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# CLONING, EXPRESSION AND CHARACTERIZATION OF AN ESTERASE GENE IN A METAGENOMIC LIBRARY OF TRADITIONAL FERMENTED FOOD

– Research paper –

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**Abstract**: Esterase is an industrial enzyme that is widely used in food, medicine, fine chemicals. The total genomic DNA was extracted from traditional fermented food in China to construct a metagenomic library that included a novel esterase gene (est_115). Sequence homology analysis showed that the highest homology with the carboxylester hydrolase from Pseudomonas lutea was 38%, indicating that esterase belongs to a new class of esterases. Then, an est_115 gene recombinant expression vector was constructed and expressed. The Est_115 had higher catalytic activity to p-nitrophenol ester, with a short acyl-carbon chain. The enzyme can maintain high catalytic activity and salt tolerance in 10%–18% NaCl, suggesting that this novel esterase can be used in processing food using high osmotic pressure.

**Keywords**: Traditional food; metagenomics; esterase; cloning; properties

## INTRODUCTION

Esterases (EC 3.1.1.X) is a general term for enzymes that catalyze the hydrolysis and synthesis of ester bonds. Catalyzed ester linkages produce glycerol and fatty acids during hydrolysis. During synthesis, the carboxyl groups of acids are dehydrated and condensed with hydroxyl groups of alcohols, yielding esters and other aroma substances (Svendsen, 2000). Esterase is a hydrolase that has been widely used in food processing and food flavor improvement, oil hydrolysis, leather/silk/yarn raw material degreasing, wastewater treatment, and the washing and pharmaceutical industries and other fields (Lehmann et al., 2014). Metagenomics is a new scientific technology that has recently emerged with advances in techniques in microbiology and modern life sciences. The essential concept is to directly extract the genomic DNA of all microorganisms in the environment, clone these into appropriate vectors, construct a metagenomic library, collect the nucleic acid information on all the microorganisms in the environment, and use sequence screening or function screening to obtain useful enzymes, antibiotics, and active substances from the library (Shestakov, 2012). Several novel biocatalysts have been successfully screened by metagenomic library technologies, including esterases, cellulases, xylanases, and β-glucosidases, generating characteristic features of novel enzymes (Yu et al., 2013).

Traditional fermented food production in China has a long history and these food items are known for its unique flavor and quality, which are closely related to their complex microbial communities. Therefore, the traditional food fermentation environment (e.g. soy sauce, bean curd, fermented blank bean) contains rich microbial resources. With the modernization and industrialization of traditional fermented food production, the development and application of microbial resources in the fermentation environment of traditional fermented foods has become a research hotspot (Nie et al., 2012). To date, no reports on the cloning of esterases from the metagenomics libraries of traditional food fermentation environment have been published. Therefore, in this study, a metagenomic library was constucted using the representative of traditional food fermentation environment in China, i.e., a high salt liquid state fermented soy sauce as a sample, from which a novel

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esterase gene from the library was selected and clones using a functional screening method, and the enzymatic properties of the expressed protein were analyzed. Our work lays the foundation for the development and utilization of microbial resources in the fermentation environment of traditional fermented foods in China.

## MATERIALS AND METHODS

### Materials and Reagents

*Escherichia coli* DH5α and BL21 (DE3) host cells were stored in our laboratory; Vector plasmid pUC118/BamHI (BAP) was purchased from TaKaRa (Dalian, Jilin, China); An expression vector pET-32 (+) was purchased from Novagen (Germany); gel recovery kits and plasmids extraction kits were purchased from QIAGEN (Germany); total DNA extraction kits were purchased from MP Biomedicals (U.S.A.); various restriction enzymes and T4 ligases were purchased from TaKaRa (Dalian, China); Other biochemical reagents were imported aliquots, purchased from Beijing Pubo Biotechnology Co., Ltd. Samples were collected from sauce mash in a soy sauce factory in Guangdong Province and stored at -80°C.

### Instruments and Equipment

The ABI Veriti gradient PCR instrument is a USA Applied Nanosystems 9700 GeneAmp® instrument; the ultrasonic cell disruptor is from US Sonics; the UV-1700 pharmespec ultraviolet (UV) and visible spectrophotometer is from Japan Shimadzu Corporation; and the Eporator electroporation instrument is from Germany Eppendorf; Eppendorf 5418 Centrifuge is from Germany Eppdendorf.

### Methods

*Extraction and purification of metagenomic DNA*

Genomic DNA was extracted from the soy sauce mash using a Fast DNA® SPIN kit for soil (MP Biomedicals), followed by a kit. DNA integrity was assessed by 0.8% agarose gel electrophoresis.

*Construction of the metagenomic library*

The obtained purified total DNA was incompletely digested with Sau3AI and then ligated into the pUC118/BamHI (BAP) vector that was previously completely digested with BamHI and dephosphorylated. The ligation product was precipitated with isopropanol, washed with 80% ethanol, re-dissolved with 10 μL of ultrapure water, electroporated into DH5α strain cells, and inoculated onto an LB plate (100 μg/mL ampicillin, 0.1 mmol/L IPTG, and 40 μg/mL X-gal), and incubated overnight at 37°C. A metagenomic library of fermented food environment samples was then constructed. Fifteen transformants were randomly selected, and the extracted plasmids were digested with *BamH*I, the size of the inserted fragments was analyzed by electrophoresis, and the diversity of the established metagenomic library was evaluated (Ye et al., 2010).

*Screening and sequence analysis of esterase genes*

The white clones from the above library were picked and spotted on a glyceryl tributyrate screening plate (100 μg/mL ampicillin, 0.1 mmol/L IPTG, 1% glyceryl tributyrate and 1% gum arabic), incubated at 30°C for 2–3 d, and observed for transparent hydrolysis circles around the colonies. The presence of a hydrolysis circle is indicative of esterase activity. Clones with hydrolyzed circles were selected, and the extracted plasmids were sequenced. The open reading frames (ORF) of the plasmids' sequences were identified using the ORF Finder on the NCBI website (http://www.ncbi.nlm.nih.gov/projects/gorf/).
In addition, homology analysis was performed on the protein sequence of the esterase using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

*Prokaryotic expression of the esterase gene*

Primers were designed based on the sequence of esterase gene est_115, and the amplification primers were designed: upstream primer F1: 5'-CGCGGATCCATGGTCCCCGCCGCCGAGTC-3' (underlined is *BamH*I cleavage site sequence); Downstream primer F2: 5'-CGGAAGCTTGGCCTGGCTGTGACATCC-3' (underlined is *Hind*III cleavage site sequence). PCR amplification was performed using the extracted plasmid pUC118-est_115 as template. The amplified product was recovered

by agarose gel electrophoresis, followed by a kit.

The PCR product of esterase gene est_115 was recovered from the gel, and the vector pET-32a (+) was double digested with HindIII and BamHI. The digested product was purified using a PCR product recovery kit. The digested products were ligated with T4 ligase and electroporated into expression host *E. coli* BL21 (DE3). Through resistance screening, positive clones were picked, and plasmid DNA was extracted for sequencing verification.

Strains containing the sequence-verified plasmids were inoculated into LB liquid medium containing 100 μg/mL ampicillin, incubated at 30°C at a speed of 250 rpm, and IPTG was added to $6\times10^{-4}$ mol/L levels when grown to an $OD_{600}$ = 0.8, incubated at 20°C for 20 h at a speed of 200 rpm, centrifuged at 12,000 rpm for 5 min, the supernatant was abandoned, the pellet was re-suspended in 20 mL of 0.05 mol/L Tri-HCl (pH 7.5), the cells were disrupted by sonication, centrifuged at 13,000 rpm at 4°C for 15 min, and the supernatant was collected to obtain a crude enzyme solution of the recombinant esterase.

The crude enzyme solution was purified using a His-tag protein purification kit (Invitrogen, USA). The purified recombinant protein was frozen in liquid nitrogen and stored at -80°C.

*Assessment of enzymatic properties*

Substrate specificity detection

Approximately 10 μL of the purified enzyme solution was added to 0.1mol/L Tris-HCl buffer (pH 7.0) with $1\times10^{-3}$ mol/L substrate, and the absorbance at a wavelength of 405 nm was measured at 35°C. The substrates to be determined were: *p*-nitrophenol acetate (C2), *p*-nitrophenol butyrate (C4), *p*-nitrophenol hexanoate (C6), *p*-nitrophenol caprylate (C8), *p*-nitrophenol palmitate (C10), and *p*-nitrophenol dodecanoate (C12).

Effect of temperature on esterase activity

Using $1\times10^{-3}$ mol/L *p*-nitrophenol butyrate as substrate, 10 μL of the purified enzyme solution was added to 0.1mol/L Tris-HCl buffer (pH 7.0). The absorbance at a wavelength of 405 nm was measured at 20°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, and 55°C, respectively.

*Effect of pH on esterase activity*

Using $1\times10^{-3}$ mol/L *p*-nitrophenol butyrate as a substrate, 10 μL of the purified enzyme solution was added. The absorbance value at a wavelength of 405 nm was measured at 35°C under different pH conditions within the ranges of pH 4.0–7.0 (0.1mol/L phosphate buffer) and pH 7.0–9.0 (Tris-HCl buffer).

*Effect of NaCl on esterase activity*

The purified enzyme solution was placed at room temperature in 5%, 10%, 15%, and 18% NaCl solution, respectively, and samples were taken to determine the enzyme activity at different time points. The enzyme activity was determined using the following method: using $1\times10^{-3}$ mol/L *p*-nitrophenol butyrate as a substrate, 20 μL of the sample (enzyme solution) was added to 0.1mol/L Tris-HCl buffer (pH 7.2), and the absorbance at a wavelength of 405 nm was measured at 35°C.

*Estimating total ester production by esterase*

The effect of esterase on total ester production was performed according to the conditions described previously (Ye et al., 2013). In a nutshell, 10 g soybean protein, 18 g NaCl, the crude protease obtained from *Koji* culture, and a certain amount of purified esterase form the reaction system (100 ml), which ensure final NaCl concentration is 18%. The reaction was carried out at 30°C for 60 h and then the total ester content in reaction mixture were measured with potentiometric titration.

## RESULTS AND DISCUSSION

### Soy Sauce Mash Metagenomic DNA Extraction and Library Construction

The kit was used to extract and prepare soy sauce mash metagenomic DNA. Most of the extracted genomic DNA was more than 25 kb in size, indicating that the genomic DNA was relatively complete, which satisfied the requirements on genomic DNA for constructing a high-quality metagenomic library.

The recovered and purified genomic DNA fragment was ligated with the vector pUC118/*BamHI* (BAP) at a molar ratio of 10:1 overnight, and the connection product was electroporated into *E. coli* DH5α cells. Fifteen single white colonies were randomly selected. After plasmid extraction, the size of the insert

was determined by enzyme digestion. The results showed that most of the digested fragments were between 2–7.5 kb in size. The positive clone rate was high, and the ligation rate was as high as 95%. These findings indicate that we successfully constructed a metagenomic library for screening esterase genes (Ye et al., 2010).

**Screening and Sequence Analysis of Esterase Genes**

A screening method for glyceryl tributyrate substrate was used to select a positive transformant with esterase activity from about 10,000 transformants of the above-mentioned soy sauce mash metagenomic library. The extracted plasmid from the positive clones was retransformed into *E. coli* DH5α cells, and the transformed clones also exhibited esterase activity, indicating that the inserted exogenous fragment was the source of esterase enzyme activity.

Positive transformants showing esterase activity were sequenced and searched for ORFs. The results showed that the insert contains a new esterase encoding gene. The gene is 948 bp long and encodes a 316-amino acid polypeptide. We named the gene as *Est_115*. The encoded protein, Est_115, was analyzed by the ClustalW software, and the results showed that the esterase contained the characteristic conserved pentapeptide sequence Gly-x-Ser-x-Gly. This functional region is an important feature of ester hydrolase proteins (Figure 1). In addition, homology analysis of the esterase Est_115 protein sequence showed that it had the highest homology with carboxylate ester hydrolase (A0A098SV65) from *Pseudomonas lutea* by 38%, followed by 37%, 37%, 37%, and 36% homology with four ester hydrolases: *Caulobacter henrici* (A0A0P0NYL4), *Methylobacterium* sp. Leaf99 (A0A0Q5CG82), *Pseudomonas syringae* CC1557 (W0N0S0), and *Pseudomonas syringae* pv. pis. (F3G4G1), showing that Esterase Est_115 belongs to a new carboxyl esterase class (Bornsch, 2002).

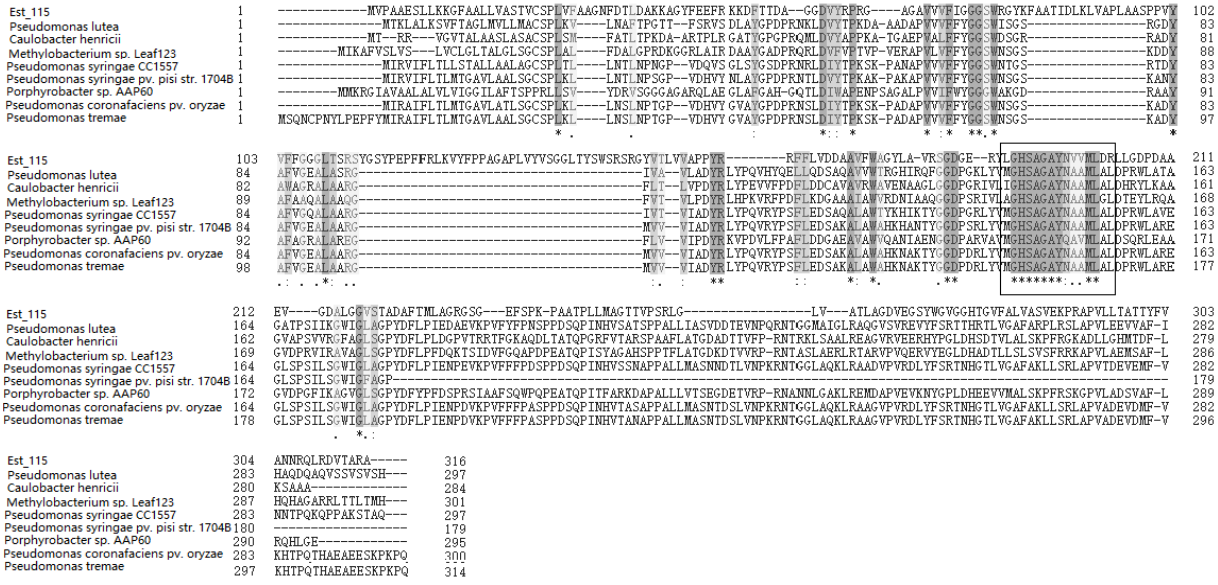


Figure 1. Alignment of protein Est_115 and other esterases.
The sequences in the box are functionally conserved regions.

**Cloning and Prokaryotic Expression of Esterase Gene *est_115***

Plasmids of positive clones were used as template, and primers F1 and F2 were used for PCR amplification. The PCR products were digested by restriction enzymes, ligated with expression vector pET-32a, and transformed into host strain BL21 cells. The transformants were randomly selected for sequencing. The sequencing results showed that the target gene sequence was completely identical to our *est_115* sequence, i.e., the recombinant strain BL21/pET-est_115 expressing the esterase gene *est_115* was obtained.

The recombinant strain BL21/pET- *est_115* was induced by IPTG and then disrupted using ultrasound. The supernatant was collected, and the effective expression of target gene was verified by enzyme activity of the crude enzyme solution. The obtained crude enzyme

solution was purified using a His-tag protein purification kit to obtain the purified recombinant esterase Est_115. The purified esterase had a single band on SDS-PAGE, with a molecular weight of approximately 50 kDa, which is similar to the theoretical analysis that was based on amino acid sequence calculations. The results are shown in Figure 2. After purification, the specific activity of esterase Est_115 reached 1,593 IU/mg protein.

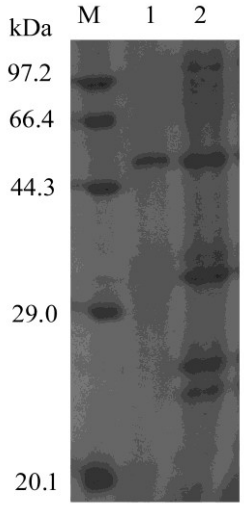


Figure 2. SDS-PAGE analysis of recombinant esterase Est_115
M: Low-molecular weight standard protein (TaKaRa); Lane 1: recombinant protein Est_115 before purification; Lane2: purified recombinant protein Est_115

**Substrate Specificity of Esterase Est_115**

The fatty acid specificity of esterases refers to the specific reactivity exhibited by fatty acids with different carbon chain lengths and saturations (Lewin et al., 2016). We determined the substrate specificity of recombinant esterase Est_115 for p-nitrophenolate ester in 0.1mol/L Tris-HCl buffer (pH 7.0). The results are shown in Figure 3. The recombinant esterase Est_115 has higher catalytic activity for the shorter p-nitrophenol esters (C2, C4, C6, C8, and C10). The substrate with the highest catalytic activity is *p*-nitrophenol butyrate (C4), followed by *p*-nitrophenol acetate (C2) and p-nitrophenol hexanoate (C6). As chain length of the ester substrate increases, its catalytic activity decreases, indicating that the enzyme prefers to hydrolyze *p*-nitrophenol ester substrate with relatively shorter acyl carbon chain, so the enzyme is a true esterase rather than a lipase (Jiang et al., 2012) (Xu et al., 2010).

**Optimum Reaction Temperature of Esterase Est_115**

As shown in Figure 4a, within a certain range, the esterase activity increased with higher temperatures, reaching the highest activity at 35°C; thereafter, enzyme activity slow decreased, but it still had high catalytic activity within a wide range (25°C–50°C) below the optimal temperature, and the residual enzyme activity was >40%. Compared to esterases from other sources (Jiang et al., 2012) (Biver and Vandenbol, 2013), the recombinant esterase Est_115 in this study showed wide temperature adaptability.

**Optimum Reaction pH of Esterase Est_115**

At a fixed temperature, buffers with different pH ranges were used to detect changes in enzyme activity with pH. As shown in Figure 4b, the optimal reaction pH of the recombinant esterase Est_115 was 7.0, and the enzyme activity was still > 60% in the acid range of pH 5.5–6.5, indicating that the esterase is a weakly acidic esterase. The environments for microbial growth are quite different. Therefore, the optimal conditions for the enzymes obtained from screening them are not the same. Several studies have shown that esterases are generally neutral proteins, and their optimal pH is generally within the neutral range of 6.5–7.5, the optimal pH of many animal and microbial esterases is close to 7.0, which is suitable for application in neutral media (Jiménez et al., 2012) (Jeon et al., 2012).

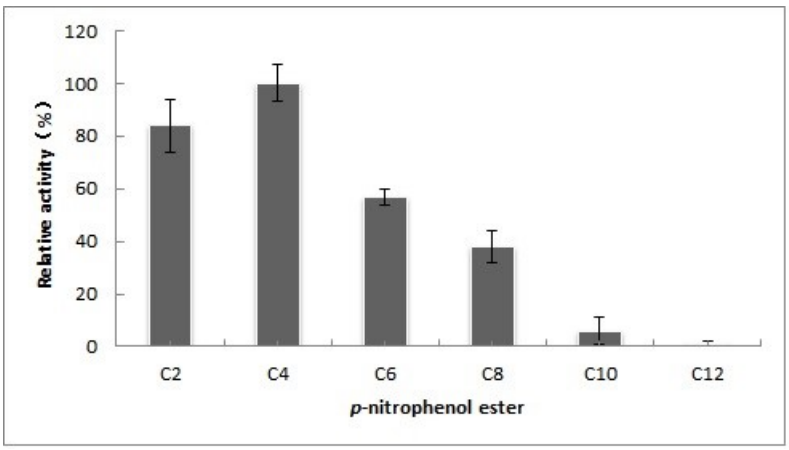


Figure 3. Substrate specificity of recombinant esterase Est_115

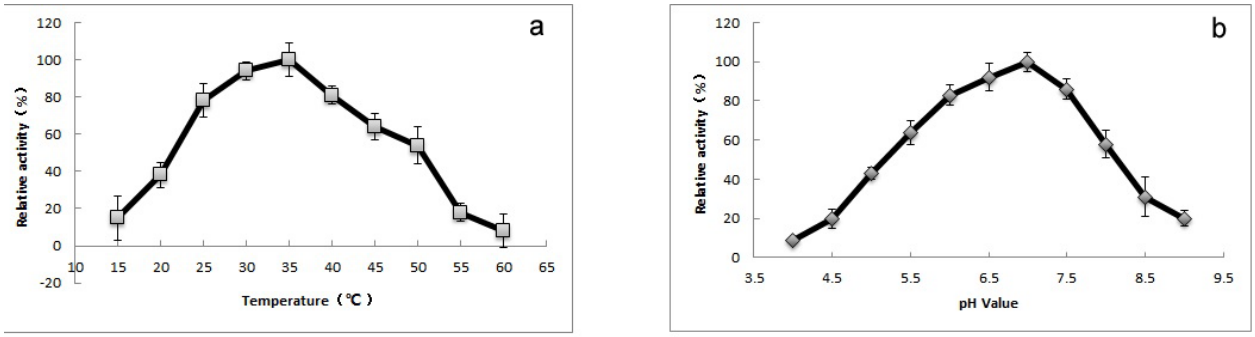


Figure 4. Effect of temperature (a) and pH (b) on the activity of Est_115

**Salt Tolerance of Esterase Est_115**

Because this esterase is derived from our traditional high-salt fermented food environment in China, we studied the changes in the activity of esterase Est_115 at different salt concentrations. A certain amount of enzyme solution and different concentrations of NaCl were stored at 25°C for a certain period of time to detect the residual activity. The results are shown in Figure 5. The recombinant esterase, Est_115, retained its 40% enzyme activity at 18% NaCl for 30 days. The results showed that the recombinant esterase Est_115 has a strong salt tolerance. To date, there have been few reports of salt-tolerant esterases. For example, esterase from *Pseudoalteromonas sp.* NJ70 has only 2.6% of enzyme activity in a 3 mol/L (about 17.5%) solution (Wang et al., 2012). When the esterase of *Psychrobacter sp*. was at a high concentration of 5.0 mol/L (approximately 29%), the activity of the enzyme remained about 80% of the original activity (Wu et al., 2013). However, neither of these has studied the stability of the enzymes with long-term treatment in high-concentration NaCl solution. Est_115 is relatively stable under high salt concentrations for a long period of time, indicating that it may be potentially applied to high-osmotic pressure environments. Therefore, the enzyme can be used in the processing of high-osmotic pressure foods and is a novel type of esterase with great potential in the food processing industry.

**Effect of purified esterase supplementation on total ester production in a model reaction of soy sauce fermentation**

The esterase is an useful enzyme additive during soy sauce fermentation, being responsible for the synthesis of ester that can improve sauce's special flavor and quality (Wei et al., 2013). So that, the effect of esterase from metagenomic library on total ester production in a model reaction of the high salt dilute fermentation of Chinese soy sauce was investigated. The results are shown in Figure 6. The total ester production of reaction mixture was higher with the addition of esterase Est_115 (15 U/g) than without. At the end of reaction, the total ester concentration of mixture with esterase was no less than 42.2 mg/L higher than that without the addition of esterase. From these results, the addition of recombinase Est_115 could accelerate and improve the production of total ester. Moreover, compared with the addition of yeast, the addition of esterase Est_115 was most

effective for the production of total ester. And esterase preparations are easier to control than yeast strains in production operation (Sluis et al., 2001). The results indicate that recombinase Est_115 is suitable for application in soy sauce liquid fermentation with high-salt process and can improve the flavor and quality of soy sauce.

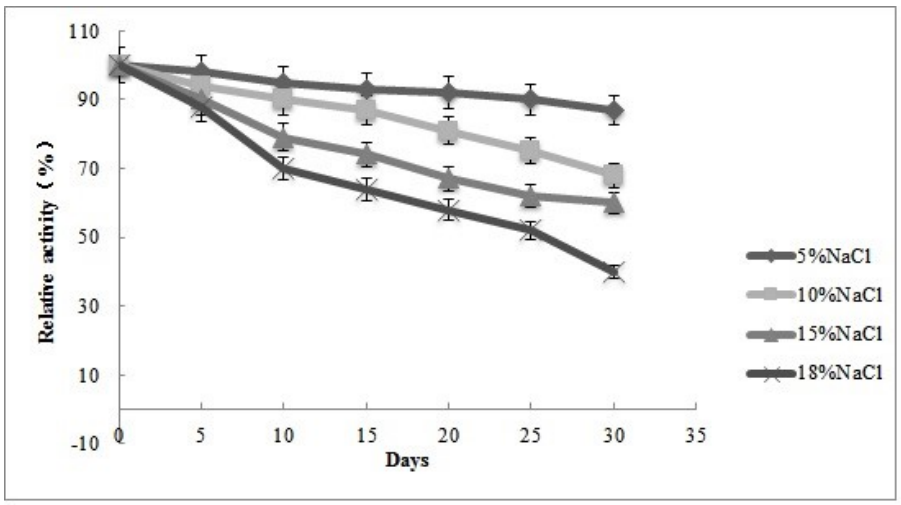


Figure 5. Effect of NaCl on the activity of Est_115

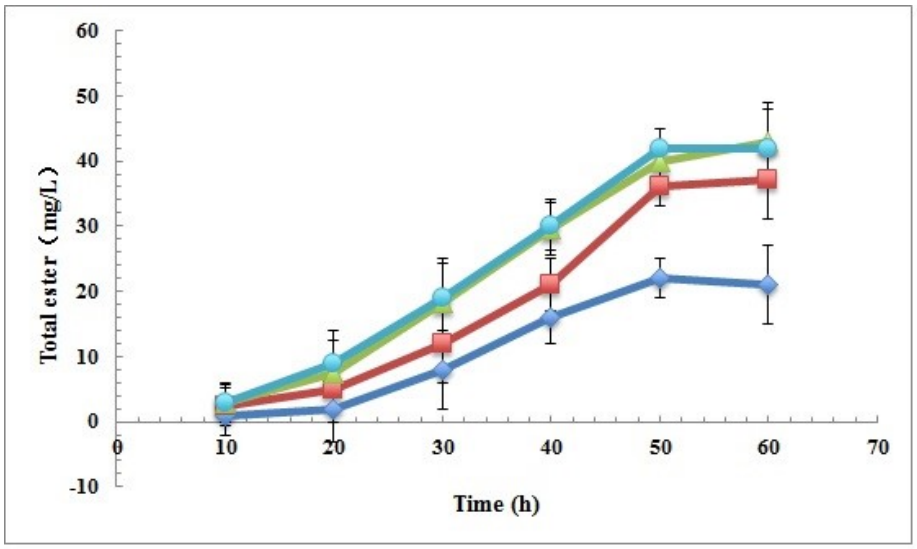


Figure 6. Effect of purified Est_115 on total ester production.
A model reaction with the addition of 20U/g Est_115 (●), with the addition of 15U/g Est_115 (▲), without the addition of 5U/g Est_115 (■) and without the addition of esterase (◆).

## CONCLUSIONS

We screened a new esterase gene *est_115* from a metagenomic library of traditional fermented food environment in China using a functional screening method, successfully cloned and expressed this in *E. coli*, and obtained purified recombinase Est_115. SDS-PAGE analysis showed that the esterase molecular weight is about 50 kDa, and substrate specificity experiments showed that the esterase has higher catalytic activity for p-nitrophenol esters with shorter acyl carbon chains. The most suitable substrate is p-nitrophenolic butyrate (C4). The optimal reaction temperature is 35°C, and the optimal pH is 7. Compared to other known esterases, the most important feature of the recombinant esterase Est_115 is that in 10%–18% NaCl, the enzyme maintains its catalytic activity and strong salt tolerance. Therefore, the enzyme can be used in the processing of high-osmotic pressure foods and is a novel type of esterase with great potential in the food processing industry. In addition, the discovery

of this enzyme is also beneficial to the expansion of microbial esterase research and the study of its salt tolerance mechanism. In the next step of this study, we will optimize the conditions for gene expression induction and further analyze its enzymatic properties and salt tolerance mechanisms to obtain better enzymes, as well as provide technical support for large-scale industrial production in the future.

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