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BIOCATALYSIS

Isolation and characterization of a thermostable esterase from a metagenomic library

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Abstract A novel esterase gene was isolated by functional screening of a metagenomic library prepared from an activated sludge sample. The gene (*est-XG2*) consists of 1,506 bp with GC content of 74.8 %, and encodes a protein of 501 amino acids with a molecular mass of 53 kDa. Sequence alignment revealed that Est-XG2 shows a maximum amino acid identity (47 %) with the carboxylesterase from *Thermaerobacter marianensis* DSM 12885 (YP_004101478). The catalytic triad of Est-XG2 was predicted to be Ser_{192}-Glu_{313}-His_{412} with Ser_{192} in a conserved pentapeptide (GXSXG), and further confirmed by site-directed mutagenesis. Phylogenetic analysis suggested Est-XG2 belongs to the bacterial lipase/esterase family VII. The recombinant Est-XG2, expressed and purified from *Escherichia coli*, preferred to hydrolyze short and medium length *p*-nitrophenyl esters with the best substrate being *p*-nitrophenyl acetate (K_m and k_{cat} of 0.33 mM and 36.21 s^{-1}, respectively). The purified enzyme also had the ability to cleave sterically hindered esters of tertiary alcohols. Biochemical characterization of Est-XG2 revealed that it is a thermophilic esterase that exhibits optimum activity at pH 8.5 and 70 °C. Est-XG2 had moderate tolerance to organic solvents and surfactants. The unique properties of Est-XG2, high thermostability and stability in the presence of organic solvents, may render it a potential candidate for industrial applications.

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Introduction

Biocatalysts are extensively used in industrial applications. Compared with chemical catalysis, enzyme-catalyzed reactions are generally more selective and run under environmentally friendly conditions with lower energy cost [4]. Lipolytic enzymes including lipases and esterases are one of the most important groups of biocatalysts applied in many industries such as food, detergent, flavors, fine chemical, cosmetic, biodiesel, and pharmaceuticals [21]. Lipases (EC 3.1.1.3) catalyze the hydrolysis and synthesis of long-chain acylglycerols with acyl chain length of more than 10 carbon atoms, with trioleoylglycerol as the standard substrate. However, esterases (EC 3.1.1.1) catalyze the hydrolysis of acylglycerols with acyl chain length of less than 10 carbon atoms, with tributyrin as the standard substrate [30]. Lipolytic enzymes are of great importance in various biotechnological applications on account of their high chemo-, regio-, and stereoselectivity, stability in organic solvents, and no requirement for cofactors [16].

Lipolytic enzymes occur in a broad range of organisms, including plants, animals, and microbes. Microbial lipolytic enzymes are more useful than those of other origins because of their high yields and ease of genetic manipulation [31]. Bacterial lipolytic enzymes have been classified into eight families based on their sequence conservation and biological properties [2]. The search for new lipolytic enzymes will increase the diversity of these enzymes and provide more suitable biocatalysts for industrial demands [8]. Thus, a great deal of efforts has been made to screen and isolate novel lipolytic enzymes

from microorganisms. The traditional method for isolating new lipolytic enzymes is culture-dependent, screening of a variety of isolated microorganisms from nature for the desired activities. However, only a small fraction (<1 %) of microorganisms in various environments are readily cultivable [18]. So, great microbial resources in environments still remain untapped. Fortunately, metagenomic approach has complemented the culture-dependent method which circumvents the cultivation of diverse microorganisms from environmental samples and involves directly accessing the global genomes of organisms in environments. Thus, metagenomic approach is regarded as one of the best methods for searching for new industrial enzymes.

Metagenome-based technology normally involves the isolation of environmental DNA, construction of a library by cloning the DNA into a small or large insert vector, and subsequent high-throughput screening [9]. Two screening approaches have been usually used for discovering the novel genes [9]. One is sequence-based screening which relies on the use of a conserved DNA sequence for designing a specific primer or probe to detect the specific sequences. However, it tends to recover sequences related to known genes and does not select for complete gene sequences [9]. The other is function-based screening, which depends on the detection of a specific phenotype expressed by the gene of interest. The limitation of this method is the difficulty associated with the enzyme production using a heterologous gene expression system because not all proteins can be successfully expressed in a heterologous host [25]. Despite the aforementioned drawbacks, several lipolytic enzymes have been discovered through the metagenomic approach from various environmental samples, including soils [33], ponds [27], lake water [29], hot springs [39], and marine sediments [15]. Some of these enzymes have potential use in biotechnological applications and have broadened our knowledge on the diversity of lipase/esterase families [8].

The wastewater from paper mills, being heavily polluted, needs to be treated to reduce possible impacts on the environment. The main treatment process is primary settlement followed by a biological treatment [38]. The conventional biological treatment is the activated sludge process where complex microorganisms utilize both intracellular and extracellular enzymes to hydrolyze the complicated natural substances in wastewater including lipids, proteins, and other chemicals [5]. Therefore, the activated sludge from paper mills is likely a valuable source for isolating novel biocatalysts. However, no lipolytic gene has been found from an activated sludge sample of a paper mill so far.

In this study, a metagenomic library was constructed using environmental DNA extracted from an activated sludge sample taken from a paper mill. An esterase gene was isolated from this library by a function-based screening method. The deduced esterase was expressed in *Escherichia coli*, purified, and characterized in terms of its substrate specificity, optimum pH and temperature, thermal stability, and organic solvent tolerance.

Materials and methods

Bacterial strains, plasmids, and chemicals

E. coli DH5α and *E. coli* BL21 (DE3) (Novagen, USA) were used as hosts for gene cloning and expression, respectively. Plasmids of pBluescript SK + (Stratagene, USA) and pET-22b (+) (Novagen, USA) were respectively used for metagenomic library construction and gene expression. *E. coli* was routinely grown in Luria–Baterni (LB) medium at 37 °C, supplemented with ampicillin (100 μg/ml). The kits for plasmid extraction and gel extraction were purchased from Omega (USA). Taq DNA polymerase, T4 DNA ligase, restriction endonucleases, and alkaline phosphatase were bought from TaKaRa (Dalian, China).

Substrates of tributyrin, *p*-nitrophenyl (*p*-NP) acetate (C2), butyrate (C4), hexanoate (C6), caprylate (C8), decanoate (C10), laurate (C12), myristate (C14), and palmitate (C16) were purchased from Sigma–Aldrich (USA). All other chemicals used were of analytical grade and were commercially available from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Metagenomic library construction and screening

An activated sludge sample was collected from a paper mill located in Xiaogan City, Hubei Province, P. R. China. Metagenomic DNA was isolated according to Zhou et al. [40] with minor modification. The sample was centrifuged at 12,000 rpm for 10 min. The pellet of 5 g was mixed with 13.5 ml of DNA extraction buffer (100 mM Tris–HCl, 100 mM sodium EDTA, 100 mM sodium phosphate, 1.5 M NaCl, 1 % CTAB) and 100 μl of proteinase K (10 mg/ml) and incubated at 37 °C for 1 h with continuous shaking at 200 rpm. Then, SDS at a final concentration of 1 % was added, and the mixture was incubated at 65 °C for 3 h with gentle shaking every 20 min. After centrifugation at 6,000 g for 20 min at room temperature, the supernatant was collected and then extracted with an equal volume of chloroform/isoamyl alcohol (24:1, v/v). The aqueous phase containing DNA was recovered by centrifugation at 12,000 rpm at 4 °C for 10 min, and the obtained DNA was precipitated by adding 0.7 volumes of isopropanol. Then, the DNA was collected by centrifugation at 10,000×*g* for 20 min, washed with cold ethanol (70 %), dried, and

re-suspended in TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). Finally, the crude DNA extract was purified by gel electrophoresis.

To construct the metagenomic library, the purified DNA was partially digested with *Hind* III. DNA fragments of 2.0–10 kb were ligated into *Hind* III-digested and dephosphorylated pBluescript SK+, and the ligated products were electroporated at 200 Ω, 25 μF, and 2.5 kV into *E. coli* DH5α using Micropulser II (Bio-Rad, USA). Then, the transformed cells were plated onto LB agar plates containing tributyrin (1 %, v/v) and incubated at 37 °C for up to 4 days for lipolytic activity screening. The lipolytic clones were selected by the formation of hydrolysis halos around individual colonies. In order to avoid false positive clones, the selected clones were retested for the ability to hydrolyze tributyrin and plasmids of which were isolated, retransformed with *E. coli* DH5α, and the resulting transformants were examined with the same type of assay for lipolytic activity. One clone (BSXG2) was finally obtained and the plasmid (pBS-est-XG2) of which was sent to Shanghai Sunny Biotechnology Company for sequencing.

Bioinformatic analysis of est-XG2

The recombinant plasmid (pBS-est-XG2) was sequenced using the primer-walking sequencing approach. Possible open reading frames (ORFs) were identified using the ORF finder at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Protein sequence similarity searches were undertaken using the NCBI online BlastP program (http://blast.ncbi.nlm.nih.gov/) [1]. Multiple sequence alignment was conducted using Clustal W, version 1.83 and presented using ESPript 2.2 (http://espript.ibcp.fr/ESPript/ESPript/) [12]. Phylogenetic analysis was conducted with MEGA 5.0 using neighbor-joining method [36]. A bootstrap analysis with 1,000 replicates was used to estimate the reliability of the tree. The 3D structure of the target protein was constructed by SWISS-MODEL (http://swissmodel.expasy.org/) [34], and presented using CCP4MG 2.5 [26]. Signal peptide was predicted using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) [24].

Cloning, expression, and purification of Est-XG2

The putative esterase gene was amplified from the plasmid of pBS-est-XG2 using primers P1 (TATCATATGGGCGAGCTCGTCGAG) and P2 (TATGCGGCCGCGAGGACGCCGTCCCAGG). Restriction sites are underlined. The PCR fragment was digested with *Nde* I and *Not* I, then ligated into pET-22b (+), which has an ampicillin resistance gene and a His-tag coding sequence at the *C*-terminus of the corresponding protein. Then, the resultant recombinant plasmid was used to transform *E. coli* DH5α. Once the sequence had been confirmed, the recombinant plasmid was transformed with *E. coli* BL21 (DE3) for overexpression.

The recombinant *E. coli* cells were grown in LB medium at 37 °C. When the culture reached an OD_{600} of approximately 0.5, protein expression was induced by adding 0.1 mM IPTG (isopropyl-D-1-thiogalactopyranoside). After 16 h of induction at 20 °C, the cells were harvested by centrifugation. The cell pellet was re-suspended in lysis buffer (20 mM Tris–HCl, 0.3 M NaCl, pH 8.0) and disrupted by using a One Shot Cell Disrupter (Constant Systems, British). Cell debris was removed by centrifugation at 12,000 rpm for 30 min at 4 °C. After being filtered with a filter membrane (Millipore, 0.22 μm), the supernatant was loaded onto a Ni Sepharose™ 6 Fast Flow resin (GE healthcare, USA) that had been previously equilibrated with washing buffer (20 mM Tris–HCl, 0.3 M NaCl, pH 8.0). The target recombinant enzyme was eluted using an imidazole concentration gradient (0, 30, 60, 100, and 200 mM) in washing buffer. Finally, the imidazole in the purified enzyme was removed by dialysis in Tris–HCl buffer (20 mM, pH 8.0) at 4 °C. Protein concentration was determined by the Bradford method [6] using bovine serum albumin (BSA) as a standard protein. The molecular mass of Est-XG2 was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12 %), and proteins were stained with Coomassie Brilliant Blue R-250.

Esterase activity assay

Esterase activity was assayed by measuring the amount of *p*-nitrophenol (*p*-NP) released from *p*-nitrophenyl ester which was dissolved in acetonitrile at a concentration of 10 mM. In a standard assay, the total reaction system of 1 ml contains 50 mM Tris–HCl buffer (pH 8.0), 200 μM *p*-NP ester, ethanol (40 %, v/v), and enzyme solution [25]. The reaction system was prewarmed at 60 °C for 5 min before the addition of enzyme solution. After incubation, the reaction was started by the addition of the purified recombinant enzyme. Following incubation at 60 °C for 5 min, the reaction was terminated by rapid cooling on ice water. The background hydrolysis of the substrate was deducted by using a reference sample with identical composition as the reaction mixture except without the enzyme. The esterase activity was determined by measuring the absorbance at 405 nm by using a spectrophotometer. One unit (U) of enzyme activity was defined as 1 μmol of *p*-NP liberated per minute.

Characterization of Est-XG2

The substrate specificity of Est-XG2 for *p*-nitrophenyl alkanoate esters was assayed using *p*-nitrophenyl (*p*-NP)

acetate (C2), butyrate (C4), hexanoate (C6), caprylate (C8), decanoate (C10), laurate (C12), myristate (C14), and palmitate (C16) as substrates under the standard assay conditions. The effect of pH on Est-XG2 was investigated at 60 °C at pH ranging from 6.0 to 10.0 in various buffers: 50 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 6.0–8.0), 50 mM Tris–HCl buffer (pH 8.0–9.0), and 50 mM glycine/NaOH buffer (pH 9.0–10.0) [23]. The optimum temperature of Est-XG2 was determined by measuring the activity at temperatures between 20 and 90 °C at pH 8.5 using *p*-NP butyrate as the substrate. To determine the thermal stability of Est-XG2, the purified enzyme was incubated in 50 mM Tris–HCl (pH 8.5) at 70, 80, and 90 °C for different periods. Subsequently, the remaining activity was analyzed at its optimum temperature. The kinetic parameters of Est-XG2 were measured in 50 mM Tris–HCl (pH 8.5) at 70 °C using the substrates of *p*-nitrophenyl esters at different concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, and 3 mM). The enzymatic kinetic parameters, K_m and V_{max}, were determined from the Lineweaver–Burk plot using the Microsoft Excel software.

Effects of additives on Est-XG2

In order to estimate the organic solvent tolerance of Est-XG2, aliquots of the recombinant enzyme were incubated in 15 or 30 % of organic solvents such as methanol, ethanol, isopropanol, acetone, and dimethyl sulfoxide (DMSO). After 1 h treatment, the residual activities of Est-XG2 were measured in 50 mM Tris–HCl buffer (pH 8.5) at 70 °C using *p*-NP butyrate as substrate. The effects of detergents (CTAB, SDS, Tween-20, and Triton X-100) on esterase activity were analyzed by measuring the remaining activity through the additions of 0.1 and 1 % (v/v) of various detergents into the reaction system. The effects of phenylmethanesulfonyl fluoride (PMSF), metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+}), and the chelating agent EDTA on esterase activity were examined by adding 1 or 10 mM of each into the reaction system. All assays were conducted three times independently under the standard assay condition, and the activity of enzyme without additives was defined as 100 %.

Site-directed mutagenesis

The Quick Change site-directed mutagenesis kit (Stratagen, USA) was used to introduce amino acid substitutions according to the standard protocol. The plasmid of pBS-est-XG2 was used as the template for the whole plasmid PCR. Mutagenic primers, designed by Primer X (http://www.bioinformatics.org/primerx/cgi-bin/DNA_1.cgi), included S192A-F: 5′-GATCTTCGGCGAGgCGGCG GGCGGCATG-3′; S192A-R: 5′-CATGCCGCCCGCCGc CTCGCCGAAGATC-3′; E313Q-F: 5′-CTACACGCGC-GACcAGTGGAAGCTCTTC-3′; E313Q-R: 5′ GAAGAG CTTCCACTgGTCGCGCGTGTAG-3′; H412L-F: 5′-CTC GGCGCCTGCCtCGGCATCGACGTG-3′; H412L-R: 5′-CACGTCGATGCCGaGGCAGGCGCCGAG-3′, respectively (modified codons are underlined). After being digested with *Dpn* I, the PCR product was transformed with *E. coli* DH5α. After sequencing, each of the three mutant plasmids (designated as pBS-est-XG2-S/A, pBS-est-XG2-E/Q, and pBS-est-XG2-H/L) was transformed with *E. coli* BL21 (DE3) for gene expression. Mutant enzymes (designated as S192A, E313Q, and H412L) were purified with hexahistidine tag and tested under standard conditions for their catalytic activities.

Hydrolysis of linalyl acetate

The reaction mixture, containing *rac*-linalyl acetate (15 mg/ml) and the purified protein [2 mg/ml in 50 mM Tris–HCl (pH 8.0)], was shaken on a rotary shaker (200 rpm) at ambient temperature. The reaction was terminated by adding four volumes of ethanol, and the insoluble material was removed by centrifugation. Samples were periodically withdrawn and analyzed by thin-layer chromatography (TLC) using Merck silica gel 60 F254 and petroleum ether/ethyl acetate (4:1) as eluent. Compounds were visualized by spraying the TLC plate with a solution of vanillin (5 g/l) in concentrated H_2SO_4 and ethanol (4:1) [17].

Nucleotide sequence accession number

The *est-XG2* nucleotide sequence obtained in this study is available in GenBank database under the accession number KC904273.

Results

Construction and screening of the metagenomic library

A metagenomic library containing approximately 40,000 clones was constructed with the environmental DNA isolated from an activated sludge sample taken from a paper mill. Restriction enzyme digestion of the plasmids extracted from 20 randomly chosen clones showed that more than 90 % of the plasmids contained inserts with an average insert of 2.5 kb (data not shown). The metagenomic library represented about 100 Mb of microbial community DNA of the activated sludge. Then the library was screened on LB agar plate containing 1 % (v/v) of tributyrin. After 48 h incubation at 37 °C, two clones (BSXG1 and BSXG2) were obtained on the basis of the formation of hydrolysis

zone. However, only plasmid (pBS-est-XG2) isolated from the clone BSXG2 resulted in the lipolytic activity of transformants after being retransformed with *E. coli* DH5α, which suggesting that the plasmid contained genes responsible for the lipolytic activity towards substrates with short acyl chains. Thus, the clone BSXG2 was finally selected for further investigation.

Sequence analysis of Est-XG2

In order to identify the genes that confer the lipolytic activity to BSXG2, the plasmid pBS-est-XG2 was sequenced. The complete insert DNA fragment of pBS-est-XG2 was about 3.0 kbp. Three ORFs were identified using ORF finder. One of these ORFs (designated as *est-XG2*) consisted of 1,506 bp with GC content of 74.8 %, and encoded a protein (designated as Est-XG2) of 501 amino acids with molecular mass and p*I* calculated to be 53,603 Da and 5.65, respectively. Subsequent BLASTP analysis using the NCBI non-redundant protein database revealed that Est-XG2 showed moderate similarity (≤47 %) to several lipolytic enzymes including the carboxylesterase (GenBank: YP_004101478) from *Thermaerobacter marianensis* DSM 12885 (identity 47 %), the esterase EstDL30 (GenBank: AEK77432) from an uncultured organism of soil (identity 44 %), and the carboxylesterase Est55 (GenBank: AAN81910) from *Geobacillus stearothermophilus* (identity 43 %), which suggests that Est-XG2 is a novel esterase. Multiple sequence alignment revealed that the catalytic triad of Est-XG2 is formed by Ser_{192}, Glu_{313}, and His_{412} with Ser_{192} in a consensus G-X_1-S-X_2-G pentapeptide (Fig. 1). Furthermore, a conserved GGG(A)X motif at the 105–108 position was detected as an oxyanion hole (Fig. 1) [11]. To classify Est-XG2, a phylogenetic tree was constructed using lipolytic enzymes representing eight different lipase/esterase families. As shown in Fig. 2, Est-XG2 belongs to family VII.

As no IPTG was added to the screening plate, the lipolytic activity detected was expected to be arise from the native promoter of the gene. However, we failed to identify the promoter sequence upstream of the ATG start codon of *est-XG2*. SignalP4.1 analysis indicated that neither the cleavage site nor the signal peptide was presented in Est-XG2.

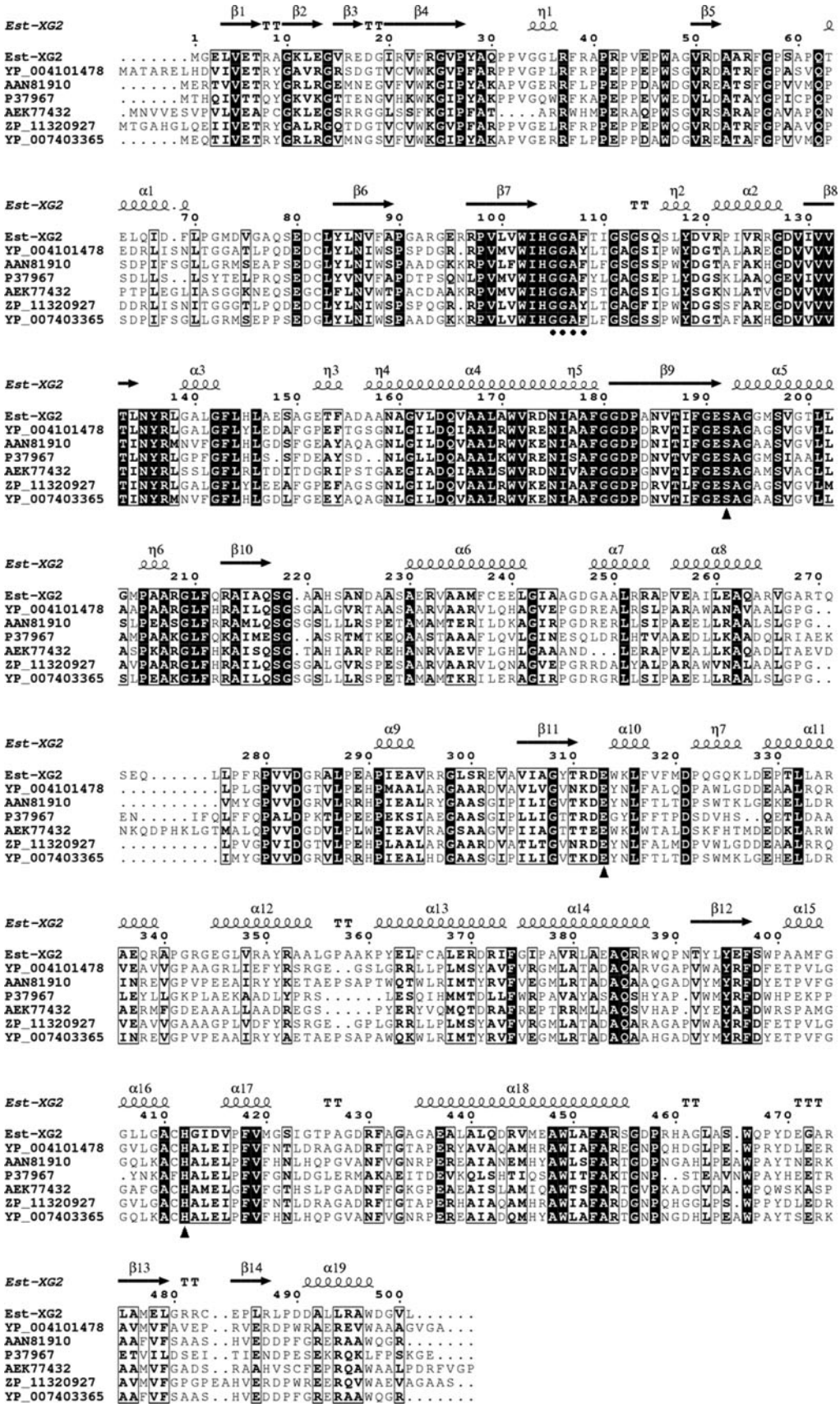
Expression and purification of Est-XG2

The enzyme expression was achieved by inducing the gene expression with 0.1 mM IPTG at 20 °C. Then, the recombinant Est-XG2 was purified from the supernatant of the cell lysate by affinity chromatography on a Ni–NTA column, and samples from different purification steps were analyzed by SDS-PAGE (Fig. 3). The results revealed that Est-XG2 expressed in *E. coli* was partially soluble (Fig. 3; lane 2, 3), and mostly eluted with the washing buffer containing 200 mM imidazole (Fig. 3; lane 8). Finally, this purification protocol for Est-XG2 resulted in 8.72-fold purification with 32.89 % recovery, and yeilded 130 mg proteins from 1L cell culture (Table 1). The purified Est-XG2 showed a single band of about 53 kDa which corresponded well with its theoretic value.

Biochemical characterization of Est-XG2

The substrate specificity of Est-XG2 was measured using various *p*-NP esters with different acyl chain lengths. The results are shown in Fig. 4a and Table 2. Est-XG2 was able to hydrolyze *p*-NP esters with acyl chain length up to 12 carbons (C12), with higher activity towards short-chain *p*-NP esters (C2, 39.60; C4, 17.71; C6, 12.23 U/mg) and much lower activity for medium length *p*-NP esters (C8, 7.19; C10, 4.85; C12, 1.26 U/mg) (Fig. 4a). No enzyme activity was detected for acyl chain length longer than C12 (Fig. 4a). Moreover, both the K_m and the catalytic efficiency (k_{cat}/K_m) values of Est-XG2 decreased gradually with the increase of the chain length from C2 to C12 (Table 2). The recombinant Est-XG2 is the most selective for *p*-NPC4, presenting the minimum K_m value of 0.24 mM, whereas it showed the highest efficiency in hydrolyzing *p*-NPC2 with the maximum k_{cat}/K_m value of 109.73 s^{-1} mM^{-1} (Table 2). On the basis of the substrate preference of Est-XG2, it can be concluded that Est-XG2 is not a lipase but an esterase [2]. In addition, the ability of Est-XG2 to hydrolyze esters with tertiary alcohols was examined using linalyl acetate as the substrate. TLC analysis showed that Est-XG2 was able to convert acetate to linalool (Fig. 5).

The optimum activity of Est-XG2 was assayed over a pH range of 5.0–10.0 and a temperature range of 20–90 °C using *p*-NP butyrates as the substrate. The optimum pH of Est-XG2 appeared was pH 8.5 (Fig. 4b). The temperature profile of Est-XG2 is presented in Fig. 4c. The results revealed that Est-XG2 was active over a wide range of temperatures from 20 to 90 °C, with the optimum temperature being 70 °C (Fig. 4c), which suggests that Est-XG2 is a themophilic esterase. The thermal stability of Est-XG2 was also determined in a temperature range of 60–90 °C. As shown in Fig. 4d, Est-XG2 was stable at 60 and 70 °C for more than 6 h. At 80 °C, Est-XG2 retained more than 80 % of its original activity after 2 h treatment (Fig. 4d). Therefore, Est-XG2 could be used over a broad temperature range of 50–70 °C and is suitable for biotechnological applications performed at high temperatures such as the removal of pitch from pulp in the paper industry [13] and the removal of subcutaneous fat in the leather industry [39].


Est-XG2
Est-XG2
YP_004101478
AAN81910
P37967
AEK77432
ZP_11320927
YP_007403365

◄ **Fig. 1** Multiple sequence alignment between Est-XG2 and other closely related lipolytic enzymes: YP_004101478, a carboxylesterase from *Thermaerobacter marianensis* DSM 12885; AAN81910, a thermostable esterase (Est55) from *Geobacillus stearothermophilus*; P37967, a carboxylesterase from *Bacillus subtilis*; AEK77432, an esterase (EstDL30) from an uncultured bacterium; ZP_11320927, a carboxylesterase from *Thermaerobacter subterraneus* DSM 13965; YP_007403365, a *para*-nitrobenzyl esterase from *Geobacillus* sp. GHH01. *Filled triangles* represent putative catalytic residues at the corresponding positions of Ser_{192}, Glu_{313}, and His_{412}. *Filled circles* represent GGG(A)X motif involved in the formation of oxyanion hole. Sequence alignment was performed with Cluster 1.83 and visualized using ESpript 2.0. The alpha helix, beta sheet, random coil, and beta turn are identical to α, β, η, and T, respectively

Effects of additives on Est-XG2

The esterase activity is frequently affected by many factors, such as organic solvents, surfactants, and metal ions [7]. Activity in organic solvents is important for biocatalysts used in organic synthesis. To study the stability of Est-XG2 in organic solvents, the residual activities after being treated in the selected organic solvents were examined (Table 3). The following organic solvents used at low concentration (10 %) had little effect on the activity of Est-XG2: methanol, isopropanol, and DMSO; whereas, ethanol and acetone decreased the activity of Est-XG2 by about 15 %. However, when a higher concentration (30 %) was used, Est-XG2 was significantly inhibited but still retained more than 60 % of its original activity. Compared with other esterases, Est-XG2 has moderate tolerance to organic solvents [17, 37]. The influence of a variety of detergents on Est-XG2 is also shown in Table 3. The detergents studied (CTAB, Tween-20, Triton X-100, SDS) had little or no effect on the activity of Est-XG2 at low concentration (1 mM), but they inhibited esterase activity at a concentration of 10 mM by around 30 % of its original activity. This activity loss might arise from the 3D conformational changes of Est-XG2 caused by the detergents [17]. This feature is different from lipases which are often activated by surfactants through increasing the access of substrates to the active center associated with the hydrophobic binding [28].

To characterize the effects of metal ions and the chelating reagent EDTA on the activity of Est-XG2, different metal ions and EDTA were added to the assay at 1 and 10 mM final concentrations (Table 4). At the former concentration, Na^+, K^+, Ca^{2+}, and Mg^{2+} showed no significant influence on the enzyme activity of Est-XG2, whereas Mn^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+} slightly decreased the activity to 94.51, 87.65, 85.07, 82.70, and 91.73 % of its original activity, respectively. However, when the metal ion concentration increased to 10 mM, Est-XG2 was strongly inhibited by Zn^{2+} (35.66 %), Ni^{2+} (49.30 %), and Co^{2+} (34.80 %); moreover, Est-XG2 was almost completely inhibited by Cu^{2+} (9.24 %). The chelating agent EDTA showed no inhibitory effect on Est-XG2, which suggests that Est-XG2 is not a metalloenzyme and metal ions may be unnecessary for the catalytic activity of Est-XG2. The enzyme activity of Est-XG2 was strongly inhibited by PMSF which further confirmed that it was a serine esterase as predicted by the presence of Ser as a catalytic residue.

Molecular structure modeling

By searching the RCSB PDB Protein Data Bank, we found that the crystal structure of the esterase Est55 (accession number, AAN81910; PDB, 2OGT) from *G. stearothermophilus* has the maximum amino acid sequence identity

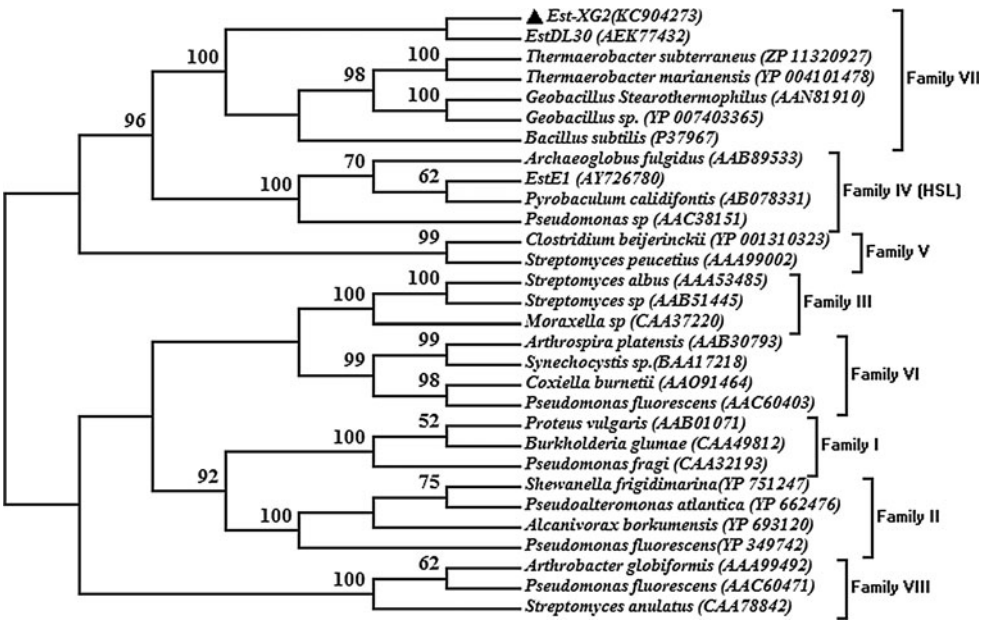


Fig. 2 Phylogenetic tree of Est-XG2 and other closely related lipolytic enzymes. The phylogenetic analysis was performed by the neighbor-joining method using MEGA 5.0. The *values at nodes* indicate the bootstrap percentage of 1,000 replicates, and only values which are above 50 % are indicated. Database accession numbers are shown in *brackets* after each enzyme

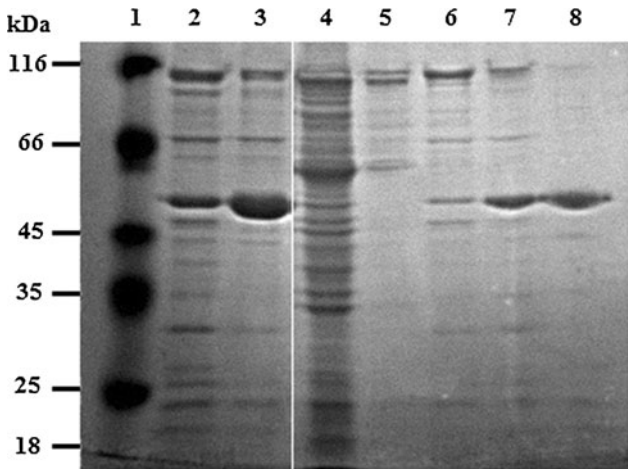


Fig. 3 SDS-PAGE analysis of the purified recombinant Est-XG2. *Lane 1* protein markers, *2* the supernatant of the induced cell lysate, *3* the pellet of the induced cell lysate, *4* fluid through Ni^{2+}-affinity chromatography column, *5* purified Est-XG2 eluted by washing buffer containing 30 mM imidazole, *6* purified Est-XG2 eluted by washing buffer containing 60 mM imidazole, *7* purified Est-XG2 eluted by washing buffer containing 100 mM imidazole, *8* purified Est-XG2 eluted by washing buffer containing 200 mM imidazole

(43 %) with Est-XG2. So it was chosen as the most appropriate template for homology modeling [22]. As validated by online PROCHECK (http://nihserver.mbi.ucla.edu/SAVES/), 84.7 % of the residues in the modeled structure are in the most favored regions, and only 3 out of 496 amino acids are in the disallowed regions. These results indicate that the model is satisfactory. Like Est 55, the final structure of Est-XG2 is composed of three domains, namely a catalytic domain, an α/β domain, and a regulatory domain, with 19 α-helices and 14 β-sheets (Fig. 6). The catalytic domain shows the typical α/β hydrolase fold with β-sheets surrounded by α-helices. The catalytic triad consists of Ser_{192}, Glu_{313}, and His_{412}, which are located on the loops between β9–α5, β1–α10, and α16–α17, respectively, and clustered together at the bottom of the active site. In order to verify this catalytic triad, Ser_{192}, Glu_{313}, and His_{412} were mutated into Ala_{192}, Gln_{313}, and Leu_{412}, respectively. The activity of each mutant enzyme was examined with different kinds of *p*-NP esters. However, none of these mutants showed hydrolytic activity towards *p*-NP esters, indicating that these amino acids were essential for esterase activity.

Discussion

To isolate new lipolytic enzymes, we constructed a metagenomic library with environmental DNA extracted from an activated sludge sample taken from a paper mill. Generally, DNA isolated from environmental samples is often contaminated with humic acid and other organic acids, which may reduce the efficiencies of polymerase, restriction endonuclease, and ligase [19]. A number of methods have been developed for the purification of environmental DNA [32]. In this study, the environmental DNA was purified with gel electrophoresis. This DNA purification procedure was simple and effective, and the resultant DNA was suitable for digestion with restriction endonucleases.

The hit rate of lipolytic gene isolation from a metagenomic library is usually between 2.86×10^{-3} and 2.08×10^{-1}/Mb [21]. In this study, the library contained about 40,000 clones with an average insert of 2.5 kb, which covered about 100 Mb of community DNA. Screening of this metagenomic library resulted in the identification of one lipolytic gene (*est-XG2*). This hit rate (1×10^{-2}/Mb) was similar to those from other metagenomic libraries, but much lower considering the lipolytic genes are widely distributed in microbes [21]. The low rate of isolating functional lipolytic genes from the metagenomic library might be due to the difficulty in expressing the lipolytic genes in *E. coli*, which is often caused by the non-recognition of regulatory elements from uncultured bacterium in *E. coli*, codon usage preference between the expressed gene and the host, and the requirement of folding or transport partner. Thus, an efficient method for protein production should be developed to improve the efficiency of discovering biocatalysts for industrial applications [35].

A novel lipolytic gene, *est-XG2*, was identified from the metagenomic library. The deduced amino acid sequence of Est-XG2 showed similarity to several known family VII members such as the esterases from *B. subtilis* (P37967) (identity 41 %), *Arthrobacter oxydans* (Q01470) (identity 42 %), *T. marianensis* DSM 12885 (YP_004101478) (identity 47 %), *T. subterraneus* DSM 13965 (ZP_11320927) (identity 47 %), and *G. stearothermophilus* (PDB: 2OGT) (identity 43 %). However, it also exhibited 32 % amino acid identity with the esterases from *Pyrobaculum calidifontis* (AB078331) [14] and a metagenomic library (AY726780) (identity 30 %) [30], which belonged

Table 1 Purification of Est-XG2 from *E. coli* BL21 (DE3)

	Concentration (mg/ml)	Volume (ml)	Activity (U/ml)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Cell lysate	2.30	15	10.44	4.54	1	100
Purified Est-XG2	0.13	10	5.15	39.60	8.72	32.89

Esterase activity was determined with *p*-nitrophenyl acetate as a substrate

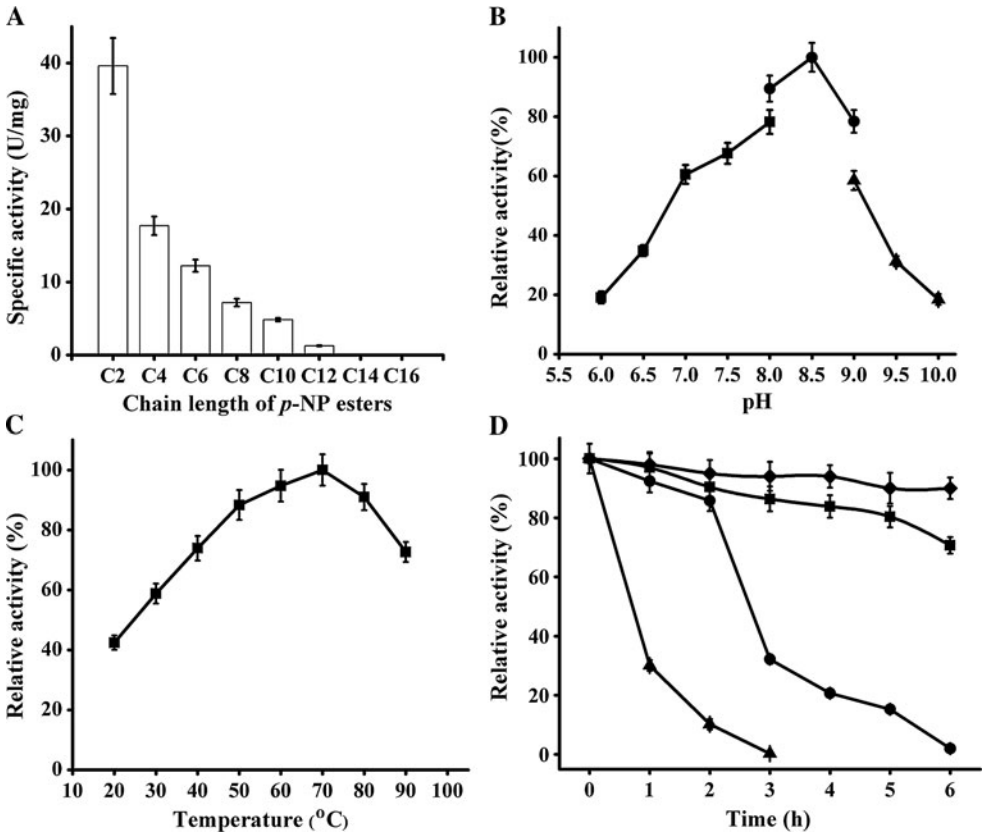


Fig. 4 Characterization of Est-XG2. **a** Activities of Est-XG2 towards *p*-NP esters of various chain lengths (C2, acetate; C4, butyrate; C6, hexanoate; C8, caprylate; C10, decanoate; C12, laurate; C14, myristate; and C16, palmitate). **b** The pH profile of Est-XG2. The enzyme activity in Tris–HCl (50 mM, pH 8.5) was taken as 100 %. Buffers used (final concentration 50 mM) were NaH_2PO_4/Na_2HPO_4 (*filled squares* pH 6.0–8.0); Tris–HCl (*filled circles* pH 8.0–9.0); glycine/NaOH (*filled triangles* pH 9.0–10.0). The values represent the means of three independent experiments (mean ± standard error). **c** Effect of temperature on esterase activity of Est-XG2. The values represent the means of three independent experiments (mean ± standard error). **d** Thermal stability of Est-XG2. The enzyme was incubated at 60 °C (*filled diamonds*), 70 °C (*filled squares*), 80 °C (*filled circles*), and 90 °C (*filled triangles*) for the indicated time. The residual activity was measured by a standard assay

Table 2 Kinetic parameters of Est-XG2

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
p-NP acetate (C2)	0.33 ± 0.01	36.21 ± 1.68	109.73 ± 4.94
p-NP butyrate (C4)	0.24 ± 0.03	16.20 ± 0.64	67.50 ± 2.57
p-NP hexanoate (C6)	0.38 ± 0.02	11.18 ± 0.44	29.42 ± 1.35
p-NP caprylate (C8)	0.62 ± 0.01	6.53 ± 0.29	10.53 ± 0.46
p-NP decanoate (C10)	0.76 ± 0.02	4.40 ± 0.19	5.79 ± 0.24
p-NP laurate (C12)	0.82 ± 0.03	1.15 ± 0.05	1.40 ± 0.06

Values are mean ± SD from three independent experiments

to lipase/esterase family IV. In the phylogenetic tree, Est-XG2 together with EstDL30 formed a distinct subgroup in family VII (Fig. 2). The moderate identities with known esterases and failure to identify the ribosome-binding site near to the start codon of *est-XG2* revealed that this DNA fragment may originate from an unknown microorganism which may not have been investigated or even cultivated. In addition, a GGG(A)X motif was identified in Est-XG2. Lipolytic enzymes containing a GGG(A)X motif in their oxyanion holes are able to catalyze the hydrolysis of certain esters with tertiary alcohols because the GGG(A)X motif increases the access of the alcohol to the active center with a broad substrate binding site [17]. In this study, Est-XG2 successfully hydrolyzed linalyl acetate, which suggested that Est-XG2 could be used in the kinetic resolution of tertiary alcohols [3].

Most thermophilic esterases come from thermophilic bacterial strains, but few have been isolated from metagenomic libraries. Est-XG2 is a thermophilic esterase with high thermostability. It showed maximum activity at 70 °C, and had high thermostability at 60 and 70 °C. This is similar to the esterase Est55 (accession number, AY186196; PDB, 2OGT) from *G. stearothermophilus* which showed optimum activity at 60 °C and retained more than 90 % of its original activity after 3 h treatment at 60 °C [10]. Like Est55, Est-XG2 may have a similar

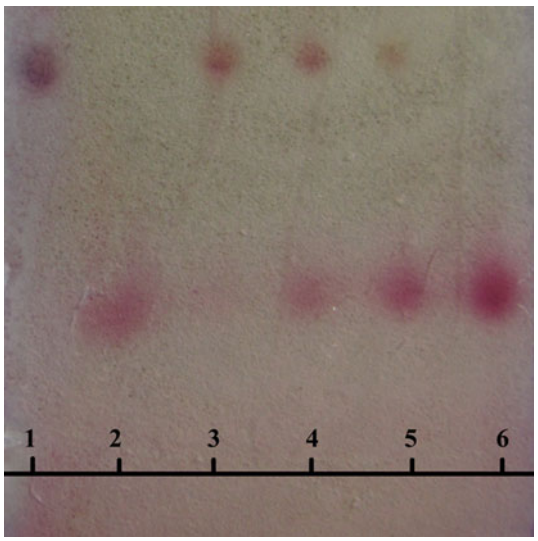


Fig. 5 TLC analysis of the enzymatic conversion of racemic linalyl acetate to linalool. *Lane 1* linalyl acetate ester, *2* linalool, *3* no enzyme added, *4* reaction time of 5 h, *5* reaction time of 10 h, *6* reaction time of 20 h

Table 3 Stability of Est-XG2 in the presence of denaturing compounds

Compounds	Relative activity (%) at concentration (%, v/v) of	
	15 %	30 %
Organic solvents		
Control	100	100
Methanol	103.14 ± 3.49	62.30 ± 2.93
Ethanol	82.36 ± 4.23	68.54 ± 3.12
Isopropanol	102.25 ± 3.88	67.68 ± 2.25
Acetone	84.36 ± 3.19	62.58 ± 2.44
DMSO	94.41 ± 4.91	83.67 ± 3.85
Surfactants		
CTAB	101.72 ± 4.58	83.14 ± 3.49
Tween-20	93.45 ± 3.64	72.67 ± 2.37
Triton X-100	96.88 ± 4.46	76.40 ± 2.70
SDS	95.49 ± 4.87	63.60 ± 2.90

Values are mean ± SD from three independent experiments

function that activates the prodrug irinotecan (CPT-11) to produce 7-ethyl-10- hydroxycamptothecin (SN-38), a topoisomerase inhibitor used in cancer therapy [22]. Esterases have a common property that distinguishes them from lipases: they do not exhibit interfacial activation phenomena at an oil–water interface because of their lack of lid structure [20]. In order to determine whether Est-XG2 has a lid structure, the effect of PMSF on Est-XG2 was analyzed. PMSF is a typical serine inhibitor and will covalently link the active serine residue if it can access it. We observed that the activity of Est-XG2 decreased

Table 4 Stability of Est-XG2 against metal ions, EDTA, and PMSF

Additives	Relative activity (%) at concentration (%, v/v) of	
	1 mM	10 mM
Control	100	100
Na^{+}	98.77 ± 4.50	103.22 ± 4.75
K^{+}	102.26 ± 4.22	103.35 ± 3.61
Ca^{2+}	104.72 ± 4.82	103.32 ± 3.93
Mg^{2+}	105.39 ± 3.95	101.39 ± 4.66
Mn^{2+}	94.51 ± 4.33	75.18 ± 3.58
Cu^{2+}	87.65 ± 3.68	9.24 ± 0.39
Zn^{2+}	85.07 ± 3.91	35.66 ± 1.50
Ni^{2+}	82.70 ± 4.22	49.30 ± 2.05
Co^{2+}	91.73 ± 4.04	34.80 ± 1.67
EDTA	102.04 ± 3.88	99.46 ± 4.08
PMSF	6.50 ± 0.52	ND

Values are mean ± SD from three independent experiments

ND not detected

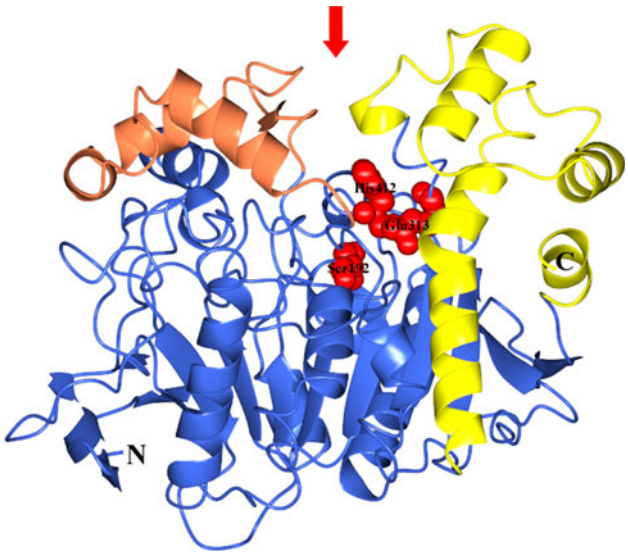


Fig. 6 3D model of Est-XG2. The catalytic domain, the α/β domain, and the regulatory domain are in *blue*, *coral*, and *yellow*, respectively. The catalytic triad (Ser_{192}, Glu_{313}, and His_{412}) is shown in *red*. *N* and *C* denote the *N* and *C* termini, respectively (color figure online)

sharply when PMSF was added. This means that PMSF could access the active site, which suggests that Est-XG2 has no lid structure. Est-XG2 was also stable in organic solvents. We found that Est-XG2, after 2 h incubation in the presence of organic solvents (30 %), retained more than 60 % of its original activity. Stability against organic solvents is important for the application of the enzymes in organic synthesis. Therefore, Est-XG2 could be a potential candidate for industrial applications, such as synthetic organic chemistry.

In conclusion, a novel esterase gene was successfully isolated from a metagenomic library prepared with the activated sludge sample taken from a paper mill, and expressed in *E. coli*. Biochemical characterization revealed that Est-XG2 is a thermophilic esterase with the ability to hydrolyze tertiary alcohol esters, and it is stable in the presence of organic solvents. All of these properties make Est-XG2 a potential candidate for biological applications. Our research also demonstrates the potential of metagenomic strategies for mining esterase genes for industrial applications and expands our knowledge on the diversity of lipase/esterase families.

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