A novel cold-adapted esterase from *Enterobacter cloacae*: Characterization and improvement of its activity and thermostability via the site of Tyr193Cys

Haofeng Gao¹,², Chanjuan Li³, Ramesh Bandikari³, Ziduo Liu³, Nan Hu²* and Qiang Yong¹*

**Abstract**

**Background:** In industries lipolytic reactions occur in insensitive conditions such as high temperature thus novel stout esterases with unique properties are attracts to the industrial application. Protein engineering is the tool to obtain desirable characters of enzymes. A novel esterase gene was isolated from South China Sea and subjected to a random mutagenesis and site directed mutagenesis for higher activity and thermo-stability compared to wild type.

**Results:** A novel esterase showed the highest hydrolytic activity against p-nitrophenyl acetate (pNPA, C2) and the optimal activity at 40 °C and pH 8.5. It was a cold-adapted enzyme and retained approximately 40% of its maximum activity at 0 °C. A mutant, with higher activity and thermo-stability was obtained by random mutagenesis. Kinetic analysis indicated that the mutant Val29Ala/Tyr193Cys shown 43.5% decrease in *K*<sub>m</sub>, 2.6-fold increase in *K<sub>c</sub>* and 4.7-fold increase in *K<sub>c</sub>/K<sub>m</sub> relative to the wild type. Single mutants V29A and Y193C were constructed and their kinetic parameters were measured. The results showed that the values of *K*<sub>m</sub>, *K<sub>c</sub>* and *K<sub>c</sub>/K<sub>m</sub> of V29A were similar to those of the wild type while Y193C showed 52.7% decrease in *K*<sub>m</sub>, 2.7-fold increase in *K<sub>c</sub>* and 5.6-fold increase in *K<sub>c</sub>/K<sub>m</sub> compared with the wild type. The 3-D structure and docking analysis revealed that the replacement of Tyr by Cys could enlarge the binding pocket. Moreover Y193C also showed a better thermo-stability for the reason its higher hydrophobicity and retained 67% relative activity after incubation for 3 h at 50 °C.

**Conclusions:** The superior quality of modified esterase suggested it has great potential application in extreme conditions and the mutational work recommended that important information for the study of esterase structure and function.

**Keywords:** Random mutagenesis, Site-direct mutagenesis, Enzymatic activity, Thermo-stability

---

**Background**

Esterases are ubiquitous enzymes widely distributed in plants, animals and microorganisms, and they represent a diverse group of hydrolases catalyzing the cleavage and formation of ester bonds [1]. In contrast to lipases, esterases hydrolyze soluble fatty acid esters without any interfacial activation [2] and display a typical Michaelis–Menten behavior [3].

In recent years, esterases have been widely used in food production, detergent products, pharmaceuticals, perfumes, degradation of pollutants and the synthesis of optically pure compounds [4, 5] owing to their broad array of substrate specificity and versatility in the reactions they catalyze [3]. Esterases with novel properties have a more and broad application prospect: cold-adapted esterases are applied to the industrial reaction at low temperature and benefit energy conservation [6–8]; salt-tolerant esterases are suitable for the reactions under...
a high salt concentration [9–11]; organic solvent-tolerant esterases/lipases are necessary for the substrates which are insoluble to water and the trans-esterification reaction in biodiesel production [5, 12, 13]. Nevertheless, there have been only a few reports available about these esterases with novel properties, implying the necessity to find more novel esterases.

Enhancing enzymatic activity of an esterase was also an important for versatility. According to previous reports, many mutations are discovered by screening functions such as activity and thermal stability [14–17]. All information of mutations can facilitate the discovery of an esterase with more application value.

In this study, a gene of a novel esterase, Lip, was cloned from the marine bacterium Enterobacter cloacae and expressed in Escherichia coli (E. coli). The esterase showed high hydrolytic activity at low temperature and tolerance to organic solvents. By random mutagenesis, we obtained a double mutant with enhanced activity, V29A/Y193C, and single mutants V29A and Y193C were individually obtained by site-directed mutagenesis. Kinetic analysis of V29A/Y193C, V29A and Y193C suggested that decrease of $K_m$ and increase of $K_{cat}$ are only attributed to Y193C. Besides, Y193C showed better thermal stability than the wild type.

Results

Cloning and sequence analysis

The esterase gene, Lip, was successfully cloned from E. cloacae with a length of 921 bp encoding 306 amino acid residues with a calculated molecular mass of 33.9 kDa. No signal peptide in this sequence was predicted by the SignalP4.1 Server. The catalytic triads and conserved motifs of Lip were displayed by the multiple sequence alignment with a new thermophilic and thermostable carboxylesterase Este1 [PDB: 2C7B_A] from a metagenomics library (identity: 31%) and a thermophilic carboxylesterase Este2 [PDB: 1EVQ_A] from Alicyclobacillus acidocaldarius (identity: 32%). The catalytic triads consisted of Ser 153, Asp 232 and His 277, and the conserved motif was Gly151-X-Ser153-X-Gly155 (Fig. 1a).

Screening of random mutant library and reverse mutation

The mutant library was screened by high-throughput screening and we acquire the 8000 clones, consequently ensure the activity of each one and the average mutational rate was shown more than 50%. Among them a mutant V29A/Y193C displayed higher catalytic efficiency than the wild type. To investigate the effect of each site, two single site mutants V29A and Y193C were constructed and analyzed.

Expression and purification of Lip and mutants

The protein Lip and mutants were expressed successfully and purified by the removal of GST Tag. The size (~33.9 kDa) was detected by SDS-PAGE and consistent with the value predicted form the deduced amino acid sequence (Fig. 1b).

Substrate specificity

Lip showed the maximum hydrolytic activity against p-NP acetate (C2), slight activity toward C4, C6, C8 and C12, and no activity toward p-NP palmitate (C16). The results indicated that Lip is esterase rather than lipase (Fig. 2a). The mutant Y193C was also similar results as the wild type (Additional file 1: Figure S1).

Biochemical characterization of Lip and mutants

The activity of Lip and mutants was the highest at 40 °C, decreased radically at the temperature above 50 °C, and maintained 40–50% relative activity at 0 °C (Fig. 2b). The data indicated that Lip was a cold-adapted esterase. V29A/Y193C showed the highest activity at pH 9.0 while Lip, V29A, Y193C showed the optimal activity at pH 8.5 (Fig. 2c). Y193C performed better in thermal stability than Lip when the temperature was less than 50 °C, retaining 67% relative activity after incubation for 3 h at 50 °C compared to 44% for the wild type. Both Lip and Y193C retained more than 50% of the maximum activity after incubation at the temperature below 50 °C for 3 h and lost activity in 30 min at 55 °C (Fig. 2d).

The effect of metal ions and reagents on activity was shown in Fig. 3a and b. Five mMNa⁺ inhibited the activity of Y193C (a decrease of 40%), but showed no effect on Lip. The activity of Lip was slightly inhibited by Mg²⁺, K⁺, Ba²⁺ and Sr²⁺, which was similar to that of Y193C. The activities of both Lip and Y193C were strongly inhibited by Mn²⁺ (retaining less than 50%) and EDTA (retaining less than 5%), and were completely undetectable by Cu²⁺. DTT could stimulate the activity of Lip (an increase of 38%), but inhibit the activity of Y193C (a decrease of 20%).

Lip presented better stability than Y193C in 10% of methanol, ethanol and acetonitrile, but Y193C showed better stability in 10% isopropanol (73% relative activity) and higher concentration of DMSO (80% relative activity). Both Lip and Y193C showed no activity in butyl alcohol, Tween-20, Tween-80, and SDS (Fig. 3c, d).

Kinetic parameters

Kinetic parameters of Lip and mutants were determined under optimal conditions (Table 1). The mutant V29A/Y193C showed a 43.5% decrease in $K_m$, a 2.6-fold increase in $K_{cat}$, and a 4.7-fold increase in $K_{cat}/K_m$. V29A
showed a similar value of $K_m$, $K_{cat}$, and $K_{cat}/K_m$ to that of the wild type. Y193C showed 52.7% decrease in $K_m$, a 2.7-fold increase in $K_{cat}$ and a 5.6-fold increase in $K_{cat}/K_m$. The results indicated that the site Y193C, rather than V29A, played a significant role in the increased catalytic efficiency of mutant V29A/Y193C.

**Homology modeling**

The 3-D models of Lip and mutants were constructed based on an alpha/beta hydrolase enzyme [PDB: 5JD4_A] from the metagenome of Lake Arreo (33.77% identity), and the structural features of Lip are shown in more detail (Fig. 4). To explore the effects of Y193C on catalytic activity, docking analysis was performed based on the homology model. It can be seen that Y193C had a larger binding pocket (Fig. 5) because of the replacement of tyrosine by cysteine, and the distance between residue 193 and residue 211 increased from 5.44 to 10.29 Å while the distance between residue 193 and residue 186 increased from 5.4 to 7 Å (Fig. 6).

**Discussion**

In this study, a novel esterase gene was successfully cloned from *E. cloacae*, expressed in *E. coli* BL21 and the protein was purified. Lip showed the catalytic activity of short chain substrate and the highest catalytic activity of p-nitrophenyl acetate (C2), revealing that Lip was an esterase instead of a lipase.

The characterization of Lip revealed that it was a cold-adapted esterase. Lip could retain approximately 50%
relative activity even at 0 °C and inactivate quickly at 55 °C (Fig. 2). These characteristics were similar to those of other reported cold-adapted esterases. For instance, a cold-adapted EstK cloned from *Pseudomonas mendelii* [18–20]. Phthalate ester hydrolase gene was identified from biofilms of a wastewater treatment plant shows activity at low temperatures [21]. Est10 from *P. pacificensis* showed the highest activity at 25 °C and retained 55% relative activity at 0 °C [22]. The optimal temperature of rEst97 was 35 °C and retained 12% relative activity at 0.5 °C [23]. MHlip displayed optimal activity at 33 °C, and maintained 20% relative activity at 4 °C [24].

The increase of conformational flexibility was usually considered as a significant factor of cold-adaptation, which appears to be obtained by a high ratio of Gly residues: less Pro and Arg and more Ser and Met [25–28]. Similarly, the decrease of the Pro content and the ratio Arg/Arg+Lys could make lipases active at low temperature [29]. Lip has obviously a higher percentage of small amino acids such as Ala (10.5%) and Gly (9.2%) than Pro (3.9%) and Arg (7.2%), which was consistent with previous reports. However, the ratio Arg/Arg+Lys of Lip (0.73) was higher than rEST97 (0.56) and MHlip (0.53), but Lip had better cold-adaptation, which seems to contradict previous report. Additionally, the majority of Arg residues were distributed on the surface of the protein (Fig. 4), and the abundance of charged residues on the surface could enhance conformational flexibility and ability of interaction with the solvent [30].

According to previous reports, mutation plays an important role in increasing catalytic properties [31–34]. In this study, a double mutant with enhanced enzymatic activity was obtained by random mutagenesis with error-prone PCR. To investigate the effect of each site, two single site mutants V29A and Y193C were constructed and analyzed. The result showed that Y193C was a positive mutant. The 3-D structure and docking analysis revealed that the enlargement of
the binding pocket was caused by the replacement of Tyr by Cys (Fig. 5). The replacement of Y193C might remodel the arrangement of residues and change the backbone and side chain, lead to the alteration of the secondary structure and the shape of the binding pocket, and finally change the catalytic activity of enzyme [35]. Besides, Tyr193 is closely located near the binding pocket, and the long amino acid side chain seems to reduce substrate access from the binding to the catalytic cavity, the side chain of Cys was much shorter than Tyr (Fig. 5). For all the reasons above, the distances between residue 193 and residue 211 and between residue 193 and residue 186 increased 4.85 and 1.6 Å respectively (Fig. 6). Accordingly, benefiting from the decrease of the steric hindrance, the substrate could enter the central binding pocket more easily and

Table 1 The kinetic parameters of the wild type and mutants

| Enzyme | Protein (μg/ml) | $K_m$ (mM) | $K_{cat}$ (s$^{-1}$) | $K_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$) |
|--------|-----------------|------------|---------------------|----------------------------------|
| Lip    | 203.41 ± 3.26   | 0.643 ± 0.03 | 9.8 ± 0.09          | 15.24 ± 0.21                     |
| V29A/Y193C | 197.29 ± 2.54   | 0.363 ± 0.03 | 25.8 ± 0.16          | 71.08 ± 1.03                     |
| V29A   | 173.23 ± 2.17   | 0.624 ± 0.05 | 10.2 ± 0.11          | 16.35 ± 0.31                     |
| Y193C  | 183.42 ± 2.91   | 0.304 ± 0.04 | 26.1 ± 0.15          | 85.87 ± 1.62                     |

Data are given as mean values ± S.D. All the assays were performed at the optimal pH and temperature for the protein being studied.
the product could release conveniently, leading to the enhanced catalytic activity of Lip. The better performance of Y193C than the wild type in thermo-stability is probably attributed to the replacement of Tyr by Cys and the replacement may increase the probability of forming a disulfide bond. According to previous reports, Cys could be classified as thermo-labile due to its tendency to undergo deamidation or oxidation at high temperature [36, 37] and protein thermo-stability could be improved by introducing disulfide bond. In addition, thermophilic protein was substantially more hydrophobic [38] because hydrophobic effect was the dominant driving force in protein folding [39]. Therefore, the replacement of Tyr by Cys increased hydrophobicity of Y193C relative to the wild type (Fig. 7), resulting in better thermal stability.

Five mM Na\(^+\) inhibited the activity of Y193C slightly while the enzyme activity was not affected by Mg\(^{2+}\), K\(^+\), Ba\(^{2+}\) and Sr\(^{2+}\). The activities of Lip and Y193C were strongly inhibited by Mn\(^{2+}\) whereas Cu\(^{2+}\) showed no activity. Similarly Mohamed [40] reported the inhibitory effect of Mn\(^{2+}\) on esterases EII and EIII. Al Khudary [41] and Metin [42] results also suggested that EstO and HBB-4 esterase were completely inhibited by addition of Cu\(^{2+}\).
The strongly impact of EDTA on catalytic activity indicate that Lip and Y193C are metal dependent enzymes. The results were consistent with EstO and Hbb-4 esterase, Metin [42] stated that some ions have a structural role rather than being involved in catalytic activity. DTT could stimulate the activity of Lip, but inhibit the activity of Y193C. While the replacement of Tyr by Cys may increase the probability of forming a disulfide bond, the disulfide bond may inhibit the activity of the enzyme.

Both Lip and Y193C showed activity in low concentration organic solvents, suggesting their potential use in organic synthesis, non-aqueous reactions and synthesis of esters [43, 44]. Meanwhile, Y193C retained 80% relative activity in 40% DMSO, whereas Lip maintained only about 29%, implying a wider application of Y193C than the wild type. The result supports the assumption that thermostable proteins tend to have high tolerance to organic solvents [45].
Conclusions
In this study, a novel cold-adapted esterase Lip from *E. cloacae* was successfully cloned and expressed. It showed not only considerable hydrolytic activity at low temperature, but also organic solvent tolerance. Moreover, we obtained a mutant Y193C with enhanced hydrolytic activity and thermostability. These results provide useful information about the relationship between structure and function of esterases.

Methods
*p*-nitrophenyl acetate (*p*NPA, C2), *p*-nitrophenyl butyrate (*p*NPB, C4), *p*-nitrophenyl hexanoate (*p*NPH, C6), *p*-nitrophenyl caprylate (*p*NPC, C8), *p*-nitrophenyl laurate (*p*NPL, C12), and *p*-nitrophenyl palmitate (*p*NPP, C16) were purchased from Sigma-Aldrich (St Louis, MO, USA). Taq DNA polymerases, DNA markers and restriction enzymes were purchased from TAKARA (Dalian, China). T4 ligase was purchased from New England Bio Labs Inc. (Singapore). The primers were synthesized by TSINGKE Co. (Wuhan, China). Gel purification kit and plasmid extraction kit were purchased from AXYGEN (USA). All the other chemicals and buffers used were of high purity and analytical grade.

Strains, vectors and medium
The marine *E. cloacae* strain ZS825 (CCTCC AB2017124) was isolated from the surface seawater in the coastal area of Fujian, China and was grown in HLB (High-salt Luria–Bertani medium, NaCl 2%, peptone 1% and yeast extract 0.5%) with shaking at 180 rpm, 28 °C. *E. coli* strains DH5α and BL21 (DE3) used as hosts for gene cloning and protein expression respectively, were grown in LB (Luria–Bertani medium, NaCl 0.5%) with shaking at 180 rpm, 37 °C. The plasmid pGEX-6P-1 (GE Healthcare, USA) was used as vector for gene cloning and protein expression.

Gene cloning
The primers of gene Lip (Accession No. MF101724) were designed by putative gene from the sequences of *E. cloacae* strain AR_0002 (Accession No. CP018814.1) were shown in (Table 2).

    | Primer                  | Sequence                                           |
    |------------------------|----------------------------------------------------|
    | Lip-F                  | CGCGGGATCCTATGGCACACTGGAAAAGGGTT (with BamHI restriction site underlined) |
    | Lip-R                  | CCGCTCGAGCTCACCCTCGCCCGGCA (with XhoI restriction site underlined)    |
    | Lip-V29A-F             | GCCGAGCAATTGAAGCCACTGAGGCA                             |
    | Lip-V29A-R             | GCCCTAAATGTCGCGAGCCGAGT                               |
    | Lip-Y193C-F            | CCGGTCTTGAAGACTGACCCCTCAAT                           |
    | Lip-Y193C-R            | CAGCTTTCAAGACTGGCCGATGGT                            |

| Table 2 Primers were used for random and site directed mutation |
|---------------------------------------------------------------|
| Lip-F: CGCGGGATCCTATGGCACACTGGAAAAGGGTT (with BamHI restriction site underlined) |
| Lip-R: CCGCTCGAGCTCACCCTCGCCCGGCA (with XhoI restriction site underlined)    |
| Lip-V29A-F: GCCGAGCAATTGAAGCCACTGAGGCA                             |
| Lip-V29A-R: GCCCTAAATGTCGCGAGCCGAGT                               |
| Lip-Y193C-F: CCGGTCTTGAAGACTGACCCCTCAAT                           |
| Lip-Y193C-R: CAGCTTTCAAGACTGGCCGATGGT                            |

The random mutant library of Lip gene was constructed by error-prone PCR [46]. The 50 μl PCR mixture contained 20 ng of the recombinant plasmid pGEX-6P-Lip, 0.2 mM dNTPs, 0.2 mM MnCl2, and 0.4 μM primers containing Lip-F and Lip-R, and 2.5 units of Taq DNA polymerase. The error-prone PCR reaction was carried out under similar conditions to those for Lip gene. The purified PCR products were digested with BamHI and XhoI and then ligated into pGEX-6P-1 with the same digestion system. The recombinant plasmid was transferred into *E. coli* DH5α, and then the cells were spread on LB agar plate containing 100 μg ml−1 ampicillin and incubated at 37 °C.

Screening of library
The colonies were grown on LB plates and picked up with sterile toothpicks. Then the colonies were grown in 96-deep well plates containing 600 μL LB medium with 100 μg ml−1 ampicillin. After incubation at 37 °C for 20 h, the mixture was supplemented with 200 μL LB medium containing 100 μg ml−1 ampicillin, 0.4 mM IPTG and T7 phage, and then incubated for another 6 h at 18 °C. Finally, the supernatant from each well was collected for activity assay.

Site-directed mutagenesis
Fast Mutagenesis System (TRANSGEN BIOTECH, China) was used for site-directed mutagenesis, and two pairs of primers (Table 2) were designed based on the result of the screening of mutant library. The wild-type recombinant plasmid pGEX-6P-Lip was used as a template. The PCR program was: 94 °C for 5 min; 25 cycles of 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s; 72 °C for 10 min. One μl DpnI was added into the PCR products for digesting the template, and after incubation at 37 °C for 1 h, the mixture was transformed into *E. coli* DH5α.
Expression and purification of Lip and mutants

The Lip and mutants were expressed in E. coli BL21, and cells were induced by adding 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) when the optical density of the culture reached 0.6 at OD₆₀₀. After incubation for another 16 h at 18 °C, the cells were collected and then washed twice with PBS buffer (0.8% NaCl, 0.02% KCl, 0.027% KH₂PO₄, 0.142% Na₂HPO₄). Further, the cells were disrupted by French pressure cell treatment and the crude enzyme was obtained by centrifuging at 4 °C. The crude enzyme was purified by Glutathione-Sepharose column (GE Healthcare, USA) and 3C protease (PreScission, Pharmacia) was added to remove GST tag. Finally, the protein was analyzed by SDS-PAGE and determined by the Bradford method [47].

Enzyme activity assay

The esterase activity was determined spectrophotometrically by measuring the production of p-nitrophenol. The reaction mixture (final volume, 200 μl) containing 188 μl of Tris–HCl buffer (50 mM, pH8.5), 2 μl of p-NP ester (10 mM) and 10 μl enzyme was incubated at 45 °C for 10 min, and the reaction without any enzyme was considered as control. The amount of released p-nitrophenol was determined from the optical density at 405 nm.

Substrate specificity

The substrate specificity of Lip was investigated with p-NP esters of different chain lengths. The substrates were p-nitrophenyl acetate (pNPA, C2), p-nitrophenyl butyrate (pNPB, C4), p-nitrophenyl hexanoate (pNPH, C6), p-nitrophenyl caprylate (pNPC, C8), p-nitrophenyl laurate (pNPL, C12), and p-nitrophenyl palmitate (pNPP, C16).

Biochemical characterization of enzyme

The optimal temperature of Lip was determined by incubating the reaction mixture in the temperature range of 0–60 °C.

The thermal stability was determined by measuring the residual activity of the enzyme after incubation at 45, 50 and 55 °C.

The optimal pH was determined from 4.0 to 10.0 (4.0–8.0 pH buffers were prepared with phosphate and citrate, and 8.5–10.0 buffers were prepared with borax and NaOH).

The effect of metal ions Na⁺, Mg²⁺, K⁺, Ba²⁺, Mn²⁺, Cu²⁺ and Sr²⁺; and inhibitory additives (EDTA, DTT) on enzyme activity were determined at the final concentration of 5 mM.

The effect of organic solvents (methanol, ethanol, acetonitrile, isopropanol, butyl alcohol, acetone, DMSO) and detergents (Tween-20, Tween-80, SDS) were examined in different final concentrations (10–40%).

Kinetic parameters

The Km and Kcat of Lip and mutants were determined by measuring the reaction rate in different substrate concentrations (5–400 μM) under optimal conditions. The Vmax and Km were estimated by the Lineweaver–Burk plot method using the Graph pad Prism software (Graph pad, San Deigo, CA). The Kcat was calculated by using the formula Kcat = Vmax/[E].

Homology modeling

The homology model of Lip and mutants was searched by SWISS-MODEL (http://swissmodel.expasy.org/) [48].

Additional file

Additional file 1: Figure S1. Substrate specificity of purified Y193C. The activity towards p-NP acetate (C2) as 100%.

Abbreviations

E. coli: Escherichia coli; C2: p-nitrophenyl acetate (pNPA); C4: p-nitrophenyl butyrate (pNPB); C6: p-nitrophenyl hexanoate (pNPH); C8: p-nitrophenyl caprylate (pNPC); C12: p-nitrophenyl laurate (pNPL); C16: p-nitrophenyl palmitate (pNPP); IPTG: isopropyl-β-d-thiogalactopyranoside; PBS: phosphate buffer saline; GST: glutathione S-transferase.

Authors’ contributions

HG designed and performed the experiment and drafted the manuscript, CL contributed to expression and purification of protein, RB executed to mutational and characterization, ZL contributed to revision of the manuscript. QT and NH are corresponding author, who conceived and supervised the experiments. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Availability of supporting data

All data supporting the conclusions of this article are included within the manuscript.

Consent for publication

All authors have read and approved this manuscript to publish.

Ethics approval and consent to participate

Not applicable.
Ethical statement

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

Funding

This work was supported by grants from National Natural Science Foundation of China (Nos. 31270162 and 3175010465).

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Accepted: 26 February 2018

Published online: 19 March 2018

References

1. Bornscheuer UT. Microbial carboxyl esterases: classification, properties and application in biocatalysis. FEMS Microbiol Rev 2002;26:73–81.

2. De Simone G, Galdiero S, Manco G, Lang D, Rossi M, Pedone C. A snapshot of a transition state analogue of a novel thermophilic esterase belonging to the subfamily of mammalian hormone-sensitive lipase. J Mol Biol 2000;303:761–71.

3. Prim N, Boffil C, Pastor FJ, Díaz P. Esterase EstA6 from Pseudomonas sp. CR-6:1 is a novel member in the utmost conserved family VI bacterial lipolytic enzymes. Biochimie 2006;88:859.

4. Gupta R, Gupta N, Rathi P. Bacterial lipases: an overview of production, purification and biochemical properties. Appl Microbiol Biotechnol 2004;64:763.

5. Rahman MA, Culsum U, Tang W, Zhang SW, Wu G, Liu Z. Characterization of a novel cold active and salt tolerant esterase from Zunongwangia profundal. Enzyme Microbiol Technol 2016;85:1–11.

6. Cavicchioli R, Siddiqui KS, Andrews D, Sowers KR. Low-temperature extremophiles and their applications. Curr Opin Biotechnol 2002;13:253–61.

7. Gerdau C, Attaele B, Mentah M, Chassa JP, Clavene P, Collins T, D’Amico S, Dumont J, Garsoux G, Georlette D, Hoyoux A, Lornhenti T, Meuwis MA, Feller G. Cold-adapted enzymes: from fundamentals to biotechnology. Trends Biotechnol 2000;18:103–7.

8. Margesin R, Schinner F. Properties of cold-adapted microorganisms and their potential role in biotechnology. J Biotechnol 1994;33:1–14.

9. Jiang X, Hua Y, Zhi X. Cloning, expression and characterization of a halotolerant esterase from a marine bacterium Pelagibacter halotolerans B2T. J Clin Microbiol 2012;46:427–33.

10. Oren A. Molecular ecology of extremely halophilic Archaea and Bacteria. FEMS Microbiol Ecol 2002;39:231–41.

11. Sellek GA, Chaudhuri JB. Biocatalysis in organic media using enzymes from extremophiles. Enzyme Microbiol Technol 1999;25:471–82.

12. Alex D, Shainu A, Pandey A, Sukumaran RK. Esterase active in polar organic solvents from the yeast Pseudozyma sp. Nl 08165. Enzyme Res 2014;49:10.

13. Ramesh B, Qian J, Baskaran R, Liu Z, Wu G. Bio-affinity mediated immobilization of lipase onto magnetic cellulose nanospheres for high yield biodiesel in one time addition of methanol. Bioreour Technol 2018;249:354–60.

14. Hang Y, Ran S, Wang X, Xiao J, Wang S, Liu Z. Mutational analysis and stability characterization of a novel esterase of lipolytic enzyme family VI from Shewanella sp. Int J Biol Macromol 2016;93:655.

15. Jiang H, Zhang S, Gao H, Hu N. Characterization of a cold-active esterase from Serratia sp. and improvement of thermostability by directed evolution. BMC Biotechnol 2016;16:7.

16. Kobayashi R, Hirano N, Kanaya S, Saito I, Haruki M. Enhancement of the enzymatic activity of Escherichia coli acetyl esterase by random mutagenesis. J Mol Catal B Enzym 2010;67:155–61.

17. Zhang S. Improved thermostability of esterase from Aspergillus fumigatus by site-directed mutagenesis. Enzyme Microbiol Technol 2014;64:11–6.

18. Boyne I, Kim J, Kang BS, Lee C, Jiang SH. Enhanced catalytic site thermal stability of cold-adapted esterase Estk by a W208Y mutation. Biochim Biophys Acta 2014;1844:1076–82.

19. Tuongvong N, Jang SH, Lee C. Flexibility and stability trade-off in active site of cold-adapted Pseudomonas mandelli esterase Estk. Biochemistry 2016;55:3542–9.

20. Hong S, Lee C, Jang SH. Purification and properties of an extracellular esterase from a cold-adapted Pseudomonas mandelli. Biotechnol Lett 2012;34:1051–5.

21. Jiao Y, Chen X, Wang X, Liao X, Xiao L, Mao A, Wu J, Yang L. Identification and characterization of a cold-active pthalate esters hydrolase by screening a metagenomic library derived from biofilms of a wastewater treatment plant. PLoS ONE. 2013;8:759–77.

22. Wu G, Wu G, Zhan T, Shao Z, Liu Z. Characterization of a cold-adapted and salt-tolerant esterase from a psychrophilic bacterial Psychrobacter pacificensis. Extremophiles. 2013;17:809.

23. Fu J, Leiros HK, de Pascale D, Johnson KA, Blencke HM, Landfeld B. Functional and structural studies of a novel cold-adapted esterase from an Arctic intertidal metagenomic library. Appl Microbiol Biotechnol. 2013;97:3965–78.

24. Berlemont R, Jacquin O, Deslaure M, La Salla M, Georis J, Verte F, Galleni M, Power P. Novel cold-adapted esterase mhlip from an antarctic soil metagenome. Biology. 2013;2:177.

25. Marshall CJ. Cold-adapted enzymes. Trends Biotechnol 1997;15:359–64.

26. Mieltynska I, Krzelewski S, Sztuzczek Z, Dobrowolski J. Metagenomic approach for the isolation of a novel low-temperature-active lipase from uncultulated bacteria of marine sediment. FEMS Microbiol Ecol 2007;59:524.

27. Siddiqui KS, Cavicchioli R. Cold-adapted enzymes. Annu Rev Biochem. 2006;75:403.

28. Zhou XX, Wang YB, Pan YJ, Li WF. Differences in amino acids composition and coupling patterns between mesophilic and thermophilic proteins. Amino Acids. 2008;34:25–33.

29. Joseph B, Ramteke PW, Thomas G. Cold active microbial lipases: some hot issues and recent developments. Biotechnol Adv. 2008;26:457–70.

30. Alquati C, De Gioia L, Bernardoni A, Alberghina L, Fantucci P, Lotti M. The cold-active lipase of Pseudomonas fragi. Heterologous expression, biochemical characterization and molecular modeling. Eur J Biochem. 2002;269:3211–8.

31. Ma BD, Kong XD, Yu HL, Zhang ZJ, Dou S, Xu YP, Ni Y, Xu JH. Increased catalyst productivity in α-hydroxy acids resolution by esterase mutation and substrate modification. ACS Catal 2014;4:1026–31.

32. Dou S, Kong XD, Ma BD, Chen Q, Zhang J, Zhou J, Xu JH. Crystal structures of Pseudomonas putida esterase reveal the functional role of residues 187 and 287 in substrate binding and chiral recognition. Biochem Biophys Res Commun. 2014;446:1145–50.

33. Chen Q, Luan ZJ, Cheng X, Xu JH. Molecular dynamics investigation of the substrate binding mechanism in carboxylesterase. Biochemistry. 2015;54:1841–8.

34. Whangsu W, Sungeereee P, Naksirisak S, Thienmgam S, Mongsri S, Loprasert S. Two endocrine disrupting dibutyl phthalate degrading esterase reveals the functional role of residues 187 and 287 in substrate binding and chiral recognition. Biochem Biophys Res Commun. 2014;446:1145–50.

35. Chen Q, Luan ZJ, Cheng X, Xu JH. Molecular dynamics investigation of the substrate binding mechanism in carboxylesterase. Biochemistry. 2015;54:1841–8.

36. Whangsu K, Sungkereee P, Nakasiri S, Thaiengam S, Mongsri S, Loprasert S. Two endocrine disrupting dibutyl phthalate degrading esterases reveal the functional role of residues 187 and 287 in substrate binding and chiral recognition. Biochem Biophys Res Commun. 2014;446:1145–50.

37. Chen Q, Luan ZJ, Cheng X, Xu JH. Molecular dynamics investigation of the substrate binding mechanism in carboxylesterase. Biochemistry. 2015;54:1841–8.

38. Haney P, Konisky J, Koretke KK, Luthey-Schulten Z, Wolynes PG. Structural mutations better? Trends Biotechnol. 2005;23:171–7.

39. Marshall CJ. Cold-adapted enzymes. Trends Biotechnol 1997;15:359–64.

40. Mieltynska I, Krzelewski S, Sztuzczek Z, Dobrowolski J. Metagenomic approach for the isolation of a novel low-temperature-active lipase from uncultulated bacteria of marine sediment. FEMS Microbiol Ecol 2007;59:524.

41. Al Khudary R, Venkatachalam R, Katzer M, Elleuche S, Antranikian G. A novel cold-adapted lipase from a cold-active psychrophilic bacterium Psychrobacter pacificensis. Extremophiles. 2013;17:809.
42. Metin K, Burcu Bakir Ateslier Z, Basbulbul G, Halil Biyik H. Characterization of esterase activity in Geobacillus sp. HBB-4. J Basic Microbiol. 2006;46:400–9.
43. Klibanov AM. Improving enzymes by using them in organic solvents. Nature. 2001;409:241.
44. Ma BD, Yu HL, Fan J, Liu JY, Ju X, Xu JH. A thermostable and organic-solvent tolerant esterase from Pseudomonas putida ECU1011: catalytic properties and performance in kinetic resolution of alpha-hydroxy acids. Bioreaour Technol. 2013;133:354–60.
45. Shiraki K. Conformational stability of a hyperthermophilic protein in various conditions for denaturation. Electrochemistry. 2001;69:949–52.
46. Cirino PC, Mayer KM, Umeno D. Generating mutant libraries using error-prone PCR. Methods Mol Biol. 2003;231:3.
47. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–54.
48. Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, Kiefer F, Gallo Cassano T, Bertoni M, Bordoli L, Schwede T. SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. Nucleic Acids Res. 2014;42:252.