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ORIGINAL PAPER

Cloning, expression and characterization of a halotolerant esterase from a marine bacterium *Pelagibacterium halotolerans* B2^T

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Abstract An esterase PE10 (279 aa) from *Pelagibacterium halotolerans* B2^T was cloned and overexpressed in *Escherichia coli* Rosetta in a soluble form. The deduced protein was 29.91 kDa and the phylogenetic analysis of the deduced amino acids sequence showed it represented a new family of lipolytic enzymes. The recombinant protein was purified by Ni–NTA affinity chromatography column and the characterization showed its optimal temperature and pH were 45 °C and pH 7.5, respectively. Substrate specificity study showed PE10 preferred short chain *p*-nitrophenyl esters and exhibited maximum activity toward *p*-nitrophenyl acetate. In addition, PE10 was a halotolerant esterase as it was still active under 4 M NaCl. Three-dimensional modeling of PE10 suggested that the high negative electrostatic potential on the surface may relevant to its tolerance to high salt environment. With this halotolerance property, PE10 could be a candidate for industrial use.

Keywords Esterase · *Pelagibacterium halotolerans* · Marine · Halotolerant · Cloning · Purification

Introduction

Enzymes are enjoying increasing popularity in the chemical industry as environmentally friendly, economical, and clean catalysts in applications ranging from laundry detergents and paper processing to fine chemical synthesis and diagnostic/research reagents (Taylor et al. 1999). Cultivation and identification of new microorganisms in different environments and screening of isolated strains for desired catalytic activities have conventionally been used to discover novel microbial enzymes (Wahler and Reymond 2001). The finding of new enzymes from microbes was focused on screening for the microbe, enzyme isolation and characterization, then cloning of the enzyme for overexpression.

Esterases (EC 3.1.1.1) represent a group of hydrolases catalyzing the cleavage and formation of ester bonds. Many of them do not require cofactors and show a wide substrate tolerance, high stereospecificity toward substrates and high stability in organic solvents (Bornscheuer 2001). These properties made esterases being attractive biocatalysts in industry. Esterases originate from various organisms, especially fungi and bacterium (Arpigny and Jaeger 1999). Now microbial esterases are more attractive in industry for their great yields, high safety in production and ease for genetic manipulation. Due to their importance and applications, esterases have been cloned, including those from several *Bacillus stearothermophilus* strains (Kim et al. 2002) and from *Pseudomonas* sp. strain S34 (Kim et al. 2003). And with the increasing demand of esterases under extreme conditions (low or high temperatures, acidic or alkaline solutions, high salt or organic solvents), the isolation of esterases from extremophiles has become a challenging task in recent years (Hess et al. 2008; Rao et al. 2009; Khudary et al. 2010). A common characteristic of these enzymes is that they contain a catalytic triad formed by Ser, His and Asp residues. The Ser residue usually appears in the pentapeptide motif GXSXG, but some do not (Akoh et al. 2004).

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Ocean covers more than two-thirds of our planet's surface; it is the habitat for many living things, especially for microbes. These microbes are a great treasure for novel enzymes. We isolated a novel member of the family *Hyphomicrobiaceae* from East China Sea. This strain, *Pelagibacterium halotolerans* B2^T, exhibited esterase activity (Xu et al. 2011) and so it was chosen for further study of esterase.

In this study, we cloned, expressed and characterized a novel esterase (PE10) from *Pelagibacterium halotolerans* B2^T. The sequence analysis showed this enzyme represents a novel family of lipolytic enzymes. Biochemical characterization revealed it was halotolerant. Halotolerant enzymes have high stability under high salt concentrations. As high salt concentration tends to greatly reduce the water activity of the medium like organic solvents do, it is quite possible for halotolerant enzymes to remain stable in organic solvents. So it is reasonable to apply halotolerant enzymes in aqueous/organic and nonaqueous media (Sellek and Chaudhuri 1999; Van den Burg 2003). For example, an extracellular protease from *Halobacterium halobium* has been exploited for efficient peptide synthesis in water/*N′*-*N′*-dimethylformamide (Kim and Dordick 1997). A homology model was build using the carboxylesterase EST2 from *Alicyclobacillus acidocaldarius* as template. The second structure alignment and the surface electrostatic potential of the two proteins were analyzed.

Materials and methods

Bacterial strains, plasmids and media

Pelagibacterium halotolerans B2^T, a novel member of the family *Hyphomicrobiaceae*, was isolated from East China Sea (125°59′24″E, 30°58′16″N) from a depth of 70 m (Xu et al. 2011), and it was cultivated in marine 2216 broth (BD, USA) at 30 °C. *E. coli* DH5α and *E. coli* Rosetta (DE3) (Novagen, Germany) used for plasmid manipulation and protein expression were grown in LB medium containing 10 g NaCl, 10 g tryptone and 5 g yeast extract per one liter at pH 7.0, and LB solid medium was supplemented with 1.5 % (w/v) agar.

Sequence analysis

The *Pelagibacterium halotolerans* B2^T genome was sequenced by the whole-genome sequencing with high-throughput technologies (Huo et al. 2012). We found a putative esterase gene (PE10), and the deduced sequence of amino acids was analyzed using blastp program (http://blast.ncbi.nlm.nih.gov/). The phylogenetic tree was constructed by neighbor-joining method (Saitou and Nei 1987) using Molecular Evolutionary Genetics Analysis 4.0 software (Tamura et al. 2007). Multiple alignments between amino acid sequences were performed using ClustalX program (Jeanmougin et al. 1998). Second structure alignment was generated by ESPript 2.2 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). The homology modeling was conducted by Robetta server (http://robetta.bakerlab.org) and the surface electrostatic potential was calculated by Discovery Studio 2.5 software (Accelrys, San Diego, CA, USA).

Cloning and expression

The genomic DNA of *Pelagibacterium halotolerans* B2^T was extracted using Genomic DNA Purification Kit (Dongsheng, China). The PE10 gene was amplified by polymerase chain reaction (PCR) using PrimeSTAR™ HS DNA Polymerase (TaKaRa, Japan). The primers used in the PCR were 5′-AGTA*CATATG*GTTGGGGCAGTTTC-3′ (*Nde*I restriction site is given in italics) and 5′-CTCT*AAGCTT*CTAGACCGAAGTGGG-3′ (*Hin*dIII restriction site is given in italics). The PCR product was digested by *Nde*I and *Hin*dIII (Takara, Japan) and cloned into the expression vector pET-28b (+) (Novagen, Germany) with the same digestion. The recombinant plasmid was transformed into *E. coli* Rosetta (DE3) (Novagen, Germany). When OD_{600}

Table 1 Comparison of PE10 with its similar relatives

Protein	Length (aa)	Accession no.	Organism	Identities (%)	*E* value
PE10	279	JF926256	*Pelagibacterium halotolerans* B2^T		
Alpha/beta hydrolase domain-containing protein	288	YP_001265205	*Sphingomonas wittichii* RW1	49	3e−64
Carboxylesterase family protein	289	YP_459014	*Erythrobacter litoralis* HTCC2594	47	1e−61
Alpha/beta hydrolase fold-3	333	YP_617284	*Sphingopyxis alaskensis* RB2256	47	1e−55
Putative hydrolase	297	YP_002870294	*Pseudomonas fluorescens* SBW25	45	3e−55
Alpha/beta hydrolase domain-containing protein	292	ZP_06860006	*Citromicrobium bathyomarinum* JL354	45	1e−54
Carboxylesterase family protein	289	NP_421101	*Caulobacter crescentus* CB15	43	2e−54

reached 0.6, 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) was added, after overnight cultivation at 25 °C, the cells were harvested by centrifugation at 5,000*g*, 5 min, 4 °C, then suspended in bufferA (500 mM NaCl, 10 mM imidazole, 20 mM Tris–HCl, pH 8.0), and disrupted by sonication. Cell debris was removed by centrifugation at

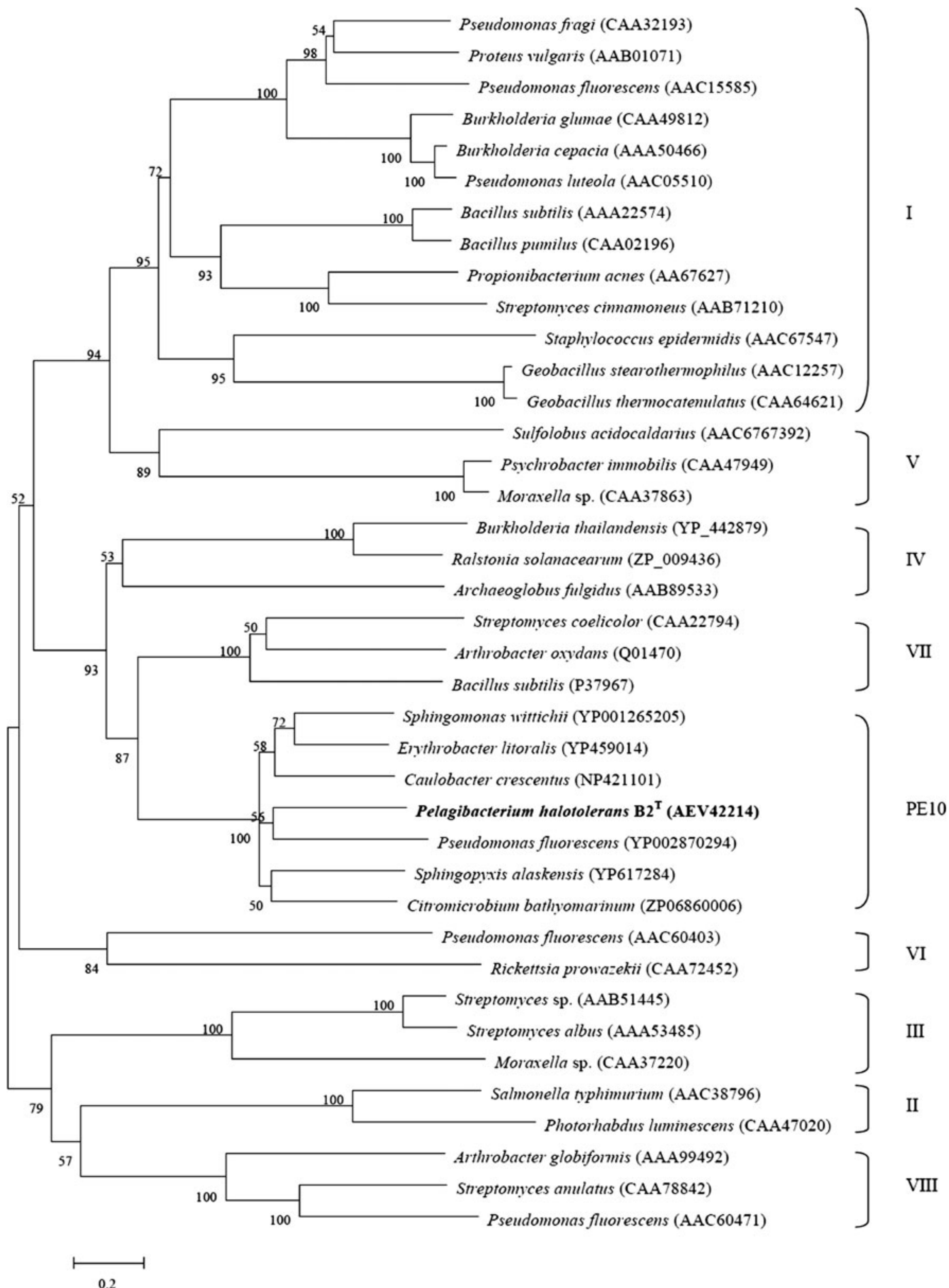


Fig. 1 Phylogenetic tree of PE10 from *Pelagibacterium halotolerans* B2^T (*bold type*) and other lipolytic enzymes. The neighbor-joining tree was constructed by the use of MEGA4.0 software, *Scale bar*, 0.2 substitutions per amino acid position

12,000*g*, 20 min, 4 °C. The resulting supernatant was purified by Ni–NTA affinity chromatography column (Qiagen, Germany) according to the manufacturer's protocol. The result of elution was tested by SDS-PAGE, using 12 % polyacrylamide gels. Protein concentration was determined by the method of Bradford (Bradford 1976), using bovine serum albumin as standard.

Enzyme assay

The standard reaction mixture contained 10 µl of acetonitrile containing 0.1 M *p*-nitrophenol acetate, 980 µl Tris–HCl (100 mM, pH 7.5) buffer and the enzyme in a final volume of 1 ml (Park et al. 2007). The activity of the enzyme was determined at 45 °C by measuring the A_{405} nm of *p*-nitrophenol anion released using a Beckman Coulter DU 800 nucleic acid/protein analyzer (Beckman, USA). All values were determined in triplicate and corrected for autohydrolysis of the substrate. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol per minute from the *p*-nitrophenyl ester.

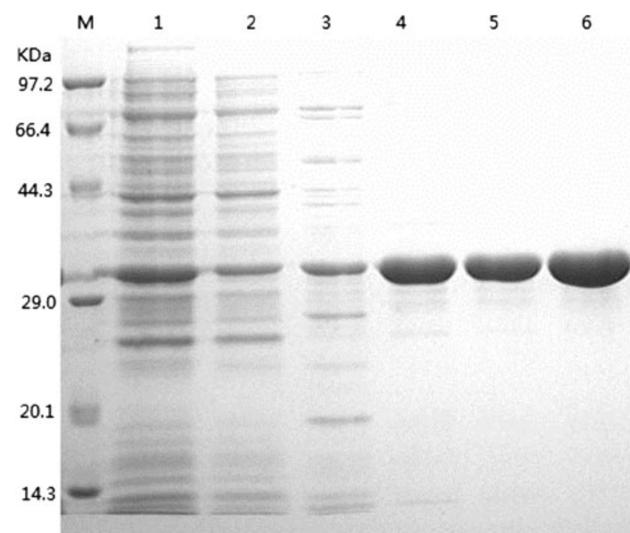


Fig. 2 SDS-PAGE of purified recombinant His-tagged PE10. *Lane M*, marker; *lanes 1–6* showed the resultant washed by Tris–HCl (pH 8.0) buffer with 10, 20, 60, 100, 100 and 250 mM imidazole, respectively

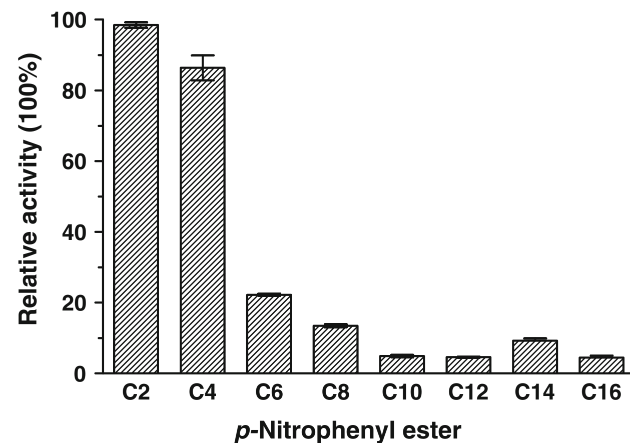
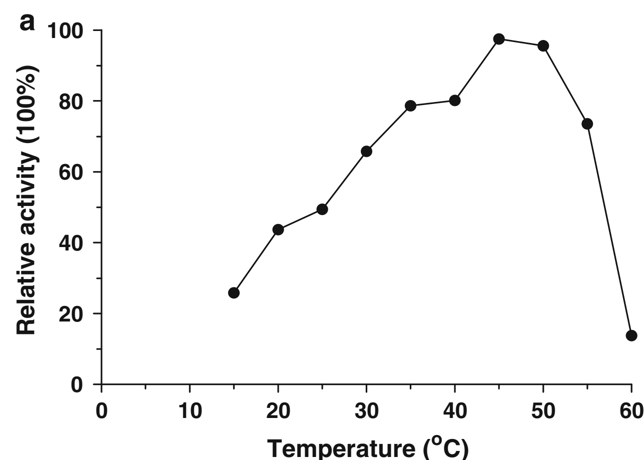


Fig. 3 Substrate specificity of PE10. The esterase activity of the purified recombinant enzyme PE10 toward various chain lengths of *p*-NP esters was assayed at 45 °C, pH 7.5. The highest level of activity with the C2 substrate was taken as 100 %

Substrate specificity

For the determination of substrate specificity, various chain lengths of *p*-nitrophenyl esters (acetate, C2; butyrate, C4;



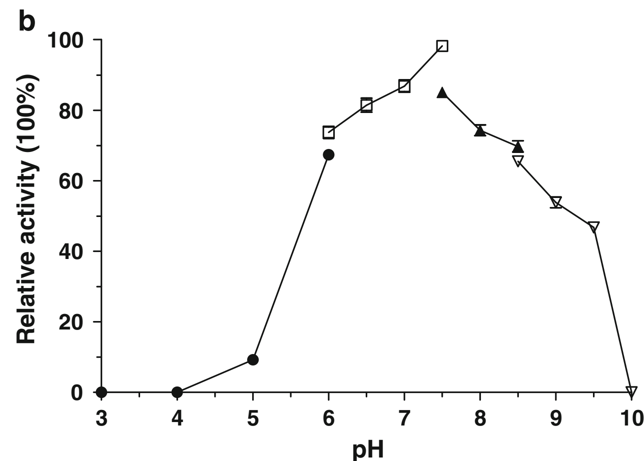


Fig. 4 Effects of temperature (**a**) and pH (**b**) on the activity of PE10. **a** The enzyme activity was measured over various temperatures at pH 7.5 with *p*-nitrophenol acetate as the substrate. The value obtained at 45 °C was taken as 100 %. **b** The activity was measured with *p*-nitrophenol acetate as the substrate. Assay was performed under 45 °C in different buffers: 100 mM citrate buffer (pH 3.0–6.0), 100 mM potassium phosphate buffer (pH 6.0–7.5), 100 mM Tris–HCl buffer (pH 7.5–8.5) and 50 mM CHES buffer (pH 8.5–10.0). The value obtained at pH 7.5 was taken as 100 %

hexanoate, C6; octanoate, C8; decanoate, C10; laurate, C12; myristate, C14; palmitate, C16) were added into the reaction mixture with the final concentration of 1 mM, the release of *p*-nitrophenol was measured at 405 nm.

Enzyme characterization

The optimum temperature of PE10 was examined from 15 to 60 °C using the standard reaction mixture. The thermostability of PE10 was investigated by incubation of the enzyme in the assay buffer without substrate at 40, 50 and 60 °C. Samples were collected at various time intervals (from 10 min to 1 h) and the activity was assayed using the standard assay.

The effect of pH on the activity of PE10 was measured over a pH range of 3.0–10.0. The buffers used were 100 mM citrate buffer (pH 3.0–6.0), 100 mM potassium phosphate buffer (pH 6.0–7.5), 100 mM Tris–HCl buffer (pH 7.5–8.5) and 50 mM CHES buffer (pH 8.5–10.0). The reaction was taken under 45 °C with *p*-nitrophenyl acetate as the substrate, and the released *p*-nitrophenol was measured under 348 nm (the pH-independent isosbestic wavelength of *p*-nitrophenoxide and *p*-nitrophenol).

The effect of NaCl on PE10 activity was determined with 0–4 M NaCl. The effects of metal ions (Co^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Sr^{2+}, Mn^{2+}, Ni^{2+} and Ba^{2+}) were examined at final concentration of 5 and 10 mM. The effect of chelating agent EDTA was examined at a final concentration of 10 mM. The effects of detergents were examined using 1 % Triton X-100, and 1 % SDS. The effects of organic solvents were examined using isopropanol, acetonitrile, alcohol, methanol, acetone, dimethyl sulfoxide (DMSO) and dimethylformamide (DMF) at a final concentration of 15 %. All the tests were performed using the standard assay, and the activity of the enzyme without additives added in the reaction mixture was defined as 100 %.

Nucleotide sequence accession number

The DNA sequence of PE10 from *Pelagibacterium halotolerans* B2^T was deposited in GenBank under accession number of JF926256.

Results

Sequence analysis

A putative ORF of 840 bp, encoding a protein of 279 amino acids (PE10), was identified from the genome sequence of *Pelagibacterium halotolerans* B2^T. Blastp of the translated protein sequences showed maximum identity (49 %) with the alpha/beta hydrolase from *Sphingomonas wittichii* RW1 (Table 1).

We constructed a phylogenetic tree with deduced amino acid sequence of lipolytic enzymes from family I–VIII and six proteins that showed similarity to PE10 (Arpigny and Jaeger 1999; Nierman et al. 2001; Oh et al. 2009; Silby et al. 2009; Lauro et al. 2009; Jiao et al. 2010; Miller et al. 2010) (Fig. 1). PE10 and its six relatives formed a unique branch in the tree, and they cannot be confidently assigned to the eight known families.

Expression and purification of PE10

For the expression of PE10, we constructed a recombinant plasmid PE10-pET28b(+) and expressed it in *E. coli*

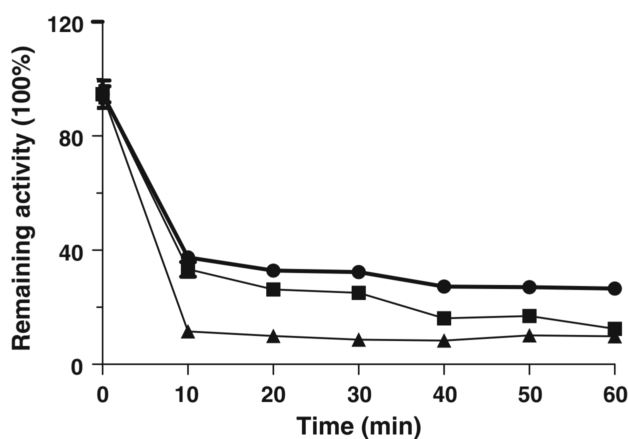


Fig. 5 Thermostability of recombinant PE10. Residual specific activity of PE10 after incubation at different temperatures: 40 °C (*filled circle*) 50 °C (*filled square*) 60 °C (*filled triangle*). Enzymatic assay was performed at 45 °C in 100 mM Tris–HCl buffer (pH 7.5) with *p*-nitrophenol acetate as the substrate

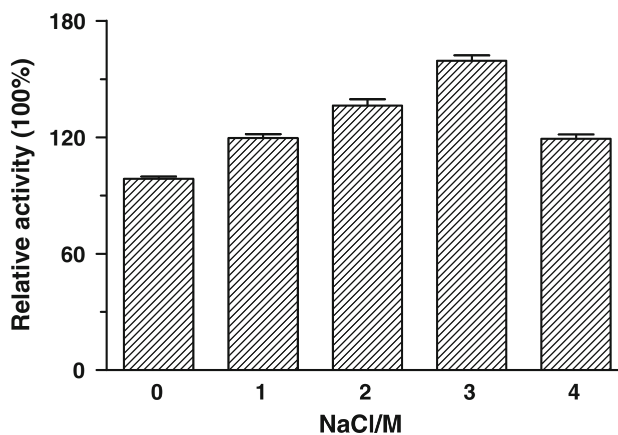


Fig. 6 Effects of NaCl concentration on the activity of PE10. Enzymatic assay was performed at 45 °C in 100 mM Tris–HCl buffer (pH 7.5) with *p*-nitrophenol acetate as the substrate. The value obtained without NaCl in the reaction mixture was taken as 100 %

Rosetta. The molecular weight of PE10 was calculated to be 29.91 kDa (http://www.expasy.org/tools/pi_tool.html). After the purification with Ni–NTA affinity chromatography column, the expected fraction was detected by SDS-PAGE (Fig. 2), and the increased molecular weight of the purified PE10 was due to its fusion with the histidine tag.

Characterization of PE10

The substrate specificity of PE10 was determined using various chain lengths of *p*-nitrophenyl esters (Fig. 3). The maximum hydrolysis activity was obtained with *p*-nitrophenyl acetate (C2) (63.10 ± 2.19 U/mg). The results indicate that PE10 was an esterase for it preferred short-chain *p*-NP esters. But it also showed low activity toward *p*-nitrophenyl myristate (C14) and *p*-nitrophenyl palmitate (C16).

The optimum activity of PE10 was measured over a pH range of 3.0–10.0 and a temperature range of 15–60 °C with *p*-nitrophenyl acetate as the substrate. PE10 showed highest activity at pH 7.5 and 45 °C (Fig. 4). Thermostability analysis of PE10 showed it was unstable and lost

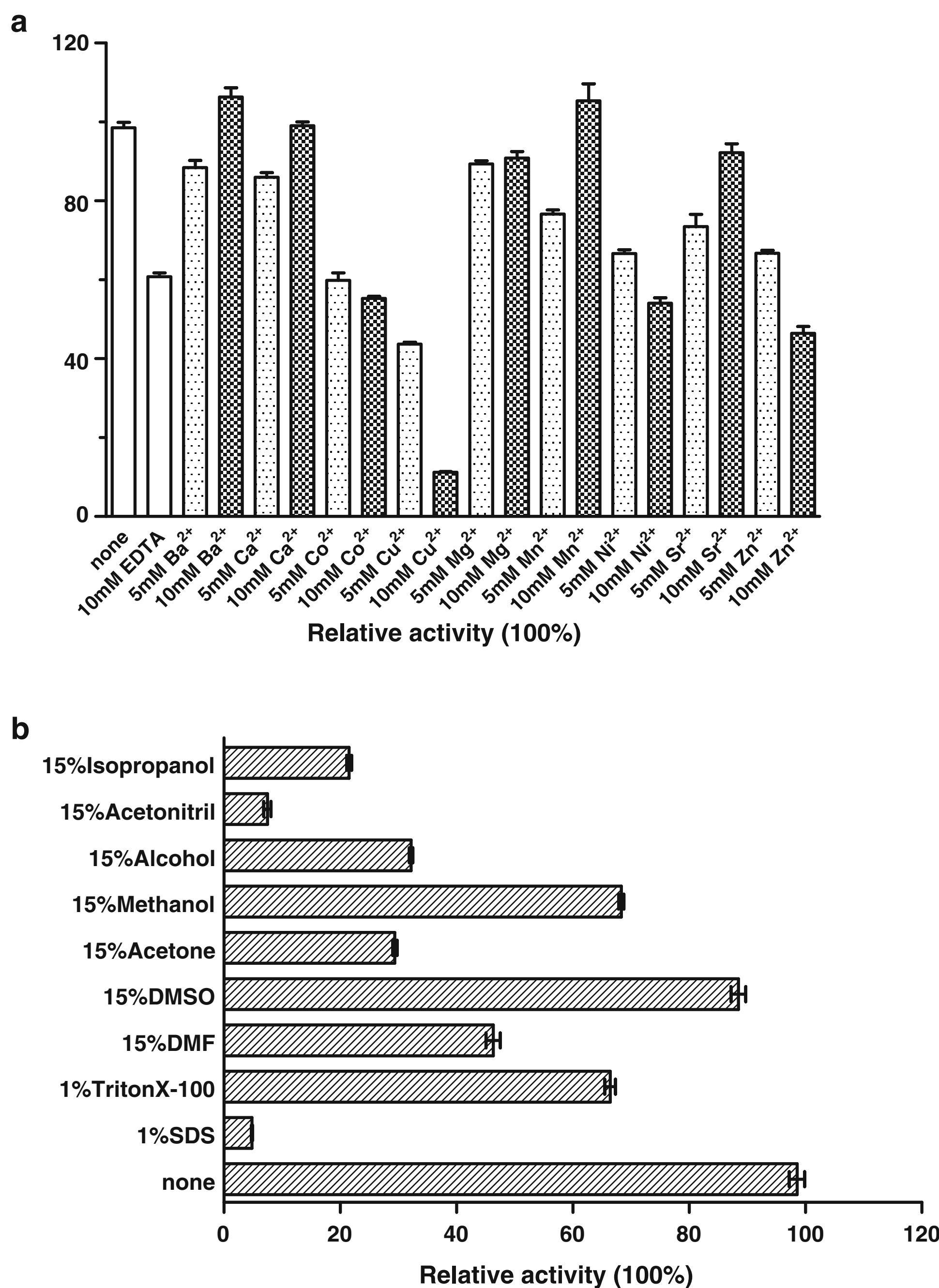


Fig. 7 Effect of metal ions (**a**), detergents and organic solvents (**b**) on the activity of PE10. Enzymatic assay was performed at 45 °C in 100 mM Tris–HCl buffer (pH 7.5) with *p*-nitrophenol acetate as the substrate. The value obtained with no additives in the reaction mixture was taken as 100 %

63 % (40 °C), 67 % (50 °C) and 89 % (60 °C) of its activity in the less than 10 min of incubation (Fig. 5).

The hydrolysis activity of PE10 was increased in the presence of NaCl and showed its maximum activity at 3 M NaCl (Fig. 6). PE10 maintained its activity under most metal ions except for high concentration of Cu^{2+} (Fig. 7a). The addition of 1 % SDS and Triton X-100 inhibited the activity of PE10. The activity of PE10 decreased in 15 % isopropanol, acetonitrile, alcohol, methanol, acetone, dimethyl sulfoxide (DMSO) and dimethylformamide (DMF) (Fig. 7b).

Homology modeling

To get insights into the structure of PE10, a homology model was generated with Robetta server using EST2 (PDB code 1QZ3; 41 % identity) as the template. The alignment of the two proteins was shown in Fig. 8. PE10 harbors the conserved pentapeptide GXSXG and the catalytic Ser139 was located at a sharp turn between strand-β5 and helix-α4, which was known as the nucleophilic elbow (Akoh et al. 2004).

The electrostatic potential of the two proteins was calculated, and the distribution of charges was displayed in Fig. 9. PE10 showed higher negative charges on the surface compared with EST2. This enrichment in negative charges on the surface was found in many proteins in halophilic archaea (Müller-Santos et al. 2009).

Discussion

Marine environment is always the vast pool of novel enzymes. Many studies were focused on the isolation and identification of novel genes and their products from marine bacterium. The traditional way of finding new enzymes from microbes was focused on functional screening of the desired activity. But nowadays, with the development in both experimental technologies and computational analyze tools, complete genome sequencing become more efficient and lower cost. Thus, searching novel genes coding for enzymes directly from genome sequence has become an important way to identify industrial biocatalysts, which was called in silico analysis

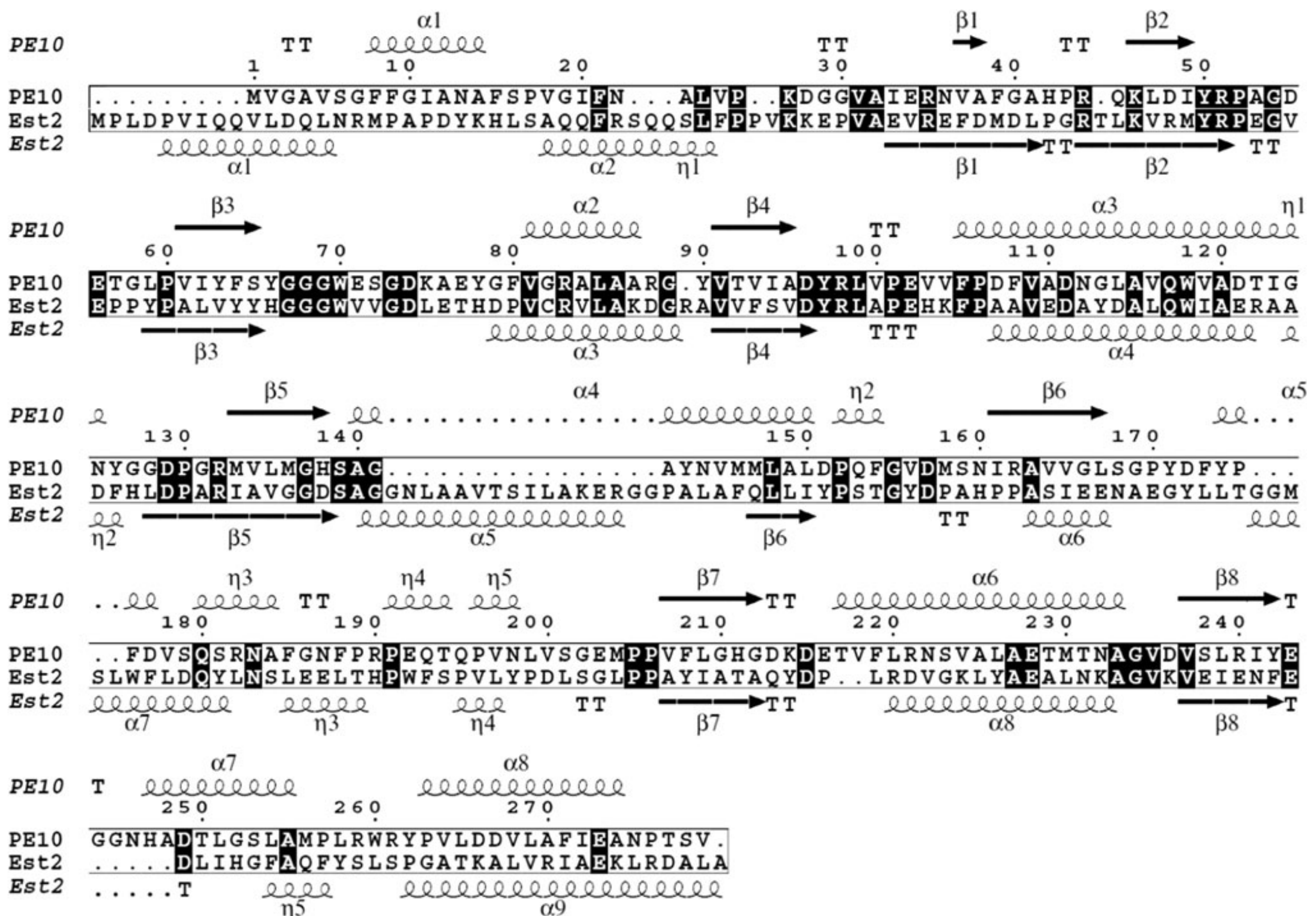


Fig. 8 Sequence alignment between PE10 and EST2. *Symbols* above and under the sequences represented the second structure, *springs* represent helices and *arrows* represent β-strands

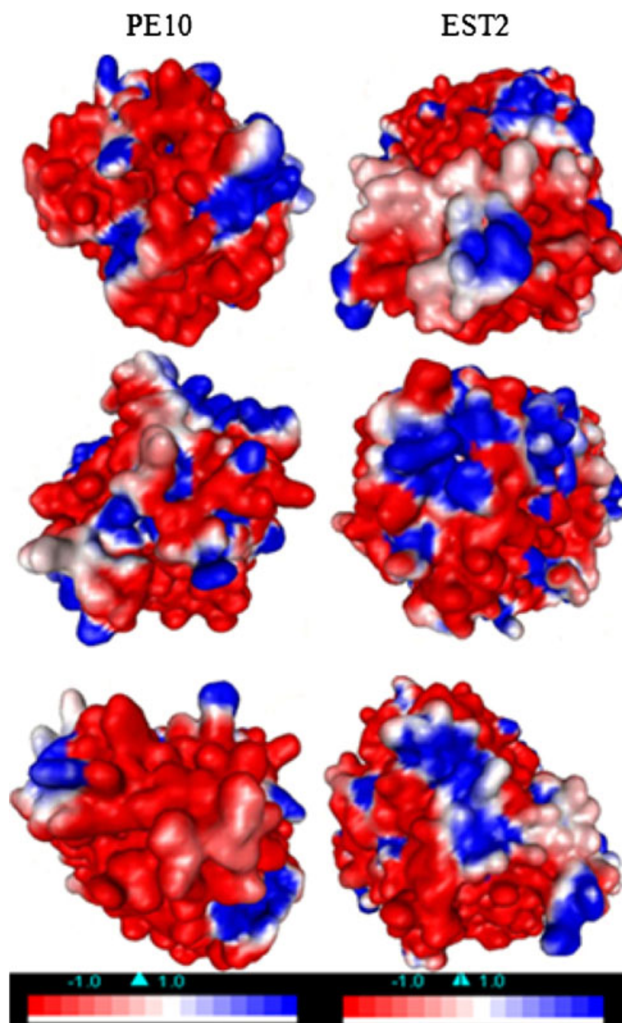


Fig. 9 Surface electrostatic potentials of PE10 and EST2 as obtained using Discovery Studio 2.5 software. The first, second and third row were seen from the *right*, *top* and *front* of the structures, respectively. The *red surface* was corresponded to negatively charged residues and the *blue surface* corresponded to positively charged residues (color figure online)

(Kwoun et al. 2004). By in silico analysis, novel family of lipolytic enzymes with special properties can be found (Pascale et al. 2008). In our study, an esterase (PE10) was identified from a marine bacteria *Pelagibacterium halotolerans* B2^T by in silico genome analysis and followed by cloning, expression and characterization of the protein.

According to the phylogentetic tree constructed by the amino acid sequences with PE10 and other lipolytic proteins, we proposed PE10 and its similar proteins can be classified into a novel family. All the six proteins that are similar to PE10 were from sequenced microbial genomes, thus none of them was characterized. PE10 was characterized biochemically in our study and can be the representation of this group of proteins. The active-site serine residue in a consensus pentapeptide GXSXG of other lipolytic enzymes is still the same in PE10.

The characterization of PE10 revealed its strong tolerance to NaCl. It showed activity from 0 to 4 M NaCl and was most active under 3 M NaCl. This unique property suggested PE10 is a halotolerant enzyme. But unlike most halophlic enzymes of halophilic archaea which are inactive under low salt concentrations (Cao et al. 2008), PE10 was still active without NaCl. *Pelagibacterium halotolerans* B2^T was isolated from sea water and can grow under 0–13 % (0–2.22 M) NaCl (Xu et al. 2011), so it is reasonable that its enzymes were halotolerance. Halophilic archaea establish an osmotic balance with their high salt environment by maintaining internal levels of salt that are isotonic with the exterior (Kushner 1988). Thus the enzymes of the halophilic archaea actually require this high concentration of salt for function and stability. However, the halotolerant bacteria do not work in this way. Instead, halotolerant bacteria accumulate low molecular weight organic solutes to effect osmoregulation (Danson and Hough 1997). Then the enzymes from these halotolerant bacteria still exhibit activity under low salt environment, like PE10 in this study.

To know more about the halotolerance property of PE10, we try to get some information from the structure of PE10. With 41 % identity to PE10, EST2 was chosen for homology modeling. EST2 is a thermostable esterase from the thermophilic eubacturium *Alicyclobacillus acidocaldarius* and it displays an optimal temperature at 70 °C toward *p*-nitrophenyl esters with an acyl chain of six to eight carbon atoms (De Simone et al. 2004). As many studies reveal that the abundance of negative charges on the surface is thought to enable the stabilization of the structure of halophilic proteins in high salt concentrations (Müller-Santos et al. 2009), we analyzed the electrostatic potential of PE10 and EST2. Comparing with EST2, a non-halotolerant esterase, PE10 showed higher negative charges on the surface. That conformed the haloterance of PE10 from the structure aspect.

PE10 showed activity at low temperature. It can maintain almost 26 % relative activity under 15 °C. The low temperature in the sea water, where *Pelagibacterium halotolerans* B2^T was isolated may contribute to this property (Xu et al. 2011).

The substrate specificity experiment revealed that PE10 was an esterase, as this enzyme preferred short-chain acylglycerols and had very low ability to hydrolyze long-chain acylglycerols (C14 and C16). In addition, PE10 was stable under many metal ions. Its activity was increased by high concentration (10 mM) of Ba^{2+}, Ca^{2+} and Mn^{2+}, and it can maintain more than 50 % of activity under most of the ions except for 10 mM Cu^{2+}. PE10 was also stable under some organic solvents and detergent by maintaining more than 50 % of relative activity (88 % under 15 % DMSO, 68 % under 15 % methanol and 66 % under 1 %

Triton X-100). With all these properties, we can suggest that PE10 is a potential candidate for industry use.

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