BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Biochemical profiles of two thermostable and organic solvent–tolerant esterases derived from a compost metagenome

Mingji Lu[1] · Amélie Dukunde[1] · Rolf Daniel[1]

Abstract

Owing to the functional versatility and potential applications in industry, interest in lipolytic enzymes tolerant to organic solvents is increasing. In this study, functional screening of a compost soil metagenome resulted in identification of two lipolytic genes, *est1* and *est2*, encoding 270 and 389 amino acids, respectively. The two genes were heterologously expressed and characterized. Est1 and Est2 are thermostable enzymes with optimal enzyme activities at 80 and 70 °C, respectively. A second-order rotatable design, which allows establishing the relationship between multiple variables with the obtained responses, was used to explore the combined effects of temperature and pH on esterase stability. The response curve indicated that Est1, and particularly Est2, retained high stability within a broad range of temperature and pH values. Furthermore, the effects of organic solvents on Est1 and Est2 activities and stabilities were assessed. Notably, Est2 activity was significantly enhanced (two- to tenfold) in the presence of ethanol, methanol, isopropanol, and 1-propanol over a concentration range between 6 and 30% (*v/v*). For the short-term stability (2 h of incubation), Est2 exhibited high tolerance against 60% (*v/v*) of ethanol, methanol, isopropanol, DMSO, and acetone, while Est1 activity resisted these solvents only at lower concentrations (below 30%, *v/v*). Est2 also displayed high stability towards some water-immiscible organic solvents, such as ethyl acetate, diethyl ether, and toluene. With respect to long-term stability, Est2 retained most of its activity after 26 days of incubation in the presence of 30% (*v/v*) ethanol, methanol, isopropanol, DMSO, or acetone. All of these features indicate that Est1 and Est2 possess application potential.

Keywords Carboxylesterases · Metagenomic library · Second-order rotatable design · Thermophilic · Organic solvent tolerance

Introduction

Extreme environments exhibiting elevated temperatures, extreme pH values, and exposure to organic solvents or high salinity are used to recover novel robust bioactive molecules that can be applied under industrial conditions (Antranikian and Egorova 2007). The targeted environments such as hot springs, compost, oil fields, and deep-sea marine sediments are reservoirs for extremophilic microorganisms that could produce potentially relevant industrial enzymes (Auernik

et al. 2008). Culture-independent metagenomic approaches are alternatives to conventional culture-based screening methods. Recently, some extremozymes, such as amylases, amidases, proteases, cellulases, and esterases, have been successfully identified through metagenomic approaches (Daniel 2005; Simon and Daniel 2011; González-González et al. 2017; Jayanath et al. 2018; Martínez-Martínez et al. 2018).

Lipolytic enzymes, which catalyze the hydrolysis and synthesis of acylglycerols, are considered as one of the most important groups of biocatalysts. Lipolytic enzymes include esterases (EC 3.1.1.1, carboxylesterases) and true lipases (EC 3.1.1.3, triacylglycerol acyl hydrolases) and are widespread in bacteria, archaea, and eukaryotes (Hasan et al. 2006). Due to their broad substrate, pH, and temperature spectra combined with high regio- and enantioselectivity, lipolytic enzymes are of interest for food, paper, medical, detergent, and pharmaceutical industries (Hita et al. 2009; Romdhane et al. 2010; Ferrer et al. 2015; Sarmah et al. 2018). In particular, lipolytic enzymes that function in non-aqueous solvents have attracted considerable attention, as they offer new possibilities for

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bioprocesses, such as shifting of thermodynamic equilibrium in favor of synthesis (esterification and transesterification), controlling substrate specificity and solubility by solvent engineering, and suppressing water-dependent side reactions (Secundo and Carrea 2002; Hun et al. 2003; Ahmed et al. 2010). However, the inhibition or inactivation of enzyme activity resulting from organic solvents has restricted the use of many lipolytic enzymes (Klibanov 2001; Jin et al. 2012). To overcome this limitation, some organic solvent–tolerant (OST) lipolytic enzymes have been isolated, including enzymes from *Bacillus licheniformis* S-86 (Torres et al. 2009), *Streptomyces coelicolor* A3(2) (Brault et al. 2012), *Psychrobacter celer* 3Pb1 (Wu et al. 2013), *Alcanivorax dieselolei* B-5(T) (Zhang et al. 2014), and *Acetomicrobium hydrogeniformans* (Kumagai et al. 2018), as well as from metagenomes of seawater (Chu et al. 2008), compost (Kang et al. 2011), lipid-contaminated soil (Glogauer et al. 2011), mountain soil (Jin et al. 2012), swamp sediment (Seo et al. 2014), and deep-sea hydrothermal vents (Yang et al. 2018). However, these enzymes only show tolerance towards specific organic solvents.

Industrially required versatile lipolytic enzymes that exhibit satisfactory activity and stability in both water-miscible and water-immiscible organic solvents are rare (Doukyu and Ogino 2010). It has been shown that the thermostability of an enzyme in water is correlated to its tolerance against organic solvents (Kumar et al. 2016). Thus, it is straightforward to screen naturally evolved OST enzymes from thermostable ones (Lotti and Alberghina 2007; Ahmed et al. 2010). Most industrial processes utilizing lipolytic enzymes are carried out at higher temperatures (above 45 °C); it is required that the enzymes exhibit activity and stability optima around 50 °C (Sharma et al. 2002). Thus, thermostable lipolytic enzymes exhibiting organic solvent tolerance are of high importance with respect to industrial applications.

Composting is the process of biological, aerobic decomposition of organic waste by microorganisms (Ryckeboer et al. 2003). During the thermophilic phase of composting, heat generated by microbial succession can raise temperatures to above 50 °C (Dougherty et al. 2012). Correspondingly, compost is a potential source for recovery of thermostable enzymes. Recently, lipolytic enzymes have been isolated from compost (Lämmle et al. 2007; Tirawongsaroj et al. 2008; Kim et al. 2010; Ohlhoff et al. 2015; Woo Lee et al. 2016), but only one (EstCS2) of the isolated enzymes was moderately thermostable (optimum 55 °C) and showed resistance to certain water-miscible organic solvents (Kang et al. 2011).

In this study, two genes encoding lipolytic enzymes (*est1* and *est2*) were identified from a thermophilic compost metagenome. The corresponding enzymes were purified and characterized. Enzyme characterizations are usually conducted as one-factor-at-a-time for comparison with reported enzyme features from other studies.

However, this methodology ignores interacting effects between factors, which may result in misleading conclusions, especially when at least two requirements must be fulfilled simultaneously. An alternative is to employ design of experiments (DOE) methodologies, i.e., second-order rotatable design approach, which use statistical and mathematical approaches to evaluate the combined effect of factors. DOE has been successfully applied in different aspects related to lipolytic enzymes such as the growth condition optimization and enzyme activity or stability measurements (Kamimura et al. 2001; Shieh et al. 2003; Benaiges et al. 2010). In this study, the combined effect of pH and temperature on the stability of Est1 and Est2 was evaluated by the second-order rotatable design approach. Analysis of the recovered two metagenome-derived enzymes showed that they are thermophilic and tolerant towards organic solvents. In addition, Est2 was remarkably resistant to both water-miscible and immiscible organic solvents.

Materials and methods

Strains and plasmids

For the construction of metagenomic plasmid libraries, *Escherichia coli* TOP10 and pFLD (Invitrogen GmbH, Karlsruhe, Germany) were used as host and vector, respectively. *Escherichia coli* BL21 Star (DE3) and the pET101/D-TOPO® vector (Invitrogen GmbH) were used for heterologous expression of the recovered lipolytic genes.

Sample collection and DNA extraction

Compost samples were collected at a composting company (Göttingen GmbH, Göttingen, Germany, 51° 34′ 25.1″ N 9° 54′ 33.0″ E). The sampling pile was the fermentation product of household waste and fresh tree branches. To ensure using mainly thermophilic microorganisms as a source for metagenomic library construction, compost at the core zone of compost pile was collected. The temperature at the sampling spot was 55 °C. The compost soil sample (50 g) was collected in sterile plastic bags and stored at − 20 °C until required.

Metagenomic DNA of the compost sample was extracted following a phenol-chloroform method according to Zhou et al. (1996). In addition, DNA was also isolated with MoBio Power Soil DNA extraction kit following the protocol of the manufacturer (MoBio Laboratories, Carlsbad, CA, USA). DNA obtained from these two methods was pooled and stored at − 20 °C until required.

Metagenomic library construction and screening for lipolytic activity

To construct a metagenomic plasmid library, DNA was sheared, and fragments from 6 to 12 kb were recovered by gel extraction with the peqGold gel extraction kit (Peqlab Biotechnologie GmbH, Erlangen, Germany). End-repaired DNA fragments and *PmlI*-digested pFLD vector were ligated by employing T4 DNA ligase at 16 °C, overnight as recommended by the manufacturer (Thermo Scientific, Bremen, Germany). To screen for lipolytic activity, metagenomic library-bearing cells were plated onto LB agar plates containing 100 μg/ml ampicillin and 1% (*v/v*) emulsified tributyrin (Sigma, Germany) and subsequently incubated at 30 °C 1 to 7 days. Lipolytic-positive clones were identified by the formation of clear zones (halos) around individual colonies. The phenotype of positive clones was confirmed by the isolation of recombinant plasmids from positive strains, transformation of the isolated plasmids into the host, and rescreening on indicator agar plates.

Sequence analysis and homology modeling

Lipolytic genes (*est1* and *est2*) were initially predicted by using the ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), and verified by Clone Manager and FramePlot analysis (Ishikawa and Hotta 1999). Similarity searches of the deduced amino acid sequences were performed by BLASTP program against the public GenBank database (Ye et al. 2006). Signal peptides were detected by using the SignalP 4.0 server (Bendtsen et al. 2004). The deduced amino acid sequences of *est1* and *est2* and reference sequences retrieved from GenBank were used to construct a phylogenetic tree with the neighbor-joining method by using MEGA version 6 (Tamura et al. 2013). Bootstrapping of 1000 replicates was used to estimate the confidence level.

Based on the deduced amino acid sequence, secondary structure and tertiary structure predictions were performed with I-TASSER (Zhang 2008). The identified structural analogs were used for multiple-sequence alignment using the Expresso webserver (Notredame et al. 2000). Figures showing secondary structure alignments were exported by ESPript3 (Robert and Gouet 2014). The analog with the highest TM score was also selected for structural superimposition.

Expression and purification of recombinant proteins Est1 and Est2

The primer pairs 5′-CACCATGCCCCTGGCCCGAGTGGA-3′ and 5′-GGCGCCCACCGGC ACCTGAGTC-3′ and 5′-CACCATGACCGAGCTGCCGGTGGGAG-3′ and 5′-GCG TCTTAGCGCGCGGTACAC-3 were used to amplify *est1* and *est2*, respectively. The resulting PCR products were purified and ligated into expression vector pET101/D-TOPO® according to the protocol of the manufacturer (Invitrogen). To produce His$_6$-tagged Est1 and Est2, recombinant plasmid DNA was transformed into *E. coli* BL21 (DE3) cells and plated on LB agar plates with 100 μg/ml ampicillin. A single colony was picked and grown overnight at 30 °C in 60-ml LB medium containing 100 μg/ml ampicillin. Subsequently, this pre-culture was added to 600-ml LB medium with 100 μg/ml ampicillin and grown with shaking at 30 °C. At an optical density (OD$_{600}$) of 0.6, isopropyl-beta-D thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. After a 6-h induction at 30 °C, cells were harvested by centrifugation (7000×*g*, 4 °C, 10 min). Cell pellets were washed with 100 ml LEW buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, pH 8) and stored at −20 °C until required.

To purify Est1 and Est2, Protino® Ni-TED 2000 packed column (Macherey-Nagel, Germany) was used following the manufacturer's protocol, however, with the modified LEW buffer (50 mM NaH$_2$PO$_4$, 1 M NaCl, 10% *v/v* glycerol, 0.05% *v/v* Triton X-100, pH 8). Protein concentration was measured by the Bradford method (Bradford 1976). Purity and molecular mass of the purified proteins were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the procedure of Laemmli (1970). Fractions derived from affinity chromatography showing a single band with the estimated molecular mass of the targeted proteins were pooled, dialyzed with 50 mM sodium phosphate buffer (pH 8), and stored in 50% (*v/v*) glycerol at −20 °C until use.

Standard enzyme assays

Esterase activity was measured by a spectrophotometric method (Jaeger et al. 1999) using *p*-nitrophenyl (*p*-NP) acyl esters (Sigma) as substrates. To minimize substrate auto-hydrolysis at high temperatures, *p*-NP caprylate (C8) was used as a standard substrate. Unless otherwise indicated, Est1 activity was measured at 80 °C in 1 ml containing 50 mM sodium phosphate assay buffer (pH 8),1 mM *p*-NP caprylate (C8), and 1% (*v/v*) isopropanol, while Est2 activity was measured at 70 °C in 1 ml containing 50 mM TAPS (3-(2, 4 dinitrostyrl)-(6R,7R-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid) assay buffer (pH 9), 1 mM *p*-NP caprylate (C8), and 1% (*v/v*) isopropanol. The assay buffer was initially incubated in a screwed-cap test tube for 10 min at assay temperature. Then, the reaction was initiated by adding enzyme and substrate to the buffer. The amount of *p*-nitrophenol released by esterase-catalyzed hydrolysis was continuously monitored at a wavelength of 410 nm against an enzyme-free blank. One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per minute. All experiments

were performed in at least triplicate. Results are shown as mean values ± standard deviation (SD).

Substrate specificities of Est1 and Est2 were checked towards the following p-NP acyl esters of different chain lengths: p-NP acetate (C2), p-NP butyrate (C4), p-NP valerate (C5), p-NP caproate (C6), p-NP caprylate (C8), p-NP caprate (C10), p-NP laurate (C12), p-NP myristate (C14), and p-NP palmitate (C16). Considering the instability of short-chain substrates, the assay temperature was decreased to 50 °C. Initial rates of reaction for p-NP butyrate and p-NP valerate were calculated by estimating Est1 and Est2 activities with different substrate concentrations ranging from 1 to 2000 μM. Values for K_m and V_{max} were determined by employing the Lineweaver-Burk plots (Lineweaver and Burk 1934). Lipolytic activity towards different triacylglycerides was also measured qualitatively by incubating Est1 and Est2 on agar plates emulsified with tributyrin (C4), tricaproin (C6), tricaprylin (C8), tricaprin (C10), trilaurin (C12), trimyristin (C14), or tripalmitin (C16). Formation of clearing zones (halos) on agar plates indicated lipolytic activity. Beta-lactamase activity of Est2 was tested spectrophotometrically at 486 nm, under standard assay conditions with 1 mM nitrocefin (E-isomer) as substrate.

Effect of temperature and pH

The effect of pH on Est1 and Est2 activities was measured at 348 nm (the pH-independent isosbestic wavelength) under standard assay conditions (Glogauer et al. 2011). The following overlapping buffer systems were used: 50 mM acetate buffer (pH 3.0 to 6.0), 50 mM sodium phosphate buffer (pH 6.0 to 8.0), 50 mM TAPS buffer (pH 8.0 to 9.0), and 50 mM CHES (N-cyclohexyl-2-aminoethanesulfonic acid) buffer (pH 9.0 to 10.0). Temperature optima for Est1 and Est2 activities were measured in a temperature range of 20 to 100 °C. Thermostability of enzyme activity was determined by incubating Est1 and Est2 in their optimal buffers at various temperatures (50 to 80 °C) for up to 6 days. Subsequently, Est1 and Est2 activities were determined under standard assay conditions.

Combined effect of pH and temperature on the stabilities of Est1 and Est2

Second-order rotatable design was applied to study the combined effect of pH and temperature on the stabilities of Est1and Est2. The design was based on five levels and two variables (Table S2). Experimental data were fitted to the empirical model using Eq. (1):

$$Z\,(\%) = b_0 + b_1X + b_2Y + b_{12}XY + b_{11}X^2 + b_{22}Y^2 \quad (1)$$

in which Z was residual relative activity, presented as the percentage of activity measured before incubation and under

standard assay conditions; X and Y were code values of pH and temperature shown in Table S2; b_0, b_1, b_2, b_{12}, b_{11}, and b_{22} were regression coefficients. Significance of regression coefficients was checked by Student's t test ($\alpha = 0.05$). Statistically non-significant coefficients were removed, and best-fit parameters were recalculated (Lazić 2004). The consistency of regression models was checked by Fisher's test ($\alpha = 0.05$). The ratios of the following mean squares were compared with the F-criterion tabular values. Based on the following mean square ratios (Box et al. 2005), models were accepted if:

$$F_1 = \text{Model/Experimental error} \qquad F_1 \geq F_{den}^{num} \quad (2)$$

$$F_2 = \text{Lack of fitting/Experimental error} \qquad F_2 \leq F_{den}^{num} \quad (3)$$

Est1 and Est2 were incubated under conditions described in Table S1 for 2 h, and residual activity was subsequently measured under the respective standard assay conditions.

Effect of miscible and immiscible organic solvents

The following organic solvents with different log p values were used in this study: water-miscible organic solvents of DMSO (− 1.3), methanol (− 0.75), ethanol (− 0.24), acetone (− 0.24), isopropanol (0.074), and 1-propanol (0.28), as well as the water-immiscible organic solvents of ethyl acetate (0.68), diethyl ether (0.85), chloroform (2.0), and toluene (2.5). The effects of water-miscible organic solvents on Est1 and Est2 activities were measured by adding each organic solvent into the assay buffer to obtain a final concentration ranging from 6 to 30% (v/v) under standard assay conditions. Enzyme activity measured in organic solvent-free assay buffer was regarded as 100%. Appropriate controls were also set to eliminate changes in extinction coefficients due to the presence of solvents.

To evaluate short-term stability towards water-miscible and water-immiscible organic, Est1 and Est2 were incubated in 100-μl aliquots with different amounts of water-miscible organic solvents (0 to 75%, v/v for Est1; 0 to 95%, v/v for Est2) or water-immiscible organic solvents (15 and 30%, v/v) at 30 °C for 2 h with vigorous shaking (300 rpm). The long-term stability towards water-miscible solvents was only measured for Est2. In the presence of 30 or 60% (v/v) organic solvents, Est2 was incubated at 30 °C with constant shaking in a screwed-cap test tube for up to 26 or 13 days, respectively. Enzyme activities in either the aqueous phase (for water-immiscible solvents) or the mixture (for water-miscible solvents) were measured. Each water-miscible organic solvent was equalized to the same final concentration in the assay buffer and the residual activity was measured under standard

assay conditions. A blank reference was prepared by using the same buffer solution without enzyme containing and the same amount and type of organic solvent (Shao et al. 2013). Residual activity was subsequently measured under the respective standard assay conditions. The activity measured at the start of the experiment was taken as 100%.

Effect of additives on Est1 and Est2 activities

The effects of metal ions on Est1 and Est2 activities were examined in 50 mM sodium phosphate buffer (pH 8) at 50 °C, in the presence of 1 mM and 10 mM KCl, $CaCl_2$, $MnCl_2$, $MgCl_2$, $ZnSO_4$, $FeSO_4$, $CuCl_2$, $NiSO_4$, $FeCl_3$, and $AlCl_3$. The inhibitory effect on enzyme activity was measured under standard assay conditions with the following known esterase effectors (each 1 mM and 10 mM): phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), and ethylenediaminetetraacetic acid (EDTA). In addition, the effect of the following detergents (each 0.1 and 1%, v/v), Triton X-100, Tween 20, Tween 80, and SDS, was determined. Furthermore, the effect of NaCl and KCl on enzyme activity in a range between 0.5 and 4 M was assessed. Activity measured in additive-free assay buffer was regarded as 100% activity, while reactions that included corresponding additive but no enzyme were used as blanks.

Accession number

The gene sequences are available at the GenBank database under accession numbers KR149567.1 (Est1) and KR149568.1 (Est2).

Results

Metagenomic library screening and analysis of two novel esterase-encoding genes

To isolate novel lipolytic enzymes, a compost sample at the thermophilic stage (55 °C) was used for constructing a metagenomic plasmid library. The library consisted of approximately 675,200 clones with an average insert size of 5.3 kb and comprised a total size of 3.58 GB. Among the 279 lipolytic-positive clones, two E. coli clones harboring the plasmids pFLD_Est1 and pFLD_Est2 showed strong lipolytic activity (large halos) on indicator plates and were selected for further characterization.

Sequence analyses of the plasmids pFLD_Est1 and pFLD_Est2 revealed that each contained one putative esterase-encoding gene, est1 (813 bp) and est2 (1170 bp), respectively. The deduced proteins comprised 270 (Est1) and 389 (Est2) amino acids. Putative signal peptides indicating extracellular localization were not detected in the deduced protein

sequences. Sequence similarity searches showed that Est1 exhibited 49% identity to a hypothetical protein from *Candidatus Entotheonella* (GenBank: ETW96815) and 43% identity to Est28 from a grassland soil metagenomic library (Nacke et al. 2011). Est2 showed 53% sequence identity to a beta-lactamase from *Streptomyces lavenduligriseus* (GenBank: WP_030784121) and 52% sequence identity to a putative esterase from *Streptomyces bottropensis* (GenBank: EMF58012).

Sequence analysis and the subsequently constructed phylogenetic tree revealed that Est1 belonged to family V and Est2 to family VIII of lipolytic enzymes (Fig. S1). The tertiary structure predicted by I-TASSER obtained the C-scores of 1.03 for Est1 and 1.27 for Est2, which indicated a significant confidence of good quality.

The two conserved family V motifs G-X-S-X-G-G and P-T-L were present in the Est1 protein sequence at amino acid positions 92 to 97 and 208 to 210, respectively (Fig. S2a). The tertiary structure of Est1 was composed of a cap domain with five α-helices (α4 to α8, Fig. S2a) and an α/β-hydrolase fold core domain (Fig. 1a). The core domain consists of six helices surrounded by eight β-strands that form parallel structures, in which Ser^{94} is located between β5 and α3, Asp^{217} after β7, and His^{268} between β8 and α10. The overall structure of Est1 superimposed on MGS-M2 (TM score 0.96; RMSD 0.94) (Alcaide et al. 2015), with a global amino acid sequence identity of 21.9% (Fig. 1c).

Due to the high sequence similarity between family VIII lipolytic enzymes and class C beta-lactamases/penicillin-binding proteins, amino acid sequences in the two categories were aligned (Fig. S2b). All the aligned sequences shared the same catalytic triad of serine, lysin, and tyrosine. Conserved family VIII motifs including S^{70}-X-X-K^{73}, G^{154}-X-X-X-X-H^{159}, and H/W^{341}-X-G^{343} were detected in Est2 sequences. However, classical β-lactamase motifs such as Y-A-N and L-S/T-G (KTG-box) were absent in the Est2 sequence (Fig. S2b). Similar to Est1, the tertiary structure of Est2 also consists of two domains: a large α/β domain and a small α-helical domain. The small α-helix domain contains four helices and two 3_{10} helices. For the large α/β domain, a central eight-stranded antiparallel β-sheet is flanked by six helices on one face and two on the opposite face (Fig. 1c). The deduced catalytic residues of Ser^{70}, Lys^{73}, and Tyr^{159} are located in the large cavity between the two domains (Fig. 1b). The overall structure of Est2 superimposed well on CcEstA (TM score 0.95; RMSD 0.46) (Woo Lee et al. 2016), with a global amino acid sequence identity of 39.6% (Fig. 1d).

Purification of Est1 and Est2 and substrate specificity towards *p*-NP acyl esters

The esterase-encoding genes est1 and est2 were successfully cloned in expression vectors and expressed in E. coli BL21(DE3). The produced gene products Est1 and Est2 were

Fig. 1 The modeled three-dimensional structure of Est1 and Est2. **a** A 3D structure model of Est1. The overall structure is composed of two domains: cap domain and catalytic domain. **b** A 3D structure model of Est2. The overall structure harbors two domains: a large α/β domain and a small α-helix domain. α-helices and β-strands are colored in cyan and magenta, respectively. The catalytic triad of Est1 (residues Ser^{94}, Asp^{215}, and His^{245}) and that of Est2 (residues Ser^{70}, Lys^{73}, and Try^{160}) are indicated in stick representation. The cap domain for Est1 and the small α-helix domain for Est2 are presented as transparent surface. **c** Structural superposition of Est1 (pink) onto its structural homolog MGS-M2 (yellow; PDB: 4Q3L). **d** Structural superposition of Est2 (pink) onto its structural homolog CcEstA (yellow; PDB:5GKV)
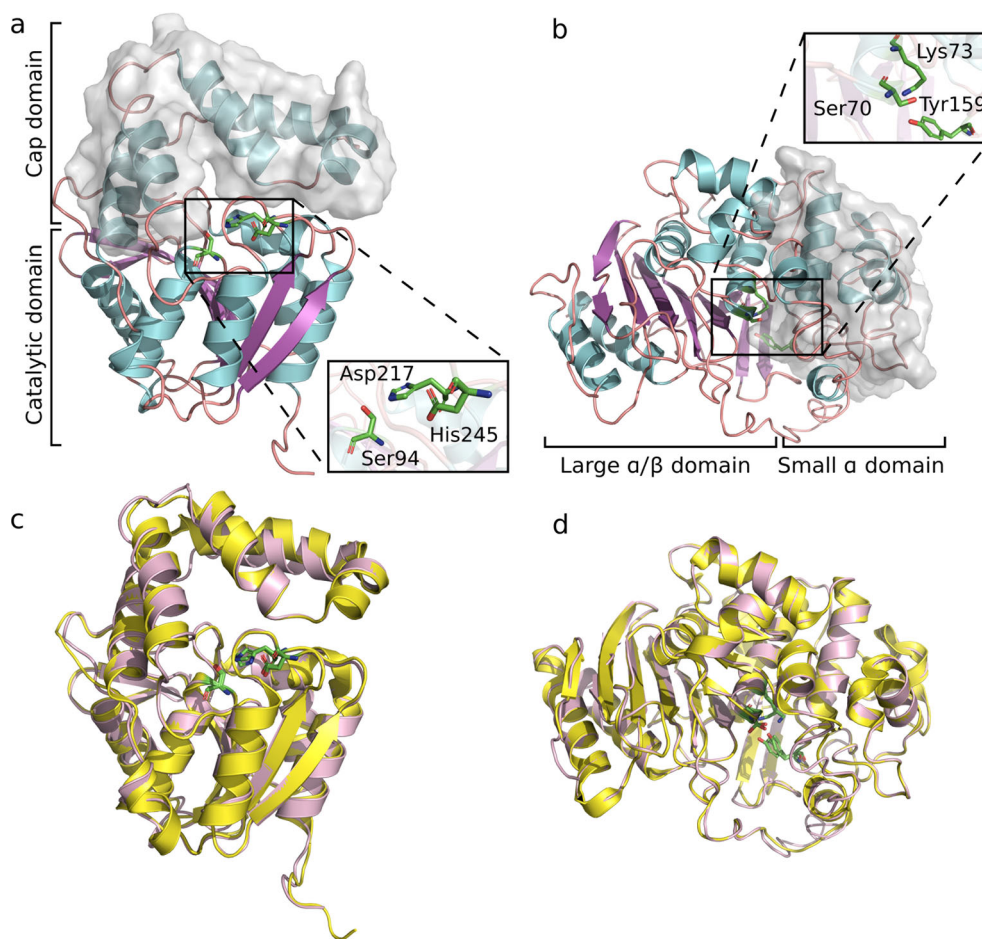
subsequently purified by Ni-TED affinity chromatography, yielding 28.9-fold and 16.9-fold purification values and specific activities of 22.2 and 7.3 U/mg, respectively (Table S2). SDS-PAGE analysis revealed single bands with molecular masses of approximately 35 kDa (Est1) and 48 kDa (Est2) (Fig. S3), which were in accordance with the calculated masses including during cloning added V5 epitope and His$_6$-tag.

Est1 and Est2 exhibited a substrate preference for esters with short-chain fatty acids. The maximal activities were detected with p-NP butyrate (C4) for Est1 and p-NP valerate (C5) for Est2 as substrates (Fig. 2). Correspondingly, p-NP butyrate and p-NP valerate were used for calculating the K_m and V_{max} values. The K_m and V_{max} values of Est1 were 3.0 μM and 31.2 U/mg, respectively, and that of Est2 2.7 μM and 19.0 U/mg, respectively. With respect to esters with long-chain fatty acids as substrates (C10 to C16), Est1 was active with p-NP caprate (C10) retaining 45% activity, but barely with other substrates. In comparison, Est2 hydrolyzes more acyl esters with long-chain fatty acids. It showed 80%, 45%, 12%, and 30% activity towards p-NP caprate (C10), laurate (C12), p-NP myristate (C14), and p-NP palmitate (C16), respectively. Chain length selectivity towards triacylglycerides

was recorded for the substrates tributyrin (C$_4$) and tricaproin (C$_6$) for both enzymes. The preference for short substrates (< C10) indicates that Est1 and Est2 are esterases, rather than "true" lipases which are commonly found in family I of lipolytic enzymes (Arpigny and Jaeger 1999). Moreover, beta-lactamase activity of Est2 with nitrocefin as substrate was tested, but no significant beta-lactamase activity was detected (data not shown).

Effect of pH and temperature on Est1 and Est2 activities

Est1 and Est2 were active over the entire tested pH range (3 to 10), with the exception of Est1 at pH 3 (Fig. 3a, d). Est1 exhibited maximal activity at pH 7 and retained more than 95% activity between pH 6 and 8. Est2 activity increased with pH and peaked at pH 9. With respect to the temperature dependence of enzyme activity, Est1 and Est2 displayed a similar ascending trend along the temperature gradient, showing a maximal activity at 80 and 70 °C, respectively. At higher temperatures (90 and 100 °C), approximately 80% activity was retained (Fig. 3b, e).
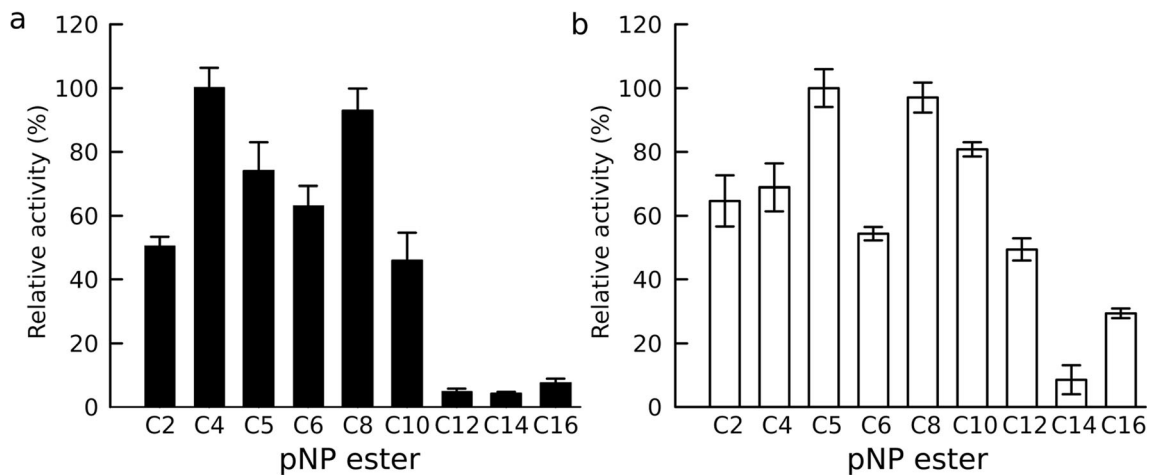
Fig. 2 Substrate specificity towards *p*-NP acyl esters with different chain lengths. **a** Est1, the maximal activity (23.3 U/mg) measured towards *p*-NP butyrate (C4) was taken as 100%. **b** Est2, the maximal activity (10.7 U/mg) measured towards *p*-NP valerate (C5) was taken as 100%

Despite that the activity maximum of Est1 was at 80 °C, stability at 70 °C was low as a rapid drop of activity to 50% was detected after 15 min of incubation. Similar results were obtained for Est2 at 80 °C with 35% residual activity after 30 min. Strikingly, Est2 showed significant stability at 70 °C, with 50% residual activity after 12 h of incubation. At lower temperatures (50 or 60 °C), the activities of both enzymes were remarkably stable for extended incubation times; nonetheless, Est1 displayed a higher stability than Est2 (Fig. 3c). Est1 retained more than 80% activity at 50 °C over the entire incubation period (7 days), whereas 52% residual activity was observed for Est2 after 5 days. When incubated at 60 °C, Est1 exhibited a half-life of 2 days, which is nearly twice as that of Est2 (Fig. 3f).
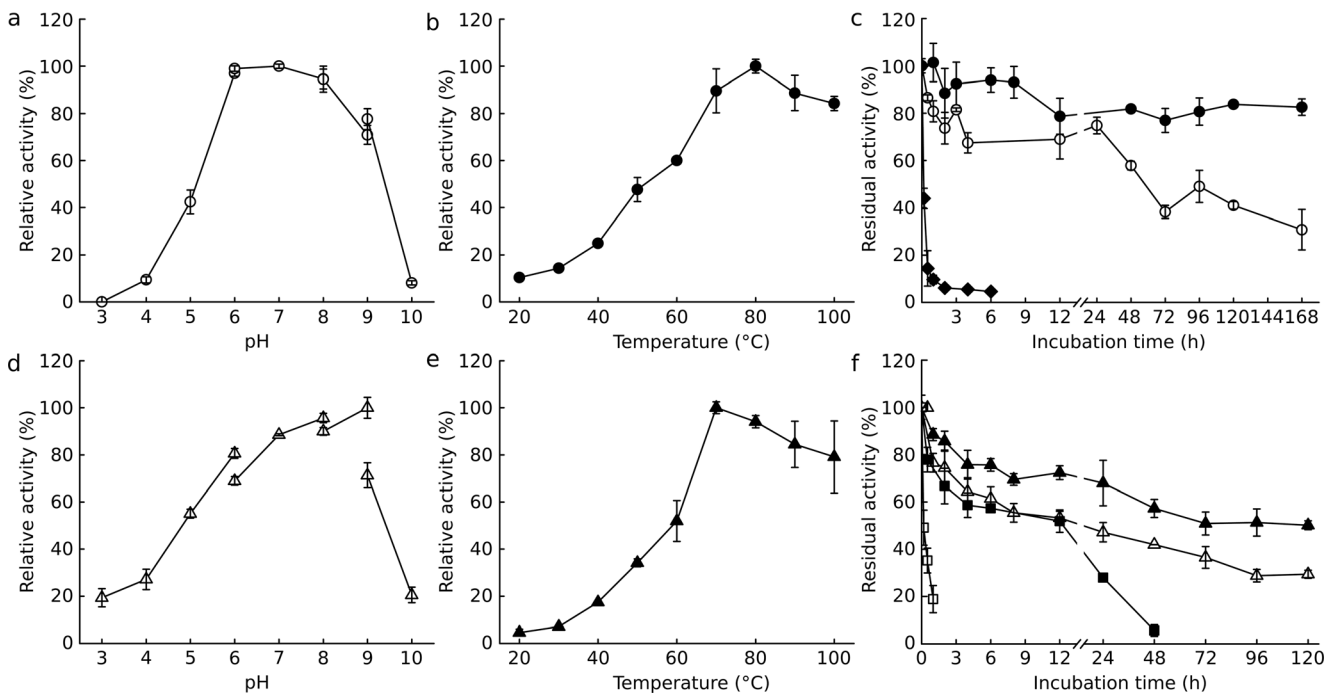

Fig. 3 Effect of pH and temperature on esterase activity. **a** Effect of pH on Est1 activity. Maximal activity at pH 7 (45.8 U/mg) was taken as 100%. **b** Effect of temperature on Est1 activity. Maximal activity at 80 °C (46.1 U/mg) was taken as 100%. **c** Thermostability of Est1 at 50 °C (closed circle), 60 °C (open circle), and 70 °C (closed diamond); activity measured before incubation (40.4 U/mg) was taken as 100%. **d** Effect of pH on Est2 activity. Maximal activity at pH 9 (14.4 U/mg) was taken as 100%. **e** Effect of temperature on Est2 activity. Maximal activity at 70 °C (15.2 U/mg) was taken as 100%. **f** Thermostability of Est2 at 50 °C (closed triangle), 60 °C (open triangle), 70 °C (closed square), and 80 °C (open square); activity measured before incubation (12.0 U/mg) was taken as 100%

Combined effect of pH and temperature on Est1 and Est2 stabilities

The combined effect of temperature and pH on the stability of Est1 and Est2 was visualized by response surfaces (Fig. 4). After removing the insignificant terms and refitting to the empirical model, the following equations for Est1 (4) and Est2 (5) stability under different conditions were obtained:

$$\text{Residual activity } (\%) = 76.33$$
$$+13.56 \text{ pH} - 31.89 \text{ } T - 11.19 \text{ pH} T - 19.09 \text{ pH}^2 - 17.82 T^2 \tag{4}$$

$$\text{Residual activity } (\%) = 84.1$$
$$+14.1 \text{ pH} - 19.3 \text{ } T - 6.3 \text{ pH}^2 - 23.8 \text{ T}^2 \tag{5}$$

Variance analysis implied that the models for Est1 and Est2 significantly fitted to the experimental data ($F_1 \geq \text{F}_{\text{den}}^{\text{num}}$, $F_2 \leq \text{F}_{\text{den}}^{\text{num}}$, $\alpha = 0.05$; Table S3). In equations (4) and (5), the negative coefficients for T, pH^2 and T^2 indicate the existence of an absolute maximum of residual activity. The calculated optimal conditions were located at pH 8.2 and 39.1 °C (Est1) and pH 8.7 and 48.9 °C (Est2). It was predicted that Est1 and Est2 retain 98.6 and 96.3% residual activity, respectively, under these conditions. Moreover, according to the elliptical contour plot on the pH–T (x–y) dimension, the pH and temperature ranges in which Est1 and Est2 retain more than 80% residual activity were pH 7.2 to 9.2 and 23.6 to 54.6 °C for Est1 and pH 7.0 to 10.4 and 37.4 to 60.4 °C for Est2 (Fig. 4). In conclusion, Est1 and Est2 are stable under thermophilic and alkaline conditions.
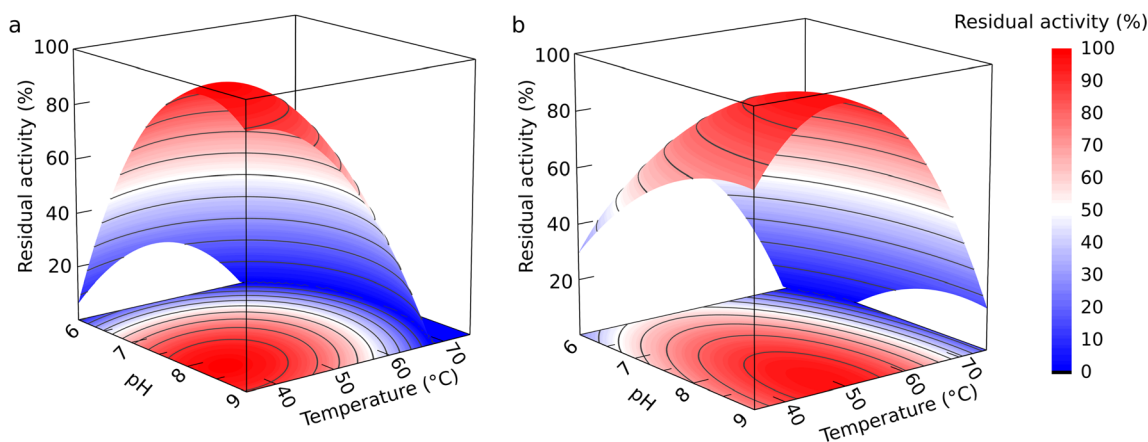
Effect of water-miscible organic solvents on Est1 and Est2 activities

Est1 activity increased to approximately 150% in the presence of low concentrations of 1-propanol (below 6%, v/v), and ethanol and isopropanol (< 12%, v/v). At higher concentration, Est1 activity decreased rapidly and was not detectable at 18% (v/v) 1-propanol, 30% (v/v) ethanol, and 24% (v/v) isopropanol (Fig. 5a). Addition of DMSO, methanol, and acetone inhibited Est1 activity over the tested concentration range, retaining 50% activity at 30% (v/v) DMSO, or less than 10% activity at 30% (v/v) methanol and acetone.

Ethanol, methanol, isopropanol, and 1-propanol had a stimulatory effect on Est2 activity almost at all tested concentrations (Fig. 5b). In the presence of ethanol and methanol, Est2 activity increased continually with raising concentrations. At 30% (v/v) ethanol and methanol, Est2 activity increased 8.8-fold and 5.4-fold, respectively. For isopropanol and 1-propanol, optimal Est2 activity was recorded at concentrations of 18 and 12% (v/v), respectively (Fig. 5b). In the presence of DMSO, Est2 retained more than 70% activity at all tested concentrations, with the maximal activity at 6% (v/v). Acetone caused an activity decrease to 34.1% at 30% (v/v).

Effect of water-miscible organic solvents on Est1 and Est2 stabilities

Est1 retained almost unchanged residual activity after 2 h of incubation in the presence of 15 and 30% (v/v) of all tested organic solvents, with the exception of 1-propanol (Fig. 6a). Est1 residual activity rapidly dropped to 17.0% at 30% (v/v) 1-propanol. Decreased tolerance was also detected after exposure to 45% (v/v) isopropanol, methanol, acetone, and DMSO for 2 h (Fig. 6a). At 60% (v/v) solvent concentration, Est1 exhibited moderate tolerance to DMSO (60.5% residual activity), but lost most of its activity (less than 10%

Fig. 4 Response surface corresponding to the combined effect of pH and temperature on Est1 (**a**) and Est2 (**b**) stabilities. Residual activity was expressed as a percentage of the initial activity measured before incubation under standard assay conditions. The elliptical contour plot on the pH–T dimension demonstrates the change of residual activity (%)

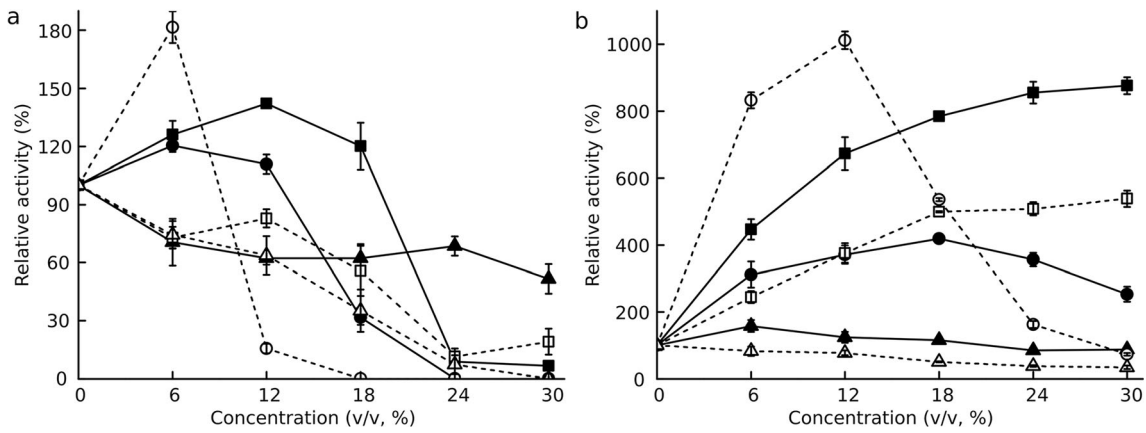**Fig. 5** Effects of water-miscible organic solvents on of Est1 (**a**) and Est2 (**b**) activities. Catalytic activity was measured under standard assay conditions in the presence of different amounts of ethanol (closed square), methanol (open square), isopropanol (closed circle), 1-propanol (open circle), DMSO (closed triangle), and acetone (open triangle). Specific activities corresponding to 100% relative activity were 21.9 U/mg (Est1) and 5.1 U/mg (Est2)

residual activity) in the presence of the other tested organic solvents. Est1 activity was almost inactivated by all the tested organic solvents at 75% (*v/v*).

In comparison with Est1, Est2 displayed higher resistance towards water-miscible solvents (Fig. 6b). In the organic solvent concentration range of 0 and 60% (*v/v*), Est2 retained its
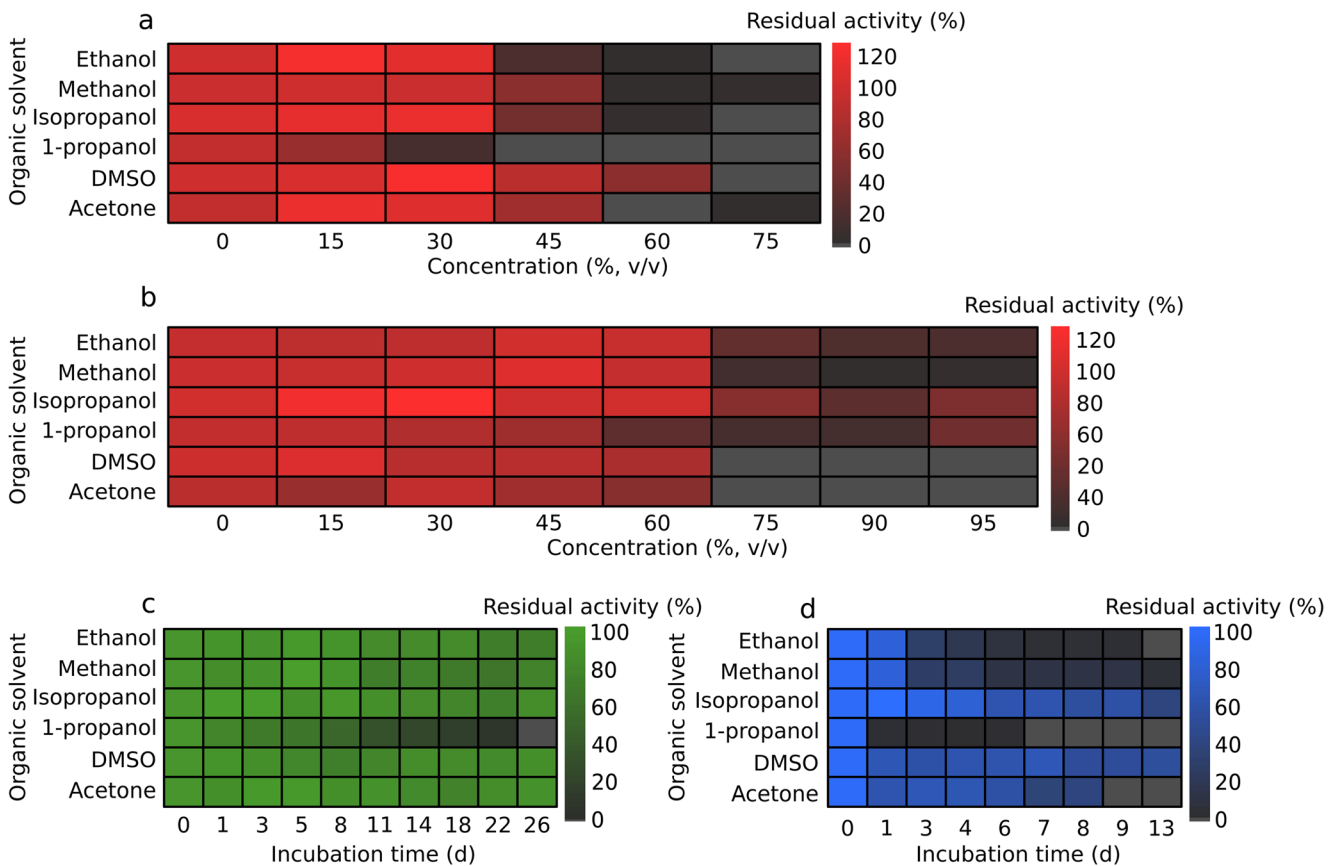

Fig. 6 Heatmap displaying the effect of enzyme stability towards water-miscible organic solvents. Short-term stability of Est1 (**a**) and Est2 (**b**) towards different concentrations of organic solvents at 30 °C for 2 h. The specific activity expressed as percentages of Est1 reference reactions (100%) are 23.3, 17.5, 24.3, 6.6, 15.3, and 13.6 U/mg for reactions in the presence of ethanol, methanol, isopropanol, 1-propanol, DMSO, and acetone, respectively, and that of Est2 are 32.7, 14.4, 17.7, 42.7, 4.34, and

3.1 U/mg, respectively. Long-term stability of Est2 towards 30% (*v/v*; **c**) and 60% (*v/v*; **d**) organic solvents for prolonged time periods at 30 °C. The specific activity values expressed as percentages of Est2 reference reactions (100%) are 14.3, 7.1, 9.2, 25.9, 5.0, and 4.1 U/mg for reactions in the presence of ethanol, methanol, isopropanol, 1-propanol, DMSO, and acetone, respectively

full activity in the presence of isopropanol, methanol, and ethanol and had a slight activity loss by the addition of DMSO and acetone. On the contrary, Est2 stability decreased along the rising concentration of 1-propanol, retaining 32.4% residual activity at 60% (v/v). Incubating with concentrations above 60% (v/v) of isopropanol, 1-propanol ethanol, or methanol led to a rapid decline of Est2 residual activity (Fig. 6b). Exposure to 75% (v/v) DMSO and acetone led to inactivation of Est2 activity.

Est2 exhibited high stability in the presence of 30% (v/v) ethanol, methanol, isopropanol, DMSO, and acetone, as its residual activity was above 70% over the extended incubation period of up to 26 days (Fig. 6c). This residual activity was even higher than that Est2 exhibited during incubation without additions (Fig. S4). However, a continuous drop in Est2 residual activity was observed when exposed to 1-propanol. Est2 also displayed decreased tolerance towards 60% (v/v) organic solvents (Fig. 6d). Incubation with 1-propanol, ethanol, and methanol reduced Est2 activity rapidly (< 10% residual activity). Nonetheless, Est2 exhibited substantial tolerance against isopropanol, DMSO, and acetone, retaining approximately 40% residual activity after the 13-day incubation.

Effect of water-immiscible organic solvents on Est1 and Est2 stabilities

Incubation with water-immiscible organic solvents of diethyl ether, chloroform, and toluene resulted in deleterious effects on Est1 enzyme activity, which was not detectable at the tested concentrations (Table 1). However, Est1 activity displayed some resistance towards ethyl acetate, with 46.6 and 21.1% residual activities at the tested concentrations of 15 and 30% (v/v), respectively. In contrast, Est2 was tolerant to ethyl acetate, diethyl ether, and toluene. Est2 retained its activity after incubation with ethyl acetate and diethyl ether. In the presence of toluene, Est2 retained 87.0 and 68.8% activities at the tested concentrations of 15 and 30% (v/v), respectively. In addition, Est2 was as Est1 inactivated by chloroform.

Effect of other additives on Est1 and Est2 activities

Metal ions, inhibitors, detergents, and salts were also analyzed for their effects on Est1 and Est2 activities. Est1 and Est2 are generally resistant to various metal ions (Table S4). The addition of tested metal ions at 1 mM concentration had minor effects on Est1 and Est2 activities as approximately 90% of the activity was retained. Moreover, both enzymes exhibited substantial tolerance (above 50% activity) towards some metal ions such as Ca^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Fe^{3+}, and Al^{3+} at a concentration of 10 mM. Enzyme activity was slightly enhanced in the presence of 10 mM Mg^{2+} (Est1) and Zn^{2+} (Est2). The presence of the chelating agent EDTA did not affect Est1 activity but decreased Est2 activity to less than

70%. The latter results indicated that Est1 activity is independent of metal ions, whereas Est2 might be a metalloenzyme (Mohamed et al. 2013).

With respect to the detergents, Est1 activity was enhanced in the presence of 0.1% (v/v) Tween 20 and 0.1 and 1% (v/v) Tween 80. Est2 activity was less resistant than that of Est1 towards the tested detergents. Est2 retained more than 70% activity after addition of 1 mM Triton-100, Tween 20, and Tween 80. The anionic detergent SDS inhibited the activity of both enzymes entirely (Table S4). The activity of Est1 and Est2 was substantially decreased at 10 mM DTT and PMSF. DEPC displayed detrimental effects on Est1 activity, while Est2 activity was almost unaffected even at a concentration of 10 mM. The inhibition of enzyme activity by SDS and PMSF indicates that Est1 and Est2 belong to the serine hydrolases (Peng et al. 2011). In addition, Est1 and Est2 were active in the presence of 0 to 4 M NaCl and KCl. Both enzymes retained more than 50% of activity up to 2.5 M NaCl and KCl, which is indicative of halotolerance (Table S5).

Discussion

Extreme environments such as compost have been used for mining biocatalysts, which are likely to be adapted to harsh industrial reaction conditions (Ryckeboer et al. 2003). In this study, compost derived from the thermophilic core of the pile was used as DNA source to construct a metagenomic library, whereby two putative genes encoding lipolytic enzymes were identified. The low identities of the deduced amino acid sequences to known proteins (Est1 43% and Est2 53%) indicate that Est1 and Est2 are novel lipolytic enzymes. Est1 is most related to the characterized esterase EstPS5, which was derived from a screening of a peat-swamp forest soil metagenome (Bunterngsook et al. 2010). Est2 shared the highest sequence identity with a beta-lactamase from *Streptomyces achromogenes*. In addition, most of the enzyme sequences similar to Est2 were derived from members of the *Streptomyces* genus. *Streptomyces* strains are predominantly found in soil and decaying vegetation and well-known as important natural sources for antibiotics (Raja and Prabakarana 2011).

Phylogenetic analyses indicate that Est1 and Est2 belong to family V and family VIII of lipolytic enzymes, respectively (Fig. S1). For most of the known lipolytic enzymes, the catalytic activity generally relies on the typical catalytic triad of Ser, Asp/Gly, and His (Ollis et al. 1992; Jaeger et al. 1999). This triad is also present in the Est1 amino acid sequence (Fig. S2a) and its tertiary structure (Fig. 1a). However, another catalytic triad consisting of Ser^{70}, Lys^{73}, and Tyr^{159} (Sakai et al. 1999) is responsible for Est2 catalytic activity (Fig. 1c, Fig. S2b). This triad is conserved among family VIII lipolytic enzymes, class C β-lactamases, and penicillin-binding proteins (Arpigny and Jaeger 1999; Hausmann and Jaeger 2010; Biver

Table 1 Short-term stabilities of Est1 and Est2 towards water-immiscible organic solvents.

Water-immiscible organic solvent (log P)	Residual activity (%)[a]		
	Concentration (v/v)	Est1	Est2
None[b]	–	100 ± 2.6	100 ± 1.5
Ethyl acetate (0.68)	15	46.6 ± 5.7	95.5 ± 4.2
	30	21.1 ± 3.0	101.5 ± 8.9
Diethyl ether (0.85)	15	ND[c]	116.0 ± 3.8
	30	ND[c]	96.5 ± 4.2
Chloroform (2.0)	15	ND[c]	ND[c]
	30	ND[c]	ND[c]
Toluene (2.5)	15	ND[c]	87.0 ± 6.0
	30	ND[c]	68.8 ± 8.0

[a] Aliquots of enzyme were shortly centrifuged; the aqueous layer was used for activity measurements under standard assay conditions

[b] Specific activity corresponding to 100% residual activities were 21.9 U/mg (Est1) and 11.2 U/mg (Est2)

[c] Not detectable

and Vandenbol 2013; Popovic et al. 2017). Despite the same catalytic triad and high amino acid sequence identity to β-lactamases, some family VIII esterases show promiscuous β-lactamase activity. Similar to Est2, the family VIII esterases EstB (Petersen et al. 2001), Lip8 (Ogino et al. 2004), Est2K (Kim et al. 2010), and Est7K (Woo Lee et al. 2016) showed negligible or no detectable β-lactamase activity, whereas EstC (Rashamuse et al. 2009), EstM-N1 (Yu et al. 2011), and PBS-2 (Boyineni et al. 2014) exhibited moderate or high β-lactamase activity. Yu et al. (2011) suggested that those promiscuous β-lactamase activities could be a result of family VIII esterases evolving from class C β-lactamases or vice versa.

According to the predicted tertiary structure (Fig. 1), the active sites of Est1 and Est2 are protected by an α-helix domain (cap domain). This domain acts as a shield for the catalytic site of many lipolytic enzymes and appears to play a key role in several functional aspects, such as activity, substrate specificity, and thermostability (Gall et al. 2014; Li et al. 2015; Kim 2017). Est2 was capable of utilizing acyl esters with long-chain fatty acids as substrate (C10, C12, and C16) (Fig. 2b). In addition, the Est2 structural homolog Est-Y29 (Ngo et al. 2013) was also reported to hydrolyze a wide variety of hydrophobic compounds, as a result of the deep hydrophobic patch between the large α/β domain in which the small α-helix domain defines a wide active site (Ngo et al. 2014).

Est1 and Est2 were generally thermoalkaline esterases (Fig. 3). In terms of thermophilicity, defined as increased enzymatic activity along a temperature gradient (Georis et al. 2000), Est1 and Est2 have great advantages in comparison with its homologs from other sources. Although esterases from *Fervidobacterium nodosum* (Yu et al. 2010), *Anoxybacillus gonensis* (Faiz et al. 2007), *Sulfolobus tokodaii*

(Angkawidjaja et al. 2012), and a compost metagenome (Riedel et al. 2015) displayed optimal activities above 70 °C, Est1 and Est2 exhibited higher activities (above 80%) at 90 and 100 °C. Moreover, Est1 and Est2 displayed unprecedented thermostability at high temperatures, with a half-life of more than 7 days at 50 °C and 2 days at 60 °C for Est1 (Fig. 4c) and 5 days at 50 °C, 1 day at 60 °C, and 12 h at 70 °C for Est2 (Fig. 4f). This remarkable feature distinguished the two enzymes from their thermophilic counterparts, such as esterases from the thermophilic microorganisms *Archaeoglobus fulgidus* (40% residual activity after 30 min at 60 °C; D'Auria et al. 2000), *Thermogutta terrifontis* (75% residual activity after 30 min at 70 °C; Sayer et al. 2015a), and *Thermoanaerobacter tengcongensis* (half-life of 2 h at 70 °C; Cook et al. 1996), as well as those from metagenomes of compost (approx. 90% residual activity after 1 h at 60 °C; Kang et al. 2011) and hot spring (half-life of 6 h at 60 °C; Zarafeta et al. 2016).

In general, thermostability is dependent on the structural rigidity, which is an accumulation of various features, including but not limited to amino acid composition, ion pairing, hydrogen bonds, hydrophobic interactions, and sulfide bridges (Sadeghi et al. 2006; Jochens et al. 2010; Ebrahimi et al. 2011; Pezzullo et al. 2013). Interestingly, the two characterized structural homologs of Est1, MGS-M2 (Alcaide et al. 2015) and TtEst (Sayer et al. 2015b), were reported as thermostable esterases (Alcaide et al. 2013; Sayer et al. 2015a). The thermostability of Est1 and Est2 is advantageous for industrial applications, as higher reactivity, stability, and process yields, as well as lower viscosity and fewer contamination problems, can be achieved at an elevated operation temperature (Lima et al. 2004; Panda and Gowrishankar 2005; Doukyu and Ogino 2010; Sood et al. 2016).

Temperature and pH play important roles in enzyme-catalyzed reactions and the two factors have to be considered together for optimization of enzyme reactions. Est1 and Est2 are most stable at conditions which are close to that of the original compost habitat (Fig. 4a, b). Generally, enzymes are stable at conditions similar to their original habitats (Elend et al. 2006; Kovacic et al. 2016), but show maximal activities at higher or lower temperatures (Hardeman and Sjoling 2007; Hu et al. 2010), which is also the case for Est1 and Est2 (Fig. 3). Est1 and Est2 were predicted to retain more than 80% residual activity over a broad temperature and pH range (Fig. 4). Thus, together with the feature of broad substrate specificity (Fig. 2), the two enzymes, particularly Est2, could be potentially utilized in detergents, in which high enzyme stability is required during washing conditions of pH 8 to 11 and temperatures of 30 to 60 °C, as well as versatile substrate specificity (Nerurkar et al. 2013; Bora 2014).

Water-miscible organic solvents are generally detrimental to enzymes. In contrast, Est2 activity was significantly enhanced by the addition of ethanol, isopropanol, methanol, and 1-propanol (Fig. 5b). The stimulated catalytic activity of esterase from *Burkholderia cepacia* was also observed in the presence of DMSO, DMF, methanol, ethanol, 2-propanol, and acetone (Takeda et al. 2006). Similar results were obtained for family VIII esterases Est2K, lpc53E1, and Est7K in the presence of isopropanol and methanol (Kim et al. 2010; Selvin et al. 2012; Woo Lee et al. 2016). The significant activating effect could be attributed to the uniform water phase formed by water-miscible solvents (Ogino and Ishikawa 2001) or the high diffusion rate of substrate in the presence of water-miscible solvents (Metin et al. 2006), which enables substrates quick and easy access to the active site. Moreover, Est2 activity generally showed a bell-shaped dependence on water-miscible organic solvent concentration (Fig. 5b), which indicated an optimal water activity for its hydrolytic activity (Léonard-Nevers et al. 2009; Adlercreutz 2013). Other esterases, including Est1 (Fig. 5a), commonly showed slightly increased (Hotta et al. 2002; Schütte and Fetzner 2007; Faulds et al. 2011; Kang et al. 2017), or decreased (Li and Yu 2013; Monsef Shokri et al. 2014; Dukunde et al. 2017) activity towards water-miscible organic solvents. For these enzymes, water-miscible solvents strip off the crucial water monolayer around the enzyme surface and compete for hydrogen bonds, which at higher solvent concentrations finally leads to denaturation (Ó'Fágáin 2003; Doukyu and Ogino 2010; Monsef Shokri et al. 2014).

In addition, Est1 was stable in the presence of 30% (v/v) water-miscible organic solvents (Fig. 6a). Similar or even lower solvent tolerance was found for most of the reported OST esterases (Doukyu and Ogino 2010; Kang et al. 2011; Brault et al. 2012; Xing et al. 2012; Zhang et al. 2014; Kang et al. 2017; Kumagai et al. 2018). Thus, in comparison, Est2 exhibited superior stability against higher concentrations of the analyzed water-miscible organic solvents (Fig. 6b). Among the

rare counterparts, a hyper-thermophilic archaeal esterase (Hotta et al. 2002) and EstB from *Alcanivorax dieselolei* B-5(T) (Zhang et al. 2014) displayed substantial stability towards certain water-miscible organic solvents at high concentrations. An increase of hydrophobic interactions and hydrogen bonds are essential in enhancing esterase tolerance against water-miscible organic solvents (Song and Rhee 2001; Kawata and Ogino 2009; Park et al. 2012). Est2 also showed considerable tolerance towards 30% (v/v) ethanol, isopropanol, DMSO, methanol, and acetone for up to 26 days (Fig. 6c) and towards 60% (v/v) isopropanol, DMSO, and acetone up to 13 days (Fig. 6d). The preservation of high esterase activity over an extended period has been rarely described previously (Sana et al. 2007; Jin et al. 2012; Li and Yu 2013). This feature could allow to apply Est2 as an immobilized biocatalyst in non-aqueous-based continuous bioprocesses (Sana et al. 2007; Yang et al. 2011).

Overall, both enhanced activity and adequate stability towards water-miscible organic solvents are pre-requisite properties for applications of esterases in organic synthesis (Panda and Gowrishankar 2005; Lopez-Lopez et al. 2014). In non-aqueous media, such enzymes could be exploited for ester synthesis and transesterification reactions and a variety of other chemical reactions (Salihu and Alam 2015; Sood et al. 2016; Sarmah et al. 2018). Thus, Est2 could be advantageous for use in biodiesel production, as the acyl acceptors methanol or ethanol are added in esterase/lipase-catalyzed transesterification reactions (Srimhan et al. 2011; Nasaruddin et al. 2014; Wang et al. 2017). In addition, Est1 and Est2 activities responded differently to the presence of polar protic solvents (methanol, ethanol, isopropanol, and 1-propanol) and polar aprotic solvents (acetone and DMSO), which further illustrates that the nature of the solvent influences esterase activity (Torres and Castro 2004). However, the relationship between the corresponding organic solvent $\log P_{ow}$ value and esterase activity/stability remains uncertain.

Comparing with water-miscible organic solvents, water-immiscible organic solvents are less deleterious for enzyme-solvent interactions (Rahman et al. 2005). However, most of the reported esterases were similar to Est1 (Table 1), showing a significant decrease of activity or inactivation after the incubation with water-immiscible organic solvents (Schütte and Fetzner 2007; Berlemont et al. 2013; Jin et al. 2012). In contrast, lipolytic enzymes of family I, also known as true lipases, are commonly resistant to water-immiscible organic solvents. In the presence of a water-solvent (hydrophobic) interface, the hydrophobic amino acid residues in the lid/flap region stabilize lipases in a flexible, open conformation (Dandavate et al. 2009; Yang et al. 2011; Kamal et al. 2013). However, the cap domain for most esterases is not intrinsically flexible and does not provide open and closed conformations (Bornscheuer 2002; Gall et al. 2014; Kim 2017). This could be the reason that water-immiscible tolerant esterases are rare. To the best of

our knowledge, only Est2 and the esterases EstC23 (Jin et al. 2012), LipBL (Pérez et al. 2012), Lpc53E1 (Selvin et al. 2012), RBest1 (Berlemont et al. 2013), Pf_Est (Mandelli et al. 2016), EST4 (Gao et al. 2016), and LipA9 (Park et al. 2018) show substantial resistance towards certain water-immiscible organic solvents. This feature further expands the application potential of Est2 to synthetic reactions in the presence of water-immiscible solvents (Gao et al. 2016; Sarmah et al. 2018).

Est1 and Est2 are to some extent resistant to metal ions (Table S4), which is an important feature in the bioremediation of environmental waste (Brault et al. 2012). Est1 and Est2 were also active at a salinity range of up to 4 M (Fig. S5), suggesting halotolerance (Jeon et al. 2012). Halotolerant enzymes are desirable in processes in which water activity is low (Delgado-García et al. 2012). In combination with the tolerance of Est1 and Est2 to organic solvents, it can be further confirmed that halotolerance is somehow positively correlated with organic solvent tolerance (Berlemont et al. 2013).

In conclusion, the characterization of Est1 and Est2 revealed that both, especially Est2, exhibit several application-relevant features, such as a broad substrate range, thermostability, halotolerance, and resistance to various organic solvents. In addition, as revealed by second-order rotatable design, Est1 and Est2 were predicted to be stable at a broad crossed range of temperature and pH. To the best of our knowledge, this is the first time to report an esterase, Est2, which simultaneously exhibits a significantly enhanced activity and unprecedented high stability towards water-miscible organic solvents and substantial tolerance towards water-immiscible organic solvents.

Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

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

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