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ORIGINAL PAPER

Characterization of esterase activity from an *Acetomicrobium hydrogeniformans* enzyme with high structural stability in extreme conditions

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Abstract

The biotechnological and industrial uses of thermostable and organic solvent-tolerant enzymes are extensive and the investigation of such enzymes from microbiota present in oil reservoirs is a promising approach. Searching sequence databases for esterases from such microbiota, we have identified in silico a potentially secreted esterase from *Acetomicrobium hydrogeniformans*, named AhEst. The recombinant enzyme was produced in *E. coli* to be used in biochemical and biophysical characterization studies. AhEst presented hydrolytic activity on short-acyl-chain *p*-nitrophenyl ester substrates. AhEst activity was high and stable in temperatures up to 75 °C. Interestingly, high salt concentration induced a significant increase of catalytic activity. AhEst still retained ~50% of its activity in 30% concentration of several organic solvents. Synchrotron radiation circular dichroism and fluorescence spectroscopies confirmed that AhEst displays high structural stability in extreme conditions of temperature, salinity, and organic solvents. The enzyme is a good emulsifier agent and is able to partially reverse the wettability of an oil-wet carbonate substrate, making it of potential interest for use in enhanced oil recovery. All the traits observed in AhEst make it an interesting candidate for many industrial applications, such as those in which a significant hydrolytic activity at high temperatures is required.

Keywords Esterase · Protein stability · Synchrotron radiation circular dichroism (SRCD) spectroscopy · Fluorescence spectroscopy · Enhanced oil recovery (EOR)

Abbreviations

AhEst	*Acetomicrobium hydrogeniformans* esterase
CD	Circular dichroism
EOR	Enhanced oil recovery
HMM	Hidden Markov models
MRW	Mean residue weight
NATA	*N*-Acetyl-tryptophanamide
*p*NA	*p*-Nitrophenyl acetate

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*p*NB *p*-Nitrophenyl butyrate
SEC Size-exclusion chromatography
SRCD Synchrotron radiation circular dichroism

Introduction

Extremophilic microorganisms are those that thrive in environments with extreme values of one or more factors such as temperature, pH, salinity, pressure, and radiation (Pikuta et al. 2007; Harrison et al. 2013; Urbieta et al. 2015). These characteristics have attracted the attention of researchers in the search for stable enzymes and other biomolecules in these extreme conditions for applications in various biotechnological/industrial processes.

Enzymes (such as cellulases, proteases, lipases, and esterases) isolated from microorganisms capable of evolving in high temperatures have been widely identified and investigated (Atomi et al. 2011; López-López et al. 2014; Elleuche et al. 2015). Many of these enzymes are used in several industrial applications mainly not only because of their high thermal stability, but also due to their broad substrate specificities, tolerance to organic solvents, and hydrolytic activity in extreme pH and/or in the presence of denaturing and/or chaotropic agents (Vieille and Zeikus 2001; Zhua et al. 2013). Several enzymes from the esterase group isolated from thermophilic microorganisms have these characteristics and, therefore, have received special attention in recent years (Levisson et al. 2009) and, more recently, also those isolated from halophiles (Schreck and Grunden 2014). Esterases (EC 3.1.1.x) are enzymes that catalyze the hydrolysis and synthesis of short-chain acyl esters (< 10 carbon atoms), do not require cofactors for their activity (Bornscheuer 2002), and have application in the chemical, food, pharmaceutical, cosmetic, and detergent industries (Panda and Gowrishankar 2005). More recently, their use has been considered in the oil industry, specifically in enhanced oil recovery (EOR) processes because they act at the oil–water–rock interfaces, which can increase the oil mobility in the reservoir and consequently the oil production. Enzymes of the lipase/esterase group, used in laboratory experiments, have shown promise in decreasing oil adhesion on rock surfaces (Nasiri et al. 2009; Khusainova et al. 2015).

In this study, a thermally stable enzyme was identified in the genome of *Acetomicrobium hydrogeniformans* which presented a striking hydrolytic activity for esters in the presence of organic solvents. *A. hydrogeniformans* is a Gram-negative, anaerobic and moderately thermophilic bacterium, which was isolated from oil production water (Maune and Tanner 2012) and initially classified as *Anaerobaculum*. *Anaerobaculum* species have recently been reclassified as *Acetomicrobium*, taking into account the available phylogenetic data (Hania et al. 2016).

The identified enzyme (named AhEst) was overexpressed in *Escherichia coli*, purified and characterized. The modulation of its enzymatic activity as a response to temperature changes and its structural properties were studied by a combination of biochemical and biophysical approaches, using enzyme activity and kinetic assays, fluorescence, and synchrotron radiation circular dichroism spectroscopies. To evaluate its potential for enhanced oil recovery (EOR) application, assays to evaluate enzyme activity in different salt concentrations, tensioactive properties and carbonate wettability alteration were also carried out.

Materials and methods

Esterase identification and DNA construct

The esterase sequence from *A. hydrogeniformans* was identified by bioinformatics analyses, performed as described in Lopes et al. (2016), using a large-scale screening of predicted proteins from 11 thermophilic bacteria genomes available at GenBank. Briefly, the search was done using the HMMER program (Eddy 2011), using Hidden Markov Models (HMM) for the esterase domain (PFAMPF00756.15). Based on the results, a synthetic gene (GenScript, New Jersey, USA) encoding the protein annotated as a dienelactone hydrolase (NCBI access number WP_009202186) was produced using the genomic sequence of *Acetomicrobium hydrogeniformans* (ACJX03000001.1). The construct excluded the region corresponding to a signal peptide at the N terminus (27 residues), which was predicted using Signal P (Petersen et al. 2011). The DNA was inserted into a pETSUMO plasmid (Novagen), allowing the production of an N-terminal fusion with a hexahistidine–SUMO protein.

Expression and purification

Escherichia coli Rosetta (DE3) cells harboring the expression vector were used to overexpress AhEst gene. The cells were grown in LB medium containing chloramphenicol (34 μg mL^{-1}) and kanamycin (50 μg mL^{-1}), then incubated at 37 °C, for 16 h, with shaking, until an absorbance of 0.6–0.8 at 600 nm was reached. Expression of AhEst was induced by 0.2 mM of IPTG. After induction, the culture was incubated at 20 °C for 16 h and then the cells were collected by centrifugation at 4 °C. The cell pellet was suspended in 40 mL of 50 mM Tris, pH 8, buffer containing 150 mM NaCl and cell lysis was done by sonication (Fisher Scientific). The lysate was centrifuged for 30 min at 15,000×*g*, at 4 °C, and the soluble fraction was applied onto a Ni–NTA Superflow (Qiagen) column, pre-equilibrated

with 50 mM Tris, pH 8.0, buffer containing 150 mM NaCl. The column was washed with 10 volumes of the same buffer, following by a second wash with 10 volumes of buffer plus 0.2% Tween-20, and then another 10 volumes of buffer containing 10 mM imidazole. The fusion protein cleavage was performed by adding 0.2 mg of Ulp-1 protease, which was maintained at 4 °C for 16 h. Elution of AhEst was done in 10 mL of 50 mM Tris, pH 8.0, buffer, containing 150 mM NaCl. For all spectroscopic studies and enzymatic assays, the protein buffer was exchanged to 10 mM sodium phosphate (pH 7.4). AhEst integrity and oligomeric state in phosphate buffer were confirmed, respectively, on SDS-PAGE 15% and size-exclusion chromatography (SEC) on a Superdex 200 10/300 GL column (GE Healthcare). The purified enzyme was lyophilized and kept at room temperature. Protein concentration was determined using the theoretical extinction coefficient of 24,410 M^{-1} cm^{-1} at 280 nm, using ProtParam tool (Gasteiger et al. 2005), available on the ExPASy server (Artimo et al. 2012).

Enzyme activity and kinetics assays

The esterase activity was determined using a colorimetric assay, measuring the amount of *p*-nitrophenol released by enzymatic hydrolysis of *p*-nitrophenyl alkanoate esters (Rhee et al. 2005; Levisson et al. 2007). The product formation was continuously monitored at 405 nm in a Cary 60 spectrophotometer (Agilent Technologies) over the first 60 s of reaction. The reaction was started with the addition of 40 µL of enzyme (1.6 µM) in 960 µL of pre-warmed sodium phosphate buffer containing the substrate. The hydrolytic activity of AhEst was assayed at 65 °C using 1 mM of the following *p*-nitrophenyl esters: acetate (C_2), butyrate (C_4), octanoate (C_8), decanoate (C_{10}), dodecanoate (C_{12}), myristate (C_{14}) and palmitate (C_{16}). Kinetic parameters were determined by the initial rate of enzymatic activity using *p*-nitrophenyl acetate (*p*NA, 7–3000 µM) and *p*-nitrophenyl butyrate (*p*NB, 7–2500 µM). The software GraphPad Prism 7 (GraphPad Software, Inc) was used to perform the analysis of the kinetic parameters.

The effects of temperature, detergent, pH, organic solvents and high salinity on AhEst activity were analyzed using 125 µM of *p*NA as substrate. The relative activity was calculated assigning the standard reaction (sodium phosphate buffer) as the maximum (100%). Reactions including the tested agents, but excluding the enzyme, were used as blanks. To find the optimum reaction temperature, the measurements were carried out from 25 to 95 °C. For thermal stability evaluation, the enzymatic activity was measured at 65 °C immediately after enzyme pre-incubation at 65, 75, 85 and 95 °C for 15 up to 180 min.

AhEst activity was also evaluated in the presence of 10, 20 and 30% of ethanol, isopropanol and acetonitrile, 1 and 2% of Tween-20, Triton X-100 and SDS. AhEst was pre-incubated for 30 min in the organic solvent solutions at room temperature. The effect of pH on the enzymatic activity of AhEst was checked over the range of pH 3.0–10.0. The buffers used were 10 mM Na_2HPO_4/citric acid (pH 3.0 and 4.0), 10 mM of sodium citrate (pH 5.5), 10 mM sodium phosphate (pH 6.0–8.0), 10 mM of 2-(cyclohexylamino) ethanesulfonic acid, CHES (pH 9.0), and 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, CAPS (pH 9.0). Additionally, esterase activity was measured at high saline concentrations to mimic oil reservoir conditions. Synthetic sea water (SSW) was used as diluent, and was composed by (in g L^{-1}) NaF (0.003), $SrCl_2{\cdot}6H_2O$ (0.020), H_3BO_3 (0.03), KBr (0.1), KCl (0.7), $CaCl_2{\cdot}2H_2O$ (1.113), Na_2SO_4 (4.0), $MgCl_2{\cdot}6H_2O$ (10.78), NaCl (23.5), $Na_2SiO_3{\cdot}9H_2O$ (0.02), Na_4EDTA (0.001), and $NaHCO_3$ (0.2), pH 8.0. Other salt concentrations were tested by adding NaCl to SSW to a final concentration of 0.6, 1.2, 1.7, 2.5 and 3 M. All esterase activity was measured at 65 °C.

Synchrotron radiation circular dichroism (SRCD) spectroscopy

The SRCD spectrum of AhEst (30 µM) in 10 mM sodium phosphate buffer (pH 7.4) was measured at the AU-CD beamline on the ASTRID2 synchrotron (ISA, Denmark) in the wavelength range from 280 to 170 nm, at 1 nm intervals, using a 0.0098 cm pathlength quartz cuvette (Hellma Scientific), at 25 °C, and collecting three individual scans of each sample. Additionally, the SRCD spectrum of a dehydrated film of AhEst (0.7 nmol) deposited on the surface of a quartz plate and kept overnight under vacuum was collected in the wavelength range from 280 to 155 nm, at 25 °C. The measurements were taken at four different rotations on the plate (0°, 90°, 180° and 270°), to assess if any linear dichroism was present. The thermal melting assays of AhEst were carried out by varying the temperature from 15 to 95 °C, in 5 °C increments, allowing 5 min equilibration at each temperature, and collecting the SRCD spectrum at each point. HT spectra (effectively, pseudo-absorbance) were measured at the same time as the CD spectra to indicate where the maximal measurable signal had been attained (Yoneda et al. 2017). After reaching 95 °C, the sample was cooled back to 15 °C, in 5 °C steps, to assess the reversibility of the changes produced by the temperature alterations. To check the effect of pH on AhEst secondary structure, the conventional CD (CD) spectra of AhEst (5 µM) were taken on a J-815 spectropolarimeter (Jasco Instruments, Japan) over the range from 270 to 190 nm, in 0.5 nm steps, as an average of five scans, using the same pH range of activity assay, after 30 min incubation, at 25 °C. Aliquots of AhEst (5.0 µM) were incubated with 10, 20 and 30% of ethanol, isopropanol or acetonitrile in phosphate buffer at 25 °C and

their respective SRCD spectra were collected. In addition, thermal melting analyses were also performed on each sample incubated with the organic solvents, by CD spectroscopy.

All CD/SRCD spectra were processed using the CDTool software (Lees et al. 2004). Processing consisted of averaging the individual scans, subtracting the respective baseline spectrum (containing the solution with all additives, except the protein), zeroing spectra in the 263–270 nm region, smoothing with a Savitzky–Golay filter, and scaled to delta epsilon ($\Delta\varepsilon$) units, using a mean residue weight (MRW) of 111. Estimations of AhEst secondary structure were performed with the DichroWeb server (Whitmore and Wallace 2008), using the SP175 database (Lees et al. 2006) and the program Continll (Sreerama and Woody 2000). To estimate the structural content of AhEst, the SRCD deconvolution was compared with the secondary structure prediction analyses performed with GOR4 software (Garnier et al. 1996), available on the ExPASy server (Artimo et al. 2012). A homology model for the three-dimensional structure of AhEst was generated with the SWISS-MODEL software (Biasini et al. 2014) using the crystal structure of a dienelactone hydrolase protein [PDB ID: 4ZI5, chain A (Colin et al. 2015)] as a template. Calculations of the structural content from the AhEst 3-D model were performed using the 2Struc server (Klose et al. 2010).

Tryptophan fluorescence spectroscopy

The emission spectrum of AhEst (2.0 µM) in 10 mM sodium phosphate buffer (pH 7.4) was measured on an ISS PC1 spectrofluorimeter using 8 nm slits for both excitation and emission and 1 cm pathlength quartz cuvettes (Hellma Inc, USA). Sample excitation was performed at 300 nm and emission spectra were recorded from 310 to 450 nm. Emission spectra were recorded at 1 nm intervals, at 25 °C, with the temperature controlled via a circulating water bath (Fisher Scientific, Pittsburgh, PA, USA). Final emission spectra were corrected for the wavelength-dependent response of the emission optics and detector.

The relative quantum yield of AhEst in phosphate buffer at 25 °C was determined on an ISS PC1 spectrofluorimeter using *N*-acetyl-tryptophanamide (NATA) (Sigma-Aldrich) as a standard ($\phi_F = 0.14$). Measurements were performed by adjusting the optical density of AhEst and NATA to <0.05 at 300 nm and using 1 cm pathlength quartz cuvettes, with 300 nm excitation with vertically polarized light, and 8 nm slits. Emission was monitored with total fluorescence intensity ($I_{/\!/}+2I_{\perp}$) collected using an excitation polarizer set parallel.

The excitation polarization spectra of AhEst in phosphate buffer were measured at 25 °C, using a 0.3 cm pathlength quartz cuvette (Hellma Inc., USA) on an ISS PC1 spectrofluorimeter (ISS Inc., Champaign, IL, USA), using calcite polarizers. Emissions at wavelengths longer than 338 nm were viewed through long-pass filters. Excitations were performed over the wavelength range from 260 to 310 nm. Fluorescence polarization (p) was determined as a function of the parallel and the perpendicular fluorescence intensities, according to the following equation:

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}.$$

Acrylamide quenching assays of AhEst Trp fluorescence were measured on phosphate buffer with the titration of acrylamide (2.5 µL aliquots of an 8.0 M stock solution) into a 2 mL cuvette containing AhEst (1.0 µM). Excitation was set at 295 nm and emission spectra were monitored from 305 to 450 nm in duplicate, through WG320 nm emission filters, at 25 °C. The Stern–Volmer quenching constants (K_{sv}) were determined from the slope of the curves using the equation below:

$$\frac{F_0}{F} = 1 + K_{sv}[Q],$$

where F_0 and F are the fluorescence intensities in the absence and in the presence of quencher (Q), respectively.

The excited-state lifetimes of AhEst were measured using frequency domain time-resolved fluorescence (Ross and Jameson 2008) on an ISS Chronos fluorometer (ISS Inc., Champaign, IL, USA) using a 300 nm LED as excitation light source, passed through a 295 nm (± 10 nm) interference filter (Semrock-Rochester, NY, USA), and *p*-terphenyl (Sigma-Aldrich) dissolved in ethanol of HPLC/spectrophotometric grade (Sigma-Aldrich) as lifetime reference (1.04 ns). The phase shifts and the relative modulations of the emitted light were monitored at 15 light modulation frequencies from 30 to 150 MHz, at 25 °C (controlled with a circulating water bath). Lifetime data of the complex decay of the Trp residues in AhEst were analyzed using discrete models (judging the goodness of fit of the measured phase and modulation data by the value of the reduced χ^2 and the phasor approach (Stefl et al. 2011; James et al. 2011). The lifetimes of Trp residues in AhEst were also measured to check its stability in the presence organic solvent tolerance of AhEst to 10, 20 and 50% of ethanol or isopropanol.

Emulsification Index (E24)

The emulsification test was done as proposed by Cooper and Goldenberg (1987), adding 2 mL of kerosene and 2 mL of AhEst protein solution in a glass tube which was sealed with a screw cap and vigorously shaken for 2 min. After 24 h incubation at room temperature, the E24 Index was calculated by dividing the height of the emulsion layer by the total height of liquid (in mm) and multiplying by 100. The enzyme solution

was prepared by dissolving the lyophilized protein in distilled water to the final concentrations of 25, 50, 100, 250 and 500 ppm. Distilled water was used as negative control.

Surface tension measurement

The direct measurement of the surface tension of the samples was carried out using an automatic tensiometer (Sigma 701 model—OneAttension) by the Du Nouy ring method, described by Bodour and Miller-Maier (1998). An aliquot of 20 mL of each AhEst solution (in the final concentration of 25, 50, 100, 250 and 500 ppm) was dispensed into a beaker, which was positioned at the base of the tensiometer. After being flame-sterilized, a platinum ring was submerged in the sample and then suspended to the liquid–air interface until its complete release from the sample. Between each measurement, the ring was rinsed with 60% hexane, dipped in acetone and flame-sterilized. The interfacial tension between AhEst solution in distilled water (100 ppm) and hexane was carried out with the same protocol described for surface tension measurement. Measurements with distilled water were taken as negative control and the chemical surfactant Tergitol 15-S-5 (Sigma-Aldrich) was used as a positive control.

Wettability alteration by flotation test

The flotation test was performed according to Wu et al. (2006). First, to simulate carbonaceous oil reservoir surface, the calcite powder ($CaCO_3$) was treated to become oil-wettable. A pre-drying of 10 g of $CaCO_3$ (in glass petri dish) was carried out at 80 °C for 16 h. After this, the temperature was then raised to 120 °C and the calcium carbonate was activated for 2 h. The powder was transferred to a glass beaker in addition to 200 mL of a 1.5% (w/w) solution of cyclohexanepentanoic acid in *n*-decane. The mixture was stirred at 200 rpm for 24 h at room temperature. After this time, the powder was decanted for a few hours and most of the supernatant was withdrawn. The carbonate was dried in the exhaust hood at room temperature for 3 days for complete removal of the *n*-decane (until reaching constant mass). The efficacy of the treatment was evaluated by adding 0.5 g of oil-wettable calcite to 10 mL of distilled water in a glass test tube. The tube was closed, sealed and shaken vigorously for 2 min, followed by standing at room temperature for 24 h. If the calcite has become more hydrophobic (oil-wet), it is expected to remain on the surface of the water. This procedure was performed with the AhEst protein in distilled water at concentrations of 25, 50 and 100 ppm. The chemical surfactant Arquad C50 (Akzo Nobel) was used as a positive control.

Results

Biophysical and biochemical characterizations of AhEst

A bioinformatics approach, based on the search of a database of proteins, from the genome of thermophilic organisms with HMM specific for esterase (PF00756) was performed using HMM tool. As a result, we found several esterases, and analyzed potential for secretion using SignalP program aiming to select secreted proteins that might present higher stability than cytoplasmic counterparts. Based on this criterion, an esterase from the genome of *Acetomicrobium hydrogeniformans*, which we named AhEst, was selected. Alignment of AhEst with the non-redundant database on NCBI resulted in the best hit with a dienelactone hydrolase from *Acetomicrobium mobile* with 64% identity. Numerous other dienelactone hydrolases also displayed a high degree of identity (~ 60% identity). Moreover, domain prediction using Pfam (Punta et al. 2012) resulted in detection of a dienelactone hydrolase family domain (PF01738), which is a more restricted family than that represented by the HMM used in our initial searches. Characterization of AhEst as a dienelactone hydrolase was further reinforced as it displayed a Gly-x-Cys-x-Gly motif in the catalytic site, which is typical of this class of enzyme, instead of the Gly-x-Ser-x-Gly commonly observed in hydrolases (Jaeger et al. 1999; Bornscheuer 2002). SDS-PAGE of overexpressed protein in *E. coli* indicated that AhEst was a soluble protein (~ 28 kDa). However, the SEC elution of AhEst gave a major peak of about ~ 53 kDa (Suppl. Material, Fig. S1a), suggesting the formation of a dimer in aqueous solution.

Bioinformatics analyses suggest that AhEst is mainly α-helical (42%), with 20% β-strand. Moreover, a homology model was constructed using a member of hydrolase superfamily, the protein dienelactone hydrolase-like promiscuous phosphotriesterase P91 from metagenomic libraries (PDB ID 4ZI5, 39% sequence identity) as a template. The homology model is comprised of α-helices (36%) and β-strands (22%) (Suppl. Material, Fig. S1b and S1c). In agreement with these findings, the CD/SRCD spectra of AhEst in aqueous solution (Fig. 1a) were typical of a mostly α-helical protein, presenting minima at 222 and 208 nm, and a large positive maximum at 192 nm (secondary structure estimations are summarized in Table 1). An additional negative peak was observed on the SRCD spectra of AhEst at 175 nm that could be attributed to the presence of β-strand conformation. These results are compatible with expected α/β hydrolase fold, as found for esterases and dienelactone hydrolases. Moreover, the SRCD spectrum of the dehydrated film of AhEst showed

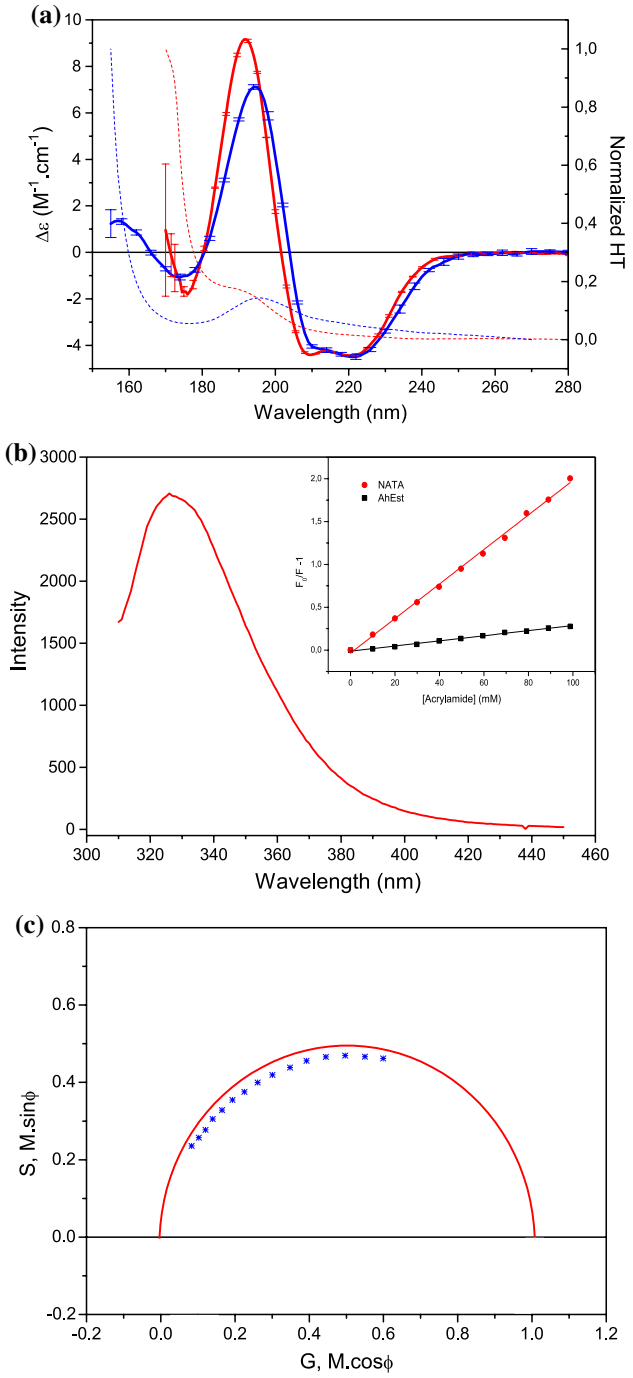

Fig. 1 AhEst secondary and tertiary structure analysis. **a** SRCD spectra (red) of AhEst in aqueous solution and on a partially dehydrated protein film (blue); error bars and HT curves (dotted lines) are shown for each measurement. **b** The corrected emission spectrum of AhEst (300 nm excitation) at 25 °C and determination of the acrylamide quenching constant (inset). **c** Phasor diagram of the excited state lifetime of AhEst in aqueous solution. Phasor points correspond to frequencies from 150 MHz (left most point) to 30 MHz (right most point)

that the enzyme was able to preserve its native globular structure after removal of bulk water, as previously shown for well-ordered globular proteins (Yoneda et al. 2017).

Table 1 Secondary structural content estimated of AhEst using bioinformatics and biophysical methods

Methods	α-Helix (%)	β-Strand (%)	Others (%)	NRMSD[a]
GOR4	42	20	38	–
Homology model	36	22	42	–
CD (25 °C)	36	19	45	0.039
SRCD (25 °C)	41	19	40	0.018

[a]The goodness of fit parameter of data to analytical fit

The enzyme AhEst contains two Trp and nine Tyr residues in its primary structure. The Trp emission spectrum of AhEst in aqueous solution at 25 °C was centered at 327 nm, typical of proteins in which the Trp residues are shielded from exposure to water molecules. In agreement with that, the value of ~ 3 M^{-1} for the Stern–Volmer constant in acrylamide quenching at this condition (this value for NATA free in aqueous solution was estimated as 20 M^{-1}) confirms the Trp residues in AhEst may be buried inside a hydrophobic core in the protein structure, not very accessible to external solvent interactions (Fig. 1b). In the native structure, the average relative fluorescence quantum yield determined for Trp residues in AhEst in aqueous solution at 25 °C was 0.22; the polarization values are ~ 0.12 in wavelengths > 290 nm, but much higher > 300 nm (Suppl. Material, Fig. S2), indicating the restricted local mobility of Trp residues in the protein native state. The ratio of the polarization upon excitation at 305 and 270 nm (305/270 ratio) is approximately 2.3, indicative of energy transfer between the tyrosine and tryptophan residues (Weber 1960). The excited state lifetimes (τ) of the Trp in AhEst were measured with time-resolved fluorescence using the frequency domain method. The intensity decay of AhEst in aqueous solution could not be well fit by a single exponential model and was fit two discrete exponentials for analysis. For AhEst at 25 °C, the decay times that fit best the curves on Fig. 1b were 4.9 and 1.5 ns, with fractional intensities of 86 and 14%, respectively. The phasor diagram for the intensity decay presented all phasor points within the universal semicircle (Fig. 1c), as expected to indicate the complex decay of the Trp residues in AhEst.

Lack of the commercial substrates for dienelactone hydrolases and our focus on esterase activity lead us to characterize the latter activity for AhEst. It has been previously described that dienelactone hydrolases display significant esterase activity (Park et al. 2010) and there is of considerable interest in enzymes with such activity. Moreover, characterization of the protein such as dienelactone hydrolase based solely on bioinformatics analysis should be seen with caution. It was previously described in cases where proteins predicted as dienelactone hydrolase actually display no such activity and instead were able to hydrolase esters with high

Table 2 Kinetic parameters of the AhEst esterase activity

Substrate	V_{max} (μM s^{-1})	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} mM^{-1})
*p*NA	7.95 ± 0.06	0.252 ± 0.008	126 ± 1	501 ± 20
*p*NB	2.59 ± 0.06	0.32 ± 0.03	41 ± 1	128 ± 13

efficiency (Park et al. 2007). The hydrolyzing activity of AhEst for various *p*-nitrophenyl esters was tested with acyl chains of different lengths (ranging from 2 to 16 carbons). AhEst exhibited preferences for substrates displaying shorter acyl chains, C_2 and C_4, and showed no significant activity with longer acyl chains (data not shown). Kinetic parameters for C_2 and C_4 acyl substrates (*p*NA and *p*NB, respectively—Table 2) demonstrated that AhEst enzyme has a preference for *p*NA.

Activity and stability in extreme conditions

The great interest in thermophilic enzymes for biotechnological applications is due to their capability to retain activities under extreme conditions. Therefore, the enzyme activity/stability of AhEst was probed under high temperature, the presence of detergents, organic solvents, and extreme pH.

High temperature

The incubation of AhEst in temperatures ranging from 25 to 55 °C did not cause changes in its secondary structure or its fluorescent properties, such as polarization (Suppl. Material, Fig. S2). The activity of AhEst increases with temperature, but above 55 °C stabilizes at an activity of approximately 150% of the observed at room temperature. The optimum temperature could not be precisely determined due to the small differences of activity in temperatures above 55 °C and the errors associated with the activity measurement (Fig. 2a). Therefore, it is possible to state that AhEst displays an optimum temperature within the range of 65–95 °C. The half-life experiments confirm that between 65 and 75 °C the enzyme was very stable and showed a small increase in activity when compared to its activity at 25 °C. At 85 and 95 °C, the enzyme showed a significant loss of activity within the first minutes of incubation (Fig. 2b). Although AhEst stability was dramatically decreased at 95 °C, it should be noted that such thermal denaturation process was highly reversible. After heating the protein to 95 °C followed by 3 h incubation at room temperature, the enzyme recovered about 90% of its activity (data not shown). The emission spectra of AhEst retained its maximum emission

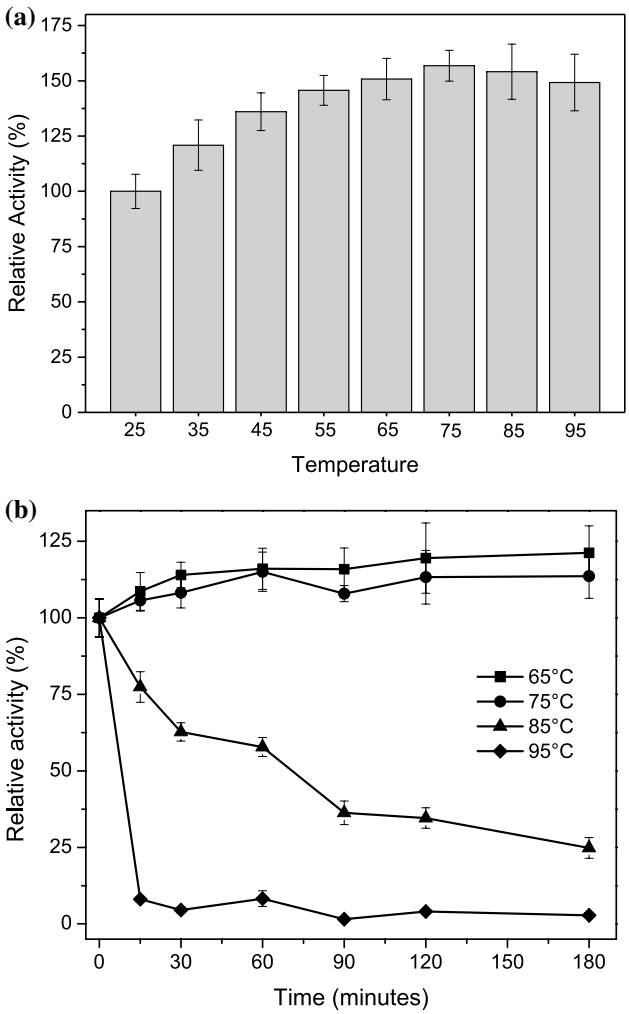

Fig. 2 Esterase activity assays. **a** Analysis of the temperature effect on AhEst activity. **b** Effect of pre-incubation at 65 (square), 75 (circle), 85 (triangle) and 95 °C (diamond) for different time lengths on the hydrolytic activity. The activity was measured at 65 °C immediately after the incubation time. Error bars represent the standard deviations in the triplicate measurements

unchanged even at high temperatures (up to 70 °C, Fig. 3a), another suggestion that the protein is quite stable in this temperature range.

The thermal melting assay revealed that AhEst was able to retain most of its secondary structure even at high temperatures, presenting only a small reduction in the intensity of the spectral bands, without peak movement (Fig. 3b). Rapid cooling of the sample back to 15 °C (after reaching 95 °C), in a single step of 10 min, allowed the peaks to reach their initial intensity magnitudes. However, a small change in the intensity ratio between 208 and 222 nm peaks was observed in the SRCD spectrum compared to native protein (Fig. 3b).

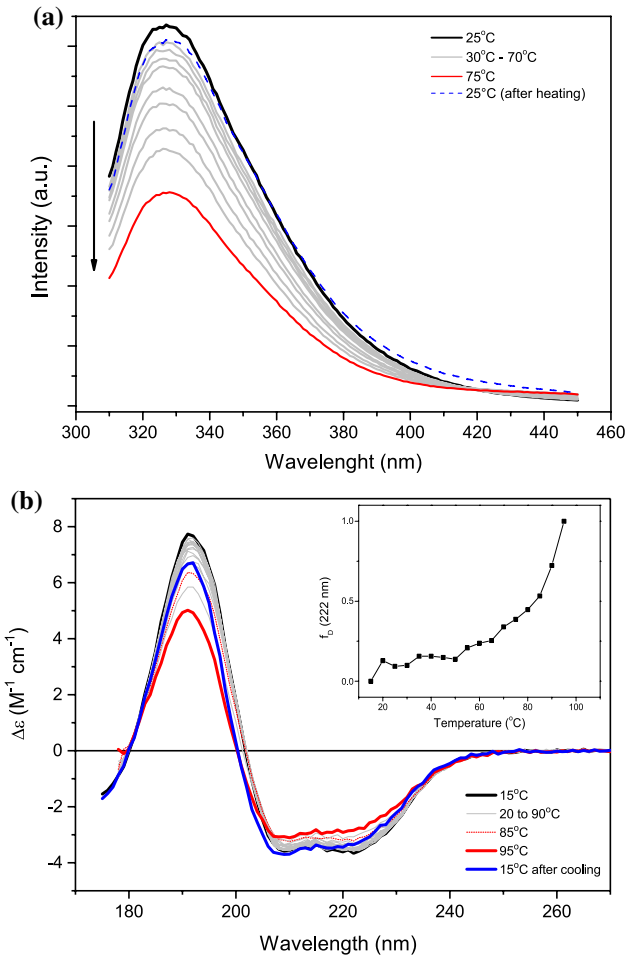

Fig. 3 AhEst thermostability. **a** Effect of temperature in the Trp emission spectrum of AhEst. **b** SRCD spectra collected upon heating AhEst from 15 (black) to 95 °C (red), in 5 °C steps (intermediate scans are in light gray), and at 15 °C after melting (blue); Inset: denaturation fraction curve is taken at 222 nm

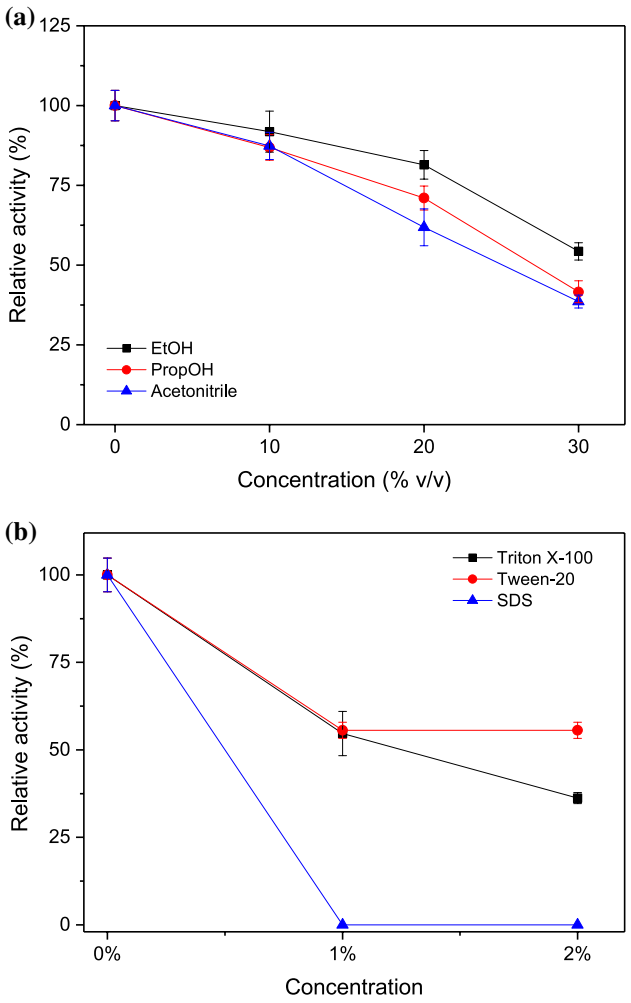

Fig. 4 Evaluation of the effect of detergents and organic solvents on the AhEst activity. **a** Relative activity in the presence of 10, 20 and 30% ethanol, isopropanol and acetonitrile. The 100% value corresponds to the enzyme activity without any organic solvent. AhEst was pre-incubated for 30 min in each condition before the activity was measured at 65 °C. **b** Relative activity in the presence of 1 and 2% of Triton X-100, Tween-20 and SDS

Effect of organic solvents and detergents

The structural stability of AhEst was evaluated in the presence of 10, 20 or 30% (v/v) of ethanol, isopropanol or acetonitrile. No significant changes were observed in the CD/SRCD spectra in those conditions compared to the native protein in solution (data not shown). Similarly, the lifetimes of the Trp residues in AhEst were not significantly affected in the presence of 10–50% of these organic solvents (Suppl. Material, Fig. S3a–b and Table S1). The conformational stability of AhEst in organic solvents suggests high tolerance of the enzyme. Activity assays in the presence of organic solvents reveal that the enzyme retained at least 90% activity when low amounts of solvent (10% v/v) were added (Fig. 4a). When 20% (v/v) of solvent was used, AhEst was still able to retain a considerable portion of its activity, ranging from 60 to 90% of relative activity (Fig. 4a). Even at 30% (v/v) of solvents, approximately 50% activity was retained.

In contrast, the AhEst activity was more sensitive to the presence of surfactants. Addition of 1 or 2% (v/v) of non-ionic surfactants, Tween 20 and Triton X-100, decreased AhEst activity by about 50% (Fig. 4b). The presence of the anionic detergent sodium dodecyl sulfate (SDS) at the same concentrations resulted in complete loss of esterase activity.

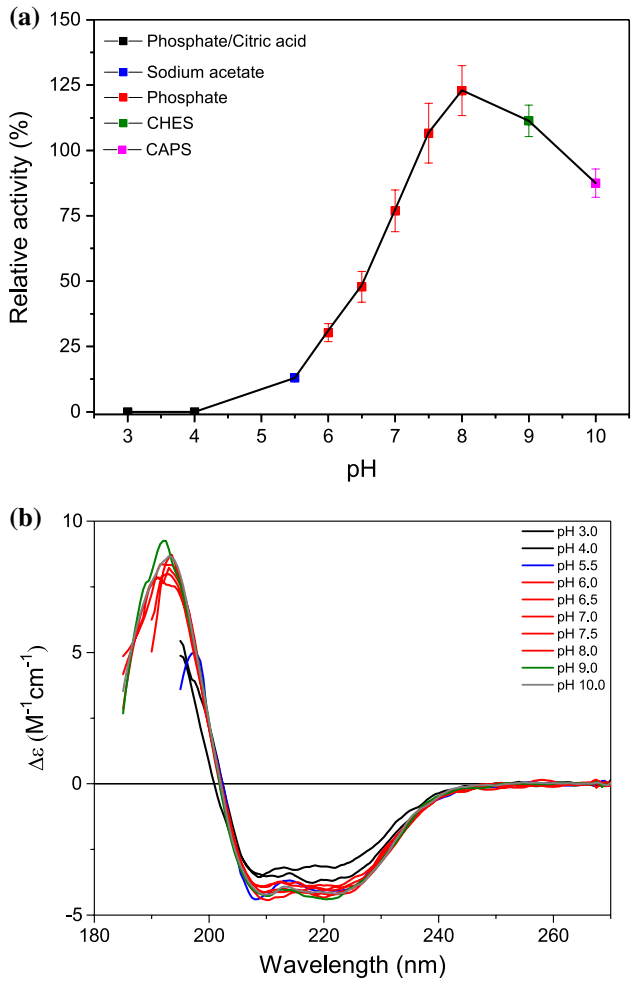

Fig. 5 Effect of the pH on AhEst activity and on its secondary structure. **a** Relative activity in different pH buffers, ranging from 3 to 10. AhEst was pre-incubated for 30 min in the indicated buffers before the activity was measured at 65 °C. **b** AhEst CD spectra after 30 min of pre-incubation in different pH buffers

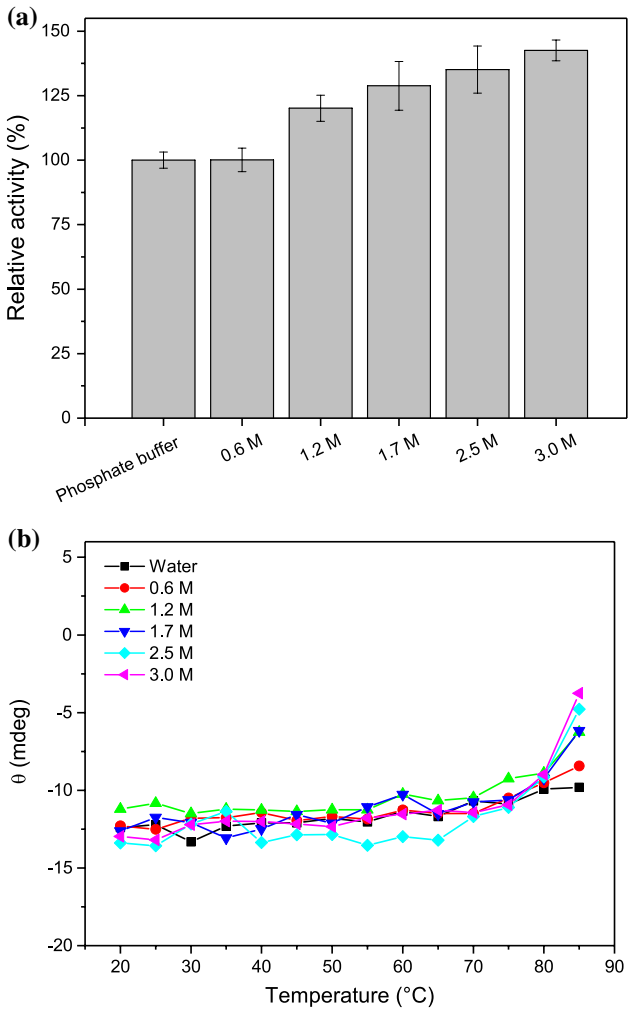

Fig. 6 Effect of high NaCl concentration on AhEst activity and structure. **a** The hydrolytic activity in increasing saline concentrations was calculated in comparison to the activity in phosphate buffer at 65 °C. **b** Ellipticity value at 222 nm on AhEst CD spectra during heating up to 85 °C in different saline concentrations

Esterase activity and pH

The activity of AhEst in a pH range of 3.0–10.0 was checked at 65 °C (Fig. 5a). The enzyme presented good activity at physiologic and alkaline pH (pH range of 7–10), with an optimal activity at pH 8.0 (Fig. 5a). On the other hand, no activity at low pH was detected (pH 3.0 and 4.0). Analyses of CD spectra revealed a decrease in the signal, together with the loss of helical content in those low pHs (Fig. 5b). This result suggests that loss of activity is associated with a partial unfolding of the protein.

AhEst enzyme for enhanced oil recovery (EOR)

Esterases and lipases have been considered in EOR processes due to their tensioactive properties, and from this perspective, some tests were carried out in this work. The AhEst activity and stability were evaluated under salinity concentrations typical of some oil reservoirs, and AhEst surfactant capacity was evaluated by the E24 Index, surface and interfacial tension activity and by the carbonate wettability alteration assay.

AhEst activity and structural stability under high-salt conditions

AhEst activity at 65 °C was evaluated in the presence of 0.6, 1.2, 1.7, 2.5 and 3 M of NaCl and in Fig. 6a it is observed that there is an increase in activity as salt is added, reaching approximately 40% increase in activity in the presence of 3 M compared to the activity in phosphate buffer. Structural stability was also evaluated at these same salt concentrations

by CD measurements. At 20 °C, the CD spectra in all salt concentrations are similar, showing AhEst has high secondary structure stability in elevated salt concentration (data not shown). Since the excess of NaCl influences the CD measurement, in the thermal analysis only the signal at 222 nm was monitored, which reflects the α-helices content of the protein (Fig. 6b). At 70 °C, the protein starts to lose secondary structure and at 85 °C, the samples differ from each other. These results show that although there is no complete thermal denaturation, conditions above 1.2 M of NaCl under heating destabilize the AhEst structure.

Tensioactive properties

AhEst presented a high E24 index for concentrations from 50 to 500 ppm, ranging from 58 to 67% (Fig. 7a). The most dense and stable emulsions were observed at concentrations of 250 and 500 ppm. The reduction of surface tension caused by AhEst was around 54 mN m^{-1} for 100, 250 and 500 ppm. For the most diluted sample (25 ppm), the surface tension almost equaled the surface tension of distilled water (Fig. 7b). Regarding interfacial tension, the AhEst solution (100 ppm) decreased it from 48 to 28 mN m^{-1}, while the positive control (Tergitol 100 ppm) reduced it to 17 mN m^{-1} (data not shown).

Carbonate wettability alteration

The qualitative test of wettability inversion of oil-wettable calcite powder by the flotation method showed that AhEst solutions at the three concentrations tested (25, 50 and 100 ppm) were able to change the wettability of a partial amount of calcite powder (Fig. 7c). The chemical surfactant Arquad C50 was used as a positive control and was able to reverse the wettability of the whole sample; seawater was used as negative control and did not lead to calcite precipitation, i.e., there was no inversion of wettability in this case.

Discussion

Esterases have been used in a diversity of industrial processes ranging from oil extraction to food and cosmetic industries. Considering the range of conditions employed in such process, the discovery of new esterases presenting singular characteristics may improve the performance of these processes. In this study, we presented the functional and structural characterization of a novel thermostable esterase identified in the genome of *A. hydrogeniformans*, named AhEst. Both sequential alignment and three-dimensional model of this enzyme showed high similarities with members of the dienelactone hydrolases family. The SRCD spectroscopy studies corroborated these findings, showing

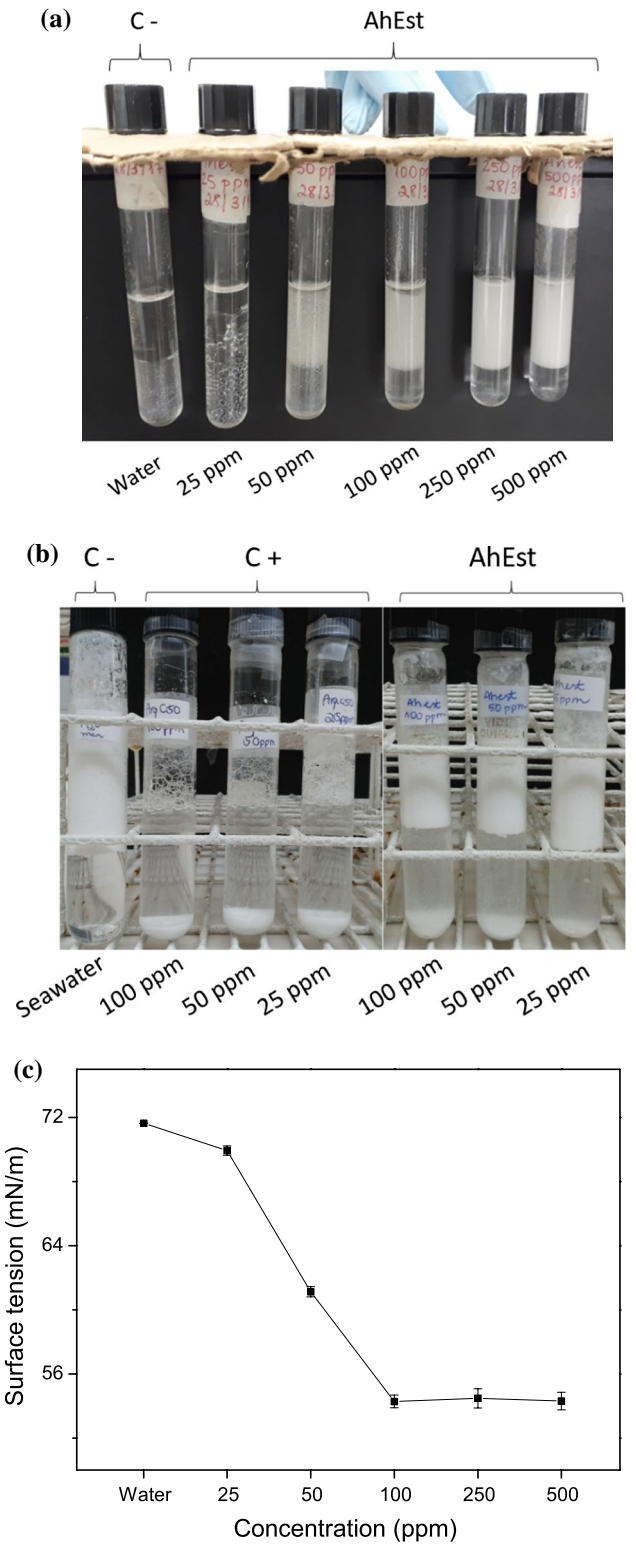

Fig. 7 AhEst E24 index, wettability and surface tension. **a** AhEst E24 index for the concentrations of 50, 100, 250 and 500 ppm (58–67%) and water as negative control (C−); **b** surface tension measurements varying AhEst concentrations. **c** AhEst flotation test, using oil-wet calcite powder, was performed with the AhEst protein diluted in distilled water at concentrations of 25, 50 and 100 ppm. The chemical surfactant Arquad C50 and sea water were used as a positive (C+) and negative controls (C−), respectively

AhEst with a typical spectrum of a mixed α-helices and β-sheet protein, corresponding with typical α/β-fold presented in both esterases and dienelactone hydrolases. In terms of hydrolytic activity, AhEst presented high catalytic activity and displayed preferences towards short-acyl-chain esters (like *p*NA). Comparison of catalytic efficiency for *p*NA of other esterases available in the BRENDA database (Schomburg et al. 2004) indicated that AhEst displays a high value, with only one of the deposited esterases from *Archaeoglobus fulgidus* presenting a higher efficiency (around three times; see Suppl. Material, Table S2). Overall, it was possible to classify AhEst as a thermophilic enzyme, displaying an optimum temperature within the range of 65–95 °C.

Moreover, AhEst showed to be structurally stable and preserved activity even under extreme conditions of temperature, pH, high salinity and the presence of chemicals and organic solvents. Despite loss of activity in few minutes, after incubation at 95 °C, AhEst recovered almost full activity after incubation at 15 °C temperature. Analysis of secondary structure by CD suggested that even at 95 °C the loss of activity is not associated with an extensive denaturation of the protein.

AhEst exhibited high tolerance to the presence of organic solvents, retaining at least 50% of its hydrolytic activity under 30% of ethanol, isopropanol or acetonitrile, which is comparable to the esterases from *Pyrobaculum calidifontis* (Hotta et al. 2002) and *Thermotoga maritima* (Levisson et al. 2012). Retention of activity of a thermostable enzyme in the presence of organic solvents, such as ethanol or isopropanol, represents potential for other industrial applications in the context of biocatalysis in organic media to be explored in future works.

Analysis of AhEst activity in the presence of surfactants suggested higher sensitivity to ionic detergents compared to non-ionic ones. Taking together that the presence of 1.2 M of NaCl under heating also destabilizes the AhEst structure, it is possible to speculate that electrostatic interactions could play an important role to maintain AhEst native conformation.

Our results also showed that AhEst is a good emulsifying agent and able to partially reverse the wettability of an oil-wet carbonate substrate. The effect of wettability alteration observed for AhEst is similar to that described for other esterases in oil recovery in displacement tests in carbonate porous medium (Nasiri 2011) or in adhesion tests on calcite substrates (Khusainova et al. 2015). It has been proposed that this effect was caused by the adsorption of the enzyme on the solid surface, replacing the oil and, therefore, interfering in the adhesion of these compounds to the surfaces, which could also be the case for AhEst. Although these results are of interest for EOR, the results from the superficial and interfacial tension assays did not show AhEst to be a good tension reducing agent. It is expected that a good biosurfactant reduces the surface tension of the water to below 30 mN m^{-1} (Das et al. 2010), but AhEst decreases to around 54 mN m^{-1}. Also, despite the decrease of the water–hexane interfacial tension (from 48 to 28 mN m^{-1}) observed for AhEst, such achievement was quite higher than what is considered suitable for EOR application ($< 10^{-2}$ mN m^{-1}) (Seethepalli et al. 2004; Sheng 2013).

Considering a possible application of this class of enzyme in processes related to the recovery of oil from reservoirs, its activity in high saline concentrations and high temperature (more similar to oil reservoirs) is a factor to be considered since in oil recovery processes brines are used as injection water (Khusainova 2016). AhEst exhibited an increased esterase activity at high salt concentrations (up to 3 M NaCl) and its structural stability at 1.2 M at high temperatures is moderate. These preliminary results are good indications of AhEst potential for use in EOR and other biotechnological processes. However, further studies of the feasibility of such use are still necessary. For example, it should be evaluated whether high salinity, which increases AhEst esterase activity, would also enhance the decrease of water–oil interfacial tension by AhEst.

Taken together, AhEst behaviour shows high plasticity concerning its activity in extreme conditions, and the enzyme reassumes an active conformation after removal of the stressful conditions. Therefore, AhEst could also be useful in industrial biotransformations that require extreme conditions involving organic solvents and high temperatures.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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