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Gene cloning and characterization of a novel esterase from activated sludge metagenome

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EDITORS NOTE Since publication of this article it has been brought to our attention that figure 3 is incorrect and the authorship of the article is in dispute. We are working with the authors to correct these issues and will publish a correction article as soon as they are resolved.

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Abstract

A metagenomic library was prepared using pCC2FOS vector containing about 3.0 Gbp of community DNA from the microbial assemblage of activated sludge. Screening of a part of the unamplified library resulted in the finding of 1 unique lipolytic clone capable of hydrolyzing tributyrin, in which an esterase gene was identified. This esterase/lipase gene consists of 834 bp and encodes a polypeptide (designated EstAS) of 277 amino acid residuals with a molecular mass of 31 kDa. Sequence analysis indicated that it showed 33% and 31% amino acid identity to esterase/lipase from *Gemmata obscuriglobus* UQM 2246 (ZP_02733109) and *Yarrowia lipolytica* CLIB122 (XP_504639), respectively; and several conserved regions were identified, including the putative active site, HSMGG, a catalytic triad (Ser92, His125 and Asp216) and a LHYFRG conserved motif. The EstAS was overexpressed, purified and shown to hydrolyse *p*-nitrophenyl (NP) esters of fatty acids with short chain lengths (≤ C8). This EstAS had optimal temperature and pH at 35°C and 9.0, respectively, by hydrolysis of *p*-NP hexanoate. It also exhibited the same level of stability over wide temperature and pH ranges and in the presence of metal ions or detergents. The high level of stability of esterase EstAS with its unique substrate specificities make itself highly useful for biotechnological applications.

Introduction

Lipolytic enzymes such as esterases (EC3.1.1.1) and lipases (EC3.1.1.3) catalyze both the fat hydrolysis and the synthesis of fatty acid esters including acylglycerides as biocatalysts [1]. Lipolytic enzymes are ubiquitous α/β hydrolyzing enzymes existed in animals, plants, and microbes, including fungi and bacteria. Microbial esterases are showing considerable industrial potential where their regiospecificity and enantioselectivity are desired characteristics [2], such as production of fine chemicals, pharmaceuticals, in the food industry and are widely used in biotechnology [2-4].

Modern biotechnology has a steadily increasing demand for novel biocatalysts, thereby prompting the development of novel experimental approaches to find and identify novel biocatalyst-encoding genes. Metagenome is the total microbial genome directly isolated from natural environments, and the power of metagenomics is the access, without prior sequence information, to the so far

uncultured majority, which is estimated to be more than 99% of the prokaryotic organisms [5-7]. In fact, the metagenomic approach was successful in searching for novel lipolytic enzymes in varied environments, and also, several genes encoding metagenomic esterases have been identified in metagenomic libraries prepared from varied environmental samples, including soils [6-9], marine sediment [10-12], pond and lake water [13-15], and tidal flat sediment [16].

Studies based on 16S rDNA library have extensively redefined and expanded our knowledge of microbial diversity in activated sludge from low-temperature aromatic wastewater treatment bioreactor, including members of various un-culturable groups (unpublished data). To the best of our knowledge, activated sludge microbial communities have not been exploited by culture-independent methods for isolation of lipolytic genes. Here, we report the isolation, sequence analysis, and enzymatic characterization of a novel esterase, EstAS, from an activated sludge derived metagenomic library. The discovery of EstAS led to the identification of a new family of bacterial lipolytic enzymes.

Materials and methods

Sampling

Activated sludge was collected from a low temperature sequencing batch bioreactor (SBR) treating nitrogen-containing aromatic wastewater in our laboratory.

Bacterial strains, plasmids, and growth conditions

The starting strains and plasmids used in this study are listed in Table 1. *E. coli* was grown at 37°C in Luria-Bertani (LB) medium supplemented with appropriate antibiotics [17], at 12.5 μg/ml for chloramphenical, 100 μg/ml for ampicillin and 25 μg/ml for kanamycin.

DNA preparation and manipulation

E. coli cells were transformed by the calcium chloride procedure [17]. Recombinant plasmid DNA was isolated by the method of Birnboim and Doly [18] or with a Tianprep Mini kit (TianGen). Restriction enzymes, T4 DNA ligase and calf intestinal alkaline phosphatases were purchased from New England Biolabs (Ipswich, USA) or Takara (Tokyo, Japan) and used according to the manufacturers' instructions.

Construction of metagenomic DNA library and sublibrary

Activated sludge DNA extraction was carried out using SDS and proteinase K treatment [19], and the removal of humic acids (HAs) prior to DNA extraction was conducted by using HAs removing buffer [20]. Approximately 100 μg of metagenomic DNA was run on a preparative pulsed-field gel (Bio-Rad CHEF DR®III; 0.1-40 s switch time, 6 V/cm, 0.5 × TBE buffer, 120° included angle, 16 h), and the appropriate size of DNA ranging from 30-50 kb was isolated, electroeluted, and dialyzed against 0.5 × TE buffer for further Fosmid library construction. The purified DNA fragments were end-repaired by End-repaired enzyme mix. After size fractionation and purification, the blunt-ended, 5'-phosphorylated DNA was ligated into the cloning-ready Copycontrol pCC2FOS vector, and the recombinant molecules were packaged *in vitro* with a MaxPlaxTM Lambda packaging kit (Epicentre Biotechnologies, Madison, Wisconsin, USA). The selected unique fosmid clone was named FosB12L1 (showing strong lipolytic activity on tributyrin plate), and purified, partially digested with *Sau* 3AI to obtain 3-5 kb size DNA, and ligated into a purified *Bam* HI/BAP pUC118 vector from Takara. Ligation products were transformed into *E. coli* TOP10 cells (Tiangen) and spread out on LB (ampicillin, 100 μg/ml) plates containing 1% (v/v) tributyrin as the indicator substrate [21].

Table 1: Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
E. coli EPI300™-T1R	*[F- e14-(McrA-) D(mcrC-mrr) (TetR) hsdR514 supE44 supF58 lacY1 or D(lacIZY)6 galK2 galT22 metB1 trpR55 l-]*	Epicentre
E. coli TOP10	*lacx74 recA1 deoR F - mcrA*Δ *(mrr-hsdRMS-mcrBC) ϕ80 lacZ*Δ*M15*Δ *araD139*Δ *(ara-leu)7697 galU galK*	TianGen
E. coli BL21(DE3)	*F-, ompT, hsdSB (rB-, mB-), dcm, gal, λ(DE3), pLysS, Cmr*	Novogen
E. coli EPI300-FosB12L1	Positive clone from Fosmid genomic library, which carries the lipolytic gene	This study
E. coli TOP10-EstAS	Positive clone from sublibrary, which carries the *EstAS* gene fragment	This study
E. coli BL21(DE3)-EstAS	Positive clone, which carries the pEstAS-His expression vector	This study
Plasmids		
pCC2FOS	Cloning vector; Cm^r	Epicentre
pUC118	Cloning vector; Ap^r	Takara
pET28a	Expression vector; Km^r	Novagen
FosB12L1	pCC2FOS, which carries the *EstAS* gene cluster (35 kb)	This study
EstAS	pUC118, which carries the complete lipolytic gene (*EstAS*)	This study
pEstAS-His	pET28a carrying amplified *Hin* dIII -*Nde* I fragment containing lipolytic gene (*EstAS*)	This study

Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Km^r, kanamycin resistant.

Identification of lipolytic clones and DNA sequence analysis

The DNA fragment obtained was sequenced with primer walking method by SinoGenoMax Co. Ltd (Chinese National Human Genome Center, Beijing). The ORFs were analyzed using DNAstar (Lynnon Biosoft) and GeneTool software (Syngene), Database searches were performed with the BLAST program via GenomeNet World Wide Web server. Peptide sequences of various enzymes or subunits were extracted from National Center for Biotechnology Information (Washington, D.C).

Phylogenetic analysis

Deduced amino acid sequences of 8 lipolytic enzymes were subjected to protein phylogenetic analysis. Sequence alignment was performed by using CLUSTAL_W program [23] and visually examined with BoxShade Server program. Phylogenetic tree was generated using the neighbor joining method of Saitou and Nei [22] with MEGA 4.0 software [24].

Protein expression and purification

For the overexpression of EstAS, the full length of the *estAS* gene was amplified using primers EstAS-f and EstAS-r (Table 2) and high fidelity PrimeSTAR™HS DNA Polymerase (code: DR010SA, Takara). The primer pairs with restriction enzyme sites (underlined) for *Hind* III and *Nde* I were designed to generate an N-terminal His-tag of the recombinant esterase. The integrity of the nucleotide sequence of all newly constructed plasmids was confirmed by DNA sequencing. The *EstAS* gene was cloned into an expression vector, pET28a(+), and the recombinant plasmid p*EstAS*-His was transformed into *E. coli* BL21 (DE3) cells. When the cell density at 600 nm reached around 0.6, 1 mM isopropylthio-β-D-galactoside was added for the induction, following a further cultivation for 4 h at 30°C. Then cells were harvested by centrifugation, resuspended in a 50 mM sodium phosphate buffer (pH 8.0) containing 10 mM imidazole, and disrupted by sonication. The protein was applied to metal-chelating chromatography using Ni-NTA affinity chromatography (Novagen) according to the manufacturer's instructions. SDS polyacrylamide gel electrophoresis was carried out according to Sambrook and Russell [17].

Characterization and biochemical properties of EstAS

The substrate specificity of the purified enzyme was analyzed using the following substrates of *p*-NP-fatty acyl esters [21,25]: acetate (C2), butyrate (C4), hexanoate (C6), caprylate (C8), decanonate (C10), laurate (C12), myristate (C14) and palmitate (C16). The enzyme was incubated with the ester derivatives (0.5 mM) in 5 ml Tris-HCl buffer (50 mM, pH 8.0) at 40°C for 10 min. The reaction was quenched by adding 5 ml trichloroacetic acid (0.5 mM) and then recovered the original pH value with 5.15 ml NaOH (0.5 mM), and the amount of released *p*-NP was determined by an absorption increase at 405 nm against an enzyme-free blank on a Biospec-1601 spectrophotometer [26,27]. One unit of esterase is defined as the amount needed to release 1 μmol *p*-NP per min under the above conditions. The highest enzyme activity on a substrate (i. e. *p*-NP-hexanoate) was defined as 100%. To determine the presence of esterase activity, the triglyceride derivative 1,2-di-*O*-lauryl-*rac*-glycero-3-glutaric acid 6'-methylresorufin ester (DGGR) (Sigma Aldrich) was used as a chromogenic substrate, and the formation of methyresorufin was analyzed spectrophotometrically at 580 nm [1,28,29]. *Candida rugosa* lipase (Sigma Aldrich) was used as a positive control.

Using *p*-NP-hexanoate (0.5 mM) as substrate, the optimal temperature and pH of purified EstAS was determined, by measuring the enzyme activity after incubation at various temperatures (10-65°C) in 50 mM Tris-HCl buffer (pH 8.0) or after incubation at 35°C for 10 min in the following buffers: 50 mM phosphate buffer (pH 5.0-7.5), 50 mM Tris-HCl (pH 8.0-10.5).

Table 2: Primers used in the study

Primer	Sequence 5'-3'	Description
HTFP061	GTACAACGACACCTAGAC	Sequencing primer for pCC2FOS™
HTRP062	CAGGAAACAGCCTAGGAA	Sequencing primer for pCC2FOS™
M13 primer RV'	CAGGAAACAGCTATGAC	Sequencing primer for pUC118
M13 primer M2	AGCTGTTCACCGAAGTGCTG	Sequencing primer for pUC118
EstAS -W1F	GGCGTCGACCGGGTGGAGGA	Genomic walking primer for *EstAS* gene
EstAS -W2F	CCCGAGATCCGAGGCGAACT	Genomic walking primer for *EstAS* gene
EstAS -W3F	TCTCGAGCACGCCCTTGAAG	Genomic walking primer for *EstAS* gene
EstAS -W4F	CGAGTGATAGACGCGATGCC	Genomic walking primer for *EstAS* gene
EstAS -f	TCAGCCAT**ATG**TCTTACCCGATCGTCCTGG *Nde*I	Forward primer for *EstAS* gene
EstAS -r	CCCAAGCTTCTACGGCAGCTCCGCCGCG *Hin*dIII	Reverse primer for *EstAS* gene

The *Nde*I and *Hin*dIII sites are underlined. The start codon is in bold.

Various metal ions ($CoCl_2$, $CaCl_2$, $ZnCl_2$, $MgCl_2$, K_2SO_4, $FeSO_4$, $CuSO_4$, $Ni(NO_3)_2$ and $MnSO_4$), and chelating agent EDTA at final concentration of 1 mM were added to the enzyme in 50 mM Tris-HCl (pH 8.0), then assayed for esterase activity after preincubation at 35°C. Effect of detergents or reductors on esterase activity was determined by incubating the enzyme for 30 min at 35°C in 50 mM Tris-HCl (pH 8.0), containing (1%, v/v) Triton X-100, Tween 20 and 80, β-mercaptoethanol, 1, 4-dithiothreitol (DTT), sodium dodecyl sulfate (SDS), cetyltrimethyl ammonium bromide (CTAB), phenylmethanesulfonyl fluoride (PMSF) and diethypyrocarbonate (DEPC), respectively. The enzyme activity without metal ions and detergents was defined as 100%.

Nucleotide sequence accession number

The DNA sequence of *EstAS* was deposited in DDBJ/EMBL/GenBank under accession number of FJ386490.

Results and discussion

Construction of a metagenomic library and screening

About 100 μg DNA was extracted from 1 g activated sludge (wet-weight), and 1.5 μg of size-selected, pulse-field gel-purified high-molecular-weight (HMW) DNA suitable for fosmid cloning was obtained. 300 ng of 30-45 kb purified metagenomic DNA was ligated into the copy control pCC2FOS vector and transfected into *E. coli* EPI300-T1R, producing a metagenomic library of more than 100, 000 fosmids with insert sizes ranging from 28 kb to 40 kb (average size of 35 kb), covering approximately 3.0 Gbp of the total metagenomic DNA. The prokaryotic origin of the library was confirmed by end-sequencing of randomly selected fosmids and comparison with known ORFs in NCBI. Expression screening of the fosmid library based on the hydrolysis of emulsified tributyrin (1%) resulted in the detection of a recombinant clone, FosB12L1, forming a clear zone on the indicator plate.

Subcloning and identification of the esterase

The DNA insert (36 kb) of fosmid B12L1 was partial digested by *Sau* 3AI and subcloned into pUC118, producing a subclone library of more than 3,000 clones with an average insert size of 3-5 kb. 300 subclones were screened for lipolytic activity. One subclone expressing extracellular lipase/esterase activity was sequenced and assembled into a contig of 3780 bp (data not shown). An ORF of 834 bp encoding a putative lipase/esterase (named EstAS) of 277 amino acid residuals was identified. Amino acid sequence alignment indicated that this EstAS showed quite low identity with other esterase/lipases, highest with the esterase/lipase from *Gemmata obscuriglobus* UQM 2246 (ZP_02733109, 33% identity), followed by the lipase from *Yarrowia lipolytica* CLIB122 (XP_504639, 31% identity), the putative lipase/esterase from *Magnaporthe grisea* 70-15 and *Saccharomyces cerevisiase* Tg12p (XP_368471, 31% identity; and NP_010343, 29% identity, respectively), members of the family of fungal hydrolases. And also, the EstAS contained a catalytic triad (Ser92, His125, and Asp249) and a LHYFRG conserved motif (starting from His36), as shown in Fig. 1, which is in close proximity to the active site contributing to the formation of the oxyanion hole that is likely to participate directly in the catalytic process [2,30,31]. Furthermore, to clarify the phylogenetic relationship of the EstAS with other esterases or lipases, a neighbour joining tree was constructed using the amino acid sequence, as shown in Fig. 2. In this tree, EstAS is located closest to the branch of esterase/lipase (accession number X53053) of strain *Moraxella* sp. TA144, and also *Streptomyces* sp. M11, *Streptomyces albus* G (accession numbers M86351 and U03114, respectively), which constitute family III lipases. This result might suggest that the EstAS is a new member of family III lipases.

Expression and purification of recombinant EstAS

To investigate the property of this EstAS, *EstAS* gene was expressed as an N-terminal His-tag fusion protein using pET-28a(+) expression system in *E. coli* BL21(DE3). SDS-PAGE analysis of the purified EstAS showed a single band corresponding to about 31 kDa (Fig. 3), quite agreement with the predicted full length of EstAS. The purity of the purified protein was more than 98% according to SDS-PAGE analysis.

Substrate specificity of EstAS

We expressed EstAS as a hexahistidine-tagged (His-tagged) protein and investigated its chain length specificity using *p*-nitrophenyl esters (Sigma). EstAS showed high activity towards short-chain fatty acids (C4, C6 and C8), while much lower towards long-chain fatty acids (>C8) (Fig. 4). In addition, EstAS showed no fluorescence on olive oil plates with rhodamine B. Moreover, the EstAS was not able to hydrolyse DGGR (data not shown), while the lipase from *Candida rugosa* (used as a positive control) was able to hydrolyse DGGR to form chromogenic product, methylresorufin. These results indicate that EstAS is an esterase but a lipase [1,25,32,33].

Effect of temperature and pH on EstAS

Purified esterase EstAS showed a broader temperature range (optimum at 35°C) than other original esterases, and retained over 65% activity at 60°C (Fig. 5). The esterase YlLip2 from *Yarrowia lipolytica* showed an optimum temperature at 40°C [34], however, it showed a poor thermostability since it lost activity just only incubation at 45°C for 4h. And also, the esterase EstAS showed activity in a rather broad pH range of 5.5-10.5. Maximal activity

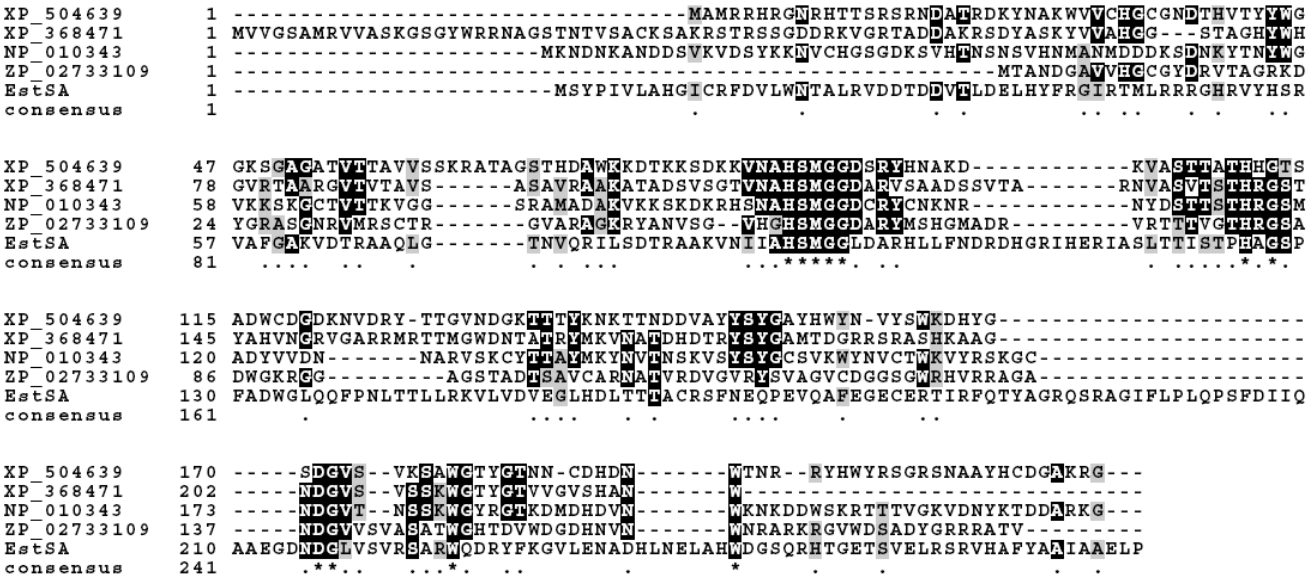

Figure 1

Conserved sequence blocks from multiple sequence alignment of EstAS from activated sludge metagenomic library and other related proteins. Sequence alignment was carried out with CLUSTAL_W [24] and BoxShade Server http://www.ch.embnet.org/software/BOX_form.html. XP_504639, esterase/lipase from *Yarrowia lipolytica* CLIB122; XP_368471, LipA from *Magnaporthe grisea* 70-15; NP_010343, esterase/lipase from *Saccharomyces cerevisiae* Tg12p; ZP_02733109, lipase from *Gemmata obscuriglobus* UQM 2246.

was observed at pH 9.0 and nearly 23% was still left at pH 10.5 (Fig. 6).

Effect of metal ions on esterase EstAS

The effect of metal ions on esterase EstAS activity is depicted in Table 3. Among metal ions tested, the activity was slightly increased by Co^{2+} (117%), Zn^{2+} (114%) and Fe^{2+} (103%), and strongly promoted by 1 mM Mn^{2+} (190%), in comparison with the control. However, it was a bit inhibited by Mg^{2+} and Ni^{2+} and almost totally inhibited by Cu^{2+} (7% residual activity). The fact that its activity was not affected by the chelating agent EDTA might suggest that this esterase is not a metalloenzyme. These results indicated that divalent metal ions, especially Mn^{2+}, are necessary for the catalytic activity of esterase EstAS, similarly to metagenomic lipase LipG [35] and esterase EstA from marine metagenome [36]. Therefore, manganese ions might carry out three distinct roles in esterase

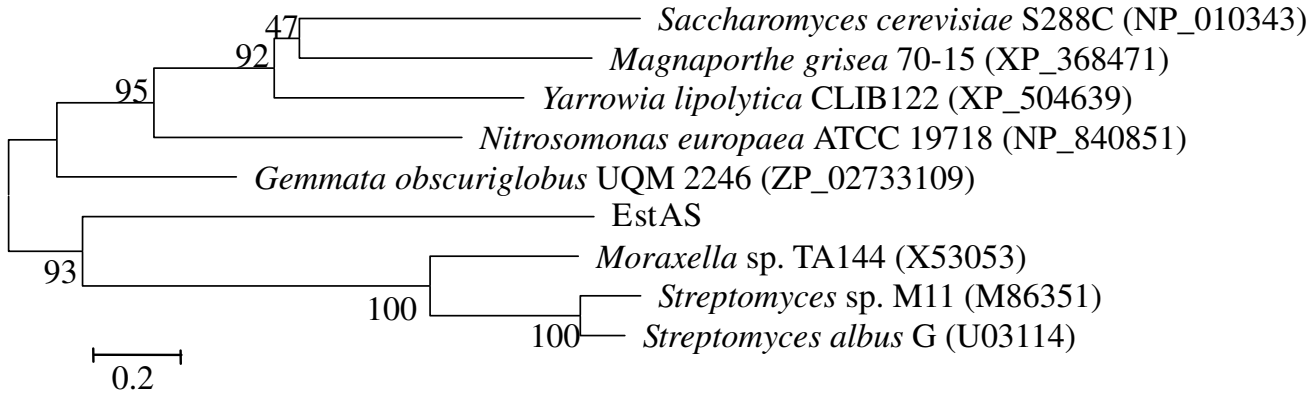

Figure 2

Phylogenetic analysis of EstAS and closely related proteins. Phylogenetic analysis was performed using the program MEGA 4.0. Except for EstAS, the protein sequences for previously bacterial lipolytic enzymes were retrieved from GenBank http://www.ncbi.nlm.nih.gov. The numbers at node indicate the bootstrap percentages of 1000 resamples.

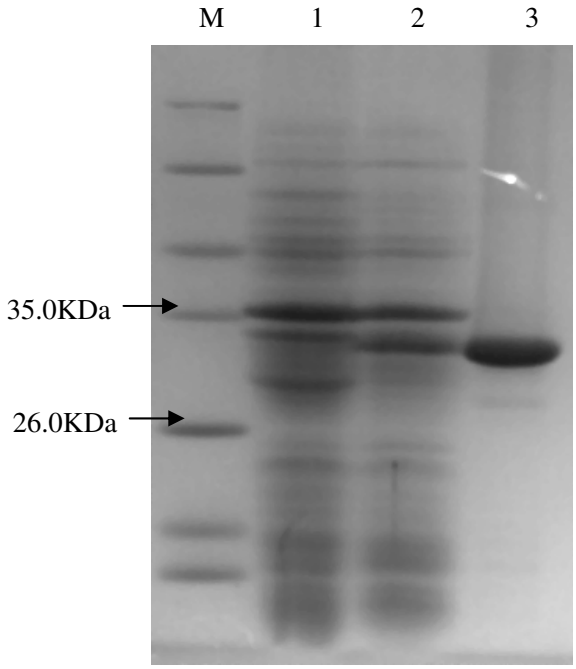

Figure 3
SDS-PAGE of overexpressed esterase EstAS in *E. coli*. Lane 1: molecular weight protein marker (Tiangen, Cat. No: MP102); lane 2, *E. coli*/pET28a: total protein extract, as negative control; lane 3: induced culture of *E. coli*/pEstAS-His, total protein extract; lane 4: purified EstAS (31 kDa).

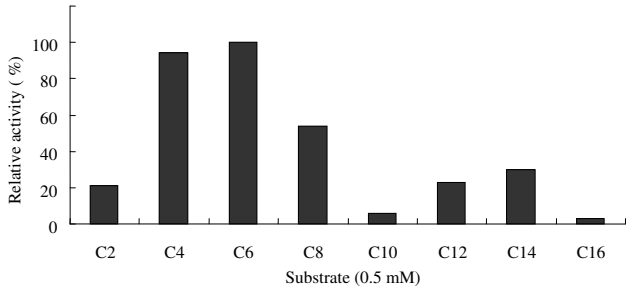

Figure 4
Substrate specificity of overexpressed and purified esterase. Relative activity was shown as the percentage of the activity towards 4-nitrophenyl hexanoate. All measurements were done in triplicate. C2, *p*-NP acetate; C4, *p*-NP butyrate; C6, *p*-NP hexanoate; C8, *p*-NP caprylate; C10, *p*-NP decanoate; C12, *p*-NP laurate; C14, *p*-NP myristate and C16, *p*-NP palmitate.

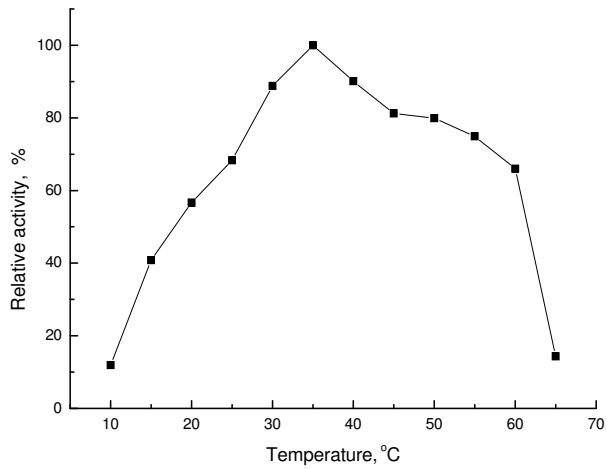

Figure 5
Apparent temperature optimum of esterase EstAS. Relative activity of *p*-NP-hexanoate hydrolysis at different temperatures by purified EstAS. The activity was determined at different temperatures at pH 8.0 in 50 mM Tris-HCl buffer. The activity at 35°C was set as 100% (4760 U/ml). All measurements were done in triplicate.

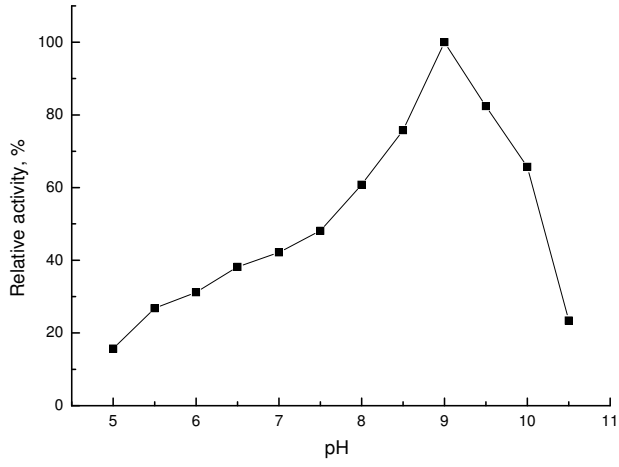

Figure 6
Effect of pH on the purified esterae EstAS. Relative activity of *p*-NP-hexanoate hydrolysis was performed in various pH buffers at 35°C (pH 5.0-7.5, 50 mM phosphate buffer; pH 8.0-10.0, 50 mM Tris-HCl buffer). The activity at pH 9.0 was set as 100% (4917 U/ml). All measurements were done in triplicate.

Table 3: Effect of metal ion on esterase activity

Compounds	Concentration (mM)	Relative activity (%)
Control	0	100.0 ± 3.7
$CoCl_2$	1	117.8 ± 2.1
$CaCl_2$	1	100.5 ± 3.4
$ZnCl_2$	1	114.7 ± 1.3
$MgCl_2$	1	81.7 ± 2.9
K_2SO_4	1	101 ± 4.1
$FeSO_4$	1	103.8 ± 1.6
$CuSO_4$	1	7.8 ± 2.3
$MnSO_4$	1	192.9 ± 3.8
$Ni(NO_3)_2$	1	46.2 ± 5.2
EDTA	1	121.7 ± 1.2

Activity without metal ions was set as 100% (5370 U/ml). All measurements were done in triplicate.

action: removal of fatty acids as insoluble Mn^{2+} salts in certain cases, direct enzyme activation acting as cofactor, and stabilizing effect on the enzyme.

Effect of detergents and reductors on esterase EstAS

The effects of detergents and reductors on esterase activity are shown in Table 4. A significant increase in lipolytic activity was observed with addition of 0.1% Tween 80 (128%), Tween 20 (135%), and 1 mM CTAB (138%), Triton X-100 (119%), after 0.5 h preincubation at 35°C. 1 mM β-mercaptoethanl, DTT did not affect the lipolytic activity (102% and 101%, respectively). However, DEPC and SDS had a strong inhibitory effect. In accordance with the esterase reported by Nawani et al. [36], a total loss of activity in the presence of SDS but an enhanced activity in the presence of Triton X-100, and Tween 20 and 80. Interesting, the esterase EstAS activity was not affected by 1 mM PMSF, suggesting it may possess a lid structure, which could eliminate the inhibition effect of PMSF, as some other esterases [10,37,38] and site-directed mutagenesis of amino acid Ser92 will be carried out to confirm the function of Ser92.

Conclusion

In conclusion, we identified a new esterase EstAS belonging to family III lipases from SBR activated sludge metagenomic library. EstAS is a very interesting enzyme with high potential for downstream biotechnological applications. This was confirmed by extensive biochemical characterization, substrate specificity, stability towards addictives including metal ions and detergents, and also, wide pH and temperature spectra. This study also demonstrated that the metagenomic approach is very useful for expanding our knowledge of enzyme diversity, especially for bacterial esterases. Accessing the metagenomic pool of lipases and esterases can be an immediate source of novel biocatalysts, or yield enzymes that can be further specialized by directed evolution.

Table 4: Effect of detergents and enzyme inhibitors on esterase activity

Compounds	Concentration	Relative activity (%)
Control	0	100.0 ± 2.1
β-mercaptoethanl	1 mM	102.7 ± 2.7
DTT	1 mM	101.9 ± 1.9
SDS	1 mM	16.2 ± 9.3
Triton X-100	0.1%	119.6 ± 4.6
Tween 80	0.1%	128.9 ± 0.8
Tween 20	0.1%	135.8 ± 3.1
CTAB	1 mM	138.3 ± 2.1
PMSF	1 mM	100.3 ± 5.2
DEPC	1 mM	48.6 ± 0.7

Activity without detergents and enzyme inhibitors was set as 100% (5290 U/ml). All measurements were done in triplicate.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TZ participated in the design of experiments, and carried out the study and drafted the manuscript. WJH carried out the SDS-PAGE experiment and sequence alignment. ZPL conceived the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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