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## **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 esters 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 are required.

**Keywords:** esterase, protein stability, synchrotron radiation circular dichroism (SRCD) spectroscopy, fluorescence spectroscopy, Enhanced Oil Recovery (EOR).

## Abbreviations:

|             |   |
|-------------|---|
| AhEst       | <i>Acetomicrobium hydrogeniformans</i> esterase |
| CD          | Circular Dichroism                              |
| EOR         | Enhanced Oil Recovery                           |
| HMM         | Hidden Markov Models                            |
| MRW         | mean residual weight                            |
| NATA        | N-acetyl-tryptophanamide                        |
| <i>p</i> NA | <i>p</i> -nitrophenyl acetate                   |
| <i>p</i> NB | <i>p</i> -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 like 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 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 of 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. In order 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 dienolactone 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) and kanamycin (50 µg/mL), then incubated at 37°C, for 16 hours, while 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 hours 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 minutes at 15,000xg, 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 hours. 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 24410 M<sup>-1</sup>cm<sup>-1</sup> 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 seconds 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<sub>2</sub>), butyrate (C<sub>4</sub>), octanoate (C<sub>8</sub>), decanoate (C<sub>10</sub>), dodecanoate (C<sub>12</sub>), myristate (C<sub>14</sub>) and palmitate (C<sub>16</sub>). Kinetic parameters were determined by the initial rate of enzymatic activity using *p*-nitrophenyl acetate (*p*NA, 7 to 3000 μM) and *p*-nitrophenyl butyrate (*p*NB, 7 to 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 maximal (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 to 10.0. The buffers used were 10 mM Na<sub>2</sub>HPO<sub>4</sub>/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): NaF (0.003), SrCl<sub>2</sub>·6H<sub>2</sub>O (0.020), H<sub>3</sub>BO<sub>3</sub> (0.03), KBr (0.1), KCl (0.7), CaCl<sub>2</sub>·2 H<sub>2</sub>O (1.113), Na<sub>2</sub>SO<sub>4</sub> (4.0), MgCl<sub>2</sub>·6H<sub>2</sub>O (10.78), NaCl (23.5), Na<sub>2</sub>SiO<sub>3</sub>·9H<sub>2</sub>O (0.02), Na<sub>4</sub>EDTA (0.001), NaHCO<sub>3</sub> (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 280–170 nm, in 1 nm intervals, using a 0.0098 cm pathlength quartz cuvette (Hellma Scientific), at 25°C, and collecting 3 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 nm to 155 nm, at 25°C. The measurements were taken at four different rotations on the plate (0, 90, 180 and 270 degrees), in order 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, in order 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  $\mu\text{M}$ ) 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 5 scans, using the same pH range of activity assay, after 30 min incubation, at 25°C. Aliquots of AhEst (5.0  $\mu\text{M}$ ) 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 ( $\Delta\epsilon$ ) units, using a mean residual 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). In order 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 diene lactone 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  $\mu\text{M}$ ) 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 in 1 nm intervals, at 25°C, with the temperature controlled via a circulating water bath (Fisher Scientific, Pittsburgh, PA). 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 ( $I_{\parallel} + 2I_{\perp}$ ) 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), 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  $\mu$ L aliquots of an 8.0 M stock solution) into a 2 mL cuvette containing AhEst (1.0  $\mu$ M). 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) using a 300 nm LED as excitation light source, passed through a 295 nm (+/- 10 nm) interference filter (Semrock-Rochester, NY), 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  $\chi^2$  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 et al. (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 a negative control and the chemical surfactant Tergitol 15-S-5 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 ( $\text{CaCO}_3$ ) was treated to become oil-wettable. A pre-drying of 10 g of  $\text{CaCO}_3$  (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 minutes, 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 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 diene lactone hydrolase from *Acetomicrobium mobile* with 64% identity. Numerous other diene lactone hydrolases also displayed a high degree of identity (~60% identity). Moreover, domain prediction using Pfam (Punta et al. 2012) resulted in detection of a diene lactone 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 diene lactone 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  $\alpha$ -helical (42%), with 20%  $\beta$ -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  $\alpha$ -helices (36%) and  $\beta$ -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  $\alpha$ -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  $\beta$ -strand conformation. These results are compatible with expected an  $\alpha/\beta$  hydrolase fold, as found for esterases and dienolactone hydrolases. Moreover, the SRCD spectrum of the dehydrated film of AhEst showed 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).

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  $\sim 3 \text{ M}^{-1}$  for the Stern-Volmer constant in acrylamide quenching at this condition (this value for NATA free in aqueous solution was estimated as  $20 \text{ M}^{-1}$ ), confirming 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  $\sim 0.12$  in wavelengths  $>290 \text{ nm}$ , but much higher  $> 300 \text{ nm}$  (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 nm 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 ( $\tau$ ) 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 considerable interest in enzymes with such activity. Moreover, characterization of the protein as dienolactone hydrolase based solely on bioinformatics analysis should be seen with caution. It was previously described cases where proteins predicted as dienolactone hydrolase actually display no such activity and instead was able to hydrolase esters with high 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,  $\text{C}_2$  and  $\text{C}_4$ , and showed no significant activity with longer acyl chains (data not shown). Kinetic parameters for  $\text{C}_2$  and

C<sub>4</sub> 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 25-55°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 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 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).

##### *- 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. S3 a-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 kept 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 nonionic 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.

#### *- Esterase activity and pH*

The activity of AhEst in a pH range of 3.0 to 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  $\alpha$ -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 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 to interfacial tension, the AhEst solution (100 ppm) decreased it from

48 mN/m to 28 mN/m, while the positive control (Tergitol 100 ppm) reduced it to 17 mN/m (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. hydrogeniformes*, named AhEst. Both sequential alignment and three-dimensional model of this enzyme showed high similarities with members of the dienolactone hydrolases family. The SRCD spectroscopy studies corroborated these findings, showing AhEst with a typical spectrum of a mixed  $\alpha$ -helices and  $\beta$ -sheet protein, corresponding with typical  $\alpha/\beta$  fold presented in both esterases and dienolactone 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 of 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 emulsifier 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 (Das et al. 2010), but AhEst decreases to around 54 mN/m. Also, despite the decrease of the water-hexane interfacial tension (from 48 mN/m to 28 mN/m) observed for AhEst, such achievement was quite higher than what is considered suitable for EOR application ( $< 10^{-2}$  mN/m) (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.

### **Compliance with ethical standards**

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

## References

Artimo P, Jonnalagedda M, Arnold K, Baratin D, Csardi G, de Castro E, Duvaud S, Flegel V, Fortier A, Gasteiger E, Grosdidier A, Hernandez C, Ioannidis V, Kuznetsov D, Liechti R, Moretti S, Mostaguir K, Redaschi N, Rossier G, Xenarios I, Stockinger H (2012). ExPASy: SIB bioinformatics resource portal. *Nucleic Acids Res* 40(W1):W597-W603. doi:10.1093/nar/gks400

Atomi H, Sato T, Kanai T (2011) Application of hyperthermophiles and their enzymes. *Curr Opin Biotech* 22: 618-626. doi:10.1016/j.copbio.2011.06.010

Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, Kiefer F, Cassarino TG, Bertoni M, Bordoli L, Schwede T (2014) SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res* 42(W1):W252-W258. doi: 10.1093/nar/gku340

Bodour AA, Miller-Maier RM (1998) Application of a modified drop-collapse technique for surfactant quantitation and screening of biosurfactant-producing microorganisms. *J Microbiol Methods* 32(3):273-280. doi:10.1016/S0167-7012(98)00031-1

Bornscheuer, UT (2002) Microbial carboxyl esterases: classification, properties and application in biocatalysis. *FEMS Microbiol Rev* 26:73-81. doi:10.1111/j.1574-6976.2002.tb00599.x

Colin PY, Kintses B, Gielen F, Miton CM, Fischer G, Mohamed MF, Hyvönen M, Morgavi DP, Janssen DB, Hollfelder F (2015) Ultrahigh-throughput discovery of promiscuous enzymes by picodroplet functional metagenomics. *Nat Commun* 6:10008. doi:10.1038/ncomms10008

Cooper DG, Goldenberg BG (1987) Surface-active agents from two *Bacillus* species. *Appl Environ Microbiol* 53(2):224-229.

Das P; Mukherjee S; Sivapathasekaran C; Sen R. (2010) Microbial surfactants of marine origin: potential and prospects. In: Sen R. (ed) *Biosurfactants - Advances in Experimental Medicine and Biology*. Landes Bioscience and Springer Science+Business Media, LLC, Austin and New York, pp 88-101.

Eddy SR (2011) Accelerated profile HMM searches. *PLoS Comput Biol* 7(10):e1002195. doi:10.1371/journal.pcbi.1002195

Elleuche S, Schäfers C, Blank S, Schröder C, Antranikian G (2015) Exploration of extremophiles for high temperature biotechnological processes. *Curr Opin Microbiol* 25: 113–119. doi:10.1016/j.mib.2015.05.011



Garnier J, Gibrat, JF, Robson B (1996) GOR method for predicting protein secondary structure from amino acid sequence. *Methods Enzymol* 266:540-553. doi:10.1016/S0076-6879(96)66034-0

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A (2005) *Protein Identification and Analysis Tools on the ExPASy Server*; In: Walker JM (ed): *The Proteomics Protocols Handbook*. Humana Press, pp 571-607.

Hania WB, Bouanane-Darenfed A, Cayol JL, Ollivier B, Fardeau ML (2016) Reclassification of *Anaerobaculum mobile*, *Anaerobaculum thermoterrenum*, *Anaerobaculum hydrogeniformans* as *Acetomicrobium mobile* comb. nov., *Acetomicrobium thermoterrenum* comb. nov., and *Acetomicrobium hydrogeniformans* comb. nov, respectively, and emendation of the genus *Acetomicrobium*. *Int J Syst Evol Microbiol* 66(3):1506-1509. doi:10.1099/ijsem.0.000910

Harrison JP, Gheeraert N, Tsigelnitskiy D, Cockell CS (2013) The limits for life under multiple extremes. *Trends Microbiol* 21: 204-212. doi:10.1016/j.tim.2013.01.006

Hotta Y, Ezaki, S, Atomi H, Imanaka T (2002) Extremely stable and versatile carboxylesterase from hyperthermophilic archeon. *Appl Environ Microbiol* 68:3925-3931

Jaeger KE, Dijkstra BW, Reetz MT (1999) Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases. *Annu Rev Microbiol* 53:315–351. doi:10.1146/annurev.micro.53.1.315

James NG, Ross JA, Stefl M, Jameson DM (2011) Application of phasor plots to *in vitro* protein studies. *Anal Biochem* 410:70-76. doi:10.1016/j.ab.2010.11.011

Khusainova A, Nielsen SM, Pedersen HH, Woodley JM, Shapiro A (2015) Study of wettability of calcite surfaces using oil–brine–enzyme systems for enhanced oil recovery applications. *J Petrol Sci Eng* 27: 53-64. doi:10.1016/j.petrol.2014.12.014

Khusainova A (2016) *Enhanced Oil Recovery with Application of Enzymes*. Ph.D thesis, Technical University of Denmark (DTU), Kongens Lyngby, Denmark.

Klose DP, Wallace BA, Janes RW (2010). 2Struc: the secondary structure server. *Bioinformatics* 26(20):2624–2625. doi:10.1093/bioinformatics/btq480

Lees JG, Smith BR, Wien F, Wallace BA (2004) CDtool-an integrated software package for circular dichroism spectroscopic data processing, analysis, and archiving. *Anal Biochem* 332(2):285–289. doi:10.1016/j.ab.2004.06.002

Lees JG, Miles AJ, Wien, F, Wallace, BA (2006) A reference database for circular dichroism spectroscopy covering fold and secondary structure space. *Bioinformatics* 22(16):1955–1962. doi:10.1093/bioinformatics/btl327

Levisson M, van der Oost J, Kengen SWM (2007) Characterization and structural modeling of a new type of thermostable esterase from *Thermotoga maritima*. *FEBS J*, 274(11):2832-2842. doi:10.1111/j.1742-4658.2007.05817.x

Levisson, M, van der Oost J, Kengen SWM (2009) Carboxylic ester hydrolases from hyperthermophiles. *Extremophiles* 13(4):567-581. doi:10.1007/s00792-009-0260-4

Levisson M, Han GW, Deller MC, Xu Q, Biely P, Hendriks S, Ten Eyck LF, Flensburg C, Roversi P, Miller MD, McMullan D, von Delft F, Kreusch A, Deacon AM, van der Oost J, Lesley SA, Elsliger MA, Kengen SW, Wilson IA (2012) Functional and structural characterization of a thermostable acetyl esterase from *Thermotoga maritima*. *Proteins* 80(6):1545-1559. doi: 10.1002/prot.24041

Lopes JLS, Yoneda JS, Martins JM, DeMarco R, Jameson DM, Castro AM, Bossolan NRS, Wallace BA, Araujo, APU (2016) Environmental factors modulating the stability and enzymatic activity of the *Petrotoga mobilis* esterase (PmEst). *PLoS One* 11(6):e0158146. doi:10.1371/journal.pone.0158146

López-López O, Cerdán ME, González-Siso, MI (2014) New extremophilic lipases and esterases from metagenomics. *Curr Protein Pept Sci* 15(5):445-455. doi:10.2174/1389203715666140228153801

Maune MW, Tanner RS (2012) Description of *Anaerobaculum hydrogeniformans* sp. nov., an anaerobe that produces hydrogen from glucose, and emended description of the genus *Anaerobaculum*. *Int J Syst Evol Microbiol* 62:832-838. doi:10.1099/ijs.0.024349-0

Nasiri H, Spildo K, Skauge A (2009) Use of enzymes to improve waterflood performance. In: International Symposium of the Society of Core Analysts, Noordwijk, Netherlands.

Nasiri H (2011) Enzymes for Enhanced Oil Recovery (EOR). Ph.D thesis, University of Bergen, Norway.

Panda T, Gowrishankar BS (2005) Production and applications of esterases. *Appl Microbiol Biotechnol* 67:160–169. doi:10.1007/s00253-004-1840-y

Park SY, Kim JT, Kang, SG, Woo JH, Lee JH, Choi HT, Kim SJ (2007) A new esterase showing similarity to putative dienelactone hydrolase from a strict marine bacterium, *Vibrio* sp. GMD509. *Appl Microbiol Biotechnol* 77:107-115. doi:10.1007/s00253-007-1134-2

- Park YJ, Yoon SJ, Lee HB (2010) A novel diene lactone hydrolase from the thermoacidophilic archaeon *Sulfolobus solfataricus* P1: purification, characterization, and expression. *Biochim Biophys Acta* 1800(11):1164-1172. doi:10.1016/j.bbagen.2010.07.006
- Petersen TN, Brunak S, von Heijne G, Nielsen, H (2011) SignalP 4.0: discriminating signal peptide from transmembrane regions. *Nat Methods*, 8(10):785-786. doi: 10.1038/nmeth.1701
- Pikuta EV, Hoover RB, Tang J (2007) Microbial Extremophiles at the Limits of Life. *Crit Rev Microbiol* 33:183–209. doi: 10.1080/10408410701451948
- Punta M, Coghill PC, Eberhard RY, Mistry J, Tate J, Boursnell C, Pang N, Forslund K, Ceric G, Clements J, Heger A, Holm L, Sonnhammer EL, Eddy SR, Bateman A, Finn RD (2012) The Pfam protein families database. *Nucleic Acids Res* 40(Database issue):D290-D301. doi:10.1093/nar/gkr1065
- Rhee JK, Ahn DG, Kim YG, Oh, JW (2005) New thermophilic and thermostable esterase with sequence similarity to the hormone-sensitive lipase family, cloned from a metagenomic library. *Appl Environ Microbiol* 71(2):817-825. doi:10.1128/AEM.71.2.817-825.2005
- Ross JA, Jameson DM (2008) Frequency domain fluorometry: applications to intrinsic protein fluorescence. *Photochem Photobiol Sci* 7(11):1301-1312. doi:10.1039/b804450n
- Schomburg I, Chang A, Ebeling C, Gremse M, Heldt C, Huhn G, Schomburg D (2004) BRENDA, the enzyme database: updates and major new developments. *Nucleic Acids Res* 32(Database issue):D431-3. doi: 10.1093/nar/gkh081
- Schreck SD, Grunden AM (2014) Biotechnological applications of halophilic lipases and thioesterases. *Appl Microbiol Biotechnol* 98:1011. doi:10.1007/s00253-013-5417-5
- Seethapalli A, Adibhatla B, Mohanty KK (2004) Wettability alteration during surfactant flooding of carbonate reservoirs. SPE/DOE Symposium on Improved Oil Recovery, 17-21 April, Tulsa, Oklahoma. SPE 89423. doi: 10.2118/89423-MS
- Sheng J. J. (2013) Comparison of the effects of wettability alteration and IFT reduction on oil recovery in carbonate reservoirs. *Asia-Pac J Chem Eng* 8:154-161. doi: 10.1002/apj.1640
- Sreerama N, Woody RW (2000) Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set. *Anal Biochem* 287:252–260. doi:10.1006/abio.2000.4880

Steffl M, James NG, Ross JA, Jameson DM (2011) Application of phasor plots to *in vitro* time-resolved fluorescence measurements. *Anal Biochem* 410:62-69. doi:10.1016/j.ab.2010.11.010

Urbietta MS, Donati ER, Chan K-G, Shahar S, Sin LL, Goh KM (2015) Thermophiles in the genomic era: Biodiversity, science, and applications. *Biotechnol Adv* 33: 633-647. doi:10.1016/j.biotechadv.2015.04.007

Vieille C, Zeikus GJ (2001) Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. *Microbiol Mol Biol Rev* 65(1):1-43. doi:10.1128/MMBR.65.1.1-43.2001

Weber G (1960) Fluorescence-polarization spectrum and electronic energy transfer in proteins. *Biochem J* 75:345-352.

Whitmore L, Wallace BA (2008) Protein secondary structure analyses from circular dichroism spectroscopy: methods and reference databases. *Biopolymers* 89:392-400. doi:10.1002/bip.20853

Wu Y, Shuler PJ, Blanco M, Tang Y, Goddard W A (2006) A Study of Wetting Behavior and Surfactant EOR in Carbonates with Model Compounds. In: *SPE/DOE Symposium on Improved Oil Recovery*, Tulsa, USA. Society of Petroleum Engineers. doi:10.2118/99612-MS

Yoneda JS, Miles AJ, Araujo APU, Wallace BA (2017) Differential dehydration effects on globular proteins and intrinsically disordered proteins during film formation. *Protein Sci* 26(4):718-726. doi:10.1002/pro.3118

Zhua Y, Li J, Cai H, Ni H, Xiao A, Hou L (2013) Characterization of a new and thermostable esterase from a metagenomic library. *Microbiol Res* 168(9):589- 597. doi:10.1016/j.micres.2013.04.004

## Figure Captions

**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 to 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.

**Fig. 2** Esterase activity assays. a) Analysis of the temperature effect on AhEst activity. b) Effect of pre-incubation at 65 (■), 75(●), 85 (▲) and 95°C (◆) 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.

**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.

**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.

**Fig. 7** AhEst E24 index, wettability and surface tension. a) AhEst E24 index for the concentrations of 50, 100, 250 and 500 ppm (58% to 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;