_2$.

All measurements were carried out in triplicate.

2.12. Combined effect of pH and Temperature on the activity and stability of the recombinant enzymes

A second-order rotatable design, based on five levels and two variables [34], was used to study the combined effect of pH and temperature on the activity and stability of E34Tt. The design consisted in 18 experiments with four (22) factorial points, four axial points to form a central composite design with $\alpha = \sqrt{2}$ and eight centre points for replication. The range and codification of the variables are shown in Table 3. Experimental data were fitted to the following empirical model with the percentage of lipolytic activity as dependent variable:

$$\text{Lipolytic activity}\ (\%) = b_0 + b_1\text{pH} + b_2T + b_{12}\text{pH}T + b_{11}\text{pH}^2 + b_{22}T^2$$

Signification of coefficients of each model was evaluated by Student's *t*-test ($\alpha = 0.05$). Non-significant parameters were removed, and best-fit parameters recalculated. Consistency of the model was tested by Fisher's *F*-test ($\alpha = 0.05$), using the following mean squares ratios:

	The model is acceptable if
F_1 = Model/Experimental error	$F_1 \geq F_{den}^{num}$
F_2 = Lack of fitting/Experimental error	$F_2 \leq F_{den}^{num}$

2.13. E600 inhibition

The enzyme solution was diluted in 50 mM phosphate buffer (pH 7) containing 150 mM NaCl and 4% (v/v) acetonitrile. Diethyl

Table 1
Summary of the purification of E34Tt from *T. thermophilus* HB27.

Purification step	Total units (U)[a]	Yield (%)	Specific activity (U/mg)	Purification (fold)
Crude extract	1069.2	100	0.83	1.0
Thermal treatment[b]	962.25	90	1.26	1.5
Ethanol/ether precipitation[c]	909.4	85.1	1.90	2.3
Butyl-Sepharose CL-4B				
Elution with CHAPS	621.2	58.1	84.16	100.8
Sephacryl S-100 HR				
E34Tt	386.5	36.2	316.21	378.7

[a] Lipolytic activity was determined using *p*-nitrophenyl laurate as substrate (65 °C, pH 8.0).
[b] Activity assayed after filtration.
[c] Activity assayed after dialysis.

p-nitrophenyl phosphate (E600), from a stock solution prepared in acetonitrile, was directly added up to a final concentration of 4 mM to ensure a large excess over the enzyme concentration (10,500-fold excess, in molar units). After 30 min incubation at 20 °C, aliquots were withdrawn and the remaining activity determined following the standard assay. Control experiments in which the inhibitor was omitted were performed.

3. Results and discussion

3.1. Identification and extraction of cell-bound lipolytic activity

Previous studies [26] and our own results [27] indicated that *T. thermophilus* produces cell-bound lipolytic activity as major fraction. We developed a methodology to isolate the periplasmic soluble proteins consisting on submitting the biomass from a 5 L-culture of *T. thermophilus* HB27, in the stationary phase, to a freeze–thaw cycle. The suspension was centrifuged to separate the periplasmic fraction from the cells. Then, several agents commonly used for the isolation of peripheral (Na_2CO_3, urea, NaCl, 2-propanol) and integral membrane proteins (cholic acid derivatives, Triton X-100, Tween 20, Brij 35) were screened to extract the cell-bound activity. Best results were achieved using detergents and particularly 1% (w/v) CHAPS dissolved in 25 mM sodium phosphate buffer (pH 7.5).

A significant proportion of the lipolytic activity produced was isolated from the cell-associated protein fraction. This extract represented a 45% (1069.2 U) of the total lipolytic activity (extracellular, intracellular, periplasmic and cell-bound), which justifies the use this fraction rather than the extracellular extract (25%). Cell-bound esterases from other organisms have been described such as *Streptomyces chrysomallus* [35], *Paenibacillus* sp. BP-23 [36], *Pseudomonas aeruginosa* [37], *Trichosporon* species (DSMZ 11829) [38].

Zymogram analysis of the CHAPS-solubilised extract indicated the presence of the same two lipolytic enzymes (34 and 62 kDa, respectively), detected in the extracellular extracts of the microorganism. In the periplasmic space, the 62-kDa protein was the predominant [22].

3.2. Purification of cell-bound lipolytic activity

Purification of the cell-bound esterases has been hampered by difficulties due to its low abundance and relative instability. In addition, the enzymes showed a very hydrophobic character and easily form aggregates during purification. To overcome these difficulties several treatments of the crude extract were assayed:

3.2.1. Precipitation steps

In a previous work, we showed the high thermostability of extracellular esterases from *T. thermophilus* [20]. As the zymogram analysis indicated that the cell-bound esterases were likely the same as those described in the extracellular extracts of the microorganism, we submitted 500 mL (1069.2 U) of a CHAPS-solubilised extract to a thermo-precipitation at high temperature (85 °C, 30 min) to promote the precipitation of contaminant proteins. The pellet formed was removed by centrifugation (35,000 × *g*, 30 min, 4 °C), followed by filtration through a Millipore nylon filter (0.2 μm). Activity recovery after this step was 90%. This treatment was followed by an ethanol/ether precipitation that yielded an 85.1% of the initial units (Table 1). A 2.3-fold increment in the purification factor was achieved after the two precipitation steps.

3.2.2. Hydrophobic interaction chromatography

900 activity units of the clarified sample obtained after the thermal treatment followed by the ethanol/ether precipitation were loaded onto a Butyl-Sepharose column. The lipolytic activity remained strongly bound to the column (99.7% of the initial activity), and was mostly eluted when the column was run with 1% CHAPS (Fig. 1). Nearly 80% of the loaded activity was recovered in this fraction but its low protein content impaired its quantification.

Table 1 summarises the results obtained in the purification starting from 500 mL of the cell-associated CHAPS-extract.

Active fractions from each peak were pooled and subjected to a zymogram analysis after SDS–PAGE. As it is shown in Fig. 1, the 62- and 34-kDa esterases identified in the crude extract, were separated in the fraction eluted with the low ionic strength buffer and with CHAPS, respectively.

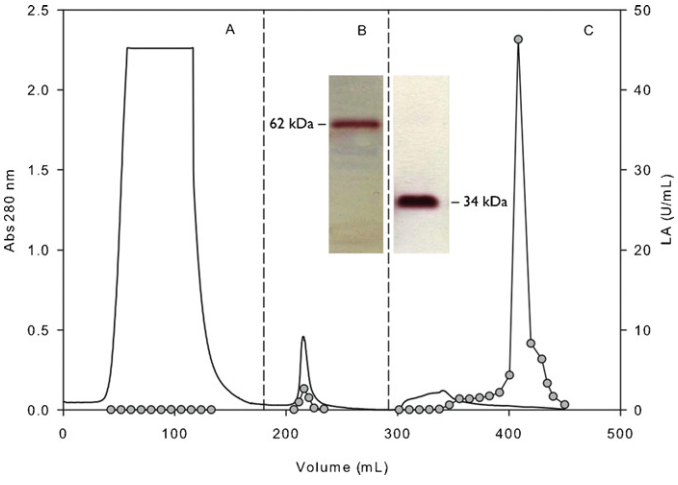

Fig. 1. Hydrophobic chromatography of membrane-solubilised extracts on a Butyl-Sepharose CL-4B column (45 mL). Sample loaded: protein extract from thermal precipitation followed by ethanol/ether precipitation (57 mL). Elution: (A) 50 mM sodium phosphate buffer (pH 7.0), (B) 1 mM sodium phosphate buffer (pH 7.0), (C) 1% (w/v) CHAPS in 1 mM sodium phosphate buffer. Flow rate: 1.5 mL/min. Fractions: 4.5 mL. Insert: zymogram analysis on SDS-polyacrylamide gels. Renaturation was done by washing for 20 min at 65 °C in 20 mM Tris–HCl buffer (pH 8.0) containing 0.5% (w/v) Triton X-100. Staining: α-naphthyl acetate followed by Coomassie Blue Brilliant R-250.

Table 2
Parametric estimations and regression coefficients of a first-order model (1) and a parallel biexponential model (2) applied to the thermal deactivation of E34Tt from *T. thermophilus* HB27, incubated at 85 °C with detergent concentrations above (1%, w/v CHAPS) and below (0.01%, w/v CHAPS) the CMC.

Incubation	Model	$t_{1/2}$ (h)	k	R	k_m (h^{-1})	k_a (h^{-1})	r^2
1% (w/v) CHAPS	(1)	2.25	0.31	–	–	–	
0.01% (w/v) CHAPS	(1)	0.45	1.51	–	–	–	0.989
	(2)	0.32	–	0.87	2.68	0.05	>0.999

3.2.3. Gel filtration chromatography

The gel filtration analysis of the concentrated CHAPS-eluted samples from the Butyl Sepharose column showed that only 27% of the loaded activity was recovered in the void volume (>1.5×10^6 Da), indicating that it was present in the form of a high molecular weight aggregates. When the pooled active fractions were mixed with CHAPS until 1% (w/v)-concentration was reached, the activity yield could be restored up to nearly 100% after 2 h incubation at room temperature. This result indicated that the aggregation involved the active site of the enzyme, and the reversible character of the phenomenon. The purification of microbial lipases is frequently a difficult process due to the presence of such aggregates, often caused by the presence of lipids used to induce the enzyme production by the microorganism [39], or simply due to hydrophobic interactions amongst the enzyme molecules [40,41].

Taking into account these observations, a new chromatography was carried out including 1%-CHAPS into the equilibration buffer. The obtained chromatographic profile is shown in Fig. 2. Nearly all the loaded activity (86%) was recovered in an elution volume corresponding to 39.5 kDa which was in agreement with that determined by denaturing electrophoresis (34 kDa) and deduced from the primary sequence (36.4 kDa), while a second protein peak was in the flow-through fraction (Table 1). The enzyme was purified 379-fold with a final specific activity of 316 U/mg.

The purity of the purified enzyme was checked by SDS–PAGE (inset in Fig. 2). Activity staining with α-naphthyl acetate and Fast Red of the SDS-polyacrylamide gels resulted in one major red band with a molecular mass of 34 kDa (lane 1). In addition, the gels showed three minor bands with high molecular masses (62, 85, and 108 kDa). These bans are probably caused by self-aggregation of the purified esterase as they mostly disappeared by increasing the concentration of the denaturant in the sample buffer (8% SDS; lane 2).

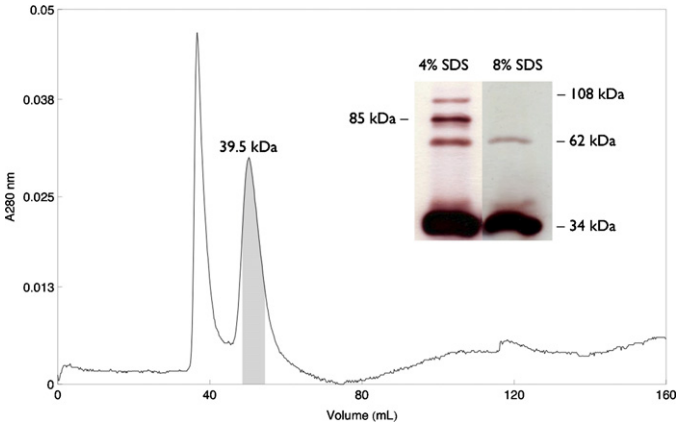

Fig. 2. Gel filtration chromatography of membrane-solubilised extracts on a Sephacryl S-100 HR column (120 mL). Sample loaded: CHAPS-eluted fraction from hydrophobic chromatography (1.2 mL). Elution: 50 mM sodium phosphate buffer (pH 7.0), containing 0.15 M NaCl and 1% (w/v) CHAPS. Flow rate: 0.5 mL/min. Fractions: 1.5 mL. Insert: zymogram analysis on SDS-polyacrylamide gels. Samples loaded with a sample buffer containing a standard SDS concentration (4%, lane1), and double SDS concentration (8%, lane 2). Renaturation was done by washing for 20 min at 65 °C in 20 mM Tris–HCl buffer (pH 8.0) containing 0.5% (w/v) Triton X-100. Staining: α-naphthyl acetate followed by Coomassie Blue Brilliant R-250.

3.3. Sequence analysis of native E34Tt

Proteins were separated by SDS–PAGE, blotted onto a polyvinylidene fluoride (PVDF) membrane and stained with Ponceau. Subsequently a piece of membrane containing a protein band at 34-kDa was excised and subjected to N-terminal sequencing. However no specific sequence was determined, suggesting the existence of a post-translational modification at the N-terminus [42]. The partially purified sample was then separated again by SDS-electrophoresis, the gel was stained with colloidal Coomassie Blue G-250, and the gel slice containing the 34-kDa protein was excised. The protein was treated with trypsin and resulting fragments were analysed by MALDI-TOF-MS. Spectra obtained were interpreted through the GPS Explorer software (Applied Biosystems, USA) with an integrated MASCOT search engine (Matrix Science, UK) that was used for protein identification.

Peptide-mass fingerprinting was carried out and data searched against NCBI databases. A total of 21 tryptic digest peptides were identified, whose masses and sequences matched to the theoretical peptides resulting from *in silico* digestion of a putative esterase from *T. thermophilus* HB27 (accession number YP_004875; gi|46199208), covering 49% of the entire sequence (329 amino acid residues).

A molecular mass of 36.445 kDa and a p*I* of 8.37 were deduced for this protein. In addition, the predicted enzyme sequence posses an N-terminal extension that has the requisites to be a transmembrane helix: highly hydrophobic and a length of 20–25 amino acid residues. Nonetheless, sequence analysis using the program SignalP 3.0 predicts a signal peptide with a probability of 1.0, the cleavage site of the signal peptide being between residues 16 (alanine) and 17 (glutamine). Phobius program, considered superior over SignalP 3.0 when it comes to prediction of transmembrane helices close to the N-terminus [31], also predicts a secretory protein.

However, we also found that once E34Tt is solubilised from membranes and purified, the detergent concentration (CHAPS) cannot be drastically decreased without loss of activity or stability. This fact strongly points to the association of this enzyme with membranes.

Another interesting aspect invoked the possibility that the membrane association of E34Tt is connected to its transport out of the cell, the membrane-bound form being only a transitory stage. In fact, we detect this enzyme by SDS-electrophoresis followed by zymogram analysis in the culture medium of the microorganism [20].

BLASTp analysis revealed similarities to other hypothetical proteins and putative esterases. The highest identities being with a putative esterase from *Thermus aquaticus* Y51MC23 (ZP_03135254, 329 aa), the hypothetical protein TTHA1268 from *T. thermophilus* HB8 (YP_144534, 214 aa), the V-type ATP synthase subunit from *T. thermophilus* HB8 (YP_144536, 142 aa), and a putative pectinacetylesterase from *Sorangium cellulosum* (YP_001611310, 365 aa). Conserved domains present in the encoded protein were also analysed using the NCBI Conserved Domain Search, which revealed a possible pectin acetylesterase family domain (PAE), despite the

putative pectinacetylesterase from *Sorangium cellulosum* presented the lower identity amongst the four high-scored sequences in the BLASTp search.

In order to further analyse the primary structural features of E34Tt, multiple sequence alignments were performed using the Geneious Pro software with the four closely related sequences (Fig. 3). The analysis revealed that the PAE domain is common to the five sequences.

The V-type ATP synthase subunit from *T. thermophilus* HB8 (YP_144536, 142 aa) showed a very high similarity (97%) with the N-terminal fragment of E34Tt (up to amino acid residue 137). Interestingly, sequence from protein TTHA1268 from *T. thermophilus* HB8 matches (94% identity) the C-terminal fragment of E34Tt (residues 133–329), starting at the end of the ATP synthase domain and goes up to the end of E34Tt protein sequence (residues 133–329); the putative esterase from the related organism *T. aquaticus* Y51MC23 showed also an identity of 85%.

Despite the fact that no homology was detected with any lipase or esterase described so far, the classical pentapeptide signature motif was identified between amino acids 157 and 161 (Gly-Cys-Ser^{159}-Ala-Gly), which contains the putative catalytic serine. To verify that E34Tt was indeed a serine hydrolase, a sample of enzyme was treated with the inhibitor E600, which binds covalently to serine residues. The experiment was conducted in molar excess of the inhibitor ([E600]/[E34Tt] = 10,500), and in the presence of 1% (w/v) CHAPS to avoid enzyme aggregation. In these conditions, a rapid and complete inhibition was observed after 30 min incubation at room temperature, which clearly indicated that a serine was involved in the catalytic mechanism.

3.4. Biochemical characterisation

3.4.1. Effect of pH and temperature on the enzyme activity

The pH and temperature dependence of E34Tt lipolytic activity were studied using *p*-nitrophenyl dodecanoate as substrate. pH–activity assays were conducted between pH 3 and 9, at 65 °C (determinations at higher pH values were hindered due to the spontaneous hydrolysis of the substrate).

Contrary to most of the known esterases, which display an optimal activity in the range 5.5–6.5, E34Tt showed a maximum at pH 8.5 (Fig. 4A), more similar to that of lipases (maximally active at 8.0–9.0) [43]. In addition, nearly 90% of the activity was still retained at pH 9. This value is lower than the optimum for the thermostable lipases from microorganisms such as *Geobacillus* sp. (pH 9.0) [44] or *Pseudomonas* sp. (pH 11.0) [45]. However, the alkalophilicity of E34Tt is similar than the reported one for the thermoalkalophilic lipases from *Bacillus thermocatenulatus* (pH 8.0–9.5) [41] and *Bacillus* sp. RSJ-1 (pH 8.0–9.0) [46], and even higher than those of some archaea such *Pyrococcus horikoshii* (pH 7.0) [47].

The temperature–activity dependence was measured in the range 40–80 °C. Results obtained (Fig. 4B) indicated an optimal temperature equal or above 80 °C (higher temperatures could not be assayed due to the elevated substrate self-hydrolysis).

The optimal catalytic temperature for E34Tt is at least 10 °C higher than the optimal temperature for the growth of *T. thermophilus* HB27 (70 °C), as previously reported by [48], and it is also higher than the optimal temperature of some other esterases from thermophilic microorganisms like *Bacillus licheniformis* (45 °C) [49], *Bacillus circulans* (60 °C) [50] or *Thermoanaerobacter tengcongensis* (70 °C) [51]. Moreover, this optimum is similar to that reported for esterases produced by hyperthermophilic organisms as *Pyrobaculum calidifontis* VA1 (90 °C) [52].

3.4.2. Thermostability

Thermal stability was assayed at 85 °C in presence of 1% (w/v) CHAPS (above the CMC [53]). In these conditions, E34Tt was extremely stable, maintaining residual activity after 10 h of incubation (Fig. 5). In order to describe the deactivation kinetic, experimental data were adjusted to a first-order model:

$$LA(t) = LA_0\, e^{-kt} \quad (1)$$

where $LA(t)$ represents the lipolytic activity at time t expressed as percentage of the initial lipolytic activity (LA_0), and k is the deactivation constant.

Deactivation in presence of 1% (w/v) CHAPS showed a good fit to the first-order model ($r^2 = 0.992$; Table 2), which predicted a half-life of 135 min. This makes E34Tt even more thermostable than some esterases from hyperthermophiles such as *Pyrococcus furiosus* ($t_{1/2}$ of 120 min at 75 °C) [54], *Thermotoga maritima* ($t_{1/2}$ of 30 min at 80 °C) [55] or *Thermococcus litoralis* ($t_{1/2}$ of 60 min at 70 °C) [56]. In addition, no changes were observed in the deactivation kinetics at 85 °C when incubations were performed in presence of 1 mM EDTA (data not shown). Thus, Ca^{2+} or equivalent ions are not likely to play a role in the thermostability of E34Tt.

To check the necessity of a micellar system to maintain the stability at high temperatures, thermal deactivation was also studied at 0.01% (w/v) CHAPS concentration (below the CMC [53]). In absence of micelles, the enzyme underwent a 70% activity loss instantly after dilution in an aqueous buffer when it was prepared from a concentrated sample containing 1% (w/v) CHAPS (17.5 U/mL *vs.* 73.5 U/mL). In other words, to maintain an active (native) conformation, the enzyme needs a micro-environment whose structural arrangement resembles the membrane bilayer. Moreover, in this case, deactivation did not follow a first-order kinetic, especially at long incubation times, in which the model (1) underestimated the residual activity.

This behaviour could be explained by the presence of aggregation forms with different activity and thermal stability, i.e., native forms (less stable in these conditions) and aggregated forms (more stable, although less active due to the inaccessibility of the substrate and/or to the scarcity of enzymes in native state being part of the aggregate). Thus, the activity measured at each time would be the sum of the residual activity of monomers and aggregates. A mathematical approach to this phenomenon can be formulated by using a parallel biexponential model based on that proposed by Aymard and Belarbi [57] for the deactivation kinetics of enzymes mixtures:

$$LA(t) = LA_0 R\, e^{-k_m t} + LA_0(1-R)\, e^{-k_a t} \quad (2)$$

where R is the *monomeric*/*total* E34Tt ratio, and k_m and k_a the deactivation constants for the monomeric and aggregated forms, respectively.

As Table 2 and Fig. 5 show, the biexponential model provided a good fitting ($r^2 > 0.999$), and the predicted half-life for E34Tt incubated with 0.01% (w/v) CHAPS was 0.32 h. Results also suggest that the aggregated form might have a thermal stability 50-fold higher than the monomer.

3.4.3. Combined effect of pH and temperature on the lipolytic activity and stability

Enzyme catalytic properties are commonly evaluated using one-factor-at-a-time experiments. This methodology is easy to understand and useful for comparative purposes (most of the reported works followed this approach). However, one-factor-at-a-time experiments ignore interactions and may lead to misleading conclusions. In this regard, experimental design methodologies [34] are more efficient than one-factor-at-a-time. Design of Experiments (DOE) uses statistical and mathematical techniques to evaluate the combined effect of factors instead of single factors at different times. These approaches have been successfully applied in the production [44,58], purification [59], characterisation [60], and application [61,62] of lipases and esterases.

```
                  1        10        20        30        40        50        60
                  |        |         |         |         |         |         |
   YP_004875      ------------------------MKRLIALVLLFALALAQGLEAFWKAVEVPG-GVCA 34
 ZP_03135254      ------------------------MKRLIALVVALALALAQGLEAFWKAVEVPG-GVCS 34
   YP_144534      ------------------------------------------------------------ 0
   YP_144536      ------------------------MKRLIALVLLFALALAQGLEAFWKAVEVPG-GVCA 34
YP_001611310      MRFKIVPPLPVQLLLCPLAVLGCGGTSAEHEDPSSEPRGGILSLPSGWSSIEPGGETTCS 60

   YP_004875      DGSPYRFYVSPGDPRKVVVDFQGGGACWDQATCGPESRTYRKRVDVQELYLAQGIYNRMS 94
 ZP_03135254      DGSPYRFYVSPGDPKKVVLDFQGGGACWDAATCGPQSQTYRKRVDVQELLLAQGIYNRMS 94
   YP_144534      ------------------------------------------------------------ 0
   YP_144536      DGSPYRFYVSPGDPRKVVIDFQGGGACWDQATCGPESRTYRKRVDVQELYLAQGIYNRMS 94
YP_001611310      RGDPFKYFVRPGTVNRLIVEFRGGGACWDATTCSFAGALFQETVG--EDALTTGIYDHEN 118

   YP_004875      VANPFFGWTHVFVPYCTGDLHVGRATVDYGGFK---VH-HQGARNAQAALEYVFRNHTNP 150
 ZP_03135254      VANPFFGWTHVFVPYCTGDLHVGRATVDYGGFK---VH-HQGARNAQAALEYVFRNHAQA 150
   YP_144534      ----------------------MCPPIKHGPLH---INPYTTARNAQAALEYVFRNHTNP 35
   YP_144536      VANPFFGWTHVFVPYCTGDLHVGRATVDYGGFK---VH-HQGARNGRVK-SYV------- 142
YP_001611310      PNNPFKDWHHVYIPYCTGDVHWGDNVATYGEGSQAVTIQHKGAVNVRAALGWIYENVPAP 178

   YP_004875      ERVFVTGCSAGAYGAVLWADKILATYKNAQIAVCGDAGVGVVTEDF--PGFTAWNPR--L 206
 ZP_03135254      ERVFVTGCSAGAYGAIFWADKVLATYKNAQVAVCGDAGVGVATPDF--PGYARWNPR--F 206
   YP_144534      ERVFVTGCSAGAYGAVLWADKILATYKNAQIAVCGDAGVGVVTEDF--PGFTAWNPR--L 91
   YP_144536      ------------------------------------------------------------ 142
YP_001611310      EKIFVTGCSAGAYGAILWSAHLREHYKSASVIEFADSGAGIITETFFRDSLPSWKPEGAY 238

   YP_004875      PE-LPGL--SSPPKVSEIYRALAQAYPKAVLAQYTTLLDGTQIYFYALMKKEAAPSEATA 263
 ZP_03135254      PE-LPGL--SARPSVSEIYLALSRAYPKAVLAQYTTLLDGTQIFFYGLMKGERTPSEATA 263
   YP_144534      PE-LPGL--SSPPKVSEIYRALAQAYPKAVLAQYTTLLDGTQIYFYALMKKEAAPSEATA 148
   YP_144536      ------------------------------------------------------------ 142
YP_001611310      PTWIPGLDPAELARLSVLYEKIGAHYPDMRLSQYNTAYDEDQIFFFNVMGGGGAQAWSDA 298

   YP_004875      REWAVAAERAVGFPASEANYTYYLAPGSQHCILPRPELYTLKVGEVSFLDWLRALAEGRT 323
 ZP_03135254      REWATEAQKAVLTPAQAENYTFYLAPGSQHCILPRPELYTLKVGEASFLDWLKALAEGKV 323
   YP_144534      REWAVAAERAVGFPASEANYTYYLAPGSQHCILPRPELYTLKVGEVSFLDWLRALAEGRT 208
   YP_144536      ------------------------------------------------------------ 142
YP_001611310      MRAEIAE-----IEASTPNFRSFLAAGTAHCILWRPEFYTLESRGARLVQWLDGLANGAP 353

   YP_004875      PPRVRP------ 329
 ZP_03135254      APRVRP------ 329
   YP_144534      PPRVRP------ 214
   YP_144536      ------------ 142
YP_001611310      PPSVACDACGSP 365
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Fig. 3. Alignment (Geneious Pro) between E34Tt predicted sequence and high-scored sequences obtained from BLASTp search. Sequences are from *T. thermophilus* HB27 putative esterase (YP_004875), *T. aquaticus* Y51MC23 putative esterase (ZP_03135254), *T. thermophilus* HB8 hypothetical protein (YP_144534), *T. thermophilus* HB8 V-type ATP synthase subunit (YP_144536), and *Sorangium cellulosum* putative pectinacetylesterase (YP_001611310). Blue-shaded characters indicate the hypothetical signal peptide. Amino acids correspondent to the PAE Conserved domain are green-shaded. Putative catalytic serine is labelled with an arrow. Bullet points indicate amino acids possibly involved in the catalytic triad. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

In this work, a central composite rotatable design for two variables at five levels [34] was used to study the combined effect of pH and temperature (T) on the lipolytic activity of E34Tt. The experimental domain and codification criteria are shown in Table 3. Considering the results obtained in the preliminary tests, temperature and pH were maintained below 70 °C and pH 8.5, respectively. Higher temperatures and pH values were not included in this study to avoid excessive substrate self-hydrolysis. The empirical model

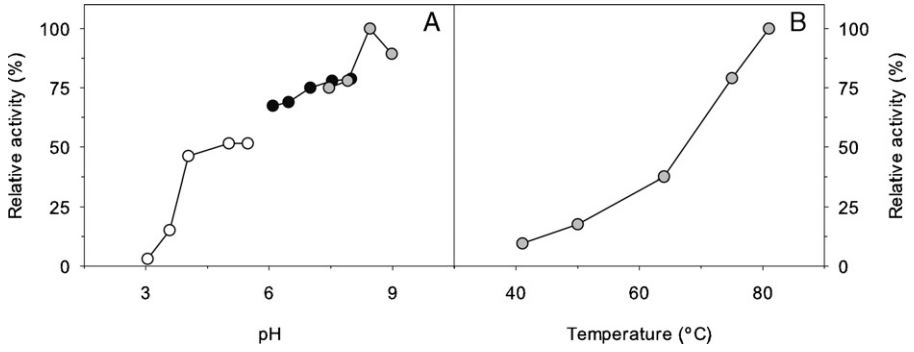

Fig. 4. Effect of pH (A) and temperature (B) on the activity of E34Tt from *T. thermophilus* HB27. Temperature in (A) was 65 °C. Buffers: (A) sodium citrate/citric acid (pH 3–5.5), Tris/maleic acid (pH 6.1–8), Tris/HCl 7.5–9; (B) Tris/HCl (pH 8.5, adjusted at each assayed temperature), all 0.1 M containing 40 mM $CaCl_2$. [Ethanol] = 10% (v/v). [*p*-Nitophenyl dodecanoate] = 2.5 mM.

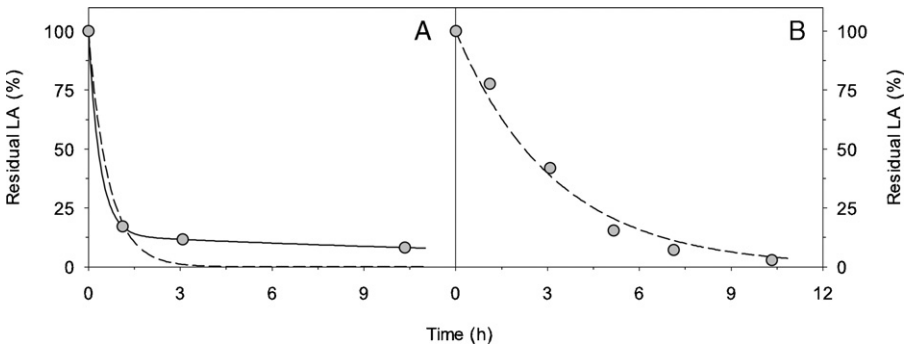

Fig. 5. Thermal deactivation kinetics of E34Tt from *T. thermophilus* HB27 incubated at 85 °C in: 50 mM sodium phosphate buffer (pH 7.0) containing (A) 0.01% (w/v) CHAPS, and (B) 1% (w/v) CHAPS. Lipolitic activity (*LA*) is expressed as percentages of the initial values, 17.5 U/mL and 73.5 U/mL in presence of 0.01% and 1% (w/v) CHAPS, respectively. Dashed and continuous lines represent, respectively, the fittings to a first-order model (1) and to a parallel biexponential model (2).

obtained for lipolytic activity (*LA*) as function of pH and temperature was:

$$LA = 14.97 + 3.43\text{pH} + 45.47T + 4.27\text{pH}T - 8.34\text{pH}^2 + 32.84T^2 \quad (3)$$

For comparative purposes, Eq. (3) was normalised by assigning 100% activity to the highest value predicted into the experimental domain (147.7 U/mL at pH 8 and 70 °C):

$$LA = 10.1 + 2.3\text{pH} + 30.8T + 2.9\text{pH}T - 5.7\text{pH}^2 + 22.2T^2 \quad (4)$$

The response surface obtained is plotted in Fig. 6A. Table 4 shows the statistical analysis of the rotatable second-order design. According to analysis of variance, the model is significant ($\alpha = 0.05$) and the adjusted r^2 value (0.798) indicates a satisfactory correlation with the experimental data. The statistical analysis indicated that all the parameters in Eq. (3) were significant. In addition, parametric analysis shows that temperature exerts greater influence than pH. An absolute maximum response could not be obtained within the experimental domain. However, lipolytic activity increased markedly with temperature. The enzyme had a very clear temperature threshold, having practically no activity below 50 °C. Above this temperature the activity increased exponentially, which indicates that the optimum of temperature for E34Tt was out of the experimental range assayed. The positive coefficient on the linear pH term and the negative sign on the quadratic term "pH^2" produced an inverse U-shaped surface with respect to the pH axis. These results suggest the existence of an optimal pH along the line from pH 7.1 – 29 °C to pH 8 – 70 °C, which is consistent with our preliminary observations.

The combined effect of pH and temperature on the stability of E34Tt was also analysed using a rotatable second-order design. The enzyme (10 μg) was incubated with detergent (1%, w/v CHAPS) under the conditions of pH and temperature showed in Table 3. After 120 min, samples were withdrawn and the residual lipolytic activity (*RLA*) was analysed following the standard method. As expected from the results of the thermal inactivation kinetics at 85 °C, E34Tt retained an elevated residual activity in all the conditions tested. Table 3 shows the results of residual activity as a percentage of the initial value (67.5 U/mL). From these data the following empirical equation was obtained:

$$RLA\ (\%) = 202.89 - 49.38\text{pH}^2 - 48.22T^2 \quad (5)$$

The best-fit model was validated by analysis of variance (Table 4). According to the F_{values}, the model was statistically significant ($\alpha = 0.05$), the lack of fit was not significant, and the adjusted r^2 was very high (0.922). Therefore, from a statistical standpoint, the model can be considered a good predictor for the experimental data. In this case, only the independent term and pure quadratic terms were significant. Both pH^2 and T^2 had negative coefficients, which indicated the existence of an absolute maximum. Optimal

Table 3
Experimental domain, codification of independent variables in the rotatable factorial designs of activity and stability, and experimental values of lipolytic activity for E34Tt.

Coded values		Natural values				Responses	
pH	*T*	Activity–pH	Activity–*T* (°C)	Stability–pH	Stability–*T* (°C)	Activity (U/L)	Residual activity (%)
1	1	8.5	64.0	8.5	79.0	62,935.7	120.6
1	−1	8.5	35.0	8.5	51.0	1464.8	102.0
−1	1	6.0	64.0	6.0	79.0	45,127.8	107.2
−1	−1	6.0	35.0	6.0	51.0	737.0	94.9
√2	0	9.0	49.5	9.0	65.0	13,337.2	102.9
−√2	0	5.5	49.5	5.5	65.0	7049.3	103.5
0	√2	7.3	70.0	7.3	84.8	18,3742.4	113.9
0	−√2	7.3	29.0	7.3	45.2	1363.8	97.2
0	0	7.3	49.5	7.3	65.0	14,885.3	228.2
0	0	7.3	49.5	7.3	65.0	13,566.2	201.1
0	0	7.3	49.5	7.3	65.0	15,139.4	205.4
0	0	7.3	49.5	7.3	65.0	14,286.2	185.3
0	0	7.3	49.5	7.3	65.0	14,401.2	205.4
0	0	7.3	49.5	7.3	65.0	15,266.5	184.8
0	0	7.3	49.5	7.3	65.0	16,210.4	228.2
0	0	7.3	49.5	7.3	65.0	16,010.7	184.8

Codification: $V_c = (V_n - V_0)/\Delta V_n$; decodification: $V_n = V_0 + (\Delta V_n \times V_c)$. V_n = natural value in the centre of the domain; ΔV_n = increment of V_n per unit of V_c. Shaded area: values corresponding to the first order design.

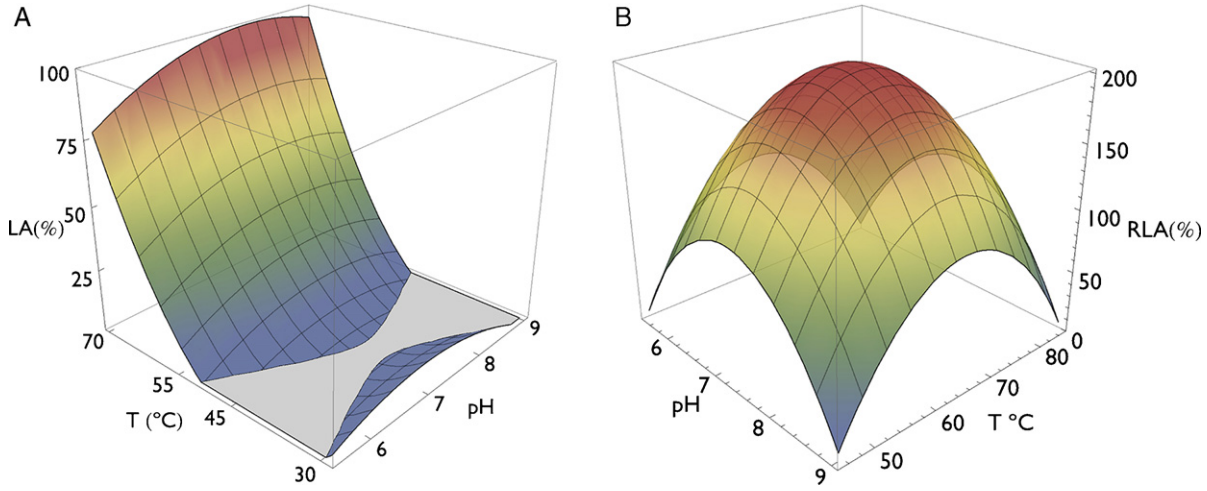

Fig. 6. (A) Response surface corresponding to the combined effect of pH and temperature on the lipolytic activity of E34Tt according to Eq. (4). Lipolytic activity was expressed as a percentage of the maximum value (147.7 U/mL). (B) Combined effect of pH and temperature on the stability of E34Tt according to Eq. (5). After 120 min incubation, lipolytic activity was measured with *p*-nitrophenyl dodecanoate at pH 8.5 and 65 °C. Residual activity was expressed as a percentage of the initial value before incubation (67.5 U/mL).

Table 4
Analysis of variance (ANOVA) for the response surface models.

Source of variation	DF[a]	SS[b]	MS[c]	r^2	r^2 adjusted	Mean square ratios[d]
Activity						
Model	5	25,893.3	5178.7	0.865	0.798	
Error	10	4041.0	404.1			$F_1 = \frac{\text{MSM}}{\text{MSE}_e} = 6582.2 > F_7^5 \ (\alpha = 0.05) = 3.97$
Experimental error	7	5.5	0.8			$F_2 = \frac{\text{MSLF}}{\text{MSE}_e} = 1709.7 > F_7^3 \ (\alpha = 0.05) = 4.35$
Lack of fit	3	4035.5	1345.2			
Stability						
Model	2	38,111.4	19,055.7	0.933	0.922	
Error	13	2751.6	211.7			$F_1 = \frac{\text{MSM}}{\text{MSE}_e} = 59.14 > F_7^2 \ (\alpha = 0.05) = 4.74$
Experimental error	7	2255.7	322.2			$F_2 = \frac{\text{MSLF}}{\text{MSE}_e} = 0.26 < F_7^6 \ (\alpha = 0.05) = 3.87$
Lack of fit	6	495.9	82.7			

[a] Degrees of freedom.
[b] Sum of squares.
[c] Mean squares.
[d] MSM, mean squares of the model; MSMLF, mean squares for the model lack of fit; MSE_e, mean squares for experimental error.

values of pH and T were located at pH 7.25 and 65 °C (Fig. 6B), that are very close to the optimal conditions of growth for *T. thermophilus* HB27 [25]. When compared to the activity, E34Tt stability presented a less dependence against pH and temperature. In fact, the mean residual activity within the experimental domain was higher than 150% after 2 h of incubation. This suggests that in a broad range of pH and temperature the enzyme lost no activity. Moreover, it appears to undergo an activation process. These results indicated that E34Tt could be successfully used in industrial applications that require robust biocatalysts.

3.4.4. Substrate specificity

The enzyme specificity was assayed using *p*-nitrophenyl esters with a chain length ranging from 10 to 16 carbon atoms (chain lengths shorter than C10 promoted a high substrate self-hydrolysis).

Results obtained (Fig. 7) showed a preference for medium chain length substrates. On this basis, E34Tt can be classified as esterase. The highest hydrolytic activity was obtained with *p*-nitrophenyl decanoate (C10). The activity towards *p*-nitrophenyl dodecanoate (C12) was 50% less, and longer chain lengths resulted in extremely low hydrolysis levels (C16 activity was 99% lower than C10). In addition, neither lipase nor phospholipase activity were detected when classical substrates for these activities such as glyceryl tributyrate, glyceryl tri(cis-9-octadecenoate), and egg yolk were assayed.

These results are in agreement with our previous studies about the substrate specificity of impure extracts from *Thermus* spp. [19], and are also in line with the reported substrate specificity for other

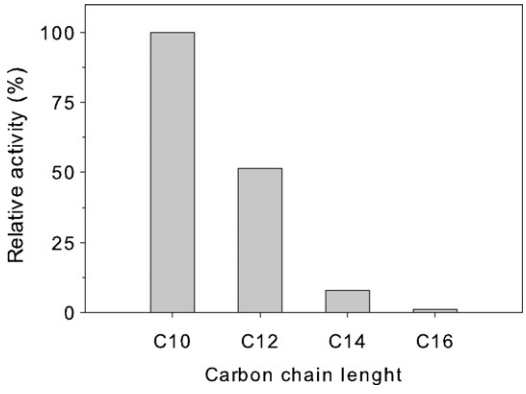

Fig. 7. Lipolytic activity of E34Tt from *T. thermophilus* HB27 towards *p*-nitrophenyl esters with different carbon chain length. Buffer: 100 mM Tris/HCl (pH 8.5) containing 40 mM $CaCl_2$. Temperature = 65 °C. [Ethanol] = 10% (v/v). [Substrate] = 2.5 mM.

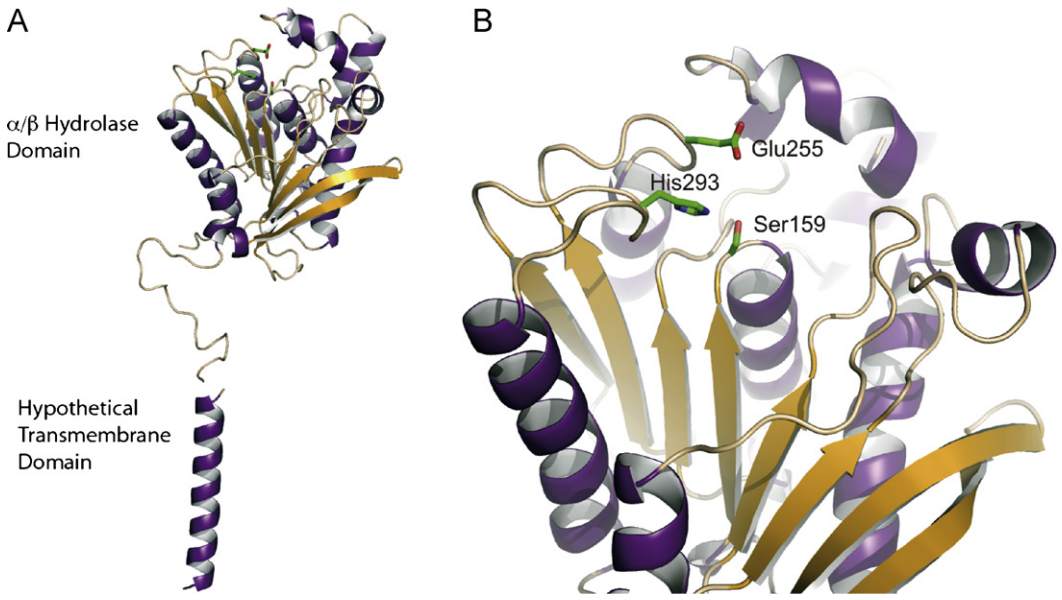

Fig. 8. 3D model of E34Tt from *T. thermophilus* HB27. (A) View of the E34Tt structure with the (α/β) hydrolase core and the hypothetical transmembrane α-helix. Secondary structure elements: α-helices, purple; β-sheets, yellow. The putative catalytic triad the active site is represented in green sticks. (B) Zoom of the active site with the putative catalytic triad labelled. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

lipolytic enzymes from thermophiles. Most of the thermophilic lipolytic enzymes characterised to date possess an optimal activity with short or medium chain-length substrates (C2–C10), e.g., EstA and EstB from *Picrophilus torridus* have shown preference for C2 esters [63], *p*-nitrophenyl decanoate (C10) was the best substrate for EST53 from *T. maritima* [55], the esterase encoded by ORF PF2001 from *P. furiosus* exhibited the maximal specificity towards 4-methylumbelliferyl-heptanoate (C7) [54], and the highest activity of the carboxylesterases from *Sulfolobus solfataricus* P1 and *Aeropyrum pernix* K1 was towards *p*-nitrophenyl octanoate (C8) [64,65].

3.5. Structural modelling

The 3D structure of E34Tt was modelled through threading methods. The search for a template using the 3D-PSSM software [33] against all protein structures deposited in the Protein Data Bank (PDB) selected the C-terminal domain (residues 431–710) of the prolyl oligopeptidase from porcine muscle (PDB entry 1QFM) as the best fit.

Prolyl oligopeptidase, involved in the hydrolysis of small peptides up to 30 amino acids [66], was previously used as template for modelling the 3D structure of other thermostable esterases, such as the *P. furiosus* esterase [54], although no protease activity was associated to the esterases neither from *P. furiosus* nor from *T. thermophilus* (measured with casein and azocasein).

Despite the low sequence identity between E34Tt and the C-terminal domain of the prolyl oligopeptidase (10%), the resulting structure has a PSSM *E*-value of 0.00112, indicating a prediction certainty higher than 95%. The final model covered almost the whole sequence of E34Tt (amino acid residues 47–325), and presents the characteristic α/β-hydrolase fold (Fig. 8A). A first secondary structural alignment indicated the residues Ser^{159}, Glu^{255} and His^{293} as the probable catalytic triad (Fig. 8B), which is usual amongst the lipase/esterase family [67,68]. These residues are conserved amongst the related sequences (Fig. 3).

This study represents the first report on the purification and characterisation of a wild-type esterase (E34Tt) from *T. thermophilus* HB27 that has no homology to any known microbial lipase or esterase. Indeed, the enzyme has a remarkable thermophilicity and an alkaline optimal pH. Experiments to overproduce E34Tt in mesophilic (*Escherichia coli*, *S. cerevisiae* [69], *K. lactis*) and thermophilic hosts (*T. thermophilus*) are currently underway. Both, wild type and recombinant enzymes are being assayed for enantioselectivity towards different substrates in order to evaluate their biotechnological applications. In addition, X-ray crystallisation studies for three-dimensional structure determination would be further developed.

Acknowledgments

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