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Identification of Novel Esterase from Metagenomic Library of Yangtze River

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A metagenomic library of surface-water microbes from the Yangtze River in China was constructed, and a novel esterase, designated as EstY, was isolated and characterized. EstY had 423 amino acids with an estimated molecular mass of 44 kDa and pI of 7.28. It hydrolyzed various *p*-nitrophenyl esters (acetate, butyrate, caprate, caprylate, laurate, myristate, and palmitate) and its best substrate was *p*-nitrophenyl caprate (C8). The optimum pH for EstY activity was 9.0 and the optimum temperature was 50°C. Metal ions, such as Mn^{2+}, Co^{2+}, Hg^{2+}, Zn^{2+}, and Fe^{3+}, strongly inhibited the activity of EstY, whereas Mg^{2+} was required for maximal activity. Activity remained in the presence of 10% alcohol, acetone, isopropanol, and dimethyl sulfoxide, respectively. An analysis of the amino acid sequence deduced from *estY* revealed that it had 7 closely related lipolytic enzymes. Moreover, a sequence analysis showed that EstY, like its 7 relatives, did not belong to any known lipolytic enzyme family.

Keywords: EstY, freshwater metagenome, lipolytic enzyme family, phylogenetic tree

The identification of novel enzymes from microorganisms is currently limited, as less than 1% of environmental microorganisms can be cultured using conventional cultivation methods [20]. However, metagenomic screening is an efficient method that can circumvent the limitations of cultivation for identifying genes encoding novel enzymes and secondary products with industrial or pharmacological value from different environments, such as soil [9, 16, 23], water [5, 19], and other environments [21]. Thus, various novel enzymes have already been identified from diverse environments based on this biotechnology, such as amylases [27], alcohol oxidoreductase [13], chitinase [3], and nitrilases [22].

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Lipolytic enzymes are an important type of enzyme that originates from various organisms, especially fungi and bacteria [1]. It has already been established that lipolytic enzymes can catalyze the hydrolysis and synthesis of ester compounds, and are mainly divided into lipases (E.C. 3.1.1.3) that hydrolyze long-chain acylglycerols (≥10), and esterases (E.C. 3.1.1.1) that hydrolyze short-chain acylglycerols (≤10) [7, 11]. Therefore, lipolytic enzymes have long been of interest to the pharmaceutical and food industries, owing to their versatile biochemical characteristics. So far, bacterial lipolytic enzymes have been classified into eight families; namely, true lipase, GDSL, hormone-sensitive lipase (HSL), and families III, V–VIII [12, 26].

However, lipolytic enzymes have been identified from microorganisms from different environments [4, 7, 9, 14, 21]. Accordingly, this study isolated and characterized EstY from a metagenomic library of surface-water microbes from the Yangtz River in China, and proposes that EstY represents a novel bacterial lipolytic enzyme family.

Materials and Methods

Library Construction and Screening

To construct the metagenomic library, microbes were collected from surface water from the Yangtze River (30°30'11.30 N, 117°04'07.70 E). The water was filtered through 8-μm nitrocellulose membranes to remove any sand and through 0.22-μm nitrocellulose membranes. The bacterial cells were then collected from the surface of the 0.22-μm nitrocellulose membranes using a 1×TE buffer, resolved in 1% low-melting-temperature agarose (Sigma-Aldrich, U.S.A.), and immediately injected into a mold (CHEF-DRIII system; Bio-Rad) to form agarose plugs. The plugs were extruded into 10 ml of a lysis buffer (10 mM Tris-Cl, pH 8.0, 50 mM NaCl, 0.1 M EDTA, 1% Sarkosyl, 0.2% sodium deoxycholate, and 1 mg of lysozyme per ml) and incubated at 37°C for 1 h, and then transferred into 20 ml of an ESP buffer (1% Sarkosyl, 1 mg of proteinase K per ml in 0.5 M EDTA) and incubated at 55°C for a further 16 h. Thereafter, the solution was replaced with a fresh ESP buffer and incubated at 55°C for an additional hour [24]. To inactive the proteinase K, the plugs were transferred into 50 mM EDTA

containing 1% PMSF at 4°C for an hour and stored in 50 mM EDTA at 4°C for the following experiments. The environmental genomic DNA was electroeluted from the plugs into a 1×TAE buffer at 110 V for 3 h. The extracted high-molecular-weight DNA was then partially digested with HindIII (TaKaRa, China). The DNA fragments (40–70 kb) were size-fractioned using pulse-field gel electrophoresis CHEF-DR II (Bio-Rad, U.S.A.) and ligated with HindIII-digested pIndigoBAC5 (Epicentre, U.S.A.). Thereafter, the ligation was electrotransformed into *E. coli* EPI300 (Epicentre, U.S.A.) according to the manufacturer's protocol (Epicentre, U.S.A.). The BAC clones were placed in 384-well plates with a freezing medium (LB+10% glycerol), incubated for 18 h, and stored at −80°C. To screen for lipolytic activity, the BAC clones were placed on LB-agar plates containing 1% tributyrin and 12.5 μg/ml chloramphenicol, and incubated at 37°C for up to 3 days. Lipolytic activity was detected by the formation of clear zones around the colonies [19]. The activity of positive clones was reconfirmed by extraction of their BAC plasmids, retransformation, and repeated screening.

Subcloning and Sequencing

Subcloning was conducted to identify the genes responsible for the lipolytic activity. In detail, the BAC plasmid was extracted from the positive clones and partially digested by Sau3AI (TaKaRa, China). About 100 ng of a 2–5 kb DNA fragment was selectively recovered through gel purification and ligated with 10 ng BamHI-digested pUC19 (TaKaRa, China). The ligation was then transformed into *E. coli* DH5α. The screening of the subclone library for lipolytic activity was conducted as described above. Sequencing of the insert in the transformants with lipolytic activity was conducted, where the sequence was identified using ORF Finder (NCBI) (http://www.ncbi.nlm.nih.gov), and the amino acid sequence of each identified open reading frame (ORF) used to find the closest match with BLAST [19]. All the ORFs containing more than 900 bp were selected for further analysis, as most genes encoding lipolytic enzymes are longer than 900 bp.

Phylogenetic Analysis

The predicted ORFs were compared with known lipolytic genes using PSI-BLAST (http://www.ncbi.nlm.nih.gov). An ORF was identified with a similarity to known enzyme genes and designated as *est*Y. The deduced protein sequence of *est*Y was aligned with representatives from different families of lipolytic enzymes to establish a phylogenetic relationship using MEGALIGN 6.1, ClustalV.

EstY Expression and Enzyme Assay

The gene was amplified using the following forward and reverse primers:

5'-ATA GGA TCC GGA CTC ACC ACA CGG AGC-3' (BamHI site underlined) and 5'-ATA CTC GAG CTT AAC CAA ATC GAA CAG GC-3' (XhoI site underlined), respectively. The PCR product was digested with BamHI and XhoI (TaKaRa, China), ligated into pET-22b (Novagen, U.S.A.) with the same digestion, and transformed into Rossetta (DE3) (Novagen, U.S.A.). The culture was grown until the OD_{600} reached 0.8, and induced with different concentrations (0.001 mM, 0.01 mM, 0.1 mM, and 1 mM) of isopropyl-β-D-thiogalactopyranoside (IPTG) at 16°C for 16 h. The purification of EstY was conducted according to the manufacturer's protocol (Novagen, U.S.A.). The protein concentration was measured using a BCA protein assay kit (Pierce, U.S.A.).

The final concentration of *p*-nitrophenyl esters (acetate, butyrate, caprate, caprylate, and laurate dissolved in 2-propanol) (Sigma-Aldrich, U.S.A.) in a reaction mixture (50 mM Tris-Cl, pH 8.0) and *p*-nitrophenyl esters (myristate and palmitate dissolved in acetonitrile) (Sigma-Aldrich, U.S.A.) in a reaction mixture (0.1% gum arabic, 0.2% deoxycholate, and 50 mM Tris-Cl, pH 8.0) was 0.8 mM. The production of *p*-nitrophenol was measured at 410 nm using a DU730 spectrophotometer (Beckman, U.S.A.).

The optimum pH for EstY activity was measured at 50°C using *p*-nitrophenyl caprate as the substrate. The assay was carried out at pHs ranging from 5.6 to 10.6. The optimum temperature was measured from 20 to 80°C in 50 mM Tris-Cl (pH 9.0) with the same substrate. The effects of metal ions and inhibitors were monitored after incubating EstY in 50 mM Tris-Cl (pH 9.0) at 50°C for 60 min using the same substrate, as was the stability of EstY in 1% (v/v) detergents, 10% (v/v) organic solvents, and 10 mM EDTA.

Results

Library Construction and Screening

Approximately 8,000 white transformants were obtained with inserted DNA ranging from 40 kb to 70 kb, and the average insert DNA size was 50 kb. Additionally, about 100 blue transformants were obtained as a result of self-ligation. One clone with lipolytic activity was detected. Therefore, the plasmid in this clone was extracted and the ~50 kb insert in the plasmid subcloned.

Subcloning and Sequencing

The subcloning of the insert produced ~1,000 clones with an average insert of ~3 kb. One clone showing strong lipolytic activity was selected, and its insert sequenced and assembled into a contig of 5,100 bp. An ORF finder (NCBI) analysis suggested three ORFs with more than 900 bp (Fig. 1). Among them, one 1,269-bp ORF was identified as a possible esterase/lipase-encoding gene (designated as *est*Y) according to the results of a PSI-BLAST search. In addition, the sequence around Ser (190) was Gly (188) - Tyr (189) - Ser (190) - Gln (191) - Gly (192) (Fig. 2), which is consistent with the characteristic G-X-S-X-G motif (X being any amino acid) conserved in lipolytic enzymes. This motif is also found in the active site of α/β hydrolase fold enzymes [1, 2, 12].

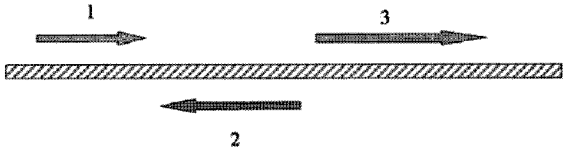

Fig. 1. Lipolytic subclone containing genes encoding esterase and other putative ORFs from a freshwater metagenomic library. 1. Putative dehydrogenase (331–1,299 bp); 2. Esterase (EstY) (1,479–2,747 bp); 3. Putative penicillin-binding protein (3,044–4,330 bp).

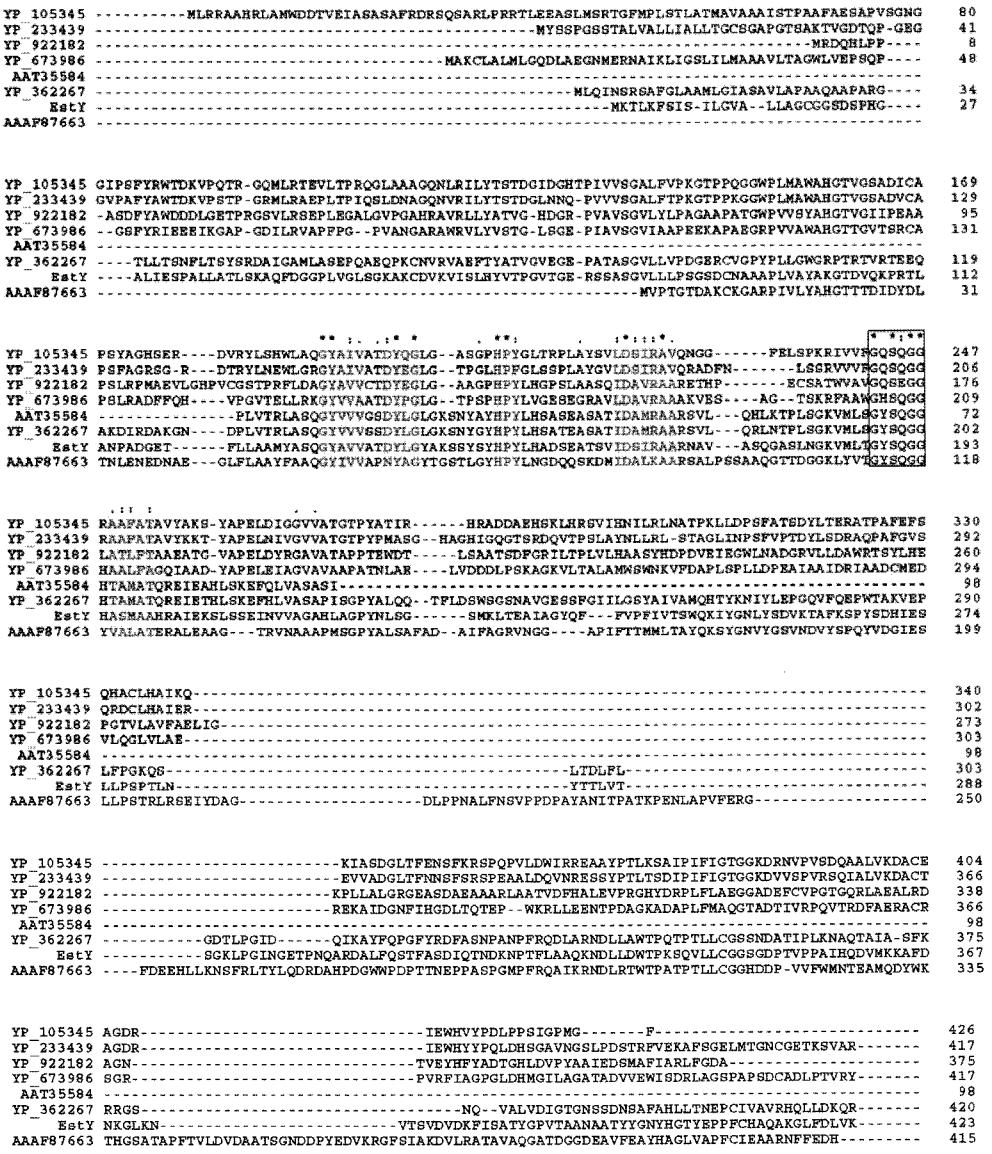

Fig. 2. Multiple sequence alignment of EstY and other closely related lipolytic enzymes using amino acid sequence analysis. These sequences share a conserved motif, G-X-S-X-G, containing a serine residue located at the putative active site (black block). The conserved motifs are shaded light grey.

Table 1. List of lipolytic enzymes in the newly proposed lipolytic enzyme family.

Protein	Source	GenBank Accession No.	%Identity/similarity	E-value	Reference
EstY	Metagenome	EU307510			This study
Putative secreted lipase	*Xanthomonas campestris* pv. *vesicatoria* str. 85-10	NC_007508	38/54	2e-52	[25]
Esterase	Metagenome	AF223646	31/46	5e-35	[8]
Lipase/esterase	*Xanthomonas oryzae* pv. *oryzae*	AY627913	55/71	8e-21	Unpublished
Secretory lipase	*Mesorhizobium* sp. BNC1	NC_008254	32/46	5e-09	Unpublished
Secretory lipase	*Pseudomonas syringae* pv. *syringae* B728a	NC_007005	32/50	5e-08	[6]
Secretory lipase family protein	*Burkholderia mallei* ATCC 23344	NC_006349	33/48	4e-08	[17]
Secretory lipase	*Nocardioides* sp. JS614	NC_008699	35/46	1e-08	Unpublished

All the proteins, sources, and GenBank accession numbers were identified by searching GenBank. The names of the proteins and bacterial strains are given as originally designated by the authors.

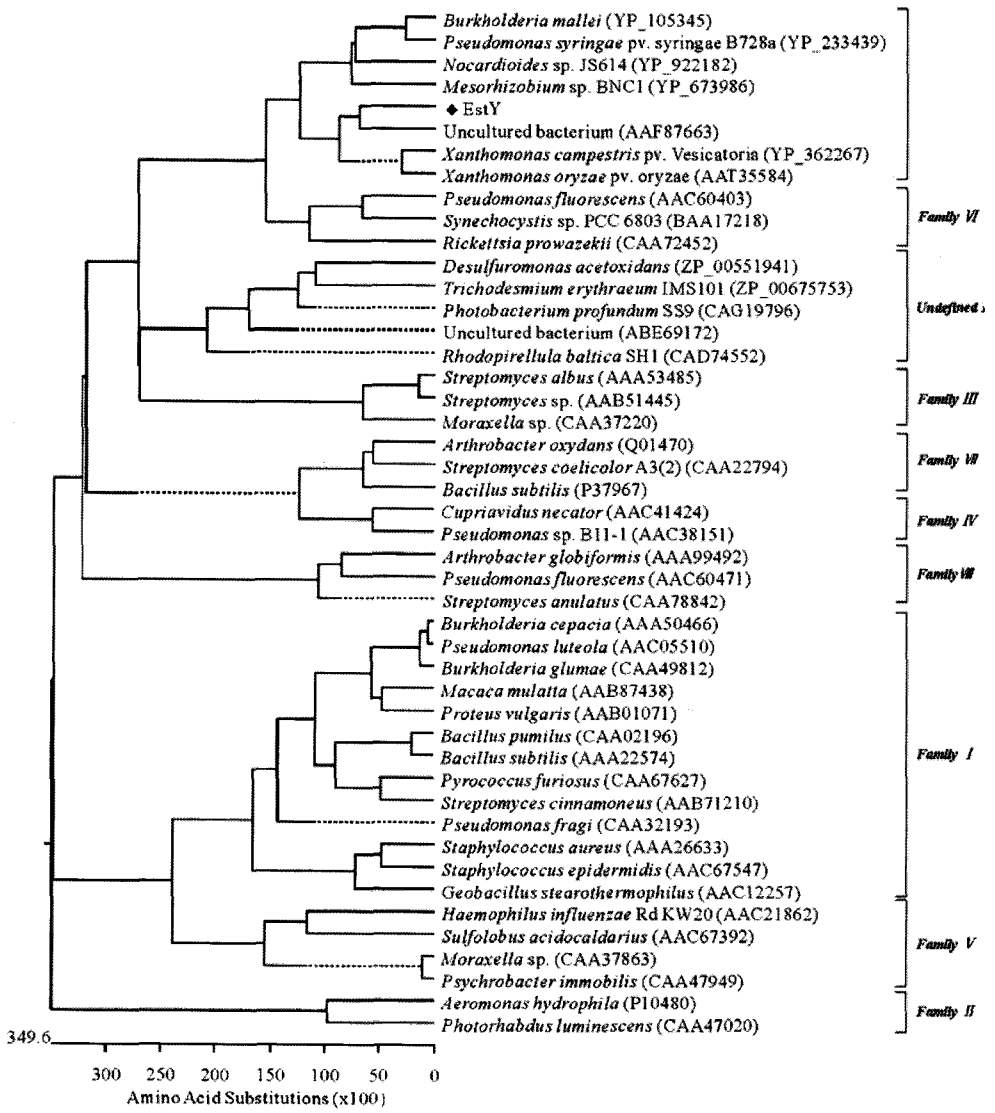

Fig. 3. Phylogenetic analysis of EstY and other lipolytic enzymes using ClustalV method.
The phylogenetic analysis was performed using MEGALIGN 6.1 (DNASTAR, Madison, WI, U.S.A.). The amino acid sequences for all previously identified families of bacterial lipolytic enzymes were retrieved from NCBI (http://www.ncbi.nlm.nih.gov), except for EstY. The units at the bottom of the tree indicate the number of substitutions. The undefined family is the newly proposed lipolytic enzyme family [15].

Phylogenetic Analysis

A PSI-BLAST search of GenBank also revealed that EstY was closely related to 7 lipolytic enzymes (Table 1). In addition, according to the classification of bacterial lipolytic enzymes [1], EstY and its 7 relatives, together with 32 bacterial lipolytic enzymes from 8 families and 6 lipolytic enzymes, appeared to form a new family [15] (Fig. 3). Clearly, EstY and its 7 lipolytic enzymes did not belong to any known lipolytic enzyme family.

EstY Expression and Enzyme Assay

The expression of EstY is displayed in Fig. 4A. When using different IPTG concentrations (0.001 mM, 0.01 mM, 0.1 mM, and 1 mM) to induce expression, EstY was only expressed in the supernatant with 0.001 mM IPTG, whereas the other IPTG concentrations led to the formation of an inclusion body (data not shown). The activity of EstY toward different substrates is showed in Fig. 4B, where one unit was defined as the amount of enzyme required to release 1 μmol *p*-nitrophenol per minute. The K_m value was 0.018 mM when using *p*-nitrophenyl caprate as the substrate. When performing a pH-stat assay with tributyrin and triolein as the substrates, respectively, EstY showed a low activity toward triolein (7% activity toward tributyrin) (data not shown). Thus, despite its ability to hydrolyze long-chain acylglycerols (≥10), EstY was considered to be an esterase.

The enzyme displayed activity at pHs ranging from 6.0 to 10.8, and the optimum pH was 9.0 (Fig. 5A). EstY also

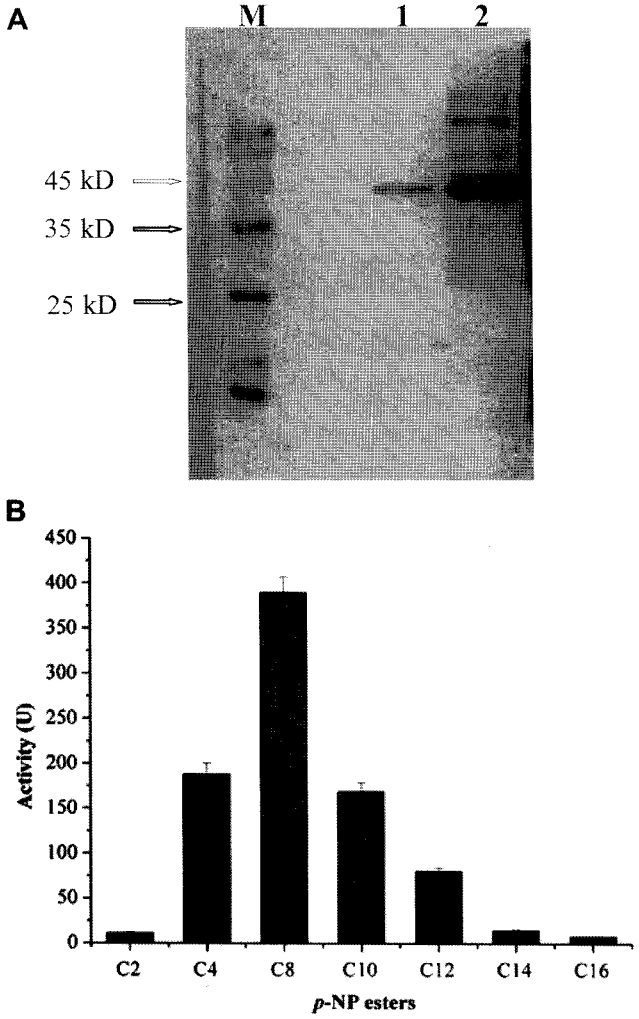

Fig. 4. A. SDS-PAGE of overexpressed and Ni-NTA-purified EstY in *E. coli* Rossetta (DE3).
Lane M, molecular standard; Lane 1, purified EstY; Lane 2, soluble fractions; **B.** Substrate specificity of EstY in 50 mM Tris-Cl, pH 8.0, at 37°C. Different *p*-nitrophenyl esters [acetate (C2), butyrate (C4), caprylate (C8), caprate (C10), laurate (C12), myristate (C14), and palmitate (C16) (Sigma)] were tested. Values are means of results of duplicate experiments.

displayed activity at temperatures ranging from 20°C to 80°C, with an optimum temperature at 50°C. In addition, EstY retained above 40% relative activity between 30°C and 60°C (Fig. 5B).

When testing the stability of EstY in the presence of metal ions, solvents, detergents, and EDTA, respectively, its activity was strongly inhibited by Mn^{2+}, Co^{2+}, Hg^{2+}, Zn^{2+}, and Fe^{3+}, yet enhanced by Mg^{2+} (Fig. 5C). Furthermore, the activity of EstY was decreased up to 50% with the addition of 10 mM EDTA and almost lost in detergents. Therefore, these results indicate that Mg^{2+} was required for maximal activity. Although the activity of EstY did not decrease in 10% alcohol, 10% acetone, or 10% isopropanol, it decreased up to 80% in 10% acetonitrile and 10% methanol (Fig. 5D).

DISCUSSION

Freshwater habitats harbor a vast and unique microbial diversity, including various unculturable microbes that are different from those found in the sea or other environments. Although many metagenomic libraries have already been constructed from soil, the ocean, and other extreme environments [4, 7, 14, 23], only two freshwater metagenomic libraries have so far been reported [5, 19]. As such, this study identified a novel esterase, EstY, derived from surface-water microbes from the Yangtze River in China.

The optimum pH and temperature for EstY did not correlate with its derived environment, where the average temperature is around 20°C and the pH is ~7. Therefore, these differences suggest that the optimal conditions for enzymes do not necessarily correspond with their derived environments [5]. Noticeably, the activity of EstY in different solvents did not obviously decrease, except in acetonitrile and methanol (Fig. 5D), which is valuable for potential biotechnological applications, such as biodiesel manufacturing [5, 11].

The phylogenetic tree (Fig. 3) revealed that EstY could not be classified into any known lipolytic enzyme family, plus the conserved motifs in EstY and its 7 relatives differed from those in the 8 known families (Fig. 2). Finally, a conserved domain was found in EstY and its 7 relatives that was similar to PFAM 03583. Lipases containing PFAM 03583 are expressed and secreted during the infection cycles of certain pathogens, such as *Candida albicans*, which includes a large number of different lipases that contribute to its persistence and virulence in human tissue [10]. Coincidently, 6 of the 7 relatives of EstY were derived from pathogenic bacteria (Table 1). For example, *Xanthomonas campestris* pv. *vesicatoria* strain 85-10 is a pathogen of many plant species [25], whereas *Burkholderia mallei* ATCC 23344 is a pathogen of many animal species [17]. Therefore, this would seem to suggest that EstY may contribute to the virulence of its native host. To date, all esterases as virulence factors belong to the GDSL family, which displays a Gly-Asp-Ser-(Leu) motif containing the active-site serine residue [1]. However, EstY can not be classified as a member of the GDSL family, as it does not include this motif. Therefore, the evidence suggests that EstY and its relative lipolytic enzymes comprise a new bacterial lipolytic enzyme family. Meanwhile, EstY and its relatives also lack the conserved Asp-His motifs among Ser-Asp-His (Fig. 2), which is a highly conserved catalytic triad in lipolytic enzymes from the α/β hydrolase superfamily [2]. Among the 7 relatives of EstY, only one was characterized from a soil metagenomic library, whereas the other 6 proteins are putative esterases/lipases whose function and structure have not yet been identified. Consequently, based on the phylogenetic analysis and conserved motifs, it is proposed that EstY and the 7

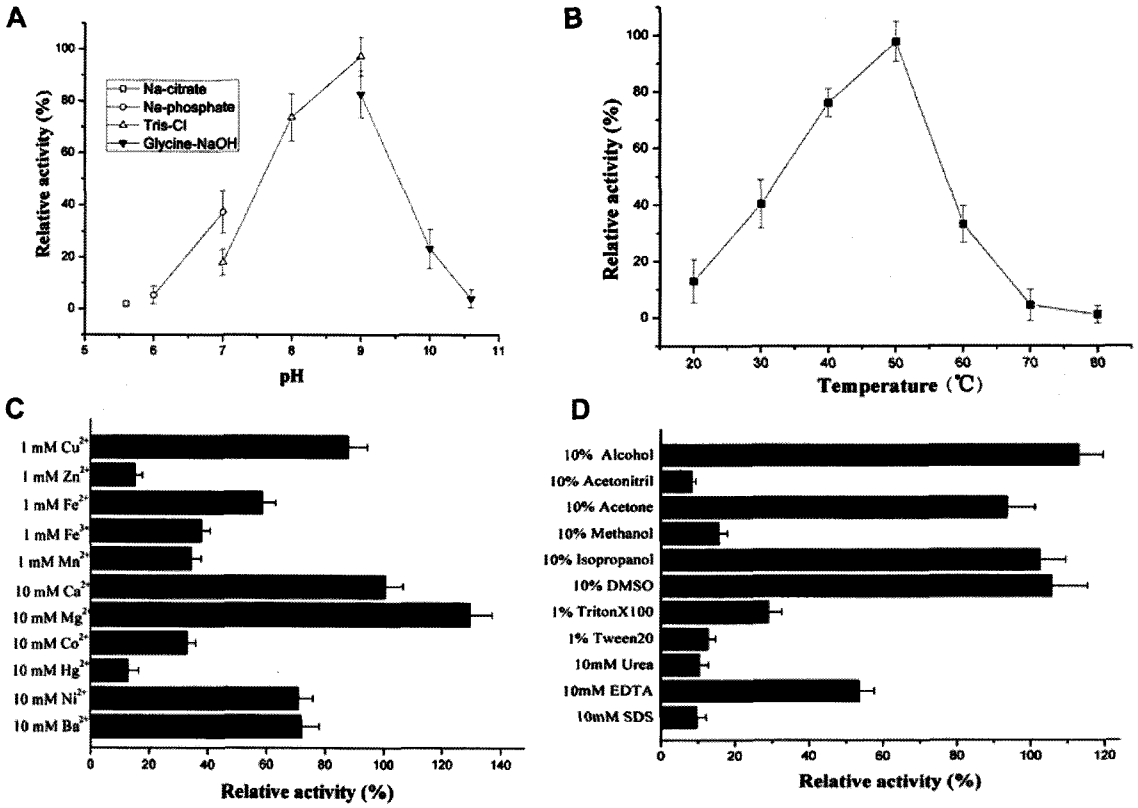

Fig. 5. A. Optimum pH for enzyme activity was measured at 50°C using a spectrophotometric method with *p*-nitrophenyl caprate as substrate. □ Na-citrate (pH 5.6), ○ Na-Phosphate (pH 6–7), △ Tris-Cl (pH 7–9), ▼ Glycine-NaOH (pH 9–10.6). **B.** Optimum temperature for enzyme activity was measured in 50 mM Tris-Cl, pH 9.0, with *p*-nitrophenyl caprate as substrate. **C.** Influence of different metal ions on EstY activity with *p*-nitrophenyl caprate as substrate in 50 mM Tris-Cl, pH 9.0. **D.** Stability of EstY activity with different solvents, detergents, and EDTA when using *p*-nitrophenyl caprate as substrate in 50 mM Tris-Cl, pH 9.0.
Values are means of results of duplicate experiments.

closely related lipolytic enzymes represent a new family of bacterial lipolytic enzymes. This is also the first experimental characterization of this proposed lipolytic enzyme family. Therefore, this work broadens current knowledge on the diversity of bacterial lipolytic enzymes and demonstrates the potential of a metagenomic library as a tool to discover valuable bioproducts from unculturable bacteria.

Nucleotide Sequence Accession Numbers

The nucleotide sequence of EstY has been deposited in the GenBank database under Accession No. EU307510, and the accession numbers for the nucleotide sequences of the other lipolytic enzymes are also from the GenBank database. The description of PFAM 03583 is from CDD in NCBI.

Acknowledgments

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